Differential genome-wide DNA methylation in association with nightshift work: From discovery to policy

Charleen Danielle Adams

A dissertation
submitted in partial fulfillment of the
requirements of the degree of

Doctor of Philosophy

University of Washington

2016

Reading Committee:

Parveen Bhatti, Chair

Wylie Burke

Timothy Randolph

Program Authorized to Offer Degree:

School of Public Health

©Copyright 2016

Charleen Danielle Adams

University of Washington

Abstract

Differential genome-wide DNA methylation in association with nightshift work: From discovery to policy

Charleen Danielle Adams

Chair of the Supervisory Committee:

Parveen Bhatti, Research Associate Professor

Department of Epidemiology

Shift work, working outside the hours of 6 a.m. to 6 p.m., is done by more than 21 million people in the United States and disproportionately done by the poor and by minorities. Many acute and chronic adverse health effects (including sleeplessness leading to injuries and accidents, cardiovascular disease, obesity, metabolic syndrome, type 2 diabetes, and cancer) have been associated with working the nightshift. But because shift work is an integral part of our economy and unlikely to go away, owing to both the necessity of some services (e.g., hospital care, police patrol, and air traffic control) and the simple demand for around-the-clock conveniences (e.g., 24-hour grocery stores and gas stations), its prevalence, health effects, and burden on vulnerable populations makes shift work not only an exacerbating social determinant of health but a pressing public health concern. Yet scientific uncertainties remain about 1) the underlying mechanisms putting shift workers at risk for these health problems, 2) biomarkers that may help

us ascertain earlier who is at greatest risk, 3) and best practices for limiting and/or mitigating shift work's harms. As such, this dissertation was undertaken to tackle some of the unknowns in each of these areas of uncertainty. The first two chapters comprise exploratory (biomarker) analyses of DNA methylation, a potential mechanism linking shift work to cancer, among day and nightshift workers from the healthcare industry in the greater Seattle area. Chapter 1 explores the main effects of shift work on genome-wide DNA methylation among shift workers with an *a priori* focus on circadian genes, and Chapter 2 investigates whether DNA methylation of the top findings from Chapter 1 depends on two modifiable factors, chronotype (preference for activity in the morning or evening) and sleep duration (derived from actigraphy). Chapter 3 explores the policy landscape of the nightshift, using what we know and what we do not know about shift work and cancer as a case study. Applying the precautionary principle to motivate shared responsibility for shift work's harms, we call for the convening of a consortium of shift work stakeholders and public deliberators to set, execute, and review the findings of a shift work research agenda aimed at speeding the discovery of mechanisms and biomarkers that can inform policy decisions to protect the lives of the many millions who work at night.

Acknowledgements

In writing this dissertation, I have benefited tremendously from the influence, support, and guidance of many people, beginning with my dissertation committee: Parveen Bhatti, Tim Randolph, Diana Buchanan, and Wylie Burke. I thank all of my coauthors—Parveen Bhatti, Timothy Randolph, Wade Copeland, Dana Mirick, Xiaoling Song, Cassandra Sather, Andres Houseman, Scott Davis, Erika Blacksher, and Wylie Burke—and especially Erika Blacksher, Wylie Burke, Wade Copeland, and Timothy Randolph, the mavens who made the ethical arguments (Erika and Wylie) and statistics (Wade and Timothy) come alive. I am grateful to Tom Vaughan and the Program in Epidemiology at the Fred Hutchinson Cancer Research Center for the training that supported the quantitative components of this project and to Bruce Weir, who funded my last two terms of writing. I owe Priya Duggal and Terri Beaty at Johns Hopkins Bloomberg School of Public Health and Mark Greene, Blanche Alter, Neelam Giri, Jennifer Loud, Mark Schiffman, Doug Stewart, and Michal Freedman at the National Cancer Institute heartfelt gratitude for launching me into genetic epidemiology—their words and research informed my choices of study at the University of Washington. I thank Steve Pinker, whose example gave me the gumption and stride to write and whose interdisciplinary work, public intellect, and integrity have been an inspiration for more than two decades and my model for what I hope to be as a person, thinker, and scientist. My daily routine during the bulk of the days I drafted this manuscript was decorated with curiosity due to Jerry Coyne, whose daily posts about science and philosophy provided me structure, humor, and insight into the human condition—that we are *determined* by our genes and environments—reinforcing the ethical motivation for this project: to understand the modifiable factors impacting shift workers and to figure out how to intervene regarding shift work, itself a social *determinant*. About figuring out

how to intervene, Lily Tomlin is said to have quipped, “I always wondered why somebody didn’t do something about that. Then I realized I was somebody.” For me, towards the end of this project, I realized that discovery and policy come together for shift work when we understand that *we* are “somebodies.” As Wylie has remarked on several occasions, quoting a colleague of hers, “ask them.” We must *ask* each other what we know, *ask* shift workers, scientists, policy makers, and the public (you and me) what we each know about shift work. And with the importance of *asking* in mind, most of all, I thank Parveen, the person I first *asked* about shift work and my mentor who opened my heart and mind to the occupational problems of the nightshift, teaching me nearly everything I know about circadian biology, methylation, and subgroup analyses and who imparted to me the official identity of Epidemiologist.

Dedication

To the many who work and have worked at night, among them my father. To the reality of our "choices" being limited by social influences, and to the "choice" (that is not a choice) to work at night. To shared responsibility.

Differential genome-wide DNA methylation in association with nightshift work: From discovery to policy

Table of Contents

Introduction **10**

Specific Aims **11**

Part A, Analysis 1—Methylation **12**

Abstract **12**
Introduction **14**
Materials and Methods **17**
Study subjects 17
Data and Biospecimen Collection 20
Laboratory Methods 20
Statistical Analysis **24**
Interpretation **26**
Results **26**
Discussion **30**
Methylation in Gene Bodies 30
CARD11 33
Additional Methylation Differences 34
Strengths and Limitations 44
Analysis I—Methylation Tables and Figures **49**
TABLE 1. Demographic data for study participants 49
TABLE 2. DNA methylation data processing procedures 50
FIGURE 1. Principal components analysis (PCoA) scree plot demonstrating the first principle component accounting for >90% of the variation in leukocyte cell mixture 51
FIGURE 2. Five (circadian) loci with the smallest unadjusted p-values and five (circadian) loci with the largest absolute effect size differences when comparing nightshift to dayshift workers 56
FIGURE 3. Five (non-circadian) loci with the smallest unadjusted p-values and five (non-circadian) loci with the largest absolute effect size differences when comparing nightshift to dayshift workers 57
FIGURE 4. Circadian autoregulatory feedback loop 62

Part A, Analysis 2—Chronotype and Sleep Quality **63**

Abstract **63**
Introduction **66**
Materials and Methods **68**
Study subjects 68
Data and Biospecimen Collection 70
Laboratory Methods 72
Data Processing 74
Statistical Analysis **75**
Results **77**
Chronotype effect modification analyses 78
Sleep duration effect modification analyses 79
Discussion **80**

Sleep duration effect modification analyses ... 82
Analysis 2—Methylation Tables and Figures ... 87
TABLE 2. Average demographic characteristics of the 361 nightshift workers and the 30 nightshift workers missing data for the chronotype and sleep duration analyses ... 88
TABLE 3. Results from shift by chronotype effect modification analysis on methylation at pre-selected differentially methylated loci among circadian genes ... 89
FIGURE 1. Shift by chronotype effect modification on methylation at cg08926642 in *PER3* ... 91
FIGURE 2. Shift by chronotype effect modification on methylation at cg17724687 in *PER3* ... 92
TABLE 4. Results from shift by chronotype effect modification analysis on methylation at pre-selected differentially methylated loci among non-circadian genes ... 93
TABLE 5. Results from shift by sleep duration effect modification analysis on methylation at pre-selected differentially methylated loci among circadian genes ... 95
FIGURE 3. Shift by sleep duration effect modification on methylation at cg27004243 in *PER1* 97
FIGURE 4. Shift by sleep duration effect modification on methylation at cg22387253 in *RORα* ... 98
TABLE 6. Results from shift by sleep duration effect modification analysis on methylation at pre-selected differentially methylated loci among non-circadian genes ... 99
FIGURE 5. Shift by sleep duration effect modification on methylation at cg01349856 in *LOXL1* ... 101

Part B—Policy ... 102
Introduction ... 102
The Precautionary Principle ... 104
Shift Work and Scientific Uncertainty ... 107
Molecular Mechanisms ... 108
FIGURE 1. Proposed pathways linking shift work and cancer ... 108
"Clocks" ... 109
Epidemiologic Studies ... 110
Justification of a Precautionary Stance ... 113
Shared Responsibility for Research and Decision-Making Through Collaboration ... 115
FIGURE 2. Shared responsibility for shift work ... 116
Research consortium ... 116
Gather evidence ... 118
Convene stakeholders for democratic deliberation ... 119
Inform policy and practice ... 120
Conclusion ... 120
Analysis III—Policy Table ... 124
TABLE 1. IARC-reviewed studies of shift work and (breast) cancer ... 124

References ... 125

Introduction

Shift work–-working outside of 6 a.m. to 6 p.m.–-is a hallmark of our 24-hour economy. Conservatively, 18% of workers in the developed world are engaged in shift work, many during their biological night (i.e. nightshift work) (McMenamin, 2007). This high prevalence of shift work is noteworthy given that sleep is normally meant to occur at night in humans. A number of acute and chronic health effects have been observed among shift workers: 32-36% of shift workers fall asleep on the job at least once a week; the risk of occupational accidents is 60% higher for shift workers compared to those who work during the day; and shift workers have an increased risk of cardiometabolic diseases and mood disturbance (S. M. W. Rajaratnam, Howard, & Grunstein, 2013). Moreover, shift work is also a probable human carcinogen, classified as such in 2007 by the International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer, 2007). This makes shift work an important public health concern, posing challenging policy questions, as the reality is that removing shift work altogether is unlikely, and policy options to mitigate and minimize harm are needed. However, it is not yet clear how interventions should be designed to mitigate harm among shift workers, as there are many outstanding uncertainties about what aspects of shift work most contribute to its potentially carcinogenic effects. In particular, a better understanding of the molecular mechanisms behind shift work's relationship to cancer could aid strategies to mitigate shift work's negative health effects, but the molecular mechanisms linking shift work to cancer are not yet completely understood. To that end, this dissertation explores changes in DNA methylation, which have been shown to play a central role in carcinogenesis (Dong, Zhao, Li, Li, & Yang, 2014; Moore, L.E., Huang, W-Y, Chung, J., Hayes, 2003) and can be modulated by environmental exposures (Suter & Aagaard-Tillery, 2009) as a potential mechanism, by comparing genome-wide DNA

methylation profiles between actively working dayshift and nightshift workers. Given that disruption of circadian rhythms (the 24-hour pattern of changes in metabolism, physiology and behavior) is a hallmark of shift work (Knutsson, 2003), we had an *a priori* focus on methylation changes in circadian genes, which govern our circadian rhythms. We also explore, for our most intriguing findings, potential modifying effects of chronotype and sleep quality, which may be used as factors to identify subgroups that may be particularly sensitive to the carcinogenic effects of shift work. The policy landscape of shift work is also discussed and a call-to-action is put forth, using the precautionary principle, to motivate shared responsibility for limiting shift work's harms.

Specific Aims

The aims of this dissertation are 1) to characterize genome-wide methylation differences between night and dayshift workers, with an *a priori* focus on the circadian genes, 2) to determine whether differences in methylation between night and dayshift workers are modified by chronotype and sleep quality, and 3) to use the precautionary principle to motivate shared responsibility for limiting shift work's harms.

Part A, Analysis 1—Methylation

Title:

Differential genome-wide DNA methylation in association with nightshift work

Authors:

Charleen D. Adams[1], Timothy Randolph[1],Wade Copeland[1], Dana K. Mirick[1], Xiaoling Song[1], Cassandra L. Sather[2], Andres Houseman[3], Scott Davis[1], Parveen Bhatti[1]

[1]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA.

[2]Genomics Resource, Fred Hutchinson Cancer Research Center, Seattle, WA, USA.

[3]College of Public Health and Human Sciences, Oregon State University, Corvallis, OR, USA

Funding / Acknowledgments:

The study was funded by grants R01 CA097090 and R01 CA116400 from the US National Cancer Institute and funds from the Fred Hutchinson Cancer Research Center.

Abstract

Introduction: Changes in DNA methylation, possibly associated with transcription, might accompany nightshift work and function as early biological markers of the negative health effects of engaging in nightshift work. However, relatively few investigations have explored DNA methylation among nightshift workers. In the largest scale investigation of nightshift work and genome-wide methylation to date, we evaluated differences in genome-wide methylation between 308 actively working nightshift and 153 actively working dayshift workers from the Seattle metropolitan area.

Methods: Methylation data were generated using the Illumina Infinium HumanMethylation450 Bead Array. For each CpG locus, the M-value was derived as the measure of methylation.

Methylation data were processed to correct for potential assay biases. Methylation levels at a total of 346 CpG loci among 12 circadian genes and 197,261 non-circadian CpG loci were compared between nightshift and dayshift workers using rank-regression adjusted for gender, age, body mass index, race, and leukocyte cell profile. Analyses at the level of gene (12 circadian and 11,598 non-circadian genes) and region within each gene [i.e., promoter (defined as: TSS1500, TSS200, 5'UTR, or first exon) gene body, and 3'UTR, including 22 circadian and 10,531 non-circadian regions] were also conducted with methylation levels across each gene and gene region modeled using kernel association tests. Only genes and regions with at least five CpG loci were included in these analyses. Analyses for the circadian and non-circadian loci, genes, and regions, were undertaken separately. For each set of analyses, the false-discovery rate (FDR < 0.05) was applied to adjust for multiple comparisons.

Results: No statistically significant differences were observed for loci in the circadian genes; the locus (cg09304381) with the lowest *p*-value was in *NR1D1* and was hypermethylated in nightshift compared to dayshift workers. The circadian locus (cg13286116) with the largest absolute effect size was in *BMAL1* and was hypomethylated in nightshift relative to dayshift workers. Among the non-circadian loci, one statistically significant difference between night and dayshift workers was observed in *CARD11* at cg03227775 (adjusted p-value=0.042); the locus was hypomethylated in nightshift compared to dayshift workers. The non-circadian locus (cg26705599) with the largest absolute difference in methylation was located upstream of LINC00368 and was hypermethylated in nightshift compared to dayshift workers. No statistically significant differences were observed at the gene or gene region levels. The lowest unadjusted *p*-value for the gene-level and gene region-level findings among the circadian genes was observed for *BMAL1* in its putative promoter region. Among the non-circadian genes, the

lowest unadjusted *p*-value for the gene-level analysis was observed for *HSPA4L*, and for the region-level analysis, the lowest unadjusted *p*-value was observed for the putative promoter of *USP42*.

Conclusion: *CARD11* is an oncogene that activates the nuclear factor–kB (NF-kB) signaling family and has been associated with the least curable form of non-Hodgkin's lymphoma (NHL) (Lenz et al., 2008). An increased risk of NHL has been reported previously in two epidemiological studies of shiftwork and cancer (Lahti, Partonen, Kyyronen, Kauppinen, & Pukkala, 2008; Parent, El-Zein, Rousseau, Pintos, & Siemiatycki, 2012). Thus, methylation changes in *CARD11* may play a role in the carcinogenicity of nightshift work. Confirmation of this finding within a larger study of nightshift work and methylation, particularly a study with cancer incidence data, is warranted. In addition, our exploratory study suggests that nightshift work may impact methylation of a variety genes, the perturbation of which may contribute to carcinogenesis. Future studies of nightshift work and methylation should also integrate expression data to explore the relationship between methylation and expression, in addition to cancer outcomes.

Introduction

Shift work—working outside of 6 a.m. to 6 p.m.—is a feature of our 24-hour economy, with 18% of workers in the United States engaged in it full-time (S. M. Rajaratnam & Arendt, 2001). This high prevalence, coupled with growing evidence for the carcinogenicity of shift work (International Agency for Research on Cancer, 2007; Jia et al., 2013; Lin et al., 2015), makes shift work a pressing public health concern. An understanding of the mechanisms behind shift work's relationship to cancer could aid strategies to mitigate its negative health effects; however, the molecular mechanisms linking shift work to cancer are incompletely understood.

Several promising mechanisms have been proposed (Bhatti, Zhang, et al., 2014; Ehrlich, 2002; Stevens, Hansen, Costa, & Rüdiger, 2011). Among these, changes in DNA methylation are compelling for two reasons: 1) Changes in DNA methylation are inducible by exogenous factors (Moore, L.E., Huang, W-Y, Chung, J., Hayes, 2003)—and 2) changes in DNA methylation occur in the early stages of carcinogenesis (Dong et al., 2014; Moore, L.E., Huang, W-Y, Chung, J., Hayes, 2003).

DNA methylation, occurring at the 5'carbon of cytosines in CpG dinucleotides, is essential for proper cellular function, and differential DNA methylation associates with transcriptional programming (Bell et al., 2011; Szyf, 2012). For example, hypomethylation in the promoter region of genes generally marks a transcriptionally permissive DNA state, while hypermethylation in the promoter region of genes generally marks a transcriptionally repressive DNA state. Likewise, inactivation of tumor-suppressor genes can accompany the hypermethylation-linked transcriptional repression of their promoters, and hypomethylation across the genome can indicate genomic instability, which contributes to cell transformation (Kulis & Esteller, 2010). Moreover, methylation may have regulatory functions outside of the proximal promoters of genes, as has been observed for enhancers (Heyn et al., 2016).

Genes that drive the circadian clock may be a particular target of differential methylation related to shiftwork. The circadian clock is the transcriptional-translational feedback loop occurring over an approximately 24-hour period, thereby producing the many daily oscillations in physiology, hormone production, metabolism, and behavior that we know as our circadian rhythms. It is well known that shift work disrupts the timing and occurrence of these rhythms, which may be at least partially driven by changes in DNA methylation (Arendt, 2010).

All major transcriptional and signal transduction pathways are influenced by the circadian clock (Sancar et al., 2015), including processes relevant to carcinogenesis, notably the cell cycle, the DNA damage response, and immunity (Gery & Koeffler, 2007; Sancar et al., 2010; X. Yu et al., 2013). Thus, transcriptional changes to the genes in the circadian clock or the genes they control might contribute to the development of cancer and/or may serve as early biological markers of shift work's negative health effects. However, little attention has been given to whether differences in methylation are detectable among those who work different shift schedules. As such, our study, which is the largest scale investigation to date of nightshift work (the overnight hours most likely to cause circadian misalignment) and methylation, explores the potential impacts of the nightshift on genome-wide DNA methylation with a particular focus on the circadian genes.

Two population-based studies have reported an association between methylation changes in circadian genes, as measured in blood, and cancer (Hoffman, Yi, et al., 2010; Hoffman, Zheng, et al., 2010), strengthening the hypothesis that changes in methylation patterns of circadian genes perhaps partially contribute to the increased risk of cancer observed among nightshift workers. Likewise, there have been two studies of DNA methylation among shift workers examining methylation of circadian genes in blood (Bhatti, Zhang, et al., 2014; Zhu et al., 2011). In a population of 117 female (19 long-term (≥10 years) nightshift workers and 98 workers without a history of working between the hours of 19:00 and 09:00), Zhu *et al*. (2011) examined promoter-specific methylation of two circadian genes (*CLOCK* and *CRY2*) and, in a subset of 10 workers, examined genome-wide DNA methylation at 27,000 CpG loci. They observed hypomethylation in the promoter of *CLOCK* and hypermethylation in the promoter of *CRY2* among long-term nightshift workers (Zhu et al., 2011). These findings are consistent with

those from the population-based studies of methylation and breast cancer in which hypermethylation of *CLOCK* was observed to decrease risk and hypermethylation of *CRY2* was observed to increase risk of breast cancer (Hoffman, Yi, et al., 2010; Hoffman, Zheng, et al., 2010). Building on the work by Zhu *et al*. (2011), our previous study (Bhatti *et al*. 2014) included both women and men, totaling 124 actively working day and nightshift workers. We examined DNA methylation at 473,800 CpG loci, including 391 loci across the 12-core circadian genes. Significant differences between nightshift and dayshift workers were observed at 16,135 of 473,800 loci, including 21 loci located in the 12-core circadian genes. Among these, one locus in *CLOCK* (cg04264638) was significant (FDR-adjusted p-value=0.02328). In contrast with Zhu *et al*. (2011), none of our loci in *CRY2* were significant. All significant differences were hypomethylated in nightshift compared to dayshift workers (Bhatti, Zhang, et al., 2014).

Because the methods for preprocessing and analyzing methylation data have advanced rapidly since our 2014 study, our current study utilizes more recent data processing methods, taking a more conservative approach than used in our previous study, and includes the 124 participants (65 dayshift and 59 nightshift) from our previous study as well as an additional 338 workers (88 dayshift and 249 nightshift) from two previous studies of nightshift work (Davis, Mirick, Chen, & Stanczyk, 2012; Mirick et al., 2013) with available DNA samples that we used to generate DNA methylation data. With 461 participants, this is the largest scale investigation of nightshift work and DNA methylation conducted to date.

Materials and Methods

Study subjects

The study was approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. Study subjects were drawn from participants of three previous studies

of shift work conducted among healthcare workers from the Seattle metropolitan area: the Female Shift Worker Study (Davis et al., 2012) (*A*), the Male Shift Worker Study (Mirick et al., 2013) (*B*) and our previous study of nightshift work and DNA methylation (Bhatti, Zhang, et al., 2014) (*C*). For the remainder of the paper, the abbreviations *A*, *B*, and *C* will be used to designate each study. For all three studies, subjects were recruited through advertisements at local area hospitals, direct mailing to Washington State Department of Health licensed and certified health care workers, and referrals from eligible and ineligible participants. To be eligible for these studies, participants had to be actively engaged in dayshift or nightshift work at the time of recruitment and during data collection. In studies *A* (recruitment and data collection from November 2003 to August 2007) and *B* (recruitment and data collection from October 2007 to May 2011), nightshift workers were required to work at least 20 hours per week (at least eight hours per shift, stopping work no earlier than 06:00) exclusively during the graveyard shift and to sleep at night during off days. Dayshift workers were required to be employed at least 20 hours per week and work exclusively during the dayshift (i.e., work at least eight hours per shift and begin work no earlier than 06:00). For study *C* (recruitment and data collection from November 2011 to November 2012), work criteria were similar, except nightshift and dayshift workers had to work their respective shifts at least 24 hours per week for the previous six months. In addition, for study *C*, all dayshift and nightshift workers were required to have schedules where they typically worked at least three consecutive days or nights, respectively, each week.

For study *A*, participants were required to be women aged 20 to 49 years. Additional eligibility criteria included: regular menstrual periods; no personal history of breast cancer, chemotherapy, or tamoxifen therapy; no pregnancy or breast feeding within the past year; no use

of supplements containing phytoestrogens or isoflavones and consumption of no more than five servings per week of soy-based foods. For study *B*, participants were required to be men aged 20 to 55 years. They could not be using medications or supplements used to treat benign prostate conditions within 30 days of participation, could not have a personal history of prostate cancer or chemotherapy, and could not have undergone general anesthesia or major surgery at least eight weeks prior to enrollment. For both studies *A* and *B*, participants were required to have a body mass index (BMI, weight in kilograms [kg] divided by the square of height in meters $[m]^2$) between 18 and 30 kg/m^2, and could not have been using hormones or supplements containing melatonin during the 30 days prior to enrollment. For study *C*, eligibility criteria included being 20 to 40 years of age, with a BMI between 18-32 kg/m^2, and no personal history of cancer, diabetes, heart disease, autoimmune disorders, or inflammatory disease. In addition, participants could not have been using melatonin supplements during the six months prior to enrollment, and women could not currently be pregnant.

Two-hundred and eighty-eight participants from *A* (129 dayshift and 159 nightshift) and 208 participants from *B* (all nightshift) had available buffy coat samples for DNA extraction or previously extracted buffy coat DNA. We restricted to those *A*/*B* participants who worked their respective shift schedules (day or night) for at least six months, who completed the study questionnaire, who provided actigraphy data from at least one sleep period (day sleep or night sleep) for sleep quality assessment, and to those who had both completed the study protocol and provided urine samples during various sleep and work periods for circulating melatonin measurements, though these samples were not used in the present study. In total, 88 dayshift and 113 nightshift workers from *A* and 137 nightshift workers from *B* were selected. One subject was excluded from study *B* due to not having questionnaire data on race. All subjects from *C* were

included in the current study (65 dayshift, 59 nightshift). The total sample size for the current study is 153 dayshift and 308 nightshift workers.

Data and Biospecimen Collection

For all eligible participants across the three studies, informed consent was obtained during either a home or work visit by trained study interviewers/phlebotomists. Structured interviews were conducted to collect information about current work and sleep schedules, work shift history, and physical activity. Height and weight measurements were also obtained during these interviews. For both *A* and *B*, blood samples (10mL EDTA vacutainer tubes) were also collected during the interviews. For the participants in *C*, blood samples (10mL ACD tube) were collected immediately after completing the third consecutive work shift. For studies *A* and *B*, data on caffeine, alcohol, tobacco, medication, and supplement intake were self-reported for the 24 hour periods preceding the day sleep period during which urine samples were collected. For study *C*, the structured interview, which was conducted on a workday, collected data on these variables for the 24 hours preceding the time of the interview.

Laboratory Methods

Specimen processing

For studies *A* and *B*, buffy coats were isolated from the whole blood samples and subsequently stored at –70°C. For study *A*, buffy coat samples were stored from seven to 11 years before DNA was extracted and utilized for the methylation assay. For study *B*, buffy coat samples were stored up to four years before DNA extraction. The extracted DNA from study *B* was subsequently stored for up to eight years, at -20°C, before being used for the methylation assay. DNA extraction for both studies was performed on the buffy coats using a salt precipitation method (SA, Dykes, & Polesky, 1988).

For study *C*, lymphocytes were isolated from the whole blood samples within 24 hours of collection. Refrigerated centrifugation was used to isolate the buffy coat, which was resuspended in RMPI 1640 + P/S. After layering the buffy coat suspension over Lymphocyte Separation Medium (Histopaque – 1077, Sigma Aldrich) and centrifugation at room temperature, the mononuclear cell layer was removed, washed, centrifuged and resuspended in freeze media (RPMI with 15% fetal calf serum and filtered 10% DMSO). After freezing at rate of -1°C/minute to -80°C, samples were transferred to a liquid nitrogen freezer for storage. DNA was extracted from cryopreserved lymphocytes (ArchivePure DNA Purification Kit, 5- Prime, Hilden, Germany).

500 ng of DNA for each participant was treated with sodium bisulfite using EZ DNA Methylation-DirectKit (Zymo Research, Irvine, CA). Treated DNA specimens were stored at –80°C, and the methylation assay was performed within two weeks.

Methylation Assay

Infinium HumanMethylation450 Bead Array (Illumina, San Diego, CA) "genotypes" sodium bisulfite-treated DNA to quantify DNA methylation for 485,577 CpG loci, capturing roughly two percent of the CpG sites throughout the human genome (Michels et al., 2013). Treatment with bisulfite converts unmethylated cytosines (C) into uracils, which are subsequently converted to thymines (T). Bisulfite-converted CpG sites can then be "genotyped" and interpreted as a proxy for methylation status. Unlike the categorical feature of genotypic calls obtained from genome-wide association studies, however, what is measured at a CpG site is the percentage of cells that are methylated at that site for the particular tissue sampled. After bisulfite-converted DNA is amplified and fragmented, it is hybridized to two probes, referred to

as Type I and Type II probes (Pidsley et al., 2013). The Type I and II probes quantify methylation differently, though they both obtain the standard index for methylation: beta (β), where [$\beta = M/(M + U)$] and M and U refer to the methylated and unmethylated signal intensities at a given CpG site.

The array measures methylation across 21,154 genes, each with an average of 17 CpGs. CpGs span various gene regions, including those located 1500 base pairs (bps) and 200 bps upstream of transcription start sites (TSS1500 and TSS200 respectively) and those located within the 5' untranslated region, first exon, gene body, and 3' untranslated region. CpGs could be positioned within or outside of CpG islands. Three hundred and ninety-one CpG loci within 12-core circadian genes are included: *CLOCK*, *BMAL1*, *NPAS2*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *RORα*, *NR1D1*, *CSNK1δ*, and *CSNK1ε*.

The DNA methylation assay for study C was previously described (Bhatti, Zhang, et al., 2014). Identical procedures were used for studies *A* and *B*. Briefly, four ml of bisulfite treated DNA was denatured and neutralized to prepare it for overnight isothemal whole-genome amplification. Next, the DNA was enzymatically fragmented for 60 min at 37°C and then precipitated with isopropanol and allowed to air dry. DNA was then resuspended in hybridization buffer. Samples were then applied to the beadchips, and the beadchips were incubated in a hybridization oven at 48°C for 16–24 h. After washing, the chip underwent extension and staining in capillary flow-through chambers. Beadchips were then scanned using the iScan+ (Illumina, San Diego, CA). Laboratory personnel were blinded to all study subject information, and specimens were identified by study ID number only. An additional 10% of DNA samples were randomly included among the study samples for quality control assurance.

Data Processing

Each CpG site represented on the array was associated with an M-value, which was calculated as the log*2* ratio of the intensities of the methylated and unmethylated probes. A series of processing steps were necessary before methylation data could be analyzed (TABLE 2). We used the "noob" method in the minfi Bioconductor package (T. J. Morris & Beck, 2015) to resolve bias in the M-values associated with differences in background fluorescence (Triche, Weisenberger, Van Den Berg, Laird, & Siegmund, 2013). Functional normalization was used to rescale the M-values, which corrects the bias from the differences in distributions of M-values from the two types of probes used in the array (Wilhelm-Benartzi et al., 2013).

For our analysis, because sex-stratified analyses would have been underpowered, we restricted to the analysis of autosomes, excluding CpG loci from the sex chromosomes. Next, we removed CpG sites measured by poor-performing probes using a two-tiered approach in the ChAMP package (T. J. Morris et al., 2014). First, a metric called a "bead count" was used to assess technical variance related to probe binding; a bead counts of <3 indicates a probe failed to bind to a bead chip. Probes with bead counts <3 were removed. Second, we used the detection statistic, which indicates the confidence that a probe's intensity is above the background level established by the negative-control probes, to remove the CpG sites associated with probes for which 20% of the samples had a p-value greater than 0.01 (T. J. Morris & Beck, 2015). 463,893 loci remained.

Since the presence of single nucleotide polymorphisms (SNPs) near a CpG locus can affect probe hybridization (N. Morris, Elston, Barnholtz-Sloan, Sun, & Comuzzie, 2015), we excluded all CpG loci that contained at least one SNP with a minor allele frequency ≥0.05 (Y. A.

Chen et al., 2013). 412,408 loci remained. We also excluded cross-reactive probes (i.e. probes that cross-hybridize to sites other than those for which they were designed) (Y. A. Chen et al., 2013). 394,851 loci remained. Further, we applied a low-variance filter to remove sites whose variance across all participants was below the median variance of the remaining 394,851 sites (Bourgon, Gentleman, & Huber, 2010), since these loci are unlikely to be informative in our analyses. After implementing these processing procedures, 346 circadian and 197,261 non-circadian CpG sites remained for analysis. Additionally, to account for any potential batch effects from combining data from three studies, we applied the COMBAT batch adjustment procedure as implemented in the sva R-package (Leek, Johnson, Parker, Jaffe, & Storey, 2012).

Statistical Analysis

The *M*-values for each CpG site were modeled as dependent variables using rank regression, which, because the *M*-values were not normally distributed, allowed us to use rank-based (non-parametric) inference for our linear models (Kloke & Mckean, 2012). Models included a variable for shift work status and adjustment variables for potential confounders: gender, continuous variables for age and BMI, race (White or Non-White), and the mixture of leukocytes that contributed DNA to each participant's sample. Leukocyte cell mixture for six major leukocyte subsets (CD8+ T-cells, CD4+ T-cells, natural killer cells, B-cells, monocytes and granulocytes) was inferred based on the method by Houseman *et al*. (2012) (Houseman et al., 2012). The six leukocyte cell-types were summarized by a single value derived from a principal component (PC) analysis (FIGURE 1). Specifically, the first PC accounted for 95% of the variation in the six cell type measures and so we used this first PC to define a single variable that was included in all statistical models to adjust for cell type. We note that cell type was strongly confounded with the study/batch and so, although we did apply a COMBAT to

normalize the data for batch/study effects, this is also taken care of by adjusting for cell type in all statistical analyses.

While alcohol consumption and smoking are potential confounders, due to differences in how these variables were collected for study *C* versus studies *A*/*B* (see Data and Biospecimen Collection), we did not include them as covariates in our analyses. Instead, for alcohol consumption, we performed a sensitivity analysis that excluded those subjects reporting consumption of alcohol to determine the potential impact of differences in alcohol consumption on study results. Because the prevalence of smoking was minimal amongst participants and given the similar prevalence of smoking between dayshift and nightshift workers, we did not conduct similar sensitivity analyses for smoking.

Analyses at the level of gene and region within gene were also conducted. The annotation file provided by Illumina was used to assess gene and gene region. For the region-level analysis, three regions were included: promoter, gene body, and 3'UTR. A locus was classified as being part of the promoter if it was located within 1500 or 200 base pairs of the transcription start site (TSS1500 or TSS200, respectively), the 5'UTR, or the first exon. This classification was chosen because loss of methylation in these regions has been previously associated with gene silencing (Brenet et al., 2011; Geybels et al., 2015; Jeronimo et al., 2011). Genes and regions within genes for which we had retained a minimum of five CpG sites were included in this analysis.

In models that included the same covariates as the locus level analyses, methylation across each gene and gene region was evaluated using a kernel association test (Zhao et al., 2015). This method was designed for testing the association of a large collection of genomic markers with a quantitative trait (Kwee, Liu, Lin, Ghosh, & Epstein, 2008; M. C. Wu et al.,

2010) and has been applied in a variety of other settings (Schaid, 2010). Briefly, the multivariate (Euclidean) distance between each pair of participants is based on all available loci in an entire gene or gene region (for genes and regions for which a minimum of five loci were available for analysis) and provides an aggregated measure of similarity between individuals' methylation profiles at the gene or gene region level. The regression framework for this variance component-based score test allows for incorporating covariates, such as age and gender, into the testing procedure.

The CpG site-, gene-, and region-level analyses were performed separately for the circadian and non-circadian tests. For each level of analysis, multiple testing was accounted for with a 5% false-discovery rate (FDR) using the Benjamini-Hochberg (BH) method (Benjamini & Hochberg, 1995).

Interpretation

To explore the regulatory landscapes and genomic features that might influence the impact of methylation, top findings were visualized within the UCSC Genome Browser (http://genome.ucsc.edu/) and the Ensembl Genomes (http://ensemblgenomes.org) interactive databases (human GRCh37/hg19 build), which have annotated tracks for various cell lines indicating chromatin states, transcription factor binding, repetitive elements, and enhancers. Within the UCSC Genome Browser, GM12878 cells (B-lymphocytes from the CEPH/Utah population) were selected as a comparison viewing group (W. Kent et al., 2002).

Results

In TABLE 1, the distribution of selected demographic variables is presented by shift status. While the distribution of nightshift workers by gender was nearly balanced, dayshift

workers tended to be female, given the unavailability of DNA from dayshift workers in study *B*, which was focused on men. Nightshift workers were younger and had a higher BMI than dayshift workers. The fraction of smokers was the same for night and dayshift workers. Alcoholic beverage consumption was greatest amongst dayshift workers, and, although we report the fraction of smokers and alcoholic beverage drinkers, the variables for these were in reference to different time periods in study *A*/*B* versus study *C* (see Data and Biospecimen Collection).

Locus-level

None of the methylation differences at CpG sites in the circadian genes were statistically significant after multiple-comparisons adjustment. The five CpG loci in circadian genes with the lowest unadjusted *p*-values were as follows [a negative shift effect (SE) indicates the locus was hypomethylated in night compared to dayshift workers]: cg09304381 (*NR1D1*; SE=0.1341), cg27004243 (*PER1*; SE=-0.0843), cg02189597 (*RORα*; SE=0.1174), cg13286116 (*BMAL1*; SE=-0.2417), and cg2287253 (*RORα*; SE=-0.0692) (Table 3). Because larger differences in methylation may be biologically meaningful, we also report the five circadian loci with the largest absolute differences in M-values when comparing nightshift workers to dayshift workers (Table 4): cg13286116 (*BMAL1*; SE=-0.2417), cg08926642 (*PER3*; SE=-0.1357), cg09304381 (*NR1D1*; SE=0.1341), cg17724687 (*PER3*; SE=-0.1178), cg02189597 (*RORα*; SE= 0.1174). cg09304381 in *NR1D1*, cg13286116 in *BMAL1*, and cg02189597 in *RORa* were among both the set of loci with the smallest p-values and the set of loci with the largest absolute differences in M-values. A volcano plot (FIGURE 2) illustrates that the loci with the five smallest p-values and the loci with the five largest absolute effect sizes are outliers relative to the remaining loci.

We interrogated 197,258 non-circadian CpG sites. Of these, nightshift work was statistically significantly associated with decreased methylation at one locus (cg03227775),

situated in an intron of *CARD11* (FDR-adjusted *p*-value=0.0421; SE=-0.27) (Table 5). In addition to cg03227775, the four non-circadian loci with lowest unadjusted p-values were: cg18439144 (closet gene is *TLE3*; SE=-0.1577), cg11480534 (*ANHAK*; SE=-0.1455), cg01349856 (*LOXL1*; SE=-0.159*)*, and cg02436272 (*RCN1*; SE=-0.1456) (TABLE 5). TABLE 6 provides results for the five non-circadian loci with the largest absolute effect size differences: cg26705599 (closest to LINC00368, long intergenic non-protein coding RNA 368; SE=0.6358), cg00993903 (*MARK3*; SE=0.4645), cg22274273 (intergenic; SE=0.4462), *RIBC2; SMC1B; SE=*0.4832), cg04246708 (*CNST*; SE=-0.4286). Both the top five most statistically significant loci and those with the five largest absolute effect size differences are displayed in a volcano plot in FIGURE 3.

Gene-level

No statistically significant differences were observed in the gene-level analysis after FDR correction, for which 12-core circadian and 12,263 non-circadian genes—each retaining a minimum of 5 CpG loci after processing—were interrogated. The circadian genes with the lowest unadjusted *p*-values are as follows: *BMAL1* (unadjusted *p*-value=0.0168), for which 14/23 CpG sites were hypomethylated (12/20 were hypomethylated in the promoter and 2/3 were hypomethylated in the gene body); followed by *NR1D1* (unadjusted *p*-value=0.1581)*,* for which 8/19 CpG sites were hypomethylated (4/12 were hypomethylated in the promoter, 3/6 in the gene body, and 1/1 in the 3'UTR); *CSNK1ε* (unadjusted *p*-value=0.3390)*,* for which 14/24 CpG sites were hypomethylated (13/22 in the promoter, 0/1 in the gene body, and 1/1 in the 3'UTR); *PER1* (unadjusted *p*-value=0.3504), for which 13/18 CpG sites were hypomethylated (9/11 were hypomethylated in the promoter, 3/6 in the gene body, and 1/1 in the 3'UTR); and *PER3* (unadjusted *p*-value=0.4001), for which 12/22 CpG sites were hypomethylated (4/8 were

hypomethylated in the promoter, 7/13 in the gene body, and 1/1 in the 3'UTR) (TABLE 7). The five non-circadian genes with the lowest unadjusted p-values were as follows*: HSPA4L* (unadjusted *p*-value=0.0003), for which 3/5 CpG sites were hypomethylated (2/2 were hypomethylated in the promoter, 0/2 in the gene body, and 1/1 in the 3'UTR); USP42 (unadjusted *p*-value=0.0006), for which 14/19 CpG sites were hypomethylated (4/5 were hypomethylated in the promoter, 7/11 in the gene body, 3/3 in the 3'UTR); *LASS3* (unadjusted *p*-value=0.0008), for which 8/8 CpG sites were hypomethylated (8/8 were hypomethylated in the promoter); *VKORC1L1* (unadjusted *p*-value=0.001), for which 7/8 CpG sites were hypomethylated (6/7 were hypomethylated in the gene body and 1/1 in the 3'UTR); *AP3B2* (unadjusted *p*-value=0.0012), for which 16/21 CpG sites were hypomethylated (4/6 were hypomethylated in the promoter and 12/15 in the gene body) (TABLE 8).

Region-level

No statistically significant differences were observed for the region-level analysis among either circadian or non-circadian genes (only those regions retaining a minimum of 5 CpG loci after processing were considered). Of the 22 regions that were tested within circadian genes, those with the lowest unadjusted p-values were as follows (smallest to largest): putative promoter region of *BMAL1* (unadjusted *p*-value= 0.0178), for which 12/20 CpG sites in the promoter were hypomethylated; followed by *NR1D1*/body (unadjusted *p*-value=0.0505), for which 4/7 CpG sites in the body were hypomethylated; *PER1*/promoter (unadjusted *p*-value=0.1407) for which 10/11 in the promoter were hypomethylated; *CSNK1ε*/promoter (unadjusted *p*-value=0.2921), for which 13/22 in the promoter were hypomethylated; and *PER3*/Body (unadjusted=*p*-value=0.3955), for which 7/13 were hypomethylated (TABLE 9). Among the 10,531 regions within non-circadian genes that were considered, the five with the

lowest p-values were as follows: *USP42*/promoter (unadjusted=*p*-value=0.0002), for which 4/5 were hypomethylated; *LOXL1*/body (unadjusted=*p*-value=0.0003), for which 2/5 in the gene body were hypomethylated; *LASS3*/promoter (unadjusted=*p*-value=0.0008), for which 8/8 in the promoter were hypomethylated; *LMO2*/promoter (unadjusted=*p*-value=0.001), for which 11/11 were hypomethylated in the promoter; and *VKORC1L1*/body, for which 6/7 were hypomethylated in the gene body (unadjusted=*p*-value=0.001) (TABLE 10).

Full tables of the results for the locus, gene, and gene region-level tests are available upon request.

Discussion

A statistically significant difference in methylation between nightshift and dayshift workers in our study was observed at the cg03227775 locus, positioned within intron 11 of the gene body of *CARD11* (caspase recruitment domain family, member 11). The locus was hypomethylated in nightshift compared to dayshift workers. But while the level of methylation in promoters is generally inversely correlated with transcription, methylation of gene bodies is less well characterized (P. A. Jones, 2012). Increases and decreases in methylation within gene bodies may correlate either positively or negatively with expression of the gene or may be associated with alternative splicing of the gene depending on the nature of the locus (P. A. Jones, 2012; Kulis et al., 2012; X. Yang et al., 2014).

Methylation in Gene Bodies

1) Transcribed regions of genes (particularly exons) have been observed to be heavily methylated with the level of methylation positively correlating with gene expression (X. Yang et al., 2014). Hypermethylation is thought to silence repetitive (transposable) elements within gene

bodies, without interfering with transcription of the host gene (P. A. Jones, 2012), whereas hypomethylation of transposable elements, contributing to genomic instability, possibly reactivates the transposable elements (D. Chen et al., 2016).

2) Recently, hypomethylation has been observed in the bodies of genes showing increased expression, particularly at locations of known and predicted enhancers and at regions marked by open chromatin. (Chromatin states can be predicted with the use of a hidden Markov model integrating ChIP-seq data for the immunoprecipitation targets of various histone marks: see more below regarding use of ChIP-seq data). In particular, Singer et al. (2016) demonstrated that hypomethylated exons of highly expressed genes are marked by the presence of histones with high levels of histone markings (Blattler et al., 2014; Kulis et al., 2012; Singer, Kosti, Pachter, & Mandel-Gutfreund, 2015).

3) Some studies report positive and some report negative correlations between methylation in the first intron of genes and transcription of their respective genes; (Lai et al., 2010; Unoki & Nakamura, 2003). While introns are generally less methylated than exons, hypomethylation of introns has been associated with increased transcription, especially at enhancers (Blattler et al., 2014; Ishii et al., 2007). It has been estimated that more than 51% of enhancers lie within gene bodies. (In fact, there are more enhancers than promoters in the genome). Though enhancers often function to modulate the transcriptional activity of genes far removed from the gene in which they reside, that is not always the case. Gene body (intragenic) enhancers can function as alternative promoters for their host genes (Kowalczyk et al., 2012). Moreover, Aran et al. (2013) observed that a large number of enhancers demonstrate a reverse correlation between methylation and expression (Aran, Sabato, & Hellman, 2013) similar to

what is observed with promoter methylation, where hypermethylation is associated with silencing and hypomethation with expression.

4) The degree of differential methylation favoring more methylation in exons over introns varies depending on GC (guanine cytosine) content across the particular exon and flanking introns. Two distinct exon-intron architectures have been reported: one with equal GC content between exons and surrounding introns and one with differential GC content between exons and introns. Differential GC content between exons and introns allows for better recognition of exons by the splicing machinery. In the architecture with the same level of GC content between introns and exons, the splicing machinery relies on introns for recognition. DNA methylation appears to mark exon boundaries (Gelfman, Cohen, Yearim, & Ast, 2013).

5) While DNA methylation does not appear to be essential for splicing (many model organisms engage in mRNA splicing and lack DNA methylation), in humans there is emerging evidence that methylation fine-tunes both splicing and alternative splicing (Maor, Yearim, & Ast, 2015). Changes in nucleosome occupancy, accompanied by DNA methylation, may influence the RNA polymerase II (Pol II) elongation rate (elongation: after initiating mRNA synthesis at the promoter, the process by which Pol II traverses the coding region; elongation rate: the rate of nucleotides added per minute (Mason & Struhl, 2005), typically negatively correlated with exon density, GC content, and methylation (Jonkers, Kwak, & Lis, 2014) and which is critically tied to mRNA production, reductions in the rate potentially leading to premature termination of Pol II from the template and transcriptional arrest (Jonkers et al., 2014; Mason & Struhl, 2005)) and cotranscriptional splicing (the splicing of introns when transcripts remain attached to DNA by Pol II) (Gelfman et al., 2013). In fact, methylation may influence the elongation rate of Pol II, which in turn influences splicing (Fong et al., 2014; Jonkers et al.,

2014). Slow and fast elongation rates influence the recognition of splice sites. An optimal rate of transcriptional elongation is thought to be required for normal splicing for both constitutive (exons typically retained in the mature mRNA) and alternatively spliced exons (exons sometimes spliced out) (Fong et al., 2014). Alternative exons have been shown to display lower methylation levels than constitutive exons, and lower levels of methylation may lead to suboptimal recognition of alternatively spliced exons by the splicing machinery (Gelfman et al., 2013). Methylation has been observed to regulate 22% of alternative exons (Maor et al., 2015). Relative to the overall level of methylation in gene bodies, hypermethylation has been reported for intronic and exonic sequences close to the 3' end of intronic acceptor sites (Malousi & Kouidou, 2012). Intron retention, one of the mechanisms of alternative splicing associated with missplicing (a splicing mistake), correlates with tumorigenesis (Dvinge & Bradley, 2015). Less methylation at sites associated with intron retention has been observed (Malousi & Kouidou, 2012), and exons reported to be sensitive to the elongation rate are misspliced in tumors (Fong et al., 2014).

CARD11

Four isoforms (transcripts) have been reported for *CARD11*: CARD11-011, CARD11-012, CARD11-013, and CARD11-014 (Aken et al., 2016). Three of the four transcripts are protein-coding, but the fourth (CARD11-014) is the product of a particular type of alternative splicing, sometimes considered to be missplicing—that is, intron retention. Moreover, use of the UCSC Genome's Chromatin State Segmentation by Hidden Markov Model (HMM) track (a computational prediction of chromatin state that integrated ChIP-seq data for nine immunoprecipitation targets—CTCF, H3K23ac, H3K27me3, H3K36me3, H3K4me1, H3K4me2, H3K4me3, H3K9ac, H4K20me1) reveals that cg03227775 (located at chr7:2972073) has been observed to have a chromatin state associated with transcriptional elongation in

GM12878 cells (W. Kent et al., 2002). This implies that features associated with cg03227775 may be important for transcription, even though cg03227775 is located in an intron and knowledge about the impact of hypomethylation in this setting is limited (Malousi & Kouidou, 2012).

A misspliced isoform would likely have negative consequences, as *CARD11* is an oncogene. *CARD11* activates the nuclear factor–kB (NF-kB) signaling family and has been associated with the least curable form of non-Hodgkin's lymphoma (NHL) (Lenz et al., 2008). An increased risk of NHL has been reported previously in two epidemiological studies of shiftwork and cancer (Lahti et al., 2008; Parent et al., 2012). Furthermore, the NF-kB pathway, which is regulated by the core circadian gene RORα (Du & Xu, 2012), is constitutively activated in another form of NHL, chronic lymphocytic leukemia (CLL) (Baldoni et al., 2013), and an increased risk for CLL has been recently documented for long-term, rotating shift workers (Costas et al., 2016). Thus, methylation changes in *CARD11* may play a role in the carcinogenicity of nightshift work.

Additional Methylation Differences

Circadian Findings

Though no other genome-wide statistically significant effects were observed, given the exploratory nature of our study, we highlight some additional methylation differences (based on unadjusted p-values or effect size estimates) that are worth exploring in future studies. The 12 genes considered to be central to the functioning of the circadian molecular clock (FIGURE 4) (Ko & Takahashi, 2006) were a particular focus of our study. Given the important regulatory role played by circadian genes in the downstream expression of genes involved in pathways related to carcinogenesis (Gery & Koeffler, 2007; Sancar et al., 2010; X. Yu et al., 2013), differences in

the methylation of circadian genes may be particularly relevant to increased risks of cancer observed among nightshift workers. Three loci (cg09304381, cg13286116, cg02189597) in the circadian genes *NR1D1, BMAL1* and *RORα* were not only among the five circadian gene loci with the lowest unadjusted p-values, but were also among the five circadian gene loci with the largest absolute differences in effect size. We observed hypermethylation of cg09304381 and cg02189597, which are located in the gene bodies of *NR1D1* and *RORα*, respectively. cg09304381 is located in exon 8 of *NR1D1* and is not associated with a known enhancer, but is weakly associated with transcription, having an HMM chromatin state 11 (weakly transcribed) (W. Kent et al., 2002). Given the location in an exon, it is likely that the hypermethylation (without association with an enhancer) among nightshift workers reflects upregulation of *NR1D1* among nightshift workers. *NR1D1* is a negative regulator of the *BMAL1*. High expression of *NR1D1* or low expression of *BMAL1* predicts poor clinical outcomes in human N-MYC-driven neuroblastomas (Altman et al., 2015). cg02189597 in the gene body of *RORα* (within either the first or second intron depending on the isoform) has an HMM chromatin state of 2 (weakly transcribed promoter). As such, the hypermethylation we observed at cg02189597 may be associated with a decrease in expression of *RORα*. *RORα* is a positive regulator of *BMAL1*; unlike *NR1D1*, which suppresses *BMAL1*, *RORα* activates *BMAL1*. Hypomethylation was observed at cg13286116 in the 5'UTR of *BMAL1*. This locus is associated with an HMM chromatin state of 2, indicating it is a weak promoter (W. Kent et al., 2002); thus hypomethylation could indicate increased *BMAL1* expression among nightshift workers, as has been previously observed (Bracci et al., 2014).

Among the five loci with the lowest unadjusted p-values, there was a second locus within the gene body of *RORα* (cg22387253), also within the first intron, but downstream of

cg02189597 by 429,034 bases, in a region associated with a predicted HMM chromatin state 13 (heterochromatin), and within a DNA transposable element: TcMar-Tigger. Although DNA transposons are considered to be fossils (immobile) in humans (Solyom & Jr, 2012), hypomethylation of repeat elements is thought to predispose towards aberrant chromosomal rearrangements (Wilson, Power, & Molloy, 2007). Transposable elements, independent of mobility, are implicated in cancer via disruption of genomic architecture through non-allelic homologous recombination between repetitive regions of transposable elements (Rebollo, Romanish, & Mager, 2012). However, while the methylation status of the TcMar-Tigger class of transposons has been shown to be environmentally modifiable (by bisphenol A) in humans (Faulk et al., 2016), scant information is available regarding the specific impact of hypomethylation of loci in DNA transposons. Thus, while down-regulation of RORα has been observed in a wide variety of tumors when compared to normal tissue (Cook, Kang, & Jetten, 2015; Kottorou et al., 2012), the hypomethylation we observed at cg22387253 requires further investigation to determine whether hypomethylation in the setting of this TcMar-Tigger promotes susceptibility to chromosomal aberrations in nightshift workers.

We observed hypomethylation of a locus (cg27004243) in the 5'UTR of *PER1*, which may associate with an increased expression among nightshift workers, as has been previously observed (Bracci et al., 2014). Increased *PER1* expression has potential anti-cancer effects, where *PER1* appears to function as a tumor suppressor through transcriptional regulation of cell-cycle control genes and by influencing the cell cycle checkpoint pathway directly (Gery et al., 2006). Contrary to our findings, *PER1* (along with *BMAL1* and *PER2*) was found to be downregulated among CLL patients with a history of nightshift work (Rana et al., 2014).

Among the five circadian gene loci for which the largest absolute differences in M-values

were observed, two loci (cg08926642 and cg17724687) were positioned within the gene body of *PER3* (both hypomethylated). cg08926642 and cg17724687 are both located in the same CpG island in exon 18 in a region of high transcription factor occupancy (W. Kent et al., 2002). While there is typically a positive correlation between exonic methylation and transcription, high transcription factor occupancy indicates that this area of *PER3* may be regulatory, potentially acting as an intragenic enhancer, though it is not yet annotated as one, and thus hypomethylation may suggest upregulation. Interestingly, cg08926642 and cg17724687 are also both predicted to have a closed (heterochromatin) state 13. However, transcription factor occupancy is a better predictor of enhancer status than histone modifications or chromatin accessibility (Dogan et al., 2015), which were used in the HMM model for predicting chromatin state. As such, the hypomethylation at cg08926642 and cg17724687 may indicate upregulation either of *PER3* itself or, if the region is an enhancer, a distal target(s). We note, however, that downregulation of *PER3* has been previously observed in nightshift workers (Bracci et al., 2014) and in both colon cancer and non-small cell lung cancer compared to normal tissue (Liu, Xu, Jiang, & Li, 2014; X. Wang et al., 2012). Patients with lower *PER3* expression had shorter survival compared to those with higher *PER3* expression (Liu et al., 2014), and breast cancer patients (especially those with ER positive, luminal or ERBB2-positive tumors) with low expression of *PER3* had poor prognosis (Climent et al., 2010). As such, should the hypomethylation we observed correlate with increased *PER3* expression, this might suggest an anti-cancer effect of the nightshift. In addition to its role in cancer, genetic variation in *PER3* has been associated with chronotype and sleep disorders. Because the magnitudes of the differences in shift effect at these two *PER3* loci are large relative to the other findings, larger differences in methylation possibly revealing biologically relevant changes in methylation, these loci make attractive candidates for

investigating the modifying impacts of chronotype and sleep quality on methylation, an analysis we plan to undertake (Dijk & Archer, 2010; Ellis, von Schantz, Jones, & Archer, 2009).

Among the circadian genes, no statistically significant associations were observed at the level of gene or gene region. While we speculated on how differences in methylation of individual loci might impact gene expression, it is unknown how the cumulative effects of a mixture of hypo- and hyper-methylated loci across a given gene or gene region would ultimately influence expression. In particular, the effect on expression is unknown for the 12/20 hypomethylated CpG loci in the promoter of *BMAL1*, the 4/7 hypomethylated CpG sites in the body of *NR1D1*, the 7/13 hypomethylated CpG sites in the gene body of *PER3*, and the 13/22 sites in the promoter of *CSNK1ε*. (We note that reduced expression of *CSNK1ε* has been associated with significantly poorer survival rates in colorectal cancer patients (Mazzoccoli et al., 2011), but it has also been suggested that *CSNK1ε* overexpression in cancer cells inhibits the tumor suppressive function of the PER proteins (W. S. Yang & Stockwell, 2008)). In contrast, 10/11 hypomethylated CpG sites in the promoter of *PER1* likely indicates that *PER1* was upregulated.

Non-circadian Findings

Though not statistically significant after multiple comparisons adjustment, the four top associations among non-circadian loci based on unadjusted p-values were all hypomethylated when comparing nightshift to dayshift workers. One of these loci, cg18439144, is not associated with a known gene. The closest gene to this locus is *TLE3* (transducin-like enhancer of split 3), a member of the Notch signaling pathway, which is constitutively activated in CLL (Baldoni et al., 2013). Three of the most statistically significant loci were found in the intronic regions of

LOXL1 (cg01349856), *AHNAK* (cg11480534) and *RCN1* (cg02436272). cg01349856 in *LOXL1* is located in an intron predicted to be a poised promoter (a promoter bound by Pol II while the gene is not yet actively transcribed; genes with poised promoters are thought to be rapidly upregulated (Beisel & Paro, 2011)), associated with open chromatin, and at a DNaseI hypersensitivity site in GM12878 cells, indicating that the hypomethylation we observed likely indicates increased expression. *LOXL1* is a copper-dependent enzyme that can modify proteins of the extracellular matrix (ECM) and whose primary function is ensuring the structural integrity of various tissues. *LOXL1* expression has been reported to be elevated in many human cancers (e.g., myeloproliferative neoplasms and prostate, colorectal, lung, pancreatic, and breast cancers). Whilst traditionally considered to be a tumor suppressor, *LOXL1* has been more recently appreciated for its role in tumor progression and metastasis (Tadmor et al., 2013; Wuest et al., 2015): *LOXL1* expression correlates with levels of tissue hypoxia and impacts breast cancer metastasis. As such, the hypomethylation we observed may reflect a tumor suppressor action of *LOXL1* in shift workers but may also mark shift workers as being at risk of cancer-related processes impacting metastasis through the non-cellular components of tumor microenvironments (the ECM and tissue hypoxia) (Wuest et al., 2015). *LOXL1* was also of note in our region-level analysis. cg11480534 in *AHNAK* is located in a region predicted to have a repressed chromatin state in GM12878 cells and is not associated with any known repetitive sequences or other genomic features providing clues to its function. However, *AHNAK* is a tumor suppressor that inhibits c-Myc and cyclin D protein expression through TGFβ signaling, mediating negative cell growth (Lee et al., 2014). As such, if the hypomethylation reflects decreased expression in this intronic setting, this could have negative consequences. cg02436272 is located in an intron of *RCN1* that is not associated with any repetitive elements, but is

predicted to have a heterochromatic state in GM12878 cells. Increased expression for *RCN1* might have negative consequences, since its activation might confer an advantage to tumors and conceivably aid tumor initiation, given that the RCN1 protein is involved in calcium binding and is located in the lumen of the endoplasmic reticulum, suggesting a role in protein synthesis (W. Kent et al., 2002). *RCN1* has been speculated to contribute to the cell viability of tumors among Wilms tumor patients (J. Kent et al., 1997).

cg04246708, which is located in an intron of the *CNST* gene and predicted to have a chromatin state associated with transcriptional elongation in GM12878 cells, was among the non-circadian loci with the largest absolute difference in M-values when comparing nightshift and dayshift workers. CNST is a connexin sorting protein (del Castillo et al., 2009) and connexins are gap junction proteins speculated to be "conditional" tumor suppressors that modulate cell proliferation, adhesion, and migration. They are deemed "conditional" tumor suppressors because their role in tumor initiation appears protective, but some reports suggest that connexins facilitate tumor invasion and metastasis (Naus & Laird, 2010). Thus, changes in expression of CNST due to differential methylation may play a role in carcinogenesis. If the hypomethylation observed at cg04246708 impacts alternative splicing, this might impair tumor suppressor effects of the *CNST*.

Among the remaining four non-circadian loci with the largest absolute effect sizes all were hypermethylated. cg22274273 was located in an intergenic region within a LINE/L2 element spanning chr6:135136126-135137801, thus hypermethylation in this setting might be beneficial for nightshift workers if the hypermethylation prevents activation of an active mobile element. cg00993903 in the first intron of *MARK3* is predicted to be a weak enhancer in GM12878 cells and is associated with the H3K4me1 (mono-methylation of lysine 4 of the H3

histone protein) mark often associated with enhancers and regions downstream of transcription start sites, indicating that the hypermethylation we observed might be associated with downregulation of *MARK3*. *MARK3* encodes a protein that phosphorylates MAP2 and MAP4. *MAP2* expression has been observed in a variety of rare central nervous system tumors (Malafronte et al., 2014), and phosphorylation of MAP4 has been reported to lead to its dysfunction, affecting cell cycle progression (Chang et al., 2001; Ou et al., 2014). Thus, downregulation of *MARK3* may be protective against carcinogenesis. The hypermethylation observed at cg01808030 corresponds to two genes, an intron in *RIBC2* (RIB43A domain with coiled-coils 2) and the TSS1500 of *SMC1B* (structural maintenance of chromosomes 1B). While little is known regarding the potential health effects of *RIBC2*, *SMC1B* belongs to the structural maintenance of chromosomes family of proteins required for chromatin cohesion and DNA recombination during meiosis and mitosis. In mitotic cells, DNA recombination provides a means to repair DNA damage (Revenkova, Eijpe, Heyting, Gross, & Jessberger, 2001). While cg01808030 is predicted to have a repressed chromatin state by the HMM track, it is also associated with open chromatin due to being in a DNaseI hypersensitivity spot in GM12878 cells, indicating the region is likely regulatory. Hypermethylation in the TSS1500 of *SMC1B* might reflect down-regulation of DNA damage repair mechanisms in our shift workers. The inability to properly repair DNA damage can lead to genomic instability and contribute to carcinogenesis (X. Li & Heyer, 2008). Hypermethylation of cg26705599 in an area predicted to have a heterochromatic state and located upstream of LINC00368, was also observed. While the function of most long intergenic non–protein coding RNAs (lincRNAs) remains unknown, long noncoding RNAs are generally thought to be regulatory (Francescatto, Vitezic, Heutink, & Saxena, 2014), and altered expression of lincRNA can result in aberrant expression of genes that

may contribute to cancer (W. Li et al., 2014). LINC00368 lies upstream of *ARHGEF7* (Rho guanine nucleotide exchange factor 7; a.k.a., *β-PIX*). *ARHGEF7* is a cytoplasmic protein that activates the Ras-like family of Rho proteins and has been shown to play a role in cancer metastasis through an impact on cell migration in lung adenocarcinoma (H. W. Yu et al., 2015). In addition, loss of *ARHGEF7* in normal mammary epithelial cells reduces Yap/Taz phosphorylation, leading to decreased expression of target genes. Yap/Taz are transcriptional regulators, which are part of the Hippo pathway that regulates tissue growth and organ size. Hippo pathway inactivation contributes to carcinogenesis through dysregulation of cell proliferation (Heidary Arash, Song, Song, Shiban, & Attisano, 2014). Thus, if the hypermethylation observed at cg26705599 promotes downregulation of *ARHGEF7* (either directly or indirectly through downregulation of LINC00368), this might comprise a novel pathway through which shift work mediates negative health effects.

USP42, *LASS3*, and *VKORC1L1* are genes that appeared among both our top five most significant gene and region-level analyses. The gene-level test for *USP42* (ubiquitin-specific peptidase 42) included 19 loci: five in the promoter (four hypomethylated), 11 in the gene body (seven hypomethylated), and three in the 3'UTR (all hypomethylated). The hypomethylation in the promoter and possibly the 3'UTR suggests that *USP42* may have been upregulated among nightshift workers. *USP42* has two important cancer-related functions: 1) it is a deubiquitylating enzyme (deubiquitylating enzymes remove ubiquitin) that can target the tumor suppressor protein p53 and help stabilize p53 in response to genotoxic stress, and 2) independently of p53, it can regulate transcription by deubiquitylating histones (Hock, Vigneron, & Vousden, 2014). Since under-expression or aberrant activity of *USP42* is oncogenic, increased expression of *USP42* due to hypomethylation, as we observed among nightshift workers, may provide anti-

cancer benefits. Our analyses included eight loci in *LASS3*, each hypomethylated and each located in the promoter, indicating that *LASS3* was likely upregulated in nightshift workers. *LASS3* synthesizes ceramides, intermediates in sphingolipid metabolism, and is found predominately in the skin and testis (Levy & Futerman, 2010). Little has been reported regarding its potential role in cancer. Similarly, eight loci were included in the analyses for *VKORC1L1*: seven in the gene body (six hypomethylated) and one in the 3'UTR (hypomethylated). *VKORC1L1* is a vitamin K epoxide reductase complex. The VKOR complex is known for being the target of the blood coagulation inhibitor, Warfarin (Rost et al., 2004), but has also been proposed to drive vitamin K-mediated intracellular anti-oxidation pathways critical to cell survival (Hammed et al., 2013). If the pattern of hypomethylation in *VKORC1L1* reflects changes in gene expression, this could represent a novel pathway through which nightshift work impacts health.

We also observed *HSPA4L* (heat shock 70kDa protein 4) and *AP3B2* (adaptor related protein complex 3 beta 2 subunit) among our top non-circadian gene-level findings. For *HSPA4L*, the five included loci spanned different gene regions and had different directions of methylation (hypomethylation in the TS1500, 5'UTR, and 3'UTR, and hypermethylation at two loci in the gene body), possibly indicating up-regulation due to the lower methylation in the promoter (TS1500 & 5'UTR) and 3'UTR. *HSPA4L* has been reported to be expressed abundantly only in normal testis tissue. However, *HSPA4L* expression is observed in leukemia cells, and heat shock proteins are generally overexpressed in malignant cells, correlating with parameters related to differentiation and cell proliferation (Takahashi et al., 2007). Twenty-one loci were included in the gene-level analysis for *AP3B2*. Fifteen of the loci were located in the gene body and 13 of these were hypomethylated. Six loci were located in the promoter, with 4/6 being

hypomethylated and in a region associated with DNaseI hypersensitivity, high transcription factor occupancy, and predicted open chromatin in GM12878 cells (W. Kent et al., 2002), implying that hypomethylation likely indicates expression. Moreover, five hypomethylated loci were located in an intragenic region with high transcription factor occupancy and predicted to have open chromatin implying the region may be an enhancer and also that hypomethylation may indicate upregulation either of *AP3B2* or a distal target (W. Kent et al., 2002). Little is known, though, about the role of *AP3B2*, a vesicle-coat protein, possibly implicated in cell-specific neurotransmitter release (Grabner, Price, Lysakowski, Cahill, & Fox, 2006).

Lastly, all 11 loci in the promoter region of *LMO2*, among our top non-circadian region-level findings, were hypomethylated, suggesting *LMO2* was upregulated among nightshift workers. *LMO2* is a gene required in normal hematopoiesis (Wiekmeijer et al., 2016). Overexpression of *LMO2* is oncogenic and causes aberrant human T-cell development. A high frequency of chromosomal arrangements involving *LMO2* have been observed in T-cell acute lymphoblastic leukemia (L. Wu et al., 2015). As such, hypomethylation of *LMO2* may play a role in the carcinogenicity of the nightshift.

Strengths and Limitations

Our findings differ from those we observed in our previous study of DNA methylation (study *C*). In study C, we observed 16,135 loci that were significantly differentially methylated between night and dayshift workers. However, in the present analysis, the only finding that was significant after FDR correction was cg03227775 in *CARD11*. In the few years since study *C* was published, rapid advances have been made in the processing of methylation data (Bourgon et al., 2010; Butcher, 2013; Leek et al., 2012; N. Morris et al., 2015; T. J. Morris & Beck, 2015; T.

J. Morris et al., 2014; Triche et al., 2013; Wilhelm-Benartzi et al., 2013). Thus, in addition to the much larger sample size, the differences in our current findings from those of our previous analysis may be explained by our updated methylation data-processing strategy, which left us with a much smaller set of CpG loci for analysis: down from 473,800 in study *C*, we included only 197,261 loci, and of the 16,135 significant loci in study C, only 8,790 were included in the set of 197,261 that we analyzed. Despite the different set of loci for analysis, we note that cg03227775 in *CARD11* was among the 3% of markers tested that were found to be statistically significant in study *C*. Likewise, the two *PER3* loci (cg08926642 and cg17724687) among our set with the largest absolute effect size differences and cg01349856 in *LOXL1* (among our top five findings with the smallest unadjusted p-values) were also statistically significant in study *C*. In addition to the differences in methylation data processing that resulted in different sets of loci to analyze, the differences in findings between the two studies might also be explained by our use of alternative analytic methods. We used rank-regression instead of statistical analysis of microarrays (SAM) (Chu, Li, Narasimhan, Tibshirani, & Tusher, n.d.), as had been used in study *C*. SAM, which is typically used in the analysis of expression microarrays, assigned a score for methylation difference at each locus relative to the standard deviation of repeated measures for that locus (Tusher, Tibshirani, & Chu, 2001), but this approach has limitations, such as the inability to directly adjust for covariates, necessitating, instead, a method for subtracting out the influence of covariates. For the present analysis, we opted to use rank-regression, which, like the score used in SAM, provides a non-parametric method to a handle non-linear response variable (our methylation data were not normally distributed). The primary advantage of rank-regression over SAM is that it permitted addition of covariates to the analytic models. Another difference in our methodology when compared to study C is the use of a kernel association test (Zhao et al.,

2015) when examining gene and region-level effects rather than simply averaging methylation across genes and regions. For example, when comparing nightshift to dayshift workers, if half of the loci in a gene were hypomethylated and the other half were hypermethylated, the average would indicate no net difference between nightshift and dayshift workers. However, measures of Euclidean distance would detect this overall difference. Unlike SAM, however, this method did not account for any potential correlation of methylation between CpG loci within a gene or gene region. In addition, because direction of effects were not considered in the gene and gene region analyses, interpretation of findings becomes difficult.

Our findings did not replicate those from the previous study of methylation and shift work by Zhu *et al*. (2011). This may be attributable to differences in the study populations and/or differences in the preprocessing methodology. In their study, Zhu *et al*. (2011) evaluated the differences in methylation between female shift workers with ≥10 years of nightshift work and female dayshift workers. They found hypomethylation in the promoter region of *CLOCK* and hypermethylation in the promoter region of *CRY2*, which we did not observe.

Ours is the largest and most comprehensive study to date on the impact of nightshift work on genome-wide DNA methylation. However, relative to the large number of loci that were examined, our sample size is small, limiting our power to detect important effects. Nevertheless, we have highlighted some compelling associations that are worth examining in future investigations. A limitation of our study is that we did not have data on long-term cigarette smoking or alcohol consumption for our study participants. Long-term cigarette smoking and alcohol consumption have been associated with differential DNA methylation (Breitling, Yang, Korn, Burwinkel, & Brenner, 2011; Zhou et al., 2011), and differences in cigarette smoking and alcohol consumption have been reported between dayshift and nightshift workers (Dorrian &

Skinner, 2012; van Amelsvoort, Jansen, & Kant, 2006). Instead, we had data on whether or not cigarettes had been used or alcohol had been consumed in one of the 24-hour periods over which data or urine specimens were collected (not the same time period across studies), which may or may not have coincided with collection of the blood sample that was ultimately assayed for genome-wide DNA methylation. Given this limitation of the variables, we did not include them as covariates in our analyses. The cigarette smoking variable would have unlikely had a significant impact on results given that the numbers of dayshift (9/153) and nightshift (17/308) workers reporting smoking were small and similar (both 6%). The numbers of dayshift and nightshift workers reporting alcohol consumption were larger and different, so we conducted sensitivity analyses in which the 70 participants that reported consuming alcohol were excluded and analyses repeated. The *CARD11* locus (cg03227775) remained the most statistically significant finding and the effect size was only modestly altered. While ranking of some of the other "top" most statistically significant associations we reported did change (results not shown), effect sizes remained mostly unchanged. Another limitation of our study is that we did not have expression data to correlate with our methylation data. Instead, we used the UCSC Genome Browser to visualize regulatory aspects of our loci with GM12878 cells, which can give an indication of the potential effects of differential methylation on gene expression among nightshift workers, but is an imperfect indicator.

Exploring the impact of shift work on DNA methylation in blood is a reasonable strategy given that shiftwork has been associated with cancer across multiple tissues types and sites (Ijaz et al., 2013; Jia et al., 2013; Lin et al., 2015; Rao, Yu, Bai, Zheng, & Xie, 2015; F. Wang et al., 2015), including hematopoietic cancers, and methylation in blood can serve as a systemic marker of methylation in other tissues (Barault et al., 2013; Ma et al., 2014). However, we are unable to

rule out the potential for confounding of our results by minor immune cell profiles not accounted for by our cell mixture adjustment. Thus, our observations may reflect differences in immune cell profile rather than direct impacts of nightshift work on DNA methylation. Such differences may have arisen due to natural diurnal variation in immune cell profile owing to differences in timing of blood collection between night and dayshift workers. For study *C*, blood samples were systematically collected after completion of work shifts. For studies *A* and *B*, blood sample collection times were not specified. Nightshift work may also directly impact the immune system through its role in sleep disruption (Costa, Haus, & Stevens, 2010; Khosro, Alireza, Omid, & Forough, 2011).

Our exploratory study suggests that nightshift work may impact methylation of genes, the perturbation of which may contribute to carcinogenesis. In particular, the association with cg03227775 in *CARD11* should be closely examined in a future study that not only has a much larger number of nightshift workers, but also prospectively collected blood samples—for analyses of the relationship between methylation and expression—and also cancer incidence data so that the relevance of differential methylation at this locus on expression and cancer can be fully assessed.

Analysis I—Methylation Tables and Figures

TABLE 1. Demographic data for study participants

	Dayshift *N* (%)	Nightshift *N* (%)
Gender		
Female	126 (82)	149 (48)
Male	27 (18)	159 (52)
Age		
22-28	32 (21)	106 (34)
29-34	41 (27)	68 (22)
35-39	45 (29)	53 (17)
>40	35 (23)	81 (26)
Body Mass Index ((lbs/in^2)*703)[#]		
14-21	38 (25)	40 (13)
21-24	39 (25)	83 (27)
24-26	37 (24)	70 (23)
26-30	39 (25)	115 (38)
Race		
White	121 (79)	222 (72)
Other	32 (21)	86 (28)
Smoking		
Yes	9 (06)	17 (06)
No	144 (94)	291 (94)
Alcohol		
Yes	45 (29)	25 (08)
No	108 (71)	283 (92)

[#]BMI categories based on quantiles among dayshift workers

TABLE 2. DNA methylation data processing procedures

	Procedure	Purpose	Package
1	Background correction	Background correction resolves bias in the M-values associated with differences in background fluorescence (Triche et al., 2013)	'noob' in minfi (T. J. Morris & Beck, 2015)
2	Functional normalization	Functional normalization was used to rescale the M-values, which corrects the bias from the differences in distributions of M-values from the two types of probes used in the array (Wilhelm-Benartzi et al., 2013)	minfi (T. J. Morris & Beck, 2015)
3	Remove sex chromosomes	Sex-stratified analyses would be underpowered	ChAMP R extension (T. J. Morris et al., 2014)
4	Remove poor-performing probes	Probes with bead counts <3 were removed since this indicates that a probe has failed to bind to a bead chip. We also used the detection p-value statistic, which indicates the confidence that a probe's intensity is above the background level established by the negative-control probes, to remove probes for which 20% of the samples had a detection p-value greater than 0.01.	ChAMP R extension (T. J. Morris et al., 2014)
5	Remove SNP loci	Since the presence of single nucleotide polymorphisms (SNPs) can affect hybridization (N. Morris et al., 2015), we excluded loci near SNPs	based on Chen's list (Y. A. Chen et al., 2013; Dedeurwaerder et al., 2014)
6	Remove cross-reacting probes	We excluded cross-reactive probes (i.e. probes that cross-hybridize to sites other than those for which they were designed) (Y. A. Chen et al., 2013)	based on Chen's list (Y. A. Chen et al., 2013; Dedeurwaerder et al., 2014)
7	Batch effect correction	The samples from our three studies were collected at different times and introduced a batch effect by study (Leek et al., 2012)	Combat with the sva R-package
8	Remove loci with the least variance	To improve power (Bourgon et al., 2010)	

FIGURE 1. Principal components analysis (PCoA) scree plot demonstrating the first principle component accounting for >90% of the variation in leukocyte cell mixture

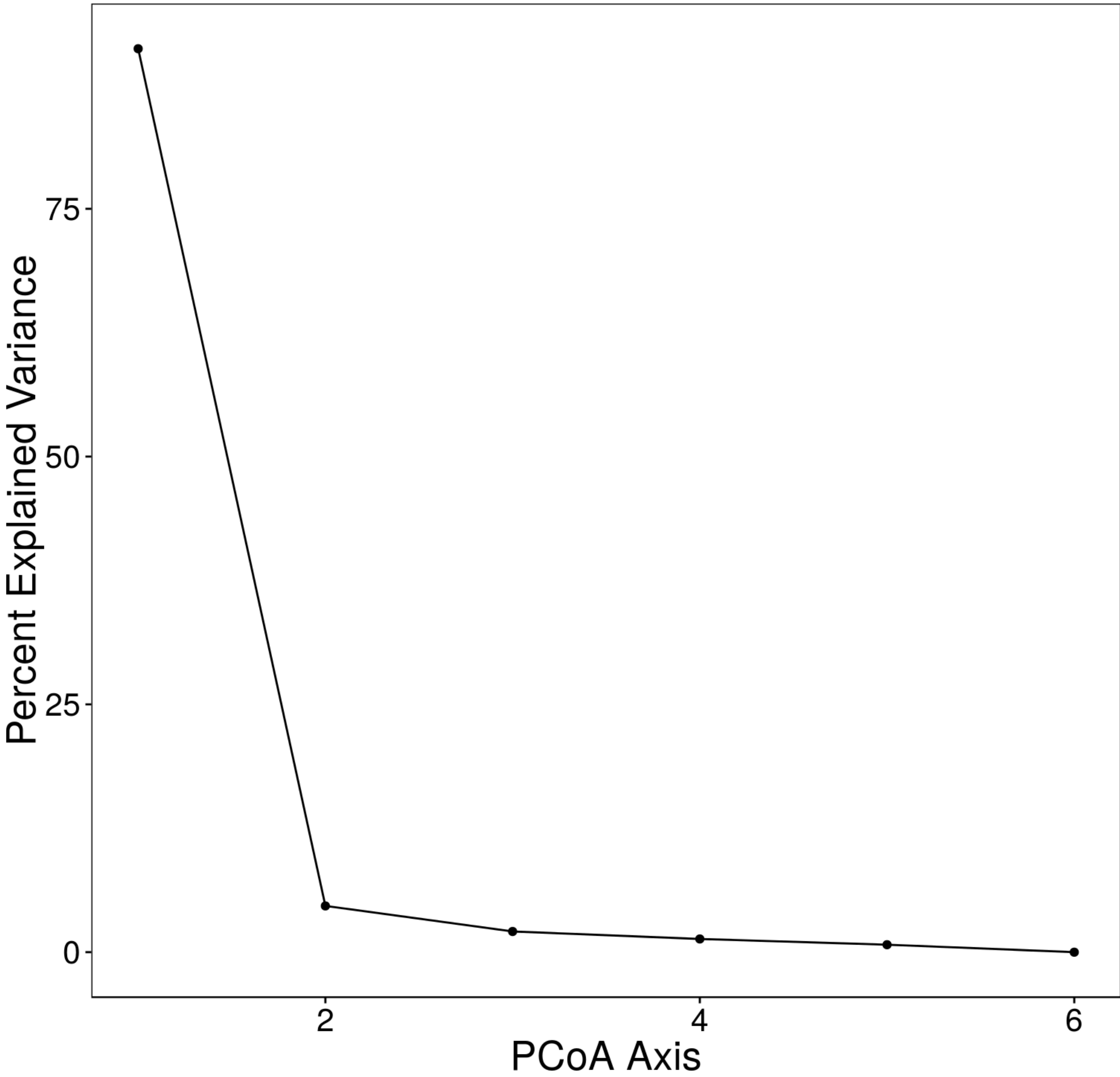


TABLE 3. Five (circadian) loci with the smallest unadjusted p-values when comparing nightshift to dayshift workers*

Gene[a]	Illumina ID[b]	Region[c]	Shift Effect[d]	Unadjusted *p*-value[e]	BH *p*-value[f]
NR1D1	cg09304381	Body	0.1341	0.0011	0.3891
PER1	cg27004243	5'UTR	-0.0843	0.0043	1
RORα	cg02189597	Body	0.1174	0.0053	1
BMAL1	cg13286116	5'UTR	-0.2417	0.0097	1
RORα	cg22387253	Body	-0.0692	0.0195	1

*Analysis included 346 CpG sites

a. Gene in which the CpG was located

b. Illumina's ID for the CpG site, as extracted from Human GRCh37/hg19

c. Functional region of gene: TSS1500=within 1500 bp of transcription start site; TSS200=within 200 bp of transcription start site; 5'UTR=5 prime untranslated region; 1st Exon=first segment of gene coding for peptide sequence; Body=within gene body; 3'UTR=3 prime untranslated region; multiple listings indicate loci belong to multiple regions because of splice variants.

d. Shift effect=difference in M-values comparing night and dayshift workers adjusted for age (continuous), gender, race (White/Other), BMI (continuous), and cell composition

e. Unadjusted p-values

f. *p*-values adjusted for multiple comparisons with the Benjamini-Hochberg (BH) method for controlling the false-discovery rate

TABLE 4. Five (circadian) loci with the largest absolute differences in M-values when comparing nightshift to dayshift workers*

Gene[a]	Illumina ID[b]	Region[c]	Shift Effect[d]	Unadjusted *p*-value[e]	BH *p*-value[f]
BMAL1	cg13286116	5'UTR	-0.2417	0.0097	0.8372
PER3	cg08926642	Body/exon 18	-0.1357	0.2752	0.9836
NR1D1	cg09304381	Body/exon 8	0.1341	0.0011	0.3891
PER3	cg17724687	Body	-0.1178	0.2939	0.9836
RORα	cg02189597	Body	0.1174	0.0053	0.6086

*Analysis included 346 CpG sites

a. Gene in which the CpG was located

b. Illumina's ID for the CpG site, as extracted from Human GRCh37/hg19

c. Functional region of gene: TSS1500=within 1500 bp of transcription start site; TSS200=within 200 bp of transcription start site; 5'UTR=5 prime untranslated region; 1st Exon=first segment of gene coding for peptide sequence; Body=within gene body; 3'UTR=3 prime untranslated region; multiple listings indicate loci belong to multiple regions because of splice variants.

d. Shift effect=difference in M-values comparing night and dayshift workers adjusted for age (continuous), gender, race (White/Other), BMI (continuous), and cell composition

e. Unadjusted p-values

f. *p*-values adjusted for multiple comparisons with the Benjamini-Hochberg (BH) method for controlling the false-discovery rate

TABLE 5. Five (non-circadian) loci with the smallest unadjusted p-values when comparing nightshift to dayshift workers*

Gene[a]	**Illumina ID**[b]	**Region**[c]	**Shift Effect**[d]	**Unadjusted *p*-value**[e]	**BH *p*-value**[f]
CARD11	cg03227775	Body/intron 11	-0.27	<0.0001	0.042
*TLE3***	cg18439144	N/A	-0.1577	<0.0001	0.1792
AHNAK	cg11480534	Body/intron 2	-0.1455	<0.0001	0.6303
LOXL1	cg01349856	Body/intron 1	-0.159	<0.0001	0.2718
RCN1	cg02436272	Body/intron 1	-0.1456	<0.0001	0.2778

*Analysis included 197,258 CpG sites
a. Gene in which the CpG was located
b. Illumina's ID for the CpG site, as extracted from Human GRCh37/hg19
c. Functional region of gene: TSS1500=within 1500 bp of transcription start site; TSS200=within 200 bp of transcription start site; 5'UTR=5 prime untranslated region; 1st Exon=first segment of gene coding for peptide sequence; Body=within gene body; 3'UTR=3 prime untranslated region; multiple listings indicate loci belong to multiple regions because of splice variants.
d. Shift effect=difference in M-values comparing night and dayshift workers adjusted for age (continuous), gender, race (White/Other), BMI (continuous), and cell composition
e. Unadjusted p-values
f. *p*-values adjusted for multiple comparisons with the Benjamini-Hochberg (BH) method for controlling the false-discovery rate
** Closest gene

TABLE 6. Five (non-circadian) loci with the largest differences in M-values when comparing nightshift to dayshift workers*

Gene[a]	Illumina ID[b]	Region[c]	Shift Effect[d]	Unadjusted *p*-value[e]	BH *p*-value[f]
LINC00368[g]	cg26705599	N/A	0.6358	<0.0001	0.278
MARK3	cg00993903	Body/intron 1	0.4645	0.0001	0.4766
	cg22274273	N/A	0.4462	0.0003	0.7038
RIBC2;SMC1B	cg01808030	Body/intron1;TSS1500	0.4832	0.0036	0.936
CNST	cg04246708	Body/intron 3	-0.4286	0.0470	0.9888

*Analysis included 197,258 CpG sites

a. Gene in which the CpG was located

b. Illumina's ID for the CpG site, as extracted from Human GRCh37/hg19

c. Functional region of gene: TSS1500=within 1500 bp of transcription start site; TSS200=within 200 bp of transcription start site; 5'UTR=5 prime untranslated region; 1st Exon=first segment of gene coding for peptide sequence; Body=within gene body; 3'UTR=3 prime untranslated region; multiple listings indicate loci belong to multiple regions because of splice variants.

d. Shift effect=difference in M-values comparing night and dayshift workers adjusted for age (continuous), gender, race (White/Other), BMI (continuous), and cell composition

e. Unadjusted p-values

f. *p*-values adjusted for multiple comparisons with the Benjamini-Hochberg (BH) method for controlling the false-discovery rate

g. cg26705599 is located near long intergenic non-protein coding RNA 368 (LINC00368) and upstream of the gene *ARHGEF7*

FIGURE 2. Five (circadian) loci with the smallest unadjusted p-values and five (circadian) loci with the largest absolute effect size differences when comparing nightshift to dayshift workers

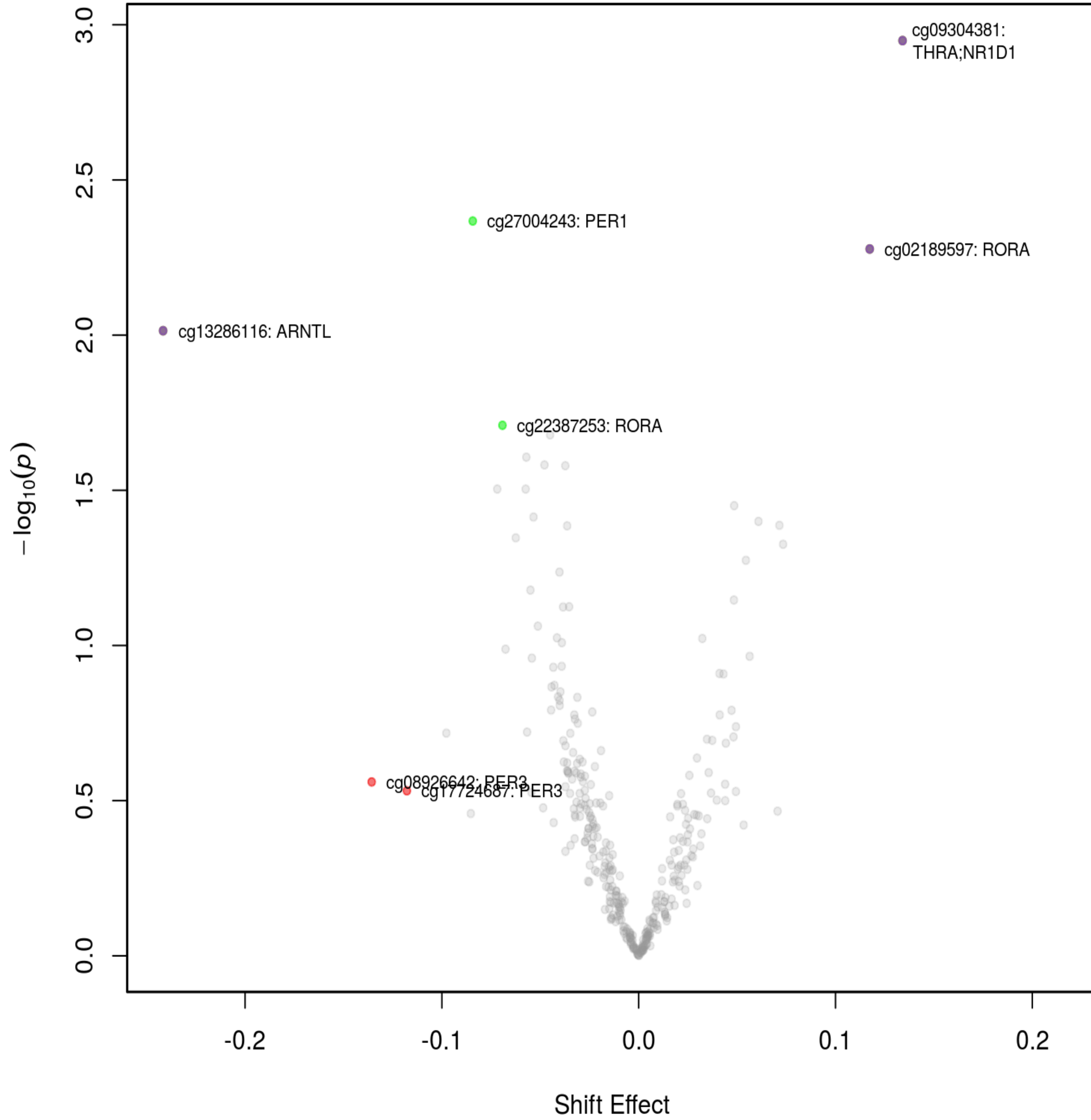


FIGURE 3. Five (non-circadian) loci with the smallest unadjusted p-values and five (non-circadian) loci with the largest absolute effect size differences when comparing nightshift to dayshift workers

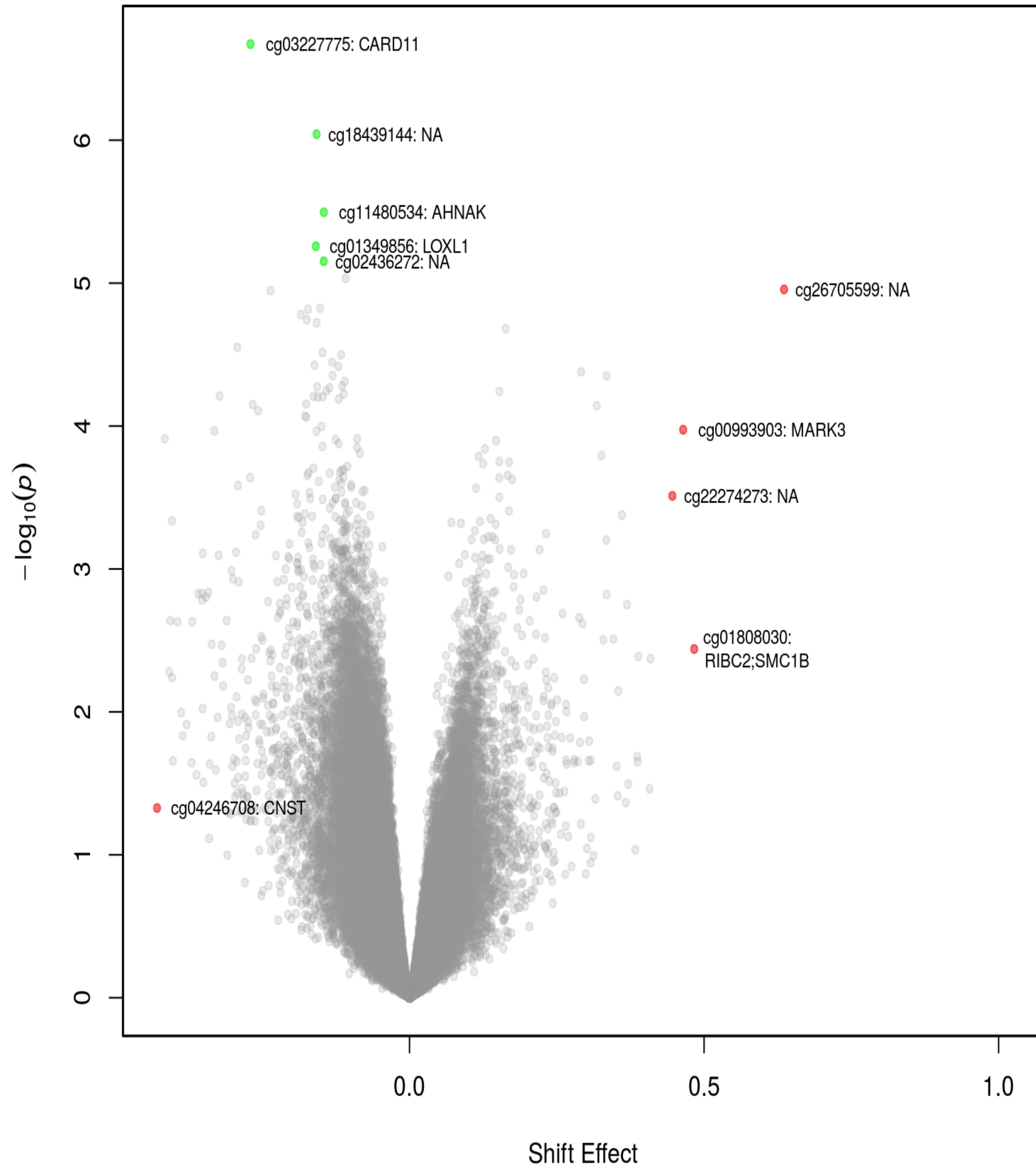


TABLE 7. Five (circadian) genes with the smallest unadjusted p-values when comparing nightshift to dayshift workers*

Gene[a]	Number of CpG loci[b]	Unadjusted *p*-value[c]	BH *p*-value[e]
BMAL1	23	0.0168	0.2015
NR1D1	24	0.1581	0.9168
CSNK1ε	24	0.3390	0.9168
PER1	18	0.3504	0.9168
PER3	22	0.4001	0.9168

*Analysis included 12-core circadian genes with a minimum of five CpG sites after preprocessing

a. Gene

b. Number of CpGs remaining in the gene after preprocessing

c. Unadjusted *p*-values for kernel tests including covariates for age (continuous), gender, race (White/Other), BMI (continuous), and cell composition

f. *p*-values adjusted for multiple comparisons with the Benjamini-Hochberg (BH) method for controlling the false-discovery rate

TABLE 8. Five (non-circadian) genes with the smallest unadjusted p-values when comparing nightshift to dayshift workers*

Gene[a]	**Number of CpG loci**[b]	**Unadjusted *p*-value**[c]	**BH *p*-value**[e]
HSPA4L	5	0.0003	0.9962
USP42	19	0.0006	0.9962
LASS3	8	0.0008	0.9962
VKORC1L1	8	0.001	0.9962
AP3B2	21	0.0012	0.9962

* Analysis included 11,598 non-circadian genes with a minimum of five CpG sites after preprocessing

a. Gene

b. Number of CpGs remaining in the gene after preprocessing

c. Unadjusted *p*-values for kernel tests including covariates for age (continuous), gender, race (White/Other), BMI (continuous), and cell composition

f. *p*-values adjusted for multiple comparisons with the Benjamini-Hochberg (BH) method for controlling the false-discovery rate

TABLE 9. Five (circadian) regions with the smallest unadjusted p-values when comparing nightshift to dayshift workers*

Gene and Region[a]	Number of CpG loci[b]	Unadjusted *p*-value[c]	BH *p*-value[e]
BMAL1 / Promoter	20	0.0178	0.3738
NR1D1 / Body	11	0.0505	0.5305
PER1 / Promoter	11	0.1407	0.9703
CSNK1ε / Promoter	22	0.2921	0.9703
PER3 / Body	13	0.3955	0.9703

* Analysis included 22 circadian regions with a minimum of five CpG sites after preprocessing
a. Gene and region: promoter (defined as CpGs located in: TSS1500=within 1500 bp of transcription start site; TSS200=within 200 bp of transcription start site; 5'UTR=5 prime untranslated region; or 1st Exon=first segment of gene coding for peptide sequence); Body=within gene body; 3'UTR=3 prime untranslated region
b. Number of CpGs remaining in the gene after preprocessing
c. Unadjusted *p*-values for kernel tests including covariates for age (continuous), gender, race (White/Other), BMI (continuous), and cell composition
f. *p*-values adjusted for multiple comparisons with the Benjamini-Hochberg (BH) method for controlling the false-discovery rate

TABLE 10. Five (non-circadian) regions with the smallest unadjusted p-values when comparing nightshift to dayshift workers*

Gene and Region[a]	Number of CpG loci[b]	Unadjusted *p*-value[c]	BH *p*-value[e]
USP42 / Promoter	5	0.0002	0.987
LOXL1 / Body	5	0.0003	0.987
LASS3 / Promoter	8	0.0008	0.987
LMO2 / Promoter	11	0.001	0.987
VKORC1L1 / Body	7	0.001	0.987

*Analysis included 10,531 non-circadian regions with a minimum of five CpG sites after preprocessing

a. Gene and region: promoter (defined as CpGs located in: TSS1500=within 1500 bp of transcription start site; TSS200=within 200 bp of transcription start site; 5'UTR=5 prime untranslated region; or 1st Exon=first segment of gene coding for peptide sequence); Body=within gene body; 3'UTR=3 prime untranslated region

b. Number of CpGs remaining in the gene after preprocessing

c. Unadjusted *p*-values for kernel tests including covariates for age (continuous), gender, race (White/Other), BMI (continuous), and cell composition

f. *p*-values adjusted for multiple comparisons with the Benjamini-Hochberg (BH) method for controlling the false-discovery rate

FIGURE 4. Circadian autoregulatory feedback loop

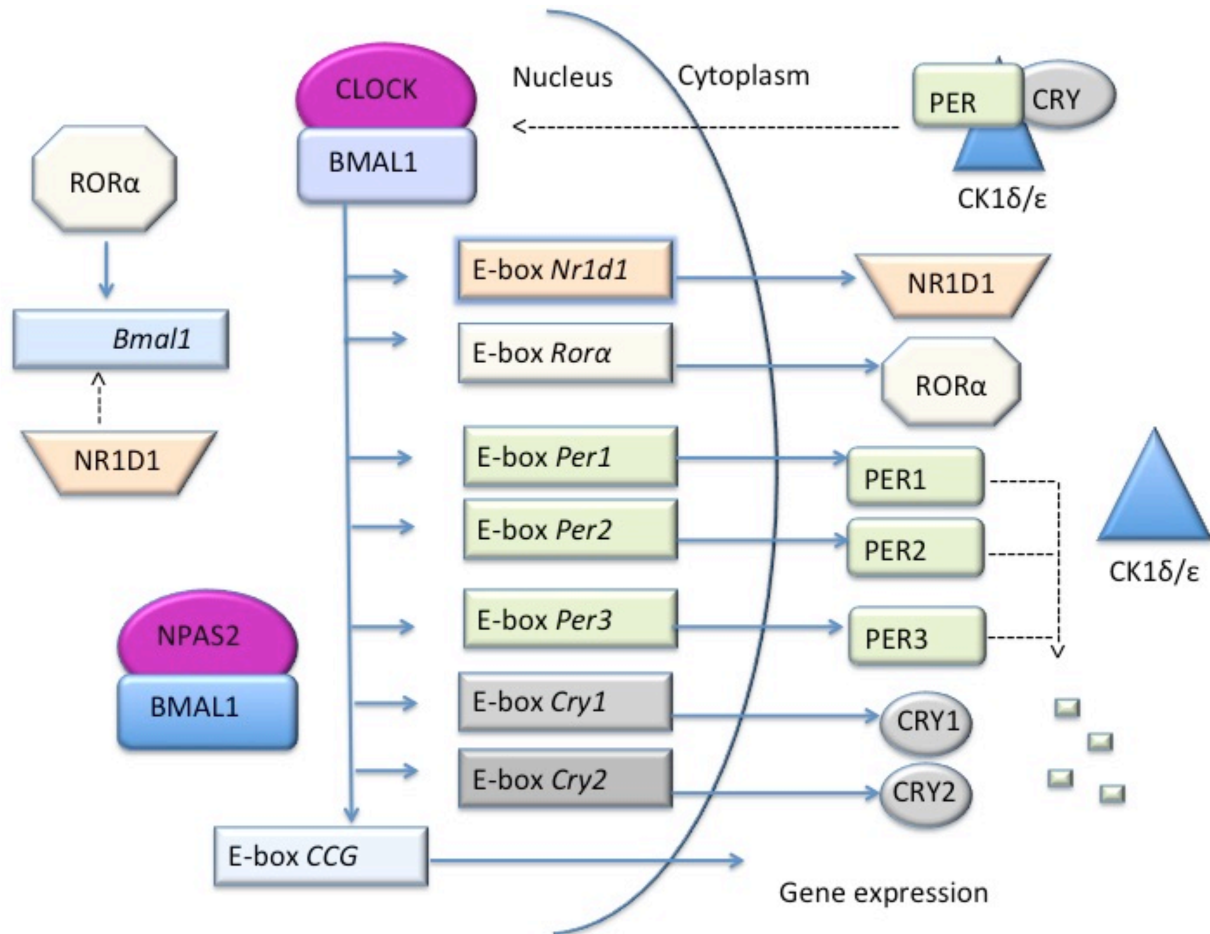


FIGURE 4 depicts the core feedback loop, which is comprised of positive and negative arms and driven by the positive (upregulating) actions of CLOCK (circadian locomotor output cycles kaput) and BMAL1/ARNTL (Aryl hydrocarbon receptor nuclear translocator-like protein 1). CLOCK and BMAL1 heterodimerize (CLOCK:BMAL1) to activate the expression of the negative arm of the clock, the cofactors, Cryptochrome (*CRY1* and *CRY2*) and the Period circadian protein homologs 1, 2, and 3 (*PER1*, *PER2*, *PER3*), as well as *RORα* (Nuclear receptor ROR-alpha) and *NR1D1/REV-ERB*α (Nuclear receptor subfamily 1 group D member 1). CSNK1δ and CSNK1ε (Casein kinase 1 δ/ε) phosphorylate the PER proteins, marking them for degradation in the cytoplasm. NPAS2 can substitute for CLOCK and also heterodimerize with BMAL1. Adapted from (Ko & Takahashi, 2006).

Part A, Analysis 2—Chronotype and Sleep Quality

Title:

The impact of chronotype and sleep quality on methylation among shift workers

Authors:

Charleen D. Adams[1], Timothy Randolph[1],Wade Copeland[1], Dana K. Mirick[1], Xiaoling Song[1], Cassandra L. Sather[2], Andres Houseman[3], Scott Davis[1], Parveen Bhatti[1]

[1]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA.

[2]Genomics Resource, Fred Hutchinson Cancer Research Center, Seattle, WA, USA.

[3]College of Public Health and Human Sciences, Oregon State University, Corvallis, OR, USA

Funding / Acknowledgments:

The study was funded by grants R01 CA097090 and R01 CA116400 from the US National Cancer Institute and funds from the Fred Hutchinson Cancer Research Center and an award from the Safeway Foundation.

Abstract

Introduction: The association between shift work and cancer, potentially mediated by the effects of methylation, may be modified by chronotype (an innate propensity for being active earlier or later in the day) and/or sleep quality. However, the impacts of chronotype and sleep quality on methylation have not been investigated among shift workers. Here, we analyze the impact of chronotype and sleep quality on previously reported differences in methylation by shift status at

8 CpG loci among circadian genes (cg09304381, cg27004243, cg02189597, cg13286116, cg22387253, cg08926642, cg09304381, and cg17724687), and 10 CpG loci among non-circadian genes (cg03227775, cg18439144, cg11480534, cd01349856, cg02436272, cg26705599, cg01808030, cg00993903, cg22274273, and cg04246708).

Methods: Our cross-sectional study included 149 actively working dayshift and 281 actively working nightshift workers from the Seattle metropolitan area from whom DNA from blood samples was assayed using the Illumina Infinium HumanMethylation450 Bead Array. To assess chronotype, participants completed the Composite Scale of Morningness. Chronotype was dichotomized at the midpoint of the scale after scoring. Sleep duration (day sleep among nightshift workers and night sleep among dayshift workers) in minutes was quantified via actigraphy and divided into tertiles. To determine if the effect of nightshift work on DNA methylation was modified by chronotype and sleep quality, interaction terms for shift schedule and chronotype and shift schedule and sleep duration were added separately to previously constructed rank-regression models of the associations between methylation at our selected loci and shift schedule, adjusted for gender, age, body mass index, race, and leukocyte cell profile.

Results: Methylation differences between nightshift and dayshift workers at two loci in *PER3* (cg08926642 and cg17724687) were statistically significantly modified by chronotype ($p_{interaction}$= 0.0141 and 0.0055, respectively), such that, compared to dayshift morning types, dayshift evening types and nightshift morning types, but not nightshift evening types, had lower average levels of methylation at both loci. Differences in methylation related to nightshift work were statistically significantly modified at three loci by sleep duration; two of the loci were in circadian genes, (cg27004243 in *PER1* and cg22387253 in *RORα*) and one was in a non-circadian gene (cg01349856 in *LOXL1).* Across all three loci, it appeared that, relative to the

lowest tertile of sleep duration, the middle and longest duration tertiles were associated with decreased methylation among dayshift workers but not nightshift workers (cg27004243 $p\text{-}_{\text{interaction middle tertile}}$= 0.001 & $p\text{-}_{\text{interaction longest tertile}}$= 0.007; cg22387253 $p\text{-}_{\text{interaction middle tertile}}$=0.0366 & p-interaction longest tertile = 0.2888; cg01349856, $p\text{-}_{\text{interaction middle tertile}}$=0.0014 & $p\text{-}_{\text{interaction longest tertile}}$=0.0400).

Conclusion: The methylation pattern observed for the chronotype effect modification analysis at *PER3* suggests that those who work out of alignment with their chronotype (dayshift evening-type chronotypes and nightshift morning-type chronotypes) have less methylation at cg08926642 and cg17724687 than do those who work in alignment with their chronotype. This pattern of methylation might reflect an anti-cancer effect among the chrono-misaligned. Alternately, due to the role of the Period genes in clock resetting, less methylation among the chrono-misaligned might mark resetting of the circadian clock. Our exploratory analysis also reveals a modifying effect of sleep duration on methylation, suggesting that, while increased sleep duration reduced methylation levels at three loci (cg27004243 in *PER1*, cg22387253 in *RORα*, and cg01349856 in *LOXL1*), this impact was restricted to dayshift workers; relative to dayshift workers, nightshift workers had reduced methylation levels at these loci that did not seem to be additionally impacted by sleep duration. At cg27004243 in *PER1*, the observed hypomethylation among nightshift workers may reflect a compromise in nightshift workers' abilities to handle DNA damage. At cg22387253 in *RORα*, the hypomethylated locus is located in a DNA transposable element, which might impact on non-allelic homologous recombination, potentially predisposing to rearrangements. The hypomethylation at cg01349856 in *LOXL1* might reflect a non-cellular pathway through which shift workers are initially protected from cancer but likewise reflect an increased risk of metastasis later, as *LOXL1* has been reported to be upregulated in many

cancers, impacting on metastasis through an influence on the tumor microenvironment. Prospective studies of shift work with methylation, expression, and cancer outcomes are needed to fully assess how these patterns of methylation relate to expression and cancer risk and to determine whether these patterns of methylation can be used to inform cancer prevention strategies.

Introduction

There is growing evidence that shift work resulting in circadian disruption is associated with increased risks of cancer among men and women (International Agency for Research on Cancer, 2007; Knutsson et al., 2013). Evaluating factors that may modify the carcinogenic effects of shift work can aid prevention efforts by identifying subgroups of workers that may need interventions because of their particular susceptibility to the carcinogenic effects of shift work and by identifying specific targets for intervention that can be modified to protect shift workers from carcinogenic effects. A lack of consideration of individual factors [such as chronotype (preference for the start of sleep in the late evening, with those having this preference being known as evening-types or "owls", or preference for the start of sleep earlier in the evening, with those having this preference known as morning-types or "larks") and sleep quality] that may modify the association between shift work and cancer has been a major limitation of previous studies (Stevens et al., 2011).

Chronotype may impact a person's ability to adapt to non-day work schedules (Erren, 2013; Saksvik, Bjorvatn, Hetland, Sandal, & Pallesen, 2011). Though chronotype changes with age, about 50% of the population has been observed to dichotomize into either preferring morning or evening activity (Paine, 2006). Given this, discovering the risk of cancer among shift

workers by chronotype could aid policy recommendations for cancer prevention, potentially steering those in the higher risk chronotype into dayshift schedules when possible.

Few studies have examined the modifying effects of chronotype on cancer (Fritschi et al., 2013; Hansen & Lassen, 2012; Papantoniou et al., 2015). Of these, two have reported statistically significant associations among shift workers by chronotype. In one study, women with a morning-type chronotype who worked the nightshift were found to have an increased risk for breast cancer compared to morning-type women who had never worked the nightshift (odds ratio (OR)= 3.91; 95% confidence interval (CI) 1.6 to 9.5); whereas, for evening-type women, the risk was increased but not significant and relatively smaller than for morning-type women (OR=2.0; 95% CI= 0.7 to 5.8) (Hansen & Lassen, 2012). Likewise, few studies have examined the modifying effects of chronotype on intermediate biomarkers that may mediate the effect of shift work on cancer (Bhatti, Mirick, & Davis, 2014; Leung et al., 2016).

Sleep quality, like chronotype, is a factor that may modify the association between shift work and cancer and could be used to inform cancer prevention strategies. Sleep is essential for overall health. Both short and long sleep durations have been associated with risk for colorectal and breast cancer, and sleep duration is dependent on circadian rhythms (Vladar, Lee, Stearns, & Axelrod, 2015a; P. Wang et al., 2015). Because shift work causes circadian disruption (a disturbance in circadian rhythms), it is conceivable that sleep duration mediates many of the negative health effects of shift work, including cancer. Therefore, strategies to improve sleep quality among shift workers may be helpful for mitigating negative health effects. To that aim, given cancer's long latency, improving our understanding of the effects of chronotype and sleep quality on biomarkers of carcinogenesis may improve our ability to detect high-risk individuals before they develop cancer. Changes in methylation are consistently observed during

carcinogenic transformation (Moore et al., 2008), and because an array of environmental factors are known to impact methylation (Hou, Zhang, Wang, & Baccarelli, 2012), methylation is an attractive biomarker with which to study shift work's early carcinogenic effects. Here we describe the first study of the effects of chronotype and sleep quality on methylation and report their impacts on the top findings from our previously reported genome-wide DNA methylation study of actively working day and nightshift workers from the Seattle metropolis.

Materials and Methods

Study subjects

The study was approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. Study subjects were drawn from participants of three previous studies of shift work conducted among healthcare workers from the Seattle metropolitan area: the Female Shift Worker Study (Davis et al., 2012) (*A*), the Male Shift Worker Study (Mirick et al., 2013) (*B*) and our previous study of nightshift work and DNA methylation (Bhatti, Zhang, et al., 2014) (*C*). For the remainder of the paper, the abbreviations *A*, *B*, and *C* will be used to designate each study. For all three studies, subjects were recruited through advertisements at local area hospitals, direct mailing to Washington State Department of Health licensed and certified health care workers, and referrals from eligible and ineligible participants. To be eligible for these studies, participants had to be actively engaged in dayshift or nightshift work at the time of recruitment and during data collection. In studies *A* (recruitment and data collection from November 2003 to August 2007) and *B* (recruitment and data collection from October 2007 to May 2011), nightshift workers were required to work at least 20 hours per week (at least eight hours per shift, stopping work no earlier than 06:00) exclusively during the graveyard shift and to sleep at night during off days. Dayshift workers were required to be employed at least 20

hours per week and work exclusively during the dayshift (i.e., work at least eight hours per shift and begin work no earlier than 06:00). For study *C* (recruitment and data collection from November 2011 to November 2012), these work criteria were similar, except nightshift and dayshift workers had to work their respective shifts at least 24 hours per week for the previous six months. In addition, for study *C*, all dayshift and nightshift workers were required to have schedules where they typically worked at least three consecutive days or nights, respectively, each week.

For study *A*, participants were required to be women aged 20 to 49 years. Additional eligibility criteria included: regular menstrual periods; no personal history of breast cancer, chemotherapy, or tamoxifen therapy; no pregnancy or breast feeding within the past year; no use of supplements containing phytoestrogens or isoflavones and consumption of no more than five servings per week of soy-based foods. For study *B*, participants were required to be men aged 20 to 55 years. They could not be using medications or supplements used to treat benign prostate conditions within 30 days of participation, could not have a personal history of prostate cancer or chemotherapy, and could not have undergone general anesthesia or major surgery at least eight weeks prior to enrollment. For both studies *A* and *B*, participants were required to have a body mass index (BMI, weight in kilograms [kg] divided by the square of height in meters $[m]^2$) between 18 and 30 kg/m^2, and could not have been using hormones or supplements containing melatonin during the 30 days prior to enrollment. For study *C*, eligibility criteria included being 20 to 40 years of age, with a BMI between 18-32 kg/m^2, and no personal history of cancer, diabetes, heart disease, autoimmune disorders, or inflammatory disease. In addition, participants could not have been using melatonin supplements during the six months prior to enrollment, and women could not currently be pregnant.

Two-hundred and eighty-eight participants from *A* (129 dayshift and 159 nightshift) and 208 participants from *B* (all nightshift) had available buffy coat samples for DNA extraction or previously extracted buffy coat DNA. We restricted to those *A*/*B* participants who worked their respective shift schedules (day or night) for at least six months, who completed the study questionnaire, who provided actigraphy data from at least one sleep period (day sleep or night sleep) for sleep quality assessment, and to those who had both completed the study protocol and provided urine samples during various sleep and work periods for circulating melatonin measurements, though these samples were not used in the present study. In total, 88 dayshift and 113 nightshift workers from *A* and 137 nightshift workers from *B* were selected. One subject was excluded from study *B* due to not having questionnaire data on race. All subjects from *C* were included in the current study (65 dayshift, 59 nightshift). The total sample size for the current study is 153 dayshift and 308 nightshift workers (Table 1). However, three of the subjects were missing chronotype questionnaire data and 31 subjects were missing the necessary actigraphy data (see below for details). Table 1 provides the demographic distribution for the study participants.

Data and Biospecimen Collection

For all eligible participants across the three studies, informed consent was obtained during either a home or work visit by trained study interviewers/phlebotomists. Structured interviews were conducted to collect information about current work and sleep schedules, work shift history, and physical activity. Height and weight measurements were also obtained during these interviews. For both *A* and *B*, blood samples (10mL EDTA vacutainer tubes) were also collected during the interviews. For the participants in *C*, blood samples (10mL ACD tube) were collected immediately after completing the third consecutive work shift. For studies *A* and *B*,

data on caffeine, alcohol, tobacco, medication, and supplement intake were self-reported for the 24 hour periods preceding sleep periods, that occurred after a typical work day, during which urine samples were collected. For study *C*, the structured interview, which was conducted on a workday, collected data on these variables for the 24 hours preceding the time of the interview.

To assess chronotype, all participants completed the Composite Scale of Morningness—a validated, 13-item questionnaire scored into a single variable from 13 to 55 points—which was dichotomized at the midpoint of the scale such that those with lower scores were indicative of evening types and those with higher scores were indicative of morning types (Smith, Reilly, & Midkiff, 1989). The choice to use the dichotomized measure of chronotype rather than the Smith et al. (1999) trichotomized scheme was made because using the trichotomous measure resulted in only 36 evening-type individuals and 47 morning-type individuals, with only 1 evening-type dayshift worker. Using the dichotomized measure provided a more balanced distribution of chronotype by shift schedule.

Participants wore an actigraph (Actiwatch-16, Mini Mitter, Bend, OR) to measure sleep duration as a metric for sleep quality. Sleep duration was derived from the logged motor activity data using a FORTRAN program that calculates the sleep/wake status of each minute of the night based on the number of detectable motions in that minute (Cole, Kripke, Gruen, Mullaney, & Gillin, 1992). For dayshift workers, sleep duration was measured during a night of sleep after completing a typical dayshift. For nightshift workers, sleep duration was measured during day sleep after completing a typical nightshift. In studies *A* and *B*, for nightshift workers, sleep duration was also measured during a night of sleep on their first day off from work during a typical workweek. To aid comparability across studies and since nightshift workers are mostly engaged in day sleep during a typical workweek, we ultimately focused our study on actigraphy

data collected during day sleep among nightshift workers.

Laboratory Methods

Specimen processing

For studies *A* and *B*, buffy coats were isolated from the whole blood samples and subsequently stored at –70°C. For study *A*, buffy coat samples were stored from seven to 11 years before DNA was extracted and utilized for the methylation assay. For study *B*, buffy coat samples were stored up to four years before DNA extraction. The extracted DNA from study *B* was subsequently stored for up to eight years, at -20°C, before being used for the methylation assay. DNA extraction for both studies was performed on the buffy coats using a salt precipitation method (SA et al., 1988).

For study *C*, lymphocytes were isolated from the whole blood samples within 24 hours of collection. Refrigerated centrifugation was used to isolate the buffy coat, which was resuspended in RMPI 1640 + P/S. After layering the buffy coat suspension over Lymphocyte Separation Medium (Histopaque – 1077, Sigma Aldrich) and centrifugation at room temperature, the mononuclear cell layer was removed, washed, centrifuged and resuspended in freeze media (RPMI with 15% fetal calf serum and filtered 10% DMSO). After freezing at rate of -1°C/minute to -80°C, samples were transferred to a liquid nitrogen freezer for storage. DNA was extracted from cryopreserved lymphocytes (ArchivePure DNA Purification Kit, 5- Prime, Hilden, Germany).

500 ng of DNA for each participant was treated with sodium bisulfite using EZ DNA Methylation- DirectKit (Zymo Research, Irvine, CA). Treated DNA specimens were stored at –80°C, and the methylation assay was performed within two weeks.

Methylation Assay

Infinium HumanMethylation450 Bead Array (Illumina, San Diego, CA) "genotypes" sodium bisulfite-treated DNA to quantify DNA methylation for 485,577 CpG loci, capturing roughly two percent of the CpG sites throughout the human genome (Michels et al., 2013). Treatment with bisulfite converts unmethylated cytosines (C) into uracils, which are subsequently converted to thymines (T). Bisulfite-converted CpG sites can then be "genotyped" and interpreted as a proxy for methylation status. Unlike the categorical feature of genotypic calls obtained from genome-wide association studies, however, what is measured at a CpG site is the percentage of cells that are methylated at that site for the particular tissue sampled. After bisulfite-converted DNA is amplified and fragmented, it is hybridized to two probes, referred to as Type I and Type II probes (Pidsley et al., 2013). The Type I and II probes quantify methylation differently, though they both obtain the standard index for methylation: beta (β), where [$\beta = M/(M + U)$] and M and U refer to the methylated and unmethylated signal intensities at a given CpG site.

The array measures methylation across 21,154 genes, each with an average of 17 CpGs. CpGs span various gene regions, including those located 1500 base pairs (bps) and 200 bps upstream of transcription start sites (TSS1500 and TSS200 respectively) and those located within the 5' untranslated region, first exon, gene body, and 3' untranslated region. CpGs could be positioned within or outside CpG islands. Three-hundred and ninety-one CpG loci within 12-core circadian genes are included: *CLOCK*, *BMAL1*, *NPAS2*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *RORα*, *NR1D1*, *CSNK1δ*, and *CSNK1ε*.

The DNA methylation assay for study C was previously described (Bhatti, Zhang, et al.,

2014). Identical procedures were used for studies *A* and *B*. Briefly, four ml of bisulfite treated DNA was denatured and neutralized to prepare it for overnight isothemal whole-genome amplification. Next, the DNA was enzymatically fragmented for 60 min at 37°C and then precipitated with isopropanol and allowed to air dry. DNA was then resuspended in hybridization buffer. Samples were then applied to the beadchips, and the beadchips were incubated in a hybridization oven at 48°C for 16–24 h. After washing, the chip underwent extension and staining in capillary flow-through chambers. Beadchips were then scanned using the iScan+ (Illumina, San Diego, CA). Laboratory personnel were blinded to all study subject information, and specimens were identified by study ID number only. An additional 10% of DNA samples were randomly included among the study samples for quality control assurance.

Data Processing

Each CpG dinucleotide represented on the array was associated with an M-value, which was calculated as the log*2* ratio of the intensities of the methylated and unmethylated probes. A series of processing steps were necessary before methylation data could be analyzed (Table 2). We used the "noob" method in the minfi Bioconductor package (T. J. Morris & Beck, 2015) to resolve bias in the M-values associated with differences in background fluorescence (Triche et al., 2013). Functional normalization was used to rescale the M-values, which corrects the bias from the differences in distributions of M-values from the two types of probes used in the array (Wilhelm-Benartzi et al., 2013).

For our analysis, because sex-stratified analyses would have been underpowered, we restricted to the analysis of autosomes, excluding CpG loci from the sex chromosomes. Next, we removed CpG sites measured by poor-performing probes using a two-tiered approach in the

ChAMP package (T. J. Morris et al., 2014). First, a metric called a "bead count" was used to assess technical variance related to probe binding; a bead counts of <3 indicates a probe failed to bind to a bead chip. Probes with bead counts <3 were removed. Second, we used the detection statistic, which indicates the confidence that a probe's intensity is above the background level established by the negative-control probes, to remove the CpG sites associated with probes for which 20% of the samples had a p-value greater than 0.01 (T. J. Morris & Beck, 2015). 463,893 loci remained.

Since the presence of single nucleotide polymorphisms (SNPs) near a CpG locus can affect probe hybridization (N. Morris et al., 2015), we excluded all CpG loci that contained at least one SNP with a minor allele frequency ≥ 0.05 (Y. A. Chen et al., 2013). 412,408 loci remained. We also excluded cross-reactive probes (i.e. probes that hybridize to sites other than those for which they were designed) (Y. A. Chen et al., 2013). 394,851 loci remained. Further, we applied a low-variance filter to remove sites whose variance across all participants was below the median variance of the remaining 394,851 sites (Bourgon et al., 2010), since these loci are unlikely to be informative in our analyses. Additionally, to account for any potential batch effects from combining data from three studies, we applied the COMBAT batch adjustment procedure as implemented in the sva R-package (Leek et al., 2012). After implementing these processing procedures, 346 circadian and 197,261 non-circadian CpG sites remained for analysis.

Statistical Analysis

The *M*-values for each CpG site were modeled as dependent variables using rank regression, which, because the *M*-values were not normally distributed, allowed us to use rank-

based (non-parametric) inference for our linear models (Kloke & Mckean, 2012). Models included a variable for shift work status and adjustment variables for potential confounders: gender, continuous variables for age and BMI, race (White or Non-White), and the mixture of leukocytes that contributed DNA to each participant's sample. Leukocyte cell mixture for six major leukocyte subsets (CD8+ T-cells, CD4+ T-cells, natural killer cells, B-cells, monocytes and granulocytes) was inferred based on the method by Houseman *et al*. (2012) (Houseman et al., 2012). The six leukocyte cell-types were summarized by a single value derived from a principal component (PC) analysis (FIGURE 1). Specifically, the first PC accounted for 95% of the variation in the six cell type measures and so we used this first PC to define a single variable that was included in the statistical models to adjust for cell type. The models for the circadian and non-circadian loci were run separately, and multiple testing was accounted for with a 5% false-discovery rate (FDR) using the Benjamini-Hochberg (BH) method (Benjamini & Hochberg, 1995). We focused effect modification analyses on those individual loci that demonstrated a compelling association with nightshift work (see Chapter 1). Specifically, we selected the five most significant CpG sites from the circadian (cg09304381, cg27004243, cg02189597, cg13286116, cg22387253) and non-circadian (cg03227775, cg18439144, cg11480534, cg01349856, cg02436272) analyses, as well as the five CpG sites with the largest absolute effect size differences for both the circadian (cg13286116, cg08926642, cg09304381, cg17724687, cg02189597) and non-circadian (cg26705599, cg01808030, cg00993903, cg22274273, cg04246708) loci. Three circadian loci (cg09304381, cg13286116, and cg02189597) met the selection criteria for inclusion in both the set of most significant loci and the set of loci with the largest absolute effect sizes. The *M*-values for these loci were modeled as dependent variables including the same set of adjustment variables as the models for the main

effects analyses in Chapter 1 but also included categorical terms for chronotype and sleep duration (based on tertiles of sleep duration among all subjects) as well as interaction terms for shift work by chronotype and shift work by sleep duration. However, the models for the modifying effects of chronotype and sleep duration were run separately. Because we performed relatively few tests (20) and our sample size limits power for effect modification analyses, p-values < 0.05 were deemed significant and results were not FDR-corrected.

While alcohol consumption and smoking are potential confounders, due to differences in how these variables were collected for study *C* versus studies *A*/*B* (see Data and Biospecimen Collection), we did not include them as covariates in our primary analysis (see Chapter 1) or the current effect modification analysis. As discussed in Chapter 1, we did perform a sensitivity analysis that excluded those subjects reporting consumption of alcohol to gauge the potential impact of differences in alcohol consumption on results of our primary analysis, and no major differences were observed. Because the prevalence of smoking was minimal amongst participants and given the similar prevalence of smoking between dayshift and nightshift workers, we did not conduct similar sensitivity analyses for smoking.

Results

In TABLE 1, the distribution of selected demographic variables is presented by shift status for the sets of shift workers included in the chronotype and sleep duration analyses. While the distribution of nightshift workers by gender was nearly balanced, dayshift workers in this pooled study were predominately female since no DNA samples were available from dayshift workers in the study of male shift workers (study *B*). Nightshift workers were younger and had a higher BMI than dayshift workers. The fraction of smokers was the same for night and dayshift

workers. Alcoholic beverage consumption was greatest amongst dayshift workers (29% of dayshift workers drank versus 8% of nightshift workers), and, although we report the fraction of smokers and alcoholic beverage drinkers, the variables for these were in reference to different time periods in studies *A*/*B* versus study *C* (see Data and Biospecimen Collection). Thirty of the subjects missing either chronotype or sleep duration data were nightshift workers. TABLE 2 compares average demographic characteristics of the total set of 361 nightshift workers to the average demographic characteristics of the 30 subjects working the nightshift who were missing chronotype and/or actigraphy data. Chi-squared computation based on the continuity of Yates was performed for categorical variables (gender, smoking, race, and alcohol) and independent 2-group t-tests were performed for continuous variables (age and BMI). No significant differences in demographic data were observed when comparing the nightshift subjects with missing chronotype/actigraphy data to the entire group of subjects.

Chronotype effect modification analyses

Chronotype was found to significantly modify differences in methylation associated with nightshift work at two CpG loci in *PER3* (cg08926642 and cg17724687) (TABLE 3). For cg08926642, those who were chrono-mismatched, who worked out-of-synch with their chronotype (dayshift evening-types and nightshift morning-types) had less methylation than those who were worked in-synch with their chronotype (dayshift morning-types and nightshift evening-types). Compared to dayshift morning-types, dayshift evening-types had M-values that were 0.31 units lower; nightshift morning-types had M-values that were 0.37 units lower, and nightshift evening-types had M-values that were 0.05 units fewer ($p_{interaction}$=0.0141) (FIGURE 1). A similar pattern of less methylation among the chrono-mismatched was observed for cg17724687. Compared to dayshift morning-types, dayshift evening-types and nightshift

morning-types had M-values that were 0.34 units lower and nightshift evening-types had M-values that were only 0.06 units lower ($p_{interaction}$=0.006) (FIGURE 2). No significant findings were observed in the effect modification analyses loci associated with non-circadian genes (TABLE 4).

Sleep duration effect modification analyses

Circadian loci

For the circadian loci, sleep duration was found to significantly modify differences in methylation associated with nightshift work at one locus in *PER1* (cg27004243) and one locus in *RORα* (cg22387253) (Table 5). For cg27004243, compared to dayshift workers that slept 38-313 minutes, dayshift workers that slept 314-377 minutes and 378-621 minutes had M-values that were 0.25 and 0.19 units lower, respectively. Among nightshift workers, M-values at this locus were 0.32 units lower among those that slept 38-313 minutes, 0.27 units lower among those that slept 314-377 minutes, and 0.27 units lower among those that slept 378-621 minutes ($p_{interaction}$ for shift by second vs. first tertile of sleep duration =0.001; $p_{interaction}$ for shift by third vs. first tertile of sleep duration =0.007). Likewise, for cg22387253, compared to dayshift workers that slept 38-313 minutes, dayshift workers that slept 314-377 minutes and 378-621 minutes had M-values that were 0.23 and 0.10 units lower, respectively. Among nightshift workers (compared to dayshift short sleepers), M-values at this locus were 0.21 units lower among those that slept 38-313 minutes, 0.25 units lower among those that slept 314-377 minutes, and 0.21 units lower among those that slept 378-621 minutes ($p_{interaction}$ for shift by second vs. first tertile of sleep duration=0.0366; $p_{interaction}$ for shift by third vs. first tertile of sleep duration=0.2888).

Non-circadian loci

For non-circadian loci, sleep duration was found to significantly modify a difference in

methylation associated with nightshift work at one locus in *LOXL1* (cg01349856) (Table 6). For cg01349856, compared to dayshift workers that slept 38-313 minutes, dayshift workers that slept 314-377 minutes and 378-621 minutes had M-values that were 0.34 and 0.28 units lower, respectively. Among nightshift workers (compared to dayshift short sleepers), M-values at this locus were 0.44 units lower among those that slept 38-313 minutes, 0.43 units lower among those that slept 314-377 minutes, and 0.49 units lower among those that slept 378-621 minutes ($p_{interaction}$ for shift by second vs. first tertile of sleep duration=0.0014; $p_{interaction}$ for shift by third vs. first tertile of sleep duration=0.0400).

Discussion

Chronotype effect modification analyses

Results from the chronotype effect modification analysis suggested that, compared to those individuals working in alignment with their chronotype (chrono-aligned), those individuals working out of alignment with their chronotype (chrono-mismatched) had less methylation at two CpG loci in *PER3* (cg08926642 and cg17724687). *PER*3 contains a variable number tandem repeat (VNTR) polymorphism and single nucleotide polymorphisms (SNPs) that have both been associated with chronotype (Dijk & Archer, 2010; Parsons et al., 2014), indicating that variation in *PER3* may partly underlie chronotype. Functionally, *Per3* has been observed to regulate the phase (timing) of the circadian system in mouse peripheral tissue (Pendergast, Niswender, & Yamazaki, 2012). Phase is related to chronotype in that morning and evening types have different phase tendencies—different preferences for the timing of their sleep-wake cycles; i.e., morning types tend to have an advanced phase compared to evening types.

In the main effects analysis referred to in Chapter 1, nightshift workers were less methylated than dayshift workers at both loci in *PER3*, and though the effects were not

statistically significant, the absolute differences in the effect sizes for the M-values between night and dayshift workers were among our largest top-five findings, indicating a potentially biologically meaningful difference in methylation. The chrono-mismatched individuals having less methylation than the chrono-aligned possibly indicates that dayshift evening-types and nightshift morning types are less able to physiologically tolerate the nightshift.

To understand the impact of methylation within the gene body of *PER3*, we used the UCSC Genome Browser, an interactive database for visualizing genomic features, such as chromatin states, transcription factor binding, repetitive elements, and enhancers (http://genome.ucsc.edu/). Specifically, we used the ENCODE/CpG Islands and Transcription Factor ChIP-seq tracks (GRCh37.p13) to explore characteristics of the genomic landscape harboring cg08926642 and cg17724687 (in GM12878 cell lines: B-lymphocytes from the CEPH/Utah, European population), which revealed that cg08926642 and cg17724687 are both located in the same CpG island in exon 18 in a region of high transcription factor occupancy (W. Kent et al., 2002). While there is typically a positive correlation between exonic methylation and transcription, high transcription factor occupancy indicates that this area of *PER3* may be regulatory, potentially acting as an intragenic enhancer, though it is not yet annotated as one. Transcription factor occupancy is a better predictor of enhancer status than histone modifications or chromatin accessibility (Dogan et al., 2015). As such, the hypomethylation among dayshift evening-types and nightshift morning-types may indicate upregulation either of *PER3* itself or the (putative) enhancer's distal target(s). Transcriptional changes at *PER3* have been observed in shift workers and in colon cancer tissue (Bracci et al., 2014; X. Wang et al., 2012), though, in the previously observed study with shift workers and in colon cancer tissue, expression was decreased in nightshift workers and in colon cancer tissue. Moreover, patients with lower *PER3*

expression have been observed to have shorter survival compared to those with higher *PER3* expression (Liu et al., 2014), and breast cancer patients (especially those with ER positive, luminal, or ERBB2-positive tumors) with low expression of *PER3* have been observed to have poor prognosis (Climent et al., 2010). As such, as mentioned in Chapter 1, should the hypomethylation we observed correlate with increased *PER3* expression, this might suggest an anti-cancer effect of the nightshift for the chrono-misaligned. Alternately, it might reflect the action of *PER3* in resetting the clock, given the function of the Period genes in clock downregulation. Because the clock controls cell cycle-related genes and other processes relevant to carcinogenesis (Matsuo et al., 2003), the differential perturbation on the clock warrants further investigation—namely, a prospective study of shift work integrating methylation, expression, and cancer outcomes to assess how this pattern of methylation in *PER3*'s gene body relates to both expression and cancer and whether it functions as a general marker of exposure to shift work among the chrono-misaligned.

Sleep duration effect modification analyses

While other measures such as sleep efficiency (ratio of total time sleep asleep to the total time in bed), sleep onset latency (the time it takes to fall asleep) and number of awakenings can be derived from the actigraphy data that we collected, we chose to focus on sleep duration as a measure of sleep quality given its wide use in previous epidemiologic studies (Gottlieb, ..., & Tiemeier, 2015; S. E. Jones et al., 2016; Liang et al., 2011; P. Wang et al., 2015).

Similar patterns of methylation differences were seen among the three loci (cg27004243 in *PER1*, cg22387253 in *RORα*, and cg01349856 in *LOXL1*) for which significant effect modification by sleep duration was observed: increased night sleep among dayshift workers was associated with reduced methylation and for nightshift workers, regardless of sleep length,

methylation was reduced compared to dayshift workers with short sleep durations.

PER1 may play a role in regulating the physiological effects of sleep disruption. In a mouse model of shift work, *Per1/2* deficient mice were protected from the physiological effects of sleep restriction on metabolic reprogramming, such as increased lipogenesis and increased secretion of adipokine leptin with food intake that were observed in *Per1/2* intact, shift-work disrupted mice (Husse, Hintze, Eichele, Lehnert, & Oster, 2012), suggesting that *PER1* is involved in mediating the metabolic effects of sleep disruption. Also in mice, *Per1/2* gene expression has been described as a molecular correlate of sleep need from studies in regions of the brain outside the SCN (Mistlberger, 2015). Thus, an increase in expression of *PER1* may reflect the need for sleep, and an increase in *PER1* expression might also mediate metabolic effects related to obesity, which is known to mediate and exacerbate the microenvironments of tumors and is likely also involved in the etiology of cancer (Sundaram, Johnson, & Makowski, 2013).

Use of the UCSC Genome Browser's ENCODE/CpG Islands, Transcription Factor ChIP-seq, Chromatin State Segmentation by HMM, and DNaseI Hypersensitivity Clusters tracks confirm that cg27004243 (located in the 5'UTR of *PER1*) is an active promoter in GM12878 cells, typically bound by a host of transcription factors and within a DNaseI hypersensitivity site, indicating open chromatin, accessible for transcription. Thus, decreased methylation (and possibly increased expression) of *PER1* in nightshift workers compared to dayshift short sleepers might reflect *PER1*'s response to the physiologic demands of the nightshift. Though, because increased sleep among dayshift workers also leads to less methylation, the impact of sleep duration on *PER1* requires future elucidation, as it appears that both nightshift work and increased sleep are associated with reduced *PER1* methylation. Nonetheless, *PER1* is a tumor

suppressor. While reduced levels of *PER1* have been reported in human cancer patients, overexpression of *PER1* has also been reported to sensitize human cancer cells to DNA damage (Gery et al., 2006). Thus, changes in *PER1* related to increased expression among nightshift workers possibly interferes with nightshift workers' abilities to handle DNA damage.

As reported in Chapter 1, cg22387253 in RORα is located within a DNA transposable element of the TcMar-Tigger class. DNA transposons are repeat elements that range from ~80-3,000 base pairs in length, span ~3% of the genome, and are thought to be immobile in humans (Solyom & Jr, 2012). Methylation of TcMar-Tigger elements has been shown to be environmentally modifiable in humans (Faulk et al., 2016). Moreover, hypomethylation of repeat elements may predispose towards aberrant chromosomal rearrangements or translocations (Wilson et al., 2007), and aberrant chromosomal events can occur, independent of mobile activity, via non-allelic homologous recombination (NAHR) between repeat sequences within repetitive elements (Rebollo et al., 2012; Shammas, 2011). While downregulation of RORα has been observed in a wide variety of tumors when compared to normal tissue (Cook et al., 2015; Kottorou et al., 2012), cg22387253 was hypomethylated in nightshift compared to dayshift workers, which might imply a susceptibility towards adverse NAHR events. Thus, for nightshift workers, regardless of sleep length, reduced methylation at cg22387253 compared to dayshift workers with short sleep durations might have negative consequences. However, it would also seem that increased night sleep among dayshift workers, also being associated with reduced methylation, also puts longer sleeping dayshift workers at risk.

As for *LOXL1*, cg01349856 in *LOXL1* is located in the first intron, predicted to be a poised promoter (a promoter bound by RNA polymerase II (Pol II) while the gene is not actively transcribed; genes with poised promoters are thought to be rapidly upregulated (Beisel & Paro,

2011)), associated with open chromatin, and at a DNaseI hypersensitivity site in GM12878 cells, indicating that the hypomethylation we observed likely indicates increased expression. As covered in Chapter 1, *LOXL1* is a copper-dependent enzyme that can modify proteins of the extracellular matrix (ECM) and whose primary function is ensuring the structural integrity of various tissues. *LOXL1* expression has been reported to be elevated in many human cancers (e.g., myeloproliferative neoplasms and prostate, colorectal, lung, pancreatic, and breast cancers). *LOXL1* expression correlates with levels of tissue hypoxia and impacts breast cancer metastasis. Because of that, whilst traditionally considered a tumor suppressor, *LOXL1* has been more recently appreciated for its role in tumor progression and metastasis (Tadmor et al., 2013; Wuest et al., 2015). As such, the hypomethylation we observed may reflect a tumor suppressor action of *LOXL1* in nightshift workers but may also mark shift workers as being at risk of cancer-related processes impacting metastasis through the non-cellular components of tumor microenvironments (i.e., the ECM and tissue hypoxia) (Wuest et al., 2015). More specifically, nightshift workers, regardless of sleep duration, and longer-sleeping dayshift workers might be at an increased risk for *LOXL1*-related increased expression.

A limitation of our findings is not having expression data to examine the correlation between methylation and expression. Instead, we relied on the UCSC Genome Browser to view how methylation correlates with expression in B-lymphocytes (GM12878 cells), only one of the leukocyte subsets from which DNA was extracted for our study. A more direct approach is needed in future studies to evaluate the biological impact of methylation among shift workers.

Our exploratory analysis of the modifying effects of chronotype and sleep duration reveals that methylation changes among shift workers are influenced by chronotype at two loci in *PER3* and that sleep duration likewise impacts the relationship between shift work and

methylation at multiple loci. Future studies should evaluate the modifying effects of chronotype on methylation in prospective studies of shift work with both expression data and cancer outcomes.

Analysis 2—Methylation Tables and Figures

TABLE 1. Demographic data for study participants [a]

	Dayshift *N* (%)	Nightshift *N* (%)
Gender		
Female	126 (82)	149 (48)
Male	27 (18)	159 (52)
Age		
22-28	32 (21)	106 (34)
29-34	41 (27)	68 (22)
35-39	45 (29)	53 (17)
>40	35 (23)	81 (26)
Body Mass Index $((lbs/in^2)*703)^{\#}$		
14-21	38 (25)	40 (13)
21-24	39 (25)	83 (27)
24-26	37 (24)	70 (23)
26-30	39 (25)	115 (38)
Race		
White	121 (79)	222 (72)
Other	32 (21)	86 (28)
Smoking		
Yes	9 (06)	17 (06)
No	144 (94)	291 (94)
Alcohol		
Yes	45 (29)	25 (08)
No	108 (71)	283 (92)

[a] Three individuals were excluded in the chronotype analyses due to not having completed all the questions for chronotype and thirty-one individuals were excluded from the sleep duration analysis due to not have actigraphy

[#]BMI categories based on quantiles among dayshift workers

TABLE 2. Average demographic characteristics of the 361 nightshift workers and the 30 nightshift workers missing data for the chronotype and sleep duration analyses

	361 subjects	**30 missing subjects**	**P-value***
Gender (% female)	0.48	0.53	0.7180
Mean Age	34.98	34.1	0.9318
Mean BMI	24.96	24.82	0.8527
Race (% White)	0.72	0.7	0.9769
Smoking (% smokers)	0.00	0.06	0.3773
Alcohol (% drank)	0.10	0.08	0.1661

*P-values for gender, smoking, and alcohol (categorical variables with small numbers) were obtained from the chi-squared computation based on the continuity of Yates; P-values for age and BMI were obtained with an independent two-group t-test

TABLE 3. Results from shift by chronotype effect modification analysis on methylation at pre-selected differentially methylated loci among circadian genes

Comparison	Gene (Region)	Difference in M-values*	P-value
Loci with the smallest p-values[a]			
cg09304381	*NR1D1* (Body)		0.9758
Dayshift Morning		Referent	
Dayshift Evening		0.04	
Nightshift Morning		0.12	
Nightshift Evening		0.16	
cg27004243	*PER1* (5'UTR)		0.0838
Dayshift Morning		Referent	
Dayshift Evening		-0.02	
Nightshift Morning		-0.13	
Nightshift Evening		-0.05	
cg02189597	*RORα* (Body)		0.6178
Dayshift Morning		Referent	
Dayshift Evening		0.06	
Nightshift Morning		0.08	
Nightshift Evening		0.18	
cg13286116	*BMAL1* (5'UTR)		0.3605
Dayshift Morning		Referent	
Dayshift Evening		-0.03	
Nightshift Morning		-0.15	
Nightshift Evening		-0.35	
cg22387253	*RORα* (Body)		0.2284
Dayshift Morning		Referent	
Dayshift Evening		0.06	
Nightshift Morning		-0.05	
Nightshift Evening		-0.06	
Loci with largest absolute effect sizes[b]			
cg13286116	*ARTNL* (5'UTR)		0.3605
Dayshift Morning		Referent	
Dayshift Evening		-0.03	
Nightshift Morning		-0.15	
Nightshift Evening		-0.35	
cg08926642	*PER3* (Body)		**0.0141**
Dayshift Morning		Referent	
Dayshift Evening		-0.31	
Nightshift Morning		-0.37	
Nightshift Evening		-0.05	
cg09304381	*NR1D1 (Body)*		0.9758
Dayshift Morning		Referent	
Dayshift Evening		0.04	

Nightshift Morning		0.12	
Nightshift Evening		0.16	
cg17724687	*PER3* (Body)		**0.0055**
Dayshift Morning		Referent	
Dayshift Evening		-0.34	
Nightshift Morning		-0.34	
Nightshift Evening		-0.06	
cg02189597	*RORα* (Body)		0.6178
Dayshift Morning		Referent	
Dayshift Evening		0.06	
Nightshift Morning		0.08	
Nightshift Evening		0.18	

*Analyzed using rank-regression and adjusted for the effects of age (continuous), gender, body mass index (continuous), race (White/Other), and cell composition; referent category is dayshift morning workers; for example, for cg09304381, dayshift evening workers had 0.04 more methylation than dayshift morning workers.

[a,b] These results were obtained from the genome-wide methylation study in Chapter 1.

FIGURE 1. Shift by chronotype effect modification on methylation at cg08926642 in *PER3*

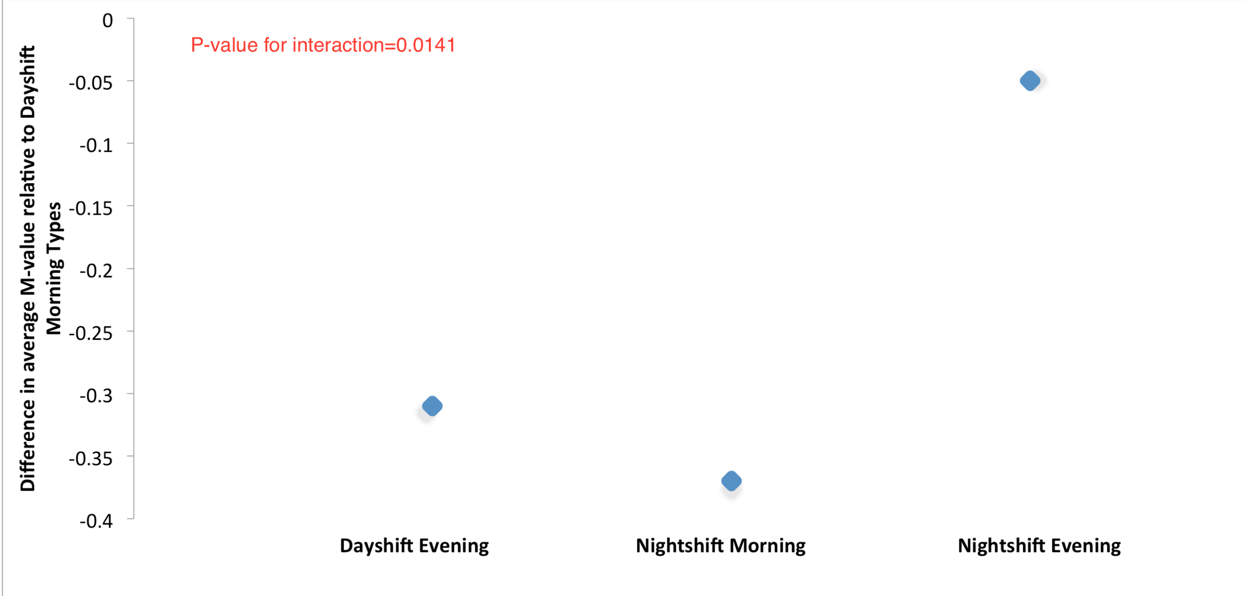


FIGURE 2. Shift by chronotype effect modification on methylation at cg17724687 in *PER3*

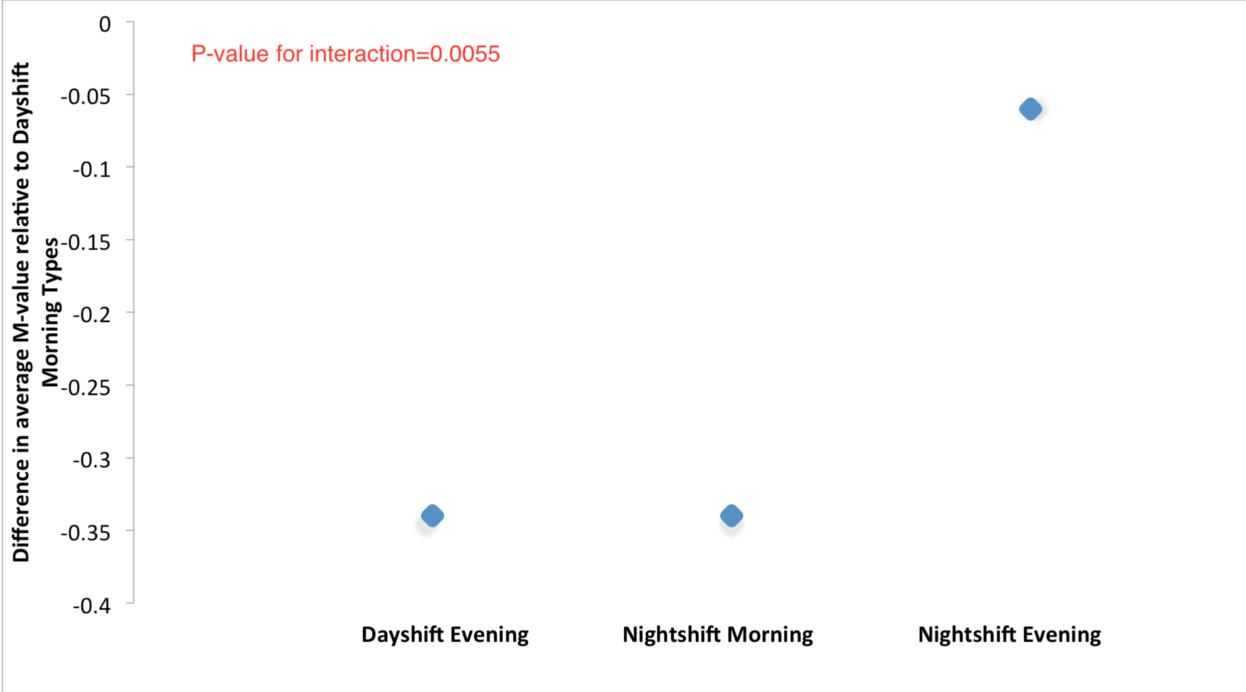


TABLE 4. Results from shift by chronotype effect modification analysis on methylation at pre-selected differentially methylated loci among non-circadian genes

Comparison	Gene (Region)	Difference in M-values*	P-value[#]
Loci with the smallest p-values[a]			
cg03227775	*CARD11* (Body)		0.6704
Dayshift Morning		Referent	
Dayshift Evening		-0.04	
Nightshift Morning		-0.24	
Nightshift Evening		-0.32	
cg18439144			0.2758
Dayshift Morning		Referent	
Dayshift Evening		0.05	
Nightshift Morning		-0.13	
Nightshift Evening		-0.15	
cg11480534	*ANNAK* (Body)		0.4153
Dayshift Morning		Referent	
Dayshift Evening		0.06	
Nightshift Morning		-0.13	
Nightshift Evening		-0.12	
cg01349856	*LOXL1* (Body)		0.0989
Dayshift Morning		Referent	
Dayshift Evening		0.11	
Nightshift Morning		-0.13	
Nightshift Evening		-0.14	
cg02436272	-		0.1919
Dayshift Morning		Referent	
Dayshift Evening		-0.05	
Nightshift Morning		-0.18	
Nightshift Evening		-0.14	
Loci with largest absolute effect sizes[b]			
cg26705599	-		0.9001
Dayshift Morning		Referent	
Dayshift Evening		0.01	
Nightshift Morning		0.66	
Nightshift Evening		0.63	
cg01808030	*RIBC2*; *SMC1B* (Body; TSS1500)		0.5065
Dayshift Morning		Referent	
Dayshift Evening		0.12	
Nightshift Morning		0.35	
Nightshift Evening		0.7	
cg00993903	*MARK3* (Body)		0.9031
Dayshift Morning		Referent	

Dayshift Evening		-0.02	
Nightshift Morning		0.45	
Nightshift Evening		0.46	
cg22274273	-		0.2139
Dayshift Morning		Referent	
Dayshift Evening		0.12	
Nightshift Morning		0.59	
Nightshift Evening		0.4	
cg04246708	*CNST* (Body)		0.1837
Dayshift Morning		Referent	
Dayshift Evening		0.83	
Nightshift Morning		-0.39	
Nightshift Evening		-0.31	

*Analyzed using rank-regression and adjusted for the effects of age (continuous), gender, body mass index (continuous), race (White/Other), and cell composition; referent category is dayshift morning workers; for example, for cg09304381, the M-value for dayshift evening workers was 0.04 units greater than dayshift morning workers, indicating that average methylation levels at this locus were higher in dayshift evening workers than dayshift morning workers.

[#]P-values <0.05 considered significant

[a,b] These results were obtained from the genome-wide methylation study in Chapter 1.

TABLE 5. Results from shift by sleep duration effect modification analysis on methylation at pre-selected differentially methylated loci among circadian genes

Comparison	Gene (Region)	Difference in M-values*	P-value[a]
Loci with the smallest p-value[b]			
cg09304381	*NR1D1* (Body)		0.3799/0.4275
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.06	
Dayshift sleep (378-621) minutes		-0.03	
Nightshift sleep (38-313) minutes		0.07	
Nightshift sleep (314-377) minutes		0.12	
Nightshift sleep (378-621) minutes		0.14	
cg27004243	*PER1* (5'UTR)		**0.0010/0.0070**[a]
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.25	
Dayshift sleep (378-621) minutes		-0.19	
Nightshift sleep (38-313) minutes		-0.32	
Nightshift sleep (314-377) minutes		-0.27	
Nightshift sleep (378-621) minutes		-0.27	
cg02189597	*RORα* (Body)		0.1446/0.1019
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.13	
Dayshift sleep (378-621) minutes		-0.09	
Nightshift sleep (38-313) minutes		-0.04	
Nightshift sleep (314-377) minutes		0.02	
Nightshift sleep (378-621) minutes		0.08	
cg13286116	*BMAL1* (5'UTR)		0.3955/0.6708
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.22	
Dayshift sleep (378-621) minutes		-0.15	
Nightshift sleep (38-313) minutes		-0.31	
Nightshift sleep (314-377) minutes		-0.28	
Nightshift sleep (378-621) minutes		-0.58	
cg22387253	*RORα* (Body)		**0.0366**/0.2888
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.23	
Dayshift sleep (378-621) minutes		-0.10	
Nightshift sleep (38-313) minutes		-0.21	
Nightshift sleep (314-377) minutes		-0.25	
Nightshift sleep (378-621) minutes		-0.21	
Loci with largest absolute effect sizes[c]			
cg13286116	*ARTNL* (5'UTR)		0.3955/0.6708
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.22	

Dayshift sleep (378-621) minutes		-0.15	
Nightshift sleep (38-313) minutes		-0.31	
Nightshift sleep (314-377) minutes		-0.28	
Nightshift sleep (378-621) minutes		-0.58	
cg08926642	*PER3* (Body)		0.5805/0.9453
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		0.14	
Dayshift sleep (378-621) minutes		0.04	
Nightshift sleep (38-313) minutes		-0.09	
Nightshift sleep (314-377) minutes		-0.17	
Nightshift sleep (378-621) minutes		-0.08	
cg09304381	*NR1D1* (Body)		0.3799/0.4275
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.06	
Dayshift sleep (378-621) minutes		-0.03	
Nightshift sleep (38-313) minutes		0.07	
Nightshift sleep (314-377) minutes		0.12	
Nightshift sleep (378-621) minutes		0.14	
cg17724687	*PER3* (Body)		0.3554/0.7233
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		0.25	
Dayshift sleep (378-621) minutes		0.07	
Nightshift sleep (38-313) minutes		0.03	
Nightshift sleep (314-377) minutes		-0.04	
Nightshift sleep (378-621) minutes		-0.02	
cg02189597	*RORα* (Body)		0.1446/0.1019
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.13	
Dayshift sleep (378-621) minutes		-0.09	
Nightshift sleep (38-313) minutes		-0.04	
Nightshift sleep (314-377) minutes		0.02	
Nightshift sleep (378-621) minutes		0.08	

*Analyzed using rank-regression and adjusted for the effects of age (continuous), gender, body mass index (continuous), race (White/Other), and cell composition; referent category is dayshift short sleep; for example, for cg09304381, dayshift medium sleep workers had 0.06 less methylation than dayshift short sleep workers.

[a] P-values correspond to the 314-377/378-621 minute categories compared to the 38-313 minutes (shortest sleep) category; p-values considered significant if <0.05.

[b,c] These results were obtained from the genome-wide methylation study in Chapter 1.

FIGURE 3. Shift by sleep duration effect modification on methylation at cg27004243 in *PER1*

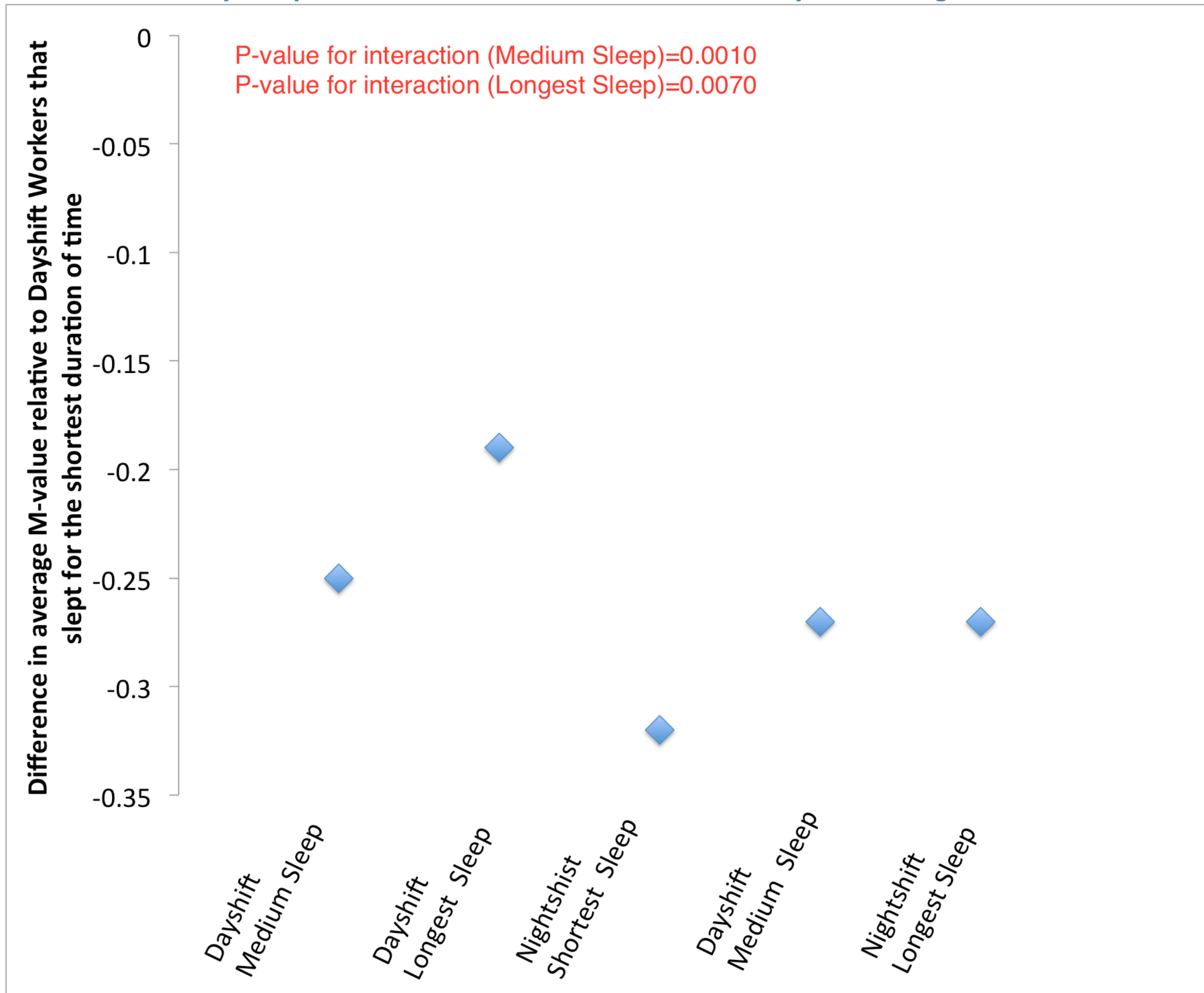


FIGURE 4. Shift by sleep duration effect modification on methylation at cg22387253 in *RORα*

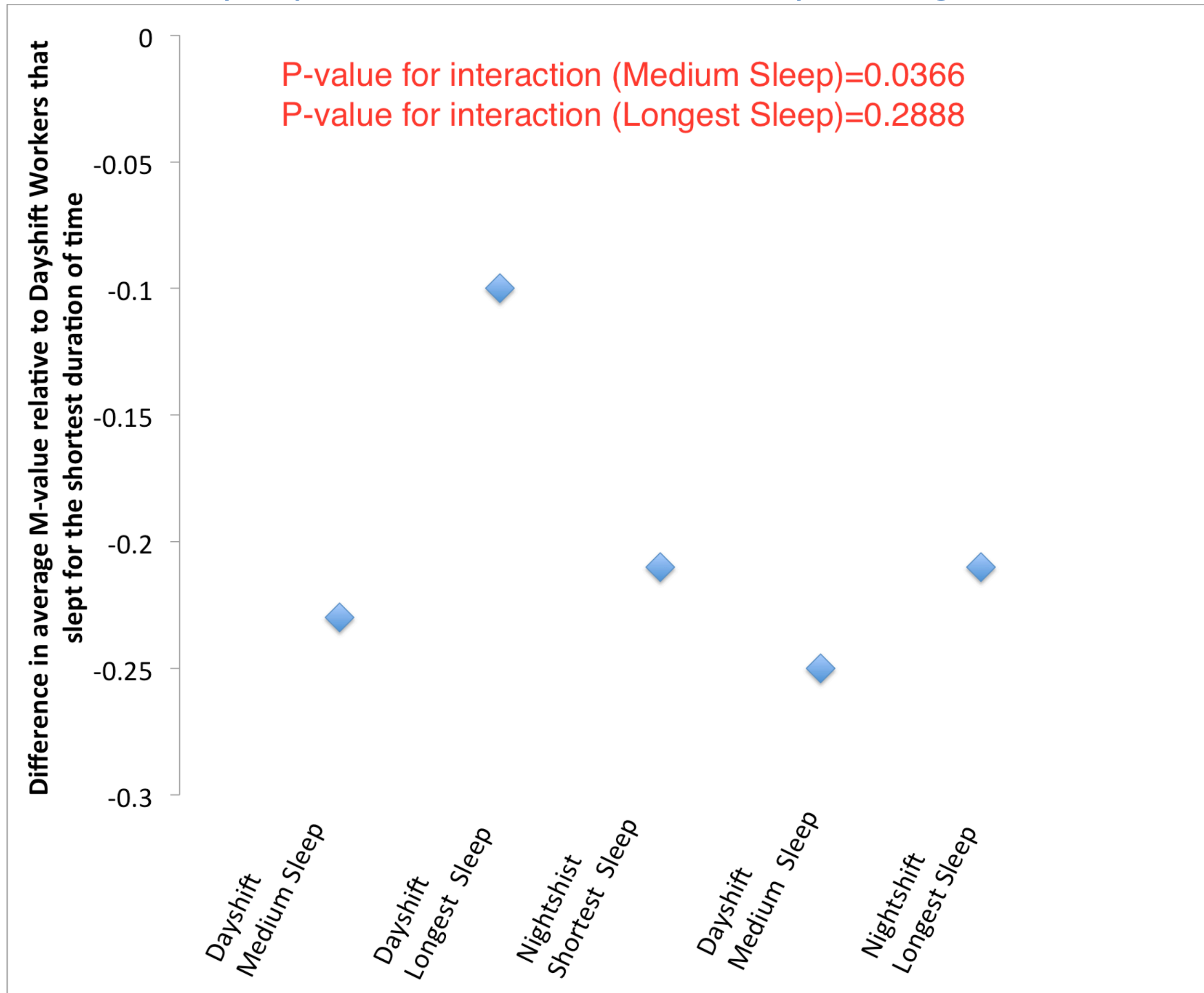


TABLE 6. Results from shift by sleep duration effect modification analysis on methylation at pre-selected differentially methylated loci among non-circadian genes

Comparison	Gene (Region)	Difference in M-values*	P-value[a]
Loci with the smallest p-values[b]			
cg03227775	*CARD11* (Body)		0.4450/0.6223
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.22	
Dayshift sleep (378-621) minutes		-0.09	
Nightshift sleep (38-313) minutes		-0.37	
Nightshift sleep (314-377) minutes		-0.47	
Nightshift sleep (378-621) minutes		-0.38	
cg18439144	-		0.7567/0.3644
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		0.03	
Dayshift sleep (378-621) minutes		0.06	
Nightshift sleep (38-313) minutes		-0.12	
Nightshift sleep (314-377) minutes		-0.06	
Nightshift sleep (378-621) minutes		-0.15	
cg11480534	*ANNAK* (Body)		0.9897/0.8459
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.05	
Dayshift sleep (378-621) minutes		-0.08	
Nightshift sleep (38-313) minutes		-0.16	
Nightshift sleep (314-377) minutes		-0.21	
Nightshift sleep (378-621) minutes		-0.1	
cg01349856	*LOXL1* (Body)		**0.0014/0.0400**[a]
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.34	
Dayshift sleep (378-621) minutes		-0.28	
Nightshift sleep (38-313) minutes		-0.44	
Nightshift sleep (314-377) minutes		-0.43	
Nightshift sleep (378-621) minutes		-0.49	
cg02436272	-		0.4035/0.6295
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.05	
Dayshift sleep (378-621) minutes		-0.04	
Nightshift sleep (38-313) minutes		-0.20	
Nightshift sleep (314-377) minutes		-0.16	
Nightshift sleep (378-621) minutes		-0.19	
Loci with largest absolute effect sizes[c]			
cg26705599	-		0.7796/0.6142
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		0.12	
Dayshift sleep (378-621) minutes		0.28	

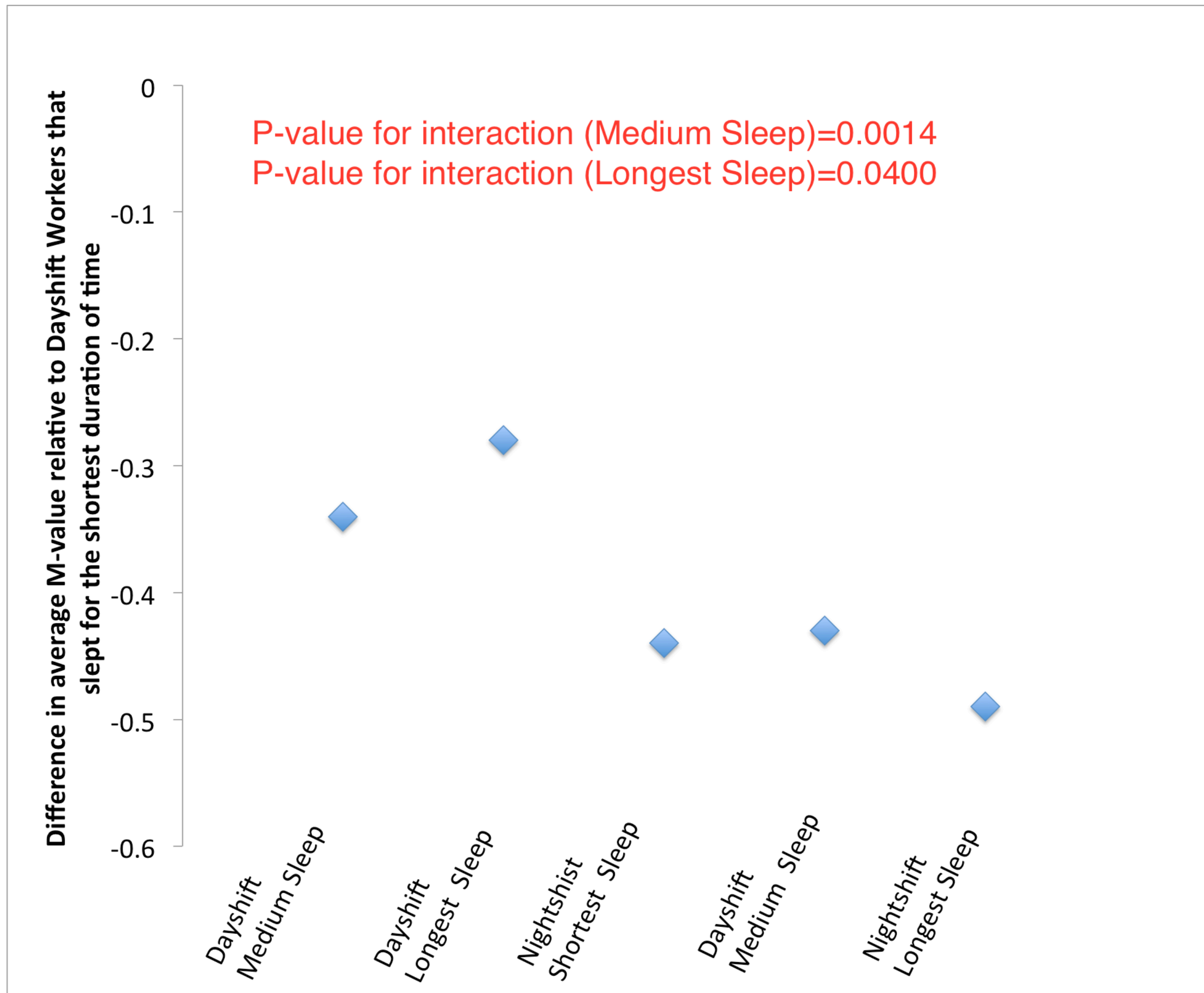
Nightshift sleep (38-313) minutes		0.82	
Nightshift sleep (314-377) minutes		0.81	
Nightshift sleep (378-621) minutes		0.87	
cg01808030	*RIBC2;SMC1B* (Body; TS1500)		0.2464/0.1383
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		-0.59	
Dayshift sleep (378-621) minutes		-0.53	
Nightshift sleep (38-313) minutes		-0.03	
Nightshift sleep (314-377) minutes		-0.01	
Nightshift sleep (378-621) minutes		0.22	
cg00993903	*MARK3* (Body)		0.8092/0.2304
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		0.23	
Dayshift sleep (378-621) minutes		0.09	
Nightshift sleep (38-313) minutes		0.63	
Nightshift sleep (314-377) minutes		0.76	
Nightshift sleep (378-621) minutes		0.24	
cg22274273	-		0.9433/0.4420
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		0.07	
Dayshift sleep (378-621) minutes		0.05	
Nightshift sleep (38-313) minutes		0.33	
Nightshift sleep (314-377) minutes		0.43	
Nightshift sleep (378-621) minutes		0.68	
cg02189597	*CNST* (Body)		0.0815/0.3430
Dayshift sleep (38-313) minutes		Referent	
Dayshift sleep (314-377) minutes		0.07	
Dayshift sleep (378-621) minutes		0.06	
Nightshift sleep (38-313) minutes		0.01	
Nightshift sleep (314-377) minutes		-1.48	
Nightshift sleep (378-621) minutes		-0.78	

*Analyzed using rank-regression and adjusted for the effects of age (continuous), gender, body mass index (continuous), race (White/Other), and cell composition; referent category is dayshift short sleep; for example, for cg03227775, dayshift medium sleep workers had 0.22 less methylation than dayshift short sleep workers.

[a] P-values correspond to the 314-377/378-621 minute categories compared to the 38-313 minutes (shortest sleep) category; p-values considered significant if <0.05.

[b,c] These results were obtained from the genome-wide methylation study in Chapter 1.

FIGURE 5. Shift by sleep duration effect modification on methylation at cg01349856 in *LOXL1*



Part B—Policy

Title:

Use of the precautionary principle to motivate shared responsibility for shift work

Authors:

Charleen Adams[1], Erika Blacksher[2], Wylie Burke[2]

[1]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA.

[2]Department of Bioethics and Humanities, University of Washington School of Medicine, Seattle, WA, USA.

Introduction

Shift work (working outside of 6 am to 6 pm) is a feature of our 24-hour economy. According to a Bureau of Labor Statistics survey, approximately 18% of workers in the United States engage in shift work, many overnight (McMenamin, 2007). However, this estimate is unlikely to capture information from those who do shift work as part of unreported employment—an important consideration. Shift work is disproportionately done by the poor and by minorities (Saenz, 2008), those most likely to be paid anonymously. Shift work, in this way, represents a highly prevalent economic and occupational social disparity.

Increasingly, evidence is accruing that shift work is associated with a broad array of adverse health effects, including cardiovascular disease, obesity, metabolic syndrome, type 2 diabetes, and cancer (Gu et al., 2014; Lin et al., 2015; Pan, Schernhammer, Sun, & Hu, 2011). In 2007, a panel of experts at the International Agency for Research on Cancer (IARC) classified shift work involving circadian disruption—the failure to coordinate biological rhythms with the daily light-dark cycle—as a *probable* (2A) carcinogen based on a review of the limited evidence from eight epidemiologic studies of shift work and breast cancer, and compelling evidence from

experimental studies of circadian-disrupting exposures in animal models, for which 80% demonstrated a link between circadian disruption and tumor incidence and growth (International Agency for Research on Cancer, 2007). This body of evidence led to calls for more epidemiologic research to better understand which subgroups of shift workers might be most at risk.

Although scientific uncertainties remain, in the decade since IARC's review, many studies have been done that further strengthen the evidence that shift work is carcinogenic, showing that the risk of cancer increases with years of shift work and that shift work is implicated in cancer sites other than breast. Additionally, the population-attributable fraction (PAF) of breast cancer due to shift work has been estimated. Should shift work be causal, its PAF in the U.S. (during 2010) was 5.7%, amounting to approximately 11,777 cases of breast cancer due to shift work in the U.S. in 2010, alone (Vladar, Lee, Stearns, & Axelrod, 2015b). This, together with the increased risks for cardiometabolic diseases and mortality observed among shift workers (Gu et al., 2014; Lin et al., 2015; Pan et al., 2011) make shift work a pressing public health concern.

In this commentary, we argue that the harms of shift work justify a precautionary approach. We draw on the precautionary principle to frame our assessment of the harms of shift work and of ways to advance responsible measures to minimize and mitigate its harms. The precautionary principle calls for proactive policies and practices to avoid harm to human health under conditions of scientific uncertainty. In the last several decades, the precautionary principle has found traction as a framework for responding to environmental and industrial health risks in the United States and abroad. It is also increasingly being recognized as an apt ethical framework for a public health focused on social determinants and the identification and elucidation of the

causes of population patterns of illness and disease, particularly in vulnerable subgroups (Jordan & O'Riordan, 2004; D. Kriebel & Tickner, 2001; Pearce, 2004; Rosner & Markowitz, 2002). The considerable complexity that characterizes population health science, the uncertainty that often accompanies findings, and calls for measures that act on upstream causes of poor health and health disparities fit the principle's orientation toward policies that anticipate and forestall harm and the principle's commitments to shared responsibility and democratic decision-making. The principle's call for inclusive and transparent processes of public deliberation and decision-making is particularly salient when addressing the health-harming effects of social determinants, such as shift work, which put disadvantaged subgroups at heightened risk of illness and disease (Saenz, 2008). For this reason, the principle empowers public health to act from a stance of social justice, casting shift work within the domain of health disparities deserving action despite scientific uncertainty.

In what follows, we describe the precautionary principle, and, utilizing what is known of the state of science around shift work and cancer, argue that shift work's potential harms represent a public health threat serious enough to warrant a precautionary stance. We interpret the principle's requirement to "shift the burden of proof to the proponents of an activity," one of its more controversial elements, as a requirement that shift work-related research and decision-making be shared by a broad coalition of parties with a stake in shift work. In a very preliminary way, we sketch what this shared responsibility might entail and who it might include, as well as some of the challenges this approach might face, particularly in the US.

The Precautionary Principle

The idea that precautionary measures should be taken to protect people from harm is an

old idea. It can be traced to the ethical principle in medicine to "first, do no harm" and to the notion of primary prevention in public health (Moure-Eraso, 2002). But scholars trace contemporary usage of the "precautionary principle" to German environmental policy in the 1970s and to the German word *Vorsorgeprinzip*, or the "forecaring" or "foresight" principle (David Kriebel et al., 2005). The principle represents a fundamental shift in policy making, from a stance of reaction to proven hazards toward a stance of anticipatory action to forestall potential serious harm (p. 3 EU). This approach to assessing and regulating potential threats to human and ecosystem health challenges basic tenets of the dominant paradigm of risk assessment, including the assumption that products and activities are safe until proven dangerous, the hegemony of professional science in science policy, and the privileging of private profit over public health and social goods (Mayer, Brown, & Linder, 2002).

For these and other reasons, the precautionary principle is the subject of considerable controversy. Nonetheless, it has in the last three decades anchored numerous national and international agreements and policy statements on the environment, health, and sustainable development, and is embodied in the European Union's system of laws (Jordan & O'Riordan, 2004). Over the years, the principle has been variously defined and elaborated, representing weaker and stronger formulations (Jordan & O'Riordan, 2004). The 1992 Rio Declaration on Environment and Development defines the principle this way:

> In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation (The United Nations Environment Program, 1992).

Another oft-cited definition of the principle comes from the Wingspread conference held at the University of Wisconsin in 1998, which asserts that when activities raise the potential for serious harm to human health, precautionary measures should be taken even in the absence of complete scientific knowledge of cause and effect (Science & Environmental Health Network, 1998). The European Union advances a more elaborated definition:

> When human activities may lead to morally unacceptable harm that is scientifically plausible but uncertain, actions shall be taken to avoid or diminish that harm. Morally unacceptable harm refers to harm to humans or the environment that is (1) threatening to human life or health, or (2) serious and effectively irreversible, or (3) inequitable to present or future generations, or (4) imposed without adequate consideration of the human rights of those affected (Montague, 2005, p.14).

These definitions, while not necessarily commensurate, all capture two interrelated realities of decision-making in health. The first relates to the complex and often uncertain nature of findings in health sciences and, more fundamentally, to the limits of scientific inquiry; the second, to the serious, sometimes global and irreversible, nature of threats to human health (Martuzzi & Tickner, 2004). These realities, in turn, point to the normative and political dimensions of science and health policy making. Determinations of what constitutes sufficient proof and of what constitutes serious harm are value-laden judgments that involve a plurality of values, including those that relate to what constitutes "sound science" and to fundamental aspects of human life, such as protection of human health and advancement of and aspiration for improved living standards now and into the future (Schettler & Raffensperger, 2004; Stirling & Gee, 2002). Recognizing the likelihood of conflict among such values and need for tradeoffs, the precautionary principle calls for an inclusive and transparent decision-making process based on

what is and is not known in the realm of science and informed by social values and public priorities. Policy-making entails social values and normative judgments that are not the sole province of experts. This is particularly true in areas of social policy and practice that involve considerable scientific uncertainty, impose potentially serious health risks, and implicate public goods and burdens, as is the case with shift work. Not only do members of the public with no experience with shift work or expertise in shift work science bring diverse perspectives and values to the discussion, they may see problems and solutions overlooked by experts and be better able to accommodate uncertainty (D. Kriebel & Tickner, 2001). Moreover, when done well, public deliberation can yield more informed, considered, civic-minded, and egalitarian discussions (Abelson et al., 2003; Carpini, Cook, & Jacobs, 2004; Davies, 2011; Gastil, Bacci, & Dollinger, 2010; Goold, Biddle, Klipp, Hall, & Danis, 2005). For these reasons, a more inclusive discussion about the harms of shift work and potential alternatives or recommendations for practices and policies may lead to better and more equitable decisions and, in turn, enhance the legitimacy of decisions and build public trust in the public and private sector. As such, the democratic foundation of the principle embodies a "certain humility about scientific knowledge and an acknowledgement of the complexity and variability of the real world" (Stirling and Gee 2002, p. 526). We turn next to discuss what is known and unknown about the potential harms of shift work and cancer risk.

Shift Work and Scientific Uncertainty

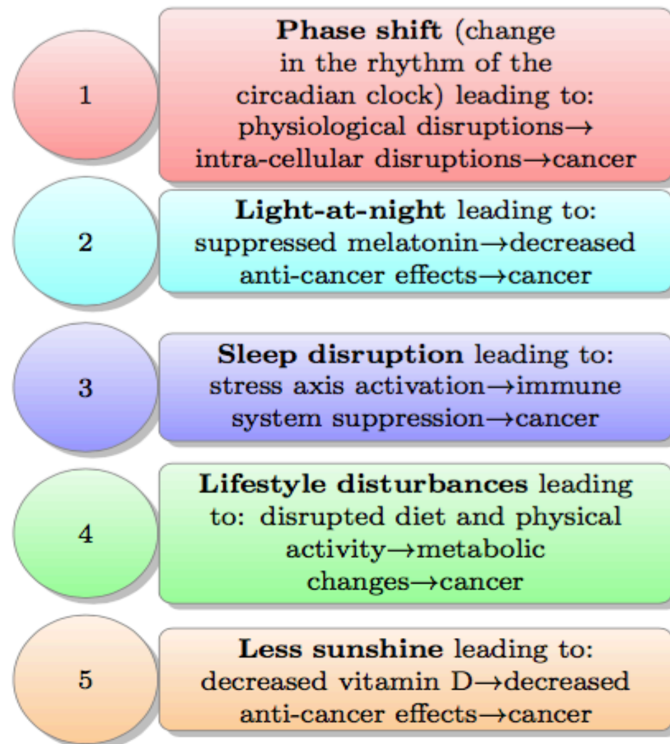
What we know about shift work is that millions of people engage in it, that it is associated with a modest risk for cancer in multiple cancer sites, and that the risk of cancer increases with years of shift work. Additionally, those most likely to do shift work are from minority and lower socioeconomic groups (Saenz, 2008), which are, in general, exposed to more

health risks and experience a greater burden of injury, illness, and disease (Braveman, Cubbin, Egerter, Williams, & Pamuk, 2010; Braveman, Egerter, & Mockenhaupt, 2011; Final Report of the Commission on Social Determinants of Health. Geneva, World Health Organization., 2008; World Health Organization, 2003). What follows is a description of what is unknown about shift work and cancer.

Molecular Mechanisms

To start, the molecular mechanism linking shift work to cancer remains unknown. At least five pathways have been proposed to conceptualize the mechanisms through which shiftwork may cause cancer (Fritschi et al., 2011; Sancar et al., 2010) (FIGURE 1, adapted from Fritschi et al., 2011).

FIGURE 1. Proposed pathways linking shift work and cancer



IARC's review of 56 studies of circadian-disrupting exposures on tumor incidence and growth in animal models was mostly focused on exposures affecting pathways 1 (Phase shift) and 2 (Light-at-night) of Fritschi's conceptual framework (International Agency for Research on Cancer, 2007). Pathways 1 and 2 converge in their impact to DNA damage: the molecular circadian clock (or simply "the clock"), which is explained in greater detail in the paragraphs that follow, is responsible for gating the DNA damage response and is disturbed (phase shifted) by shift work (Sancar et al., 2010); and light-at-night suppresses melatonin, a hormonal output of the clock which has anti-carcinogenic properties, some mediated through the DNA damage response (Santoro et al., 2013). Fundamentally, both pathways 1 and 2 derive from the exposure of light-at-night, as it is light-at-night that signals the phase shift, the change in the timing of the signals from the brain that potentially dysregulate the clocks in peripheral tissues—those outside of the superchiasmatic nucleus (SCN), in other locations in the brain and in other parts of the body—and also signals a change in the primary output of the pineal gland, melatonin.

"Clocks"

The molecular circadian clock is a transcriptional-translational feedback loop within each nucleus-containing cell in the body. It is composed of a set core of transcription factors (CLOCK, BMAL1, PER1-3, and CRY1-2), whose interactions and activity produce a near 24-hour (endogenous) rhythm—one that continues its activity even in the absence of signals from the outside world—and whose function is to self-regulate their own daily rhythms as well as the daily rhythms of the genes they control, leading to daily outputs in metabolism, hormone production, energy balance, and cellular homeostasis—our circadian rhythms. This is accomplished by CLOCK and BMAL1 binding together and acting as transcription factors for *PER* and *CRY*, which themselves bind together and down regulate *CLOCK* and *BMAL1*.

Likewise, CLOCK:BMAL1 act as transcription factors for 3-30% of genes throughout the genome, depending on the tissue (Aguilar-Arnal, Sassone-Corsi, & Pfaff, 2015).

While the molecular circadian clock is endogenous, the external light/dark signal permits the clocks in the SCN to coordinate their timing with that of the outside world and to use their coordinated timing as a signal for the rest of the body. Absent this coordination, clocks endogenously free run about 24 hours, but the signals from the SCN (and the pineal output of melatonin) get the clocks in the rest of the body to synch up. Thus, a disturbance in the timing of light (such as by light-at-night or traveling across time zones) can lead to phase shifting that interferes with the timing of the clock's signals to peripheral tissues. This is largely what is meant by the term chronodisruption or circadian disruption. We experience this as jet lag and malaise, but at the molecular level, this means that the clocks in various tissues have changed their own timing and the timing of the genes they control. For instance, the *XPA* gene, involved in nucleotide excision repair, is under clock control. Some of these clock-controlled genes are involved in the cell cycle and the DNA damage response (Sancar et al., 2010). The DNA damage response helps maintain cellular and genetic stability and is necessary to stave off carcinogenesis (Negrini, Gorgoulis, & Halazonetis, 2010). Thus, changing the timing of light impacts circadian genetics throughout the body, potentially impacting pathways related to cancer. Eighty percent of the circadian-disrupting animal studies IARC reviewed had significant findings—contributing to IARC's interpretation of the total evidence that shift work is probably carcinogenic—but the exact underlying mechanism, though implicating DNA damage, has yet to be elucidated.

Epidemiologic Studies

The evidence in humans for the carcinogenicity of shift work is suggestive but

incomplete. Six of the eight shift work and breast cancer studies IARC examined in 2007 demonstrated a statistically significant (albeit modest) increase in the risk for breast cancer (see TABLE 1), mostly among long-term shift workers. A meta-analysis of these studies confirmed the increased risk of breast cancer (Relative Risk (RR)=1.51, 95% CI: 1.36–1.68) (Megdal, Kroenke, Laden, Pukkala, & Schernhammer, 2005). However, the specific aspects of shift work that contributed to cancer were un-discernable in part because of the variable definitions of shift work used in these studies. Specifically, in the two Nurses' Health Studies (NHS), nurses who worked full-time at night were included in the unexposed population on the hypothesis that permanent night shift work was comparable to dayshift work or less disruptive than rotating night and day shifts (Schernhammer, E.S., Laden, F., Speizer, F. E., Willett, W.C., Hunter, D., J., Kawachi, I., Colditz, 2001; Schernhammer, Kroenke, Laden, & Hankinson, 2006). But the classification of rotating shift work as three or more nights per month plus day and evening shifts was arbitrary. By contrast, Davis *et al*. (2001) defined shift work as the graveyard shift (beginning work after 19:00 and leaving work before 09:00 on a permanent basis without rotating to days) and analyzed ever/never having worked a graveyard shift, the number of years working the graveyard shift at least once a week, and the average hours per week on the graveyard shift over a 10-year period (Davis, Mirick, & Stevens, 2001). Omitting shift system altogether, Tynes *et al*. (1996) viewed shift work as "work at night with exposure to artificial light" without reference to rotating or permanent shift classification (Tynes, Hannevik, Andersen, Vistnes, & Haldorsen, 1996). For O'Leary *et al*. (2006), shift work was any evening or overnight shift, where "evening" could have started in the afternoon, ending as late as 2:00 a.m. and "overnight" could have started as early as 7:00 p.m., ending anytime the following morning (O'Leary et al., 2006). The Hansen (2001), Lie *et al*. (2006), and Schwartzbaum *et al*.

(2007) studies used job-exposure matrices to classify individuals as shift workers. Those individuals working occupations in which a pre-defined percentage of respondents (e.g. 60%) indicated engaging in shift work were classified as shift workers. As such, what counted as circadian-disrupting shift work varied considerably between the studies available to IARC.

Since 2007, there have been approximately 12 new studies of shift work and breast cancer and five meta-analyses (Ijaz et al., 2013; Jia et al., 2013; Kamdar, Tergas, Mateen, Bhayani, & Oh, 2013; Lin et al., 2015; F. Wang et al., 2013). Their overall picture is similar to the studies before 2007 with the additional strength that some revealed a dose-effect: the risk of cancer increases with years of shift working. In addition, two meta-analyses of the association of shift work and cancer at sites other than breast have been performed—one for prostate and one for colorectal cancer—also revealing modest risks and dose effects (Rao et al., 2015; F. Wang et al., 2015). Generally, small risks are observed for breast, prostate, and colorectal cancers, and the risks increase with years of shift working.

Despite the growing evidence of shift work's carcinogenic effects, the data are insufficient to assess whether the effect of shift work on cancer varies by individual characteristics, such as chronotype (innate preference for engaging in activity earlier or later in the day) and sleep quality, factors that could steer prevention guidelines. Likewise, few studies have been performed in non-European populations, leaving unanswered questions about possible racial/ethnic differences (International Agency for Research on Cancer, 2007). Similarly, epidemiological studies have not been able to consistently capture which aspects of shift work potentially most dispose towards cancer. As such, more studies of shift work and cancer are needed to capture domains of shift schedules more precisely. To that end, in concert with recommendations by Stevens *et al*. (2011), future studies still need to better capture detailed shift

work data such as shift system (e.g. rotating, permanent), years on a particular non-day shift schedule, and (3) shift intensity (frequency of shift working; days off between shifts) (Stevens et al., 2011). Additionally, most studies of shift work have been unable to examine potentially mitigating social factors, such as the social conditions that allow for undisrupted sleep when not working or access to various services, such as gyms and child care, factors that could affect shift worker's abilities to cope with the demands of working at night.

Justification of a Precautionary Stance

On balance, we think the evidence suggests that the harms associated with shift work represent a pressing public health concern that warrants a precautionary stance. Three features of shift work support our position. First, as already described, ~18% of the wage and salary workers in the United States or about 21 million people engage in shift work (McMenamin, 2007), and its effects represent serious and sometimes irreversible threats to health. In addition to cancer, around which this report is focused, shift workers are at an increased risk for common chronic morbidity, such as cardiovascular events (Vyas et al., 2012) and type 2 diabetes (Pan et al., 2011). Moreover, the acute risks of sleep deprivation put those who work the nightshift at an increased risk for accidents: 32-36% of shift workers fall asleep on the job at least once a week; the risk of occupational accidents is 60% higher for shift workers compared to those who work during the day (S. M. W. Rajaratnam et al., 2013). Together these shift-work related health risks constitute a serious set of maladies to which a large fraction of the work force is exposed.

Second, some forms of shift work, representing essential public safety functions in law enforcement, health care, and some public utilities (e.g., air traffic control), cannot be eliminated. Other less essential forms of shift work may be considered so integral to social goals in modern

24-hour economies that they might be difficult to eliminate, such as 24-hour food stores. They might make major contributions to the economy or modern conveniences and thus be highly valued by some segments of the public at large and by some segments of shift workers. Thus, shift work's impacts, both its potential burdens and benefits, are broad, affecting many types of services and segments of society.

Third, the health risks of shift work fall disproportionately on members of minority and low socioeconomic groups, who are exposed to an array of additional health risks and who experience a disproportionate incidence of preventable morbidity and premature death. These groups may be exposed to health risks associated with, for example, resource-poor neighborhoods that have high levels of pollution and toxins and of violence and crime, institutional and interpersonal discrimination, and inadequate health care, all of which put them at heightened risks of poor health (Marmot, 2005; Waitzman & Smith, 1998). Additionally, these groups may have few employment options beyond shift work due to low educational attainment, language barriers, and discriminatory employment practices. These considerations suggest that these groups may be particularly vulnerable to the effects of shift work and also raise concerns about social justice. Although public health and precautionary decision-making are often guided by utilitarian aims to maximize the good of the population at large, concerns about a fair distribution of burdens and benefits are also important in these analyses (Brock, 2000; Comba, Martuzzi, & Botti, 2004; Powers & Faden, 2006). Taken together, these considerations—the broad impact of potential harms, the inability to eliminate all shift work, and the disproportionate impact on socially disadvantaged groups—provide considerable support for making a fundamental shift in how to think about responsibility for shift work-related research and decision-making.

Shared Responsibility for Research and Decision-Making Through Collaboration

Perhaps the most contentious aspect of the precautionary principle is the requirement to shift the burden of proof to proponents of the activity in question (Pearce, 2004). What a 'shift in burden of proof' entails, however, has been variously interpreted. Some commentators suggest it means that an activity's proponents must establish that "an activity is safe rather than for its opponents to prove that it is unsafe," an interpretation that leaves the principle vulnerable to criticisms that it would stifle innovation and waste resources (David Kriebel et al., 2005; Pearce, 2004). Others suggest that a shift in burden of proof means not that the activity must cease until proven safe, which may be impossible, but rather requires that proponents of the activity take on obligations such as testing and monitoring potential harms, publicly disclosing information about potential harms, and making restorations for damage done (Schettler & Raffensperger, 2004).

We find the latter interpretation compelling in the context of shift work. Recasting this requirement as a set of responsibilities for ongoing research, public engagement, and assurances to those harmed recognizes broad moral and social dimensions of shift work, already described. For these reasons, in the case of shift work we interpret the precautionary principle's requirement to "shift the burden of proof to the proponents of an activity" as a *shared responsibility* to press for more and better research about its potential harms and for inclusive deliberation and transparent decision-making about alternatives and measures to mitigate and minimize harms. We envision a process of shared responsibility for shift work that entails the following phases: convening a research consortium, gathering evidence, convening stakeholders for deliberation, and using the evidence and deliberative output to inform policy recommendations and practice guidelines (FIGURE 2).

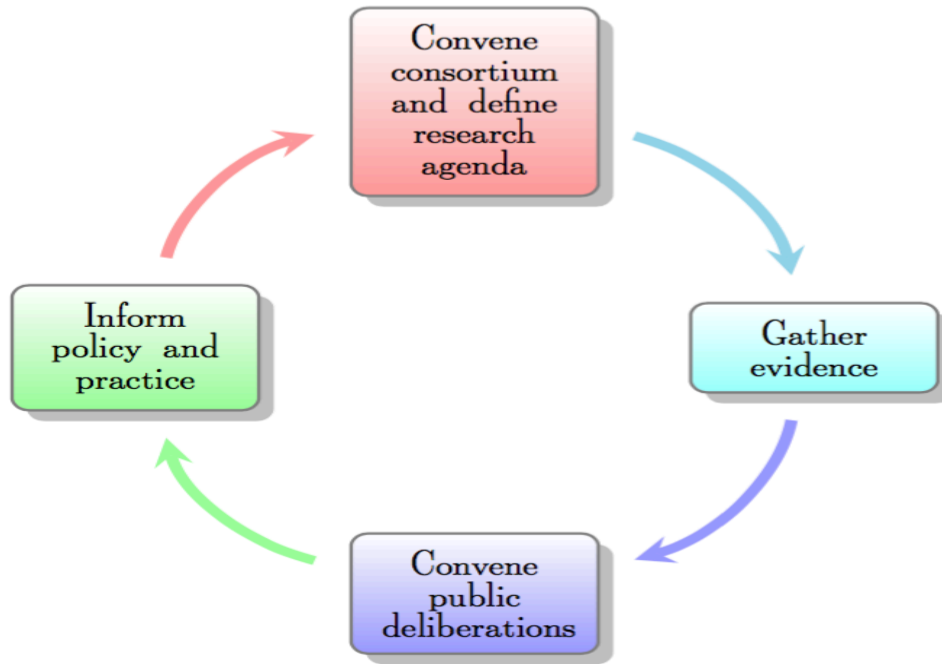


FIGURE 2. Shared responsibility for shift work

Research consortium

Specifically, we would propose the formation of a broad-based consortium comprised of diverse stakeholders to take up a long-term research and action agenda to increase the safety of shift work. This effort would include investing in research that contributes to knowledge about potential harms, harmonizing definitions of shift work so that data are comparable across settings, publicly disclosing research results, ensuring that shift workers have access to good healthcare, and convening various publics to weigh the evidence and deliberate alternatives and options to mitigate harms. The National Institute for Occupational Safety and Health (NIOSH), the agency within Centers for Disease Control and Prevention (CDC) that has responsibility for assuring research and making recommendations for worker safety, would be an ideal body to convene this consortium. NIOSH could then oversee the research process and function as a hub for public deliberation. The knowledge gained from this process could be used to inform an

Occupational Safety and Health Administration (OSHA) standard to regulate the nightshift, which currently does not exist.

Our conception of those with a stake in shift work is broad. It includes people who represent public and commercial sectors that employ shift workers, researchers who study the health effects of shift work, government agencies who set safety standards for occupations and public health, and shift workers themselves. Given that some forms of shift work represent essential public health safety functions (e.g., law enforcement and air traffic control), may be highly valued by the public (e.g., 24-hour access to retail, services, and technology), and that its costs may be borne in some ways by the public (e.g., in lost productivity and health care costs), members of public also have a stake in shift work.

Shift workers should have a special role in the consortium, acting as experts in their own right. There should be ample and regular opportunities for workers to share their insights into the real world conditions of shift work and to discuss their observations about its effects. Shift workers may be the first to recognize its harmful effects and may have suggestions for mitigating its harms that are missed by policy makers and scientists (Stirling & Gee, 2002). In addition, the consortium should foster dialogue among employers and others with the power to effect changes to shift worker schedules about barriers to and opportunities for modifying schedules, as well as for brainstorming realistic avenues for risk assessment on the job in different types of occupational settings.

Pairing the voices of the workers and employers with those of scientists, policy makers, and others will enable the consortium to surface a research agenda that identifies the types of research projects that are needed to uncover potential alternatives, policies, and best practices for

shift work. For instance, chronotype is a factor that may impact how workers cope with circadian disruption, and it has been hypothesized that people who work a schedule out-of-synch with their chronotype (for example: people with an evening chronotype who do dayshift work and people with a morning chronotype who do nightshift work) may be less able to tolerate shift work than those who work in alignment with their chronotype (Erren, 2013). In fact, an increased risk of breast cancer for morning-type nightshift workers was observed in a nested case-control study of 218 breast cancer cases and 899 controls within the Danish military [Odds ratio: 3.9 (95% confidence interval: 1.6 to 9.5)] (Hansen & Lassen, 2012). However, it is unknown whether this association would remain in a prospectively followed group of shift workers, whether it holds for workers of different occupations, whether there are factors that could mitigate the modifying effect of chronotype, and whether chronotype has a measureable impact on biomarkers of disease processes among shift workers. As such, research related to chronotype is an agenda item that the consortium could consider, though other factors that affect worker's abilities to adapt to the demands of the nightshift or to get quality sleep on days off would likely surface once the members of the consortium start talking to each other.

Gather evidence

During this phase, the consortium would plan how to get the data and do the research. Broadly, we envision the consortium doing the following: identifying funding sources (e.g., one potential funder is the National, Heart, Lung, and Blood Institute (NHLBI), which convened a group of experts in circadian biology and sleep medicine in 2015 and issued recommendations for the development of biomarkers to assess circadian function in population-based research; another, given the question of carcinogenicity, is the National Cancer Institute); coordinating funding and activities between the stakeholders at the various research organizations;

harmonizing definitions of shift work; setting standards for data acquisition; and doing the research. As the research progresses, it will inevitably produce findings with policy, social, and ethical implications that can benefit from a broader deliberative public discussion.

Convene stakeholders for democratic deliberation

We imagine deliberative engagement of diverse stakeholders taking place at critical moments in research process. Continuing with the example of chronotype, if an increased risk for cancer is verified for nightshift workers who have a morning chronotype, the finding would raise difficult questions about what action to recommend. Approximately 25% of the population is thought to be morning-type, 25% evening-type, and the remainder of the population intermediate chronotype (Paine, 2006). What, if any, policy recommendations should be made? A recommendation that morning-types avoid the nightshift or that employers avoid hiring morning-types for nightshift work may result in a reduced risk of cancer (and other chronic diseases) among these workers, but it would also place all the risk on evening and intermediate-type shift workers. It might also eliminate or greatly reduce employment options for populations that have few employment options, and who might value the work despite its risks. Should low-risk populations bear the entire burden of shift work? Should high-risk populations be disallowed from working nightshifts? Should testing for chronotype be mandatory? More broadly, what obligations does an employer have to shift workers to monitor health status or to provide health care, should they develop cancer or other maladies? These sorts of questions are value-laden and likely to be highly controversial, making them well suited to public deliberation. By drawing on the values and insights of diverse segments of the public, including those who engage in shift work, shared social values can be identified to inform best shift work practices.

Numerous approaches to public deliberation have been developed, from citizen panels to deliberative polling, and much has been written about the design and implementation of deliberative forums (Gastil & Levine, 2005). Given the complexity and uncertainty that surrounds the science of shift work, we stress here the need for balanced, plain-language materials to serve as an information base. Although experts often contend that non-experts will not be able to understand scientific topics, empirical studies of deliberations that have taken up complex topics suggest otherwise (Carman et al., 2015).

Inform policy and practice

After the findings from research on shift work have been publicly deliberated, we envision the consortium formulating and publishing a set of guidelines and recommendations. Ideally, NIOSH and other governmental bodies (e.g., the National Highway Traffic Safety Administration) would work jointly to disseminate the findings and guidelines broadly throughout the public sector, working closely with state and county-level public health agencies and key figures in the shift work industry to deliver the message to shift workers and stakeholders within industry (e.g., heads of hospitals and managers).

Conclusion

We have argued that evidence of health harms associated with shift work justify a precautionary stance. The harms associated with shift work are serious, sometimes irreversible, affect millions of people, and fall disproportionately on minorities and the poor, who are exposed to an array of health risks that include but are not limited to shift work. This evidence in combination with the inability of some groups to avoid shift work and of society to eliminate some forms of shiftwork (i.e., essential public health and safety functions) support a

precautionary stance. We also have argued that responsibilities associated with increasing the body of knowledge about shift work's potential harms and advancing policies and practices to mitigate and minimize those harms should be shared across a consortium of diverse stakeholders and the public at large.

The idea of shared responsibility for research and action on shift work comports with the very definition of public health—"what society does collectively to assure the conditions for people to be healthy" (Institute of Medicine, 2002, p. xiv). Yet, admittedly it faces considerable obstacles in the US, which has a political culture that favors individual responsibility over shared responsibility and privileges private industry over public health and social goods. US history is rife with examples of industries—from tobacco and lead paint to asbestos and automakers—actively blocking information about their products' harms or otherwise shirking responsibility for them (Kurland, 2002). But there have been success stories, and those examples often work through collaborative cross-sectoral models that draw on the talents, energies, perspectives, and commitments of many social sectors and the public at large. One such success story involves bringing together unlikely stakeholders—the National Rifle Association, the Second Amendment Foundation, those interested in gun suicide and injury prevention, and mental health advocates—leading to new law in Washington state to develop gun suicide prevention messaging and training for gun businesses and pharmacies (Stuber, 2016). Similarly, we believe having a broad range of stakeholders with diverse views, each with a valued vote and a unique contribution, helps protect against the "bystander effect" (Thomas, Freitas, Descioli, & Pinker, 2016), the dilemma of many knowing about a problem but each person thinking someone else will deal with it, as the challenges of shift work belong to us all. By pooling our unique perspectives, by listening to shift workers, business owners, regulators, and scientists, we can stride towards

reducing the harms of shift work.

Analysis III—Policy Table

TABLE 1. IARC-reviewed studies of shift work and (breast) cancer

Study	Type	Risk Estimate* (Extreme group *vs*. referent)	95% CI	Shift work Definition
Schernhammer *et al*. (2001)	Prospective cohort	1.36	1.04–1.78	Rotating (≥3 nights/month + days)
Schernhammer *et al*. (2006)	Prospective cohort	1.79	1.06–3.01	Rotating (≥3 nights/month + days)
Tynes *et al*. (1996)	Nested case-control	1.5	1.1–2.0	Work at night with exposure to artificial light
O'Leary *et al*. (2006)	Case-control	1.04	0.79–1.38	Any evening or overnight work
Davis *et al*. (2001)	Case-control	2.3	1.0–5.3	"Graveyard" (either permanent or rotating)
Hansen (2001)	Nested case-control	1.5	1.3–1.7	Night work assigned for trades for which >60% of women estimated to work at night
Lie *et al*. (2006)	Case-control	2.21	1.10–4.45	Years of night work imputed based on nursing jobs outside of hospitals
Schwartzbaum *et al*. (2007)	Retrospective cohort	0.94 (SIR[#])	0.74–1.18	Night work assigned for job titles for which >40% of staff worked at night

* Odds ratios and relative risks; [#]SIR=standardized incidence ratio

References

Abelson, J., Forest, P., Eyles, J., Smith, P., Martin, E., & Gauvin, F. (2003). Deliberations about deliberative methods: Issues in the design and evaluation of public participation processes. *Social Science & Medicine*, *57*(2), 239–51. doi:http://dx.doi.org/10.1016/S0277-9536(02)00343-X

Aguilar-Arnal, L., Sassone-Corsi, P., & Pfaff, D. W. (2015). Chromatin landscape and circadian dynamics: Spatial and temporal organization of clock transcription. *Proc Natl Acad Sci USA*, *112*(22), 6863–70. doi:10.1073/pnas.1411264111

Aken, B. L., Ayling, S., Barrell, D., Clarke, L., Curwen, V., Fairley, S., … Searle, S. M. J. (2016). The Ensembl gene annotation aystem. *Database*, 1–19. doi:10.1093/database/baw093

Altman, B. J., Hsieh, A. L., Sengupta, A., Krishnanaiah, S. Y., Stine, Z. E., Walton, Z. E., … Dang, C. V. (2015). MYC disrupts the circadian clock and metabolism in cancer cells. *Cell Metabolism*, *22*(6), 1009–1019. doi:10.1016/j.cmet.2015.09.003

Aran, D., Sabato, S., & Hellman, A. (2013). DNA methylation of distal regulatory sites characterizes dysregulation of cancer genes. *Genome Biology*, *14*(3), 1–14. doi:10.1186/gb-2013-14-3-r21

Arendt, J. (2010). Shift work: Coping with the biological clock. *Occup Med (Lond)*, *60*(1), 10–20. doi:10.1093/occmed/kqp162

Baldoni, S., Sportoletti, P., Del Papa, B., Aureli, P., Dorillo, E., Rosati, E., … Di Ianni, M. (2013). NOTCH and NF-κB interplay in chronic lymphocytic leukemia is independent of genetic lesion. *Int J Hematol.*, *98*(2), 153–7.

Barault, L., Ellsworth, R. E., Harris, H. R., Valente, A. L., Shriver, C. D., & Michels, K. B. (2013). Leukocyte DNA as surrogate for the evaluation of imprinted Loci methylation in mammary tissue DNA. *PLoS One*, *8*(2), e55896. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23409079

Beisel, C., & Paro, R. (2011). Silencing chromatin: comparing modes and mechanisms. *Nat Rev Genet*, *12*(2), 123–35. doi:10.1038/nrg2932

Bell, J. T., Pai, A. a, Pickrell, J. K., Gaffney, D. J., Pique-Regi, R., Degner, J. F., … Pritchard, J. K. (2011). DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. *Genome Biology*, *12*(1), 1–13. doi:10.1186/gb-2011-12-1-r10

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. B*, *57*, 289–300.

Bhatti, P., Mirick, D. K., & Davis, S. (2014). The impact of chronotype on melatonin levels among shift workers. *Occup Environ Med*, *71*(3), 195–200. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/24399070

Bhatti, P., Zhang, Y., Song, X., Makar, K. W., Sather, C. L., Kelsey, K. T., … Wang, P. (2014). Nightshift work and genome-wide DNA methylation. *Chronobiol Int*, 1–10.

Blattler, A., Yao, L., Witt, H., Guo, Y., Nicolet, C. M., Berman, B. P., & Farnham, P. J. (2014). Global loss of DNA methylation uncovers intronic enhancers in genes showing expression changes. *Genome Biology*, *15*(9), 1–16. doi:10.1186/s13059-014-0469-0

Bourgon, R., Gentleman, R., & Huber, W. (2010). Independent filtering increases power for detecting differentially expressed genes. *Proc Natl Acad Sci USA*, *107*(2), 9546–9551.

Bracci, M., Manzella, N., Copertaro, A., Staffolani, S., Strafella, E., Barbaresi, M., … Santarelli, L. (2014). Rotating-shift nurses after a day off: peripheral clock gene expression, urinary

melatonin, and serum 17-β-estradiol levels. *Scand J Work Environ Health*, *40*(3), 295–304. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/24402410

Braveman, P. A., Cubbin, C., Egerter, S., Williams, D. R., & Pamuk, E. (2010). Socioeconomic disparities in health in the united States: What the patterns tell us. *Am J Public Health*, *100*(SUPPL. 1), S186–S196. doi:10.2105/AJPH.2009.166082

Braveman, P. A., Egerter, S. A., & Mockenhaupt, R. E. (2011). Broadening the focus: The need to address the social determinants of health. *Am J Prev Med*, *40*(SUPPL. 1), S4–S18. doi:10.1016/j.amepre.2010.10.002

Breitling, L. P., Yang, R., Korn, B., Burwinkel, B., & Brenner, H. (2011). Tobacco-smoking-related differential DNA methylation: 27K discovery and replication. *Am J Hum Genet*, *88*(4), 450–457. doi:10.1016/j.ajhg.2011.03.003

Brenet, F., Moh, M., Funk, P., Feierstein, E., Viale, A. J., Socci, N. D., & Scandura, J. M. (2011). DNA methylation of the first exon is tightly linked to transcriptional silencing. *PLoS ONE*, *6*(1), 1–12. doi:10.1371/journal.pone.0014524

Brock, D. (2000). Broadening the bioethics agenda. *Kennedy Institute of Ethics Journal*, *10*(1), 21–38.

Butcher, L. (2013). Illumina450ProbeVariants.db: Annotation Package combining variant data from 1000 Genomes Project for Illumina HumanMethylation450 Bead Chip probes. R package version 1.4.0.

Carman, K. L., Mallery, C., Maurer, M., Wang, G., Garfinkel, S., Yang, M., … Siu Chao, A. (2015). Effectiveness of public deliberation methods for gathering input on issues in healthcare: Results from a randomized trial. *Soc Sci Med*, *133*, 11–20. doi:10.1016/j.socscimed.2015.03.024

Carpini, M. X. D., Cook, F. L., & Jacobs, L. R. (2004). Public deliberation, discursive participation, and citizen engagement: A review of the empirical literature. *Annu. Rev. Polit. Sci.*, *7*(1), 315–344. doi:10.1146/annurev.polisci.7.121003.091630

Chang, W., Gruber, D., Chari, S., Kitazawa, H., Hamazumi, Y., Hisanaga, S., & Bulinski, J. C. (2001). Phosphorylation of MAP4 affects microtubule properties and cell cycle progression. *J Cell Sci*, *114*(Pt 15), 2879–2887.

Chen, D., Zhang, X., Zhang, Y., Zhang, L., Ma, J., You, W., & Pan, K. (2016). Hypomethylation of repetitive elements in blood leukocyte DNA and risk of gastric lesions in a Chinese population. *Cancer Epidemiol*, *41*, 122–128. doi:10.1016/j.canep.2016.02.004

Chen, Y. A., Lemire, M., Choufani, S., Butcher, D. T., Grafodatskaya, D., Zanke, B. W., … Weksberg, R. (2013). Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. *Epigenetics*, *8*(2), 203–209. doi:10.4161/epi.23470

Chu, G., Li, J., Narasimhan, B., Tibshirani, R., & Tusher, V. (n.d.). SAM - Significance Analysis of Microarrays - Users guide and technical document, 1–42. doi:10.1261/rna.1473809

Climent, J., Perez-Losada, J., Quigley, D. A., Kim, I. J., Delrosario, R., Jen, K. Y., … Balmain, A. (2010). Deletion of the PER3 gene on chromosome 1p36 in recurrent ER-positive breast cancer. *J Clin Oncol*, *28*(23), 3770–3778. doi:10.1200/JCO.2009.27.0215

Cole, R. J., Kripke, D. F., Gruen, W., Mullaney, D. J., & Gillin, J. C. (1992). Automatic sleep/wake identification from wrist activity. *Sleep*, *15*, 461–469.

Comba, P., Martuzzi, M., & Botti, C. (2004). The precautionary principle in decision-making: The ethical values. In *The precautionary principle: Protecting public health, the environment and the future of our children* (pp. 85–91).

Cook, D., Kang, H., & Jetten, A. (2015). Retinoic acid-related orphan receptors (RORs): Regulatory functions in immunity, development, circadian rhythm, and metabolism. *Nucl Receptor Res.*, *2*, 1–40. doi:10.1007/978-1-4614-5915-6

Costa, G., Haus, E., & Stevens, R. (2010). Shift work and cancer – considerations on rationale, mechanisms, and epidemiology. *Scand J Work Environ Health*, *36*(2), 163–179. doi:10.5271/sjweh.2899

Costas, L., Benavente, Y., Olmedo-Requena, R., Casabonne, D., Robles, C., Gonzalez-Barca, E.-M., … de Sanjosé, S. (2016). Night shift work and chronic lymphocytic leukemia in the MCC-Spain case-control study. *Int J Cancer*, *139*(9), 1994–2000. doi:10.1002/ijc.30272

Davies, C. (2011). Participatory citizens - Desirable? Possible? Practical? *Sociology*, *4*, 1134–9.

Davis, S., Mirick, D. K., Chen, C., & Stanczyk, F. Z. (2012). Night shift work and hormone levels in women. *Cancer Epidemiol Biomarkers Prev*, *21*(4), 609–618. doi:10.1158/1055-9965.EPI-11-1128

Davis, S., Mirick, D. K., & Stevens, R. G. (2001). Night shift work, light at night, and risk of breast cancer. *J Natl Cancer Inst*, *93*(20), 1557–1562.

Dedeurwaerder, S., Defrance, M., Bizet, M., Calonne, E., Bontempi, G., & Fuks, F. (2014). A comprehensive overview of Infinium HumanMethylation450 data processing. *Briefings in Bioinformatics*, *15*(6), 929–941. doi:10.1093/bib/bbt054

del Castillo, F. J., Cohen-Salmon, M., Charollais, A., Caille, D., Lampe, P. D., Chavrier, P., … Petit, C. (2009). Consortin, a trans-Golgi network cargo receptor for the plasma membrane targeting and recycling of connexins. *Hum Mol Genet*, *19*(2), 262–275. doi:10.1093/hmg/ddp490

Dijk, D. J., & Archer, S. N. (2010). PERIOD3, circadian phenotypes, and sleep homeostasis. *Sleep Med Rev*, *14*(3), 151–160. doi:10.1016/j.smrv.2009.07.002

Dogan, N., Wu, W., Morrissey, C. S., Chen, K.-B., Stonestrom, A., Long, M., … Hardison, R. C. (2015). Occupancy by key transcription factors is a more accurate predictor of enhancer activity than histone modifications or chromatin accessibility. *Epigenetics Chromatin*, *8*(1), 1–21. doi:10.1186/s13072-015-0009-5

Dong, Y., Zhao, H., Li, H., Li, X., & Yang, S. (2014). DNA methylation as an early diagnostic marker of cancer (Review). *Biomedical Reports*, *2*(3), 326–330. doi:10.3892/br.2014.237

Dorrian, J., & Skinner, N. (2012). Alcohol consumption patterns of shiftworkers compared with dayworkers. *Chronobiol Int*, *29*(5), 610–8. doi:10.3109/07420528.2012.675848

Du, J., & Xu, R. (2012). RORa, a potential tumor suppressor and therapeutic target of breast cancer. *Int J Mol Sci*, *13*(12), 15755–15766. doi:10.3390/ijms131215755

Dvinge, H., & Bradley, R. K. (2015). Widespread intron retention diversifies most cancer transcriptomes. *Genome Med*, *7*(1), 1–13. doi:10.1186/s13073-015-0168-9

Ehrlich, M. (2002). DNA methylation in cancer: too much, but also too little. *Oncogene*, *21*, 5400–5413. doi:10.1038/sj.onc

Ellis, J., von Schantz, M., Jones, K. H. S., & Archer, S. N. (2009). Association between specific diurnal preference questionnaire items and PER3 VNTR genotype. *Chronobiol Int*, *26*(3), 464–73. doi:10.1080/07420520902820970

Erren, T. C. (2013). Shift work and cancer research: can chronotype predict susceptibility in night-shift and rotating-shift workers? *Occup Environ Med*, *70*(4), 283–4. doi:10.1136/oemed-2012-100984

Faulk, C., Kim, J., Anderson, O. S., Nahar, M. S., Jones, T., Sartor, M., & Dolinoy, D. C. (2016). Detection of Differential DNA Methylation in Repetitive DNA of Mice and Humans

Perinatally Exposed to Bisphenol A. *Epigenetics*, *11*(7), 489–500. doi:10.1080/15592294.2016.1183856

Final Report of the Commission on Social Determinants of Health. Geneva, World Health Organization. (2008). CSDH. Closing the gap in a generation: health equity through action on the social determinants of health.

Fong, N., Kim, H., Zhou, Y., Ji, X., Qiu, J., Saldi, T., … Bentley, D. L. (2014). Pre-mRNA splicing is facilitated by an optimal RNA polymerase II elongation rate. *Genes Dev*, *28*(23), 2663–2676. doi:10.1101/gad.252106.114

Francescatto, M., Vitezic, M., Heutink, P., & Saxena, A. (2014). Brain-specific noncoding RNAs are likely to originate in repeats and may play a role in up-regulating genes in cis. *Int J Biochem Cell Biol*, *54*, 331–337. doi:10.1016/j.biocel.2014.06.014

Fritschi, L., Erren, T. C., Glass, D. C., Girschik, J., Thomson, A. K., Saunders, C., … Heyworth, J. S. (2013). The association between different night shiftwork factors and breast cancer: a case–control study. *Br J Cancer*, *109*(9), 2472–2480. doi:10.1038/bjc.2013.544

Fritschi, L., Glass, D. C., Heyworth, J. S., Aronson, K., Girschik, J., Boyle, T., … Erren, T. C. (2011). Hypotheses for mechanisms linking shiftwork and cancer. *Med Hypotheses*, *77*(3), 430–436. doi:10.1016/j.mehy.2011.06.002

Gastil, J., Bacci, C., & Dollinger, M. (2010). Is deliberation neutral? Patterns of attitude change during "The deliberative polls(TM)." *Journal of Public Deliberation*, *6*(2), 2010. Retrieved from
http://psu.summon.serialssolutions.com/2.0.0/link/0/eLvHCXMwY2AwNtIz0EUrE4wNjVKMkoxTzNPMDQ3Tkk3TEi1SkkDr3w3NTFOMjBJhu8fAy6GQy0I3IQam1DxRBjk31xBnD11YcRmfkpMTbwya5TIxBrYZjMUYWIC95VQJBgULI0uTpJSk5FSzxCQTYPWamGhsYmlomWiaam5ham5pJMkgjsMQAFjbLoA\nwww.summon.com

Gastil, J., & Levine, P. (Eds.). (2005). *The Deliberative Democracy Handbook: Strategies for Effective Civic Engagement in the 21st Century*. San Francisco: Jossey-Bass.

Gelfman, S., Cohen, N., Yearim, A., & Ast, G. (2013). DNA-methylation effect on cotranscriptional splicing is dependent on GC architecture of the exon – intron structure DNA-methylation effect on cotranscriptional splicing is dependent on GC architecture of the exon – intron structure. *Genome Research*, *25*(5), 789–799. doi:10.1101/gr.143503.112

Gery, S., & Koeffler, H. P. (2007). The role of circadian regulation in cancer. *Cold Spring Harb Symp Quant Biol*, *72*, 459–464. doi:10.1101/sqb.2007.72.004

Gery, S., Komatsu, N., Baldjyan, L., Yu, A., Koo, D., & Koeffler, H. P. (2006). The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. *Mol Cell*, *22*(3), 375–382. doi:10.1016/j.molcel.2006.03.038

Geybels, M. S., Zhao, S., Wong, C.-J., Bibikova, M., Klotzle, B., Wu, M., … Stanford, J. L. (2015). Epigenomic profiling of DNA methylation in paired prostate cancer versus adjacent benign tissue. *The Prostate*, *1950*(August), 1941–1951. doi:10.1002/pros.23093

Goold, S. D., Biddle, A. K., Klipp, G., Hall, C. N., & Danis, M. (2005). Choosing healthplans all together: a deliberative exercise for allocating limited health care resources. *Journal of Health Politics, Policy and Law*, *30*(4), 563–601. doi:10.1215/03616878-30-4-563

Gottlieb, D. J., ..., & Tiemeier, H. (2015). Novel Loci Associated with Usual Sleep Duration: The CHARGE Consortium Genome-Wide Association Study. *Mol Psychiatry*, *20*(10), 1232–1239. doi:10.1038/mp.2014.133.Novel

Grabner, C. P., Price, S. D., Lysakowski, A., Cahill, A. L., & Fox, A. P. (2006). Regulation of large dense-core vesicle volume and neurotransmitter content mediated by adaptor protein

3. *Proc Natl Acad Sci USA*, *103*(26), 10035–40. doi:10.1073/pnas.0509844103
Gu, F., Han, J., Laden, F., Pan, A., Caporaso, N. E., Stampfer, M. J., … Schernhammer, E. S. (2014). Total and Cause-Specific Mortality of U.S. Nurses Working Rotating Night Shifts. *Am J Prev Med*, *48*(3), 241–252. Retrieved from http://dx.doi.org/10.1016/j.amepre.2014.10.018
Hammed, A., Matagrin, B., Spohn, G., Prouillac, C., Benoit, E., & Lattard, V. (2013). VKORC1L1, an enzyme rescuing the vitamin K 2,3-epoxide reductase activity in some extrahepatic tissues during anticoagulation therapy. *J Biol Chem*, *288*(40), 28733–28742. doi:10.1074/jbc.M113.457119
Hansen, J., & Lassen, C. F. (2012). Nested case-control study of night shift work and breast cancer risk among women in the Danish military. *Occup Environ Med*, *69*(8), 551–556. doi:10.1136/oemed-2011-100240
Heidary Arash, E., Song, K. M., Song, S., Shiban, A., & Attisano, L. (2014). Arhgef7 promotes activation of the Hippo pathway core kinase Lats. *EMBO J*, *33*(24), 2997–3011. doi:10.15252/embj.201490230
Heyn, H., Vidal, E., Ferreira, H. J., Vizoso, M., Sayols, S., Gomez, A., … Esteller, M. (2016). Epigenomic analysis detects aberrant super-enhancer DNA methylation in human cancer. *Genome Biology*, *17*(1), 1–16. doi:10.1186/s13059-016-0879-2
Hock, A., Vigneron, A., & Vousden, K. (2014). Ubiquitin-specific peptidase 42 (USP42) functions to deubiquitylate histones and regulate transcriptional activity. *J Biol Chem*, *289*(50), 34862–34870. Retrieved from http://doi.org/10.1074/jbc.M114.589267
Hoffman, A., Yi, C., Zheng, T., Stevens, R., Leaderer, D., Zhang, Y., … Zhu, Y. (2010). CLOCK in breast tumorigenesis: genetic, epigenetic, and transcriptional profiling analyses. *Cancer Res*, *70*(4), 1459–1468. doi:10.1158/0008-5472.CAN-09-3798
Hoffman, A., Zheng, T., Yi, C., Stevens, R., Ba, Y., Zhang, Y., … Zhu, Y. (2010). The core circadian gene cryptochrome 2 influences breast cancer risk, possibly by mediating hormone signaling. *Cancer Prev Res (Phila)*, *3*(4), 539–548. doi:10.1158/1940-6207.CAPR-09-0127
Hou, L., Zhang, X., Wang, D., & Baccarelli, A. (2012). Environmental chemical exposures and human epigenetics. *Int J Epidemiol*, *41*(1), 79–105. doi:10.1093/ije/dyr154
Houseman, E. A., Accomando, W. P., Koestler, D. C., Christensen, B. C., Marsit, C. J., Nelson, H. H., … Kelsey, K. T. (2012). DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics*, *13*(1), 86. doi:10.1186/1471-2105-13-86
Husse, J., Hintze, S. C., Eichele, G., Lehnert, H., & Oster, H. (2012). Circadian Clock Genes Per1 and Per2 Regulate the Response of Metabolism-Associated Transcripts to Sleep Disruption. *PLoS ONE*, *7*(12). doi:10.1371/journal.pone.0052983
Ijaz, S., Verbeek, J., Seidler, A., Lindbohm, M.-L., Ojajarvi, A., Orsini, N., … Neuvonen, K. (2013). Night-shift work and breast cancer - a systematic review and meta-analysis. *Scand J Work Environ Health*, *39*(5), 431–447. doi:10.5271/sjweh.3371
Institute of Medicine. (2002). The future of the public's health in the 21st century, 1–510.
International Agency for Research on Cancer. (2007). *IARC monographs on the evaluation of carcinogenic risks to humans, volume 98. Painting, firefighting, and shiftwork* (Vol. 98). Lyon, France. Retrieved from iarc2010
Ishii, T., Kohu, K., Yamada, S., Ishidoya, S., Kanto, S., Fuji, H., … Arai, Y. (2007). Up-regulation of DNA-methyltransferase 3A expression is associated with hypomethylation of intron 25 in human testicular germ cell tumors. *Tohoku J Exp Med*, *212*(2), 177–90.

doi:10.1620/tjem.212.177
Jeronimo, C., Bastian, P. J., Bjartell, A., Carbone, G. M., Catto, J. W. F., Clark, S. J., … Shariat, S. F. (2011). Epigenetics in prostate cancer: Biologic and clinical relevance. *Eur Urol*, *60*(4), 753–766. doi:10.1016/j.eururo.2011.06.035
Jia, Y., Lu, Y., Wu, K., Lin, Q., Shen, W., Zhu, M., … Chen, J. (2013). Does night work increase the risk of breast cancer? A systematic review and meta-analysis of epidemiological studies. *Cancer Epidemiol*, *37*(3), 197–206. doi:10.1016/j.canep.2013.01.005
Jones, P. A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet*, *13*(7), 484–492. doi:10.1038/nrg3230
Jones, S. E., Tyrrell, J., Wood, A. R., Beaumont, R. N., Ruth, K. S., Tuke, M. A., … Weedon, M. N. (2016). Genome-wide association analyses in >119,000 individuals identifies thirteen morningness and two sleep duration loci. *PLoS Genet*, *8*, 031369. doi:10.1101/031369
Jonkers, I., Kwak, H., & Lis, J. T. (2014). Genome-wide dynamics of Pol II elongation and its interplay with promoter proximal pausing, chromatin, and exons. *eLife*, *2014*(3), 1–25. doi:10.7554/eLife.02407
Jordan, A., & O'Riordan, T. (2004). The precautionary principle: A legal and policy history. In M. Martuzzi & J. Tickner (Eds.), *The precautionary principle: Protecting public health, the environment and the future of our children* (pp. 31–48). Retrieved from http://www.euro.who.int/__data/assets/pdf_file/0003/91173/E83079.pdf
Kamdar, B. B., Tergas, A. I., Mateen, F. J., Bhayani, N. H., & Oh, J. (2013). Night-shift work and risk of breast cancer: a systematic review and meta-analysis. *Creaset Cancer Res Treat*, *138*(1), 291–301. doi:10.1007/s10549-013-2433-1
Kent, J., Lee, M., Schedl, A., Boyle, S., Fantes, J., Powell, M., … Bickmore, W. a. (1997). The reticulocalbin gene maps to the WAGR region in human and to the Small eye Harwell deletion in mouse. *Genomics*, *42*(2), 260–7. doi:10.1006/geno.1997.4706
Kent, W., Sugnet, C., Furey, T., Roskin, K., Pringle, T., Zahler, A., & Haussler, D. (2002). The human genome browser at UCSC. *Genome Res*, *12*(6), 996–1006.
Khosro, S., Alireza, S., Omid, A., & Forough, S. (2011). Night work and inflammatory markers. *Indian J Occup Environ Med*, *15*(1), 38–41.
Kloke, J. D., & Mckean, J. W. (2012). Rfit: Rank-based estimation for linear models rank-regression. *The R Journal*, *4*(December), 57–64.
Knutsson, A. (2003). Health disorders of shift workers. *Occup Med (Lond)*, *53*(2), 103–108. doi:10.1093/occmed/kqg048
Knutsson, A., Alfredsson, L., Karlsson, B., Åkerstedt, T., Fransson, E. I., Westerholm, P., & Westerlund, H. (2013). Breast cancer among shift workers: Results of the WOLF longitudinal cohort study. *Scand J Work Environ Health*, *39*(2), 170–177. doi:10.5271/sjweh.3323
Ko, C. H., & Takahashi, J. S. (2006). Molecular components of the mammalian circadian clock. *Hum Mol Genet*, *15 Spec No*(2), R271–7. doi:10.1093/hmg/ddl207
Kottorou, A. E., Antonacopoulou, A. G., Dimitrakopoulos, F. I. D., Tsamandas, A. C., Scopa, C. D., Petsas, T., & Kalofonos, H. P. (2012). Altered expression of NFY-C and RORA in colorectal adenocarcinomas. *Acta Histochemica*, *114*(6), 553–561. doi:10.1016/j.acthis.2011.10.005
Kowalczyk, M. S., Hughes, J. R., Garrick, D., Lynch, M. D., Sharpe, J. A., Sloane-Stanley, J. A., … Higgs, D. R. (2012). Intragenic Enhancers Act as Alternative Promoters. *Mol Cell*, *45*(4), 447–458. doi:10.1016/j.molcel.2011.12.021

Kriebel, D., & Tickner, J. (2001). Reenergizing public health through precaution. *Am J Public Health*, *91*(9), 1351–1355. doi:10.2105/AJPH.91.9.1351

Kriebel, D., Tickner, J., Epstein, P., Lemons, J., Levins, R., Loechler, E., … Stoto, M. (2005). The precautionary principle in environmental science. In M. Martuzzi & J. Tickner (Eds.), *The precautionary principle: Protecting public health, the environment and the future of our children,* (pp. 145–166).

Kulis, M., & Esteller, M. (2010). DNA methylation and cancer. *Adv Genet*., *70*, 27–56.

Kulis, M., Heath, S., Bibikova, M., Queirós, A., Navarro, A., Clot, G., … Martín-Subero, J. (2012). Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. *Nat Genet*, *44*(11), 1236–1242. doi:10.1038/ng.2443

Kurland, J. (2002). The heart of the precautionary principle in democracy. *Public Health Reports*, *117*(6), 498–500. doi:10.1016/S0033-3549(04)50194-2

Kwee, L. C., Liu, D., Lin, X., Ghosh, D., & Epstein, M. P. (2008). A powerful and flexible multilocus association test for quantitative traits. *Am J Hum Genet*, *82*(2), 386–397. doi:10.1016/j.ajhg.2007.10.010

Lahti, T. A., Partonen, T., Kyyronen, P., Kauppinen, T., & Pukkala, E. (2008). Night-time work predisposes to non-Hodgkin lymphoma. *Int J Cancer*, *123*(9), 2148–2151. doi:10.1002/ijc.23566

Lai, A. Y., Fatemi, M., Dhasarathy, A., Malone, C., Sobol, S. E., Geigerman, C., … Wade, P. a. (2010). DNA methylation prevents CTCF-mediated silencing of the oncogene BCL6 in B cell lymphomas. *J Exp Med*., *207*(9), 1939–1950. doi:10.1084/jem.20100204

Lee, I. H., Sohn, M., Lim, H. J., Yoon, S., Oh, H., Shin, S., … Bae, Y. S. (2014). Ahnak functions as a tumor suppressor via modulation of TGFβ/Smad signaling pathway. *Oncogene*, *33*(38), 4675–84. doi:10.1038/onc.2014.69

Leek, J. T., Johnson, W. E., Parker, H. S., Jaffe, A. E., & Storey, J. D. (2012). The SVA package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics*, *28*(6), 882–883. doi:10.1093/bioinformatics/bts034

Lenz, G., Davis, R. E., Ngo, V. N., Lam, L., George, T. C., Wright, G. W., … Staudt, L. M. (2008). Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science*, *319*(5870), 1676–9. doi:10.1126/science.1153629

Leung, M., Tranmer, J., Hung, E., Korsiak, J., Day, A. G., & Aronson, K. J. (2016). Shiftwork, chronotype and melatonin patterns among female hospital employees on day and night shifts. *Cancer Epidemiol Biomarkers Prev*, (613). doi:10.1158/1055-9965.EPI-15-1178

Levy, M., & Futerman, A. H. (2010). Mammalian ceramide synthases. *IUBMB Life*, *62*(5), 347–356. doi:10.1002/iub.319.Mammalian

Li, W., Zheng, J., Deng, J., You, Y., Wu, H., Li, N., … Zhou, Y. (2014). Increased levels of the long intergenic non-protein coding RNA POU3F3 promote DNA methylation in esophageal squamous cell carcinoma cells. *Gastroenterology*, *146*(7), 1714–1726. doi:10.1053/j.gastro.2014.03.002

Li, X., & Heyer, W. D. (2008). Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res*., *18*(1), 99–113. doi:10.1038/cr.2008.1.Homologous

Liang, G., Schernhammer, E., Qi, L., Gao, X., De Vivo, I., & Han, J. (2011). Associations between rotating night shifts, sleep duration, and telomere length in women. *PloS One*, *6*(8), e23462. doi:10.1371/journal.pone.0023462

Lin, X., Chen, W., Wei, F., Ying, M., Wei, W., & Xie, X. (2015). Night-shift work increases morbidity of breast cancer and all-cause mortality: A meta-analysis of 16 prospective cohort

studies. *Sleep Med*, *16*(11), 1381–1387. doi:10.1016/j.sleep.2015.02.543

Liu, B., Xu, K., Jiang, Y., & Li, X. (2014). Aberrant expression of Per1, Per2 and Per3 and their prognostic relevance in non-small cell lung cancer. *Int J Clin Exp Pathol*, *7*(11), 7863–7871.

Ma, B., Wilker, E. H., Willis-Owen, S. A., Byun, H. M., Wong, K. C., Motta, V., … Liang, L. (2014). Predicting DNA methylation level across human tissues. *Nucleic Acids Res*, *42*(6), 3515–3528. doi:10.1093/nar/gkt1380

Malafronte, P. J., LePage, E. M., Hatanpaa, K. J., Burns, D. K., White, C. L., & Raisanen, J. (2014). Expression of MAP 2 by haemangioblastomas: an immunohistochemical study with implications for diagnosis. *Pathology*, *46*(5), 450–1. doi:10.1097/PAT.0000000000000138

Malousi, A., & Kouidou, S. (2012). DNA hypermethylation of alternatively spliced and repeat sequences in humans. *Mol Genet Genomics*, *287*(8), 631–642. doi:10.1007/s00438-012-0703-y

Maor, L. G., Yearim, A., & Ast, G. (2015). The alternative role of DNA methylation in splicing regulation. *Trends in Genetics*, *31*(5), 274–280. doi:10.1016/j.tig.2015.03.002

Marmot, M. (2005). Public Health Social determinants of health inequalities. *Lancet*, *365*, 1099–1104. doi:10.1016/S0140-6736(05)71146-6

Martuzzi, M., & Tickner, J. (2004). Executive summary. In M. Martuzzi & J. Tickner (Eds.), *The precautionary principle: Protecting public health, the environment and the future of our children* (p. 1).

Mason, P. B., & Struhl, K. (2005). Distinction and relationship between elongation rate and processivity of RNA polymerase II in vivo. *Mol Cell*, *17*(6), 831–840. doi:10.1016/j.molcel.2005.02.017

Matsuo, T., Yamaguchi, S., Mitsui, S., Emi, A., Shimoda, F., & Okamura, H. (2003). Control mechanism of the circadian clock for timing of cell division in vivo. *Science*, *302*(5643), 255–259. doi:10.1126/science.1086271

Mayer, B., Brown, P., & Linder, M. (2002). Moving further upstream: from toxics reduction to the precautionary principle. *Public Health Reports*, *117*(6), 574–586. doi:10.1093/phr/117.6.574

Mazzoccoli, G., Panza, A., Valvano, M. R., Palumbo, O., Carella, M., Pazienza, V., … Piepoli, A. (2011). Clock gene expression levels and relationship with clinical and pathological features in colorectal cancer patients. *Chronobiol Int.*, *28*(10), 841–51. doi:10.3109/07420528.2011.615182

McMenamin, T. M. (2007). A time to work: recent trends in shift work and flexible schedules. *Monthly Labor Review*, *130*(December), 3–15. Retrieved from http://www.bls.gov/opub/mlr/2007/12/art1full.pdf

Megdal, S. P., Kroenke, C. H., Laden, F., Pukkala, E., & Schernhammer, E. S. (2005). Night work and breast cancer risk: a systematic review and meta-analysis. *European Journal of Cancer*, *41*(13), 2023–32. doi:10.1016/j.ejca.2005.05.010

Michels, K. B., Binder, A. M., Dedeurwaerder, S., Epstein, C. B., Greally, J. M., Gut, I., … Irizarry, R. a. (2013). Recommendations for the design and analysis of epigenome-wide association studies. *Nat Methods*, *10*(10), 949–955. doi:10.1038/nmeth.2632

Mirick, D. K., Bhatti, P., Chen, C., Nordt, F., Stanczyk, F. Z., & Davis, S. (2013). Night shift work and levels of 6-sulfatoxymelatonin and cortisol in men. *Cancer Epidemiol Biomarkers Prev*, *22*(6), 1079–87. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23563887

Mistlberger, R. (2015). Sleep and circadian rhythms: Reciprocal partners in the regulation of

physiology and behavior. In C. S. Colwell (Ed.), *Circadian Medicine* (pp. 57–80). New Jersey: John Wiley & Sons, Inc.
Montague, P. (2005). The precautionary principle: World Commission on the Ethics of Scientific Knowledge and Technology (COMEST), 1–52.
Moore, L.E., Huang, W-Y, Chung, J., Hayes, R. B. (2003). Epidemiologic considerations to assess altered DNA methylation from environmental exposures in cancer. *Ann N Y Acad Sci*, *983*, 181–196.
Moore, L. E., Pfeiffer, R. M., Poscablo, C., Real, F. X., Kogevinas, M., Silverman, D., … Malats, N. (2008). Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study. *Lancet Oncol*, *9*(4), 359–366. doi:10.1016/S1470-2045(08)70038-X
Morris, N., Elston, R. C., Barnholtz-Sloan, J. S., Sun, X., & Comuzzie, A. G. (2015). Novel approaches to the analysis of family data in genetic epidemiology. *Front Genet*, *6*(27), 1–2. doi:10.3389/fgene.2015.00027
Morris, T. J., & Beck, S. (2015). Analysis pipelines and packages for Infinium HumanMethylation450 BeadChip (450k) data. *Methods*, *72*(C), 3–8. doi:10.1016/j.ymeth.2014.08.011
Morris, T. J., Butcher, L. M., Feber, A., Teschendorff, A. E., Chakravarthy, A. R., Wojdacz, T. K., & Beck, S. (2014). ChAMP: 450k Chip Analysis Methylation Pipeline. *Bioinformatics*, *30*(3), 428–430. doi:10.1093/bioinformatics/btt684
Moure-Eraso, R. (2002). Primary prevention and precaution in hazard identification in the NIEHS/NTP: body in the morgue approach. *Public Health Reports*, *117*(6), 564–573.
Naus, C. C., & Laird, D. W. (2010). Implications and challenges of connexin connections to cancer. *Nat Rev Cancer*, *10*(6), 435–441. doi:10.1038/nrc2841
Negrini, S., Gorgoulis, V. G., & Halazonetis, T. D. (2010). Genomic instability — an evolving hallmark of cancer. *Nat Rev Mol Cell Biol*, *11*(March), 220–228. doi:10.1038/nrm2858
O'Leary, E. S., Schoenfeld, E. R., Stevens, R. G., Kabat, G. C., Henderson, K., Grimson, R., … Leske, M. C. (2006). Shift work, light at night, and breast cancer on Long Island, New York. *Am J Epidemiol*, *164*(4), 358–366. doi:10.1093/aje/kwj211
Ou, Y., Zheng, X., Gao, Y., Shu, M., Leng, T., Li, Y., … Hu, H. (2014). Activation of cyclic AMP/PKA pathway inhibits bladder cancer cell invasion by targeting MAP4-dependent microtubule dynamics. *Urol Oncol*, *32*(1), 47.e21–47.e28. doi:10.1016/j.urolonc.2013.06.017
Paine, S.-J. (2006). The epidemiology of morningness/eveningness: Influence of age, gender, ethnicity, and socioeconomic factors in adults (30-49 Years). *J Biol Rhythms*, *21*(1), 68–76. doi:10.1177/0748730405283154
Pan, A., Schernhammer, E. S., Sun, Q., & Hu, F. B. (2011). Rotating night shift work and risk of type 2 diabetes: Two prospective cohort studies in women. *PLoS Medicine*, *8*(12).
Papantoniou, K., Castaño-Vinyals, G., Espinosa, A., Aragonés, N., Pérez-Gómez, B., Burgos, J., … Kogevinas, M. (2015). Night shift work, chronotype and prostate cancer risk in the MCC-Spain case-control study. *Int. J. Cancer*, *00*, n/a–n/a. doi:10.1002/ijc.29400
Parent, M. É., El-Zein, M., Rousseau, M. C., Pintos, J., & Siemiatycki, J. (2012). Night work and the risk of cancer among men. *Am J Epidemiol*, *176*(9), 751–759. doi:10.1093/aje/kws318
Parsons, M. J., Lester, K. J., Barclay, N. L., Archer, S. N., Nolan, P. M., Eley, T. C., & Gregory, A. M. (2014). Polymorphisms in the circadian expressed genes PER3 and ARNTL2 are associated with diurnal preference and GNβ3 with sleep measures. *J of Sleep Res*, *23*(5),

595–604. doi:10.1111/jsr.12144

Pearce, N. (2004). Public health and the precautionary principle. In M. Martuzzi & J. Tickner (Eds.), *The precautionary principle: Protecting public health, the environment and the future of our children* (pp. 49–62).

Pendergast, J. S., Niswender, K. D., & Yamazaki, S. (2012). Tissue-specific function of period3 in circadian rhythmicity. *PLoS ONE*, *7*(1), 1–5. doi:10.1371/journal.pone.0030254

Pidsley, R., Y Wong, C. C., Volta, M., Lunnon, K., Mill, J., & Schalkwyk, L. C. (2013). A data-driven approach to preprocessing Illumina 450K methylation array data. *BMC Genomics*, *14*(293), 1–10. doi:10.1186/1471-2164-14-293

Powers, M., & Faden, R. (2006). *Social justice: The moral foundations of public health and health policy*. (J. Harris & S. Holm, Eds.). Oxford: Oxford University Press.

Rajaratnam, S. M., & Arendt, J. (2001). Health in a 24-h society. *Lancet*, *358*(9286), 999–1005. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11583769

Rajaratnam, S. M. W., Howard, M. E., & Grunstein, R. R. (2013). Sleep loss and circadian disruption in shift work: health burden and management. *Med J Aust*, *199*(8), 11–15. doi:10.5694/mja13.10561

Rana, S., Munawar, M., Shahid, A., Malik, M., Ullah, H., Fatima, W., … Mahmood, S. (2014). Deregulated expression of circadian clock and clock-controlled cell cycle genes in chronic lymphocytic leukemia. *Mol Biol Rep*, *41*(1), 95–103. doi:10.1007/s11033-013-2841-7

Rao, D., Yu, H., Bai, Y., Zheng, X., & Xie, L. (2015). Does night-shift work increase the risk of prostate cancer? A systematic review and meta-analysis. *OncoTargets and Therapy*, *8*, 2817–2826. doi:10.2147/OTT.S89769

Rebollo, R., Romanish, M. T., & Mager, D. L. (2012). Domesticated transposable element gene products in human cancer. *Mob Genet Elements*, *46*(5), 21–42. doi:10.4161/mge.26693

Revenkova, E., Eijpe, M., Heyting, C., Gross, B., & Jessberger, R. (2001). Novel meiosis-specific isoform of mammalian SMC1. *Mol Cell Biol*, *21*(20), 6984–6998. doi:10.1128/MCB.21.20.6984-6998.2001

Rosner, D., & Markowitz, G. (2002). Industry challenges to the principle of prevention in public health: The precautionary principle in historical perspective. *Public Health Reports*, *117*(6), 501–512. doi:10.1016/S0033-3549(04)50195-4

Rost, S., Fregin, A., Ivaskevicius, V., Conzelmann, E., Hörtnagel, K., Pelz, H.-J., … Oldenburg, J. (2004). Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature*, *427*(6974), 537–41. doi:10.1038/nature02214

SA, M., Dykes, D., & Polesky, H. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*, *16*(3), 55404–55404.

Saenz, R. (2008). A demographic profile of U.S. workers around the clock. Retrieved January 1, 2016, from http://www.prb.org/Publications/Articles/2008/workingaroundtheclock.aspx

Saksvik, I. B., Bjorvatn, B., Hetland, H., Sandal, G. M., & Pallesen, S. (2011). Individual differences in tolerance to shift work - A systematic review. *Sleep Med Rev*, *15*(4), 221–235. doi:10.1016/j.smrv.2010.07.002

Sancar, A., Lindsey-Boltz, L. A., Gaddameedhi, S., Selby, C. P., Ye, R., Chiou, Y.-Y., … Ozturk, N. (2015). Circadian clock, cancer, and chemotherapy. *Biochemistry*. doi:10.1021/bi5007354

Sancar, A., Lindsey-Boltz, L. A., Kang, T.-H., Reardon, J. T., Lee, J. H., & Ozturk, N. (2010). Circadian clock control of the cellular response to DNA damage. *FEBS Lett*, *584*(12), 2618–2625. doi:10.1016/j.surg.2006.10.010.Use

Santoro, R., Mori, F., Marani, M., Grasso, G., Cambria, A. M., Blandino, G., … Strano, S. (2013). Blockage of melatonin receptors impairs p53-mediated prevention of DNA damage accumulation. *Carcinogenesis*, *34*(5), 1051–1061. doi:10.1093/carcin/bgt025

Schaid, D. J. (2010). Genomic similarity and kernel methods I: Advancements by building on mathematical and statistical foundations. *Human Heredity*, *70*(2), 109–131. doi:10.1159/000312641

Schernhammer, E.S., Laden, F., Speizer, F. E., Willett, W.C., Hunter, D., J., Kawachi, I., Colditz, G. A. (2001). Rotating night shifts and risk of breast cancer in women participating in the Nurses' Health Study. *J Natl Cancer Inst*, *93*(20), 1563–1568.

Schernhammer, E. S., Kroenke, C. H., Laden, F., & Hankinson, S. E. (2006). Night work and risk of breast cancer. *Epidemiology*, *17*(1), 108–111. doi:10.1097/01.ede.0000190539.03500.c1

Schettler, T., & Raffensperger, C. (2004). Why is a precautionary approach needed? In M. Martuzzi & J. Tickner (Eds.), *The precautionary principle: Protecting public health, the environment and the future of our children*. (pp. 63–84).

Science & Environmental Health Network. Wingspread Conference on the Precautionary Principle. (1998). Retrieved January 1, 2016, from http://www.sehn.org/wing.html

Shammas, M. a. (2011). Repetitive sequences, genomic instability and Barrett's esophageal adenocarcinoma. *Mob Genet Elements*, *1*(3), 208–212. doi:10.4161/mge.1.3.17456

Singer, M., Kosti, I., Pachter, L., & Mandel-Gutfreund, Y. (2015). A diverse epigenetic landscape at human exons with implication for expression. *Nucleic Acids Res*, *43*(7), 3498–3508. doi:10.1093/nar/gkv153

Smith, C. S., Reilly, C., & Midkiff, K. (1989). Evaluation of three circadian rhythm questionnaires with suggestions for an improved measure of morningness. *J Appl Psychol*, *74*(5), 728–738.

Solyom, S., & Jr, H. H. K. (2012). Mobile elements in the human genome: implications for disease. *Genome Med*, *4*(12), 1–9.

Stevens, R. G., Hansen, J., Costa, G., & Rüdiger, H. W. (2011). Considerations of circadian impact for defining "shift work" in cancer studies: IARC Working Group Report. *Occup Environ Med*, *68*, 154–162.

Stirling, A., & Gee, D. (2002). Science, precaution, and practice. *Public Health Reports*, *117*(December), 521–533.

Stuber, J. (2016). My husband died by suicide. Here's what happened during my awkward call with the NRA. *The Washington Post*. Retrieved from https://www.washingtonpost.com/opinions/i-made-an-awkward-call-to-the-nra-and-found-that-we-had-common-ground/2016/04/07/4214bed2-fb67-11e5-886f-a037dba38301_story.html?utm_term=.5b3e9e843c78

Sundaram, S., Johnson, A., & Makowski, L. (2013). Obesity, metabolism and the microenvironment: Links to cancer. *J Carcinog*, *12*(19).

Suter, M. A., & Aagaard-Tillery, K. M. (2009). Environmental influences on epigenetic profiles. *Semin Reprod Med*, *27*(5), 380–390. doi:10.1055/s-0029-1237426

Szyf, M. (2012). DNA methylation signatures for breast cancer classification and prognosis. *Genome Med*, *4*(26), 1–12.

Tadmor, T., Bejar, J., Attias, D., Mischenko, E., Sabo, E., Neufeld, G., & Vadasz, Z. (2013). The expression of lysyl-oxidase gene family members in myeloproliferative neoplasms. *Am J Hematol.*, *88*(5), 355–358. doi:10.1002/ajh.23409

Takahashi, H., Furukawa, T., Yano, T., Sato, N., Takizawa, J., Kurasaki, T., … Aizawa, Y. (2007). Identification of an overexpressed gene, HSPA4L, the product of which can provoke prevalent humoral immune responses in leukemia patients. *Exp Hematol*, *35*(7), 1091–1099. doi:10.1016/j.exphem.2007.03.015

Thomas, K. A., Freitas, J. De, Descioli, P., & Pinker, S. (2016). Recursive mentalizing and common knowledge in the bystander effect recursive mentalizing and common knowledge in the bystander effect. *J Exp Psychol Gen.*, *145*(5), 621–9. doi:10.1037/xge0000153

Triche, T. J., Weisenberger, D. J., Van Den Berg, D., Laird, P. W., & Siegmund, K. D. (2013). Low-level processing of Illumina Infinium DNA Methylation BeadArrays. *Nucleic Acids Res*, *41*(7), 1–11. doi:10.1093/nar/gkt090

Tusher, V. G., Tibshirani, R., & Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA*, *98*(9), 5116–21. doi:10.1073/pnas.091062498

Tynes, T., Hannevik, M., Andersen, A., Vistnes, A., & Haldorsen, T. (1996). Incidence of breast cancer in Norwegian female radio and telegraph operators. *Cancer Causes Control*, *7*(2), 197–204.

Unoki, M., & Nakamura, Y. (2003). Methylation at CpG islands in intron 1 of EGR2 confers enhancer-like activity. *FEBS Lett*, *554*(1-2), 67–72. doi:10.1016/S0014-5793(03)01092-5

van Amelsvoort, L. G. P. M., Jansen, N. W. H., & Kant, I. (2006). Smoking among shift workers: More than a confounding factor. *Chronobiol Int*, *23*(6), 1105–1113. doi:10.1080/07420520601089539

Vladar, E. K., Lee, Y. L., Stearns, T., & Axelrod, J. D. (2015a). Sleep as a potential fundamental contributor to cardiovascular health disparities. *Ann Rev Public Health*, *36*, 37–54. doi:10.1016/bs.mcb.2015.01.016.Observing

Vladar, E. K., Lee, Y. L., Stearns, T., & Axelrod, J. D. (2015b). The proportion of cancer attibutable to occupational exposures. *Ann Epidemiol.*, *25*(3), 188–192. doi:10.1016/bs.mcb.2015.01.016.Observing

Vyas, M. V., Garg, A. X., Iansavichus, A. V., Costella, J., Donner, A., Laugsand, L. E., … Hackam, D. G. (2012). Shift work and vascular events: systematic review and meta-analysis. *BMJ*, *345*, 1–11.

Waitzman, N. J., & Smith, K. R. (1998). Phantom of the area: Poverty-area residence and mortality in the United States. *Am J Public Health*, *88*(6), 973–976. doi:10.2105/AJPH.88.6.973

Wang, F., Yeung, K. L., Chan, W. C., Kwok, C. C. H., Leung, S. L., Wu, C., … Tse, L. A. (2013). A meta-analysis on dose-response relationship between night shift work and the risk of breast cancer. *Ann Oncol*, *24*(11), 2724–2732. doi:10.1093/annonc/mdt283

Wang, F., Yeung, K. L., Chan, W. C., Kwok, C. C., Leung, S. L., Wu, C., … Tse, L. A. (2015). A meta-analysis on dose-response relationship between night shift work and the risk of colorectal cancer. *Oncotarget*, *6*(28), 25046–25060. doi:10.1093/annonc/mdt283

Wang, P., Ren, F. M., Lin, Y., Su, F. X., Jia, W. H., Su, X. F., … Ren, Z. F. (2015). Night-shift work, sleep duration, daytime napping, and breast cancer risk. *Sleep Med*, *16*(4), 462–468. doi:10.1016/j.sleep.2014.11.017

Wang, X., Yan, D., Teng, M., Fan, J., Zhou, C., Li, D., … Peng, Z. (2012). Reduced expression of PER3 is associated with incidence and development of colon cancer. *Ann Surg Oncol*, *19*(9), 3081–8. doi:10.1245/s10434-012-2279-5

Wiekmeijer, A.-S., Pike-Overzet, K., Brugman, M. H., van Eggermond, M. C. J. A., Cordes, M.,

de Haas, E. F. E., … Staal, F. J. T. (2016). Overexpression of LMO2 causes aberrant human T-Cell development in vivo by three potentially distinct cellular mechanisms. *Exp Hematol*. doi:10.1016/j.exphem.2016.06.002

Wilhelm-Benartzi, C., Koestler, D., Karagas, M., Flanagan, J., Christensen, B., Kelsey, K., … Brown, R. (2013). Review of processing and analysis methods for DNA methylation array data. *British Journal of Cancer*, *109*(10), 1394–1402. doi:10.1038/bjc.2013.496

Wilson, A. S., Power, B. E., & Molloy, P. L. (2007). DNA hypomethylation and human diseases. *Biochim Biophys Acta*, *1775*(1), 138–162. doi:10.1016/j.bbcan.2006.08.007

World Health Organization. (2003). The social determinants of health: The solid facts.

Wu, L., Xu, Y., Wang, Q., Ruan, C., Drexler, H. G., Wu, D., … Chen, S. (2015). High frequency of cryptic chromosomal rearrangements involving the LMO2 gene in T-cell acute lymphoblastic leukemia. *Haematologica*, *100*(6), 100:e233–100:e236. doi:10.3324/haematol.2014.108258

Wu, M. C., Kraft, P., Epstein, M. P., Taylor, D. M., Chanock, S. J., Hunter, D. J., & Lin, X. (2010). Powerful SNP-set analysis for case-control genome-wide association studies. *Am J Hum Genet*, *86*(6), 929–942. doi:10.1016/j.ajhg.2010.05.002

Wuest, M., Kuchar, M., Sharma, S. K., Richter, S., Hamann, I., Wang, M., … Löser, R. (2015). Targeting lysyl oxidase for molecular imaging in breast cancer. *Breast Cancer Res*, *17*, 107. doi:10.1186/s13058-015-0609-9

Yang, W. S., & Stockwell, B. R. (2008). Inhibition of casein kinase 1-epsilon induces cancer-cell-selective, PERIOD2-dependent growth arrest. *Genome Biology*, *9*(6), 1–13. doi:10.1186/gb-2008-9-6-r92

Yang, X., Han, H., DeCarvalho, D., Lay, F., Jones, P., & Liang, G. (2014). Gene body methylation can alter gene expression and is a therapeutic target in cancer. *Cancer Cell*, *26*(4), 577–590. doi:10.1016/j.ccr.2014.07.028

Yu, H. W., Chen, Y. Q., Huang, C. M., Liu, C. Y., Chiou, A., Wang, Y. K., … Kuo, J. C. (2015). B-PIX controls intracellular viscoelasticity to regulate lung cancer cell migration. *J Cell Mol Med*, *19*(5), 934–947. doi:10.1111/jcmm.12441

Yu, X., Rollins, D., Ruhn, K. A., Stubblefield, J. J., Green, C. B., Kashiwada, M., … Hooper, L. V. (2013). TH17 cell differentiation is regulated by the circadian clock. *Science*, *342*(8), 727–730.

Zhao, N., Chen, J., Carroll, I. M., Ringel-Kulka, T., Epstein, M. P., Zhou, H., … Wu, M. C. (2015). Testing in microbiome-profiling studies with MiRKAT, the microbiome regression-based kernel association test. *Am J Hum Genet.*, *96*(5), 797–807. doi:10.1016/j.ajhg.2015.04.003

Zhou, F. C., Balaraman, Y., Teng, M., Liu, Y., Singh, R., & Nephew, K. P. (2011). Alcohol alters DNA methylation patterns and inhibits neural stem cell differentiation. *Alcohol Clin Exp Res*, *35*(4), 735–746. doi:10.1111/j.1530-0277.2010.01391.x

Zhu, Y., Stevens, R. G., Hoffman, A. E., Tjonneland, A., Vogel, U. B., Zheng, T., & Hansen, J. (2011). Epigenetic impact of long-term shiftwork: Pilot evidence from circadian genes and whole-genome methylation analysis. *Chronobiol Int*, *28*(10), 852–61.