



Chronobiology International The Journal of Biological and Medical Rhythm Research

ISSN: 0742-0528 (Print) 1525-6073 (Online) Journal homepage: http://www.tandfonline.com/loi/icbi20

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To cite this article: Parveen Bhatti, Yuzheng Zhang, Xiaoling Song, Karen W. Makar, Cassandra L. Sather, Karl T. Kelsey, E. Andres Houseman & Pei Wang (2015) Nightshift work and genome-wide DNA methylation, Chronobiology International, 32:1, 103-112, DOI: 10.3109/07420528.2014.956362

To link to this article: http://dx.doi.org/10.3109/07420528.2014.956362

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ORIGINAL ARTICLE

Nightshift work and genome-wide DNA methylation

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The negative health effects of shift work, including carcinogenesis, may be mediated by changes in DNA methylation, particularly in the circadian genes. Using the Infinium HumanMethylation450 Bead Array (Illumina, San Diego, CA), we compared genome-wide methylation between 65 actively working dayshift workers and 59 actively working nightshift workers in the healthcare industry. A total of 473 800 loci, including 391 loci across the 12 core circadian genes, were analyzed to identify methylation markers associated with shift work status using linear regression models adjusted for gender, age, body mass index, race, smoking status and leukocyte cell profile as measured by flow cytometry. Analyses at the level of gene, CpG island and gene region were also conducted. To account for multiple comparisons, we controlled the false discovery rate (FDR ≤0.05). Significant differences between nightshift and dayshift workers were found at 16135 of 473 800 loci, across 3769 of 20164 genes, across 7173 of 22721 CpG islands and across 5508 of 51 843 gene regions. For each significant loci, gene, CpG island or gene region, average methylation was consistently found to be decreased among nightshift workers compared to dayshift workers. Twenty-one loci located in the circadian genes were also found to be significantly hypomethylated among nightshift workers. The largest differences were observed for three loci located in the gene body of PER3. A total of nine significant loci were found in the CSNK1E gene, most of which were located in a CpG island and near the transcription start site of the gene. Methylation changes in these circadian genes may lead to altered expression of these genes which has been associated with cancer in previous studies. Gene ontology enrichment analysis revealed that among the significantly hypomethylated genes, processes related to host defense and immunity were represented. Our results indicate that the health effects of shift work may be mediated by hypomethylation of a wide variety of genes, including those related to circadian rhythms. While these findings need to be followed-up among a considerably expanded group of shift workers, the data generated by this study supports the need for future targeted research into the potential impacts of shift work on specific carcinogenic mechanisms.

Keywords: Circadian genes, DNA methylation, shift work

INTRODUCTION

The biology underlying observed associations between nightshift work and cancer has yet to be fully elucidated. A number of promising mechanisms have been investigated, including melatonin suppression leading to increased circulating sex hormones and increased DNA damage (Costa et al., 2010). However, relatively little attention has been given to epigenetic mechanisms that may impact gene expression, particularly of circadian genes. Twelve genes central to the regulation of the normal 24-h circadian clock have been identified. These core circadian genes are also involved in various mechanisms important to carcinogenesis, including cell cycle control, DNA damage response (Gery & Koeffler, 2007) and immunity (Baumann et al., 2013; Nguyen et al., 2013; Yu et al., 2013). Thus, differential expression of these genes, through epigenetic modulation, may be a mechanism by which nightshift workers are at an increased risk of developing cancer.

DNA methylation, which occurs at the 5' carbon of cytosines in CpG dinucleotides throughout the genome, is an epigenetic mechanism essential for proper cellular function. Differential methylation at CpG loci has been closely linked to differential expression of the genes with

Submitted June 18, 2014, Returned for revision August 14, 2014, Accepted August 17, 2014

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which they are associated (Ball et al., 2009; Bell et al., 2011; Rauch et al., 2009). For example, increased methylation of promoter regions of genes has been associated with a decreased transcription of those genes (Bell et al., 2011). Exposures to various exogenous agents including smoking and air pollution have been shown to modulate DNA methylation (Baccarelli & Bollati, 2009; Baccarelli et al., 2009; Bollati et al., 2007; Breitling et al., 2011; Moore et al., 2003). Modulation of DNA methylation by environmental exposures, particularly those with no demonstrable genotoxic effects, may induce carcinogenesis (Suter & Aagaard-Tillery, 2009).

In population-based studies, methylation changes in circadian genes have been associated with cancer risk (Hoffman et al., 2010a, b), but only a single study among women has previously evaluated whether nightshift work may specifically modulate circadian gene methylation in blood (Zhu et al., 2011). In addition to evaluating promoter-specific levels of DNA methylation in two circadian genes, this previous study compared methylation levels at each of ~27 000 CpG loci across the genome between female nightshift and dayshift workers. To expand on this previous work, we explored methylation differences at ~450 000 CpG loci, including a total of 391 loci across the 12-core circadian genes in blood, between a group of actively working female and male nightshift and dayshift workers in the healthcare industry.

MATERIALS AND METHODS

Study population

The study was approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center and meets the ethical standards of the journal (Portaluppi et al., 2010). The study population consisted of women and men who were currently employed as health care workers in the Seattle metropolitan area. 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. Eligibility criteria included being 20 to 40 years of age, with a body mass index (BMI) between 18 and 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 in the last six months, and women could not currently be pregnant. Dayshift workers were required to be exclusively working the dayshift for the past 6 months at least 24 h per week with the dayshift beginning no earlier than 06:00 h. Dayshift workers must not have worked any nights in the past 6 months. Nightshift workers could be currently engaged in some day work, but were required to be primarily working the nightshift for the past 6 months at least 24 h per week with the nightshift ending no earlier than 06:00 h. All dayshift and nightshift workers were required to have schedules where they typically worked at least three consecutive days or nights, respectively, each week. A total of 106 dayshift and 94 nightshift workers were screened. Seventy dayshift and 69 nightshift workers were deemed eligible for the study. Two dayshift workers and four nightshift workers decided not to enter the study, and an additional three dayshift and six nightshift workers dropped out during the course of the study, giving a final sample size of 65 dayshift and 59 nightshift workers.

Data and biospecimen collection

After a brief telephone screening interview, potentially eligible subjects were visited at home or work to further describe the study and to obtain informed consent. The interviewer also collected height and weight measurements and scheduled a subsequent appointment for blood sample collection, which was to be completed only after the subject worked his/her respective shift schedule each day for three consecutive days. Blood samples were collected (10 mL ACD tube) within two hours of completing the work shift on the third day (07:00-09:00 h for nightshift workers and 17:00-19:00 h for dayshift workers). At the time of blood collection, a structured interview was administered to collect data about physical activity, current work and sleep schedules, reproductive and menstrual history (for female participants) and current medication, supplement, caffeine, alcohol and tobacco use.

Laboratory methods

Lymphocyte isolation and cryopreservation

The ACD blood samples were processed within 24 h 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, St. Louis, MO) 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/min to -80° C, samples were transferred to a liquid nitrogen freezer for storage.

Flow cytometry

Cryopreserved PBMCs were thawed and counted using Trypan blue. A total of 200 000 cells were resuspended in staining buffer (130-221-091, Miltenyi Biotec, San Diego, CA) and stained with the following antibodies: CD14-APC-Cy7 (557831, BD Biosciences, San Jose, CA), CD45-AF700 (304024 Biolegend, San Diego, CA), CD3-VioBlue (130-094-363 Miltenyi Biotec, San Diego, CA), CD19-APC (130-091-248 Miltenyi Biotec, San Diego, CA), CD19-APC (130-091-248 Miltenyi Biotec, San Diego, CA), CD56-PE (130-00-755 Miltenyi Biotec, San Diego, CA), CD4-FITC (300505 Biolegend, San Diego, CA), CD8-PE-Cy7 (560917 BD Biosciences, San Jose, CA). Cells were incubated for 15 min on ice in the dark, washed with staining buffer and resuspended in fixation buffer (420801 Biolegend, San Diego, CA). Flow cytometric analysis was carried out on an LSR-II (BD Biosciences, San Diego, CA) in the FHCRC Flow Cytometry Shared Resource. Data analysis was performed using FlowJo (Tree Star, Ashland, OR) software to determine the relative percentages of monocytes, granulocytes, B cells, NK cells, CD4+ T cells and CD8+ T cells within each sample following the gating strategies previously described (Calvelli et al., 1993).

DNA extraction and bisulfite conversion

DNA was extracted from remaining cryopreserved lymphocytes (ArchivePure DNA Purification Kit, 5-Prime, Hilden, Germany) after cells were removed for flow cytometric analysis. 500 ng of DNA was then treated with sodium bisulfite using EZ DNA Methylation-DirectKit (Zymo Research, Irvine, CA). Sodium bisulfite converts unmethylated cytosine into uracil. Treated DNA specimens were stored at -80 °C and assayed within two weeks.

Methylation assay

The Infinium HumanMethylation450 Bead Array (Illumina, San Diego, CA) was used to interrogate 485 577 carefully chosen methylation sites per sample. The CpG loci were chosen by an expert consortium and covers 21154 genes, with an average of 17 CpG sites per gene distributed across various regions including the first exon, 3' and 5' untranslated regions, the gene body and close proximity to transcription start sites and may be associated with informatically determined elements, such as enhancers and promoters. Within each gene, CpGs can also occur within or near CpG islands. The array included 391 CpG loci distributed across the 12-core circadian genes: CLOCK, ARNTL, NPAS2, PER1, PER2, PER3, CRY1, CRY2, RORA, NR1D1, CSNK1D and CSNK1E. 4 µl of bisulfite treated DNA was denatured and neutralized to prepare it for overnight isothermal wholegenome 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). Methylation data were normalized for any potential dye-bias by using the "methylumi" package in R (Davis et al., 2014). Each CpG dinucleotide represented on the microarray was associated with an *M*-value which was calculated as the log₂ ratio of the intensities of the methylated and unmethylated probes. A detection p value was also generated for each locus (i.e. tests that the target signal was distinguishable from negative controls). Laboratory personnel were blinded to all study subject information, and specimens were identified by study ID number only. Twelve blind

duplicate DNA samples were randomly included among the study samples.

Statistical analysis

The M-values for each CpG site were modeled as dependent variables using linear regression that included a variable for shift work status, gender, age, BMI, race (White or Non-White) and current smoking status (yes/no). To account for changes in DNA methylation due to variation in leukocyte cell mixture, we also included in our regression models variables for the proportions of each of six major leukocyte subsets (CD8+ T-cells, CD4+ T-cells, natural killer cells, B-cells, monocytes and granulocytes) in each sample measured using flow cytometry. In addition to conducting analyses at the level of each CpG site, analyses at the level of CpG island and region within gene were conducted when three or more loci occurred within that CpG island or gene region (gene, CpG island and gene region were assessed using annotation data provided by Illumina). Any loci located at the "shelf" or "shore" of a particular CpG island were included as part of that CpG island. Any CpG loci that occurred in more than one CpG island or gene region were included in multiple CpG islands or gene regions for the purposes of these analyses. For each gene, CpG island or region within gene, the average M-values of CpG sites associated with that gene, CpG island or region within gene were calculated, and those average values were used to fit linear regression models evaluating associations with shift work status.

In order to account for multiple comparisons, we applied the Significant Analysis of Microarray (SAM) method (Tusher et al., 2001) implemented in the *R* package "samr" to control for the false discovery rate (FDR) within each level of analysis (locus level, genomic context level and gene level), such that on average only 5% of those differences deemed statistically significant were false positives. Specifically, we first subtracted other covariate effects from the *M*-values, and then tested the association between the adjusted *M*-values and shift work status using "samr". The average ratio of adjusted *M*-values among nightshift workers versus dayshift workers was reported as an estimate of the percent change in methylation.

For the gene-level analyses, we used the on-line Gene Ontology enRIchment anaLysis and visualization tool (GOrilla) to determine if the genes found to be significantly differently methylated belonged to various Gene Ontology processes more often than predicted by chance (Eden et al., 2009). All genes with $q \leq 0.05$ were submitted and ranked from largest to smallest according to the difference from unity of the nightshift versus dayshift ratio of methylation observed across each gene.

RESULTS

In Table 1, the distribution of selected demographic variables is presented by shift status. Nightshift workers

	Dayshift	Nightshift
	1V (70)	IV (70)
Gender		
Female	38 (58)	36 (61)
Male	27 (41)	23 (39)
Age		
22–29	22 (34)	32 (54)
30-34	20 (31)	15 (25)
35-40	23 (35)	12 (20)
Body Mass Index (kg/n	n ²)	
<21.7	16 (25)	15 (25)
≥ 21.7	14 (22)	18 (31)
≥ 24.7	24 (37)	6 (10)
≥ 26.9	11 (17)	20 (33)
Race		
White	47 (73)	48 (81)
Asian	12 (18)	7 (12)
Black	2 (3)	3 (5)
Other	4 (6)	1 (2)
Current smoker		
Yes	3 (5)	2 (3)
No	62 (95)	57 (97)
Months engaged in cur	rent shift schedule	
≤ 12	13 (20)	15 (25)
>12-24	18 (28)	18 (31)
>24-48	24 (37)	11 (19)
>48	10 (15)	15 (25)
Proportions of blood co	ell types (Means)	
B-cells	0.08	0.08
CD4+ T-cells	0.4	0.4
CD8+ T-cells	0.2	0.2
granulocytes	0.03	0.04
monocytes	0.08	0.07
NK cells	0.12	0.09

TABLE 1. Demographic data and blood cell profile among dayshift and nightshift study participants.

tended to be younger and have a higher BMI than dayshift workers. Also dayshift workers tended to be engaged in their current shift schedule slightly longer than nightshift workers (median 30 versus 24 months, respectively). Mean proportions of six major blood cell types measured by flow cytometry are also provided; no notable differences by shift status were observed.

The correlation coefficients between *M*-values of the 12 replicate pairs ranged from 0.96 to 0.99. Those 11 648 loci associated with sex chromosomes, as indicated by the annotation file provided by Illumina, were excluded since sex-stratified analyses would be underpowered. An additional 129 loci which were undetected (detection *p* value >0.05) in \geq 20% of study samples were also excluded from analyses, leaving a total of 473 800 loci, including 391 loci across the 12 circadian genes.

Significant associations (FDR ≤ 0.05) were found at 16 135 loci, and at each locus, methylation levels were significantly lower among nightshift workers (Supplementary Table 1). Seventy-five percent of these loci were located within known genes, which was identical to the proportion of loci occurring within known genes in the entire dataset. Eighteen percent of these loci were associated with a single nucleotide polymorphism (SNP present within probe ≤ 10 base

pairs from query site or >10 base pairs from query site as indicated by Illumina), a similar proportion to the overall dataset. The proportion of significant loci located within CpG islands (65%), 200 base pairs of a transcription start site (11%), 1500 base pairs of a transcription start site (17%), 3' untranslated regions (4%), 5' untranslated regions (13%), 1st Exons (7%) and gene bodies (38%) were comparable to respective proportions in the overall dataset. The largest loci-specific methylation effect was a 33% lower level of methylation at the cg05704942 locus in the *MRTO4* gene (Table 2) among nightshift compared to dayshift workers.

At the level of gene, significant associations (FDR ≤ 0.05) were observed across 3769 of 20164 genes and average methylation levels across each of these genes were significantly lower among nightshift workers (Supplementary Table 2). The largest gene-level effect was an 11% lower average methylation level across the two loci belonging to the *PPIAL4A/PPIAL4B* gene (Table 2). The probes used to assay one of the loci in this gene contained a SNP (rs76383089). The minor allele frequency (MAF) of this SNP was not available through National Center for Biotechnology Information dbSNP (http://www.ncbi.nlm.nih.gov/snp/); a high MAF would indicate that the occurrence of the SNP may have influenced results.

CpG loci were found to occur within 24592 CpG islands, with 1871 of these being represented by only 1 or 2 CpG loci. When restricting to those CpG islands containing three or more CpG loci (n=22721), average methylation was found to be significantly decreased among nightshift workers compared to dayshift workers across 7173 of these CpG islands ($q \le 0.05$) (Supplementary Table 3). The largest CpG-island level effects were 11% lower levels of methylation across the 13 CpG loci within a CpG island in the *DUSP22* gene (chr6:291948-292839) and across the 8 CpG loci within a CpG island in the *ZNF714* gene (chr19:21265164-21265433). Nine of these *DUSP22* loci were associated with a promoter region.

CpG loci were grouped into 89461 gene-specific regions, with 37618 of these being represented by only 1 or 2 CpG loci. When restricting to those gene regions containing three or more CpG loci (n = 51843), average methylation was found to be significantly reduced among nightshift workers compared to dayshift workers across 5508 of these gene regions (FDR ≤ 0.05) (Supplementary Table 4). In the gene-region analyses, the largest effect was observed for the 1st Exon/5' UTR region of *DUSP22*. This region, made up of three loci, exhibited a 15% lower level of DNA methylation among nightshift workers compared to dayshift workers.

Among the loci with FDR \leq 0.05, 21 were located in the circadian genes, including *CLOCK*, *CSNK1D*, *CSNK1E*, *NPAS2*, *PER2*, *PER3* and *RORA* (Table 3). The largest effects were seen for three loci within close proximity of each other in the body of the *PER3* gene that were 8% less methylated in nightshift workers as

TABLE 2. DNA methylation differences between nightshift and dayshift workers.

	Number		.			Gene	Enhancer/	D of f	
Gene	of Loci	Illumina ID	Location	SNPs (MAFs) ⁵	CpG Island	Region	Promoter	Ratio	FDR [®]
MRTO4	24							0.98	< 0.001
		cg05704942	19454129		chr1:195778849-19578708 ^h	Body ⁱ		0.66	< 0.001
PPIAL4A/4B	2	0						0.88	< 0.001
		cg22106265	146421956	rs76383089 (N/A)		1st Exon		0.97	0.05
		cg23350716	146423368			TSS1500		0.80	< 0.001
DUSP22	36							0.96	0.01
		cg07332563	236687		chr6:291948-292839 ^j	TSS1500 ^k	Promoter	0.90	0.05
		cg21548813	236882		chr6:291948-292839	TSS1500	Promoter	0.84	0.02
		cg03395511	236903		chr6:291948-292839	TSS200	Promoter	0.86	0.03
		cg15383120	236909		chr6:291948-292839	TSS200	Promoter	0.87	0.03
		cg18110333	237329	rs12525419 (N/A)	chr6:291948-292839	1stExon; 5′UTR ¹	Promoter	0.78	0.02
		cg05064044	237385		chr6:291948-292839	1stExon; 5′UTR	Promoter	0.89	0.03
		cg11235426	237522		chr6:291948-292839	1stExon; 5′UTR	Promoter	0.89	0.05
		cg01516881	237596		chr6:291948-292839	Body ^m	Promoter	0.85	0.02
		cg26668828	237823	rs815596 (0.50)	chr6:291948-292839	Body	Promoter	0.82	0.03
		cg01171360	238285	rs7767281 (0.05); rs72838722 (0.002)	chr6:291948-292839	Body		0.90	0.05
ZNF714	9							0.91	0.01
		cg01483656	21056736		chr19:21265164-21265433 ⁿ	TSS200	Promoter	0.80	0.01
		cg17751872	21056822		chr19:21265164-21265433	5′UTR°	Promoter	0.87	0.01
		cg15174294	21057104		chr19:21265164-21265433	5′UTR		0.86	0.02
		cg06238316	21057204	rs8101412 (0.26)	chr19:21265164-21265433	5′UTR		0.85	0.02
		cg09352518	21057261		chr19:21265164-21265433	5′UTR		0.81	0.01

^aLocation in NCBI Genome Build 36.

^bSingle nucleotide polymorphisms associated with probe and minor allele frequencies provided by NCBI dbSNP; N/A = Not Available; (http://www.ncbi.nlm.nih.gov/snp/).

^cAssociated CpG island.

^dFunctional 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; 1stExon = First segment of gene coding for peptide sequence; Body = Within gene body; 3'UTR = 3 prime unstranslated region; multiple listings indicate loci belong to multiple regions because of splice variants.

^eAssociated with informatically determined enhancer or promoter elements.

^tRatio of average methylation at loci among nightshift workers compared to dayshift workers.

^gFalse Discovery Rate.

^h0.98-fold difference across 22 loci of this CpG island in the *MRTO4* gene (q < 0.001).

¹0.97-fold difference across 15 loci of this gene body in the *MRTO4* gene (q < 0.001).

¹0.89-fold difference across 13 loci of this CpG island in the *DUSP22* gene (q = 0.01).

^k0.91-fold difference across 3 loci of this TSS1500 region in the *DUSP22* gene (q = 0.01).

¹0.85-fold difference across 3 loci of this 1stExon/5'UTR region in the *DUSP22* gene (q = 0.01).

^m0.98-fold difference across 25 loci of this gene body in the *DUSP22* gene (q=0.05).

ⁿ0.89-fold difference across 8 loci of this CpG island in the *ZNF714* gene (q < 0.001).

°0.87-fold difference across 5 loci of this 5'UTR in the ZNF714 gene (q = 0.01.)

compared to dayshift workers. The largest number of significantly differentially methylated loci were located in the CSNK1D gene; nine loci were found to be significantly hypomethylated (3-4%) in nightshift workers compared to dayshift workers. Eight of the nine loci were located within the same CpG island and seven were located immediately upstream of the gene transcription start site. Five of these loci were also associated with an enhancer element. At the level of gene, both PER3 and CSNK1E demonstrated the largest effects among the circadian genes; both were 2% less methylated in nightshift workers as compared to dayshift workers. Among CpG islands in the circadian genes, the largest effect was observed in a CpG island of the PER3 gene (5% lower in nightshift workers). The largest generegion effect (3% less methylation in nightshift workers)

was observed near the transcription start site of the *CSNK1E* gene.

In the gene ontology enrichment analysis, 3691 of the 3769 gene names that were submitted were recognized, and 3170 of the genes were associated with a Gene Ontology term. Table 4 lists the 11 Gene Ontology processes, many related to host defense and immunity, found to be significantly represented among the submitted genes.

DISCUSSION

All statistically significant findings in our study entailed decreased DNA methylation in the blood of nightshift workers compared to dayshift workers. In fact, 65% of the 473 800 loci tested were hypomethylated among

Gene	Number of loci	Illumina ID	Location ^a	SNPs (MAFs) ^b	CpG island ^c	Region ^d	Enhancer/ Promoter ^e	Ratio ^f	FDR ^g
ARNTL ^h	24							0.99	0.09
CLOCK	16							0.99	0.03
		cg04264638	56105895		chr4:56411993-56413357 ⁱ	5′UTR ^j		0.96	0.02
CRY1	14							1.00	>0.1
CRY2	24							1.00	>0.1
CSNK1D ^k	45							0.99	0.03
CSNK1E	24							0.98	0.01
		cg14055351	37018667			3′UTR	Enhancer	0.97	0.03
		cg13110239	37041213		chr22:38712684-38713333 ¹	5′UTR ^m	Enhancer	0.97	0.02
		cg02516101	37041837		chr22:38712684-38713333	5′UTR		0.97	0.05
		cg26449680	37044218		chr22:38712684-38713333	TSS1500 ⁿ ;		0.97	0.03
		0				TSS200°			
		cg19781870	37044231		chr22:38712684-38713333	TSS1500		0.97	0.02
		cg06748147	37044233		chr22:38712684-38713333	TSS1500		0.97	0.03
		cg10142237	37044341		chr22:38712684-38713333	TSS1500	Enhancer	0.96	0.02
		cg01441777	37044362		chr22:38712684-38713333	TSS1500	Enhancer	0.97	0.05
		cg06991495	37044412	rs79995406 (N/A)	chr22:38712684-38713333	TSS1500	Enhancer	0.96	0.02
NPAS2	37							1.00	0.09
		cg26574325	100951299			Body		0.97	0.02
		cg27051778	100960753	rs58248145 (0.02)		Body	Enhancer	0.97	0.02
NR1D1	26							1.00	>0.1
PER1 ^p	20							0.99	0.03
PER2	24							0.99	0.05
		cg13469320	238826861			Body		0.98	0.05
		cg23072629	238860276		chr2:239196923-239197900	5′UTR ^q	Enhancer	0.97	0.02
		cg24831107	238861878		chr2:239196923-239197900	1st Exon; 5′UTR		0.97	0.02
PER3 ^r	26							0.98	0.01
		cg17328665	7809786		chr1:7887198-7887476 ^s	Body		0.92	0.01
		ag04705100	7000050		abr1.7007100 7007470	Dedr		0.04	0.05

PER1 ^p	20							0.99	0.03
PEN2	24	cg13469320	238826861			Body		0.99	0.05
		cg23072629	238860276		chr2:239196923-239197900	5'UTR ^q	Enhancer	0.97	0.02
		cg24831107	238861878		chr2:239196923-239197900	1st Exon; 5′UTR		0.97	0.02
PER3 ^r	26							0.98	0.01
		cg17328665	7809786		chr1:7887198-7887476 ^s	Body		0.92	0.01
		cg04725166	7809858		chr1:7887198-7887476	Body		0.94	0.05
		cg17724687	7809933		chr1:7887198-7887476	Body		0.92	0.02
		cg08926642	7810042	rs228696 (0.03)	chr1:7887198-7887476	Body		0.92	0.03
		cg09168692	7810147		chr1:7887198-7887476	Body		0.97	0.03
RORA ^t	111	-				-		1.00	>0.1
		cg20006924	59309268	rs77221633 (N/A)	chr15:61519621-61520031 ^u	TSS1500		0.97	0.02

^aLocation of loci in NCBI Genome Build 36.

^bSingle nucleotide polymorphisms associated with probes and minor allele frequencies provided by NCBI dbSNP; N/A=Not Available; (http://www.ncbi.nlm.nih.gov/snp/).

^cAssociated CpG island.

^dFunctional 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; 1stExon = First segment of gene coding for peptide sequence; Body = Within gene body; 3'UTR = 3

prime unstranslated region; multiple listings indicate loci belong to multiple regions because of splice variants. ^eAssociated with informatically determined enhancer or promoter elements.

^fRatio of average methylation across gene among nightshift workers compared to dayshift workers.

^gFalse Discovery Rate.

^h0.99-fold difference across 14 loci of chr11:13298796-13300735 CpG island (q = 0.04) in the ARNTL gene.

ⁱ0.99-fold difference across 8 loci of this CpG island (q = 0.01) in the CLOCK gene.

^j0.99-fold difference across 9 loci of this 5'UTR (q = 0.03) in the CLOCK gene.

^k0.98-fold difference across 6 loci of chr17:80202799-80203289 CpG island (q = 0.02); 0.99 fold difference across 8 loci of chr17:80206689-80206927 CpG island (q = 0.03); 0.99 fold difference across 13 loci of chr17:80231019-80231820 CpG island (q = 0.03); 0.98 fold difference across 6 loci of 3'UTR (q = 0.02) of CSNK1D gene.

¹0.98-fold difference across 23 loci of chr22:38712684-38713333 CpG island (q=0.01) of CSNK1E gene.

^m0.99-fold difference across 14 loci of 5'UTR (q = 0.03) in the CSNK1E gene.

ⁿ0.97-fold difference across 10 loci of TSS1500 region (q=0.01) of CSNK1E gene.

^o0.98-fold difference across 9 loci of TSS200 region (q = 0.01) of CSNK1E gene.

^p0.99-fold difference across 15 loci of chr17:8054550-8055835 CpG island (q = 0.01); 0.98 fold difference across 4 loci of TSS1500 region (q=0.03) of *PER1* gene.

^q0.99-fold difference across 5 loci of 5'UTR (q = 0.03) in the *PER2* gene.

^r0.99-fold difference across 4 loci of chr1:7843460-7843693 CpG island (q=0.04); 0.99 fold difference across 5 loci in 5'UTR (q=0.03) of PER3 gene.

^s0.95-fold difference across 9 loci of this CpG island (q < 0.001) in the *PER3* gene.

^t0.99-fold difference across 10 loci in chr15:61520423-61521716 CpG island (q = 0.04); 0.99 fold difference across 20 loci of chr15:60883298-60885350 CpG island (q = 0.04) in RORA gene.

^u0.99-fold difference across 8 loci in this CpG island (q = 0.02) in the RORA gene.

GO term	Description	<i>p</i> Value	Number of genes related to GO term
GO:0098542 GO:1901685 GO:1901687 GO:0042742 GO:0002446 GO:0002444 GO:0051707 GO:0009617 GO:0006749	Defense response to other organism Glutathione derivative metabolic process Glutathione derivative biosynthetic process Defense response to bacterium Neutrophil mediated immunity Myeloid leukocyte mediated immunity Response to other organism Response to bacterium Glutathione metabolic process	$2 \times 10^{-5} 9 \times 10^{-5} 9 \times 10^{-5} 1 \times 10^{-4} 2 \times 10^{-4} 2 \times 10^{-4} 2 \times 10^{-4} 2 \times 10^{-4} 4 \times 10^{-4} $	60 8 8 36 3 3 77 39
GO:0043901 GO:0016337	Negative regulation of multi-organism process Cell–cell adhesion	5×10^{-4} 9×10^{-4}	11 11 89

TABLE 4. Gene ontology terms associated with genes across which significant differences in methylation were observed between nightshift and dayshift workers.

nightshift workers. If methylation changes in blood are a surrogate for other tissues, this may indicate nightshift work is associated with a general systemic loss of methylation, which may also lead to increased genomic instability (Ehrlich, 2002) and has been associated with an increased cancer risk (Choi et al., 2009; Gao et al., 2012; Hsiung et al., 2007; Liao et al., 2011; Moore et al., 2008; Pufulete et al., 2003). In terms of gene expression, the effect of this loss of methylation would be dependent on the genomic context. While hypomethylation in upstream regions, such as transcription start sites has been associated with an increased gene expression, gene body hypomethylation may be associated with decreased gene expression or may impact gene splicing (Jones, 2012).

Overall, the most significant reductions in methylation were observed in the MRTO4, PPIAL4A/PPIAL4B, DUSP22 and ZNF714 genes. Little information is available on the specific role of the MRTO4 gene, though in one study, mitochondrial levels of the MRTO4 protein, which appears to be involved in mRNA turnover, were associated with aggressive ovarian cancer (Wang et al., 2011). The two significantly hypomethylated loci in the PPIAL4A/PPIAL4B gene, which codes for a propyl isomerase involved in the folding of proteins, occurred in the first exon and near the transcription start site, and, as such, may be associated with an increased expression of this gene. No studies suggesting a biological impact of increased PPIAL4A/PPIAL4B expression were identified. For both the DUSP22 and ZNF714 genes, significant hypomethylation was observed in CpG islands that included known promoter regions so it is likely that this would result in an increased expression of these genes among nightshift workers. While little is known about the function of ZNF714 gene, DUSP22 is a gene involved in the MAPK signaling pathway and overexpression of this gene has been observed in chronic lymphocytic leukemia (Lewintre et al., 2009).

Statistically significant hypomethylation was observed among the core circadian genes, though the effects were relatively small in magnitude and, as such, their potential to impact gene expression is uncertain. The hypomethylation observed in the gene body of

PER3 could be associated with decreased gene expression. Decreased expression of PER3 has previously been observed in the lymphocytes of shift workers (Bracci et al., 2014). Reduced PER3 expression has been associated with increased risks of colon cancer and mortality from the disease (Wang et al., 2012). The hypomethylation observed in the upstream CpG islands of PER1 and PER2 that occurred in the 5'UTR or near the transcription start site could lead to an increased expression of these genes among nightshift workers, which has been previously observed (Bracci et al., 2014). Increased expression of these genes may, in fact, be associated with anticancer effects since studies of *PER1* and PER2 knockout mice have shown increased susceptibility to tumor development and deregulated cell division and apoptosis (Fu et al., 2002; Gery & Koeffler, 2007; Lee, 2006). Hypomethylation of CSNK1E occurred near the transcription start site within a CpG island and known enhancer element, so this could lead to an increased expression of the gene among nightshift workers. CSNK1E overexpression has been observed in cancer tissues and its effects on cancer may be mediated through down-regulation of the PER genes (Yang & Stockwell, 2008). Given that the three significantly hypomethylated CpG islands within CSNK1D spanned the transcription start site, gene body and 3'UTR, the overall potential impact on expression is uncertain.

The hypomethylated promoter-associated CpG island in the CLOCK gene may lead to an increased expression of the gene among nightshift workers. Increased CLOCK gene expression has been previously observed in the lymphocytes of shift workers (Bracci et al., 2014), and hypomethylation of the *CLOCK* promoter, as measured in blood, has been associated with an increased risk of breast cancer (Hoffman et al., 2010a). A CpG island in ARNTL occurring near the transcription start site was also found to be hypomethylated and may, thus, be overexpressed among nightshift workers. Increased ARNTL expression has been observed among shift workers (Bracci et al., 2014), but the impact of this overexpression on carcinogenesis has not been previously described. Two loci in the body of the NPAS2 gene were found to be significantly hypomethylated, which

may be associated with a decreased expression of the gene among nightshift workers, though a previous study indicated an increased expression of *NPAS2* among shift workers (Bracci et al., 2014). Knockdown of *NPAS2* has been associated with a reduced DNA repair capacity in a previous *in vitro* study (Hoffman et al., 2008).

Hypomethylation observed at the single locus near the transcription start site in *RORA* and the CpG island with which it is associated may lead to an increased expression of the gene among nightshift workers. Increased expression of *RORA* may be beneficial, since it has been associated with the DNA damage response (Shi et al., 2012), and RORA has been shown to be down-regulated in colorectal tumors (Kottorou et al., 2012).

Though we observed no differences in the number of major leukocyte cell types by shift status, we cannot rule out differences in minor leukocyte subsets not captured by flow cytometry analysis. Thus, rather than an indication of direct systemic effects of shift work on DNA methylation, our observations may indicate immunerelated differences between night and dayshift workers. This notion is supported by results of the gene ontology analysis which revealed that processes related to host defense and immunity were represented among the significantly hypomethylated genes in our study. In addition, the largest CpG island/gene region effects were observed for DUSP22, a gene involved in T-cell receptor signaling (Li et al., 2014). Differences observed in the circadian genes may also be indicative of differences in immune cell profile since circadian genes have regulatory functions in these cells (Baumann et al., 2013; Nguyen et al., 2013; Yu et al., 2013).

Differences in immune cell profile between night and dayshift workers could be attributable to natural diurnal variation of immune cells since blood samples for dayshift workers were collected between 07:00 and 09:00 h and for nightshift workers they were collected between 17:00 and 19:00 h. However, previous studies suggest that shift work has direct impacts on immune function. An increased prevalence of common infections and higher levels of circulating inflammatory markers have been observed among shift workers (Khosro et al., 2011; Mohren et al., 2002), and sleep disruption, which is common among shift workers (Kronholm et al., 2006; Pilcher et al., 2000), has been associated with alterations in the balance of T-helper cells (Costa et al., 2010), which would not be apparent from our flow cytometry data. Given the key role of the immune system in cancer etiology (Grivennikov et al., 2010), the potential impact of shift work on immune cell profile is, in itself, of tremendous importance, and DNA methylation markers may offer an efficient method by which to detect immune effects. There is also a possibility that, within individual cell types, differences in DNA methylation between night and dayshift workers reflect natural diurnal variation in DNA methylation

responsible for diurnal changes in gene expression, particularly for circadian genes. While an impact of DNA methylation on circadian rhythms of gene expression has not been observed in the liver (Azzi et al., 2014; Vollmers et al., 2012), similar data have not been reported for blood cells.

Glutathione derivative processes were also represented among the significantly hypomethylated genes in gene ontology analysis. For example, *GSTM5* was found to be significantly hypomethylated among nightshift workers compared to dayshift workers (Supplementary Table 2), including multiple loci near its transcription start site. GSTM5 is involved in detoxification of reactive oxygen species, and an increased expression of this gene as a result of transcription start site hypomethylation may be a response to the increased levels of oxidative stress that have been observed among shift workers (Faraut et al., 2013).

A previous study of 150 male chemical plant workers evaluated the association of rotating shift work with DNA methylation (Bollati et al., 2010). Specifically, the study examined blood-based DNA methylation levels in the promoters of the glucocorticoid receptor (GCR), tumor necrosis factor alpha (TNFA), and interferongamma (IFNG) genes, as well as across Alu and LINE-1 repetitive regions as surrogates of global changes in DNA methylation. While no differences between rotating and dayshift workers were observed, when restricting the analysis to rotating shift workers, increased work years (>15 years) were significantly associated with a decreased methylation of Alu and of the promoter region of IFNG gene. We did not observe significant effects on *IFNG* in our study, but our finding of a general decreased state of methylation among nightshift workers supports the previous finding of decreased Alu methylation with duration of rotating shift work. A comparable analysis of the impact of shift work duration was not possible in our study given the relatively short durations of shift work in our population (median = 2 years).

Another previous study evaluated the impact of longterm shift work (i.e. ≥ 10 years of working a job that involved night work) on blood-based DNA methylation (Zhu et al., 2011). Among 19 long-term female shift workers, promoter methylation levels in the CLOCK and CRY2 genes were found to be decreased and increased, respectively, when compared to 98 female dayshift workers (i.e. never worked the nightshift). While we observed no effect on CRY2, we did observe significantly decreased methylation across CLOCK's upstream CpG island and 5'UTR, which is in support of these previous findings. In that previous study, 5408 of 27 578 CpG sites captured by the Infinium HumanMethylation 27K BeadChip (Illumina, San Diego, CA) were found to be significantly differentially methylated between 10 longterm nightshift and 10 dayshift workers after adjusting for multiple comparisons. Unlike our study, the majority of these CpG sites (66%) were hypermethylated. This

may be related to study population differences since the previous study evaluated long-term shift workers that may or may not have been engaged in shift work at the time of blood sample collection, while our study focused on actively employed shift workers who were engaged in nightshift work at the time of sample collection.

Despite the limited sample size, the comprehensive evaluation of genome-wide DNA methylation was a major strength of the study. In addition, issues of exposure misclassification were avoided by evaluating subjects that were actively employed in night or dayshift work, though given that increased risks of cancer have been associated with long-term shift work, shift workrelated methylation differences that are most relevant to carcinogenesis may not have been detectable among our subjects with their relatively limited cumulative durations of nightshift work. While nightshift workers may have also been actively engaged in day work, they were required to be primarily engaged in night work, and blood samples were collected only after three consecutive nights had been worked. Our data were limited in that we did not collect more specific information on the typical work schedules that subjects were engaged in (e.g. exact number of consecutive nights, frequency of dayshift work). While verification of our major findings using methods such as a quantitative pyrosequencing to eliminate false positives would have been ideal, inclusion of such a component was beyond the scope of our study, and previous studies have consistently demonstrated a high-degree of congruence between Illumina Infinium HumanMethylation450 Bead Array and quantitative pyrosequencing results (Almgren et al., 2014; Glossop et al., 2013; Roessler et al., 2012; Shenker et al., 2013). We were unable to entirely rule out the potential for confounding of our results by diurnal variation of minor immune cell profiles given the differences in the timing of blood sample collection between day and nightshift workers. Future studies should collect multiple blood samples throughout the day to account for this variation and should evaluate a much more comprehensive panel of leukocyte cell types to better understand the potential contribution of immunity to observed differences in DNA methylation. These future studies should also consider the potential modifying effects of gender and diurnal preference. Results of our study may not be generalizable to workers on a regularly rotating shift pattern, which should also be specifically evaluated in future studies.

Our exploratory study revealed a number of intriguing differences in DNA methylation between nightshift and dayshift workers. Our findings certainly need to be followed-up among a considerably expanded number of shift workers, but they suggest that the negative effects of shift work may be partly mediated by hypomethylation of a wide variety of genes, including those related to circadian rhythms. Our study also suggests that nightshift work may impact oxidative stress and immune function, and, as such, provides data to support future targeted research into the potential impacts of shift work on these mechanisms.

DECLARATION OF INTEREST

The authors have no conflicts of interest to declare. The study was supported by funds from the Fred Hutchinson Cancer Research Center and an award from the Safeway Foundation.

REFERENCES

- Almgren M, Schlinzig T, Gomez-Cabrero D, et al. (2014). Cesarean delivery and hematopoietic stem cell epigenetics in the newborn infant: Implications for future health? Am J Obstet Gynecol. http//dx.doi.org/10.1016/j.ajog.2014.05.014.
- Azzi A, Dallmann R, Casserly A, et al. (2014). Circadian behavior is light-reprogrammed by plastic DNA methylation. Nat Neurosci. 17:377–82.
- Baccarelli A, Bollati V. (2009). Epigenetics and environmental chemicals. Curr Opin Pediatr. 21:243–51.
- Baccarelli A, Wright RO, Bollati V, et al. (2009). Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med. 179:572–8.
- Ball MP, Li JB, Gao Y, et al. (2009). Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol. 27:361–8.
- Baumann A, Gönnenwein S, Bischoff SC, et al. (2013). The circadian clock is functional in eosinophils and mast cells. Immunology. 140:465–74.
- Bell JT, Pai AA, Pickrell JK, et al. (2011). DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome Biol. 12:R10.
- Bollati V, Baccarelli A, Hou L, et al. (2007). Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res. 67:876–80.
- Bollati V, Baccarelli A, Sartori S, et al. (2010). Epigenetic effects of shiftwork on blood DNA methylation. Chronobiol Int. 27: 1093–104.
- Bracci M, Manzella N, Copertaro A, et al. (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. 40:295–304.
- Breitling LP, Yang R, Korn B, et al. (2011). Tobacco-smokingrelated differential DNA methylation: 27K discovery and replication. Am J Hum Genet 88:450–7.
- Calvelli T, Denny TN, Paxton H, et al. (1993). Guideline for flow cytometric immunophenotyping: A report from the national institute of allergy and infectious diseases, division of AIDS. Cytometry. 14:702–15.
- Choi J-Y, James SR, Link PA, et al. (2009). Association between global DNA hypomethylation in leukocytes and risk of breast cancer. Carcinogenesis. 30:1889–97.
- Costa G, Haus E, Stevens R. (2010). Shift work and cancer Considerations on rationale, mechanisms, and epidemiology. Scand J Work Environ Health. 36:163–79.
- Davis S, Du P, Bilke S, et al. (2014). Methylumi: Handle illumina methylation data. Version 2.6.1.
- Eden E, Navon R, Steinfeld I, et al. (2009). GOrilla: A tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics. 10:48.
- Ehrlich M. (2002). DNA methylation in cancer: Too much, but also too little. Oncogene. 21:5400–13.
- Faraut B, Bayon V, Léger D. (2013). Neuroendocrine, immune and oxidative stress in shift workers. Sleep Medicine Rev. 17: 433–44.

- Fu L, Pelicano H, Liu J, et al. (2002). The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell. 111:41–50.
- Gao Y, Baccarelli A, Shu XO, et al. (2012). Blood leukocyte Alu and LINE-1 methylation and gastric cancer risk in the Shanghai Women's Health Study. Br J Cancer. 106:585–91.
- Gery S, Koeffler HP. (2007). The role of circadian regulation in cancer. Cold Spring Harb Symp Quant Biol. 72:459–64.
- Glossop JR, Nixon NB, Emes RD, et al. (2013). Epigenome-wide profiling identifies significant differences in DNA methylation between matched-pairs of T- and B-lymphocytes from healthy individuals. Epigenetics. 8:1188–97.
- Grivennikov SI, Greten FR, Karin M. (2010). Immunity, inflammation, and cancer. Cell. 140:883–99.
- Hoffman AE, Yi C-H, Zheng T, et al. (2010a). CLOCK in breast tumorigenesis: Genetic, epigenetic, and transcriptional profiling analyses. Cancer Res. 70:1459–68.
- Hoffman AE, Zheng T, Ba Y, Zhu Y. (2008). The circadian gene NPAS2, a putative tumor suppressor, is involved in DNA damage response. Mol Cancer Res. 6:1461–8.
- Hoffman AE, Zheng T, Yi C-H, et al. (2010b). The core circadian gene Cryptochrome 2 influences breast cancer risk, possibly by mediating hormone signaling. Cancer Prev Res (Phila). 3:539–48.
- Hsiung DT, Marsit CJ, Houseman EA, et al. (2007). Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev. 16:108–14.
- Jones PA. (2012). Functions of DNA methylation: Islands, start sites, gene bodies and beyond. Nat Rev Genet. 13:484–92.
- Khosro S, Alireza S, Omid A, Forough S. (2011). Night work and inflammatory markers. Indian J Occup Environ Med. 15:38–41.
- Kottorou AE, Antonacopoulou AG, Dimitrakopoulos F-ID, et al. (2012). Altered expression of NFY-C and RORA in colorectal adenocarcinomas. Acta Histochem. 114:553–61.
- Kronholm E, Härmä M, Hublin C, et al. (2006). Self-reported sleep duration in Finnish general population. J Sleep Res. 15: 276–90.
- Lee CC. (2006). Tumor suppression by the mammalian Period genes. Cancer Causes Control. 17:525–30.
- Lewintre EJ, Martín CR, Montaner D, et al. (2009). Analysis of chronic lymphotic leukemia transcriptomic profile: Differences between molecular subgroups. Leukemia Lymphoma. 50:68–79.
- Li J-P, Yang C-Y, Chuang H-C, et al. (2014). The phosphatase JKAP/ DUSP22 inhibits T-cell receptor signalling and autoimmunity by inactivating Lck. Nat Commun. 5:3618.
- Liao LM, Brennan P, van Bemmel DM, et al. (2011). LINE-1 methylation levels in leukocyte DNA and risk of renal cell cancer. PLoS One. 6:e27361.
- Mohren DCL, Jansen NWH, Kant IJ, et al. (2002). Prevalence of common infections among employees in different work schedules. J Occup Environ Med. 44:1003–11.
- Moore LE, Huang W-Y, Chung J, Hayes RB. (2003). Epidemiologic considerations to assess altered DNA methylation from environmental exposures in cancer. Ann N Y Acad Sci. 983:181–96.

- Moore LE, Pfeiffer RM, Poscablo C, et al. (2008). Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: A case-control study. Lancet Oncol. 9:359–66.
- Nguyen KD, Fentress SJ, Qiu Y, et al. (2013). Circadian gene Bmal1 regulates diurnal oscillations of Ly6Chi inflammatory monocytes. Science. 341:1483–8.
- Pilcher JJ, Lambert BJ, Huffcutt AI. (2000). Differential effects of permanent and rotating shifts on self-report sleep length: A meta-analytic review. Sleep. 23:155–63.
- Portaluppi F, Smolensky MH, Touitou Y. (2010). Ethics and methods for biological rhythm research on animals and human beings. Chronobiol Int. 27:1911–29.
- Pufulete M, Al-Ghnaniem R, Leather AJM, et al. (2003). Folate status, genomic DNA hypomethylation, and risk of colorectal adenoma and cancer: A case control study. Gastroenterology. 124:1240–8.
- Rauch TA, Wu X, Zhong X, et al. (2009). A human B cell methylome at 100-base pair resolution. Proc Natl Acad Sci USA. 106:671–8.
- Roessler J, Ammerpohl O, Gutwein J, et al. (2012). Quantitative cross-validation and content analysis of the 450k DNA methylation array from Illumina, Inc. BMC Res Notes. 5:210.
- Shenker NS, Polidoro S, van Veldhoven K, et al. (2013). Epigenomewide association study in the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) identifies novel genetic loci associated with smoking. Hum Mol Genet. 22: 843–51.
- Shi Y, Cao J, Gao J, et al. (2012). Retinoic acid-related orphan receptor- α is induced in the setting of DNA damage and promotes pulmonary emphysema. Am J Respir Crit Care Med. 186:412–19.
- Suter MA, Aagaard-Tillery KM. (2009). Environmental influences on epigenetic profiles. Semin Reprod Med. 27:380–90.
- Tusher VG, Tibshirani R, Chu G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A. 98:5116–21.
- Vollmers C, Schmitz RJ, Nathanson J, et al. (2012). Circadian oscillations of protein-coding and regulatory RNAs in a highly dynamic mammalian liver epigenome. Cell Metab. 16:833–45.
- Wang X, Yan D, Teng M, et al. (2012). Reduced expression of PER3 is associated with incidence and development of colon cancer. Ann Surg Oncol. 19:3081–8.
- Wang Y, Dong L, Cui H, et al. (2011). Up-regulation of mitochondrial antioxidation signals in ovarian cancer cells with aggressive biologic behavior. J Zhejiang Univ Sci B. 12:346–56.
- Yang WS, Stockwell BR. (2008). Inhibition of casein kinase 1-epsilon induces cancer-cell-selective, PERIOD2-dependent growth arrest. Genome Biol. 9:R92.
- Yu X, Rollins D, Ruhn KA, et al. (2013). TH17 cell differentiation is regulated by the circadian clock. Science. 342:727–30.
- Zhu Y, Stevens RG, Hoffman AE, et al. (2011). Epigenetic impact of long-term shiftwork: Pilot evidence from circadian genes and whole-genome methylation analysis. Chronobiol Int. 28:852–61.

Supplementary material available online Supplementary Tables 1–4