胎児期有機フッ素化合物（PFASs）曝露の臍帯血 DNA 網羅的エピゲノム解析
An epigenome-wide study of cord blood DNA methylations in relation to prenatal perfluoroalkyl substance exposure

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研究要旨

Background: Prenatal exposure to perfluoroalkyl substances (PFASs) influences fetal development and later in life.

Objective: To investigate cord blood DNA methylation changes associated with prenatal exposure to PFASs.

Methods: We assessed DNA methylation in cord blood samples from 190 mother-child pairs from the Sapporo cohort of the Hokkaido Study (discovery cohort) and from 37 mother-child pairs from the Taiwan Maternal and Infant Cohort Study (replication cohort) using the Illumina HumanMethylation 450 BeadChip. We examined the associations between methylation and PFAS levels in maternal serum using robust linear regression models and identified differentially methylated positions (DMPs) and regions (DMRs).

Results: We found four DMPs with a false discovery rate below 0.05 in the discovery cohort. Among the top 20 DMPs ranked by the lowest *P*-values for perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) exposure, four DMPs showed the same direction of effect and *P*-value < 0.05 in the replication assay: cg16242615 mapped to *ZBTB7A*, cg21876869 located in the intergenic region (IGR) of *USP2-AS1*, cg00173435 mapped to *TCP11L2*, and cg18901140 located in the IGR of *NTN1*. For DMRs, we found a region associated with PFOA exposure with family-wise error rate < 0.1 located in *ZFP57*, showing the same direction of effect in the replication cohort. Among the top five DMRs ranked by the lowest *P*-values that were associated with exposure to PFOS and PFOA, in addition to *ZFP57*, DMRs in the *CYP2E1*, *SMAD3*, *SLC17A9*, *GFPT2*, *DUSP22*, and *TCERG1L* genes showed the same direction of effect in the replication cohort.

Conclusion: We suggest that prenatal exposure to PFASs may affect DNA methylation status at birth. Longitudinal studies are needed to examine whether methylation changes observed are associated with differential health outcomes.

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A．研究目的

Perfluoroalkyl substances (PFASs) are synthetic compounds ubiquitously distributed in the environment that can disrupt endocrine system functions (Lau et al. 2007). PFASs have long half-lives in human: 5.4 years for perfluorooctane sulfonate (PFOS) and 3.8 years for perfluorooctanoic acid (PFOA) (Olsen et al. 2007). Furthermore, PFASs can pass through the placental barrier (Inoue et al. 2004). Consequently, fetuses can be exposed to PFASs via maternal circulation, which suggests a possibility of PFAS negative effects on embryonic and fetal development. Epidemiological studies have shown that prenatal exposure to PFASs has been associated with various health outcomes, including birth size reduction, disruption of hormone balance, obesity, neurodevelopmental problems, and immune function impairment (Apelberg et al. 2007; Chen et al. 2013; Grandjean et al. 2012; Halldorsson et al. 2012; Kishi et al. 2017; Olsen et al. 2009). However, the mechanisms underlying these associations are not clear. One hypothesis is that prenatal exposure to PFASs might lead to health outcomes in the offspring through epigenetic alterations *in utero* because epigenetics (i.e., chemical modification of DNA) is an *intrinsic* biological mechanism that can be affected by *extrinsic* environmental factors in humans.

DNA methylation is an epigenetic modification that plays a role in embryonic development and cellular differentiation (Breton et al. 2017). It occurs by the addition of a methyl group to a cytosine mostly at cytosine-guanine dinucleotide (CpG) loci and acts like a gene expression switch (Hackett and Surani 2013). Human epidemiological studies, including genome-wide approaches, have indicated that environmental factors such as diet, hormones, stress, drugs, or toxicants (e.g., lead, mercury, or tobacco smoke) during prenatal development influence DNA methylation patterns in children (Breton et al. 2017). Despite a significant impact of PFASs on health outcomes, there were few epidemiological studies of epigenetic effects of PFAS exposure *in utero*. Guerrero-Preston et al. (2010) observed that cord blood PFOA concentrations negatively correlated with cord serum global DNA methylation levels. We also reported that prenatal PFOA exposure was associated with reduced *IGF2* methylation in cord blood, which could predict infant ponderal index at birth (Kobayashi et al. 2017).

Genome-wide methylation analyses allow a hypothesis-free assessment of epigenetic alterations in relation to the environmental factors (Christensen and Marsit 2011). To our knowledge, only one study showed an association between maternal PFOA levels and genome-wide DNA methylation using 44 cord blood samples (Kingsley et al. 2017). The objective of the present study was to investigate cord blood DNA methylation changes in association with prenatal exposure

to PFASs using the genome-wide approach and to determine CpG loci epigenetically vulnerable to prenatal PFAS exposure.

B．研究方法

450K DNA methylation analysis. We assessed DNA methylation in cord blood samples from 190 mother-child pairs from the Sapporo cohort of the Hokkaido Study (discovery cohort) and from 37 mother-child pairs from the Taiwan Maternal and Infant Cohort Study (replication cohort) using the Illumina HumanMethylation 450 BeadChip. After quality control (Aryee et al. 2014), signal intensities were normalized using functional normalization (Fortin et al. 2014). We applied the ComBat method to adjust methylation data for sample plate to reduce a potential bias due to batch effects (Leek et al. 2012). Beta-values were calculated from signal intensities and used for the subsequent data analyses by using the following equation (Bibikova et al. 2011): β = methylated / (methylated + unmethylated + 100).

Exposure assessment. PFOS and PFOA levels were measured in maternal serum by using column-switching liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Lien et al. 2011; Okada et al. 2012; Washino et al. 2009).

Data analysis. Cord blood cell proportion was estimated by the method implemented in the R/Bioconductor package *minfi* (Bakulski et al. 2016). Using *limma* package in R, robust linear regression analyses (Fox and Weisberg 2011) and empirical Bayesian methods (Smyth 2004) were applied to determine the associations of β-value at each CpG site with either PFOS or PFOA $\log_{10}$-transformed concentration, adjusted for maternal age, parity, maternal educational levels, maternal blood sampling period, maternal pre-pregnancy BMI, maternal smoking during pregnancy, gestational age, infant sex, and cord blood cell estimates. Due to the small sample size of the replication cohort, we used only maternal age, infant sex, and cord blood cell estimates as covariates. For multiple comparisons, *P*-values were adjusted by the false discovery rate (FDR) to obtain *q*-values. Successful replication for differentially methylated positions (DMPs) was defined as having the same direction of effect with those observed in the discovery cohort and *P*-value < 0.05. We also identified differentially methylated regions (DMRs) associated with PFAS exposures using *bumphunter* function in R/Bioconductor (Jaffe et al, 2012) and the same models as those in the linear regression analyses. *P*-values were adjusted by the family-wise error rate (FWER). Statistical analyses were performed using *minfi*, *sva*, and *limma* packages in R ver. 3.3.2 and Bioconductor ver. 3.3.

Gene ontology analysis. We identified the enrichment of genes corresponding to the DMPs with *P*-value < 0.001 in Kyoto Encyclopedia Genes and Genomes (KEGG) pathways (Kanehisa et al. 2002) using *missMethyl* package in R/Bioconductor (Phipson et al. 2016)

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The study was conducted with the informed consent of all subjects in the written form. The institutional Ethical Board for human gene and genome studies at the Hokkaido University

Graduate School of Medicine and the Hokkaido University Center for Environmental and Health Science approved the study protocol. The Human Ethics Committee of the National Health Research Committee of Taiwan approved the study of the replication cohort.

C．研究結果

Epigenome-wide association study in the discovery cohort. Maternal and infant characteristics and their relationship to PFOS and PFOA concentrations are described in Table 1. Median (25^{th} to 75^{th} percentiles) of PFOS and PFOA concentrations in maternal blood were 5.2 ng/mL (3.8 to 7.1) and 1.4 ng/mL (0.9 to 2.1), respectively. The average (± SD) age of the mothers was 29.7 ± 4.8 years. Of the 190 newborns, 84 (44.2%) were male. We observed statistically significant differences in both PFOS and PFOA levels by parity, maternal blood sampling periods, and smoking during pregnancy. Additionally, PFOA level was significantly higher among mothers with male infants, and PFOS levels were marginally affected by the educational level.

Figure 1 shows the results of genome-wide analyses of the association between cord blood DNA methylation and prenatal PFOS (Figure 1A) or PFOA (Figure 1B) exposure. The volcano plots (Figure 1) showed imbalance in positive versus negative methylation changes, suggesting global methylation shifts due to PFAS exposure.

Differentially methylated positions (DMPs). We found epigenome-wide significant associations (FDR *q*-value < 0.05) between PFOS exposure and DNA methylation for two CpGs: one located in the intergenic region (IGR) of *CXADRP3* (cg02044327), and another mapped to *SNAPIN* (cg25705526). In addition, significant associations between PFOA exposure and DNA methylation for another two CpGs were found: one located in the IGR of *AC002480.3* (cg11260715), and another mapped to *GPR126* (cg04461802). Top 20 DMPs ranked by the lowest *P*-value for the association with exposure to PFOS and PFOA are shown in Table 2. Among them, four DMPs met the criteria for replication that showed the same direction of effect and *P*-value < 0.05 in the replication assay: cg16242615 mapped to *ZBTB7A* for PFOS; cg21876869 located in the IGR of *USP2-AS1*, cg00173435 mapped to *TCP11L2*, and cg18901140 located in the IGR of *NTN1* for PFOA.

Differentially methylated regions (DMRs). Next, we assessed DMRs associated with prenatal PFAS exposures using *bumphunter* function (Jaffe et al, 2012). We found one region associated with PFOA exposure with FWER < 0.1 that was located in the IGR of *ZFP57* and included 21 CpGs. We showed top five regions for PFOS and PFOA exposures ranked by the smallest *P*-value (Table 3). We also compared the direction of methylation changes in the discovery and replication cohorts (Table 4), in which we averaged methylation levels of each site because those were highly correlated (data not shown). A DMR in *CYP1E2* was observed for both PFOS and PFOA exposures. Eight of the ten regions showed the same direction of methylation changes in the replication cohort.

Gene ontology analysis. Lastly, we tested for the enrichment of KEGG pathways (Kanehisa et al. 2002) among the genes with annotated CpGs showing *P*-value < 0.001. Among the 323 pathways analyzed, 31 and 26 KEGG pathways were significantly enriched after Bonferroni correction among the genes affected by for PFOS and PFOA exposures, respectively. Gene Ontology analyses of the data obtained using 450K chip are known to be biased for cancer-related genes (Haper et al. 2013). Human disease pathways, including cancer, were therefore excluded from the list of the pathways affected by PFAS exposures (Figure 2). Enrichments in the pathways involved in signal transduction and signal molecules and interactions were observed among the genes affected by both PFOS and PFOA exposures

D．考察

Few studies have focused on the epigenetic effects of prenatal exposure to PFASs. In this study, median concentrations of PFOS and PFOA were 5.2 and 1.4 ng/mL, respectively, which were lower than those reported in the United States (PFOS: 8.2, PFOA: 2.9 ng/mL) (Stein et al. 2012), Canada (PFOS: 16.6, PFOA: 2.1 ng/mL) (Monroy et al. 2008), Denmark (PFOS: 21.5, PFOA: 3.7 ng/mL) (Huang et al. 2012), Norway (PFOS: 13, PFOA: 2.2 ng/mL) (Starling et al. 2014), South Korea (PFOS: 9.3, PFOA: 2.6 ng/mL) (Lee et al. 2013), and China (PFOS: 6.7, PFOA: 4 ng/mL) (Jiang et al. 2014). Despite the low levels of exposure, we showed suggestive evidence for the presence of CpGs epigenetically vulnerable to PFAS exposure *in utero*.

We observed potential global methylation shifts resulting from prenatal PFAS exposure (see volcano plots in Figure 1): up-methylation for PFOS exposure and down-methylation for PFOA exposure. This was consistent with previous reports for prenatal PFOA exposure (Guerrero-Preston et al. 2010; Kingsley et al. 2017). Two studies in adult populations have suggested a possibility of PFAS exposure effect on global methylation (Leter et al. 2014; Watkins et al. 2014).

We then focused on the changes at specific regions and found four DMPs with FDR < 0.05: cg02044327 (*CXADRP3*), cg25705526 (*SNAPIN*), cg11260715 (*AC002480.3*), and cg04461802 (*GPR126*) (Figure 1), although these DMPs did not meet the criteria for replication (Table 2). Among 20 DMPs with lowest *P*-values for PFOS and PFOA exposures (Table 2), four DMPs were replicated: cg16242615 (*ZBTB7A*), cg21876869 (*USP2-AS1*), cg00173435 (*TCP11L2*), and cg18901140 (*NTN1*). *ZBTB7A* (Zinc finger and BTB domain containing 7A) encodes a proto-oncogenic transcription factor that interacts directly with MBD3 (methyl-CpG-binding domain protein 3) in the nucleus (Choi et al. 2013). *TCP11L2* (T-Complex 11 Like 2) codes for the TCP11 like protein. TCP11 plays a role in the process of sperm capacitation and acrosome reactions. *USP2-AS1* (USP2 Antisense RNA 1) belongs to the non-coding RNAs. Netrin 1 (NTN1) is a secreted laminin-like protein identified as an axon guidance molecule.

Next, we explored DMRs that are potentially more informative than individual

CpG sites (Solomon et al. 2017). We found one down-methylated region with FWER < 0.1, which was located in the IGR of *ZFP57* (ZFP57 Zinc Finger Protein) (Table 4). ZFP57 is necessary for maintaining repressive epigenetic modifications at imprinting control regions (Riso et al. 2016). We observed down-methylation of this region in the replication cohort (Table 4). In addition to a DMR in *ZFP57*, we reported six DMRs in *CYP2E1*, *SMAD3*, *SLC17A9*, *GFPT2*, *DUSP22*, and *TCERG1L* that showed the same direction of methylation change in the replication cohort (Table 4). Among them, methylation of *SMAD3* (SMAD Family Member 3) at birth has been previously linked to asthma in children of asthmatic mothers (DeVries et al. 2016). Cord blood DNA methylation of *GFPT2* (Glutamine-Fructose-6-Phosphate Transaminase 2) was associated with adiposity in childhood (Kresovich et al. 2017). Furthermore, methylations of *CYP2E1* (Cytochrome P450 Family 2 Subfamily E Member 1), *DUSP2* (Dual Specificity Phosphatase 2), and *TCERG1L* (transcription elongation regulator 1-like) were associated with rheumatoid arthritis (Mok et al. 2017) and colon tumors (Bae et al. 2014). Additionally, PFOS inhibited the oxidation reaction of CYP2E1 *in vitro* (Narimatu et al. 2011).

Gene ontology analysis showed that differentially methylated genes were enriched in multiple KEGG pathways (Figure 2), including signal transduction, signaling molecules and interaction, endocrine system, and immune system. However, in this assay we used genes with annotated CpGs with *P*-value < 0.001, i.e., not achieving epigenome-wide significance. It remains to be seen whether identified DNA methylation changes are functionally relevant.

Methylation changes derived from the exposures to PFOS and PFOA were different. We have reported that PFOS and PFOA differentially affected health outcomes related to these pathways. Prenatal exposure to PFOS, but not PFOA, was negatively associated with the levels of maternal fatty acids (Kishi et al. 2015), possibly disrupted both maternal and infant thyroid hormone levels (Kato et al. 2016), and showed an inverse relationship with cord blood levels of glucocorticoids (Goudarzi et al. 2017). Dehydroepiandrosterone level was positively associated with the exposure to PFOS and negatively associated with the exposure to PFOA (Goudarzi et al. 2017). PFOS and PFOA showed both positive and inverse associations with the levels of several reproductive and steroid hormones (Ito et al. 2016). The observed different potencies and modes of action may partly account for the distinct patterns of methylation changes. The differences in PFOS and PFOA concentrations and/or placental permeability could be an alternative explanation.

E．結論

In this epigenome-wide study, we suggested that even relatively low levels of prenatal exposure to PFASs impacted DNA methylation status at birth. Further study is needed to examine the persistence of DNA methylation changes due to prenatal exposure throughout life, and the associations of these changes with health outcomes causally linked to PFAS exposure in longitudinal studies.

F．研究発表

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G. 知的財産権の出願・登録状況

該当なし

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Table 1 Characteristics of study population of the discovery cohort (*n*=190).

		n	Mean ±SD (%)	PFOS (ng/mL) Median (25th, 75th) or correlation[a]	P	PFOA (ng/mL) Median (25th, 75th) or correlation[a]	P
Concentration in maternal blood							
		190		5.2 (3.8, 7.1)		1.4 (0.9, 2.1)	
Maternal characteristics							
Maternal age (year)[a]		190	29.7 ± 4.8	ρ= −0.087	0.233	ρ= −0.041	0.579
Pre-pregnancy BMI (kg/m^2)[a]		190	21.2 ± 3.1	ρ= −0.018	0.803	ρ= −0.056	0.444
Parity (times)[b]							
	0	104	54.7	5.7 (4.2, 8.0)	0.002	1.6 (1.2, 2.4)	<0.001
	≥ 1	86	45.2	4.7 (3.1, 6.2)		1.0 (0.7, 1.5)	
Blood sampling period[c]							
	< 28 weeks	74	38.9	5.8 (4.6, 7.5)	<0.001	1.7 (1.2, 2.3)	0.004
	28–36 weeks	47	24.7	5.6 (4.0, 8.5)		1.3 (0.8, 1.8)	
	≧ 36 weeks	69	36.3	4.6 (2.8, 5.7)		1.2 (0.8, 1.8)	
Educational level (year)[b]							
	≤ 12	89	46.8	5.2 (4.1, 7.0)	0.966	1.3 (0.8, 1.8)	0.072
	> 12	101	53.2	5.3 (3.6, 7.4)		1.5 (1.0, 2.3)	
Annual household income (million yen)[c]							
	< 3	38	20	5.4 (3.9, 8.0)	0.878	1.4 (0.8, 2.2)	0.541
	3–5	95	50	5.1 (3.5, 7.0)		1.4 (0.9, 1.8)	
	5–7	40	21.1	5.4 (4.2, 6.9)		1.4 (0.9, 2.1)	
	> 7	15	7.9	5.1 (3.0, 8.8)		2.3 (0.8, 2.4)	
	missing	2	1.1	7.8 (4.5, 11.1)			
Smoking during pregnancy[b]							
	No	157	82.6	5.3 (4.0, 7.3)	0.039	1.4 (0.9, 2.2)	0.011
	Yes	33	17.4	4.3 (2.5, 6.8)		1.0 (0.7, 1.6)	
Alcohol consumption during pregnancy[b]							
	No	130	68.4	5.2 (3.9, 7.2)	0.954	1.4 (0.9, 2.1)	0.821
	Yes	60	31.6	5.3 (3.7, 7.1)		1.4 (0.9, 2.2)	
Infant characteristics							
Sex[b]	Male	84	44.2	5.1 (3.3, 7.0)	0.117	1.6 (1.0, 2.4)	0.025
	Female	106	55.8	5.4 (4.1, 7.5)		1.3 (0.8, 1.9)	
Gestational age (week)[a]		190	39.9 ± 1.0	ρ= 0.031	0.675	ρ= 0.093	0.203
Birth weight (g)[a]		190	3131 ± 330	ρ= −0.122	0.095	ρ= −0.118	0.104

[a]Spearman's correlation test (ρ), [b]Mann-Whitney U-test, [c]Kruskal-Wallis test

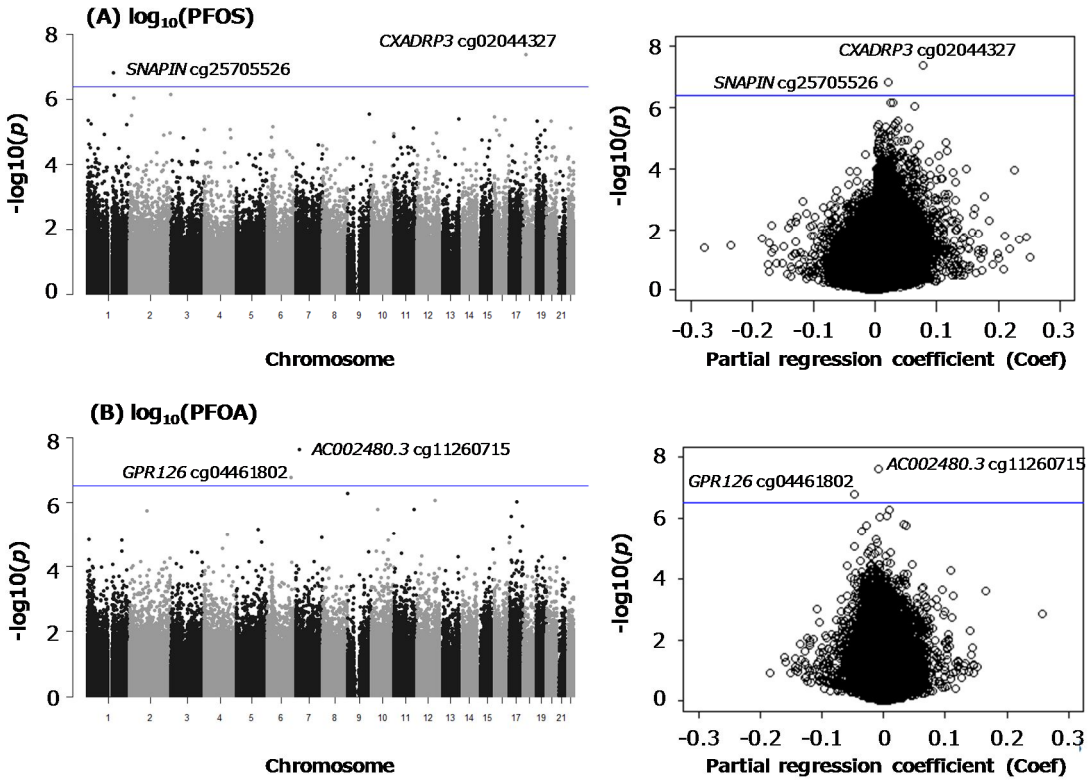


Figure 1. Manhattan (left panels) and volcano plots (right panels) of the genome-wide associations of DNA methylation with prenatal exposure to PFOS (A) or PFOA (B) in the discovery cohort.

Left panels: Manhattan plots of *P*-value for the associations between prenatal PFAS exposures and DNA methylation across chromosomes. Right panels: Volcano plots showing *P*-values versus the magnitude of effect (Coef) on DNA methylation associated with prenatal PFAS exposures. Horizontal lines represent the significance threshold of a FDR < 0.05.

Table 2. Top 20 CpGs ranked by the smallest *P*-value from the epigenome-wide analysis of the association between prenatal PFAS exposure and cord blood DNA methylation.

Probe ID	Gene	Chr	Feature[a]	Discovery cohort		Replication cohort		Replicated[d]
				Coef[b]	P-Value	Coef[b]	P-Value	
log_{10}(PFOS)								
cg25705526	*SNAPIN*	1	TSS200	0.020	1.48E-07[FDR, d]	-0.006	0.425	
cg04928693	*MTX1*	1	TSS1500	0.024	7.19E-07	-0.003	0.737	
cg18155888[c]	*MORN1*	1	Body	0.017	4.32E-06	0.023	0.277	
cg17086204	*PLA2G5*	1	3'UTR	0.025	5.56E-06	0.000	0.955	
cg10504365	*TRIM67*	1	TSS1500	0.032	5.84E-06	-0.022	0.003	
cg01889773[c]	*GPC1*	2	Body	0.030	6.92E-07	0.008	0.538	
cg16845265	*KLHL29*	2	IGR	0.065	8.73E-07	-0.019	0.528	
cg25808157	*GREB1*	2	Body	0.034	3.13E-06	-0.002	0.926	
cg21969395	*LRAT*	4	TSS200	0.016	8.23E-06	-0.001	0.870	
cg22953687	*ZFYVE28*	4	Body	0.025	8.25E-06	-0.018	0.117	
cg12215478	*SRPK1*	6	Body	0.008	6.93E-06	-0.002	0.421	
cg14369981[c]	*LMX1B*	9	IGR	0.037	2.81E-06	0.007	0.705	
cg17918227[c]	*CADM1*	11	Body	0.012	7.56E-06	0.005	0.336	
cg14120075[c]	*TFDP1*	13	Body	0.031	3.94E-06	0.008	0.626	
cg03097541[c]	*ZNF213*	16	5'UTR	0.006	3.38E-06	0.002	0.131	
cg01718742	*CDH8*	16	TSS200	0.003	4.06E-06	-0.001	0.651	
cg02044327[c]	*CXADRP3*	18	IGR	0.078	4.06E-08[FDR, d]	0.036	0.142	
cg16242615[c]	*ZBTB7A*	19	5'UTR	0.037	4.54E-06	0.035	0.028	✓
cg15815607	*HM13*	20	Body	0.053	4.57E-06	-0.008	0.645	
cg12700033	*YWHAH*	22	TSS200	0.006	7.41E-06	0.000	0.958	
log_{10}(PFOA)								
cg23049737	*RERE*	1	3'UTR	0.011	1.43E-05	-0.009	0.056	
cg00567854[c]	*BTG2*	1	TSS1500	-0.025	1.48E-05	-0.009	0.214	
cg10403518	*SLC9A4*	2	Body	0.038	1.75E-06	-0.014	0.083	
cg22325921	*DUX2*	4	IGR	-0.020	9.90E-06	0.002	0.719	
cg05158146[c]	*MIR4460*	5	IGR	-0.010	6.75E-06	0.000	0.931	
cg23917868[c]	*MIR145*	5	TSS200	-0.020	1.76E-05	-0.006	0.289	
cg04461802	*GPR126*	6	5'UTR	-0.046	1.65E-07[FDR, d]	0.008	0.456	
cg11260715[c]	*AC002480.3*	7	IGR	-0.008	2.32E-08[FDR, d]	-0.002	0.247	
cg12105980[c]	*EN2*	7	IGR	-0.023	1.24E-05	-0.008	0.220	
cg13951074	*DMRT2*	9	IGR	0.010	5.09E-07	0.000	0.888	
cg07661167[c]	*ZNF33BP1*	10	IGR	0.032	1.62E-06	0.015	0.065	
cg01486146	*PAX2*	10	IGR	-0.014	1.50E-05	0.005	0.088	
cg21876869[c]	*USP2-AS1*	11	IGR	-0.027	1.64E-06	-0.024	0.000	✓
cg17114584	*IRF7*	11	Body	-0.047	9.01E-06	0.001	0.928	
cg00173435[c]	*TCP11L2*	12	TSS200	0.006	8.52E-07	0.003	0.001	✓
cg16475925[c]	*SPG7*	16	TSS200	0.003	1.82E-05	0.002	0.095	
cg00897875	*MAP3K14*	17	5'UTR	-0.006	9.16E-07	0.004	0.077	
cg18901140[c]	*NTN1*	17	IGR	-0.036	2.66E-06	-0.029	0.000	✓
cg01426818[c]	*CBX4*	17	IGR	-0.012	5.20E-06	-0.002	0.720	
cg18002862[c]	*RAP1GAP2*	17	Body	-0.008	1.22E-05	-0.002	0.146	

Abbreviations: Chr, chromosome; IGR, intergenic region; TSS, transcription start site; TSS200, 200 bases from TSS; TSS1500, 1500 bases from TSS; body, gene body; UTR, untranslated region.
[a]Gene feature category of the methylation locus.
[b]Partial regression coefficient; the magnitude of the effect on DNA methylation.
[c]CpG that showed the same direction of effect in both the discovery and replication cohorts.
[d]Genome-wide significance threshold (FDR q < 0.05).
[e]Successful replication defined as having the same direction of effect and a *P*-value < 0.05 in the discovery cohort.

Table 3. TOP5 of differentially methylated regions indicated by the bumphunting method.

Gene	Chr	Start	End	Number of Probes	Features[a]	CGI[b]	P-value
$\log_{10}$(PFOS)							
CYP2E1	10	135342560	135343280	6	Body	island/shore	3.75E-04
KLHL35	11	75139390	75139736	4	Body	island/shore	8.86E-04
SMAD	15	67356310	67356942	5	TSS1500/IGR	shore	1.09E-03
HOOK2	19	12876846	12877188	4	Body	island/shore	1.65E-04
SLC17A9	20	61590751	61591209	4	Body	island/shore	4.83E-04
$\log_{10}$(PFOA)							
GFPT2	5	179740743	179741120	4	Body	island	2.03E-03
ZFP57	6	29648225	29649084	21	IGR	open sea	1.00E-04[FWER,c]
DUSP22	6	291687	293285	10	Body	island	7.87E-04
CYP2E1	10	135342560	135343280	6	Body	island/shore	3.72E-04
TCERG1L	10	132910868	132911152	4	Body	open sea	1.52E-03

Abbreviations: Chr, Chromosome; body, gene body; IGR, intergenic region; TSS, transcription start site; TSS1500, 1500 bases from TSS, FWER, family-wise error rate
[a]Gene feature category of the methylation locus.
[b]Relation to CpG island.
[c]FWER < 0.1

Table 4. The average partial regression coefficient of TOP5 DMRs in the discovery and replication cohorts.

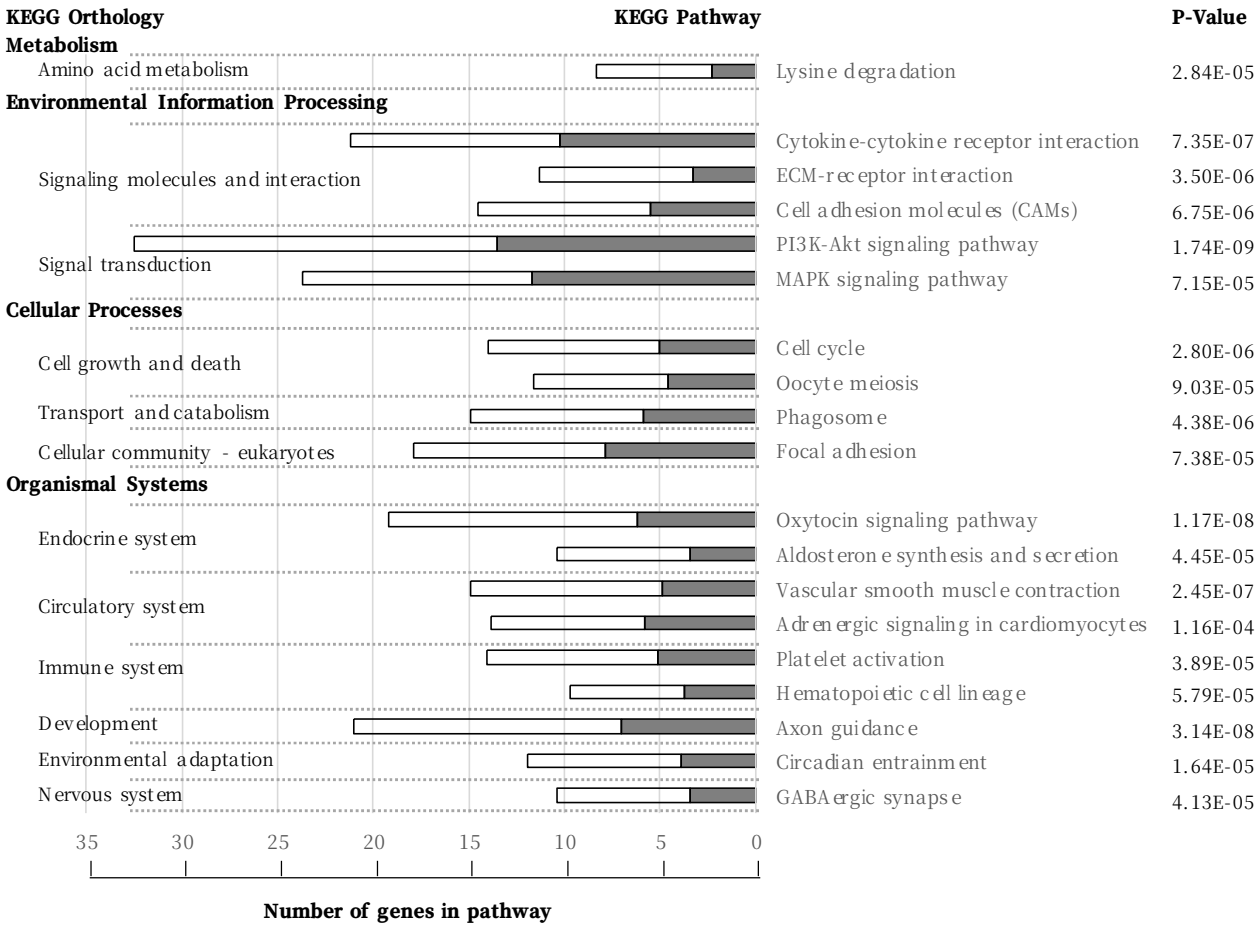
Gene	Chr	Discovery cohort		Replication cohort	
		Average Coef[a]	Direction[b]	Average Coef[a]	Direction[b]
$\log_{10}$(PFOS)					
CYP2E1	10	0.040	+	0.059	+
KLHL35	11	0.134	+	-0.127	–
SMAD	15	0.128	+	0.027	+
HOOK2	19	-0.212	–	0.290	+
SLC17A9	20	0.165	+	0.163	+
$\log_{10}$(PFOA)					
GFPT2	5	-0.100	–	-0.035	–
ZFP57	6	-0.112	–	-0.052	–
DUSP22	6	-0.030	–	-0.113	–
CYP2E1	10	0.112	+	0.007	+
TCERG1L	10	0.025	+	0.009	+

Abbreviations: Chr, Chromosome.

[a]Average partial regression coefficient at CpG sites in the region.

[b]direction of methylation change: +, up-methylated, –, down-methylated.

(A) PFOS


KEGG Orthology
KEGG Pathway
P-Value
Metabolism
Amino acid metabolism
Lysine degradation
2.84E-05
Environmental Information Processing
Signaling molecules and interaction
Cytokine-cytokine receptor interaction
7.35E-07
ECM-receptor interaction
3.50E-06
Cell adhesion molecules (CAMs)
6.75E-06
Signal transduction
PI3K-Akt signaling pathway
1.74E-09
MAPK signaling pathway
7.15E-05
Cellular Processes
Cell growth and death
Cell cycle
2.80E-06
Oocyte meiosis
9.03E-05
Transport and catabolism
Phagosome
4.38E-06
Cellular community - eukaryotes
Focal adhesion
7.38E-05
Organismal Systems
Endocrine system
Oxytocin signaling pathway
1.17E-08
Aldosterone synthesis and secretion
4.45E-05
Circulatory system
Vascular smooth muscle contraction
2.45E-07
Adrenergic signaling in cardiomyocytes
1.16E-04
Immune system
Platelet activation
3.89E-05
Hematopoietic cell lineage
5.79E-05
Development
Axon guidance
3.14E-08
Environmental adaptation
Circadian entrainment
1.64E-05
Nervous system
GABAergic synapse
4.13E-05
35
30
25
20
15
10
5
0
Number of genes in pathway

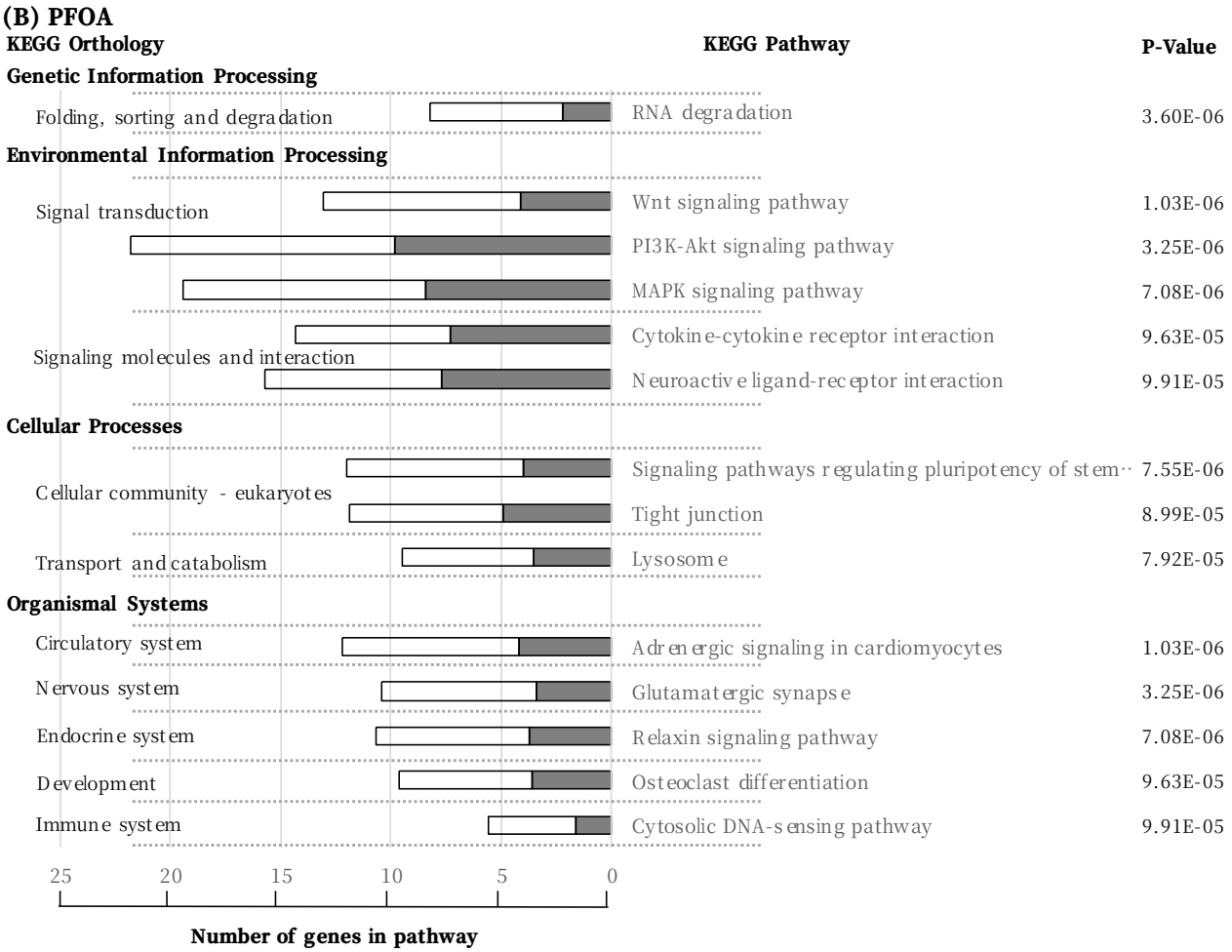


Figure 2. Significantly enriched pathways among the genes with differentially methylated CpGs associated with the exposures to PFOS (A) and PFOA (B).
White bars, observed number of genes; black bars, expected number of genes in each pathway. $P <$ 0.001 vs. the expected number of genes.