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Supplemental Material

Longitudinal Effects of Developmental Bisphenol A Exposure on Epigenome-Wide DNA Hydroxymethylation at Imprinted Loci in Mouse Blood

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Figure S3. Enhanced reduced representation bisulfite sequencing (ERRBS) DNA methylation data from identified *Klf14* differentially hydroxymethylated region (DHMR). Using the *RnBeads* R package, ERRBS data was visualized for the CpG sites contained within the identified *Klf14* DHMR. DNA methylation level is represented by intensity of color, with zero % methylation represented by white and 100% methylation represented by dark red. ERRBS data from Control mice is shown on the left, while ERRBS data from BPA exposed mice is shown on the right. Blood sample collection age (2 months, 4 months, or 10 months old) is indicated in the left column. Mean methylation across all CpGs with data is represented by line plots below the raw data. Line plots are colored according to age group. At the *Klf14* DHMR, there were no significant differentially methylated CpGs by BPA exposure, although comparisons were difficult due to limited coverage at some CpG.

Figure S4. Additional differential imprinted gene 5-hydroxymethylcytosine (5-hmC) peaks by bisphenol A (BPA) exposure. 5-hmC coverage was visualized at six additional imprinted loci with significant BPA-related differentially hydroxymethylated regions (DHMRs): A) *Airn*, B) *Cmah*, C) *Ppp1r9a*, D) *Kcnq1*, E) *Phactr2*, and F) *Pde4d*. 5-hmC levels are shown for matched 2 month, 4 month, and 10 month blood samples, as indicated by y-axis labels. Blue and red peaks represent forward and reverse strand 5-hmC enrichment, respectively. *Cmah*, *Ppp1r9a*, and *Kcnq1* DHMRs occurred on the forward strand. *Airn*, *Phactr2*, and *Pde4d* DHMRs occurred on the reverse strand.

Figure S5. 5-hydroxymethylcytosine (5-hmC) peaks at the *Igf2*/*H19* imprinted loci. Based on results at the *Plagl1* locus, which is a regulator of *Igf2* and *H19*, longitudinal 5-hmC patterns across the *Igf2* and *H19* imprinted genes were also visualized. 5-hmC peaks at both loci appear stable across individuals and adulthood stage in longitudinal mouse blood (2, 4, and 10 months old). Regions of non-significant bisphenol A (BPA)-related differential 5-hmC are indicated in red boxes for both genes. Despite not being detected as significant differentially hydroxymethylated regions (DHMRs) in *csaw*, these bisphenol A (BPA)-related changes in 5-hmC were stable at all three measured time points.

Table S1. Real-time quantitative polymerase chain reaction (RT-qPCR) primers for self-designed gene assays.

Gene	Full Name	GenBank Number	Strand	Primers (5' to 3')	Tm (°C)	Amplicon Size
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	NM_008084.3	FWD	GCCTGCTTCACCACCTTCTT	59.96	98
			RVS	CATGGCCTTCCGTGTTCCTA	59.07	
Gnas	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	NM_201618.2	FWD	CTGCCATCATCTTCGTGGTG	58.99	193
			RVS	GATTTGCCAGCGAGGACTTT	58.83	

Forward and reverse primer sequences for the *Gapdh* and *Gnas* RT-qPCR assays are listed, along with melting temperatures and amplicon size. All assays were designed using the online Genscript Real-time PCR Primer Design software (https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool). FWD = forward. RVS = reverse. Tm = melting temperature.

Table S2 – Enhanced reduced representation bisulfite sequencing (ERRBS) differentially methylated CpGs (DMCs) annotated to top hit imprinted genes.

BPA-related *Gnas/Nespas* DMCs					
Chr	**Position**	**Location**	**Δ % Methylation†**	**Gene ID**	**Gene name**
chr2	**174295388**	**1to5kb:6242**	**-17.1%**	**14683**	***Gnas***
chr2	**174295403**	**1to5kb:6241**	**-87.2%**	**14683**	***Gnas***
chr2	**174299808**	**exon:51698**	**-10.6%**	**14683**	***Gnas***
chr2	174305903	intron:45418	23.1%	14683	*Gnas*
chr2	174329842	5UTR:7653	-7.7%	14683	*Gnas*
chr2	174330525	intron:45422	-12.7%	14683	*Gnas*
chr2	174330995	intron:45434	9.9%	14683	*Gnas*
chr2	174335278	intron:45423	29.8%	14683	*Gnas*
chr2	174284270	intron:64744	-20.8%	56802	*Nespas*
chr2	174284281	intron:64744	-35.9%	56802	*Nespas*
chr2	174284283	intron:64740	-14.1%	56802	*Nespas*
chr2	174291169	intron:64746	26.3%	56802	*Nespas*
BPA-related *Grb10* DMCs					
Chr	**Position**	**Location**	**Δ % Methylation†**	**Gene ID**	**Gene name**
chr11	11967584	exon:321631	20.7%	14783	*Grb10*
chr11	12037132	intron:281448	44.8%	14783	*Grb10*
BPA-related *Plagl1* DMCs					
Chr	**Position**	**Location**	**Δ % Methylation†**	**Gene ID**	**Gene name**
chr10	13060418	promoter:34649	12.9%	22634	*Plagl1*
chr10	13077185	intron:242831	25.8%	22634	*Plagl1*
chr10	13080633	intron:242831	19.4%	22634	*Plagl1*
chr10	13090048	promoter:34650	37.0%	22634	*Plagl1*
chr10	13127241	exon:277501	-6.4%	22634	*Plagl1*
BPA-related *Klf14/Mest* DMCs					
Chr	**Position**	**Location**	**Δ % Methylation†**	**Gene ID**	**Gene name**
chr6	30732802	promoter:19692	47.6%	17294	*Mest*
chr6	30742815	exon:163519	-7.1%	17294	*Mest*
BPA-related *Pde10a* DMCs					
Chr	**Position**	**Location**	**Δ % Methylation†**	**Gene ID**	**Gene name**
chr17	8541336	intron:376679	20.4%	23984	*Pde10a*
chr17	8550528	intron:376679	35.3%	23984	*Pde10a*
chr17	8551487	intron:376679	71.0%	23984	*Pde10a*
chr17	8556028	intron:376679	25.3%	23984	*Pde10a*
chr17	8569240	intron:376679	19.3%	23984	*Pde10a*
chr17	8580177	intron:376679	22.8%	23984	*Pde10a*
chr17	8612493	intron:376679	20.9%	23984	*Pde10a*
chr17	8613629	intron:376679	-12.5%	23984	*Pde10a*
chr17	8724732	intron:376679	31.9%	23984	*Pde10a*

chr17	8831508	intron:376680	30.6%	23984	*Pde10a*
chr17	8875353	intron:376723	7.6%	23984	*Pde10a*
chr17	8880832	intron:376723	24.3%	23984	*Pde10a*
chr17	8955545	intron:376734	11.5%	23984	*Pde10a*
chr17	8969673	exon:430628	6.3%	23984	*Pde10a*
chr17	8972335	intron:376739	36.0%	23984	*Pde10a*
BPA-related *Snrpn* DMCs					
Chr	**Position**	**Location**	**Δ % Methylation†**	**Gene ID**	**Gene name**
chr7	60155536	intron:186662	-6.4%	20646	*Snrpn*
chr7	60289904	intron:186663	-12.3%	20646	*Snrpn*
chr7	60289948	intron:186663	25.2%	20646	*Snrpn*
chr7	60439916	intron:186665	19.5%	20646	*Snrpn*

†Change in average % methylation from control to BPA samples

In our differential methylation analyses of the ERRBS data by bisphenol A (BPA) exposure, we identified a number of differentially methylated CpGs annotated to each of the top six imprinted loci that had differentially hydroxymethylated regions (DHMRs). All significant DMCs annotated to the six imprinted genes with visualized DHMRs are listed in the table. Each gene is split into a sub-table. Of note, we identified three CpG sites within the *Gnasxl/Nespas* ICR that demonstrated significant hypomethylation with BPA exposure. None of the identified DMCs at the other top imprinted genes – *Grb10*, *Plagl1*, *Pde10a*, *Klf14*, and *Snrpn* – fell in known murine imprinting control regions (ICRs). **BOLD** = Located in *Nespas/Gnasxl* domain ICR (Coombes et al. 2003; Williamson et al. 2006). Chr = chromosome number.

Table S3 – Number of cytosine-phosphate-guanine (CpG) and cytosine-H-guanine (CHG; where H = adenine or thymine) sites at imprinted gene differentially hydroxymethylated regions (DHMRs).

Gene Name	Location	Chr	Start	End	CpG Sites	CHG Sites
Gnas	intronic	chr2	174315451	174315750	0	21
Grb10	intronic	chr11	12004801	12005100	0	13
Plagl1	exonic	chr10	13130651	13131050	0	17
Klf14	exonic	chr6	30957751	30958050	23	11
Pde10a	intronic	chr17	8746901	8747050	1	10
Airn	intronic	chr17	12828001	12828550	4	25
Cmah	intronic	chr13	24335001	24335150	0	3
Snrpn	intronic	chr7	60207401	60207600	0	0
Ppp1r9a	intronic	chr6	5079501	5079850	1	31
Kcnq1	intronic	chr7	143348051	143348350	5	13
Phactr2	intronic	chr10	13402051	13402250	1	1
Pde4d	intronic	chr13	108963951	108964200	1	5

The number of CpG and CHG sites contained within the twelve bisphenol A (BPA)-related imprinted gene DHMRs were quantified using the mm10 reference genome (https://www.ncbi.nlm.nih.gov/nuccore). The number of CpG sites varied by region, with five of the DHMRs showing zero CpG sites. Chr = chromosome number.

Table S4 - Enriched bisphenol A (BPA)-related differentially hydroxymethylated region (DHMR) pathways.

Hypo-hydroxymethylated BPA-related DHMR Pathways			
Pathway Type	**Description**	**FDR**	**Genes**
Gene Ontology Biological Process	hippocampus development	0.007896	12568, 12570, 19280, 22062, 22416
Gene Ontology Biological Process	spinal cord association neuron differentiation	0.013765	18505, 21349, 22416

Hyper-hydroxymethylated BPA-related DHMR Pathways			
Pathway Type	**Description**	**FDR**	**Gene IDs**
Gene Ontology Molecular Function	L-ascorbic acid binding	0.006355	18484, 320452
Gene Ontology Molecular Function	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.006355	13105, 18484, 232174, 320452, 60527
Gene Ontology Cellular Component	nucleolar part	0.02366	12578, 68147
Gene Ontology Biological Process	positive regulation of establishment of protein localization to plasma membrane	0.028313	56212, 76686
Gene Ontology Molecular Function	isoprenoid binding	0.041794	225467, 232174

Pathway analysis for DHMRs was performed using the ChIP-enrich analysis tool (http://chip-enrich.med.umich.edu/). Within the ChIP-enrich tool, the gene set filter was set to 2000, peak threshold was set to 1, and adjustment for mappability of gene locus regions was set to false. The genome used for pathway analyses was mm10, and the ChIP-enrich method was used for enrichment testing. DHMRs were split into hypo- and hyper-methylated regions prior to separate analyses. Only regions <5kb from transcription start site (TSS) were included in ChIP-enrich analysis. All pathway analyses included gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genome (KEGG) pathways. After correcting for multiple testing, only pathways with false discovery rate (FDR) <0.05 were considered significant. Significant pathways for hypo- and hyper-methylated DHMRs are listed in separate tables; there was no overlap between significant pathways for these separate analyses. None of the significant pathways included any imprinted genes.

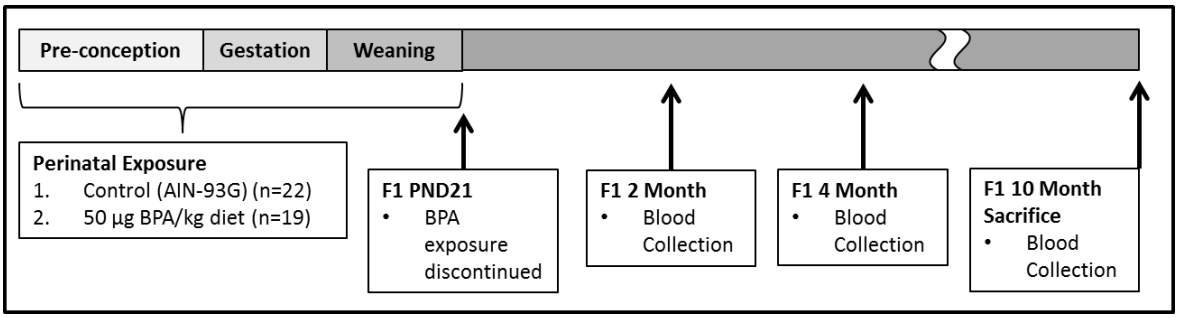
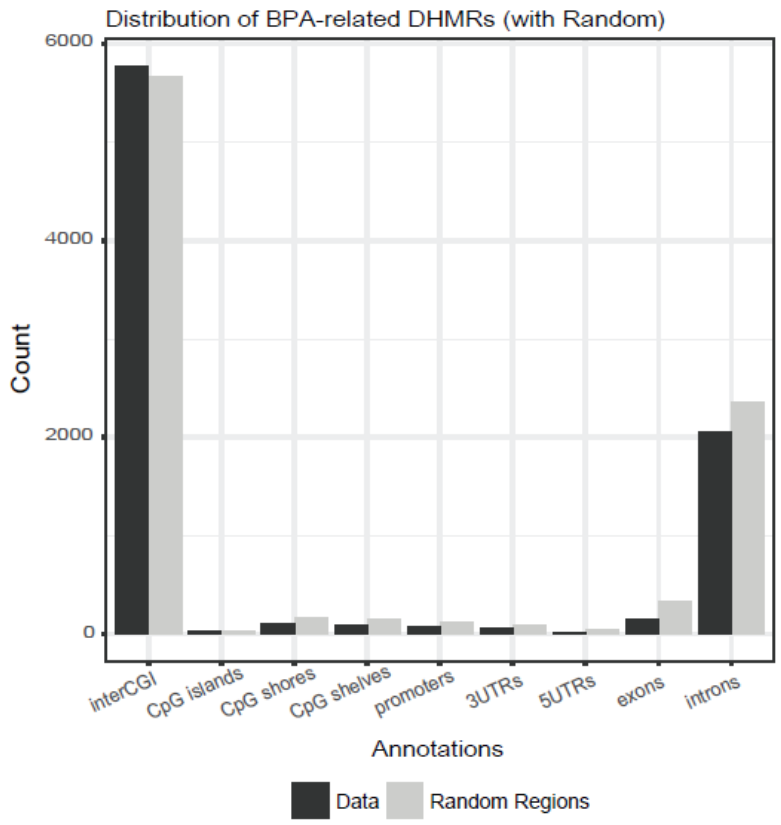


Figure S1. Exposure paradigm and blood collection time points to measure longitudinal 5-hydroxymethylcytosine (5-hmC) patterns. Two weeks prior to mate-pairing with A^{vy}/a males, six week old wild type *a/a* dams were placed on one of two experimental diet groups: (1) Control (modified AIN-93G), (2) Control + 50 µg bisphenol A (BPA)/kg diet. Exposure continued through pregnancy and lactation, ending for offspring at postnatal day (PND) 21. Matched blood samples were collected from wildtype *a/a* offspring at 2 months, 4 months, and 10 months of age.



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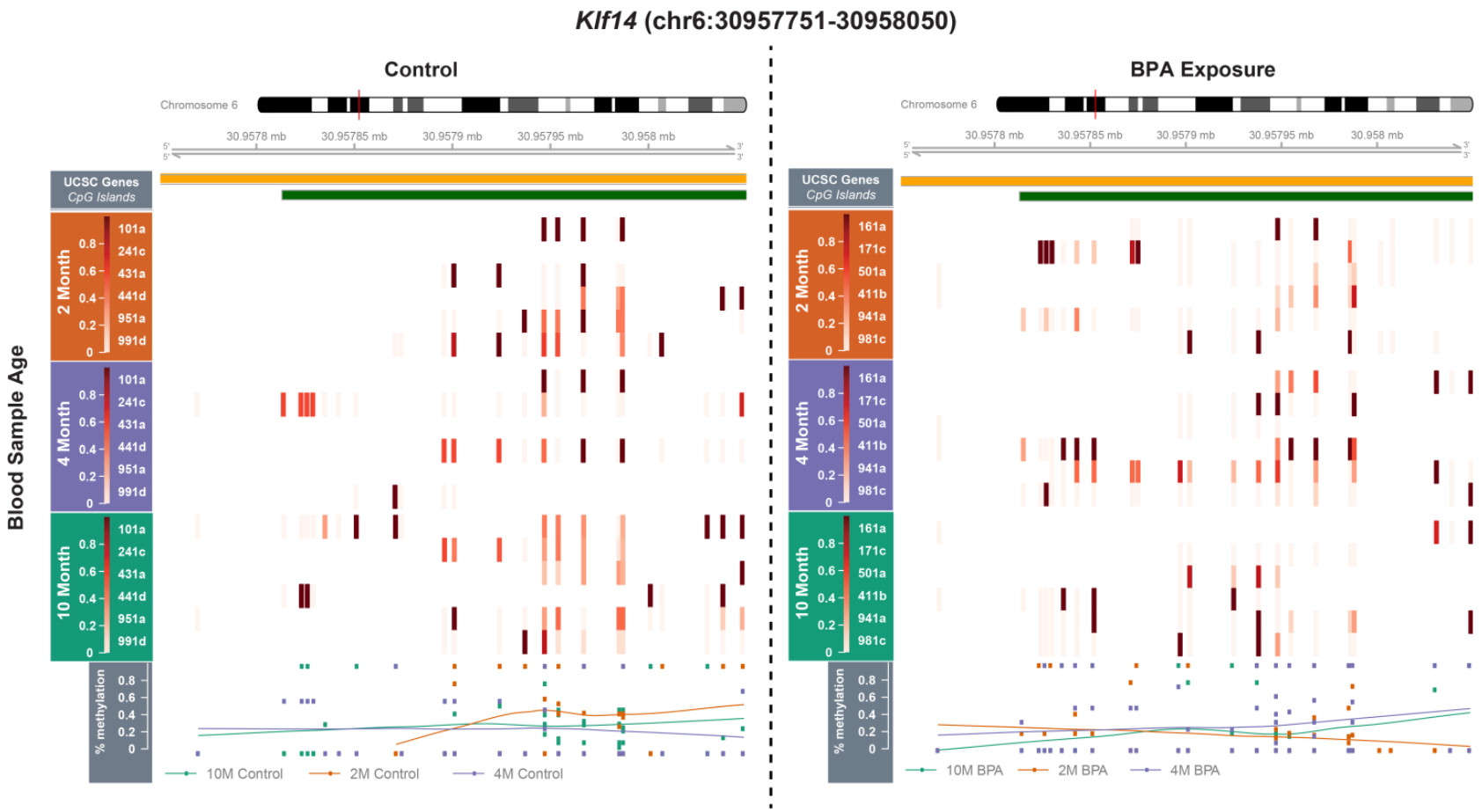

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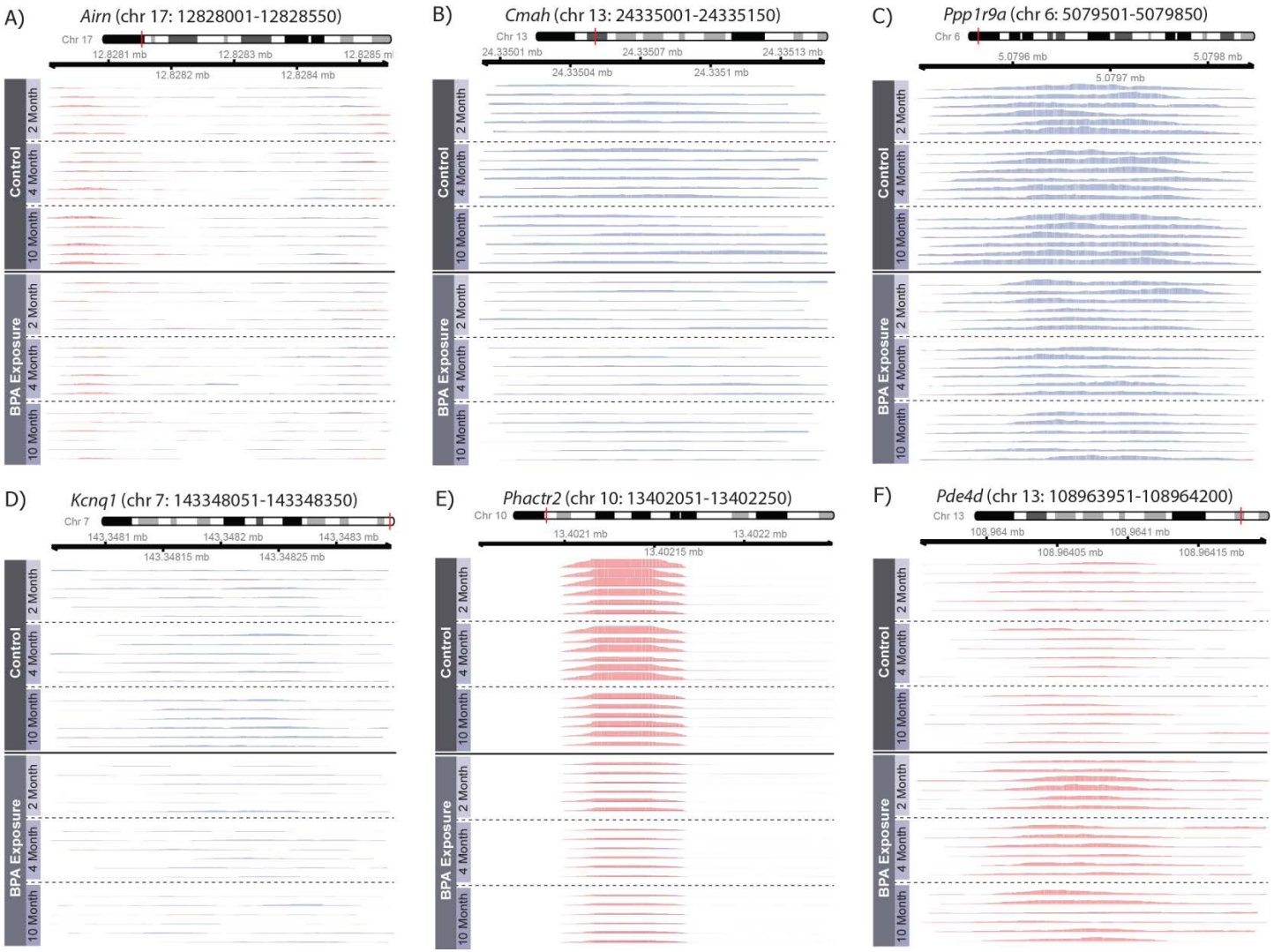

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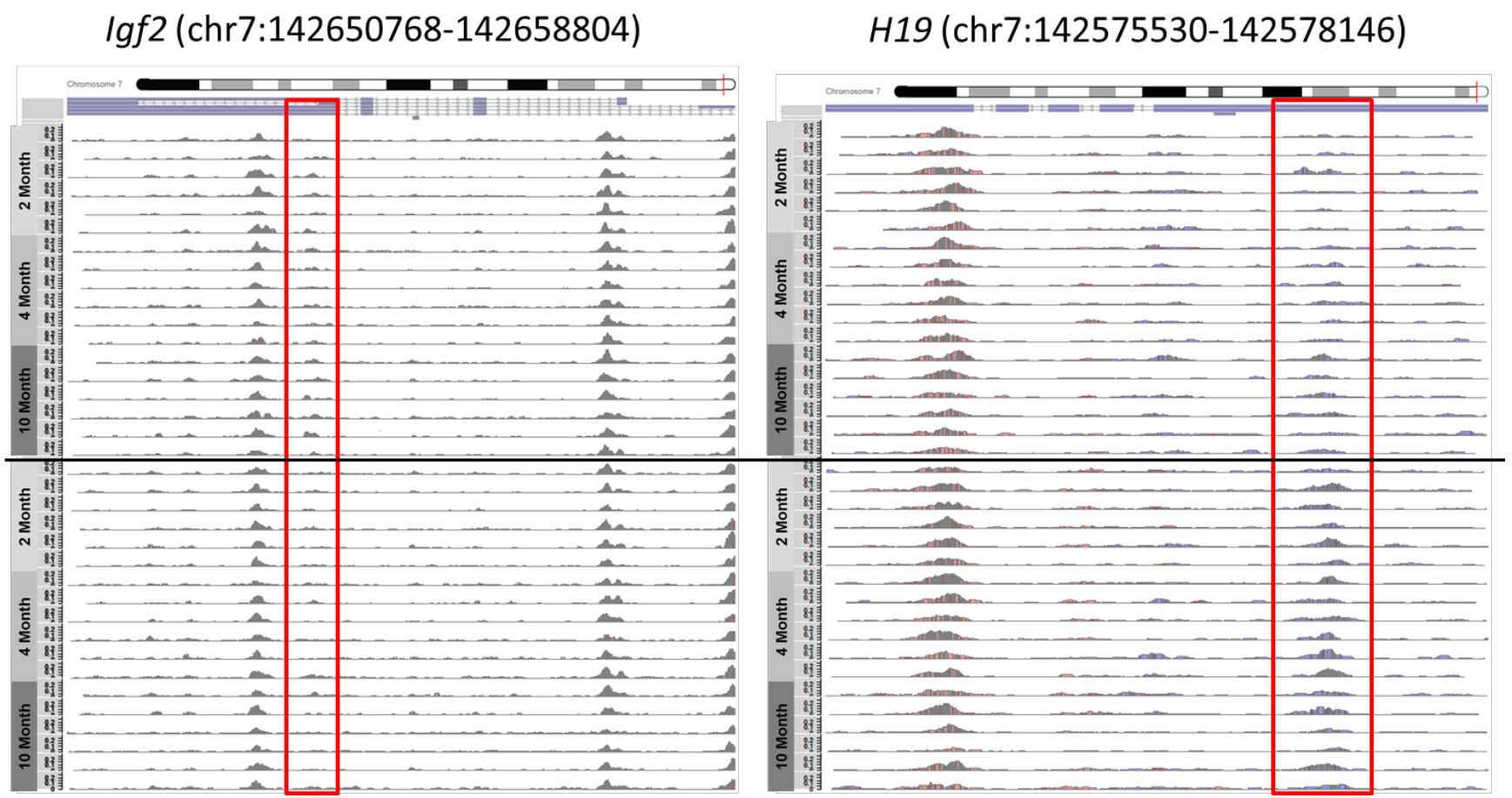

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