Published in final edited form as:
Epigenomics. 2013 October ; 5(5): . doi:10.2217/epi.13.49.

Early-life lead exposure results in dose- and sex-specific effects on weight and epigenetic gene regulation in weanling mice

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Abstract

Aims—Epidemiological and animal data suggest that the development of adult chronic conditions is influenced by early-life exposure-induced changes to the epigenome. This study investigates the effects of perinatal lead (Pb) exposure on DNA methylation and bodyweight in weanling mice.

Materials & methods—Viable yellow agouti (A^{vy}) mouse dams were exposed to 0, 2.1, 16 and 32 ppm Pb acetate before conception through weaning. Epigenetic effects were evaluated by scoring coat color of A^{vy}/a offspring and quantitative bisulfite sequencing of two retrotransposon-driven (A^{vy} and CDK5 activator-binding protein intracisternal A particle element) and two imprinted (*Igf2* and *Igf2r*) loci in tail DNA.

Results—Maternal blood Pb levels were below the limit of detection in controls, and 4.1, 25.1 and 32.1 μg/dl for each dose, respectively. Pb exposure was associated with a trend of increased wean bodyweight in males ($p = 0.03$) and altered coat color in A^{vy}/a offspring. DNA methylation at A^{vy} and the CDK5 activator-binding protein intracisternal A-particle element was significantly different from controls following a cubic trend ($p = 0.04$; $p = 0.01$), with male-specific effects at the A^{vy} locus. Imprinted genes did not shift in methylation across exposures.

Conclusion—Dose- and sex-specific responses in bodyweight and DNA methylation indicate that Pb acts on the epigenome in a locus-specific fashion, dependent on the genomic feature hosting the CpG site of interest, and that sex is a factor in epigenetic response.

Keywords

developmental origins of health and disease; DNA methylation; environmental epigenomics; epigenetics; lead; metastable epiallele; plasticity; viable yellow agouti

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

This work was supported by the University of Michigan (UM) NIEHS/EPA Children's Environmental Health Formative Center (P20 ES018171/RD834800), as well as the UM NIEHS Core Center (P30 ES017885). C Faulk was supported by the UM NIEHS Core Center (T32 ES007062). JM Goodrich was supported by the UM NIH National Center for Advancing Translational Sciences grant (2UL1TR000433). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
No writing assistance was utilized in the production of this manuscript.
The authors would like to thank BN Sanchez, Z Zhang and KE Peterson for their technical and intellectual assistance with this project.

The 'early origins' hypothesis postulates that nutrition and other environmental factors during prenatal and early postnatal development influence developmental plasticity, thereby, altering susceptibility to adult chronic diseases, including obesity [1,2]. However, relatively little research has considered the mechanisms by which perinatal environmental exposures may influence physical growth and development during sensitive periods in childhood. For example, animal and human data have established that exposure to lead (Pb) *in utero* affects birth size, neurodevelopment and the development of adult chronic conditions including high blood pressure and cardiovascular disease [3–7]. Despite these associations, no studies to date have robustly assessed the role of environmentally induced epigenetic gene alterations as a mechanism linking *in utero* Pb exposure to later in life disease risk.

Pb is commonly found throughout the environment as it is used in the manufacture of automotive batteries, paints, glazes, ammunition and piping, and in some parts of the world as an additive to gasoline. Thus, air, soil, water, old paint and food are all avenues for Pb uptake via ingestion, inhalation, and dermal absorption [8]. Since 1991, the US CDC have established a blood Pb level (BLL) of 10 μg/dl as the level of concern and recently lowered this to 5 μg/dl, yet research continues to find adverse health effects at even lower doses [9]. Transfer of Pb from mother to offspring is more efficient transplacentally and lactationaly than by oral ingestion in adults [10]. In mice, early-life Pb exposure acts as an obesogen, and even very low *in utero* doses can result in early puberty [11,12]. Mechanisms of health effects following early-life exposure are not well established, and studies of epigenetic modifications induced by developmental exposure to Pb are limited.

Epigenetic patterns in offspring, including DNA methylation and histone modifications, are known to be influenced by maternal nutrition, behavior, stress and toxins, causing changes that can persist into adulthood long after the acute developmental exposure has ceased [13,14]. Recent findings have confirmed this hypothesis following exposure to several organic toxicants [15], and here we explore the effects of the heavy metal Pb. Previously, our group has shown global and gene-specific changes in weanling DNA methylation induced by fetal exposure to bisphenol A [15]. Similarly, exposure to heavy metals has been shown to correlate with gene specific and global epigenetic effects for several metals including arsenic, nickel, chromium, cadmium and mercury, in combination [16]. To address the long-term stable epigenetic alterations resulting from early Pb exposure, we used the viable yellow agouti (A^{vy}) mouse model as a biosensor to expose mice to multiple physiologically relevant doses of Pb *in utero* and in early life.

The epigenetics of the A^{vy} mouse model are well established [17]. Interindividual variation in DNA methylation at this locus is visually reflected by the coat color of carrier mice, where increased DNA methylation levels yield darker coat colors in contrast to decreased DNA methylation levels yielding lighter yellow coat colors. The average level of DNA methylation, and consequently coat color, among a litter of mice is somewhat normally distributed; however, the distribution can be shifted towards more or less DNA methylation both nutritionally and toxicologically [15,18,19]. A^{vy}'s DNA methylation status is stochastically established in development, and remains fixed thereafter, so it is considered a metastable epiallele – a locus whose DNA methylation status varies among genetically identical individuals. In addition to A^{vy} the CDK5 activator-binding protein intracisternal A particle element (IAP; $Cabp^{IAP}$) metastable epiallele has been shown to be sensitive to environmental exposures, making them ideal for evaluating the effect of perinatal environmental exposures on the epigenome [20]. Both the A^{vy} and $Cabp^{IAP}$ metastable epialleles are the result of an inserted IAP retrotransposon repetitive element, and the variable methylation of CpG sites within their long terminal repeats drives the variable expression of nearby genes (Figure 1A). By contrast, imprinted genes harbor epigenetic

marks based upon the sex of the parent transmitting each copy [21]. Because methylation profiles at imprinted genes are allele specific and are often well characterized due to their importance in growth and development, they have frequently served as biomarkers of environmental exposure [22–28]. The *Igf2* and its receptor (*Igf2r*) are imprinted in mice and contain differentially methylated regions (Figure 1B). Both have previously shown varying or stable responses to environmental perturbations and are measured here as potential biomarkers.

Here, using a well-established mouse model of perinatal environmental exposures, we evaluate the effects of three physiologically relevant concentrations of Pb, 2.1, 16 and 32 ppm, on bodyweight and epigenetic patterning by observing weaning weight, coat color shift and concurrent change in DNA methylation at the A^{vy} locus. We also evaluate DNA methylation at a second IAP-driven metastable epiallele $Cabp^{IAP}$, and at two imprinted genes, *Igf2* and *Igf2r*. To our knowledge, this is the first study of early-life Pb exposure and its effects on DNA methylation and weaning weight in the A^{vy} mouse model.

Methods

Animals & diet

A^{vy} mice were obtained from a colony that has been maintained with sibling mating and forced heterozygosity of the A^{vy} allele through the male line for over 220 generations, resulting in a genetically invariant background [29]. To minimize the effects of parity and age, virgin *a*/*a* (wild-type) dams, of 6–8 weeks of age, were randomly assigned to one of four Pb treatment groups and fed a phytoestrogen-free AIN-93G diet (diet 95092 with 7% corn oil substituted for 7% soybean oil; Harlan Teklad, WI, USA). Pb (II) acetate trihydrate (Sigma-Aldrich, MO, USA) was dissolved in a single batch for each concentration level in distilled water, and the concentration was verified by inductively coupled plasma mass spectrometry (ICPMS; NSF International, MI, USA). Prior to study commencement, the distilled diluent water was confirmed as Pb free by ICPMS, and the same batch dilution of Pb-acetate-treated water was used throughout the study for all mice and administered using standard 8 oz gravity bottles that were changed at least weekly. Thus, exposure groups were as follows: distilled water (n = 11 litters, 78 total surviving offspring, 39 surviving A^{vy}/a offspring); distilled water supplemented with 2.1 ppm Pb acetate (n = 12 litters, 75 total surviving offspring, 41 surviving A^{vy}/a offspring); distilled water supplemented with 16 ppm Pb acetate (n = 12 litters, 86 total surviving offspring, 48 surviving A^{vy}/a offspring); distilled water supplemented with 32 ppm Pb acetate (n = 14 litters, 75 total surviving offspring, 42 surviving A^{vy}/a offspring). Blood was collected in a subset of dams at weaning by cardiac puncture and tested at the Michigan Department of Community Health (MI, USA), via ICPMS, with a limit of detection of 1.3 μg/dl, as summarized in Figure 2.

Following 2 weeks on their respective Pb-acetate water, dams were mated with A^{vy}/a males of 7–10 weeks of age. The animals were provided with access to diet and water *ad libitum.* Pb-acetate water was provided to dams throughout pregnancy and lactation. All offspring were weaned, tail tipped and weighed at postnatal day 22 (d22). Furthermore, A^{vy}/a offspring were scored for phenotype as follows: a single observer visually allocated all A^{vy}/a mice into five categories depending on the proportion of brown fur: yellow (<5% brown fur), slightly mottled (between 5 and 40% brown), mottled (~50% brown), heavily mottled (between 60 and 95% brown) and pseudoagouti (>95% brown).

The mice in this study were monitored by the University of Michigan Unit for Laboratory Animal Medicine (MI, USA) and treated humanely during the study and sacrifice. The guidelines for the care and use of laboratory animals were followed when maintaining the

mice. The protocol for the study was reviewed and approved by the University of Michigan Committee on Use and Care of Animals.

DNA isolation & methylation analyses

Using a standard phenol–chloroform–isoamyl alcohol protocol, total genomic DNA was isolated from d22 tail tissue (<3 mm) of all *a*/*a* and A^{vy}/*a* offspring [30]. The Qiagen Epitect kit (Qiagen, Venlo, The Netherlands) automated on the Qiagen QIAcube® purification system, was utilized for bisulfite conversion. Sodium bisulfite was added to approximately 1 μg of genomic DNA, thereby converting unmethylated cytosines to uracil, which are then replaced by thymine during PCR; methylated cytosines remained unchanged [31]. After bisulfite conversion, amplification of candidate gene regions of interest was performed using HotStarTaq master mix (Qiagen), forward primer (50 pmol), and reverse primer (50 pmol) in a 30 μl PCR and subsequently resolved by gel electrophoresis. The reverse primer was biotinylated in all assays. The PCR conditions, forward, reverse and sequencing primers and biotin labeling for all assays are shown in Table 1.

Pyrosequencing technology using PyroMark MD (Qiagen) was used to quantify DNA methylation of CpG sites of interest. To determine the percentage of methylation, PyroMark software calculates the fraction of 5-methylated cytosines among the total sum of methylated and unmethylated cytosines. Duplicate runs of all samples were performed, and these duplicates were averaged to determine the mean CpG site methylation to be used in statistical analysis. Figure 1 lists the mm9 chromosomal position, primer location, CpG sites, and sequences to analyze for pyrosequencing runs. The four CpG sites considered at the A^{vy} allele can be found at nucleotide positions 306, 319, 322 and 334 of GenBank accession number AF540972.1 and the insertion exists at mm9 genomic position chromosome (chr)2:154776947 (complementary strand) [29]. The four CpG sites considered at the $Cabp^{IAP}$ allele can be found at nucleotide positions 44, 57, 60 and 72 of GenBank accession number BB842254 or mm9 genomic position chr2:154179960–154180168 (complementary strand) [32]. The eight CpG sites considered at the *Igf2* allele can be found at nucleotide positions 1227, 1229, 1234, 1240, 1264, 1270, 1273 and 1279 of GenBank accession number AY849922 or mm9 genomic position chr7:149839707–149839926 (complementary strand) [33]. The five CpG sites considered at the *Igf2r* allele can be found at nucleotide positions 1070, 1076, 1091, 1106 and 1108 of GenBank accession number L06446 or mm9 genomic position chr17: 12935154–12935313 (complementary strand) [34].

Statistical analysis

The influence of perinatal Pb exposure on sex ratio, genotypic ratio and pup survival rate significance were determined by Fisher's exact test comparing exposure groups with control. Litter size variation across exposure groups was tested via analysis of variance with a Tukey's honestly significant difference *post hoc* adjustment to determine intergroup significance. Coat color distribution variation across exposure levels was performed using the χ^2 goodness-of-fit test with the control coat color distribution representing the expected distribution. The margin of error for maternal BLL was calculated at the 95% confidence level. Weaning weight and candidate gene methylation analyses were carried out by fitting to linear mixed models in the R statistical package, version 2.13.2 (package lme4) with weaning weight or gene methylation and exposure as fixed effects in each model and a random effect component to account for within-litter effects. P-values were calculated by Markov Chain Monte Carlo resampling (obtained by the pvals.fnc function). Pb exposure was an ordered factor, therefore linear, quadratic and cubic trends were fitted. Statistical significance for all analyses was defined as $p < 0.05$.

Results

Maternal BLLs for the control group on distilled water were all below the analytical limit of detection (1.3 μg/dl). While levels for the 2.1 ppm group ranged from 2.0 to 5.8 μg/dl (mean: 4.1), levels for the 16 ppm group ranged from 13 to 40 μg/dl (mean: 25.1), and levels for the 32 ppm group ranged from 16 to 60 μg/dl (mean: 32.1) (Figure 2). In comparison with control offspring, perinatal exposure to Pb acetate at 2.1, 16 or 32 ppm in drinking water did not significantly alter litter size, sex ratio or genotype ratio of *a*/*a* to A^{vy}/*a* offspring (Table 2). Sex ratio, as compared with control, reached near significance in the 16 ppm group ($p = 0.06$), with 64% male offspring. Survival rate to weaning was significantly altered between the control and 2.1 ppm concentration ($p = 0.01$), and was the result of poor maternal care in several litters in this group, causing a disproportionate loss of pups. The control group had a 96% survival rate, while the 2.1, 16 and 32 ppm groups had rates of 82, 96 and 90%, respectively (Table 2).

Bodyweight

Pb exposure did not result in a significant linear trend of altered wean bodyweight by exposure concentration for all animals ($p = 0.15$; $n = 314$); however, when females were excluded, we found a male-specific increase in the trend ($p = 0.03$; $n = 177$) (Table 3). When weaning weight analysis was restricted to *a*/*a* males, the significance was still observed ($p = 0.04$; $n = 80$) (Figure 3). In A^{vy}/*a* males, weaning weight was higher and showed a similar trend with increasing exposure, but did not reach significance across exposure groups. Female mice did not trend towards weight increase overall ($p = 0.93$) or in any exposure or genotype subgroup ($p > 0.79$).

Coat color effects

A^{vy}/*a* mice ($n = 170$; 54% of the total offspring) were evaluated at weaning for coat color classification, a preliminary visual indication of DNA methylation status at the A^{vy} metastable epiallele. Perinatal exposure to Pb acetate in drinking water shifted the coat color distribution of the A^{vy}/*a* offspring in a dose-dependent fashion (Figure 4). Maternal exposure to 32 ppm of Pb acetate shifted offspring coat color towards yellow (19% of control offspring characterized as yellow compared with 7% of control offspring; $p = 0.01$). The 16 ppm group did not significantly differ in color distribution from the control ($p = 0.64$). However, given the similarity in maternal BLLs among the 16 and 32 ppm groups, shown in Figure 2, we repeated the coat color classification analysis with these groups combined and found a significant shift in coat color from control ($p = 0.04$; $n = 90$). Interestingly, the 2.1 ppm exposure level resulted in a small decrease in yellow mice compared with control and a relatively large increase in pseudoagouti mice, 12% in control and 23% in the 2.1 ppm exposure group, suggesting a non-monotonic effect at this locus between low-level and the higher levels of exposure.

IAP-associated DNA methylation

As expected in this mouse strain, DNA methylation levels at the locus underlying the coat color, A^{vy}, corresponded with coat color with yellow mice displaying low levels of methylation compared with higher levels in mottled and pseudoagouti animals (data not shown). The levels of methylation at A^{vy} in Pb-exposed offspring were significantly different from control offspring following a cubic trend ($p = 0.04$; $n = 145$). The 2.1 ppm exposure group displayed a 10.9% increase in A^{vy} methylation, while minimal changes or a slight decrease in average DNA methylation were noted in the two higher exposure groups, compared with controls (Figure 5). Sex-specific analysis indicated that this trend was driven by male offspring ($p = 0.02$; $n = 83$) (Table 4). Similarly, DNA methylation patterns at a second murine metastable epiallele, $Cabp^{IAP}$, were significantly different in all exposed

animals as compared with control animals following a cubic trend (p = 0.01; n = 285). At this locus, both males and females displayed significant differences (p = 0.05 and 0.05; n = 166 and 119, respectively) (Table 4). When divided by genotype subgroups, the *a*/*a* offspring had a significant cubic trend (p = 0.01; n = 132), driven by the females (p = 0.01; n = 58), which is opposite to the results seen at the A^{vy} locus. Neither male nor female A^{vy}/*a* animals showed a significant trend in $Cabp^{IAP}$ methylation differences compared with the exposure group.

Imprinted gene DNA methylation

Two murine imprinted genes were assessed for methylation, *Igf2* and *Igf2r*. Unlike the IAP-associated loci, neither imprinted gene revealed consistent trends in methylation shifts across exposures or genotype groups. For *Igf2*, only *a*/*a* males reached near significance (p = 0.07; n = 173) in cubic trend. No subgroup of female or A^{vy}/*a* pups was significantly altered in methylation across exposures. In *Igf2r*, neither the group of mice as a whole, nor any subgroup of animals responded with a statistically significant cubic trend (Table 5) or linearly (data not shown).

Discussion

In the current study, we report a dose- and sex-dependent response in bodyweight and tail DNA methylation levels in weanling mice following perinatal exposure to three levels of Pb acetate in drinking water. First, we observed a significant linear trend increase in bodyweight at weaning with sex-specific effects. When segregated by sex, males exposed to 2.1, 16 and 32 ppm of Pb acetate exhibited increased weight compared with control offspring; however, effects on wean bodyweight were not observed for females. This association was stronger when analysis was restricted to *a*/*a* males and is not evident in *a*/*a* females. The A^{vy}/*a* males exhibited higher weaning weight trending with exposure, but did not reach significance across exposure groups, likely due to the confounding effect of the varying levels of methylation at the A^{vy} locus, which is known to affect bodyweight [35]. Weight gain in mice exposed to moderate levels of Pb during gestation has also been associated with male-specific increases in bodyweight at 1 year of age [11]. No other obvious health effects were evident in mice at this age, however, persistent effects throughout the life course should be determined. Rodent studies suggest Pb's effect on bodyweight may have a dual site of action: at the level of the hypothalamic pituitary unit, and directly at the level of gonadal steroid biosynthesis [36]. Among animals, Pb is believed to act on the hypothalamic–pituitary–gonadal axis by blocking the release of GnRH, thus decreasing puberty-related hormones such as LH, IGF-1 and estradiol [37–41]. At the gonadal steroid biosynthesis level, Pb has been shown to impair Leydig cell and Sertoli cell functions [42, 43]. Our measurement here was limited to overall weanling bodyweight rather than in changes in fat and lean body mass. Such measurements, particularly taken over the life course after early-life Pb exposure, will be important in defining human health relevance and toxicant influences on obesity.

Second, we observed that perinatal exposure to Pb acetate in drinking water shifted the coat color distribution of the A^{vy}/*a* offspring, a preliminary visual indication of altered DNA methylation status, in a dose-dependent fashion. When candidate IAP-associated and imprinted genes were assayed by quantitative bisulfite sequencing, IAP loci were associated with altered DNA methylation with exposure, following a cubic trend, while candidate imprinted genes were not associated with altered DNA methylation. The two IAP-associated metastable epialleles studied here, A^{vy} and $Cabp^{IAP}$, both vary in methylation at the site of an inserted transposable IAP long terminal repeat element. While IAP elements do not exist in human genome and are not directly relevant to human health, their frequency of

occurrence and activity in the mouse genome serves as a good proxy for changes in similar elements in humans. Although these insertions share 98.5% sequence identity [44], their shifts in methylation in response to Pb are subtly different. $Cabp^{IAP}$ is more highly methylated on average, and has a smaller methylation range. While both $Cabp^{IAP}$ and A^{vy} show a significant cubic trend with increased methylation levels in the 2.1 ppm group, the results for A^{vy} are sex-specific, driven by male offspring, and the results for $Cabp^{IAP}$ are significant among both male and female offspring. Sex-specific epigenetic effects have been observed following diverse environmental exposures, including in A^{vy} mice from radiation [45] and in mice exposed to altered nutrition [28,46], bisphenol A exposure [47], and with stress in rats [48]. Furthermore, in rats, multidose Pb exposure at two time points prior to weaning showed significant changes in *Dnmt1*, *Dnmt3a* and *MeCP2* expression, with differences seen even at the lowest concentration correlated to both sex and developmental window of exposure [49]. Interestingly, the hormetic response reported in Bernal *et al*. to low-dose radiation is similar to our low-level Pb exposure (2.1 ppm) increase in both coat color and directly tested DNA methylation levels at the IAP-associated loci [45].

Imprinted genes have been targeted as potentially more environmentally labile than other genomic regions [50,51]. Here, we studied two murine imprinted genes, *Igf2* and *Igf2r*, which have been previously shown to exhibit altered methylation upon various environmental exposures. Both are associated with differences in growth rate and present good targets for any correlation between weight and DNA methylation shifts [27,33,52]. In contrast, we found both genes to be stable in methylation after exposure to Pb *in utero* and during lactation. In our study, we assayed mouse tail tissue at d22, collected at weaning, whereas previous studies finding alterations have examined other tissues during different life stages, such as whole embryo, pancreas, fetal germ cells, muscle and liver. Similar to our results, several studies have also found stable methylation at these loci in tissues such as fetal gut, embryo and sperm. We consider the tail as a useful proxy tissue for imprinting methylation analyses since methylation changes early in development can be expected to propagate throughout the body. A literature survey of mouse exposure studies where our candidate genes were also subject to methylation analysis is summarized in Table 6. In light of these other studies, our data reinforce the notion that loci can react differently to various environmental exposures, suggesting tailored epigenetic reactions rather than a universal response. Pb has also been shown to act as both a gene-specific as well as global hypomethylation agent in rat pheochromocytoma cells, increasing the expression of amyloid precursor protein, suggesting a link between DNA methylation changes and disease etiology [53]. Together, these data suggest that Pb acts in a locus-specific fashion, potentially dependent on the genomic feature hosting the CpG site of interest (i.e., transposon, promoter or differentially methylated region), and that sex is a factor in the epigenetic response to Pb.

The toxic effects of Pb, especially in relation to neuropathology and cardiovascular disease risk have been studied for decades, resulting in direct human benefits across a range of outcomes (i.e., removal of Pb from gasoline and paint) [54,55]. Despite these efforts, Pb is still present in the environment and in consumer products, and with a half-life of years to decades, can be mobilized from bone during pregnancy and lactation. Thus, although the epigenetic effects of early-life Pb exposure are subtle, they may serve as an underlying mechanism linking exposure to later-in-life disease risk. Here we measured both the actual Pb content of the drinking water supplied to all mice in the study from a single dilution and the resulting BLLs in dams at weaning. Previous mouse studies involving Pb acetate exposure in drinking water range from exposures of 0.02 [12] to 4000 ppm [56] with widely varying resulting BLLs. Many factors may account for the differences in resulting BLLs between studies, including impurities in the water source, Pb acetate source and pH, among others. Thus, it is crucial to report both the diluted Pb water supply and the resulting BLLs in future studies. At the 2.1 ppm level, we found significant epigenetic effects, from a blood

level of approximately 4.1 μg/dl, corresponding to human relevant doses. The impact of *in utero* Pb exposure on the epigenome and downstream health implications for today's youth is relatively unknown but could be substantial. In a Mexican cohort recruited in the 1990s, over 75% of new mothers had a BLL greater than 5 μg/dl [57]. In the US population from 1988–1994, 5.8% of women of reproductive age had BLLs greater than 5 μg/dl while 25.6% of children between 1 and 5 years of age had a BLL above this level [58,59]. Therefore, low-level Pb exposure, particularly during the epigenetically sensitive developmental period, impacts a significant percentage of the human population and understanding its epigenetic consequences may provide direct health benefits.

Future perspective

An increasing understanding of environmental impacts on the epigenome is resulting in the recognition that the context and makeup of genes may govern their epigenetic response to toxic exposures. Likewise, more sensitive studies are revealing that effects of early-life exposures can reverberate throughout the life course. The continuing global Pb exposure risk combined with internal Pb sequestration in bone and subsequent release into blood, including during pregnancy, warrants an understanding of epigenetic effects established in early life that may have direct bearing on disease risk in later life.

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Executive summary

Background

- Chronic health conditions have been associated with early-life exposures to lead (Pb).
- Epigenetic mechanisms such as DNA methylation at CpG sites may mediate the connection between early-life exposure and later health effects.

Materials & methods

- The viable yellow agouti mouse (A^{vy}) varies in coat color according to DNA methylation at a single locus, and was exposed to Pb at concentrations of 0, 2.1, 16 and 32 ppm.
- Weaning weight was measured in both A^{vy} allele carriers (A^{vy}/a) and noncarriers (a/a [wild-type]).
- DNA methylation was assayed via pyrosequencing at two transposon-associated genes, A^{vy} and $Cabp^{IAP}$, and at two imprinted genes, *Igf2* and *Igf2r*.

Results

- Pb exposure was associated with increased weaning weight in a dose-dependent fashion in males only ($p = 0.03$).
- Coat color distribution in agouti offspring was shifted towards the hypomethylated yellow phenotype, and found to be significant between the control and highest dose group (32 ppm).
- In three dose groups, methylation at the transposon-associated genes A^{vy} and $Cabp^{IAP}$ was significantly shifted in a cubic trend, compared with controls ($p = 0.04$ and $p = 0.01$, respectively), with male-specific effects predominating at the A^{vy} locus.
- Imprinted gene methylation at *Igf2* and *Igf2r* was not significantly affected by Pb exposure.

Discussion

- Our findings suggest that the epigenome is affected by Pb in a dose- and sex-specific manner, and that sex is a predictor of physiological and epigenetic response to exposure.
- The epigenetic effects of Pb are specific and suggestive of a toxicant-specific response in development that can have long-term consequences for adult health.

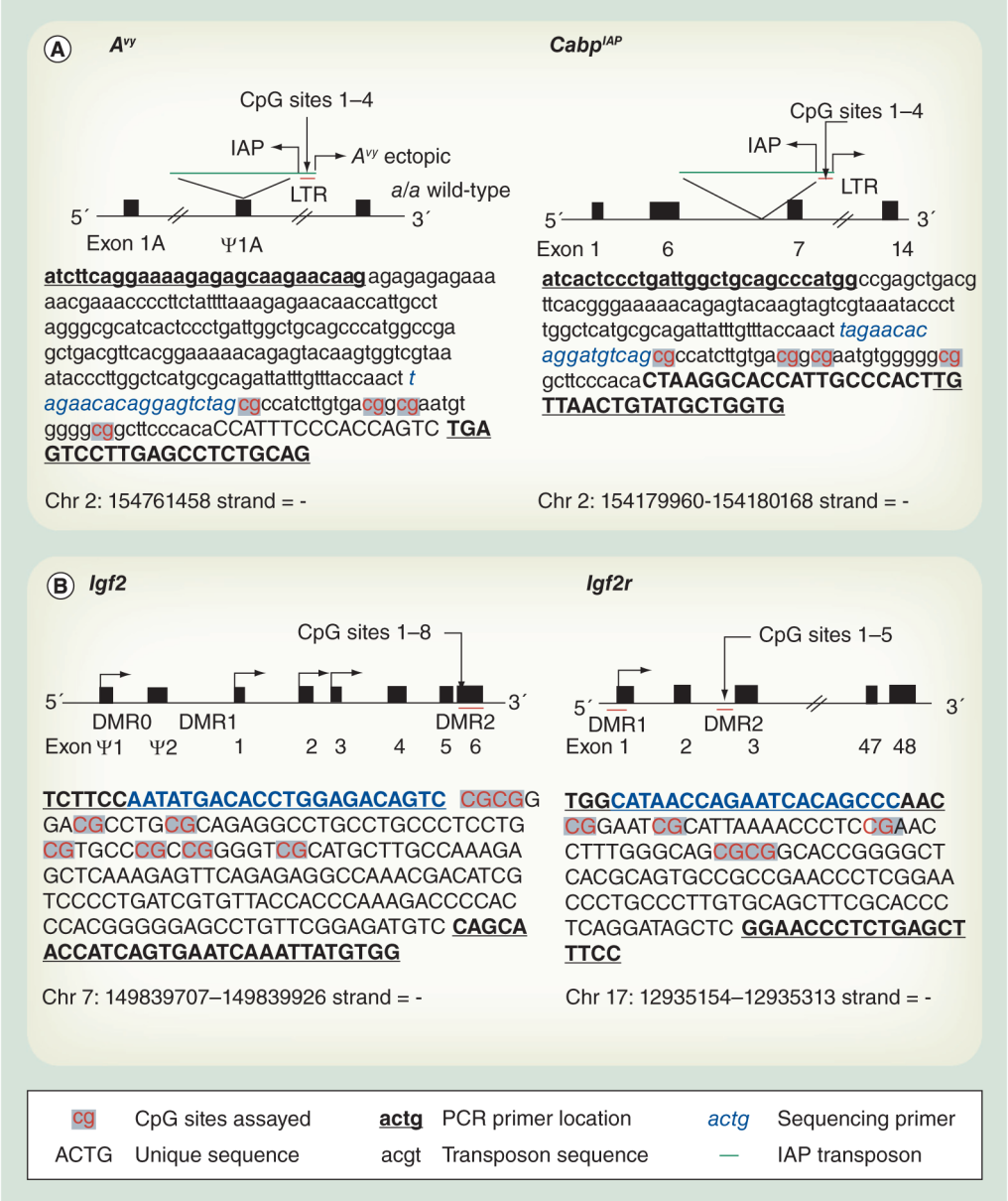

Figure 1. Metastable and imprinted genic regions

(A) Metastable epiallele loci, *A*^{vy} and *Cabp*^{IAP}, are caused by the insertion of an IAP element (green lines) containing variably methylated CpGs (sites labeled 1–4) in the LTR region (red lines). **(B)** Imprinted loci, *Igf2* and *Igf2r*, contain multiple differentially methylated regions (red lines) whose methylation status is dependent on the parent of origin. Assayed CpG sites are in red text. Primer locations are underlined; see Table 1 for primer sequences for bisulfite converted DNA. The sequencing primer location is in blue text. *A*^{vy}: Viable yellow agouti; *Cabp*^{IAP}: CDK5 activator-binding protein intracisternal A particle element; Chr: Chromosome; DMR: Differentially methylated region; IAP: Intracisternal A particle; LTR: Long terminal repeat.

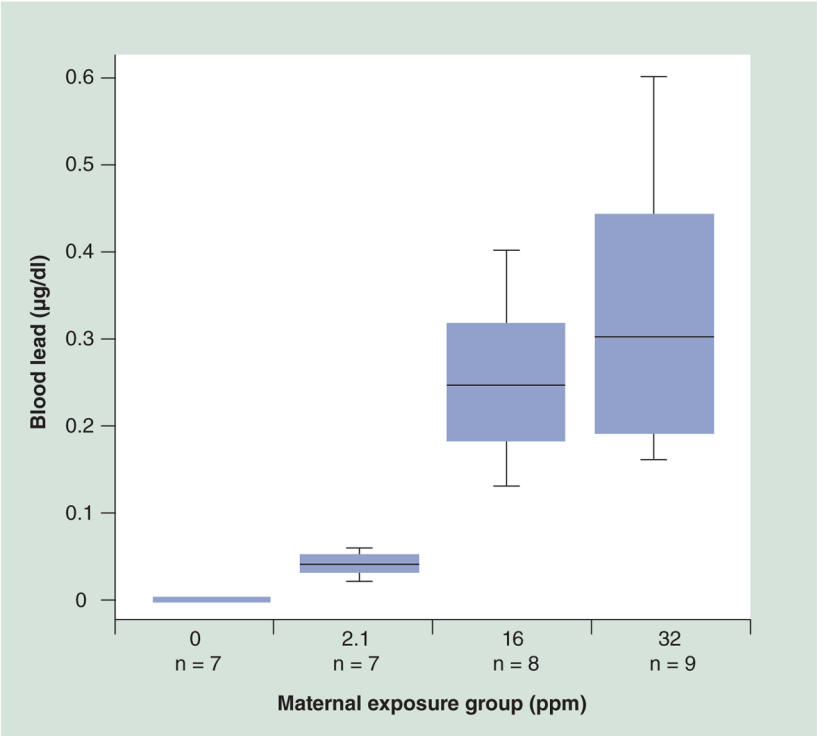

Figure 2. Maternal blood lead levels at 8 weeks of exposure

At weaning (day 22), blood lead levels were measured for 7–9 days from each exposure group. All controls (0 ppm) measured below the detection limit (1.3 μg/dl), the 2.1 ppm group averaged 4.1 μg/dl (± 1.3), the 16 ppm group averaged 25 μg/dl (± 7.3) and the 32 ppm group averaged 32 μg/dl (± 11.4).

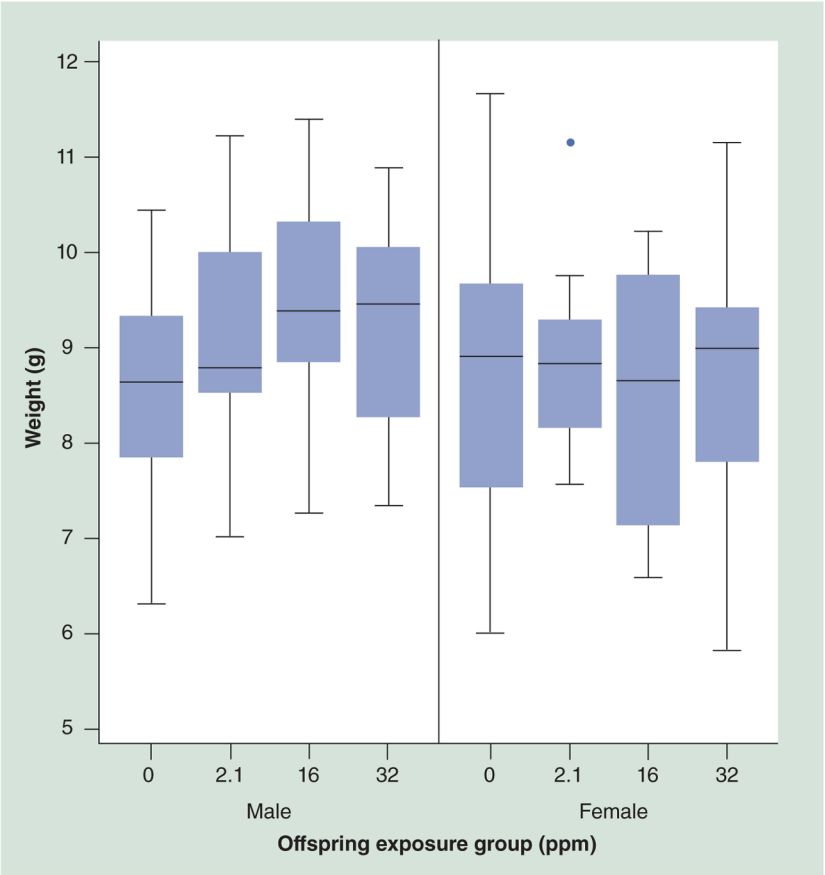

Figure 3. Day 22 weight in *a*/*a* (wild-type) animals across exposure groups

A linear increase in mean weight with increasing lead exposure was found in males (p = 0.04) but not females (p = 0.83) in *a*/*a* (wild-type) animals (n = 144). Among males, the mean weight for 0 ppm was 8.55 g (1.10 standard deviation [SD]), the 2.1 ppm group measured 9.14 g (1.15 SD), the 16 ppm group measured 9.51 g (1.22 SD) and the 32 ppm group measured 9.22 g (1.16 SD). Among females, the mean weight for 0 ppm was 8.60 g (1.48 SD), the 2.1 ppm group measured 8.74 g (0.99 SD), the 16 ppm group measured 8.35 g (1.37 SD) and the 32 ppm group measured 8.63 g (1.45 SD). The dot represents an outlier.

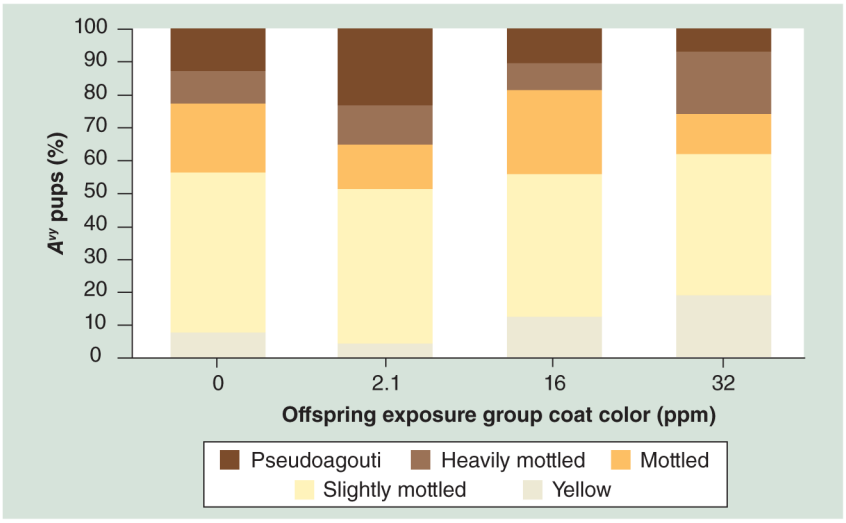

Figure 4. Coat color distribution by exposure

The percentage of animals with each coat color across five categories for all A^{vy}/a animals (n = 170) separated by lead exposure group is shown. A significant increase in yellow offspring between control and 32 ppm exposure (p = 0.01) reflects a decrease in methylation of the A^{vy} allele. At the 2.1 ppm concentration, the percentage of pseudoagouti mice increased from 12 to 23%, indicating an increased frequency of highly methylated A^{vy} offspring in this group and suggesting a non-monotonic effect at this locus with low lead exposure.

A^{vy}: Viable yellow agouti.

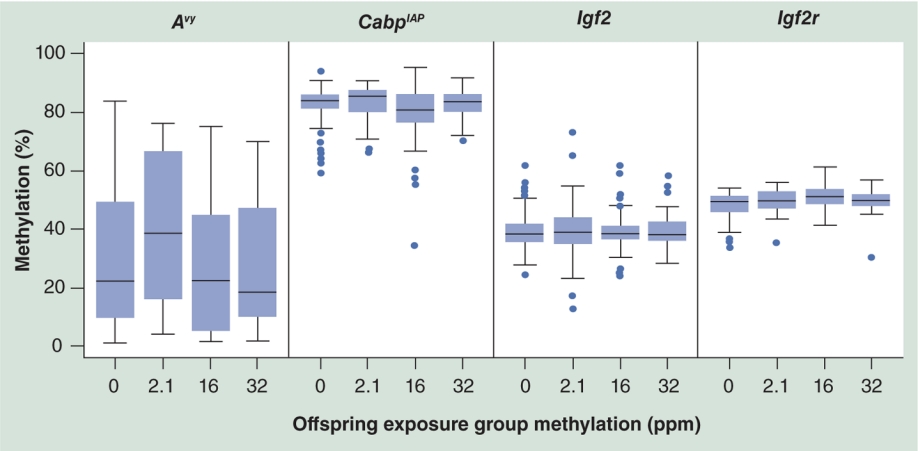

Figure 5. DNA methylation levels at metastable and imprinted loci

Day 22 tail tissue methylation assayed via pyrosequencing reveals a cubic trend of methylation in A^{vy} (p = 0.04; all A^{vy} animals) and $Cabp^{IAP}$ (p = 0.01; all A^{vy}/a and a/a [wild-type] animals) loci, resulting in an increased methylation level at the 2.1 ppm level and decreased methylation at higher levels. The imprinted loci, *Igf2* and *Igf2r*, do not show a significant shift in methylation levels across dosage in either linear or cubic trends for any subgroup of animals, suggesting that methylation response is dependent on the genetic features at each locus. A^{vy} naturally exhibits a wide range of variation from near 0 to 85% in this population, and the range is slightly reduced as dosage increases. By contrast, $Cabp^{IAP}$ has a smaller range and higher median methylation. The dots represent outliers.
A^{vy}: Viable yellow agouti; $Cabp^{IAP}$: CDK5 activator-binding protein intracisternal A particle element.

Table 1

Primers (5′ to 3′) and sequences to analyze DNA methylation quantification via pyrosequencing for candidate genes.

Primer/sequence to analyze	*A^{vy}* assay	*$Cabp^{IAP}$* assay	*Igf2* assay	*Igf2r* assay
Strand	Chr2: 154761458 strand = reverse	Chr2: 154179960–154180168 strand = reverse	Chr7: 149839707–149839926 strand = reverse	Chr17: 12935154–12935313 strand = reverse
Forward PCR primer	ATTTTTAGGAAAAGAGAGTAAGAAGTAAG	ATTATTTTTTGATTGGTTGTAGTTTATGG	TTTTTTAATATGATATTTGGAGATAGTT	TGGTATAATTAGAATTATAGTTTAAT
Reverse PCR primer	Biotin-CTACAAAAACTCAAAAACTCA	Biotin-CACCAACATACAATTAACA	Biotin-CCACATAATTTAATTCACTAATAATTACTA	Biotin-AAAAAACTCAAAAAATTCC
Sequencing primer	TAGAATATAGGATGTTAG	TAGAATATAGGATGTTAG	AATATGATATTTGGCGATAGTT	ATAATTAGAATTATAGTTTA
Sequence to analyze	YGTTATTTTGTGAYGGYGAATGTGGGGGYGGTT	YGTTATTTTGTGAYGGYGAATGTGGGGGYGGTT	YGYGGGAYGTTTGYGTAGAGGTTTGTTTGTTTTTTTGYGTGTTYGTYGGGGTYGT	ATYGGAATYGTATTAAAATTTTTYGAATTTTTGGGTAGYG
Amplicon length (bp)	294	209	220	127
Temperature (°C)	53	47	56	52
Cycles (n)	42	40	50	50

A^{vy}: Viable yellow agouti; $Cabp^{IAP}$: CDK5 activator-binding protein intracisternal A particle element; Chr: Chromosome.

Table 2

Litter parameters: offspring litter size, survival rate, genotype and sex ratio across exposure groups.

Exposure (ppm)	Litter (n)	Pups (n)	Mean pups (n)	Pup survival rate	*a/a* offspring (%)	Male offspring (%)
Control	11	81	7.4	0.96	50	49
2.1	12	91	7.6	0.82*	45	56
16	12	90	7.5	0.96	44	64**
32	14	83	5.9	0.90	44	57
Total	–	345	–	–	–	–
Survived	–	314	–	–	–	–

* $p < 0.05$ compared with control exposure group.

** $p < 0.10$ compared with control exposure group.

a/a: Wild-type.

Table 3

Offspring weaning weight from maternal lead exposure.

Exposure (ppm)	All live pups; g (SD)	All male; g (SD)	All female; g (SD)	*a*/*a* pups; g (SD)	*a*/*a* males; g (SD)	*a*/*a* females; g (SD)	A^{vy}/*a* pups; g (SD)	A^{vy}/*a* males; g (SD)	A^{vy}/*a* females; g (SD)
0	8.68 (1.4)	8.73 (1.4)	8.64 (1.4)	8.57 (1.3)	8.55 (1.1)	8.6 (1.5)	8.8 (1.5)	8.97 (1.7)	8.68 (1.3)
2.1	8.78 (1.5)	8.93 (1.6)	8.55 (1.4)	8.97 (1.1)	9.14 (1.1)	8.74 (1.0)	8.61 (1.8)	8.76 (1.9)	8.37 (1.7)
16	9.02 (1.4)	9.37 (1.3)	8.41 (1.4)	8.93 (1.4)	9.51 (1.2)	8.35 (1.4)	9.09 (1.4)	9.29 (1.3)	8.5 (1.6)
32	9.11 (1.5)	9.39 (1.3)	8.72 (1.6)	8.99 (1.3)	9.22 (1.2)	8.63 (1.5)	9.2 (1.6)	9.54 (1.5)	8.78 (1.7)
Linear trend p-value	0.15	0.03*	0.89	0.30	0.04*	0.83	0.16	0.15	0.79

*$p < 0.05$ compared with control exposure group.

a/a: Wild-type; A^{vy}: Viable yellow agouti; SD: Standard deviation.

Table 4

Percentage methylation summary for A^{vy} and $Cabp^{IAP}$ loci.

Exposure (ppm)	All live pups; % (SD)	All male; % (SD)	All female; % (SD)	*a/a* pups; % (SD)	*a/a* males; % (SD)	*a/a* females; % (SD)	A^{vy}/*a* pups; % (SD)	A^{vy}/*a* males; % (SD)	A^{vy}/*a* females; % (SD)
$Cabp^{IAP}$ (A^{vy}/a *and* a/a *offspring) mean methylation across four CpG sites*									
0	82.2 (6.7)	82.4 (7.1)	82 (6.3)	82.7 (5.4)	84.2 (4.8)	80.6 (5.7)	81.7 (7.7)	80 (8.9)	82.9 (6.7)
2.1	83.5 (5.7)	82.8 (6.5)	84.3 (4.3)	83.9 (6.7)	83.3 (8.1)	84.7 (4.6)	83 (4.5)	82.3 (4.7)	83.9 (4.1)
16	80.1 (9.4)	79.7 (10.3)	80.9 (7.6)	79.2 (7.6)	79.6 (7.7)	78.8 (7.6)	80.7 (10.7)	79.7 (11.6)	83.7 (6.9)
32	82.7 (4.6)	83.2 (4.8)	82 (4.4)	81.2 (4.2)	81.4 (4.5)	80.9 (3.9)	83.9 (4.7)	84.5 (4.6)	83 (4.8)
Cubic trend p-value	0.01*	0.05*	0.05*	0.01*	0.26	0.01*	0.11	0.12	0.96
A^{vy} (A^{vy}/a *offspring) mean methylation across four CpG sites*									
0	–	–	–	–	–	–	29.5 (24.8)	24.1 (26.1)	33 (23.9)
2.1	–	–	–	–	–	–	40.4 (24.8)	41.3 (21.2)	39.2 (29.4)
16	–	–	–	–	–	–	27.1 (23.1)	24.8 (23.1)	34 (23.1)
32	–	–	–	–	–	–	26.9 (21.8)	29.4 (24.1)	23.4 (18.5)
Cubic trend p-value	–	–	–	–	–	–	0.04*	0.02*	0.85

* $p < 0.05$ for the cubic trend.

a/a: Wild-type; A^{vy}: Viable yellow agouti; $Cabp^{IAP}$: CDK5 activator-binding protein intracisternal A particle element; SD: Standard deviation.

Table 5

Percentage methylation summary for *Igf2* and *Igf2r* imprinted loci.

Exposure (ppm)	All live pups; % (SD)	All male; % (SD)	All female; % (SD)	*a/a* pups; % (SD)	*a/a* males; % (SD)	*a/a* females; % (SD)	A^{vy}/a pups; % (SD)	A^{vy}/a males; % (SD)	A^{vy}/a females; % (SD)
Igf2 (A^{vy}/a *and* a/a *offspring) mean methylation across eight CpG sites*									
0	39.3 (6.9)	38.9 (7.4)	39.7 (6.3)	40.6 (7.1)	40.9 (7.5)	40.2 (6.5)	37.9 (6.5)	36 (6.4)	39.3 (6.3)
2.1	39.8 (9.8)	40.4 (10.0)	39.1 (9.6)	41.1 (9.0)	42.9 (8.1)	38.9 (9.8)	38.6 (10.5)	38.1 (11.2)	39.2 (9.8)
16	39.2 (5.9)	39.1 (6.5)	39.3 (4.8)	39.6 (4.7)	38.4 (4.5)	40.9 (4.6)	38.9 (6.8)	39.6 (7.5)	36.8 (4.0)
32	39.2 (5.1)	39.6 (5.7)	38.6 (4.2)	39.9 (4.8)	40.3 (5.5)	39.4 (3.7)	38.6 (5.4)	39.1 (6.0)	38.1 (4.5)
Cubic trend p-value	0.51	0.37	0.76	0.50	0.07*	0.38	0.97	0.85	0.45
Igf2r (A^{vy}/a *and* a/a *offspring) mean methylation across five CpG sites*									
0	48.0 (5.1)	48.2 (5.9)	47.9 (4.0)	49.1 (4.2)	49.2 (5.0)	49 (2.8)	46.8 (5.9)	46.8 (7.1)	46.7 (4.9)
2.1	49.4 (4.5)	49.1 (5.3)	49.6 (3.8)	49.9 (3.7)	52.6 (NA*)	49.4 (3.9)	49.2 (4.8)	48.8 (5.4)	49.8 (4.0)
16	51.0 (4.3)	51.7 (4.5)	49.2 (3.3)	50.9 (4.8)	52.1 (5.1)	48.9 (4.2)	51.1 (4.1)	51.6 (4.4)	49.5 (2.6)
32	49.6 (4.4)	48.9 (2.5)	50.3 (5.6)	50.2 (3.4)	48.2 (2.4)	52.2 (3.1)	49.2 (5.0)	49.3 (2.6)	49.1 (6.6)
Cubic trend p-value	0.62	0.19	0.50	0.80	0.96	0.52	0.59	0.37	0.56

*$p < 0.10$ for the cubic trend.

a/a: Wild-type; A^{vy}: Viable yellow agouti; $Cabp^{IAP}$: CDK5 activator-binding protein intracisternal A particle element; NA: Not applicable; SD: Standard deviation.

Table 6

Literature review of imprinted and metastable gene methylation shifts in mice.

Study (year)	Gene	Exposure	Methylation status	Tissue	Disease/condition	Ref.
McKay *et al.* (2011)	*Igf2*	Low maternal folate	Stable	Fetal gut	–	[23]
Downing *et al.* (2011)		*In utero* alcohol	Stable	Embryonic	Teratogenesis	[22]
Ding *et al.* (2012)		–	Hypermethylation	Pancreatic	Gestational diabetes mellitus	[24]
Susiarjo *et al.* (2013)		*In utero* BPA	Hypermethylation	Embryonic	Abnormal placentation	[27]
Zhang *et al.* (2012)	*Igf2r*	*In utero* BPA	Hypermethylation	Fetal germ cells	–	[25]
Somm *et al.* (2013)		*In utero* TCDD	Stable Hypermethylation	Sperm Muscle and liver	Decreased sperm count	[26]
Gallou-Kabani *et al.* (2010)		High-fat maternal diet	Sexual dimorphism	Placental	Altered nutrient transfer from the placenta to the fetuses	[28]
Dolinoy *et al.* (2006)	*Cabp*^{IAP}	*In utero* moderate-dose BPA	Hypomethylation	Liver	–	[19]
Anderson *et al.* (2012)		*In utero* low-dose BPA	Hypermethylation	Tail	Decreased weaning weight	[15]
Dolinoy *et al.* (2006)	*A*^{vy}	*In utero* moderate-dose BPA *In utero* BPA followed by genistein	Hypomethylation Stable	Tail	–	[19]
Anderson *et al.* (2012)		*In utero* low-dose BPA	Hypomethylation	Tail	Decreased weaning weight	[15]
Kaminen-Ahola *et al.* (2010)		*In utero* ethanol	Hypermethylation	Tail	Skull deformation	[60]
Bernal *et al.* (2013)		*In utero* radiation	Hypermethylation	Liver	–	[45]
Waterland *et al.* (2006)		*In utero* methyl donor diet	Hypermethylation	Tail	–	[18]
Dolinoy *et al.* (2006)		*In utero* genistein	Hypermethylation	Tail	–	[61]

A^{vy}: Viable yellow agouti; BPA: Bisphenol A; Cabp^{IAP}: CDK5 activator-binding protein intracisternal A particle element; TCDD: Tetrachlorodibenzo-p-dioxin.