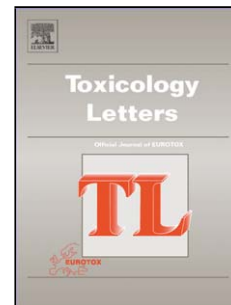


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Ontogenic Expression of Hepatic Ahr mRNA is associated with Histone H3K4 Dimethylation during Mouse Liver Development

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Running Title: Epigenetic Regulation of Ahr Ontogeny

Key words: Ahr; ontogeny; histone methylation; DNA methylation; liver

ABBREVIATIONS

Ahr, aryl hydrocarbon receptor; ARNT, Ahr nuclear translocator ; bDNA assay, branched DNA amplification technology; ChIP, chromatin immunoprecipitation; H3K4Me2, histone H3 dimethylation at lysine-4; H3K27Me3, histone H3 trimethylation at lysine-27; HSP90, heat shock protein 90; IGB, integrated genome browser; RLU, relative light units; TAS, Affymetrix Tiling Analysis Software; TSS, transcription start site

ABSTRACT

The aryl hydrocarbon receptor (Ahr) is a xenobiotic sensor that regulates the expression of a battery of drug-metabolizing genes. However, Ahr is also important for normal liver development. The purpose of the present study was to examine the ontogeny of Ahr mRNA in mouse liver, and determine the epigenetic mechanisms regulating Ahr gene transcription during postnatal liver development. There was a 224% increase in hepatic Ahr mRNA from 2 days before birth to 45 days after birth. ChIP-on-chip analysis demonstrated that DNA methylation and histone H3K27 tri-methylation (H3K27Me3), two epigenetic marks for suppression of gene transcription, were consistently low around the *Ahr* gene locus. In contrast, enrichment of histone H3K4 di-methylation (H3K4Me2), a hallmark for gene activation, increased 182% from prenatal to young adult period around the *Ahr* gene locus. Regression analysis revealed a strong correlation between enrichment of H3K4Me2 and Ahr mRNA ($r=0.91$). In conclusion, postnatal H3K4Me2 enrichment positively associates with Ahr mRNA in developing mouse liver, providing a permissive chromatin state allowing *Ahr* gene transactivation in postnatal liver development.

(171 words)

INTRODUCTION

The aryl hydrocarbon receptor (Ahr) is well recognized as a ligand-activated transcription factor for aromatic hydrocarbons, including TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and polycyclic aromatic hydrocarbons. In the absence of a ligand, the Ahr is sequestered in the cytosol by two molecules of heat-shock protein 90 (HSP90). Upon ligand binding, the Ahr is released from HSP90, translocates into the nucleus, and dimerizes with the Ahr nuclear translocator (ARNT). The Ahr-ARNT heterodimer then binds to the xenobiotic response element of target genes, which results in gene transcription (Li et al., 1994; Ma et al., 1995).

In mice, Ahr is also most enriched in lung, and is expressed at intermediate levels in liver and the gastrointestinal tract (Petrick and Klaassen, 2007). In liver, Ahr regulates the expression of a large battery of drug-metabolizing genes, including the prototypical target genes cytochrome P450 1A1, 1A2, and 1B1 (Rowlands and Gustafsson, 1997), several liver aldehyde dehydrogenases in mice (Alnouti and Klaassen, 2008b), some liver UDP glucuronosyltransferases in rats (Shelby and Klaassen, 2006) and mice (Buckley and Klaassen, 2009), the organic anion transporting polypeptides 2b1 and 3a1 (Cheng et al., 2005), and some multidrug resistance-associated protein efflux transporters in mice (Cheng et al., 2005; Maher et al., 2005).

In addition to its role in drug metabolism, the Ahr is also important for normal liver development. Ahr-null mice have been engineered, either by deleting Ahr exon 1 (Fernandez-Salguero et al., 1995; Gonzalez and Fernandez-Salguero, 1998) or exon 2 (Schmidt et al., 1996; Harstad et al., 2006). Common phenotypes of the two lines of Ahr-null mice include a marked decrease in liver size per gram of body weight, moderate hepatic portal fibrosis, and decreased constitutive expression of certain xenobiotic-metabolizing enzymes, such as Cyp1a2 (Lahvis and Bradfield, 1998). In addition to the common liver phenotypes, Ahr-null mice with exon 1 deletion also have increased mortality within the first 2 weeks of age, hyper-proliferative blood vessels in the portal areas of the liver, glycogen depletion in liver, inflammation of bile ducts, and adenocarcinomas with aging (Gonzalez and Fernandez-Salguero, 1998). In contrast, Ahr-null mice with exon 2 deletion are viable and fertile, but exhibit a spectrum of hepatic defects including transient microvesicular fatty metamorphosis, prolonged extramedullary hematopoiesis, and portal hypercellularity with thickening and fibrosis (Schmidt et al., 1996).

Although the Ahr plays important roles in both drug metabolism and normal development, the mechanisms underlying the developmental regulation of the Ahr gene *in vivo* are poorly characterized. Recently, it has become increasingly evident that developmental gene regulation

is controlled by epigenetic mechanisms (Jaenisch and Bird, 2003; Kiefer, 2007). DNA methylation and histone modifications are the ultimate regulatory epigenetic mechanisms of gene expression during development. In general, changes in DNA methylation profiles and chromatin structure determine whether there is a permissive chromatin environment for the transcription machinery to access gene promoter regions and initiate transcription. DNA methylation is a covalent modification that results in stable silencing of the function of methylated DNA sequences during development, either by interfering directly with transcription factor binding to response elements of target genes, or by recruiting corepressor complexes and reinforcing gene silencing (Bird, 2002; Reik, 2007).

Histone modifications can be divided into those that correlate with activation and those that correlate with repression. Acetylation, methylation, phosphorylation, and ubiquitination have been implicated in activation, whereas methylation, ubiquitination, sumoylation, deamination, and proline isomerization have been implicated in repression (Kouzarides, 2007). Formerly, it was thought that histone acetylation was the most important epigenetic modification regarding the ability for genes to be transcribed. However, it is now known that histone acetylation is not only involved in transcription, but also in DNA repair, replication, and condensation (Kouzarides, 2007). Histone H3 phosphorylation has also been shown to correlate with gene trans-activation. However, like histone acetylation, phosphorylation of histone is not very specific for gene transcription, because it correlates with entry into mitosis as well (Cheung et al., 2000). Only recently, by analyzing on a genome-wide scale chromatin landmarks and transcription initiation of most promoters in human ES cells, several classes of genes with various histone methylations have been identified (Guenther et al., 2007). The majority of actively transcribed genes have histone methylation at the K4 position around the transcription start site. Histone H3 lysine 4 di-methylation (H3K4Me₂) is found in a broad range of promoters, enhancers, and long-range regulatory elements, and it also strongly associates with tissue-specific gene regulation (Bernstein et al., 2005). In addition, it has also been shown that the co-occurrence between certain types of histone acetylation and methylation of H3K4 is high (Bernstein et al., 2005; Pokholok et al., 2005). Until now, vast amount of data have confirmed that H3K4me₂ is related to initiation of gene transcription on a genome-wide scale, building a strong argument that H3K4Me₂ is important in gene transcriptional activation (Bernstein et al., 2005; Kim et al., 2005; Roh et al., 2006). In contrast, H3 lysine-27 tri-methylation (H3K27Me₃) is associated with suppression of gene transcription, because H3 lysine-27 tri-methylated histone is a target for the chromodomain protein Polycomb, that silences genes by yet unknown mechanisms (Boyer et al., 2006; Lee et al., 2006; Kiefer, 2007).

Little is known of the ontogeny of the *Ahr* mRNA in mouse liver during development, nor the epigenetic mechanism of *Ahr* gene transcriptional activation during development. Therefore, the purpose of the present study was to reveal the ontogeny of hepatic *Ahr* mRNA expression in mice, and determine the epigenetic mechanisms mediating the *Ahr* mRNA expression during liver development. Because alterations of chromatin structure by epigenetic modifications is a critical mechanism to regulate gene expression, it is hypothesized in the present study that specific epigenetic marks associate with changes in *Ahr* mRNA expression during liver development in mice.

METHODS

Animals. Eight-week old C57BL/6 breeding pairs were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed according to the American Animal Association Laboratory Animal Care guidelines, and were bred under standard conditions at the University of Kansas Medical Center. Breeding pairs were established at 4:00 pm, and separated the following day at 8:00 am. The body weight of females was recorded each day to monitor pregnancy status. Livers from offspring were collected at the following four ages: day -2, 1, 5, and day 45, representing 3 different developmental periods: prenatal (day -2), neonatal

(day 1 and 5), and young adulthood (day 45). Due to the smaller liver size, from two days before birth to 5 days of age, livers from male and female offspring (same litter) were pooled at each age to achieve the minimum amount of liver needed for subsequent experiments. Due to variations caused by estrous cycle in sex-maturing adult female mice, only male livers were used at day 45 of age. Livers were frozen immediately in liquid nitrogen, and stored at -80°C . All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at KUMC.

RNA Isolation. Total RNA was isolated using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) per the manufacturer's protocol. RNA concentrations were quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at a wavelength of 260 nm. The integrity of the total RNA samples was evaluated by formaldehyde-agarose gel electrophoresis, and confirmed by visualization of 18S and 28S rRNA bands.

Branched DNA Amplification (bdNA) Technology. The mRNA expression of Ahr was determined by bdNA assays (QuantiGene bdNA signal amplification kit, Panomics, Fremont, CA). Multiple oligonucleotide probe sets for Ahr (including capture, label, and blocker probes) were designed using ProbeDesigner Software v1.0 (Bayer Corp., Diagnostics Div.) as previously described (Petrick and Klaassen, 2007). Ten μg of total RNA was added to each well of a 96-well plate ($n=5$ per age). The mRNA was captured by specific probe sets and attached to a branched DNA amplifier. Enzymatic reactions occur upon substrate addition and the luminescence for each well is reported as Relative Light Units (RLU). Statistical significance compared to day 45 expression levels were considered at $p<0.05$ (one way ANOVA followed by Duncan's multiple range post hoc test (SPSS program, Chicago, IL)).

Western blotting Analysis. AhR protein was quantified by western blotting analysis during liver development (day -2 and day 1: $n=2$; day 5 and day 45: $n=3$). Protein concentrations from total tissue homogenate were determined using Pierce protein assay reagents according to the manufacturer's recommendations (Pierce Biotechnology, Rockford, IL). Briefly, $60\mu\text{g}$ of total protein was loaded per lane and separated on 7.5% sodium dodecyl sulfate–polyacrylamide gels. Proteins were transferred overnight at 4°C to polyvinylidene difluoride membranes. Membranes were blocked for 2h in blocking buffer (1% nonfat dry milk with 0.5% Tween 20). All primary and secondary antibodies were diluted in blocking buffer. Primary antibody (rabbit polyclonal) against mouse AhR protein (SA-210) was purchased from BIOMOL (Plymouth Meeting, PA). Protein levels of mouse β -actin were used as a loading control (Primary antibody: ab8227, Abcam, Cambridge, MA). Lung from Adult C57/BL6 male mice was used as a positive control for AhR protein expression. Liver from adult male Ahr-null male mice was used as a negative control. AhR-null mice were purchased from Jackson laboratories (stock # 002831) as previously described (Schmidt et al., 1996). Blots were subsequently incubated with a goat anti-rabbit horseradish peroxidase–conjugated secondary antibody for 1 h. Protein-antibody complexes were detected using an ECL chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and exposed to Fuji Medical X-ray film (Fisher Scientific, Springfield, NJ).

In silico analysis of CpG island localization. CpG islands are defined as DNA sequences at least 200bp in length, with a GC percentage greater than 50% and an observed/expected CpG ratio that is greater than 60%. An *in silico* analysis of CpG islands within 10kb upstream plus 1kb downstream of the Ahr promoter region was performed using the Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA).

ChIP-on-chip assay of DNA methylation and histone methylation. Genpathway's ChIP-on-chip assays (San Diego, CA) using Affymetrix GeneChip Mouse Tiling 2.0R E array, were used to determine the following epigenetic profiles of Ahr on mouse chromosome 12: DNA methylation, histone H3K4 di-methylation, and histone H3K27 tri-methylation as described previously (Li et al., 2009).

Briefly, for DNA methylation, genomic DNA from mouse livers was isolated and sonicated to an average length of 300-500 bp. An antibody against 5-methyl-cytosine was used for

immunoprecipitation (ab51552, Abcam, Cambridge, MA). DNA without immunoprecipitation was used as a control for background hybridization. The specificity of the immunoprecipitation was validated by quantitative PCR reactions with positive and negative control primers. Both the immunoprecipitated and control DNA were amplified by random priming. The amplified DNA was purified, quantified, and tested by quantitative PCR at the same genomic regions as the original immunoprecipitated DNA to assess quality of the amplification reactions. Amplified DNAs were fragmented and labeled using the DNA Terminal Labeling Kit (Affymetrix, Santa Clara, CA), and then hybridized to Affymetrix GeneChip Mouse Tiling 2.0R E Array at 45°C overnight. Arrays were washed and scanned by a GeneChip HT Array Plate Scanner.

For histone modifications, liver homogenates were fixed with 1% formaldehyde and quenched with glycine. Chromatin containing DNA cross-linked by formaldehyde was isolated and sonicated to an average length of 300-500 bp. Methylated histone H3 proteins at lysine 4 and 27 were immunoprecipitated with polyclonal antibodies (Millipore 07-030 for H3K4Me2, and Millipore 07-449 for H3K27Me3) (Millipore, Billerica, MA). Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinking was reversed by incubation overnight at 65°C, and DNA fragments were purified by phenol-chloroform extraction, precipitated by ethanol, labeled, and then hybridized to the Affymetrix GeneChip Mouse Tiling 2.0R E arrays. Arrays were scanned with a GeneChip HT Array Plate Scanner.

Data from the arrays were analyzed using the Affymetrix Tiling Analysis Software (TAS), which generated BAR files that contain the intensities for all probes on the arrays. The intensities are expressed as fold enrichment of ChIP signals vs. input signals. The data image was viewed by the Integrated Genome Browser (IGB) (Affymetrix, Santa Clara, CA). The boundaries of detection were set from 10kb upstream of the transcription start site, to 10kb downstream from the end of the entire gene locus. Based upon recommendations by Affymetrix and known positive and negative genomic regions, thresholds were set as >3-fold enrichment of the probe intensities for DNA methylation, and >4-fold enrichment for histone methylations. Enrichments above the recommended thresholds reflect a false discovery rate below 2%. Intervals were defined as regions of a sequence > 200bp with signals higher than the threshold values, and active regions are genomic regions that contain one or more intervals in close proximity to each other (with intervals overlapping by at least 1 bp to be added to the same active region) at any given age.

Regression analysis of Ahr mRNA expression with DNA and histone methylations. The mRNA expression of Ahr during liver development and the patterns of the three epigenetic marks (DNA methylation, histone H3K4Me2 and H3K27Me3) were analyzed using Sigma Plot 10.0 (Systat Software Inc., San Jose, CA).

RESULTS

Ahr mRNA expression during mouse liver development. The ontogenic expression of mouse liver Ahr mRNA is shown in Fig. 1A. Ahr mRNA expression was low before birth, increased after birth (216% of prenatal levels at day 1, and 162% at day 5 of age), and peaked at 45 days of age (293%). Compared to levels at day 45 of age, Ahr mRNA expression was significantly lower at day -2 and 5. The Ahr mRNA expression at 1 day of age also appeared lower than adult levels, although the difference was not statistically significant. Corresponding to the Ahr mRNA expression, western blotting analysis demonstrated that the AhR protein was low two days before birth, but was up-regulated in liver right after birth, and remained high till day 45 of age (Fig.1B). Because it has been demonstrated that AhR protein expression in lung of rodents is high (Pollenz et al. 1998; Petrick and Klaassen, 2007), adult lung from wild-type mice was used as a positive control, whereas adult liver from Ahr-null mice was used as a negative control as described in METHODS.

DNA Methylation of the *Ahr* gene during liver development. The *Ahr* gene is approximately 36.9 kb long on chromosome 12. Within 10kb upstream and 1kb downstream of the *Ahr* gene promoter region, *in silico* analysis identified one CpG island slightly upstream of the TSS site, which is 1635bp in length. However, despite the presence of a GC-rich region, DNA-methylation signal enrichment within the CpG island did not reach the threshold value of 3.0-fold for positive DNA methylation, and there was also no enrichment in DNA methylation throughout the *Ahr* gene locus (Fig. 2, upper panel). In addition, there was no DNA methylation enrichment upstream of the *Ahr* gene transcription start site or downstream of the end of the *Ahr* gene (10kb up and down). The fold changes were all below the threshold of 3-fold (day -2, 1.12-fold; day 1, 1.27-fold; day 5, 1.28-fold; and day 45, 1.14-fold). In summary, *Ahr* DNA methylation signals were consistently low in the developing mouse liver.

Di-methylation at lysine-4 of histone H3 (H3K4Me2) of the *Ahr* gene during liver development. Based on the threshold (4.0), four active regions for H3K4Me2 were found around the *Ahr* gene locus, which are all in the gene, and are around 1.5, 3.3, 5.4, and 32.9kb downstream of the TSS. There was no H3K4Me2 enrichment upstream of the *Ahr* gene transcription start site or downstream of the *Ahr* gene locus (10kb up and down). The fold change of H3K4Me2 was lower than the threshold at day -2 of age with no positive H3K4Me2 regions of the *Ahr* gene locus (Fig. 3, upper panel) (fold change at day -2: 2.3-fold, Fig. 3, lower panel). At 1 day of age, there was an increase in the fold change of H3K4Me2 of the *Ahr* gene, with one interval identified in the gene (fold enrichment: 6.03), which is located 1350 bp downstream of the promoter region (Fig. 3, upper panel), although the overall average fold changes still did not reach the 4.0 threshold (3.3-fold, Fig. 3, lower panel). At 5 days of age, there was another positive H3K4Me2 region identified in the gene, which is 1405 bp downstream of the promoter region (fold enrichment: 6.02, Fig. 3, upper panel), but still, the overall average fold change was below the threshold (3.7-fold, Fig. 3 lower panel). At 45 days of age, there was a strong enrichment of histone H3K4 di-methylation that exceeded the threshold, with four active regions in the *Ahr* gene locus, at 1522 bp (8.46-fold), 3271 bp (5.33-fold), 5374 bp (6.21-fold), and 32.85kb (5.86-fold) downstream of from the TSS, respectively (Fig. 3, upper panel), and the overall average fold change was as high as 6.5-fold (Fig. 3, lower panel). In summary, there was an enrichment in the histone H3K4 di-methylation of the *Ahr* gene locus from prenatal to the young adult period in mouse liver, and the value in young adults was 182% higher than the prenatal value.

Tri-methylation at lysine-27 of histone 3 (H3K27Me3) at the *Ahr* gene in liver during development. There was no H3K27Me3 enrichment upstream of the *Ahr* gene transcription start site or downstream of the end of the *Ahr* gene (10kb up and down). As shown in the upper panel, the H3K27Me3 average values for the *Ahr* gene locus gradually decreased during development, however, all fold changes were below the 4.0-fold at any age examined (-2, 1, 5, and 45 days of age). The average fold changes of H3K27Me3 around the *Ahr* gene locus (including 10kb up and down) were computed and shown in the lower panel of Fig. 4. The histone H3K27 tri-methylation values for *Ahr* were approximately 1.23-fold at day -2 of age, followed by 1.08-fold at day 1, 0.99-fold at day 5, and 0.87-fold at day 45. In summary, the histone H3K27 tri-methylation signals of *Ahr* gradually decreased and were lower than threshold throughout liver development.

Regression analysis of *Ahr* mRNA expression and epigenetic marks. Regression analysis demonstrated no correlation between DNA methylation of the *Ahr* gene locus and *Ahr* mRNA ($R = 0.06$), whereas histone H3K4Me2 and H3K27Me3 each exhibited a strong correlation with *Ahr* mRNA expression ($R = 0.91$ and -0.86 , respectively). However, histone H3K4Me2 of the *Ahr* gene locus was the only methylation profile to exceed the threshold value (4.0-fold). In summary, among the three epigenetic marks, H3K4Me2 is the only enriched mark above the threshold that strongly correlated with *Ahr* mRNA expression during mouse liver development.

DISCUSSION

The present study revealed a postnatal increase in the *Ahr* mRNA expression in mouse liver during development, and demonstrated the role of histone H3K4 di-methylation in triggering the postnatal increase of *Ahr* mRNA during mouse liver development.

The methylation of DNA at cytosine residues is a well-established epigenetic mechanism that regulates tissue-specific gene expression. DNA methylation usually silences gene transcription, by preventing the recruitment of the transcription complex, or by indirect mechanisms involving changes in chromatin structure (Jaenisch and Bird, 2003). It has been suggested that the interplay between methylation and demethylation dictates the distinct DNA methylation patterns of genes and consequently influences their transcriptional activity. Much attention has been paid to the association between DNA methylation patterns and *Ahr* target genes. For example, mouse *Cyp1a2* gene expression coincides well with the methylation status of DNA during liver development (Jin et al., 2004). However, little is known of the role of DNA methylation in the ontogenic expression of mouse *Ahr* in liver. The present study is the first to demonstrate that despite the presence of a CpG island, DNA methylation is consistently low at the *Ahr* gene locus from 2 days before birth to 45 days of age, and therefore does not appear to play a significant role in regulating *Ahr* mRNA expression in liver development.

Recently, a large body of data has been generated for histone marks on the genomes of various organisms, primarily focusing on yeast (Hawkins and Ren, 2006). A current area of research is to understand how these histone modifications correlate and/or regulate transcriptional activity. A remarkable pattern has emerged for histone H3K4 di-methylation of actively transcribed genes, and H3K27 tri-methylation of silenced genes. Among the various modifications, histone methylations are more stable and the enzymes that catalyze the histone methylation are implicated in playing essential roles in the function of the human genome (Barski et al., 2007). High-resolution profiling of histone methylations in the entire human genome has demonstrated that active genes are characterized by high levels of H3K4Me₂, and in contrast, inactive genes are characterized by low or negligible levels of H3K4 methylation of the promoter regions, and high levels of H3K27 tri-methylations (Barski et al., 2007). Therefore, histone methylations were selected in the present study rather than other types of histone modifications. It has been shown that the H3K4Me₂ signals are usually localized to the vicinity of the transcription start site, providing a permissive chromatin environment to trigger gene transcription (Barski et al., 2007). In the present study, the strong postnatal enrichment of H3K4Me₂ in the close vicinity of the *Ahr* gene promoter indicates histone H3K4 di-methylation is likely a mechanism to trigger the increase in *Ahr* gene activation during liver development in mice. In contrast, although the gene suppression mark H3K27Me₃ gradually decreases during development around the *Ahr* gene locus, which correlates with *Ahr* mRNA increase, the signals of H3K27Me₃ are well below threshold at all ages. Therefore, it is difficult to ascertain the importance of the H3K27Me₃ profile with regard to *Ahr* mRNA expression despite the strong correlation between H3K27Me₃ and hepatic *Ahr* mRNA expression.

Interestingly, it appears that *Ahr* target genes also undergo regulation by histone modifications. For example, it has been shown in the mouse Hepa-1 cell line that the chromatin structure plays an essential role in *Cyp1a1* gene transcription. Specifically, *Cyp1a1* gene induction by the *Ahr*/ARNT complex is strongly associated with modifications of specific chromatin marks of *Cyp1a1*, including hyperacetylation of histone H3K14 and H4K16, tri-methylation of histone H3K4, and phosphorylation of H3S10 (Schnekenburger et al., 2007). Taken together, analyzing distinct histone epigenetic signatures around the *Ahr* gene locus and its target genes might become an essential approach in future research on *Ahr*-mediated drug metabolism and disposition. However, lack of specific inhibitors for histone modification enzymes makes it more challenging to establish a causative role of histone modifications in regulating *Ahr* gene transcription. Nevertheless, the strong association between the increase in

Ahr gene expression and H3K4 di-methylation enrichment provides a direction for future studies that may explore specific upstream factors regulating Ahr gene expression during development.

Taken together, the present study suggests that epigenetic modifications are a probable mechanism facilitating a permissive chromatin state that activates *Ahr* gene transcription during liver development in mice. The lack of all epigenetic marks, as observed in the *Ahr* gene locus in fetal liver, associate with a low basal expression of Ahr mRNA. In contrast, high levels of H3K4Me2 in the absence of suppressor signals of H3K27Me3 and DNA methylation triggers the increase in Ahr expression in adult mouse liver. Moreover, the increase in Ahr mRNA correlates with the dynamic enrichment of H3K4Me2 during liver development. Epigenetic regulation of Ahr gene transcription by histone H3K4 di-methylation is a strong candidate to be considered in the developmental programming of Ahr expression in mouse liver. Future studies will determine the causative mechanisms of the ontogeny of Ahr by altering the epigenetic signatures during liver development.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1A. Ontogenic expression of Ahr mRNA in mouse liver. Ahr mRNA ontogenic expression was determined by bDNA assay as described in MATERIALS AND METHODS (n=5). Data were expressed as RLU / 10 μ g total RNA. Asterisks (*) indicate statistical significance compared to day 45 levels (p<0.05, one way ANOVA followed by Duncan's multiple range post hoc test). Figure. 1B. Ontogenic expression of AhR protein from liver homogenates. Day -2 and day 1 of age: n=2; day 5 and day 45 of age: n=3. β -actin was used as a loading control; adult lung from wild-type mice (Ahr+/+) was used as a positive control for AhR protein expression, whereas adult liver from Ahr-null (Ahr-/-) mice was used as a negative control. Figure 2. DNA methylation status of *Ahr* gene during mouse liver development. Upper panel: browser view of fold changes of DNA methylation at the *Ahr* gene locus at day -2, 1, 5, and 45 of age (equal amounts of pooled samples from n=5 at each age). Solid lines through the signal enrichment peaks indicate the threshold value (3.0) for positive DNA methylation. Lower panel: Fold enrichment of DNA methylation at day -2, 1, 5, and 45 of age. The dashed line indicates the threshold value (3.0-fold) for enriched intervals.

Figure 3. Di-methylation of histone H3 at lysine-4 (H3K4Me2) at the *Ahr* gene locus during mouse liver development. Upper panel: browser view of fold changes of histone H3K4Me2 at the *Ahr* gene locus at day -2, 1, 5, and 45 of age (equal amount of pooled samples from n=5 at each age). Solid lines through the signal enrichment peaks indicate the threshold value (4.0) for enriched intervals. Bars under the peaks of each age indicate the existence and length of active regions for H3K4Me2. Asterisks (*) indicate the peak center. Lower panel: Fold enrichment of H3K4Me2 at day -2, 1, 5, and 45 of age. The dashed line indicates the threshold value (4.0-fold) for enriched intervals.

Figure 4. Tri-methylation of histone H3 at lysine-27 (H3K27Me3) at the *Ahr* gene locus during mouse liver development. Upper panel: browser view of fold changes of histone H3K27Me3 at the *Ahr* gene locus at day -2, 1, 5, and 45 of age (equal amount of pooled samples from n=5 at each age). Solid lines through the signal enrichment peaks indicate the threshold value (4.0) for enriched intervals. Lower panel: Fold enrichment of H3K27Me3 at the *Ahr* gene locus at day -2,

1, 5, and day 45 of age. The dashed line indicates the threshold value (4.0-fold) for enriched intervals.

Figure 5. Regression analysis of the correlation (R) between Ahr mRNA and the fold changes of the three epigenetic marks (DNA and histone di- and tri-methylations) at day -2, 1, 5, and 45 of age during liver development in mice.

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CONFLICT OF INTEREST STATEMENT

All authors declared no conflict of interest.

Figure 1

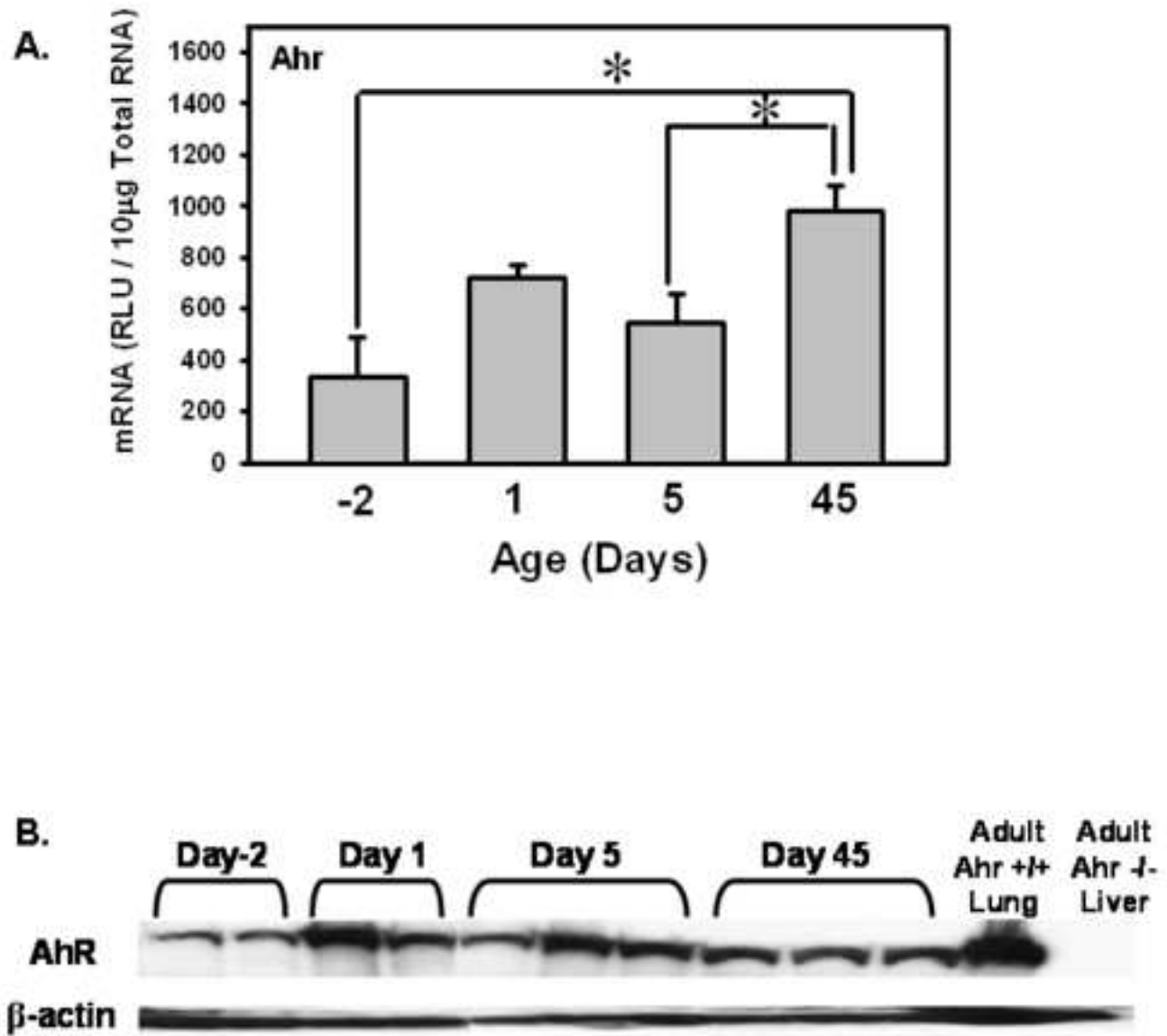


Figure 2. DNA Methylation

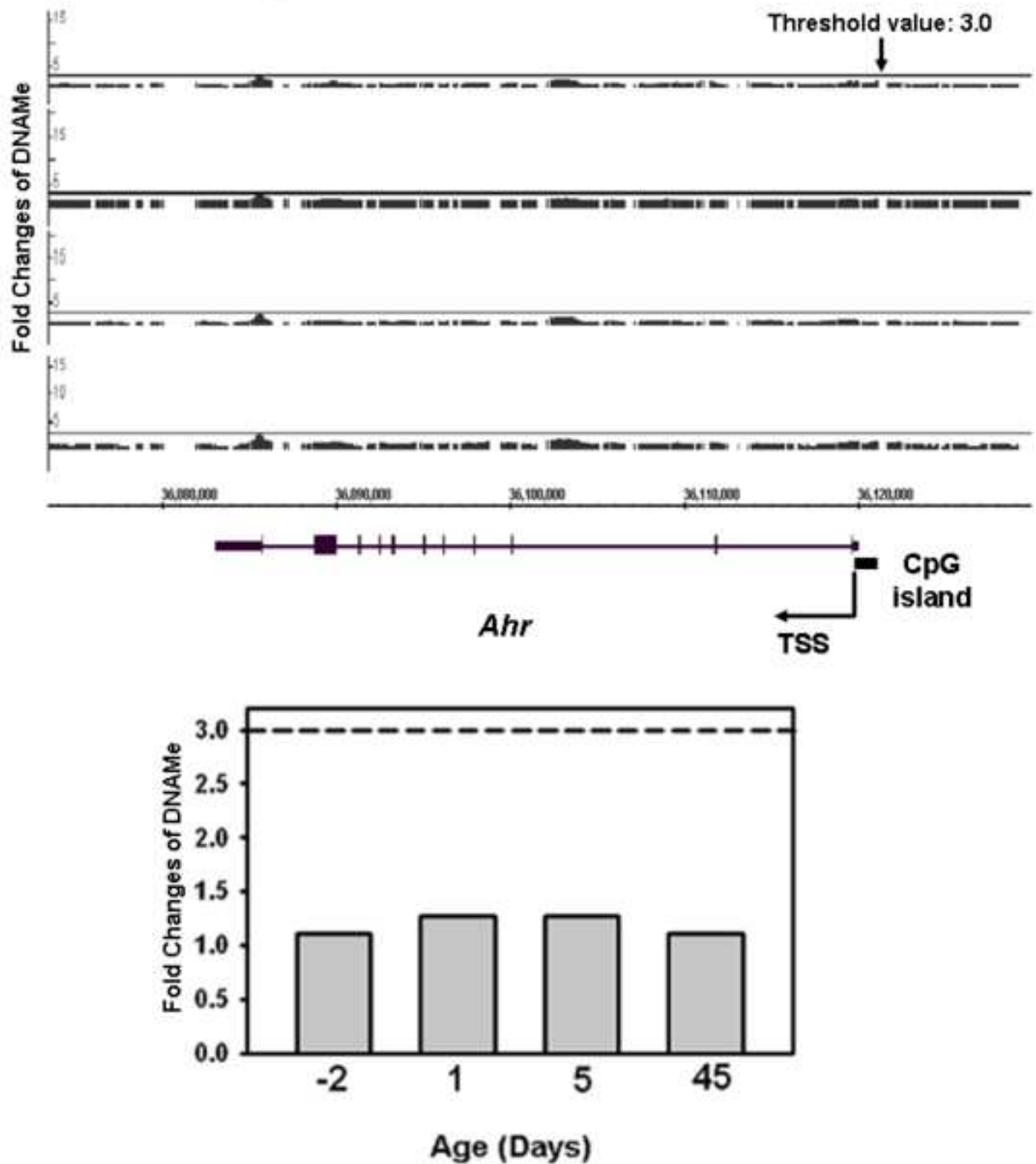


Figure 3. Histone H3K4 Di-methylation

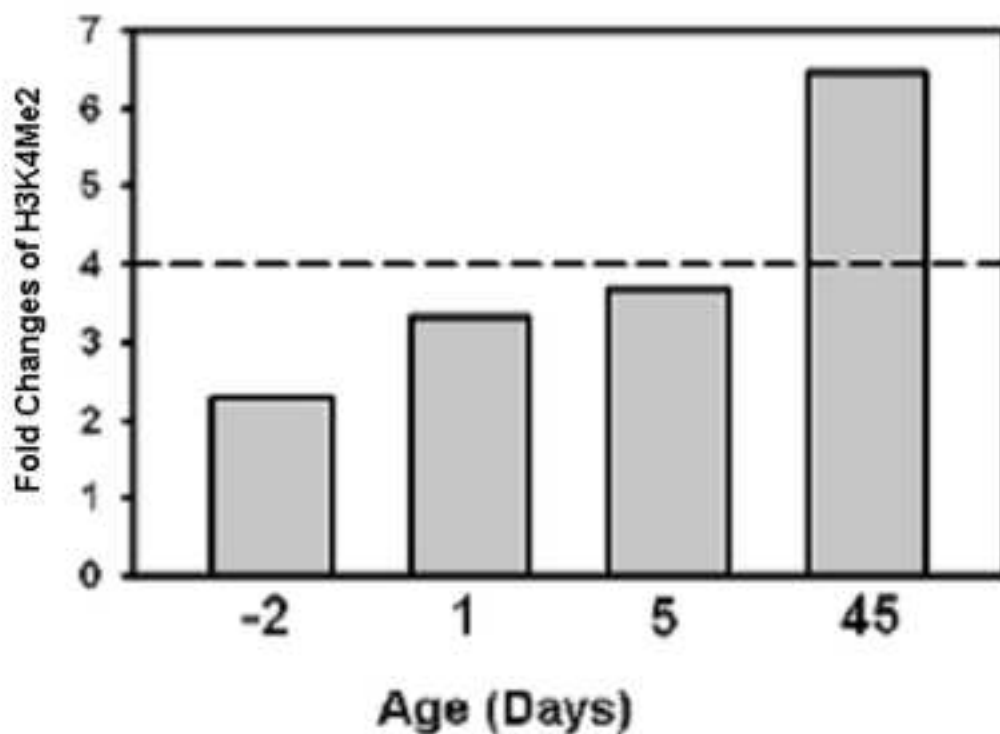
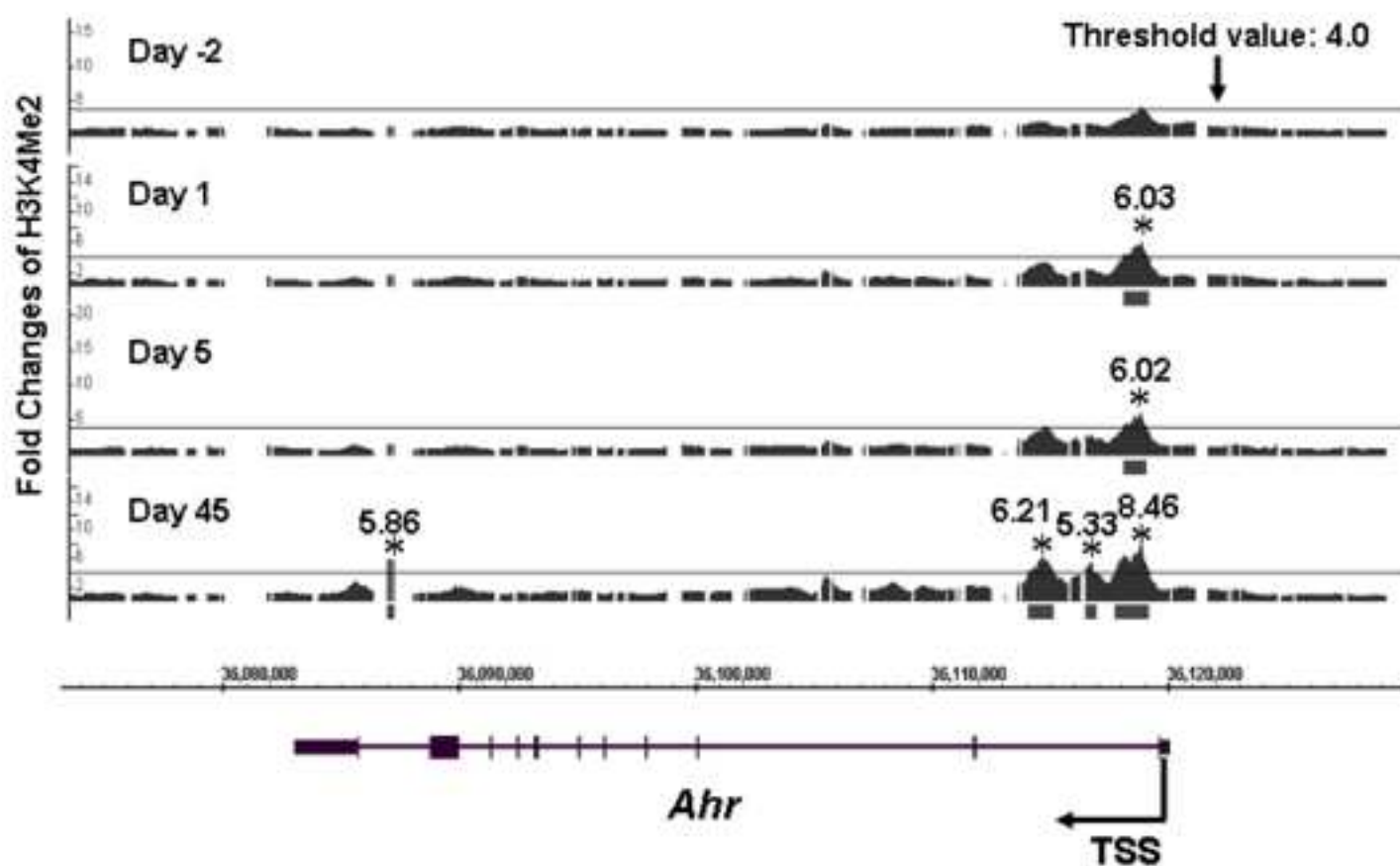


Figure 4. Histone H3K27 Tri-methylation

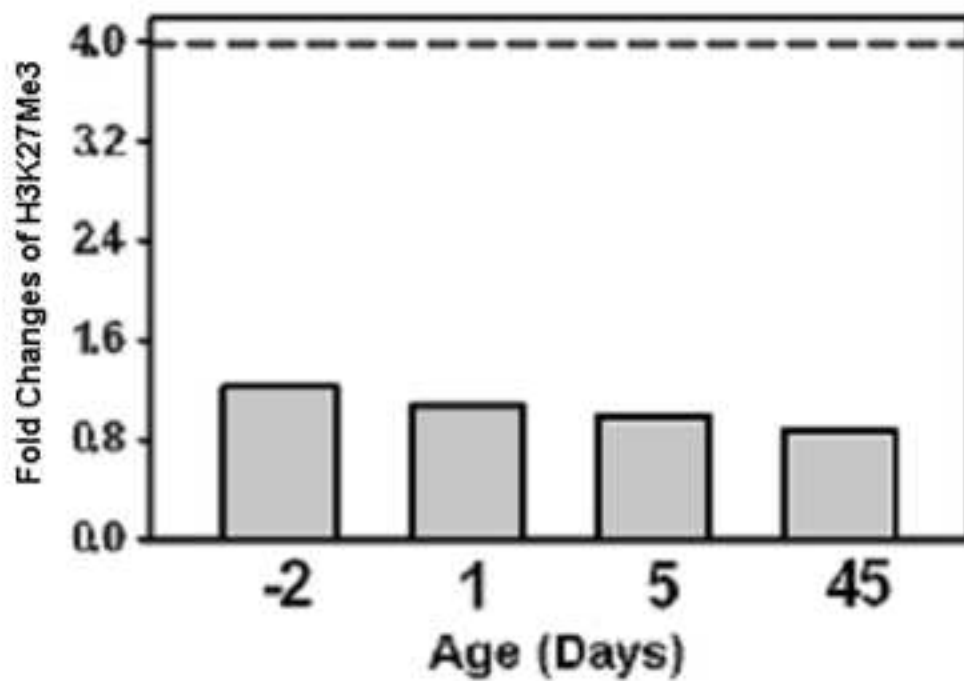
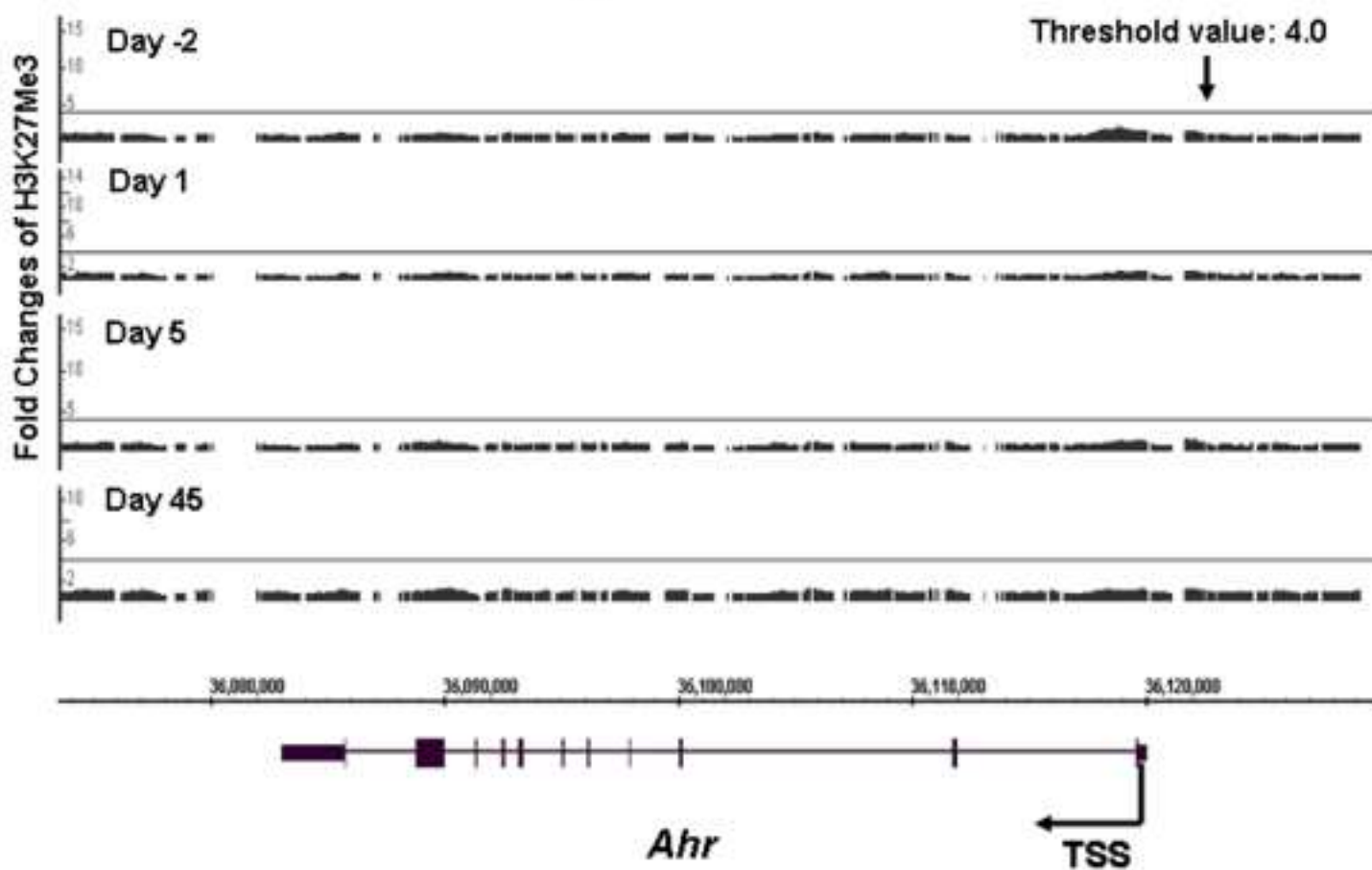


Figure 5

