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The STORM 2.0 Project Next Generation Hurricane Data Collection

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CHARACTERIZATION OF AEBP2 MUTANT MICE

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ABSTRACT

AEBP2 (Adipocyte Enhancer Binding Protein 2) is an evolutionarily well-conserved DNA binding protein, which acts as an allosteric cofactor for Polycomb Repressive Complex II (PRC2) responsible for trimethylating lysine 27 on histone H3 (H3K27me3). The *Aebp2* gene has three alternative promoters: P1, P2 and P3. In order to study the roles and function of *Aebp2 in vivo*, Dr. Kim's lab generated a line of mutant mice in which the expression of *Aebp2* is disrupted via the insertion of a β-geo gene trap vector. This gene trap vector also allowed us to perform β-gal staining to ascertain the expression pattern of *Aebp2*. According to our results, it appears to be highly expressed in the embryo and in the hippocampus of adult brains. Breeding experiments and phenotypic analyses show that mice that are homozygote for the mutant allele are embryonic lethal, whereas the heterozygotes are viable and fertile, albeit with a host of disease phenotypes. Here we focus on late-onset tonic-clonic seizures which affect about 35% of the *Aebp2*$^{+/\beta\text{-geo}}$ mice. Most seizures tend to develop when the mice are around 7-8 months of age, with females being nearly twice as susceptible to developing tonic-clonic seizures as males. There is much variation in the severity and duration of seizures displayed the *Aebp2*$^{+/\beta\text{-geo}}$ mice, which may be due to inter-individual differences in the level of *Aebp2* expression, as suggested by β-gal staining and Quantitative Real-Time PCR (qRT-PCR). In order to gain a better understanding of the differential expression levels of *Aebp2*, we studied methylation at the three promoters and assayed P1 for being potentially imprinted in *Aebp2*$^{+/\beta\text{-geo}}$ mice. While our imprinting tests remain inconclusive, we find mosaic methylation at the P1 promoter and indications that it may be a metastable epiallele. *Aebp2* P2 and P3 are completely unmethylated in WT tissues; however, P3 shows higher levels of methylation in *Aebp2*$^{+/\beta\text{-geo}}$ mouse tissues. Interestingly, we find that P1 and P3 may be more methylated in *Aebp2*$^{+/\beta\text{-geo}}$ seizure mouse

tissue samples than WT or normal *Aebp2*$^{+/\beta\text{-geo}}$ mouse samples. Based on this, we propose that differential methylation at P1 and P3 between individuals could affect the level of gene expression from these promoters (and also from P2) due to promoter competition and transcription factor activity. Mice which have higher levels of methylation at P1 and P3 are expected to have lower levels of AEBP2, which could affect epigenetic settings through PRC2 early in embryonic development, resulting in seizures later in life. Overall, this study highlights the potential role of epigenetic modifications, especially DNA methylation and PRC2 activity, in epileptogenesis and further identifies novel hypotheses for future projects.

CHAPTER ONE

BACKGROUND

Epigenetics

Since Conrad Waddington first coined the term in 1942 to explain the role of environment in development, the field of epigenetics has come a long way. The term "epigenetics" has been broadened to not only encompass the field of developmental biology but has also opened new avenues of research for many disease states including cancer (Jones and Baylin, 2007), epilepsy (Lubin, 2012) and autism spectrum disorders (Schanen, 2006). Four main epigenetic mechanisms have been identified by which gene expression may be regulated: DNA methylation, histone modification, RNA interference and chromatin remodeling. Of these four, DNA methylation is perhaps the best understood (Feinberg, 2010); however histone modification is increasingly being recognized as being important in the field of epigenetics. As such, these two mechanisms will be briefly discussed below in this chapter.

DNA Methylation

DNA methylation in mammals involves addition of a methyl (CH_3) group on a cytosine directly next to a guanine (CpG), converting it to 5-methyl-cytosine**.** The key enzymes that carry out DNA methylation in mammals are DNMT3A and DNMT3B, which are the *de novo* DNA methyltransferases (Okano *et al.*, 1999) and DNMT1, the "maintenance DNA methyltransferase," which is responsible for methylating hemi-methylated DNA during DNA replication in the S phase of the cell cycle (Sharif *et al.*, 2007). CpG sites often occur in GC-rich clusters called CpG islands near gene promoter elements in mammals. CpG islands are defined by their unusually high CpG content in the sequence. Given four possible base pairs, the likelihood of the CpG dinucleotide occurring in the genome is 1/16 or 0.0625. However in mammalian genomes, the frequency of CpG is much lower due to spontaneous deamination of

methylated cytosines and the subsequent conversion of CpG to TpG (or CpA on the opposite strand). Yet at CpG islands, we see the expected 0.0625 frequency of occurrence of CpG, which is one the major identifying markers of a CpG island (Goldman, 2001). Canonically, unmethylated CpG islands near promoters have been associated with transcriptionally actives genes whereas methylated CpG has been a sign of transcriptional silencing (Goll and Bestor, 2005).

DNA methylation is also the molecular mechanism for genomic imprinting in mammals (Li *et al.*, 1993). Imprinting is the process of allele-specific silencing, by which only one of the two parental alleles is expressed in an organism. *Peg3* is an example of a paternally expressed gene in which the promoter region of the maternal allele is heavily methylated and silenced, while that of the paternal allele is unmethylated and expressed (Kuroiwa, *et al*., 1996). DNA methylation is also among the most stable epigenetic marks that can be acquired with the error rate of maintaining methylation at approximately 1% per division, with no erasure at CpG islands during normal development (Reik, 2007). However, DNA methylation at CpG sites of differentially methylated regions (DMRs) on imprinted genes are actively erased in the primordial germ cells so that gender- and allele-specific methylation may be imposed to retain proper imprinting in the next generation (Bao, *et al.*, 2001; Lee, *et al.*, 2002). Imprinted genes do, however, tend to escape the second genome-wide epigenetic reprogramming which occurs soon after fertilization in the pre-implantation stage (Reik, 2007). Recent research suggests that a protein, STELLA may bind to specific sequences and protect from demethylation. Imprinted genes and some transposon families such as intracisternal A particles (IAPs) are expected to be regulated and their methylation preserved through this mechanism, but further research is needed to confirm these hypotheses (Nakamura, *et al.*, 2007; Reik, 2007).

Histone Modifications: Polycomb Repressive Complex II (PRC2)

Histone modifications as a major epigenetic regulator was first proposed by Thomas Jenuwein and David Allis as the "histone code" in 2001 (Jenuwein and Allis, 2001). In recent years, many different histone marks have been identified, although the best characterized marks are acetylation and methylation (Kouzarides, 2007). While histone acetylation almost universally signals transcriptional activation, histone methylation can be more complex depending on the location of the methyl group and the particular histone being modified. Trimethylation of lysine 27 on histone H3 (H3K27me3), which is the functional outcome of PRC2 (Polycomb Repressive Complex II), is one of the best studied histone methylation marks. H3K27 methylation by the Polycomb group (PcG) proteins in PRC2 is critical for proper embryonic development and have been implicated in the regulation of *hox* (*homeobox*) genes in *Drosophila* (Schuettengruber, *et al.*, 2007). The PRC2 complex consists of four core evolutionarily-conserved proteins, EED (Embryonic Ectoderm Development), EZH2 (Enhancer of Zeste Homolog 2), SUZ12 (Suppressor of Zeste Homolog 12) and RBAP48 (also called RBBP4, Retinoblastoma Binding Protein 4) (Ciferri *et al.*, 2012). A fifth protein, AEBP2 (Adipocyte Enhancer Binding Protein 2) has also been repeatedly co-purified along with the PRC2 complex (Cao, *et al.*, 2002; Cao and Zhang, 2004). It has been suggested that AEBP2, being a DNA-binding protein that has been consistently co-immunoprecipitated with the complex, may be a potential targeting protein for PRC2 (Kim, *et al.*, 2009). Notably, AEBP2 appears to significantly stimulate the enzymatic activity of PRC2 and it has been proposed it may act as an allosteric cofactor which stabilizes the complex by binding to it at a central hinge point (Cao and Zhang, 2004; Ciferri, *et al.*, 2012).

In recent years, the PcG genes have received much interest from the scientific community because of their role in cancer and tissue regeneration from stem cells. Several proteins that comprise PRC2, when aberrantly or insufficiently expressed, have been shown to impair the capability of an embryonic stem cell to properly differentiate or adult stem cells to renew themselves (Richly *et al.*, 2012). Moreover, some of these proteins, such as EZH2, have been found to be overexpressed in many types of cancer. In fact, it has been suggested that misregulation of PcG complexes may lead to the formation of cancer stem cells, which have the ability to self-renew, metastasize, form heterogeneous tumors and be chemo-resistant. Needless to say, these factors have targeted PcG genes like Ezh2 as major avenues for potential cancer treatment and H3K27 methylation has been proposed to be used as a prognosis marker (Crea, *et al.*, 2011). Therefore characterizing the proteins involved in the two PcG complexes are currently of great interest to the scientific as well as medical community. With this in mind, I have focused on the PcG gene, *Aebp2*, which may act as a targeting protein for PRC2 as well as stabilize the complex allosterically, and have attempted to characterize mutant mice in which the expression of this gene is disrupted.

Adipocyte Enhancer Binding Protein 2 (AEBP2)

AEBP2 is an evolutionarily conserved DNA-binding protein with three zinc fingers units. It was initially discovered for its binding capability to the promoter region of the fatty-acid binding protein adipocyte P2 (aP2), hence its name (He, *et al.*, 1999). However, multiple studies in our lab have linked it to embryonic development through epigenetic means, specifically as a potential targeting protein for Polycomb Repressive Complex II (Kim, *et al.*, 2009). Interestingly, the homolog for *Aebp2* in *Drosophila*, JING, has also been shown to interact with PcG proteins (Culi, *et al.*, 2006; McClure and Schubinger, 2008). Additionally, jing has been

shown to be involved in border cell migration (Liu and Montell, 2001) and central nervous system (CNS) development (Sedaghat, *et al.*, 2002) as well.

The AEBP2 protein contains six domains: acidic (glutamic and aspartic acid-rich), neutral (glycine and serine-enriched), serine-rich, zinc-finger (with three zinc finger units), basic and lysine-rich **(Fig. 1.1A)**. Of these six domains, the zinc-finger and basic domains appear to be the most conserved, with a high degree of sequence similarity between mammals, fish and insects. The C-terminus lysine-rich domain, on the other hand, seems to be found only in mammals but is highly conserved within mammalian species (Kim, *et al.*, 2009). The same study by Kim, *et al.* identified two main isoforms of the protein. A 31 kDa form was found to be expressed highly in embryonic tissue, which likely includes only the zinc-finger and basic domains. A larger, 52 kDa, form was found to be more or less ubiquitously expressed, which also includes acidic and neutral domains along with the zinc-finger and basic domains (**Fig. 1.1B)**. Several experiments previously done in our lab and reported by Kim, *et al.* have been able to show that AEBP2 is indeed a DNA-binding protein with a predicted DNA-binding motif having a bipartite structure: $CTT(N)_{15\text{-}23}$cagGCC (with lower case meaning less critical).

The *Aebp2* gene is located on mouse chromosome 6 and consists of 11 exons. There are two known alternative promoters for the gene (P1 and P2) and three different potential START and STOP codons each (**Fig. 1.1C**), allowing for a total of nine possible isoforms. The two main isoforms detected likely arise from the activity of the alternative promoters and different START codons (Kim, *et al.*, 2009). The 52 kDa form is expected to arise from the P2 promoter using exon 1b as the first exon, and the 31 kDa form could use promoter P1 and exon 1a (**Fig. 1.1C**). However, the smaller isoform could also result from alternative splicing or post-translational modification on the larger isoform. A third alternative promoter (P3) has been recently

discovered for *Aebp2*, which is expected to give rise to the 31 kDa isoform of the protein as well (Kim, *et al.*, unpublished results), however this promoter is still in process of being characterized.

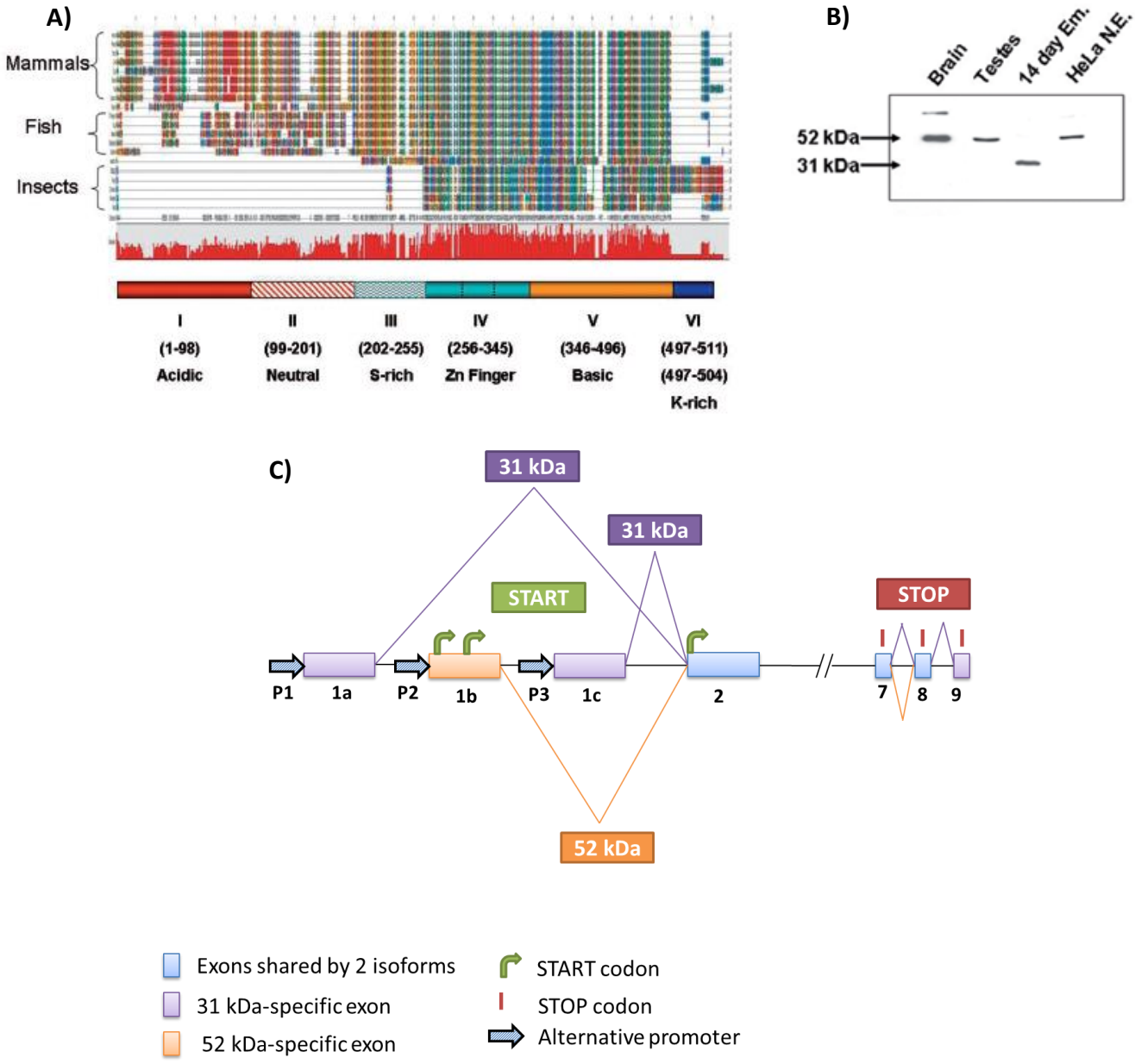
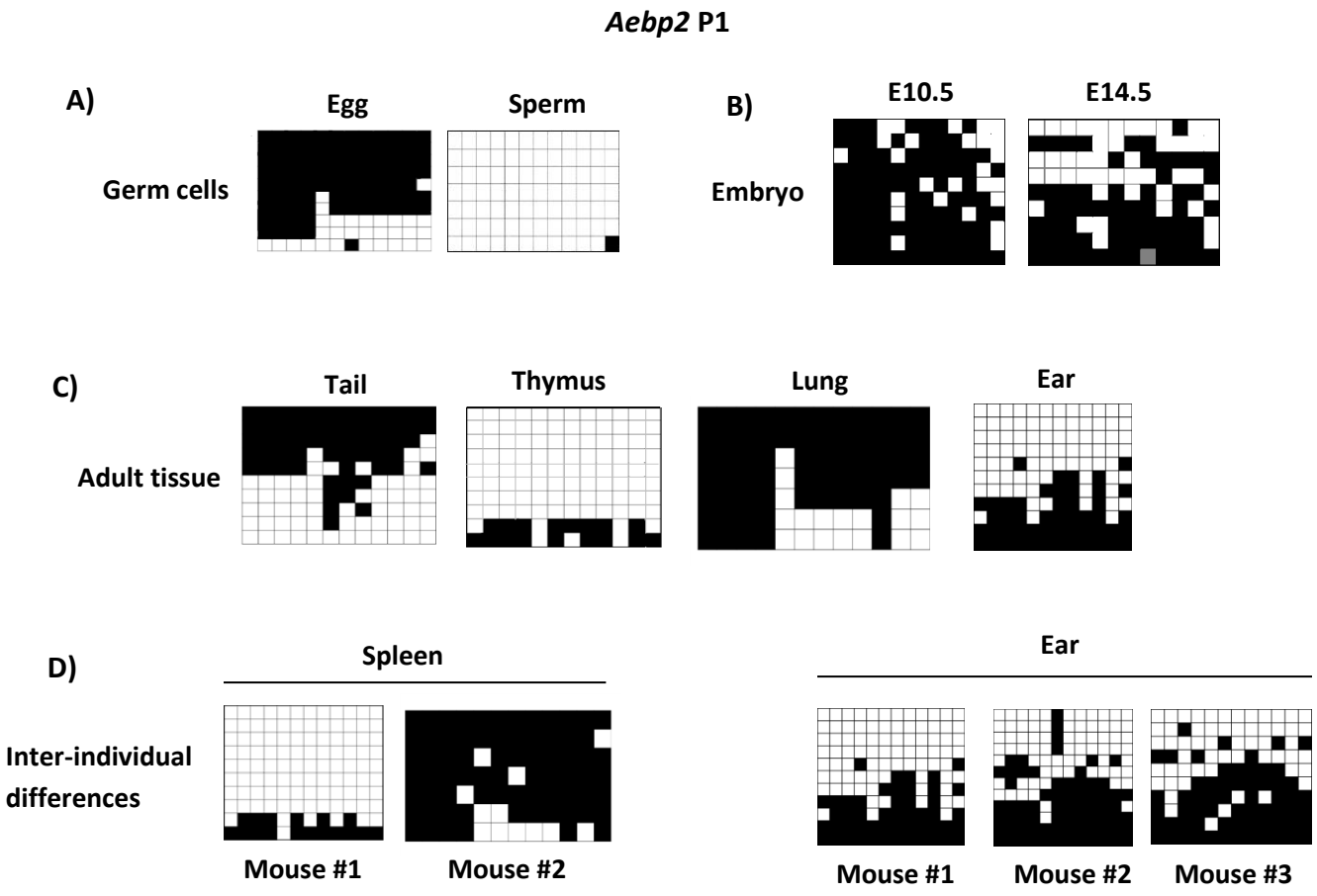


Figure 1.1. AEBP2: protein and gene structure. (A) Evolutionary conservation of the six domains in AEBP2 protein (Kim, *et al.*, 2009). **(B)** Two main isoforms of AEBP2 – 52 kDa somatic form and 31 kDa embryonic form (Kim, *et al.*, 2009). **(C)** Schematic of the *Aebp2* gene structure showing the three alternative promoters, alternative START and STOP codons and the main splicing variants arising from the three promoters that give rise to the two main isoforms.

Interestingly, the first promoter (P1) is a SINE element (short interspersed element) which is conserved in mice and rats. We are currently characterizing the unusual DNA methylation pattern on this promoter. Transposable elements (TEs) are most often completely methylated so as to prevent their replication and transposition in the genome ("genome defense hypothesis," proposed by Yoder, *et al.*, 1997). However, it has been posited that insertion of TEs in certain specific loci may lead to their exaptation as gene regulatory elements which could offer a selective advantage to the organism ("exaptation hypothesis," first proposed by Brosius and Gould, 1992; for a comparative study between the "exaptation" and "genome defense" hypotheses, see Huda, *et al.*, 2010). In such a case, exapted TEs are expected to be unusually conserved between species, as seen in the case of the SINE element which acts as an alternative promoter for *Aebp2*. In many instances, TEs which become a functional unit of the genome escape complete methylation (repression) and may acquire active histone modifications (Huda, *et al.*, 2010). Our preliminary results suggest similarly in the case of P1 in that it seems sporadically methylated in most tissues, including tail, lung, spleen, ear and whole 10.5-dpc (days post coitum) and 14.5-dpc embryos (**Fig. 1.2B, C**). This random derepression of the SINE element is reminiscent of the stochastic methylation pattern observed in metastable epialleles, which describe genes which are variably expressed in genetically identical individuals and may exist stably yet differentially in various epigenetic states (see review by Rakyan, *et al.*, 2002). Although P1 appeared to be more derepressed in certain tissues more so than others, such as spleen and thymus, inter-individual differences may exist, at least in spleen, as would be expected if this SINE element was indeed a metastable epiallele (**Fig. 1.2C, D**).

An alternative functional outcome of retrotransposon insertion could be genomic imprinting, as in the case of *PEG10* which is a retrotransposon-derived paternally-expressed

gene (Suzuki, *et al.*, 2007). Interestingly, while P1 appears to be stochastically methylated in many somatic tissues, it shows a vastly different methylation pattern in the germ cells. In fact, P1 appears to be significantly more methylated in the egg than sperm of mice from B6/129 mixed background which indicates that P1 may be imprinted (**Fig. 1.2A**). This prompted us to conduct an imprinting test on P1 which is described in further detail in chapter three. In contrast, the second and the third promoters (P2 and P3) which are not retrotransposon-derived are completely unmethylated in the wild type (WT) tissues we tested: dentate gyrus and ear respectively for P2 and P3 (**Fig. 1.2E, F**).



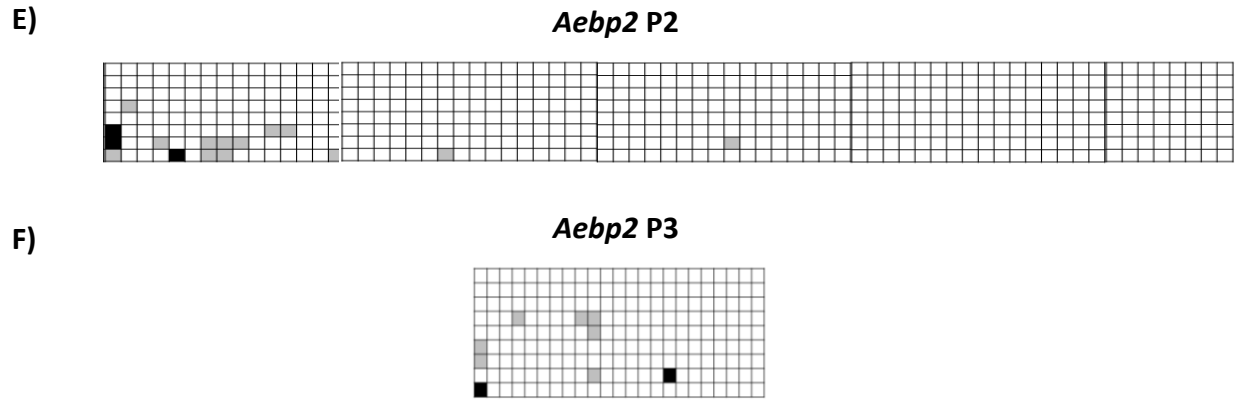


Figure 1.2. DNA methylation on the three *Aebp2* promoters. Filled black boxes indicate methylated CpG sites whereas unfilled boxes indicated unmethylated CpG sites. Grey boxes indicate unresolved CpG sites. **(A)** Methylation pattern of *Aebp2* P1 in germ cells (egg and sperm). **(B)** *Aebp2* P1 DNA methylation at two embryonic stages (E10.5 and E11.5). **(C)** *Aebp2* P1 DNA methylation in four adult tissues (spleen, thymus, lung and ear). **(D)** Inter-individual differences between DNA methylation patterns in spleen and ear. **(E)** *Aebp2* P2 DNA methylation in adult dentate gyrus tissue. **(E)** DNA methylation of *Aebp2* P3 in adult ear tissue.

In the light of *Aebp2*'s interesting protein, exon and promoter structure, our lab has developed a line of mutant mice in which the expression of *Aebp2* is disrupted in order to investigate its roles *in vivo* (Kim, *et al.*, 2011). This was accomplished by the shotgun insertion of β-geo gene trap vector in the first intron of *Aebp2*, as previously reported by Kim, *et al.* (2011). The inserted vector contains a β-geo fusion cassette followed by a polyadenylation signal, a splice acceptor site, and two FRT and loxP sites each (**Fig. 1.3A**). Through qRT-PCR and western blot, it was confirmed that that *Aebp2* expression in *Aebp2*$^{+/\beta\text{-geo}}$ mice was reduced in WT mice (**Fig. 1.3B, C**). Lacking its own promoter, the β-geo fusion protein expression is driven by the endogenous *Aebp2* promoter (**Fig. 1.4A**). This allowed us to assay the expression pattern of *Aebp2* in multiple tissues by β-galactosidase (β-gal) staining (**Fig. 1.4B-G**). In the 6.5-dpc embryo, high *Aebp2* expression was observed in the embryonic ectoderm (Ect) and primitive streak (PS) and moderate levels in the allantois (Al) and chorion (Ch) of the embryo (**Fig. 1.4B**).

From embryonic day (E) 9.5 to 14.5, strong β-gal staining was observed in the midbrain section, neural tube and along the somites (**Fig. 1.4C-E**). In the sagittal section of E15.5 embryo, strong expression was found in tissues derived from neural crest cells (NCC), including the dorsal root ganglia, and the cranial-facial neural crest cells (**Fig. 1.4F, G**) (Kim, *et al.*, 2011). As a part of this current study, we attempted to characterize *Aebp2* expression in adult tissues and have found it to be highly expressed in the brain. A detail spatial expression of *Aebp2* in the brain is included in chapter two.

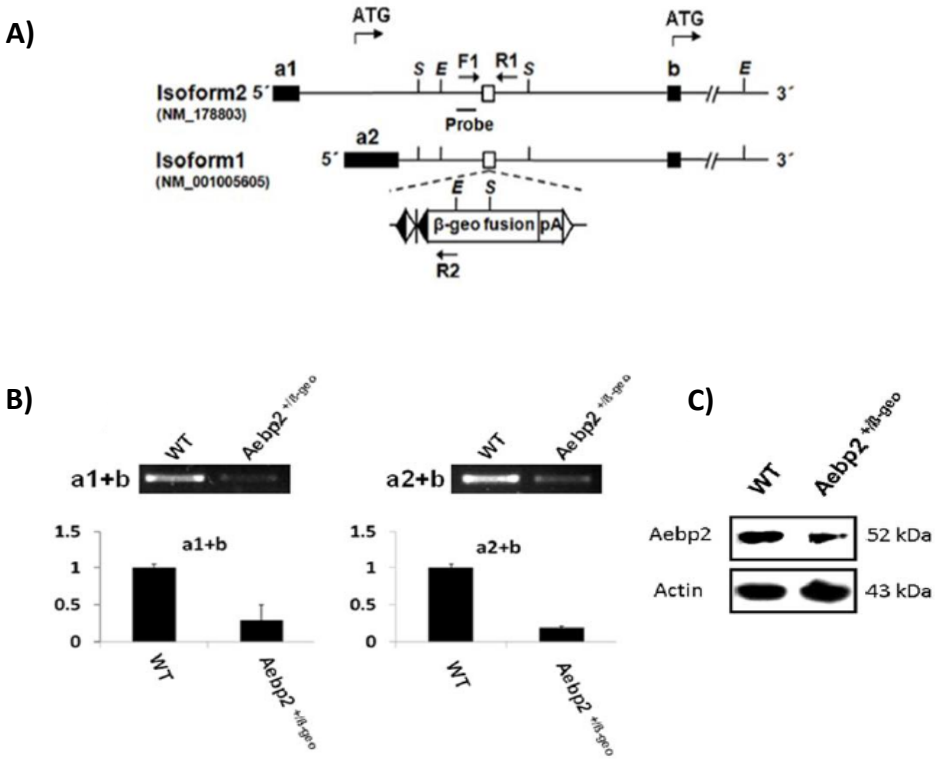


Figure 1.3. Disruption of *Aebp2* coding region by β-geo gene trap vector (Kim, *et al.*, 2011). (A) Schematic showing the location of the inserted vector. Empty triangles represent FRT sites and black triangles represent loxP sites. The gene trap vector is also flanked by a splice acceptor site, represented by a vertical line, and a polyA (pA) tail. Two START codons (ATG) have been represented with arrows. Three primers (F1, R1 and R2) have been used to genotype for the *Aebp2* mutant allele. Primers F1 and R1 amplify a 570 bp region in the WT allele, whereas F1 and R2 produce a 304 bp product from the mutant allele. **(B)** qR-T PCR performed on total RNA extracted from neonatal brain from WT and mutant mice show proper truncation of all *Aebp2* mRNA by the gene trap vector. **(C)** Western blot performed on protein extracted from neonatal brain shows reduced levels of AEBP2 in mutant mice.

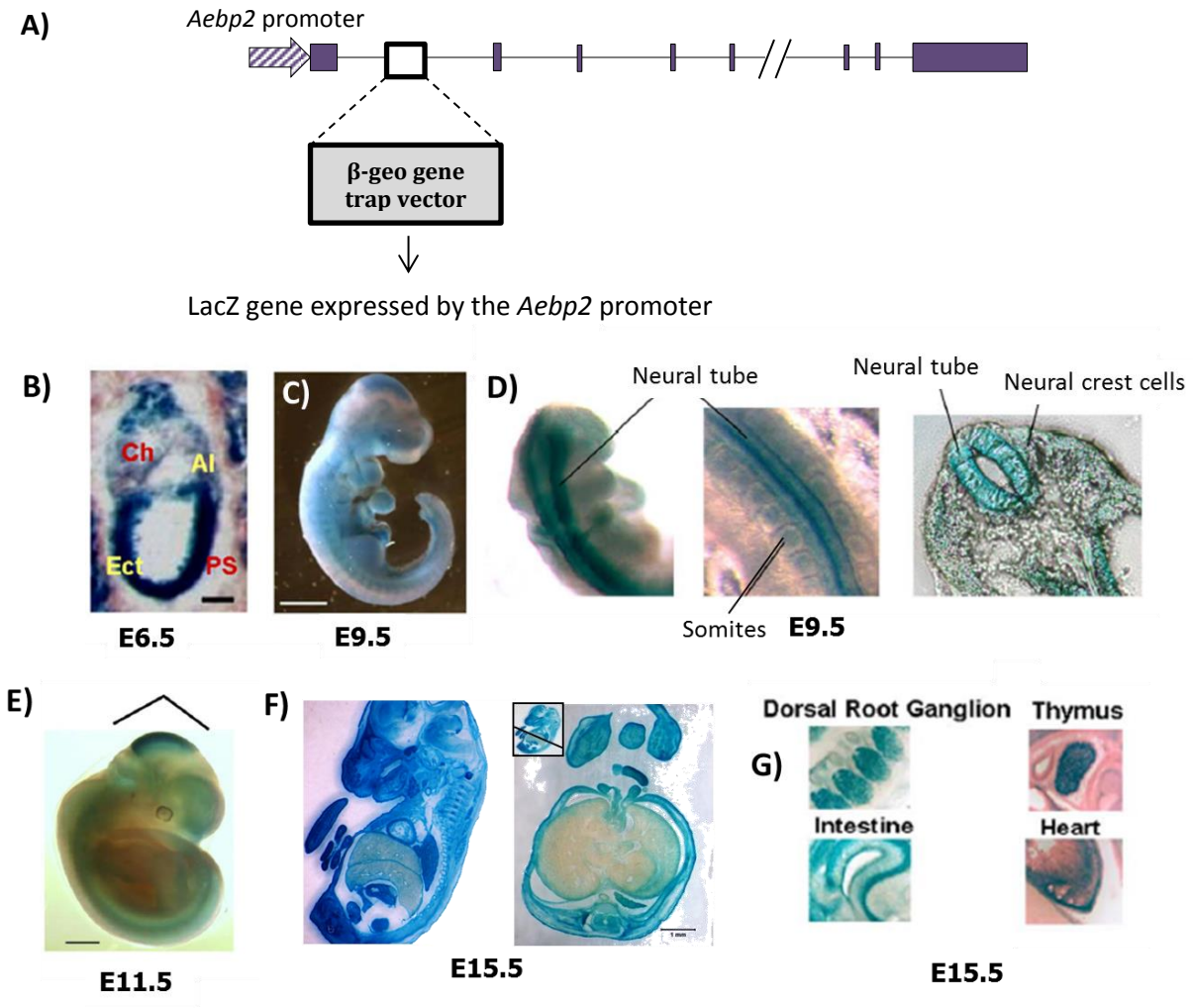


Figure 1.4. Spatial and temporal expression pattern of *Aebp2*. (A) Schematic showing the principle of β-gal staining representing the spatial expression pattern of *Aebp2*. **(B)** *Aebp2* is highly expressed in the embryonic ectoderm (Ect) and primitive streak (PS) and at moderate levels in the allantois (Al) and chorion (Ch) in 6.5-dpc embryo (Kim, *et al.*, 2011). **(C-E)** From 9.5 to 11.5-dpc, strong *Aebp2* expression is seen along the neural tube, somites and midbrain (Kim, *et al.*, 2011). **(F)** At the 15.5-dpc stage, *Aebp2* is highly expressed in cell derived from neural crest cells, including the dorsal root ganglia (DRG) and cranial-facial neural crest cells. **(G)** Close-up of *Aebp2* expression in the DRG, thymus, intestine and heart of a 15.5-dpc embryo (Kim, *et al.*, 2011).

Similar to when many PcG genes are disrupted (Richly, *et al.*, 2011), the homozygotes for the mutant allele for *Aebp2* were found to be embryonic lethal (Kim, *et al.*, 2011). Mice that were heterozygote for the mutation were viable and fertile, albeit with several disease

phenotypes such as hearing defects, enlarged colon and missing melanocytes (**Fig. 1.5A-B**), reminiscent of human patients with Hirschsprung's Disease and Waardenburg Syndrome Type 4, which are disorders associated with NCC migration defects (Kim, *et al.*, 2011). Along with the strong expression of *Aebp2* in NCC-derived cell lineages, this was a strong indication that *Aebp2* may play an important role in the regulation of neural crest cells. This prediction was further supported by ChIP (Chromatin Immunoprecipitation) and qRT-PCR data showing *Aebp2* binding at known NCC-associated loci and affecting the expression of at least one of them, *Sox10*, in *Aebp2* haploinsufficient mice (Kim, *et al.*, 2011). The fact that EZH2 and H3K27me3 ChIP also yielded significant enrichment at these NCC-associated loci further indicated that *Aebp2* may regulate neural crest cells epigenetically through PRC2-mediated histone modifications.

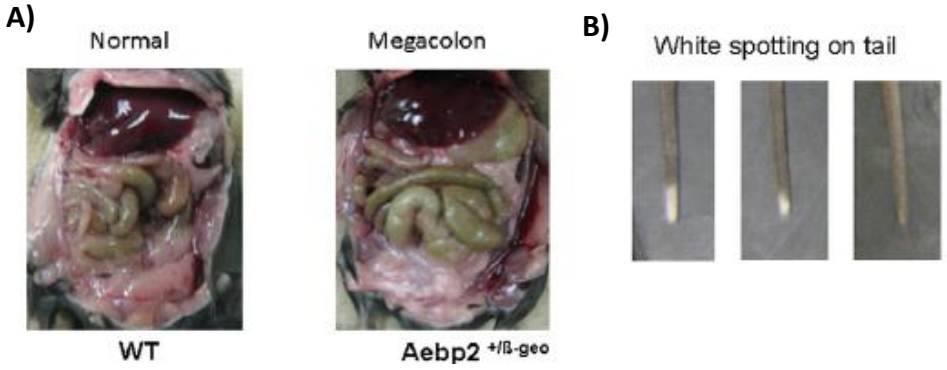


Figure 1.5. Disease phenotypes in *Aebp2* mutant mice (Kim, *et al.*, 2011). (A) Enlarged green colons (megacolon) can be observed in *Aebp2*$^{+/\beta\text{-geo}}$ mice compared to normal WT mice, which is one of the symptoms associated with Hirschsprung's disease resulting from improper neural crest cell migration. **(B)** Lack of melanocytes seen as white tail tips of varying lengths in *Aebp2*$^{+/\beta\text{-geo}}$ mice.

This thesis chronicles the characterization of another interesting phenotype displayed by *Aebp2*$^{+/\beta\text{-geo}}$ mice, which is late-onset tonic-clonic seizure due to *Aebp2* haploinsufficiency (chapter two). Based on various lines of evidence, this study proposes that *Aebp2*, in connection with PRC2-led H3K27 methylation, may have an important role in proper brain development. In

order to explain the incomplete penetrance of the seizure phenotype and as a part of an ongoing project to further characterize the *Aebp2* gene, *Aebp2* promoter P1 (**Fig. 2-D**) was studied in terms of its potentially imprinted status in *Aebp2*$^{+/\beta\text{-geo}}$ mice (chapter three). Based on our analysis of P1 methylation, we propose that dynamic regulation of *Aebp2* via its alternative promoters at critical developmental stages may contribute to seizure formation due to aberrant epigenetic settings during early embryogenesis (chapter four). Overall, this thesis highlights the importance of epigenetics, specifically in the case of *Aebp2* as a Polycomb group protein, in proper mammalian development and should contribute to the growing repertoire of studies which link epigenetics to various disease phenotypes, especially complex polygenic diseases such as epilepsy.

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CHAPTER TWO

SEIZURE PHENOTYPE BY HAPLOINSUFFICIENCY OF AEBP2

Abstract

AEBP2 is an evolutionarily well-conserved DNA-binding protein whose functions have been largely uncharacterized, despite repeated associations with PRC2. In an effort to elucidate the roles of *Aebp2 in vivo*, we have been studying a line of mutant mice in which the expression of *Aebp2* has been disrupted. Previous results from our lab indicate that the mice homozygous for the mutant allele are embryonic lethal; however, the heterozygotes are viable and fertile, albeit with an array of disease phenotypes. Here we describe late-onset tonic-clonic seizures in *Aebp2*$^{+/\beta\text{-geo}}$ mice with a maximum penetrance of 35%. The severity of seizures was variable but females showed a higher tendency to develop tonic-clonic seizures than males. The seizures varied in duration depending on the individual with most lasting about one minute. We also studied the spatial expression pattern of *Aebp2* in the brain and found that *Aebp2* was ubiquitously expressed in the brain, with especially high expression in the CA1 and dentate gyrus regions of the hippocampus and the purkinje cells of the cerebellum. In the light of other studies with PRC2 protein haploinsufficiency models which show gross brain morphological abnormalities, we propose that *Aebp2* and PRC2 may play an important role in brain development, and aberrant epigenetic settings during early embryogenesis could lead to CNS developmental defects which could ultimately result in seizures later in life. Further research in this area should be of interest to the medical field since the inherently flexible nature of the H3K27me3 mark should make it an attractive target for translational studies with regards to designing novel drugs for managing intractable epilepsy. Further, studying a developmental epigenetic regulator such as PRC2 and its components could yield clues regarding the developmental and epigenetic basis of epilepsy, which would be useful in designing treatments in pediatric cases of epilepsy and also idiopathic cases in adults.

Introduction

Epilepsy is generally recognized as a neurological disorder which is most commonly marked by seizures. While in many cases the seizure may be accompanied by abnormalities of brain structure, there are instances of "idiopathic" epilepsies which may have a genetic basis (Anderman, 1982). Clinical research has supported the idea of epilepsy being a neurodevelopmental disorder which may be influenced strongly by genetic factors. However, most kinds of epilepsy are not monogenic and there are often several genes involved which makes it harder to identify the causative roles of any single gene (Pal and Greenberg, 2012). Despite this inherent difficulty, several genes associated with CNS development and neuronal excitability have been identified as causative factors and abnormalities in their spatial and temporal expression is suspected to lead to deregulated neuronal circuits and a tendency for aberrant electrical activity (Qureshi and Mehler, 2010). Recently, the focus has shifted more towards understanding the epigenetic mechanisms which may be playing a role in seizure formation as aberrant epigenetic regulation has been shown to be a factor in many CNS diseases (Lubin, 2012). DNA methylation and histone modification are the two main epigenetic mechanisms which have been shown to affect epileptogenesis so far (Lubin, 2012).

In this study we describe seizures in mice with half dosage of the gene *Aebp2*. As one of the proteins comprising Polycomb Repressive Complex II (PRC2), it may play an important role in embryonic development (Cao, *et al.*, 2002; Cao and Zhang, 2004; Schuettengruber, *et al.*, 2007). AEBP2 has been proposed to be the targeting protein for PRC2 (Kim, *et al*., 2009) and is known to enhance PRC2 activity (Cao and Zhang, 2004; Ciferri, *et al.*, 2012). As such it is interesting to note that mice which are homozygote for the mutation which disrupts *Aebp2* expression are embryonic lethal and the heterozygotes display an array of disease phenotypes

(Kim, *et al.*, 2011), including late-onset generalized tonic-clonic seizures (this study). PRC2 has many important developmental regulatory functions that have been identified and recently, it has also been proposed that PcG proteins may promote neurogenesis through regulating transiently expressed genes (Popkova, *et al.*, 2012). Therefore, it is interesting to present *Aebp2* as one of the PcG genes whose haploinsufficiency results in seizures.

Materials and Methods

Seizure tests

All *Aebp2* mutant mice were of B6/129 mixed background and were housed in a controlled environment. Seizures were induced by moving the mouse into a different cage in order to change their immediate environment. All mice were studied for one minute to check for the development of seizure, after which they were returned to their original cages. Over 200 mice, including wild-type individuals, were checked in this manner for seizure development twice a week for over nine months, starting when the mice were at least three months old and up to eight months of age. Additionally, these tests were conducted during normal daylight hours without disrupting the day-and-night cycle. All the experiments related to mice were performed in accordance with National Institutes of Health guidelines for care and use of animals, and also approved by the Louisiana State University Institutional Animal Care and Use Committee (IACUC), protocol #10-071.

β-galactosidase staining

Mouse brains were isolated from three month old adult mice and fixed in fixing solution (0.2% paraformaldehyde, 0.1 M PIPES buffer pH 6.9, 2 mM $MgCl_2$, 5 mM EGTA) at 4℃. They were then cryopreserved in a PBS solution containing 30% sucrose and 2mM $MgCl_2$ at 4℃ until

the tissue sank to the bottom. They were then embedded in OCT and frozen at -80℃ before sectioning them 50 microns thick on a cryotome (Leica CM1850) and placing on poly-L-lysine coated slides. The staining protocol involved fixing the tissues an additional 10 minutes in 0.2% paraformaldehyde solution as described above, washing in PBS solution with 2 mM $MgCl_2$ for 10 minutes before the detergent rinse for another 10 minutes. Finally, the sections were stained in a staining solution with 40 μg/mL X-gal (5-bromo-4-chloro-indolyl-galactopyranoside) at 37℃ overnight.

Before staining whole mount, adult mouse brains were cut along the coronal plane in five parts for better penetration of the staining solution through the dense tissue. The fixed tissue was then washed in PBS solution for 30 minutes, detergent rise for 60 minutes and stained overnight at 37°C in a staining solution with 40 μg/mL X-gal (5-bromo-4-chloro-indolyl-galactopyranoside). Tissue sections and whole-mount brains were visualized using a dissecting light microscope (Leica MZ75) and images were captured using a digital camera (Model #4.2 Color Mosaic, Diagnostic Instruments Inc.).

Statistical analysis

Fisher's exact test was used as a statistical significance test to test for association between gender and seizure susceptibility, where the null hypothesis was that both genders are equally susceptible to developing seizures. A two-tailed p-value of less than 0.05 was considered significant enough to reject the null hypothesis. All calculations were performed on GraphPad Software QuickCalcs.

Results

Tonic-clonic seizures in *Aebp2*$^{+/\beta\text{-geo}}$ mice

As a part of the effort to elucidate the roles of *Aebp2 in vivo*, we characterized several *Aebp2*$^{+/\beta\text{-geo}}$ mice over a period of nine months. One of the most striking phenotypes observed was late-onset seizures induced by a change in environment, most likely due to stress. The seizures were determined to be of the tonic-clonic type (formerly called grand mal) (**Fig. 2.1**), similar to the handling-induced seizures observed in the epilepsy mouse model SWXL-4 (Frankel, *et al.*, 1994). The tonic phase involved backward flexation of the neck, stiffening and tonic extension of limbs (**Fig. 2.1A and B**). Forelimbs would often flex up, extend in and remain rigid (**Fig. 2.1D**). Tonic extension of hindlimbs was variable, but it invariably resulted in loss of postural control (**Fig. 2.1E**). The clonic phase was marked by a series of jerking motions due synchronous muscle contraction and relaxation. Salivation and tonic extension of the tail occurred in many cases as well (**Fig. 2.1C, F, G**). The tonic phase typically preceded the clonic phase, as seen in the timeline of images (**Fig. 2.1A-C)**, although the duration of two phases remained variable depending on the individual.

The likelihood of a mouse developing seizure appeared to increase with age. At four months of age, approximately 2% mice were found to have seizures, whereas approximately 35% mice had seizures past seven months of age. The maximum penetrance of the seizure phenotype was determined to be 35% mice when the mice were 7-8 months old (**Fig. 2.2A**). Female mice were found to have a significantly greater chance of developing seizures than male mice. In fact, the penetrance of the seizure phenotype in females was twice that of males (**Fig. 2.2B**). Out of the 134 mice tested for gender bias for over nine months, 64 were males and 70

were females. Despite males being fewer than females in our population, this difference was not significant. While only 10 out of 64 males developed seizures (15%), 25 out of the 70 females displayed tonic-clonic seizures (33%, p=0.01, Fisher's exact test) (**Fig. 2.2B**).

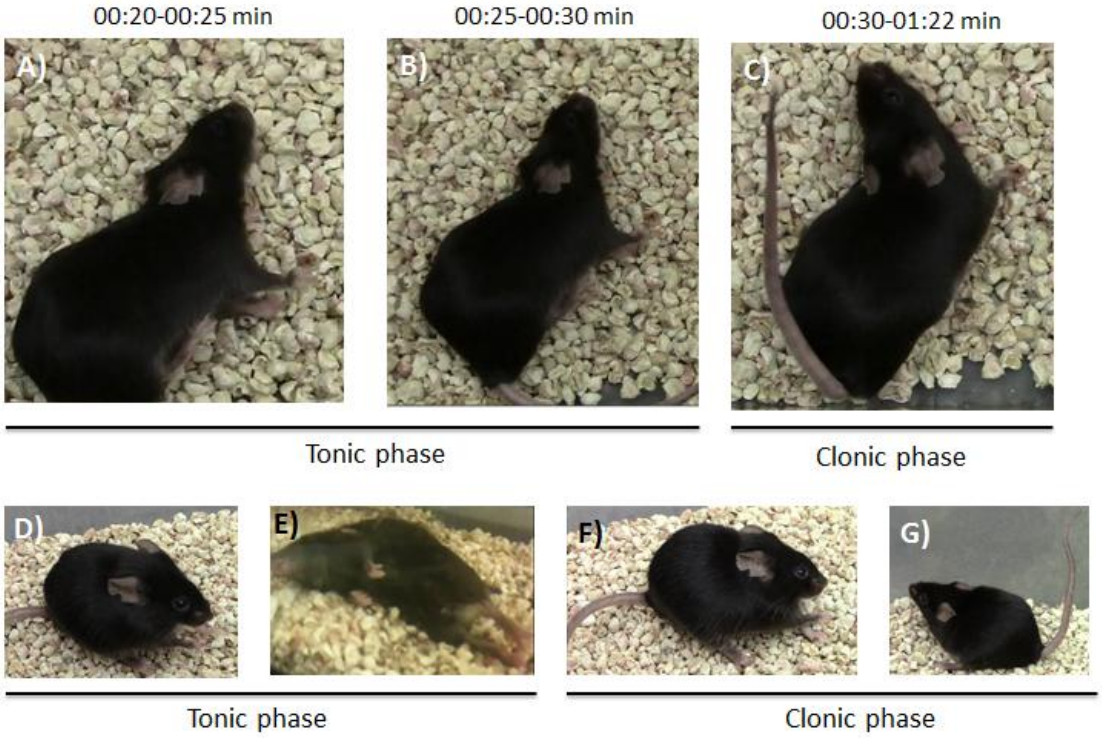

Figure 2.1. ***Aebp2*$^{+/\beta\text{-geo}}$ mice display tonic-clonic seizures. (A-C)** Timeline of seizure activity, which started 20 seconds after initiation of seizure test and lasted for about one minute. Tonic phase typically precedes the clonic phase, however the duration of each phase is subject to variation. **(D-G)** Different postures of mice when they exhibit the tonic and clonic phases during tonic-clonic seizures.

We also characterized the seizures in terms of duration and severity. Most seizures, up to 47%, lasted for about one minute. Approximately 13% seizures lasted half a minute, whereas the remaining 40% lasted longer than a minute (**Fig. 2.1A-C, 2.2C**). Severity of seizure was arbitrarily defined into four categories: high, moderate-high, moderate and low (**Fig. 2.2D**). High severity seizures were tonic-clonic seizures with all symptoms, including salivation, which lasted

one minute or more. About 18% of the seizures analyzed fell into this category. Approximately 10% of seizures were categorized as moderate-high severity where all criteria for high severity applied, except no salivation was observed and the duration was not more than one minute. A moderately severe seizure was defined as a tonic-clonic seizure with all symptoms lasting significantly less than one minute, or seizures where only a prolonged tonic phase was observed with a very mild or no clonic phase. Approximately 12% seizures observed were relegated to this category. A fourth category of low intensity "seizures" was created to accommodate another 60% of mice who did not develop tonic-clonic seizures, however showed signs of muscle tone irregularities such as a mild, short-lived tonic phase with limb and neck flexations and hyper-excitability (**Fig. 2.2D**). Because characterizing this fourth group of seizures is inherently difficult without EEG screening, these cases were not included in any other statistical analysis and will not be discussed further in this chapter.

Aebp2 expression in the brain

In order to determine the spatial expression pattern of *Aebp2* in the brain, we took advantage of the promoterless β-geo gene trap vector which allows the endogenous *Aebp2* promoters to drive the expression of β-galactosidase (**Fig. 2.3A**). The spatial expression pattern was determined by β-galactosidase (β-gal) staining performed on an adult brain cryo-sectioned along the sagittal plane. We found *Aebp2* to be expressed ubiquitously in the brain, with especially high expression levels in the hippocampus and cerebellum (**Fig. 2.3B-C)**. Particularly high levels of staining was observed at the CA1 (Cornu Ammonis area 1) and dentate gyrus regions of the hippocampus (**Fig. 2.3B**) and purkinje cells of the cerebellar cortex (**Fig. 2.3C**). In view of the fact that the dentate gyrus gives rise to the migratory granule cell precursors (Altman and Bayer, 1990) and purkinje cells are known to migrate on radial glia during neuronal

development (Miyata, *et al*, 2010), it is intriguing to note *Aebp2* expression in multiple migratory cell populations and its potential role in cell migration, as previously suggested by Kim, *et al.* (2011).

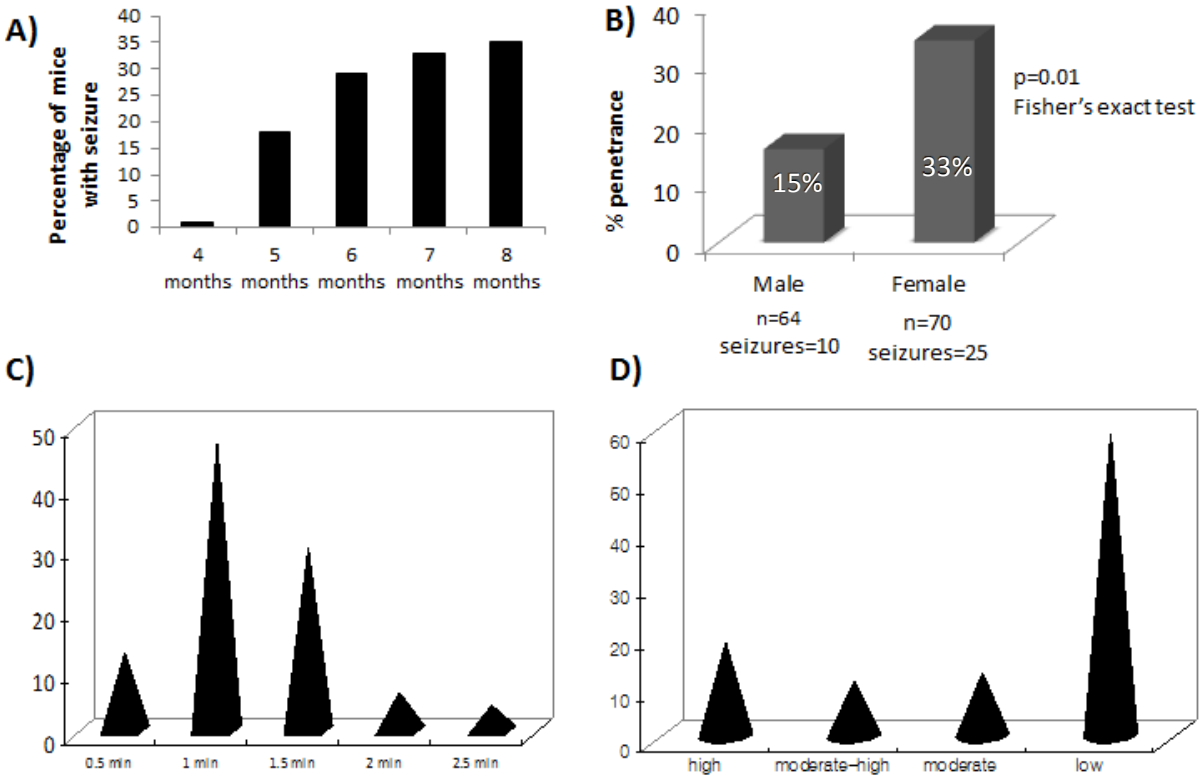

Figure 2.2. Characterization of the seizure phenotype. (A-B) Penetrance of the seizure phenotype. **(A)** Tonic-clonic seizures are more likely to occur in older mice, with the maximum penetrance of 35% when the mice are 7-8 months old. **(B)** Females have been found to display the seizure phenotype significantly more than male heterozygote mice. **(C)** Average duration of seizures. Most seizures in *Aebp2*$^{+/\beta\text{-geo}}$ mice lasted approximately one minute though there was some variation observed. **(D)** Average severity of seizures, organized in four arbitrarily defined categories. High, tonic-clonic seizures with salivation lasting 1 minute or more. Moderate-high, tonic-clonic seizures without salivation lasting approx. one minute. Moderate, tonic-clonic seizures lasting less than 1 minute or prolonged tonic phase with mild or no clonic phase. Low, hyperexcitability and mild tonic phase with neck and limb flexation.

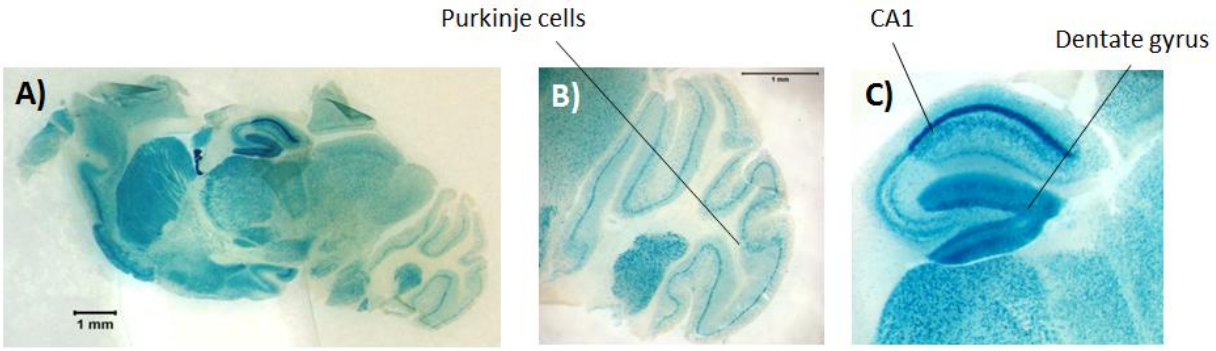

Figure 2.3. Expression of *Aebp2* in the brain. (A) β-gal stained images of the saggital section of adult mouse brain. **(B)** *Aebp2* is highly expressed in the purkinje cells of the cerebellar cortex and **(C)** CA1 and dentate gyrus regions of the hippocampus.

Discussion

Despite repeated connections to PRC2, *Aebp2* has remained a largely uncharacterized gene. So far, it has been proposed that *Aebp2* may be the targeting protein for PRC2 (Kim, *et al.*, 2009) and may play a crucial role in in neural crest cell migration (Kim, *et al.*, 2011). Here we describe late-onset tonic-clonic seizures of varying severity in mice (**Fig. 2.1, 2.2A,D**) that are haploinsufficient for *Aebp2* and a strong expression pattern of *Aebp2* in the brain, especially in the hippocampus (**Fig. 2.3A-C**). Interestingly, we found that females appear to be twice as likely to develop severe tonic-clonic seizures as males (**Fig. 2.2B**). There was also much inter-individual variation observed in the severity of seizure which may be due to differences in epigenetic regulation. However, the specific type of epilepsy displayed by *Aebp2*$^{+/\beta\text{-geo}}$ mice and the underlying molecular mechanisms remain to be identified. Potential points of future interest have been briefly discussed in this section below based on unconfirmed results.

In human patients with temporal lobe epilepsy, it has long been known that seizures seem to originate in the hippocampus (Thom, *et al.*, 2010). Thus it is intriguing to find high expression

of *Aebp2* in that region through β-gal staining. Moreover, preliminary results from morphological analysis of mouse brains that have experienced seizures suggest granule cell loss and/or dispersion in the hippocampus, especially in the CA1 region (data not shown), which is reminiscent of human patients with temporal lobe epilepsy with hippocampal sclerosis (TLE-HS) (Thom, *et al.*, 2010; Haas, *et al.*, 2002). It is believed that granule cell dispersion may be a result of misregulation of the migration process since granule cells in the hippocampus retain the capacity for neurogenesis postnatally (Haas, *et al.*, 2002). In that regard, it is intriguing to propose the role of *Aebp2* as one of the Polycomb genes which has been suggested to play a role in cell migration (Kim, *et al.*, 2011), as a potential factor whose misexpression or downregulation could result in aberrant neuronal migration. In classic TLE that involves the hippocampus, however, males are found to be more likely than females to develop tonic-clonic seizures, according to a study in humans by Janszky, *et al.* (2004). Our results suggest the opposite gender bias, indicating that the seizures associated with *Aebp2* haploinsufficiency may be more nuanced. Further studies involving EEG screening would be able to better identify the type of epilepsy experienced by the haploinsufficient mice.

Moreover, based on unsubstantiated Chromatin Immunoprecipitation (ChIP) data from whole brain (data not shown), we have identified that *Aebp2* downstream genes may be involved in central nervous system development ($p=5.162 \times 10^{-9}$, visible node enrichment, value generated by EGAN using default parameters) and neurogenesis ($p=6.558 \times 10^{-9}$, visible node enrichment, value generated by EGAN using default parameters) (Paquette and Tokuyasu, 2010). Quantitative RT-PCR analyses suggest that *Aebp2* downstream genes are further downregulated in heterozygous seizure mice compared to heterozygous mice. We saw such significant downregulation of genes with known neuronal functions such as Tbr1 and Mecp2. Further

analysis of confirmed ChIP results from brain regions with high *Aebp2* expression, such as the hippocampus or the dentate gyrus specifically, may provide more clues as to the molecular events underlying seizure formation in mice haploinsufficient for *Aebp2*. If the unconfirmed results are true, *Aebp2* downstream genes could provide clues as to the genetic basis of epileptogenesis at least in this instance.

Interestingly, haploinsufficiency of Suz12, another one of the PcG proteins comprising PRC2, also seems to result in brain malformations and hippocampal aberrations (Miró, *et al.*, 2009). Although there has been no seizure or epileptic activity reported in these mice, it makes it tempting to hypothesize that H3K27 methylation may have a major role in brain development, and thereby seizure formation as a result of aberrations in brain development. Recently, there have been many connections made to epigenetic modifiers in TLE patients; significantly DNMT1, DNMT3A (Zhu, *et* al., 2012) and HDAC2 (Huang, *et al.*, 2012) expressions have been found to be upregulated in brains of human patients with TLE. Reelin expression, on the other hand, was downregulated most likely due to hypermethylation of the Reelin promoter, which correlated with increased granule cell dispersion in TLE cases (Kobow, *et al.*, 2009). In several other models of hippocampal seizures, anomalies in histone acetylation and phosphorylation were also observed (Lubin, 2012). Although there has been no connection that has yet been shown between epilepsy and H3K27 methylation, studies such as this and Suz12 haploinsufficiency studies encourage more exploration in that area. The inherently reversible nature of H3K27me3 would make it an attractive target for translational studies in the future if it is indeed shown to have important roles in brain development and epileptogenesis.

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CHAPTER THREE

IMPRINTING STATUS OF AEBP2

Abstract

Aebp2 is an evolutionarily well conserved gene with repeated associations to Polycomb Repressive Complex II (PRC2), which has nonetheless remained largely uncharacterized. In an ongoing effort to characterize *Aebp2*, here we study the differential expression of *Aebp2* in 11.5-dpc embryos as determined by β-gal staining, based on parental transmission of β-gal. Embryos with paternally transmitted β-gal appeared to stain with higher intensity suggesting higher levels of expression from the paternal allele than the maternal allele. This suggested that *Aebp2* may be an imprinted gene. In an effort to find the differentially methylated region (DMR), we focused on the two documented promoters for the gene, named P1 and P2 based on their order on the chromosome. Bisulfite sequencing analysis in WT mice (chapter one) and COBRA results (this chapter) have suggested that P2 is completely unmethylated in all tissues tested; however, P1 is partially methylated in several adult tissues, with a very distinct methylation pattern in the germ cells. The P1 locus in the sperm is completely unmethylated whereas in the egg, it appears mostly methylated, suggesting preferential expression from the paternal allele. Interestingly, P1 is also a SINE-derived promoter. Since genomic imprinting may have evolved to silence retrotransposons and the main mechanism for it is DNA methylation, we tested P1 for differential methylation based on parent-of-origin (imprinting). Our results for this imprinting test remained inconclusive; however, it allowed us to make some interesting observations which suggested that P1 may act as a metastable epiallele. While we are unable to conclusively link P1 to the differential staining pattern observed in 11.5-dpc embryos, it is intriguing to propose that if P1 is indeed acting as a metastable epiallele, it could contribute to some parent-of-origin effects.

Introduction

Genomic imprinting is a specialized form of non-Mendelian expression of genes in a parent-of-origin specific manner which evolved in placental mammals in the animal kingdom. Most genes in the diploid mammalian genome have an equal chance of getting expressed from either the paternal or the maternal chromosome. However, a small subset of these genes is expressed solely from one of two parental chromosomes. Such genes are called imprinted genes. There are two major classes of imprinted genes: paternally expressed genes (*Peg*) and maternally expressed genes (*Meg*) (Allis, *et al.*, 2007). Paternally expressed genes are silenced on the maternal chromosome (called maternal imprinting) and the reverse is true for maternally expressed genes, where the paternal allele is silenced (or imprinted). The main molecular mechanism for genomic imprinting is DNA methylation (Li, *et al.*, 1993) which establish differentially methylated regions (DMRs) which act as imprinting control regions (ICRs) by regulating imprinting for several imprinted genes in a cluster (Reik, 2007). During oogenesis and spermatogenesis, there is an epigenetic resetting event during which DNA methylation is erased, including those at DMRs, and genomic imprinting is reestablished in a germ cell specific manner. However, imprinted genes escape the second epigenetic reprogramming which occurs soon after fertilization, thus maintaining their integrity as genes whose expression is restricted to only one parental allele in the developing embryo (Reik, 2007). In fact, it has been proposed that imprinted genes evolved in placental mammals as a regulatory mechanism which balances the interests of the paternal and maternal genomes during embryogenesis. It is in the interest of the paternal genome to promote growth in offsprings so that the paternal alleles are well represented in the next generation. However, since in placental mammals, the embryo develops within the uterine cavity of the female, it is in the interest of the maternal genome to inhibit such growth

factors so that maternal resources may be well distributed amongst each embryo (Moore and Haig, 1991). This theory is supported by the discovery that over 50% of paternally expressed genes tend to be growth factors whereas maternally expressed genes are growth inhibitors (Allis, *et al.*, 2007).

While this "parental conflict" theory may provide a reasonable working hypothesis for the functional relevance for the evolution of imprinted genes, it leaves open the question of origin of imprinted genes. The currently accepted hypothesis suggests that retrotransposons may have been adapted (or exapted) by the mammalian genome to serve new functions which enabled it to diversify via placenta formation. Retrotransposons are a major target for epigenetic modifications, such as DNA methylation, which are required to prevent their movement in the genome. When such a transposable element is inserted near the promoter region of a gene, it could be used to serve regulatory functions because of its tendency to acquire epigenetic marks. Over time, this may have led to the evolution of differentially methylated regions and imprinted genes. The exaptation hypothesis was first proposed by Brosius and Gould in 1992 and is discussed extensively in a review article by Kaneko-Ishino and Ishino (2010) in the context of a retrotransposon-derived imprinted gene *Peg10*. Here we study a promoter of the Polycomb group gene *Aebp2* (P1) which is derived from a SINE element. It has been established that the egg and sperm show differential methylation at this promoter, with the locus being significantly more methylated in egg than sperm (**Fig. 1.2A**), suggesting that the gene may be paternally expressed. According to our results, bisulfite-based imprinting tests for this locus remain inconclusive; however we make some interesting observations regarding the promoter which open up further avenues of research into the role of exaptation of transposons in gene regulation in mammals.

Materials and Methods

β-galactosidase staining

Embryos were harvested at 11.5-dpc stage (E11.5) from timed mated mice and fixed in 0.2% paraformaldehyde (PFA) fixing solution (0.2% paraformaldehyde, 0.1 M PIPES buffer pH 6.9, 2 mM $MgCl_2$, 5 mM EGTA) at 4℃ for at least 48 hours. They were then transferred to a PBS solution with 2mM $MgCl_2$ and incubated at room temperature for 1 hour. Next, we transferred the embryos to a detergent rinse for another 1 hour at room temperature, before adding 5 mL staining solution with 40 μg/mL X-gal (5-bromo-4-chloro-indolyl-galactopyranoside). The embryos were then incubated at 37℃ for 2.5 hours. All embryos from the same litter were stained in together in one 15 mL tube. All stained embryos were visualized using a dissecting light microscope (Olympus SZX7) and images were captured using a digital camera (Olympus DP70).

Bisulfite conversion

Genomic DNA was isolated from the following tissue samples: (1) 11.5-dpc embryos, fixed in 0.2% PFA immediately after harvest, (2) dentate gyrus from brains of 8 month old male mice, and (3) ear tissue from 17 month old female mice. Tissues samples from ear and E11.5 were incubated at 65℃ overnight in lysis buffer (50mM Tris-Cl pH 8.0, 100mM EDTA [ethylenediaminetetraacetic acid] pH 8.0, 1% SDS [sodium dodecylsulfate] and 250 mM NaCl) and genomic DNA was isolated by phenol-chloroform extraction using UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Life Technologies). Genomic DNA from dentate gyrus was isolated using Trizol (Invitrogen) DNA fraction followed by phenol-chloroform extraction. Approximately 2 μg of this DNA was used for bisulfite conversion using

the EZ DNA Methylation Kit (Zymo Research). Specifically, DNA samples were incubated in freshly prepared CT Conversion Reagent (provided in kit) for exactly 16 hours and final eluted DNA was suspended in 20 µL HPLC water. This kit provides a modified version of bisulfite conversion protocol first established by Clark, *et al.* (1994).

Bisulfite-DNA PCR

Aebp2 P1 and P2 loci were amplified using 2 µL of eluted DNA using the following PCR protocol under standard ionic and pH conditions: 95℃ for 4 minutes, 40 cycles of 95℃ (30 seconds), 60℃ (30 seconds) and 72℃ (30 seconds), 72℃ for 7 minutes. Primers used to amplify P1 for COBRA (combined bisulfite restriction analysis): **Aebp2-ef-bF1** – GAAGTTTAGGAA GATGTAATGTTTTTTTAGTTG; **Aebp2-ef-bR1** – TAAATCCTAAAACTTCTAATAAAATC TAAAATAACAC. Primers used to amplify P2 for COBRA: **Aebp2-sf-bF1** –TTTGTAGAAGT TTAGGGTTGTGAAAATTT; **Aebp2-sf-bR1** – CTACTACTACCCACCAAACTCTC. For bisulfite sequencing, *Aebp2* P1 was amplified by nested PCR using the protocol described above with the some changes. The first PCR reaction was carried out under standard conditions, 20 cycles with 55℃ annealing temperature. 2 µL of this DNA was then used undiluted for the second PCR reaction, which was also carried out for 20 cycles, 55℃ annealing temperature. First PCR primer set: **Aebp2-E-promoter-imp-bs-F** – TGTTTGGGGATAGGGAGGAGTTTT TTATTAG; **Aebp2-E-promoter-imp-bs-R** – TAAATCCTAAAACTTCTAATAAAATCTAA AATAACAC. Nested PCR primer set: **Aebp2-E-promoter-imp-bs-F.1** – TGTTTGGGGATAGG GAGGAGTTTTTTATTAGTAG; **Aebp2-E-promoter-imp-bs-R.1** – ATCTAAAATAACACTTA TAAACAAACTAAA.

Combined Bisulfite Restriction Analysis (COBRA)

Bisulfite-converted P1 and P2 DNA samples were digested with *Taq*^{a}I (TCGA) for 6-7 hours at 65℃ using NEBuffer 4 (New England Biolabs). Bisulfite-converted P2 DNA was also digested with *Fok*I (GGATG) using NEBuffer 2 (New England Biolabs) for 6-7 hours at 37℃. Restriction enzyme digestion products were then mixed with dye mix and run on 1.5% w/v agarose gel at 100V for 40 minutes. Gel images were captured using Gel Doc XR+ System and Quantity One software (BioRad).

Bisulfite sequencing

Aebp2 P1 DNA amplified using Aebp2-E-promoter-imp-bs primers was run on a 1.5% gel to ascertain the presence of bands, which were then cloned for sequencing. Purification of DNA extracted from agarose gel was conducted using MEGA-spin Agarose Gel Extraction Kit (Intron). The eluted DNA was further precipitated using ethanol precipitation overnight at -20℃. Precipitated DNA was used for cloning into the pGEM T-easy vector (Promega) using company-suggested protocol. The ligation product was then transformed into *E. coli* JM107 competent cells and plated using IPTG (Isopropyl β-D-1-thiogalactopyranoside) and X-gal for blue/white screening. White colonies were then inoculated in LB-broth with 100 mg/mL ampicillin and incubated at 37℃ for 16 hours. Plasmid DNA was isolated using DNA-spin Plasmid DNA Purification Kit (Intron) and sequenced using the Big-Dye protocol. Maternal and paternal alleles in hybrid (PWD x B6) embryos were separated a three base pair (GAG) deletion in the P1 locus of PWD which otherwise matched very well with the B6 sequence. Bisulfite sequencing results for each parental allele were then analyzed using BiQ Analyzer (Bock, *et al.*, 2005) which

checked for inverted sequences, presence of clones, at least 80% sequence similarity and at least 90% bisulfite conversion rate.

Results

Parent-of-origin dependent staining of *Aebp2*$^{+/\beta\text{-geo}}$ embryos

As part of an ongoing effort to characterize *Aebp2* as a gene and a protein, in this study we focused on the spatial expression pattern of *Aebp2* as determined by β-gal staining in 11.5-dpc embryos from timed-mated *Aebp2*$^{+/\beta\text{-geo}}$ (♀) x *Aebp2*$^{+/\beta\text{-geo}}$ (♂) breeding scheme (**Fig. 3.1A**). We observed two distinct patterns of staining in these embryo: first, the wild-type (WT) embryo showed no β-gal staining as would be expected due to lack of the β-geo gene trap vector (**Fig. 3.1A**, embryo 1), and among the *Aebp2*$^{+/\beta\text{-geo}}$ embryos, some embryos stained darker than others (**Fig. 3.1A**, embryo 2-6). While all embryos appear to globally express β-galactosidase, the embryos numbered 3, 5 and 6 stain visibly darker compared to 2 and 4, especially in the body and limb buds of the embryos. Staining in the midbrain region also appears to be stronger for embryos 3 and 6 compared to 2 and 4, however we were unable to determine whether this was significant because embryo 5, which also stained darker globally like 3 and 6, showed midbrain staining at an intensity comparable to embryos 2 and 4, which stained lighter globally. Genotyping these embryos for the β-geo gene trap vector which disrupts *Aebp2* expression in mutant mice confirmed the presence of only one WT embryo, which ruled out the possibility that the lighter stained embryos may be due to background staining of WT embryos (**Fig. 3.1B**). Next, we genotyped these embryos for the Y-chromosome gene *Sry* to test whether the differential staining pattern is based on gender. According to our results, this is unlikely to be the

case since embryos numbered 3, 4 and 6 were all determined to be females, and embryos 3 and 6 stain darker than embryo 4 (**Fig. 3.1C**).

These results prompted us to test whether staining patterns may be allele-specific. We performed this test by timed mating two reciprocal crosses: WT (♀) x *Aebp2*$^{+/\beta\text{-geo}}$ (♂) (paternal transmission of β-gal) and *Aebp2*$^{+/\beta\text{-geo}}$ (♀) x WT (♂) (maternal transmission of β-gal). Our results from β-gal staining of embryos harvested at embryonic day (E) 11.5 demonstrate that all embryos from the individual crosses stain similarly and no differential staining is observed when β-gal transmission is restricted to one or the other parental allele (**Fig. 3.1D**). Moreover, it appeared as though the stronger staining pattern may correlate with paternal transmission of β-gal. While global staining is observed in maternal as well as paternal transmission embryos at E11.5 stage, the embryos tend to stain darker when β-gal is transmitted paternally (**Fig. 3.1D**). Midbrain staining was also found to be stronger in all paternal transmission embryos compared to maternal transmission embryos. Moreover, the staining intensity for paternal transmission embryos in **Fig. 3.1D** appears similar to embryos 3, 5, and 6 in **Fig. 3.1A**. Conversely, the staining intensity for maternal transmission embryos (**Fig. 3.1D**) matches that of embryos 2 and 4 (**Fig. 3.1A**). In all instances, the WT embryos showed no staining as expected, thus serving as appropriate negative controls for staining artifacts. Taken together, the correlation of allele specificity with the intensity of staining, strongly suggested genomic imprinting at the *Aebp2* locus, at least in *Aebp2*$^{+/\beta\text{-geo}}$ mice.

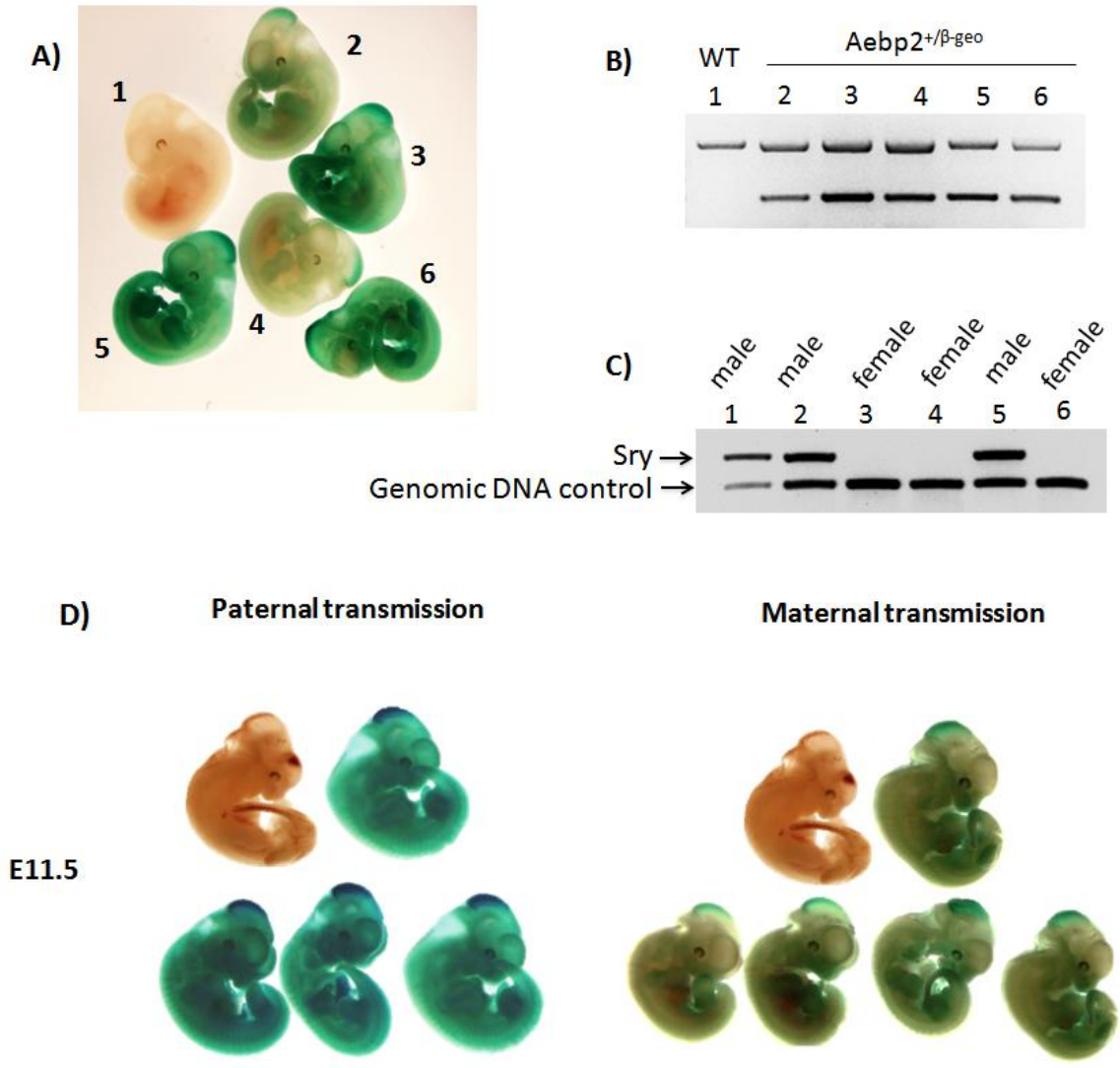
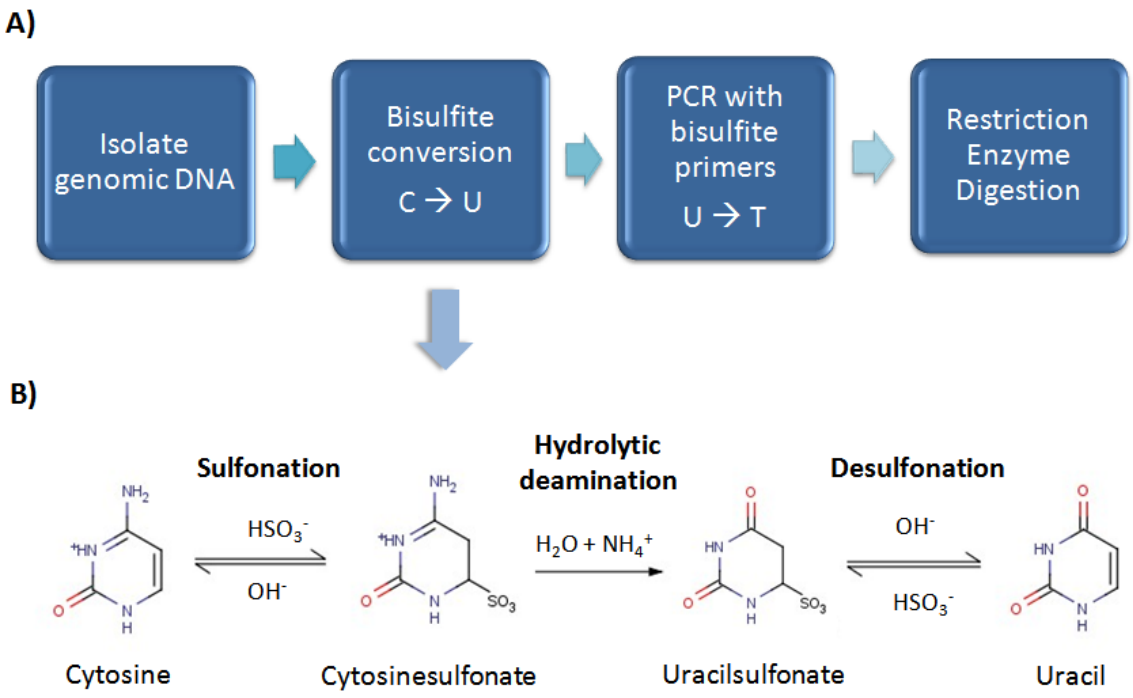


Figure 3.1. Differential spatial expression pattern of β-gal is dependent on parent of transmission. **(A)** β-gal staining of 11.5-dpc embryos from *Aebp2*$^{+/\beta\text{-geo}}$ x *Aebp2*$^{+/\beta\text{-geo}}$ cross (1-6). **(B)** Genotyping for *Aebp2* in 11.5-dpc embryos (1-6). **(C)** Sex-typing 11.5-dpc embryos (1-6) with Sry. **(D)** β-gal staining of 11.5-dpc embryos from crosses yielding maternal and paternal transmission of β-gal.

<u>*Aebp2* P1 may be a target for allele-specific silencing</u>

Since DNA methylation is the best characterized of molecular mechanisms used to establish genomic imprinting (Li *et al.*, 1993), we used COBRA (**Fig. 3.2A, B**) and bisulfite sequencing (**Fig. 3.2C**) in order to study allele-specific methylation at the *Aebp2* locus. The

genomic regions amplified by PCR for DNA methylation analysis are portrayed as labeled black bars (*Aebp2*-P1 and *Aebp2*-P2 in **Fig. 3.3A**). We considered the two documented *Aebp2* promoters (P1 and P2) as potential targets for differential methylation, with special emphasis on the SINE-derived P1 which is anomalously conserved between rat and mouse (Conservation: rat, SINE in **Fig. 3.3A**). The degree of conservation of this SINE element suggests that the initial transposon inserted may have been exapted to offer a selective advantage, thus warranting its evolutionary conservation in the mouse-rat lineage. Exapted TEs often perform epigenetic regulatory functions by being the target of epigenetic modifications which then affects nearby genes (Huda, *et al.*, 2010). Genomic imprinting, too, could have its roots in transposon silencing (Suzuki, *et al.*, 2007). Thus given the unusual conservation of the SINE element in P1, we predicted that it may be the target of allele-specific silencing.



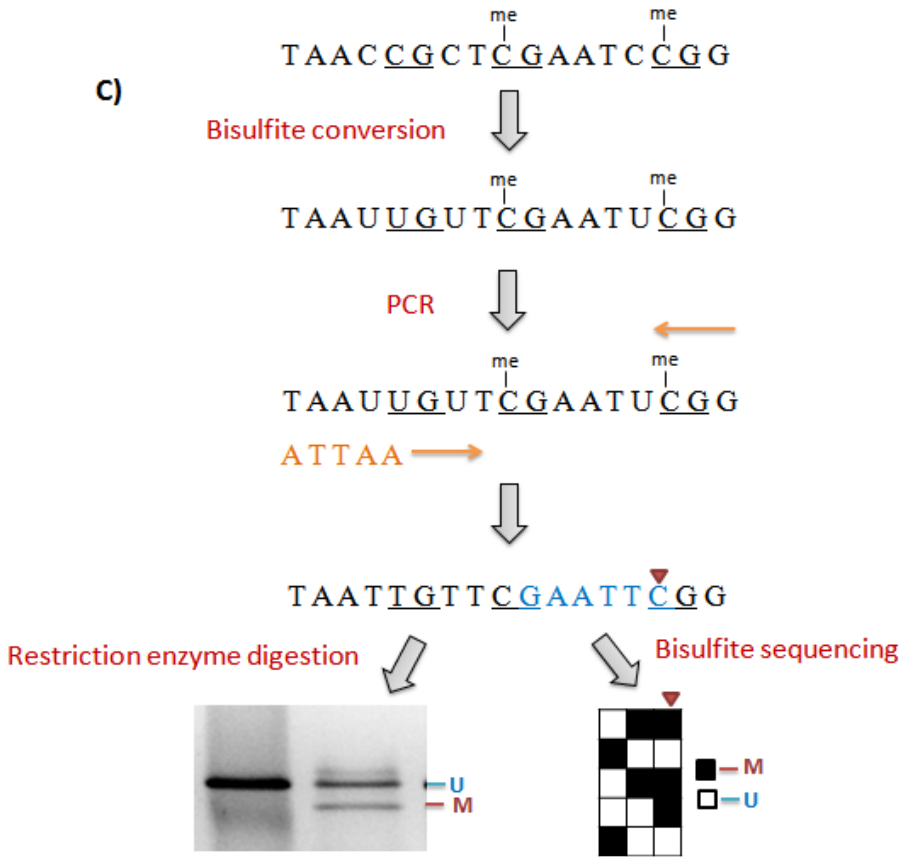


Figure 3.2. Analysis of DNA methylation using COBRA and bisulfite sequencing. (A) Schematic outline of COBRA protocol. **(B)** Chemical reaction yielding cytosine-to-uracil conversion, dubbed bisulfite conversion. **(C)** Overview of protocol used to analyze genomic DNA methylation.

We independently assayed three tissues using the COBRA protocol demonstrated in **Fig. 3.2C** – whole 11.5-dpc embryo, dentate gyrus (DG) and ear – in three littermates each (**Fig. 3.3B**). Digestion of bisulfite converted *Aebp2* P1 DNA with *Taq*$^{\alpha}$I, which is expected to digest methylated DNA, showed two bands signifying partial methylation at the P1 locus in all tissues tested. While P1 appears mostly methylated in the DG, faint bands are observable for unmethylated DNA upon closer observation (**Fig. 3.3B**). Interestingly, a visual comparison of the band densities of the "methylated" and "unmethylated" PCR bands (M:U) in E11.5 suggests a ratio of 1:1, which would be expected in the case of allele-specific methylation, where roughly

50% of total DNA (i.e. DNA from one parental allele) would be expected to be methylated and the other 50%, from the opposite parental allele, unmethylated. This pattern of partial methylation at P1 was repeatedly observed in COBRA performed on WT and *Aebp2*$^{+/\beta\text{-geo}}$ tissues (data not shown), suggesting it is indeed the behavior of the endogenous gene promoter and is not caused by the insertion vector. In contrast, P2 was found to be completely unmethylated, as demonstrated by complete digestion of bisulfite converted DNA by *Fok*I, which is expected to digest unmethylated DNA, and no digestion by *Taq*$^{\alpha}$I. Overall, these COBRA results are consistent with what would be expected based on bisulfite sequencing results discussed in chapter one and confirm our initial prediction that P1 may be a more likely candidate for allele-specific silencing by methylation than P2.

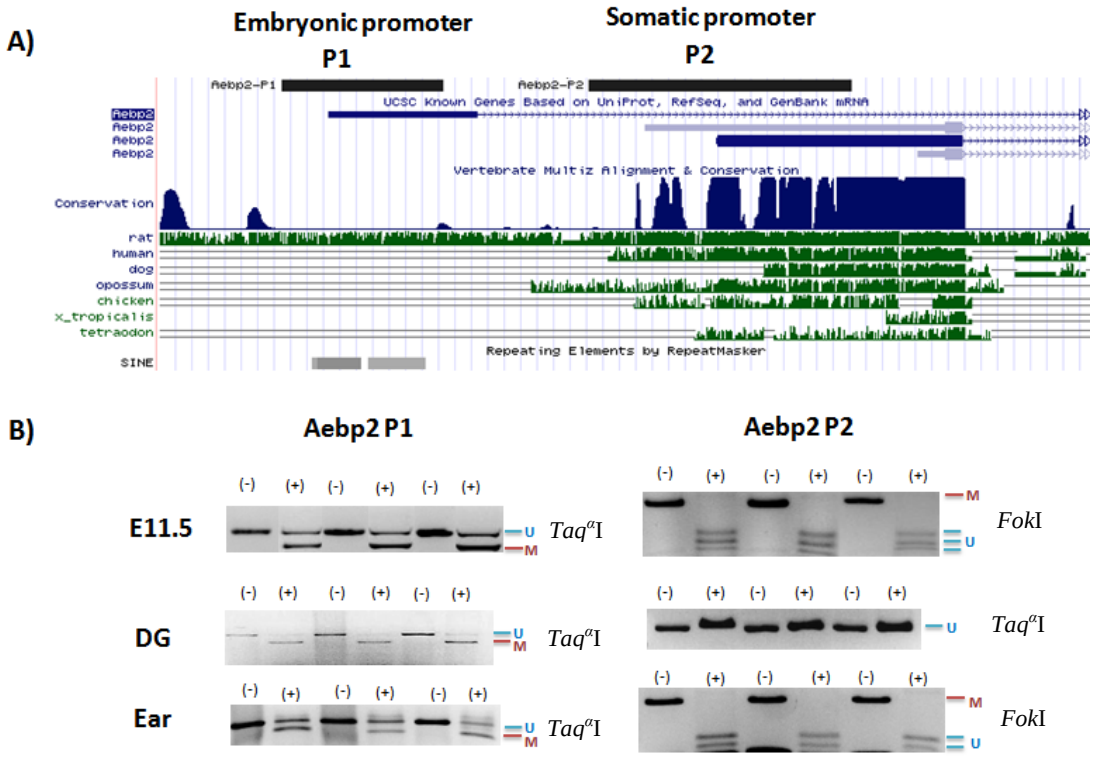


Figure 3.3. Partial methylation of SINE-element derived *Aebp2* P1. (A) Screenshot from UCSC Genome Browser showing genomic regions amplified by PCR (*Aebp2*-P1 and *Aebp2*-P2 as black bars) for promoter methylation analysis. **(B)** COBRA results demonstrating partial methylation at *Aebp2* P1 (using *Taq*$^{\alpha}$I) whereas P2 is completely unmethylated (digested with *Fok*I and *Taq*$^{\alpha}$I).

Bisulfite-based imprinting test on P1

In order to determine whether the partial methylation pattern was due to allele specificity, as visually indicated by the 1:1 (M:U) band density ratio, we performed bisulfite-based imprinting test on B6/129 x PWD hybrid mice. Tail DNA from hybrid offsprings of two reciprocal crosses were studied for this experiment: first, B6/129 (♀) x PWD (♂) and second, PWD (♀) x B6/129 (♂) (**Fig. 3.4A-B**). The maternal and paternal alleles were distinguished based on a 3 base pair (GAG) deletion in the P1 locus of PWD which otherwise matched very well with the B6 sequence. In **Fig. 3.4 (A-B)**, replicates (clones) from bisulfite-DNA library are stacked on the horizontal axis, whereas the twelve columns in the table represent twelve CpG sites in the sequence amplified. According to our results, both the maternal and paternal alleles in both crosses are each approximately 50% methylated on the whole, with a large variation in methylation level between clones (**Fig. 3.4**). Overall, we were unable to conclude that P1 is differentially methylated between maternal and paternal alleles in an imprinting-like fashion (where one allele is 100% silenced whereas the other 100% expressed) from either cross by Fisher's exact test for statistical significance. With $p>0.1$ (two-tailed) for the first cross (**Table 3.1**) and $p>0.9$ (two-tailed) for the second (**Table 3.2**), we cannot reject our null hypothesis which states that there is no difference in methylation between maternal and paternal alleles. However, the bisulfite-sequencing results demonstrate that within one parental allele, reestablishment of DNA methylation after epigenetic reprogramming during embryogenesis may be a stochastic event, giving rise to different levels of methylation between different cells, as evidenced by the mosaic methylation pattern for individual parental alleles. Thus while our results for the imprinting status of *Aebp2* P1 remain inconclusive at the moment, we cannot rule

out the possibility that P1 may nonetheless play a role in the differential staining of *Aebp2*$^{+/\beta\text{-geo}}$ embryos.

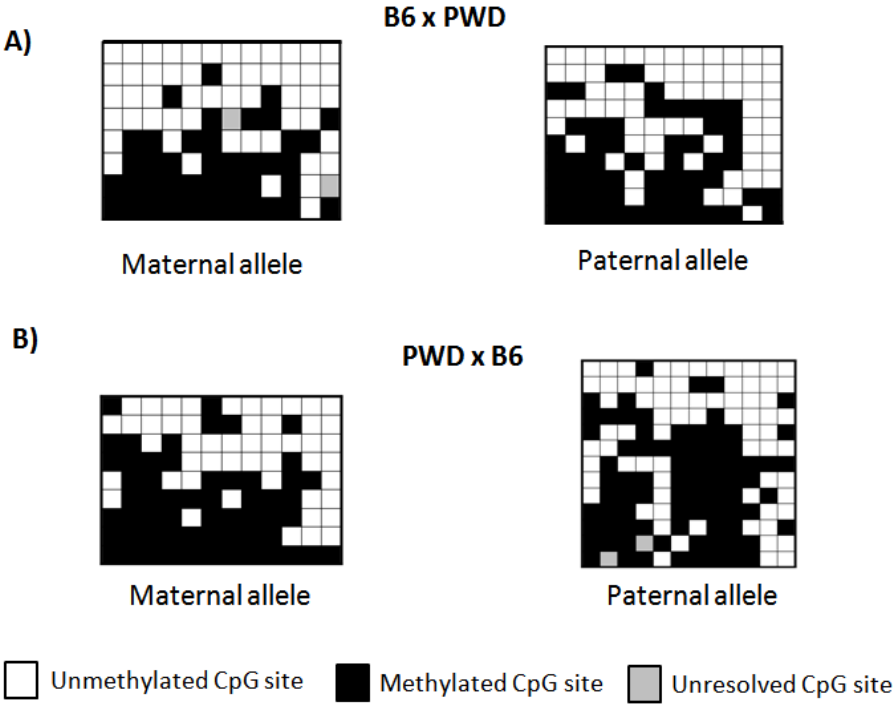


Figure 3.4. Bisulfite-based imprinting test conducted on P1. (A) DNA methylation on maternally and paternally derived alleles in B6/129 x PWD offspring tail DNA. **(B)** DNA methylation on maternally and paternally derived alleles in PWD x B6/129 offspring tail DNA.

Table 3.1. Statistical analysis of methylation at the 12 CpG sites in the *Aebp2* P1 sequence shows no significant corelation between overall percent methylated and the parental allele in **B6/129 (♀) x PWD (♂)** offspring tail DNA.

CpG	1	2	3	4	5	6	7	8	9	10	11	12	Total
Maternal allele	16.7	41.7	41.7	50	25	75	58.3	58.3	66.7	58.3	8.3	9.1	42.7
Paternal allele	75	75	75	75	37.5	50	62.5	50	50	50	12.5	25	53.1
P-value	0.0194	0.1968	0.1968	0.3729	0.6424	0.3563	1	1	0.6479	1	1	0.5459	0.1154

Table 3.2. Statistical analysis of methylation at the 12 CpG sites in the *Aebp2* P1 sequence shows no significant corelation between overall percent methylated and the parental allele in **PWD (♀) x B6/129 (♂)** offspring tail DNA.

CpG	1	2	3	4	5	6	7	8	9	10	11	12	Total
Maternal allele	66.7	77.8	66.7	66.7	33.3	77.8	55.6	55.6	44.4	66.7	22.2	11.1	53.7
Paternal allele	53.8	58.3	69.2	69.2	15.4	61.5	69.2	84.6	69.2	38.5	15.4	30.8	52.9
P-value	0.6740	0.6424	1	1	0.6090	0.6478	0.6619	0.1778	0.3842	0.3870	1	0.3602	0.9008

Discussion

In this study, we have attempted to study the differential expression pattern of *Aebp2* as determined by β-gal staining in 11.5-dpc embryos derived from *Aebp2*$^{+/\beta\text{-geo}}$ (♀) x *Aebp2*$^{+/\beta\text{-geo}}$ (♂) cross. While the WT embryos showed no staining (as expected), *Aebp2*$^{+/\beta\text{-geo}}$ embryos were found to stain at two levels of intensity which we dubbed "light" and "dark" (**Fig. 3.1A**). Embryos harvested at 11.5-dpc from two separate crosses where β-gal transmission was restricted to only one parent of origin showed uniform staining with no inter-individual differences between littermates, but differences in staining intensity were observed between embryos derived from maternally and paternally transmitted β-gal (**Fig. 3.1D**). This suggested genomic imprinting at the *Aebp2* locus, where one parental allele was preferentially expressed over another. While unlike canonical imprinted genes, the difference in expression was not all-or-none, it is interesting to note there have been other genes found on chromosome 6, 7 and 12 which are "partially imprinted," where expression from one parental allele is favored over another (Clark, *et al.*, 2002; Tsai, *et al.*, 2002; Ono, *et al.*, 2003). Through sequence and COBRA

analysis (**Fig. 3.3**), we identified the first *Aebp2* promoter (P1) to be a more likely target for differential methylation compared to P2. These results correlated well with the methylation patterns of P1 observed in egg and sperm (**Fig. 1.2A**). The P1 promoter in the sperm appeared almost completely unmethylated whereas the egg was more methylated. However, the P1 promoter in egg was not completely methylated, and some eggs were distinctly more methylated than others. Yet, if the P1 locus was undergoing genomic imprinting, we would expect it be to be paternally expressed and maternally imprinted, since the locus was more unmethylated in sperm than egg and paternal transmission of β-gal inserted into the *Aebp2* locus yielded more expression of β-gal than when it was maternally transmitted. However, a bisulfite-sequencing based imprinting test on tail DNA extracted from two reciprocal crosses B6/129 (♀) x PWD (♂) and PWD (♀) x B6/129 (♂) yielded inconclusive results regarding the imprinting status of P1. It did, however, point to a stochastic nature of reestablishment of epigenetic marks in an individual at that locus after the epigenetic reprogramming event which occurs at embryogenesis. Also, the mosaicism in methylation of P1 between individuals was hinted at by the egg methylation results where some eggs were completely methylated whereas other eggs were completely unmethylated.

This observation supports the possibility that P1 may be a "metastable epiallele" as first described by Rakyan, *et al.* in 2002. Metastable epialleles have been known to have a labile nature, such that environmental influences and stochastic changes in protein levels may influence their level of DNA methylation. This may result in different levels of methylation, and therefore expression, in tissues between individuals depending on environmental factors. Moreover, metastable epialleles have also been known to show parent-of-origin effects. These effects are inherently different from what is observed in genomic imprinting because the level of

methylation of each allele can only be expressed as a probability, as opposed to the predictable nature of imprinted genes (either paternally or maternally expressed). Also, parent-of-origin effects arising from metastable epialleles can "switch" alleles – in other words, while the metastable epiallele may be maternally expressed in certain individuals, it may be paternally expressed in others, and biallelic in yet another set of individuals (Rakyan, *et al.*, 2002). This is possible in the case of *Aebp2* P1 locus when the egg methylation data is considered. While some eggs show complete methylation, some eggs show intermediate methylation, whereas others show almost no methylation. If an egg with complete methylation at P1 underwent a fertilization event with a completely unmethylated sperm, it would result in imprinting-like effects in *Aebp2* expression from that promoter. Therefore, while we cannot conclude that the differential staining pattern of the 11.5-dpc embryos (**Fig. 3.1A**) is due to genomic imprinting of *Aebp2* through the parent-of-origin dependent methylation of P1 because of inconclusive imprinting test results, we cannot exclude the possibility that P1 may contribute to some parent-of-origin effects in these embryos if it is indeed proven to behave as a metastable epiallele.

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CHAPTER FOUR

SUMMARY AND DISCUSSION

Summary

In this thesis, I have attempted to chronicle our studies at characterizing mice that are mutant for the *Aebp2* gene. In the first chapter, I have presented a short overview of my major topics of discussion, namely epigenetics as a field of study and two main epigenetic mechanisms known so far, DNA methylation and histone modification. In terms of histone modification, the main focus has been the trimethylation of lysine 27 on histone H3 (H3K27me3) because it is established by the Polycomb Repressive Complex II (PRC2), which has been directly associated to our gene of interest in many previous studies. AEBP2 is known to stabilize the histone modifying complex by binding to its hinge region (Ciferri, *et al.*, 2012) and enhancing its activity (Cao and Zhang, 2004), and has also been proposed to be the targeting protein *in vivo* (Kim, *et al.*, 2009). Given the importance of PRC2 in embryonic development (Schuettengruber, *et al.*, 2007), it is not surprising that AEBP2 is an evolutionarily conserved DNA-binding protein (Kim, *et al.*, 2009). Two main isoforms of the protein have been detected so far: 52 kDa (somatic form) and 31 kDa (embryonic form). The two isoforms could arise from the activity of multiple alternative promoters, which we have here named P1, P2 and P3, based on their order on the chromosome. The larger somatic form (52 kDa) arises from the P2 promoter, whereas P1 and P3 give rise to the smaller (31 kDa) form (**Fig. 1.1**). The latter promoter, P3, has only been recently discovered and is still in the process of being characterized. However, later in this chapter, we will consider its potential role briefly in the context of our studies.

In order to understand the role of *Aebp2 in vivo*, Dr. Kim's lab has generated a line of mutant mice in which the expression of *Aebp2* is disrupted (**Fig. 1.3**). This was accomplished via the insertion of a β-geo gene trap vector which drives the production of β-galactosidase from the

endogenous *Aebp2* promoter (Kim, *et al.*, 2011). This vector also allowed us to study the expression of *Aebp2* through β-gal staining. According to the results, *Aebp2* is highly expressed in the 9.5-dpc embryo (E9.5), especially in neural tube. In E11.5, very high level of expression was observed in the midbrain region of the embryo. By 15.5-dpc, high *Aebp2* expression could be observed in cells derived from neural crest cells such as the dorsal root ganglia and cranial-facial neural crest cells (**Fig. 1.4)**. During the course of the present study, we also studied *Aebp2* expression in the adult brain and found it to be expressed ubiquitously with especially high levels of expression in the hippocampus, specifically the dentate gyrus and CA1 regions, and the purkinje cells of the hippocampus. Further, a detailed analysis of mutant phenotypes was conducted, and some phenotypes, such as enlarged colon and lack of melanocytes, associated with neural crest cell migration disorders have been documented (Kim, *et al.*, 2011). In an ongoing effort to characterize mutant phenotypes, here we focus on late-onset tonic-clonic seizures displayed by about 35% of *Aebp2*$^{+/\beta\text{-geo}}$ mice (**Fig. 2.1**). Maximum penetrance of the seizure phenotype (35%) appears to be when the mice are 7-8 months old, and seizures are typically triggered by stress when the mice are moved to a new environment. There is wide inter-individual variation in terms of severity and duration of seizure; however, we find females tend to be nearly twice as susceptible to developing seizures as males (**Fig. 2.2**).

In this chapter, we will discuss some lines of potential interest which correlate DNA methylation at the *Aebp2* promoters P1 and P3 with the seizure phenotype. These are the two promoters which can give rise to the 31 kDa embryonic form of AEBP2. DNA methylation on P1 appears to be probabilistic with differences in methylation levels between cell types and also between individuals (**Fig. 1.2**). An imprinting test on P1 remained inconclusive; however, along with other tissue methylation data, it pointed to the fact that P1 may act as a metastable epiallele.

As a metastable epiallele, its methylation is expected to be probabilistic, potentially leading to differences in P1 activity between individuals. Moreover, it may also contribute to some parent-of-origin effect (although unlike imprinting) as a metastable epiallele. In fact, these epialleles have been known to "switch" alleles of expression – that is to say, while it may be paternally expressed in some individuals, it may be maternally expressed or biallelic in others – because of the probabilistic nature of methylation at these loci (Rakyan, *et al.*, 2002). P3, on the other hand, was found to be completely unmethylated in the WT tissues tested and bisulfite sequencing results from ear tissue have been presented in **Fig. 1.2 (F)**.

Discussion and Future Directions

Differential expression of *Aebp2* in *Aebp2*$^{+/\beta\text{-geo}}$ brains

β-gal staining of multiple brains (whole-mount) from 3 month old female mice arising from *Aebp2*$^{+/\beta\text{-geo}}$ x *Aebp2*$^{+/\beta\text{-geo}}$ breeding, showed differential staining depending on the individual **(Fig. 4.1A-B**). None of the mice had experienced seizure by the time they were sacrificed; therefore, a differential expression of *Aebp2* is unlikely to be an effect of the seizure. While one brain (first from left in **Fig. 4.1A and B**) appeared to stain ubiquitously along the coronal plane, other brains showed much sparser staining, with one brain (fourth from left in **Fig. 4.1A**) showing a very defined staining pattern only in the hippocampus. We independently assayed *Aebp2* expression in the dentate gyrus of the hippocampus in four male *Aebp2*$^{+/\beta\text{-geo}}$ mice from the same breeding scheme (not litter mates) because of the high staining intensity in the hippocampus. Quantitative RT-PCR data confirmed an inter-individual variation in the expression of *Aebp2* in the hippocampi in male mice, similar to what was observed through β-gal staining in female mouse brains (**Fig. 4.1C**). Interestingly, upon comparing β-gal stained

Aebp2$^{+/\beta\text{-geo}}$ seizure brains with normal *Aebp2*$^{+/\beta\text{-geo}}$ brains, we noticed that seizure brains tend to stain darker than their normal counterpart (data not shown), showing much of the "light" and "dark" staining pattern discussed in the context of 11.5-dpc embryos in chapter three. Overall, these results suggest much inter-individual variation in the level of *Aebp2* expression in *Aebp2*$^{+/\beta\text{-geo}}$ mice.

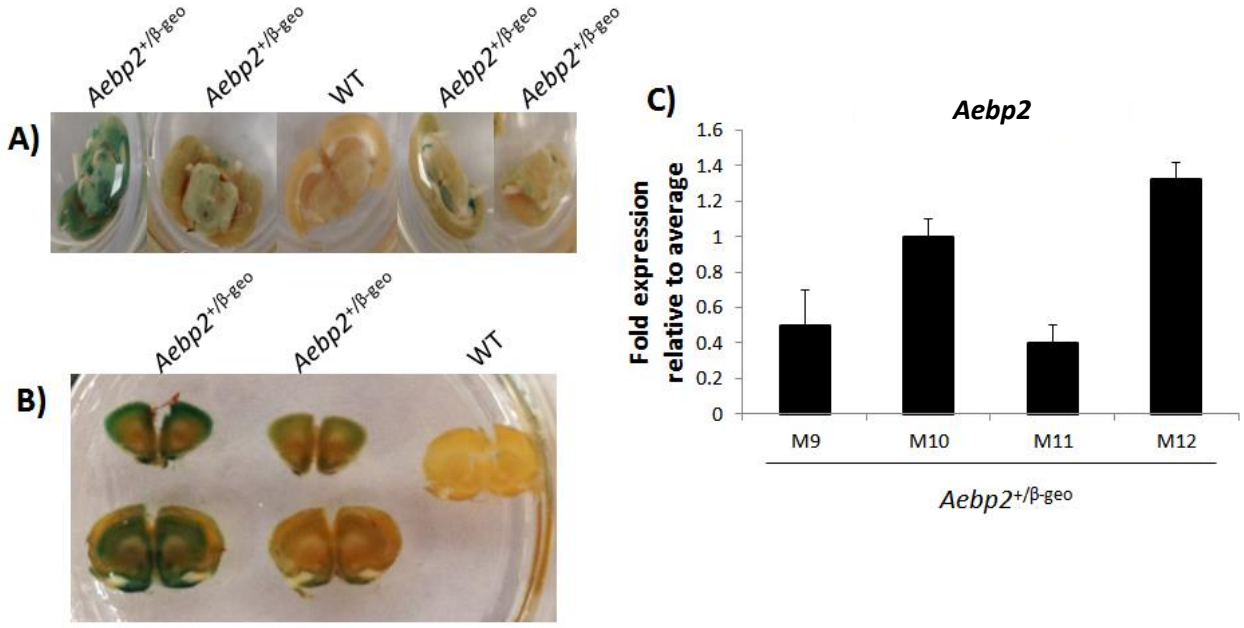

Figure 4.1. Differential expression of *Aebp2* in *Aebp2*$^{+/\beta\text{-geo}}$ mice. (A-B) β-gal staining images of female *Aebp2*$^{+/\beta\text{-geo}}$ brains showing differential staining between different brains, indicating differential expression of *Aebp2* among different individuals. **(C)** qRT-PCR results showing fold differences in *Aebp2* expression in four male *Aebp2*$^{+/\beta\text{-geo}}$ mice.

<u>*Aebp2* P1 may be significantly more methylated in *Aebp2*$^{+/\beta\text{-geo}}$ seizure mice</u>

In an effort to understand the inter-individual differences observed in the staining and by real-time PCR, we studied methylation pattern of P1 in three female littermates of the following genotypes: WT, *Aebp2*$^{+/\beta\text{-geo}}$ (normal) and *Aebp2*$^{+/\beta\text{-geo}}$ seizure. *Aebp2* P1 was our primary locus of interest because of previous indication that it may be a metastable epiallele, which is indeed

expected to show different levels of DNA methylation in different individuals, which would then result in differential expression of *Aebp2*. If P1 was indeed a metastable epiallele, we would expect its methylation pattern to be conserved in tissues derived from the three germ layers – ectoderm, mesoderm and endoderm. We therefore aimed to analyze DNA methylation in ectoderm-derived ear tissue because of its ease of access and because it does not affect survivability in mice. Moreover, in the event that we find a correlation between P1 methylation and seizure susceptibility, our aim was to test the tendency to develop seizure quickly and efficiently, without sacrificing the mouse. In this manner, *Aebp2* downstream gene expression studies could be conducted before and after seizure events, which could lend clues towards understanding the underlying molecular mechanism of epilepsy in the *Aebp2*$^{+/\beta\text{-geo}}$ mice.

According to our preliminary results, *Aebp2* P1 in the WT mouse ear was approximately 38% methylated, though there was a large amount of variation observed between clones (**Fig. 4.2**). While some clones were completely methylated, others were completely unmethylated with several clones showing intermediate levels of methylation. P1 was more methylated in *Aebp2*$^{+/\beta\text{-geo}}$ mice, with the normal mouse showing about 50% methylation and the seizure mouse approximately 58%. We were unable to confirm whether the difference in methylation level between the WT and normal *Aebp2*$^{+/\beta\text{-geo}}$ mouse was significant ($p=0.05$, Fisher's exact test); however, interestingly, we found that P1 in *Aebp2*$^{+/\beta\text{-geo}}$ seizure mice are significantly more methylated than in WT mice ($p=0.0012$, Fisher's exact test) (**Fig. 4.2**). This appears to support our initial hypothesis that P1 methylation level in non-invasively obtained tissues may correlate to seizure susceptibility. However, many more mice need to be tested against this hypothesis before conclusions can be made. This initial data point, incidentally, also supports our earlier prediction that P1 may be a metastable epiallele, because we were able to demonstrate that there

are significant differences in P1 methylation level in different individuals. However, whether or not this directly correlates to a phenotypic outcome is in the scope of a future study. Moreover, in order to prove P1 is a metastable epiallele, we would need to test its methylation in multiple tissues arising from the three germ layers in one mouse, and compare the results to those obtained similarly from other mice. If P1 is indeed a metastable epiallele, we would expect its methylation level to not significantly differ between germ layers in the same mouse, but show inter-individual differences between littermates.

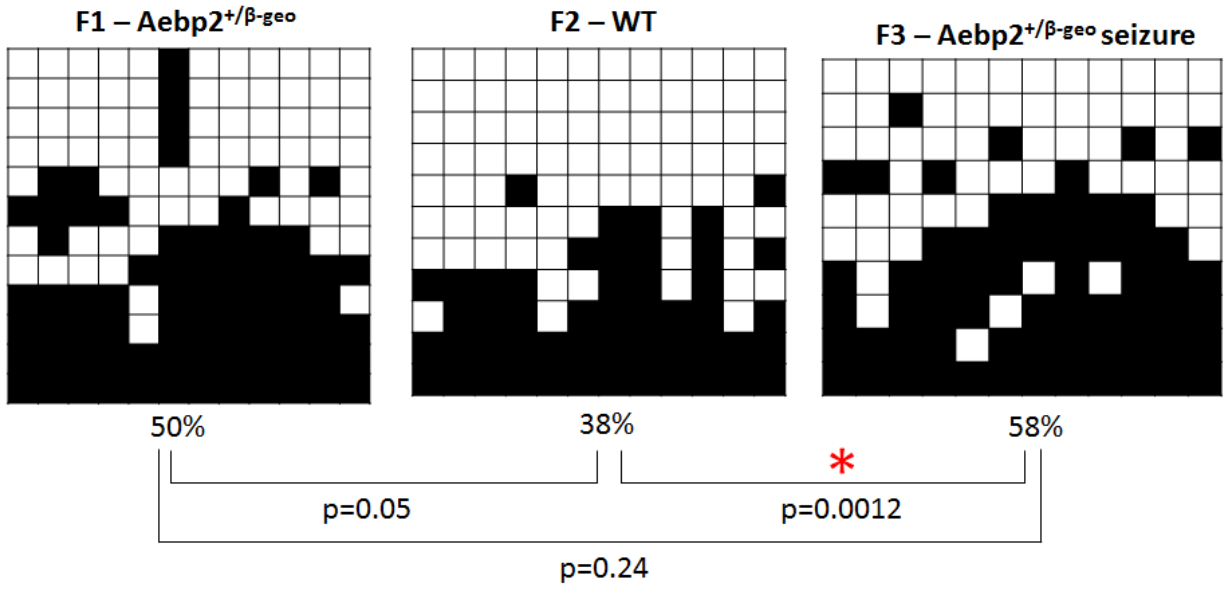

Figure 4.2. *Aebp2* P1 is significantly more methylated in *Aebp2*$^{+/\beta\text{-geo}}$ mouse ear tissue. Inter-individual differences in P1 methylation can also be observed. All analyses performed on ear tissue from 19 months old female mice.

Based on these results, it is interesting to reconsider the egg methylation data for *Aebp2* P1 (**Fig. 1.2A**). Each sequenced clone represents DNA isolated from one egg which is stacked on the x-axis, whereas the columns refer to the individual CpG position within each strand of DNA. Keeping in mind that each egg will contribute to one individual, it is intriguing to note the wide

variation in the level of methylation between eggs. While some eggs are completely methylated, some are completely unmethylated, while yet others show variable intermediate levels of methylation. *Aebp2* is an early developmental gene which is highly expressed in the egg and stem cells. If the level of P1 methylation is indeed correlated to the level of *Aebp2* expression, the completely methylated eggs would have the least expression of *Aebp2*, whereas the completely unmethylated eggs would have similar expression levels as the WT. Having low levels of AEBP2 has been shown to cause an increase in H3K27 methylation by PRC2 (Kim, *et al.*, 2011). Having minimal levels at early developmental stages could then upset epigenetic regulation of various genes at critical time points, which could result in gross malformations of the neuronal structures and result in seizures later in life. While this idea is currently in its nascent stage, it would be plausible to test it in conditional knock-out mice where *Aebp2* is completely deleted only in the neuronal cell lineages by assaying them for seizure development. By reducing or completely eliminating *Aebp2* expression at different time points and assaying the mice for seizures, we can gain important clues regarding the role of *Aebp2* in the timeline of brain development and the genes which are active and potentially affected at that stage.

<u>*Aebp2* P3 may be more methylated in *Aebp2*$^{+/\beta\text{-geo}}$ seizure mice than normal mice</u>

In order to explore the role of DNA methylation at the promoter regions better in the context of seizures in *Aebp2*$^{+/\beta\text{-geo}}$ mice, we also studied methylation at P2 and P3. *Aebp2* P2 was completely unmethylated regardless of genotype; however, we found some interesting differences between WT, *Aebp2*$^{+/\beta\text{-geo}}$ normal and *Aebp2*$^{+/\beta\text{-geo}}$ seizure mice at the P3 locus. According to our COBRA results from the same ear tissue as what was assayed for P1 methylation, P3 appears to be almost completely unmethylated in WT but much more methylated

in *Aebp2*$^{+/\beta\text{-geo}}$ mice. Interestingly, even among *Aebp2*$^{+/\beta\text{-geo}}$ mice, *Aebp2*$^{+/\beta\text{-geo}}$ seizure ear tissue showed a stronger "methylated" band than normal mice, suggesting that P3 may be more methylated in seizure mice than normal mice (**Fig. 4.3A**). We were able to confirm these COBRA results to some extent by bisulfite sequencing, however, no conclusions regarding the significance of the data could be made because of the lack of an appropriate number of clones from each genotype (**Fig. 4.3B**).

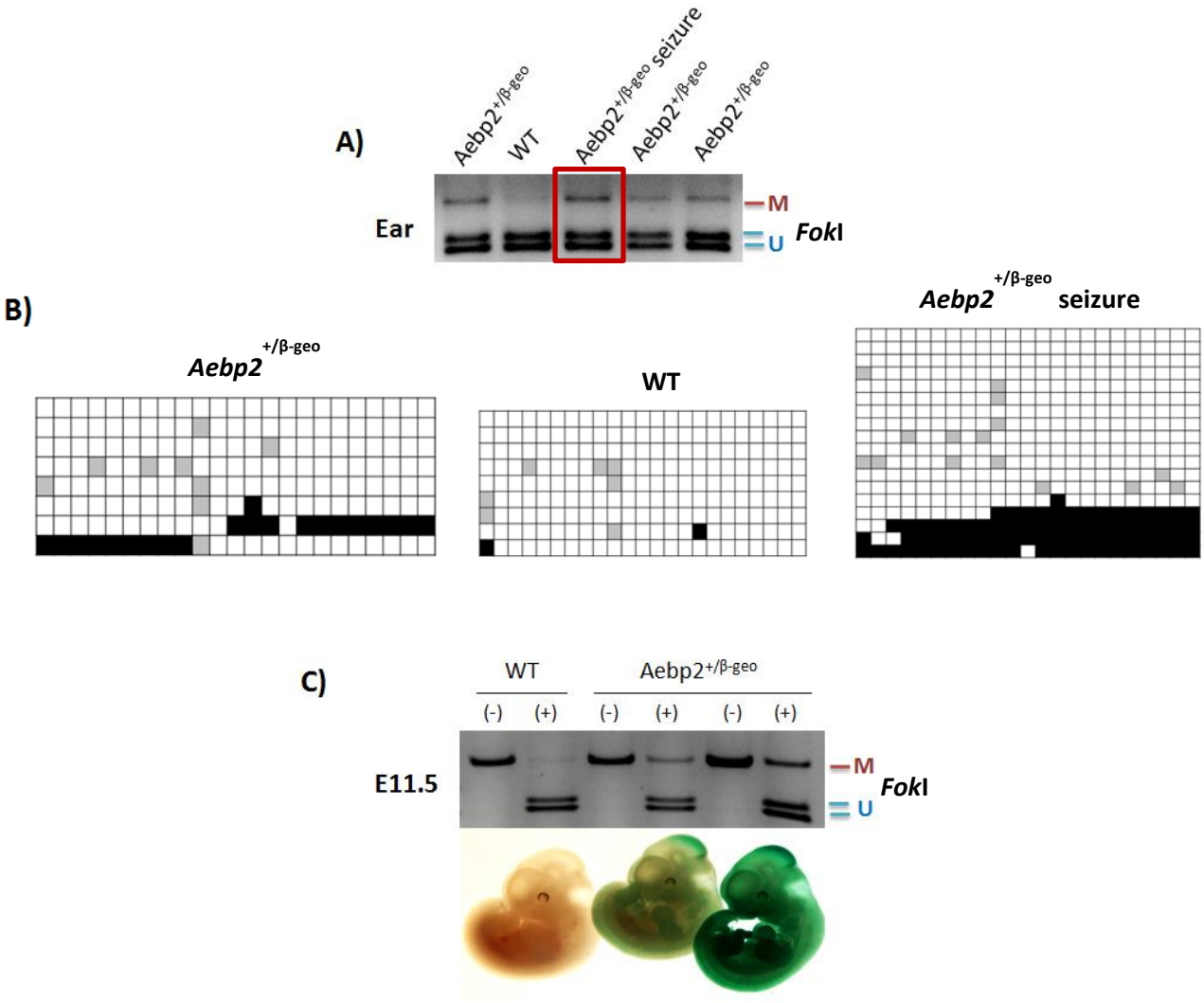

Figure 4.3. ***Aebp2*** **P3 may be more methylated in seizure mice and dark-staining embryos. (A)** COBRA and **(B)** Bisulfite sequencing on 19 month old female ear samples showing seizure samples may be more methylated than non-seizure and WT samples. **(C)** COBRA on 11.5-dpc decrosslinked embryos shown in figure 3.1A showing P3 may be more methylated in darker stained embryos.

To test the effects of P3 methylation on *Aebp2* expression in *Aebp2*$^{+/\beta\text{-geo}}$ mice *in vivo*, we decrosslinked a representative set of the 11.5-dpc embryos in **Fig. 3.1A –** WT (no staining), *Aebp2*$^{+/\beta\text{-geo}}$ (light green) and *Aebp2*$^{+/\beta\text{-geo}}$ (dark green). In keeping with the methylation data from ear, we found almost no methylation at P3 in WT tissue from the E11.5 COBRA results. In contrast, *Aebp2*$^{+/\beta\text{-geo}}$ embryos had a much more distinct "methylated" band. Further, we observed that the third embryo from the left, which stained dark green, had a stronger "methylated" band than the light green *Aebp2*$^{+/\beta\text{-geo}}$ embryo (**Fig. 4.3C**). The higher intensity staining pattern was reminiscent of the *Aebp2*$^{+/\beta\text{-geo}}$ seizure brains which also stained more strongly than normal *Aebp2*$^{+/\beta\text{-geo}}$ brains. This suggests that P3 may indeed contribute to the imprinting-like staining pattern observed in *Aebp2*$^{+/\beta\text{-geo}}$ embryos at 11.5-dpc stage, as discussed in chapter three. Moreover, methylation at this promoter could also play a role in seizure formation and/or susceptibility. Due to variable levels of methylation at P1 and P3 in *Aebp2*$^{+/\beta\text{-geo}}$ mice, there may be some interesting dynamics at play which regulate promoter competition during transcription. This could have an overall effect of the level of *Aebp2* expression and thus gene regulation that could ultimately lead to seizures. This promising hypothesis is, however, yet to be tested before any conclusive comments regarding the role of P3 in *Aebp2* expression level or seizure formation can be made. Transgenic mice with inserted gene trap vectors can often show higher levels of methylation near these inserted vectors because these vectors essentially get treated as transposable elements by the genome, which are silenced by DNA methylation. The DNA methylation can then spread to nearby regions and produce interesting phenotypes, such as, imprinting-like effects (Sapienza, *et al.*, 1987). Therefore, a conditional knock-out mouse would be essential in determining whether the methylation at P3 is an artifact of the

insertion vector or if it is an effect produced due to the downregulation of *Aebp2*, and study its role *in vivo*, especially in the context of epileptogenesis.

In conclusion, this thesis outlines the seizure phenotype displayed by mice which are haploinsufficient for *Aebp2* and focuses on the variable severity aspect of it. Finally, we propose that epigenetic mechanisms such as DNA methylation at the three *Aebp2* alternative promoters could regulate the expression level of *Aebp2*. We find differential expression of *Aebp2* among different *Aebp2*$^{+/\beta\text{-geo}}$ mice, which opens up a new avenue of research in this project, testing whether lower levels of *Aebp2* result in more severe seizures. These mice may have higher levels of methylation at their P1 and P3 loci than their normal counterparts, which could result in lower levels of *Aebp2* expression. Expression levels of both the embryonic and somatic forms may be affected due to promoter competition and transcription factor activities. We propose that the subsequent downregulation of *Aebp2* could result in major aberrations in global epigenetic regulation through PRC2, which would impair the proper establishment of neuronal networks and may eventually lead to seizures.

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APPENDIX

LETTERS OF PERMISSION

January 28, 2013

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Colin Dixon
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29 January 2013

VITA

Arundhati “Runa” Bakshi was born to Mr. Amitava Bakshi and Mrs. Alakananda Bakshi in Jamshedpur, India. She attended Sacred Heart Convent School in Jamshedpur from kindergarten until tenth grade, and graduated with the Indian Certificate of Secondary Education in 2007. For her final two years of high school, she attended Sinhgad College of Arts, Science and Commerce in Pune, India. She graduated twelfth grade with the Maharashtra Higher Secondary School Certificate in 2009. After high school, she attended Louisiana State University, Baton Rouge, where she pursued a Biological Sciences major and Chemistry minor through the College of Science in association with the Honors College. There she engaged in her Honors Thesis under the mentorship of Dr. Joomyeong Kim. She will graduate a summa cum laude university medalist with a B.S. (Hons.) in Biological Sciences in May 2013. Her future plans involve attending graduate school and obtaining her Ph.D. before embarking on an academic career.