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Division of Human Genetics

School of Pathology



*The Effect of Alcohol on the Methylation Status of the
Imprinting Control Regions Contained within Three
Developmentally Significant Loci*

Jaysen Knezovich

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Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree in
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Declaration

I, Jaysen Knezovich, hereby declare this dissertation to be my own work. It is being submitted for the degree of Master of Science (Medicine) at the University of the Witwatersrand, Johannesburg. It has not been previously submitted for any degree or examination at this, or any other university.

Signed this 6th day of the month of November, 2009.

Dedication

To loving and supportive friends and family.

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Abstract

Imprinted loci are critical in foetal development and most are regulated by the methylation-specific CTCF binding protein which binds imprinting control regions (ICRs). The ICR is located between two genes that comprise imprinted loci, which are reciprocally expressed in a parent-of-origin specific manner. Maternally hypomethylated ICRs allow CTCF binding, creating a boundary element which prevents downstream enhancers from acting on the paternally expressed gene upstream of the ICR. Conversely, the hypermethylated (imprinted) paternal ICR prevents CTCF binding, allowing downstream enhancers to act on the gene upstream of the ICR, while suppressing the downstream maternally expressed gene. Alcohol and its metabolites are able to reach the testes via the blood supply and are known to reduce global DNA methylation by disrupting the folate, methyl group and homocysteine pathway. This may therefore affect gene expression at imprinted loci, whose parental alleles are discriminated by the imprinting status at the ICR.

The effect of pre-conception paternal alcohol exposure on the DNA methylation of three paternally imprinted ICRs (*H19*, *Rasgrf1*, *IG-DMR*) as well as the maternally imprinted *Snrpn* ICR was examined in mouse sperm and their offspring. Male mice were gavaged with ethanol or sucrose. DNA was extracted from sperm of treated males and tail biopsies from offspring. Samples were bisulphite modified and the ICRs PCR amplified. DNA methylation patterns of ICRs were analysed by sequencing and quantitatively via pyrosequencing. Sperm samples of ethanol treated males did not show significant demethylation when compared to sucrose treated mice, with the exception of *H19* CpG 7, *Rasgrf1* CpG 26 and *Snrpn* CpG 10 ($p=0.024$, 0.014 and

0.002, respectively). However, a significant reduction in DNA methylation at the *H19* CTCF 1 ($p=0.0017$) and CTCF 2 ($p=0.0007$) binding sites in the offspring of ethanol treated sires was observed. A lower growth rate trend was observed in female and male offspring sired by ethanol treated males. At days 35 and 42 ($p<0.05$), offspring sired by ethanol treated males had a significantly lower weight than controls. These data suggest that excessive paternal exposure to ethanol prior to conception has the ability to negatively influence the epigenetic state of developmentally significant imprinted genes in offspring. Decreased DNA methylation profiles of the *H19* CTCF1 and CTCF 2 regions in the offspring of ethanol treated sires were significantly correlated with reduced postnatal growth at days 35 and 42 ($p<0.05$). Furthermore, the effect of treatment (ethanol) on growth (retardation) was shown to be mediated to a limited extent through (a decrease in) DNA methylation in offspring.

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Glossary of Terms

µg	Microgram
µl	Microlitre
A	Adenosine
AS	Angelman syndrome
BWS	Beckwith-Weidemann syndrome
C	Cytosine
cfu	Colony forming units
cm	Centimetre
CO ₂	Carbon dioxide
CTCF	CCCTC-binding factor
ddH ₂ O	Deionised distilled water
dH ₂ O	Distilled water
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferase protein
DTT	Dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i> bacterium
ESC	Embryonic stem cell
EtOH	Ethanol
FAS	Foetal alcohol syndrome
FASD	Foetal alcohol spectrum disorders
g	Gram

G	Guanine
GSH	Glutathione
HAT	Histone acetyltransferase
HDAC	Histone deacetylase protein
HMTases	Histone methyltransferases
ICF	Immunodeficiency, centromeric instability and facial anomaly
ICR	Imprinting control region
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IUPAC	International Union of Pure and Applied Chemistry
K	Lysine residue
L	Litre
LA	Luria agar
LB	Luria broth
MAT	Methionine adenosyltransferase
MBD	Methyl-CpG binding domain protein
MeCP	Methyl-CpG-binding protein
mg	Milligram
min	Minutes
ml	Millilitre
mm	Millimeter
MS	Methionine synthase
ng	Nanogram
°C	Degrees Celsius
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PGC	Primordial germ cell
PWS	Prader-Willi syndrome
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
s	Seconds
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulphate
SRS	Silver-Russell syndrome
T	Thymine
Ta°C	Annealing temperature
<i>Taq</i> pol	<i>Thermus aquaticus</i> polymerase
U	Uracil
Xce	X-chromosome controlling element
XCI	X-chromosome inactivation
xg	Gravitational force
X-gal	5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside
XIC	X-inactivation centre
<i>Xist</i>	<i>X-inactive specific transcript</i> mouse gene

Chapter 1

1. Introduction

1.1. In High Spirits – Foetal Alcohol Spectrum Disorder

1.1.1. In the beginning

The angel of the Lord suddenly appeared to Manoah's childless wife and proclaimed, "Behold now, thou art barren, and barest not: but thou shalt conceive, and bear a son. Now therefore beware, I pray thee, and drink not wine nor strong drink..." (Judges, 13: 3 – 4). This passage taken from Judges tells the story of the conception of the legendary hero, Samson, and accounts for one of the earliest Biblical references to the connection between alcohol consumption and adverse effects on foetal outcome (Abel, 1997). This connection has also been traced as far back as the Greek philosopher, Aristotle, who wrote "Foolish, drunken, and harebrained women, most often bring forth children like unto themselves, morose and languid" (Abel, 1990). In fact, these are just two of many early references that indicate that although historically the connection between prenatal alcohol consumption and subsequent developmental abnormalities was not completely understood, the link between the two was indeed noted.

Recently, a closer inspection was taken by the medical profession into the effects of alcohol exposure to unborn children. During the early 18th century the price of gin dropped dramatically in England, resulting in levels of consumption soaring to 11 million gallons in 1750 (Abel and Bilitzke, 1990). The gin epidemic was rife and concerns mounted regarding the negative impacts of such intake on exposed fetuses (Friedler, 1996). This prompted the College of Physicians to draft a letter to parliament stating that “the fatal effects of the frequent use of several sorts of distilled spirituous liquors upon great numbers of both sexes rendering them diseased, not fit for business, poor, a burthen to themselves and neighbours and too often the cause of weak, feeble, and distempered children” (Abel, 1990).

Recognition of the adverse effects of alcohol on offspring development began to peak in the mid 1900s, with a number of publications describing some characteristics of children born of alcoholics (Lemoine et al., 1968). However, it was not until 1973 that the full spectrum of developmental defects associated with *in utero* alcohol exposure was explored in individuals who were severely affected by maternal alcohol abuse, and the term “foetal alcohol syndrome” (FAS) was coined (Jones et al., 1973). These findings by Jones and Smith were important in emphasizing the teratogenic properties of alcohol, and provoked much investigation into its effect on the developing human body (Sulik et al., 1981).

1.1.2. Foetal alcohol syndrome

Prolonged levels of maternal alcohol exposure during pregnancy have been implicated in a range of unfavourable foetal outcomes, collectively known as Foetal Alcohol Spectrum Disorder or FASD (Burd et al., 2003), with the most severe of these outcomes being Foetal Alcohol Syndrome (FAS). FAS can manifest variably resulting in diverse combinations of craniofacial, growth and neurobehavioral abnormalities associated with a multitude of symptoms and psycho-social problems. These include attention deficit-hyperactivity disorder (ADHD), learning disabilities, behavioural disorders (Burd et al., 2003), facial dysmorphia, reduced body growth and central nervous system abnormalities, of which mental retardation is the most prevalent (O'Leary, 2004). The quintessential characteristics of FAS which all diagnoses are based upon are prenatal and postnatal growth retardation, mental disability and the distinct craniofacial abnormalities, which include a thin upper lip, smooth filtrum and a flat nasal bridge.

The Centre for Disease Control has reported prevalence rates of 0.2 to 1.5 cases per 1000 live births in the United States (Cordero et al., 2004). Roughly 15% of American women of childbearing age are regarded as moderate to heavy drinkers, and nearly 2% to 3% are at risk of dangerously exposing their unborn child to the adverse effects of alcohol (Cordero et al., 2004). While an average FAS rate of 0.97 per 1000 children has been reported globally, a staggering rate of 65.2 - 89.2 per 1000 children – the highest

world-wide – has been reported in the Western Cape, South Africa (May et al., 2000; Viljoen et al., 2005; May et al., 2007). This statistic emphasizes the importance, if not the necessity of foetal alcohol related research, especially in South Africa.

Although FAS research has been primarily focused on maternal drinking during pregnancy, it is surprising to note that there is substantial and compelling evidence of a paternal contribution. As early as 1621, Gellius in the *Anatomy of Melancholy* noted that “if a drunken man get a child, it will never likely have a good brain” (Friedler, 1996), that is, it was observed that children of alcoholic men had “weak” brains. It is of significant interest to note that even before the potential adverse effects of excessive paternal alcohol consumption were formally acknowledged, in 1911, Forel cites an account by Schweighofer where a woman marries a man, who both do not abuse alcohol, and have three children of good health. The man dies and the woman re-marries a drunkard. She bears more three children: one became a drunkard; another had “infantilism”; and the third was a social degenerate and a drunk. Two of these children also had tuberculosis, which hadn’t been seen in the family. The woman then marries a third time to a sober man, and produces children of good health once again.

Research on the influence of alcohol on mammalian offspring began in 1888, where Mairet and Cambemale (Stockard, 1913) treated a dog with absinthe, which was mated with a normal bitch. She gave birth to twelve pups – two

were still-born, three died after 14 days, and the rest died later due to intestinal complications and tuberculosis. In 1916 Charles Stockard, later joined by George Papanicolaou, made some fascinating observations about the transmission of deformities in descendents of alcoholised male guinea pigs (Stockard and Papanicolaou, 1916a). For over seven years their research tracked the development of offspring sired by males exposed to alcohol fumes through successive generations. They noted that the effect of alcohol on the male was of such a nature that an exposed male almost invariably “begets defective offspring even when mated with a vigorous normal female” (Stockard and Papanicolaou, 1916a). These defects primarily involved the central nervous system and special sense organs, including opaque lenses and cornea of the eye, and in some cases even complete absence of eyes. Other abnormalities included low birth weights and limb deformities. Subsequently, experimentation and reports in the 1970s exposed the adverse effects of not only maternal, but paternal alcohol consumption on their offspring (Sulik et al., 1981).

The potential contribution of the father to FASD aetiology has remained of interest to researchers and is receiving increased attention. Lemoine et al. (1968) reported the first case of infants born with characteristic FASD symptoms to mothers who had not consumed alcohol during pregnancy, but whose fathers were alcoholics (Lemoine et al., 1968). It was later noted that 75% of children with FAS had biological fathers who were heavy drinkers or alcoholics (Abel et al., 1983). This evidence opened the prospect that a

number of FAS cases attributed to maternal alcohol abuse during pregnancy may have in fact been a result or partly due to excessive paternal drinking (Abel, 2004). The possibility of paternal preconception alcohol abuse on foetal development prompted further investigation, and studies using rat models found evidence that paternal exposure to alcohol had significant effects on the neurobehaviour of subsequent offspring (Jamerson et al., 2004). Others have reported congenital malformations, low birth weights, growth retardation and neonatal mortality associated with preconception paternal alcohol intake (Friedler, 1996), providing convincing evidence for the potential for paternally mediated foetal alcohol effects.

1.2. Paternally conceivable

The link between paternal consumption of alcohol and subsequent alcohol-related phenotypic effects in offspring is supported in the literature, but how can one provide a plausible explanation for these effects? After all, the alcohol consumed during paternal drinking cannot come into direct contact with a developing foetus, as is credible in the case of maternal alcohol intake during pregnancy. In order to begin to grasp the concept of paternal alcohol effects on sired offspring, we need to focus on the very essence of foetal alcohol syndrome... the alcohol itself.

1.2.1. The biological effects of alcohol

Alcohol is a well known teratogen and able to cause developmental abnormalities in the foetus (Randall, 1987; Randall et al., 1994; Boehm et al., 1997; Ogawa et al., 2005; Camarillo and Miranda, 2008). These teratogenic effects have been studied in both humans and in animal models. As described previously, researchers have shown that foetal exposure to excessive levels of alcohol can result in a wide variety of neurobehavioural deficits, primary of which include central nervous system dysfunction, growth deficiencies and distinct facial anomalies, with varying degrees of severity. But mechanistically, how is alcohol able to induce these effects?

Many mechanisms have been proposed in an attempt to explain the teratogenic effects of alcohol on the developing foetus. In addition to the fact that alcohol itself has the ability to act as a direct cytotoxic agent (De Vito et al., 2000), the metabolic products that arise from its subsequent breakdown in the body play a significant role in alcohol's deleterious effects (Poggi et al., 2003; Fernandez-Checa, 2003). This therefore highlights the fact that there is no single mechanism that accounts for the range and extent of phenotypes evident in children born with foetal alcohol effects as a result of being exposed to significantly high levels of alcohol. As the focus of this research is on paternal effects of excessive alcohol intake, the direct effects of this teratogen on the developing foetus will not be discussed further, but the emphasis will be on the metabolic aspects of alcohol and its potential effect on male

gametogenesis.

1.2.2. The Effects of Alcohol Metabolism

Recent reviews (Halsted et al., 2002b; Schalinske and Nieman, 2005) have shown that the metabolism of alcohol and the interplay of its metabolites on the folate, methionine and homocysteine pathways. The metabolism of these three compounds is intricately connected and is vital for the maintenance of optimal health (Wagner, 1995). It has been shown that folate deficiency is a common clinical sign of chronic alcohol abuse and has been implicated in the development of alcoholism-related complications, such as alcoholic liver disease (Eichner et al., 1971). Researchers have also found that disruption of this pathway has been associated with various disorders including, among others, birth defects and neurological abnormalities (Boyles et al., 2006).

To understand the biological importance and therefore pathologies associated with disrupted folate, methionine and homocysteine metabolism, a closer inspection of these processes needs to be considered. As a coenzyme, folate is required to supply methyl groups for S-adenosylmethionine (SAM)-dependent transmethylation reactions. In the folate-dependent pathway, the B₁₂-dependent enzyme methionine synthase (MS) is responsible for transferring the methyl group contained within the 5-methyl-tetrahydrofolate compound to homocysteine (Figure 1.1), which ultimately generates methionine (Friso et al., 2002). This is followed by the conversion of methionine to SAM by methionine

adenosyltransferase (MAT), which is used for the methylation of DNA. As an essential amino acid, methionine produced via both folate-dependent and folate-independent pathways, provides the necessary methyl groups for biological DNA methylation.

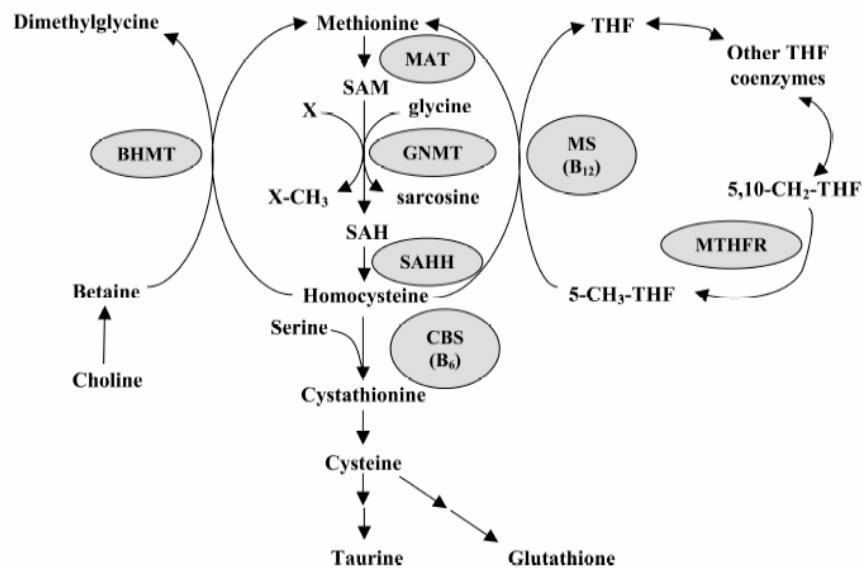


Figure 1.1: Folate, methyl group and homocysteine metabolism. BHMT = betaine homocysteine S-methyltransferase; CBS = cystathionine β -synthase; GNMT = glycine N-methyltransferase; MAT = methionine adenosyltransferase; MS = methionine synthase; MTHFR = 5,10-methylenetetrahydrofolate reductase; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; SAHH = SAH hydrolase; THF = tetrahydrofolate (Schalinske and Nieman, 2005).

As early as 1974, research on alcohol-fed rats described reduced MS activity and subsequent reduction of the by-products methionine and SAM (Finkelstein, 1974; Barak et al., 1987; Trimble et al., 1993). It has also been shown that ethanol appears to enhance the catabolism and loss of methyl groups, which in turn disrupts subsequent SAM-dependent transmethylation reactions (Schalinske and Nieman, 2005). Cancer related research has demonstrated that perturbations to these reactions are known to result in hypomethylation of hepatic DNA (Wilson et al., 1984; Bhawe et al., 1988; Wainfan et al., 1989; Pogribny et al., 2006)

These findings suggest that alcohol has a direct negative effect on the folate pathway, but how does it exert this effect? The answer lies in the liver.

1.2.3. Destructive Free Radicals

Folate and methionine metabolism occur in the liver and coincidentally, so does that of alcohol. Although the means by which alcohol is able to alter SAM levels, is not yet clear, several pathways have been proposed. According to the hypothesis by Trimble and colleagues (Trimble et al., 1993), alcohol metabolism results in a catabolic pathway whereby homocysteine is degraded in order to replenish glutathione levels. They suggest that the oxidation of alcohol to acetaldehyde is accompanied by the release of free radicals, which stimulates the cell to redirect homocysteine to a catabolic pathway to replenish glutathione, thereby diminishing SAM (Figure 1.1 and Figure 1.2).

Alternatively, it has been suggested that the increase in reactive-oxygen species (ROS) produced by the oxidation of acetaldehyde to acetate leads to the production of superoxide. The superoxide free radical is then able to interact with carbohydrates, proteins, lipids, and nucleic acids, which in turn can lead to damage of biomacromolecules involved in the folate pathway (Goodlett et al., 2005). Despite the controversies around the exact mechanism, it is unanimously agreed that it is the by-products of alcohol metabolism that result in the adverse effects on methionine production, whereby the concurrent events of both folate and alcohol metabolism within the liver allow for the action of free radicals to exert a destructive effect on the folate pathway.

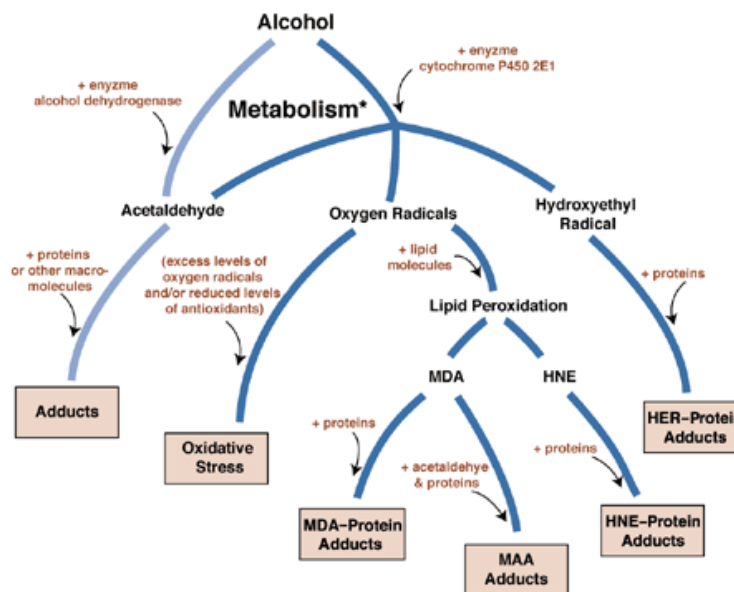


Figure 1.2: Alcohol metabolism. The metabolism of alcohol results in the production of acetaldehyde, oxygen (free) radicals and hydroxyethyl radicals (www.niaaa.nih.gov).

1.2.4. The Free Radical Theory of Development

The free radical theory of development was first conceptualized between 1985 – 1986 (Sohal and Allen, 1985; Sohal et al., 1986a; Sohal et al., 1986b), and hypothesized a role of oxygen free radicals in cellular differentiation. Subsequently, in 1989 Allen and Balin developed this hypothesis further and formalized the free radical theory of development (Allen and Balin, 1989). The central premise of this theory states that “changes in oxygen gradients and an alteration in the balance between the formation and the removal of metabolically generated oxidants can elicit developmental events” which form complex tissues and organ systems (Hitchler and Domann, 2007). They went on to suggest that altered gene expression during development can be mediated through oxygen gradients and an altered redox state in cells.

In 2007, Hitchler and Domann provided insight into how the redox status of a cell could possibly influence development by affecting the production of SAM, the cofactor responsible for DNA (and histone) methylation reactions. Central to the free radical theory of development is that in order for cells to differentiate, the redox potential of a cell must change from a reducing to an oxidizing state (Hitchler and Domann, 2007). Glutathione (GSH) is a naturally occurring antioxidant found in plants and animals and serves to protect cells against harmful oxidizing agents, and thereby ensures the proper functioning of various proteins. Thus, if overproduced, GSH is able to inhibit the conversion of an oxidative redox state to a reduced state during cellular

differentiation and thereby impeding development.

In mammals, elevated levels of GSH production are required during critical points in development. These include periods during gametogenesis, after fertilization and the interval between fertilization and blastogenesis (Hitchler and Domann, 2007). These increased levels of GSH are required to protect the developing embryo from oxidative stress. However, the metabolic cycles of GSH and SAM are connected (Figure 1.1). Homocysteine is commonly recycled in the methionine pathway where it is converted back into methionine via MS. Thus, when increased GSH is required during times of oxidative stress or during development, homocysteine's entry into the transsulfuration pathway is favoured, producing GSH (Hitchler and Domann, 2007). This in turn would dramatically decrease the level of SAM, and as SAM is pivotal in transmethylation reactions, genome-wide DNA hypomethylation occurs (Lertratanangkoon et al., 1996; Lertratanangkoon et al., 1997a; Lertratanangkoon et al., 1997b).

1.2.5. An integrated model of alcohol metabolism and the free radical theory of development

As described previously, alcohol is a well studied teratogen, and one of the means by which it can exert these effects is via its metabolism in the body. These metabolites have been shown to directly interfere with the folate pathway, which is essential for providing the methionine required for

transmethylation reactions. The means by which alcohol metabolism achieves this is through the release of free radicals. This idea has been supported by the free radical theory of development whereby the folate/methionine pathway is redirected to produce GSH instead of SAM (the methionine group donor) in order to counteract the oxidative stress brought on by free radicals. This therefore significantly reduces the methionine available for methylation, causing wide-spread DNA hypomethylation.

This has been supported by Garro et al. (1991) who highlighted the concept of the indirect effect of alcohol on a developing organism through disruption of the cellular environment. They found that maternal ingestion of alcohol during pregnancy inhibited foetal DNA methylation in mice. Since DNA methylation plays an important role in the regulation of gene expression during embryogenesis, ethanol-associated alterations in fetal DNA methylation may contribute to the developmental abnormalities seen in FAS. Thus, when considering the potential effects of excessive paternal alcohol consumption on offspring outcome, it is clear that paternal ingestion of alcohol prior to conception cannot exert a direct effect on sired progeny. Instead, the indirect action of alcohol's metabolites on vital transmethylation pathways that guide DNA methylation must be investigated.

1.3. The slippery slope of development

If every cell in our body contains the same genetic information, then why

don't we grow hair on our eyes and have brains at our finger-tips? The answer lies in a precise orchestration of gene regulation that is tissue specific in both a spatial and temporal manner. But how is this achieved? If every cell is genetically identical, what is controlling the expression of their genes? The most important mediators that turn genes "on" or "off" involve sequence-specific transcription factors. However, chromatin based regulatory mechanisms, in addition to transcription factors, have been implicated as vital components in establishing and maintaining transcriptional systems (Mohn and Schubeler, 2009). By implication, these programs must therefore be functioning independently of the DNA sequence itself. This idea of genetic control via external forces was first coined by Conrad Waddington in 1938 as "epigenetics" – "the science concerned with the causal analysis of development" (Waddington, 1952). His idea was conceptualized in the form of a "genetic landscape" (Figure 1.3), whereby his theory of development follows the passage of a fertilized egg or cell down a "landscape". The unique path that each cell follows through the landscapes canals will ultimately determine its developmental state – a process known as "canalization".

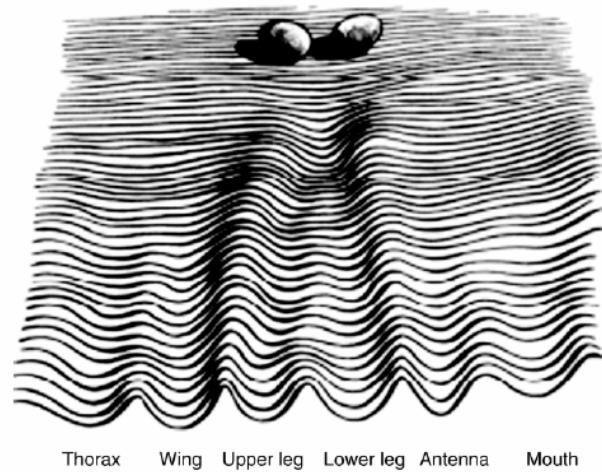


Figure 1.3: Conrad Waddington's depiction of an epigenetic landscape. The developmental possibilities of the genotype follow the passage of a fertilized egg or cell down a landscape. The unique path that each cell follows through the landscapes canals will ultimately determine its developmental state (Waddington, 1956).

The term "epigenetics" has subsequently been redefined by Robin Holliday as the "unfolding of the genetic program for development" (Holliday, 2006), and more recently by Adrian Bird as the "structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird, 2007).

1.3.1. DNA methylation as a form of epigenetics

"Epigenetics" is an umbrella term that encompasses all of the factors independent of the DNA sequence itself which are responsible for the regulation of gene expression. There are two main epigenetic mechanisms by

which the tissue-specific gene expression patterns are established and maintained. These include chromatin modification and chromatin remodeling. Chromatin modification and chromatin remodeling is achieved through the interaction of various proteins to establish epigenetic marks that define active or inactive chromatin conformations. Such epigenetic marks include DNA methylation and histone modifications.

As early as 1975, two independent research groups put forward the suggestion that DNA methylation was responsible for the stable expression of genes throughout subsequent meiotic cell divisions (Riggs, 1975; Holliday and Pugh, 1975). Genetic regulation through DNA methylation occurs via a number of distinct mechanisms. DNA methylation is able to directly prevent transcription regulatory factors from binding to their target sequences, and so inhibiting transcription. Secondly, in some instances, instead of being repulsive, DNA methylation promotes the binding of methyl-CpG binding domain proteins (MBDs) and the methyl-CpG-binding protein MeCP2 (Jaenisch and Bird, 2003). Together, these methyl-binding proteins function as transcriptional silencers. The final mechanism through which DNA methylation is able to regulate gene activity is by altering the chromatin structure itself.

Eukaryotic genomes are organized into chromatin, a DNA-protein complex that packages the DNA into a highly compact form. 146 base pairs of DNA are wrapped around an octamer of histone proteins to form a nucleosome. The octamer core consists of two copies of each of the histones, H2A, H2B, H3

and H4. Chromatin may consist of various combinations of core histone variants, or histones that have undergone post-translational modifications, which results in diverse regulation of associated genetic material. These modifications include histone acetylation, methylation and phosphorylation.

Histone acetyltransferases (HATs) are enzymes that are able to acetylate the N-terminal tails of the core histones. This chemical modification leads to the neutralization of positively charged histones, which promotes an open chromatin conformation, and thereby transcriptional activation (Kuo and Allis, 1998). Histone terminal tails are also targets of phosphorylation. In particular, phosphorylation of serine 10 in the H3 histone has been associated with gene activation in mammalian cells. Although the mechanism of phosphorylation is not fully understood, HAT activity appears to be linked with phosphorylation. It has been noted that acetyltransferases have increased HAT activity on serine 10 phosphorylated substrates (Lo et al., 2000). This suggests that phosphorylation may contribute to transcriptional activation by stimulation of HAT activity on the same histone tail (Grant, 2001).

Conversely, the methyl-CpG-binding protein MeCP2 that DNA methylation is able to recruit, has been shown to associate with histone deacetylase proteins (HDACs). These HDACs deacetylate histone residues associated with the nucleosome, which forces the chromatin into a closed conformation, inducing transcriptional repression (Heinzel et al., 1997). This interaction with HDACs has also been postulated to occur with DNA methyltransferases (DNMTs) to

form transcriptionally repressive states (Nowak-Imialek et al., 2008).

1.3.2. DNMTs establish DNA methylation

Methylation occurs primarily at CG dinucleotide sequences (CpG islands), where approximately 60% – 90% of the cytosines in these islands are modified in mammals, accounting for about 3% - 8% of the total number of cytosine residues (Jeltsch, 2002). CpG islands cluster in promoter and first exon regions of many genes, where the advent of cytosine methylation effectively silences gene expression. Abnormal methylation patterns within regions of regulatory importance may therefore result in the irregular expression of their associated genes (Jones and Takai, 2001).

DNMTs are the only known enzymes that catalyze the methylation of cytosine nucleotides in mammals and are critical modulators of foetal development (Li et al., 1992). DNMTs make use of the methionine produced via the folate pathway for DNA methylation reactions, which involve the addition of a methyl group to the 5' carbon of a cytosine nucleotide (Rodenhiser and Mann, 2006).

Various publications have reported that Dnmts are essential for embryonic development in mice (Lee et al., 2001; Jeltsch, 2002; Robertson, 2005). Li et al. (1992) have shown that inhibiting DNA methyltransferase gene expression causes increased embryonic lethality in mice, while reduced levels of these

enzymes result in abnormal embryonic phenotypes associated with growth and neural defects (Stoger et al., 1997). Okano et al. (1999) provided supporting evidence for the vital role of Dnmt3a and Dnmt3b for *de novo* methylation in embryonic development. In their studies, they generated Dnmt3-deficient mice from multiple embryonic stem cell lines heterozygous for either *Dnmt3a* or *Dnmt3b* mutations. *Dnmt3a*^{-/-} mice developed to term, but later became runted and died at about four weeks of age, while *Dnmt3b*^{-/-} embryos showed multiple developmental abnormalities including growth impairments and neural tube defects (Okano et al., 1999). Thus, through the action of DNMTs, DNA methylation provides a mechanism for maintaining a specific state of gene expression and genome stability during development (Stoger et al., 1997; Robertson, 2005).

To date there have been three active DNMTs classified in mammals, namely, Dnmt1, Dnmt3a and Dnmt3b. Dnmt2 has been identified as a candidate protein, but has not been shown to be catalytically active (Jeltsch, 2002). Similarly, Dnmt3L has homology to the other members of the Dnmt3 family but is missing crucial residues in catalytic motifs, and has not been shown to possess enzymatic activity in vitro (Bourc'his et al., 2001). Dnmt3a and Dnmt3b contain a domain called PWWP that occurs in proteins known to play a role in cell growth and differentiation (Jeltsch, 2002). These two DNMTs have been highlighted as the *de novo* methyltransferases, and are therefore vital components for establishing methylation patterns. Dnmt3L interacts with Dnmt3a and Dnmt3b, co-localising these enzymes in cellular nuclei enabling

effective establishment of genomic imprints (Hata et al., 2002). *Dnmt3L* is highly expressed during gametogenesis and contributes to the establishment of paternally imprinted regions during spermatogenesis (Webster et al., 2005). *Dnmt1* has been implicated in the maintenance of DNA methylation during DNA replication ensuring the propagation of specific methylation patterns after each round of cell division, including those of imprinted regions (Leonhardt et al., 1992).

1.3.3. Imprinting

In the early 1980's, a puzzling question shrouded the field of developmental biology – why didn't parthenogenetic embryos develop to term? By activating an ovulated oocyte and repressing either the second meiotic or first mitotic division, researchers were successfully able to restore the diploid state of the oocyte with a full set of chromosomes (Solter, 2006). However, these maternally diploid "parthenogenetic" embryos failed to develop, despite having a normal number of chromosomes. One hypothesis to explain this phenomenon suggested that the "sperm makes an essential physical (non-genetic) or physiological contribution without which development cannot be completed" (Solter, 2006).

In 1984 McGrath and Solter made use of fertilized zygotes to construct zygotes that contained either two male (androgenones) or two female (gynogenones) pronuclei through pronucleus exchange. This experiment

satisfied any possible non-chromosome contributions from the sperm and it was therefore expected that both androgenones and gynogenones would develop normally. Surprisingly, this was not the case. In both instances, development failed soon after implantation – the androgenones showed poor embryo proper development, while the gynogenones displayed poor development of the extraembryonic membranes, as was the case with the parthenogenones (McGrath and Solter, 1984). This group therefore proposed that the maternal and paternal genomes are functionally different from one another and that both are essential for development. Furthermore, they suggested that specific genes are inherited in such a way that one form is functional, while the other non-functional, and that this is conditioned during gametogenesis. Surani and colleagues (Surani et al., 1984) called this “imprinting”, and this has remained as the term used to refer to the process whereby the two parental alleles are rendered functionally different.

1.3.4. Evolution of imprinting

To gain insight into the evolution of imprinting, both the molecular events that gave rise to imprinted alleles and the selective advantages conferred by this advent need to be considered (Wood and Oakey, 2006). A number of theories have been put forward in an attempt to explain the phenomenon of genomic imprinting. The most popular of these are the ovarian time bomb hypothesis and the conflict hypothesis.

The ovarian time bomb hypothesis states that imprinting arose as an attempt to prevent the development of unfertilized oocytes – a process known as parthenogenesis. The importance of imprinting according to this theory was demonstrated by Kono et al. (2004) who produced viable mouse offspring that were borne through the parthenogenesis of oocytes from mice carrying a deletion of the *Igf2/H19* imprinting control region (Kono et al., 2004). As parthenogenones often display abnormalities, such as malignant trophoblastic disease, imprinted genes that prevent parthenogenesis have obvious advantages in mammalian development, and have thereby been selected for.

The most prominent theory that explains the evolution of genomic imprinting is the kinship theory, more commonly known as the conflict hypothesis (Haig and Graham, 1991). This hypothesis states that there is a “conflict” between the contributions of each parental allele to the development of offspring. That is, the paternal allele favours offspring development at the expense of maternal resources; while the maternal allele serves to maximize the reproductive potential of the mother’s reproductive lifespan by conserving her resources. Evidence for this theory is strongly supported by mouse knockout models, which show dramatic over-growth and restricted-growth phenotypes (Reik et al., 2003; Ono et al., 2006; Tycko, 2006; Smith et al., 2006).

Imprinted genes play a significant role in the development of the placenta (Coan et al., 2005), which suggests that the acquisition of imprinting may have been vital for its evolution (Kaneko-Ishino et al., 2003; Reik and Lewis,

2005). It has been speculated that the earliest form of mammalian placentation evolved over 150 million years ago at the time when early placental mammals diverged from the egg-laying monotremes, and coincided with the first genes that acquired imprinting (Killian et al., 2001).

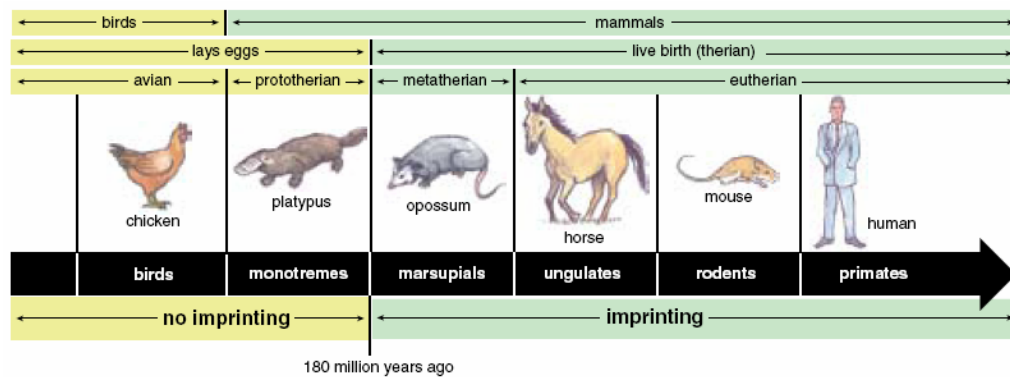


Figure 1.4: Evolution of genomic imprinting. Genomic imprinting and the practice of live birth coevolved in primitive mammals approximately 180 million years ago. The most ancient of mammals is the platypus, an egg-laying monotreme that does not exhibit genomic imprinting. The first example of genomic imprinting occurred in an extinct relative of marsupials and eutherian (placental) mammals (Jirtle and Skinner, 2007).

Imprinting is the mechanism by which X-chromosome inactivation (XCI) is achieved in the placenta and embryo of female (XX) marsupials. As marsupials are the most primitive of the eutherian mammals, it has been proposed that imprinting was the ancestral mechanism by which early eutherians achieve sex chromosome dosage compensation (Figure 1.4). The evolutionary acquisition of random XCI in the embryonic tissues of eutherian mammals was accompanied by mechanistic changes in the maintenance of monoallelic silencing, which appeared after the divergence from marsupials (Wood and Oakey, 2006).

1.3.5. Dosage compensation

The sex of many organisms is determined by a difference in their sex chromosomes. This heterogametic sex determining system, however, poses a problem. A difference in genetic material translates into dosage differences between sexes. Thus, a dosage compensation system is required in such organisms to eliminate these dosage discrepancies, and thereby avoiding any possible phenotypic manifestations associated with disparities of genetic information between sexes (Hendrich and Willard, 1995).

In mammals, XCI is the mechanism responsible for compensating for X chromosome differences between males (XY) and females (XX). XCI is achieved by transcriptionally inactivating one of the two X chromosomes present in a normal mammalian female during early embryogenesis. Two X-linked loci are known to influence XCI. Firstly, the X inactivation centre (XIC) is a *cis* acting regulatory element that is critical for X inactivation, and secondly, the X chromosome controlling element (Xce) ensures that XCI occurs randomly. Within the XIC, the *X-inactive specific transcript* or *Xist* gene is known to code for an untranslated RNA, which has been implicated in the control of XCI (Hendrich and Willard, 1995). At the start of XCI, *Xist* transcription is up-regulated and coats the future inactive X chromosome. This process converts the active, euchromatic X chromosome into a transcriptionally silent and highly condensed heterochromatic state, known as the Barr body. However, *Xist* RNA is not essential for the maintenance of the

inactive X chromosome. The stable repressive state of this chromosome is achieved through DNA methylation and histone deacetylation in somatic cells (Li, 2002).

The untranslated RNA transcribed from the *Xist* locus that is required for X chromosome silencing, and thereby dosage compensation of X-linked genes, is similar amongst epigenetic systems involving imprinted loci, such as *H19*. The notion has been put forward that both XCI and autosomal imprinting evolved out of the necessity to regulate the expression of growth-related genes in early eutherian mammals (Wood and Oakey, 2006).

Autosomal dosage compensation in eutherian mammals is achieved through genomic imprinting. Imprints are established during gametogenesis (discussed in detail later) in a parent-of-origin specific manner, effectively silencing the imprinted (hypermethylated) allele. As in XCI, autosomal genomic imprinting thereby essentially renders imprinted genes functionally hemizygous and dosage compensation is attained.

1.3.6. Epigenetic Reprogramming

Upon fertilisation of an egg, the early embryo has the ability to differentiate into all the tissues required for embryonic development. At the point of fertilisation, the parental genomes are in different stages of the cell cycle and

possess very different epigenetic marks and chromatin organisation. A process known as epigenetic reprogramming removes established epigenetic marks present in the sperm and the oocyte genomes without altering the DNA sequence itself, returning the early zygote to an unprogrammed, pluripotent state. Subsequently, this process resets these marks which establish specific gene expression programmes essential for cellular differentiation and embryonic development. This phenomenon involves the reprogramming of both histone modifications and DNA methylation.

In mammals, reprogramming DNA methylation is a bimodal event (Figure 1.5), occurring during embryo preimplantation and gametogenesis (Reik et al., 2001). Upon fertilisation, the protamines present in the sperm genome are replaced with the histones H3 and H4, while the maternal genome completes meiosis. Following the deposition of histones, the paternal genome undergoes a genome wide loss of DNA methylation (Santos et al., 2002). This process occurs before the initiation of DNA replication and importantly, excludes paternally methylated imprinted genes (Olek and Walter, 1997).

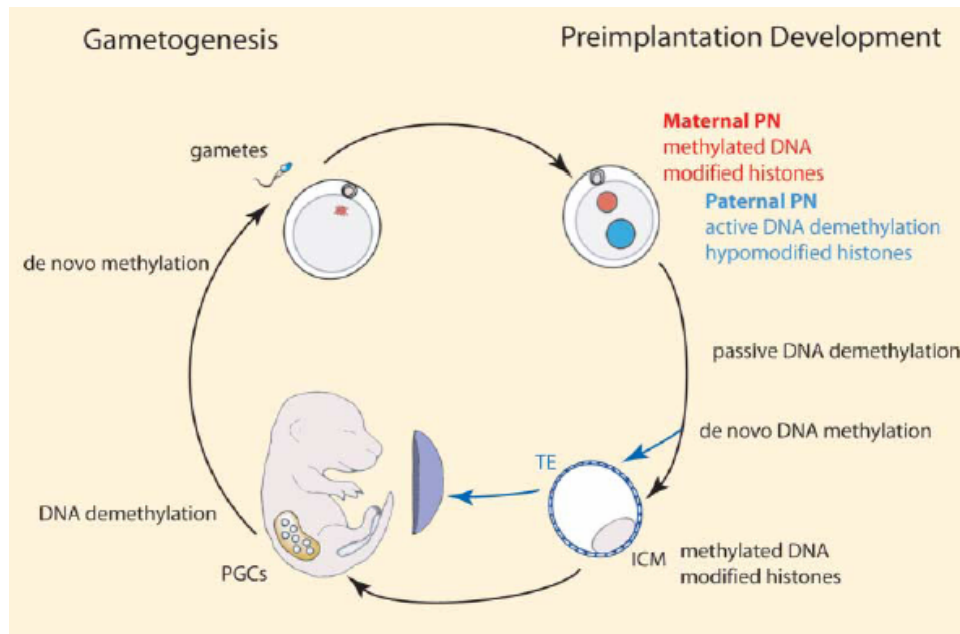


Figure 1.5: Epigenetic reprogramming cycle. Epigenetic reprogramming occurs in two phases during gametogenesis and preimplantation development. Primordial germ cells (PGCs) arise from somatic tissue and give rise to mature gametes. This is followed by genome-wide DNA demethylation and includes imprinted genes. Subsequently, gametes are methylated *de novo* and imprints are re-established. Upon fertilisation, during preimplantation a second reprogramming event actively and later passively (during cell cycles) demethylates the paternal genome but excludes imprinted regions. During the differentiation of the first two cell lineages, *de novo* methylation occurs. PN = pronucleus; TE = trophectoderm; ICM = inner cell mass (Morgan et al., 2005).

Further changes in global DNA methylation and histone modifications occur once the fertilised egg starts to divide. From the one cell stage of the mouse blastocyst, DNA methylation is reduced through a passive mechanism where successive rounds of cellular division and DNA replication result in unequally methylated sister chromatids. However, imprinted regions escape passive demethylation and retain their methylation status in a parent-of-origin specific manner and are maintained throughout embryonic development. At the morula stage of embryogenesis lineage allocation occurs and the

trophectoderm and inner cell mass lineages are defined. At this point, global differences in DNA methylation between extraembryonic (placenta) and embryonic lineages become apparent.

During embryonic development, primordial germ cells (PGCs) derived from epiblast cells migrate to the genital ridge by embryonic day 11.5 (E11.5). These PGCs contain epiblast-derived epigenetic marks which are subsequently erased through active demethylation by E12.5. Unlike in embryonic somatic tissue, genome-wide erasure of DNA methylation in PGCs includes imprinted regions. Reprogramming of primordial germ cells is critical as it renders the epigenetic state of germ cells of both sexes equivalent. Male PGCs enter mitotic arrest until birth when spermatogonial stem cells resume mitosis. Subsequently, during gametogenesis where spermatids mature to spermatozoa, a wave of *de novo* methylation by Dnmts re-establishes paternal-specific imprints.

The biological purpose of this reprogramming involves the erasure and re-establishment of parental genomic imprints in germ cells, but is also a vital process that erases and corrects deleterious aberrations of epigenetic marks, termed epimutations.

1.3.7. Imprinted genes

The first three imprinted genes were discovered in 1991 in short succession (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991). Barlow et

al. demonstrated that the *insulin-like growth factor-2 receptor (Igf2r)* gene is only expressed from the maternal chromosome. The *insulin-like growth factor II (Igf2)* gene was then reported by De Chiara et al., who showed that only the paternal copy of this gene was expressed. The third imprinted gene, *H19*, was suspected to be imprinted as it lies in an imprinted locus on chromosome 7, and indeed Bartolomei et al. demonstrated that *H19* was maternally expressed. To date there have been over 80 mammalian imprinted genes identified.

Imprinted genes are critical regulators of embryonic growth and development. The embryos of both mice and humans exhibit a number of imprinted genes, some of which are involved in common regulatory pathways (Feil and Berger, 2007). Chromosomal regions that demonstrated parent-of-origin phenotypic effects when inherited uniparentally, were identified using a mouse model carrying translocation chromosomes (Choi et al., 2005). The phenotypes observed ranged from early embryonic lethality to postnatal effects on growth and development. These phenotypes were attributed to the misexpression of imprinted genes present within the uniparentally inherited or parent-specific duplicated region (Cattanach and Kirk, 1985).

Genes that are imprinted are “tagged” for reduced expression by means of cytosine methylation. Imprinting occurs via allele-specific methylation while the genomes are separated in their respective germ lines and is parent-of-origin specific (Lucifero et al., 2002). This means that only one of the parental alleles

is imprinted, while the other remains relatively unmethylated. This results in the reciprocal expression of imprinted genes, that is, if a gene is paternally imprinted, it is maternally expressed and *vice versa*.

Although methylation is possible at any CG dinucleotide, DNA methylation of imprinted regions occurs at specific regions known as CpG islands. CpG islands are genomic regions of approximately 1kb in length which contain a series of repeating CG dinucleotides that designate potential methylation sites (Robertson, 2005). These islands are commonly found in the promoters (Jones and Takai, 2001) of tissue specific genes and if methylated, prevent the binding of transcription factors, causing strong and heritable transcriptional inhibition (Stoger et al., 1997). The CpG islands of imprinted alleles can occur in what are known as differentially methylated regions (DMRs) (Robertson, 2005).

DMRs can be divided into two classes, namely, primary and secondary DMRs (Kobayashi et al., 2006). During either spermatogenesis or oogenesis, primary DMRs acquire gamete-specific methylation and maintain this allelic methylation difference throughout development. Conversely, secondary DMRs establish differential methylation patterns after fertilization. The term imprinting control region (ICR) is given to a primary DMR when the loss of that primary DMR results in the aberrant expression of its associated imprinted genes (Kobayashi et al., 2006). In the hypomethylated state, CpG-islands contained within ICRs act as binding targets for proteins, which may result in

either transcription or repression of the gene (Rodenhiser and Mann, 2006). This form of repression is fairly complex and involves the methylation-dependent binding of CTCF proteins (Burns et al., 2001).

1.3.8. Locus regulation by CTCF proteins

The CCCTC-binding factor (CTCF) is a zinc finger protein that is able to specifically bind nucleotide sequences resulting in either the activation or silencing of associated genes. Initially, CTCF was identified as a transcriptional repressor in chickens (Baniahmad et al., 1990). Subsequently, the role of CTCF has been implicated in enhancer-blocking activity of several vertebrate boundary or insulator elements (Bell et al., 1999), where it has been shown to be ubiquitously expressed and highly conserved (Gray and Coates, 2005). The CTCF consensus sequence is able to bind the insulator protein, which isolates downstream enhancers from upstream promoters, creating a boundary element, resulting in transcriptional repression.

The insulating property of CTCF is a vital element by which most imprinted loci define monoallelic expression of imprinted genes in a parent-of-origin specific manner. CTCF binding sites are commonly found in ICRs of imprinted loci and bind the protein in a methylation-dependent manner. That is, in a methylated state, CTCF is unable to bind to its target sequence abrogating its insulator function. Conversely, an unmethylated state allows for CTCF binding, preventing communication between gene promoters and downstream

enhancers. It is important to note that CTCF binding is not a simple on/off switch. While the bound state (unmethylated CTCF binding site) of CTCF blocks the interaction of downstream enhancers with the promoter of one gene, it allows the enhancer to interact with the promoter of another gene in the imprinted cluster. The interaction of the enhancer is reversed in the unbound (methylated) state, i.e. the previously suppressed gene is favourably acted upon, while the corresponding gene in the cluster is now silenced (Figure 1.6). In this vein, the two genes comprising the imprinted cluster are reciprocally expressed depending on the methylation state of the parental ICR.

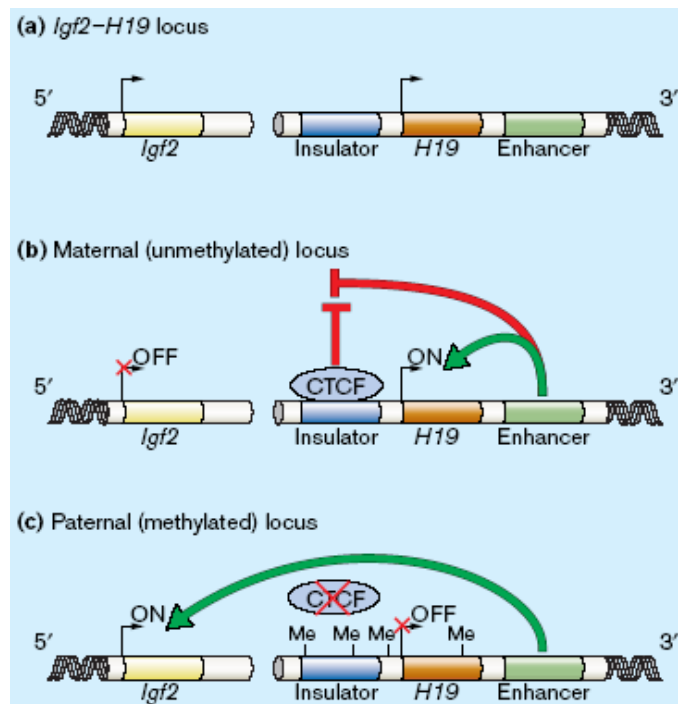


Figure 1.6: Regulation of imprinted loci. This example of the *Igf2*/*H19* locus demonstrates how CTCF regulates most imprinted loci. (a) The *Igf2* and *H19* locus. Arrows indicate transcription start sites. The positions of the imprinted control region (ICR; insulator) and downstream enhancer are shown. (b) In the hypomethylated maternal state, the ICR binds CTCF, preventing the downstream enhancer from acting on the upstream *Igf2* gene, while activating *H19*. (c) The paternal ICR is hypermethylated (Me), preventing CTCF binding. This allows the enhancer to act on the upstream *Igf2* gene, while *H19* is repressed (Wolffe, 2000).

1.3.9. Imprinting Disorders

Due to their monoallelic expression, imprinted genes are effectively haploid and therefore vulnerable to recessive mutations and epigenetic changes. Epimutations that arise from altered methylation or DNA mutations can bring about an aberrant change in the genetic regulation of a locus, manifesting in developmental disorders and human diseases.

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are two developmental disorders associated with the same imprinted region on human chromosome 15. PWS is inherited paternally and is associated with short stature, hypogonadism, obesity and behavioural abnormalities (Knoll et al., 1989). Conversely, AS is maternally inherited and is characterised by mental retardation, gait ataxia and an inappropriately “happy” demeanour. In both cases, there is an imbalance of the inherited imprinted parental signatures of the 15q11 – q13 region. This region is maternally imprinted and paternally hypomethylated. In PWS, deletion of the paternal 15q11 – q13 region results in the loss of genes expressed exclusively from the paternal chromosome, allowing for only the expression of genes expressed exclusively from the methylated maternal allele. Reciprocally, deletion of this region on the maternal allele leads to expression of only the paternal genes within this locus, resulting in AS. In addition to this large deletion, PWS may also be the result of maternal uniparental disomy (UPD), where both copies of chromosome 15 are maternally inherited; and in some cases aberrant methylation of the paternal

chromosome (Polychronakos and Kukuvtis, 2002).

The incorrect establishment of DNA methylation can occur as a result of mutations in the *DNMT* genes. In patients with ICF (immunodeficiency, centromeric instability and facial anomaly), reactivation of genes on the inactive X chromosome occurs due to inadequate methylation as a result of deleterious mutations in *DNMT3B*, a vital *de novo* methyltransferase (Okano et al., 1999; Hansen et al., 2000). Aberrantly methylated promoter-region CpG islands have been associated with cancer development, where hypermethylation of promoter regions silences tumour suppressor genes in rodent tumours, lung cancer and metastasis. Beckwith-Weidemann syndrome (BWS) is an imprinting disorder associated with a high risk of embryonal Wilm's tumours as a result of the loss of an unidentified tumour suppressor gene (Polychronakos and Kukuvtis, 2002). In paternal UPD cases, the paternally expressed gene *IGF2*, which codes for a foetal growth factor, is expressed at levels twice that of the normal state explaining the overgrowth and cancer phenotypes associated with BWS.

Hypomethylation of the *H19* ICR has also been associated with the features of asymmetry and growth retardation seen in Silver-Russell syndrome (SRS) patients (Bliek et al., 2006). These aberrations at the ICR have been associated with reduced methylation and increased CTCF binding and therefore enhancer-blocking activity, which results in the biallelic expression of *H19* (Gicquel et al., 2005).

1.4. An epigenetic model of FAS

DNA methylation is a vital regulator of gene expression. At imprinted loci, it determines the binding of the insulator protein CTCF, which in turn regulates the expression of associated genes within the locus in a parent-of-origin-specific manner. Aberrations to the imprints, and in particular ICRs, have been associated with developmental disorders, including AS, PWS and BWS, which are associated with abnormal growth phenotypes and neurological deficits.

The cause of aberrant DNA methylation at ICRs, such as that of *H19* in SRS, remains largely unknown. However, several environmental factors have been associated with decreased methylation. Heavy metals (Aina et al., 2004), pesticides (Anway and Skinner, 2006), cigarette smoke (Kaplan et al., 2003), dietary folate deficiencies (Friso and Choi, 2002) and alcohol have all been associated with DNA methylation disruption. Alcohol and dietary folate intake have been linked with cancer, where alcohol-fed rats displayed disruption of the folate, methyl group and homocysteine metabolism pathway, with subsequent reduction of the by-products essential for DNA methylation (Finkelstein, 1974; Barak et al., 1987; Trimble et al., 1993). Perturbations to these metabolic pathways result in hypomethylation of hepatic DNA, and is associated with hepatomas in the rat liver (Bhave et al., 1988; Wainfan et al., 1989). Low folate/high alcohol intake has been associated with sporadic colorectal cancer (Giovannucci et al., 1995; van Engeland et al., 2003), upper alimentary and liver cancer (Poschl and Seitz, 2004).

Not only does alcohol affect methylation in cancers, but also alters methylation involved in developmental pathways. Prolonged levels of maternal alcohol exposure during pregnancy have been implicated in a range of unfavourable foetal outcomes, such as FAS. Investigations using a mouse model into the effects of alcohol on the methylation signatures of imprinted genes have revealed that excessive exposure resulted in global hypomethylation of fetal DNA, providing a link between loss of methylation and FAS (Garro et al., 1991). The implication of this and other similar studies is significant as DNA methylation plays a vital role in the regulation of gene expression during embryogenesis, particularly at imprinted loci.

Epigenetic reprogramming during gametogenesis provides a vulnerable window for environmental factors to exert their effect. In particular, alcohol is able to decrease the methylation available for transmethylation reactions vital for establishing imprints. When paternally ingested, alcohol is able to exert a negative effect on the establishment of such imprints at paternally imprinted loci, where it was shown that chronic alcohol use in men was correlated with demethylation of normally hypermethylated regions in sperm DNA (Ouko et al., 2009).

To date, three paternally methylated DMRs/ICRs have been described (Kobayashi et al., 2006). The *H19* ICR, *Rasgrf1* ICR and *IG*-DMR (ICR) are primary DMRs whose methylation is established during gametogenesis and are

representative of three separate loci that contain genes vital to embryonic/fetal development.

Developmentally significant genes are typically organized into functional pairs and are co-expressed at specific loci (Takada et al., 2000). Located distally on mouse chromosome 7, the *Igf2/H19* locus contains two genes and is regulated by the *H19* ICR. *Igf2* encodes an embryonic mitogen, while *H19* produces a non-coding RNA of unknown function. *Igf2* is strongly expressed in the mouse placenta and in homologous structures of the human placenta, where it promotes placental and fetal growth (Redline et al., 1993).

The *IG* (intergenic)-DMR is encompassed in the *Dlk1/Gtl2* locus on the distal portion of mouse chromosome 12. *Dlk1* has been reported to be homologous to the Notch-Delta family of developmentally regulated signalling molecules (Takada et al., 2002). The Delta-Notch signalling pathway is essential in determining cell fate, proliferation, and apoptosis of the vascular system in embryo and adult tissue. This pathway also plays a crucial role in the vertebrate nervous system through the regulation of molecular signals that promote cellular differentiation in a spatial and temporal manner (Edlund and Jessell, 1999). *Dlk1* encodes a transmembrane protein that is extensively expressed in the developing embryo, while *Gtl2* encodes an untranslated RNA (Schmidt et al., 2000). *DLK1* is thought to be involved in development and differentiation of the adrenal medulla, adipocytes, haemopoietic progenitor cells and osteoblasts (Fukuzawa et al., 2005).

Ras protein specific guanine nucleotide-releasing factor 1 or *Rasgrf1*, is located distally on mouse chromosome 9. *Rasgrf1* is paternally imprinted at a domain 30kb 5' of its transcription start site. *Rasgrf1* is exclusively expressed in the neonatal brain where it functions to couple Ras protein activation to signal transduction processes mediated by Ca^{2+} (Plass et al., 1996; Yoon et al., 2005). *Rasgrf1* knock-out mouse models have shown that *Rasgrf1* deficiency is highly correlated with reduced postnatal growth and memory defects (Brambilla et al., 1997; Fernández-Medarde et al., 2002).

Given the importance of these imprinted genes in foetal development and the negative impact of alcohol on DNA methylation, this study aims to investigate the effect of excessive paternal alcohol intake on the imprinting status of these three paternally imprinted loci in mice. It is hypothesised that alcohol will decrease methylation at these paternally methylated ICRs in sperm DNA, particularly at the CTCF binding sites. Moreover, we aim to investigate whether any DNA methylation aberrations observed in these imprinted regions will be inherited by the offspring sired by ethanol treated males and whether the treatment of these sires will impact on the outcome of their progeny.

Chapter 2

2. Materials and Methodology

Preparation of solutions marked with * are available in Appendix B.

2.1. Sample collection

2.1.1. Male mouse model for excessive paternal alcohol consumption

A group of 15 male C57BL/6JOLA^{Hsd} mice ranging from seven to eight weeks of age were selected for the study. Ethics clearance was obtained from the Animal Ethics Committee, University of the Witwatersrand (Appendix A). Mice were imported by the National Health Laboratory Service (Sandringham) from Harlan UK Limited (originally from the Jackson Labs). The weights of the male mice ranged from 24g – 28g, and were monitored throughout the experiment. Eight male mice were selected for treatment with 7.5µl/g of a 50% solution of ethanol*. This concentration was specifically chosen as it has been shown to significantly inhibit DNA methylation (Garro et al., 1991) and results in excessively high blood alcohol concentrations (Livy et al., 2004). The control group consisted of seven males treated with an isocaloric solution of 7.5µl/g of a 0.704g/ml solution of sucrose*. Both the treatment and control

groups were orally gavaged with a blunt-ended needle and syringe. Oral gavaging was carried out over a five week period, during which the frequency of feeding was progressively increased to allow the mice to become accustomed to the new diet. A five week gavaging period was chosen as this encompasses a complete round of spermatogenesis in the mouse (Abel and Moore, 1987). Male mice were orally gavaged three times during the first week (every second day from Monday to Friday); for five days consecutively during the second week (Monday to Friday); and daily from week three to five. Mice were housed at the Central Animal Service (CAS), situated at the University of the Witwatersrand's Medical School, for the duration of the experiment. All health and dietary needs were attended to by the CAS veterinary and support staff. Food and water were given *ad lib* for the duration of the experiment.

2.1.2. Mating of alcohol fed males

Once the five week treatment regimen of the male mice was complete, all males were separated into separate cages with two untreated C57BL/6 females. Mating was allowed for a period of 24 hours. Males were subsequently removed and females were monitored for the formation of vaginal plugs. Once vaginal plugs and/or pregnancies were confirmed by veterinary staff at the CAS, females were placed in separate cages, where they were allowed to give birth and care for their young. Both male and female mice were tagged with metal ear tags with unique identification numbers.

2.1.3. Harvesting of sperm

Male mice were anaesthetised in a CO₂ chamber and their testes were removed surgically immediately following the 24 hour mating period. All male mice had their testes surgically removed at this point, regardless of whether the presence of vaginal plugs was confirmed in their mates. Briefly, the body cavity was opened by using an incision from the abdomen into the testicle region. Each testis was pulled from the body cavity using forceps. Using a pair of scissors, each testicle and associated epididymus was removed and placed in phosphate buffered saline (PBS)*. The epididymus was then punctured several times and transferred to tube containing 1ml PBS. This “swim out” protocol allows the mature motile spermatozoa to swim out of the epididymus into the PBS. The swim out selection was carried out for 60 – 90 minutes at 30°C to facilitate sperm motility. The sperm was then separated from the cellular debris by centrifuging for 5 minutes at 2000xg. Following this, a wet-mount slide was prepared to check for successful swim out, indicated by the abundance of motile sperm (Figure 1.7a). Once the presence of sperm was confirmed, the sperm solution was placed in a cryo-tube and flash frozen using liquid nitrogen (stored at -80°C).

2.1.4. Offspring measurements

Offspring sired by alcohol treated and control (sucrose treated) males were monitored from birth. Offspring weights and anatomical measurements were

recorded at two days of age, and reassessed every two days subsequently over a period of 30 days. Thereafter, weights and anatomical measurements were recorded weekly for a further five weeks. The measurements taken for each offspring included crown-rump length, head length and the distance between the eyes.

Offspring were not assessed from birth as it is common for females to cannibalize new-born offspring (Gandelman et al., 1971), which is exacerbated by human contact (Marques-de-Araujo and Cardoso, 1999). Pups were uniquely marked with a non-toxic felt tip pen on their backs using a scoring system, and later a ringing system was used to mark their tails when bodily hair started developing. When the pups were four weeks old, the ear punch method was used to permanently mark each pup.

2.1.5. Offspring tail biopsies

Tail biopsies were obtained from 13 of the 17 offspring sired by EtOH treated sires, and 23 of the 29 offspring sired by control males at four weeks of age. In instances of still-births and partly cannibalised pups, tail biopsies were obtained immediately post-mortem. Four-week old (viable) offspring were anaesthetized with Isofor and 1cm of tail was removed using a scalpel. After the biopsy, the tip of each mouse's tail was quarterised so as to prevent further bleeding. The tail biopsies were placed in 500µl PBS and stored at -20°C.

2.2. DNA extractions

2.2.1. Sperm DNA extraction

In order to extract DNA from sperm obtained from the “swim out” protocol, it is essential to separate the contaminating epithelial cells from the desired spermatozoa. The Differex™ System (Promega) served to enrich for sperm cells in the presence of an excess of epithelial cells (Gill et al., 1985).

The Differex™ System makes use of Proteinase K digestion in conjunction with phase separation and differential centrifugation in order to separate sperm from epithelial cells. Proteinase K* digests epithelial cells and sperm tails, while the resilient sperm head remains intact. Through density gradient centrifugation, cellular DNA and debris are differentially separated from the intact sperm heads, from which DNA is later extracted.

Sperm samples stored at -80°C were rapidly thawed to 37°C. The Differex™ System was then carried out according to the manufacturer’s protocol from steps 1 – 11. The only change to this protocol occurred at step 3, where the incubation at 56°C was extended to two hours. This digests the contaminating epithelial cells, along with the sperm tails, but leaves the sperm heads intact (Figure 1.7b). At this point, separation of the sperm heads from the epithelial cells is complete.



Figure 1.7a: Undigested sperm with head (arrow) and tail intact.

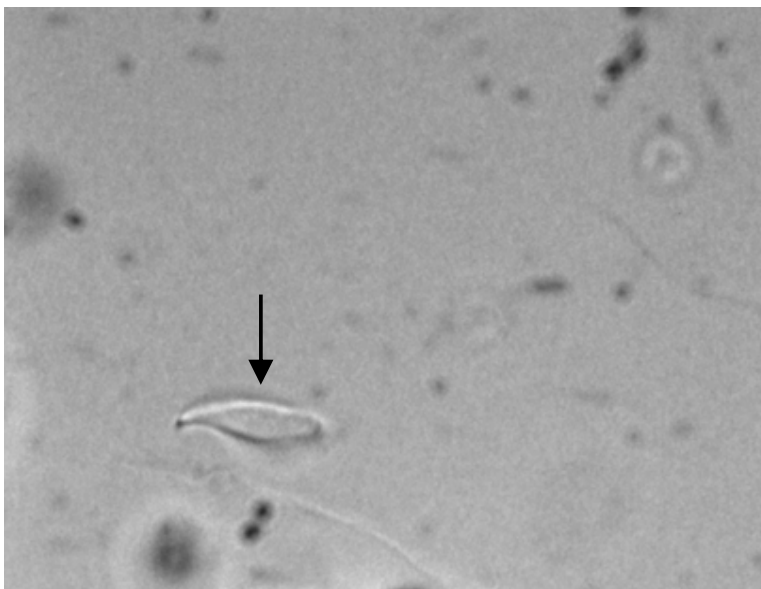


Figure 1.7b: Image of intact sperm head after digestion with Proteinase K. Note that only the head is intact (denoted by the black arrow), while the tail and surrounding epithelial cells have been digested.

An intermediate protocol bridges the Differex™ System with the extraction of DNA from the sperm heads using QIAamp DNA Micro Kit (QIAGEN) protocol. This was obtained via personal communication with Dr Tsukada (Appendix C). Steps 9 – 14 of the QIAamp DNA Micro Kit Handbook were followed according to the manufacturer's protocol. Sperm DNA was eluted in 50µl of buffer AE. This was concentrated down to a volume of about 25µl due to very low initial DNA concentrations. The final concentrations of the sperm DNA ranged from 1ng/µl to 30ng/µl and were stored at -20°C.

2.2.2. Tail biopsy DNA extraction

The QIAamp DNA Micro Kit (QIAGEN) was used to extract DNA from offspring tail biopsies. The protocol used was based on isolation of genomic DNA from tissues. 5mm of mouse tail was used for each extraction. The protocol was followed as per the manufacturer's specifications. DNA was eluted in 50µl of Buffer AE and stored at -20°C. DNA yields ranged from 268ng/µl to 1160ng/µl.

2.3. Bisulfite modification

In principle, bisulfite modification results in the deamination of all unmethylated cytosine bases and converts them to uracil bases, while methylated cytosines (5-methylcytosines) remain unchanged (Figure 1.8). Thus,

the sequence of the treated DNA will differ from that of its original composition at unmethylated cytosines. During subsequent PCR reactions, uracil bases are complemented with adenosine bases, which are in turn used as a template for thymine complementation. Thus CpG dinucleotides of the bisulphite modified sample strand are converted to TpG. Primers for PCR are specifically designed based on the chemically-modified sequence, which can be further analysed by sequencing.

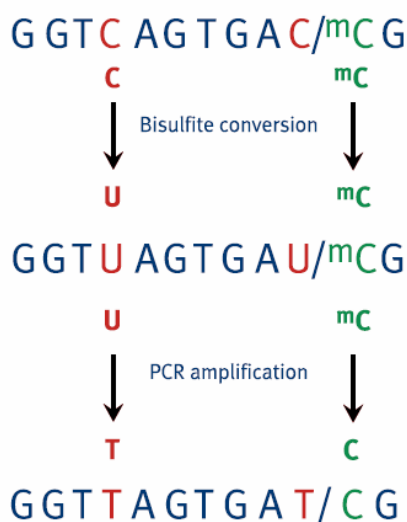


Figure 1.8: DNA treatment with sodium bisulfite. Methylated cytosines (^mC) remain as Cs, while unmethylated Cs are converted to uracil (U) and subsequently to thymine (T) during PCR (England and Petterson, 2005).

DNA samples were bisulfite modified in duplicate using the EpiTect® Bisulfite Kit (QIAGEN) as per the manufacturer's specifications. The protocol used was based on the sodium bisulfite conversion of unmethylated cytosines in DNA from solutions with low concentrations of DNA. 500ng of DNA was used for the bisulfite conversion of tail DNA samples, while the maximum volume of

DNA allowed by the kit was used for conversion of the low-concentration sperm DNA.

2.4. Polymerase chain reaction (PCR)

All PCR amplification protocols were optimized and performed using nested PCR. Nested PCR involves two consecutive rounds of amplification, where the first round uses external primers, and the second uses internal or nested primers. The first round amplifies the target region as in a typical PCR reaction, while in the second round, the nested primers anneal to sequences internal to the product of the first round PCR. The rationale behind this method is to maximize amplification of the highly degraded bisulfite modified genomic DNA and/or DNA of relatively low quantity (as in sperm DNA).

Primer sets were obtained/designed to amplify a specific region within the ICR of each of the four imprinted loci under investigation (*A19-Rasgrf1*, *Igf2-H19*, *Dlk1-Gtl2* and *Snrpn*; Table 1.1). These ICRs are located between two genes within each locus, and are referred to in terms of the gene downstream of the ICR. That is, the ICRs of *Rasgrf1*, *H19* (also *H19* DMR), *IG* (intergenic region of *Dlk1/Gtl2*; also *IG*-DMR), and *Snrpn*. The *Rasgrf1* ICR amplified region is located on contig AC102545.9 and contains 29 CpG sites. This region also contains a CTCF binding site that spans CpG sites 20 – 27. The *H19* ICR region locates to contig AC13548.13 and contains 16 CpG sites. The entire *H19* DMR contains four CTCF binding sites. The region amplified in the present study

contained two of these CTCF binding sites. These two CTCF sites were selected as small changes to DNA methylation at these binding sites are able to switch the *Igf2/H19* domain from a paternally hypermethylated epigenotype to a maternally hypomethylated genotype (Engel et al., 2004). The first CTCF site contains six CpGs, and the second contains five. The *IG*-DMR (or ICR) maps to contig AC107681.15 and the region amplified contains 29 CpG sites. However, this ICR does not contain a CTCF binding site.

As the aim of the present study is to investigate methylation patterns of the hypermethylated paternal allele, it is essential that potentially contaminating somatic DNA (containing the maternally imprinted allele) is ruled out. The *Snrpn* ICR is hypomethylated on the paternal allele, and methylated or hypermethylated on the maternal allele. This imprinted region was therefore included in this study as a control region to detect maternal allele contamination. The amplified region of this ICR contains 16 CpGs, located on contig AC167813.4 with no reported CTCF binding sites.

Table 1.1: Primers used for nested PCR.

<i>Primer</i>	<i>Sequence 5' → 3'</i>	<i>Amplicon size</i>	<i>Reference</i>
Rasgrf1 F	TAGAGAGTTTATAAAGTTAG	284bp	(Yoon et al., 2002)
Rasgrf1 R	ACTAAAACAAAAACAACA		
H19 out F	GAGTATTTAGGAGGTATAAGAATT	474bp	(Tremblay et al., 1997)
H19 out R	ATCAAAAATAACATAAACCCCT		
H19 in F	GTAAGGAGATTATGTTATTTTGG	423bp	
H19 in R	CCTCATAAAACCCATAACTAT		
IG out F	GTTGYGGTATTGTTAYGTGGT	997bp	This study

IG R	TAATCCCATTCCCAATCTATAA		
IG in F	GGAGATGTGTTGTGGATT	466bp	(Li et al., 2004)
IG R	TAATCCCATTCCCAATCTATAA		
Snrpn out F	TATGTAATATGATATAGTTTAGAAATT AG	529bp	
Snrpn out R	AATAAACCCAAATCTAAAATATTTTA ATC		
Snrpn in F	AATTTGTGTGATGTTTGAATTATTG G	420bp	
Snrpn in R	ATAAAATACACTTTCACACTAAAAT CC		

Out = external or outer primer; in = internal or inner primer; F = forward primer; R = reverse primer.

Both the *H19* DMR and the *Snrpn* ICR were PCR amplified using conventional nested PCR. However, due to the high thymine content of bisulfite modified DNA it was not possible to design nested PCR primers for both the *IG*-DMR and the *Rasgrf1* ICR. An external and internal forward primer and a single degenerate reverse primer were designed for the *IG*-DMR. One set of primers was used for the amplification of the *Rasgrf1* ICR, which were used in two successive rounds of PCR using the first round product as the starting template for the second round.

All PCR reactions were performed in duplicate using a GeneAmp 2720 thermal cycler (Applied Biosystems). The annealing temperatures (T_a) for amplification of the ICRs were as follows: *IG*-DMR, 59°C; *H19*, 55°C; *Rasgrf1*, 59°C; and *Snrpn*, 59°C. The reaction conditions for all PCR reactions were performed as per Table 1.2.

Table 1.2: PCR reaction conditions.

Reagents	Round 1	Round 2	Cycle (IG-DMR)	
ddH ₂ O	30.8µl	29.8µl	95°C	5min
Buffer 10x	5µl	5µl	95°C	15s
MgCl ₂	4µl	4µl	Ta°C	30s
dNTP Mix*	5µl	5µl	72°C	15s
Primer F (10µM)	2µl	2µl	72°C	5min
Primer R (10µM)	2µl	2µl	4°C	∞
Taq pol (5U/µl)	0.2µl	0.2µl		
DNA	1µl	2µl* ^{round 1}		
	50µl	50µl		

Note: *^{round 1} = PCR product from first round of PCR.

2.5. PCR product cloning

PCR products obtained from duplicate nested PCRs were cloned using the pGEM[®]-T Easy Vector System (Promega) in conjunction with DH5α competent *Escherichia coli* (*E.coli*) bacterial cells. Cloning was only performed on the sperm DNA of the treated males.

2.5.1. pGEM[®]-T Easy Vector ligations

Following successful PCR amplification, products were ligated into pGEM[®]-T Easy Vectors in preparation for cloning. This vector system contains a multiple cloning site within the coding region of the β-galactosidase enzyme, which is able to metabolize the colourless substrate X-gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside), converting the substrate to a bright blue colour.

When a PCR product is incorporated (ligated) into the multiple cloning site β -galactosidase gene expression is interrupted preventing the conversion on the chromogenic substrate X-gal from white to blue. Therefore, vectors that have successfully incorporated a PCR fragment (insert) will result in colonies that remain white, while those that have not incorporated an insert will convert the colourless X-gal blue. This method is known as blue-white colony selection/screening. The ampicillin resistance gene contained within the pGEM[®]-T Easy Vector aids in colony selection. Vector ligations were setup and performed as per the pGEM[®]-T Easy Vector System protocol. Ligation reactions were incubated over night at 4°C to increase ligation efficiency.

2.5.2. Preparation of competent *E.coli* cells

Competent cells were prepared from DH5 α *E.coli* stocks (Appendix D).

2.5.3. Transformations

Once PCR products were successfully ligated, competent DH5 α *E.coli* cells were used for transformations (Appendix E). These *E.coli* cells are high efficiency competent cells with a transformation efficiency of $>1 \times 10^8$ cfu/ μ g DNA, and are compatible with blue/white colony screening and standard ampicillin selection.

2.5.4. Bacterial minipreps

After transformed bacterial cells were plated and grown overnight at 37°C, individual white colonies were selected for miniprep cultures. Colonies were individually picked from agar plates with a pipette tip and placed into a centrifuge tube containing Luria broth* (1µl/ml ampicillin* (50mg/ml). This was allowed to grow overnight at 37°C at 200rpm in a shaking incubator. Tubes were left half a turn open so as to allow for gaseous exchange.

2.5.5. Isolation and purification of plasmid DNA from *E.coli*

Isolation and purification of plasmid DNA from *E.coli* was carried out according to the Plasmid Quickprep protocol (Schneitz Lab, University of Zurich Institute of Plant Biology (Sambrook et al., 1989) (Appendix F). The presence of an insert was determined by digesting plasmid DNA using *EcoRI*.

2.5.6. Cycle sequencing

Prior to capillary electrophoresis, plasmid DNA samples were cycle sequenced using BigDye® (ABI) and an M13 Reverse primer (tcatagctgtttcctgtgtga). The Montage SEQ₉₆ Sequencing Reaction Cleanup Kit (Millipore) (Appendix H) was used for cycle sequencing product clean up and preparation. Cycle sequencing was optimized using the 1/2 reaction as per Table 1.3.

Table 1.3: Cycle sequencing conditions.

1/2 Reaction		Cycle	
Plasmid DNA (400-1000ng/μl)	5μl	96°C	30s
BigDye®	1μl	50°C	15s
BigDye® Buffer	1.5μl	60°C	4min
Primer (M13 Reverse; 10μM)	1μl	24°C	∞
ddH ₂ O	1.5μl		
	10μl		

Note: BigDye® is light sensitive and samples must be placed in a black bag or container after cycle sequencing.

2.5.7. Sequencing of sperm DNA clones

Sequencing was performed on an Applied Biosystems (ABI) 3130xl Genetic Analyser. ABI sequence files were converted to FASTA format using ABI 2 FASTA Converter (Heracle Software).

2.5.8. DNA sequence analysis

DNA methylation data from bisulfite sequenced clones was analysed from FASTA sequence files using BiQ Analyzer (Bock et al., 2005). This programme aligns clones using a clustal W alignment. Inclusion criteria for clone sequence data included: 90% bisulfite conversion or greater; and 80% sequence identity or greater with the reference sequence. To avoid inclusion of identical/same clone sequences, all sequences are required to differ in at least

one of the genomic sequence's aligned C positions. Sequences that are identical are excluded from further analysis.

2.6. PyrosequencingTM for quantitative DNA analyses

PyrosequencingTM technology can be adopted as a sensitive method for the analysis of CpG methylation. This technology makes use of a light-emitting reaction that allows allele quantification at individual CpG sites. In this case, the alleles will either be a C (^mCpG) or T (TpG) for each CpG site that has the potential to be either methylated or unmethylated.

Briefly, a sequencing primer is hybridized to a single-stranded DNA fragment and incubated with an enzyme cocktail containing DNA polymerase, ATP sulfurylase, luciferase and apyrase, together with the substrates adenosine 5' phosphosulfate and luciferin. dNTPs are then added in a pre-defined dispensation order and via DNA polymerase catalysis, are incorporated sequentially into the growing strand. When a dNTP is incorporated, pyrophosphate is released in a quantity that is equimolar to the amount of incorporated nucleotide. Through the interaction of the accompanying enzymes, a visible light reaction is generated. This light emission is detected by a charge coupled device camera and recorded as a peak in a PyrogramTM, where the peak height is proportional to the number of nucleotides incorporated. Apyrase finally degrades unincorporated dNTPs and stops the light-emitting reaction, and the dNTP incorporation cycle is continued.

2.6.1. Pyrosequencing assay design

Pyrosequencing assays were designed using PSQ Assay Design Software (Biotage). This software specifically designs an assay to amplify a region of interest that contains a number of single nucleotide polymorphisms (SNPs). In this instance, the cytosine nucleotide contained within a CpG dinucleotide, that will either remain as a cytosine if methylated, or converted to a thymine (through bisulfite modification) if unmethylated, is in essence the “polymorphism”, being C/T (IUPAC code, Y). However, in the case of DNA methylation, the C (methylated):T (unmethylated) ratio at a given CpG within a specific DNA sample may vary, and is therefore unlike a traditional SNP where a sample is simply heterozygous or homozygous. Thus, the C and T alleles are quantified and subsequently expressed as a percentage.

Once the reference sequence containing the IUPAC code for each CG (YG) has been imported and a target region of approximately 100bp selected, the assay design software generates a series of potential primer sets to amplify the region. In addition to this, a sequencing primer is also generated for each primer set that will be used during the Pyrosequencing reaction.

Primer sets were obtained for each of the four ICRs under investigation (Table 1.4). 100bps is the optimal target region length and was centred on the CTCF binding sites of those ICRs where this is present. As mentioned previously, the *H19* ICR has two CTCF binding sites, where both were analysed by

pyrosequencing; and the *Rasgrf1* region contains a single binding site. As the *Snrpn* ICR does not contain a CTCF binding site, a random 100bp site was selected containing 7 CpG sites.

The technique of pyrosequencing in conjunction with the Pyro Q-CpG methylation software (Biotage) has a distinct advantage over cloning in that it is highly sensitive, accurate and therefore reproducible (Huse et al., 2007) and quantifies the DNA methylation of a sample by analysing all amplicons within a pooled PCR sample, and is therefore regarded as being more accurate. Selecting colonies for sequencing introduces selection bias and cloning limits the number of clones that can be selected for sequencing due to financial and time constraints.

Table 1.4: Primers sets used for pyrosequencing.

ICR	Primer sequence 5' → 3'	Amplicon region length	Annealing Temperature
<i>H19</i> (CTCF 1) F R(Bio) S	AGGAGGTATAAGAATTTGTAAGG TTAAATAACCCACAACATTACCAT GGAATTAGTTGTGGGGTT	180bp	60.3°C
<i>H19</i> (CTCF 2) F R(Bio) S	TTGTGTGTAAAGATTAGGGTTGTT AACTAACATAAACCCCTAACCTCA GTGTAAAGATTAGGGTTGTT	146bp	66°C
<i>Snrpn</i> F R(Bio) S	TGGTAGTTGTTTTGGTAGGATA TCCACAAACCCAACTAACCTT GTGTAGTTATTGTTGGGA	240bp	66°C
<i>Rasgrf1</i> F R(Bio) S	GTTATAGTTGAGTTATAATTATTA ACTAAACAAAAACAACACTACAAC CCAAATACCACAACAACAA	121bp	50.1°C

Note: F= forward primer; R = reverse primer; S = sequencing primer; Bio = 5' biotin label. Amplicon lengths exclude the 23bp 5' biotin label.

2.6.2. Pre-pyrosequencing PCR

Pre-pyrosequencing PCR utilised first round PCR products as a starting template for a second PCR using the primers designed by the Assay Design Software. The method of pyrosequencing employed here requires the addition of a 5' biotinylated tag on either the forward or reverse pre-pyrosequencing PCR primer (Table 1.4). To eliminate the need for a unique biotin-labelled primer for each primer set, a universal biotin-labelled primer was used to generate labelled DNA fragments (Colella et al., 2003). The pre-pyrosequencing PCR primer that has been designated to be biotin labelled (Bio-; Table 1.4) requires a complimentary tail or tag off of which the universal primer can prime. This is achieved by adding the 23bp complimentary tag, 5'-GACGGGACACCGCTGATCGTTTA -3', to the 5' end of the specified primer. Thus, each pre-pyrosequencing PCR contained three primers – a normal, tagged (tail), and universal primer (Table 1.5).

Table 1.5: Pre-pyrosequencing reaction conditions.

<i>Reagents</i>	<i>Volume</i>	<i>Cycle</i>	
ddH ₂ O	33.1µl	95°C	5min
Buffer 10x	5µl	95°C	15s
MgCl ₂	4µl	Ta°C	30s
dNTP Mix*	2.5µl	72°C	15s
Primer Normal (10 µM)	1µl	72°C	5min
Primer Tagged (1 µM)	1µl	4°C	∞
Primer Universal (10 µM)	1µl		
Taq pol (5U/µl)	0.4µl		
DNA (~100ng)	2µl		
	50µl	Ta°C = annealing temperature	

2.6.3. Pyrosequencing preparation and run

Pyrosequencing was performed using the PSQ 96MA System (Biotage) (Appendix G). Before commencing with pyrosequencing, pre-pyrosequencing PCR samples were loaded into a 96-well PCR plate. Briefly, through the interaction with the biotin-labelled universal primer, the pre-pyrosequencing PCR product is immobilized on streptavidin-coated Sepharose beads. Subsequently, the Sepharose beads are captured on the 96 probe Vacuum Prep Tool. The beads (bound to the PCR product) are then washed in 70% ethanol, Denaturation solution, Washing buffer, and ddH₂O for twelve seconds each. This process separates the DNA strands, leaving only the biotin-labelled strand attached to the captured Sepharose bead. Once the wash steps are completed, the Vacuum Prep Tool probes are lowered into a PSQ 96 Plate Low pre-filled with the sequencing primer and Annealing buffer, releasing the Sepharose beads. The PSQ 96 Plate Low is then heated to 80°C and allowed to cool, allowing for the sequencing primer to anneal. A reagent cartridge is then filled with the required amount of enzyme, substrate and dNTPs. The PSQ 96 Plate Low is then placed into the PSQ 96MA instrument along with the reagent cartridge, and the run is commenced.

2.6.4. Pyrosequencing data analysis

Pyrosequencing data was analysed using the Pyro Q-CpG Software (Biotage).

2.7. Statistical analyses

2.7.1. Descriptive statistics

To assess the methylation status of the paternal ICRs, each CpG dinucleotide was scored as being either “methylated” or “unmethylated”, represented by either a CG or TG, respectively, in the bisulphite modified genomic sequence.

With respect to clone data, each CpG site is scored to determine the overall methylation status of individual clones. Every methylated site is scored as ‘1’, while unmethylated sites are scored as ‘0’. In this binary fashion, the total number of methylated sites per clone are calculated and divided by the total number of CpG sites and recorded as a percentage as the “total methylation” of the clone. In this manner, the total methylation of clones obtained from the treated males’ sperm samples was calculated.

The conversion rate of the bisulphite modification process is important as unmethylated cytosines that are not correctly converted to thymines will give rise to false methylation positive sites. The bisulphite conversion error rates were calculated for each DNA sample using the BiQ Analyzer software (Bock et al., 2005), where clones with non-converted non-CpG cytosine content >5% were discarded.

In conjunction with clone data, pyrosequencing data was collected for the treated male sperm samples, while only pyrosequencing data was collected for the offspring. Pyrosequencing assays were designed for *H19*, *Rasgrf1* and *Snrpn*, and concentrated on a smaller number of CpG sites than those obtained for their corresponding clone data. Two assays were designed for *H19*, centred on the two CTCF binding sites.

The descriptive statistics data for the treated male sperm DNA and offspring tail DNA methylation levels are reported. These data are described in terms of central tendency (mean levels) and variation (standard deviation) and presented graphically in the form of box-plots.

2.7.2. Hypothesis testing

In this present study, it is predicted that excessive ingestion of ethanol by a male mouse will (1) cause a reduction in sperm DNA methylation at ICRs, (2) will be inherited paternally as an epimutation by sired offspring, (3) will cause growth retardation in these offspring. To measure these effects, Mann-Whitney U tests, Wilcoxon two sample tests and hierarchical linear modelling were used to test predictions (1) and (2). Mediation was used to test prediction (3). *P*-values of 0.05 and lower were considered to be significant, while *p*-values significant at the 10% level ($p < 0.1$) were regarded as showing a trend.

2.7.2.1. Mann-Whitney U tests and one-way ANOVAs

The nature of the data presented here is continuous and distributed into two treatment groups, i.e. EtOH and sucrose. Traditionally, this type of data would be analysed using the t-test. However, the t-test assumes that the data is normally distributed and usually requires a sample size ≥ 20 .

Due to the small sample size of male sperm samples ($n=13$) and the lack of normal distribution, the non-parametric Mann-Whitney U and One-way ANOVA (Wilcoxon) tests, which do not assume normality, were employed for sperm DNA methylation analyses. Furthermore, one-tailed tests were used, as it was predicted that EtOH will cause a reduction in DNA methylation. Similarly, the DNA methylation profiles of offspring sired by EtOH treated males were compared with those sired by the sucrose treated males using the one-tailed Mann-Whitney U and Wilcoxon tests, as our hypothesis predicts that EtOH will cause growth retardation.

Mann-Whitney U tests were performed using GraphPad Prism[®] version 5 (GraphPad Software, Inc.), while one-way ANOVA Wilcoxon two sample tests were performed in Enterprise Guide 4 (SAS Institute Inc.).

2.7.2.2. Correlations between treatment, DNA methylation and weight

The direct relationships between treatment, DNA methylation and weight were assessed non-parametrically using the Spearman's correlation. These correlations determine whether one variable outcome is associated with that of another in a relationship that is either positive or negative. For example, whether treatment is associated/correlated with a decrease in offspring weight; if treatment is correlated with reduced DNA methylation; and if DNA methylation is correlated with offspring weight. These analyses were performed using SAS 9.1 (SAS Institute Inc.).

2.7.2.3. Mediating effect of DNA methylation on the relationship between treatment and offspring weight

According to the hypothesis of this study, it is predicted that EtOH will cause a reduction in DNA methylation in the sperm, which will subsequently be inherited by their offspring, whose DNA methylation will be reduced (when compared to the control group), which in turn will result in growth retardation. Thus, the only manner by which EtOH can exert this negative effect on the offspring would be indirectly through the sperm. Therefore, to

test this indirect effect, mediation was employed to determine whether the effect of treatment on offspring weight is mediated by DNA methylation. The amount of mediation that is brought about by the mediator is called the indirect effect.

The mediation model below (Figure 1.9) describes the relationship between an independent variable (IV), the mediator variable (M) and the dependent variable (DV or outcome variable). In this study, the IV is treatment; M is DNA methylation; and the DV is offspring weight.

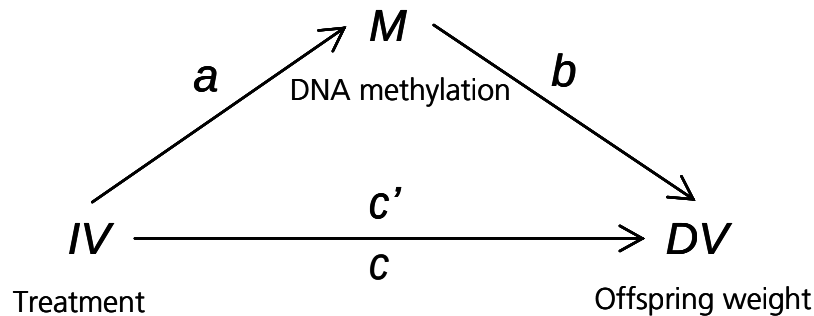


Figure 1.9: Mediation model. This model determines the amount of mediation that the mediator variable (M – DNA methylation) exerts on the relationship between the independent variable (IV – treatment) and the dependent variable (DV – offspring weight). The correlation between two variables is determined by paths *a*, *b* and *c*; while path *c'* establishes the mediating effect of IV on DV, controlling for M.

Before one can assess the degree of mediation by M, the paths *a*, *b*, *c* and *c'* must be estimated (Figure 1.9). These paths initially test correlations between the three variables (IV, DV and M). The first step is to show that treatment is

correlated with offspring weight (path c); secondly, a correlation of treatment with DNA methylation (path a) must be shown; thirdly, show that DNA methylation affects offspring weight (path b); and lastly, establish that DNA methylation mediates the relationship between treatment and offspring weight (path c'). These analyses were performed in SAS 9.1 using the least trimmed squares (LTS) method of robust regression (SAS Institute Inc.) (for script details see Appendix J).

2.7.2.4. Multilevel/hierarchical regression modelling

The mediation model above does not consider the hierarchical structure of the data of this study; that is, offspring are nested within mothers, and mothers are nested within males. These observations are therefore not independent. That is, offspring that come from the same male are related. Multilevel/hierarchical regression modelling was performed in STATA 10 (StataCorp) for two time periods: day 30-39 and day 40-63 where the treated males/sires were used as the grouping variable. Furthermore, two analyses were performed for each time period at the region specific level (*H19* CTCF 1, *H19* CTCF 2, *Rasgrf1* CTCF and *Snrpn*) and at the level individual CpG sites using pyrosequencing data. The purpose of this was to determine which CpG sites and/or regions contributed to the overall relationship between treatment and offspring weight, the significance of which was assessed by Wald chi-square tests.

Chapter 3

3. Results

The primary objective of this study was to assess the effect of excessive ethanol ingestion on the methylation status of paternally imprinted genes, where it is predicted to (1) reduce DNA methylation profiles of these genes in mouse sperm (paternal allele); (2) be inherited by sired offspring; and (3) result in growth retardation of offspring. To test these predictions, the methylation status of the three paternal ICRs – *H19*, *IG-DMR* and *Rasgrf1* – were analysed from sperm DNA of treated (EtOH and sucrose) males. Subsequently, methylation profiles of these imprinted regions were analysed in the tail DNA of sired offspring. Weight data of offspring was analysed over a 63 day period.

3.1. Mouse model

A total of 13 (six EtOH treated, seven sucrose treated) C57BL/6 male mice were used who, when mated with two female C57BL/6 mice each, sired a total of 46 offspring (17 sired by EtOH treated males, 29 sired by sucrose treated males).

3.1.1. Mouse identification numbering system

Unique identification numbers were assigned to each mouse used in the study, and is employed as the standard nomenclature from here forth. The 13 males and their respective treatment group are shown (Table 3.1a). Although each treated male was mated with two females, only those females that produced offspring were recorded. Each offspring is uniquely identified by the number assigned to their mother (also used as the “litter” number), and a number ranging from one to nine. For each offspring, the sex and treatment of the father was also recorded (Table 3.1 b). In cases where offspring died shortly after birth or were cannibalised by their mothers, sex could not be determined (indicated as “?”). Offspring sired by EtOH or sucrose treated males are referred to as “EtOH offspring” and “sucrose offspring”, respectively. Male mice 64 and 67 showed adverse effects to alcohol ingestion during the feeding regime and were omitted from the study. From here on, treated male mice/fathers are referred to as “sires”, and female mates/mothers are referred to as “dams”.

Table 3.1a: Identification numbering system for male mice.

<i>Sire</i>	<i>Treatment</i>
60	EtOH
61	Sucrose
62	EtOH
63	EtOH
65	EtOH
66	EtOH
68	EtOH
69	Sucrose
70	Sucrose
71	Sucrose

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72	Sucrose
73	Sucrose
74	Sucrose

Table 3.1 b: Identification numbering system for dams and offspring.

<i>Dam</i>	<i>Offspring</i>	<i>Sire / Treatment</i>	<i>Offspring Sex</i>
82	1	63 / EtOH	F
	2	63 / EtOH	M
	3	63 / EtOH	M
	4	63 / EtOH	F
	5	63 / EtOH	M
83	1	63 / EtOH	F
	2	63 / EtOH	F
	3	63 / EtOH	M
	4	63 / EtOH	M
	5	63 / EtOH	M
87	1	66 / EtOH	?
89	1	68 / EtOH	?
	2	68 / EtOH	?
	3	68 / EtOH	?
	4	68 / EtOH	M
	5	68 / EtOH	F
	6	68 / EtOH	F
90	1	70 / Sucrose	F
	2	70 / Sucrose	M
	3	70 / Sucrose	M
	4	70 / Sucrose	M
	5	70 / Sucrose	M
	6	70 / Sucrose	F
91	1	71 / Sucrose	F
	2	71 / Sucrose	F
	3	71 / Sucrose	F
	4	71 / Sucrose	M
	5	71 / Sucrose	F
95	1	72 / Sucrose	?
	2	72 / Sucrose	?
	3	72 / Sucrose	?
98	1	74 / Sucrose	F
	2	74 / Sucrose	F
	3	74 / Sucrose	F
	4	74 / Sucrose	F
	5	74 / Sucrose	F
	6	74 / Sucrose	F
100	1	72 / Sucrose	F
	2	72 / Sucrose	M
	3	72 / Sucrose	F
	4	72 / Sucrose	M
	5	72 / Sucrose	M
	6	72 / Sucrose	M

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	7	72 / Sucrose	?
	8	72 / Sucrose	?
	9	72 / Sucrose	?

Note: M = male; F = female; ? = sex unknown.

3.2. CpG methylation analyses of the three paternally imprinted ICRs

Analysis of ICR DNA methylation profiles of the three paternally imprinted genes (*H19*, *Rasgrf1* and *IG*) and the maternally imprinted gene *Snrpn* were conducted at three levels. Firstly, “total methylation analyses” assessed methylation of the entire ICR containing up to 31 CpG sites. These analyses are based exclusively on clone sequence data obtained from sperm DNA samples of the sires (Appendix I and Appendix M). Secondly, methylation was analysed at the level of a selected region which contained a defined number of CpG sites. In the case of *H19* and *Rasgrf1*, the selected regions encompassed CTCF binding sites, while seven consecutive CpG sites were selected in the *Snrpn* ICR. These analyses were based on pyrosequencing data and performed in duplicate for sperm samples and in triplicate for offspring samples, with the exception of the *Snrpn* ICR, which was performed in duplicate for both sperm and offspring samples. In some cases additional pyrosequencing data was obtained and was included in the analyses (Appendix K). The third analysis assessed DNA methylation at the level of individual CpG sites and made use of both clone sequences and pyrosequencing data. CpG site numbers are consistent across clone and

pyrosequencing data.

3.2.1. Sperm DNA methylation analyses of sires

A total of 600 clone sequences were obtained for the four ICRs from sire sperm samples (*H19* n=168, *Rasgrf1* n=159, *IG-DMR* n=119, *Snrpn* n=154). Clone data could not be obtained for *H19* ICR for mouse 60 (*H19* 60), and the *IG-DMR* of mouse 60 and mouse 61 (*IG-DMR* 60 and 61). Furthermore, despite having sufficient numbers of clones for analyses, the stringent BiQ Analyzer inclusion criteria excluded all clone sequences for *IG-DMR* 69, 70 and 74. After satisfying all sequence data inclusion criteria, a total of 278 sequences remained for subsequent analyses (*H19* n=92, *Rasgrf1* n=85, *IG-DMR* n=51, *Snrpn* n=50).

3.2.1.1. Detection of somatic DNA contamination in sperm samples

The *Snrpn* ICR was initially assessed to detect the presence of somatic cell (reflected by the presence of a maternally imprinted allele) contamination in sperm samples. Samples that exceeded 10% mean total methylation were regarded as contaminated. According to clone sequence data, none of the sperm samples exceeded this threshold (Figure 3.1). However, the number of clone sequences obtained for each sample ranged from 2 to 11, with a mean

number of 3.8 clones per sample. Considering the small sample size, there is a strong possibility that this may not be a true representation of the total methylation for these samples.

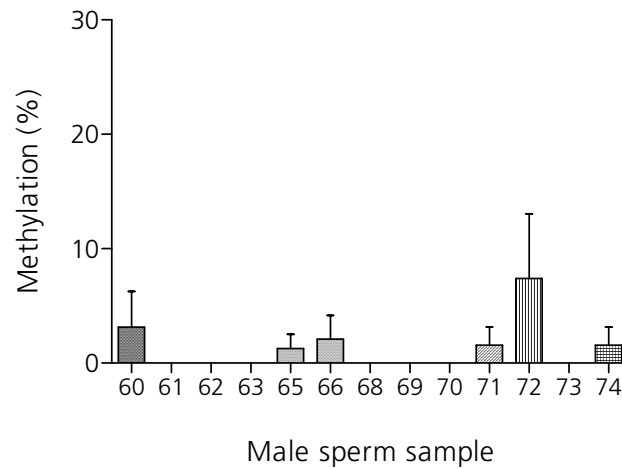


Figure 3.1: Clone sequence data of the total methylation of sperm samples at the *Snrpn* ICR.

Pyrosequencing revealed that sample *Snrpn* 69 exceeded the 10% threshold (Figure 3.2), and considering the sensitivity of pyrosequencing when compared with cloning, was excluded from further sperm DNA methylation analyses. After excluding all clones pertaining to sperm sample 69, a total of 261 clones were available for subsequent analyses (*H19* n=87, *Rasgrf1* n=79, *IG-DMR* n=51, *Snrpn* n=44) (Figure 3.3).

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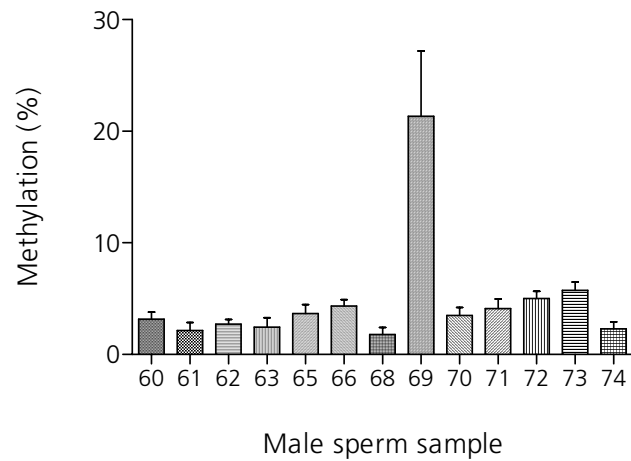


Figure 3.2: Pyrosequencing data of the total methylation of sperm samples at the *Snrpn* ICR.

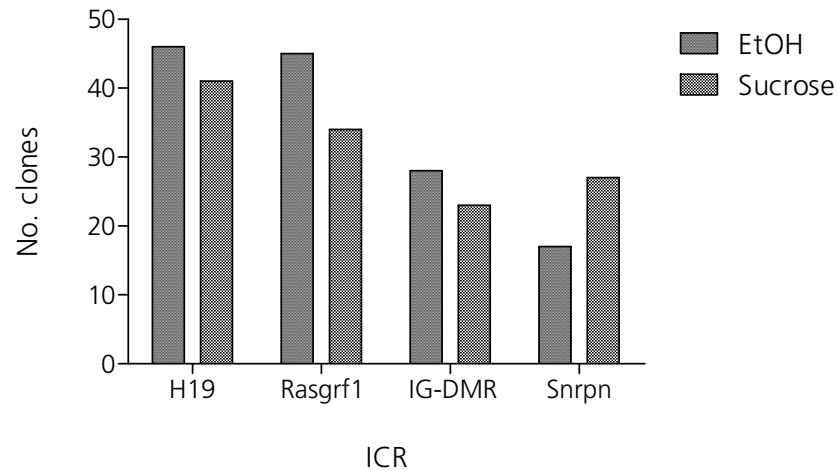


Figure 3.3: Distribution of clone sequences among the four ICRs under investigation.

3.2.1.2. Total methylation analyses of sperm DNA

Clone sequence data obtained from the sire sperm samples revealed no statistically significant differences between the two treatment groups with

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respect to the three paternally imprinted ICRs and the maternally imprinted *Snrpn* ICR (Figure 3.4).

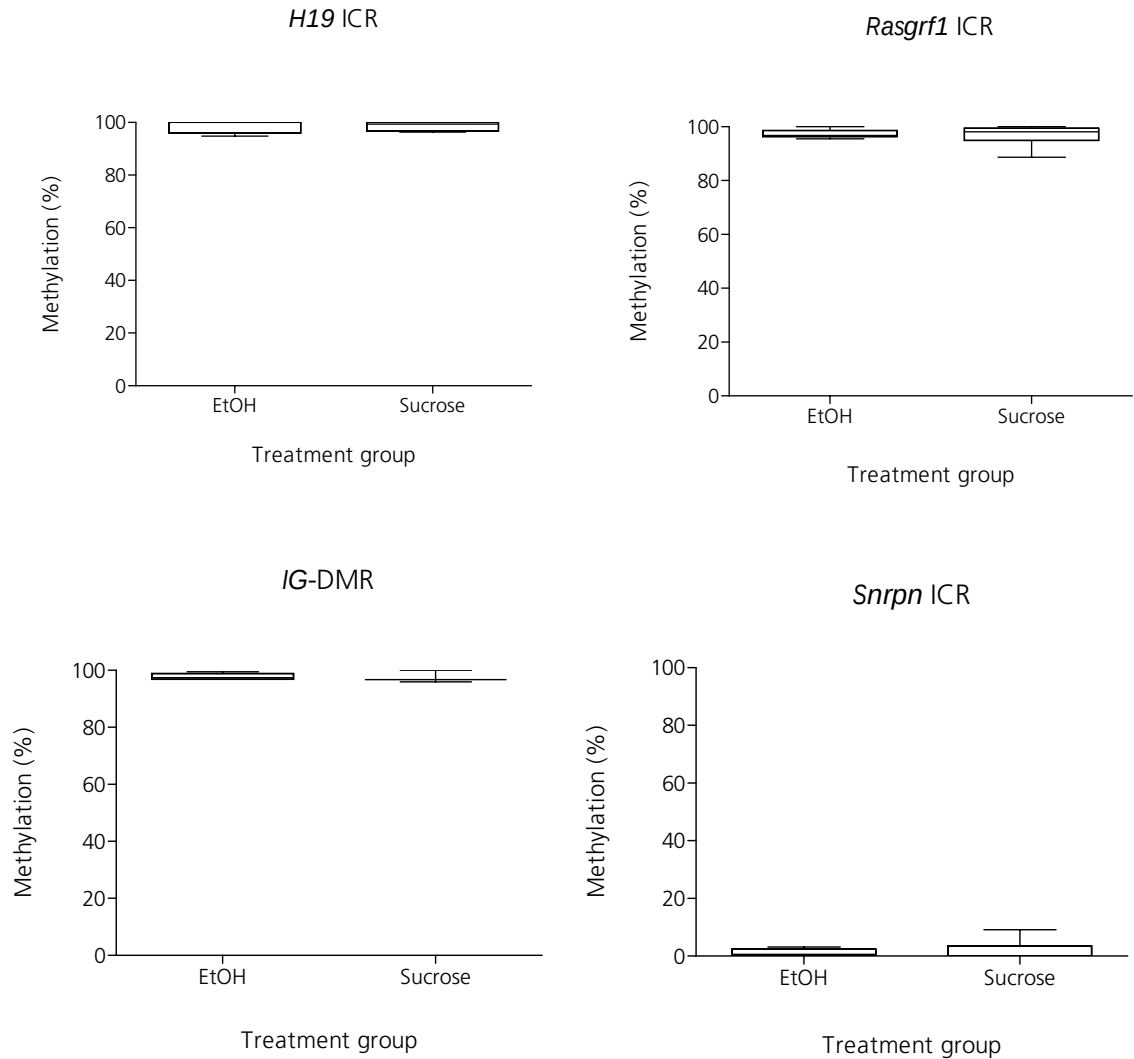


Figure 3.4: Total methylation of ICRs from sire sperm DNA clone data. The total methylation of each sire sperm sample was quantified for the three paternally methylated ICRs (*H19* n=87, *Rasgrf1* n=79, *IG* n=51). Total methylation was quantified for the maternally imprinted ICR (*Snrpn* n=44), which served as a control for somatic contamination.

3.2.1.3. Region specific analyses of sperm DNA

Clone and pyrosequencing data were analysed to assess whether there was evidence of region-specific reduction of DNA methylation at the *H19* CTCF binding sites (CTCF 1 and 2), the *Rasgrf1* CTCF binding site and the seven-CpG-site region within the *Snrpn* ICR (Table 3.2). No significant differences in total DNA methylation were observed in both data sets between the two treatment groups for all regions, with the exception of *H19* CTCF 1, which was significant at the 1% level.

Table 3.2: Region-specific sperm DNA methylation analyses.

<i>Imprinted Region</i>	<i>Clone / Pyrosequencing data</i>	<i>CpG Sites</i>	<i>Ethanol Median (%)</i>	<i>Sucrose Median (%)</i>	<i>Mann- Whitney U</i>
H19 ICR	Clone	1 - 16	100.0	99.4	14.5
H19 CTCF 1	Clone	2 - 7	100.0	100.0	14.0
H19 CTCF 1	Pyro		96.28	97.3	8.5 ^a
H19 CTCF 2	Clone	11 - 15	100.0	100.0	14.0
H19 CTCF 2	Pyro		97.65	98.10	17.5
Rasgrf1 ICR	Clone	1 - 29	96.8	98.2	13.5
Rasgrf1 CTCF	Clone	20 - 26	96.92	99.11	13.0
Rasgrf1 CTCF	Pyro		85.92	87.62	14.0
IG-DMR	Clone	1 - 31	97.4	96.7	5.0
Snrpn ICR	Clone	1 - 16	0.6	0.0	17.5
Snrpn	Pyro	6 - 12	2.7	3.7	13.5

^a $p < 0.1$

3.2.1.4. Analysis of individual CpG sites in sperm DNA

In addition to region specific analyses, methylation levels of individual CpG sites were assessed (Table 3.3 and 3.4). No significant findings were observed at any of the CpG sites in the *H19* ICR clone sequence data. However, a trend was noted at sites 6 and 11 where the EtOH group demonstrated lower methylation profiles. Pyrosequencing data revealed a significant reduction in methylation at CpG site 7 ($p=0.0244$) in the *H19* CTCF 1 region of the EtOH group, but no significant differences in CTCF 2. Clone sequence data obtained for the *IG*-DMR revealed no significant findings, and observations were inconsistent as some CpG sites showed reduced methylation in the EtOH group, while a number of CpG sites showed lower methylation levels in the sucrose group. The *Rasgrf1* ICR clone sequence data revealed a hypomethylation trend at CpG sites 17 and 22 ($p<0.1$) in the EtOH group, while pyrosequencing data for the *Rasgrf1* CTCF region showed a significant reduction in the EtOH group at CpG 26 ($p=0.014$) and no significant observations in CpGs 20 – 25. As expected, *Snrpn* ICR CpG methylation was low in the clone sequence data, since this region is maternally imprinted, while pyrosequencing data from the seven-CpG-site region showed CpG site 10 to be significantly less methylated in the EtOH group ($p=0.003$). Furthermore, a definite trend was observed in the pyrosequencing data at all *Snrpn* CpG sites (6 – 12) which showed reduced methylation in the EtOH group, with sites 6 and 9 approaching significance ($p=0.090$ and 0.063 , respectively).

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Table 3.3: Clone data of sperm samples.

Region	EtOH Mean (%)	Sucrose Mean (%)	Wilcoxon Standardised Statistic
clone_H19CpG1	100.00	98.33	0.730
clone_H19CpG2	100.00	97.22	0.730
clone_H19CpG3	100.00	96.67	0.730
clone_H19CpG4	100.00	100.00	0.000
clone_H19CpG5	100.00	98.33	0.730
clone_H19CpG6	86.06	95.00	-0.529
clone_H19CpG7	100.00	100.00	0.000
clone_H19CpG8	100.00	100.00	0.000
clone_H19CpG9	98.34	96.67	0.583
clone_H19CpG10	100.00	100.00	0.000
clone_H19CpG11	93.34	100.00	-0.913
clone_H19CpG12	100.00	98.33	0.730
clone_H19CpG13	100.00	98.33	0.730
clone_H19CpG14	100.00	100.00	0.000
clone_H19CpG15	96.66	100.00	-0.913
clone_H19CpG16	98.34	100.00	-0.913
clone_Rasgrf1CpG1	100.00	97.92	0.833
clone_Rasgrf1CpG2	100.00	100.00	0.000
clone_Rasgrf1CpG3	97.92	91.25	0.632
clone_Rasgrf1CpG4	100.00	100.00	0.000
clone_Rasgrf1CpG5	100.00	100.00	0.000
clone_Rasgrf1CpG6	98.33	100.00	-0.833
clone_Rasgrf1CpG7	100.00	86.25	1.787 *
clone_Rasgrf1CpG8	100.00	97.22	0.833
clone_Rasgrf1CpG9	100.00	100.00	0.000
clone_Rasgrf1CpG10	83.33	95.13	0.315
clone_Rasgrf1CpG11	98.33	97.22	0.000
clone_Rasgrf1CpG12	96.67	97.22	0.000
clone_Rasgrf1CpG13	98.33	100.00	-0.833
clone_Rasgrf1CpG14	100.00	97.92	0.833
clone_Rasgrf1CpG15	100.00	100.00	0.000
clone_Rasgrf1CpG16	100.00	97.22	0.833
clone_Rasgrf1CpG17	91.97	100.00	-1.354 ^a
clone_Rasgrf1CpG18	98.33	100.00	-0.833
clone_Rasgrf1CpG19	95.83	100.00	-0.833
clone_Rasgrf1CpG20	100.00	96.67	0.833
clone_Rasgrf1CpG21	97.92	100.00	-0.833
clone_Rasgrf1CpG22	81.25	100.00	-1.354 ^a

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clone_Rasgrf1CpG23	100.00	97.22	0.833
clone_Rasgrf1CpG24	95.83	100.00	-0.833
clone_Rasgrf1CpG25	96.97	97.92	0.000
clone_Rasgrf1CpG26	100.00	97.22	0.833
clone_Rasgrf1CpG27	94.88	97.22	-0.525
clone_Rasgrf1CpG28	96.25	95.13	0.191
clone_Rasgrf1CpG29	100.00	97.22	0.833
clone_SnrpnCpG1	5.55	0.00	0.833
clone_SnrpnCpG2	0.00	0.00	0.000
clone_SnrpnCpG3	0.00	4.17	-0.833
clone_SnrpnCpG4	11.67	19.32	-0.095
clone_SnrpnCpG5	0.00	0.00	0.000
clone_SnrpnCpG6	0.00	0.00	0.000
clone_SnrpnCpG7	0.00	1.52	-0.833
clone_SnrpnCpG8	0.00	0.00	0.000
clone_SnrpnCpG9	0.00	1.52	-0.833
clone_SnrpnCpG10	0.00	1.52	-0.833
clone_SnrpnCpG11	0.00	0.00	0.000
clone_SnrpnCpG12	0.00	0.00	0.000
clone_SnrpnCpG13	0.00	0.00	0.000
clone_SnrpnCpG14	0.00	0.00	0.000
clone_SnrpnCpG15	0.00	0.00	0.000
clone_SnrpnCpG16	0.00	0.00	0.000
clone_IGCpG1	100.00	100.00	0.000
clone_IGCpG2	100.00	100.00	0.000
clone_IGCpG3	100.00	100.00	0.000
clone_IGCpG4	90.00	95.83	0.000
clone_IGCpG5	100.00	100.00	0.000
clone_IGCpG6	96.00	87.50	-0.390
clone_IGCpG7	97.78	100.00	0.516
clone_IGCpG8	100.00	91.67	-1.033
clone_IGCpG9	100.00	100.00	0.000
clone_IGCpG10	100.00	100.00	0.000
clone_IGCpG11	100.00	95.83	-1.033
clone_IGCpG12	96.00	87.50	-0.390
clone_IGCpG13	92.00	83.33	-0.390
clone_IGCpG14	100.00	100.00	0.000
clone_IGCpG15	100.00	100.00	0.000
clone_IGCpG16	100.00	100.00	0.000
clone_IGCpG17	92.22	100.00	0.976
clone_IGCpG18	80.00	100.00	0.516
clone_IGCpG19	93.34	100.00	0.516
clone_IGCpG20	97.78	100.00	0.516

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clone_IGCpG21	100.00	100.00	0.000
clone_IGCpG22	100.00	100.00	0.000
clone_IGCpG23	97.78	100.00	0.516
clone_IGCpG24	100.00	100.00	0.000
clone_IGCpG25	100.00	100.00	0.000
clone_IGCpG26	100.00	95.83	-1.033
clone_IGCpG27	100.00	100.00	0.000
clone_IGCpG28	100.00	100.00	0.000
clone_IGCpG29	100.00	100.00	0.000
clone_IGCpG30	97.78	87.50	-0.390
clone_IGCpG31	100.00	100.00	0.000

^a $p < 0.1$, $*p < 0.05$

Table 3.4: Pyrosequencing data of sperm samples.

Region	EtOH Mean (%)	Sucrose Mean (%)	Wilcoxon Standardised Statistic
sire_H19CpG2	95.33	94.92	-0.518
sire_H19CpG3	98.83	99.00	-0.604
sire_H19CpG4	100.00	100.00	0.000
sire_H19CpG5	96.92	97.50	-0.407
sire_H19CpG6	94.67	98.83	-0.804
sire_H19CpG7	91.92	93.67	-1.974 *
sire_CTCF1	96.28	97.32	-1.446 ^a
sire_H19CpG11	99.00	95.58	-0.417
sire_H19CpG12	96.25	94.33	1.557 ^a
sire_H19CpG13	97.42	95.75	0.739
sire_H19CpG14	96.58	99.08	-0.982
sire_H19CpG15	96.50	98.25	-0.809
sire_CTCF2	97.15	96.60	0.000
sire_Rasgrf1CpG20	90.25	93.25	-0.887
sire_Rasgrf1CpG21	84.75	91.42	-0.643
sire_Rasgrf1CpG22	69.00	79.33	-1.044
sire_Rasgrf1CpG23	83.75	75.75	1.205
sire_Rasgrf1CpG24	99.58	99.58	0.315
sire_Rasgrf1CpG25	75.50	74.00	0.000
sire_Rasgrf1CpG26	98.58	100.00	-2.197 *
sire_Rasgrf1	85.92	87.62	-0.561
sire_SnrpnCpG6	2.11	3.14	-1.363 ^a

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sire_SnrpnCpG7	5.42	5.75	-0.646
sire_SnrpnCpG8	4.94	5.22	-0.241
sire_SnrpnCpG9	1.25	2.06	-1.529 ^a
sire_SnrpnCpG10	0.72	2.92	-2.666 **
sire_SnrpnCpG11	3.72	4.86	0.000
sire_SnrpnCpG12	2.53	2.69	-0.081
sire_Snrpn	2.96	3.81	-0.721

^a $p < 0.1$, * $p < 0.05$, ** $p < 0.01$

3.2.2. Offspring tail DNA methylation analyses

The methylation statuses of the *H19* CTCF binding sites 1 and 2, the *Rasgrf1* CTCF binding site and the *Snrpn* seven-CpG-site region were quantified using pyrosequencing from the 46 offspring tail biopsy samples (EtOH offspring n=17, sucrose offspring n=29). Pyrosequencing was performed in triplicate for each sample. "Average methylation per CpG site" was calculated by averaging the triplicate values for each site within a given sample. "Average methylation per CpG site" values for each CpG were then averaged across sites to give the total methylation of the sample. A non-parametric one-way ANOVA was performed to determine differences in DNA methylation levels of the four ICRs under investigation between the EtOH and sucrose treated offspring groups.

3.2.2.1. Region specific DNA methylation analyses in offspring

Region specific DNA methylation analyses were conducted for offspring samples

using pyrosequencing data (Table 3.5). The total methylation of the *H19* CTCF 1 of the EtOH offspring was significantly lower than those of the sucrose offspring ($p=0.0017$). Similarly, the *H19* CTCF 2 region was highly significant ($p=0.0007$), with the EtOH offspring demonstrating a reduced DNA methylation profile. No differences in total DNA methylation were observed at the *Rasgrf1* CTCF binding site and *Snrpn* seven-CpG-site region between the two treatment groups. However, Wilcoxon tests revealed that the *Snrpn* region is approaching significance ($p=0.08$). Contrary to findings in the *H19* CTCF binding sites, the *Snrpn* region appeared to be more methylated in the EtOH group.

Table 3.5: Pyrosequencing data of mean offspring DNA methylation of the three ICRs.

<i>Region</i>	<i>EtOH Mean (%)</i>	<i>Sucrose Mean (%)</i>	<i>Wilcoxon Standardised Statistic</i>	
<i>H19 CTCF1</i>	68.61	75.51	-2.92	**
H19CpG2	66.89	73.84	-2.95	**
H19CpG3	68.90	75.95	-3.03	**
H19CpG4	76.07	82.08	-2.45	**
H19CpG5	67.04	73.41	-2.54	**
H19CpG6	68.95	75.75	-2.79	**
H19CpG7	63.83	72.04	-2.79	**
<i>H19 CTCF2</i>	75.23	83.87	-3.20	***
H19CpG11	71.84	84.06	-2.82	**
H19CpG12	70.59	81.28	-3.04	**
H19CpG13	66.08	81.51	-3.12	***
H19CpG14	84.46	86.69	-0.56	
H19CpG15	83.16	85.82	-1.46	^a
<i>Rasgrf1</i>	68.08	65.36	0.82	
Rasgrf1CpG20	74.91	72.62	0.94	
Rasgrf1CpG21	71.84	65.33	0.98	
Rasgrf1CpG22	52.38	49.66	0.31	
Rasgrf1CpG23	54.50	53.48	-0.42	

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Rasgrf1CpG24	86.84	80.95	1.17	
Rasgrf1CpG25	49.00	46.95	1.30	^a
Rasgrf1CpG26	87.06	88.53	-0.95	
<i>Snrpn</i>	63.42	60.65	1.38	^a
SnrpnCpG6	60.71	59.21	1.10	
SnrpnCpG7	64.62	62.41	1.31	^a
SnrpnCpG8	78.74	74.03	2.28	*
SnrpnCpG9	50.00	53.41	-1.69	*
SnrpnCpG10	61.74	58.72	1.06	
SnrpnCpG11	66.53	59.48	2.43	**
SnrpnCpG12	61.65	57.26	1.22	

^a $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

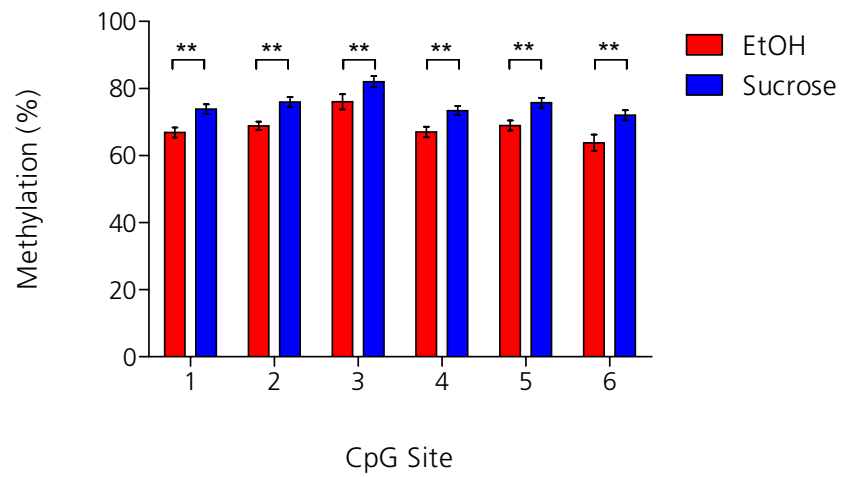
3.2.2.2. DNA methylation analysis of individual CpG sites of offspring

Analysis of individual CpG sites (Table 3.5, Figure 3.5) of the *H19* CTCF 1 revealed that all of the sites (1 – 6) were significantly less methylated in the EtOH offspring ($p < 0.01$). A similar analysis of the *H19* CTCF 2 showed that CpG sites 11 and 12 were significantly different ($p = 0.0024$ and 0.0012 , respectively) and site 13 was highly significant ($p = 0.0009$), while sites 14 and 15 were not statistically different. However, these sites in the EtOH offspring showed a lower methylation trend, with site 15 tending towards significance ($p = 0.072$). Analysis of the *Rasgrf1* CTCF binding site revealed only CpG site 25 to be reaching significance ($p = 0.096$). The seven-CpG-site region of the *Snrpn* ICR revealed significant differences at CpG sites 8, 9 ($p = 0.011$ and 0.0459 , respectively) and CpG 11 was highly significant ($p = 0.0077$). However, mixed observations were

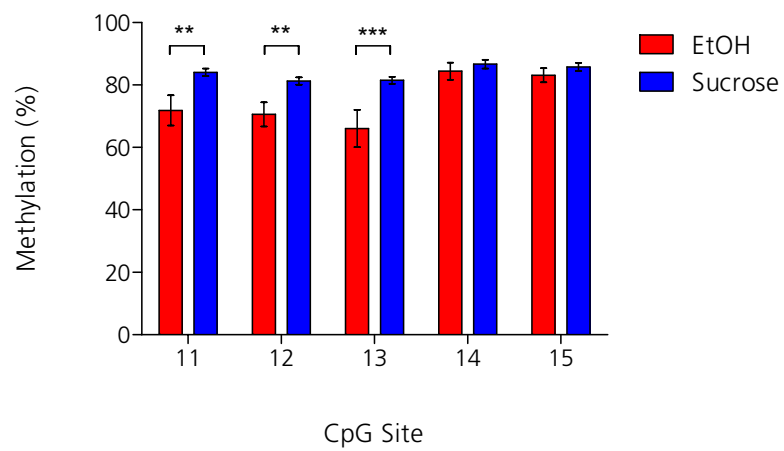
- Results -

noted at these CpG sites. DNA methylation at CpG 9 was decreased in the EtOH group, while CpG sites 8 and 11 showed increased DNA methylation in the EtOH offspring.

H19 ICR CTCF 1



H19 ICR CTCF 2



- Results -

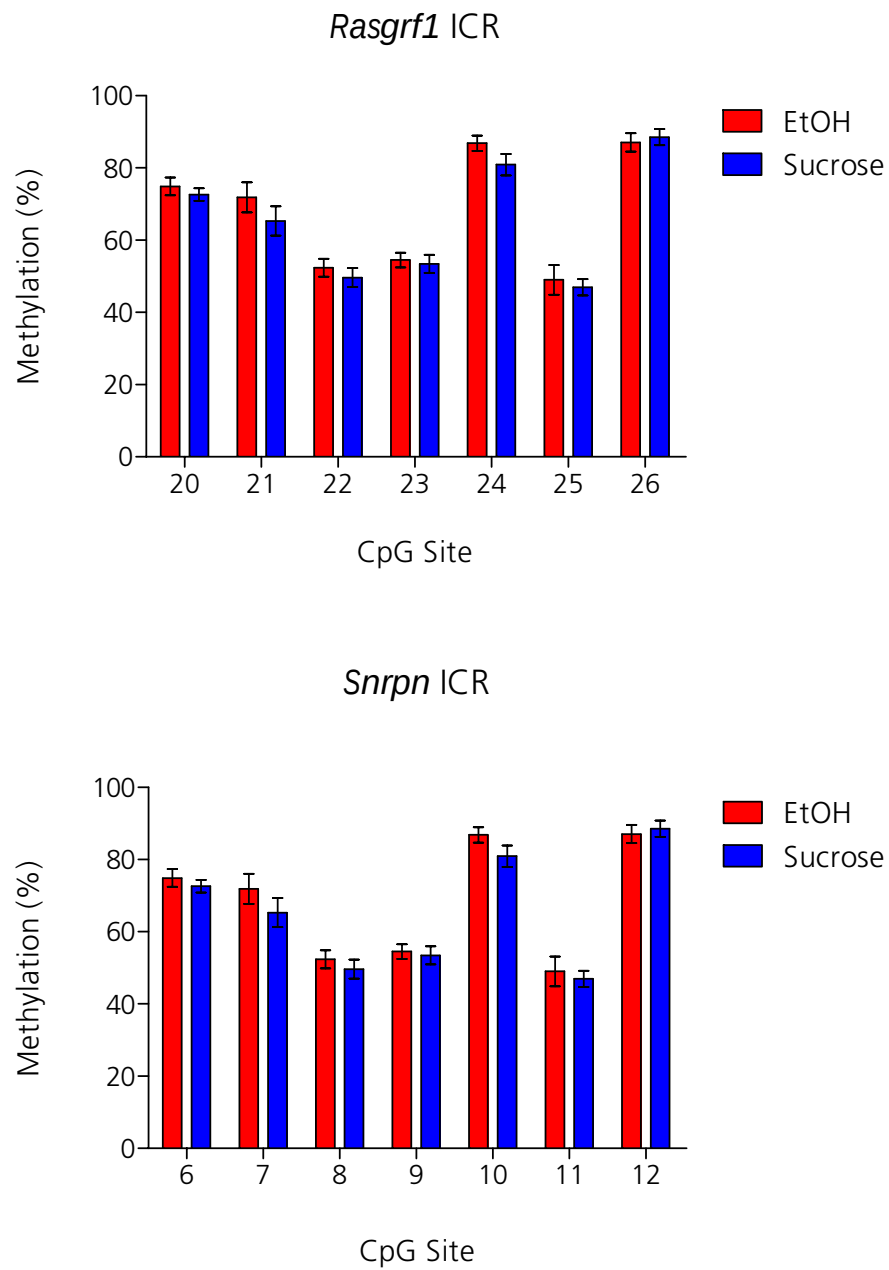


Figure 3.5: DNA methylation of individual CpG sites of offspring from pyrosequencing data.

3.3. Parturition analyses

To ascertain whether the treatment of each sire had an impact on the pregnancy outcome of mated dams, pregnancy success rates (Table 3.6); total number of offspring (Table 3.7); litter sizes (Table 3.8) and offspring sex ratios (Table 3.9) were assessed.

3.3.1. Pregnancy success rate

To assess the success rate of impregnation, each sire was allocated a score out of two, representative of the potential pregnancies of the two dams mated (Table 3.6). A successful impregnation was indicated by the presence of a vaginal plug, and successful fertilisation was indicated by pregnancy and birth of offspring.

The EtOH treated males ($n = 6$) had an overall impregnation success rate of 33.3% or four pregnancies of a possible 12. Similarly, the sucrose treated males ($n = 7$) showed an overall success rate of 35.71% or five of a potential 14 pregnancies.

Table 3.6: Overall success rates of pregnancies by treated males.

<i>Sire</i>	<i>Treatment</i>	<i>No. successful pregnancies (n = 2 females)</i>	<i>Total no. pregnancies</i>	<i>Overall success rate</i>
60	EtOH	0	4	33.33%
62	EtOH	0		
63	EtOH	2		
65	EtOH	0		
66	EtOH	1		

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68	EtOH	1		
61	Sucrose	0		
69	Sucrose	0		
70	Sucrose	1		
71	Sucrose	1	5	35.71%
72	Sucrose	2		
73	Sucrose	0		
74	Sucrose	1		

3.3.2. Total number of sired offspring

The total number of offspring sired in each treatment group was calculated for each successful pregnancy, and included those offspring that were cannibalised after birth. The four pregnancies of the EtOH treated males produced a total of 17 offspring, while the five pregnancies of the sucrose treated males totalled 29 (Table 3.7).

Table 3.7: Total number of offspring sired by treated males.

<i>Sire</i>	<i>Treatment</i>	<i>No. offspring sired</i>	<i>Total no. offspring sired per treatment group</i>
63	EtOH	10	
66	EtOH	1	17
68	EtOH	6	
70	Sucrose	6	
71	Sucrose	5	29
72	Sucrose	12	
74	Sucrose	6	

3.3.3. Litter sizes

The average litter size of the EtOH treated males was 4.25, which was marginally

smaller than that of the sucrose treated group with an average of 5.8 pups/offspring per litter (Table 3.8) and not statistically significant (Wilcoxon two-sample test; $p=0.1874$).

Table 3.8: Average size of litters sired by treated males.

<i>Sire</i>	<i>Treatment</i>	<i>Dam</i>	<i>Litter size</i>	<i>Average litter size</i>
63	EtOH	82	5	4.25
63	EtOH	83	5	
66	EtOH	87	1	
68	EtOH	89	6	
70	Sucrose	90	6	5.8
71	Sucrose	91	5	
72	Sucrose	95	3	
72	Sucrose	100	9	
74	Sucrose	98	6	

3.3.4. Sex ratios of sired offspring

To determine whether the treatment of each male group had an impact on the sex ratio of male and female offspring, the number of each sex in each group was analysed (Table 3.9). These data excluded cannibalised pups as their sex could not be determined. The EtOH group had a male to female sex ratio of 1.6:1; while that of the sucrose group was 1:2.3, respectively. The total number of each sex was not significant between the two treatment groups (Wilcoxon two-sample test; $p=0.4715$).

Table 3.9: Sex ratios of sired offspring.

Sire	Treatment	Dam	Sex ratio		Total ratio	
			M	F	M	F
63	EtOH	82	3	2		
63	EtOH	83	3	2	8	5
68	EtOH	89	2	1		
70	Sucrose	90	4	2		
71	Sucrose	91	1	4	7	16
72	Sucrose	100	2	4		
74	Sucrose	98	0	6		

M=male; F=female

3.4. Mouse weight data

3.4.1. Offspring growth rates

The growth rates of offspring sired by the treated (EtOH and sucrose) male mice were analysed over a 63 day period. Initially weight measurements were taken every second day until 30 days of age, and recorded weekly thereafter (20 time points).

3.4.1.1. Overall growth rates

The overall growth rate of offspring sired by EtOH treated males (n=17) was compared with that of those sired by sucrose treated males (n=29) over the 63 day period. No significant difference was observed between these two groups ($p=0.9461$; Mann-Whitney U test; Figure 3.6).

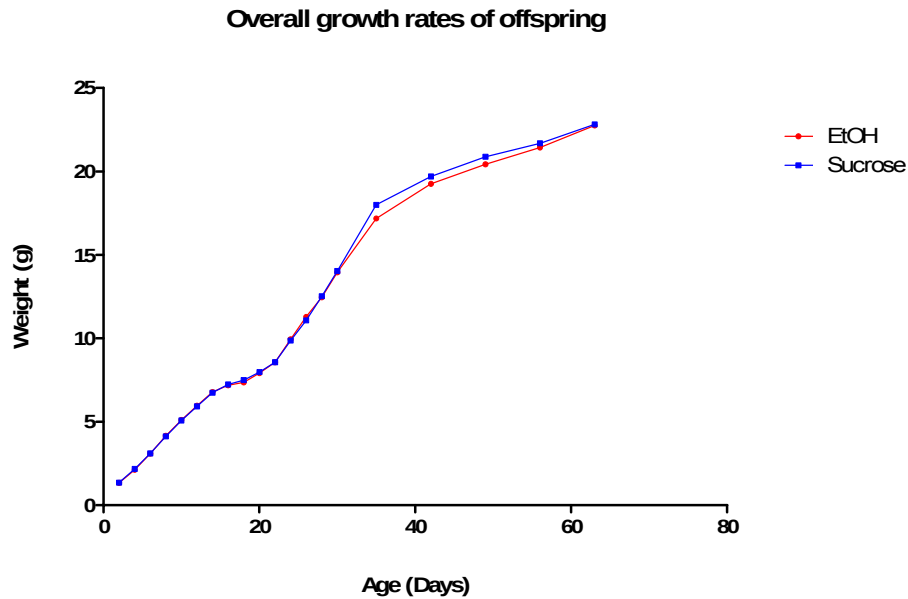


Figure 3.6: Growth rate of EtOH and sucrose offspring over the 63 day period.

3.4.1.2. Male pup vs. female pup growth rates

The growth rates and adult weights of male and female C57BL/6 mice vary greatly, where male mice grow more rapidly and weigh more as adults. Thus, pooling the growth rates of the two pup sexes would inaccurately represent such data, which is skewed when sex bias is present within a sample. Taking this into consideration, the growth rate and weight at the 20 time points of each sex in both treatment groups was compared separately using a non-parametric one-way ANOVA (Table 3.10).

No significant difference in growth rates between the two female offspring

groups was observed, while that of the male offspring tends towards significance ($p=0.0844$). However, there was a notable trend between these groups with both EtOH male and female offspring exhibiting a lower growth rate compared to their sucrose counterparts (Figure 3.7). This trend is most pronounced after day 30. Upon further investigation of offspring weight on a day-by-day basis revealed that the divergence of sucrose male offspring from EtOH male offspring was significant at days 35, 42 and 49 ($p=0.0221$, 0.0356 and 0.0356 , respectively), with day 56 approaching significance ($p=0.0503$). Similarly, female EtOH offspring showed lower weights than their sucrose counterparts at days 35 and 42 ($p=0.0196$ and 0.0196 , respectively), with days 49 and 56 approaching significance ($p=0.052$ and 0.087 , respectively).

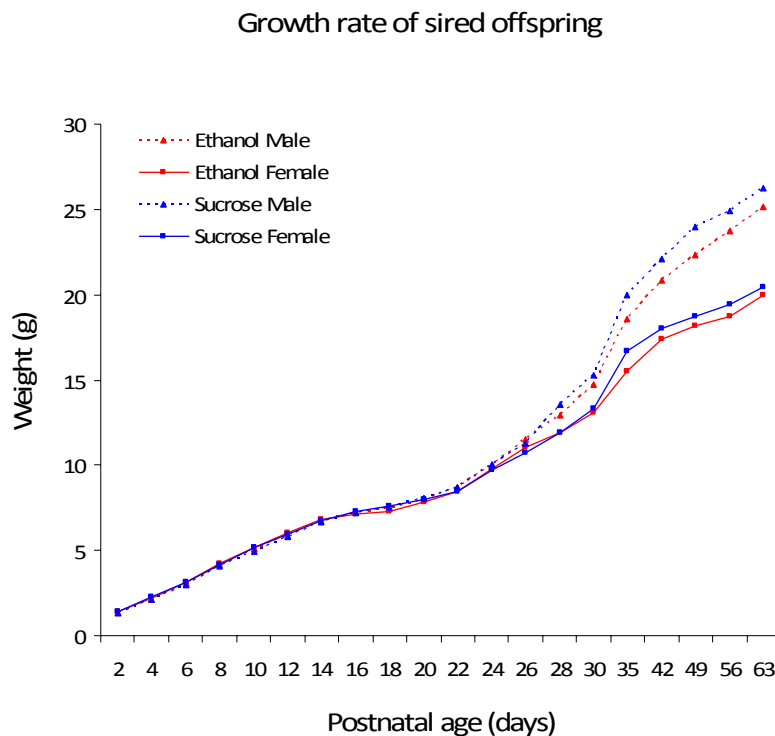


Figure 3.7: Growth rate of EtOH and sucrose offspring separated by sex over the 63 day period.

- Results -

Table 3.10: Comparison of offspring growth rate and weight over the 63 day period.

<i>Offspring Sex</i>	<i>Growth variable</i>	<i>EtOH Mean Weight (g)</i>	<i>Sucrose Mean Weight (g)</i>	<i>Wilcoxon Standardised Statistic</i>
Male	Growth_Rate	0.38	0.40	-1.38 ^a
	Day2	1.32	1.33	-0.11
	Day4	2.09	2.11	0.00
	Day6	3.07	2.99	0.11
	Day8	4.12	4.09	-0.32
	Day10	5.06	4.94	0.00
	Day12	5.90	5.81	-0.42
	Day14	6.73	6.66	-0.53
	Day16	7.20	7.18	-0.42
	Day18	7.42	7.56	-0.85
	Day20	7.96	8.06	-0.74
	Day22	8.68	8.72	-0.85
	Day24	10.06	10.00	-0.37
	Day26	11.50	11.29	0.42
	Day28	12.93	13.53	-1.06
	Day30	14.71	15.29	-0.69
	Day35	18.59	19.95	-2.01*
	Day42	20.87	22.11	-1.80*
	Day49	22.36	23.96	-1.80*
	Day56	23.73	24.89	-1.64 ^a
	Day63	25.17	26.27	-1.27
Female	Growth_Rate	0.29	0.30	-0.66
	Day2	1.37	1.38	-0.33
	Day4	2.16	2.25	-0.78
	Day6	3.13	3.17	-0.62
	Day8	4.21	4.12	0.29
	Day10	5.16	5.14	0.04
	Day12	6.03	5.96	-0.08
	Day14	6.83	6.77	-0.37
	Day16	7.16	7.30	-0.37
	Day18	7.28	7.60	-0.62
	Day20	7.85	7.97	-0.87
	Day22	8.44	8.47	-0.78
	Day24	9.80	9.69	0.04
	Day26	11.04	10.73	0.87
	Day28	11.90	11.93	-0.33
	Day30	13.08	13.28	-0.37
	Day35	15.53	16.85	-2.06*
	Day42	17.37	18.03	-2.06*

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Day49	18.17	18.73	-1.63 ^a
Day56	18.76	19.46	-1.36 ^a
Day63	19.95	20.41	-0.75

^a $p < 0.1$, * $p < 0.05$

3.4.1.3. Litter analyses

Offspring from smaller litters generally weigh more at birth than those born from larger litters (Reading, 1966). If litter size is a significant underlying factor, it may confound data analyses.

In order to assess whether this bias was present within the seven litters analysed in this study (the remaining two litters from mothers 87 and 89 were cannibalised shortly after birth and therefore could not be included) and whether this influenced offspring growth rates, the “birth” weight (weight at first time point, day 2) and growth rate of each litter was correlated with litter size. Analyses were further grouped by sex and treatment. Spearman’s correlations revealed no significant correlations in the sucrose male, EtOH male and EtOH female offspring groups ($p > 0.05$). However, a significant negative correlation with the sucrose female offspring group was observed (Spearman correlation coefficient = -0.69754; $p = 0.0055$).

3.5. Correlation analyses – direct relationships between DNA methylation and offspring weight

The relationship between DNA methylation and offspring weight was analysed in a correlation matrix. Analyses were conducted on offspring DNA methylation profiles at the region specific level (including the *H19* CTCF binding sites 1 and 2, *Rasgrf1* CTCF, the *Snrpn* seven-CpG-site region) and at the level of individual CpG sites, which were correlated with offspring growth rate and weight at each of the 20 time points (day 2 – day 63) (Appendix N).

DNA methylation at the *H19* CTCF 1 region was significantly correlated with offspring weight at day 35 ($p=0.0348$), and approaching significance at day 42 ($p=0.0966$). At the level of individual CpG sites, a general pattern of significance was observed for all CTCF 1 CpG sites (2 – 7) around days 30 – 42 ($p<0.05$). The *H19* CTCF 2 region was significantly correlated with offspring weight at days 14 – 22 ($p<0.05$) and again at days 30 – 56 ($p<0.05$). However, at the CpG level, no significant correlations were seen at days 14 – 22 for any of the sites, while significance was only noted for all CpG sites (11 – 15) at day 35 ($p<0.05$).

DNA methylation at the *Rasgrf1* CTCF region was significantly correlated with offspring weight at day 6 ($p=0.0193$), with days 2 and 4 approaching significance ($p=0.0877$ and 0.0633 , respectively). Correlation at these days was a general trend for all CpG sites (20 – 27), with the exception of CpG 25, which showed no correlation with offspring weight.

The DNA methylation profile of the seven-CpG-site *Snrpn* region showed a weak correlation with the overall growth rate of the offspring ($p=0.0854$), and significant correlations at days 35 and 56 ($p=0.0477$ and 0.038 , respectively). Similarly, CpG sites 9 and 10 were significantly correlated with offspring growth rate ($p=0.0171$ and 0.0454 , respectively), with sites 7 and 12 approaching significance ($p=0.0665$ and 0.0516 , respectively). Significant correlations were noted at days 35 for CpG sites 9, 10 and 12, and day 56 for sites 7, 9, 10 and 12.

The correlations described above were all found to be positive. That is, the relationship between DNA methylation and offspring weight was directly proportionate – when DNA methylation increases, weight tends to increase, and *vice versa*.

Overall, significant correlations between DNA methylation and offspring weight occurred in unique block-like patterns. That is, DNA methylation profiles of *H19* (CTCF 1 and 2) were highly correlated with offspring weight at days 28 – 56, *Rasgrf1* CTCF with days 2 – 6, and *Snrpn* with days 35 and 56. These patterns seemed to be maintained in a strict day-specific manner, and significant observations rarely occurred outside of these well defined “correlation blocks”.

3.6. Mediation analyses

The relationship between DNA methylation and offspring weight has thus far been

established, where correlations were observed in strict blocks. Furthermore, it has been shown that EtOH exposure (through the sire) is able to decrease both methylation and weight of the offspring. It is now required to determine whether treatment (EtOH) of the offspring sires is responsible for the variation in offspring weight, but mediated by DNA methylation, otherwise known as the “indirect effect” of treatment (Appendix O).

Direct effect and indirect effect (PATHAB when DNA methylation of each region was controlled for - PATHBEST) of treatment on offspring weight was calculated at days 30 – 56. When these two effects are added, the total effect of treatment on average offspring weight is calculated.

Analyses revealed only two regions that significantly mediate the effect of EtOH on decreased offspring weight, albeit it at the 10% level. The direct effect of treatment (EtOH) decreased the average mouse weight by 1.5g and the indirect of EtOH decreased average mouse weight by 0.36g when mediated through the *H19* CTCF 1 region at day 35, therefore decreasing the average weight of offspring by an overall of 1.90g (1.54g + 0.36g). Similarly, the direct effect of EtOH on average offspring weight is a decrease of 1.75g, and an indirect effect of a 0.13g decrease when mediated through *H19* CpG12 at day 35, giving a total decreased effect on average offspring weight of 1.88g. Although not significant at the level of mediation (PATHAB), the total effect of treatment (EtOH) at all CpGs/regions was negative, except for *H19* CpG 3.

Results of the mediation analyses also revealed that the direct effect of EtOH at all methylation regions was negative; that is, that the effect of treatment of the sire with EtOH reduced the average offspring weight at all CpG sites. Furthermore, day 35 predominantly ranked among the top 10 total effects in decreasing average offspring weight. These included *H19* CTCF 2, along with the CpG sites which comprise this region that were shown to be significantly demethylated in the EtOH offspring group (CpG 11, 12, 13 and 15). *H19* CTCF 1 demonstrated high reductions in average offspring weight at days 30, 35, 42 and 49, which is consistent with findings of significantly reduced growth in offspring around this period.

Despite demonstrating a decreased direct effect on average offspring weight at all five days (days 30, 35, 42, 49 and 56), the mediating effect (PATHAB) of treatment through *Snrpn* CpG sites showed a trend in increasing average offspring weight (PATHAEST) and/or DNA methylation (PATHBEST) at all five days.

3.7. Multilevel modelling

In order to determine the effect of treatment on offspring weight in a hierarchical fashion, multilevel regression was performed (Appendix L). This was assessed using offspring weight as the outcome variable once each region/CpG site was controlled for in the model. Analyses revealed that when the data is hierarchically structured, the effect of sire treatment with EtOH (treatment~1) decreases the average offspring weight (indicated by a negative coefficient (Coef.) value) by approximately 1.2g and 1g at the day 30 – 39 and day 40 – 63 period, respectively at both the region specific

and individual CpG site level. These results were insignificant, with the period day 40 – 63 at the region specific level approaching significance ($p < 0.01$).

Region specific analyses confirmed that females (sex_1) weighed significantly less than males at both time periods. Females were 2.4g and 4.8g lighter at day 30 – 39 ($p = 0.001$) and day 40 – 63 ($p < 0.001$), respectively.

However, all regions and individual CpG sites insignificantly affected average offspring weight. At the region specific level, treatment with EtOH marginally decreased the average offspring weight (day 30 – 39 = 0.01g, day 40 – 63 = 0.03g) when the *H19* CTCF 1 (ctcf1) region was controlled for, while other regions seemed to marginally increase average offspring weight (indicated by a positive coefficient value). At the level of individual CpG sites, similar conflicting results were noted, with some CpGs within each region demonstrating a slight decrease in average offspring weight, while others seemed to increase offspring weight.

Chapter 4

4. Discussion

4.1. Outline of discussion

The findings of this study are consistent with the prediction that treatment of male mice with EtOH has an adverse effect on sired offspring. This includes both a reduction in DNA methylation and growth retardation.

At the level of offspring DNA methylation, strong evidence was observed at the *H19* ICR, at both CTCF binding sites, while the *Rasgrf1* CTCF binding site and the *Snrpn* ICR demonstrated less dramatic effects. Furthermore, pronounced growth retardation was observed in offspring of EtOH treated sires from approximately days 35 to 56. In addition, strong positive correlations of offspring DNA methylation at the *H19* CTCF 1 and CTCF 2 binding sites with offspring weight were evident around days 35 to 56. Although sire treatment was significantly correlated with offspring DNA methylation and weight, mediation analyses revealed that offspring DNA methylation was not a significant mediator of the relationship between sire treatment and offspring weight. That is, the growth retardation observed in the EtOH offspring group was not shown to be mediated through the reduction in offspring DNA methylation.

These findings would suggest that treatment of male mice with EtOH prior to conception alters sperm in an unknown manner, postulated as possibly an alteration to DNA methylation, which in turn is inherited by their offspring, accounting for the observed reduction in offspring DNA methylation within this group. Surprisingly however, no significant reduction in sperm DNA methylation profiles was evident in the EtOH treated male group.

The discussion that follows explores the implications of each of these findings. Firstly, the evidence (or lack thereof) of decreased DNA methylation in sperm and offspring as a result of excessive paternal EtOH exposure will be assessed in accordance with the hypothesis of this study. Secondly, possible mechanisms accounting for the altered epigenome evident in the EtOH offspring group and their implications will be explored. Thirdly, the relationships between sire treatment, offspring DNA methylation, and offspring growth are explored. And lastly, the implications of the loss of imprinting observed in the offspring of EtOH exposed sires in conjunction with their adverse outcome will be explored in terms of epigenetic control of imprinted loci.

4.2. Rationale for inclusion of the maternally imprinted *Snrpn* ICR in methylation analyses

Despite including maternally imprinted *Snrpn* in this study for the sole purpose of

detecting somatic DNA contamination in sperm samples, the *Snrpn* region was analysed along with the paternally imprinted regions of the offspring. The reason for this was because during analyses for somatic DNA contamination detection (primarily based on pyrosequencing data), it was noted that this ICR was not completely devoid of methylation on the paternal allele, but instead was hypomethylated. That is, a low level of “basal” methylation was detected. Moreover, on closer inspection, when the levels of “basal” *Snrpn* DNA methylation on the paternal allele were compared between the two treatment groups, there seemed to be a trend for lower levels of methylation across the EtOH group. As this finding seemed to support the hypothesis of the current study, it was decided to include the *Snrpn* ICR in subsequent analyses.

4.3. The effect of excessive EtOH exposure on sperm DNA methylation

In the present study, the exposure of male mice to 7.5µl/g of a 50% EtOH solution over a period of five weeks did not appear to cause dramatic effects in sperm DNA methylation profiles. Sequence data showed that of all the CpG sites contained within the four ICRs (*H19*, *IG*, *Rasgrf1* and *Snrpn*), only three out of the 92 showed significantly altered DNA methylation, all of which were contained within the *Rasgrf1* ICR. In accordance with our hypothesis, *Rasgrf1* CpGs 17 and 22 showed a reduction in DNA methylation in the EtOH group ($p < 0.1$). However, contrary to the current hypothesis, the sucrose group demonstrated a lower level

of methylation at *Rasgrf1* CpG 7, where the largest difference in the level of DNA methylation was observed ($p < 0.05$). These conflicting findings may be explained by the fact that the number of clones analysed were very limited. The reduced level of methylation at *Rasgrf1* CpG 7 is primarily derived from three sperm samples (61, 71 and 73). In particular, sample 61 showed a 50% level of methylation at this CpG. However, this value was quantified from only six clone sequences. Similarly, CpG 7 methylation levels of samples 71 and 73 were 80% and 87.5%, respectively, but were the result of only a single clone in each sample being unmethylated. Thus, the dramatic decreases in DNA methylation observed in these samples was merely due to limited clones, where a single unmethylated site within one clone could result in a 20% decrease in DNA methylation at a CpG for a particular sample (e.g. 71). This could therefore be argued not to accurately represent the level of DNA methylation. The same argument could be made for the reduced levels of methylation observed at CpG 22. However, the decrease in methylation at CpG 17 could be an accurate representation of the sample, as this level of methylation was quantified from a larger number of clones ($n=22$). There therefore seems only to be reliable evidence for a reduction in the level of DNA methylation at *Rasgrf1* CpG 17 in the EtOH treated group based on sequence (clone) data.

Visual inspection of sequence data did not appear to show any distinct patterns of reduced DNA methylation at linked CpGs. According to the literature discussed previously, EtOH (through the metabolite, acetaldehyde) reduces the availability of the methyl group donor (SAM) required for DNA methylation reactions by the

Dnmt enzymes (Halsted et al., 2002a). We might therefore expect that if transmethylation reactions were hampered, large “blocks” of unmethylated regions would be observed in sequence data of aligned clones, where a number of consecutive CpGs would have been unsuccessfully methylated. This was not observed in our data. Instead, the very few CpGs that were unmethylated seemed to be distributed randomly and it was very rare to observe two consecutively unmethylated CpGs, while more than this was never seen.

Similar to the sequence data, pyrosequencing data revealed only a select number of CpGs with significantly reduced levels of methylation. Although more than two consecutive significantly unmethylated CpGs were not observed, affected CpG sites did tend to cluster, often being within one or two CpGs from each other. Although this trend is based on very few observations, it is more consistent with a model where EtOH disrupts Dnmt activity, resulting in stretches of unmethylated CpG sites.

However, unlike the sequence data, reduced methylation was observed across all three of the ICRs (pyrosequencing data was not obtained for the *IG*-DMR), including the *Snrpn* ICR. Due to the robust quantification of pyrosequencing, these data probably reflect the DNA methylation profiles of the three ICRs more accurately. Thus, the decreased level of methylation observed at *H19* CpG 12 in the sucrose group is likely to be genuine and is therefore contrary to our hypothesis. However, this is an isolated CpG site and on its own does not provide evidence compelling enough to disregard the validity of the hypothesis put

forward in this study.

In summary, large-scale demethylation induced by excessive EtOH exposure was not evident in the sperm of treated male mice. However, significant effects were noted at the level of individual CpG sites which generally showed lower levels of methylation in the EtOH treated male group, which agrees with previous findings (Ouko et al., article in press). Moreover, there was some evidence that affected CpG sites tended to cluster, suggesting possible disruption in the DNA transmethylation pathway as a result of EtOH exposure.

4.3.1. Ethanol-induced DNA demethylation may be masked in sperm samples

Based on the empirical findings of this study where no significant, large-scale differences were observed in the paternal sperm DNA methylation profiles as was anticipated, one can only speculate on potential mechanisms that could account for the growth retardation phenotype and significant reductions in DNA methylation observed in the EtOH sired offspring.

Although a trend at the *H19* CTCF 1 region and significantly lower levels of DNA methylation at *H19* CpG 7 and *Rasgrf1* CpG 26 were observed in the EtOH group, it is interesting to note that the *Snrpn* region contained three CpG sites significantly affected by EtOH. Although two of these sites were only significant at the 1% level (CpGs 6 and 9), *Snrpn* CpG 10 displayed the most dramatic

decrease in methylation ($p < 0.004$) of all the CpGs of the three ICRs in sperm DNA. The implications of this finding may suggest that because unlike *H19* and *Rasgrf1*, the *Snrpn* ICR is maternally imprinted and therefore hypomethylated (not imprinted) in male germ cells, it may escape mechanisms that ensure the correct establishment of paternal imprints during spermatogenesis. Being unchecked, the effects of EtOH may be more pronounced at this region, accounting for the higher frequency of altered CpG methylation levels at the *Snrpn* ICR, as well as the highly significant reduction observed at *Snrpn* CpG 10. By implication, this theory also suggests that if these regulatory mechanisms that ensure the correct establishment of paternal imprints during spermatogenesis do exist, then aberrations (losses) in paternal DNA methylation at imprinted loci as a result of EtOH exposure may be corrected at later stages of spermatogenesis and would therefore be unobserved in mature spermatozoa. Alternatively, this regulatory mechanism may make these regions more resistant to changes in DNA methylation. By way of one or both of these scenarios this regulatory mechanism would act to safe-guard and maintain the integrity of paternal imprints in spermatozoa. This theory may explain the lack of observed significant, widespread decreases in DNA methylation in the sperm of male mice exposed to EtOH in this study.

In addition, the sperm samples obtained from the treated males contain a population of mature spermatozoa that have been exposed to EtOH metabolites, but would also be representative of individual spermatozoa that have been affected to varying degrees. Thus each sperm could potentially represent a

uniquely altered epigenetic profile, which may be unobservable when quantifying the “total” or average methylation for a particular sperm sample which is comprised of a milieu of potentially divergent epigenomes. Furthermore, the negative effects of EtOH on DNA methylation may only impact on a fraction of the total sperm population, while the vast majority of the spermatozoa maintain “normal” DNA methylation profiles, masking the altered epigenomes. This argument may account for the lack of reduced DNA methylation observed in the EtOH treated male sperm samples.

4.4. The effect of paternal treatment on offspring outcome and development

The difficulty with studying the effect of alcohol on spermatozoa is that treatment often renders the sperm inactive or damages it to such an extent that it is unable to penetrate and fertilize the ovum (Stockard, 1913). However, these severe effects are noted almost exclusively in chronic alcoholics who have been abusing alcohol over many years, resulting in atrophy of the testicles and spermatogonia (Stockard, 1913). This is an important consideration as the aim of this study was to determine the effect of alcohol on sperm DNA methylation, an epigenetic factor, and not its cytotoxic effects. The male mice used in this study were treated with either alcohol or sucrose over a 35 day period. This ensured that treatment was performed for the duration of a single complete cycle of mouse spermatogenesis. The dosage of alcohol administered mimicked chronic

alcoholism in humans, but over a period that was short enough so as to avoid severe cytotoxic effects (Stockard, 1913).

4.4.1. The effect of sire treatment on fecundity

4.4.1.1. Pregnancy success rates

The results of this study demonstrated very low pregnancy success rates of dams by both EtOH and sucrose treated sire groups (33.33% and 35.71%, respectively). Contrary to these findings, previous studies on paternal preconception alcohol exposure have reported rates of approximately 20 - 44% and 90 - 100% in EtOH and sucrose groups, respectively (Stockard, 1913; Anderson and Willis, 1981; Abel and Tan, 1988; Cicero et al., 1994a). However, in agreement with the findings of this study Cicero (1990) and Abel (1989) did not find significant differences in success rates between the two treatment groups.

4.4.1.2. Litter sizes

The average litter sizes of the EtOH and sucrose dam groups were relatively low in this study, with no significant differences between the two (4.25 and 5.8, respectively). The average litter size of female C57BL/6 mice is around seven pups. However, a female mouse's first pregnancy generally yields a smaller litter

than subsequent pregnancies, which may account for the findings of this study as nulliparous females were used. Whether the findings of the current study agree with those of others is difficult to determine as varying concentrations of EtOH, exposure periods and rodent species were used, which is further confounded with often opposing results in studies with similar methodologies. However, both rodent models (mouse and rat) seem to suggest that paternal exposure to EtOH (despite being administered at different concentrations and exposure periods) does not affect litter size (Anderson and Willis, 1981; Abel and Tan, 1988; Abel and Lee, 1988; Abel, 1989b; Abel, 1993). Evidence to the contrary stems from studies using only rat models (Cicero et al., 1990; Cicero et al., 1994a), and may reflect species specific differences. Paternal alcohol exposure may have paradoxical effects in mice and rats (Abel and Bilitzke, 1990) as the offspring of alcohol exposed male mice were more immobile than the offspring of male rats exposed to alcohol, which showed decreased immobility in swimming tests. The opposite behavioural phenotype observed in this study provides evidence for species specific paternally-mediated effects. This may, in part, explain the findings of our study where a mouse model was employed.

4.4.1.3. Total number of offspring

The effect of preconception paternal alcohol exposure on the total number of offspring sired by treated males differs between rodent models. Findings of this study suggest that despite a relatively equal pregnancy success rates and litter sizes between the two treatment groups, far fewer offspring were obtained from

the EtOH group when compared to the sucrose control group (n=17 and 29, respectively). Livey et al. (2004) found no differences in the total number of offspring between the two treatment groups using a C57BL/6 mouse model. However, this study used a 3g/kg dosage of alcohol (equivalent to 6µl/g), while the current study used a higher dosage (7.5µl/g). Findings of the present study agree with those of Cicero (1994), who exposed male rats to alcohol doses of 5g/kg (equivalent to 10µl/g) and observed a significant decrease in total offspring in the EtOH group (n=30) when compared to the control group (n=60). It could be argued that the alcohol concentration used in this study is closer to that of Livey (2004) – only 1.5µl/g higher – while Cicero (1994a) used a concentration 2.5µl/g higher than the present study. However, Livey (2004) exposed male mice for 60 days with no effect, while the present study exposed males for only 35 days with a significant effect on offspring numbers. This suggests that an alcohol dose of 6µl/g may be insufficient in mediating a significant effect, while doses of 7.5µl/g and 10µl/g are significant, even over a shorter exposure period. It is also important to note that Cicero (1994) observed significant reduction in total offspring numbers by only exposing male rats to a single dose of 10µl/g 24 hours prior to conception. This once-off, high dose of alcohol is synonymous to binge-like behaviour observed in human alcoholics.

4.4.1.4. Postnatal mortality rates

Stockard (1913) observed that male guinea pigs chronically treated with alcohol gave rise to defective embryos or weakened offspring that died shortly after birth.

This has been corroborated by Cicero (1994a) who noted a 2.5% postnatal mortality rate in the control group, while this was doubled in the EtOH group. In the present study, similar postnatal mortality rates in both offspring groups were observed (sucrose = 20.7%; EtOH = 19.0%). In agreement with this study, others observed equal postnatal mortality rates in both groups (Anderson and Willis, 1981; Abel and Moore, 1987; Abel and Lee, 1988; Abel, 1989a; Cicero et al., 1990; Abel, 1993).

4.4.1.5. Sex ratios

The effect of paternal alcohol exposure on sex ratios has been observed in a number of studies. Findings have been mixed with studies showing significantly decreased male to female ratios in litters sired by alcohol exposed fathers (Abel, 1993), while others demonstrate dramatic increases in females (Anderson et al., 1978; Anderson and Willis, 1981). However, the majority of findings report no changes in sex ratios between the two groups (Klassen and Persaud, 1978; Randall et al., 1982; Leichter, 1986; Abel and Tan, 1988; Abel, 1989a; Cicero et al., 1990). Contrary to all of these findings, the present study observed relatively equal sex ratios in the EtOH group (M:F = 8:5), while more than double the number of females were present in the control group (M:F = 7:16). This unusual finding may be a consequence of a combination of factors resulting in a highly skewed sex ratio. Firstly, the total number of offspring obtained for the sucrose group was relatively small in comparison to previous studies that obtained roughly 60 offspring (Anderson and Willis, 1981) or approximately 25 litters for

analyses. Secondly, the sex of six offspring from the sucrose group could not be determined; accounting for 20% of the group, and thus male offspring might have been overlooked. Lastly, considering the small sample size, sex ratio would have been dramatically biased by litter number 98, where all six pups were female. Thus, the control group may reflect an unrealistic representation of sex ratio. Furthermore, due to the smaller sample size in the EtOH group and missing sex data for four pups (23.4% of the total EtOH offspring), sex ratio in this group may also not be truly representative.

4.4.1.6. Offspring growth

Although there was no significant difference in the growth rates of offspring sired by EtOH or sucrose treated sires observed, there was a definite trend demonstrating the growth rates of pups sired by EtOH treated males to be weaker than those sired by sucrose treated males. This trend was observed from approximately day 28 onwards, where days 35 to 56 showed significantly reduced weights of EtOH offspring when compared to controls.

It has been shown that a single dose of alcohol orally administered to a male 24 hours prior to conception tends to result in lower body weights of their sired offspring (Cicero et al., 1994b). Longer exposure periods (three months) of males to alcohol prior to breeding have also reported decreased birth weights in offspring (Ledig et al., 1998). However, no significant differences were observed in birth weight in our study, even when separated by sex. A number of studies

using rodent models support our findings (Anderson and Willis, 1981; Abel and Tan, 1988; Abel and Lee, 1988; Abel, 1989a; Abel, 1993), which has also been reported in humans (Passaro et al., 1998).

The growth rate data of this study agrees with that of Stockard and Papanicolaou (Stockard, 1913; Stockard and Papanicolaou, 1916b; Stockard and Papanicolaou, 1916a) who observed that the growth rate of the control offspring (sired by sucrose treated males) was superior to that of offspring sired by alcohol treated males. Studies on alcoholic men also noted significant decreases in the postnatal growth of their children (Abel and Sokol, 1983; Little and Sing, 1987)

4.4.1.7. Physical development

It was found that male guinea pigs who were treated with alcohol fumes and subsequently mated with healthy females, gave rise to offspring that were defective (Stockard and Papanicolaou, 1916b). These defects were present from the F_1 generation (the first litter sired by the alcohol treated males) and included increased mortality rates and deformities of the eyes and brain. In the present study, anatomical measurements (crown-rump length, head length and the distance between the eyes) as well as visual observations of eye condition were recorded for each pup from day 2 to day 63.

No gross abnormalities of the eyes or any other organ were observed, while anatomical measurements were difficult to record accurately and were thus

unreliable (data not shown). However, no obvious anatomical differences were noted between the offspring of the two treatment groups. This agrees with previous findings which reported no significant difference in the crown-rump length (Livy et al., 2004) and the lack of gross abnormalities in offspring of EtOH exposed sires (Anderson and Willis, 1981; Cicero et al., 1994a). Although the findings of this study are not consistent with those of Stockard and Papanicolaou (1916a) it must be noted that these researchers used a larger sample size (90 matings, producing over 50 litters).

4.4.2. Summary of findings of the outcome offspring sired by EtOH treated males

Together, these findings suggest that frequency of exposure and alcohol dosage (volume intake) prior to conception are significant factors that can influence offspring outcome. Furthermore, binge-like behaviour can have dramatic and perhaps a more pronounced deleterious effect on offspring.

Data from the current study revealed that exposure of male mice for five weeks preconception does not have a significant impact on the birth weight of offspring, but does result in significantly fewer offspring who show significant growth retardation from approximately days 35 to 56. The implication of this finding suggests that the deleterious effects of paternal alcohol exposure prior to conception affect genetic or epigenetic elements in the sperm that are active during postnatal development, particularly during the five to eight week period.

4.5. The effect of paternal alcohol exposure on offspring imprints

Despite the absence of large-scale, alcohol-induced demethylation at imprinted loci in the sperm of males, the offspring of the EtOH treated sires displayed significantly reduced DNA methylation at paternally imprinted regions. In particular, the *H19* CTCF binding sites appeared to be most affected. Overall, the methylation content of *H19* CTCF 1 was significantly reduced ($p < 0.05$), which was echoed at each of the six CpG sites that comprise the region (CpG sites 2 – 7). *H19* CTCF 2 was most affected ($p < 0.001$), but inspection of individual CpGs revealed that only CpG 11 to 13 were significantly hypomethylated when compared to the sucrose control group. Conversely, although significance was observed at *Rasgrf1* CpG 25, *Rasgrf1* CTCF region appeared to be largely unaffected in offspring.

4.5.1. Gain of methylation at the paternally hypomethylated *Snrpn* ICR

Interestingly, although site-specific reductions in DNA methylation were observed at the *Snrpn* seven-CpG-site region in sperm, methylation levels were increased on the paternal allele in offspring. This finding was further confirmed in mediation data, where treatment (EtOH) caused increases in offspring DNA methylation at *Snrpn* CpG sites. Similar findings have been reported (Haycock

and Ramsay, 2009) which found gains in DNA methylation on the maternal allele at the *H19* ICR when embryos were exposed to EtOH during the preimplantation period. As discussed previously, regulation of imprinted loci involves the interplay of the CTCF insulator protein with its binding site. When bound, CTCF maintains a hypomethylated state by actively preventing DNA methylation (Fedoriw et al., 2004). Therefore, loss of methylation at paternally methylated CTCF binding sites would increase demands for CTCF, reducing the pool of factors available for normally unmethylated maternal alleles. This in turn may result in decreased binding of CTCF on hypomethylated ICRs, allowing for *de novo* methylation (Engel et al., 2006). In conjunction with our findings of significantly reduced methylation at the paternal *H19* ICR, this may explain the gain of methylation observed at the normally hypomethylated *Snrpn* region. However, despite the plausibility of this mechanism, a CTCF binding site has not been reported in the *Snrpn* region, but may suggest the presence of a putative CTCF binding site.

4.5.2. Evidence of inherited reduced methylation in offspring

A significant reduction of DNA methylation in EtOH sired offspring was observed at the *H19* CTCF 1 region, *H19* CpG 7 and *H19* CpG 13. This was also evident at these regions in the sperm of EtOH treated sires. This correlation suggests that the loss of methylation induced by EtOH exposure in the sperm may be inherited as an altered epimutation by sired offspring.

However, although DNA methylation at *Rasgrf1* CpG 26 was significantly reduced in the sperm, a significant reduction was not observed at the corresponding site in offspring. Furthermore, one would expect that CpG sites/regions in the offspring showing the most significant reductions in DNA methylation to be correlated in the sperm, even if at lower significance. This was observed at *H19* CpG 12, but not at the *H19* CTCF 2 region and *H19* CpG 13, which showed a highly reduced level in the offspring, but was not significant in the sperm.

These findings show an imbalance of reduced DNA methylation at corresponding sites in the sperm and sired offspring. Sperm profiles show only a few regions of significant losses in DNA methylation, which for the most part, are also significant in the offspring. Conversely, offspring show highly significant reductions at regions that are insignificant in the sperm.

As previously proposed, the presence of a minor pool of epigenetically altered spermatozoa may be undetected in the overall sperm sample due to the presence of unaffected/normal sperm which make up the majority. It would therefore be plausible that a single sperm, whose epigenome has been altered as a consequence of EtOH exposure, could fertilize an oocyte and give rise to an embryo/offspring that would inherit the “mutant” paternal epigenotype. Thus, when DNA methylation is quantified at a particular locus in both the sire and offspring, the profile of the father’s sperm sample may appear unaffected, while the offspring show reduced DNA methylation (as observed in this study). This is

supported by Abel (1993) who found an increased frequency of runts sired by EtOH exposed male rats, suggesting that alcohol had an effect on individual sperm as opposed to affecting entire sperm production.

However, according to the above argument, it would follow that if only a fraction of the sperm population would be affected by EtOH exposure and that this effect would be limited to such an extent that it is undetected in sperm samples; then only a very small proportion of the offspring would have inherited the altered epigenotype. Considering the small sample size of the EtOH sired offspring in this study, this effect would be virtually undetected. On the contrary, reduced DNA methylation profiles in the offspring of the EtOH treated males were observed at significant levels. While the few CpG sites that were altered in the sperm seemed to correlate with significantly reduced levels at the corresponding site in the offspring, the majority of CpGs in the sperm showed no significant changes in DNA methylation despite their corresponding sites in the offspring being significantly altered. Because a reduction in offspring DNA methylation isn't always correlated with a reduction in the corresponding site in the sperm, it suggests that something other than DNA methylation itself influences offspring. What other paternal factors could account for the observed effect in EtOH sired offspring?

4.6. Alternative mechanisms of epigenetic inheritance

Sequence data obtained from sperm samples confirmed that EtOH did not alter

the DNA sequence itself or methylation of the DNA. Thus, the paternal factors inherited by EtOH sired offspring that resulted in an altered pheno- and epigenotype would most likely be epigenetic in nature. Besides DNA methylation, two other epigenetic mechanisms exist in mammals that regulate gene expression: RNA species and chromatin remodelling through histone modifications.

4.6.1. RNA species

The mature sperm head is commonly thought to contain the tightly condensed haploid paternal genome which is transcriptionally inert. However, several RNA species have been found in spermatozoa that are both known and unknown protein-coding and non-coding RNAs. It is argued that the RNA species in spermatozoa are so few in number when compared to the abundance of RNAs found in oocytes, that they may be considered remnants and unlikely to play a significant role in embryonic development. However, the transmission of these RNAs from the sperm to the oocyte has been confirmed (Ostermeier et al., 2004), and raises the possibility that these and other spermatozoal RNAs do in fact play a unique role in zygotic development.

Transcripts unique to male germ cells play a role in embryonic stem cell differentiation. mRNA transcripts have been identified in sperm that are known to participate in fertilization and embryonic development, but which are absent in the oocyte (Geijsen et al., 2004). In addition to mRNAs, approximately 68 siRNAs

have been identified in human spermatozoa, with 13 of these showing similarities with siRNAs known to function in gene regulation (Ostermeier et al., 2005). This implies the possibility of early regulation of parental genes through selective RNA degradation. Human and mouse sperm is also enriched with microRNAs (miRNAs) that have the capacity to epigenetically alter the phenotype of offspring through “paramutation”. Disruption of the *Kit* gene in a mouse model yielded a non-lethal white tail phenotype. However, the mutated allele was not detected by genotyping. Instead, varied transcript lengths of the *Kit* gene were observed in later spermatogenic cells. Injection of this RNA into fertilised oocytes yielded the paramutation phenotype, as did exogenous miRNAs targeting the *Kit* gene. This further supported the idea that sperm RNA can possess a function, and furthermore, that it can act as an epigenetic modifier in the early embryo (Rassoulzadegan et al., 2006).

4.6.2. Influence of histones on gene expression

Somatic RNAs are associated with the nuclear matrix that lies interior to the nuclear envelope. The nuclear matrix dynamically organises DNA into loop domain structures on the matrix (Ward et al., 1989), which provides a platform for transcription and replication (Tsutsui et al., 2005). The integrity of this nuclear matrix is vital in mouse fertility (Ward et al., 1999; Ward et al., 2000). Like somatic RNAs, the RNA contained within spermatozoa is integrated into the nuclear matrix. The retention and preferential packaging of sperm RNA at the nuclear matrix is paralleled in the sperm nuclear DNA. Central to the formation of

the mature male gamete is the replacement of histones with protamines to form a highly condensed inert conformation. The replacement of histones with protamines was thought to be a complete process. However, emerging evidence has revealed that the haploid sperm genome is not entirely protamine-bound, with some regions of the genome retaining and associating with core histones (Ooi and Henikoff, 2007). In humans, approximately 15% of sperm DNA is retained in histone bound structures (Gatewood et al., 1987).

Like RNA packaging at the nuclear matrix, differential packaging of sperm DNA with histones suggests functional significance. The β -globin gene family analysed in human sperm demonstrated that gene products active in the embryonic sac preferentially associated with histones, while those that remain silent in the yolk sac associate with protamines (Gardiner-Garden et al., 1998). The maternally imprinted *IGF2* gene has also been confirmed to be differentially packaged with histones (Wykes and Krawetz, 2003). These studies suggest that histone retention is not only a non-random event, but is linked with gene activity. Furthermore, packaging of DNA with protamines results in a conformation that is more tightly compact than that with histones, and it is hypothesised that the less tightly condensed genetic regions associated with histones may allow genes important in development to be rapidly transcribed during early embryonic stages.

The extent of histones in regulating gene expression extends beyond their association with genes that are merely active in early embryo development to those specifically associated with imprinted regions. Comparative genome

hybridisation has shown preferential packaging of DNA with histones with CTCF factor binding sites (Lalancette et al., 2008). As mentioned previously, CTCF sites are found extensively in the ICRs of imprinted regions, which through binding of the CTCF protein, acts as an insulator, regulating imprinted gene expression in a parent-of-origin specific manner. CTCF is a nuclear matrix associated factor and the nuclear matrix is the site at which histone bound DNA (and RNA) containing genes actively transcribed in early embryogenesis localises. From this, it has been speculated that CTCF may play a role in coordinating chromatin repackaging, so that the 15% histone content in sperm is 'reserved' for coding and regulatory elements of the paternal genome (Lalancette et al., 2008). The question raised here is why there is a need to segregate the coding from the non-coding regions of the paternal genome if protamines are rapidly replaced with histones after fertilisation. Again, this group speculated that by excluding most genes from protamine packaging, the paternal genome delivers an epigenetic blue-print to the oocytes of all coding genes to ensure compatibility. This may be critical for imprinted genes for example, which are monoallelically expressed, and biallelic expression of these genes results in deleterious phenotypes. Lalancette et al. (2008) go on to suggest that the RNA present in spermatozoa may act to suppress transcription of those regions that 'escape' compact protamine conformations through an RNA-mediated mechanism similar to RNAi.

It has also been suggested that the resistance of vital genomic imprinting regions to genome-wide demethylation of the paternal genome may be mediated by histone modifications (Ooi and Henikoff, 2007). Furthermore, the role of histones

in maintaining gene expression is exemplified by evidence that a 60% reduction in DNA methylation had no influence on gene expression.

4.6.3. The effect of ethanol on histone modifications and RNA species

4.6.3.1. Ethanol disrupts H3K9 trimethylation

As described previously, chromatin is comprised of nucleosomes which consist of DNA wrapped around an octamer of histone proteins. These histones can be chemically modified to open and close the chromatin structure, regulating transcription and thereby gene activity. Histone modifications include, among others, acetylation and methylation of lysine (K) residues of the histone N-terminal tail. Histone methylation is mediated by histone methyltransferases (HMTases). Identical to DNMTs, HMTases make use of the methyl group donated by S-adenosyl-methionine (SAM). During cellular differentiation or metabolism, changes in gene expression are achieved through global epigenetic modifications that include histone acetylation and methylation, and DNA methylation (Shukla et al., 2008). Methylation of histone lysines H3K9, H3K27 and H4K20 are related to gene silencing, whereas H3K4 and H3K79 are associated with gene activation.

EtOH metabolism has been shown to cause significant biological effects by altering histone modifications (Shukla et al., 2008). Studies have revealed

selective acetylation of H3K9 in rat hepatocytes (Park et al., 2003; Kim and Shukla, 2005), as well as the lung, spleen and testes (Kim and Shukla, 2006) as a consequence of EtOH exposure. As a result of H3K9 acetylation, a decrease in methylation at the same lysine residue was observed. Concurrently, H3K4 methylation increased, and was associated with increased gene expression (Pal-Bhadra et al., 2007). Furthermore, altered histone H3K9 methylation has been shown to result in the epigenetic silencing of tumour necrosis factor α (TNF α), which is suspected to be involved in endotoxin-mediated inflammatory processes (El Gazzar et al., 2007).

As evidenced in the present study, although no differences were observed in sperm DNA methylation, a decrease in DNA methylation was observed in the EtOH offspring. If EtOH had induced site specific modifications of sperm DNA histones in this study but not caused a significant decrease in sperm DNA methylation, a role for histones in mediating DNA methylation may be implied.

Evidence for the interaction between DNA methylation and histone methylation was first observed in the fungus, *Neurospora crassa* (Tamaru and Selker, 2001). Mutations in the *dim-5* gene that encodes a H3K9 methyltransferase resulted in a complete loss of all DNA methylation. Similar findings demonstrated that mutations in the *Arabidopsis thaliana* H3K9 methyltransferase, *kryptonite*, abolished DNA methylation of CpNpG sites (Jackson et al., 2002). This is thought to be regulated by the *A.thaliana* heterochromatin protein 1 (HP1), which binds to methylated H3K9 and recruits a DNA methyltransferase to target CpNpG sites.

This mechanism is also evident in mouse embryonic stem cells (Lehnertz et al., 2003). Pericentric heterochromatin in mice is characterised by H3K9 trimethylation and high levels of DNA methylation. H3K9 trimethylation is mediated by the histone methyltransferases Suv39h1 and Suv39h2 via heterochromatin protein 1 isoforms HP1 α and HP1 β to stabilise heterochromatic domains. Lehnertz et al. (2003) showed that Suv39h-mediated H3K9 trimethylation recruits Dnmt3b (a *de novo* Dnmt) to heterochromatic regions, guiding DNA methylation (Figure 4.1). Furthermore, increased histone acetylation and decreased DNA methylation are associated with increased gene expression, indicating a reciprocal relationship between these two modifications.

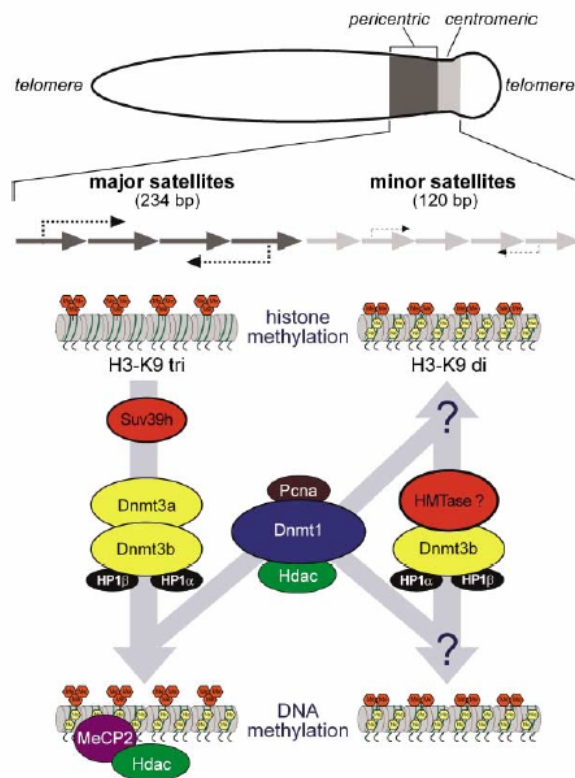


Figure 4.1: Histone and DNA methylation pathways at mammalian heterochromatin. The pathways of histone and DNA methylation pathways at satellite regions in the mouse are depicted. The putative Dnmt3a/Dnmt3b-HP1 α /HP1 β complex is recruited via Suv39h-dependent H3 K9 trimethylation to direct DNA methylation at satellite regions. There also exists a Suv39h-independent pathway where the Suv39h-unrelated HMTase which is hypothesized to mediate H3 K9 dimethylation, and interacts with Dnmt3b to establish a silent chromatin domain. Dnmt1 is also speculated to play a role in directing DNA methylation independently of H3 K9 methylation in a currently unknown manner (Lehnertz et al., 2003).

4.6.3.2. Ethanol induces differential microRNA expression

MicroRNAs comprise approximately 1% of mammalian genes (Berezikov et al., 2005) and are estimated to regulate the expression of 30% of the human genome (Lewis and Redrup, 2005) and are therefore abundant regulators of gene expression. They are vital in embryonic development (Yu et al., 2007) and perturbations to miRNAs have been associated with disease states and birth abnormalities.

Based on prenatal ethanol exposure models, the effects of ethanol on miRNAs have only been recently explored with seemingly conflicting results. A sub-set of miRNAs have been identified in the cortical neurons of mice *in vitro* that are aberrantly down-regulated as a consequence of EtOH exposure (Sathyan et al., 2007). A bioinformatic analysis of global gene expression data obtained from ethanol exposed embryos was used to detect potential *cis*-acting elements that may underlie the biological effects of alcohol (Wang et al., 2008). In contrast to the findings of Sathyan et al. (2007), analyses revealed that alcohol treatment stimulated expression of miRNAs, as detected by increased levels of miRNA binding sites (3'-UTRs). Wang et al. (2009) used a similar *in utero* mouse model and analysed the expression of 509 miRNAs in foetus brains using a miRNA microarray, which was verified using northern blot and quantitative real-time PCR techniques. They found that of the miRNAs screened, six were found to be up-regulated, while eight were down-regulated. These findings were congruent with significant decreases in litter size and weight observed in the ethanol exposed

foetuses (Wang et al., 2009).

Together, these findings demonstrate that ethanol has the ability to both up- and down-regulate miRNAs. Considering the vast number, the diverse functions and intricate mechanisms of miRNA expression and gene regulation, these results may not be surprising or in conflict. What is important however is that ethanol has a significant impact on the regulation of miRNAs in biological models based on prenatal ethanol exposure.

4.7. Implications of altered epigenetic mechanisms

The effect of EtOH to decrease miRNA levels may have a direct link with a loss in DNA methylation. RNA-directed DNA methylation was first observed in plants, where RNA virus infection triggered methylation at identical DNA sequences (Wassenegger et al., 1994). Similarly, RNA species in mammals are able to direct DNA methylation. X inactivation in the female mouse relies on the Xist RNA to coat the X chromosome to be inactivated in *cis*. This association has been demonstrated to trigger cytosine methylation (Heard et al., 2001; Lee, 2003). By implication, the loss of Xist would result in the inability to inactivate the X chromosome through RNA-directed DNA methylation.

The apparently minimal EtOH-induced loss of sperm DNA methylation yet pronounced decreases observed in offspring in this study may be explained by disruption of miRNA in the sperm prior to conception. As in X chromosome

inactivation, miRNA species responsible for directing DNA methylation at imprinted loci may be down-regulated during spermatogenesis as a consequence of alcohol exposure. Upon fertilisation, a deficiency of sperm-specific miRNAs responsible for RNA-directed DNA methylation may be introduced to the embryo, with subsequent failure to establish correct imprinting marks.

Ethanol's ability to decrease H3K9 methylation provides another mechanism that could account for the dramatic loss of DNA methylation observed in offspring. During spermatogenesis, EtOH-induced increased acetylation and subsequent loss of H3K9 histone methylation in mature spermatozoa may be present at significant levels globally, and thereby at regions of DNA whose methylation/imprints have already been established. Thus, normal imprinting marks would be present at these regions, but concurrently harbouring altered histone modifications. These aberrant histone modifications would be conveyed through the affected sperm to the fertilised oocyte. During the onset of DNA replication initiation, H3K9 acetylation marks would actively prevent DNA methylation, repressing imprint establishment.

The proposed "conflict" chromatin mark model, whereby chromatin harbours both DNA methylation (a repressive chromatin mark) and histone acetylation (an active chromatin mark) has not been extensively explored in the literature. However, investigations into methylation mechanisms in *Neurospora* identified an unmethylated DNA region that was histone H4 hypoacetylated {selker 2002}. As active chromatin is conventionally associated with unmethylated DNA and

hyperacetylation of histone H4, this finding supports evidence a “conflicting” chromatin state. Furthermore, the erythroid-specific carbonic anhydrase II (CAII) promoter has been shown to possess active histone modifications () localised around the transcription start site, while concurrently harbouring high levels of CpG methylation directly upstream of these active marks {Brinkman 2007}. This “bipartite” epigenetic code, in part, also appears to support evidence of a chromatin state that contains opposing marks.

However, the mechanisms of reduced miRNAs and altered histone modifications in sperm accounting for losses of DNA methylation in subsequent offspring still require much investigation.

4.8. Epigenetic switching: a mechanism for growth retardation

In the original hypothesis of the current study, it is proposed that exposure of male mice to alcohol would induce a loss of DNA methylation at the *H19*, *IG* and *Rasgrf1* ICRs in sperm DNA. The implications of this would have a significant impact on the parent-of-origin reciprocal imprinting of these loci, in which the maternal allele is normally hypomethylated and the paternal allele hypermethylated. The loss of methylation at a paternal ICR would be carried as an aberrant methylation imprint in the nucleus of mature sperm. Subsequently, each

of these loci would become actively regulated in the embryo after fertilization. According to the proposed model by which imprinted loci are regulated, the hypomethylated state of an ICR allows for the binding of the CTCF protein, as in the maternal allele. Binding of CTCF to the maternal ICR insulates the downstream common enhancer from accessing the upstream paternally-expressed gene promoter, while the downstream maternally-expressed gene promoter is selectively acted upon.

The data of this study confirm the ability of ethanol to reduce DNA methylation to some extent in the sperm of exposed males, but significantly impacts imprinted regions of the paternal allele in their offspring. This loss of imprinting on the paternal allele, especially at the *H19* ICR (CTCF 1 and 2), would allow CTCF binding at the ICR, essentially switching the allele to a maternal epigenotype. This would result in the expression of the normally silent maternal *H19* gene from the paternal (sperm) allele, while silencing the paternally expressed *Igf2* gene. Concurrently, the maternal allele still possesses normal regulation and gene expression, resulting in double expression of the maternally expressed *H19* gene and repression of the paternal *Igf2* gene from both alleles. Support for this stems from findings whereby the inhibition of Dnmts resulted in decreased methylation at the *Igf2/H19* locus and subsequent activation of *H19* from the paternal allele (Li et al., 1992).

This has significant implications according to the conflict hypothesis, which states that paternally expressed genes from imprinted regions use maternal resources to

promote embryonic growth and development; while maternally expressed genes endeavour to conserve maternal resources. As mentioned previously, the *Igf2/H19* locus is vital in embryonic development. The paternally expressed *Igf2* gene is a potent mitogen promoting offspring growth while the maternally expressed *H19* gene negatively regulates *Igf2* expression.

The observed switch in epigenotype from a heavily methylated (imprinted) paternal allele to a hypomethylated “maternal” allele state, as observed in the offspring at the *H19* ICR, has significant implications on embryonic growth. Suppression of the paternally expressed growth-promoting *Igf2* gene and enhanced activity of the maternally expressed *H19* gene would in effect hamper offspring growth.

4.9. The three-way tryst – treatment, DNA methylation and offspring development

Thus far, it has been shown that paternal exposure to EtOH influences both the weight and DNA methylation profiles of sired offspring. The question that must be asked is whether treatment (EtOH) causes the observed growth retardation in offspring through decreases in DNA methylation. In other words, does the EtOH-induced decrease in offspring DNA methylation (via the sire) dysregulate imprinting control and establishment and thereby cause aberrant regulation of its associated gene, resulting in the stunted growth phenotype.

Correlation analyses of the level of offspring DNA methylation at particular regions/CpG sites with offspring weight at each of the 20 time points were assessed. The findings of this study demonstrated that the level of methylation at the *H19* regions (CTCF 1 and 2) were highly correlated with days 28 to 56. Furthermore, these correlations appeared in a “block”, where correlations were rarely observed outside of this. Similarly, but not as obvious, *Rasgrf1* CTCF methylation correlated with days 2, 4 and 6 while *Snrpn* correlated primarily with days 35 and 56. This demonstrates that the level of DNA methylation is correlated with offspring weight at specifically grouped days in a positive relationship. That is, low levels of DNA methylation correlate with low weights (and *vice versa* for higher levels of methylation). This therefore suggests a functional relationship between DNA methylation and offspring weight.

To investigate this relationship further, mediation analyses and multilevel modelling were conducted to establish whether treatment influenced offspring weight via DNA methylation. Multilevel modelling revealed insignificant results at all regions and individual CpG sites investigated. Furthermore, results seemed to be in conflict as some regions and CpG sites appeared to marginally decrease average offspring weight, while others slightly increased offspring weight. This conflict and lack of significance can be attributed to the fact that a small male sample size was employed (n=13, two males excluded from the study). All offspring data is nested within females/dams, which in turn are nested within the 13 males. This hierarchical structure therefore decreases the sample size used for

analyses to essentially 13 data points, which is further separated into the two treatment groups. This would therefore provide inadequate power to detect significant differences.

Mediation analyses revealed only two regions that significantly mediated a decrease in offspring weight (CTCF 1 and *H19* CpG12) at day 35. In conjunction with the significant decrease in offspring DNA methylation at these regions and the strong correlation of these regions with decreased offspring weight at day 35, these mediating effects strongly support a functional role of DNA methylation in offspring growth.

4.10. Strengths and limitations of the present study

4.10.1. Limitations

The limited sample size of both treated males and subsequently the number of offspring obtained were a significant limitation to this study in terms of statistical analyses. Primary of which included mediation and hierarchical/multilevel modelling analyses, which failed to detect significance due to lack of power as a consequence of undersized samples. Furthermore, mediation analyses were not based on a hierarchically structured model and thus analyses may not have been accurate in terms of a nested data set structure.

The current study focused on a single maternal ICR, namely *Snrpn*. Conclusions regarding the observed demethylation and gain of methylation in the sperm and offspring, respectively, cannot therefore be generalised to other maternally imprinted regions.

Although pyrosequencing is a highly effective method of quantifying DNA methylation, it has the distinct disadvantage in that it does not allow for qualitative assessment. Qualitative analyses, such as clonal bisulfite sequencing, enable the assessment of DNA methylation at a site-specific level of individual clones. This analysis allows for patterns in the loss of DNA methylation to be observed (as performed in sire sperm analyses). That is, one can assess whether losses of DNA methylation at individual CpG sites occur in “blocks”, or whether they are randomly distributed across clones. These “patterns” may have important implications for the binding efficiency of certain proteins, such as CTCF, and the proposed allele-switching mechanism. As this study only examined DNA methylation in offspring tail biopsies quantitatively via pyrosequencing, the resolution of DNA methylation could not be analysed qualitatively at the level of individual clones.

Lastly, the lack of significant EtOH-induced loss of DNA methylation observed in the sperm of treated males suggests that the treatment regimen employed in this study may not have been performed over a sufficient period of time. Greater EtOH concentrations and/or longer exposure of males to EtOH may be more appropriate to detect EtOH-induced epigenetic aberrations at imprinted loci.

Furthermore, significant decreases in DNA methylation in the offspring of EtOH treated males suggest that other epigenetic mechanisms are affected by EtOH exposure. These include RNA species and histone modifications, which have been shown to be altered by EtOH, but were however beyond the scope of this study.

4.10.2. Strengths

The major strength of this study was that it included all three of paternal ICRs known to date that spanned separate and distinct imprinted loci. Without this range, it may have been concluded that imprinting loci in offspring sired by EtOH treated males are significantly demethylated based on findings of the *H19* ICR. However, as evidenced in the present study, the extent of aberrant methylation varies significantly between loci.

Another clear strength of this study was the inclusion of the *Snrpn* ICR. This locus not only served to determine somatic (maternal allele) contamination, but provided further evidence of EtOH-induced loss of methylation in sperm DNA and increased levels of methylation in offspring.

4.11. Future studies

As outlined in the study limitations above, future studies are required to focus primarily on the functional implications of loss of imprinting. This would provide

further insight into the role of imprinting as a mechanism of EtOH-induced growth retardation in offspring. Such studies would assess the status of methylation at imprinted loci in relation to the relative expression of their associated genes. Furthermore, it may be advisable to explore various tissue types as some imprinted genes show tissue-specific expression, where they may be biallelically expressed in some tissues, and monoallelically expressed in others (Wilkins, 2006). Tissues that demonstrate strict imprinting and monoallelic expression of imprinted genes include epithelial tissues (such as those obtained from tail biopsies), hepatic, muscle and neural tissues. Quantification of gene expression could be achieved using quantitative real-time PCR or gene expression arrays. In addition, techniques such as chromatin immunoprecipitation should be employed to quantify the level of CTCF binding at ICRs and correlate these findings with levels of methylation and gene expression.

The role of primary DMRs (ICRs) in determining the methylation status of secondary DMRs and promoter regions would require further studies to expand the repertoire of imprinted loci to include paternal secondary differentially methylated regions, such as the *Igf2* DMR and the *H19* promoter. As demonstrated by the loss and gain of methylation at the *Snrpn* ICR in sperm and offspring, respectively, maternally differentially methylated regions should also be investigated.

As highlighted previously, the duration and dosage of the EtOH regimen may have significant impacts on offspring development and methylation at imprinted

loci. It is therefore suggested that future studies investigate the effects of paternal EtOH exposure over varied time periods and include a range of EtOH concentrations.

Lastly, the effect of EtOH exposure on other epigenetic mechanisms, such as regulation of gene expression via RNA species and histone modifications should be explored in both sperm and in sired offspring. A variety of techniques can be employed to assess histone modifications, including western blotting and immunofluorescence. However, these immunoassay methods are often fraught with problems, including cross-reactivity, variable specificity and epitope occlusion. For these reasons, mass spectrometry is often favoured to quantify histone modifications (Garcia et al., 2007). RNA array platforms (including micro RNA arrays), in conjunction with quantitative reverse transcription PCR (qRT-PCR) enable the detection of RNAs, and facilitate the analysis of global RNA expression profiles (Tang et al., 2006). These findings should be further correlated with levels of methylation and gene expression of associated regions.

Chapter 5

5. Conclusion

The findings of this study suggest that exposure of sires to EtOH for a five week period prior to conception has a limited ability to reduce DNA methylation at ICRs in sperm DNA but has the ability to negatively influence the epigenetic state of these ICRs of developmentally significant imprinted genes in sired offspring.

Findings of EtOH-induced loss of methylation in sperm DNA were confined to specific CpG sites which spanned all four ICRs, albeit to a very limited degree. This agreed only in part with our hypothesis, where it was predicted that this loss would occur on a larger scale. The minimal decrease in DNA methylation observed in sperm DNA of EtOH-exposed males suggests that exposure time of males to EtOH and/or the dosage utilised may not have been sufficient to induce significant epigenetic effects. It is also suggested that methylation in sperm DNA may be more resistant to the deleterious effects of EtOH. However, this mechanism can only be hypothesised as no such mechanism has been scientifically explored or identified to date.

In contrast, a severely reduced methylation status was observed at the *H19* ICR, which included both the CTCF binding site regions. This was in accordance with the prediction that treatment of sires with EtOH would adversely affect the

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methylation pattern of imprinted loci in offspring.

Due to the large effects noted at the *H19* ICR in the offspring of the EtOH-exposed males and the limited significant findings in sire sperm, it is proposed that other epigenetic mechanisms are more susceptible to the effects of EtOH or other teratogenic agents. These epigenetic alterations may involve histone modifications and/or aberrant effects on RNA species which are transferred through the mature spermatozoa to the oocyte.

It was further noted that the offspring of EtOH-treated males displayed growth retardation during postnatal development. Correlation analyses revealed that the reduced levels of DNA methylation at the *H19* ICR in EtOH offspring coincided with this period of growth retardation. Furthermore, the effects of EtOH on reduced offspring weight were shown to be mediated through DNA methylation, supporting the predication that lowered levels of DNA methylation (as a consequence of paternal EtOH exposure) in the offspring would negatively affect their growth.

Overall, the excessive exposure of male mice prior to conception for at least the duration of one cycle of spermatogenesis impacts negatively on offspring DNA methylation at imprinted loci and weight. Furthermore, the effect of EtOH on offspring growth retardation appears to be mediated through reduced levels of DNA methylation at the *H19* ICR. The growth retardation phenotype observed in the offspring was proposed to involve an ethanol-induced epigenetic switch. It is

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proposed that reduction in the DNA methylation patterns of the *H19* ICR on the paternal allele switches the epigenotype to a hypomethylated maternal allele state. This in turn, promotes the binding of the CTCF enhancer-blocking protein at the paternal *H19* ICR reducing expression of *Igf2* and consequently resulting in offspring growth retardation.

In conclusion, EtOH-induced demethylation of paternally methylated ICRs in the sperm of treated males was not significant. However, large-scale reduction in DNA methylation was observed in their offspring, demonstrating that excessive exposure of male mice to EtOH impacts on the epigenetic state of their progeny, and is associated with a growth retardation phenotype. This mechanism may have important implications in the aetiology of FAS, where pre- and postnatal growth retardation is observed. Although the effect of paternal treatment with EtOH on growth retardation appears to be mediated through DNA methylation, further studies are required to determine the effect of reduced methylation at the *H19* ICR on the expression levels of *H19* and *Igf2*.

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Software references

ABI 2 FASTA Converter version 1.0.0.14 (2002) Heracle Software, <http://www.DNABaser.com>

GraphPad Prism version 5 for Windows (GraphPad Software, Inc., San Diego California USA)

PSQ Assay Design Software version 1.0.6 (Biotage AB, Kungsgatan, Uppsala, Sweden)

Pyro Q-CpG version 1.0.9 (Biotage AB, Kungsgatan, Uppsala, Sweden)

SAS Enterprise Guide (SAS Institute Inc. Cary, USA)

SAS v9.1 (SAS Institute Inc. Cary, USA)

StataCorp (2007) Stata Statistical Software: Release 10. College Station, TX: StataCorp LP)

Appendices

Appendix A

Ethics Approval

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2007/41/03

APPLICANT: Mr J Knezovich

SCHOOL: Human Genetics

DEPARTMENT:

LOCATION: NHLS

PROJECT TITLE: The effect of Alcohol on the Methylation Status of the imprinting Control Regions Contained within Three Developmentally Significant Loci in mice

Number and Species

15 Male Mus – mice

26 Female Mus Musculus - mice

Approval was given for the use of animals for the project described above at an AESC meeting held on 20070529. This approval remains valid until 20070529.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

- i. Add "in mice" to title
- ii. Confirm the number of mice required as applications says 30 but the table adds up to 41(confirm with CAS).
- iii. Justify and give references for the alcohol dosages which appear to be excessive.
- iv. Prove to CAS Vet that you can do gavage in mice.
- v. Keep exact records of pups.

Signed: _____

(Chairperson, AESC)

Date: _____

26/6/07

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____

(Registered Veterinarian)

Date: _____

26/06/07

cc: Supervisor: #



Appendix B

Solution Preparations

dNTP Mix

dATP (10mM)	10µl
dTTP (10mM)	10µl
dCTP (10mM)	10µl
dGTP (10mM)	10µl
ddH ₂ O	760µl
	800µl

dNTPs are commercially available from Roche Diagnostics

3% Agarose Gel

Agarose	12g
1 x TBE*	400ml
EtBr* (10mg/ml)	12µl

EtBr (Ethidium Bromide: 3µl EtBr/100ml 1 x TBE)

10 x TBE

Tris	216g
Boric acid	110g
EDTA	14.88g

Make up to 2 litres with dH₂O

TE Buffer (10mM Tris-HCl, pH7.4; 0.1mM EDTA)

2M Tris-HCl (pH7.4)*	0.5ml
EDTA (pH 8.0)*	20µl
dH ₂ O	99.48ml
	100ml

Autoclave and store at room temperature

2M Tris-HCl pH7.4

Tris base	242.2g
dH ₂ O	700ml
Final volume (H ₂ O)	1L

pH was adjusted by adding ~140µl concentrated HCl and autoclaved

- Appendices -

0.5M Ethylenediamine Tetra-acetic Acid (EDTA)

Na ₂ EDTA.2H ₂ O	93.05g
dH ₂ O	<u>300ml</u>
Final volume (H ₂ O)	<u><u>500ml</u></u>

pH adjusted to 8.0 with 10M NaOH* and autoclaved

Luria Broth

NaCl	6g
Tryptone	6g
Yeast extract	<u>3g</u>
Final volume (dH ₂ O)	<u><u>1L</u></u>

pH was adjusted to 7 using 5M NaOH, autoclaved and stored at 4°C

Luria Agar Plates

dH ₂ O	500ml
NaCl	5g
Tryptone	5g
Yeast extract	2.5g
Agar	10g

pH was adjusted to 7.0 using 5M NaOH*, autoclaved and 500µl (50mg/ml) ampicillin was added
This was then poured into 100mm petri-dishes

Ampicillin (50mg/ml)

10M NaOH

NaOH pellets	4g
dH ₂ O	10ml

Caution: this is an extremely caustic base

Lysis Solution (freshly prepared)

ddH ₂ O	7.35ml
50% Glucose	2ml
0.25M EDTA	0.4ml
1M Tris.HCl (pH8.0)	<u>0.25ml</u>
	<u><u>10ml</u></u>

Glucose solution was filter sterilized using a 0.45µm filter prior to use and stored at 4°C

- Appendices -

Alkaline SDS

ddH ₂ O	8.33ml
10% SDS	1ml
3M NaOH	0.67ml
	<u>10ml</u>

High Salt Solution (3M NaOAc)

NaOAc	4.1g
ddH ₂ O	50ml

pH corrected to 5.2 with concentrated HCl

RNase A Solution

ddH ₂ O	500μl
RNase A stock (7000u/ml)	10μl
	<u>510μl</u>

Ethidium Bromide (EtBr)

EtBr powder	10mg
dH ₂ O	1ml

EtBr is light sensitive and must be stored in a dark bottle.

This reagent is also commercially available from Sigma Aldrich

100 bp Molecular Weight Marker (100ng/μl)

Ficoll*	1μl
100bp DNA stock (1μg/μl)	1μl
ddH ₂ O	8μl
	<u>10μl</u>

100bp molecular weight marker is available from Invitrogen

Proteinase K (10mg/ml)

Proteinase K stock (100mg/ml)	100mg
ddH ₂ O	10ml

Proteinase K stock is available from Roche Diagnostics

Ficoll-bromophenol Blue Loading Dye (Ficoll)

50% Sucrose crystals	50g
0.5M EDTA (pH 7.0)	0.1ml
0.1% Bromophenol blue dye	0.1g
10% Ficoll powder	<u>10g</u>

- Appendices -

Final volume (ddH2O)	<u>100ml</u>
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Phosphate Buffered Saline

Tablet	x 1
ddH2O	100ml

Allow tablet to dissolve in water

Note: PBS tablet Oxid Cat. No. BR14a)

70% Ethanol (EtOH)

100% Ethanol	70ml
ddH2O	<u>30ml</u>
	<u>100ml</u>

90% Ethanol

100% Ethanol	90ml
ddH2O	<u>10ml</u>
	<u>100ml</u>

50% Ethanol

100% EtOH	50ml
ddH2O	<u>50ml</u>
	<u>100ml</u>

TFB I Buffer (per 200ml)

Potassium acetate	0.588g
Rubidium chloride	2.42g
Calcium chloride	0.294g
Manganese chloride	2.0g
Glycerol	30ml

Make up to 200ml with ddH2O

Adjust pH to 5.8 with dilute acetic acid and filter sterilise

TFB II Buffer (per 100ml)

MOPS	0.21g
Calcium chloride	1.1g
Rubidium chloride	0.121g
Glycerol	15ml

Make up to 100ml with ddH2O

Adjust pH to 6.5 with 5M NaOH and filter sterilise

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Sucrose solution (0.704g/ml)

dH₂O

1000ml

Sucrose

704g

Dissolve sucrose in dH₂O at RT

Appendix C

Differex™ System to QIAamp DNA Micro Kit bridging protocol

1. Add 100µl of Buffer ATL to the Separation Solution to obtain a final volume of approximately 200µl.
2. Add 7µl of 1M DTT*, and add 20µl of Proteinase K*.
3. Close the lid and mix by pulse-vortexing, and briefly spin down the solution.
4. Add 200µl of Buffer AL and mix by pulse-vortexing for 15s.
5. Place the tube into an orbital incubator at 56°C at 900 rpm overnight.
6. Spin down and add 200µl of 100% EtOH, close the lid and mix thoroughly by pulse-vortexing for 15s.
7. Incubate for 3 min at RT.
8. Spin down and place entire contents of tube into a QIAamp spin column.

Appendix D

Preparation of competent DH5 α *E.coli* cells

1. 20 μ l of stock DH5 α *E.coli* was plated onto a LA* plate containing no antibiotic, and left to incubate overnight at 37°C.
2. A single colony was then removed from the plate and inoculated into 5ml of LB medium* in a 50ml flask and incubated overnight at 37°C.
3. 1ml of this overnight culture was then inoculated into a 500ml flask containing 100ml LB, and incubated at 37°C with aeration to $A_{600} = 0.48$.
4. Once the required absorbance reading was achieved, the flask was immediately placed on ice for 15min. Note: cultured cells were kept cold from this step forward.
5. 50ml of the culture medium was then placed in a 50ml centrifuge tube and pelleted at 3500rpm for 5min.
6. The resulting supernatant was then discarded and 0.4 volume of TFB I buffer* was added to gently re-suspend the pellet. This was then incubated on ice for a further 15min.
7. Cells were then pelleted at 3500rpm for 5min.
8. Once again, the supernatant was discarded and the pellet re-suspended in 0.04 volume of TFB II* (0.04 of the culture volume). The solution was then kept on ice for 15min.
9. 500 μ l aliquots of the bacterial solution were prepared and flash frozen in liquid nitrogen and stored at -80°C.

Appendix E

Transformation protocol

1. Competent DH5 α *E.coli* cells were stored at -80°C and thawed on ice immediately prior to use.
2. 25 μ l of DH5 α *E.coli* cells was added to a 1.5ml microfuge tube for each sample.
3. 5 μ l of (~200ng/ μ l) the ligation product was then added to the cells. The remaining 5 μ l was stored at -20°C as backup stock.
4. The transformation solution was then placed in ice for 15min.
5. Subsequently, the solution was heat-shocked for 90s at 42°C.
6. This was then placed on ice for a further 25min.
7. Following this, 700 μ l of luria broth (LB) was added to each sample. Note: this is a "rescue" LB, which contains no antibiotics.
8. Samples were then incubated at 37°C for 1 hour. This time allows the bacteria to initiate expression of the ampicillin resistance gene.
9. After the 1 hour incubation, each sample was centrifuged for 1 min at 3500 rpm. This pellets the transformed bacterial cells.
10. The bacterial pellet was then pipetted out along with 500 μ l of the LB medium. This was then plated on luria agar (LA) plates containing 1 μ l/1ml Ampicillin (Amp; 50mg/ml).
11. Plates were left to absorb the 500 μ l of LB culture medium for 1 hour at 42°C.
12. Plates were then inverted and incubated overnight at 37°C.

Note: plates were prepared for blue/white screening an hour prior to use by spreading 50 μ l 100mM IPTG* and 50 μ l 2% X-gal* onto each LA plate.

Appendix F

Protocol for isolation and purification of plasmid DNA from *E.coli*

1. Overnight minipreps were spun down at 4 000 rpm for 1min in a microfuge to pellet the bacterial cells. 1ml of culture along with the bacterial pellet was removed and pipetted into a 1.5ml microfuge tube. This was spun for 30-40s in a centrifuge at 14 000 rpm to re-pellet the cells. Note: this step was modified from the original protocol. Miniprep cultures were pelleted as opposed to being resuspended (as stated in the original protocol). This was performed so as to obtain a greater number of bacterial cells for higher yields of plasmid DNA.
2. Supernatant was decanted and the pellet was resuspended in 100µl Lysis Solution*.
3. 200µl Alkaline SDS* was added to each tube and vortexed well.
4. Tubes were then incubated in an ice water bath for 5min.
5. 150µl of High Salt Solution* was then added to each sample and vortexed well.
6. Tubes were then incubated in the ice water bath for 10min.
7. These were spun for 10min at 14 000 rpm.
8. 400µl of the supernatant from each sample was removed and transferred to a new 1.5ml microfuge tube pre-filled with 400µl 100% isopropanol.
9. Samples were vortexed well and kept in the ice water bath for 10min.
10. Tubes were spun at 14 000rpm for 10min.
11. The supernatant was then discarded.
12. 500µl 70% EtOH* was added to each tube and briefly vortexed to wash the pellet.
13. Samples were then re-pelleted for 3min at 14 000 rpm.
14. EtOH was removed by inverting each tube onto clean paper towel.
15. Tubes were then placed in a heating block at 50°C for two hours to dry the pellets of excess EtOH.
16. 50µl ddH₂O (not 100µl TE* as per protocol) with 4µg/ml RNase A* was added to each

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sample to resuspend the pellet, and incubated at 42°C for 20min.

17. 1µl of this was used for restriction enzyme digestion.
18. Plasmid DNA was stored at -20°C.

Appendix G

Pyrosequencing preparation protocol

The following protocol was been taken from Biotage's sample preparation guidelines: Sample preparation guidelines for PSQ™ 96 and PSQ 96MA systems.

1) Immobilization of PCR product to beads:

- For all samples, run 5µl of pre-pyrosequencing PCR on a 3% agarose gel to check for strong PCR products.
- Place samples in a 96-well PCR plate.
- Vigorously shake the bottle of streptavidin Sepharose beads until a homogenous solution is obtained.
- Make up a master mix of Sepharose and Binding buffer by adding 3µl of Sepharose and 40µl of Binding buffer for each sample.
- Aliquot 43µl of the Sepharose/binding buffer mix to each sample.
- Place the 96-well plate on a shaker for 10min so as to ensure that the beads remain in solution and don not settle.
- Prepare the PSQ 96 Plate Low well plate with the sequencing primer and Annealing buffer. For each sample add 1.6µl of 10µM sequencing primer to 38.4µl of Annealing buffer. Make a master mix if required and aliquot 40µl into each well of the PSQ 96 Plate Low.

2) Strand separation of products:

- Place the four troughs on the Vacuum Prep Workstation in the order shown in the diagram in the manual pg. 5.
- Fill each trough up with the following:
 - 1) 70% Ethanol – 180ml
 - 2) Denaturation Buffer – 120ml
 - 3) Washing Buffer – 180ml
 - 4) ddH₂O – 180ml
- Turn the vacuum pump on and apply vacuum. Capture the beads and immobilized templates on the filter probes by lowering the Vacuum Prep Tool into the 96-well PCR plate. (Note: this must be done within 3min of agitation terminated so that beads do not settle. Also, re-suspend the samples with a pipette after agitation on the shaker.)

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- Make sure all the Sepharose beads have been captured onto the filter probes. Place the Vacuum Prep Tool for 12sec into: 1) 70% ethanol 2) Denaturation buffer 3) Washing buffer. Hold the vacuum prep tool up at 90° to allow for all the liquid to completely drain from it for a few seconds and return to horizontal position.
- Turn the pump off to release the vacuum.
- Place the PSQ 96 Plate Low plate on the workstation and release the beads from the filter probes onto the plate by shaking the vacuum prep tool while allowing the filter probes to rest on the bottom of the wells.

4. Primer Annealing:

- Heat the PSQ 96 Plate Low plate with the sequencing primer and Annealing buffer on a heating block at 80°C for 3min.
- Allow to cool to room temperature.

6. Cartridge preparation:

- Add the required volume of enzyme, substrate, and dNTPS into the reagent cartridge as specified by the software. This volume is sample number and assay design-dependent.
- Place the PSQ 96 Plate Low and cartridge into the PSQ 96MA instrument and start the run.

Appendix H

Montage SEQ₉₆ Sequencing Reaction Cleanup Kit protocol

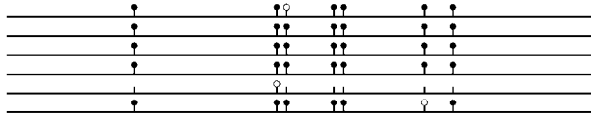
1. Cycle sequencing samples were diluted with 20µl Injection Solution (provided) and mixed gently by pipetting up and down 3-5 times.
2. Diluted samples were then transferred into the bottom of a SEQ₉₆ plate wells.
3. The SEQ₉₆ plate was then placed on the vacuum manifold (-15.in Hg) for 8min.
4. After the vacuum is turned off, the bottom of the plate is blotted dry with paper towel.
5. 30µl of Injection Solution was the added to each well and the SEQ₉₆ plate placed back on the vacuum manifold.
6. The vacuum was applied for a further 8min.
7. Once the vacuum was switched off, the bottom of the plate was once again blotted dry.
8. 30µl of Injection Solution was added to each samples well of the SEQ₉₆ plate.
9. The plate was then placed on an orbital shaker for 5min at 200rpm, to ensure that samples were sufficiently resuspended.
10. Subsequently, samples were resuspended again by pipetting up and down 20 times, and transferred to a 96 well injection plate.

Appendix I

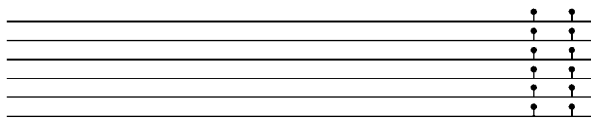
Clone sequence data

H19 61 (Sucrose)

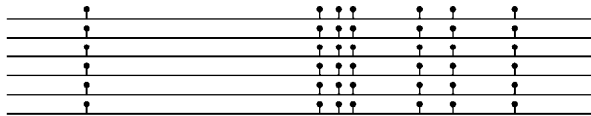
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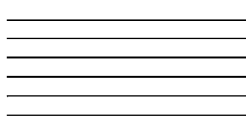
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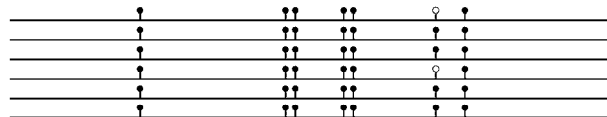


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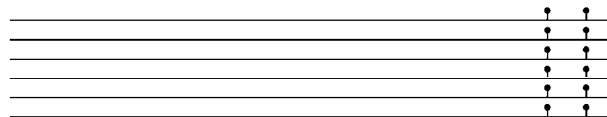


H19 62 (EtOH)

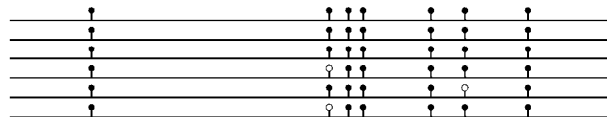
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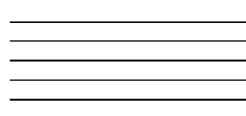
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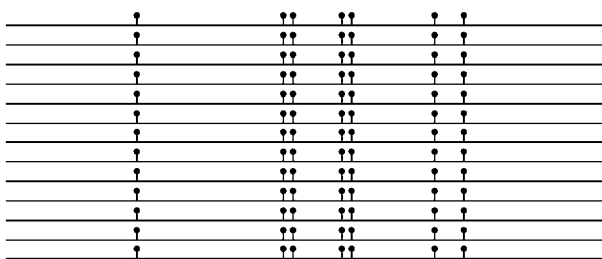
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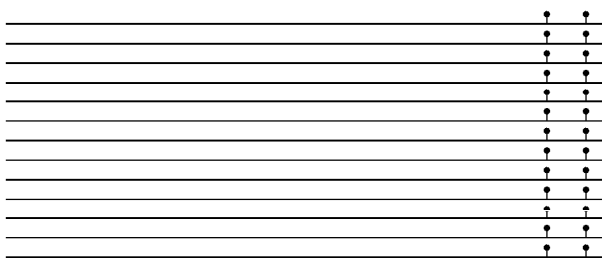
- Appendices -

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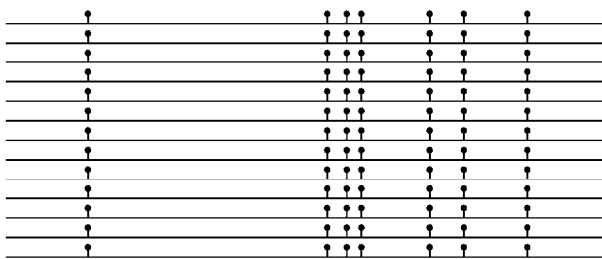
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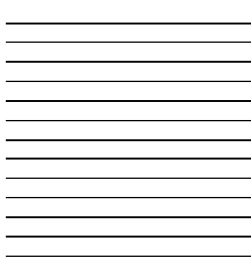
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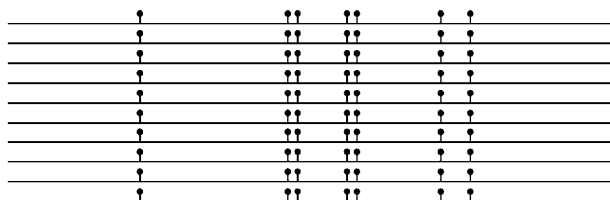


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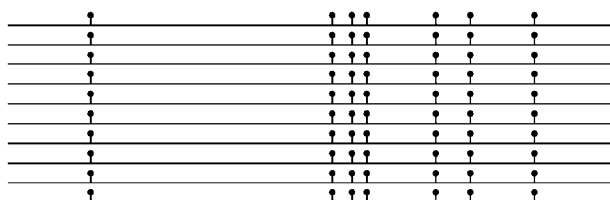
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H19 68 (EtOH)

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[23]_Clone#90_D07_2008-07-21		↑	↑↑	↑	↑
[24]_Clone#91_E07_2008-07-21		↑	↑↑	↑	↑
[25]_Clone#92_F07_2008-07-21		↑	↑↑	↑	↑
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[3]_Clone#83_E06_2008-07-21					
[4]_Clone#84_F06_2008-07-21					
[5]_Clone#85_G06_2008-07-21					
[6]_Clone#86_H06_2008-07-21					
[7]_Clone#87_A07_2008-07-21					
[8]_Clone#88_B07_2008-07-21					
[9]_Clone#89_C07_2008-07-21					
[10]_Clone#90_D07_2008-07-21					
[11]_Clone#91_E07_2008-07-21					
[12]_Clone#92_F07_2008-07-21					

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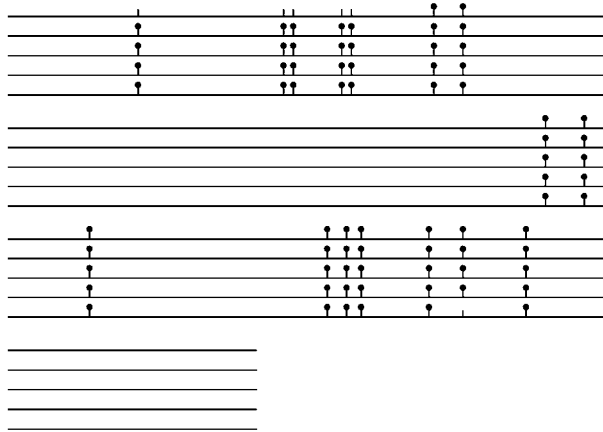
H19 69 (Sucrose)

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 [6]_Clone#278_D04_2008-07-31

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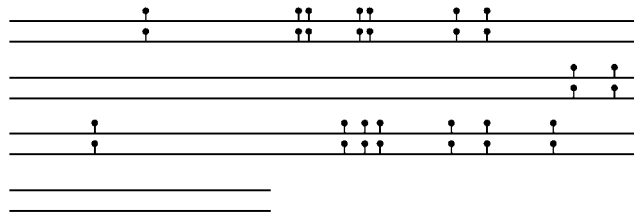
H19 70 (Sucrose)

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 [3]_Clone#290_H05_2008-07-31

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 [3]_Clone#290_H05_2008-07-31

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 [3]_Clone#290_H05_2008-07-31

[2]_Clone#285_C05_2008-07-31
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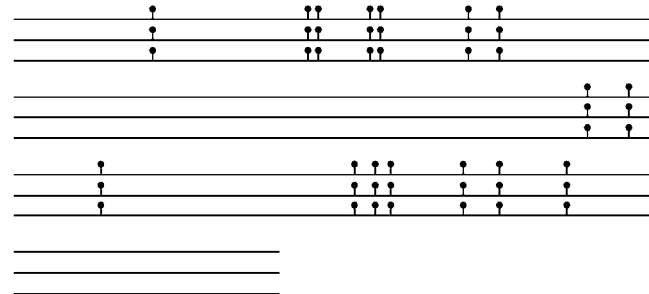
H19 71 (Sucrose)

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 [3]_Clone#655_A03_2008-10-29
 [4]_Clone#660_F03_2008-10-29

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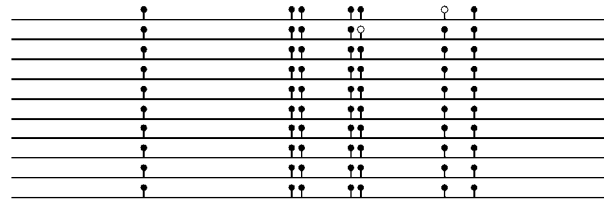
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H19 72 (Sucrose)

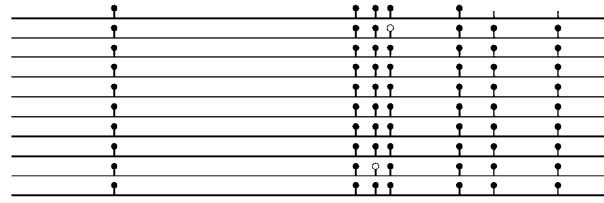
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[11]_Clone#308_B08_2008-07-31



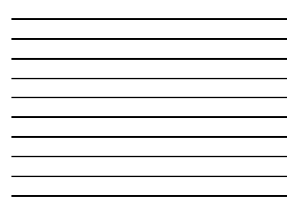
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[8]_Clone#305_G07_2008-07-31
[9]_Clone#306_H07_2008-07-31
[10]_Clone#307_A08_2008-07-31
[11]_Clone#308_B08_2008-07-31



[2]_Clone#298_H06_2008-07-31
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[3]_Clone#299_A07_2008-07-31
[4]_Clone#300_B07_2008-07-31
[5]_Clone#301_C07_2008-07-31
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[7]_Clone#303_E07_2008-07-31
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[9]_Clone#306_H07_2008-07-31
[10]_Clone#307_A08_2008-07-31
[11]_Clone#308_B08_2008-07-31



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H19 73 (Sucrose)

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 [7]_Clone#315_A09_2008-07-31
 [8]_Clone#316_B09_2008-07-31
 [9]_Clone#317_C09_2008-07-31
 [10]_Clone#318_D09_2008-07-31
 [11]_Clone#319_E09_2008-07-31

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 [9]_Clone#317_C09_2008-07-31
 [10]_Clone#318_D09_2008-07-31
 [11]_Clone#319_E09_2008-07-31

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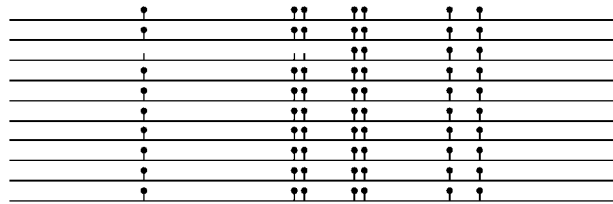
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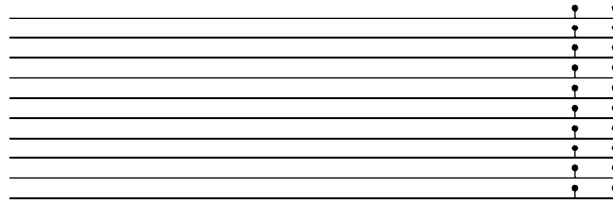
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 [9]_Clone#317_C09_2008-07-31
 [10]_Clone#318_D09_2008-07-31
 [11]_Clone#319_E09_2008-07-31

H19 74 (Sucrose)

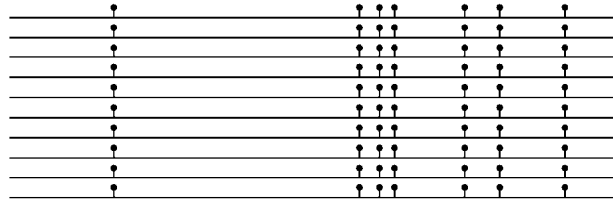
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[7]_Clone#647_A02_2008-10-29
[8]_Clone#792_B08_2008-10-30
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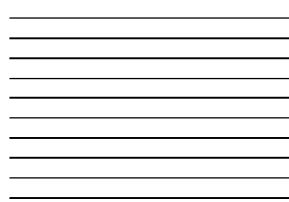
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[7]_Clone#647_A02_2008-10-29
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[11]_Clone#800_B09_2008-10-30



[2]_Clone#632_E09_2008-09-30
[3]_Clone#634_G09_2008-09-30
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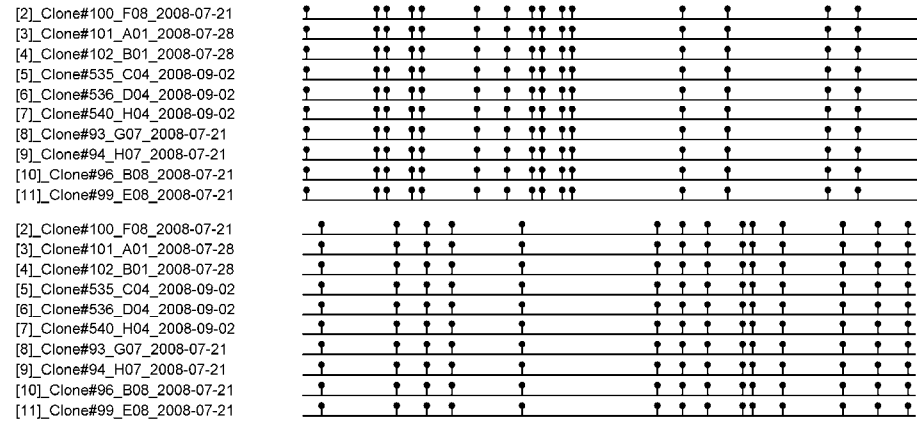


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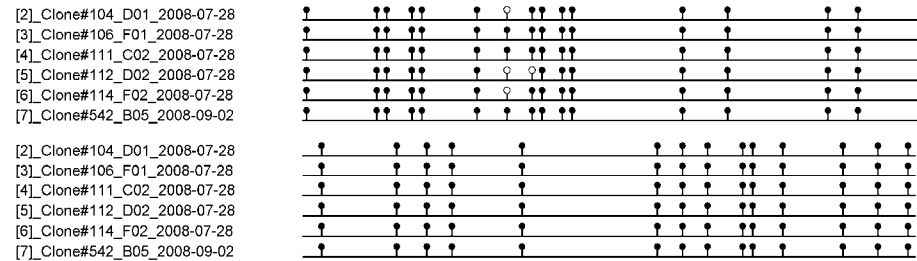


Rasgrf1 ICR

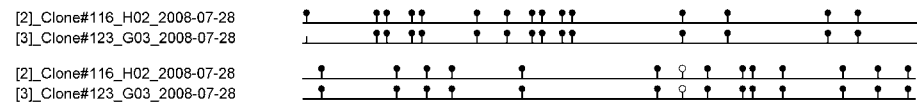
Rasgrf1 60 (EtOH)



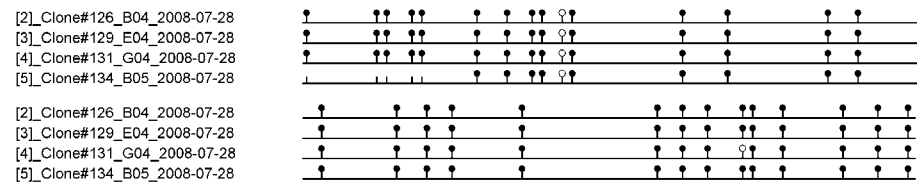
Rasgrf1 61 (Sucrose)



Rasgrf1 62 (EtOH)

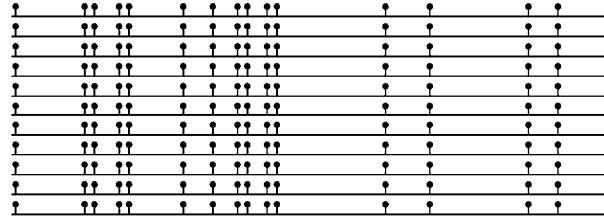


Rasgrf1 63 (EtOH)

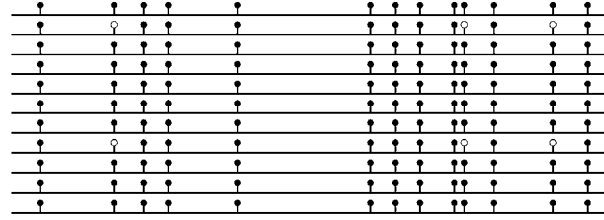


Rasgrf1 65 (EtOH)

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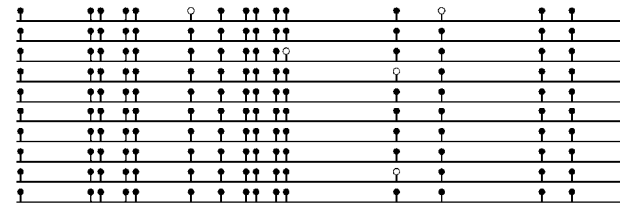


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[11]_Clone#147_G06_2008-07-28
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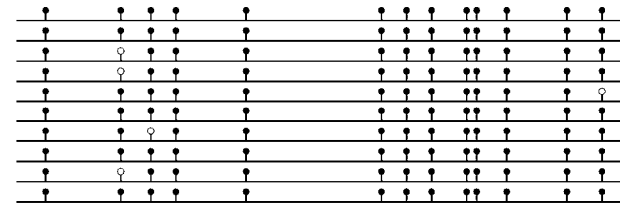


Rasgrf1 66 (EtOH)

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[6]_Clone#152_D07_2008-07-28
[7]_Clone#153_B03_2008-09-09
[8]_Clone#154_C03_2008-09-09
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[10]_Clone#156_B01_2008-07-31
[11]_Clone#549_A06_2008-09-02

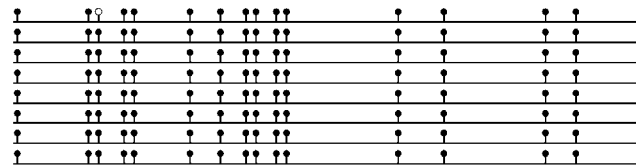


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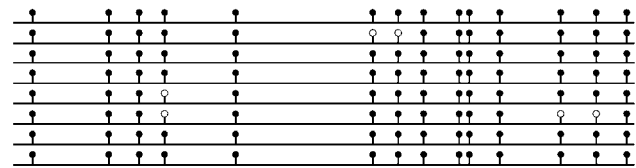


Rasgrf1 68 (EtOH)

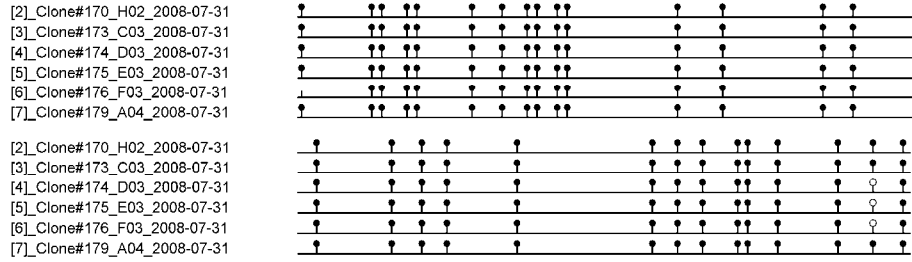
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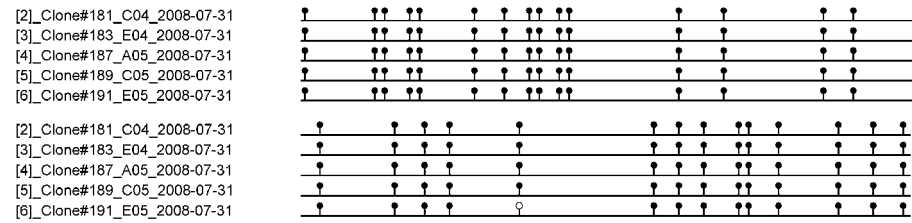
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[9]_Clone#169_G02_2008-07-31



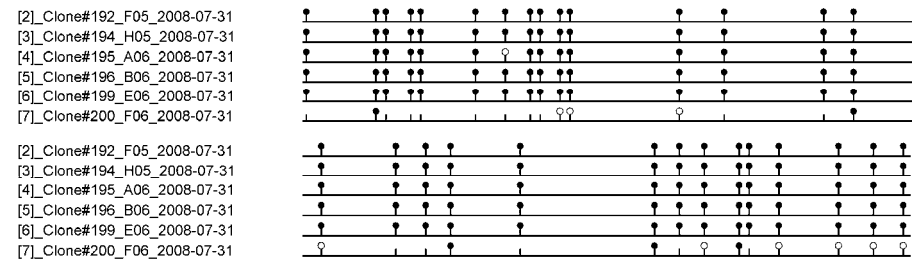
Rasgrf1 69 (Sucrose)



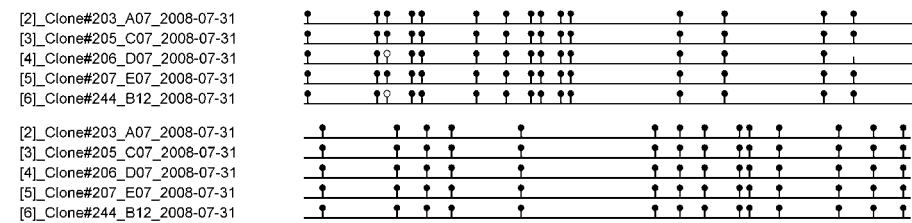
Rasgrf1 70 (Sucrose)



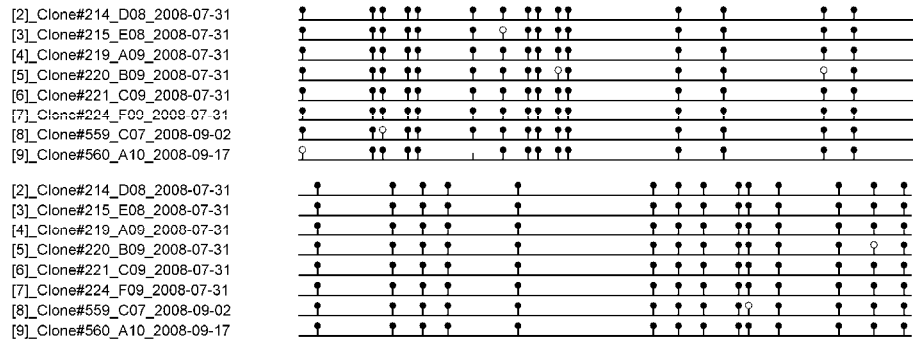
Rasgrf1 71 (Sucrose)



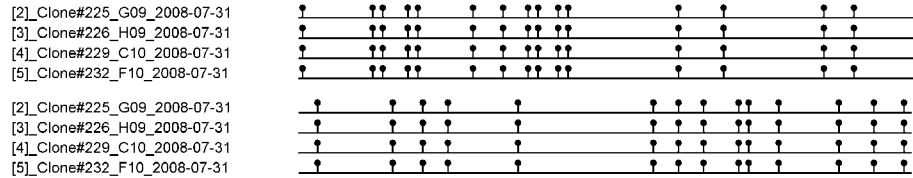
Rasgrf1 72 (Sucrose)



Rasgrf1 73 (Sucrose)

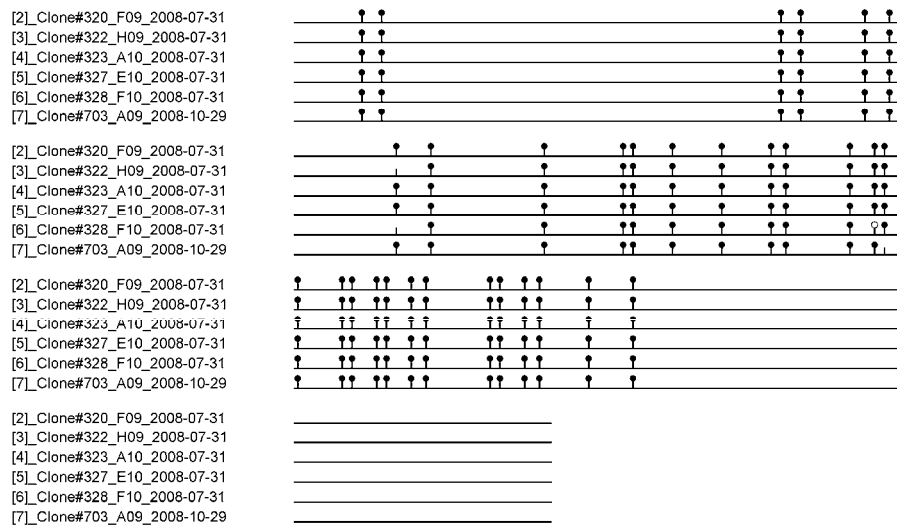


Rasgrf1 74 (Sucrose)



IG-DMR

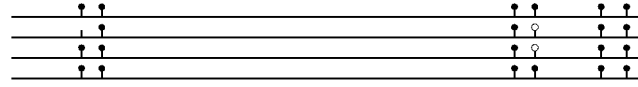
IG-DMR 62 (EtOH)



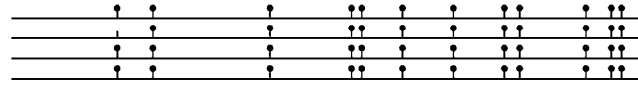
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IG-DMR 63 (EtOH)

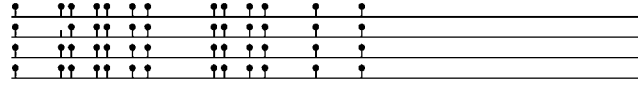
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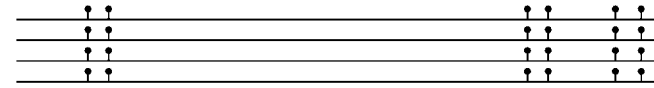


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IG-DMR 65 (EtOH)

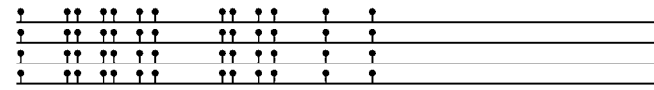
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[5]_Clone#349_C01_2008-08-10



[2]_Clone#342_D12_2008-07-31
[3]_Clone#344_F12_2008-07-31
[4]_Clone#345_G12_2008-07-31
[5]_Clone#349_C01_2008-08-10



[2]_Clone#342_D12_2008-07-31
[3]_Clone#344_F12_2008-07-31
[4]_Clone#345_G12_2008-07-31
[5]_Clone#349_C01_2008-08-10



[2]_Clone#342_D12_2008-07-31
[3]_Clone#344_F12_2008-07-31
[4]_Clone#345_G12_2008-07-31
[5]_Clone#349_C01_2008-08-10

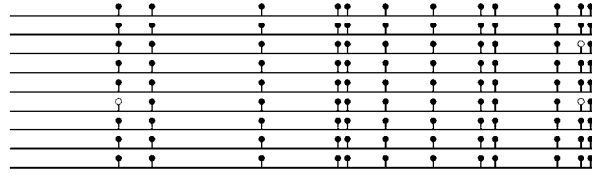


IG-DMR 66 (EtOH)

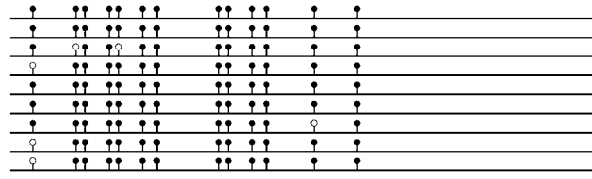
[2]_Clone#353_G01_2008-08-10
[3]_Clone#354_H01_2008-08-10
[4]_Clone#355_A02_2008-08-10
[5]_Clone#357_C02_2008-08-10
[6]_Clone#358_D02_2008-08-10
[7]_Clone#359_C09_2008-08-10
[8]_Clone#360_F02_2008-08-10
[9]_Clone#399_E07_2008-08-10
[10]_Clone#401_G07_2008-08-10



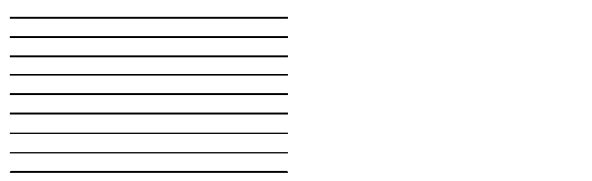
[2]_Clone#353_G01_2008-08-10
[3]_Clone#354_H01_2008-08-10
[4]_Clone#355_A02_2008-08-10
[5]_Clone#357_C02_2008-08-10
[6]_Clone#358_D02_2008-08-10
[7]_Clone#359_C09_2008-08-10
[8]_Clone#360_F02_2008-08-10
[9]_Clone#399_E07_2008-08-10
[10]_Clone#401_G07_2008-08-10



[2]_Clone#353_G01_2008-08-10
[3]_Clone#354_H01_2008-08-10
[4]_Clone#355_A02_2008-08-10
[5]_Clone#357_C02_2008-08-10
[6]_Clone#358_D02_2008-08-10
[7]_Clone#359_C09_2008-08-10
[8]_Clone#360_F02_2008-08-10
[9]_Clone#399_E07_2008-08-10
[10]_Clone#401_G07_2008-08-10

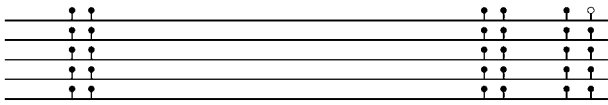


[2]_Clone#353_G01_2008-08-10
[3]_Clone#354_H01_2008-08-10
[4]_Clone#355_A02_2008-08-10
[5]_Clone#357_C02_2008-08-10
[6]_Clone#358_D02_2008-08-10
[7]_Clone#359_C09_2008-08-10
[8]_Clone#360_F02_2008-08-10
[9]_Clone#399_E07_2008-08-10
[10]_Clone#401_G07_2008-08-10

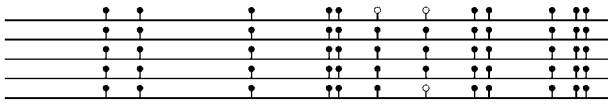


IG-DMR 68 (EtOH)

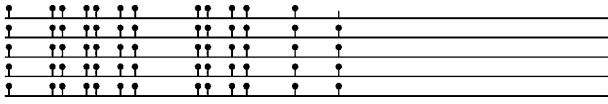
[2]_Clone#415_C01_2008-09-17
[3]_Clone#491_C03_2008-10-04
[4]_Clone#492_H06_2008-09-17
[5]_Clone#493_A11_2008-09-02
[6]_Clone#494_B07_2008-09-17



[2]_Clone#415_C01_2008-09-17
[3]_Clone#491_C03_2008-10-04
[4]_Clone#492_H06_2008-09-17
[5]_Clone#493_A11_2008-09-02
[6]_Clone#494_B07_2008-09-17



[2]_Clone#415_C01_2008-09-17
[3]_Clone#491_C03_2008-10-04
[4]_Clone#492_H06_2008-09-17
[5]_Clone#493_A11_2008-09-02
[6]_Clone#494_B07_2008-09-17



[2]_Clone#415_C01_2008-09-17
[3]_Clone#491_C03_2008-10-04
[4]_Clone#492_H06_2008-09-17
[5]_Clone#493_A11_2008-09-02
[6]_Clone#494_B07_2008-09-17



- Appendices -

IG-DMR 71 (Sucrose)

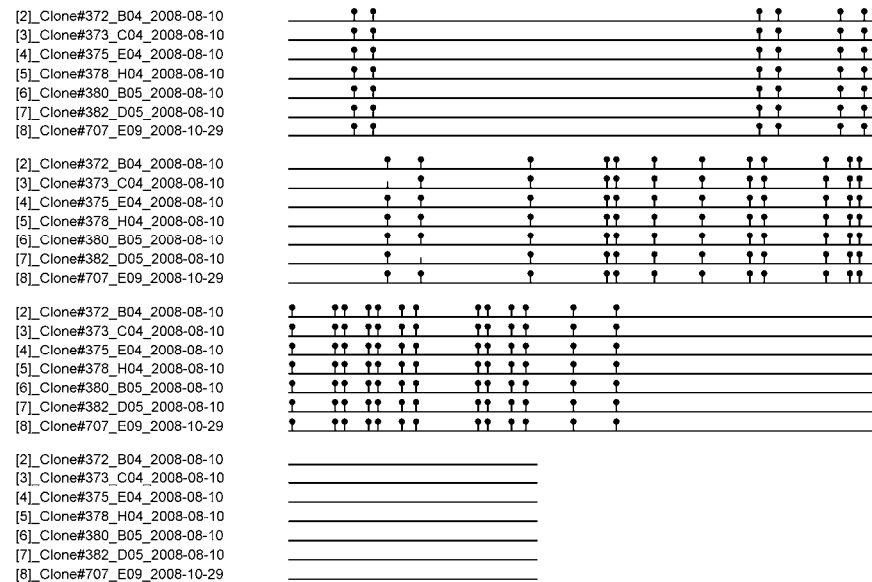
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IG-DMR 72 (Sucrose)

[2]_Clone#362_D09_2008-08-10	↑	↑																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
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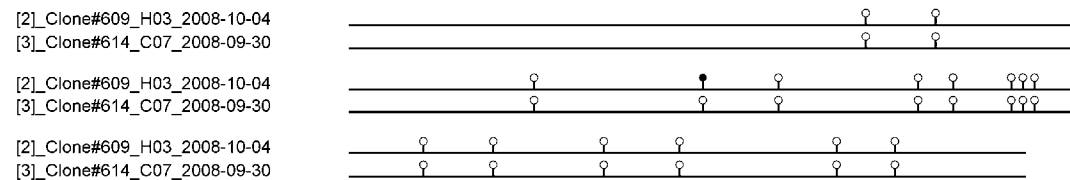
- Appendices -

IG-DMR 73 (Sucrose)

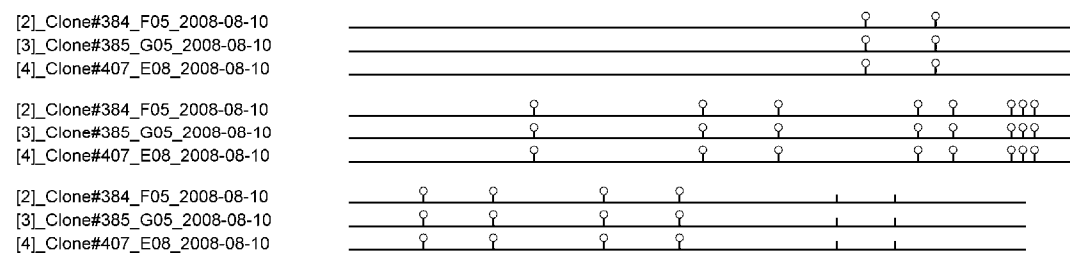


Snrpn ICR

Snrpn 60 (EtOH)



Snrpn 61 (Sucrose)

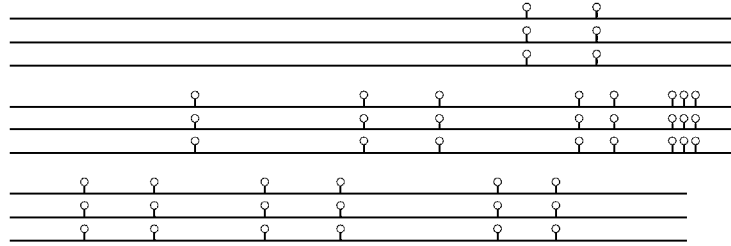


Snrpn 62 (EtOH)

[2]_Clone#387_A06_2008-08-10
 [3]_Clone#390_D06_2008-08-10
 [4]_Clone#411_A09_2008-08-10

[2]_Clone#387_A06_2008-08-10
 [3]_Clone#390_D06_2008-08-10
 [4]_Clone#411_A09_2008-08-10

[2]_Clone#387_A06_2008-08-10
 [3]_Clone#390_D06_2008-08-10
 [4]_Clone#411_A09_2008-08-10

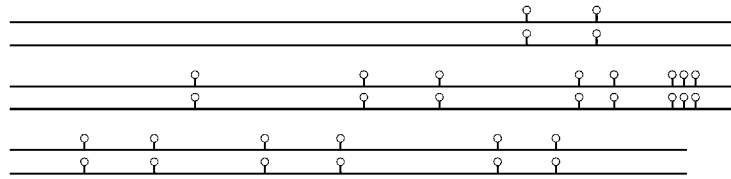


Snrpn 63 (EtOH)

[2]_Clone#391_E06_2008-08-10
 [3]_Clone#393_G06_2008-08-10

[2]_Clone#391_E06_2008-08-10
 [3]_Clone#393_G06_2008-08-10

[2]_Clone#391_E06_2008-08-10
 [3]_Clone#393_G06_2008-08-10

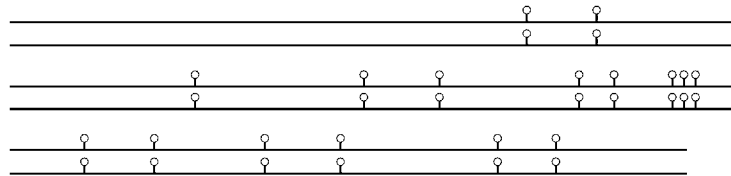


Snrpn 65 (EtOH)

[2]_Clone#458_F06_2008-09-02
 [3]_Clone#746_D02_2008-10-29

[2]_Clone#458_F06_2008-09-02
 [3]_Clone#746_D02_2008-10-29

[2]_Clone#458_F06_2008-09-02
 [3]_Clone#746_D02_2008-10-29

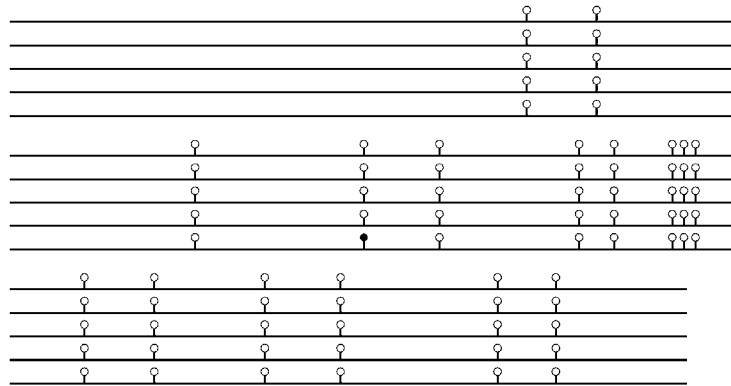


Snrpn 66 (EtOH)

[2]_Clone#444_H04_2008-09-02
 [3]_Clone#447_C05_2008-09-02
 [4]_Clone#731_E12_2008-10-29
 [5]_Clone#732_F12_2008-10-29
 [6]_Clone#734_H12_2008-10-29

[2]_Clone#444_H04_2008-09-02
 [3]_Clone#447_C05_2008-09-02
 [4]_Clone#731_E12_2008-10-29
 [5]_Clone#732_F12_2008-10-29
 [6]_Clone#734_H12_2008-10-29

[2]_Clone#444_H04_2008-09-02
 [3]_Clone#447_C05_2008-09-02
 [4]_Clone#731_E12_2008-10-29
 [5]_Clone#732_F12_2008-10-29
 [6]_Clone#734_H12_2008-10-29

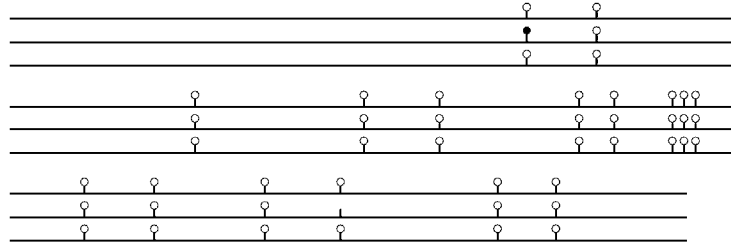


Snrpn 68 (EtOH)

[2]_Clone#433_E03_2008-09-02
 [3]_Clone#441_E04_2008-09-02
 [4]_Clone#773_G05_2008-10-29

[2]_Clone#433_E03_2008-09-02
 [3]_Clone#441_E04_2008-09-02
 [4]_Clone#773_G05_2008-10-29

[2]_Clone#433_E03_2008-09-02
 [3]_Clone#441_E04_2008-09-02
 [4]_Clone#773_G05_2008-10-29

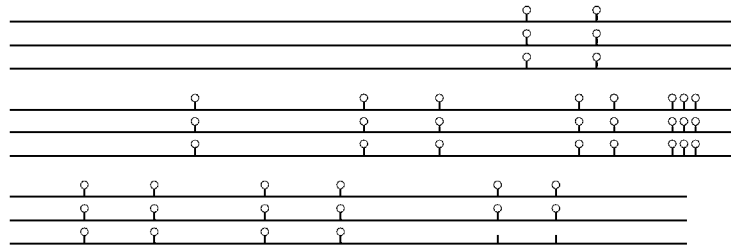


Snrpn 70 (Sucrose)

[2]_Clone#530_F03_2008-09-02
 [3]_Clone#532_E08_2008-09-17
 [4]_Clone#534_F08_2008-09-17

[2]_Clone#530_F03_2008-09-02
 [3]_Clone#532_E08_2008-09-17
 [4]_Clone#534_F08_2008-09-17

[2]_Clone#530_F03_2008-09-02
 [3]_Clone#532_E08_2008-09-17
 [4]_Clone#534_F08_2008-09-17

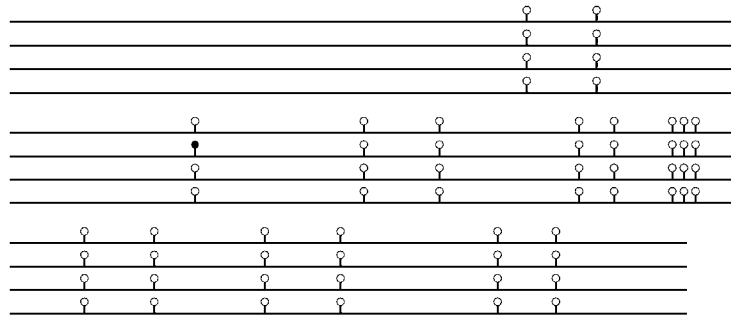


Snrpn 71 (Sucrose)

[2]_Clone#471_C08_2008-09-02
 [3]_Clone#779_E06_2008-10-29
 [4]_Clone#780_F06_2008-10-29
 [5]_Clone#782_H06_2008-10-29

[2]_Clone#471_C08_2008-09-02
 [3]_Clone#779_E06_2008-10-29
 [4]_Clone#780_F06_2008-10-29
 [5]_Clone#782_H06_2008-10-29

[2]_Clone#471_C08_2008-09-02
 [3]_Clone#779_E06_2008-10-29
 [4]_Clone#780_F06_2008-10-29
 [5]_Clone#782_H06_2008-10-29

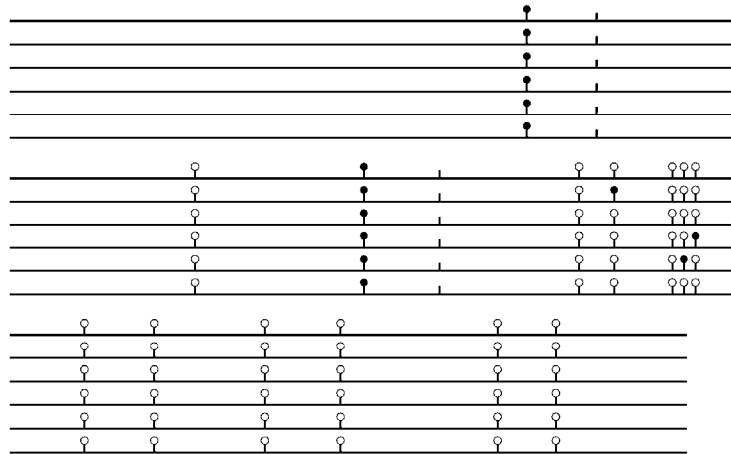


Snrpn 72 (Sucrose)

[2]_Clone#480_D09_2008-09-02
 [3]_Clone#481_E09_2008-09-02
 [4]_Clone#484_H09_2008-09-02
 [5]_Clone#485_A10_2008-09-02
 [6]_Clone#488_D10_2008-09-02
 [7]_Clone#490_F10_2008-09-02

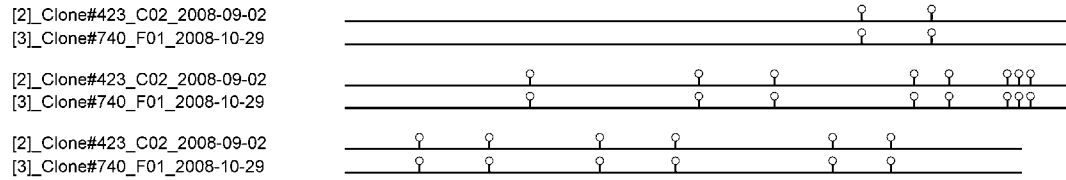
[2]_Clone#480_D09_2008-09-02
 [3]_Clone#481_E09_2008-09-02
 [4]_Clone#484_H09_2008-09-02
 [5]_Clone#485_A10_2008-09-02
 [6]_Clone#488_D10_2008-09-02
 [7]_Clone#490_F10_2008-09-02

[2]_Clone#480_D09_2008-09-02
 [3]_Clone#481_E09_2008-09-02
 [4]_Clone#484_H09_2008-09-02
 [5]_Clone#485_A10_2008-09-02
 [6]_Clone#488_D10_2008-09-02
 [7]_Clone#490_F10_2008-09-02

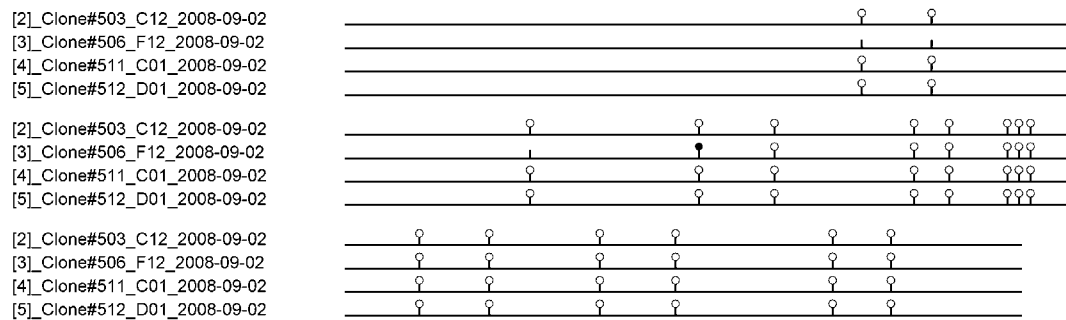


- A p p e n d i c e s -

Snrpn 73 (Sucrose)



Snrpn 74 (Sucrose)



Open lollipops (o) = unmethylated CpG

Filled lollipops (●) = methylated CpG

Absent lollipops = data missing

Appendix J

LTS robust regression macro script

```
DATA changed;
SET alldata.nosire;

RUN;
%MACRO ROBUSTMOUSE (DATA=, DV=, IV=, M=, CONTROLS=);
TITLE 'ROBUST LTS Base';
PROC ROBUSTREG data=&DATA /*outest=mouse1*/ FWLS METHOD=LTS;
Base: MODEL &DV=&CONTROLS/DIAGNOSTICS LEVERAGE;
RUN;
TITLE 'ROBUST LTS IV_DV';
PROC ROBUSTREG data=&DATA FWLS METHOD=LTS;
IV_DV: MODEL &DV=&CONTROLS &IV/DIAGNOSTICS LEVERAGE;
RUN;
ODS LISTING CLOSE;
ODS OUTPUT PARAMETERESTIMATESF=PATHA;
TITLE 'ROBUST LTS IV_M';
PROC ROBUSTREG data=&DATA FWLS METHOD=LTS;
IV_M: MODEL &M=&CONTROLS &IV/DIAGNOSTICS LEVERAGE;
RUN;
DATA PATHA;
SET PATHA(WHERE=(PARAMETER='Treatment'));
Keep ESTIMATE STDERR;
LABEL ESTIMATE='PATHAEST';
RENAME ESTIMATE=PATHAEST;
LABEL STDERR='STDERRA';
RENAME STDERR=STDERRA;

ODS OUTPUT PARAMETERESTIMATESF=PATHB;
TITLE 'ROBUST LTS IV_M_DV';
PROC ROBUSTREG data=&DATA FWLS METHOD=LTS;
IV_M_DV: MODEL &DV=&CONTROLS &M &IV/DIAGNOSTICS LEVERAGE;
RUN;
DATA PATHB;
SET PATHB(WHERE=(PARAMETER='/*Region/CpG of interest*/'));
KEEP ESTIMATE STDERR;
LABEL ESTIMATE='PATHBEST';
RENAME ESTIMATE=PATHBEST;
LABEL STDERR='STDERRB';
RENAME STDERR=STDERRB;
DATA PATHAB;

MERGE PATHA PATHB;
PATHAB=PATHAEST*PATHBEST;
SEAB=SQRT(PATHAEST*PATHAEST*STDERRB*STDERRB+PATHBEST*PATHBEST*STDERRA*STDERRA);
TSTAT=PATHAB/SEAB;
RUN;
PROC PRINT DATA=PATHAB;
RUN;
ODS OUTPUT CLOSE;
ODS LISTING;
%MEND ROBUSTMOUSE;
%ROBUSTMOUSE (DATA=CHANGED, DV=DAY/*day of interest*/, IV=TREATMENT, M=/*region/CpG of interest*/, CONTROLS=SEX LITTER_SIZE)
```

Appendix K

Pyrosequencing data

Male	Treatment	H19 CTCF 1 methylation (%)						Average methylation (%)
		CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	
60	EtOH	94	99	100	98	100	93	97.42
		96	98	100	98	100	93	
		95.00	98.50	100.00	98.00	100.00	93.00	
61	Sucrose	96	99	100	98	100	94	97.83
		96	99	100	98	100	94	
		96.00	99.00	100.00	98.00	100.00	94.00	
62	EtOH	95	97	100	95	82	90	94.00
		96	99	100	98	82	94	
		95.50	98.00	100.00	96.50	82.00	92.00	
63	EtOH	96	100	100	97	100	94	97.25
		94	98	100	96	100	92	
		95.00	99.00	100.00	96.50	100.00	93.00	
65	EtOH	95	99	100	97	91	88	95.58
		96	100	100	100	92	89	
		95.50	99.50	100.00	98.50	91.50	88.50	
66	EtOH	95	99	100	94	94	91	95.58
		95	99	100	94	95	91	
		95.00	99.00	100.00	94.00	94.50	91.00	
68	EtOH	96	99	100	98	100	94	97.83
		96	99	100	98	100	94	
		96.00	99.00	100.00	98.00	100.00	94.00	
69	Sucrose	96	100	100	98	100	94	97.75
		95	99	100	98	100	93	
		95.50	99.50	100.00	98.00	100.00	93.50	
70	Sucrose	95	99	100	97	100	93	97.67
		96	100	100	98	100	94	
		95.50	99.50	100.00	97.50	100.00	93.50	
71	Sucrose	96	99	100	97	100	94	97.50
		95	99	100	97	100	93	
		95.50	99.00	100.00	97.00	100.00	93.50	
72	Sucrose	92	100	100	97	99	94	96.83
		91	99	100	97	99	94	
		91.50	99.50	100.00	97.00	99.00	94.00	
73	Sucrose	95	98	100	96	93	93	96.17
		96	98	100	97	95	93	
		95.50	98.00	100.00	96.50	94.00	93.00	
74	Sucrose	96	99	100	99	100	95	97.92
		95	99	100	99	100	93	
		95.50	99.00	100.00	99.00	100.00	94.00	

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Dam	Pup	Sire / Treatment	H19 CTCF 1 methylation (%)					Average methylation (%)	4	63 / EtOH	64	64	73	65	68	67			
			CpG2	CpG3	CpG4	CpG5	CpG6				CpG7	64	65	71	65	68	66		
82	1	63 / EtOH	67	69	71	68	67	66	68.28	5	63 / EtOH	65	64	71	66	85	66	66.24	
			68	69	73	68	68	65				66	59	74	67	73	62		
			68	69	72	69	66	66				64	65	72	64	67	60		
			67.67	69.00	72.00	68.33	67.00	65.67				64	65	69	62	68	60		
			63	63	68	64	65	61				64.29	63.57	71.14	64.71	70.57	63.14		
	2	63 / EtOH	67	70	76	67	69	33	63.67	87	1	66 / EtOH	63	65	69	63	65	64	64.39
			67	69	77	67	67	31					63	64	66	63	63	59	
			68	71	78	68	69	?					65	65	71	64	63	64	
			67.33	70.00	77.00	67.33	68.33	32.00					63.67	64.67	68.67	63.33	63.67	62.33	
			77	79	83	76	79	76					76.00	77.00	95.00	78.00	80.00	74.33	
	3	63 / EtOH	77	79	83	75	78	74	78.28	89	1	68 / EtOH	75	77	94	79	79	75	80.06
			77	79	83	75	78	74					77	76	95	77	82	74	
			79	79	84	77	78	76					69	72	80	72	71	71	
			77.67	79.00	83.33	76.00	78.33	75.33					69	71	83	72	71	71	
			61	65	68	60	64	62					70	72	82	72	72	70	
	4	63 / EtOH	61	64	69	60	63	62	63.39	2	68 / EtOH	69.33	71.67	81.67	72.00	71.33	70.67	72.78	
			63	64	68	60	64	63				61	65	72	61	65	59		
			61.67	64.33	68.33	60.00	63.67	62.33				60	63	72	63	65	60		
			76	71	79	76	?	75				61	65	72	?	65	61		
			76	72	80	75	77	75				60.67	64.33	72.00	62.00	65.00	60.00		
5	63 / EtOH	76	72	80	75	77	75	76.08	3	68 / EtOH	64	67	71	66	63	62	64.00		
		78	73	81	78	76	75				64	62	75	66	67	60			
		76.67	72.00	80.00	76.33	76.50	75.00				66	67	73	67	67	63			
		62	67	70	64	67	58				64.67	65.33	73.00	66.33	65.67	61.67			
		68.33	72.00	76.67	69.33	73.33	67.00				72	73	97	70	75	72			
83	1	63 / EtOH	73	77	86	72	81	74	71.11	4	68 / EtOH	74	75	98	73	73	73	77.33	
			68.33	72.00	76.67	69.33	73.33	67.00				73	77	99	72	73	73		
			48	59	65	52	62	55				73.00	75.00	98.00	71.67	73.67	72.67		
			?	?	49	45	46	61				63	75	69	62	58	57		
	2	63 / EtOH	65	69	81	63	70	60	59.47	5	68 / EtOH	64	65	71	62	60	60	63.67	
			56.50	64.00	65.00	53.33	59.33	58.67				63.33	68.00	70.00	62.00	59.67	59.00		
			65	69	76	68	74	65				58	62	64	59	62	58		
			67	70	75	70	71	66				58	60	66	59	64	58		
	3	63 / EtOH	69	71	77	69	71	68	70.06	6	68 / EtOH	62	62	66	62	66	61	61.50	
			59.33	61.33	65.33	60.00	64.00	59.00				59.33	61.33	65.33	60.00	64.00	59.00		
			67.00	70.00	76.00	69.00	72.00	66.33				66	70	79	70	68	68		
			66	69	79	70	68	67				66	69	79	70	68	67		
			67	71	80	71	68	68				66.33	70.00	79.33	70.33	68.00	67.67		
			66	69	79	70	68	67				66	69	79	70	68	67		
			67	71	80	71	68	68				66.33	70.00	79.33	70.33	68.00	67.67		

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2	70 / Sucrose	80	78	84	69	76	66	74.17	2	72 / Sucrose	83	85	87	84	85	80	84.50	
		80	77	76	71	73	65				84	85	88	84	86	79		
		74	76	81	65	75	69				84	86	89	85	85	82		
		78.00	77.00	80.33	68.33	74.67	66.67				83.67	85.33	88.00	84.33	85.33	80.33		
3	70 / Sucrose	69	72	77	72	72	69	71.67	3	72 / Sucrose	79	82	90	84	82	76	82.28	
		68	71	78	70	72	67				79	81	86	79	84	73		
		71	71	78	72	73	68				83	83	91	84	84	81		
		69.33	71.33	77.67	71.33	72.33	68.00				80.33	82.00	89.00	82.33	83.33	76.67		
4	70 / Sucrose	71	76	85	72	7	74	76.08	98	1	74 / Sucrose	65	66	71	67	64	65	66.28
		70	74	86	73	76	74					63	65	73	66	68	63	
		72	76	87	74	77	76					63	65	71	66	68	64	
		71.00	75.33	86.00	73.00	76.50	74.67					63.67	65.33	71.67	66.33	66.67	64.00	
5	70 / Sucrose	63	66	66	63	65	60	64.56	2	74 / Sucrose	74	76	78	75	73	71	75.00	
		63	66	66	65	64	61				73	75	80	76	72	74		
		65	67	69	65	66	62				75	76	80	76	72	74		
		63.67	66.33	67.00	64.33	65.00	61.00				74.00	75.67	79.33	75.67	72.33	73.00		
6	70 / Sucrose	78	78	81	67	77	74	76.11	3	74 / Sucrose	67	68	75	69	68	63	69.39	
		78	78	81	66	79	74				67	69	74	70	71	61		
		79	79	83	65	79	74				69	70	77	73	71	67		
		78.33	78.33	81.67	66.00	78.33	74.00				67.67	69.00	75.33	70.67	70.00	63.67		
91	1	71 / Sucrose	68	72	80	67	70	68	71.56	4	74 / Sucrose	72	73	79	71	74	69	72.89
			69	71	81	72	70	68				70	74	78	71	72	69	
			71	71	80	71	70	69				72	74	80	71	72	71	
			69.33	71.33	80.33	70.00	70.00	68.33				71.33	73.67	79.00	71.00	72.67	69.67	
2	71 / Sucrose	61	64	66	61	67	58	63.61	5	74 / Sucrose	73	75	81	74	73	71	75.11	
		62	63	68	63	68	59				74	74	80	75	74	71		
		63	64	65	63	68	62				77	74	82	76	75	73		
		62.00	63.67	66.33	62.33	67.67	59.67				74.67	74.33	81.00	75.00	74.00	71.67		
3	71 / Sucrose	73	75	81	71	78	73	75.22	6	74 / Sucrose	69	77	91	81	80	79	79.83	
		72	74	80	72	77	73				70	76	90	82	79	81		
		73	75	82	73	78	74				71	76	92	82	80	81		
		72.67	74.67	81.00	72.00	77.67	73.33				70.00	76.33	91.00	81.67	79.67	80.33		
4	71 / Sucrose	79	85	88	80	74	78	80.50	100	1	72 / Sucrose	65	59	68	60	62	59	68.17
		81	84	85	81	73	78					67	69	75	69	74	62	
		78	83	89	80	74	79					73	70	82	68	72	73	
		79.33	84.00	87.33	80.33	73.67	78.33					68.33	66.00	75.00	65.67	69.33	64.67	
5	71 / Sucrose	74	81	85	80	81	7	79.31	2	72 / Sucrose	86	87	98	86	85	83	88.00	
		74	80	85	79	81	74				85	86	100	86	86	84		
		76	80	86	81	81	75				86	87	100	87	87	85		
		74.67	80.33	85.33	80.00	81.00	74.50				85.67	86.67	99.33	86.33	86.00	84.00		
95	1	72 / Sucrose	75	79	82	77	79	73	77.83	2	72 / Sucrose	86	87	98	86	85	83	88.00
			76	79	82	76	80	72				85	86	100	86	86	84	
			78	79	83	77	79	75				86	87	100	87	87	85	
			76.33	79.00	82.33	76.67	79.33	73.33				85.67	86.67	99.33	86.33	86.00	84.00	

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3	72 / Sucrose	62	66	71	63	62	?	66.00
		63	66	73	65	77	64	
		64	66	72	64	62	64	
		63.00	66.00	72.00	64.00	67.00	64.00	
4	72 / Sucrose	67	70	81	70	70	67	71.56
		68	70	84	72	69	69	
		68	71	83	71	70	68	
		67.67	70.33	82.67	71.00	69.67	68.00	
5	72 / Sucrose	71	73	74	66	69	60	68.89
		71	72	75	70	?	58	
		71	71	75	68	69	58	
		71.00	72.00	74.67	68.00	69.00	58.67	
6	72 / Sucrose	78	83	85	76	79	75	79.72
		79	83	86	77	77	76	
		81	82	88	77	77	76	
		79.33	82.67	86.33	76.67	77.67	75.67	
7	72 / Sucrose	89	96	100	64	93	91	88.94
		89	96	100	65	92	91	
		90	96	100	64	95	90	
		89.33	96.00	100.00	64.33	93.33	90.67	
8	72 / Sucrose	90	95	100	88	94	88	93.39
		91	93	100	93	96	88	
		92	94	100	93	96	90	
		91.00	94.00	100.00	91.33	95.33	88.67	
9	72 / Sucrose	79	76	80	80	81	72	79.06
		79	76	82	80	81	78	
		81	76	82	80	82	78	
		79.67	76.00	81.33	80.00	81.33	76.00	

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Dam	Pup	Sire / Treamnet	H19 CTCF 2 methylation (%)					Average methylation (%)	5	63 / EtOH	85	76	76	77	81				
			CpG11	CpG12	CpG13	CpG14	CpG15												
82	1	63 / EtOH	79	77	76	80	81	79.27	87	1	66 / EtOH	77	76	82	83	81			
			81	79	80	81	82					79	77	76	78	77			
			79	78	76	81	79					80.33	76.33	78.00	79.33	79.67			
			87	69	71	?	95					67	68	71	71	70			
			72	77	75	78	78					75.33	71.33	72.33	74.50	81.00			
			84	81	82	90	83					82	77	76	77	78			
	2	63 / EtOH	83	83	72	100	87	83.67				89	1	68 / EtOH	84	79	80	81	80
			84.00	80.33	78.00	92.33	83.67								82	78	76	78	80
			90	83	84	93	89								82.67	78.00	77.33	78.67	79.33
			91	87	86	91	90								72	69	70	100	72
			96	?	?	100	90								75	72	73	78	76
			92.33	85.00	85.00	94.67	89.67								75	70	67	73	70
3	63 / EtOH	73	70	71	71	74	89.33	2	68 / EtOH	68 / EtOH	74.00				70.33	70.00	83.67	72.67	
		76	74	75	75	75					78				74	74	75	74	
		72	72	70	73	73					81				75	79	80	79	
		73.67	72.00	72.00	73.00	74.00					79				75	76	80	78	
		85	82	83	85	83					79.33				74.67	76.33	78.33	77.00	
		85	85	85	82	82					81				78	84	100	86	
83	1	63 / EtOH	87	83	83	88	84				84.13	4	68 / EtOH	68 / EtOH	78	80	80	91	86
			85.67	83.33	83.67	85.00	83.00								85	80	78	92	79
			76	78	14	100	100								81.33	79.33	80.67	94.33	83.67
			14	61	15	100	100								76	73	73	19	49
			30	53	20	100	100								78	72	76	76	79
			40.00	64.00	16.33	100.00	100.00								76.67	72.67	74.00	57.33	69.33
	2	63 / EtOH	18	?	3	100	100	64.07	6	68 / EtOH	68 / EtOH				80	75	72	77	76
			14	17	8	100	100								82	79	79	80	77
			?	23	6	?	?								66	62	74	89	85
			16.00	20.00	5.67	100.00	100.00								76.00	72.00	75.00	82.00	79.33
			29	58	21	100	100								80	80	79	80	81
			40	32	*52	100	100								84	82	82	84	82
3	63 / EtOH	56	43	29	100	100	62.20	2				70 / Sucrose	70 / Sucrose	83	80	80	82	80	
		41.67	44.33	25.00	100.00	100.00								82.33	80.67	80.33	82.00	81.00	
		81	75	74	*100	80								79	76	69	87	82	
		80	79	79	81	78								82	78	74	88	83	
		87	81	77	83	84								79	77	69	92	82	
		82.67	78.33	76.67	82.00	80.67								80.00	77.00	70.67	89.00	82.33	
4	63 / EtOH	82	81	77	83	84	80.07		3	70 / Sucrose	70 / Sucrose			82	75	77	76	82	
		82.67	78.33	76.67	82.00	80.67								80	78	80	81	80	
		79	77	74	80	78								79	77	74	80	78	
		80.33	76.67	77.00	79.00	80.00								80.33	76.67	77.00	79.00	80.00	
		78.60																	

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4	70 / Sucrose	89	83	83	90	87	87.20	98	1	74 / Sucrose	95	87	90	88	88	89.60
		89	85	85	94	90					93	88	91	87	89	
		86	84	84	93	86					93	88	88	88	91	
		88.00	84.00	84.00	92.33	87.67					93.67	87.67	89.67	87.67	89.33	
5	70 / Sucrose	77	72	77	71	73	75.07	2	74 / Sucrose	74 / Sucrose	79	71	75	73	100	77.73
		76	75	81	75	75					79	76	78	77	76	
		76	74	78	73	73					77	74	74	79	78	
		76.33	73.67	78.67	73.00	73.67					78.33	73.67	75.67	76.33	84.67	
6	70 / Sucrose	82	83	85	78	86	83.40	3	74 / Sucrose	74 / Sucrose	84	77	86	87	89	85.20
		83	85	88	79	86					83	78	85	92	87	
		83	85	84	80	84					86	81	86	91	86	
		82.67	84.33	85.67	79.00	85.33					84.33	78.67	85.67	90.00	87.33	
91	1	71 / Sucrose	81	77	75	83	80.20	4	74 / Sucrose	74 / Sucrose	82	74	75	98	79	79.87
			82	79	75	83					82	74	76	84	78	
			84	80	76	85					83	74	77	83	79	
			82.33	78.67	75.33	83.67					82.33	74.00	76.00	88.33	78.67	
2	71 / Sucrose	82	75	77	76	75	78.80	5	74 / Sucrose	74 / Sucrose	80	76	78	76	100	79.27
		86	79	81	79	80					80	76	78	78	77	
		80	78	78	78	78					83	75	77	78	77	
		82.67	77.33	78.67	77.67	77.67					81.00	75.67	77.67	77.33	84.67	
3	71 / Sucrose	83	68	*61	84	85	83.60	6	74 / Sucrose	74 / Sucrose	85	79	82	81	89	83.13
		84	81	85	87	88					83	80	83	82	89	
		86	81	83	89	86					85	80	76	82	91	
		84.33	76.67	84.00	86.67	86.33					84.33	79.67	80.33	81.67	89.67	
4	71 / Sucrose	95	90	90	94	88	91.67	100	1	72 / Sucrose	82	79	79	88	78	81.93
		95	92	91	97	87					82	80	80	92	80	
		93	90	90	94	89					82	81	80	90	76	
		94.33	90.67	90.33	95.00	88.00					82.00	80.00	79.67	90.00	78.00	
5	71 / Sucrose	90	85	86	86	89	87.67	2	72 / Sucrose	72 / Sucrose	?	86	67	100	100	83.30
		90	87	87	88	88					?	?	?	100	100	
		89	86	87	89	88					71	61	77	100	100	
		89.67	86.00	86.67	87.67	88.33					71.00	73.50	72.00	100.00	100.00	
95	1	72 / Sucrose	88	84	85	83	86.73	3	72 / Sucrose	72 / Sucrose	88	87	83	100	91	90.27
			90	85	86	86					86	85	79	100	92	
			89	86	86	91					94	90	89	100	90	
			89.00	85.00	85.67	86.67					89.33	87.33	83.67	100.00	91.00	
2	72 / Sucrose	70	89	86	97	96	88.53	3	72 / Sucrose	72 / Sucrose	76	72	71	80	75	75.33
		67	92	87	99	96					75	72	74	80	73	
		71	92	90	100	96					77	76	73	82	74	
		69.33	91.00	87.67	98.67	96.00					76.00	73.33	72.67	80.67	74.00	

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4	72 / Sucrose	83	78	79	80	88	82.27
		84	80	79	83	86	
		84	81	80	86	83	
		83.67	79.67	79.33	83.00	85.67	
5	72 / Sucrose	84	75	75	77	77	78.07
		85	76	77	78	76	
		85	77	76	77	76	
		84.67	76.00	76.00	77.33	76.33	
6	72 / Sucrose	86	81	86	100	88	86.60
		87	82	87	83	87	
		90	82	88	83	89	
		87.67	81.67	87.00	88.67	88.00	
7	72 / Sucrose	95	94	94	100	100	95.20
		91	93	86	100	100	
		95	92	88	100	100	
		93.67	93.00	89.33	100.00	100.00	
8	72 / Sucrose	99	92	95	88	97	94.47
		96	93	94	91	99	
		97	94	95	87	100	
		97.33	93.00	94.67	88.67	98.67	
9	72 / Sucrose	85	88	76	95	88	87.47
		87	89	81	90	89	
		89	89	82	97	87	
		87.00	88.67	79.67	94.00	88.00	

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Male	Treatment	Rasgrf1 CTCF methylation (%)							Average methylation (%)
		CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26	
60	EtOH	84	86	72	78	100	72	100	84.93
		92	89	72	74	100	76	94	
		88.00	87.50	72.00	76.00	100.00	74.00	97.00	
61	Sucrose	91	90	76	77	100	79	100	88.07
		93	93	78	80	100	76	100	
		92.00	91.50	77.00	78.50	100.00	77.50	100.00	
62	EtOH	77	91	34	100	100	73	98	81.86
		77.00	91.00	34.00	100.00	100.00	73.00	98.00	
63	EtOH	100	96	77	80	98	79	94	92.43
		100	98	75	100	97	100	100	
		100.00	97.00	76.00	90.00	97.50	89.50	97.00	
65	EtOH	100	92	77	76	100	61	100	85.43
		80	91	76	80	100	63	100	
		90.00	91.50	76.50	78.00	100.00	62.00	100.00	
66	EtOH	100	93	77	79	100	76	100	88.43
		94	85	81	78	100	76	99	
		97.00	89.00	79.00	78.50	100.00	76.00	99.50	
68	EtOH	92	51	78	80	100	79	100	82.43
		87	54	75	80	100	78	100	
		89.50	52.50	76.50	80.00	100.00	78.50	100.00	
69	Sucrose	100	51	35	66	100	74	100	74.50
		89	53	35	64	100	76	100	
		94.50	52.00	35.00	65.00	100.00	75.00	100.00	
70	Sucrose	91	89	79	76	98	76	100	88.14
		92	90	80	83	99	81	100	
		91.50	89.50	79.50	79.50	98.50	78.50	100.00	
71	Sucrose	91	91	78	76	100	81	100	88.50
		90	98	76	80	98	80	100	
		90.50	94.50	77.00	78.00	99.00	80.50	100.00	
72	Sucrose	100	100	100	72	100	72	100	92.00
		100.00	100.00	100.00	72.00	100.00	72.00	100.00	
73	Sucrose	80	83	69	70	100	66	100	80.93
		91	84	71	65	100	54	100	
		85.50	83.50	70.00	67.50	100.00	60.00	100.00	
74	Sucrose	100	90	75	80	100	76	100	88.07
		100	89	70	78	100	75	100	
		100.00	89.50	72.50	79.00	100.00	75.50	100.00	

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Dam	Pup	Sire / Treatment	Rasgr1l CTCF methylation (%)							Average methylation (%)											
			CpG20	CpG21	CpG22	CpG23	CpG24	CpG25	CpG26												
82	1	63 / EtOH	87	55	48	42	93	38	100	65.36	90	1	70 / Sucrose	81	80	64	69	79	63	99	72.71
			77	83	56	34	90	56	87					79.00	81.50	60.00	51.50	84.50	59.50	93.00	
			82	55	44	45	93	33	100					48	45	28	30	60	25	49	
	2	63 / EtOH	84.50	55.00	46.00	43.50	93.00	35.00	100.00	80.14	2	70 / Sucrose	46	44	23	28	57	29	47	39.93	
			85	93	64	68	96	64	100				47.00	44.50	25.50	29.00	58.50	27.00	48.00		
			88	92	60	65	98	63	86				59	60	48	58	82	46	99		
	3	63 / EtOH	*no data available*							69.14	3	70 / Sucrose	63	56	42	54	89	42	94	63.71	
			61.00	58.00	45.00	56.00	85.50	44.00	96.50				76	72	61	63	94	52	95		
			78.50	72.00	53.00	54.00	87.00	50.00	89.50				82	85	57	62	88	60	85		
	83	1	63 / EtOH	82	80	68	63	96	62	95	73.93	4	70 / Sucrose	79.00	78.50	59.00	62.50	91.00	56.00	90.00	73.71
				64	82	58	46	84	60	95				57	57	50	54	67	51	78	
				73.00	81.00	63.00	54.50	90.00	61.00	95.00				58	57	45	47	71	49	74	
2		63 / EtOH	83	85	55	63	89	54	90	73.00	5	70 / Sucrose	57.50	57.00	47.50	50.50	69.00	50.00	76.00	58.21	
			82	78	52	59	95	51	86				76	29	26	55	44	46	91		
			82.50	81.50	53.50	61.00	92.00	52.50	88.00				75	26	21	58	44	32	84		
3		63 / EtOH	80	80	63	70	89	63	95	73.07	6	70 / Sucrose	75.50	27.50	23.50	56.50	44.00	39.00	87.50	50.50	
			70	75	55	56	87	55	85				66	64	36	47	69	51	93		
			75.00	77.50	59.00	63.00	88.00	59.00	90.00				74	49	35	40	51	47	82		
4		63 / EtOH	74	69	53	59	76	55	78	64.14	91	1	71 / Sucrose	70.00	56.50	35.50	43.50	60.00	49.00	87.50	57.43
			67	69	49	51	72	50	76					82	87	63	66	97	59	95	
			70.50	69.00	51.00	55.00	74.00	52.50	77.00					67	81	53	61	79	53	100	
87	1	66 / EtOH	44	45	33	34	59	11	56	40.14	2	71 / Sucrose	74.50	84.00	58.00	63.50	88.00	56.00	97.50	74.50	
			42	47	27	31	69	9	55				79	71	42	56	100	41	89		
			43.00	46.00	30.00	32.50	64.00	10.00	55.50				73	70	39	54	100	42	97		
	2	63 / EtOH	70	64	56	55	83	54	86	65.36	3	71 / Sucrose	76.00	70.50	40.50	55.00	100.00	41.50	93.00	68.07	
			63	66	50	48	88	54	78				82	83	67	70	96	65	95		
			66.50	65.00	53.00	51.50	85.50	54.00	82.00				82	80	61	59	95	57	100		
	3	68 / EtOH	88	27	44	64	100	14	100	60.36	4	71 / Sucrose	82.00	81.50	64.00	64.50	95.50	61.00	97.50	78.00	
			68	65	26	36	97	33	83				77	97	58	61	96	59	98		
			78.00	46.00	35.00	50.00	98.50	23.50	91.50				84	?	?	?	?	?	?		
	89	1	68 / EtOH	72	53	36	48	77	34	68	58.71	5	71 / Sucrose	80.50	97.00	58.00	61.00	96.00	59.00	98.00	78.50
				81	33	44	60	100	16	100				76	28	49	67	100	22	100	
				76.50	43.00	40.00	54.00	88.50	25.00	84.00				63	40	51	56	88	35	100	
2		68 / EtOH	74	81	58	62	81	55	95	71.29	95	1	72 / Sucrose	69.50	34.00	50.00	61.50	94.00	28.50	100.00	62.50
			76	77	50	56	88	55	90					78	65	41	43	89	33	95	
			75.00	79.00	54.00	59.00	84.50	55.00	92.50					73	65	40	53	88	34	100	
3		68 / EtOH	77	85	59	54	83	61	79	69.86	2	72 / Sucrose	75.50	65.00	40.50	48.00	88.50	33.50	97.50	64.07	
			72	77	57	52	80	57	85				69	63	54	47	89	36	100		
			74.50	81.00	58.00	53.00	81.50	59.00	82.00				77	77	58	60	84	60	96		
4		68 / EtOH	77	100	66	66	97	63	96	76.93	3	72 / Sucrose	73.00	70.00	56.00	53.50	86.50	48.00	98.00	69.29	
			74	98	55	60	82	56	87				82	79	58	65	80	60	96		
			75.50	99.00	60.50	63.00	89.50	59.50	91.50				81	75	63	63	85	62	93		
5	68 / EtOH	81	80	66	69	94	67	89	75.07	90	1	68 / EtOH	81.50	77.00	60.50	64.00	82.50	61.00	94.50	74.43	
		79	81	54	51	90	62	88					77	83	56	34	90	56	87		
		80.00	80.50	60.00	60.00	92.00	64.50	88.50					79.00	81.50	60.00	51.50	84.50	59.50	93.00		

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98	1	74 / Sucrose	71	84	62	52	82	50	94	72.29
			73	75	65	62	83	64	95	
			72.00	79.50	63.50	57.00	82.50	57.00	94.50	
	2	74 / Sucrose	77	98	62	63	83	59	90	75.71
			74	95	61	65	90	54	89	
			75.50	96.50	61.50	64.00	86.50	56.50	89.50	
	3	74 / Sucrose	78	75	53	58	87	49	88	68.36
			77	80	49	59	77	44	83	
			77.50	77.50	51.00	58.50	82.00	46.50	85.50	
	4	74 / Sucrose	70	66	59	50	79	48	88	64.93
			79	62	57	47	72	51	81	
			74.50	64.00	58.00	48.50	75.50	49.50	84.50	
100	5	74 / Sucrose	78	78	63	61	90	57	95	73.21
			78	82	55	53	90	57	88	
			78.00	80.00	59.00	57.00	90.00	57.00	91.50	
	6	74 / Sucrose	64	61	56	56	76	51	95	64.00
			69	57	51	46	74	51	89	
			66.50	59.00	53.50	51.00	75.00	51.00	92.00	
	1	72 / Sucrose	71	74	49	54	86	53	89	66.71
			71	68	47	53	84	51	84	
			71.00	71.00	48.00	53.50	85.00	52.00	86.50	
	2	72 / Sucrose	80	14	12	9	80	12	100	43.57
			79	15	13	9	77	10	100	
			79.50	14.50	12.50	9.00	78.50	11.00	100.00	
	3	72 / Sucrose	77	71	57	59	81	52	86	68.79
			74	72	54	54	80	52	94	
			75.50	71.50	55.50	56.50	80.50	52.00	90.00	
	4	72 / Sucrose	87	64	56	69	100	41	100	72.93
			85	62	54	64	100	39	100	
			86.00	63.00	55.00	66.50	100.00	40.00	100.00	
	5	72 / Sucrose	78	75	53	58	87	49	88	73.21
			88	78	59	65	100	47	100	
			83.00	76.50	56.00	61.50	93.50	48.00	94.00	
	6	72 / Sucrose	63	63	60	42	78	48	79	60.93
			61	66	58	44	69	45	77	
			62.00	64.50	59.00	43.00	73.50	46.50	78.00	
	7	72 / Sucrose	60	84	54	62	86	48	83	71.93
			80	82	66	63	87	63	89	
			70.00	83.00	60.00	62.50	86.50	55.50	86.00	
	8	72 / Sucrose	74	85	60	68	82	55	64	72.21
			87	84	71	73	96	65	47	
			80.50	84.50	65.50	70.50	89.00	60.00	55.50	
	9	72 / Sucrose	48	0	24	30	34	31	76	33.79
			56	17	13	16	27	20	81	
			52.00	8.50	18.50	23.00	30.50	25.50	78.50	

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Male	Treatment	Snrpn methylation (%)							Average methylation (%)
		CpG6	CpG7	CpG8	CpG9	CpG10	CpG11	CpG12	
60	EtOH	5	5	5	0	0	5	0	2.93
		5	5	4	3	0	4	?	
		5.00	5.00	4.50	1.50	0.00	4.50	0.00	
61	Sucrose	6	7	0	0	0	0	0	2.14
		0	3	5	0	5	4	0	
		3.00	5.00	2.50	0.00	2.50	2.00	0.00	
62	EtOH	2	4	3	0	3	0	4	2.71
		0	2	3	0	2	2	3	
		5	5	6	4	0	5	4	
63	EtOH	2.33	3.67	4.00	1.33	1.67	2.33	3.67	2.43
		2	3	3	0	0	0	2	
		0	7	9	0	0	0	8	
65	EtOH	1.00	5.00	6.00	0.00	0.00	0.00	5.00	3.57
		0	12	7	2	2	4	4	
		2	12	7	2	2	3	?	
66	EtOH	3	5	6	0	0	0	0	4.31
		1.67	9.67	6.67	1.33	1.33	2.33	2.00	
		2	6	6	2	0	7	3	
68	EtOH	2	5	5	2	2	8	4	1.79
		4	9	7	3	2	8	?	
		2.67	6.67	6.00	2.33	1.33	7.67	3.50	
69	Sucrose	0	3	3	0	0	9	0	21.33
		0	2	2	2	0	2	2	
		0.00	2.50	2.50	1.00	0.00	5.50	1.00	
70	Sucrose	8	8	7	4	4	80	13	3.48
		11	18	22	10	0	85	20	
		18	12	17	12	0	86	13	
71	Sucrose	12.33	12.67	15.33	8.67	1.33	83.67	15.33	3.93
		6	10	8	5	0	0	0	
		0	2	3	0	0	0	3	
72	Sucrose	8	4	5	0	6	5	8	4.93
		4.67	5.33	5.33	1.67	2.00	1.67	3.67	
		2	4	5	2	3	3	4	
73	Sucrose	0	2	2	2	2	2	0	6.07
		9	14	12	5	5	?	?	
		3.67	6.67	6.33	3.00	3.33	2.50	2.00	
74	Sucrose	3	6	4	3	6	9	4	2.29
		2	7	5	2	5	9	?	
		2.50	6.50	4.50	2.50	5.50	9.00	4.00	
75	Sucrose	2	5	6	2	3	11	5	6.07
		3	8	8	5	0	13	8	
		4	8	9	4	5	?	?	
76	Sucrose	3.00	7.00	7.67	3.67	2.67	12.00	6.50	2.29
		4	3	3	3	3	0	0	
		0	5	7	0	0	4	0	
77	Sucrose	2.00	4.00	5.00	1.50	1.50	2.00	0.00	2.29

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Dam	Pup	Sire / Treatment	Snrpn methylation (%)							Average methylation (%)										
			CpG6	CpG7	CpG8	CpG9	CpG10	CpG11	CpG12											
82	1	63 / EtOH	61	62	73	54	63	65	63	62.43										
			59	62	70	55	60	65	62											
			60.00	62.00	71.50	54.50	61.50	65.00	62.50											
			61	71	85	50	67	72	64											
	2	63 / EtOH	61	71	83	52	68	72	61	67.00										
			61.00	71.00	84.00	51.00	67.50	72.00	62.50											
			64	64	81	50	63	71	61											
			65	64	83	52	62	71	62											
	3	63 / EtOH	64.50	64.00	82.00	51.00	62.50	71.00	61.50	65.21										
			60	63	83	46	59	69	61											
			59	64	81	48	60	69	60											
			59.50	63.50	82.00	47.00	59.50	69.00	60.50											
83	5	63 / EtOH	66	68	79	56	68	66	66	67.14										
			65	70	78	59	67	65	67											
			65.50	69.00	78.50	57.50	67.50	65.50	66.50											
			60	67	82	46	61	69	62											
	1	63 / EtOH	61	67	81	50	63	71	60	64.29										
			60.50	67.00	81.50	48.00	62.00	70.00	61.00											
			59	59	72	49	62	62	60											
			59	61	69	53	57	63	57											
	2	63 / EtOH	59.00	60.00	70.50	51.00	59.50	62.50	58.50	60.14										
			65	68	83	50	62	70	64											
			64	68	81	53	65	70	66											
			64.50	68.00	82.00	51.50	63.50	70.00	65.00											
87	4	63 / EtOH	63	65	77	54	66	70	63	65.29										
			60	64	87	53	67	67	58											
			61.50	64.50	82.00	53.50	66.50	68.50	60.50											
			55	60	73	48	55	60	58											
	5	63 / EtOH	56	60	69	51	56	59	55	58.21										
			55.50	60.00	71.00	49.50	55.50	59.50	56.50											
			59	71	83	43	60	68	54											
			58	70	81	45	69	65	50											
89	1	66 / EtOH	58.50	70.50	82.00	44.00	64.50	66.50	52.00	62.57										
			61	64	85	46	60	71	64											
			59	64	83	50	61	69	66											
			60.00	64.00	84.00	48.00	60.50	70.00	65.00											
		68 / EtOH	61	64	85	46	60	71	64	64.50										
			59	64	83	50	61	69	66											
			60.00	64.00	84.00	48.00	60.50	70.00	65.00											
			61	64	85	46	60	71	64											
		68 / EtOH	59	64	83	50	61	69	66	64.50										
			60.00	64.00	84.00	48.00	60.50	70.00	65.00											
			61	64	85	46	60	71	64											
			59	64	83	50	61	69	66											

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95	5	71 / Sucrose	68	71	80	58	67	70	70	69.36	6	72 / Sucrose	61	74	77	64	71	66	62	67.64													
			66	72	77	64	69	69	70				61	72	79	65	71	63	61														
			67.00	71.50	78.50	61.00	68.00	69.50	70.00				61.00	73.00	78.00	64.50	71.00	64.50	61.50														
	1	72 / Sucrose	58	62	78	45	53	62	57	59.57	7	72 / Sucrose	69	72	81	66	69	68	66	69.43													
			58	63	71	51	56	64	56				69	69	81	66	65	66	65														
			58.00	62.50	74.50	48.00	54.50	63.00	56.50				69.00	70.50	81.00	66.00	67.00	67.00	65.50														
	2	72 / Sucrose	62	55	73	42	49	51	49	54.79	8	72 / Sucrose	67	66	68	62	63	69	67	65.43													
			57	59	73	46	49	51	51				66	65	67	61	62	66	67														
			59.50	57.00	73.00	44.00	49.00	51.00	50.00				66.50	65.50	67.50	61.50	62.50	67.50	67.00														
	3	72 / Sucrose	63	63	71	52	60	61	63	61.36	9	72 / Sucrose	85	87	95	77	84	81	75	83.71													
			59	64	68	56	56	61	62				86	87	97	77	84	82	75														
			61.00	63.50	69.50	54.00	58.00	61.00	62.50				85.50	87.00	96.00	77.00	84.00	81.50	75.00														
98	1	74 / Sucrose	55	57	66	51	55	59	56	57.00																							
			57	56	67	49	57	57	56																								
			56.00	56.50	66.50	50.00	56.00	58.00	56.00																								
	2	74 / Sucrose	53	50	80	51	51	47	43	55.00																							
			54	63	81	55	49	44	49																								
			53.50	56.50	80.50	53.00	50.00	45.50	46.00																								
	3	74 / Sucrose	48	54	71	38	48	39	41	48.21																							
			41	51	82	34	44	45	39																								
			44.50	52.50	76.50	36.00	46.00	42.00	40.00																								
	4	74 / Sucrose	61	66	73	56	63	65	59	61.93																							
			59	64	73	54	58	60	56																								
			60.00	65.00	73.00	55.00	60.50	62.50	57.50																								
5	74 / Sucrose	69	67	87	59	63	68	63	67.07																								
		69	64	80	58	63	67	62																									
		69.00	65.50	83.50	58.50	63.00	67.50	62.50																									
6	74 / Sucrose	78	77	100	65	69	75	68	74.79																								
		75	76	100	63	65	70	66																									
		76.50	76.50	100.00	64.00	67.00	72.50	67.00																									
100	1	72 / Sucrose	57	59	63	51	56	57	54	56.14																							
			55	56	63	50	55	56	54																								
			56.00	57.50	63.00	50.50	55.50	56.50	54.00																								
	2	72 / Sucrose	64	65	79	63	72	67	68	67.36																							
			61	66	73	66	68	66	65																								
			62.50	65.50	76.00	64.50	70.00	66.50	66.50																								
	3	72 / Sucrose	53	59	61	52	57	57	54	55.93																							
			53	57	62	51	55	58	54																								
			53.00	58.00	61.50	51.50	56.00	57.50	54.00																								
	4	72 / Sucrose	60	61	72	54	59	62	57	59.86																							
			58	60	70	52	58	60	55																								
			59.00	60.50	71.00	53.00	58.50	61.00	56.00																								
5	72 / Sucrose	71	74	81	73	71	73	68	71.86																								
		70	74	79	68	69	72	63																									
		70.50	74.00	80.00	70.50	70.00	72.50	65.50																									

CpG site specific analyses

Period: day 30-39

Mixed-effects REML regression Number of obs = 69

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
mse	35	1	2.0	2
sire	35	1	2.0	2

Log restricted-likelihood = -186.30781	Wald chi2(29)	=	10.49
	Prob > chi2	=	0.9994

	weight	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]
_I	treatmen~1	-1.286406	4.31192	-0.30	0.765	-9.737613 7.164801
_I	sex_1	-2.593695	2.057701	-1.26	0.207	-6.626715 1.439325
	h19cpg1	.0715712	.7317518	0.10	0.922	-1.362636 1.505778
	h19cpg2	.3732926	.526723	0.71	0.479	-.6590655 1.405651
	h19cpg3	.1110855	.455746	0.24	0.807	-.7821603 1.004331
	h19cpg4	-.1719754	.435407	-0.39	0.693	-1.025357 .6814066
	h19cpg5	-.0279914	.5813911	-0.05	0.962	-1.167497 1.111514
	h19cpg6	-.1930399	.2737113	-0.71	0.481	-.7295042 .3434245
	h19cpg11	-.2568221	.5842693	-0.44	0.660	-1.401969 .8883248
	h19cpg12	.0965403	.4095353	0.24	0.814	-.706134 .8992147
	h19cpg13	.1056471	.2546755	0.41	0.678	-.3935077 .6048019
	h19cpg14	-.0428082	.1928243	-0.22	0.824	-.4207368 .3351203
	h19cpg15	-.0041308	.2520058	-0.02	0.987	-.4980531 .4897915
rasgrf1	cpg20	-.0198742	.2197475	-0.09	0.928	-.4505714 .4108229
rasgrf1	cpg21	.0746328	.1563451	0.48	0.633	-.2317979 .3810635
rasgrf1	cpg22	.212412	.4260954	0.50	0.618	-.6227198 1.047544
rasgrf1	cpg23	-.0276157	.1471397	-0.19	0.851	-.3160044 .2607729
rasgrf1	cpg24	-.1142225	.2148275	-0.53	0.595	-.5352766 .3068316
rasgrf1	cpg25	-.2323963	.2199385	-1.06	0.291	-.6634679 .1986753
rasgrf1	cpg26	.0155868	.1944886	0.08	0.936	-.3656039 .3967774
	snrpncpg6	.2999133	.543293	0.55	0.581	-.7649214 1.364748
	snrpncpg7	-.1634216	.581732	-0.28	0.779	-.9767522 .6477522
	snrpncpg8	.0685574	.2668933	0.26	0.797	-.4545438 .5916586
	snrpncpg9	-.2433805	.2883526	-0.84	0.399	-.8085413 .3217803
	snrpncpg10	-.3055619	.4257494	-0.72	0.473	-1.140015 .5288915
	snrpncpg11	-.4103058	.637474	-0.64	0.520	-1.659732 .8391202
	snrpncpg12	.7258726	.7165462	1.01	0.311	-.6785323 2.130277
	_cons	23.07388	39.27387	0.59	0.557	-53.90149 100.0493

Period: day 40-63

Mixed-effects REML regression Number of obs = 559

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
mse	35	15	16.0	16
sire	35	15	16.0	16

Log restricted-likelihood = -1668.4292	Wald chi2(29)	=	2.24
	Prob > chi2	=	1.0000

weight	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
_Itreatmen~1	-.9821916	3.433995	-0.29	0.775	-7.712698	5.748315
_Isex_1	-.6568974	1.642706	-0.40	0.689	-3.876541	2.562746
h19cpg1	.122442	.5808451	0.21	0.833	-1.015993	1.260877
h19cpg2	.1463721	.4158907	0.35	0.725	-.6687587	.9615028
h19cpg3	.1110418	.3626125	0.31	0.759	-.5996655	.8217492
h19cpg4	-.189572	.3457058	-0.55	0.583	-.867143	.487999
h19cpg5	-.1106162	.4635065	-0.24	0.811	-1.019072	.7978398
h19cpg6	-.0951105	.2185617	-0.44	0.663	-.5234836	.3332625

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h19cpg11	-.1368006	.4663982	-0.29	0.769	-1.050924	.777323
h19cpg12	.1089556	.3270006	0.33	0.739	-.5319538	.7498649
h19cpg13	.0325164	.2026719	0.16	0.873	-.3647132	.4297459
h19cpg14	.013797	.1538475	0.09	0.929	-.2877386	.3153325
h19cpg15	-.0304492	.2010716	-0.15	0.880	-.4245423	.363644
rasgrf1cpg20	.030434	.1751112	0.17	0.862	-.3127777	.3736456
rasgrf1cpg21	.0128896	.1236685	0.10	0.917	-.2294962	.2552754
rasgrf1cpg22	.1854026	.3389164	0.55	0.584	-.4788614	.8496666
rasgrf1cpg23	-.0443129	.1165129	-0.38	0.704	-.2726739	.1840481
rasgrf1cpg24	-.0783701	.1715434	-0.46	0.648	-.4145889	.2578487
rasgrf1cpg25	-.1310222	.1755458	-0.75	0.455	-.4750856	.2130413
rasgrf1cpg26	-.0139374	.1553025	-0.09	0.928	-.3183248	.29045
snrpncpg6	.1915629	.4336135	0.44	0.659	-.6583039	1.04143
snrpncpg7	-.1143765	.4602383	-0.25	0.804	-1.016427	.787674
snrpncpg8	.0780861	.2130066	0.37	0.714	-.3393991	.4955713
snrpncpg9	-.1254523	.230242	-0.54	0.586	-.5767183	.3258138
snrpncpg10	-.1968681	.3399688	-0.58	0.563	-.8631947	.4694585
snrpncpg11	-.2664288	.5089723	-0.52	0.601	-1.263996	.7311385
snrpncpg12	.4346117	.5714447	0.76	0.447	-.6853992	1.554623
_cons	13.83798	31.3347	0.44	0.659	-47.57691	75.25287

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Appendix M

Clone data of sperm samples

H19 ICR

Treatment	Male	No. clones analysed	Avg. methylation of sample	Avg. methylation per site															
				1	CTCF binding site # 1						8	9	10	CTCF binding site # 2					16
					2	3	4	5	6	7				11	12	13	14	15	
EtOH	60	* unable to obtain data *																	
	62	6	94.82%	100%	100%	100%	100%	100%	67%	100%	100%	100%	100%	67%	100%	100%	100%	83%	100%
	63	13	100.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	65	10	100.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	66	12	96.90%	100%	100%	100%	100%	100%	64%	100%	100%	92%	100%	100%	100%	100%	100%	100%	92%
	68	5	100.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Sucrose	61	6	96.27%	100%	83%	80%	100%	100%	80%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	69	5	100.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	70	2	100.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	71	3	100.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	72	10	96.79%	100%	100%	100%	100%	90%	90%	100%	100%	90%	100%	100%	90%	90%	100%	100%	100%
	73	10	98.76%	90%	100%	100%	100%	100%	100%	100%	100%	90%	100%	100%	100%	100%	100%	100%	100%
	74	10	100.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

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IG-DMR

Treatment	Male	No. clones analysed	Avg. methylation of sample	Avg. methylation per site																															
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
EtOH	60	* unable to obtain data *																																	
	62	6	99.45%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	83%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		
	63	4	98.30%	100%	100%	100%	50%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		
	65	4	96.78%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	0%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	
	66	9	96.78%	100%	100%	100%	100%	100%	100%	89%	100%	100%	100%	100%	100%	100%	100%	100%	78%	100%	67%	89%	100%	100%	89%	100%	100%	100%	100%	100%	100%	100%	89%	100%	
	68	5	97.36%	100%	100%	100%	100%	100%	80%	100%	100%	100%	100%	100%	80%	60%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	
Sucrose	61	* unable to obtain data *																																	
	69	* unable to obtain data *																																	
	70	* unable to obtain data *																																	
	71	8	95.96%	100%	100%	100%	100%	100%	63%	100%	100%	100%	100%	100%	63%	50%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	
	72	8	96.74%	100%	100%	100%	88%	100%	100%	100%	75%	100%	100%	88%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	88%	100%	100%	100%	63%	100%	
	73	7	100.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	
	74	* unable to obtain data *																																	

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Rasgrf1 ICR

Treatment	Male	No. clones	Avg. methylation of sample	Avg. methylation per site																													
																							CTCF binding site										
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
EtOH	60	10	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	
	62	2	96.50%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	0.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	
	63	4	95.53%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	0.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	
	65	11	98.13%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	
	66	10	96.56%	100.00%	100.00%	100.00%	100.00%	100.00%	90.00%	100.00%	100.00%	100.00%	100.00%	90.00%	80.00%	90.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	70.00%	90.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	90.00%	100.00%	
	68	8	97.00%	100.00%	100.00%	87.50%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	
	61	6	97.72%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	50.00%	83.30%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	
	69	6	98.27%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	50.00%	100.00%
Sucrose	70	5	99.32%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	
	71	6	88.72%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	80.00%	100.00%	100.00%	83.30%	83.30%	83.30%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	83.30%	83.30%	83.30%	83.30%
	72	5	98.60%	100.00%	100.00%	60.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	
	73	8	96.98%	87.50%	100.00%	87.50%	100.00%	100.00%	100.00%	100.00%	87.50%	100.00%	100.00%	87.50%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	87.50%	100.00%	100.00%	
	74	4	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	

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Treatment	Male	No. clones	Avg. methylation of sample	Avg. methylation per site															
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>EtOH</i>	60	2	3.10%	0.00%	0.00%	0.00%	50.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	62	3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	63	2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	65	2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	66	5	1.24%	0.00%	0.00%	0.00%	20.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	68	3	2.23%	33.30%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
<i>Sucrose</i>	61	3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	?	?
	69	6	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	70	3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	71	4	1.55%	0.00%	0.00%	25.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	72	11	9.10%	0.00%	0.00%	0.00%	90.90%	0.00%	0.00%	9.10%	0.00%	9.10%	9.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	73	2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	74	4	0.01925	0.00%	0.00%	0.00%	25.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Regions in grey blocks show CTCF binding regions

? = no data

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Appendix N

Correlation Table

Spearman Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

Key: a $p < 0.1$
** $p < 0.05$
*** $p < 0.001$

Genetic region	Growth Rate	Offspring Weight (g)																			
		Day2	Day4	Day6	Day8	Day10	Day12	Day14	Day16	Day18	Day20	Day22	Day24	Day26	Day28	Day30	Day35	Day42	Day49	Day56	Day63
CTCF1	0.0836	0.0085	0.0647	0.0939	-0.0348	0.1138	0.0456	0.1192	0.0756	0.1395	0.1021	0.1159	0.1021	0.0787	0.1631	0.1779	0.3579	0.2853	0.1694	0.2208	0.0810
CTCF1	0.6329	0.9608	0.7075	0.5859	0.8405	0.5088	0.7919	0.4887	0.6614	0.4171	0.5536	0.5007	0.5536	0.6484	0.3419	0.2993	0.0348	0.0966	0.3306	0.2025	0.6438
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG2	0.2708	-0.0883	-0.0160	0.0353	-0.0307	0.0880	0.0703	0.1490	0.1693	0.2099	0.1910	0.2443	0.2222	0.1953	0.3033	0.3765	0.5181	0.4458	0.3152	0.4220	0.2689
H19CpG2	0.1156	0.6088	0.9263	0.8381	0.8591	0.6099	0.6838	0.3857	0.3237	0.2191	0.2644	0.1511	0.1928	0.2537	0.0721	0.0236	0.0014	0.0073	0.0651	0.0116	0.1183
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG3	0.2339	-0.0275	0.0622	0.0976	0.0372	0.1443	0.1124	0.1719	0.1709	0.1914	0.1988	0.2497	0.2292	0.2049	0.3200	0.3716	0.5026	0.4276	0.2932	0.3866	0.2372
H19CpG3	0.1762	0.8735	0.7185	0.5711	0.8294	0.4013	0.5138	0.3160	0.3190	0.2636	0.2451	0.1420	0.1787	0.2306	0.0571	0.0257	0.0021	0.0104	0.0874	0.0218	0.1701
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG4	0.2568	0.0481	0.1474	0.1669	0.1417	0.2302	0.2050	0.2867	0.2907	0.2963	0.3053	0.3333	0.2674	0.2241	0.3560	0.3952	0.5214	0.4563	0.3269	0.4052	0.2693
H19CpG4	0.1364	0.7806	0.3910	0.3308	0.4096	0.1768	0.2305	0.0900	0.0855	0.0793	0.0702	0.0470	0.1149	0.1889	0.0331	0.0170	0.0013	0.0059	0.0552	0.0158	0.1178
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG5	0.1677	0.1068	0.2289	0.2194	0.1138	0.2702	0.1670	0.2729	0.2533	0.2625	0.2866	0.3208	0.2860	0.2491	0.3309	0.3580	0.4748	0.3692	0.2910	0.2756	0.1920
H19CpG5	0.3357	0.5353	0.1794	0.1986	0.5087	0.1110	0.3304	0.1073	0.1360	0.1220	0.0902	0.0564	0.0908	0.1429	0.0487	0.0320	0.0040	0.0290	0.0899	0.1091	0.2691
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG6	0.1684	-0.0813	0.0003	0.0390	-0.0319	0.1217	0.0724	0.1603	0.1662	0.1684	0.1973	0.2587	0.2506	0.2287	0.3047	0.3577	0.4929	0.3519	0.2398	0.3127	0.1712
H19CpG6	0.3334	0.6375	0.9988	0.8215	0.8533	0.4796	0.6746	0.3503	0.3326	0.3262	0.2488	0.1276	0.1405	0.1797	0.0708	0.0322	0.0026	0.0382	0.1652	0.0674	0.3253
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG7	0.1027	0.0567	0.1779	0.1544	0.0885	0.2263	0.1859	0.2838	0.3118	0.3000	0.2757	0.2647	0.2364	0.1881	0.2699	0.2563	0.3862	0.3255	0.1818	0.2312	0.1164

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H19CpG7	0.5572	0.7425	0.2992	0.3687	0.6080	0.1844	0.2776	0.0935	0.0641	0.0754	0.1037	0.1187	0.1651	0.2719	0.1114	0.1313	0.0219	0.0564	0.2960	0.1815	0.5054
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
CTCF2	0.2723	0.0215	0.1572	0.2003	0.1481	0.2413	0.2814	0.3336	0.3757	0.3583	0.3110	0.3485	0.2744	0.2585	0.3359	0.3828	0.5012	0.3913	0.3351	0.3549	0.2866
CTCF2	0.1136	0.9010	0.3600	0.2415	0.3885	0.1562	0.0964	0.0468	0.0239	0.0319	0.0649	0.0373	0.1053	0.1280	0.0452	0.0212	0.0022	0.0201	0.0491	0.0364	0.0951
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG11	0.2533	0.0327	0.1648	0.1744	0.0551	0.1426	0.1393	0.1642	0.1649	0.1623	0.1523	0.2089	0.1487	0.1708	0.2770	0.3441	0.4699	0.3977	0.3509	0.3540	0.2648
H19CpG11	0.1420	0.8500	0.3369	0.3089	0.7494	0.4067	0.4179	0.3385	0.3366	0.3444	0.3753	0.2215	0.3868	0.3192	0.1020	0.0399	0.0044	0.0180	0.0388	0.0369	0.1242
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG12	0.2840	-0.0684	0.0843	0.1085	0.0528	0.1614	0.1957	0.2133	0.2392	0.2343	0.1957	0.2157	0.1611	0.1784	0.2882	0.3281	0.4506	0.4353	0.3333	0.3743	0.2858
H19CpG12	0.0983	0.6919	0.6249	0.5286	0.7598	0.3469	0.2527	0.2116	0.1600	0.1690	0.2527	0.2065	0.3478	0.2979	0.0882	0.0507	0.0066	0.0090	0.0504	0.0268	0.0960
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG13	0.2129	0.1225	0.2212	0.2453	0.1317	0.2502	0.2607	0.2675	0.3047	0.3286	0.2564	0.2387	0.2078	0.2001	0.2643	0.2897	0.4015	0.3896	0.2740	0.2809	0.2200
H19CpG13	0.2196	0.4767	0.1948	0.1494	0.4438	0.1411	0.1246	0.1148	0.0708	0.0504	0.1312	0.1610	0.2240	0.2419	0.1193	0.0866	0.0168	0.0207	0.1112	0.1021	0.2041
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG14	0.2271	-0.1173	-0.0411	-0.0592	-0.0172	0.0112	-0.0368	0.0823	0.1296	0.0471	0.1703	0.2783	0.2424	0.2924	0.3529	0.4053	0.4005	0.1860	0.2395	0.2326	0.2429
H19CpG14	0.1896	0.4957	0.8117	0.7317	0.9209	0.9482	0.8312	0.6334	0.4513	0.7848	0.3207	0.1003	0.1543	0.0835	0.0348	0.0142	0.0171	0.2848	0.1659	0.1788	0.1597
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
H19CpG15	0.1373	-0.1169	0.0004	-0.0654	-0.0728	-0.0539	-0.1357	0.0018	0.1129	0.0691	0.1314	0.2104	0.2160	0.2633	0.3198	0.3620	0.3645	0.1807	0.0937	0.1772	0.1376
H19CpG15	0.4317	0.4972	0.9982	0.7048	0.6731	0.7551	0.4299	0.9917	0.5122	0.6887	0.4448	0.2180	0.2059	0.1208	0.0573	0.0300	0.0313	0.2988	0.5924	0.3086	0.4307
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
Rasgrf1	-0.1867	0.2929	0.3173	0.3936	0.2755	0.2681	0.1880	0.2066	0.1436	0.1674	0.0793	0.1540	0.1730	0.1226	-0.0822	-0.0067	-0.0605	-0.1432	-0.1839	-0.1592	-0.1643
Rasgrf1	0.2903	0.0877	0.0633	0.0193	0.1091	0.1194	0.2795	0.2337	0.4106	0.3364	0.6507	0.3772	0.3205	0.4830	0.6389	0.9694	0.7338	0.4192	0.2979	0.3684	0.3532
	34.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	34.0000	34.0000	34.0000	34.0000	34.0000
Rasgrf1CpG20	-0.0861	0.1143	0.2232	0.1646	0.0941	0.1392	0.0485	0.0791	0.0508	0.1681	0.1248	0.1588	0.1339	0.0894	-0.0494	0.0465	-0.1326	-0.1067	-0.0993	-0.1000	-0.0843
Rasgrf1CpG20	0.6283	0.5131	0.1975	0.3448	0.5909	0.4252	0.7820	0.6517	0.7722	0.3346	0.4751	0.3622	0.4432	0.6097	0.7780	0.7906	0.4547	0.5479	0.5764	0.5736	0.6356
	34.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	34.0000	34.0000	34.0000	34.0000	34.0000
Rasgrf1CpG21	-0.2753	0.3020	0.2591	0.3714	0.2804	0.2998	0.1938	0.2090	0.1753	0.1512	0.0701	0.1511	0.1958	0.1523	-0.0810	-0.0752	-0.1067	-0.2169	-0.2517	-0.2339	-0.2459
Rasgrf1CpG21	0.1151	0.0779	0.1329	0.0280	0.1028	0.0801	0.2647	0.2282	0.3139	0.3859	0.6889	0.3864	0.2596	0.3824	0.6437	0.6675	0.5481	0.2179	0.1511	0.1831	0.1609
	34.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	34.0000	34.0000	34.0000	34.0000	34.0000
Rasgrf1CpG22	-0.1904	0.3417	0.3244	0.4484	0.3223	0.3328	0.2336	0.2566	0.1959	0.1794	0.1200	0.1820	0.2344	0.2013	-0.0325	-0.0269	-0.0314	-0.0698	-0.1888	-0.1699	-0.1669
Rasgrf1CpG22	0.2807	0.0445	0.0573	0.0069	0.0590	0.0508	0.1769	0.1367	0.2594	0.3025	0.4924	0.2954	0.1754	0.2463	0.8528	0.8780	0.8599	0.6951	0.2849	0.3368	0.3455
	34.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	34.0000	34.0000	34.0000	34.0000	34.0000
Rasgrf1CpG23	-0.0399	0.2380	0.2900	0.3301	0.2216	0.1515	0.1127	0.1095	0.0136	0.0781	0.0397	0.1356	0.1423	0.1031	0.0782	0.1579	0.1267	-0.0289	-0.0292	0.0010	-0.0278

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Rasgrf1CpG23	0.8227	0.1687	0.0911	0.0528	0.2008	0.3850	0.5193	0.5314	0.9382	0.6557	0.8207	0.4373	0.4149	0.5557	0.6552	0.3648	0.4752	0.8711	0.8697	0.9955	0.8759
	34.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	34.0000	34.0000	34.0000	34.0000	34.0000
Rasgrf1CpG24	-0.0150	0.2894	0.3989	0.4274	0.3088	0.3242	0.2760	0.3284	0.2459	0.3019	0.2494	0.2882	0.2835	0.2372	0.0493	0.1774	0.0424	-0.0844	-0.0677	-0.0766	-0.0026
Rasgrf1CpG24	0.9330	0.0918	0.0176	0.0104	0.0711	0.0574	0.1086	0.0541	0.1545	0.0779	0.1485	0.0932	0.0989	0.1700	0.7784	0.3080	0.8120	0.6352	0.7035	0.6668	0.9884
	34.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	34.0000	34.0000	34.0000	34.0000	34.0000
Rasgrf1CpG25	-0.2584	0.1180	0.1127	0.2313	0.1536	0.1385	0.0832	0.0852	0.0477	0.0093	-0.0700	-0.0095	0.0549	0.0416	-0.1743	-0.1557	-0.1495	-0.1983	-0.2978	-0.2522	-0.2382
Rasgrf1CpG25	0.1401	0.4995	0.5193	0.1813	0.3783	0.4276	0.6347	0.6265	0.7858	0.9579	0.6894	0.9567	0.7539	0.8123	0.3166	0.3718	0.3988	0.2609	0.0872	0.1501	0.1749
	34.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	34.0000	34.0000	34.0000	34.0000	34.0000
Rasgrf1CpG26	0.0729	0.1202	0.3827	0.3560	0.2250	0.3229	0.3391	0.4128	0.3625	0.4265	0.3945	0.3842	0.3476	0.2949	0.2092	0.2599	0.2032	0.1241	0.0308	0.0648	0.0753
Rasgrf1CpG26	0.6822	0.4914	0.0233	0.0358	0.1938	0.0585	0.0463	0.0137	0.0324	0.0106	0.0190	0.0227	0.0408	0.0854	0.2278	0.1316	0.2491	0.4843	0.8628	0.7157	0.6721
	34.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	35.0000	34.0000	34.0000	34.0000	34.0000	34.0000
Snrpn	0.2950	-0.1330	-0.1452	-0.0454	-0.0471	0.0587	-0.0539	0.0075	0.0263	-0.0109	0.1016	0.1639	0.1801	0.2529	0.2241	0.2739	0.3370	0.2762	0.2511	0.3521	0.2815
Snrpn	0.0854	0.4394	0.3982	0.7925	0.7850	0.7339	0.7548	0.9655	0.8792	0.9495	0.5556	0.3396	0.2933	0.1367	0.1889	0.1060	0.0477	0.1083	0.1457	0.0380	0.1014
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
SnrpnCpG6	0.1711	-0.1395	-0.1165	-0.0508	-0.0441	0.0633	-0.0643	0.0032	0.0446	-0.0081	0.0923	0.1465	0.1437	0.2254	0.1660	0.1794	0.2315	0.1454	0.1262	0.2045	0.1634
SnrpnCpG6	0.3258	0.4171	0.4987	0.7684	0.7986	0.7139	0.7095	0.9851	0.7963	0.9628	0.5925	0.3940	0.4031	0.1862	0.3331	0.2952	0.1809	0.4046	0.4699	0.2386	0.3484
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
SnrpnCpG7	0.3136	-0.0951	-0.1284	-0.0446	-0.0556	0.0626	-0.0491	0.0043	0.0430	-0.0072	0.0967	0.1675	0.1665	0.2304	0.1779	0.2478	0.3198	0.2689	0.2706	0.3544	0.3050
SnrpnCpG7	0.0665	0.5811	0.4555	0.7964	0.7473	0.7169	0.7763	0.9804	0.8033	0.9667	0.5747	0.3288	0.3319	0.1764	0.2994	0.1450	0.0611	0.1182	0.1160	0.0367	0.0749
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
SnrpnCpG8	0.0292	0.0703	0.0653	0.1582	0.0345	0.2023	-0.0323	0.0205	-0.0251	-0.0476	0.0461	0.1268	0.2310	0.3008	0.1419	0.1714	0.1004	0.0533	0.0943	0.0782	0.0339
SnrpnCpG8	0.8679	0.6837	0.7051	0.3567	0.8415	0.2368	0.8518	0.9056	0.8844	0.7827	0.7897	0.4612	0.1752	0.0746	0.4092	0.3175	0.5660	0.7611	0.5902	0.6553	0.8466
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
SnrpnCpG9	0.4005	-0.1136	-0.1144	-0.0619	-0.0722	-0.0066	-0.0223	0.0560	0.0730	0.0761	0.1924	0.2077	0.1581	0.1666	0.3060	0.3153	0.4551	0.4093	0.3827	0.4604	0.3809
SnrpnCpG9	0.0171	0.5096	0.5065	0.7199	0.6755	0.9696	0.8972	0.7458	0.6723	0.6591	0.2609	0.2243	0.3570	0.3314	0.0695	0.0610	0.0060	0.0146	0.0233	0.0054	0.0240
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
SnrpnCpG10	0.3404	-0.2388	-0.2481	-0.1361	-0.0924	-0.0085	-0.0743	-0.0229	-0.0012	-0.0322	0.0888	0.1853	0.1944	0.2686	0.2583	0.3022	0.3859	0.3303	0.3044	0.4134	0.3234
SnrpnCpG10	0.0454	0.1607	0.1446	0.4287	0.5921	0.9608	0.6666	0.8944	0.9946	0.8524	0.6067	0.2793	0.2559	0.1132	0.1283	0.0733	0.0220	0.0527	0.0754	0.0136	0.0581
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
SnrpnCpG11	0.2039	-0.2311	-0.2292	-0.1476	-0.1517	-0.0508	-0.1632	-0.1434	-0.1289	-0.1730	-0.0695	0.0113	0.0649	0.1807	0.0924	0.1811	0.1638	0.1759	0.1434	0.2490	0.1853
SnrpnCpG11	0.2400	0.1751	0.1788	0.3904	0.3770	0.7688	0.3416	0.4042	0.4536	0.3131	0.6871	0.9480	0.7070	0.2915	0.5921	0.2905	0.3471	0.3122	0.4111	0.1492	0.2866
	35.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	36.0000	35.0000	35.0000	35.0000	35.0000	35.0000
SnrpnCpG12	0.3316	-0.2072	-0.1935	-0.0748	-0.0427	0.0611	-0.0026	0.0296	0.0255	0.0153	0.1042	0.1584	0.1937	0.2659	0.2584	0.2831	0.3385	0.2932	0.2738	0.3486	0.3185

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SnrpnCpG12	0.0516	0.2254	0.2581	0.6646	0.8049	0.7235	0.9881	0.8638	0.8826	0.9293	0.5454	0.3560	0.2576	0.1170	0.1282	0.0943	0.0467	0.0874	0.1115	0.0402	0.0623
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Appendix O

Mediation analyses

<i>Region</i>	<i>Day</i>	<i>Direct effect of EtOH (g)</i>	<i>PATHAEST</i>	<i>STDERRA</i>	<i>PATHBEST</i>	<i>STDERRB</i>	<i>PATHAB</i>	<i>SEAB</i>	<i>TSTAT</i>	<i>Total effect (g)</i>
CTCF1	35	-1.5416	-6.5797	2.1734	-0.0553	0.0224	0.36369	0.19013	1.91289 ^a	-1.90529
H19CpG12	35	-1.75	-4.881	1.9467	-0.0262	0.0102	0.12781	0.07133	1.79178 ^a	-1.87781
H19CpG11	35	-1.3766	-5.6523	1.7507	-0.0179	0.0109	0.1011	0.069288	1.4591	-1.4777
CTCF1	49	-1.0772	-6.5797	2.1734	-0.0472	0.0248	0.31065	0.19297	1.60983	-1.38785
H19CpG13	35	-1.3207	-5.3146	1.8412	-0.0117	0.009	0.062102	0.052256	1.18842	-1.3828
CTCF2	35	-1.2484	-5.4252	2.1327	-0.0151	0.0206	0.081724	0.11651	0.70146	-1.33012
CTCF1	42	-1.1171	-6.5797	2.1734	-0.0273	0.022	0.17957	0.15652	1.1473	-1.29667
CTCF1	30	-0.9527	-6.5797	2.1734	-0.0452	0.0242	0.29742	0.18692	1.59112	-1.25012
H19CpG4	49	-0.9767	-7.9719	2.5353	-0.0169	0.024	0.1345	0.19587	0.68666	-1.1112
H19CpG15	35	-1.2026	-2.2159	2.9757	0.0516	0.0172	-0.11443	0.1583	-0.72286	-1.08817
H19CpG12	49	-0.9185	-4.881	1.9467	-0.0193	0.0158	0.094186	0.085693	1.0991	-1.01269
H19CpG2	49	-0.8526	-5.735	2.3385	-0.0258	0.0312	0.14778	0.18883	0.78264	-1.00038
H19CpG5	49	-0.8524	-6.9471	2.5031	-0.0196	0.027	0.13636	0.19374	0.7038	-0.98876
CTCF2	49	-0.8686	-5.4252	2.1327	-0.0195	0.025	0.10559	0.142	0.74357	-0.97419
H19CpG11	49	-0.8993	-5.6523	1.7507	-0.0129	0.0132	0.072962	0.077993	0.9355	-0.97226
SnrpnCpG9	35	-0.9947	1.6162	2.9376	-0.0192	0.0221	-0.03098	0.066645	-0.4648	-0.96372
H19CpG6	49	-0.8286	-5.8555	2.2447	-0.0212	0.0324	0.12406	0.19553	0.63449	-0.95266
H19CpG14	35	-0.9167	0.3017	3.9271	0.04	0.0163	0.012077	0.15727	0.07679	-0.90462

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H19CpG5	35	-0.8845	-6.9471	2.5031	-0.0025	0.0293	0.017534	0.20372	0.086071	-0.90203
H19CpG13	49	-0.8472	-5.3146	1.8412	-0.0081	0.0106	0.043203	0.058421	0.73951	-0.8904
H19CpG7	49	-0.815	-5.6764	2.4144	-0.013	0.019	0.073957	0.11258	0.65692	-0.88896
Rasgrf1CpG24	35	-0.8848	0.1385	3.4798	0.0096	0.0135	0.001328	0.033417	0.039753	-0.88347
SnrpnCpG12	35	-0.8893	0.3908	2.2802	-0.021	0.0209	-0.00819	0.048482	-0.16896	-0.88111
H19CpG11	42	-0.8249	-5.6523	1.7507	-0.0081	0.0109	0.04559	0.063011	0.72352	-0.87049
CTCF1	56	-0.7104	-6.5797	2.1734	-0.0216	0.0295	0.14231	0.19988	0.712	-0.85271
Rasgrf1CpG22	35	-0.8823	3.1981	3.0615	0.0106	0.0137	0.033926	0.054499	0.62251	-0.84837
H19CpG12	42	-0.8004	-4.881	1.9467	-0.0089	0.0131	0.043419	0.066294	0.65494	-0.84382
Rasgrf1	35	-0.8625	2.3071	2.6578	0.0115	0.0164	0.026616	0.048697	0.54657	-0.83588
SnrpnCpG12	42	-0.8333	0.3908	2.2802	0.0176	0.0182	0.006876	0.040739	0.16878	-0.82642
Rasgrf1CpG23	35	-0.8272	-1.4357	2.8161	0.0019	0.0146	-0.00272	0.021659	-0.12572	-0.82448
Rasgrf1CpG26	35	-0.8271	-2.5837	2.1284	0.0024	0.0151	-0.00615	0.039439	-0.15597	-0.82095
H19CpG3	49	-0.7608	-6.0626	2.1291	-0.0094	0.0328	0.056824	0.20009	0.284	-0.81762
H19CpG11	56	-0.7418	-5.6523	1.7507	-0.0125	0.0136	0.070439	0.079651	0.88435	-0.81224
H19CpG13	42	-0.783	-5.3146	1.8412	-0.0046	0.0087	0.024316	0.04719	0.51528	-0.80732
H19CpG4	30	-0.6986	-7.9719	2.5353	-0.0134	0.0273	0.1066	0.21989	0.48481	-0.8052
SnrpnCpG7	35	-0.9554	6.834	2.0319	-0.0227	0.0268	-0.15536	0.18893	-0.8223	-0.80004
Rasgrf1CpG21	35	-0.8985	8.1327	7.9456	0.0124	0.0094	0.10123	0.12515	0.8089	-0.79727
H19CpG7	30	-0.7793	-5.6764	2.4144	-0.0023	0.0207	0.013187	0.11745	0.11228	-0.79249
Rasgrf1CpG25	35	-0.8504	5.9818	2.6455	0.0116	0.0136	0.069473	0.08677	0.80066	-0.78093
SnrpnCpG11	42	-0.8161	3.174	2.6795	0.012	0.0184	0.038097	0.06672	0.571	-0.778
H19CpG2	35	-0.8237	-5.735	2.3385	0.0094	0.0306	-0.05366	0.17672	-0.30364	-0.77004
Rasgrf1CpG20	35	-0.7983	4.6627	2.9813	-0.0074	0.0173	-0.03458	0.083654	-0.41334	-0.76372
H19CpG12	56	-0.6988	-4.881	1.9467	-0.0133	0.0164	0.06492	0.084057	0.77233	-0.76372
SnrpnCpG12	56	-0.7671	0.3908	2.2802	0.0288	0.0225	0.011252	0.066235	0.16988	-0.75585

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SnrpnCpG10	42	-0.7675	1.636	2.4809	0.0097	0.0198	0.0159	0.040446	0.39311	-0.7516
Rasgrf1	30	-0.7596	2.3071	2.6578	0.008	0.0172	0.018348	0.045036	0.4074	-0.74125
H19CpG13	56	-0.6969	-5.3146	1.8412	-0.0081	0.0109	0.043257	0.059784	0.72356	-0.74016
SnrpnCpG9	42	-0.7688	1.6162	2.9376	0.0178	0.0195	0.028786	0.061093	0.47119	-0.74001
Rasgrf1CpG23	30	-0.7388	-1.4357	2.8161	0.0013	0.0153	-0.00182	0.022302	-0.08174	-0.73698
H19CpG6	35	-0.8095	-5.8555	2.2447	0.0129	0.0333	-0.07555	0.19709	-0.38331	-0.73395
CTCF2	42	-0.7191	-5.4252	2.1327	-0.0024	0.0207	0.013018	0.11237	0.11585	-0.73212
SnrpnCpG8	35	-0.8042	3.7843	2.5247	-0.0203	0.0218	-0.07673	0.097088	-0.7903	-0.72747
Rasgrf1CpG22	30	-0.734	3.1981	3.0615	-0.002	0.0147	-0.00653	0.047583	-0.13728	-0.72747
Rasgrf1CpG21	30	-0.7352	8.1327	7.9456	-0.0014	0.0098	-0.01113	0.080221	-0.1387	-0.72407
H19CpG5	56	-0.6324	-6.9471	2.5031	-0.0125	0.0309	0.087079	0.21671	0.40183	-0.71948
SnrpnCpG12	49	-0.7063	0.3908	2.2802	0.0014	0.0226	0.000534	0.009354	0.05705	-0.70577
SnrpnCpG11	56	-0.7715	3.174	2.6795	0.0213	0.0212	0.067551	0.088151	0.7663	-0.70395
H19CpG14	42	-0.7043	0.3017	3.9271	0.0222	0.0152	0.006698	0.087307	0.076722	-0.6976
H19CpG14	49	-0.7007	0.3017	3.9271	0.0162	0.019	0.004878	0.063758	0.076516	-0.69582
H19CpG7	56	-0.6309	-5.6764	2.4144	-0.0113	0.0217	0.064006	0.12612	0.50751	-0.69491
SnrpnCpG7	49	-0.6945	6.834	2.0319	-0.0003	0.0288	-0.00199	0.19661	-0.01014	-0.69251
SnrpnCpG8	42	-0.694	3.7843	2.5247	-0.0016	0.0195	-0.00599	0.073802	-0.08113	-0.68801
CTCF2	56	-0.6329	-5.4252	2.1327	-0.0099	0.0258	0.053494	0.14174	0.37742	-0.68639
SnrpnCpG10	56	-0.7264	1.636	2.4809	0.0251	0.0243	0.041103	0.073942	0.55589	-0.6853
H19CpG15	49	-0.7094	-2.2159	2.9757	0.0109	0.0231	-0.02424	0.060693	-0.39942	-0.68516
Snrpn	42	-0.785	6.9352	2.5099	0.0146	0.022	0.10117	0.15713	0.64385	-0.68383
SnrpnCpG9	30	-0.6982	1.6162	2.9376	-0.0105	0.0257	-0.01705	0.051864	-0.32864	-0.68116
SnrpnCpG6	30	-0.7091	7.5643	2.3092	-0.0043	0.0271	-0.0326	0.20513	-0.15894	-0.6765
H19CpG15	42	-0.7242	-2.2159	2.9757	0.0217	0.0186	-0.04798	0.076495	-0.62728	-0.67622
SnrpnCpG10	49	-0.6795	1.636	2.4809	-0.0023	0.0241	-0.0037	0.039906	-0.09277	-0.6758

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SnrpnCpG6	42	-0.7652	7.5643	2.3092	0.012	0.021	0.090765	0.16113	0.56331	-0.67444
Rasgrf1CpG24	42	-0.671	0.1385	3.4798	0.0015	0.0129	0.000213	0.005647	0.037753	-0.67079
Rasgrf1CpG23	42	-0.6668	-1.4357	2.8161	-0.0018	0.0141	0.002615	0.020837	0.1255	-0.66941
SnrpnCpG7	42	-0.7521	6.834	2.0319	0.0122	0.024	0.083694	0.16564	0.50527	-0.66841
SnrpnCpG8	49	-0.6853	3.7843	2.5247	-0.0045	0.0236	-0.01694	0.089968	-0.1883	-0.66836
Rasgrf1	42	-0.6707	2.3071	2.6578	0.0023	0.0159	0.005263	0.037143	0.1417	-0.66544
Rasgrf1CpG21	42	-0.6769	8.1327	7.9456	0.0016	0.0087	0.013351	0.072244	0.1848	-0.66355
H19CpG3	35	-0.7767	-6.0626	2.1291	0.019	0.0321	-0.11497	0.19867	-0.57868	-0.66173
Rasgrf1CpG20	42	-0.662	4.6627	2.9813	-0.0004	0.0166	-0.00183	0.077558	-0.0236	-0.66017
Rasgrf1CpG22	42	-0.6654	3.1981	3.0615	-0.0018	0.0126	-0.00568	0.04074	-0.13933	-0.65972
H19CpG7	35	-0.7525	-5.6764	2.4144	0.0166	0.0194	-0.09426	0.11698	-0.80574	-0.65824
SnrpnCpG9	49	-0.6671	1.6162	2.9376	-0.0072	0.0241	-0.01159	0.044284	-0.26176	-0.65551
H19CpG5	42	-0.6729	-6.9471	2.5031	0.0036	0.0248	-0.02476	0.17279	-0.14328	-0.64814
Rasgrf1CpG24	30	-0.6485	0.1385	3.4798	0.0259	0.0112	0.003594	0.090288	0.039809	-0.64491
H19CpG2	30	-0.6999	-5.735	2.3385	0.0097	0.0313	-0.05536	0.18064	-0.30649	-0.64454
Rasgrf1CpG24	49	-0.6398	0.1385	3.4798	0.0021	0.0144	0.000287	0.007488	0.038369	-0.63951
Rasgrf1CpG26	49	-0.6329	-2.5837	2.1284	-0.0015	0.016	0.003806	0.041558	0.091584	-0.63671
Rasgrf1CpG25	42	-0.6496	5.9818	2.6455	-0.0035	0.0126	-0.02103	0.075829	-0.27727	-0.62858
Rasgrf1CpG23	49	-0.6268	-1.4357	2.8161	0.0016	0.0157	-0.00226	0.02295	-0.09847	-0.62454
SnrpnCpG9	56	-0.6527	1.6162	2.9376	0.0268	0.0242	0.043299	0.087909	0.49255	-0.6094
SnrpnCpG6	35	-0.7419	7.5643	2.3092	-0.018	0.0253	-0.13612	0.19559	-0.69596	-0.60578
H19CpG11	30	-0.5546	-5.6523	1.7507	-0.009	0.0161	0.050768	0.092583	0.54834	-0.60537
Snrpn	49	-0.6535	6.9352	2.5099	-0.0071	0.027	-0.04897	0.18837	-0.25998	-0.60453
Rasgrf1CpG26	42	-0.6419	-2.5837	2.1284	0.0153	0.0133	-0.03944	0.047243	-0.83492	-0.60246
H19CpG7	42	-0.6403	-5.6764	2.4144	0.0075	0.0175	-0.04278	0.1009	-0.42401	-0.59752
H19CpG12	30	-0.5425	-4.881	1.9467	-0.0112	0.0194	0.054745	0.097401	0.56206	-0.59725

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SnrpnCpG11	49	-0.6198	3.174	2.6795	-0.0072	0.021	-0.0227	0.069288	-0.32755	-0.59711
H19CpG5	30	-0.674	-6.9471	2.5031	0.0117	0.0288	-0.08097	0.20192	-0.40098	-0.59303
Rasgrf1	49	-0.602	2.3071	2.6578	-0.0088	0.0176	-0.02029	0.04693	-0.43235	-0.58171
H19CpG14	30	-0.5908	0.3017	3.9271	0.0327	0.0151	0.009869	0.12853	0.07678	-0.58093
SnrpnCpG6	56	-0.8432	7.5643	2.3092	0.0348	0.0305	0.26316	0.24417	1.07777	-0.58004
SnrpnCpG10	35	-0.6201	1.636	2.4809	-0.0275	0.024	-0.04504	0.078795	-0.57164	-0.57506
H19CpG15	30	-0.6526	-2.2159	2.9757	0.0404	0.0179	-0.08953	0.12663	-0.70701	-0.56307
SnrpnCpG8	30	-0.555	3.7843	2.5247	0.0011	0.0229	0.004109	0.08665	0.047425	-0.55089
H19CpG6	30	-0.5649	-5.8555	2.2447	0.0029	0.0293	-0.01725	0.17163	-0.10053	-0.54765
H19CpG14	56	-0.5529	0.3017	3.9271	0.0252	0.0192	0.007595	0.099021	0.076697	-0.54531
H19CpG13	30	-0.5127	-5.3146	1.8412	-0.0053	0.0129	0.028423	0.069468	0.40916	-0.54112
Rasgrf1CpG20	30	-0.6012	4.6627	2.9813	0.0148	0.0159	0.068836	0.086351	0.79717	-0.53236
H19CpG4	35	-0.7457	-7.9719	2.5353	0.0268	0.0224	-0.21388	0.19079	-1.121	-0.53182
Snrpn	56	-0.6903	6.9352	2.5099	0.0242	0.0274	0.16756	0.19933	0.84063	-0.52274
Rasgrf1CpG21	49	-0.5805	8.1327	7.9456	-0.0073	0.0093	-0.05916	0.094934	-0.62312	-0.52135
H19CpG15	56	-0.5759	-2.2159	2.9757	0.0247	0.0233	-0.05467	0.089805	-0.6088	-0.52123
SnrpnCpG8	56	-0.5307	3.7843	2.5247	-0.0062	0.0241	-0.02353	0.092726	-0.25373	-0.50717
Rasgrf1CpG22	49	-0.5566	3.1981	3.0615	-0.0195	0.0133	-0.06233	0.073237	-0.85103	-0.49427
Rasgrf1CpG26	30	-0.5716	-2.5837	2.1284	0.0366	0.0212	-0.09467	0.095341	-0.99301	-0.47693
Rasgrf1CpG24	56	-0.4757	0.1385	3.4798	0.0018	0.0147	0.000253	0.006667	0.037917	-0.47545
Snrpn	35	-0.6629	6.9352	2.5099	-0.0272	0.0269	-0.18864	0.19854	-0.95016	-0.47426
SnrpnCpG7	56	-0.6803	6.834	2.0319	0.0302	0.029	0.20612	0.20713	0.9951	-0.47418
Rasgrf1CpG20	56	-0.4717	4.6627	2.9813	0.0012	0.0189	0.005675	0.088014	0.064476	-0.46603
Rasgrf1	56	-0.4565	2.3071	2.6578	-0.0033	0.018	-0.00755	0.042449	-0.17786	-0.44895
Rasgrf1CpG25	30	-0.5097	5.9818	2.6455	-0.0106	0.0119	-0.06347	0.076351	-0.83134	-0.44623
Rasgrf1CpG23	56	-0.4546	-1.4357	2.8161	0.0067	0.0159	-0.00964	0.029653	-0.32514	-0.44496

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Rasgrf1CpG25	49	-0.5615	5.9818	2.6455	-0.0198	0.013	-0.11856	0.093649	-1.26597	-0.44294
SnrpnCpG6	49	-0.5858	7.5643	2.3092	-0.0196	0.0256	-0.14804	0.19862	-0.74536	-0.43776
H19CpG6	42	-0.5664	-5.8555	2.2447	0.0224	0.0275	-0.13095	0.16848	-0.77723	-0.43545
SnrpnCpG12	30	-0.4321	0.3908	2.2802	-0.0155	0.0216	-0.00604	0.036232	-0.16667	-0.42606
Rasgrf1CpG20	49	-0.4687	4.6627	2.9813	-0.0099	0.0179	-0.046	0.088547	-0.51952	-0.4227
Rasgrf1CpG22	56	-0.4393	3.1981	3.0615	-0.0054	0.0151	-0.01726	0.051065	-0.33793	-0.42204
Rasgrf1CpG26	56	-0.446	-2.5837	2.1284	0.0095	0.0162	-0.0246	0.046554	-0.52851	-0.4214
SnrpnCpG7	30	-0.5168	6.834	2.0319	-0.0148	0.0249	-0.10119	0.17274	-0.5858	-0.41561
Rasgrf1CpG21	56	-0.4186	8.1327	7.9456	-0.0059	0.0099	-0.04829	0.092989	-0.51931	-0.37031
H19CpG2	42	-0.5275	-5.735	2.3385	0.0281	0.0281	-0.16104	0.17387	-0.92622	-0.36646
SnrpnCpG10	30	-0.4017	1.636	2.4809	-0.0224	0.0203	-0.03664	0.064758	-0.56583	-0.36506
Snrpn	30	-0.4626	6.9352	2.5099	-0.0142	0.0261	-0.09873	0.18448	-0.53519	-0.36387
SnrpnCpG11	35	-0.4602	3.174	2.6795	-0.0327	0.02	-0.10371	0.10811	-0.95935	-0.35649
H19CpG2	56	-0.4453	-5.735	2.3385	0.0175	0.033	-0.10008	0.19347	-0.51732	-0.34522
Rasgrf1CpG25	56	-0.4178	5.9818	2.6455	-0.0124	0.0141	-0.07403	0.090478	-0.81816	-0.34377
H19CpG6	56	-0.4376	-5.8555	2.2447	0.0184	0.0343	-0.1077	0.20533	-0.52454	-0.3299
SnrpnCpG11	30	-0.346	3.174	2.6795	-0.0183	0.02	-0.05797	0.080249	-0.72234	-0.28803
CTCF2	30	-0.3194	-5.4252	2.1327	0.0105	0.0305	-0.05723	0.16708	-0.34251	-0.26217
H19CpG4	56	-0.4202	-7.9719	2.5353	0.0213	0.0242	-0.16981	0.2004	-0.84735	-0.25039
H19CpG4	42	-0.4981	-7.9719	2.5353	0.0339	0.0186	-0.27034	0.17111	-1.57994	-0.22776
H19CpG3	56	-0.3657	-6.0626	2.1291	0.0299	0.0334	-0.18129	0.2124	-0.85353	-0.18441
H19CpG3	42	-0.4173	-6.0626	2.1291	0.0452	0.028	-0.27375	0.195	-1.40386	-0.14355
H19CpG3	30	-0.0164	-6.0626	2.1291	0.066	0.0381	-0.40033	0.27053	-1.47978	0.38393

^ap<0.1