THE ROLE OF GENOMIC IMPRINTS IN PLACENTAL BIOLOGY

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Genomic imprinting is a process by which heritable epigenetic marks at a subset of genomic loci are established in a sex-specific manner in parental gametes and then maintained in nascent offspring. This study probes the poorly understood function of genomic imprints in placental biology. Genomic imprints are responsible for the regulation of parent-of-origin specific monoallelic expression of clusters of imprinted genes. The primary epigenetic mark that distinguishes parental alleles at imprinted loci is 5-methylcytosine in the context of cytosine-guanine (CpG) dinucleotides within differentially methylated domains (DMDs). The *Dnmt1* gene encodes the maintenance DNA methyltransferase, an enzyme responsible for replicating CpG methylation that is critical throughout the process of genomic imprinting. Genetic disruption of the oocyte specific isoform of *Dnmt1* (*Dnmt1o*) results in partial and wide-spread loss of DMD methylation during preimplantation development and has strong effects on embryonic and extraembryonic development. In this dissertation the morphology of DNMT1o-deficient placentas is examined and their abnormal phenotypes correlated with loss of methylation at specific DMDs. A strong association between loss of methylation at the *Kcnq1* DMD and accumulation of trophoblast giant cells was made. In addition, an association between loss of methylation at the *Peg10* DMD and loss of fetal viability and placental labyrinthine volume was made. In conjunction with my study of the $Dnmt1^{\Delta 1o}$ model, I have engineered a novel targeted deletion of the imprinted *Klf14* gene and found it has an effect on placental growth. My thesis unambiguously shows that genomic imprints are essential for placental development.

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PREFACE

The researched presented in this dissertation contains published work from the following peer reviewed journal manuscripts on which I am an author.

Erik Koppes, Katherine Himes, J Richard Chaillet. 2015. Partial loss of genomic imprinting reveals important roles of Kcnq1 and Peg10 imprinted domains in placental development. PLoS ONE 10(8): e0135202

K.P. Himes, A. Young, E. Koppes, D. Stolz, Y. Barak, Y. Sadovsky and J.R. Chaillet. 2015. Loss of inherited genomic imprints in mice leads to severe disruption in placental lipid metabolism. Placenta **36**:389-396

K.P. Himes, E. Koppes and J. Richard Chaillet. 2013. Generalized disruption of inherited genomic imprints leads to wide-ranging placental defects and dysregulated fetal growth. Developmental Biology **373**:72-82

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LIST OF ABBREVIATIONS

Abbreviation	Full Nomenclature
BGS	Bisulfite Genomic Sequencing
CGI	CpG Island
CpG	Cytosine Guanine Dinucleotide
C-TGCs	Channel Trophoblast Giant Cell
DMD	Differentially Methylated Domain
Dnmt1s	Somatic Isoform of DNA-Methyltransferase-1
Dnmt1o	Oocyte-specific isoform of DNA-Methyltransferase-1
EPC	Ectoplacental Cone
ESC	Embryonic Stem Cell
EXE	Extraembryonic Ectoderm
GC	Glycogen Cell
H&E	Hematoxylin and Eosin
ICE	Imprint Control Element
ICM	Inner Cell Mass
ICR	Imprinting Control Region
IHC	Immunohistochemistry
iPSC	Induced Pluripotent Stem Cell
ISH	In Situ Hybridization
JZ	Junctional Zone
LZ	Labyrinthine Zone
matUPD	Maternal Uniparental Disomy
Mir	micro RNA gene
miRNA	micro RNA
ncRNA	Non-Coding RNA
P-TGC	Parietal Trophoblast Giant Cell
patUPD	Paternal Uniparental Disomy
PAS	Periodic Acid-Schiff
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S-TGCs	Sinusoidal Trophoblast Giant Cell
snoRNA	Small Nucleolar RNA
SpT	Spongiotrophoblast
SynT	Syncytiotrophoblast
TE	Trophectoderm
TGC	Trophoblast Giant Cell
TSC	Trophoblast Stem Cell
UPD	Uniparental Disomy

1.0 INTRODUCTION

1.1 GENOMIC IMPRINTING

1.1.1 Discovery of genomic imprints

In 1984 two different research groups firmly established that maternal and paternal genomes are non-equivalent in their developmental potential (*1-4*). These studies used pronuclear transfer techniques to remove one pronucleus and replace it with another to generate either bimaternal (gynogenetic) or bipaternal (androgenetic) genomes. They found that gynogenetic eggs are capable of developing into a 25-somite fetus but failed to properly generate extraembryonic tissue, whereas androgenetic eggs develop ample extraembryonic tissues but poorly developed fetuses. These results supported earlier research in mice that neither parthenogenetic (activated oocytes) nor gynogenetic conceptuses can survive (*5-7*). In addition, these results confirmed the clinical findings that parthenogenesis is not tolerated in human fetal development, and that androgenetic, dispermic conception results in molar pregnancies with excessive extraembryonic growth (*6, 8, 9*). In short, these studies found that the potential for embryonic development of male and female pronuclear genomes are different, with maternal genomes capable of initiating fetal but not extraembryonic development, and paternal genomes favoring extraembryonic growth.

Around the same time mouse geneticists using different recombination driven models including uniparental disomies (UPDs), reciprocal translocations (symmetrical rearrangement of non-homolgous chromosomes) and Robertsonian translocations (fusion of two acrocentric chromosomes) identified chromosomes that are responsible for parent-of-origin effects (*10, 11*). Research on phenotypes observed from inheritance of Robertsonian reciprocal translocations, in which offspring had either two maternal or two paternal copies of a chromosomal segment, are particularly informative (*12, 13*). These studies recognized mouse chromosomes 2, 6, 7, 11 and 17 as having parent of origin effects on fetal and/or placental growth (*10*). For example, in regards to chromosome 6, maternal uniparental disomy (matUPD) is lethal whereas paternal uniparental disomy (patUPD) is viable (*10*). Further studies into the early 90s using finer translocation breakpoints and chromosomal deletions narrowed down parent-of-origin effect regions (imprinted loci) to specific chromosomal bands (*14, 15*). This research led to speculation that these chromosomal regions have unique epigenetic marks that imprinted functionality based on which germline they passage through (*16*).

DNA methylation was determined to be the primary heritable epigenetic modification at endogenous genomic imprints in experiments using transgenes expressed from a single paternal allele under the control of upstream regulatory elements (*17-22*). It was found that DNA methylation at these transgene regulatory elements is established in germ cells (*ie*. oocytes and spermatocytes) in a sex-specific manner and that germline passage is associated with the allelic expression pattern in resultant offspring. For example, the *RSVIgmyc* transgene is a cloned DNA fragment that contains the RSV long terminal repeat (LTR) from the Rouse sarcoma virus (RSV) and the *c-myc* gene transposed within the immunoglobulin (Ig) locus. In this context, when integrated on an autosome, *c-myc* is expressed exclusively in myocardial tissue only when

paternally inherited; when maternally inherited it is not expressed. It was found that DNA methylation of the *RSVIgmyc* transgene occurs when the transgene is transmitted through the maternal germline but is erased (or not established) when transmitted through the paternal germline. Not only did this research establish DNA methylation as the key epigenetic mark in genomic imprinting, but it also provided evidence for erasure and establishment of imprinted DNA methylation during both male and female gametogenesis.

Through a combination of biochemical and genetic experiments endogenous genomic imprints were conclusively shown to be heritable DNA methylation marks at discrete loci scattered throughout the genome. Each chromosomal region identified as having parent-of-origin effects (*10*) was subsequently found to harbor one or more imprinted genes expressed from a single parental allele that are regulated by DNA elements methylated on one parental allele: mouse chromosome 2 harbors the *Gnas* and *Nnat* imprinted loci; mouse chromosome 6 harbors maternal imprints at the *Peg10, Nap1l5* and *Mest* loci; mouse chromosome 7 contains three maternal imprints at the *Kcnq1*, *Snrpn* and *Peg3* loci and one paternal imprint at *H19*; mouse chromosome 11 contains maternal imprints at the *Grb10* and *Zrsr1* loci; and mouse chromosome 17 has a maternal imprint at the *Igf2r* locus. A summary of all known and putative imprinted loci can be found at the online mouse book resource (*23*).

Imprinted regulatory DNA sequence regions have been named differentially methylated domains (DMDs), and alternatively called differentially methylated regions (DMRs), imprinting centers (ICs) and imprinting control regions (ICRs) in the scientific literature (*24*). There are subtle differences in the meanings of these terms; ICRs refer directly to the sequences controlling imprinted gene expression and often overlap with DNA binding sites for the insulator protein CTCF, whereas DMDs and DMRs refer to the larger imprinted methylated region including the

ICR. Confirmation of the role of DMDs was provided by the result of experimental insertion of *Snrpn* DMD sequences in place of the RSV sequences in the *RSVIgmyc* transgene; this *SnrpnIgmyc* transgene is consistently maternally imprinted in mice (*25*). Each set of imprinted genes and their regulatory DMD can be considered a single epigenetic unit termed an imprinted gene cluster. There are 24 imprinted clusters in the mouse with the majority of these conserved in humans (*26*). In section 1.5 I have reviewed 15 of the most functionally relevant clusters to placental biology.

1.1.2 Establishment and maintenance of imprinted DNA methylation

The DNA methyltransferase class of enzymes (DNMTs) catalyze the addition of methyl groups to the 5′ carbon of cytosine nucleosides (reviewed by (*27*)). This class of enzymes is divided into two groups. So called "*de novo*" DNA methyltransferases establish methylation in the absence of prior methylation and include the DNMT3a, DNMT3b and the non-catalytic DNMT3l. In contrast, the "maintenance" methyltransferase, DNMT1, along with its essential binding partner UHRF1, perpetuate CpG methylation after each round of DNA replication by recognizing and acting on hemi-methylated DNA templates. The DNMT2 enzyme, despite its homology and designated nomenclature, does not function to methylate DNA but rather acts on tRNA and other non-genomic substrates. The boundary between *de novo* and maintenance methyltransferases has recently become blurred, as they often work in tandem to establish and maintain DNA methylation.

The mammalian genome is methylated at roughly 70-80% of all CpGs in adult somatic tissues (*28*). However, during embryogenesis genomic methylation is dynamic as it is depleted during preimplantation and germ cell development and is then reacquired (*29-32*). DNA

methylation occurs at CpGs throughout the genome including intergenic regions, transposable elements, CpG islands (CGIs), gene bodies, inactivated X-chromosomes and imprinted DMDs (*28, 33*). Intergenic regions including microsatellites and other repetitive elements are sparsely populated with CpGs but are heavily methylated where they occur (*28, 33*). DNA methylation also occurs at endogenous retroviral elements and acts to suppress transcription and possibly transposition (*34, 35*). CpG islands are an interesting feature of the mouse genome because they are CpG rich sequences found at 70% of gene promoters but also within gene bodies, or at genic termini (*36*). They are largely unmethylated (at both active and inactive genes) except in some repressive heterochromatin contexts during development (*31, 36*). The majority of gene bodies outside of CGIs are highly methylated (*33*). In female conceptuses paternally inherited X chromosomes are inactive due to the absence of DNA methylation at the X inactivation center located at the promoter of the *Xist* gene (reviewed by (*37*)). The imprinted X-chromosome state is maintained in extraembryonic tissues (*e.g.* trophoblast) but is undone in the embryonic (epiblast) lineages of rodents prior to random X-inactivation (*37*).

DNA methylation at imprinted DMDs occurs in a monoallelic manner based on allele parent-of-origin and is stably inherited through nearly all cell types (*24*). DMDs have many of the same features as CGIs and are located within gene promoters, introns and intergenic regions (*36*). However unlike non-imprinted CGIs and other types of genomic methylation, imprinted DMDs do not undergo the same dynamic changes in their methylation levels during development (*30-32*). Not surprisingly imprinted DMD methylation, unlike other types of genomic methylation, follows a unique developmental dynamic cycle during which DMD methylation is erased in primordial germ cells (PGCs), reestablished in a sex-specific manner within gametes, and then faithfully perpetuated throughout embryonic and postnatal development (*24, 38*).

Although DNA methylation is the primary epigenetic mark at DMDs, imprinted loci contain histone modifications on methylated alleles (*e.g.* H3K9me3) and on unmethylated alleles (*e.g.* H3K4me3) that are acquired during the normal genomic imprinting process (*39, 40*).

The experiments previously discussed using imprinted *RSVIgmyc* transgenes firmly established that imprinted methylation marks are erased in PGCs. There has been recent controversy as to whether this demethylation event is through passive or active mechanisms (*41, 42*). PGCs are initially established in the proximal-posterior epiblast at embryonic day 6.25 (E6.25) of mice and then proliferate and migrate up the genital ridges toward their somatic sex organ niches between E8.5 and E12.5 (*43, 44*). Both imprinted and non-imprinted CpGs are markedly demethylated in PGCs at E12.5 (*44-47*). The initial handful of cells, estimated to be approximately 6-32 in number at E7 undergo numerous rounds of cell division to establish the gametic stem cell pool. During this time maintenance DNA methylation activity does not occur, leading to passive loss of DNA methylation (*41*). It is postulated that an active de-methylation mechanism involving the cytosine deaminase AID, the glycosylases TDG and MBD4, and components of the base excision repair pathway PARP1, XRCC1 and APE1 are also involved to insure complete erasure of methylation patterns (*48*, *49*). *Aid1* homozygous null PGCs have three times more DNA methylation than controls (*49*). In addition, the 5-methylcytosine dioxygenase TET1 is present in PGCs leading to elevated levels of 5-hydroxymethyl-cytosine (5hmC) in PGC genomes (*48*). It is unclear if 5hmC is involved in active demethylation, as it is not a substrate for glycosylases, although both the 5hmC deamination product 5-hydroxymethyluracil and the TET catalyzed carboxylated nucleoside 5-carboxylcytosine are TDG substrates (*41*, *48*, *50-52*). Alternatively, it has been suggested that 5hmC and bound TET proteins may act to inhibit DNMT1 activity thereby leading to passive loss (*42*).

The timing of DNA methylation acquisition in gametes, a process mediated by the *de novo* methyltransferases, differs between male and female germlines (*53*). The *RSVIgmyc* transgene experiments suggested that imprinted DNA methylation occurs during spermatogenesis by the pachytene stage prior to meiosis I, whereas in the female germline methylation is observed after meiosis I in mature (MII) oocytes (*18*). During spermatogenesis the *H19* DMD is methylated on the paternally inherited allele in spermatogonia and acquires methylation on the maternally inherited allele by the pachytene stage (*54*). DNA methylation at various CGIs and repetitive elements also increases during transition between spermatogonia and pachytene spermatocytes (*55*). During oogenesis the *Snrpn* DMD is methylated gradually during postnatal development, increasing from minimal methylation at postnatal day 0, to 50% methylation at postnatal day 10 and full methylation in MII oocytes at maturity (*56*). Genome wide methylation analysis has confirmed that in female mice oocyte genomic methylation gradually increases from very low levels in immature (postnatal day 5) oocytes through the germinal vesicle (GV) and MII mature stages (*32*). The overall level of DNA methylation is higher in sperm than female pronuclei, however the paternal pronuclear genome is rapidly demethylated within hours of fertilization (*30, 57*). Methylation at DMDs is known to shift, expand and recede at different loci and is evident in the comparison of gametic and mid-gestation embryonic DMD sizes (*58*). There are roughly 1600 CGIs (including imprinted and non-imprinted sequences) that are differentially methylated between sperm and oocytes, and roughly half of these are resistant to erasure during preimplantation development (*59*).

Maintenance of genomic imprinting during preimplantation development prior to zygotic *Dnmt1* activation is dependent on maternally inherited DNMT1 protein that is stored in oocytes (*60-63*). Oocytes produce somatic (DNMT1s) and oocyte-specific (DNMT1o) isoforms that

function in the zygote. Oocyte produced DNMT1s is 1620 amino acids in length, is sequestered in ooplasm but migrates to the zygotic nucleus, and functions to maintain imprints during the first and second zygotic cell divisions (*60, 61*). *Dnmt1* transcription from an upstream oocyte-specific promoter generates the mRNA template for the DNMT1o isoform, which is shortened by 118 amino acids at the N-terminus, and is sequestered in the ooplasm until the 4th mitotic division (*60, 62, 63*). The combination of maternal DNMT1s and DNMT1o maintains imprints up to the 32 cell stage when zygotically produced DNMT1s acquires the full maintenance responsibility (*60, 61*).

Imprinted DMD methylation is perpetuated on one parental allele of each genomic DMD during early mammalian development despite fluctuating levels of genomic methylation (*29-32*). The genome of mature spermatocytes contains higher overall levels of CpG methylation than oocytes (*30, 57*). Immediately following fertilization, the 5-methylcytosine sites within the male pronulclear genome are converted to 5hmC by the 5-methyl-cystosine dioxygenase TET3 (*64-68*). The female pronuclear genome, and a minority of CpG sites within male pronuclei are protected by maternal and zygotic stores of DPPA3 bound to dimethylated histone 3 lysine 9 (H3K9me2) chromatin (*69, 70*). Maternal deletion of *Dppa3* results in increased 5hmC abundance in the maternal pronuclear genome (*69, 70*). Both parental genomes are demethylated during preimplantation at the majority of sites (except at imprinted DMDs), however the effect is more pronounced in male pronuclear genomes due to their higher initial DNA methylation levels and rapid reduction (*30, 32, 64, 67*). In addition to the mechanism involving DPPA3 it is thought that DMDs are also protected from demethylation, through the recruitment of DNA-binding protein ZFP57 and TRIM28 (*71-76*). Maternal-zygotic deletion of *Zfp57* results in mid-gestation embryonic lethality with loss of methylation at the *Snrpn*, *Peg1*, *Peg3* and *Nnat* DMDs but

normal methylation at the *H19* and *Dlk1* DMDs and repetitive *Line1* and *Iap* elements (*71, 72*). *Trim28* homozygous null embryos have a loss of methylation at the *H19* and *Snrpn* but not *Peg3* DMDs (*74*). In ES cells *Zfp57* deletion results in loss of DMD methylation whereas *Trim28* deletion results in decreased H3K9me3 (*75*). These results combined with recent biochemical and structural data provide a model whereby ZFP57 binds methylated DMDs at the hexanucleotide TGCCGC, recruits TRIM28 via its KRAB domain, which promotes heterochromatin formation through the recruitment of the H3K9 methyltransferase EHMT2, DNMT1 and DNMT3 isoforms (*72-76*).

This preimplantation wave of demethylation occurs simultaneously with the phenomenon known as nuclear reprogramming whereby the zygotic genome transitions from a totipotent epigenetic state to a pluripotent state to be able to generate the many cell types required for embryonic and extraembryonic development (Recently reviewed in (*77-79*)). During cellular differentiation DNA methylation at various promoter CGIs is modulated (e.g. *Oct4*, *Nanog* and *Ets5*) thereby altering the expression of key components of important developmental programs (*31, 80, 81*). During the many waves of *de novo* methylation during the process of differentiation imprinted DMDs remain methylated only on one parental allele, indicating that there are mechanisms that prevent the establishment of methylation at unmethylated DMDs. The processes by which both *de novo* and maintenance DNA methyltransferases are regulated are the subjects of ongoing research in the field of epigenetics.

1.1.3 DNA methyltransferase mouse models

Targeted mutation of members of the *Dnmt* gene family have major effects due to loss of DNA methyltransferase activity at critical times during gametogenesis and preimplantation development (reviewed by (*27, 82*)). All three classes of DNMTs contain a catalytic C-terminal S-adenyl-methionone (SAM)-dependent methyltransferase domain. *Dnmt1* encodes a 1620 amino acid protein with an extensive N-terminal domain containing a nuclear localization, replication foci targeting sequence (RFTS), a CXXC zinc finger that recognizes umethylated DNA and a tandem bromo-adjacent homology (BAH) domains (*83-85*). DNMT1 is understood to function as homodimer, mediated through interactions with its N-terminus, at hemimethylated DNA (*86*). The *de novo* methyltransferase genes *Dnmt3a* and *Dnmt3b* encode much shorter proteins (908 and 776 amino acids respectively) with a N-terminal DNA binding PWWP motif and an unmethylated H3K4 binding plant homeodomain like (PHD) domain (*87-89*). The *Dnmt3l* gene encodes a catalytically inactive methyltransferase lacking an N-terminal PHD domain that interacts, stimulates and co-localizes with DNMT3a and DNMT3b at heterochromatin loci (*87, 90, 91*). DNMT3A interacts with DNMT3L to form tetramers that can bind CpGs with a periodicity of 8-10 base pairs (*92*).

Targeted mutations of the genes encoding *de novo* DNMTs (i.e. *Dnmt3a*, *Dnmt3b* and *Dnmt3l*) have profound developmental effects based on the failure to establish DNA methylation. Heterozygous null *Dnmt3a* and *Dnmt3b* as well as compound heterozygotes are normal and can be intercrossed to generate homozygous null offspring (*93*). *Dnmt3a* homozygous offspring die at 4 weeks after birth and are growth restricted but have normal imprinted and non-imprinted genomic methylation (*93*). *Dnmt3b* null mice die in late gestation and have reduced IAP and centromeric satellite DNA methylation similar to the loss-of-function

DNMT3B mutations associated with ICF syndrome (*93*). Compound homozygous *Dnmt3a*/*Dnmt3b* null mice die around gastrulation and have loss of IAP and centromeric DNA methylation (*93*). Methylation in each of these models does not affect imprints and is higher at repetitive elements than observed in *Dnmt1* null models indicating that imprints and some genomic methylation established prior to implantation (*i.e*. in the germline) is maintained (*93*).

Dnmt3l homozygous null mutants survive to adulthood but are infertile (*91, 94*). In males absence of DNMT3L results in germ cell mitotic catastrophe, lack of spermatocytes and infertility, and a lack of DNA methylation at the *H19* paternal DMD and repetitive DNA elements (*91, 95, 96*). Female homozygous *Dnmt3l* dams generate oocytes that lack all maternal imprinted and non-imprinted DNA methylation and yield progeny that fail to develop past E9.5 (*91, 94, 97-99*). This embryonic lethality is thought to be caused by extraembryonic defects including failure of chroio-allantoic fusion and trophoblast cell differentiation defects that are reviewed in comparison to my results in section 3.5.4 (*94, 97, 99*). Conditional germ cell deletion of *Dnmt3a*, but not *Dnmt3b*, recapitulates the many of the same phenotypes of *Dnmt3l* null models including a maternal effect from the maternal germline and mitotic catastrophe from the paternal germline providing definitive evidence that DNMT3A and DNMT3L work in concert (*100*).

Targeted inactivation of *Dnmt1* is severely detrimental to mouse embryonic development. The $Dnmt1^{n}$ allele is a N-terminal partial loss-of-function mutation (*101*). Homozygous $Dnmt1^{n}$ mice have a 70% reduction in total genomic methylation including at imprinted DMDs and arrest development at 9.5 primarily due to failure of hematopoiesis (*101, 102*). Two complete loss of function null alleles that target the replication targeting region ($Dnmt1^{s}$) and the catalytic C-terminus ($Dnmt1^{c}$) respectively have even lower methylation levels (95% loss) and arrest

development in homozygous embryos at E8.5 (*103*). Residual genomic methylation is likely due to *de novo* methyltransferase activity (*103*). Intriguingly, despite the drastic loss of DNA methylation, homozygous *Dnmt1* null embryonic stem cells are viable and proliferate until they are induced to differentiate (*101, 103*). This suggests that DNA methylation is not vital for stem cell survival and proliferation but is required for complex mammalian development. These *Dnmt1* models are indicative of the importance of DNA methylation but cannot distinguish which types of genomic methylation targets are critical for mammalian development.

Maternal homozygous deletion of the oocyte specific promoter of *Dnmt1* (*Dnmt1o*) eliminates DNMT1o protein from resulting oocytes and preimplantation embryos and results in partial loss of DMD methylation, but normal non-DMD methylation levels (*104*). These conceptuses are mosaic for loss of imprinting and some survive to term (*104*). Many DNMT1o-deficient mutants have severe fetal phenotypes, and preliminary findings also indicate placental defects (*105*). A detailed introduction of the $Dnmt1^{\Delta 1o}$ model is provided in Section 2.2. The $Dnmt1^{\Delta 1o}$ model is superb for investigating the direct effects of loss of DMD methylation because it specifically results in primary epigenetic mutations only at imprinted DMDs. The $Dnmt1^{v}$ allele constitutively expresses the *Dnmt1o* isoform in all tissues (*106*). Homozygous $Dnmt1^{v/v}$ mice are normal, however maternal-zygotic lethality is observed in compound $Dnmt1^{v}$ and *Dmap1* null embryos (*106, 107*). This epistatic interaction is likely direct because the N-terminus of DNMT1, which the DNMT1o isoform lacks, contains a DMAP binding motif, and their interaction is eliminated in compound mutant offspring (*107*).

How DNMT1 is able to distinguish between imprinted and non-imprinted sequences during times of dynamic methylation changes is an important biological question. An interesting finding in homozygous $Dnmt1^{c}$ ESCs is that the majority of DNA methylation, but not imprinted

DMD methylation, returns after transfection of a functional *Dnmt1* minigene, further validating that imprints require germline passage to be established (*108, 109*). Building on this finding a *Dnmt1* TET-Off allele was engineered to suppress expression of *Dnmt1 in vitro* with addition of tetracycline to culture media, and then allow recovery of expression after replacement with tetracycline depleted media (*110, 111*). Similar to the minigene system genomic methylation largely recovers (*110, 111*). However imprints and a select group of promoter CGIs do not recover their methylation (*110, 111*). Many of these CGIs are located at developmentally important regulators, indicating that DNMT1 is required for maintenance of imprint-like sequences that are established in heterologous contexts.

The DNMT1 protein contains an intrinsically disordered domain from amino acids 100-400 that is a strong candidate for autoregulation of DNMT1 activity and its interaction with other proteins (*110*). This region has no predicted structure, is rich in charged amino acids (making it hydrophilic) and contains multiple proline residues that block secondary structures such as alpha helices (*110*). Amino acids 190-350 of DNMT1 within the disordered domain, like the process of genomic imprinting, is specific to mammals (*110*). Although deletion of the whole mammalian specific region has no effect on genomic methylation smaller deletions have distinct effects in ESCs (*110*). Deletion of the coding region for amino acids 255-291 results in loss of DMD and non-DMD methylation, deletion of amino acids 191-324 results in loss of DMD but not non-DMD methylation and deletion of amino acids 297-309 results in loss of non-DMD methylation but normal DMD methylation (*110*). Furthermore replacement of murine *Dnmt1* codons 328-333 with a rat specific sequence, produces a hypomorphic allele with lower overall levels of methylation and embryonic growth restriction (*112*). These studies indicate that the mammalian

specific DNMT1 region is involved in distinguishing DMD and non-DMD sequences and has species-specific features.

Because DNMT1 is able to distinguish between imprinted and non imprinted sequences during dynamic developmental processes it is speculated that it must be regulated in some allosteric manner. The structure of DNMT1 in the absence of DNA reveals that the RFTS domain is situated in the catalytic DNA interacting region (*85*). In complex with unmethylated DNA a DNMT1 autoinhibitory loop between the CXXC and BAH domains blocks catalytic domain access to CpG dinucleotides and a linker between the BAH2 and the catalytic domains blocks target recognition (*83, 84*). These allosteric auto-inhibitory conformations are relaxed at hemimethylated DNA and are likely modulated by interactions with multimeric heterochromatin protein complexes. DNMT1 interacts with multiple proteins including UHRF1, DMAP, ZFP57, TRIM28, DNMT3 isoforms and others (*75, 76, 107*). Mutations in the genes encoding the DNMT1 interactome not surprisingly alter genomic methylation. A better understanding of the complexes DNMT1 is part of will likely provide clues to how DNMT1 has differential activity at DMD and non-DMD CpG sites during periods of development when there is upheaval of genomic methylation patterns.

1.2 GENOMIC IMPRINTS IN HUMAN DEVELOPMENTAL DISORDERS

One of the reasons it is important to study genomic imprints is because the etiologies of multiple severe congenital developmental disorders are based on genetic and epigenetic changes at imprinted genomic loci (Table 1). The Beckwith-Wiedemann (BWS), Silver-Russel (SRS),

Prader-Willi (PWS), and Angelman (AS) syndromes are all classified as imprinting disorders. In addition both Pseudohypothyroidism Type I (PHP1a) and transient neonatal diabetes mellitus (TNDM) are metabolic diseases caused by loss of imprinting at other loci. It is clear from these disorders that the dosage of imprinted genes is critical, and that neither loss nor gain of imprinted gene expression is well tolerated. Because these disorders can be diagnosed and in some cases manifest symptoms prior to parturition it is plausible that the symptoms are in part based on placental dysfunction. It is my hope that some of my dissertation research on the role of genomic imprints in mouse placental biology will provide insight into the prenatal disease mechanisms of these disorders.

1.2.1 Beckwith Wiedemann Syndrome

Beckwith-Wiedemann Syndrome (BWS; OMIM 130650) is a neonatal overgrowth syndrome. It is characterized by the major traits of overgrowth (macrosomia), large tongue (macroglossia), protusion of the umbilical cord and/or gut (exomphalos), enlarged internal organs (visceromegaly), renal abnormalities and an increased incidence of embryonic tumors (*e.g.* Wilms tumor) (*113, 114*). Minor traits associated with BWS include enlarged placenta (placentamegaly), certain facial structures (e.g. anterior earlobe creases) and neonatal hypoglycemia (*113, 114*). The overgrowth seen in BWS children is often considered hemihyperplasitic and hemitrophic because overgrowth is confined to certain internal organs and uneven in extremeties. It is thought that hemihyperplasia can be caused by the development of mosaic early embryos composed of epigenetically normal and imprint-defective cells.

Both the clinical phenotypes and etiology of BWS are heterogeneous. The majority of BWS cases are sporadic with no apparent familial history (*114, 115*). The genomic region

involved in BWS is a large imprinted region on 11p15.5 that includes both the *KCNQ1* and *H19* imprinting clusters (*115-121*). The rare heritable cases are usually attributed to maternal inheritance of loss of function mutations within the paternally imprinted *CDKN1C* gene (*122*). The majority of sporadic cases of BWS are caused by loss of DNA methylation on the maternal allele of the *KCNQ1* DMD (clinically referred to as IC2 or KvDMR) (*114*). Gain of methylation on the maternal allele of the *H19* DMD (clinically referred to as IC1 or H19DMR) is responsible for about 5% of sporadic BWS cases. Infrequent translocations, duplications and inversions of this region are also causative mutations of BWS. The loss of imprinting and paternalization of the 11p15.5 region results in loss of expression of one (*e.g. CDKN1C*) or more paternally imprinted genes within the *KCNQ1* cluster and increased expression of the maternally imprinted *IGF2* in the *H19* cluster, manifesting in the abnormal growth phenotypes (*115*). The biallelic overexpression of *IGF2,* a potent mitogen, is a major factor in the overgrowth phenotype as well as the enhanced prevalence of fetal and infantile Wilms tumor in BWS.

1.2.2 Silver-Russell syndrome

The major clinical phenotypes of Silver-Russell syndrome (SRS; OMIM 180860) are intrauterine growth restriction and diminished postnatal growth along with minor traits such as facial disproportion, body asymmetry, syndactly and short stature. There is a broad spectrum of SRS and SRS-like clinical manifestations that include growth restriction to varying degrees in compilation with one or more minor traits (*123, 124*). Birth weights and postnatal growth greater than two standard deviations less than the mean are typical for SRS patients, and growth hormone is the standard treatment for this growth disorder (*123, 124*). Intriguingly the 11p15.5 region (*H19* DMD) implicated in the overgrowth syndrome BWS is also involved in SRS. The

majority (estimated at 31-55%) of SRS patients have loss of methylation at the *H19* DMD, or maternal UPD11p15 (~4%), with subsequent downregulation of *IGF2* (*125-129*). The abundance of *IGF2* in BWS and its paucity in SRS demonstrates the strong influence of IGF2 levels on fetal growth. Although SRS and BWS are opposing imprinting disorders based at 11p15.5 (*H19* DMD), SRS appears to be a multi loci disorder with alternative etiologies based on molecular abnormalities on chromosome 7 in 7-10% of cases (*125, 130*).

Maternal UPD7 is the genetic eitology of a minority of Silver-Russell patients (*125, 130*). Human chromosome 7 has three distinct imprinting centers at the *GRB10* (7p12.1), *PEG10* (7q21.3) and *MEST* (7q32.2) loci. Reports of matUPD7p21 including *GRB10* and the non-imprinted insulin binding protein genes *IGBP1* and *IGBP3* in SRS patients is parsimonious given their role in IGF2 signaling (*131, 132*). Maternal duplication of the 7q31qter inclusive of the *MEST* imprinting locus has also been identified as a cause of SRS however it is unclear which imprinted genes within this cluster are directly involved (*133*).

One explanation for the heterogeneity of clinical phenotypes and molecular etiologies in SRS and SRS-like cases is that it is a multigenic syndrome involving imprinting loci that co-regulate key growth pathways. It has been shown that some SRS patients have tissue-specific epigenotypes at imprinted loci, suggesting that mosaic loss of methylation may occur early in development and perhaps modulate the SRS phenotypic outcome (*134, 135*). Quantitative methylation studies have been informative in making correlations between specific epigenetic loci involved in SRS and phenotypic traits (*129*). Hypomethylation at 11p15.5 is associated with the classical SRS phenotypes of facial asymmetry, 5^{th} finger clindactyl, and congenital defects; whereas matUPD7 is associated with a triangular facial structure, global developmental delay and need for speech therapy (*129*). However, the degree of loss of methylation is not indicative

of the severity of SRS phenotypes (*129*). This study is proof that correlation of DMD methylation can be predictive of quantitative traits.

1.2.3 Prader-Willi syndrome and Angelman syndrome

Prader-Willi syndrome (PWS; OMIM 176270) and Angelman syndrome (AS; OMIM 105830) are reciprocal developmental disorders whose molecular etiologies are based on genetic or epigenetic abnormalities in the *SNRPN* imprinted cluster at 15q11.2 (reviewed by (*136, 137*)). PWS is characterized by prenatal fetal inactivity, neonatal muscular hypotonia, growth hormone insufficiency, hypogonadism and juvenile onset obesity. Hyperphagia-induced obesity in PWS is associated with elevated ghrelin orexigenic hormone levels that lower satiety sensitivity, and can be treated through a combination of recombinant growth hormone and behavioral therapy (*137*). Patients with AS have severe mental retardation with deficiencies in motor, balance, speech and language (*136*). Although both PWS and AS are primarily recognized as postnatal disorders, they appear to have fetal origins, therefore it is worthwhile to investigate whether the *SNRPN* cluster has a role in placenta development (*136, 137*).

While PWS is based on the lack of expression of paternally expressed genes within the *SNRPN* locus, AS is caused by lack of expression of adjacent maternally expressed genes within the same genetic locus (*136, 137*). The molecular etiologies of both diseases range from *de novo* inherited deletions, UPD15q11-13, imprinting disorders and single gene mutations (*136, 137*). The majority (~70%) of PWS and AS cases are due to inherited *de novo* deletions of a 5-6 MB region encompassing the 2MB *SNRPN* imprinting cluster (*136, 137*). PWS patients inherit this deletion on the maternal allele whereas AS patients inherit the deletion from the paternal allele (*136, 137*). The large PWS and AS deletions arise from common recombination breakpoints

centromeric of the PWS paternally expressed genes and a series of three telomeric breakpoints telomeric to the AS genes (*136, 137*). Maternal UPD15q11-13 is the cause of approximately 20% of PWS cases whereas paternal UPD15q11-13 is a cause of approximately 1-2% of AS cases (*136, 138, 139*).

A minority of PWS (~1-3%) and AS (~2-4%) cases result from loss of imprinting at the *SNRPN* locus (*136, 137*). This is primarily due to alteration of the genomic methylation patterns at the *SNRPN* DMD (*136, 137*). A small fraction (~15%) of loss of imprinting cases in PWS and AS have interstitial deletions ranging from a 10kb to 800bp that abolish imprinted expression patterns (*140-143*). These loss of imprinting deletions have identified non-overlapping IC sequences that are causative of PWS (when paternally inherited), or AS (when maternally inherited) (*140-143*). The PWS IC overlaps *SNRPN* exon 1, and the AS IC is centered at *SNRPN* upstream exon u5 (*140-143*). Normally these regions are oppositely methylated with the PWS IC methylated on the maternal allele and the AS IC methylated on the paternal allele.

Single gene mutations of members of the *SNRPN* imprinted gene cluster are also causes of PWS and AS. Maternal inheritance of genetic mutation at *UBE3A* is a cause of 2-5% of AS cases (*136, 144, 145*). *UBE3A* encodes a ubiquitin ligase responsible for marking proteins for degradation that is up-regulated in neuronal tissues (*144, 145*). Roughly 10-15% of AS cases have unknown genetic etiologies suggesting the involvement of additional genes (*136*). Paternally inherited mutations in the maternally expressed small nucleolar RNA (snoRNA) gene 116 (*SNORD116*) within the *SNRPN* locus has recently been identified as an etiology of a small minority of PWS and PWS-like cases (*146, 147*). The *SNRPN* locus is discussed in more detail in section 1.5.8 with emphasis on PWS and AS models that target the syntenic *Snrpn* region in mouse.

1.2.4 Pseudohypothyroidism type I

Pseudohypothyroidism type Ia and Ib (PHP Ia; OMIM 103580 and PHP Ib; OMIM 603233) have etiologies based on alteration of the imprinted *GNAS* cluster at 20q13.32 (*148*). PHP Ia, also known as Albright hereditary osteodystrophy, manifests in patients as short stature, obesity, round faces, sub-cutaneous ossification, brachydactyl and other skeletal abnormalities. These features arise due to resistance to parathyroid hormone (PTH), thyroid stimulating hormone (TSH) and gonadotropins. PHP Ia is caused by maternal inheritance of loss-of-function mutations in the gene encoding a variant guanine nucleotide exchange factor alpha subunit (*149*). In an opposite manner genetic mutation of paternally inherited *GNAS* allele is the cause of progressive osseous heteroplasia (POH; OMIM 166350) and pseudopseudohypothyroidism (PPHP; OMIM) which show many of the same muscular and skeletal symptoms of PHPIa but without endocrine abnormalities (*150*).

PHP Ib is characterized by obesity and resistance to parathyroid hormone within the renal proximal tubule. This results in hypocalcemia and hyperphophatemia, and compensatory increase in PTH levels. PHP Ib is caused by epigenetic mutation (loss of methylation) of the maternal exon 1a *GNAS* DMD, resulting in loss of *GNAS-XL* expression in a tissue specific manner (*151*). These results indicate that some tissues are more stringent in their exclusive parent-of-origin monoallelic imprinted gene expression.

1.2.5 Transient neonatal diabetes mellitus Type 1

Transient neonatal diabetes mellitus type I (TNDM1; OMIM 601410) is characterized by intrauterine growth restriction and hyperglycemic infancy that resolves in half of patients within

a few months but can reoccur in adulthood (*152*) . It is caused by loss of imprinting at 6q24 including patUPD 6, 6q24 paternal duplications and maternal hypomethylation at the *PLAGL1* DMD (*152-154*). These changes result in a double dosage of the normally paternally expressed *PLAGL1*, a gene encoding a zinc finger transcription factor that controls a network of genes regulating insulin sensitivity (*155*). The prenatal and postnatal clinical phenotypes of TNDM1 indicate that this disease may be in part modulated by the role of *PLAGL1* in the placenta.

Imprinted Disease	Clinical Phenotypes	Genomic Loci & Molecular Etiologies	DMDs
Beckwith-Wiedemann Syndrome (BWS; OMIM 130650)	Macrosomia, hemihyperplasia, visceromegaly, macroglossia, exomphalos, , embryonic tumors placentamegaly, neonatal hypoglycemia, facial characteristics	11p15.5 Genetic and epigenetic mutations; *CDKN1C* genetic mutations, loss of *KCNQ1* DMD methylation, gain of *H19* DMD methylation, paternal UPD of 11p15.5,	*KCNQ1*, *H19*
Silver-Russell Syndrome (SRS;OMIM 180860)	Intrauterine and neonatal growth restriction, body asymmetry, syndactyl, triangular face, short stature	11p15.5, 7q21.3, 7q32.2 Loss of *H19* methylation, matUPD11q15, matUPD7	*H19*, *GRB10*, *MEST*
Prader-Willi Syndrome (PWS; OMIM 176270)	Fetal inactivity, neonatal hypotonia, juvenile onset obesity, hyperphagia, low GH levels, elevated ghrelin, short stature, hypogonadism, small hands and feet	15q11.2 Paternal deletion of *SNRPN* imprinted cluster, matUPD15, *SNRPN* imprinting mutations, *SNORD116* mutations	*SNRPN*
Angelman Syndrome (AS; OMIM 105830)	Mental retardation, motor and balance deficiency, language and speech problems	15q11.2 Maternal deletion of *SNRPN* imprinted cluster, patUPD15, *SNRPN* imprinting mutations, *UBE3A* mutations	*SNRPN*
Pseudohypothyroidism Type Ia (Albright hereditary osteodystrophy; PHPIA OMIM 103580)	Short stature, obesity, PTH and TSH resistance, round faces, subcutaneous ossification, brachydactyl, skeletal abnormalities	20q13.32 Maternal inheritance of non-functional *GNAS* allele	*GNAS*
Pseudohypothyroidism Type Ib (PHPIB; OMIM 603233)	Obesity, PTH resistance in renal PT, hypocalcemia, hyperphosphatemia	20q13.32 Loss of *GNAS* exon 1a DMD methylation	*GNAS*
Transient Neonatal Diabetes (TNDM1; OMIM 601410)	Intrauterine growth restriction, infantile hyperglycemia	6q24 PatUPD6, paternal 6q24 duplications, loss of *PLAGL1* DMD methylation	*PLAGL1*

Table 1. Syndromes and disorders associated with genomic imprints. See section 1.2 for references

1.3 PLACENTAL INFLUENCE ON PREGNANCY OUTCOME

1.3.1 Requirements for prenatal development

Normal fetal development and pregnancy outcome depends on the genetic integrity of the fetus, health of the mother and function of the placenta. Adverse pregnancy outcomes include miscarriages, malformations, pre-term delivery and low birth weight. Genomic disorders can be inherited or arise spontaneously in the germline and affect single genes to whole chromosomes (*156*). Chromosome imbalance (aneuploidy) in mammals originates from oocyte meiosis I non-disjunction events, it affects roughly 5% of all pregnancies and usually results in miscarriage although a handful of trisomies are viable to birth including trisomy 13 (Patau syndrome), trisomy 18 (Edwards syndrome) trisomy 21 (Down syndrome) and sex chromosome trisomy XXY (Klinefelter syndrome), XXX and XYY (*157-159*).

The fetal genome has a large contribution to pregnancy outcome, however it is not completely deterministic. It has been shown that birth weights of half siblings are more correlated when there is a common mother than when the father is common indicating that the maternal intrauterine environment is a major factor in fetal growth ((*160*) and references therein). Diet, obesity, insulin-resistance, hypertension, smoking, alcohol consumption and drug use are all strong maternal influences on pregnancy outcome. The placenta is the interface between mother and fetus and is critical for the exchange of nutrients, wastes and as an immunological barrier. The proper development and function of the placenta is therefore critical for optimal fetal

health. Intrauterine growth restriction, preeclampsia and gestational diabetes mellitus are three common pregnancy complications that if left untreated lead to poor outcomes.

1.3.2 Intrauterine growth restriction

Intrauterine growth restriction (IUGR) is a term synonymous with fetal growth restriction (FGR) describing reduced growth of a fetus during pregnancy. IUGR often leads to pre-term delivery and small for gestational age (SGA; lowest 10^{th} percentile of birth weights) babies. The causes of IUGR include conditions such as hypoxia, preeclampsia, genetic determinants and placental insufficiency (*161*). Maternal smoking causes placental hypoxia and IUGR without preeclampsia (*162*). Smoking during pregnancy also increases the risk for ectopic pregnancies, spontaneous abortions, teratogenesis, pre-term birth, and sudden infant death syndrome and is also associated with postnatal childhood obesity and diabetes mellitus and psychological problems (*162*). Fetal alcohol syndrome is a neurological and developmental disorder with IUGR (*163*). Illicit drug use also increases the risk of IUGR, pre-term birth and low birth weight (*164*). Many genetic IUGR determinants encode components of the insulin like growth factor (*e.g. IGF1*, *IGF2*, *IGF2R* and *IGFBP1*), placental growth factor (*i.e. PGF*), stress related cortisol (*e.g. CRH* and *11bHSD*) and growth hormone (*i.e. GH)* signaling pathways (*165*). However, only *IGF2* has been directly linked through genetic and epigenetic (*i.e. H19* loss of methylation in SRS) mutations to cause IUGR (*166*).

1.3.3 Preeclampsia

Preeclampsia (PE) is a maternal vascular disease associated with poor placental perfusion that occurs in roughly 3-5% of all pregnancies (*167-169*). Clinically, PE is defined by gestational hypertension and proteinuria after 20 weeks gestation. Risk factors for PE include a number of metabolic, coronary, genetic and environmental conditions. PE can have severe effects on fetal and neonatal morbidity and mortality and accounts for 30-40% of IUGR pregnancies, 25% of SGA babies and 15% of pre-term births. If left untreated PE can transition into maternal eclamptic seizures. It is thought that factors released from the placenta and the lack of trophoblast induced vascular remodeling and vascularization combined with maternal influences (*e.g.* obesity) results in maternal vasoconstriction and hypertension (*170*). Endothelial cellular signaling pathways influence PE including vascular endothelial growth factor and angiotensin signaling (*171*). In addition, there is evidence that fetal genetic determinants (*e.g. STOX1*, *MMP-9* and *ANG*) are predicative of PE pregnancies (*171*).

1.3.4 Gestational diabetes mellitus

Metabolic disease is a common occurrence in post-industrial societies and encompasses obesity, insulin resistance, dyslipidaemia and accompanying hypertension (*172*). During normal pregnancy maternal insulin levels generally rise and can induce maternal diabetes mellitus (GDM). Obese mothers (BMI>30) are at greater risk for the pregnancy complications gestational diabetes mellitus (GDM) and PE (*172*). An increase in fetal and neonatal body mass due to adipose deposition is observed in offspring from mothers with GDM (*172*). Although GDM

resolves after partition both the mother and offspring are at increased risk for metabolic disorders later in life (*172*).

1.3.5 Fetal origins of adult diseases

The period of prenatal development is a critical time that has great influence on the overall health of individuals throughout their lifetime (*173-175*). This concept of the fetal origins of adult diseases has a strong backing of evidence. For example, low birth weight is tightly correlated with hypertension and coronary-based mortality (*173, 174*). The placenta is the organ at the maternal-fetal interface and thus is intimately involved in fetal development and therefore the lifelong health of individuals (*176*). Placental characteristics such as weight, length and breadth have been linked with systolic blood pressure, and the adult diseases hypertension, asthma and colo-rectal cancer (*176*). It is therefore extremely important to understand how the placenta functions to support normal fetal growth and optimize the lifetime health of offspring.

1.3.6 Maternal influences on placental function

The placenta functions as a physical and immunological barrier between mother and fetus, as a transporter of nutrients, gasses and wastes as well as a site of active metabolism and hormone production (*177, 178*). Maternal physiology is known to influence placental development through blood flow, nutrition and toxicology. For example, obese mothers have increased placental triglycerides, malondialdehyde, carbonyl protein, and reduced glutathione, as well as increased placental oxidative stress response (*179*). Maternal smoking induces hypoxic responses (*e.g.* vascularization) within the placenta thereby decreasing the risk of PE, but increasing the

risk for placentaprevia and placenta abruptia (*162*). Alcohol exposure, a cause of IUGR in humans, has been shown in rats to modulate placental insulin like growth factor signaling (*180*).

1.4 PLACENTAL DEVELOPMENT AND FUNCTION

1.4.1 Comparison of human and mouse placental structure and development

The placenta is a temporary organ that sustains and supports fetal growth during gestation and is a defining feature of mammalian development (*177*, *178*). It is made up of cells derived from maternal, embryonic and extraembryonic origins. The primary extraembryonic cell lineage in the placenta is trophoblast. Human and mouse placentas are both defined as hemochorial because maternal blood is in direct contact with trophoblast. In both species during pregnancy, at the site of implantation, the uterine endometrium becomes the highly vascularized decidua basalis and bathes the placenta with maternal blood The human and mouse placenta share common genetic networks that regulate placental development. However, the placental structures of the two species are distinct. The human placenta consists of microvilli that branch from the chorionic plate and are bathed in maternal blood. Each microvillous consists of an outer syncytiotrophoblast (SynT) layer, a diploid columnar epithelium, and innermost fetal vessels and supporting mesenchyme. The mouse placenta on the other hand consists of three distinct layers with unique functions. The labyrinthine zone (LZ) layer is the site of nutrient and waste exchange while the junctional zone (JZ) and trophoblast giant cell (TGC) layers have endocrine functions.

Cytotrophoblast and SynT are two types of trophoblastic cells that are common between mouse and human placentas (*177, 178*). The cytotrophoblast are diploid single nucleated cells and are called spongiotrophoblast (SpT) in mouse. In humans there are three types of cytotrophoblast: the villous-cytotrophoblast separate the SynT from the villous mesenchyme and fetal endothelium; the extravillous-cytotrophoblast invade the maternal decidua; and the columnar-cytotrophoblast, which reside at the tips of the microvilli and abutting the maternal decidua serve as a source of precursors to other cytotrophoblasts and SynT cell types. The spongiotrophoblast in mouse similarly resides between the decidua and the labyrinth within the so called junctional zone (JZ), however they do not serve as precursors to SynT. In the mouse placenta the SpT performs many of the endocrine functions delegated to SynT in human placenta (*178*).

The syncytiotrophoblast are multi-nucleated cells formed from cell fusion which funnel and contain maternal blood pools, transport nutrients and have active metabolism. There is only a single layer of SynT in human microvilli but a bilaminar layer in the mouse labyrinth. A third type of trophoblasts, trophoblast giant cells are polyploid and found only in mouse, they have similar function as extra-villous cytotrophoblast in induction of decidualization and maternal vascular remodeling. Arterial blood vessels extending from the umbilical cord into the chorionic plate (base of the placenta) and branching adjacent to SynT line maternal blood are composed of extraembryonic mesoderm derived fetal endothelial cells in both species.

The common cell types and developmental processes make the mouse a superb model for placental research (*177, 178*). The genetic networks important in trophoblast differentiation and function are highly conserved in human and mouse (*181, 182*). Placental gene expression in human and mouse is greatly conserved, particularly for those genes with known placental

phenotypes associated with gene mutations (*181*). Intriguingly, both rodent-specific and primate-specific genes, many of which originated from gene duplication, are enriched for preferential placental expression, indicating an organ undergoing recent evolutionary adaptation (*182*). The short generation time, and ease of genetic and physiological manipulations, are major reasons why the mouse is a superb system to study placental development. Targeted genetic studies have unearthed the central pathways in early trophoblast differentiation and placental function. However, there will also be incongruities in comparing placental development across mammalian species, and this must be kept in mind when extrapolating experimental data from mouse to discern the function of genes in human.

1.4.2 Preimplantation development

The period of time from fertilization to implantation is a critical time for the establishment and differentiation of trophoblast lineages (reviewed by (*183-188*)). After fertilization of an egg the maternal and paternal pronuclei replicate their haploid genomes, fuse and then undergo cell division (*183, 189*). Early rounds of mitotic cleavage are symmetric and produce totipotent cells (*183*). The earliest cell fate specification event, which distinguishes the trophectoderm (TE) from the inncer cell mass (ICM), occurs at the 8 and 16 cell stages (*183*). In the 8-cell embryo the outer apical surface of each blastocyst tightens and these cells become polarized, and divide into distinct outer and inner daughter cells (*183*). Expression of the TE specific transcription factor *Cdx2* begins at the 8-cell stage in the outer cells concomitantly with expression of the ICM specific transcription factor *Oct4* within the inner cells (*190, 191*). This initial fate specification event is mediated by the cell-contact dependent hippo signaling pathway kinases STK3 and LATS2, that suppress YAP1 through phosphorylation (*192*). YAP1 is the coactivator for the

transcription factor TEAD4, which is the master regulator of TE differentiation (*192*). The TEAD4/YAP1 complex promotes a cascade of transcription factors beginning with activation of the *Cdx2*, *Gata3*, *Eomes* and *Tfap2a/c* genes that define TE lineages, followed by transcriptional activation of genes that maintain TE fate including *Ets2*, *Elf5*, *Essrb*, *Foxd3* and *Sox2*/3, and subsequently transcription factors such as *Gcm1*, *Ascl2*, *Hand1* and *Stra13* that promote specific trophoblastic lineage differentiation (*183-185, 193, 194*).

At the 32 cell morula stage, the embryo begins to hollow out and compact to one pole to form a blastocyst by E4.5 (*189*). The blastocyst is a cellular sphere consisting of the outer TE layer, the ICM clumped at one pole and a cavity called the blastocoel (Figure 1). The ICM is subdivided into the epiblast, which generates the fetus, and the primitive endoderm, a layer of cells between the epiblast and the blastocoel that gives rise to the parietal and visceral endoderm as well as the yolk sac (*183*). The polar TE overlying the ICM is the progenitor of most trophoblast lineages, whereas the mural TE encasing the blastocoel generates only TGCs upon implantation (*183, 185, 189*). The TE, epiblast and primitive endoderm are comprised of pluripotent cells expressing distinctive lineage markers; they are called trophoblast stem cells (TSCs; marked by *Cdx2*), embryonic stem cells (ESCs; marked by *Oct4*, *Nanog* and *Sox2*) and extraembryonic stem cells (XEN; marked by *Gata6*) respectively (*195*). TSCs are a unique cell type that can be cultured from blastocyst and ectoplacental cone extracts up to E7.5 on inactivated fibroblasts in ESC conditioned media replete with serum and infused with FGF4 (*186, 196*).

Expression of the growth factors *Fgf4* and *Nodal* by the ICM sustain TSCs pluripotency and identity (*186, 196-198*). Following TE specification and up through postimplantation development FGF4 is required for the maintenance of TSCs (*196*). FGF4 is produced by ESCs in

the ICM, binds to the FGFR2 receptor on TSCs, thereby activating the RAS-MAPK signaling pathway promoting proliferation and inhibiting differentiation (*186, 197, 198*). Induced activation of *Ras* (iRas) can convert ESCs to TSC like states (*198, 199*). However, their trophoblast developmental potential is limited, indicating that earlier specification events trigger epigenetic barriers to full TSC induction (*198, 199*). The nodal signaling pathway is also required for TSC maintenance. *Nodal* is expressed by the ICM and is activated by proteases secreted by the TE (*197*). Preimplantation embryos with deletion of *Spc1* and *Spc4,* two genes encoding nodal proteases secreted by TE, have a reduced population of TSCs and diminished expression of early trophoblast differentiation markers (*197*). The combined activity of Fgf4 and Nodal signaling on TE prevents terminal differentiation (*197*).

1.4.3 Implantation

At the time of implantation the polar TE is subdivided into the extraembryonic ectoderm (EXE) that is proximal to the ICM, and the ectoplacental cone (EPC) that is distal to the ICM (Figure 1) (*185-188*). The EXE is the default state of polar TE and expresses a transcription factor network including *Tead4, Cdx2, Eomes, Tcfap2c, Gata3, Essrb, Elf5* and *Ets2* (*185-188*). The EPC differentiates from this basal state and represses transcription of *Cdx2* and *Eomes* while increasing expression of *Tead4*, *Ets2* and *Gata3,* and gaining expression of *Ascl2* and *Tpbpa* (*185-188*). The EXE is the progenitor of the chorion and syncytiotrophoblast, where as the EPC generates TGCs and SpT. Fgf4 and Nodal signaling continues to act on both the EXE and EPC to maintain TS populations in both through E7.5 (*185-188*).

Progesterone secreted by the corpus luteum following ovulation induces decidualization, a process by which the maternal uterine endometrium prepares for implantation by increasing

glandular secretion, vascularity and vascular permeability (*200*). Blastocyst implantation into the receptive maternal uterine endometrium (decidua) is mediated by the primary outermost parietal-TGCs (P-TGCs) that have differentiated from the EPC and the mural TE. These P-TGCs integrate into the extracellular matrix in an interstitial manner and further remodel the uterine environment. Some TGCs (SpA-TGCs) invade into the maternal decidua and embedd within the spiral arteries where they remodel the vasculature to be dilated and fenestrated to increase placental perfusion. Prior to implantation the fetus survives on internal energy stores but postimplantation subsistence is dependent on the transfer of nutrients and wastes between fetus and mother as mediated by the placenta. Therefore it is not surprising that many targeted gene deletions are embryonic lethal immediately following implantation (~E9.5) due to placental defects (*201, 202*).

1.4.4 Labyrinthine zone development

The placental labyrinthine zone (LZ or labyrinth) layer is composed of cells derived from both the TE lineages and epiblast derived extraembryonic mesoderm fetal vessels extending from allantois (umbilical cord) (*185, 201, 203-205*). The mouse placental labyrinth is analogous to the single syncytial layer and vascular components of the human microvillous placental structure, and both function to mediate the exchange of nutrients and wastes between maternal and fetal circulation (*177, 204, 205*). Syncytiotrophoblast (SynT) are the primary trophoblast cell type found in the labyrinth layer. They are post-senescent, elongated and multinucleated cells formed from the fusion of multiple diploid EXE precursors (*206*). The transcription factor *Gcm1* is a marker and master regulator for SynT lineages (*188, 203-205*). Targeted deletion of *Gcm1* results in greatly diminished branching of the SynT lined network of maternal blood passages

(*207-209*). Diminished *GCM1* expression in humans is associated with an increase in fetal vascular tissue in microvilli (and a corresponding decrease in SynT), which is a placental state indicative of preeclampsia (*209*). The *SynA* and *SynB* genes, derived from retroviral envelope genes, code for coopted factors that enable the fusion of neighboring diploid EXE cells (*210, 211*). The syncytiotrophoblast forms a bilaminar layer that channels and pools maternal blood. SynT layer I lines the maternal blood pools, whereas SynT layer II is in contact with fetal vasculature (*204*). Sinusoidal trophoblast giant cells (S-TGCs) and channel TGCs (C-TGCs) , derived from the EXE, form a thin layer adjacent SynT layer I at the borders of maternal blood pools and channels (*204*).

The fetal vasculature within the murine placenta is derived from the allantois (*212*). The allantois is formed from the extraembryonic mesoderm at the posterior end of the primitive streak. It extends upwards from the dorsal aorta forming the umbilical cord and then fuses with the chorion at E8.5 and afterwards integrates into the chorionic plate and branches upwards to vascularize the placenta. Many of the genes involved in angiogenesis are involved during this process including members of the VEGF signaling pathway (e.g. *Vegf, Flt, Flk*) and other extra cellular matrix proteins and signal transducers (e.g. *Fibronectin, Cathepsin, Mek1, Mek2*) (*213*). The allantois and chorion also have limited hematopoietic properties (*214*).

Because SynT is intimately involved in transferring nutrients across the maternal-fetal interface it expresses a multitude of facilitated and passive transporters along the polarized microvillus and basal membranes. Circulating levels of IGF2 and insulin among other effectors modulate the expression of glucose, amino acid and fatty acid transporters within the SynT (*215*). Trans-placental fatty acid transport is a complex process mediated by SynT involving uptake of triacylglycerides from maternal circulation, intracellular transport and lipolysis

(Reviewed by (*216*)). Free fatty acids generated by lipolysis undergo β-oxidation in SynT mitochondria to supply acetyl-CoA for the TCA cycle, or can be stored within intracellular lipid droplets and eventually exported to fetal circulation (*216*).

The peroxisome proliferator-activated receptor (PPAR) family of proteins is vital to placental labyrinth development (*217*). These nuclear receptors bind small molecule ligands and interact with co-activator nuclear factors (e.g. RXR, RAR and LCOR) to modulate gene expression patterns (*217*). Homozygous null *Pparγ* mutation is embryonic lethal at E10.5 due to lack of fetal vasculature and ruptured maternal blood sinusoids within the placental labyrinth in combination with fetal heart defects (*218*). One key SynT gene that PPARγ and RXR regulate is *Muc1*, which encodes a protein that coats the lining of the maternal blood pools; the lack of its expression may in part explain the hemorrhaging of these spaces in *Pparγ* homozygous null placentas (*219*). In addition to *Pparγ*, *Pparδ* and *Rxrα* are crucial to early placental development (*217*). The lethality of targeted *Pparδ* alleles is strain dependent. Targeted *Pparδ* mutation is embryonic lethal due to failure of full chorio-allantoic fusion at E8.5 and diminished JZ development on pure B6 and FvB strains but not on hybrid backgrounds (*220*). Homozygous *Rxrα* null placentas manifest labyrinth abnormalities at E14.5 with excessive pooling of maternal blood, thickened SynT lamina and thinner and necrotic fetal vessels (*221*). Both *Pparγ* and *Rxrα* null mice display a reduced amount of lipid droplets in SynT layers I and II (*218, 221*).

The cell types within the labyrinth layer are marked by expression of different genes. Early in labyrinth development the chorion and early SynT are marked by the transcription factors *Gcm1* and *Tfeb* expression. Later in development, at E12.5 and beyond, the leptin receptor (*LepR*) is strongly expressed in SynT. The markers of C-TGCs and S-TGCs are discussed in section 1.3.7. The fetal vasculature within the placenta, like most arterial vessels, is

marked by CD31 (VE-Cadherin). Interestingly, expression of the imprinted genes *Mest* and *Dlk1* are both highly expressed in fetal vessels and can be used as *in situ* lineage markers. Many targeted imprinted gene deletions have phenotypes that occur within the labyrinth including *Peg10* and *Rtl1*, which are discussed in detail within section 1.5.

1.4.5 Junctional zone development

The junctional zone layer (JZ or spongiotrophoblast) is composed almost entirely of spongiotrophoblast (SpT), a diploid cell type derived from the ectoplacental cone (*188, 203*). It is structurally analogous to the columnar cytotrophoblast of human placenta that forms epithelium at the tips of each microvillus (*177, 178*). Expression of *Ascl2* and *Tpbpa* is used to delineate SpT by *in situ* hybridization. The SpT lineage marker *Tpbpa* encodes a secreted peptide of the cathepsin family, and is expressed ubiquitously throughout gestation in all SpT subtypes (*188, 203*). *Ascl2* is an imprinted gene strongly expressed in the EPC and during early SpT differentiation. It encodes a basic helix-loop-helix transcription factor which inhibits the default EPC differentiation pathway leading to TGCs (*222, 223*). Deletion of *Ascl2*, results in placentation based prenatal lethality with pronounced reduction in both LZ and JZ layers and excessive accumulation of TGCs (*222*). Insertional mutagenesis of the *Nodal* gene has a similar early embryonic lethality as the *Ascl2* null, with loss of SpT and expansion of TGCs, indicating the importance of the nodal signaling pathway in SpT specification (*224*).

The junctional zone has strong endocrine functions that support pregnancy through the secretion of members of the prolactin family, lactogens and other cytokines that stimulate corpus luteum progesterone expression and other maternal physiological responses (*178, 225*). Secretion of a soluble anti-angiogenic isoform of the VEGF receptor *(s-Flt1*), and the vasoinhibin

precursor proliferin (*Plf1*; *Prl2c2*) by SpT also inhibits maternal endometrial vascularization into the placenta (*226, 227*).

Glycogen cells (GCs) are a specialized subtype of SpT that contain large glycogen stores and are marked by the expression of *Pcdh12* and *Igf2* (*228, 229*). Glycogen cells are detectable as early as E7.5, start accumulating glycogen by E10.5, proliferate rapidly at E12.5 and then migrate into the neighboring decidua around E14.5 (*228, 229*). By E17.5 GCs within the decidua undergo lysis and release their glycogen content into lacunae with maternal blood; this is thought to provide a burst of energy for the end stages of fetal growth and partition (*229*). Mouse GCs are analogous to human columnar cytotrophoblasts closest to the decidua, and interstitial decidua embedded cytotrophoblast both of which contain glycogen vacuoles (*177*). The imprinted genes *Igf2, Igf2r, Ascl2, Cdkn1c* and *Phlda2* are known to influence SpT and GC development and are discussed in detail in section 1.5 (*230*).

1.4.6 Trophoblast giant cells

Trophoblast giant cells (TGCs) are polyploid cells formed via genomic amplification without cell division (endoreduplication) of diploid precursors (reviewed by (*231, 232*)). The polyploidy genomes of TGCs are also polytene, having distinct chromosome bands of both highly active and highly repressed chromatin. TGCs have extensive rough endoplasmic reticulum indicative of their elevated protein secretion pathways. In these respects, TGCs are similar to hepatocytes. TGCs are the default fate of TE development. In the absence of FGF4 and conditioned media TSCs differentiate within 4-5 days into TGCs (*196*). The expression of *Ascl2* within the EPC (and in TSCs) suppresses the TGC differentiation programing. ASCL2 antagonizes the activity of HAND1, a basic helix-loop-helix transcription factor that promotes TGC differentiation,

either through transcriptional repression or competition for common promoters (*233, 234*).Tthe interacting HAND1 and STRA13 transcription factors negate FGF4 suppression of trophoblast terminal differentiation and promote differentiation into TGC (*233-235*).

There are four subtypes of TGCs found in the mouse placenta (reviewed by (*231, 232*)). They can be distinguished by their expression of different members of the prolactin/placental lactogen and prolactin-like gene family. Each of the 23 members of this family is a 22-33 kDa peptide related to growth hormone (*225, 227*). The earliest TGCs are formed from the mural TE and EPC just prior to implantation. These cells are parietal TGCs (P-TGCs) because they form the outermost surface of the placenta. P-TGCs express proliferin (*Prl*; *Plf1*; *Prl2c2*) and prolactin-1 (*Pl1*; *Prl3d1*) at E9.5, and then transition to primarily prolactin-2 (*Pl2*; *Prl3b1*) at E12.5 and later gestational time points (*232*). These cells are vital to implantation and decidualization. P-TGCs express a multitude of integrins that interact with maternal uterine ECM components fibronectin, laminin, vitronectin and collagen (*231*). Spiral artery TGCs (SpA-TGCs) integrate into the maternal spiral arteries to remodel the vascular blood flow. These cells phenocopy many of the functions of normal endothelial cells and are analogous to human endovascular cytotrophoblast (*201*). The trophoblast decidual invasion is shallower in mice than in human pregnancy, and some of the vascular remodeling is thought to be instigated by uterine natural killer cells in mice (*177*).

SpA-TGCs are present by E10.5 and express *Prl2c2*. The chorion gives rise to the C-TGCs and S-TGCs starting at E10.5, these are marked by expression of *Prl3b1* but can be distinguished by expression of *Prl2c2* in C-TGCs and *Ctsq* in S-TGCs (*231, 232*). The expression of the prolactin gene family, which is found in large cluster of duplicated genes on mouse chromosome band 13qA1, is both a defining and functional feature of TGCs (*227, 236*).

Prolactins are secreted proteins with both paracrine functions that modulate decidualization and have endocrine functions that alter maternal physiology and behavior (*225*, *227*). In the polypolid P-TGCs there is overamplification of genomic regions harboring gene clusters of the prolactin (including *Prl2c2*, *Prl3b1*, and Prl2d1), Cathepsin (including Ctsq1, and Tpbpa), Serpin (intracellular serine proteases) and NK/Clec (Natural-killer/C-type lectins) families (*237*). Although the human placenta does not have TGCs the extravillous cytotrophoblast provide many of the same functions during implantation and the more invasive cell types are polypoloid, although not to the same extent as mouse TGCs (*238*).

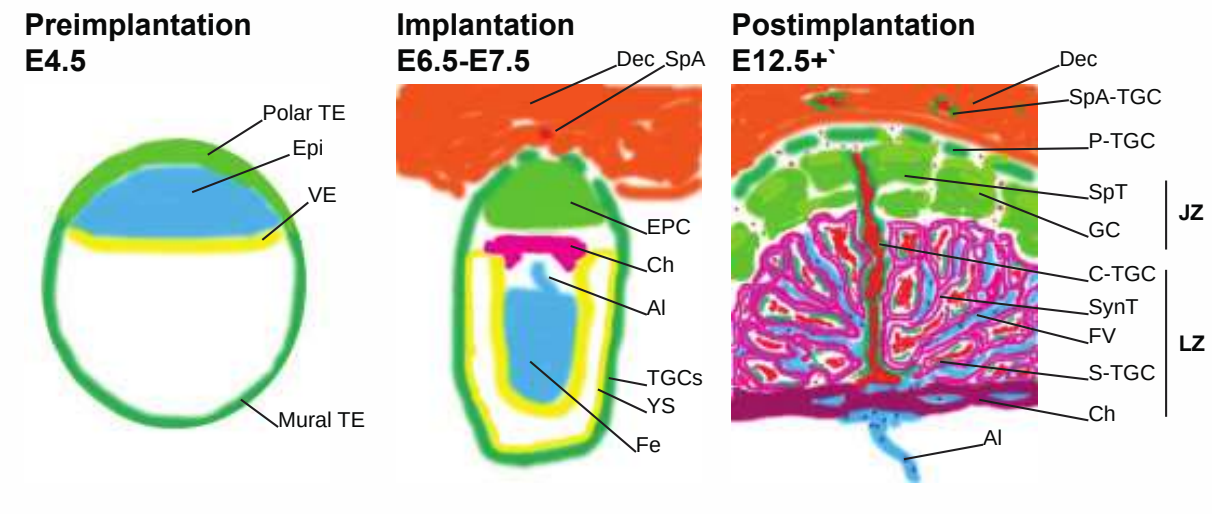


Figure 1. Mouse placental development from preimplantation to E12.5+. Abbreviations: Trophectoderm (TE), Epiblast (Epi), Visceral Endoderm (VE), Decidua (Dec), Spiral Artery (SpA), Ecto Placental Cone (EPC), Chorion (Ch), Allantois (Al), Trophoblast Giant Cells (TGCs), Yolk Sac (YS), Fetus (Fe), Spiral Artery TGCs (SpA-TGCs), Parietal TGCs (P-TGCs), Sinusoidal TGCs (S-TGCs), Spongiotrophoblast (SpT), Glycogen Cells (GCs), Syncytiotrophoblast (SynT), Fetal Vessels (FV). In each stage cell populations are color coded based on lineage origin. At the preimplantation stage the Polar TE is light green, the mural TE is dark green, the Epi is blue and the VE is yellow. At the implantation stage Dec is orange, TGCs are dark green, the EPC is light green, the Ch is purple, the YS is yellow and the Fe and Al are blue. Postimplantation all TGCs are dark green (irrespective of origin), SpT is light green, GC are gray-green, SynT is purple and magenta, Fv are light blue and the chorion is Purple. Maternal and fetal blood (unlabeled are red and dark blue respectively.

1.5 GENOMIC IMPRINTS WITH POSSIBLE PLACENTAL FUNCTIONS

I chose to study 15 out of the 24 known genomic imprints based on their known involvement in prenatal fetal and placental phenotypes. These genomic imprints and the genes within the clusters they regulate are discussed in detail below. Many of these imprinted gene clusters harbor genes that exhibit parent-of-origin specific monoallelic expression exclusively in extraembryonic tissues. Different types of mouse models have been instrumental in providing insight the role of each genomic imprint and the imprinted genes they regulate. The study of mouse embryos with UPDs and robertsonian translocations revelaed parent-of-origin specific prenatal developmental effects attributable to specific imprinted genomic regions. Targeted mouse genetic approaches including gene deletions and transgenic duplications identified the role of single imprinted genes. Similarly, targeted deletion of DMDs in mice has been used to investigate imprinting mechansism and their functional significance in development. Although the research reviewed herein has brought to light unique placental functions of imprinted loci, the integrated role of individual imprinted clusters and imprinted DNA methylation *per se* is largely unknown.

1.5.1 *Nnat*

The neuronatin (*Nnat*) imprinted gene cluster is a microimprinted domain found on mouse chromosome band 2qH4 with a syntenic region on human chromosome 20q11.2-12 (*239*-*242*). The *Nnat* gene resides within the single intron of the much larger *Blcap* (*239*). The *Nnat* DMD is located at the *Nnat* promoter, which transcribes in reverse orientation relative to *Blcap* (*239*).

Expression of *Nnat* is silenced on the methylated maternal allele, whereas the unmethylated paternal allele is transcriptionally active (*239*). Mice with matUPD of distal chromosome 2 are deficient in *Nnat,* expression, are growth restricted and have decreased cerebral folding (*243*). The expression of neither *Blcap* nor any other gene in the local vicinity is imprinted (*239*). Hypomethylation of the *Nnat* DMD and resultant overexpression of *Nnat* is known to occur in Wilms Tumor, similar to the effects on the *H19* DMD and *Igf2* expression (*244*). There is evidence that the *Nnat* DMD is hypermethylated in term mouse placenta (*245*).

The NNAT protein is a plasma membrane bound proteolipid cation channel (*240*). It is involved in both neural development and regulation of metabolic pathways including gluconeogenesis and glucose transport (recently reviewed in (*246*)). In pigs (*Sus domesticus*), *NNAT* is paternally expressed during gestation in uterine glandular and luminal epilethelial cells, and in the placental chorionic plate (*247*). The expression of porcine *NNAT in utero* is co-regulated with many components of metabolic pathways (*e.g. GLUT1*, *AKT*, *IRS1*, *MTOR* and *PI3K*) indicating either a downstream or congruent regulation (*247*). Based on the parent-of-origin growth effects of proximal chromosome 2, the expression profile of *Nnat* and potential role in placental metabolism it would be of interest to determine if loss of the *Nnat* imprint influences mouse placental development.

1.5.2 *Gnas*

The *Gnas* imprinting cluster resides on mouse distal chromosome band 2qH4 within the region identified in recombination models as having parent-of-origin effects. A homologous region is located on human chromosome band 20q13. The *Gnas* transcriptional unit produces three unique isoforms: the biallelically expressed (except in kidney proximal tubule) *Gnas*, the paternally

expressed *Gnas-xl* and the maternally expressed *Nesp* from unique promoters that share a common exon 2 (*248*). Additionally, *Nesp* has an antisense transcript (*Nespas*) that initiates transcription from a promoter that is adjacent and in opposite orientation to the *Gnas-xl* promoter (*249*). *Gnas* encodes a guanine exchange factor α-subunit (*250*). The *Gnas-xl* isoform also encodes the cell membrane bound ALEX protein from an alternative open reading frame (*250*). The *Nesp* gene encodes a neuropeptide of unknown function (*248*).

Several targeted mutations at the *Gnas* locus have been generated to model PHPIa and PHPIb. Targeted deletion of maternally inherited *Gnas-xl* results in reduced viability, with the few surviving pups having lower adiposity, increased lean mass, increased glucose tolerance, low insulin levels and altered sympathetic neuronal activity (*251*, *252*). Heterozygous deletion of the *Gnas* exon 2 has different effects based on parental inheritance (*253*). Similar to the difference in PHPIa and PPHP patients, maternal null mouse pups have both skeletal defects and are PTH resistant, whereas paternal null pups have skeletal defects, do not suckle, but are not PTH resistant (*253*). Further parental inheritance experiments established that *Gnas* is imprinted in a tissue specific manner in the kidney (*254*). In the distal tubule and collecting ducts *Gnas* is not imprinted and has no parent-of origin-effects when *Gnas* exon 2 is deleted (*254*). However, in the proximal tubule *Gnas* is imprinted and PTH resistance occurs with maternal but not paternal inherited *Gnas* exon 2 deletion (*254*). Two models of Exon1a deletion manifest in postnatal growth retardation when paternally inherited (*255*). The Ex1a-T model truncates transcripts originating from both *Gnas* exon 1a and *Gnas-xl* promoters has along-term phenotype with hyperactive metabolism and decreased bone growth, whereas a mutation of *Gnas* exon 1a eliminates all transcription from that promoter but enables both *Gnas* exon 1 and full length

Gnas-xl transcription, which results in transient postnatal growth retardation during the first 6-10 weeks after birth (*255*).

The *Gnas* cluster harbors three imprinted DMDs. A paternally imprinted DMD is found overlapping the *Nesp* promoter, a maternally imprinted DMD overlapping the *Nespas* and *Gnas-xl* promoter, and a maternally imprinted DMD at *Gnas* exon1a (*256-259*). The DMD region overlapping the *Nespas* promoter is the primary germline DMD, the *Gnas-xl* promoter and *Gnas* exon 1a DMDs acquire methylation secondarily in the germline, and the *Nesp* promoter DMD is only imprinted during postimplantation development in certain somatic tissues (*256*). Paternal inheritance of a *Nespas* DMD deletion increases *Gnas* Exon 1a methylation and decreases *Nesp* DMD methylation, mimicking the maternal allele's epigenetic state and resulting in increased transcription from the *Nesp* and *Gnas* exon 1 promoters (*259*). A compound mutation of maternally inherited *Gnas* loss-of-function and paternally inherited *Nespas* DMD deletion partially rescued neonatal edema observed in the former (*259*). The role of the *Nespas* non-coding RNA (ncRNA) is important for the establishment of the *Nesp* DMD, as shown genetically by paternally inherited truncation of *Nespas* results in loss of *Nesp* DMD methylation and in increase in its transcription (*260*). Paternal inherited deletion of the Exon1a DMD results in derepression of *Gnas* exon 1 expression and can rescue the metabolic and neonatal growth defects of maternally inherited *Gnas* mutations in compound heterozygotes (*257, 258*). Because of the importance of *Gnas* to metabolic and endocrine regulation it is plausible, although previously unsubstantiated, that *Gnas* plays a role in placental hormone production and metabolism during prenatal development and is worthwhile to study the effects of loss of imprinting at this locus on placental development.

1.5.3 *Mest*

The long arm of mouse chromosome 6 has multiple imprinted loci. Reciprocal translocations involving chromosome arm 6q from centromere to the 6G3 telomeric band are embryonic lethal in matUPD6q offspring but support viable development in patUPD6q offspring (*10*). The parent-of-origin specific region was narrowed to the region between the centromere and a breakpoint in chromosomal 6qB3 and is now known to harbor the *Peg10, Mest* (also known as *Peg1*), and *Nap1l5* imprinting clusters (*261*). Furthermore bimaternal inheritance of the region between the centromere and a proximal breakpoint at 6qA3.2 between the *Mest* and *Peg10* loci is viable, demonstrating that the lethal matUPD6q phenotype cannot be attributed to the sub-proximal *Mest* and therefore is likely attributable to the *Peg10* cluster (*262*). A fetal growth restriction phenotype is associated with matUPD of the sub-proximal region between 6qA3.2 and 6qC2 including *Mest* and *Nap1l5*, whereas fetal overgrowth is associated with patUPD of the same region, suggesting the 6q sub-proximal imprinting region is a regulator of fetal growth (*262, 263*). Placental weights were normal in both sub-proximal matUPD and patUPD offspring (*262, 263*). In humans, matUPD7 and *Mest* DMD hypermethylation are causes of a minority of SRS cases (*125, 264*).

The *Mest* DMD is located at a 550bp CGI overlapping *Mest* exon 1 as well as 157bp of 5′ promoter and 120bp of 3′ intron 1 DNA sequence (*265*). The *Mest* DMD is maternally methylated on the inactive allele; therefore in this context DNA methylation is repressive (*265, 266*). The full DMD extends in both 5′ and 3′ direction to cover 2.4kb in total in embryonic tissues (*265*). Imprinted methylation patterns at the *Mest* DMD control the monoallelic expression of a cluster of imprinted genes including the paternally expressed *Mest*, and the maternally expressed *Copg2*, *Cpa4* and *Klf14* (*265-270*).

The *Mest* (mesoderm expressed transcript) gene is as a transcript enriched in fetal mesodermal tissues in both mouse and man (*271, 272*). It is located at mouse chromosomal band 6qB1 and human chromosome band 7q32 (*271-273*). The MEST protein is a putative beta-hydrolase with unknown substrates, although it is speculated to function as a lipid hydrolase (*268*). A targeted mutation of *Mest*, in which an IRES-βGeo cassette is inserted in place of exons 3-8, maintains normal methylation patterns but reduces viability (*274*). Fetuses and placentas of paternal null conceptuses at E18.5 are growth restricted by approximately 15% (*274*). Maternal behavior of placentaphagia and nesting are decreased in paternal *Mest* null F1 female mothers (*274*). These growth restriction phenotypes are similar to the sub-proximal translocation mouse with loss of *Mest* expression, however no maternal behavioral changes were observed in that model (*262*). The placental growth restriction phenotype is particularly interesting given that *Mest* is expressed within extraembronic mesoderm lineages within the placenta including the chorionic plate, fetal vessels and hemangioblast precursors (*275*). In human placenta *Mest* is also expressed in cytrophoblasts (*275*). *Mest* isoform 2 (which encodes a protein shorter by 7 amino acids) transcribed from upstream exon 1a is the predominant form expressed in the human placenta and has a promoter CGI that is maternally methylated specifically in the placenta; a similar genetic structure suggests a homologous mechanism in mouse (*276*). Stochastic loss of *Mest* imprinting (biallelic expression) is correlated with increased body, spleen and kidney weight in interspecific hybrids of *Mus musculus and M. spretus*, suggesting not only that *Mest* is involved in somatic growth, but also that maintenance of imprints is not as robust in inter-species crosses (*277*).

Copg2 (nonclatharin coat protein γ 2) is paternally imprinted in adult and late gestation fetal brain (*278*). Its transcription is directionally opposed and abutting *Mest*. Neuronal

expression of *Mest-xl*, an alternatively polyadenylated *Mest* transcript that incorporates a 3′ UTR that runs through *Copg2* intron 20, occludes paternal *Copg2* expression via transcriptional interference (*278*). Additionally, *Mest* contains an intronic (between exon1a and exon1) paternally expressed antisense transcript emanating from the *Mest* DMD as well as harboring the microRNA (miRNA or Mir) gene *Mir335* downstream of exon1. Upstream of *Mest* resides a series of carboxypeptidase paralogs (*Cpa1, 2, 4* and *5*), of which only *Cpa4* is imprinted (paternally) (*267, 269*). Roughly 21kb downstream from *Mest* resides the paternally imprinted *Klf14* (Krüppel-like factor 14). *Klf14* is a parent-of-origin specific expression quantitative triat loci (eQTL) that modulates diabetes mellitus and elevated HDL risk (*279-281*). A handful of single nucleotide polymorphsims (SNPs) upstream of the *Klf14* promoter decrease *Klf14* expression and increase risk for the above metabolic diseases when maternally inherited (*279-281*). The same SNPs also regulate a network of metabolic genes *in trans* (*282, 283*). These findings implicate the KLF14 transcription factor as a master regulator of metabolic pathways (*282, 283*). A more thorough introduction to *Klf14* and results from a novel targeted deletion model are presented in Chapter 4. The evidence presented here indicates that the *Mest* cluster is involved in the regulation of fetal growth, and deserving of further study of its function in prenatal development with a particular focus on placental function.

1.5.4 *Nap1l5*

The maternally imprinted *Nap1l5* resides 30Mb distal to *Mest* in mouse chromosomae band 6qB3 (*263, 284*). It is located within the same sub-proximal breakpoint region as *Mest* that is associated with matUPD growth restriction and patUPD overgrowth (*263*). The *Nap1l5* promoter is a maternally methylated DMD that represses *Nap1l5* transcription, mechanistically resulting in

either double or zero dosage of *Nap1l5* transcription in sub-proximal 6q matUPD and patUPD mice respectively (*263, 285*). *Nap1l5* is an intronless retrotransposed copy of a gene encoding nucleosome assembly protein-1 (*Nap1*) (*263*). It is located within an intron of the *Herc3* host gene. (*284*) While *Herc3* is not imprinted *per se*, it does undergo allele specific polyadenylation through transcriptional interference (*286*). *Herc3*, which encodes a ubiquitin ligase, is transcribed into a non-functional truncated isoform (*Herc3b*) from the unmethylated paternal allele, and a full length isoform (*Herc3a*) from the methylated allele (*286*). A syntenic homologous region is found on human chromosome 7 near *MEST*. Mouse embryonic neuronal and heart tissues express *Nap1l5*, but its expression has not been studied in placenta (*263, 284*). Due to its location in the sub-proximal breakpoint region the *Nap1l5* imprinted locus must be considered a candidate for the regulation of placental and fetal growth.

1.5.5 *Peg10*

Mouse chromosome band 6qA1 and human chromosome 7q21.3 harbor the *Peg10* imprinting cluster (*287, 288*). A maternally methylated imprinted DMD is found at this shared promoter and regulates monoallelic gene expression of *Peg10, Sgce*, as well as the downstream maternally expressed genes *Pon2*, *Pon3*, and *Ppp1r9a* (*288*).The gene order of the syntenic *Peg10/PEG10* regions in mouse and man is identical (*288*). *Peg10* is a intronless endogenous retrovirus derived gene of the suchi-ichi family (*289, 290*). It encodes *gag* and *pol* retroviral derived transcripts from two distinct open reading frames (*291*). Transcription of *Peg10* runs adjacent and in opposite orientation from a shared promoter of the paternally expressed sarcoglycan protein epsilon gene (*Sgce*) (*288*). *Peg10* is expressed in trophoblast lineages early within the ectoplacental cone and chorion and later in both P-TGCs and S-TGCs (*292*). Paternally inherited

deletion of *Peg10* is embryonic lethal at E9.5 due to failure of LZ and JZ layer development (*293*). The lethality of matUPD of proximal chromosome arm 6q may be attributed to loss of *Peg10* expression (*262, 293*). The role of imprinted genes within the *Peg10* cluster and the function of *Peg10* DMD methylation in placental biology is worthwhile to study further.

1.5.6 *H19*

The subtelomeiric chromosomal band 7qF5 in mouse has parent-of-origin effects derived from both bimaternal and bipaternal inheritance. This region is homologous with 11p15.5 in humans that contains the *H19* and *Kcnq1* imprinting clusters. Bipaternal inheritance of distal chromosome arm 7q results in fetal overgrowth, whereas bimaternal inheritance in growth restriction (*294*). The *H19* imprinting cluster consists primarily of maternally expressed ncRNA *H19* and the paternally expressed insulin-like growth factor 2 (*Igf2*). Differential DNA methylation has been observed at the *H19* gene body and promoter and at the *Igf2* promoter, however it is paternally inherited methylation at the *H19* 5′ upstream flanking regions that is the primary genomic imprint (*295, 296*). Deletion of the *H19* DMD within the 5′ flanking region relieves strict monoallelic expression (*297*). Paternal inheritance of the *H19* DMD deletion activates *H19* expression and attenuates *Igf2* expression, whereas maternal inheritance has the reciprocal effect of decreasing *H19* expression and increasing *Igf2* expression (*297*). Adulteration of the CTCF binding site within the *H19* DMD has a similar effect of *Igf2* activation when maternally inherited (*298*). Based on these genetic models a mechanistic explanation of the *H19* imprinting cluster has been proposed by which DNA methylation at the *H19* DMD blocks chromatin access of the insulator protein CTCF thereby protecting *Igf2* from being spooled into a transcriptionally inactive chromatin loop (*299*).

The *H19* imprinting cluster has strong influences on prenatal growth. Paternally inherited targeted deletion of *Igf2* causes severe postnatal growth restriction, and explains the growth restriction associated with bimaternal inheritance of distal chromosome arm 7q (*300*). Transgenic overexpression of *Igf2* results in prenatal overgrowth and phenocopies many of the changes observed in BWS (*301*). Paternally inherited deletion of the placenta specific *Igf2p0* isoform, normally expressed within the SynT, results in both fetal and placental growth restriction (*302*). The efficiency of placental transport is decreased in these placenta (*302*). A study of both the *Igf2* and *Igf2p0* paternal null placentas indicate that the *Igf2* deletion has a disproportionate decrease of JZ volume whereas the *Igf2p0* deletion decreases LZ and JZ volume proportionately (*303*). In contrast, only the *Igf2p0* knockout altered placental diffusion transport (*303*). In a targeted model of *H19* in which 5kb of the *H19* gene body and 10kb of upstream region were deleted, IGF2 expression was increased and placentas were overgrown with an abundance of GCs (*304*, *305*). The *H19* ncRNA primary transcript is processed into *Mir675* in late gestation placenta but not in embryonic tissues (*306*). A 3kb deletion of the *Mir675* region induces placental overgrowth indicating that *H19* itself is a negative regulator of growth mediated through repression of identified *Mir675* targets including *Igfr1* (*306*). In addition to *H19* and *Igf2*, imprinted expression of *Ins2* and the ncRNAs *Igf2os* and *Mir483* (both antisense to *Igf2*) have been reported in extraembryonic tissues (*307*). Given the strong placental and fetal phenotypes of the various *H19* and *Igf2* genetic models it is valuable to study the direct effects of loss of imprinting at this cluster.

1.5.7 *Kcnq1*

The *Kcnq1* imprinting cluster is roughly 500Mb distal to *H19* within subtelomeric band 7qF5 in mouse. It is one of the largest and most important imprinting clusters in mammalian development. The *Kcnq1* cluster includes the paternally expressed *Kcnq1ot* and several maternally expressed genes including *Aslc2, Cdkn1c*, and *Phlda2* (*118, 120, 125, 308*). The regulation of a greater number of imprinted genes has been reported as specific to placental and extraembryonic lineages including *Th, Tspan32, Cd81, Tssc4*, *Nap1l4*, and *Osbpl5* (*118, 309*). The *Kcnq1* DMD resides at the promoter of *Kcnq1ot,* an antisense ncRNA transcript emanating from within *Kcnq1* intron 5 (*308, 310*). An allele in which the *Kcnq1* DMD is deleted mimics the maternally methylated imprinted state (*310*). Paternal inherited deletion of the *Kcnq1* DMD deletion results in fetal growth restriction and overexpression of *Ascl2, Cdkn1c* and *Phlda2* (*310*). These phenotypes are opposite of those observed in BWS cases where there is loss of *Kcnq1* DMD methylation (*310*).

The importance of the *Kcnq1* cluster in placenta biology is most evident in targeted single imprinted gene deletion mouse models. Maternally inherited targeted deletion of *Ascl2* is embryonic lethal at E10.5 due to failure of placental formation (*222*). Trophoblast lineage differentiation is strikingly abnormal in these placenta with a vast proliferation of TGCs at the expense of JZ and LZ development (*222*). Embryonic *Ascl2* maternal null lethality can be rescued by tetraploid complementation, indicating placental maldevelopment was independent of fetal development (*222*). A hypomorphic *Ascl2* allele is viable but growth restrictive and results in expansion of TGCs at the expense of LZ development (*311*). Similar placental phenotypes to *Ascl2* maternal null mice are observed in and *Dnmt3l* maternal effect offspring that lack all maternally imprinted DMD methylation (including at *Kcnq1*), suggesting that *Ascl2* is an

imprinted gene at the focal point of placental development (*94*, *97*, *99*). Maternal inherited deletion of either the *Phlda2* or *Cdkn1c* genes, which also reside in the *Kcnq1* cluster, results in placental overgrowth (*312*, *313*). In contrast, transgenic over-expression of either *Phlda2* or *Cdkn1c* results in poor placental growth (*314*-*316*). Deletion of *Phlda2* has recently been shown to increase placental glycogen cell abundance and glycogen content suggesting that *Phlda2* may limit maternal resource allocation (*317*). Based on the aforementioned studies it would be of interest to determine if loss of *Kcnq1* DMD methylation has similar effects on placental development.

1.5.8 *Snrpn*

The central region of mouse chromosome 7 (7qB5-7qC) has a parent-of-origin postnatal lethality phenotype when two maternal copies are inherited (*318*). This genetic locus is homologous to a syntenic region on human chromosome 15q11.2 implicated in the imprinting disorders PWS and AS (reviewed in section 1.2.3). The *Snrpn* cluster contains the maternally expressed *Ube3a*, and the paternally expressed *Snrpn*, *Magel2*, *Mkrn3*, *Peg12* and a cluster of C/D snoRNAs genes (*Snord64*, *107*, *114*, and *116*) (*319*). The imprinted genes within the *Snrpn* cluster are regulated by a maternally inherited imprinted methylation mark at the promoter and first exon of *Snrpn* (*320*). The *Snrpn* gene is a bicistronic transcript encoding the mRNA splicing factor SmN and a neuropeptide encoded by *Snurf* from an uprstream reading frame (*321*). *Snrpn*, the *Snord* cluster and *Ipw* are part of a contiguous transcriptional unit that runs from the *Snrpn* promoter through *Ube3a* in an anti-sense orientation (*319*). The components of the *Snrpn* cluster are involved in numerous cellular processes including alternative splicing, transcription and neurogenesis. A

cluster of *Mir334* transcripts are also found within the cluster but have not been firmly established as imprinted.

An array of targeted genetic mouse models have been generated to study the *Snrpn* imprinting cluster with a focus on understanding PWS and AS (recently reviewed by (*319*)). Deletion of the *Snrpn* DMD replicates the growth restriction and hypotonia traits observed in PWS cases with imprinting mutations (*320*). Paternal inherited deletion of the *Snrpn* DMD results in loss of expression of the maternally imprinted *Ndn*, *Ipw* and *Mkrn3* genes, indicating that *Snrpn* DMD deletion imitates the maternally methylated state, and that the paternally unmethylated *Snrpn* DMD acts as a bidirectional promoter (*320*). A 6.8MB paternal deletion of the *Snprn* region encompassing the totality of the imprinted cluster as well as some genes upstream of *Ube3a* results in fetal and neonatal growth restriction, and abnormal endocrine pancreatic structure and function that culminates in in neonatal lethality (*322, 323*). The loss of *Snord* products in this model alters alternative splicing patterns (*324*). Targeted deletion of the *Snord116* cluster in mouse results in both growth restriction and hyperphagia, two hallmarks of PWS (*325*). Although no specific role has been ascribed for any member of the *Snrpn* cluster in placenta biology, it is plausible they are involved in placental function based on their ubiquitous expression, the prenatal phenotypes found in *Snrpn* deletion models, and the endocrine functions the placenta has during *in utero* development

1.5.9 *Peg3*

The *Peg3* imprinting cluster resides on proximal mouse chromosome 7 in chromosomal band 7qA1 (*326*). A homologous region is found on human chromosome 19q13.4 (*327, 328*). The *Peg3* cluster consists of paternally expressed *Peg3*, *Peg3os*, *Usp29* and *Zfp264*, and the

maternally expressed *Zim1*, *Zim2* and *Zim3* (*329*). *Peg3* and *Zim1-3* encode zinc finger transcription factors, whereas *Usp29* encodes a ubiquitin-specific protease (*328, 330, 331*). The PEG3 protein is a transcriptional repressor containing an N-terminal KRAB domain and 12 C-terminal C2H2 Krüppel-type zinc finger DNA binding motifs (*329, 332*). The germline *Peg3* DMD resides in the bidirectional promoter between *Peg3* and *Usp29*. A secondary somatic DMD is established during preimplantation development at the bidirectional promoter of *Zim3* and *Zfp64* (*333*). *Peg3* and the majority of imprinted genes in this cluster are highly expressed in human ovary, placenta, testis and hypothalamus (*328*). Based on gene expression patterns and the phenotypes described below, it is evident that this locus is involved in reproductive, metabolic and hormonal pathways and placental function.

Multiple genetic models have been made to study the function of the *Peg3* cluster in mouse (most recently reviewed by (*329*)). The first reports of targeted deletion of *Peg3* showed that paternal inheritance of the null allele leads to growth retardation from E17.5 to adult, and that paternal null dams have poor maternal behavior that results in partial perinatal lethality (*334*). In addition, paternal null offspring have increased abdominal subcutaneous fat, hypophagia, elevated circulating leptin, resistance to leptin, reduced metabolic rate and resistance to cold. These changes are concomitant with alterations in hypothalamic gene expression, indicating the involvement of *Peg3* in metabolic regulation (*335*). A second targeted *Peg3* deletion model has slightly different phenotypes with perinatal lethality, reduced suckling and altered fetal brain gene expression (*336*). Intriguingly, the brain of paternal null fetuses have altered expression of metabolic genes (*e.g. Clec2d*, *Cidea* and *Pparg*), and express placenta-specific genes of the prolactin (*ie. Prl3b1, Prl2b1*), cathepsin (*ie. Ctsj and Ctsq*) and ceacam (*ie. Ceacam11* and Ceacam12) families (*336*). Based on the derepresion of many genes in the *Peg3*

null model concomitant with reduction in repressive H3K9me3 suggests that the PEG3 KRAB domain interacts with TRIM28 and SETDB1 and/or EHMT2 to modulate chromatin structure at target genes (*329*).

A deletion of the *Peg3* DMD, when paternally inherited, results in fetal growth restriction and partial embryonic lethality (*337*). Gene expression is altered in these mice with increased *Zim1* and reduced *Peg3* expression indicating the *Peg3* DMD null recapitulates a maternal (methylated) allele (*337*). Based on these results, the effects of direct loss of *Peg3* DMD methylation may have an opposite phenotype as the *Peg3* DMD deletion, with fetal overgrowth rather than growth restriction. It would be interesting to examine placental phenotypes in the absence of the *Peg3* imprint given the expected increase in *Peg3* expression and its known involvement in placental gene regulation.

1.5.10 *Plagl1*

Plagl1, previously called *Zac1*, is an imprinted gene on mouse chromosome band 10qA2 and human chromosome 6q24 that is a single imprinted gene cluster (reviewed by(*338*)). A maternally imprinted DMD found 5′ of the *Plagl1* gene body regulates the expression of the paternally expressed *Plagl1* and *Hymai* transcripts (*338*). *Plagl1* encodes a zinc finger transcription factor with multiple alternative splicing isoforms (*338*). *Hymai* is a ncRNA with possible epigenetic function transcribed in the same direction as *Plagl1* and sharing the same first exon (*338*). The boundaries of the *Plagl1* imprinted locus are defined by CTCF sites and exclude the neighboring genes *Phactr2* and *Stx11* (*339*).

Loss of *PLAGL1* imprinting via patUPD6, pat6q24 duplication or mat6q24 hypomethylation causes TNDM1 with associated fetal pancreatic glucose insensitivity and IUGR

(*152-154*). Transgenic over-expression of *Plagl1* in mouse captures many of the phenotypes of TNDM1 (*340*). Paternal inherited deletion of *Plagl1* in mouse results in fetal but not placental growth restriction and reduced neonatal survival (*341*). Based on meta-analysis of co-expressed genes in micro-array data, PLAGL1 is part of a network of imprinted genes which regulate embryonic growth, including members of the *H19*, *Peg3*, *Kcnq1*, *Mest*, *Gnas*, and *Dlk1* clusters (*341*). Neither *PLAGL1* DMD methylation nor imprinted expression is altered in term placentas from IUGR pregnancies (*155*). However, overall expression levels of *HYMAI* is increased in IUGR placentas and *PLAGL1* is decreased in female IUGR placentas (*155*). Chromatin immuno-preicipitation has identified *PLAGL*1 binding to the *H19* enhancer and DMD, as well as to the promoters of metabolic regulators *GLUT4, TCF4* and *PPARG1* in human term placentas (*155*). Given these finding it is important to determine the direct effects of loss of imprinting at the *PLAGL1* locus on placental development.

1.5.11 *Grb10*

Robertsonian translocations involving proximal chromosome 11 have parent-of-origin specific effects. A homologous imprinted region on human chromosome 7q11.2-12 is a candidate SRS locus (*342*). Proximal chromosome 11 matUPD neonates are growth restricted whereas patUPD neonates are larger (*10*). These growth abnormalities occur prenatally in E12.5-E17.5 mid gestation placentas and fetuses, and likely begin at E7.5 (*343*). The *Grb10* gene at 11qA1 within this region is maternally expressed in fetuses and placentas, but is paternally expressed in adult brain tissues from an alternative promoter (*344*). The genes flanking *Grb10, Ddc* and *Cobl,* are imprinted in an isoform and tissue specific manner (*309, 345*). *Ddc* is preferentially paternally expressed from exon 1 in yolk and liver, and from exon 1a in whole embryo and neonate heart

(*345*). *Cobl* is preferentially maternally expressed in yolk (*345*). The GRB10 protein is an adaptor for receptor tyrosine kinases including the insulin and insulin-like growth factor receptors, and is an intermediate between receptor-ligand binding and downstream signaling (*342*).

Similar to the proximal UPD11 phenotypes, targeted deletion of the *Grb10* gene results in altered embryonic growth. Maternal inheritance of the *Grb10* null allele eliminates embryonic *Grb10* expression in non-brain tissues, and results in both fetal and placental overgrowth (*346*). Placental overgrowth in *Grb10* maternal null placentas is a direct result of LZ expansion (*347*). This phenotype is independent of the *Igf2* pathways as evidenced by the partial rescue of the *Igf2* null growth restriction in compound heterozygotes (*346*). At maturity maternal *Grb10* null mice are lean (reduced adiposity and greater muscle mass) and have enhanced insulin sensitivity and glucose responsiveness (*348*). These effects are likely mediated through increased insulin receptor signaling (*348*). These results indicate that in the mouse fetus, placenta and adult the maternal *Grb10* allele has growth restricting functions mediated through its inhibition of insulin receptor signaling.

The maternally methylated *Grb10* DMD resides adjacent its brain specific promoter (Exon 1b) (*349, 350*). The brain specific promoter is downstream of the major promoter (Exon 1a) and splices into conserved exons 2-17. Exons 1a and 1b encode unique 3′ UTRs. A mouse specific region overlapping the *Grb10* DMD contains CTCF binding sites and may be responsible for an insulator mechanism of imprinting by regulating promoter access to downstream enhancers (*350*). Neuronal expression from the paternal Exon 1a promoter is driven by tissue specific epigenetic changes. The maternal *Grb10* allele in neurons has an expanded DMD extending across exon 1b (*349*). On the paternal allele, bivalent chromatin (with activating

H3K4me2 and repressive H3K27me3) at the *Grb10* 1b promoter resolve into active chromatin during neuronal differentiation (*351, 352*).

Genetic ablation of the *Grb10* DMD effectively makes the allele analogous to the maternally methylated state (*345*). Paternal inheritance of the *Grb10* DMD deletion allele results in biallelic expression of *Grb10* from the major promoter, decreased *Ddc* expression and increased (biallelic) *Cobl* expression (*345*). These mice are fetal and placental growth restricted from E10.5 onwards (*345*). Adult paternal *Grb10* DMD deletion mice are growth restricted and do not recover (*345*). The results from these studies show the maternal *Grb10* imprint regulates expression of a small cluster of imprinted genes critical for prenatal development and warrants further study.

1.5.12 *Zrsr1*

Located at mouse chromosome 11qA3.2 the *Zrsr1* gene contains a maternally methylated DMD at its promoter (*353, 354*). *Zrsr1,* formerly known as *U2af1-rs1*, is a retrotransposed copy of an auxiliary alternative splicing factor inserted in an antisense orientation within intron 1 of the *Commd1* (*Murr1)* gene (*353, 354*). This transposition event is specific to rodents and is not found at the non-imprinted human *COMMD1* locus at 2p15 (*355*). The maternally methylated *Zrsr1* allele is transcriptionally silenced, whereas the unmethylated paternal allele is active in preimplantation embryos, embryonic and adult neuronal tissue and all other adult mouse tissues analyzed (*353, 354*). *Commd1* is exclusively maternally expressed in adult neuronal tissue and preferentially maternally expressed in other adult tissues (*356*). In contrast, fetal tissues do not show *Commd1* parental allelic expression bias (*354, 356*). It is speculated that the paternal

imprinting of *Commd1* expression is due to anti-sense transcriptional interference from *Zrsr1* (*356*, *357*).

Imprinted expression of neither *Zrsr1* nor *Commd1* within the placental compartment has been confirmed. However, homozygous deletion of *Commd1* in mouse is embryonic lethal at E9.5 in part due to failure of placental vasculogenesis and over activation of *Hif1a* (*358*). The COMMD1 protein is critical for copper metabolism and other cellular processes (*359*). *Commd1* is expressed in the mouse allantois and chorionic plate as well as in the human chorionic villi (*358*). The *Commd1* null allele was generated by insertion of a *neo* cassette in place of exon 2, and has no effect on *Zrsr1* expression (*358*). There are currently no genetic models targeting *Zrsr1* or the *Zrsr1* DMD specifically. It would be interesting to see if loss of *Zrsr1* DMD methylation influences placental development.

1.5.13 *Dlk1*

Whole and distal chromosome 12 disomies have parent-of-origin effects on mouse development (*360*, *361*). A syntenic imprinted region found at human 14q32-31 is thought to be responsible for clinical developmental phenotypes associated UPD14 (*362-364*). In mice, maternal UPD12 offspring have fetal and placental growth restriction starting at E15.5, reduced skeletal muscle fiber thickness, and 50% neonatal lethality (*360*). Paternal UPD12 progeny have placental overgrowth at E18.5, increased skeletal muscle fiber thickness, 50% late gestation (E18.5) lethality, and 100% lethality at birth (*360*). The phenotypes of maternal and paternal distal UPD12, revealed from studies of reciprocal translocation T(4;12)47H, collaborate the whole chromosome UPD12 findings (*361*). Maternal distal UPD12 results in fetal and placental growth restriction at E15.5 onwards together with decreased fetal skeletal muscle fiber widths and

delayed bone ossification (*361*). Paternal distal UPD12 is non-viable past E15.5 and shows a gradual decline in fetal:placental ratio from E13.5 when it is higher than control littermates, to E15.5 where it substantially lower than control littermates (*361*).

Genetic studies have attributed the UPD12 parent-of-origin effects to the large *Dlk1* imprinted gene cluster at distal chromosomal band 12qF1 (*365*). This cluster contains the paternally expressed protein coding genes *Dlk1, Rtl1* and *Dio3* and numerous maternally expressed ncRNAs including *Meg3* (also called *Gtl2*), *Rtl1as*, *Meg8* (*Rian*) and *Meg9* (*Mirg*) (*366*). The *Dlk1* imprinting cluster is regulated by an intergenic DMD (IG-DMR; herein called *Dlk1* DMD) between the *Dlk1* and *Meg3* promoters and secondarily by differentially methylated CGIs at the *Meg3* and *Dlk1* promoter (*364, 365, 367*). Imprinting of the *Dlk1* cluster arose during eutherian evolution with the insertion of *Rtl1*, ncRNAs and intergenic DMD sequences between the ancestral *Dlk1-Dio3* locus found in more primitive vertebrates (*368*).

Dlk1 encodes a transmembrane protein with EGF like extracellular domains similar to the juxtacrine signaling Notch ligand Delta in *Drosophila*, but can also undergo proteolytic cleavage to produce a soluble extracellular ligand involved in intercellular signaling (*369*). *Dlk1* is expressed at sites of branching morphogenesis during embryonic development (*e.g*. lungs, liver, and adrenal cortex) as well as connective tissues and skeletal muscle (*370*). Within extraembryonic lineages expression of *Dlk1* is detected in the fetal endothelium of the placental labyrinth and yolk-sac blood pools (*370*). Genetic ablation of *Dlk1* results in late gestation (E18.5) growth restriction and partial neonatal lethality in both homozygous and paternal null offspring (*371*). These mice also have postnatal growth retardation, increased adiposity and increased circulating lipid metabolites (*371*). Conditional deletion of *Dlk1* within myoblasts reduces body mass, muscle fiber count and myocyte differentiation, whereas overexpression of

Dlk1 in myoblasts promotes differentiation indicating DLK1 has muscle-specific functions (*372*).

Rtl1, like *Peg10,* is a paternally expressed endogenous retrovirus of the suchi-ichi family (*289, 290, 373*). However it is encodes a larger protein complete with retroviral derived pol, gag and env domains that have been subverted to mammalian function (*289, 290, 373*). An antisense transcript, *Rtl1as,* is transcribed in opposite orientation to *Rtl1* and encodes miRNAs *Mir431*, *Mir434*, *Mir432* and *Mir136* that directly overlap the *Rtl1* coding region (*374*). Paternal inherited deletion of *Rtl1* results in prenatal growth restriction and neonatal lethality (*374*). At E15.5 placental morphological abnormalities are observed including fractured SynT basement membranes, lysosomes in SynT layer II, clogged fetal capillaries and phagocytic uptake of fetal endothelium by SynT leading to placental infarction (*374*). Maternal deletion on the other hand results in 150% placentomegaly with expanded fetal capillary spaces, and vacuoles in SynT layer II that are evidence of trophoblast starvation (*374*). These results display the opposing effects of *Rtl1* and *Rtl1as* on placental development.

A plethora of maternally expressed ncRNAs arise from the 12qF1 imprinted region from a continuous transcript including, from 5′ to 3′, the *Meg3*, *Rtl1as*, *Meg8* and *Meg9* genes (*375, 376*). No fewer than 65 Refseq miRNA genes are found within this region, as well as a series of snoRNAs that have not yet been annotated within *Meg8* (*376*). A model in which *Meg3* exons 1-5 are deleted along with the *Meg3* promoter CGI, reveals a strong developmental role for this cluster (*377*). The maternal knockout offspring die within 0-4 weeks due to lung and liver defects and have decreased *Meg8* and *Meg9* expression (and lower levels of the miRNA and snoRNAs they encode), but increased *Rtl1* expression (*377*). No changes in *Dlk1* or *Dio3* expression are observed in *Meg3* maternal null offspring (*377*). In contrast, paternal inheritance of the *Meg3*

null allele results in 50% perinatal lethality and 50% postnatal lethality, with one quarter surviving to a viable and fertile but growth restricted adulthood (*377*). Expression of *Dlk1, Dio3* and *Rtl1* are decreased in the paternal null offspring. Surprisingly homozygous null offspring, generated from F1 hybrid crosses of viable paternal knockout offspring, survive but have lower birth weights (*377*). Because the *Meg3* null mutation does not affect methylation at the *Dlk1* DMD it is unlikely that there is *de facto* loss of imprinting (*377*). It is hypothesized that maternal *Meg3* promoter CGI deletion results in direct loss of ncRNA products and derepression of *Rtl1* in *trans* (*377*). However, paternal inheritance of the *Meg3* null allele is thought to lead to repression of *Dlk1* and *Dio3 in cis* due to ablation of the secondary DMD (*377*). There is a need for finer mutations targeting individual miRNAs and snoRNAs to better understand the function of ncRNAs in this cluster.

Deletion of the *Dlk1* DMD that resides between the 3′ end of *Dlk1* and the *Meg3* promoter, results in developmental defects based on parental inheritance (*367*). Maternal *Dlk1* DMD null conceptuses have late gestation lethality (~E16) with increased expression of *Dlk1*, *Rtl1* and *Dio3*, and decreased *Meg3*, snoRNA and miRNA expression (*367*). However, paternal inheritance results in normal transcriptional profiles and viable offspring (*367*). These results indicate that the *Dlk1* DMD is the primary germline-DMD regulating the 12qF1 cluster, and that its deletion effectively creates a paternal (methylated) allele (*367*)

Additional research on the importance of the *Dlk1* imprinting cluster in fetal and placental development is warranted. The *Dlk1* cluster has been implicated in animal husbandry research as the genetic determinant of the *calligpye* allele in sheep, which has parent of origin effects that result in lean muscle hypertrophy (*378*). In humans, maternal and paternal UPD14 cases can also be caused by *Dlk1* DMD deletion and hypomethylation (*362, 363*). Intriguingly,

one of the phenotypes associated with UPD14 is placentomegaly (*363*). The fetal and placental phenotypes observed in the *Dlk1*, *Rtl1*, *Meg3* and DMD deletions discussed above demonstrate that the *Dlk1* cluster has a critical role in prenatal development. Lastly, there are only 2 paternal imprinted DMDs shared in mouse and human (*H19* and *Dlk1*), and both are required for embryonic development from induced pluripotent stem cell and bimaternal embryonic development in mouse (*379-381*). These disparate but important phenomenon may be related to modulation of placental function by the *Dlk1* imprinted loci.

1.5.14 *Igf2r*

The insulin receptor growth factor type 2, *Igf2r*, is maternally expressed and resides within the larger *Tme* locus that when deleted in maternal lineages is lethal at E15.5 (*10, 382*). *Igf2r* and its human homolog are found on chromosomal bands17qA1 and 6q25.3 respectively. The *Igf2r* DMD is maternally methylated in both species, however in humans and other primates *Igf2r* is biallelically expressed in the majority of individuals ((*383*) and references therein). A maternally methylated DMD sequence within intron 2 of *Igf2r* can confer imprinting of YAC transgenes in mice (*384, 385*). The *Igf2r* DMD is situated at the promoter of *Airn* (antisense *Igf2r* RNA), a long antisense ncRNA (*385, 386*). Transcription of *Airn* occurs on the unmethylated paternal allele that is associated with a silenced *Igf2r* (*385, 386*). Truncation of the *Airn* transcript by insertion of poly-adenylation sequences mitigates *Igf2r* silencing independent of DNA methylation (*387, 388*). It is therefore hypothesized that the act of *Airn* transcription is directly responsible for *Igf2r* maternal inactivation (*387*). However, it has also been noted that in the placenta full length but not truncated *Airn* ncRNA associates with the *Slc22a3* promoter and recruits the H3K9 methyltransferase EHMT2 to invoke lineage-specific imprinting (*389*).

In the majority of embryonic and adult tissues imprinting at the *Igf2r* locus is limited to the expression of *Igf2r* and *Airn*. However, in the placenta and extra embryonic visceral yolksac the downstream *Slc22a2* and *Slc22a3* transcripts, encoding molecular transporters of unknown function, are preferentially expressed from the maternal allele (*307, 309, 390*). The expansion of the imprinted locus in extraembryonic tissues is similar to that found at other loci including *Kcnq1, Igf2, Peg10* and *Grb10* (*309*). The IGF2R protein is a receptor for IGF2 expressed in SynT as both a plasma membrane and cleaved soluble form (*391*). IGF2R exerts negative inputs to IGF2 signaling by acting to bind and clear excess IGF2, but may also have signaling properties (*391*). Targeted deletion of *Igf2r* (deletion of exons 13-18) when maternally inherited results in fetal and placental overgrowth (*392, 393*). Maternal *Igf2r* null conceptuses have elevated levels of IGF2 and IGF2-binding proteins (*392*). Deletion of the *Igf2R* DMD generates an allele that effectively mimics the maternal methylated state, and when paternally inherited results in increased *Igf2r* expression and embryonic and neonatal growth restriction (*394*). Due to increased *Igf2r* expression, paternally inherited the *Igf2r* DMD deletion can rescue maternally inherited *Tme* mutations (*394*). Based on these studies *Igf2r* is a growth suppressor. Based on the importance of the *Igf2r* locus in growth and its expanded boundaries in extraembryonic tissues it is important to determine the effects of loss of imprinting at the *Igf2r* DMD in placental development

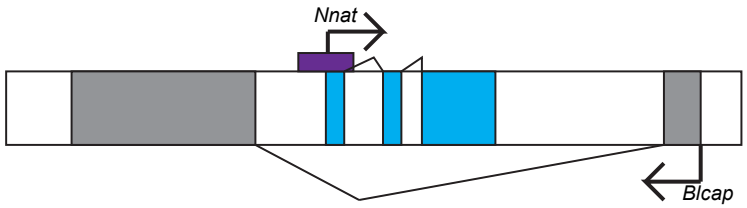
1.5.15 *Impact*

The *Impact* gene resides on chromosomal band 18qA1 and is equated with parent-of-origin specific embryonic developmental delays observed with patUPD in studies of Robertsonian translocations involving chromosome 18 (*395, 396*). The paternally imprinted and ubiquitously

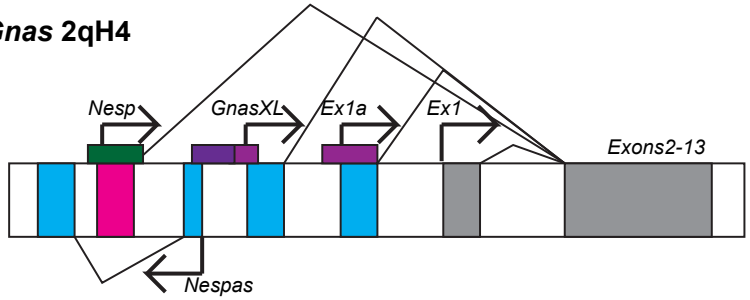
expressed *Impact* contains 11exons, and has a maternally methylated DMD within its first intron (*397*). *Impact* is adjacent to the non-imprinted proximal *Osbpl1* and the distal *Hrh4* (*398*). In humans, *IMPACT* is found in the same genomic context on chromosome band 18q11.2-12.1 but is not imprinted and lacks an intronic DMD (*397, 398*). Intriguingly a similar arrangement of *Osbpl2* and *Hrh3* but lacking an *Impact* paralog is found between the tandem duplicated *Lama3* and *Lama5* genes on mouse chromosome 2qH4 near the *Nnat* and *Gnas* clusters, and within a syntenic region on human chromosome 20q13.3 (*398*). Expression of *Impact* is much higher in rodent species where it is imprinted (*e.g.* mouse, rat and rabbit) than in pigs and primates where it is not (*399*). This suggests that the regulation of *Impact* gene dosage can explain the difference in imprinting status between mammalian clades (*399*).

The IMPACT protein is homologous to the ancestral yeast protein YIH1 involved in nutrient starvation responses (*395, 397*). In fact, IMPACT participates in the same translational regulation molecular pathways as its yeast homolog. IMPACT binds to GCN1 and inhibits the activity of GCN2 inactivating phosphorylation of Ser51 on the eukaryotic translational initiation factor 2 (eIF2α) (*400*). Unphosphorylated eIF2α inhibits translation of many proteins and activates translation of a few stress response genes (*400*). High levels of IMPACT in the hypothalamus and low levels of phosphorylated eIF2α are thought to be involved in nutrient deprivation and feeding behavior stress responses (*400*). In addition, IMPACT associates with ribosomes in post-differentiation neurons to promote neuritogenesis (*401*). Given the expression of *Impact* within the placenta and its role in mediating nutrient stress response it is plausible this gene has important functions within this essential organ.

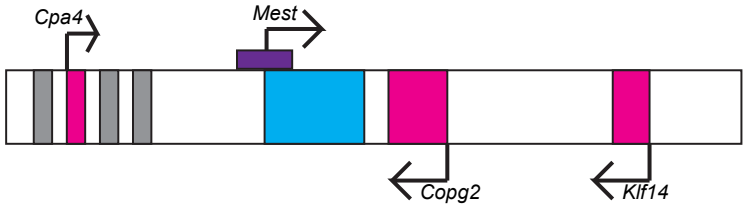
A. *Nnat* 2qH4



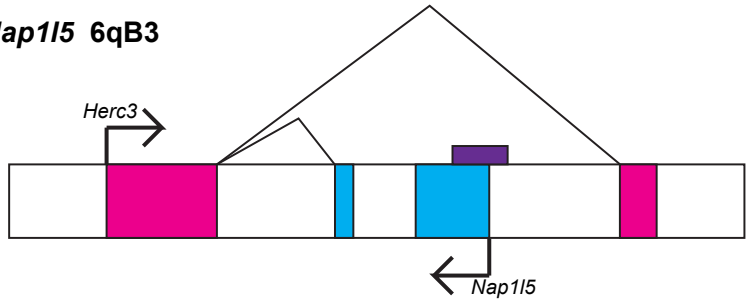
B. *Gnas* 2qH4



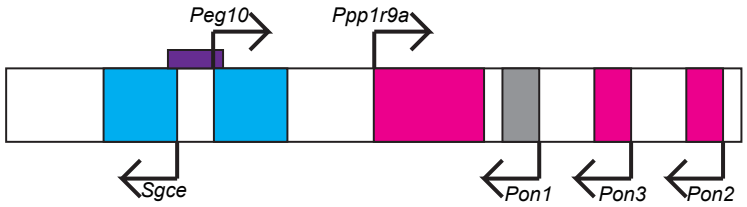
C. *Mest* 6qB3



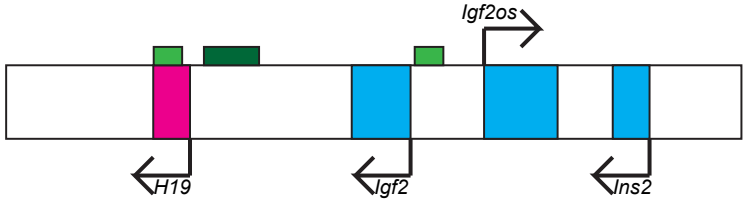
D. *Nap1l5* 6qB3



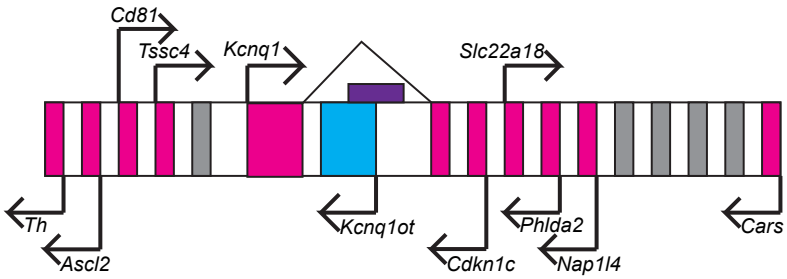
E. *Peg10* 6qA1



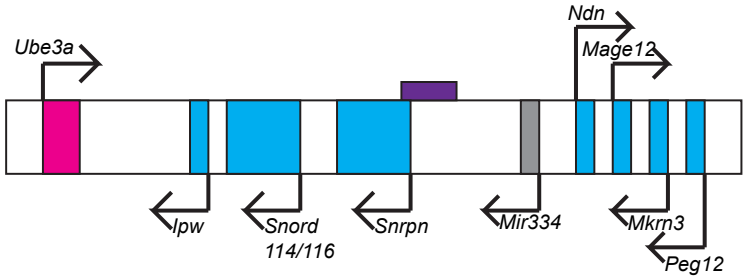
F. *H19* 7qF5



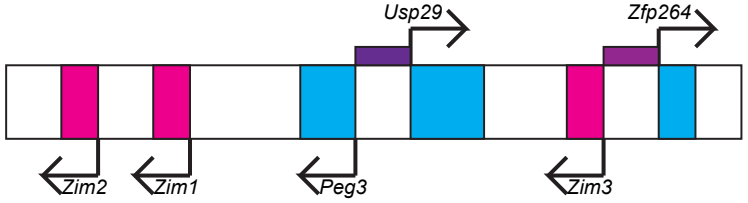
G. *Kcnq1* 7qF5



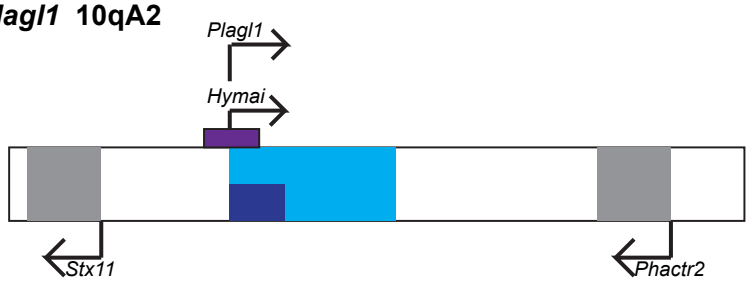
H. *Snrpn* 7qB5-7qC



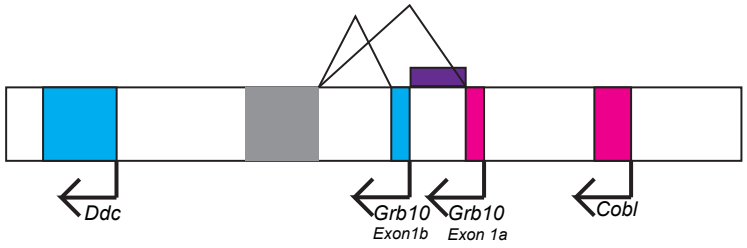
I. *Peg3* 7qA1



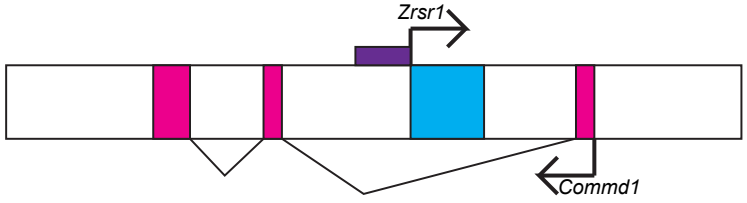
J. *Plagl1* 10qA2



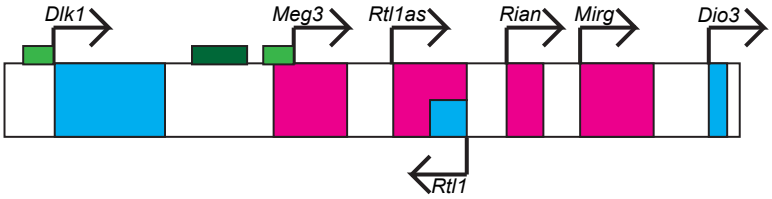
K. *Grb10* 11qA1



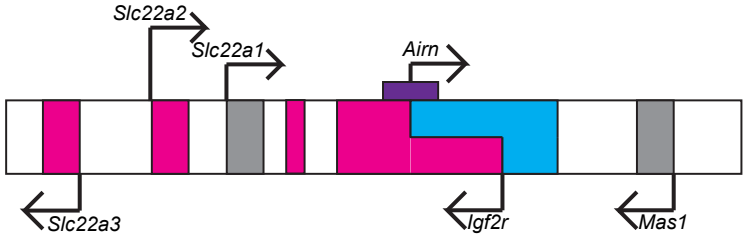
L. *Zrsr1* 11qA3.2



M. *Dlk1* 12qF1



N. *Igf2r* 17qA1



O. *Impact* 18qA1

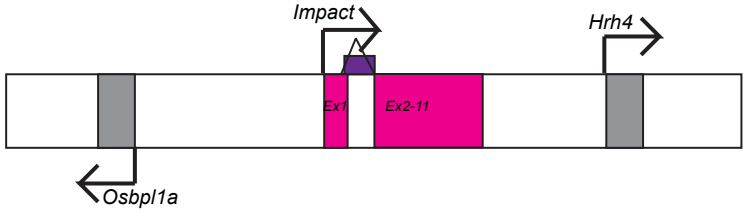


Figure 2. 15 Imprinted gene clusters. (A-O) 15 imprinted loci implicated in placental development are labeled by mouse chromosomal bands and refseq gene names. Each gene is represented by a filled rectangle and arrows give transcriptional direction. The color of each gene box indicates its imprinting status as maternally expressed (Pink), paternally expressed (Blue) or biallelic (Gray). DMDs are indicated as rectangle of lesser height outside above the gene diagram and are colored as to indicate their DNA methylation as maternal (purple) or paternal (green) in origin with secondary DMDs in lighter shades. Triangulated lines indicate alternative splicing, where relevant to genomic imprinting. This figure is the summation of the numerous works cited within section 1.5.1-15. Figure not drawn to scale.

1.6 SPECIFIC AIMS

My dissertation research tests the hypothesis that genomic imprints are essential during placental development and aims to define their importance in this fundamental mammalian organ. In my introduction I have described the importance of genomic imprinting in mammalian development and human disease. I have discussed the dynamics of methylation changes during germ cell and preimplantation development with a focus on imprinted DMD methylation. In addition, I have reviewed the function and development of the mouse placenta highlighting aspects relevant to modeling the human placenta. Herein, I have also described in detail fifrteen imprinted gene clusters with possible involvement in placental development. The placental function of imprinted gene clusters as an integrated unit, including both imprinted genes and the DMD methylation that controls their parent-of-origin specific expression, are incompletely understood. My thesis research builds off the knowledge presented within the introduction and seeks to identify the roles of genomic imprints in placental development by studying placental development in their absence.

In Chapters 2 and 3 of my dissertation I address the first two aims of my dissertation which examine the effects of partial loss of genomic imprints on placental development using the

Dnmt1$^{\Delta 1o}$ maternal effect model. The first aim of my dissertation research is to determine which placental processes are influenced by genomic imprinting. To these ends I described the range of placental morphological and molecular phenotypes of *Dnmt1*$^{\Delta 1o}$ maternal effect (DNMT1o-deficient) placentas across murine gestation from E9.5 to E17.5. Considerable emphasis was placed on quantifying changes in the following attributes: cellular composition, fetal viability, labyrinth vascularity, glycogen and lipid deposition, and gene expression. This section reveals many aspects of placental development are dependent on normal genomic imprinting as a whole.

The second aim of my thesis is to identify specifically which imprints are responsible are responsible for placental phenotypes associated with loss of imprinting. I started by describing the spectrum of mosaic loss of genomic imprinting in DNMT1o-deficient placentas by assaying imprinted gene expression and DMD methylation levels Then I tested for associations between placental phenotypes quantified in Chapter 2 and loss of imprints at specific DMDs using linear regression analysis. These results enabled the direct interpretation of functional significance of individual imprinted clusters in placental biology.

My third, and final dissertation research aim is to generate and describe a novel targeted deletion of the imprinted *Klf14* gene. This allele was designed and engineered using recombineering and was used to test the maternal-specific expression of *Klf14* in the placenta, and to record any phenotypes associated with its loss of expression. These results aide in the understanding of the role of the *Mest* imprinting cluster in its entirety in placental biology. The sum of my thesis aims is to provide pillars of evidence that unequivocally demonstrate the importance of genomic imprinting in placental development and identify specific placental functional and developmental processes that genomic imprints regulate.

2.0 PLACENTAL PHENOTYPES OBSERVED IN THE *DNMT1$^{\Delta 1o}$* MOUSE MODEL

2.1 SUMMARY

The *Dnmt1$^{\Delta 1o}$* maternal effect model produces DNMT1o-deficient embryos with partial loss of genomic imprints and grossly abnormal morphology (*60, 104, 105, 402*). As a consequence, DNMT1o-deficient fetuses do not survive until birth except on rare occasion. It is suspected that the poor developmental outcome of DNMT1o-deficient embryos is in part due to the failure of normal placental development. In this chapter I show that DNMT1o-deficient placentas exhibit variable abnormal phenotypes throughout gestation. Early in gestation *Dnmt1$^{\Delta 1o}$* maternal effect mutant placentas were found to have considerable loss of LZ development and an increased abundance of TGCs. In mid to late gestation DNMT1o-deficient placentas were found to have ectopic SpT extensions into the LZ, dilated fetal blood vessels, SynT lipid droplet accumulation and increased glycogen cell abundance. The balance of placental and fetal growth was dysregulated in late gestation *Dnmt1$^{\Delta 1o}$* maternal effect placentas. In addition, placental gene expression patterns were altered revealing that the imprinted gene network regulates important transcriptional pathways including oxidative stress response, molecular transport, lipid transport, and lipid metabolism. This evidence that placental development is disrupted in the *Dnmt1$^{\Delta 1o}$* maternal effect model is interpreted as support for the hypothesis that genomic imprints regulate key processes in trophoblast differentiation and placental development.

2.2 INTRODUCTION

2.2.1 The *Dnmt1*$^{\Delta 1o}$ model

The *Dnmt1* oocyte-specific isoform deletion (*Dnmt1*$^{\Delta 1o}$) model is a unique genetic system in which absence of the maternal effect DNMT1o protein in oocytes results in offspring with partial and mosaic loss of imprinted DMD methylation. It is unique in that its primary genetic mutation compromises the integrity of epigenetic inheritance of genomic imprints. This model is particularly useful because it yields a myriad of loss of imprinting epigenotypes unlike other available genetic models used to study genomic imprinting. Inactivating mutations of *Dnmt1*, which eliminate maintenance methyltransferase activity throughout development, are embryonic lethal and erase all imprinted DMD methylation as well as other genomic methylation (*101-103*). In addition, offspring of the *Dnmt3l* null maternal effect model are homogenous in their complete loss of maternal imprints and early embryonic lethality at E9.5 due to placental failure (*94, 97, 99*). Furthermore, nearly all DMD deletion models (reviewed in section 1.5) acquire the imprinted state of the methylated allele. The *Dnmt1*$^{\Delta 1o}$ model provides a means to directly study the effects of loss of DMD methylation on embryonic and placental development.

2.2.2 Identification of *Dnmt1o*

DNA methylation is perpetuated during each mitotic S-phase by the maintenance DNA methyltransferase DNMT1 at replication foci (*403*). The DNMT1 protein contains the modular

RFTS, CxxC Zn finger, BAH, and methyltransferase catalytic domains from N- to C-terminal as well as a disordered domain between amino acids 100 and 400 (*27, 83-85, 110*). DNMT1 is a component of multimeric complexes that dynamically modulate the epigenome through its interactions with other epigenetic regulators including DMAP, ZFP57, TRIM28, and DNMT3 isoforms (*75, 76, 107*). At least three mRNA isoforms of *Dnmt1* are produced from transcription of alternative promoters yielding different 5′ exons (*62*). The mouse somatic, *Dnmt1s* isoform is transcribed in the majority of tissues and encodes a 1620 amino acid protein (DNMT1s) with a molecular weight of 190kDa. Two sex-specific isoforms are generated from alternative promoters of *Dnmt1* in germ cells (*62*). Spermatagonia at the pachytene stage transcribe *Dnmt1p* from a downstream promoter immediately adjacent to the *Dnmt1* exon 1s 3′ splice juncture (*62*). The *Dnmt1p* exon 1p contains a much weaker translation initiation sequence and does not produce DNMT1 protein (*62*). The *Dnmt1o* promoter initiates approximately 7.5kb upstream from the *Dnmt1s* promoter and yields a protein product that is shortened by 118 amino acids at the N-terminus (*62*)

The oocyte specific DNMT1o isoform was first identified by the discovery of a truncated DNMT1 protein isoform in oocytes and preimplantation embryos that had a molecular weight of approximately 175kDa (*404*). The truncated N-terminus of DNMT1o as compared with DNMT1s ablates the sequences required for interaction with DMAP but enables the interaction of the N-terminus with annexin V (ANXA5) (*62*). The importance of the interaction between DNMT1 and DMAP is particularly important based on the epistatic lethality of the $Dnmt1^{v}$ (constitutive *Dnmt1o* expression) and *Dmap1* null alleles (*107*). ANXA5 is a phospholipid binding protein at the plasma membrane of mature oocytes, and has been shown to interact with DNMT1o but not DNMT1s, suggesting that the longer N-terminus of DNMT1s may block this

interaction (*62, 405*). The ANXA5-DNMT1o interaction is likely responsible for the sequestration of DNMT1o to the cytoplasm and plasma membrane in oocytes until it translocates to the nucleus at the 8-cell stage (see section 2.2.4) and may explain differences in maternally produced DNMT1s and DNTM1o stability and activity in preimplantation embryos (*62, 63, 104*). The research presented in the remaining introduction ascribes the primary function of DNMT1o to maintain imprinted DMD methylation during the rapid preimplantation genomic demethylation event.

2.2.3 Targeted deletion of *Dnmt1o*

The *Dnmt1*$^{\Delta 1o}$ allele was generated using homologous recombination in ESCs to eliminate a 260bp region spanning *Dnmt1* exon 1o and part of the 5′ 1o promoter (*104*). The targeting construct contained a loxp-neo-TK-loxp cassete in place of the 260bp to be deleted. Homologous recombinant ESCs were positively selected using neomycin and then transfected with a CRE expressing plasmid to induce deletion and then negatively selected for using the thymidine kinase substrate ganciclovir. These ESCs were used to derive a line of chimeric males that passed the *Dnmt1*$^{\Delta 1o}$ allele through the germline to establish a colony that was backcrossed onto the 129Sv background. Heterozygous and homozygous offspring of crosses between heterozygous parents develop normally, however homozygous females had progeny that could not survive, indicating a maternal effect.

Howell *et al.*(2001) studied DNMT1o-deficient embryos, derived from the *Dnmt1*$^{\Delta 1o}$ maternal effect model, for changes in their DNA methylation patterns (*104*). Methylation sensitive Southern blot analysis showed that global methylation was unchanged. Furthermore, both γ-satellite and IAP repetitive elements were proven to be normally methylated by

methylation sensitive Southern blotting and bisulfite sequencing in DNMT1o-deficient embryos. Genomic imprints on the other hand were not normally methylated in the *Dnmt1*$^{\Delta 1o}$ maternal effect offspring.

A severe reduction in genomic imprinting was observed in DNMT1o-deficient embryos (*104*). A single nucleotide polymorphism extension assay using conceptuses derived from crosses between the C57/B6 *mus musculus* homozygous *Dnmt1*$^{\Delta 1o}$ females and *mus castenous* wild-type males provided evidence of biallelic *H19* and *Snrpn* expression in an example DNMT1o deficient embryo. Using the same interspecific crosses an approximately fifty percent loss of methylation was observed at the *H19, Snrpn* and *Peg3* DMDs by bisulfite genomic sequencing. Imprinting in oocytes was shown to be normal, indicating a failure to maintain imprints in the zygote rather than a defect in the establishment of maternal imprints. Transplantation of DNMT1o-deficient pro-nuclei into wild-type oocytes resulted in full rescue of the methylation defect. The majority of *Dnmt1*$^{\Delta 1o}$ maternal effect progeny die between E12.5 and term and very few survive through the neonatal period of development.

2.2.4 Localization of DNMT1 in preimplantation embryos

Early studies on DNMT1 using the PATH-52 anti-DNMT1 antibody found that oocytes and preimplantation embryos express a shorter form (later named DNMT1o) than somatic cells (*404*). Immunoflourescense of oocytes and preimplantation embryos using the PATH-52 antibody showed that DNMT1 was concentrated in the subcortical cytosplasm in oocytes and 2-cell embryos, in cytoplasmic granules at the 4-cell stage and nuclear at the 8-cell stage (*62, 404*). Further studies using C-terminal truncated *Dnmt1*-β-galactosidase fusion genes identified regions required for cytoplasmic sequestration C-terminal to the RFTS domain (*406*).

Two new anti-DNMT1 antibodies, UPTC21 and UPT82, enabled a clearer picture of DNMT1 localization during preimplanation development (*63*). These antibodies have greater sensitivity than PATH52, and UPT82 can distinguish between full length somatic DNMT1 and the shorter DNMT1o. UPTC21 is an antiserum raised against DNMT1 amino acids 636-1108 and recognizes all DNMT1 isoforms, whereas UPT82 is an antiserum raised against the DNMT1 118 N-terminal amino acids and thus recognizes the longer somatic form of DNMT1 (DNMT1s) but not DNMT1o. The presence of a UPT-82 band in western blot analysis of oocytes showed that oocytes express DNMT1s in addition to the shorter DNMT1o isoform identified with the PATH-52 isoform. Immunofluorescence revealed UPT82 signal within ooplasm and 2-cell nuclei indicating maternal DNMT1s present and likely to be active early in zygotic development. UPTC21 immunoflourescene was detected in ooplasm and in the zygote cytoplasm up until the 8-cell stage, results interpreted to mean that DNMT1o is sequestered within the cytoplasm during early cleavage events. The amount of maternal effect DNMT1o protein far exceeds maternal DNMT1s in oocytes and preimplantation embryos, and provides an explanation for observations of the apparent absence of DNMT1s in the nucleus of 2- and 4- cell embryos using pan DNMT1 antibodies. Furthermore replacement of the 1o exon with the 1s coding sequence increased the amount of DNMT1s in 4-cell embryos. Reverse transcription PCR was also used to confirm expression of both *Dnmt1s* and *Dnmt1o* isoforms in the mouse oocyte (*63*).

The *Dnmt1*$^{\Delta 1o}$ and *Dnmt1*^{v} models provided an excellent confirmation of the above immunofluorescence assays (*60*). Wildtype embryos from 2 to 8-cells had nuclear UPT82 staining, whereas embryos from homozygous *Dnmt1*^{v} dams showed no UPT82 staining at the 1-cell stage and only gradually increased through the morula stage. Additionally, heterozygous *Dnmt1*^{v} females crossed with homozygous *Dnmt1*^{v} males all had nuclear UPT82 signal in the 1-

cell embryo, but only half had maintained UPT82 signal beyond that point. These results indicate that maternally derived DNMT1s is a nuclear protein during early cleavage prior to zygotic *Dnmt1s* transcriptional activation. Furthermore, loss of DMD methylation at *H19* and *Snrpn* DMDs could be prevented in *Dnmt1*$^{\Delta 1o}$ maternal effect offspring by transgenic overexpression of *Dnmt1s* in occytes driven by the zona pelucida *Zp3* promoter. This result was interpreted as evidence that there may not necessarily be anything inherently different between maternally acquired DNMT1o and DNMT1s recognition of methylation targets. Rather, DNMT1o is more effectively stored in oocytes, thus ensuring accurate maintenance of imprints (DMDs) in 8-cell embryos.

2.2.5 Preimplantation function of DNMT1o

The role of DNMT1o during preimplantation development is to maintain the integrity of genomic imprints. The initial report of loss of DMD methylation in *Dnmt1*$^{\Delta 1o}$ maternal effect offspring showed a fifty percent loss at three different DMDs in the analysis of a handful of samples and speculated that loss of methyltransferase activity at the 8-cell stage and random chromosomal assortment at the 5th mitosis would result in mosaic partial loss of genomic imprinting (*104*). A more thorough analysis confirmed these findings and found a profound level of epigenetic mosaicisim in *Dnmt1*$^{\Delta 1o}$ maternal effect offspring (*402*). Loss of *Snrpn* and *H19* DMD methylation could be detected as early as the morula stage by allele-specific bisulfite genomic sequencing and ranged from 13% to 65% methylation on the normally fully methylated parental alleles. The TR2+3 /*Igmyc* maternally imprinted transgene was crossed onto *Dnmt1*$^{\Delta 1o}$ females and used to determine that loss of methylation occurs as early as the 8-cell embryo and resolves to approximately 51% methylation at the blastocyst stage (*402*). Combined bisulfite

sequencing and restriction analysis (COBRA) was used to further validate that different DNMT1o-deficient ESC derived lines had varying levels of *H19* and *Snrpn* DMD methylation. At E9.5 loss of imprinted methylation at the *H19* and *Snrpn* DMD in DNMT1o-deficient embryos and placentas was observed alongside loss of monoallelic imprinted gene expression patterns. These studies firmly established the role of DNMT1o in preventing epigenetic variation at imprinted loci during preimplantation development and provided the framework for our current understanding of the *Dnmt1*$^{\Delta 1o}$ maternal effect model (Figure 3).

2.2.6 Fetal development of *Dnmt1*$^{\Delta 1o}$ maternal effect offspring

It is not surprising that the epigenetic mosaic predicted in the maternal effect model results in profound and variable embryonic morphology. A study of DNMT1o-deficient embryos focusing on fetal development at E9.5 revealed that mutant fetuses scored lower in nearly all morphological developmental features as compared to wild-type (*105*). DNMT1o-deficient fetuses were developmentally delayed on average 12hrs, had shorter crown-rump lengths and decreased number of somites. Although there were DNMT1o deficient fetuses that were morphologically normal, others had features such as failure of axial rotation, failure of anterior neuropore closure, poor heart morphogenesis and some that were in states of disintegration and reasbsorption. This varied and mosaic nature of DNMT1o-deficient fetal defects were in line

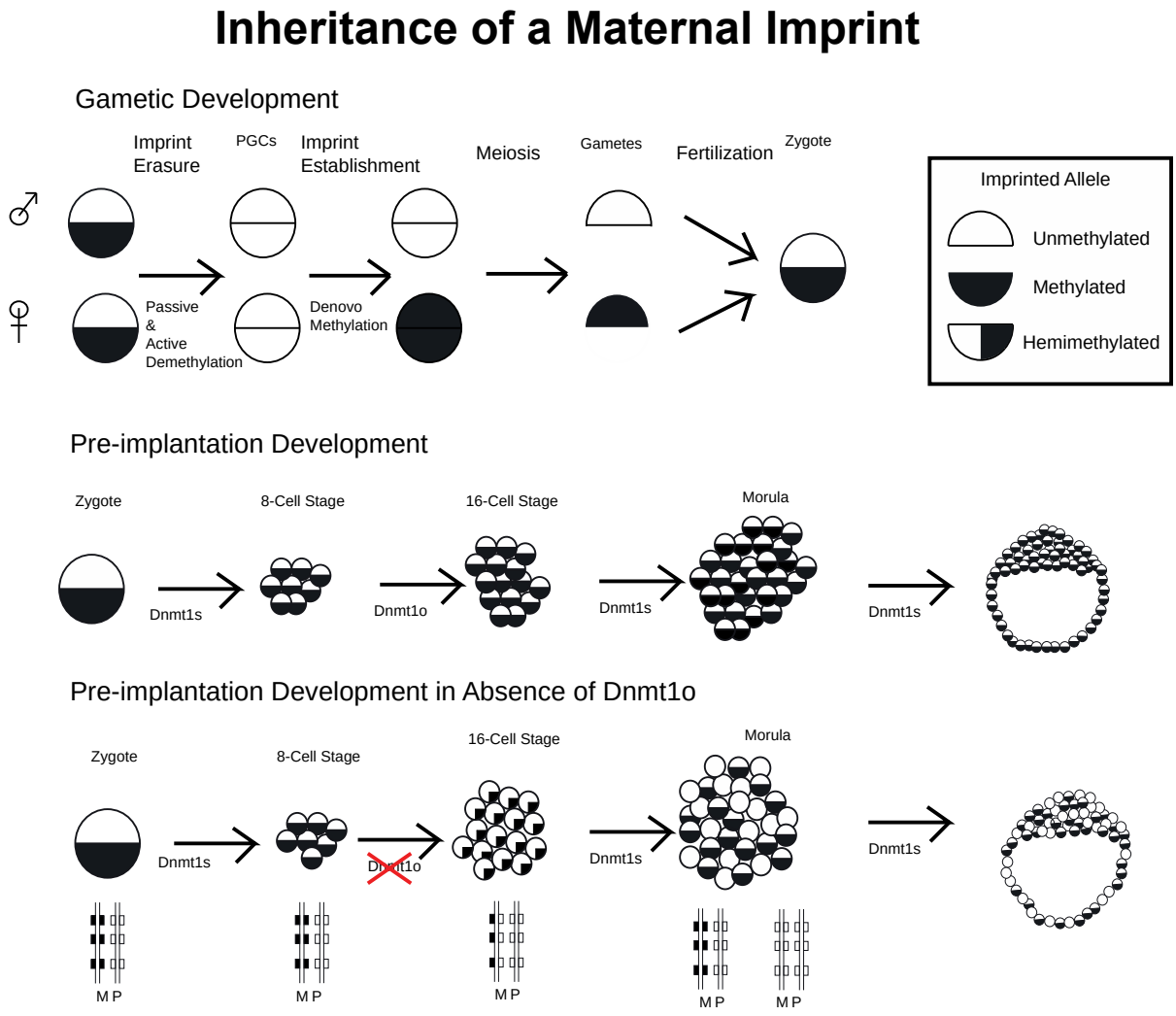


Figure 3. Inheritance of a maternal imprint in normal and *Dnmt1*$^{\Delta 1o}$ maternal effect embryos. During gametic development genomic imprints are erased in primordial germ cells and then reestablished in a sex-specific manner, such that in this example the maternal gamete contains a methylated imprint. In the wild-type zygote the combined action of maternally inherited DNMT1s, DNMT1o and zygotic DNMT1s account for maintenance of imprinted DMD methylation during preimplantation development. In DNMT1o-deficient conceptuses the lack of DNMT1o at the 4th mitotic event generates hemimethylated DNA that is then replicated, generating a mosaic of cells with half completely lacking the maternal imprint. Because this occurs at 24 different imprints scattered on various independently segregating autosomes, a great number of partial loss of imprinting permutation of can occur. Therefore, each DNMT1o-deficient 32-cell morula contains 16-normal cells and a mosaic of 16 cells with unique loss of imprinting epigenotypes.

with the molecular model of partial and varied loss of genomic imprinting and suggest a numerous roles for imprinted loci in early fetal development.

2.2.7 Placental development of *Dnmt1$^{\Delta 1o}$* maternal effect offspring

Initial reports also suggest that the placental compartment is affected by loss of imprinting in the *Dnmt1$^{\Delta 1o}$* maternal effect model. DNMT1o-deficient fetal and placental epigenomes are not always congruent. In fact, in a small set of E9.5 DNMT1o-deficient embryos, neither *H19* nor *Snrpn* DMD methylation was eqivalent in corresponding fetal and placental DNA samples (*402*). Preliminary research on placental morphology in E9.5 *Dnmt1$^{\Delta 1o}$* maternal effect offspring revealed that many DNMT1o-deficient placentas had shell shaped placentas draped around the conceptuses suggestive of trophoblast hyperplasia (*407*). This phenotype was more common in female than male conceptuses. Further studies examining loss of X-chromosome imprinting in extraembryonic lineages and placental trophoblast hyperplasia and diminished LZ development were performed by McGraw and colleagues (2013) contemporaneously with my own dissertation work (*408*).

2.2.8 Chapter 2 aims

The aim of this section is to utilize the *Dnmt1$^{\Delta 1o}$* maternal effect model of partial loss of imprinting to better understand the role of genomic imprints in placental biology. The *Dnmt1$^{\Delta 1o}$* maternal effect model is based on a genetic mutation that manifests loss of DMD methylation as the primary defect. This model produces a mosaic of epigenetic mutants that provide an opportunity to study the effects of loss of imprinting as a whole on placental development. In this

chapter I have tested the functional significance of genomic imprints in placental biology and tried to determine what layers and cell lineages are affected by describing DNMT1o-deficient placentas across gestation from E9.5 to E17.5. To assess placental efficiency and the balance of fetal and placental growth wet weight measurements of DNMT1o-deficient placentas and fetuses were made. To examine placental layer and cell type composition DNMT1o-deficient placentas were histologically analyzed using an array of tissue staining techniques. Placental metabolism in DNMT1o-deficient placentas was studied by triglyceride quantification and qualitatively by lipid and glycogen histological staining. The description and analyses of DNMT1o deficient placentas has been performed to delineate which placental processes genomic imprints are involved in and to lay the foundation for further research and modeling of the role of genomic imprints in placental development.

2.3 MATERIALS AND METHODS

2.3.1 Animal husbandry

All mice were maintained and used in accordance with guidelines from the Institutional Care and Use Committee (IACUC) at the University of Pittsburgh. The *Dnmt1*$^{\Delta 1o}$ allele was maintained on a 129Sv (Taconic) background by backcrossing heterozygous females to 129Sv males. Placentas from crosses between heterozygous *Dnmt1*$^{\Delta 1o}$ females and 129Sv males were used as wild-type (wt) controls. Crosses between homozygous *Dnmt1*$^{\Delta 1o}$ females and 129Sv males generated DNMT1o-deficient (*Dnmt1*$^{\Delta 1o}$ maternal effect) mutant (mt) conceptuses.

2.3.2 Genotyping

Genotyping was carried out on DNA isolated by phenol-chloroform extraction and ethanol precipitation of tails snipped at weaning or during embryonic collection. In addition, the genotype of pregnant mouse dams that were dissected for placental analysis was confirmed using DNA isolated from tail and spleen extracted at the time of dissection. *Dnmt1*$^{\Delta 1o}$ genotyping PCR used 350ng of starting genomic DNA and used the following thermocylcer program with Taq polymerase (Invitrogen): 95°C 5′ denaturing followed by 35 cycles of 95°C 30″, 56°C 30″; 72°C 45″, a 72°C 10′ final extension and indefinite hold at 4°C. PCR products were run on a 1 or 1.5% agarose gel to look for the presence of a wild-type (380bp) or mutant (120bp) band. Sex genotyping of embryos was performed using primers specific to the *Zfy* gene on the male sex chromosome. PCR conditions were as follows: 95°C 5′ denaturing followed by 35 cycles of 95°C 30″, 56°C 30″; 72°C 30″, a 72°C 7′ final extension and indefinite hold at 4°C. PCR products from the *Zfy* reaction were run on a 2% agarose gel to look for the presence of a 200bp band indicating male identity. The PCR primer sequences used for genotyping are given in Appendix A.

2.3.3 Collection of placentas

Embryonic day 0.5 (E0.5) was established based on the presence of a post-copulatory vaginal plug. Fertilization was assumed to have occurred at 12am based on the nocturnal nature of mating in *Mus* species. Pregnant females were sacrificed by CO_2 asphyxiation and conceptuses were collected at embryonic day 9.5, 12.5, 15.5 and 17.5 (E9.5, E12.5, E15.5 and E17.5). Dissections were carried out under low magnification (0.8X-2.5X) with a 350MZ dissection

microscope (Leica) using fine 8mm forceps. The whole uterus of sacrificed pregnant dams was isolated following cesarean section. Incision at the fat pad of each ovary and at the cervix facilitated removal of the uterus, which was placed in a 10cm petri dish of phosphate buffer saline (PBS) pH 7.5.

Each individual conceptus was removed from the uterine myometrium and placed in a 60mm petri dish with PBS leaving the embryo, yolk sac, placenta and decidua and inner myometrium intact. Whole E9.5 conceptuses were immediately placed in 4% paraformaldehyde (4% PFA). E12.5, E15.5 and E17.5 placentas were collected by the below protocol. The inner myometrium was carefully removed leaving the embryonic tissues and decidua intact. The yolk sac was excised away from the base of the placenta leaving the embryo exposed. The umbilical cord was pinched for 20″ to stop blood flow and stimulate clotting to stem leakage from both the fetus and placenta. The umbilical cord was then cut at the base of the chorionic plate and fetal abdomen. Each placenta and fetus was then weighed to the nearest tenth of a milligram. A small tail piece was cut and used for embryonic sex genotyping. Placentas were immersed in PBS again, then paced on a glass microscope slide with labyrinth facing down and cut in half with a sterile razor blade. One half was then placed back in fresh PBS and the other in 4% PFA for fixation. The half immersed in PBS was then cleaned of the decidua cap and any remaining yolk-sac and allantois. The E12.5 half samples were placed in RNA later, whereas the E15.5 and E17.5 samples were cut into two quarters, one placed in RNA later and the other snap frozen on dry ice for lipid extraction. Samples at each gestational age were labeled by litter (Letter A-G) and individual embryo (Number 1-8) for those used in DMD methylation analysis in Chapter 3.

2.3.4 Cryo-histology

Following fixation in 4% PFA, placental halves were suspended through a sucrose gradient up to 20% weight per volume, and then embedded in Tissue-Tek O.C.T compound (Sakura). Placental cryosections of 5μm and 10μm thickness were cut with a CM1850 cryostat (Leica) for histological analysis. Regressive hematoxylin and eosin (H&E; Sigma or Leica) staining was performed on a series of 5 micron meridian placental sections. In brief cryosections were fixed with O-Fix (Leica) for 30″, flushed with tap water for 30″, placed in hematoxylin for 45″ then washed for 1′ in water, placed in bluing reagent for 1′, water for 30″ and then 70% EtOH for 7-dips, alcoholic eosin for 16″, 95% EtOH for 7-dips, two washes in 100% EtOH of 7-dips and two washes in xylene of 7-dips before being mounted in a xylene based mounting medium. Periodic Acid-Schiff (PAS; Sigma) staining was carried out on E15.5 samples using standard instructions with the addition of a 5′ methyl-green (0.5%) nuclear staining prior to dehydration steps. In brief, PAS staining was carried out in coplin jars by placing cryosections in O-Fix for 1′, washing with water for 1′, placing in periodic acid solution for 5′, washing with water for 1′,, placing in Schiff's reagent for 15′, washing with water for 5′, placing in distilled deionized water for 1′, in 5′ methyl-green (0.5%) for 5′, rinse in water for 1′, and then run through 95-100-100% EtOH dehydration steps of 7-dips and then cleared in xylene for 7-dips twice and coverslipped in xylene based medium. All E9.5 and E12.5 histology was performed on cryosections, however some E15.5 and E17.5 H&E, PAS and immunocytochemistry was performed on paraffin embedded samples with the assistance of the MWRI histology core.

2.3.5 *In situ* hybridization

A series of 10μm sections were stained by *in situ* hybridization (ISH) with Digoxigenin-11-dUTP (Roche) labeled antisense RNA probes. ISH probes of the placental marker genes *Tpbpa, Tfeb, LepR, Pchdh12, Mest, Prl2c2, Prl3b1 and Prl3d1* were in-vitro transcribed (Promega) from cDNA cloned into pBluescript, and used to identify the labyrinth (*Tfeb*), SpT (*Tpbpa*), SynT (*LepR*), GCs (*Pchdh12*), fetal vascular (*Mest*) and TGCs (*Prl2c2, Prl3b1 and Prl3d1*) respectively. ISH probes of the imprinted genes *Ascl2, Phlda2* and *Igf2* were similarly generated. RNA probes were purified using a G50 column (GE Healthcare) to remove unincorporated nucleotides, ammonium acetate ethanol precipitation, and dissolved in 30μl of water. RNA concentration was then determined by using a nanovue spectrometer (GE Healthcare) and diluted in ISH hybridization buffer (50% Formamide, 5X SSC, 5X Denhardt's, 0.25mg/ml tRNA, 0.5mg/ml herring sperm DNA) to a concentration of 200ng/μl.

Cryosections were stored at -70°C and warmed to 30°C to dry before starting ISH processing. Slides were fixed in 4% PFA for 10′, washed with PBS 3x for 3′ and digested with proteinase K for 5′. Following digestion slides were placed back into 4% PFA for 5′, washed 3x with PBS and then placed in an acetylation reaction (200ml aqueous solution of 0.1M triethanolamine and 0.2M HCl, initiated with 500μl of acetic anhydride immediately prior to use) for 10′. Slides were then washed with PBS 3x for 5′, tissue sections outlined with a pap-pen and were pre-incubated with ISH buffer for 2h at room temperature. At the end of 2h ISH probes were heated to 75-80°C for 5′ on hot beads, placed on ice for 3′ and spun down and then diluted (10μl probe +140μl ISH buffer per section). 125μl of diluted probe was added to each slide, a cover slip placed on top, the slides placed in a humidified slide box wrapped with paraffin tape and incubated overnight at 65°C. The following day slides were washed with 5x SSC to remove

coverslips, and incubated with 0.2X SSC for 2h at 70°C. After cooling down to room temperature samples were incubated with buffer B1 (0.1M Tris pH7.5 and 0.15M NaCl) for 5′ then incubated with B2 (B1 +5% Fetal Goat Serum) for 1 hour and then with B1 + anti-DIG alkaline phosphatase (AP) conjugated antibody (1:5000) overnight at 5°C. On the third day slides were washed three times with B1 and then equilibrated with B3 (0.1M Tris pH9.5, 0.1M NaCl and 50mM $MgCl_2$) for 5′. Slides were then incubated in the dark with B4 (B3+levamisole and NBT/BCIP) for 3-24 hours depending on strength of probe. AP reactions were stopped using TE buffer, the slides air-dried, passed through an ethanol dehydration gradient and xylene clearing step and cover slipped with a xylene based media.

2.3.6 Immunohistochemistry

E17.5 10μm paraffin sections were stained by immunohistochemistry (IHC) using an anti-CD31 antibody. Briefly, samples were processed according to IHC kit instructions (Pierce): Slides were deparaffinized and rehydrated using xylene and ethanol washes, then were washed with exposed 3% hydrogen peroxide for 10′, washed with PBST (PBS + 1% Tween 20) blocked with horse serum, incubated overnight with 1:200 rabbit polyclonal anti-CD31 (Vector Labs) at 4°C, washed, incubated with biotinylated secondary, washed and then stained using the Vectastain ABC reagent (Pierce), and counter stained with hematoxylin.

2.3.7 Immunofluorescence

E17.5 cryosections were stained with a combination of DAPI, LipidTOX (Invitrogen) and anti-CD31 fluorescent reagents. Briefly samples were washed with PBST, blocked with 5% goat

serum, incubated with primary anti-CD31 (Jackson ImmunoResearch Labs) overnight at 4°C, washed with PBST, incubated with 1:200 alexa-555 or -488 goat anti-rat IgG (BD) for 1h at room temperature, washed with PBST, stained with 1:250 LipidTOX for 30′ in the dark, washed with PBST and then coverslipped with DAPI containing medium. E12.5 samples were stained with DAPI and coverslipped to count TGCs.

2.3.8 Stereology and morphometrics

All images of placental tissue sections were taken using a DMI4000B inverted microscope (Leica) using 1.6x, 5x, 10x, 20x or 40x objectives and a 10x multiplier. Morphometric area measurements were made using the Image J (NIH) grid tool. The area of JZ and LZ layers were determined using random grid sampling within 2-3 central 50x or 16x fields of view of H&E stained sections for E12.5 and E15.5 placentas respectively. H&E images were overlaid with a random offset grid with 0.050 or 0.125mm^2 per point respectively based on image magnification. The placental cell layer of each point (or upper left quadrant at boundary areas) was determined based on cell morphology of JZ (compact diploid SpT layer) or LZ (integrated SynT, fetal vasculature and maternal blood spaces). The chorionic plate was excluded from LZ measurements. Areas were integrated across the known distance (~250μm) between serial sections to generate a central LZ and JZ volume metric. Area and volume measurements were confirmed by analysis of adjacent slides stained by ISH of lineage markers. P-TGCcount measurements were obtained from 2-3 central 10μm DAPI stained sections. The polyploidy P-TGC nuclei were readily distinguished from the diploid nuclei of the neighboring decidua and SpT cells at greater than 100x total magnification. The average cell count per 10μm section was

used as the reported metric. The identity of trophoblast giant cells was confirmed with ISH of adjacent sections.

2.3.9 Lipid extraction

Placenta samples cleaned of maternal decidua were stored at -70C for up to 3 months prior to lipid extraction. Samples were weighed and reduced to 25mg total weight. Samples were then placed in a dounce homogenizer with 250µl of deionized water with 5% NP40 and completely homogenized. Samples were then transferred with a Pasteur pipette to a clean glass test tube and cycled through a beaker of hot (80-100°C) water and room temperature two times to precipitate cell debris. The samples were then transferred to microcentrifuge tubes and centrifuged at 13000rpm for 5′ to pellet debris. Supernatant was transferred to a clean glass test tube, covered with paraffin film and stored for up to 1 week at 5°C. A triglyceride assay kit (Biovision) was used to quantify lipid content using a colorimetric assay in 96 well flat bottom plates. Duplicate standard controls of 10, 20, 30, 40 and 50 ng/well were used to generate a standard curve. Samples were plated in triplicate using 10µl of placental triglyceride extract and 40µl of sample buffer per well. Samples and controls were incubated with 2µl of lipase for 20′ at room temperature and then incubated with a reaction mix containing 46µl of buffer, 2µl of triglyceride probe and 2µl of enzyme mix per well and incubated for 1 hour at RT with gentle shaking. Absorbance at 570nm was the colormetric readout. Sample concentrations were back calculated as nmol triglyceride per mg of tissue using the standard curve and known mg per volume of sample.

2.3.10 Biostatistics

Mean mutant and wild-type phenotypic averages were calculated for all quantitative traits. The phenotypic data was also subdivided into dead/alive and male/female subgroups to determine the influence of fetal viability and sex on placental phenotypes. The distribution of each data set was tested for normality using the Kolmogorov-Smirnov, Shapiro-Wilk and Anderson-Darling tests of normality. When distributions approximated normality the students t-test was used to compare the distribution of mutant and wild-type phenotypes as well as the phenotypes observed in subgroups. Likewise, where the data was non-normally distributed the Mann-Whitney U (Rank-sum) test was used to compare the sample averages. Phenotypic data is most often displayed in charts showing mean + SEM.

2.3.11 Microarray analysis

E17.5 placental halves cleaned of maternal decidua were used to analyze global gene expression. Four wild-type controls, four low E/P DNMT1o-deficient and four high E/P DNMT1o-deficient placentas were analyzed. An even number of males and females were included in each group to minimize sex-specific expression differences. RNA was extracted from placental samples using a DNA/RNA combined kit (Qiagen). Illumina mouse WG-6-V2.0 Expression bead chips were used for whole genome expression profiling of 45,200 transcripts. Data was normalized and then mean expression values were determined for each subgroup. Ingenuity pathway analysis (IPA) was used to determine which biological pathways had altered gene expression patterns in low and high E/P ratio DNMT1o-deficient placentas.

2.4 RESULTS

2.4.1 *Dnmt1*$^{\Delta 1o}$ maternal effect embryonic viability

DNMT1o-deficient placentas exhibited a large spectrum of phenotypic abnormalities in the placental compartment across gestational development from E9.5 to E17.5. The number of live DNMT1o-deficient embryos decreased across gestation such that a greater number of litters was required late to collect the same number of placental samples for each gestational age (Table 2). These results revealed a strong selection event between E9.5 and E12.5 that reduced the number of viable embryos. The results reported here differ slightly from those I published in Developmental Biology (*409*) and PLoS One: Epigenetics (*410*) because of less stringent viability criteria at E9.5 used herein.

A diverse array of placental phenotypes was observed in the *Dnmt1*$^{\Delta 1o}$ maternal effect across gestation from E9.5 to E17.5. Early in development I observed decreases in the central volume of JZ and LZ layers and expansion of trophoblast giant cells. While placentas were smaller than average at E12.5, those recovered at E17.5 were overgrown. At mid (E15.5) and late (E17.5) gestational time points DNMT1o-defiicent placentas had reduced fetal vascular surface area, labyrinth hemorraghing, increased GCs abundance, increased lipid content and a prevalence of SpT extensions within the LZ. In addition, the balance of fetal and placental growth was disrupted

Gestational Age (dpc)	# Litters	# Placentas(a)	#Live Embryos(b)
E9.5	4	30	29
E12.5	3	24	10
E15.5	4	23	11
E17.5	5	23	14

Table 2. Survival of DNMT1o-deficient embryos and placentas. (a) Only intact placentas that were not necrotic, reabsorbing or hydatidiform moles were counted. (b) Based on structural integrity at E9.5 and presence of active circulation at E12.5 and later.

2.4.2 E9.5 Phenotypes

I performed histological analysis on E9.5 whole mount cryo-embedded conceptuses from 3 *Dnmt1*$^{\Delta 1o}$ maternal effect litters. A single example of a DNMT1o-deficient litter with 7 conceptuses (M1-M7) is shown in Figure 4 alongside an exemplary wild-type (wt) control. Abnormalities in placental layers were identified in central placental H&E stained sections. Central sections were determined based on presence of the maternal spiral artery, a triangular shaped maternal artery at the apex of trophoblast implantation. Maternal and fetal blood was distinguished by the presence of hematoxylin stained nuclei in fetal erythrocytes and absence in erythrocytes of maternal origin. In wild-type placenta a large maternal artery was observed for all samples and each placental layer was intact (wt; Figure 4). Not all mutant placentas observed had a dilated maternal artery, indicating a defect in implantation (DNMT1o-deficient mutants M2, M5 and M7; Figure 4). Poor structural integrity of the labyrinth and expansion of trophoblast giant cells was noticeable in many of the mutants. DNMT1o-deficient placentas M2, M3, M4, M5 and M7 had underdeveloped LZ. DNMT1o-deficient placentas M2 and M7 had an easily discernible overabundance of trophoblast giant cells.

To confirm the cell identities and phenotypes interpreted from H&E histology I performed in situ hybridization with a set of trophoblast lineage markers (Figure 4). The prolactin family member *Prl2c2* was used as a TGC marker. Expansion of the TGC layer from 1-

3 cells thick to 5-6 cells was observed in DNMT1o-deficient mutants M2 and M7 (Figure 4). An antisense probe to the transcription factor *Tfeb* cDNA was used to identify EXE derived chorionic plate and SynT progenitors. DNMT1o-deficient mutant M2, M3, M4, M5 and M7 showed decreased expression and less structural development of maternal blood spaces (Figure 4). In wild type placenta *Tfeb* had a complex spatial expression pattern of raised and convoluted chorionic trophoblast, whereas the abnormal DNMT1o-deficient placentas displayed flattened labyrinth chorionic trophoblast. An antisense probe for the ectoplacental cone specific transcription factor *Tpbpa* was used to identify SpT and progenitors. In wild-type placentas *Tpbpa* marks an apex area around the maternal spiral artery. DNMT1o-deficient mutants M3 and M4 have a marked decrease in *Tpbpa* expression.

In conjunction with ISH of placenta lineage markers I probed adjacent E9.5 whole-mount DNMT1o-deficient placenta with antisense probes to the imprinted genes *Ascl2, Phlda2* and *Igf2* (Figure 4). We expected and observed a decrease in the spatial expression pattern for each imprinted genes. *Ascl2* is expressed in the EPC in wild-type E9.5 placentas whereas *Phlda2* is expressed primarily in the labyrinthine EXE derived lineages. Loss of methylation at the *Kcnq1* DMD should have the predicted effect of decreased *Aslc2* and *Phlda2*. DNMT1o-deficient mutants M3 and M4 had severely restricted expression of both *Aslc2* and *Phlda2* (Figure 4). *Igf2* is ubiquitously expressed in placental and embryonic tissues. Loss of *H19* DMD methylation is expected to result in diminished *Igf2* expression. The majority of DNMT1o-deficient mutants had minimal *Igf2* staining, with the exceptions being M6 and M7 (Figure 4). Discordant *Igf2* expression patterns were observed between placenta and fetus and also between EPC (SpT and TGC) and EXE (chorion and SynT) derived trophoblast lineages. These results indicate that the

DNMT1o-maternal effect model has early wide-ranging phenotypes in placental structure and imprinted gene expression.

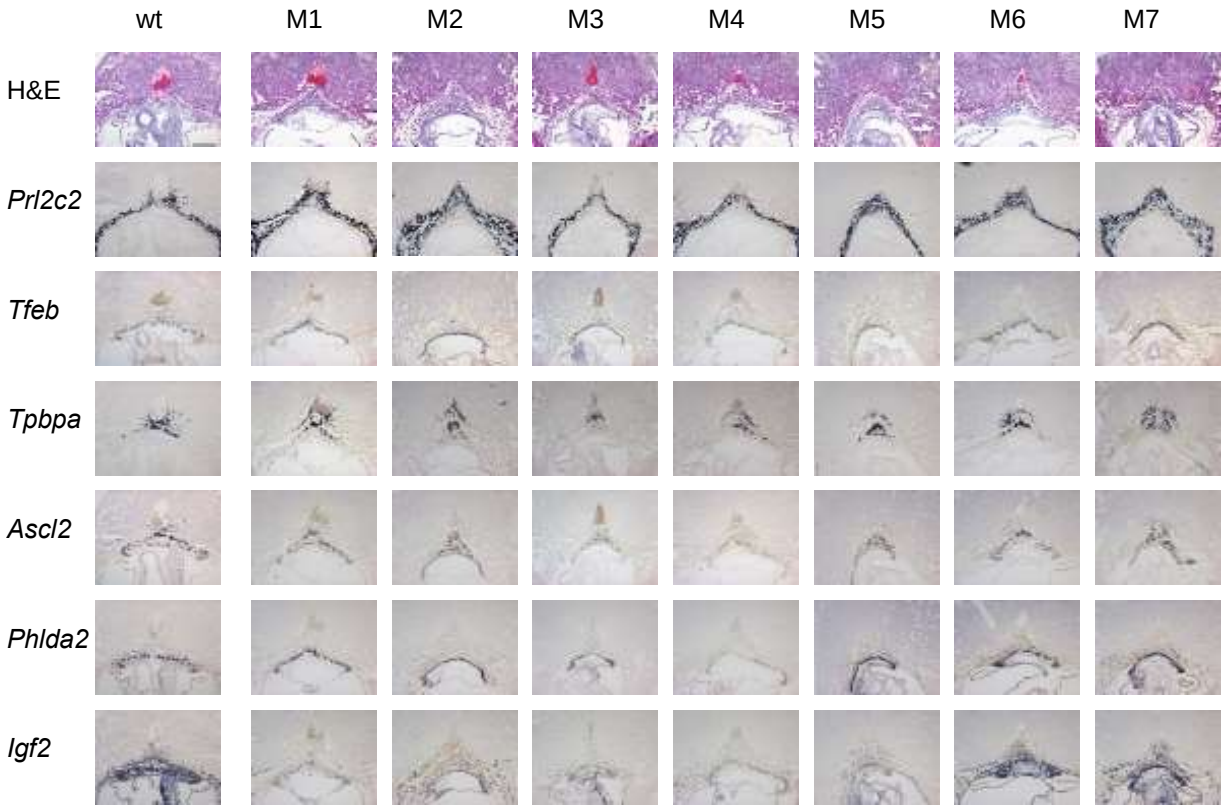


Figure 4. E9.5 Histology of wild-type (wt) and DNMT1o-deficient placentas (M1-M7). A broad range of morphologic and gene expression abnormalities is present in an exemplary single litter of E9.5 DNMT1o-deficient placentas. H&E staining and ISH of adjacent central placental sections are shown. ISH probes are listed on the left bar include placenta lineage markers Prl2c2 (TGCs), Tpbpa (EPC), Tfeb (Chorion) and imprinted genes Ascl2, Phlda2 and Igf2. Scale bar 500um.

2.4.3 E12.5 Phenotypes

I performed histological analysis on 24 E12.5 whole mount cryo-embedded conceptuses from 3 *Dnmt1*$^{\Delta 1o}$ maternal effect litters (E12.5 Litters A-C). Two wild-type litters, with a total of 16 conceptuses were used as controls. H&E staining was used to observe overall abnormalities and

in situ hybridization in order to confirm cell type identities. DNMT1o-deficient and wild-type placentas differed in many ways at E12.5, particularly in weight, central JZ volume, central LZ volume and number of TGCs (Figure 5). There was a trend toward decreased placental weight in DNMT1o-deficient placentas ($P<0.05$; Figure 5A). Additionally, there were significant decreases in measured central LZ volume ($P<0.005$; Figure 5B) and central JZ volume ($P<0.005$; Fig 5B) in the DNMT1o-deficient placentas compared to wild-type controls. A marked increase in the number of TGCs per central section was measured in the E12.5 DNMT1o-deficient placentas compared to wild-type controls ($P<0.01$; Figure 5C). These findings are in line with my previous findings of distorted placental layer development at E9.5 (Section 2.4.2; (*409*)).

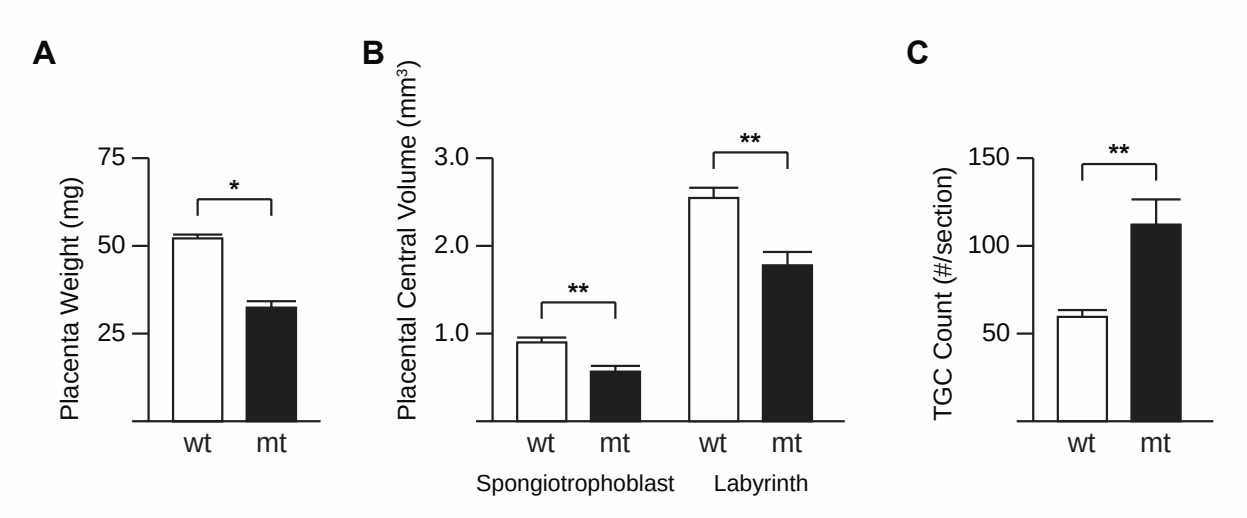


Figure 5. Phenotypic comparison of wild-type (wt) and DNMT1o-deficient (mt) placentas at E12.5. (A) Measurements of wet placenta weight, (B) Spongiotrophoblast and Labyrinth central volume, and (C) the number of TGCs per slide of a cohort of wt and mt placentas are displayed as open and filled bars respectively. Data are plotted as mean +SEM. *($P<0.05$) and **($P<0.005$) denote significant differences between wt and mt averages by the Rank-sum test, wt n=21 for placental weights and wt n=12 for layer volumes, mt n=24 for all.

To determine the effects of fetal viability and sex on placental phenotypes in the *Dnmt1*$^{\Delta 1o}$ maternal effect mouse model we compared live/dead and male/female mutant and wild-type cohorts (Figures 6 and 7). We compared the phenotypes of DNMT1o-deficient

placentas that harbored live and dead fetuses and found that those placentas that did not support a viable fetus had less labyrinth volume than those that did support a live fetus (Figure 6). In a sex comparison of DNMT1o-deficient placentas we discovered that female placentas on average had smaller central LZ volumes than mutant males ($P<0.05$; Figure 7). In addition, DNMT1o-deficient females had significant differences from wild-type counterparts at all measured phenotypes whereas mutant males only differed from wild-type males in LZ central volume and TGC number (Figure 7). These results are in line with preliminary reports earlier placental phenotypes that are more prominent in female than male *Dnmt1*$^{\Delta 1o}$ maternal effect offspring (*407*).

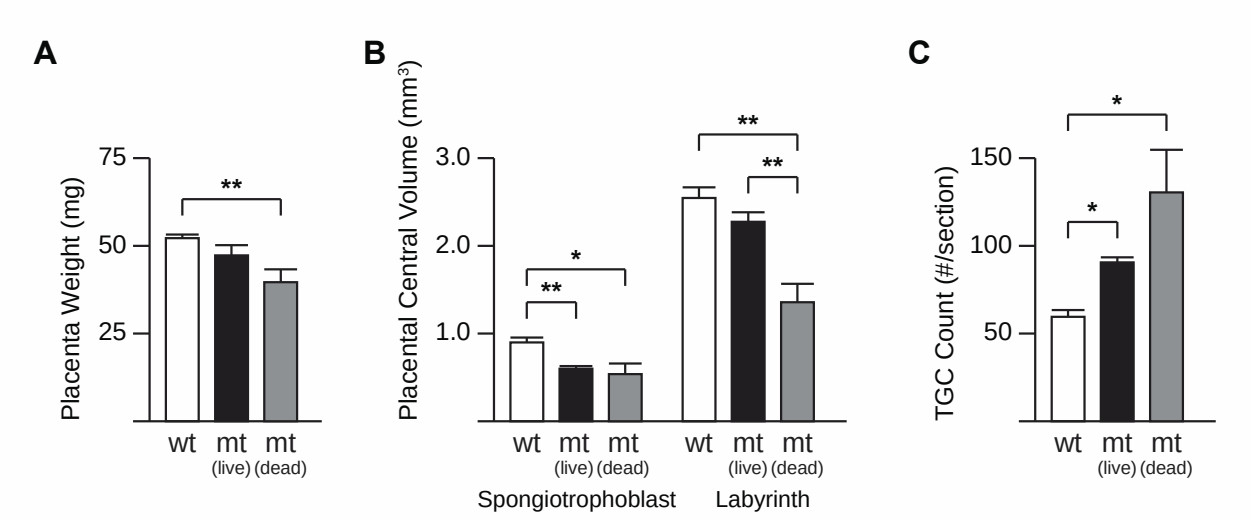


Figure 6. Phenotypic comparison of wild-type (wt) and DNMT1o-deficient (mt) live and dead placentas at E12.5. (A) Measurements of wet placenta weight, (B) Spongiotrophoblast and Labyrinth central volume, and (C) the number of TGCs per slide of a cohort of wt and mt-Live and mt-Dead placentas are displayed as white, black and gray bars respectively. Data are plotted as mean + SEM. *($P<0.05$) and **($P<0.005$) denote significant differences between wt, mt-live and mt-dead averages by the Rank-sum test. wt n=21 for placental weights, n=12 for layer volumes, mt(live) n= 11, mt(dead) n=13.

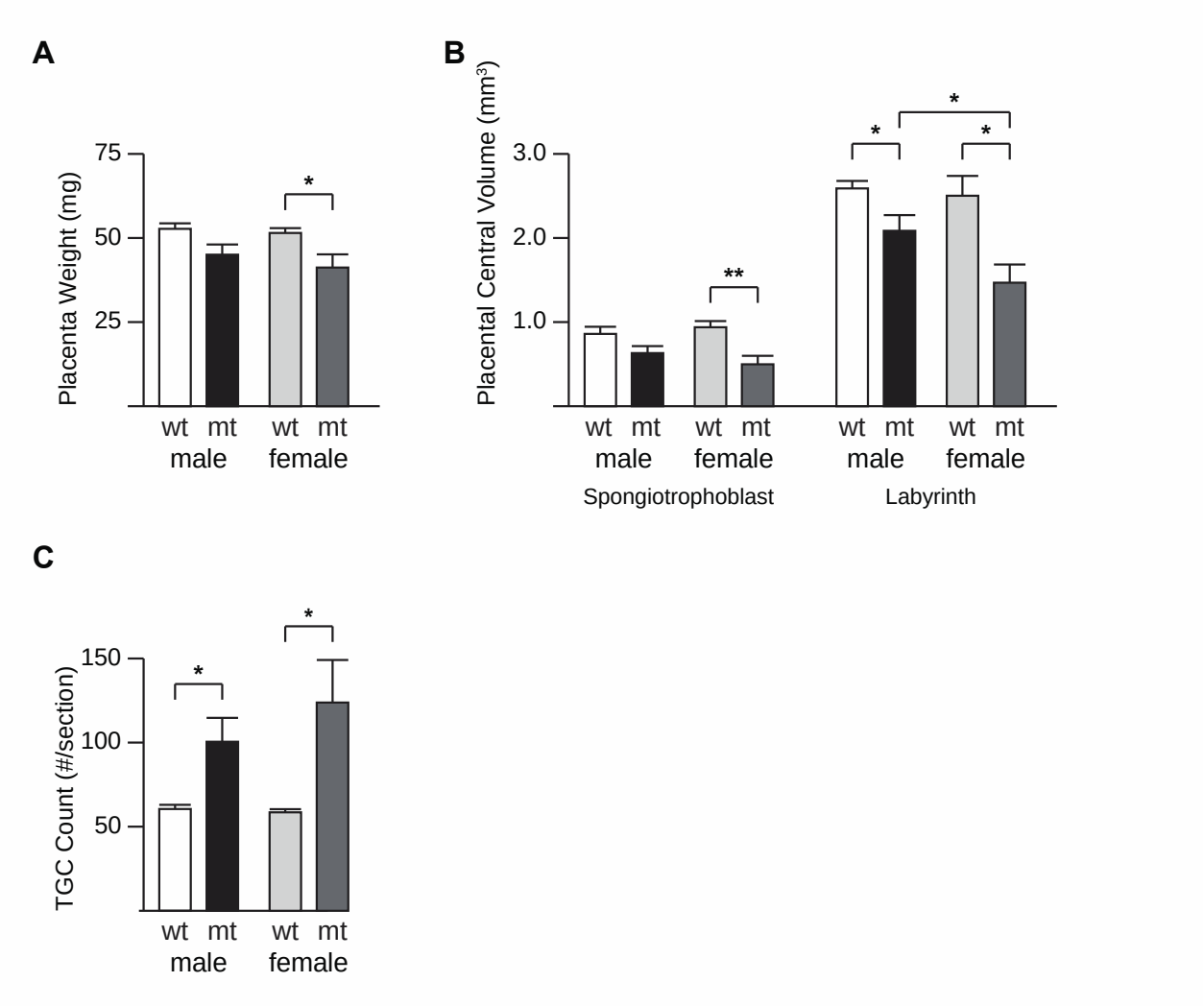


Figure 7. Phenotypic comparison of wild-type (wt) male and female, and DNMT1o-deficient (mt) male and female placentas at E12.5. (A) Measurements of wet placenta weight, (B) Spongiotrophoblast and Labyrinth central volume, and (C) the number of TGCs per slide of a cohort of wt-male, mt-male, wt-female and mt-female placentas are displayed as white, black, light-gray and dark-gray bars respectively. Data are plotted as mean + SEM. *($P<0.05$) and **($P<0.005$) denotes significant differences between wt-male, wt-female, mt-male and mt-female averages by the Rank-sum test. wt-male n=11 and wt-female n=10 for placental weights, wt-male n=6 and wt-female n=6 for layer volumes, mt-male n=12, mt-female n=12.

Labyrinth morphology was noticeably abnormal with hemorrhaging of maternal blood pools and poor fetal vasculature development (see Figure 37 in section 3.4.5 for H&E staining of DNMT1o-deficient placentas associated with loss of *Peg10* DMD methylation). To confirm the poor vascular development in DNMT1o-deficient samples ISH with an RNA probe antisense to

the *Mest* transcript was used to identify fetal vessels. Although *Mest* is an imprinted gene, its expression is expected to increase rather than decrease with loss of methylation at the *Mest* DMD. Therefore, any reduction in expression observed should be due to loss of fetal vasculature and not loss of imprinting at the *Mest* locus. In one maternal effect litter of eight (Figure 8 C1-C8), four have normal fetal vessel branching (C1, C5, C6 and C7), two have stumped and underdeveloped vasculature (C2 and C3) and two completely lacked vasculature (C4 and C8). Not surprisingly the placentas with diminished or no vasculature did not support a viable fetus. Although these results indicate imprinting is important for placental fetal vasculature development it does not tell us whether it is imprinting within the fetal derived vascular endothelium or trophoblast components of the placenta that is critical.

The spongiotrophoblast layer was examined by ISH expression analysis of *Tpbpa* and *Ascl2* (Figures 9 and 10). In wild type placentas a distinct JZ layer was present at the boundary of the LZ and maternal decidua consisting of SpT that stained strongly for *Tpbpa* (WT; Figure 9). ISH staining for *Tpbpa* in DNMT1o-deficient placentas ranged from normal (C2 and C7; Figure 9) to abnormal, showing ectopic expression in the LZ or decidua (C1, C6, C7 and C8; Figure 9) and/or diminished and dispersed signal (C1, C3 and C4 and C8; Figure 9). Expression of *Ascl2* marks SpT but does not stain as strongly in wild-type placentas at E12.5 than at E9.5 (wt, Figures 4 and 10). However, at E12.5 *Ascl2* is also detected in C-TGCs and S-TGCs and remaining TSC populations embedded in the LZ (WT; Figure 10). DNMT1o-deficient mutant placentas exhibited a wide spectrum of *Ascl2* spatial expression patterns (C1-8; Figure 10). *Dnmt1*$^{\Delta 1o}$ maternal effect mutants showed near normal (C1), partial loss (C3, C6, C8) or complete loss (C4) of *Ascl2* expression, consistent with varying degrees of partial loss of *Kcnq1* DMD methylation. Intriguingly, DNMT1o-deficient placenta C3 has partial loss of *Ascl2*

expression on the same junctional zone side that lacks *Tpbpa* expression, and has stunted vascular development; whereas DNMT1o-deficient placenta C4 with complete loss of *Ascl2* has neither *Tpbpa* expression nor vascular development. Some DNMT1o-deficient placentas had stronger than normal *Ascl2* staining with the LZ (C1, C5 and C7) suggesting that undifferentiated EPC progenitors, C-TGCs or S-TGCs may be accumulating.

Excessive abundance of TGCs is shown in three examples of DNMT1o-deficient placentas by DAPI nuclear staining (Figure 11; also See Figure 38 in section 3.4.5) for H&E and in situ analysis of TGC accumulation in E12.5 placentas with loss of *Kcnq1* DMD methylation). DAPI TGC nuclei were counted for 2-3 central sections and averaged for each placenta. Wild-type and $Dnmt1^{\Delta 1o}$ maternal effect mutant cohort TGC count averages were compared with extended analysis of live/dead and male/female subgroubs (Figures 5C, 6C, and 7C). Accumulation of TGCs in DNMT1o-deficient placentas was most evident in placentas lacking a viable fetus, and was significant in both male and female conceptuses. DNMT1o-deficient placentas C1, C3 and C8 had elevated TGC counts and abnormal (although not absent) *Ascl2* and *Tpbpa* expression. These results suggest that the maldevelopment of each layer is interdependent in DNMT1o-deficient placentas.

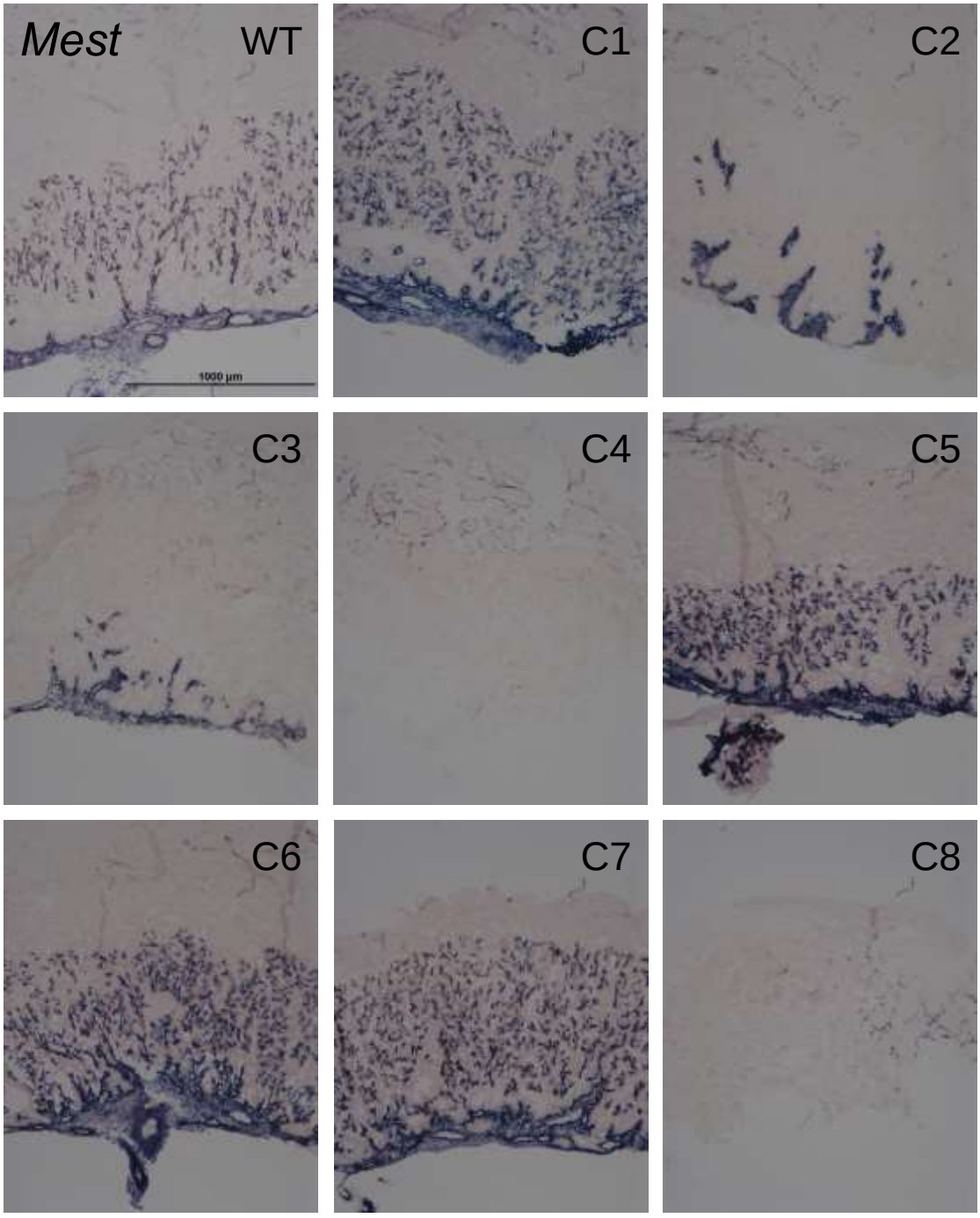


Figure 8. E12.5 *Mest* ISH of a Wild-type (WT) and a litter of eight DNMT1o-deficient (C1-C8) placentas. *Mest* expression marks fetal endothelium and chorionic plate. Note the normal fetal vessel branching patterns in WT and C1, C5, C6, and C7, stumped in C2 and C3, and lack of in C4 and C8. 4x objective Scale bar is 1000um.

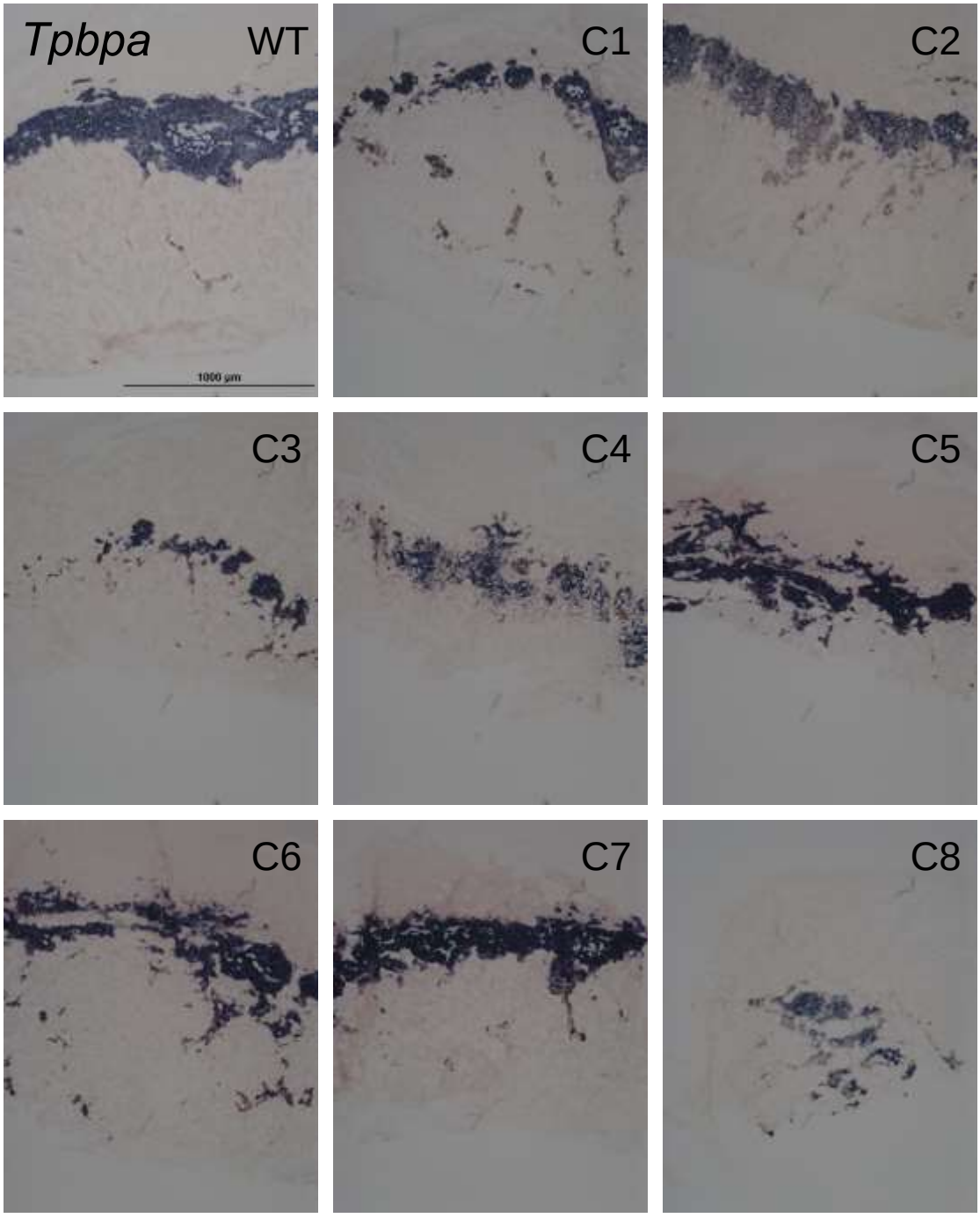


Figure 9. E12.5 *Tpbpa* ISH of a Wild-type (WT) and a litter of eight DNMT1o-deficient (C1- C8) placentas. *Tpbpa* expression marks the spongiotrophoblast. Notice the strong staining and compact layer in WT and mutant C2. However, other DNMT1o-deficient placentas show Ectopic (C6) diffuse (C1, C3, C4 and C8) or ectopic (C5, C6 and C7) expression. 4x objective Scale bar is 1000um

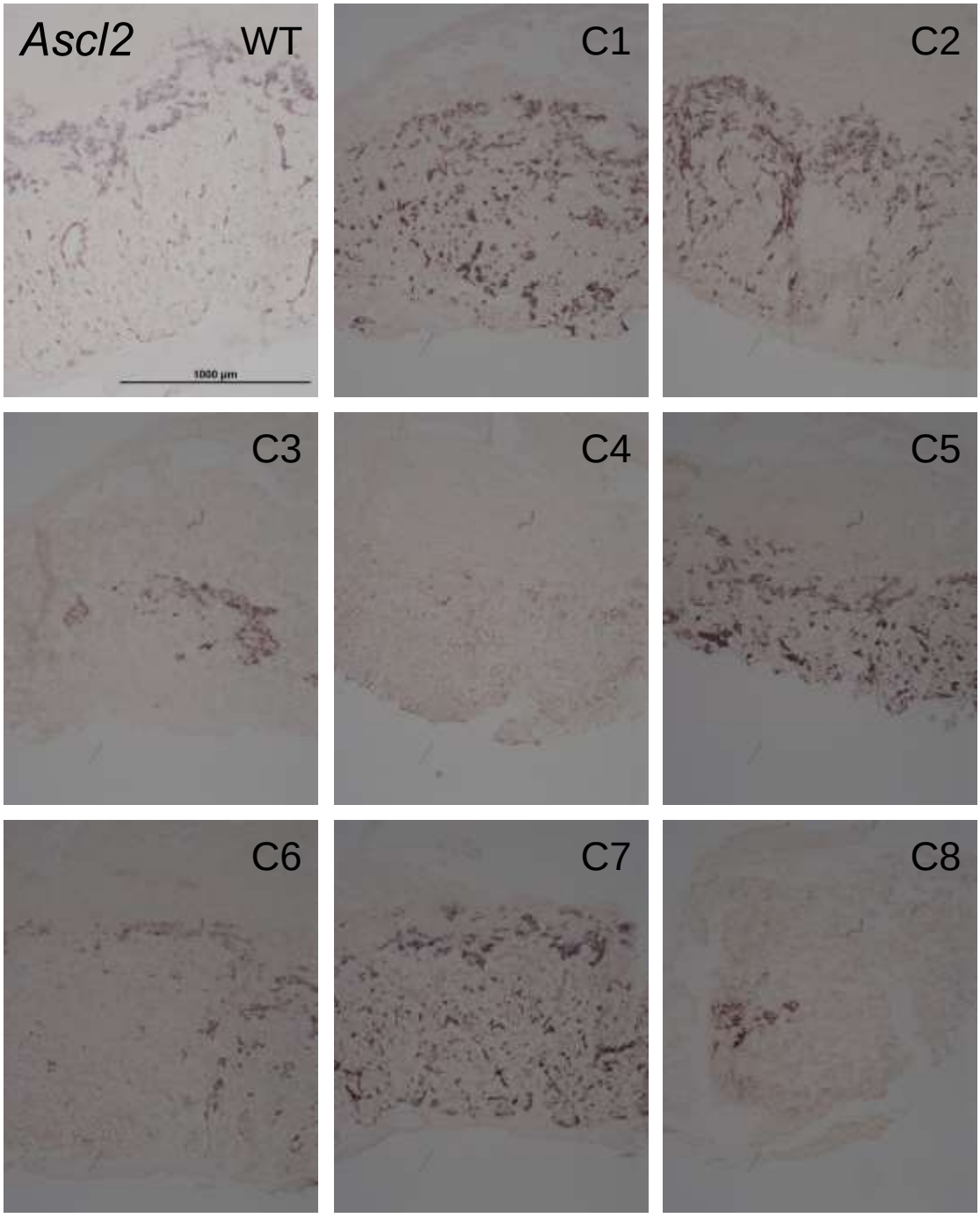


Figure 10. E12.5 *Ascl2* ISH of a Wild-type (WT) and a litter of eight DNMT1o-deficient (A-H) placentas. *Ascl2* marks primarily the spongiotrophoblast and scattered S-TGCs and C-TGCs in the labyrinth Notice the compact SpT layer in WT and DNMT1o-deficient placentas C1, C2, 5 and C7; whereas placentas C3, C4, C6 and C8 have have a range of loss of Ascl2 expression from partial to complete. 4X objective, scale is 1mm.

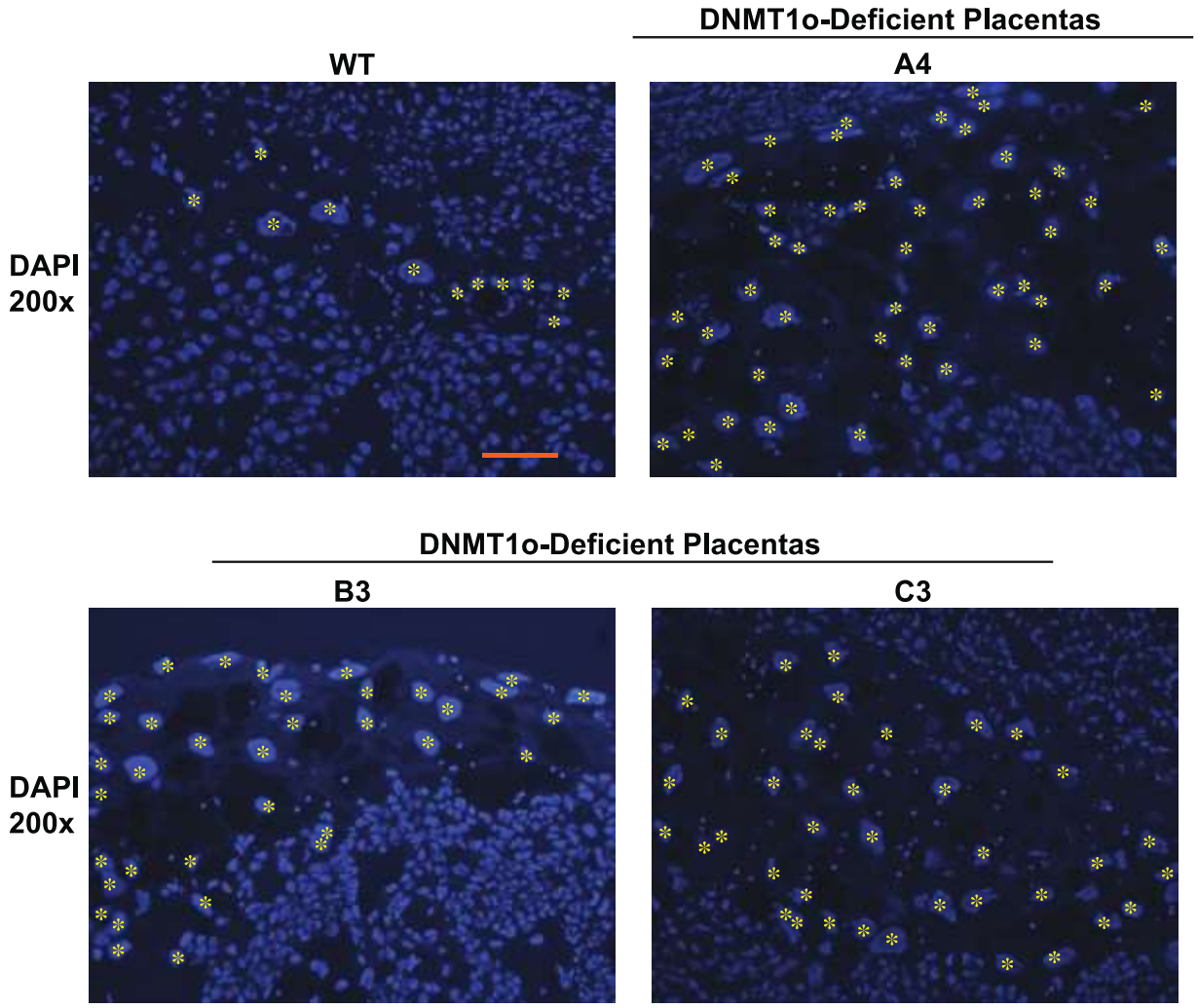


Figure 11. DAPI nuclear staining of TGCs in a wild-type (WT) and three DNMT1o-deficient E12.5 placentas. A yellow asterisk highlights the nucleus of each TGC. 20X objective, orange scale bar is 100μm.

2.4.4 E15.5 phenotypes

The variability in phenotypic metrics observed at E15.5 was smaller than that seen at E12.5.

Specifically, at E15.5 neither JZ central volume nor LZ central volume phenotypic metrics

significantly differed between wild-type and mutant cohorts (Figure 12C). However, there was an increase in both placental and fetal weights in DNMT1o-deficient placentas compared to gestational age matched controls (Figures 12A and 12B). In the comparison of live and dead mutants, viable DNMT1o-deficient placentas and fetuses were overgrown ($P<0.005$; Figures 13A and 13B). Mutant female placentas weighed more than wild-type females ($P<0.05$) but males did not differ from their wild-type counterparts (Figure 14A).

Although there was no quantitative difference in JZ central volume between wild-type and DNMT1o-deficient placentas (Figure 12C), there were noticeable qualitative changes. Wild-type *Tpbpa* ISH staining in E15.5 controls showed a single compact layer of JZ (WT; Figure 15). DNMT1o-deficient placentas ranged from having a thin JZ layer (C3; Figure 15), to having an increased JZ layer (C6 and D2; Figure 15). Many of the E15.5 $Dnmt1^{\Delta 1o}$ maternal effect placentas exhibited extensions of *Tpbpa* positive SpT within the LZ. Much of the SpT including the extensions were rich in GCs. Three examples of GC rich placentas are shown with PAS staining to highlight glycogen content (Figure 16). These findings suggest that volumetric estimates may not be indicative of the full phenotypic spectrum of placental layer development.

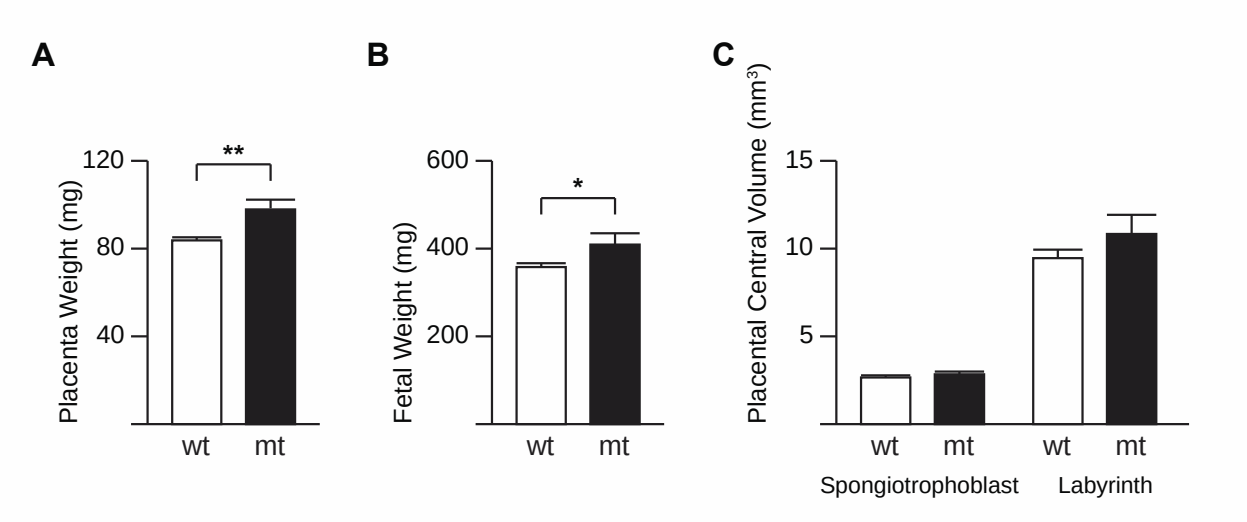


Figure 12. Phenotypic comparison of wild-type (wt) and DNMT1o-deficient (mt) placentas and fetuses at E15.5. (A) Wet placental weight, (B) Wet fetal weight, (C) Spongiotrophoblast volume and labyrinth zone volume of a cohort of wt and mt samples are displayed as open and filled bars respectively. Data are displayed as mean + SEM. * ($P<0.05$) and **($P<0.005$) denote significant differences between wt and mt averages by the Rank-sum test. wt n=27 for placental and fetal weights, wt n=9 for layer fractions, and mt n=21 for all measurements

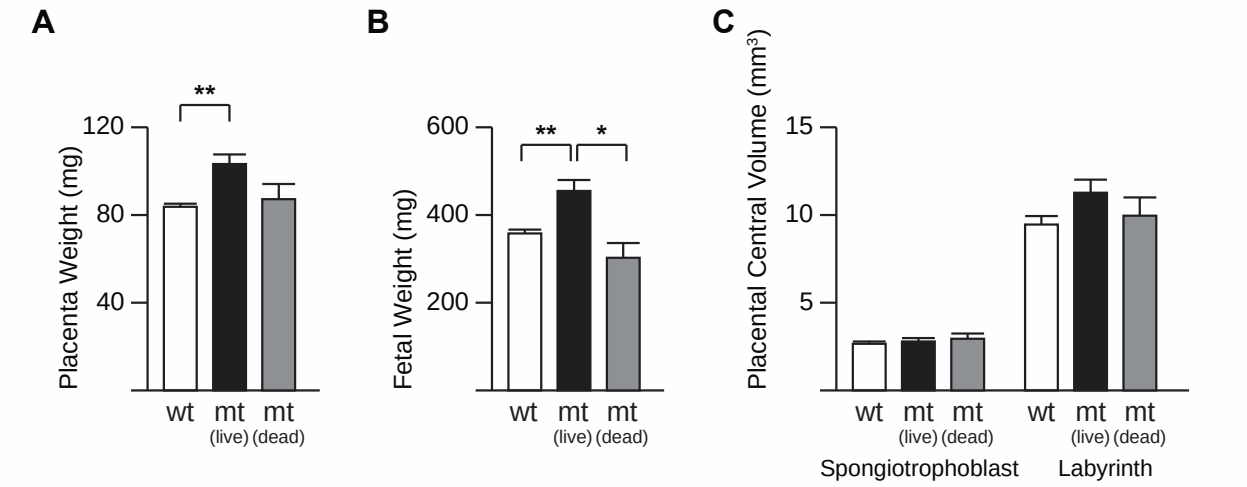


Figure 13. Phenotypic comparison of wild-type (wt) and DNMT1o-deficient (mt) live and dead placentas at E15.5. (A) Measurements of wet placenta weight, (B) Wet fetal weight and (C) Spongiotrophoblast and Labyrinth central volume of a cohort of wt and mt-live and mt-dead placentas are displayed as white, black and gray bars respectively. Data are plotted as mean + SEM. *($P<0.05$) and **($P<0.005$) denote significant differences between wt, mt-live and mt-dead averages by the Rank-sum test. wt n=27, mt-live n= 14 and mt-dead n=7.

The concentration of triacylcglycerides (lipids) within E15.5 DNMT1o-deficient placentas was measured. This analysis compared four wild-type and twenty *Dnmt1*$^{\Delta 1o}$ maternal effect placentas. Wild-type E15.5 placentas had an average triacylglyceride concentration of 5.70 nmol/mg compared to the DNMT1o-deficient placental average of 7.36 nmol/mg ($P>0.05$; Figure 17A). Additionally, neither fetal viability nor embryonic sex significantly differed from wild-type nor within mutant subgroups ($P>0.05$; Figures 17B and 17C). This data is interpreted as evidence that there may be changes to trophoblast lipid accumulation at E15.5 but that elevated levels may not occur to a great enough degree or in a sufficient fraction of samples to reach significance in a comparison of means. LipidTOX fluorescence of E15.5 DNMT1o-deficient placenta shows that lipid droplets primarily accumulate in SynT rather than CD31 positive fetal endothelium (Figure 18).

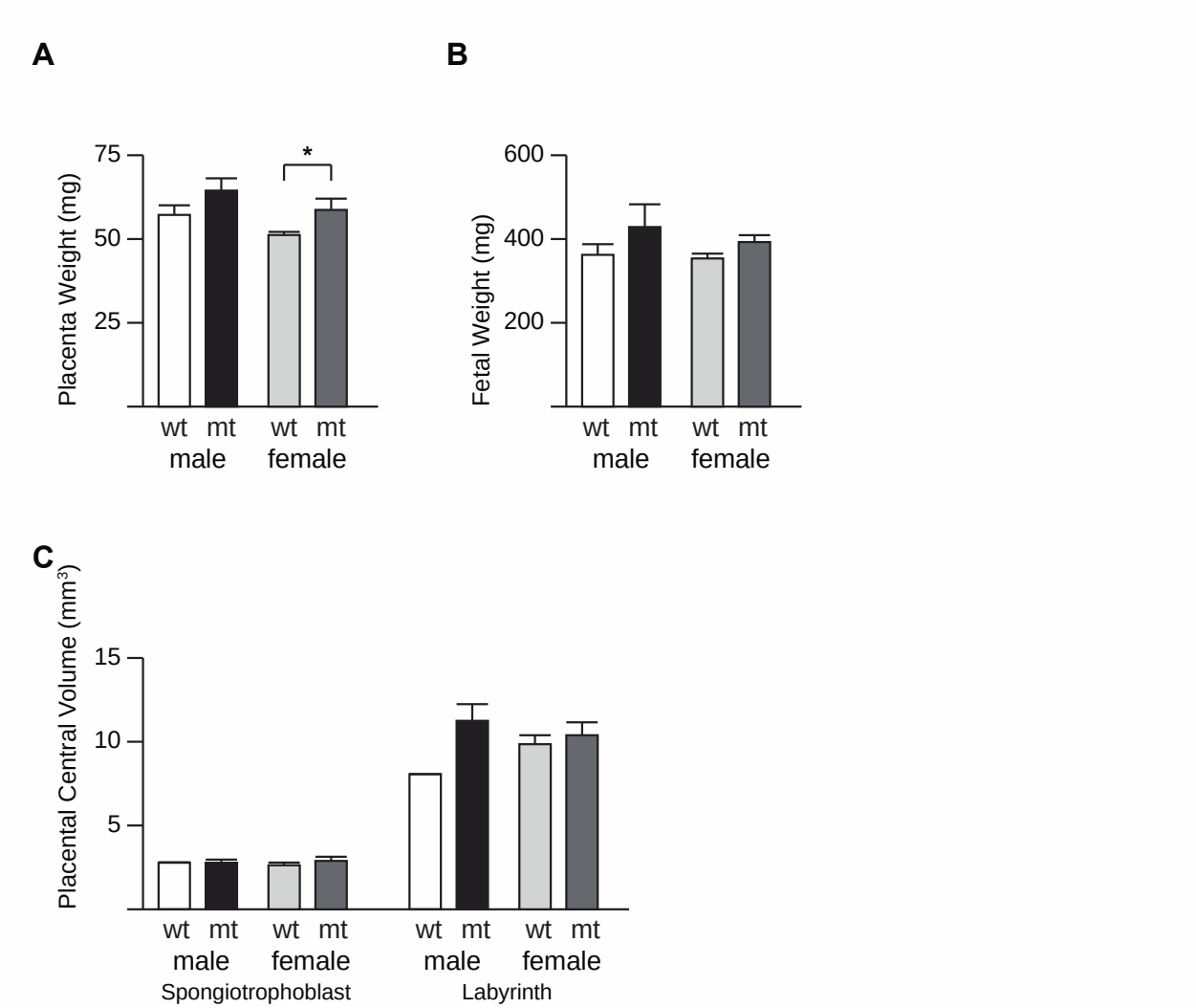


Figure 14. Phenotypic comparison of wild-type (wt) male and female, and DNMT1o-deficient (mt) male and female placentas at E15.5. (A) Measurements of wet placenta weight, (B) wet fetal weight and (C) spongiotrophoblast and labyrinth central volume of a cohort of wt-male, mt-male, wt-female and mt-female placentas are displayed as white, black, light-gray and dark-gray bars respectively. Data are plotted as mean + SEM. *($P<0.05$) and **($P<0.005$) denotes significant differences between wt-male, wt-female, mt-male and mt-female averages by the Rank-sum test. wt-male n=4 and wt-female n=17 for placental and fetal weights, wt-male n=2 and wt-female n=7 for layer fractions, mt-male n=9 and mt-female n=12 for all measurements.

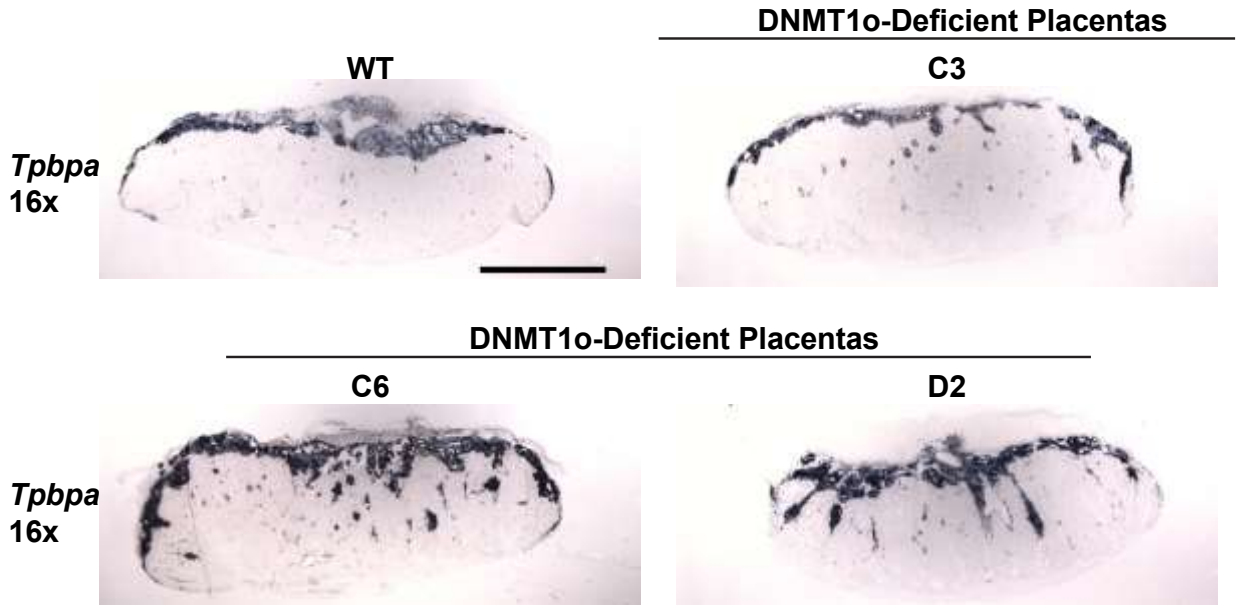


Figure 15. *Tpbpa* ISH of wild-type (WT) and DNMT1o-deficient E15.5 placentas. *Tpbpa* is a marker of spongiotropbhoblast, which is present as a compact layer in WT but ranges from a thin layer to thickened and having projections in to the labyritnth in DNMT1o-deficient mutant placentas. Scale bar is 2mm. 1.6X objective.

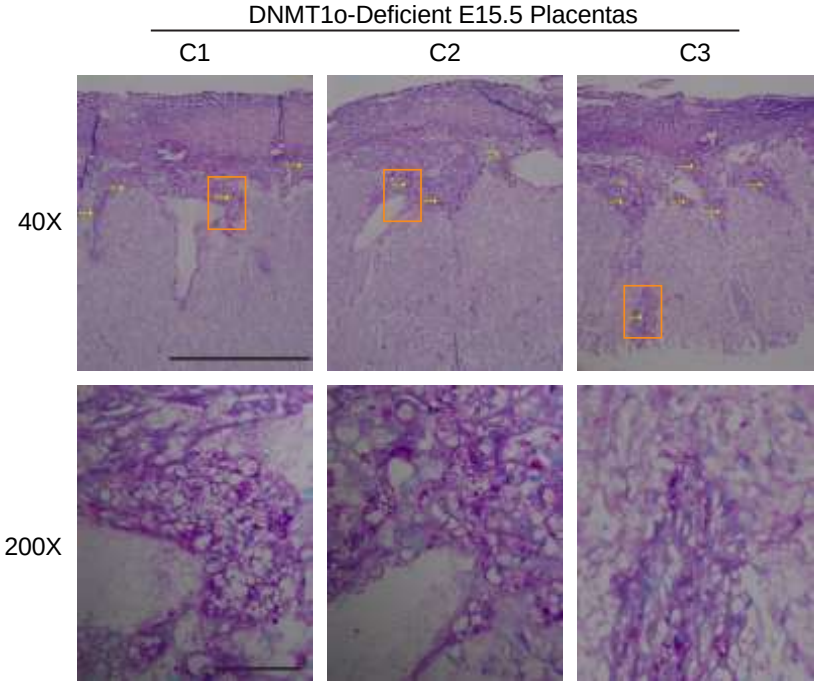


Figure 16. PAS in E15.5 DNMT1o-deficient placentas. Vacuolated and glycogen rich (purple) GCs indicated by Yellow arrows and zoomed in areas in orange boxes. Scale bars 1mm and 100μm.

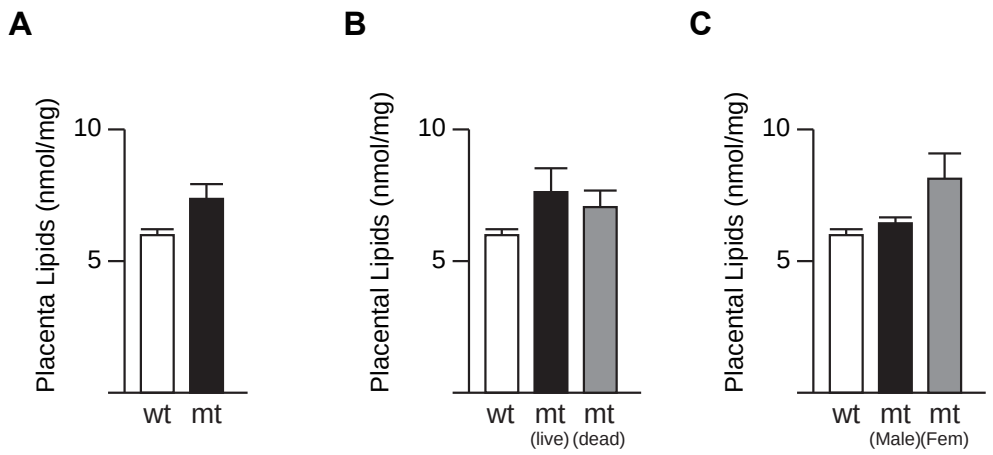


Figure 17 E15.5 triacylglyceride Levels. (A) Comparison of wild-type (wt) and DNMT1o-deficient (mt) placental lipid concentrations. (B) Comparison of wt and mt-live and mt-dead placentas. (C) Comparion of wt and mt-male and mt-female placentas. Mean and SEM of each sample distribution are shown. All comparions were not siginificant (P>0.05) by rank sum test. wt n=4, mt n=20, mt-live n=13, mt-dead n=7, mt-male n=9, mt-female n=11

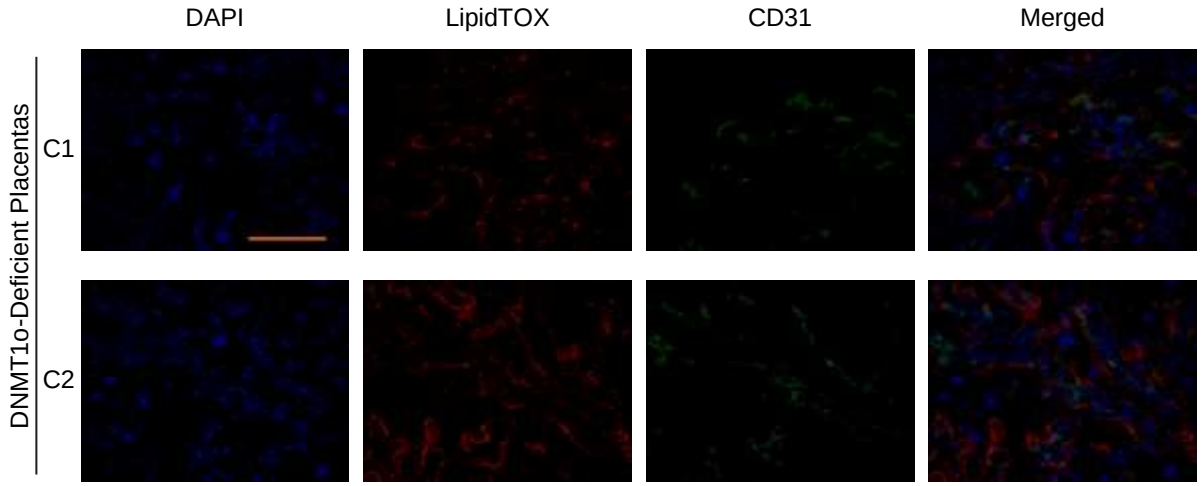


Figure 18. Fluorescent imaging of E15.5 DNMT1o-deficient placentas. Nuclei are shown in blue (DAPI), lipid droplets in red (LipdTOX), fetal vasculature in green (CD31) and composite RBG merged image. Note that lipid droplets occur primarily in SynT that are negative for CD31 staining. Images taken with 40X objective, orange scale bar is 100μm.

2.4.5 E17.5 placental phenotypes

Placental and fetal weights at of DNMT1o-deficient placentas recovered at E17.5 were greater than wild-type controls ($P<0.005$; Figure 19A). Conceptuses supporting live fetuses harbored heavier placentas ($P<0.005$) and fetuses ($P<0.005$) than those that were not viable (Figures 20A and 20B). Both male and female mutant placentas were heavier than wild-type controls ($P<0.005$ and $P<0.05$; Figure 21A). These results suggest that DNMT1o-deficient placentas that survive into late-gestation are overgrown.

The ratio of embryonic to placental weight (E/P) is a standard metric of placental efficiency as it directly measures the placental capacity to support fetal growth normalized to the amount of placenta present. An analysis of fetal and placental weights from 17 wild-type and 62 DNMT1o-deficient placentas revealed that mutant placentas are widely variable in their placental efficiency. Wild-type placentas had an average an E/P ratio of 9.99 and when plotted cluster into a tight group (Figures 22A and 22B). DNMT1o-deficient placentas showed a wide range in E/P ratios with a significantly lower than wild-type average of 6.08 and when plotted show a scattered distribution ($P<0.001$; Figures 22A and 22B). Not surprisingly mutant placentas that did not support growth had a lower E/P ratio than those that could (E/P of 3.13 versus 8.36), and formed distinct groups when plotted (Figures 22A and 22B).

To determine if the wild-type, mutant-live and mutant-dead samples formed distinct E/P groups the Kmeans clustering algorithm was applied to all E17 embryo and placenta data. The number of clusters (K) was set to 5, because the data as plotted in Figure 22B appeared to have one wild-type placenta group and four DNMT1o-deficient groups: normal placenta with large embryo, large placenta with large embryo, normal placenta with small embryo, and small placenta with small embryo. Kmeans clustering returned these expected groups (Figure 22C). Cluster 2

included all wild-type samples, along with one live mutant and one dead mutant and had a centroid E/P ratio of 9.62, close to that of wild-type samples (Figure 22C). Cluster 1 contained 18 live mutants and one dead mutant, and had a centroid E/P ratio of 8.78 with enhanced fetal growth (Figure 22C). Cluster 3 contained 14 live mutants with a centroid E/P ratio of 8.5 with large placentas and fetuses (Figure 22C). Cluster 4 had 7 dead mutants and a centroid E/P ratio of 2.9; these samples had extremely small placentas and fetuses (Figure 22C). Lastly, Cluster 5 contained 18 deceased mutants and one live mutant with a centroid E/P ratio of 2.7; these samples had normal placental weights but small fetuses and therefore were very inefficient (Figure 22C). These results highlight that DNMT1o-maternal effect placentas without a viable fetus are very inefficient, whereas those that survive are slightly less efficient than normal but can be extremely overgrown. The range of fetal weights found in cluster 1 show that within the live-mutant population that have slightly above average placental weights some placentas are more efficient (supporting higher fetal weight) than others (supporting lower fetal weight). Placentas within cluster 1 with high and low E/P are compared in transcriptional analysis described at the end of this results section.

In a cohort of E17.5 DNMT1o-deficient placentas substantial phenotypic variance was unearthed. The volume fraction of JZ increased whereas the volume fraction of the LZ layer decreased ($P<0.05$; Figures 23B and 23C). Strong qualitative phenotypes were also noticed. The JZ layer of many placentas had ectopic extensions of SpT into the LZ visible on H&E and *Tpbpa* ISH histological sections (left column; Figure 24). Much of the excess SpT was positive for the glycogen cell marker *Pcdh12* (data not shown) and contained glycogen vacuoles visible in PAS staining (right columns; Figure 24).

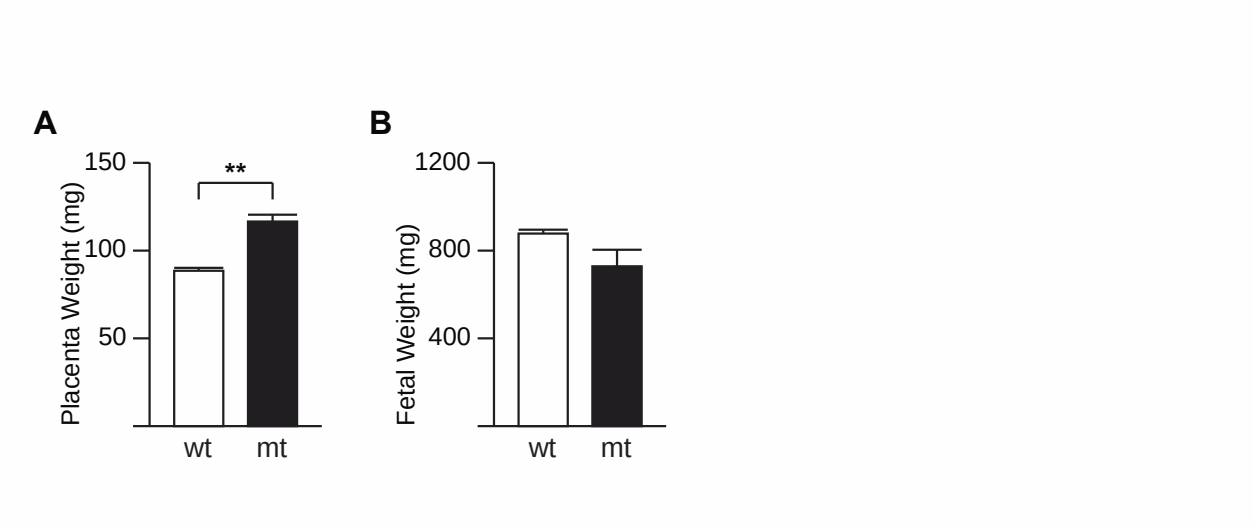


Figure 19. Comparison of wild-type (wt) and DNMT1o-deficient placental and fetal weights at E17.5. (A) Wet placental weights and (B) wet fetal weights of wt and mt cohorts are shown as open and filled bars respectively. Data are displayed as mean +SEM. ** (P<0.001) by the Rank-Sum test, wt n=17, mt n=23.

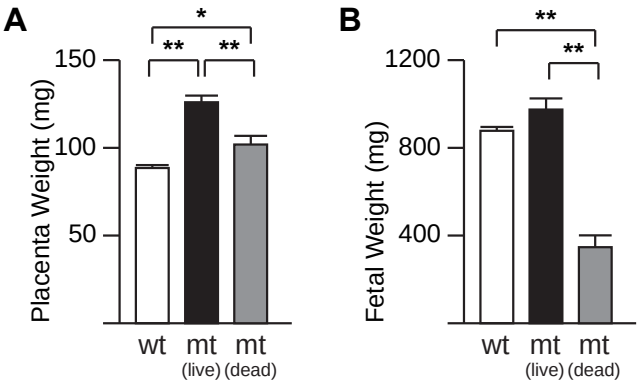


Figure 20. Phenotypic comparison of wild-type (wt) and DNMT1o-deficient (mt) live and dead placentas at E17.5. (A) Measurements of wet placenta weight and (B) Wet fetal weights of wt and mt cohorts are shown as open and filled bars respectively. Data are displayed as mean +SEM. ** (P<0.001) by the Rank-Sum test. wt n=17, mt-live n=14 and mt-dead n=9.

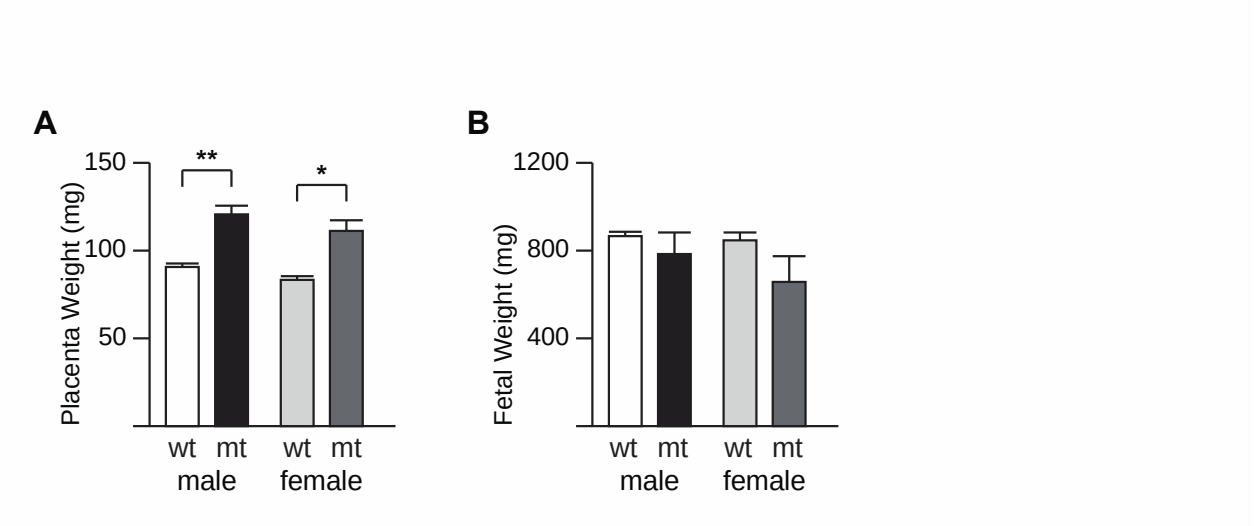


Figure 21. Phenotypic comparison of wild-type (wt) male and female, and DNMT1o-deficient (mt) male and female placentas at E17.5. (A) Measurements of wet placenta weight and (B) Wet fetal weights of wt and mt cohorts are shown as open and filled bars respectively. Data are displayed as mean +SEM. ** ($P<0.001$) by the Rank-Sum test. wt-male n=12, wt-female n=5, mt-male n=13 and mt-female n=10.

The labyrinth layer of E17.5 DNMT1o-deficient placentas was also abnormal. Expression of SynT marker *Lepr* was sporadic or diminish to varying degrees in mutant placentas (left column; Figure 25). IHC staining with antibodies against the fetal vascular endothelium marker CD31(VECAM) enabled detailed resolution of both fetal vessels and maternal blood pools (right column; Figure 25). This IHC analysis showed that many DNMT1o-deficient placentas have dilated maternal blood sinuses (asterisks; Figure 25). However, it was of note that unlike at E12.5 all placentas at E17.5 had intact fetal vasculature, indicating that those showing severe labyrinth defects at E12.5 cannot survive to E17.5. Taken together this evidence demonstrates that DNMT1o-deficient placentas exhibit variable abnormal labyrinth morphology.

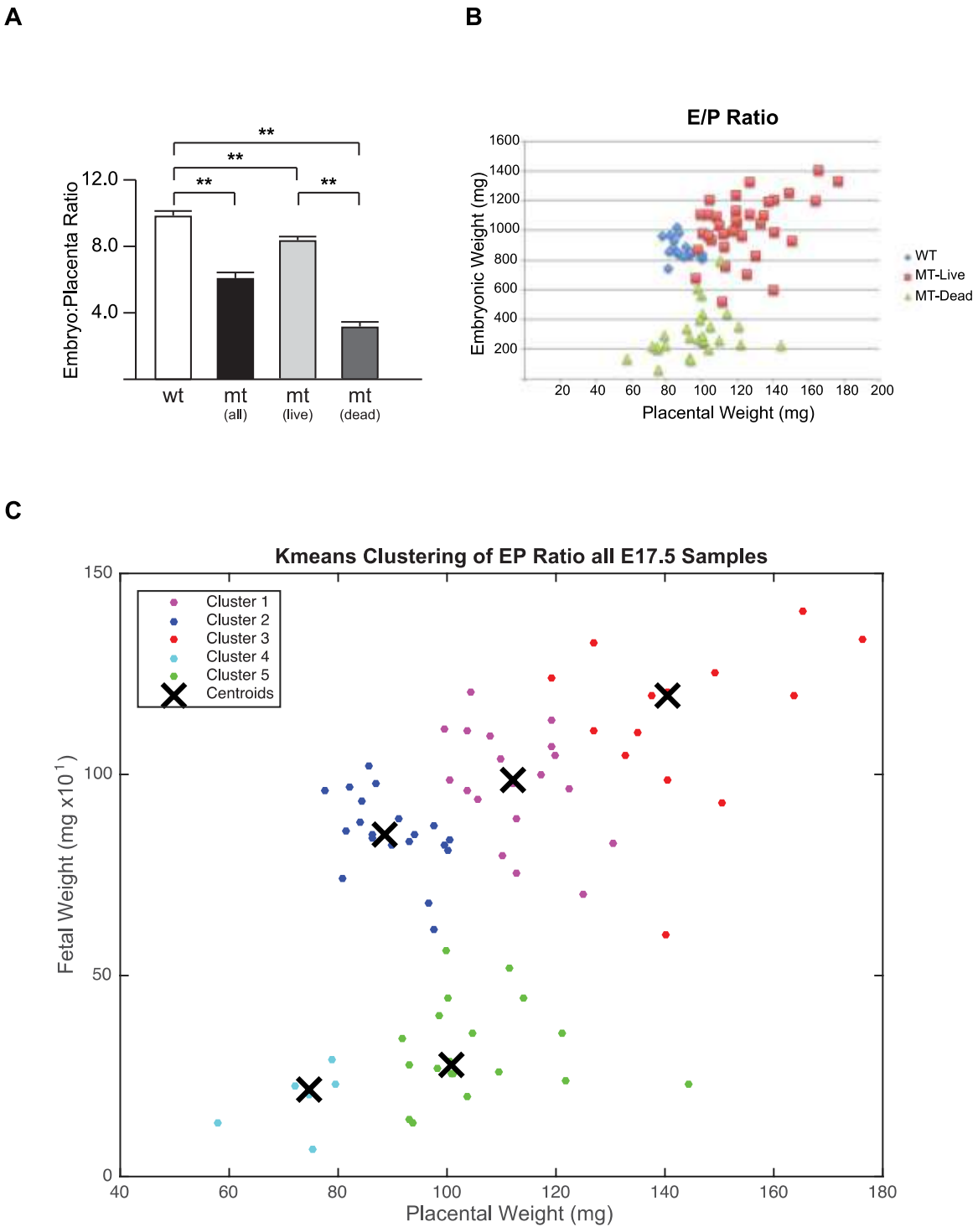


Figure 22. Analysis of E/P ratio in wild-type and DNMT1o-deficient E17.5 offspring. (A) Bar chart of E/P ratio in wild-type, mutant live and mutant dead samples. (B) Plot of E/P ratio for each subgroup: wt n=17, mt-live n=67, mt-dead n=34. (C) Kmeans clustering with K=5. **P<.001 with two tailed unequal variance T-test.

Histological analysis of E17.5 DNMT1o-deficient placentas by ORO staining revealed an abundance of lipids in the LZ (Figure 26A-D). There was a wide range in the staining from extremely abundant staining to similar levels as wild-type. Quantification of triaclyclgeride content revealed an overabundance of lipids in DNMT1o-deficient placentas that was statistically significant (P<0.0 Figure 26E). The robust lipid deposition combined with the overabundance of GCs is evidence that the metabolic states of late gestation DNMT1o-deficient placentas are altered.

A microarray gene expression analysis was performed to determine what cellular pathways are altered in the $Dnmt1^{\Delta 1o}$ maternal effect model in E17.5 placentas with high and low E/P ratios (Figure 27). Hierarchical clustering analysis of all samples revealed a structured hierarchy in which wild-type samples were highly similar, and high and low E/P samples separated into distinct mutant sub-groups (Figure 27A). Of the genes down-regulated 1.3 fold or more there was significant overlap in the low and high E/P groups (Figure 27B); and these were enriched in genes involved in oxidative response (Figure 27C). On the other hand there was less overlap in genes up-regulated by both low and high E/P ratio DNMT1o-deficient placentas placentas (Figure 27B). Genes involved in lipid metabolism were up-regulated by both groups. In contrast, only high E/P ratio had up-regulated genes enriched for molecular transport and lipid metabolism pathways. Overall, my description of DNMT1o-deficient at E17.5 and throughout mid-gestation demonstrate a large gamut of placental developmental and structural defects.

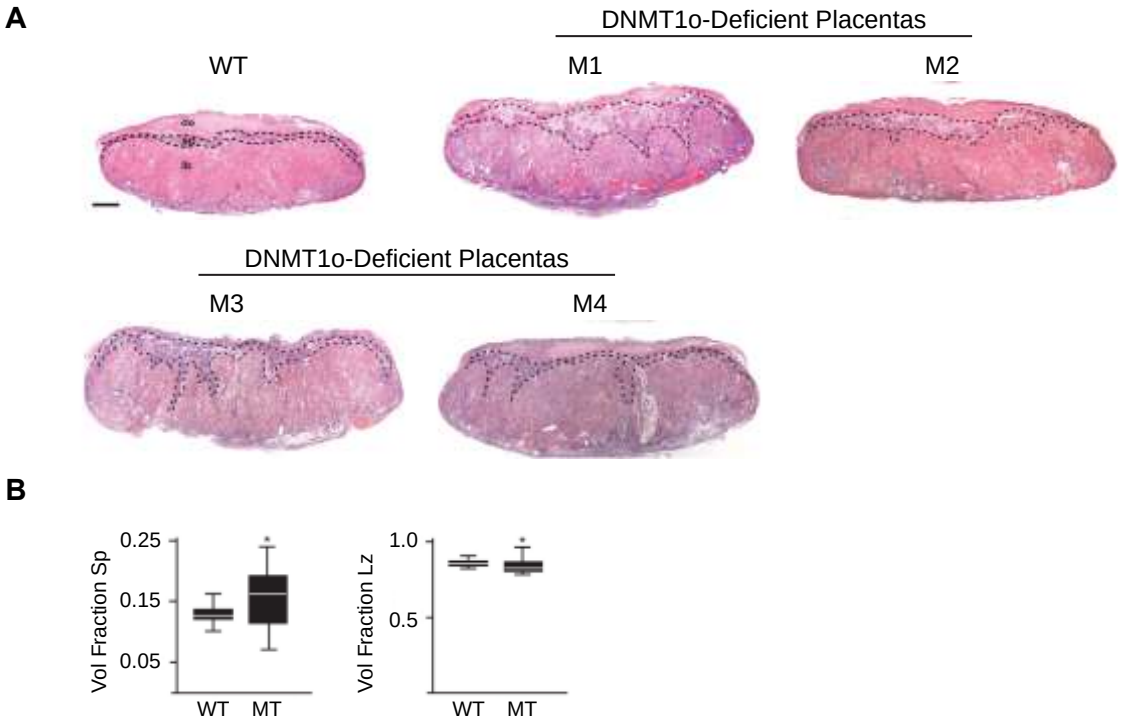


Figure 23. H&E Histology and volume fraction measurements of E17.5 DNMT1o-deficient placentas. (A) Histology of H&E stained paraffin embedded wild-type (WT) and DNMT1o-deficient (M1-M4) placentas. (B) Box plots showing spongiotrophoblast and labyrinthine wild-type and DNMT1o-deficient volume fractions medians and upper and lower quartiles. Abbreviations de decidua; sp spongiotrophoblast; lb labyrinthine.1.6X ojective, Scale is 400μm *P<0.05 Kruskal-Wallis

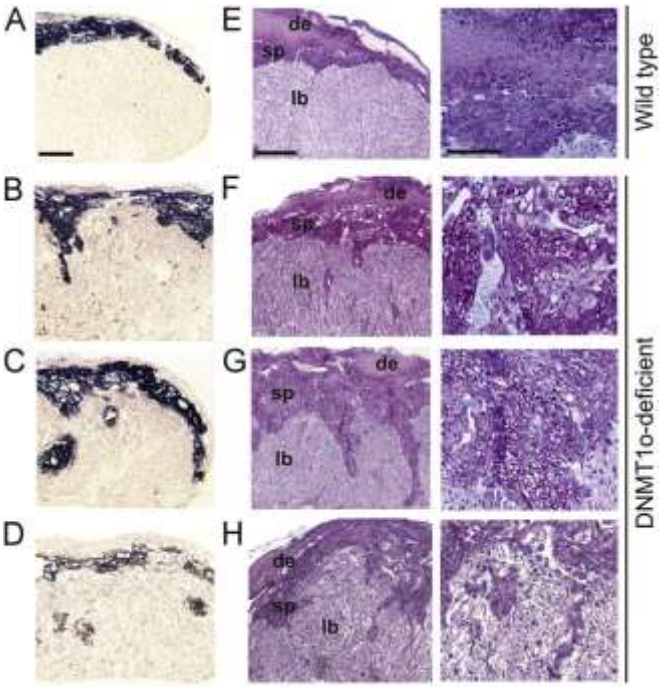


Figure 24. *Tpbpa* ISH and PAS staining of a wild-type and three E17.5 DNMT1o-deficient placentas. (A-D) *Tpbpa* ISH of a wild-type and 3 representative DNMT1o-deficient placentas. (E-H) Adjacent sections from the same samples with PAS glycogen staining. Abbreviations: de decidua; sp spongiotrophoblast; lb labyrinthine. Left and center panels 5x objective, scale bar is 400μm; Right panel 10X objective scale bar is 100μm.

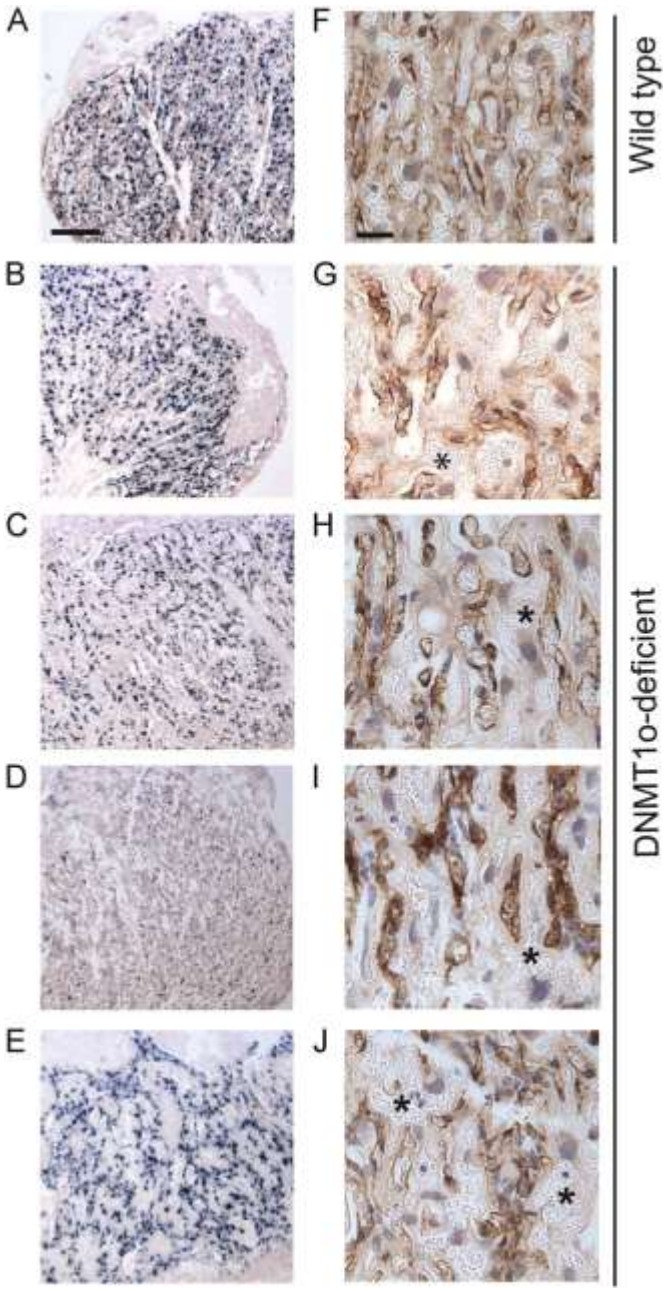


Figure 25. *Lepr* ISH and CD31 IHC histology of DNMT1o-deficient E17.5 placentas. (A-E) *Lepr* ISH staining of SynT in a wild-type and four representative DNMT1o-deficient placentas. (F-J) CD31 IHC staining of fetal vessels in a different set of representative DNMT1o-deficient placentas. Left panel 5X objective, scale bar is 400μm. Right panel 40X objective, scale bar is 50μm. * dilated maternal blood pools.

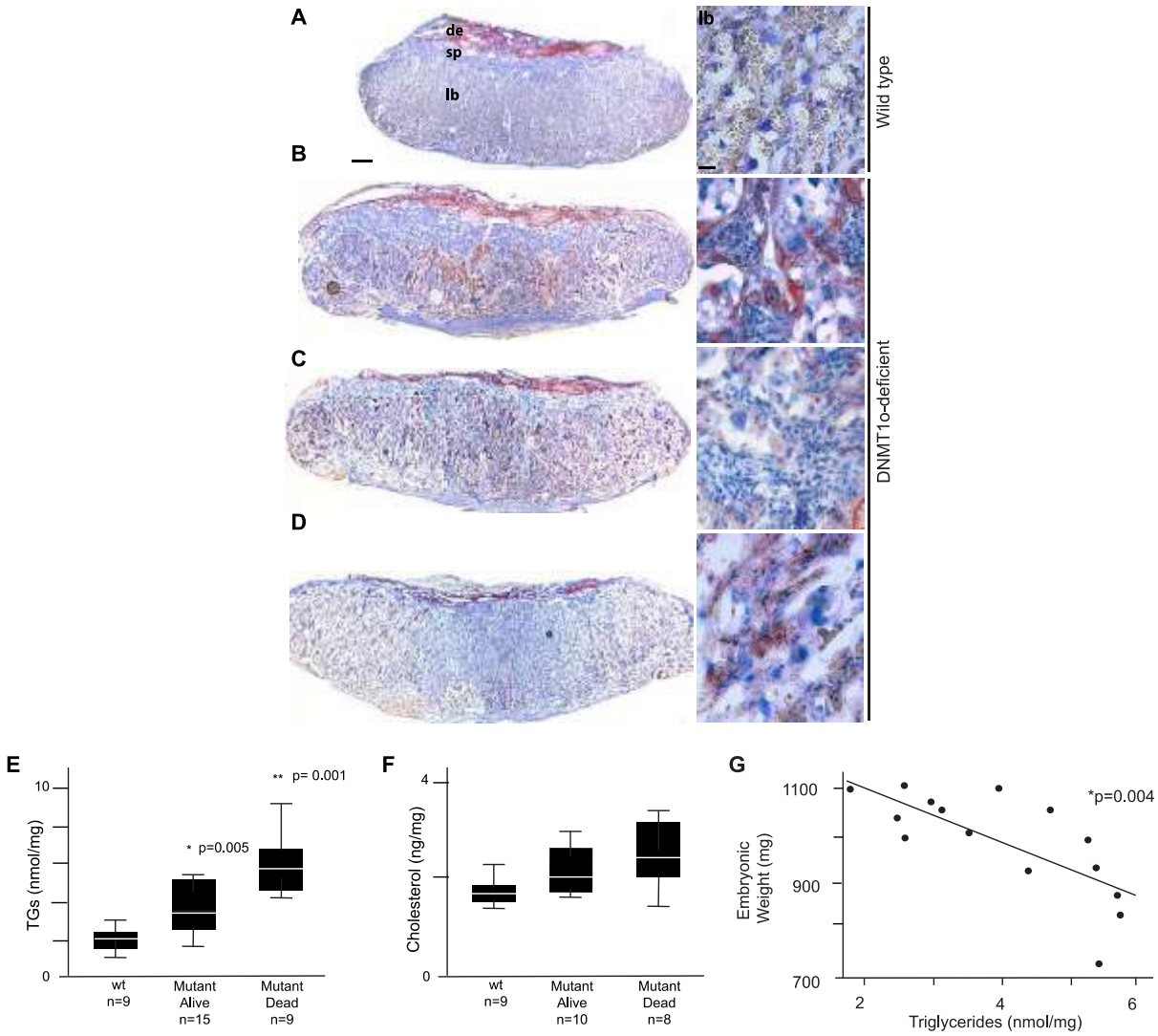


Figure 26. Lipid accumulation in E17.5 DNMT1o-deficient placentas. (A-D) Staining with Oil-Red-O in a single wild type and three DNMT1o-deficient placentas. (E) Quantification of triaclyglyceride concentrations. (F) Quantification of cholesterol concentrations. (G) Inverse relationship of triglyceride concentration and embryonic weight revealed by linear regression. Abbreviations: TGs Trigylcerides; wt Wild-type; de decidua; sp spongiotrophoblast; lb labyrinthine. Left panel 1.6X objective, scale is 400um. Right panel 40x objective, scale is 100μm. * P-values from Kruskal-Wallis test.

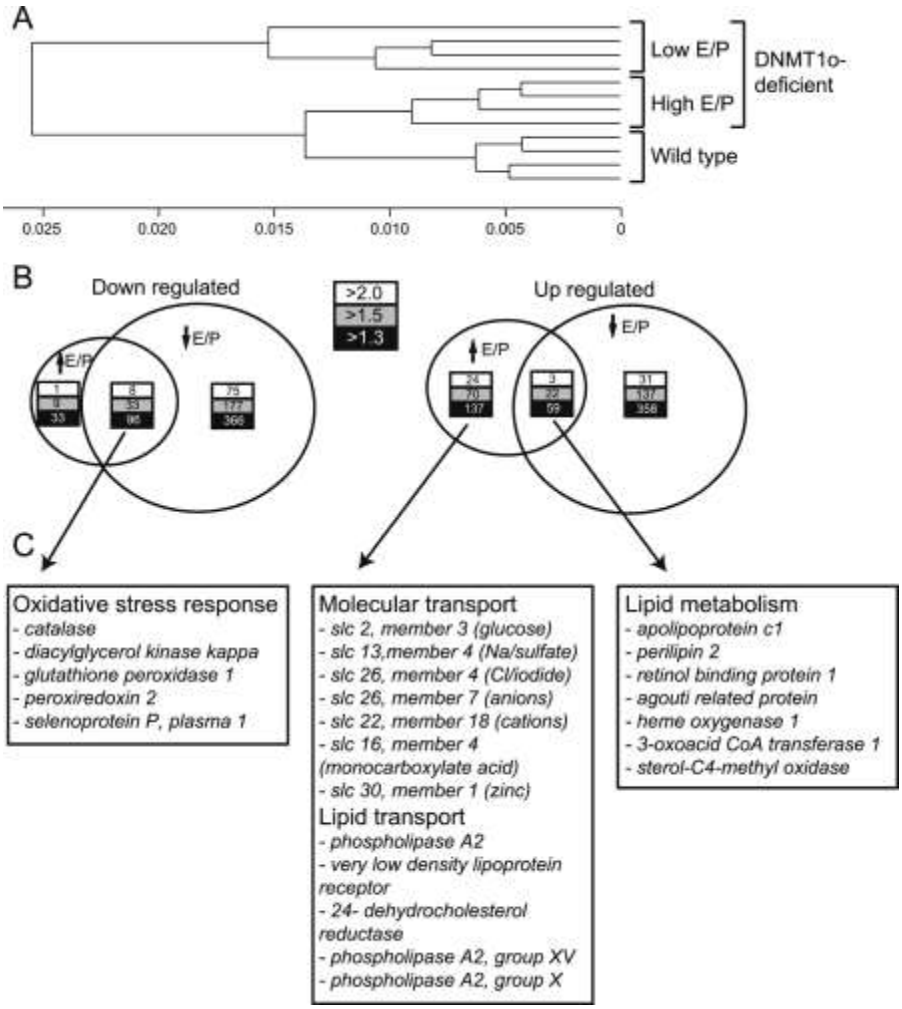


Figure 27. E17.5 microarray analysis of DNMT1o-deficient placentas. (A) Hierachical clustering derived dendrogram describing genome-wide expression patterns. The X-axis is 1 minus the correlation coefficient. (B) Ven diagrams showing overlapping subsets of genes common to low and high E/P ratio DNMT1o-deficient placentas and (C) enriched gene ontology pathways determined by Ingenuity Pathway Analysis.

2.5 DISCUSSION

2.5.1 Fetal viability and growth

Partial mosaic loss of imprinting in the *Dnmt1*$^{\Delta 1o}$ maternal effect model has a profound impact on placental development and fetal growth that progresses across gestation. The most prominent and obvious phenotype in DNMT1o-deficient placentas was the inability to support a viable fetus. At E9.5 the majority of embryos are alive, at E12.5 roughly half of each litter is deceased, and by E15.5 and E17.5 only a fraction of normal litter sizes are recovered (Table 2). This gradual mid-gestation lethality points to a temporal juncture between E9.5 and E12.5 in genomic imprinting that is critical for proper development. While the embryonic lethality observed in the *Dnmt1*$^{\Delta 1o}$ maternal effect model is likely due to loss of imprinting in both the fetal and placental compartment, given the severe placental abnormalities and similar stage of gestational developmental arrest in known placental lethal mutants (*e.g. Ascl2*, *Rtl1*, *Peg10*, *Pparδ* and *Pparγ*) it is likely that placental development is the major factor.

At E12.5 *Dnmt1*$^{\Delta 1o}$ maternal effect litters were similar in size to wild-type litters in having 8 conceptuses. The partial embryonic lethality observed at E12.5 was an opportunity to observe placental abnormalities associated with loss of fetal viability. Two distinct groups of placentas were observed in a representative E12.5 DNMT1o-deficient litter that were either able to support a viable fetus (C1, C5, C6 and C7; Figures 8, 9 and 10) or were unable to (C2, C3, C4, C8; Figures 8, 9 and 10). Notably expression of the fetal endothelium marker *Mest* was normal to

slightly expanded in placenta with viable fetuses but stunted or nearly absent placenta from non-viable conceptuses (Figure 8). Likewise, three out of four non-viable placentas had diminished *Ascl2* staining and abnormal *Tpbpa* spatial gene expression patterns (C3, C4, and C8; Figures 9 and 10). I interpret these results to indicate that fetal vasculature in the labyrinth is an absolute requirement for fetal development. In addition, I interpret these results as evidence that that loss of placental *Ascl2* imprinting resulting in lower expression may predispose embryonic lethality through abnormal JZ and LZ layer development. However, these results do not enable the determination as to whether the absence of fetal vasculature is the cause or effect of loss of imprinting in the fetus proper, extraembryonic mesoderm derived fetal vessels or trophoblast derived cellular subtypes.

The parental conflict hypothesis, put forth by Haig and Moore suggests that genomic imprinting evolved to balance maternal health and fetal growth (*411*). The majority of mammalian species are not monogamous, therefore having multiple less taxing litters over a long lifetime increases maternal reproductive fitness by increasing the total number of offspring, whereas having vigorous fecund offspring with little regard to maternal resources and post-nurturing maternal health increases male reproductive fitness by ensuring robust and viable offspring. Imprinted genes generally follow the canonical dogma of the parental conflict hypothesis with maternally expressed genes restricting placental and fetal growth (e.g. *Phlda2*, *Igf2r*) and paternally expressed genes enhancing placental and fetal growth (e.g. *Igf2*, *Mest*, *Grb10*). Based on this theory, a widespread disruption of genomic imprinting such as predicted in the $Dnmt1^{\Delta 1o}$ maternal effect model, should result in a broad range of placental and fetal weights.

In fact, this is exactly what was observed in DNMT1o-deficient placentas (Figures 22). At E12.5 DNMT1o-deficient placentas were on average growth restricted, particularly those associated with either non-viable or female conceptuses (Figures 5A, 6A and 7A). At E15.5 DNMT1o-deficient placentas and fetuses were overgrown, particularly with those associated with either a viable or female conceptus (Figures 12A, 12B, 13A, 13B and 14A and 14B). However, at E17.5 only placental weight, but not fetal weight was significantly increased in DNMT1o-maternal effect placenta (Figures 19A, 19B, 20A, 20B, 21A and 21B). At E17.5 placenta efficiency (E/P ratio) trended lower and showed great variation between samples (Figure 22). It was lowest in conceptuses associated with a non-viable fetus (Figure 22). The E/P ratio of wild-type samples was clustered in a tight group but in DNMT1o-deficient mutants formed expansive clusters segregated by fetal viability (Figures 22B and 22C). In addition, we found specific gene ontology pathways (i.e. oxidative stress response and lipid metabolism) that were altered in high and low E/P placentas and others that were specific to high E/P placenta (molecular transport and lipid metabolism) suggesting that loss of placental imprinting has major effects on placental metabolism (Figure 27). These findings were further corroborated in analysis of placental glycogen and lipid content in the JZ and LZ layers. A number of imprinted genes have been implicated in regulating placental efficiency including *Igf2* (enhances efficiency) and *Grb10* (represses efficiency) (*347, 412, 413*). Overall these results suggest that the balance of fetal and placental growth is disrupted in the $Dnmt1^{\Delta 1o}$ maternal effect partial loss of imprinting model.

2.5.2 Placental labyrinth phenotypes

In early gestation DNMT1o-deficient placentas exhibit under development of the placenta LZ. At E9.5 the labyrinth marker *Tfeb* as well as the imprinted gene *Phlda2* show a decreased and less convoluted labyrinth structure in the majority of DNMT1o-deficient samples (M2, M3, M4, M5 and M7; Figure 4). While the chorionic plate appears intact, there is poor integration of the EXE derived chorionic trophoblast and fetal derived vessels with the EPC, resulting in less definition of maternal blood spaces and diminished percolation of maternal blood. At E12.5 the phenotype is pronounced as evidence of the overall decrease in placental LZ central volume and decreased penetration and branching of fetal vessels (Figures 5B and Figure 8). The decrease in LZ central volume is most pronounced in female DNMT1o-deficient placentas (Figure 7B). These findings of an exacerbated phenotype in female DNMT1o-deficient placenta are similar to those made by a group led by Trasler (*408*). In their study a coarse scoring system was used to show that female placentas had reduced LZ development (*408*). In addition they found female placentas to have placental hyperplasia at E9.5 indicative of TGC proliferation (*408*). Many of the E9.5 and E12.5 DNMT1o-deficient placentas studied herein had a combination of diminished labyrinth development and expanded TGCs, indicating that these two phenotypes are connected.

Late in gestation DNMT1o-deficient placentas that lacked fetal vasculature were not recovered. In contrast E15.5 placentas had nearly normal LZ central volumes and E17.5 placentas displayed a large range of LZ volume fractions that were on average slightly lower than wild-type. However strong qualitative differences were observed in the structure of the LZ. For instance, many DNMT1o-deficient placentas had engorged maternal blood spaces and dilated fetal vessels. Furthermore, ultrastructural SEM of DNMT1o deficient placentas, performed by colleagues, showed that SynT basement membrane was thickened (*414*). The

findings presented here demonstrate that loss of genomic imprinting is detrimental to both LZ structural development and suggest that the placental SynT development is more robust and less of an absolute requirement than fetal vessels in the partial absence of genomic imprints.

In addition to the structural defects both E15.5 (not significant) and E17.5 ($P<.001$) had increased triacylglyceride content (Figures 18 and 26E). These triglycerides accumulated in SynT within lipid droplet organelles as seen in both LipidTOX and ORO staining (Figures 18 and 26B-D). A broader metabolomics survey of DNMT1o-placental tissue performed by my colleague Katherine Himes, MD., revealed accumulation of mitochondrial metabolites (*414*). Excess carnitine derivatives in DNMT1o-deficient placentas indicated disrupted β-oxidation of triacylglycerols. Reduced acontinase activity and accumulation of the TCA cycle intermediate citrate suggested mitochondrial oxidative stress. These results were interpreted as evidence that triacylglycerides accumulate in lipid droplets because their catabolism by β-oxidation and the TCA cycle is blocked. In combination with ultrastructual findings that mitochondria were bloated, Dr. Himes has hypothesized that the mitochondria of DNMT1o-deficient placenta are dysfunctional. Furthermore, the transcriptional changes to molecular transport and lipid metabolism genes in high and low E/P placenta provide two additional lines of evidence that genomic imprints directly regulate fetal and placental growth through metabolic pathways (Figure 27).

The disorganized and abnormal labyrinth structure seen in DNMT1o-deficient placentas is similar to targeted deletions of both imprinted and non-imprinted genes. For example, *Peg10*, *Ascl2* and *Rtl1* genes are imprinted genes that when deleted have detrimental consequences on LZ placental development and embryonic survivorship. *Peg10* is an imprinted gene within a cluster of imprinted genes on chromosome 6 (Section 1.5.5) that is expressed in many

trophoblast cell types. When *Peg10* is paternally deleted the resulting phenotype is embryonic lethal at E9.5-E10.5 due to vastly diminished LZ development as well as a near complete absence of JZ (*293*). However, the connection with the DNMT1o-deficient phenotype is counterintuitive because loss of *Peg10* DMD methylation on the maternal allele should result in an increase in *Peg10* expression rather than a decrease. *Ascl2* is a maternally expressed transcription factor within the *Kcnq1* imprinting cluster (Section 1.4.7) that is expressed in the EPC, and when maternally deleted results in diminished LZ and JZ layers and an expanded TGC population (*222*). *Rtl1* is a paternally expressed gene within the *Dlk1* cluster (Section 1.4.13) that is expressed in the fetal endothelium (*374*). Paternal deletion of *Rtl1* results in a trophoblast phagocytic response to invading fetal endothelium, placental infarction, and late-gestation growth restriction and lethality (*374*). Based on my results in combination with the information garnered from individual imprinted gene deletions it is evident that imprinted genes are important both for early SynT differentiation and LZ development as well as normal late gestation LZ integrity.

It is also of note that some DNMT1o-deficient placentas exhibited phenotypes similar to targeted deletion of ***Pparγ***, *Pparδ* and *Rxrα* (Section 1.3.5). Deletion of ***Pparγ*** is embryonic lethal at E10.5 due to maternal blood space hemorrhaging and lack of fetal vasculature, two phenotypes commonly observed in E12.5 DNMT1o-deficient placentas (*218*). The *Pparδ* null model shows attenuated chorio-allantoic fusion, which is also observed in many E9.5 DNMT1o-deficient placentas (*220*). Lastly, both ***Pparγ*** and *Rxrα* mutants display an accumulation of lipid droplets within the SynT (*218, 221*). I interpret these common phenotypes to indicate that loss of genomic imprinting may alter the genetic pathways vitally important for placental development

2.5.3 Placental junctional zone phenotypes

Early in placental development at E9.5 and E12.5 *Dnmt1*$^{\Delta 1o}$ maternal effect placentas exhibited mosaic, patchy and sporadic SpT layers that often extended into the LZ. At E9.5 *in situ* hybridization with the SpT marker *Tpbpa*, and the imprinted EPC marker *Ascl2* revealed that many placentas had decreased SpT with corresponding decreases of both markers (Figure 4). At E12.5 these phenotypes progressed into overt structural abnormalities. On average E12.5 DNMT1o-deficient placentas had a diminished SpT central volume (Figure 6B). E12.5 placentas stained with *Tpbpa* showed very distinct patterns with some having minimal SpT development (C3 and C8; Figure 9), others having a sporadic staining patterns (C4; Figure 9), while others had expanded SpT with LZ extensions (C2, C6 and C7; Figure 9). The staining pattern for *Tpbpa* matched the pattern of sporadic *Ascl2* (Figure 10). I interpret these results to indicate that reduction in *Ascl2* expression, as modulated by loss of imprinting at the *Kcnq1* cluster, has a direct impact on SpT development. However, it is also plausible that the changes in *Ascl2* spatial expression patterns are passive secondary changes associated with abnormal SpT development due to loss of imprinting at other loci.

In mid- to late-gestation, DNMT1o-deficient placentas had either a thinner layer of SpT or an expanded layer of SpT with LZ extensions and accumulation of GCs (Figures 9, 15, 16 23, 24). A transition from diminished JZ central volume at E12.5 to no difference at E15.5 to an increase in JZ volume fraction at E17.5 occurs in DNMT1o-deficient cohorts. This indicates that those DNMT1o-deficient placentas with severely diminished SpT layers either compensate during mid-gestation or do not survive. While some E12.5 DNMT1o-deficient placentas show SpT extensions into the LZ, it is much more pronounced at E15.5 and E17.5 (Figures 9, 15, 23A, 24A-D). Both the junctional zone as well as the ectopic extensions are enriched in GCs and

glycogen content (Figures 16, 24E-H). There generally appears to be a correlation between GC enrichment and SpT volume, although this has not been quantified. These results, in combination with the previously discussed accumulation of lipid droplets, suggest that loss of genomic imprinting alters placental energy storage.

The diminished spongiotrophoblast observed herein is similar to phenotypes observed in *Ascl2* and *Peg10* targeted deletion models that disrupt both JZ and LZ development, indicating that early placental layer developent are dependent on each other (*222, 293*). *Igf2* and *Igf2p0* null placentas also have decreased SpT development (*303*). The mid- to late-gestation SpT and GC expansion phenotypes observed in DNMT1o-deficient placentas have similarities and differences with *Phlda2* deletion and overexpression models (*316, 317*). Ablation of *Phlda2* expression promotes placental and fetal overgrowth with excessive SpT and GCs (*317*). Given the endocrine role of spongiotrophoblast one interpretation is that expansion of this layer and increased glycogen stores due to loss of genomic imprinting enhances fetal growth. In contrast, overexpression of *Phlda2* in mouse results in growth restriction with reduced SpT and GCs, however protrusion of SpT into the LZ, similar to that seen in the $Dnmt1^{\Delta 1o}$ maternal effect model does occur (*316*). It has been suggested by Tunster *et al*., that these ectopic SpT and GCs within the late gestation LZ are due to failure of these cells to migrate (*316*).

2.5.4 Trophoblast giant cell phenotypes

Early in development at E9.5 and E12.5 there was a marked increase in the number of P-TGCs found between the JZ and decidua (Figures 4 and 5C). The accumulation of P-TGCs was often accompanied by a lack of LZ development. However, at E15.5 and E17.5 none of the recovered placentas displayed an abundance of P-TGCs (data not shown). These findings suggest a

selection event against placentas with an overabundance of P-TGCs early in development. These results can also be interpreted to indicate that TGC accumulation is a common response to failing embryonic development and may facilitate reabsorption. The similarities to placental development in the absence or muted *Ascl2* expression implies a direct role of this imprinted gene in the dysregulation of TGCs in DNMT1o-deficient placentas (*222*, *311*). In addition, the accumulation of TGCs was the most prominent phenotype in *Dnmt3l* maternal effect placentas at E9.5, suggesting that this is the strongest early phenotype caused by loss of maternal imprints (*95*, *97*, *99*).

2.5.5 Conclusion

The wide range of placental phenotypes observed in DNMT1o-deficient placentas begs the question of what are the causative epigenetic changes that drive these abnormalities. Previous work in the Chaillet lab has shown that the *Dnmt1*$^{\Delta 1o}$ maternal effect model generates a mosaic of conceptuses with partial loss of genomic imprinting (*60*, *402*), however no prior attempts to link these alterations with phenotypic measurements have been made. Herein I have quantified placental phenotypic metrics in across mid to late-gestation which will enable using quantitative measurement of imprinted gene expression and DMD methylation the association of specific phenotypes and epigenotypes. The array of distinct phenotypes ranging from early lethality to overgrowth, as well as specific placental layer disruptions fits in with the model in which each placenta of a litter has a unique set of DMDs with loss of imprinting. While it may be difficult to make 1:1 associations between loss of imprinted DMD methylation and placental metrics in order to better understand the role of genomic imprinting in placental biology.

3.0 LOSS OF GENOMIC IMPRINTING IN DNMT1o-DEFICIENT PLACENTAS IS ASSOCIATED WITH SPECIFIC PLACENTAL ABNORMALITIES

3.1 SUMMARY

My dissertation results in Chapter 2 revealed that the *Dnmt1*$^{\Delta 1o}$ maternal effect model produces varied and abnormal placentas. Previous studies, focusing primarily on the fetus, determined that the *Dnmt1*$^{\Delta 1o}$ maternal effect model generates a mosaic and partial loss of imprinting. In this section I have confirmed that DNMT1o-deficient placentas are also subjected to this mosaic partial loss of imprinting by measuring imprinted gene expression by quantititative RT-PCR, by allele specific DMD methylation analysis by bisulfite genomic sequencing, and by quantifying imprinted methylation at a broad array of DMDs using EpiTYPER-mass array technology. Imprinted gene expression was altered at many imprinted gene clusters examined but did not always fit the pattern expected to be associated with loss of DMD methylation late in gestation. Regression analysis revealed a direct association between *Mest* expression and E/P ratio, and an inverse association between *Ascl2* expression and E/P ratio. Allele specific methylation analysis revealed partial loss of methylation on the normally methylated allele of the *H19, Snrpn, Peg1*, and *Kcnq1* DMDs in some but not all DNMT1o-deficient placentas, and provided no evidence of compensatory methylation of the normally unmethylated allele. EpiTYPER DNA methylation analysis of 15 DMDs in E12.5, E15.5 and E17.5 DNMT1o-deficient placental cohorts revealed a

mosaic partial loss of genomic imprinting. Methylation of some imprinted DMDs, most notably *Dlk1*, was nearly normal in mid-gestation DNMT1o-deficient placentas, consistent with the notion that cells having lost methylation on these DMDs do not contribute significantly to placental development. Most imprinted DMDs however showed a wide range of methylation loss among DNMT1o-deficient placentas. Furthermore, I have revealed significant associations between strong placental phenotypes and loss of methylation at specific imprinted loci. At E12.5 two striking associations were observed. First, loss of DNA methylation at the *Peg10* imprinted DMD associated with decreased embryonic viability and decreased LZ volume. Second, loss of methylation at the *Kcnq1* imprinted DMD was strongly associated with TGC expansion. I conclude that the *Peg10* and *Kcnq1* ICRs are key regulators of mid-gestation placental function.

3.2 INTRODUCTION

3.2.1 Loss of genomic imprinting in DNMT1o-deficient placentas

The *Dnmt1*$^{\Delta 1o}$ maternal effect model generates offspring with a mosaic and partial loss of genomic imprinting due to the absence of DNMT1o maintenance methyltransferase activity during preimplantation development (Section 2.2 for full review). This has previously been confirmed at a small subset of DMDs in the embryonic (*H19*, *Snrpn*, *Mest* and *Dlk1*) and placental (*H19* and *Snrpn* only) compartments. DNMT1o-deficient placentas have profoundly abnormal phenotypes (Section 2.3 for description). At E9.5 and E12.5 attenuated LZ development and TGC accumulation is common in DNMT1o-deficient placentas. Later in

gestation, at E15.5 and E17.5, SpT extensions, dilated fetal vessels, and triacylgylceride accumulation are prevalent features. It is of note that *Dnmt1*$^{\Delta 1o}$ maternal effect offspring have sporadic phenotypes with large variation within and between litters that is similar to the predicted mosaic nature of imprinting in this model. In this chapter, I test the hypothesis that the wide-ranging phenotypes are associated with loss of imprinted methylation at specific DMDs in DNMT1o-deficient placentas.

Loss of genomic imprinting in the *Dnmt1*$^{\Delta 1o}$ maternal effect model has previously been validated by DNA methylation and gene expression analysis at a limited set of imprinted loci. Bisulfite genomic sequencing, bisulfite converted restriction analysis, and methylation Southern blots provided the original evidence that the *Dnmt1*$^{\Delta 1o}$ maternal effect model generates partial loss of methylation at DMDs but not other genomic loci (*e.g. Iap* and *Line1* repetitive elements) (*104*, *402*). Restriction fragment polymorphisim analysis of RT-PCR products of imprinted genes within the *Kcnq1* cluster of *Dnmt1*$^{\Delta 1o}$ maternal effect trophoblast and embryo displayed biallelic maternally biased expression of the normally exclusively paternally expressed *Kcnq1ot* but had no effect on allelic bias of maternally expressed genes within the cluster (*415*). Quantitative RT-PCR gene expression analysis of *Kcnq1ot* and seven maternally expressed genes (*Osbpl5*, *Phlda2*, *Cdkn1c*, *Kcnq1*, *Tssc4*, *Cd81* and *Ascl2*) within the *Kcnq1* cluster revealed that in DNMT1o-deficient E10.5 trophoblast *Kcnq1ot* expression was increased, while the expression of the maternally expressed genes decreased (*415*). Intriguingly, despite this transcriptional repression, the H3K4me3 active chromatin mark levels at these maternally imprinted genes was not statistically altered in DNMT1o-deficient trophoblast (*415*). In addition, loss of *H19*, *Igf2* and *Snrpn* imprinting has been observed in a handful of *Dnmt1*$^{\Delta 1o}$ maternal

effect placentas (*402*). However, transcriptional analysis of the majority of imprinted gene clusters has not been performed in either DNMT1o-deficient embryonic or placental lineages.

3.2.2 Transcriptional analysis of imprinted genes

Out of the 24 confirmed genomic imprints in mouse there are reasons to suspect at least 15 of them harbor imprinted genes that are integral to placental development (Section 1.5). There is particularly strong genetic evidence derived from targeted gene deletion mouse models for the involvement of imprinted genes in the *Kcnq1*, *H19, Peg10, Mest, Grb10*, *Dlk1* and *Igf2r* clusters in placental developmental pathways (*222, 274, 293, 302, 312, 313, 347, 374, 392*). In this chapter, quantitative RT-PCR was used to test the hypothesis that *Dnmt1*^{41o} maternal effect placentas have loss of imprinted gene expression, with some transcripts increasing in expression (presumably biallelic), while others becoming fully repressed. Specifically, imprinted gene expression from the following clusters were analyzed: *Peg10* (*Peg10*, *Sgce* and *Pon2*), *Dlk1* (*Dlk1*, *Meg3*, and *Rtl1*), *H19* (*H19* and *Igf2*), *Igf2r* (*Igf2r* and *Slc22a2*), *Mest* (*Mest* and *Klf14*), *Kcnq1* (*Phlda2* and *Ascl2*) and *Grb10* (*Grb10*).

3.2.3 Imprinted DMD methylation analysis

The exact genomic coordinates of DMDs in oocyte and embryonic genomes are overlapping but significantly different, suggesting that the physical extent of differential methylation is dynamic during embryonic development (*58*). Both gametic and embryonic DMD boundaries are defined by biallelic methylated regions (*58*). It is unclear if the exact DMD coordinates in the genome of trophoblast lineages are closer to those in gametes or embryos. In my dissertation research I have

examined imprinted DNA methylation at sequences that are common to both germline and embryonic DMDs. Bisulfite genomic sequencing was used to confirm loss of imprinting in the placenta compartment. A survey of DNA methylation at 15 DMDs across gestation was performed using EpiTYPER technology to quantify DMD methylation at 15 different loci in cohorts of mid-gestation DNMT1o-deficient placentas and to correlate loss of methylation with placental phenotypic metrics.

Imprinted DNA methylation patterns have been analyzed over the years using an array of techniques. Early studies performed methylation sensitive Southern blots that utilized isoschizomeric restriction enzyme pairs in which one enzyme is methylation-sensitive (*e.g.* HpaII) and the other is methylation-insensitive (*e.g.* MspI) and sequence specific genomic probes. This approach analyzes a handful of CpG dinucleotides at a time. The use of sodium bisulfite ($NaSO_4$) based techniques enabled methods of DNA methylation analysis that can provide a readout of multiple CpGs at specific sequences (*416*). High sodium bisulfite concentrations at elevated (85°C) temperatures and low pH (5.0) deaminate unmethylated cytosine nucleosides to uracil (*416*). PCR amplification of bisulfite treated genomic DNA using targeted primers replicates the ribonucleoside uracil into the deoxy-ribonucleoside thymidine (*416*). These bisulfite treated DNA amplicons can be analyzed by DNA sequencing (BGS: bisulfite converted genomic sequencing) or by restriction analysis (COBRA: combined bisulfite restriction analysis) to infer the ratio of unmethylated to methylated CpG methylation by the amount of C→T converted and unconverted product (*416, 417*). Both BGS and COBRA have their limitations: BGS requires the sequencing of many amplicons to obtain a good representation of the allelic population; and the COBRA assay, similar to methylation sensitive Southern blotting, only analyzes a handful of CpGs at a time (*416, 417*).

EpiTYPER analysis is a novel technique that provides a readout of many CpGs at a time from the whole allelic population of a sample (*418*). Ehrich and colleagues (2005) invented EpiTYPER DNA methylation analysis as a high-throughput bisulfite conversion based method to identify methylation differences of targeted sequences in normal and neoplastic lung tissue (*418*). In brief, EpiTYPER analysis is performed as follows: DNA is treated with bisulfite reagent; targeted genomic regions are PCR amplified using sequence specific primers with a T7 polymerase promoter attached; ssRNA is in vitro transcribed from the bisulfite PCR amplicon templates; the ssRNA is then cut with a nucleotide specific RNase; and the digestion products are analyzed by mass spectrometry to determine the sizes of the products (which are different if they incorporated a C or a T) (*418*). This technique gives a readout of 8-25 informative mass spectrometry fragments containing 1-4 CpGs each across amplicons ranging from 200-800bp (*418*).

Bisulfite converted DNA has also been used to measure genome wide methylation patterns using reduced representation bisulfite genomic sequencing (RRBS; a technique that cuts DNA with methylation insensitive MspI, and sequences 200-250bp fragments enriched in CpG islands) and by microarray analysis (*e.g.* Illumina 450k human array), however these approaches are not as cost efficient for detailed analysis of small sets of genomic sequences such as imprinted loci which represent a minute fraction of the genome (*419, 420*).

3.2.4 Association of loss of imprinting and placental phenotypes

In the results of Chapter 2 (Section 2.3) I quantified phenotypic metrics in cohorts of mid-gestation *Dnmt1*$^{\Delta 1o}$ maternal effect placentas. Herein, the DMD methylation profiles of individual placentas from the same DNMT1o-deficient cohorts were quantified by EpiTYPER

analysis. A separate cohort of E17.5 DNMT1o-deficient placentas had both phenotypic measurements and quantified imprinted gene expression. Linear regression was used to examine the relationship between E/P ratio and expression of imprinted genes in those E17.5 placentas. Having the phenotypic and DMD methylation data sets enabled me to test for meaningful phenotype-epigenotype associations. Logistic regression analysis was used to determine if fetal viability, a binary variable, was associated with methylation at any individual DMD. Likewise, linear regression analysis was used to test for meaningful associations between continuous placental metrics (*e.g.* weight, layer volumes, TGC counts and triacylglyceride content) and loss of methylation at specific DMDs. It was expected that only the strongest phenotype-epigenotype associations would emerge due to the complications of epigenetic pleiotropic and epistatic interactions associated with mosaic loss of DMD methylation in the *Dnmt1*$^{\Delta 1o}$ model.

3.2.5 Chapter 3 aims

In this chapter I have analyzed placental DMD methylation patterns in the *Dnmt1*$^{\Delta 1o}$ maternal effect model. This analysis aimed to confirm loss of imprinting in DNMT1o-deficient placentas consistent with prior observations in the embryo and limited preliminary extraembryonic analysis using a combination of quantitative RT-PCR and BGS. The extent of epigenetic mosaicism in DNMT1o-deficient placentas was probed using EpiTYPER analysis of 15 different DMDs. This enabled logistic regression analysis of which epigenotypes are viable and which are not. Ultimately, I attempted to connect loss of DNA methylation at specific DMDs to quantitative placental phenotypic metrics using linear regression analysis. The overarching goal of this section was to gain insight into the collective and individual role of genomic imprints in placental biology and identify those imprints most crucial for normal placental development.

3.3 MATERIALS AND METHODS

3.3.1 DNA and RNA purification

Genomic DNA was extracted from placenta tissues cleaned of maternal decidua contamination and with minimal umbilical cord and yolksac membranes. Samples were stored in RNA later (Sigma-Adlrich) prior to extraction. Whole (E9.5) half (E12.5), or quarter (E15.5 and E17.5) placenta samples weighing <30mg were minced with a razor on a glass microscope slide and placed in 600ml of RLT buffer pre mixed with 6ul of 2'mercaptoethanol in a 2ml microcentrifuge tube with homogenization microbeads, and then homogenized using two 45second cycles with a mini-beadbeater (Biospec). Nucleic acids were then extracted using the DNA/RNA combined extraction mini kit (Qiagen) or the RNeasy kit (Qiagen) following the manufacturer's protocols.

3.3.2 Imprinted gene quantitative real-time RT-PCR

Quantitative real-time reverse transcription PCR (qPCR) was carried out on purified RNA samples from E9.5, E12.5, E15.5 and E17.5 wild-type and DNMT1o-deficient placentas using the standard methods described below and gene specific primers (Appendix B). Contaminating DNA was digested either on column during purification (with the RNeasy kit) or using an RQ1 RNase free DNase digest before cDNA preparation. cDNA was prepared from 1μg of RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). qPCR was

performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) in total reaction volume of 10µl on the 7900HT Fast Real-Time PCR machine. Dissociation curves were run on all reactions to ensure amplification of a single product. A control without RT (-RT) was run for each sample and a control without template (-template) was run for each primer set. Samples were analyzed using the ΔΔct method method relative to the *Rpl32* housekeeping gene. Five wild-type placentas were analyzed at each gestational age (*421*).

3.3.3 Bisulfite genomic sequencing

Genomic bisulfite conversion was carried out using an EpiTect bisulfite conversion kit (Qiagen). 500ng to 1µg of genomic DNA was converted based the amount extracted from each sample. The DNA was diluted with water to 20µl volume, and mixed with 85µl of bisulfite conversion mix and 35µl of DNA protect buffer. Conversion was carried out in 200µl PCR tubes using the following thermocycler program: 99°C 5′, 60°C 25′, 99°C 5′, 60°C 85′, 99°C 5′, 60°C 90′, 20°C hold. Columns and reagents provided by the EpiTect bisulfite kit were used per manufacturers instruction to purify converted DNA in 100µl of elution buffer. Nested bisulfite converted genomic PCR was used to amplify targeted DMDs (primers in Appendix C). The first round of nested PCR used 125ng of bisulfite converted DNA and in a 25 µl Taq Polymerase (Invitrogen) reaction with the following thermocycler program: Precycle: 94°C 4′, 55°C 2′ , 72°C 2′, 94°C 4′, 55°C 2′, 72°C 2′; followed by 35 amplification cycles: 94°C 1′, 55°C 2′, 72°C 2′; and a short 72°C 30″ extension and indefinite hold at 4°C. The second round of nested PCR was primed from 2.5 or 5µl of the first PCR and used a standard thermocycler program: 95°C 5′ denaturing followed by 35 cycles of 95°C 30″, 60°C 30″; 72°C 30″, a 72°C 7′ final extension and indefinite hold at 4°C. Bisulfite PCR reactions were run on 1.5% agarose gels at 80V and the appropriate

sized bands excised and gel purified using Qiaquick gel extraction kits (Qiagen). PCR amplicons were cloned using the TOPO-TA cloning kit (Invitrogen), transformed into competent *E.coli* cells, and plated onto LB + ampicillin agar plates coated with β-galactose and IPTG. 15-30 white opaque colonies were picked and sequenced in both directions using M3 and T7 promoter primers. Sequences were viewed and shortened to include only relevant genomic (and not plasmid) sequence and positioned in the same DNA strand orientation using CLC sequence viewer (CLC Bio). Bisulfite PCR amplicons from each placenta were aligned to fully converted DMD sequences with the Clustalomega multi-sequence alignment program and then exported to a text document where CG dinucleotides were underlined to easily identify converted and unconverted CpGs. Maternal and paternal DMD alleles were determine by use of SNPs between *Mus musculus domesticus* (maternal) and *M.m castenous* (paternal) strains. Results were transcribed into original figures in adobe illustrator.

3.3.4 Epityper analysis

EpiTYPER DNA methylation was used as a high-throughput bisulfite conversion based method to identify DMD methylation differences between wild-type and DNMT1o-deficient placentas (See section 3.1.3 for review). Genomic bisulfite conversion, bisulfite converted genomic PCR, and EpiTYPER (TM – Sequenom) mass-array DNA methylation analysis was performed at the Center for Genetics and Pharmacology at the Roswell Park Cancer Institute. Pre-validated bisulfite PCR primers for imprinted DMD genomic regions were used for the imprinted methylation analysis (Appendix D). All bisulfite amplcion sequences overlapped known primary imprinted DMDs ((*26*), and references therein). Bisulfite converted PCR amplification primers for all but *H19* were chosen from a publicly available mouse imprinted panel (Sequenom). *H19*

primer sequences were originally published by McGraw et al. (2013) (*408*). Each EpiTYPER amplicon was further validated by internal control wild-type placenta DNA (50% imprinted DMD methylation), *Dnmt1*-null (*Dnmt1*$^{c/c}$) ES cell DNA (0% imprinted DMD methylation) and 1:2 (16.6% imprinted DMD methylation) and 2:1 (33.3% imprinted DMD methylation) mixtures of the two. Only amplicons that produced a linear relation between control genomic DNA expected and observed methylation fractions were selected for use in this study

3.3.5 Biostatitics and bioinformatics

EpiTYPER absolute methylation levels were calculated as the unweighted average CpG methylation fraction across each individual imprinted DMD amplicon. Overall imprinted DMD methylation was determined from 12 non-redundant DMD EpiTYPER amplicons (Appendix D) To determine if the wild-type and mutant sample methylation levels were normally distributed Kolmogorov-Smirnov, Shapiro-Wilk and Anderson-Darling tests of normality were applied to the data in Matlab (Mathworks). Because the mutant data were non-normally distributed we compared distributions using a Mann-Whitney U (Rank-Sum) test. In addition Fisher's exact test was used to compare the number of low DMD methylation (less than 0.75 or 0.5 wild-type levels) placentas for each individual DMD across gestation. Bar graphs and scatter plots of overall and individual imprinted DMD methylation levels were originally generated with Matlab and then adapted into Adobe Illustrator.

To display the variability in DMD methylation intrinsic to the *Dnmt1*$^{\Delta 1o}$ maternal effect model we constructed heat maps. Mutant imprinted DMD methylation levels were normalized to wild-type by dividing each sample's imprinted DMD absolute methylation fraction by the average wild-type methylation level for that imprinted DMD and gestational age. The relative

methylation levels were then log2 transformed and clustered using the clustergram function in Matlab. Each clustergram was adapted into a grayscale Adobe Illustrator file.

To associate individual placental DMD methylation defects with particular placental phenotypic abnormalities I performed regression analyses in Matlab. Logistic regression was performed to find associations between individual imprinted DMD methylation levels and the binary fetal viability variable. Bivariate linear regression analysis was used to associate imprinted DMDs with the continuous phenotypic metrics for LZ volume, JZ volume, trophoblast giant cell count and fetal/placental weights. Similarly, P/E ratio and aberrant imprinted gene expression was tested for meaningful associations using linear regression analysis. Stepwise forward linear regression modeling was performed to generate models that explain the *Dnmt1*$^{\Delta 1o}$ maternal effect phenotypic variation based on DNA methylation of the least number of significant DMDs. Phenotypes with strong associations ($P<0.05$) identified by bivariate regression were plotted against DMD methylation or imprinted gene expression.

3.4 RESULTS

3.4.1 Loss of imprinted gene expression

The epigenotypes of DNMT1o-deficient placentas showed a wide-range of loss of imprinting across gestation. Expression of genes from 7 imprinted loci (*Peg10*, *Dlk1*, *H19*, *Igf2r*, *Mest*, *Kcnq1* and *Grb10*) were analyzed by qPCR with the expectation that loss of DMD methylation at any imprinted cluster would cause expression of some imprinted genes to increase and others to

decrease. The observed average imprinted gene expression patterns of cohorts of DNMT1o-deficient placentas are shown in Figure 28. The expected loss of imprinting gene expression patterns are displayed at the bottom of Figure 28. Abnormal imprinted gene expression patterns were observed by qPCR as early as E9.5 and continued through mid (E12.5 and E15.5) and late (E17.5) gestation. Early in gestation many imprinted genes behaved in the manner expected with loss of methylation at neighboring ICRs. For example, placental expression of *Pon2, Igf2, Igf2r, Klf14* and *Grb10* were down regulated in DNMT1o-deficient placentas relative to wild type at E9.5 (Figure 28). However, by E17.5 the expected loss of imprinted gene expression patterns was not as encompassing. At E17.5 DNMT1o-deficient placentas showed loss of imprinted gene expression within the *Mest* cluster congruent with expected results; with *Mest* expression up regulated and *Klf14* down regulated. In contrast, within the *Peg10* cluster, *Sgce* and *Pon2* were up regulated in a manner incoherent with loss of *Peg10* DMD methylation (Figure 28). Unexpected upregulation was also observed for *Dlk1, Phlda2* and *Ascl2* but did not reach significance compared to wild-type (Figure 28). This transcriptional analysis is evidence that the partial loss of imprinting observed in the *Dnmt1*$^{\Delta 1o}$ maternal effect model changes across gestation.

Linear regression was used to determine if the expression of any imprinted gene was associated with the E/P ratio placental efficiency metric in DNMT1o-deficient placentas. Both *Ascl2* and *Mest* expression were significantly associated with E/P ratio ($P<0.01$; Figure 29). Expression of *Ascl2* had an inverse association with with E/P ratio (Figure 29A), whereas expression of *Mest* had a direct association (Figure 29B). Furthermore, in multivariate linear regression modeling the expression of *Ascl2* and *Mest* accounted for 82% of the variance in E/P

ratio. These relationships were maintained when controlling for gender ($P<0.001$; data not shown).

3.4.2 Bisulfite genomic sequencing

DMD methylation patterns were also disrupted in DNMT1o-deficient placentas. Allele specific loss of DMD methylation as measured by BGS was observed at E9.5 and E17.5 (Figures 30 and 31). Strict parent-specific DMD methylation was observed for both paternally imprinted (*H19*) and maternally imprinted (*Kcnq1*, *Snrpn*, and *Mest*) DMDs in a wild-type placentas. In contrast, significant deviations from parent-specific methylation were observed in DMDs of all DNMT1o-deficient placentas analyzed. For example, at E9.5 nearly complete loss of *Mest* DMD methylation was observed in all DNMT1o-deficient placentas (M1-M3; Figure 30). A large range of variation in the extent of methylation loss at individual DMDs was observed across placental samples. For example, whereas M1 showed a pattern of strict parent-specific methylation for the *Snrpn* DMD, M2 showed a near complete loss. In any individual E9.5 placenta there was no correlation among the four different DMDs for the extent of methylation loss. For example, M1 showed nearly complete loss of DMD methylation of the *H19* and *Mest* DMDs, yet normal methylation of the *Snrpn* DMD, whereas M2 showed nearly complete loss of DMD methylation

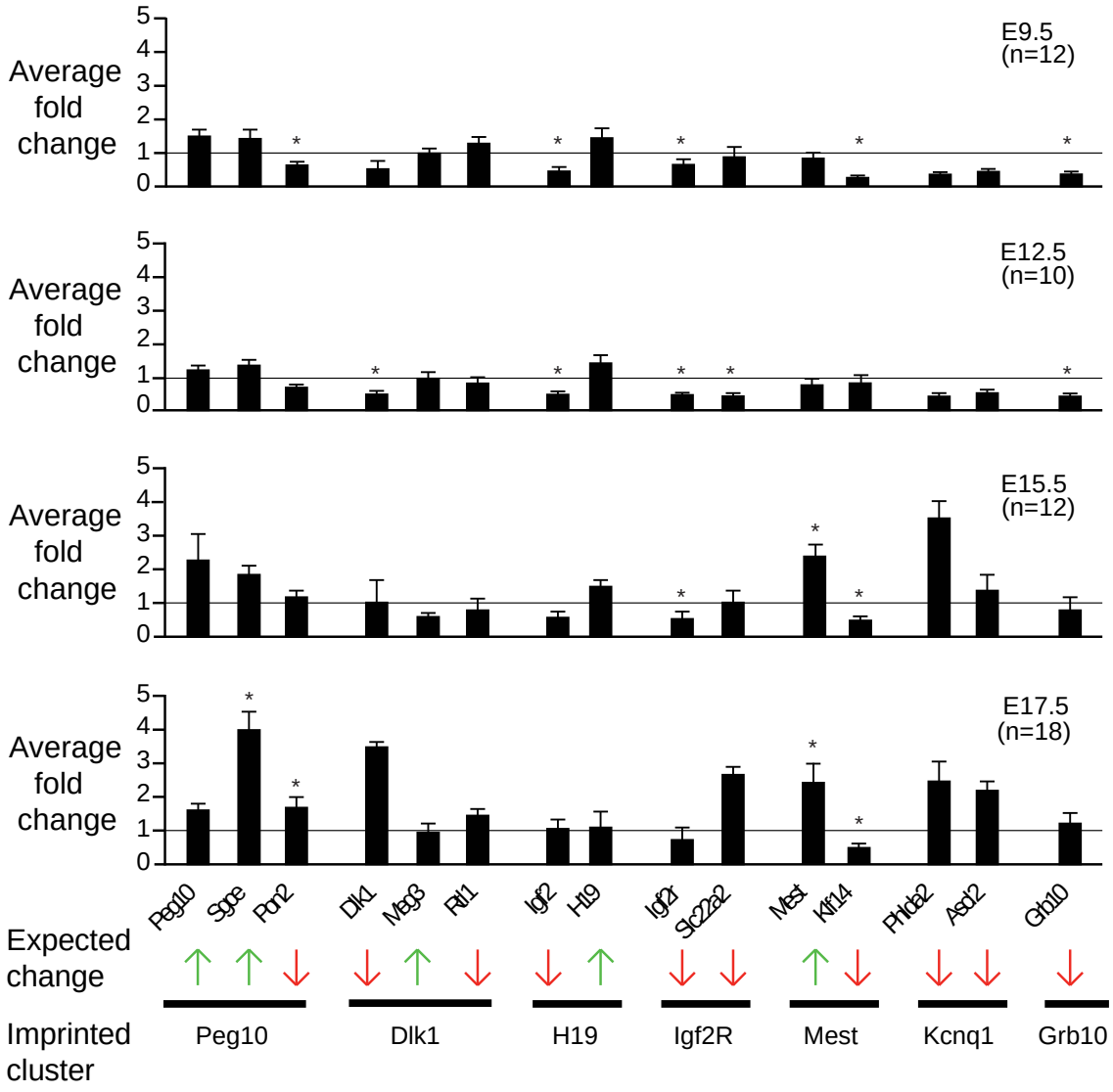


Figure 28. Imprinted gene expression patterns in DNMT1o-deficient placentas across gestation. Bar graph columns show mean expression (+SEM) of 15 imprinted genes in DNMT1o-deficient placentas compared to wild-type (n=5). Normalization to the Rpl32 gene using the ΔΔct method. (*n* = the number of DNMT1o-deficient placentas studied). * Denotes significant (P<0.05) differences in expression between DNMT1o deficient and wild-type ascertained by Kruskal Wallis test. Expected expression changes with loss of DMD methylation for each imprinted gene cluster. Green up arrows indicates expected increase in expression; Red down arrows represent expected decreased expression.

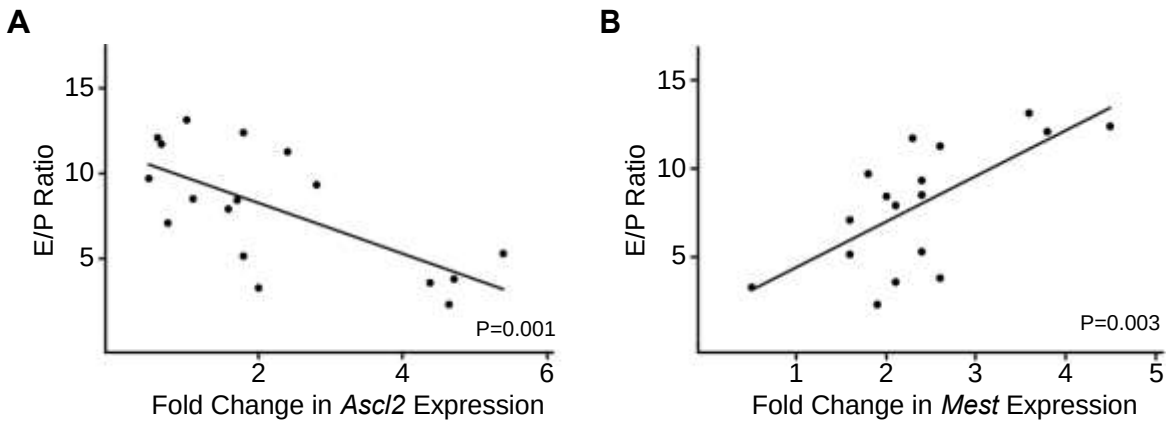


Figure 29. Linear regression of E/P Ratio and imprinted gene expression in E17.5 DNMT1o-deficient placentas. (A) Negative association between *Ascl2* expression and E/P ratio. (B) Positive association between *Mest* expression and E/P ratio.

of the *Mest*, *Kcnq1*, and *Snrpn* DMDs, yet near normal methylation of the *H19* DMD. Finally, there was no evidence in wild-type or in DNMT1o-deficient placentas of methylation on the normally unmethylated parental allele. In summation, these results indicate that DNMT1o-deficient placentas develop as cellular epigenetic mosaics with loss of DMD methylation during the first half of gestation.

At E17.5, the type and extent of DMD methylation changes were distinct from those obtained from E9.5 DNMT1o-deficient placentas (Figure 31). Loss of methylation at the *Snrpn* DMD was observed at a single DNMT1o-deficient placenta (M1) whereas the *Mest* DMD was variable in all three mutants analyzed (M1-M3; Figure 31). The *H19* and *Kcnq1* DMDs did not significantly lose methylation in the 3 analyzed E17.5 DNMT1o-deficient placentas. This finding is notably different than the variation in loss of *H19* and *Kcnq1* DMD methylation observed in E9.5 DNMT1o-deficient placentas. There was no evidence for gain of methylation on the normally unmethylated allele at any of the four DMDs. Based on these results, I conclude that while DMD epigenetic mosaicism is found in late gestaional DNMT1o-deficent placentas, the

precise makeup of mosaics transforms during the second half of gestation such that it is uncommon to recover certain patterns of DMD methylation.

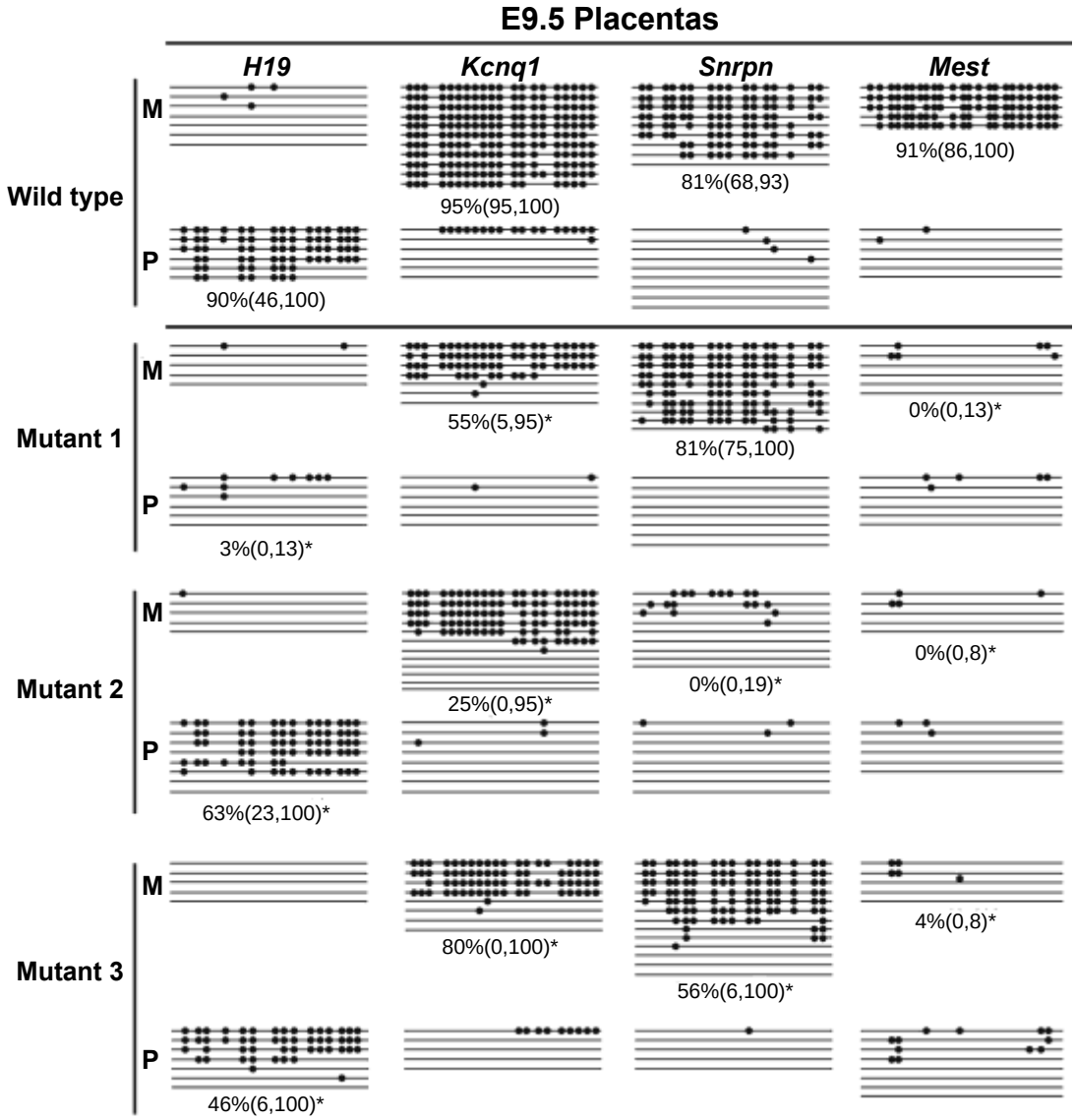


Figure 30. Variable loss of DMD methylation in E9.5 DNMT1o-deficient placentas. Bisulfite genomic sequencing of the H19, Kcnq1, Snrpn and Mest DMDs in a wild-type and three DNMT1o-deficient placentas. Abbreviations: M Maternal allele, P Paternal allele. Position of methylated CpGs are indicated as filled circles. Parantheses indicate intraquartile range. (*) significantly lower methylation than wild-type by Kruskal Wallis ($P<0.05$).

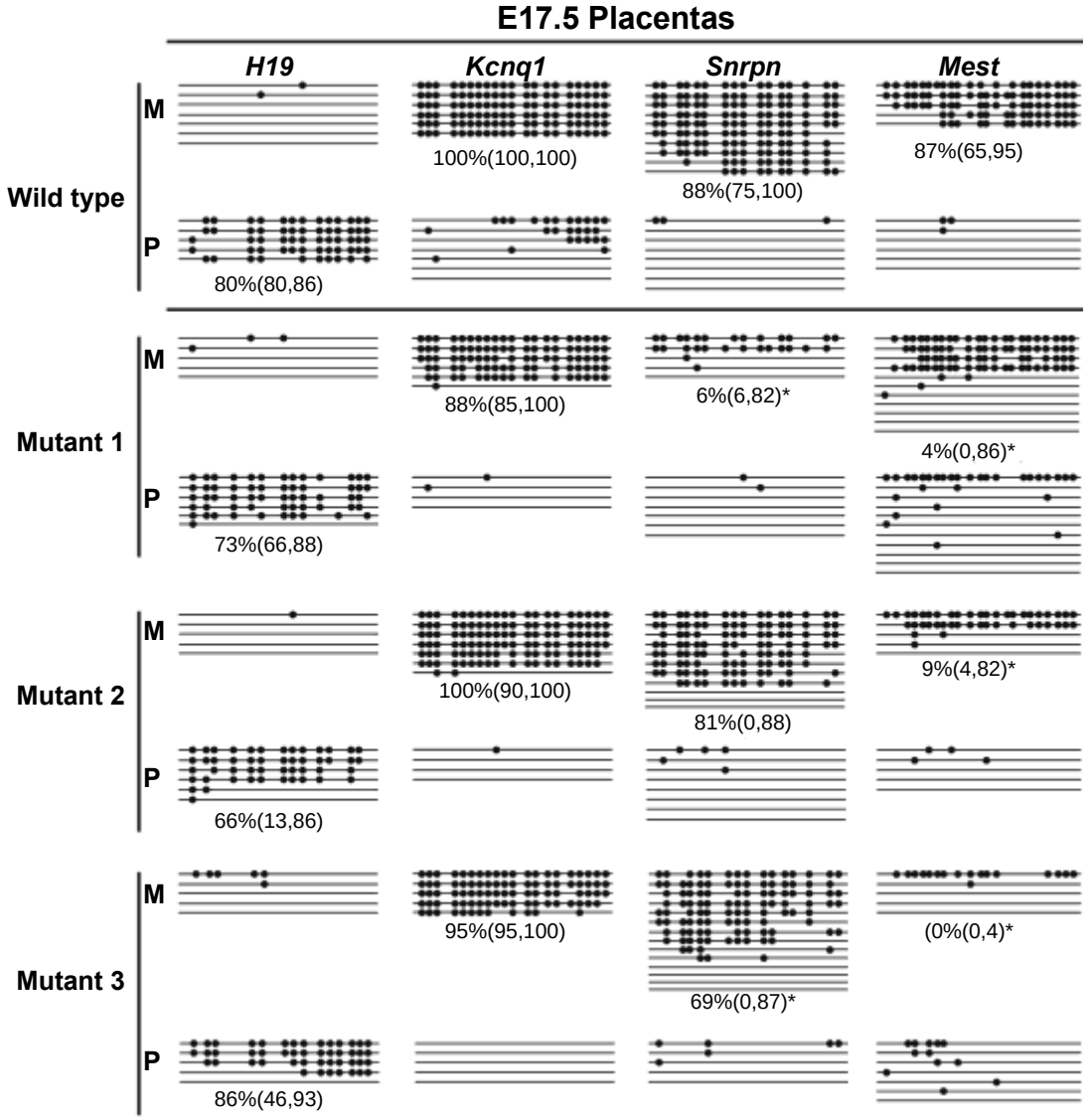


Figure 31. Variable loss of DMD methylation in E17.5 DNMT1o-deficient placentas. Bisulfite genomic sequencing of the H19, Kcnq1, Snrpn and Mest DMDs in a wild-type and three DNMT1o-deficient placentas. Abbreviations: M Maternal allele, P Paternal allele. Position of methylated CpGs are indicated as filled circles. Parantheses indicate intraquartile range. (*) significantly lower methylation than wild-type by Kruskal Wallis ($P<0.05$).

3.4.3 EpiTYPER imprinted DMD methylation analysis

To understand the role of imprinted methylation on the wide-range of placental abnormalities seen in the *Dnmt1*$^{\Delta 1o}$ model, DNA methylation was measured at 14 imprinted DMDs at three times during the latter half of gestation. The average methylation fraction across 12 non-redundant DMD EpiTYPER amplicons was calculated for both wild-type and DNMT1o-deficient placental samples at each time point. Methylation was reduced in DNMT1o-deficient placentas at E12.5, E15.5 and E17.5 (Figure 32A). At E12.5 there was a significant decrease in the average methylation across all DMDs ($P<0.001$) from 0.388 for wild-type to 0.232 for mutant placentas. In a collection of 23 E15.5 DNMT1o-deficient placentas, the average DMD methylation was 0.283, significantly lower than the wild-type average of 0.382 ($P<0.001$). Similarly in a collection of 24 E17.5 placentas average DMD methylation was 0.272, significantly lower than the wild-type average of 0.407 ($P<0.001$). There was a trend toward mutants approaching wild-type levels of imprinted DMD methylation levels as gestation progressed; average DMD methylation increased from E12.5 to E15.5 ($P<0.01$) and from E12.5 to E17.5 ($P<0.001$) but not from E15.5 to E17.5 (not significant). These findings show that total DMD methylation levels in DNMT1o-deficient placentas do not remain constant across gestation but rather resolve to more normal levels, suggesting selection against low DMD methylation epigenotypes that do not support placental development and function.

There was a large range in loss of DMD methylation at most, but not all, individual imprinted DMDs in DNMT1o-deficient placentas. The hypergeometric distribution was used to categorize DMDs as being enriched, depleted or within the expected range of loss of methylation (<0.75 wild-type levels) at E12.5 as compared to the total E12.5 DMD measurements. The *Grb10, Plag1, Igf2r, Kcnq1,* and *H19* DMDs had a number of low methylation samples within

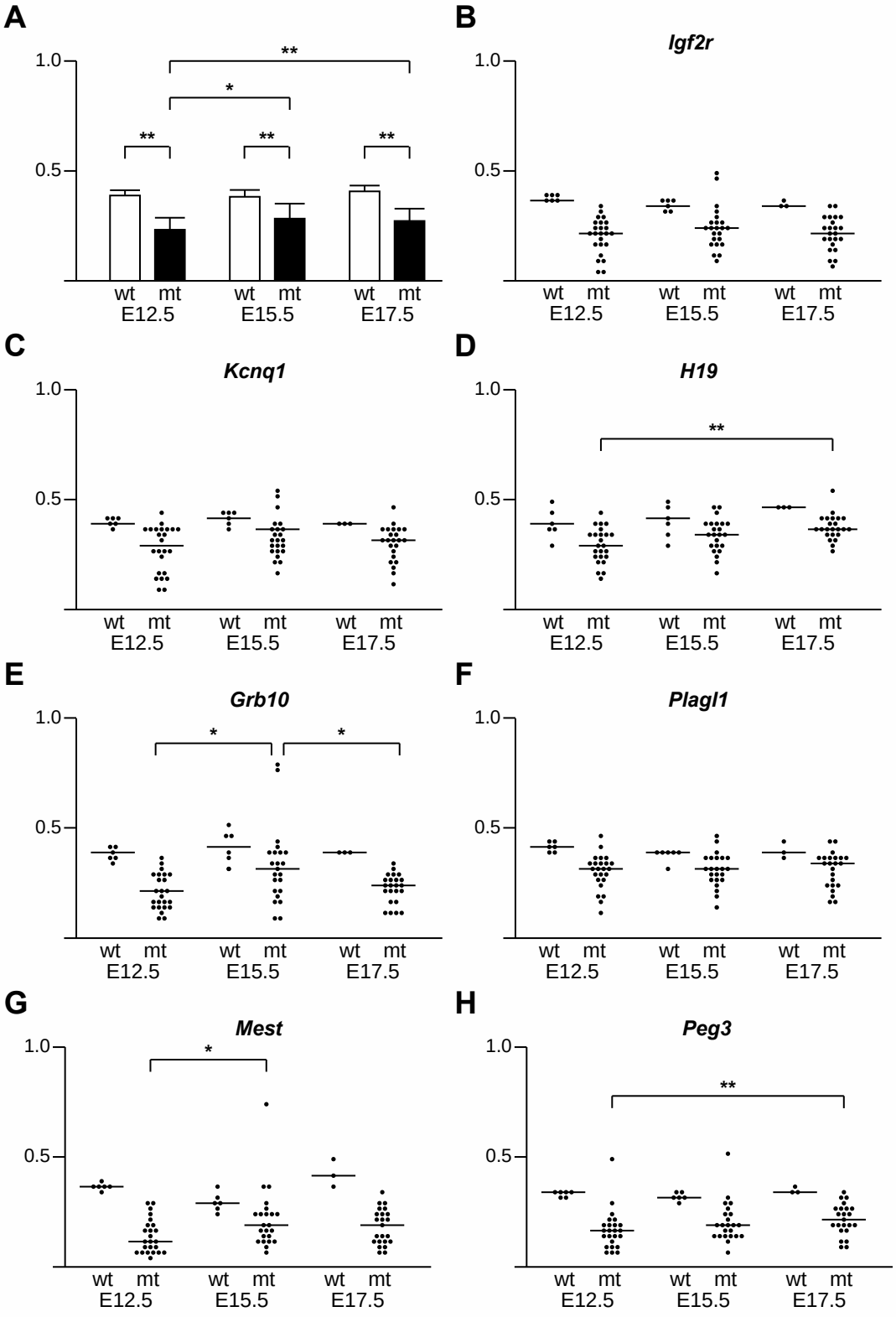
the expected range (Figures 32B-F). Other DMDs including *Peg10*, *Peg1*, *Peg3*, and *Impact* were hyper-variable and had a greater number of samples with low methylation values (Figures 32G-K). A third group of DMDs was hypovariable and depleted for low methylation values, these included *Dlk1*, *Nespas*, and *Snrpn* (Figures 32L-P). Adjacent DMD amplicons were analyzed to confirm the hypervariable *Impact* and hypovariable *Nespas* and *Dlk1* measurements. These findings are interpreted as evidence for early selection against loss of *Nespas, Snrpn* and *Dlk1* DMD methylation in trophoblast.

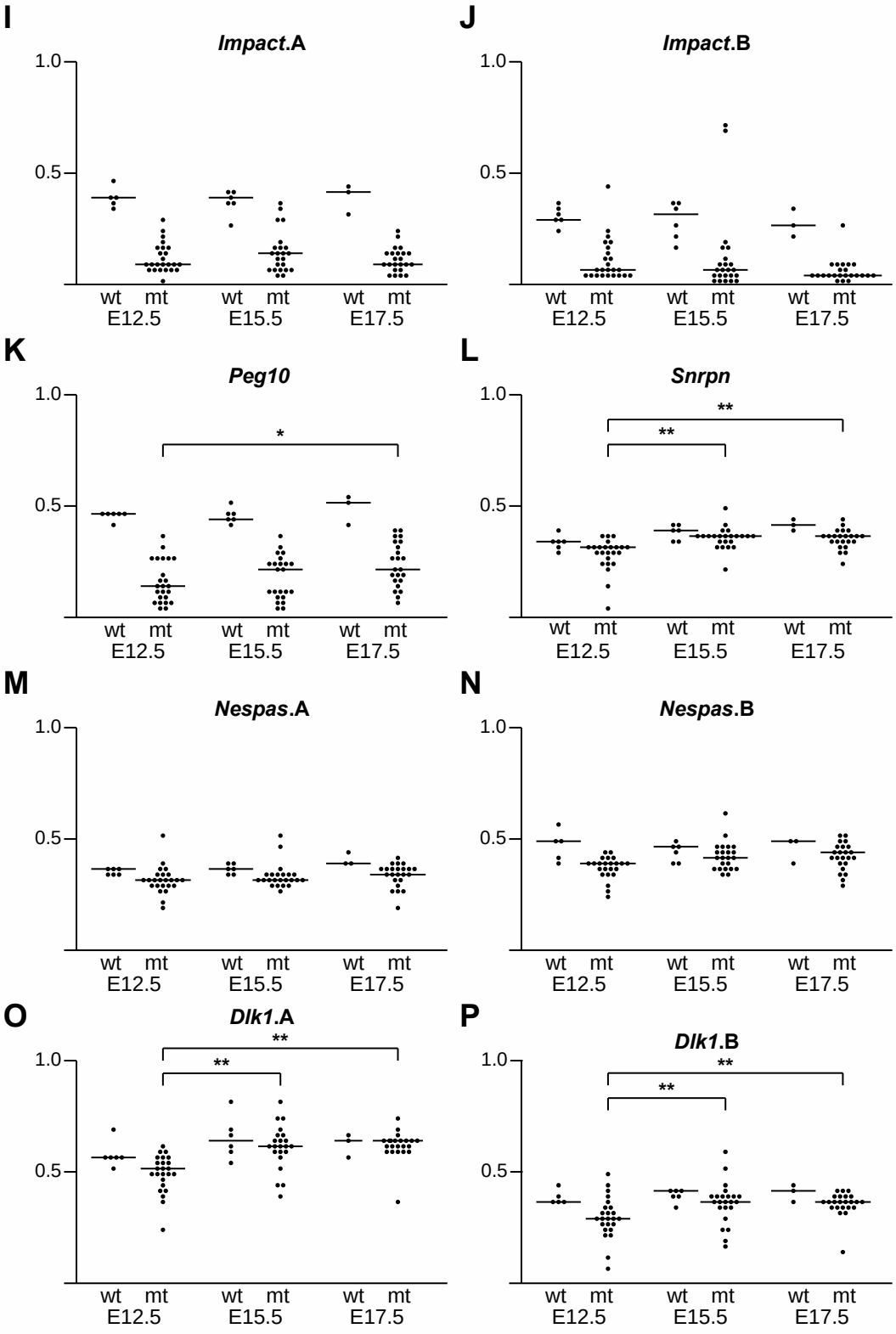
Concomitant with the increasing average imprinted DMD methylation from E12.5 to E15.5 in DNMT1o-deficient placentas (Figure 32A), I observed a steady reduction in the range of methylation among the examined placentas for many but not all individual DMDs (Figures 32B-P). Based on this, three distinct temporal patterns of methylation were defined by comparing median methylation levels across gestation for each DMD with the Rank-sum test. A group of five DMDs had higher average methylation at E15.5 than at E12.5 (*Mest*, *Snrpn*, *Dlk1*.A, *Dlk1*.B, and *Nespas*.B; $P<0.025$; Figures 32 G, L, O and P). Other DMDs had a more gradual increase in methylation from E12.5 to E17.5 (*Peg10*, *H19* and *Peg3*; $P<0.025$; Figures 32D, H and K). Five DMDs comprise a third DMD class that did not significantly change their average methylation across gestation in DNMT1o-deficient placentas (*Igf2r, Kcnq1 Plagl1*, *Impact*.A, *Impact*.B, *Nespas*.A, and *Nespas*.B; Figures 32B, C, F, I, J, M and N). Out of the three imprinted DMDS for which duplicate adjacent EpiTYPER amplicons were selected (*Dlk1*, *Impact* and *Nespas*), only *Nespas* showed a discordant trend with *Nespas*.A not differing between gestational cohorts and *Nespas*.B transitioning to higher average methylation between E12.5 and E15.5 (Figures 32M and N). Additionally the *Grb10* DMD displayed opposing

changes from E12.5 to E15.5 and E15.5 to E17.5, and did not significantly differ between E12.5 and E17.5 (Figure 32E).

Fisher's exact test was used to confirm these temporal patterns by comparing the number of low methylation placentas using a cutoff of <0.75 or <0.5 wild-type levels. These findings were slightly different. Using a low methylation cutoff of <0.5 wild-type the Fisher's exact test revealed that *Mest* and *Kcnq1* DMDs had significantly fewer low methylation samples at E15.5 than at E12.5, and the *Peg3* DMD had a significant gradual decrease in low methylation samples from E12.5 to E17.5. With a less stringent low methylation cutoff of <0.75 the Fisher's exact test revealed that *Nespas* and *Mest* had a significant decrease in low methylation samples between E12.5 and E15.5, and *Dlk1*.B had a significant decrease in low methylation samples between E12.5 and E17.5. No significant changes were unearthed within the E15 to E17 transition using Fisher's exact test with either cutoff. These results indicate selective pressure against loss of methylation of the *Mest*, *Kcnq1*, *Nespas*, *Peg3* and *Dlk1* DMDs during mid-gestation.

Three additional imprinted DMDs were examined in E12.5 and E15.5 cohorts (*Zrsr1*, *Nnat* and *Nap1l5*), and only *Zrsr1* had a significant difference between wild-type and the *Dnmt1*$^{\Delta 1o}$ mutant average methylation levels (Figures 32Q-S). Each of these DMDs is within a putative microimprinted domain (Section 1.5.1, 1.5.4 and 1.5.12; Figures 2A, D and L). In DNMT1o-deficient placentas *Zrsr1* remained variable and did not differ between E12.5 and E15.5. Overall, the observed trends in DMD methylation during gestation suggest that there are strong biological influences blocking the loss of imprints at specific DMDs during mid-gestation.


A
B
Igf2r
C
Kcnq1
D
H19
E
Grb10
F
Plagl1
G
Mest
H
Peg3
1.0
0.5
wt mt
E12.5
E15.5
E17.5
*
**


I
Impact.A
J
Impact.B
K
Peg10
L
Snrpn
M
Nespas.A
N
Nespas.B
O
Dlk1.A
P
Dlk1.B
1.0
0.5
wt mt
E12.5
E15.5
E17.5
*
**

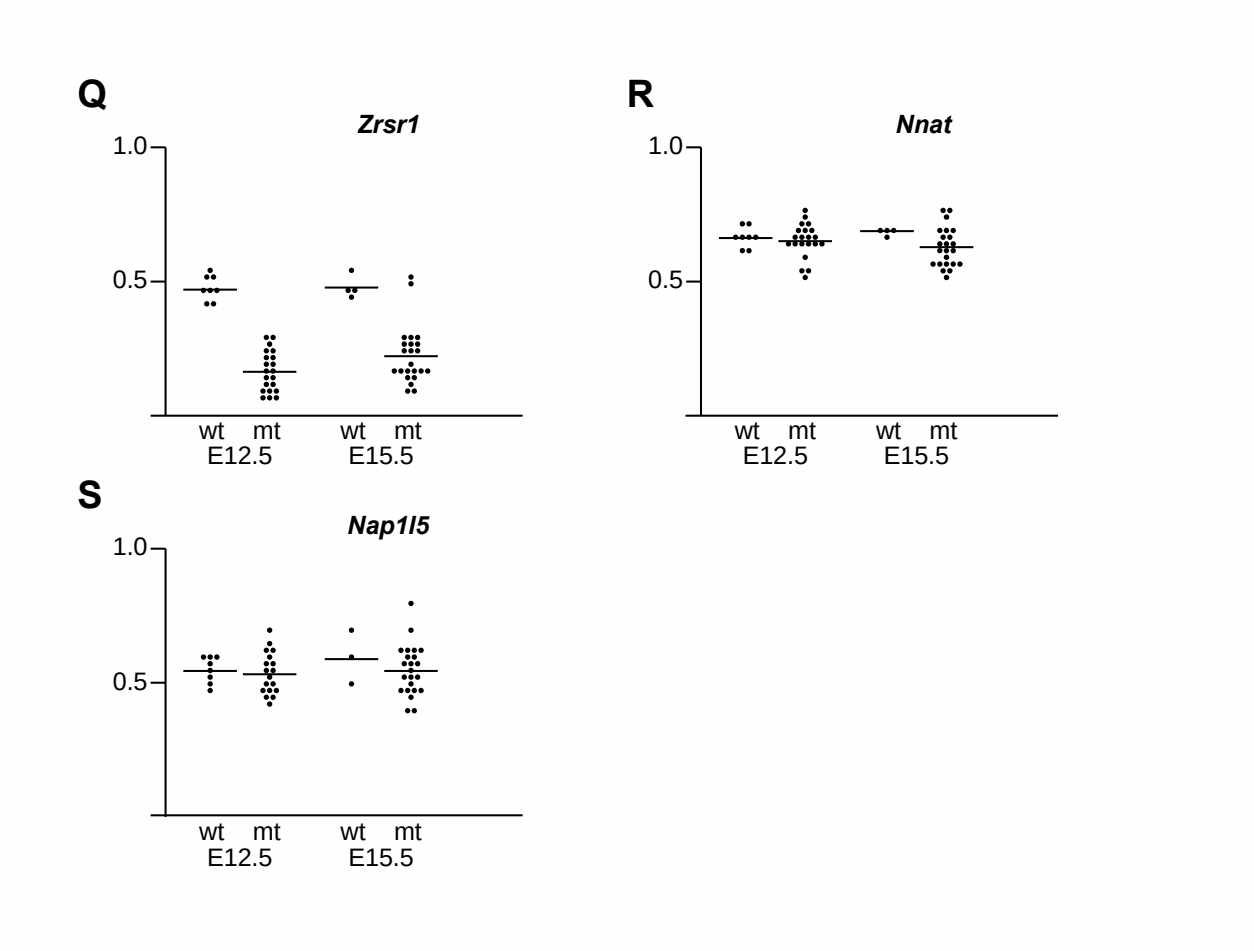


Figure 32. Imprinted DMD methylation levels in wild-type (wt) and DNMT1o-deficient (mt) placentas across mid gestation (E12.5, E15.5 and E17.5). (A) Bar graphs showing average mean and standard deviation of total imprinted DMD methylation of wt (open bars) and mt (filled bars) across mid-gestation. (B-S) Binned scatter plot showing individual wt and mt placentas across mid-gestation and the sample mean for the following imprinted DMDs: (B) *Igf2r*, (C) *Kcnq1*, (D) *H19*, (E) *Grb10*, (F) *Plagl1*, (G) *Mest* (H) *Peg3* (I) *Impact.*A, (J) *Impact.*B, (K) *Peg10*, (L) *Snrpn*, (M) *Nespas.*A, (N) *Nespas.*B, (O) *Dlk1*.A, (P) *Dlk1*.B, (Q) *Zrsr1*, (R) *Nnat*, and (S) *Nap1l5*. Small brackets indicate significant differences between gestational age matched sample populations of wt and mt DMD methylation medians. Larger brackets indicate significant differences between mutant DMD methylation medians at different gestational ages. * ($P<0.01$), and ** ($P<0.001$) denote significant differences of mutant median imprinted DMD methylation compared to wild type, or between gestational ages of mutant sample population by the Rank-sum test. DMDs are organized by hypergeometric distribution as having a number of low methylation samples falling within the expected range (B-F), greater than the expected range (G-K) or less than expected range (L-P).

The spectrum of methylation among 12 DMDs for each individual E12.5 DNMT1o-deficient placenta is displayed in the form of a heat map clustergram (Fig 33). Among the 24 E12.5 placentas represented in this manner, the majority of placentas have a unique DMD methylation profile not found in other placentas, although there are a few cases of high similarity. For example placentas A8 and B2 have identical DMD methylation profiles. Placentas A4 and B5 differ only at *Grb10, Kcnq1* and *H19* DMDs, and placentas A3 and C1 are unique at only the *Plagl1* DMD. Clustering of the DMDs at E12.5 indicate the genetically linked *Peg10* and *Mest* DMDs as well as the linked *Kcnq1* and *Snrpn* DMDs vary in conjunction. Although there is a trend toward more normal DMD methylation levels at E15.5 and E17.5, each DNMT1o-deficient placenta at these stages still has a unique imprinted epigenotype (Figures 33 and 34). These comparisons among placentas across the latter half of gestation point out the intrinsic power of the $Dnmt1^{\Delta 1o}$ maternal effect model to produce diverse and abnormal patterns of imprinted DMD methylation.

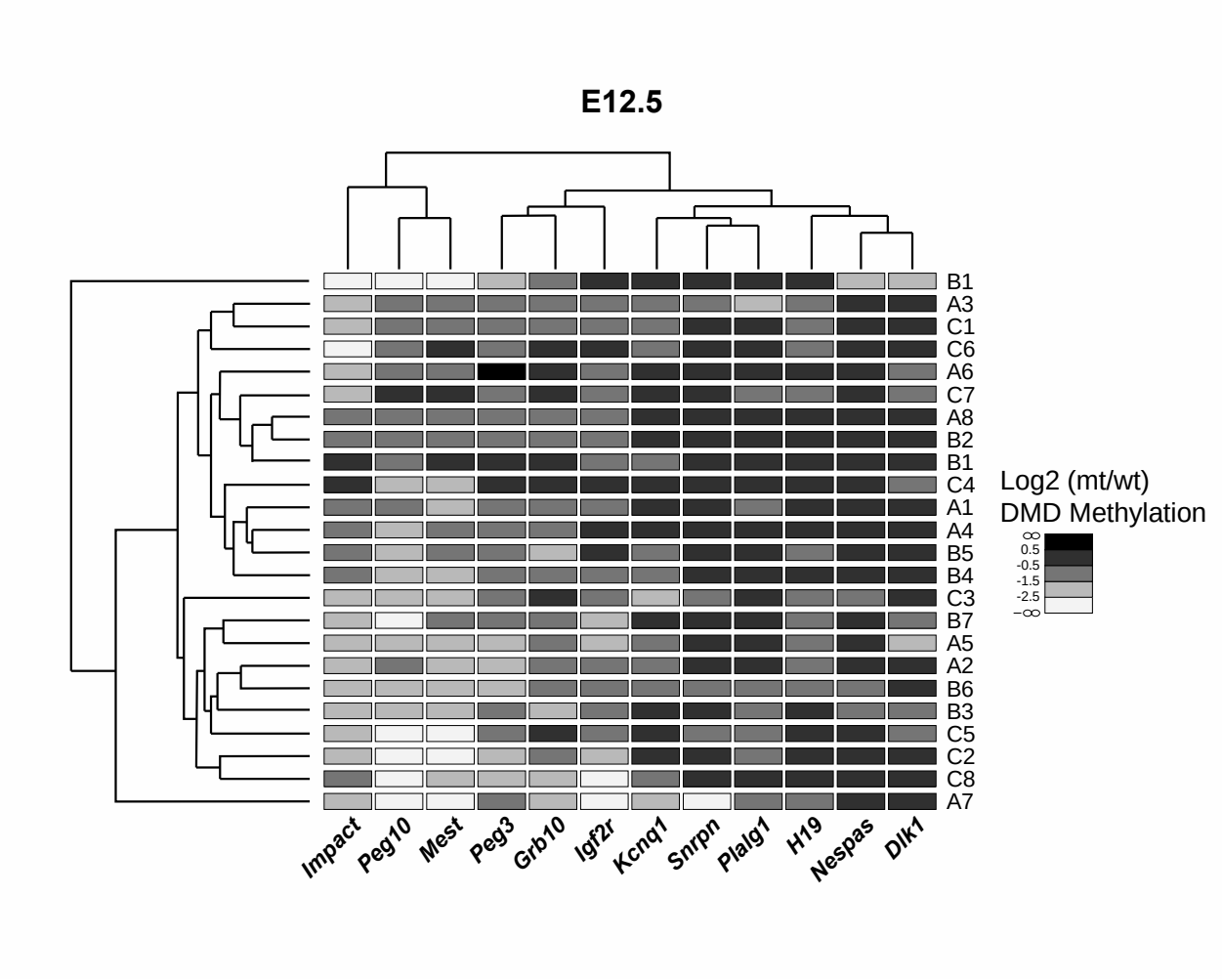


Figure 33. Hierarchical clustering of 24 E12.5 DNMT1o-deficient placentas based on DMD methylation. Data is shown as the log2 transformed ratio of mt:wt DMD methylation. The heat map displays normally methylated DMDs as dark boxes whereas loss of methylation is indicated by lighter shades. The upper and side dendrograms display linkage between imprinted DMDs and DNMT1o-deficient samples respectively. Imprinted DMDs are labeled across the bottom axis. DNMT1o-deficient samples are labeled down the right hand side by cohort litter (Letters A-C) and conceptus (Numbers 1-8).

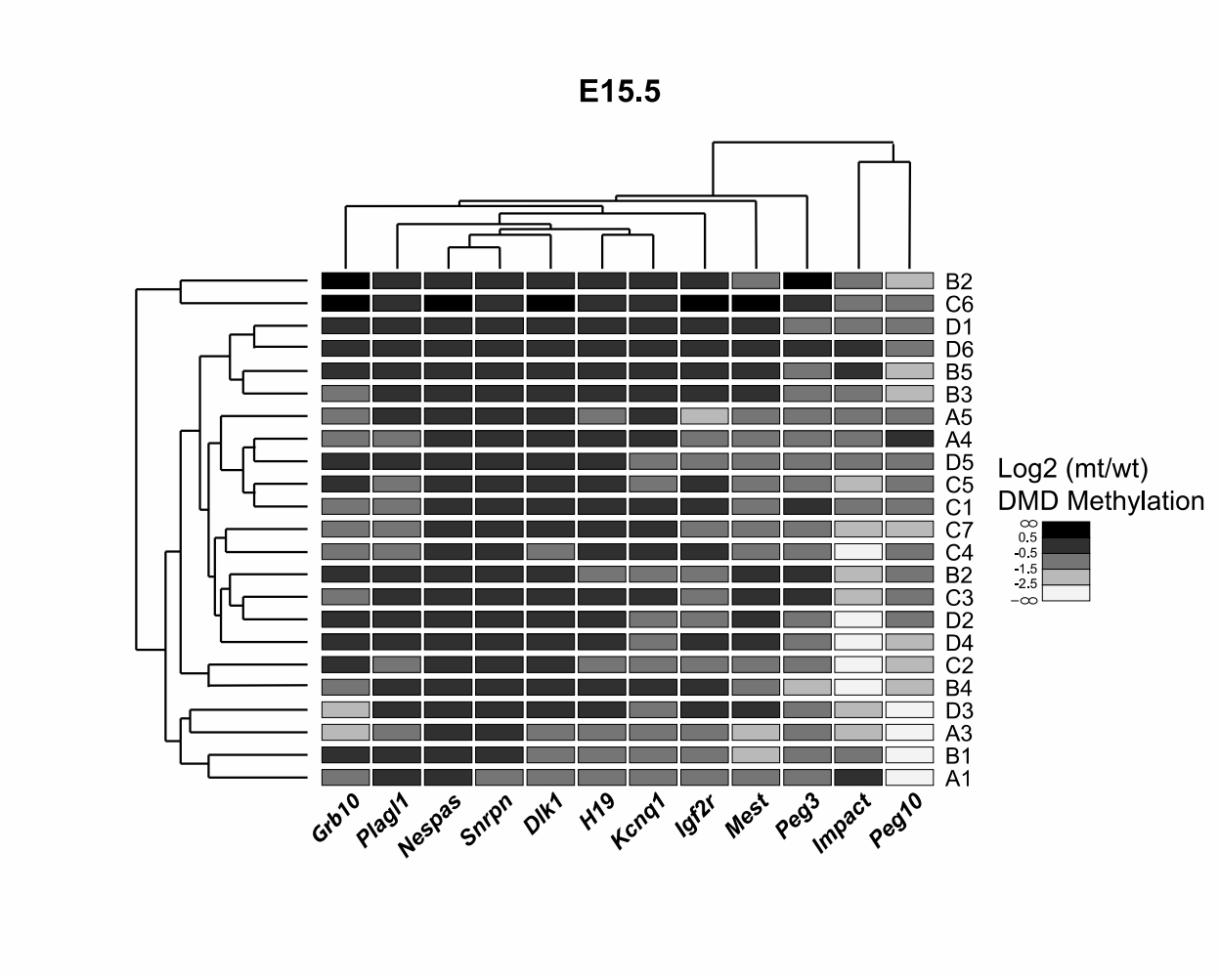


Figure 34. Hierarchical clustering of 23 E15.5 DNMT1o-deficient placentas based on DMD methylation. Data is shown as the log2 transformed ratio of mt:wt DMD methylation. The heat map displays normally methylated DMDs as dark boxes whereas loss of methylation is indicated by lighter shades. The upper and side dendrograms display linkage between imprinted DMDs and DNMT1o-deficient samples respectively. Imprinted DMDs are labeled across the bottom axis. DNMT1o-deficient samples are labeled down the right hand side by cohort litter (Letters A-D) and conceptus (Numbers 1-8).

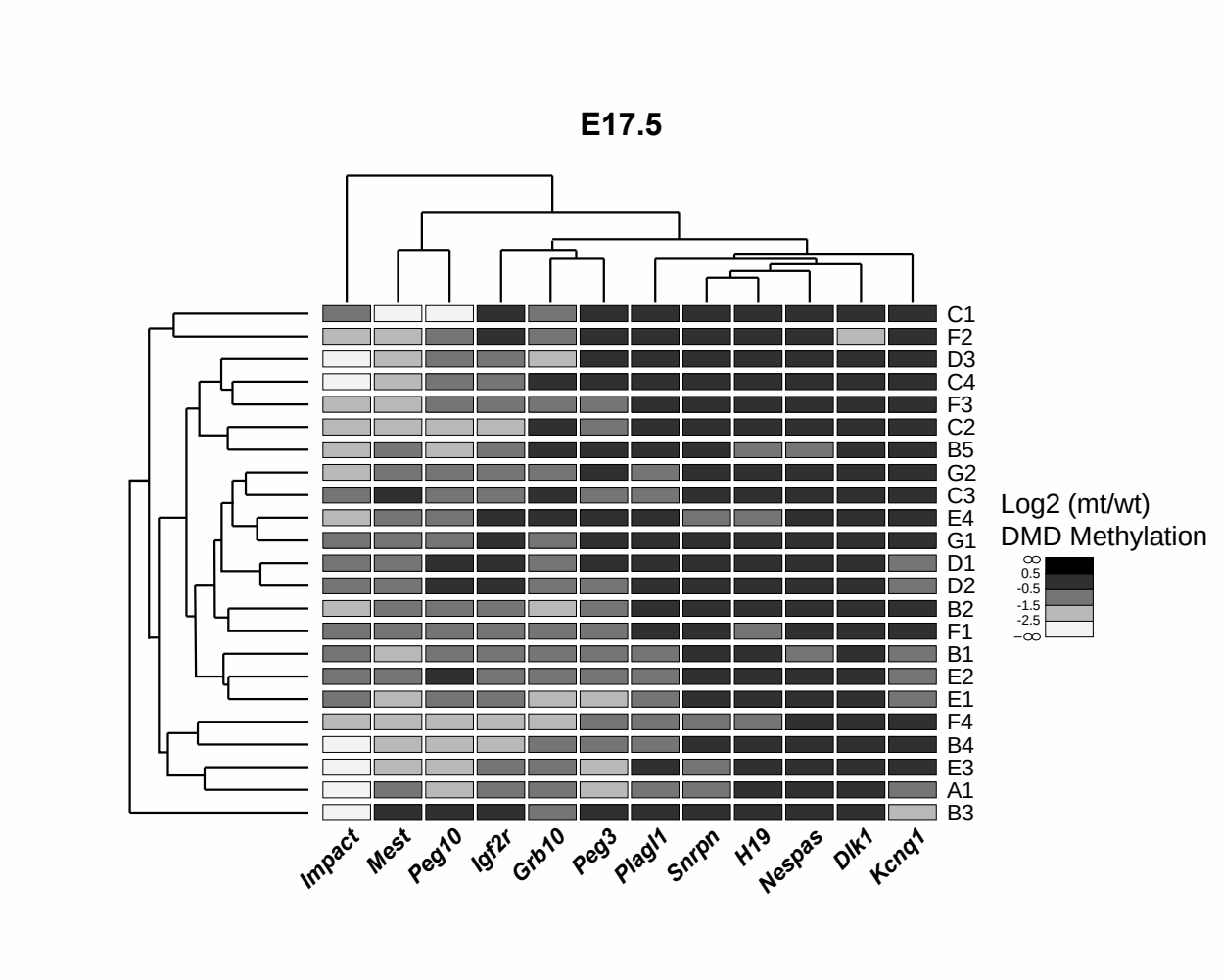


Figure 35. Hierarchical clustering of 23 E17.5 DNMT1o-deficient placentas based on DMD methylation. Data is shown as the log2 transformed ratio of mt:wt DMD methylation. The heat map displays normally methylated DMDs as dark boxes whereas loss of methylation is indicated by lighter shades. The upper and side dendrograms display linkage between imprinted DMDs and DNMT1o-deficient samples respectively. Imprinted DMDs are labeled across the bottom axis. DNMT1o-deficient samples are labeled down the right hand side by cohort litter (Letters A-G) and conceptus (Numbers 1-8).

3.4.4 Decreased fetal viability is associated with loss of *Peg10* DMD methylation

Logistic regression was used to identify those imprinted DMDs that exerted the greatest influence on fetal viability at E12.5 through placental imprinting (Table 3). The logistic regression coefficient (logit) is reported as a measure of the effect of DMD methylation levels on the odds ratio of fetal survival. A positive association was discovered between *Peg10* DMD methylation and fetal viability at E12.5 ($P<0.05$) indicating that placentas with loss of the *Peg10* methylation imprint are less likely to support a viable fetus. A negative association between *Nnat* DMD methylation and fetal viability was observed ($P<0.05$). The only significant association identified between imprinted DMD methylation and fetal viability at either E15.5 or E17.5 was a negative association between *Nespas*.B DMD methylation and viability at E15.5 ($P<0.05$; Table 3). These findings suggest that in the context of the *Dnmt1*$^{\Delta 1o}$ maternal effect mouse model, nearly normal *Nnat* and *Nespas* imprinting may decrease viability.

Gestational Age	DMD	Logit	P-Value
E12.5 (n=24)	*Peg10*	+2.17	3.25E-02
	Nnat	-2.49	4.12E-02
E15.5 (n=23)	*Nespas*.B	-1.05	3.69E-02
E17.5[a] (n=23)			

Table 3. Logistic regression of E12.5, E15.5 and E17.5 DNMT1o-deficient placentas based on DMD methylation and fetal viability. Only significant ($P<0.05$) associations established by logistic regression analysis between dependent fetal viability and independent imprinted DMD methylation values are shown. The log odds ratio(logit) is the coefficient indicating the direction and strencth of the relationship. ([a]) no significant associations were found at E17.5.

3.4.5 Placental abnormalities are associated with loss of DMD methylation

Because of the broader range of abnormal DMD methylation, histomorphological abnormalities and effects on fetal viability at E12.5 I focused primarily on phenotype-epigenotype regression analysis at the E12.5 time point. Bivariate linear regression analysis was used to determine which imprinted DMDs underlie the observed E12.5 placental abnormalities. The most significant ($P<0.05$) DMD associations for each phenotype are displayed in Table 3 and Figure 36. The regression coefficient (β) is reported as the change in phenotype associated with modulation of the DMD methylation fraction (0 to 1.0). Placenta weight is negatively associated with DMD methylation at *Nespas*.A (Table 4 and Figure 36A) although not at *Nespas*.B. For each 1% decrease in *Nespas*.A DMD methylation (0.01 methylation fraction) placental weight increased by a corresponding 1.275 milligrams (95% CI: 0.648, 1.902). Spongiotrophoblast volume was negatively associated with both analyzed *Nespas* regions as well as the *H19* DMD (Table 4 and Figures 36B-D). Each 1% decrease in DMD methylation at *Nespas*.A, *Nespas*.B and *H19* increased JZ volume by 0.0399 (95% CI: 0.0258, 0.054), 0.0266 (95% CI: 0.035, 0.0497) and 0.0170 (95% CI: 0.0017, 0.0323) mm^3 respectively.

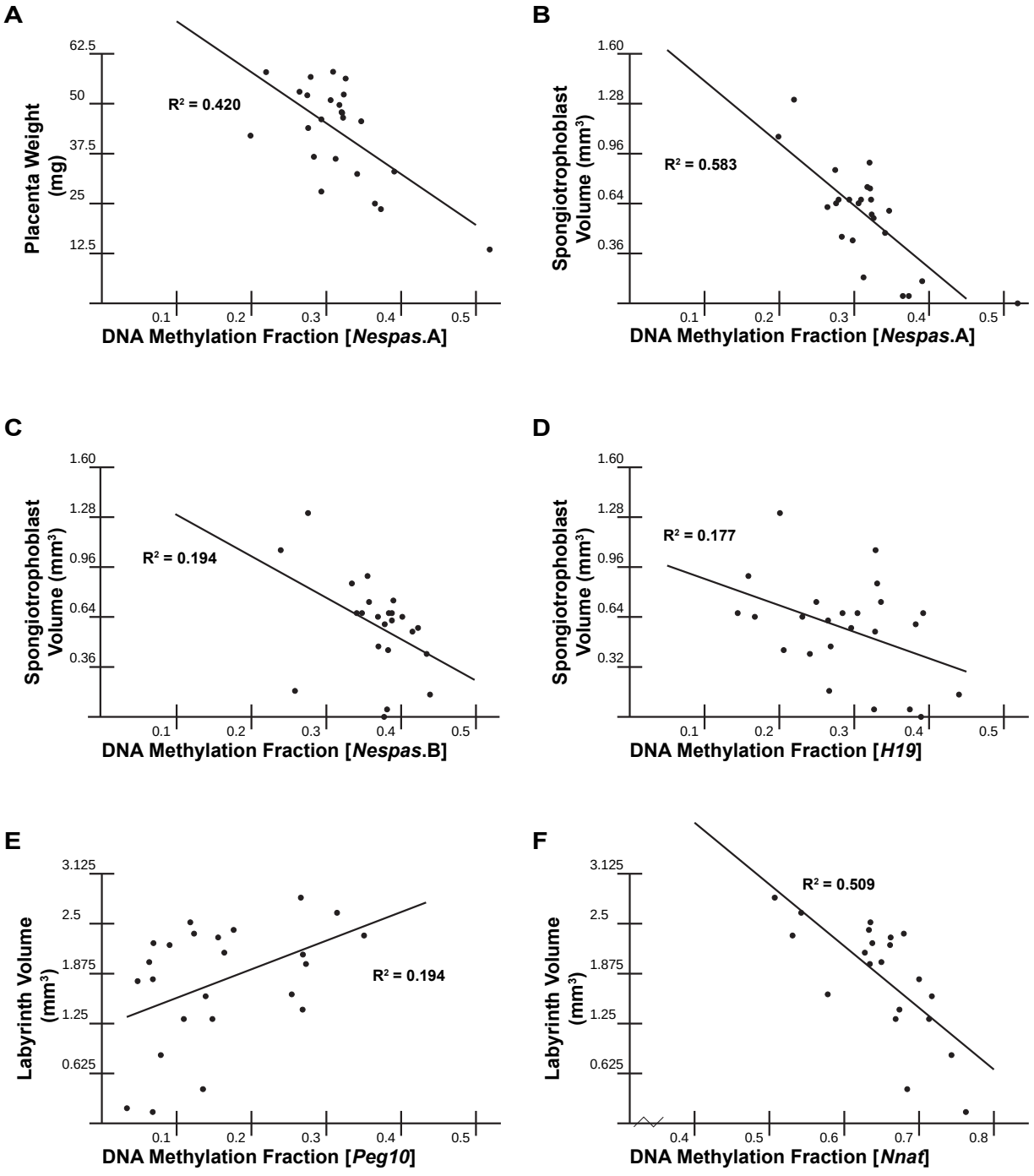
Linear regression analysis revealed a strong association between *Peg10* DMD methylation and LZ volume (Table 4 and Figure 36E). Diminishment of *Peg10* DMD methylation by 1% corresponds to a 0.0217 (95% CI: 0.002, 0.066) mm^3 decrease in LZ central volume. Labyrinth structures in three DNMT1o-deficient placentas with low *Peg10* DMD methylation are shown in Fig 4. Labyrinths in these samples are noticeably smaller, disorganized and hemorrhagic. Notably, methylation of the *Nnat* DMD is negatively associated with LZ volume (Table 4 and Figure 36F), counter to the observed trend of decreased LZ in DNMT1o-

deficient placentas; a 1% decrease in *Nnat* DMD methylation resulting in a 0.0249 (95% CI: 0.043, 0.111) mm^3 increase in LZ central volume.

Placental Phenotype	DMD	β	P-Value
Placental Weight (mg)	*Nespas*.A	-127.5	6.21E-04
Spongiotrophoblast Central Volume (mm^3)	*Nespas*.A	-3.99	1.41E-05
	Nespas.B	-2.65	3.54E-02
	H19	-1.7	4.08E-02
Labyrinth Central Volume (mm^3)	*Peg10*	+3.58	3.12E-02
	Nnat	-7.72	2.82E-04
Trophoblast Giant Cell Count (#/section)	*Kcnq1*	-508	1.87E-05
	Snrpn	-674	2.31E-04
	Plagl1	-438	1.97E-02
	Nespas.B	-575	4.38E-02

Table 4. Bivariate regression analysis of 24 E12.5 DNMT1o-deficient placentas based on DMD methylation and placental phenotypes. Only significant ($P<0.05$) associations established by bivariate regression analysis between dependent placental phenotypes and independent imprinted DMD methylation values are shown. β is the linear regression coefficient.

Bivariate regression analysis revealed a significant negative association between *Kcnq1* DMD methylation and accumulation of TGCs (Table 3 and Figure 36G). A 1% decrease in *Kcnq1* DMD methylation corresponds to an increase of 5.08 (95% CI: 3.25, 6.91) TGCs per histological section. Representative H&E and *ISH* stained histological sections of wild-type and DNMT1o-deficient placentas with very low *Kcnq1* DMD methylation and pronounced expansion of parietal TGCs bordering the JZ are displayed in Figure 5. Positive *ISH* staining for the pan-TGC transcripts *Prl2c2* and *Prl3b1* was observed in both parietal TGCs and JZ layers (Figure 5). Intriguingly, the early TGC marker Prolactin-1 (*Prl3d1*) was ectopically expressed in the parietal TGCs of DNMT1o deficient placentas with low *Kcnq1* DMD methylation, where as it should be restricted to TGCs embedded within maternal spiral arteries by E12.5.


A
Placenta Weight (mg)
62.5
50
37.5
25
12.5
R² = 0.420
0.1
0.2
0.3
0.4
0.5
DNA Methylation Fraction [Nespas.A]
B
Spongiotrophoblast Volume (mm³)
1.60
1.28
0.96
0.64
0.36
R² = 0.583
0.1
0.2
0.3
0.4
0.5
DNA Methylation Fraction [Nespas.A]
C
Spongiotrophoblast Volume (mm³)
1.60
1.28
0.96
0.64
0.36
R² = 0.194
0.1
0.2
0.3
0.4
0.5
DNA Methylation Fraction [Nespas.B]
D
Spongiotrophoblast Volume (mm³)
1.60
1.28
0.96
0.64
0.32
R² = 0.177
0.1
0.2
0.3
0.4
0.5
DNA Methylation Fraction [H19]
E
Labyrinth Volume (mm³)
3.125
2.5
1.875
1.25
0.625
R² = 0.194
0.1
0.2
0.3
0.4
0.5
DNA Methylation Fraction [Peg10]
F
Labyrinth Volume (mm³)
3.125
2.5
1.875
1.25
0.625
R² = 0.509
0.4
0.5
0.6
0.7
0.8
DNA Methylation Fraction [Nnat]

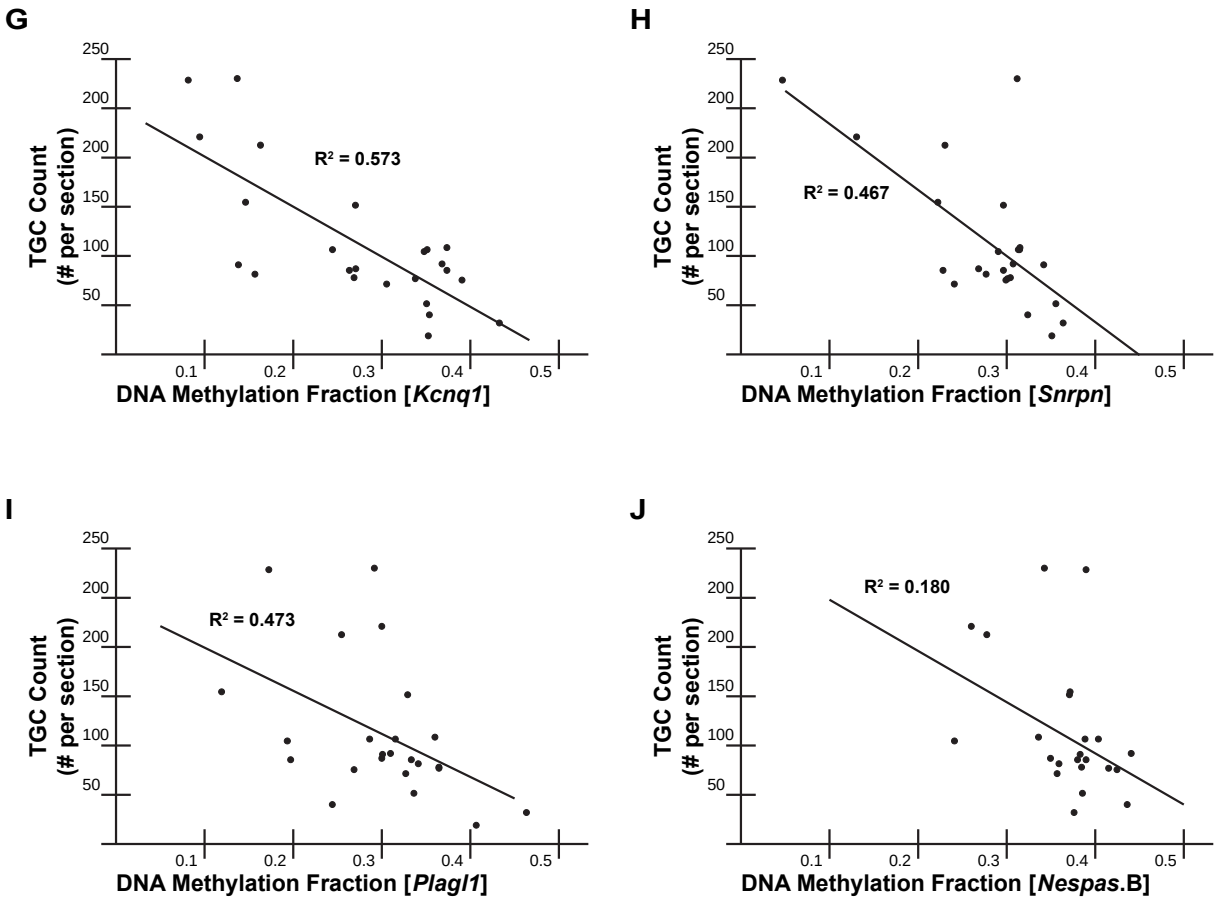


Figure 36. Linear regression plots of imprinted DMD methylation versus placental phenotypic metrics in a cohort of E12.5 DNMT1o-deficient placentas. (A) Negative association between *Nespas*.A DMD methylation and placental weight. (B) Negative association between *Nespas*.A DMD methylation and spongiotrophoblast volume. (C) Negative association between *Nespas*.B DMD methylation and spongiotrophoblast volume. (D) Negative association between *H19* DMD methylation and spongiotrophoblast volume. (E) Positive association between *Peg10* DMD methylation and labyrinth volume. (F) Negative association between *Nnat* DMD methylation and labyrinth volume. (G) Negative association between *Kcnq1* DMD methylation and TGC counts. (H) Negative association between *Snrpn* DMD methylation and TGC counts. (I) Negative association between *Plagl1* DMD methylation and TGC counts. (J) Negative association between *Nespas*.B DMD methylation and TGC counts. R^2 is unadjusted R-square value.

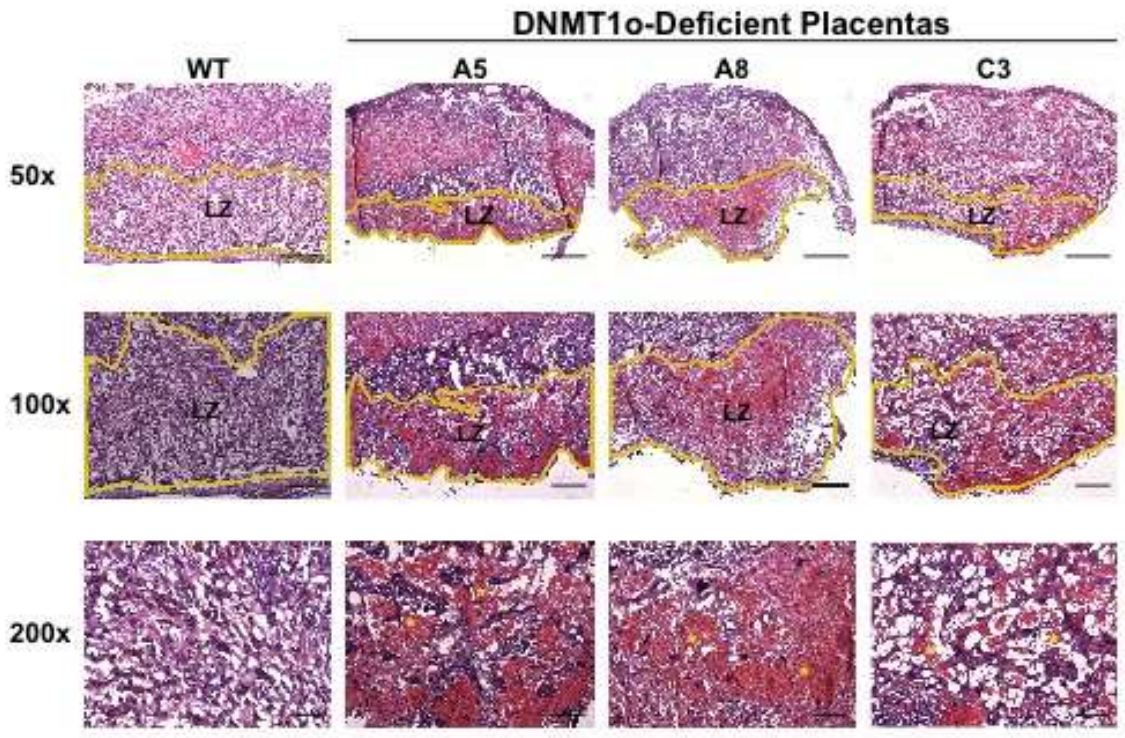


Figure 37. Histology of hematoxylin and eosin (H&E) stained labyrinth of one wild-type (wt) and three DNMT1o-deficient low-*Peg10* DMD methylation placentas. The scale bars for 50X, 100X and 200X magnification are 500, 200 and 100 μm respectively. Yellow lines in 50x and 100x magnification images outline the labyrinthine zone (LZ).

DNA methylation at the genetically linked *Snrpn* DMD (both *Kcnq1* and *Snrpn* DMDs are on mouse chromosome 7) also inversely associated with TGC accumulation (Table 3 and Figure 36H). For every 1% decrease in *Snrpn* DMD methylation there is a corresponding increase of 6.74 (95% CI: 3.73, 9.75) TGCs per section. A weaker inverse association between both *Plagl1* and *Nespas*.B DMD methylation and TGC number was also identified (Table 3 and Figures 36I and 36J). Decreases of 1% methylation at *Plagl1* and *Nespas*.B modulate an increase in TGCs per section of 4.38(95% CI: 0.970, 7.79) and 5.75(95% CI: 0.500, 11.0) respectively. Imprinted DNA methylation at the *Peg3* DMD, which like *Kcnq1* and *Snrpn* is a maternally derived methylation imprint on mouse chromosome 7, was not significantly associated with TGC

accumulation (P=0.40). Linear regression model building confirmed the major DMD methylation influence of TGC accumulation to that of just the *Kcnq1* and *Snrpn* DMDs (S2 Table).

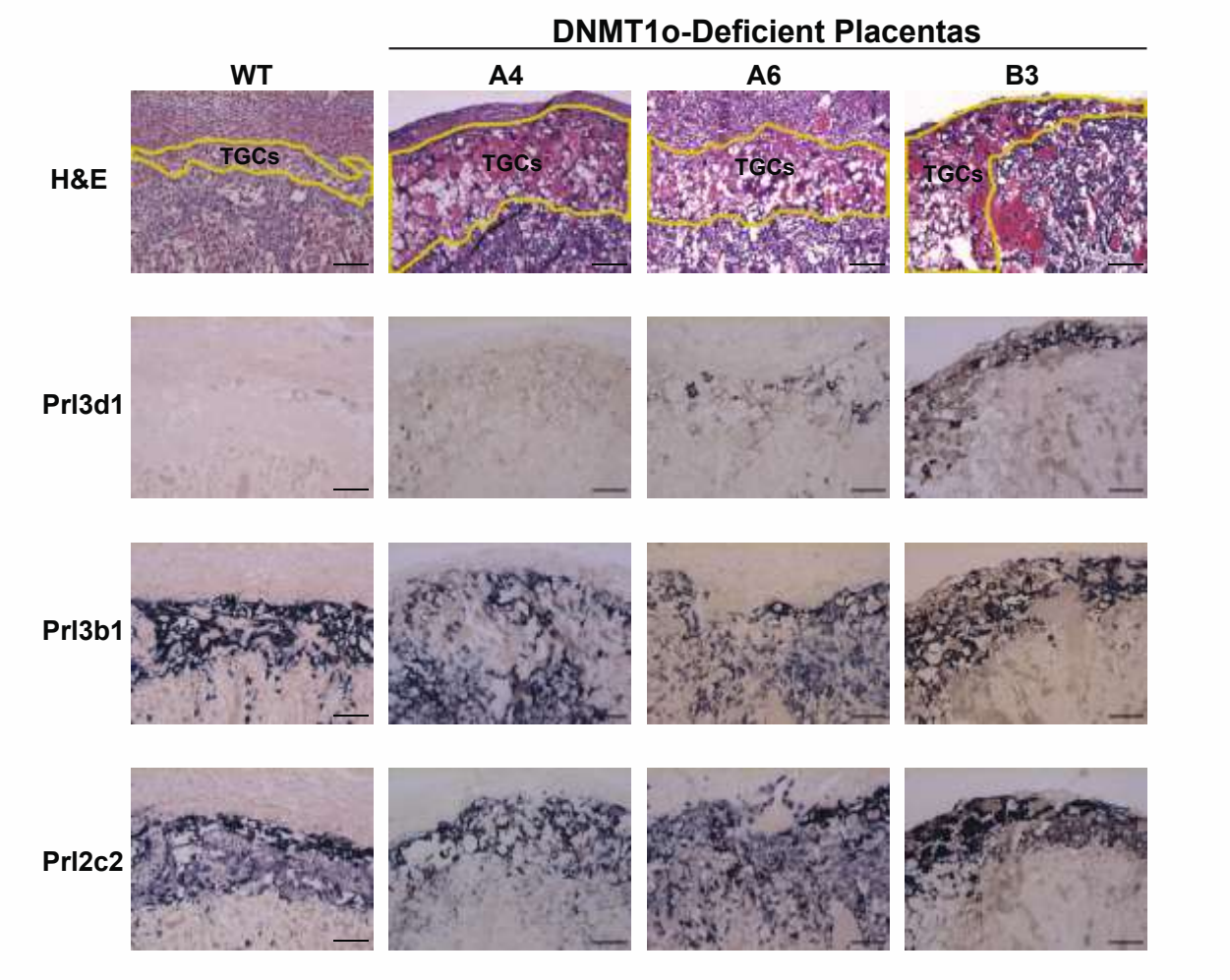


Figure 38. In situ hybridization analysis of TGCs in E12.5 wild-type and DNMT1o-deficient placentas with low *Kcnq1* DMD methylation. All images were taken at 100X magnification. The scale bar is 100μm. Yellow lines delineate the layer containing trophoblast giant cells (TGCs) in the top row displays histology of hematoxylin and eosin (H&E) stained sections. *ISH* for the prolactin gene family members *Prl3d1*, *Prl3b1* and *Prl2c2* on adjacent sections to H&E are shown in the lower three rows.

Parameter	Estimate	SE	P-Value
Intercept	+310	36.8	3.54E-08
Kcnq1 Regression Coefficient (β)	-364	109	3.11E-03
Snrpn Regression Coefficient (β)	-350	160	4.01E-02

Table 5. Stepwise forward linear regression analysis of associations between imprinted DMD methylation and TGC accumulation in E12.5 DNMT1o-deficient placentas. N=24, df=21, model P-value=1.54×10^{-5}

I performed bivariate linear regression to determine if there were any phenotype-epigenotype associations at E15.5 and E17.5. Spongiotrophoblast central volume inversely associated with *Impact*.B and *Mest* methylation at E15.5 ($P<0.05$; Table 6). Each 1% decrease in *Impact*.B and *Mest* methylation increased JZ central volume by 0.0717 (95% CI: 0.043, 0.1004) and 0.0449 (95% CI: 0.0275, 0.0623) mm^3 respectively. Using a relaxed significance threshold only three meaningful phenotype-epigenotype associations were found at E17.5 ($P<0.075$; Table 7). Placental weight was positively associated with *Dlk1*.A methylation: each 1% decrease in *Dlk1*.A methylation corresponded to a 1.166 (95% CI: 0.591, 1.741) milligram decrease in placental weight. Fetal weight was associated with placental methylation at the *Igf2r* and *Mest* DMDs: for each 1% decrease in *Igf2r* and *Mest* methylation fetal weight decreased by 20.40 (95% CI: 11.17, 29.63) and 16.81 (95% CI: 8.05, 25.57) milligrams respectively.

Placental Phenotype	DMD	β	P-Value
Spongiotrophoblast Central Volume (mm^3)	*Impact*.B	-7.72	1.45E-02
	Mest	-4.49	1.87E-02

Table 6. Bivariate Regression analysis of 21 E15.5 DNMT1o-deficient placentas based on DMD methylation and placental phenotypes. Only significant ($P<0.05$) associations established by bivariate regression analysis between dependent placental phenotypes and independent imprinted DMD methylation values are shown. β is the linear regression coefficient.

Placental Phenotype	DMD	β	P-Value
Placental Weight (mg)	*Dlk1*.A	+116	5.52E-02
Fetal Weight (mg)	*Igf2r*	+2041	3.82E-02
	Mest	+1668	7.07E-02

Table 7. Bivariate regression analysis of 23 E17.5 DNMT1o-deficient placentas based on DMD methylation and placental phenotypes. Only significant (P<0.075) associations established by bivariate regression analysis between dependent placental phenotypes and independent imprinted DMD methylation values are shown. β is the linear regression coefficient.

Linear regression analysis revealed an association between loss of *Mest* DMD methylation and triacylglyceride accumulation in E17.5 DNMT1o-deficient placentas (Figure 29B). *Mest* was the only DMD that associated with lipid accumulation. In contrast to the prior regression analysis the methylation variable used in this regression analysis was normalized as the ratio of mutant:wild-type. In section 3.3.1 I showed that imprinted gene expression within the *Mest* imprinting cluster is altered at E17.5 in a manner indicative of loss of imprinting. Both *Mest* and *Klf14* encode factors with functions in lipid metabolism as a putative lipid hydrolase and as a key metabolic transcription factor respectively. However, these results leave open the possibilities that loss of *Mest* DMD methylation causes placental lipid accumulation in a direct fashion through abnormal *Mest* and *Klf14* imprinted gene expression or via an indirect developmental defect (*e.g.* poor SynT or fetal vessel development).

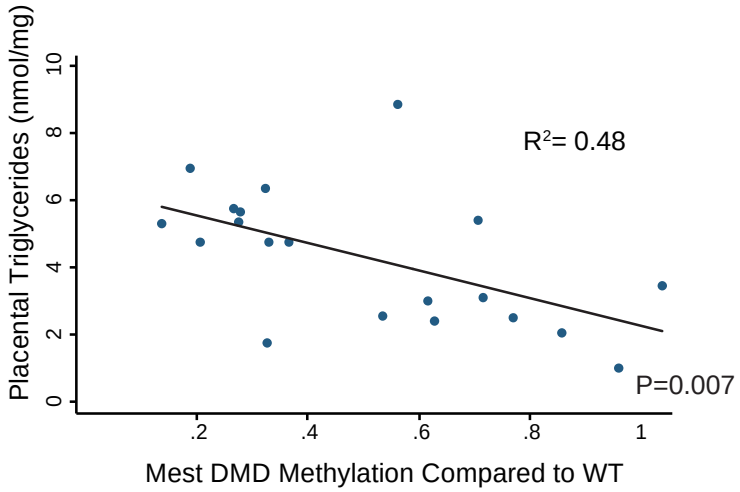


Figure 39. Linear regression plot of *Mest* DMD methylation and placental triglycerides at E17.5. Loss of methylation at *Mest* is associated with elevated placental triacylglycerol concentrations.

3.5 DISCUSSION

3.5.1 A broad spectrum of loss of imprinting is revealed in DNMT1o-deficient placentas

In the results of Chapter 3 I expected and found a large range of loss of imprinting between DNMT1o-placentas and individual DMDs underscoring the mosaicism of this model. Analysis of imprinted gene expression from 7 different imprinted loci revealed many of the expected patterns of loss of imprinting that yielded increased (biallelelic) or reduced (diminished monoallelic) expression at E12.5 (Figure 28). However, at E15.5 and to a greater extent at E17.5, imprinted gene expression was not as expected with certain genes behaving in unexpected and opposite ways (Figure 28). I reconcile these results to indicate that the primary effects of loss of DMD methylation in the *Dnmt1*$^{\Delta 1o}$ maternal effect model are manifested directly on gene

expression early in development, but become increasingly influenced by secondary factors such as trophoblast layer distribution and undefined compensatory mechanisms. In addition, novel associations were revealed between the expression of the *Mest* and *Ascl2* genes with E/P ratio (Figure 29). DMD methylation was also highly variable but not static between gestation ages. My initial BGS results show variable methylation levels at all DMDs analyzed in at least some DNMT1o-deficient placentas at E12.5 but not for every DMD at E17.5, suggestive of an changing population of surviving DNMT1o-deficient placentas in late gestation in terms of both imprinted gene expression and DMD methylation (Figure 30 and 31). These results are further corroborated by EpiTYPER analysis of 14 DMDs in larger cohorts of DNMT1o-deficient mid- and late- placentas.

Quantitative EpiTYPER DNA methylation analysis was used to ascribe placental functions for DMDs in two ways: by identifying nearly normal DMD methylation in DNMT1o-deficient placentas; and by correlating highly variable DMD methylation with placental phenotypes. We expected total wild-type placental DMD methylation to be approximately 50%, but found the wild-type average to be just under 40% at each time point. These results are consistent with the slightly lower levels of DMD methylation found in control placentas than embryos in prior studies (*408*). E12.5 Wild-type placentas showed a large range of methylation across individual DMDs with *Peg3* (32.7%) on the low end and *Dlk1*.A (57.7%) on the high. Based on the current understanding of DNMT1o action it is predicted that on average a 50% loss of methylation at each DMD should be observed in cohorts of DNMT1o-deficient placentas (*60, 104, 402*). However, using the hyper-geometric distribution, I found that the *Dlk1*, *Nespas* and *Snrpn* DMDs were hypovariable and near normal in their methylation levels in E12.5 DNMT1o-deficient placentas (Figures 32G-K). These findings suggest that many epigenotypes with these

DMDs poorly methylated may be incompatible with early trophoblast survival and/or proliferation resulting in selection against specific epigenotypes at the cellular and organismal level.

Interrogation of the association of DMD methylation and placental phenotypes by regression analysis confirmed the importance of DMD methylation in placental development and function. Significant associations were observed between diminished imprinted methylation of the DMDs *Peg10*, *Kcnq1*, *H19* and *Nespas*, and specific placental phenotypes in DNMT1o-deficient E12.5 placentas (Tables 3, 4 and 7; Figure 36). Additional associations were found between the *Impact*.B, *Mest*, *Dlk1*, and *Igf2r* DMDs and placental phenotypes at E15.5 and E17.5 (Tables 6 and 7; Figure 39). Importantly, this approach using the *Dnmt1*$^{\Delta 1o}$ maternal effect model to gain insight into the role of imprinted genes in placental development and function is fundamentally different in two significant ways from genetic approaches that either inactivate single imprinted genes or remove ICRs. First, the *Dnmt1*$^{\Delta 1o}$ maternal effect model produces epigenetic mutant offspring with loss of DMD methylation, while retaining the genetic sequence of ICRs and imprinted genes. Second, the *Dnmt1*$^{\Delta 1o}$ maternal effect model produces broadly variable methylation effects across many DMDs. This permits DMD methylation to be treated as a continuous variable in a quantitative trait analysis, thus revealing strong associations between loss of methylation at particular DMDs and histo-morphological placental phenotypes. The recognition of these associations offers new insights into the integral role of genomic imprints on placenta development.

3.5.2 Association between *Ascl2* and *Mest* gene expression with E/P ratio

Overall my findings of wide-ranging E/P ratios in DNMT1o-deficient conceptuses supports the idea that a subset of imprinted genes is vitally important in placental function. Expression of *Ascl2* and *Mest* accounts for a significant proportion of the observed variation in E/P ratio observed in DNMT1o-deficient offspring. There was a direct correlation between *Mest* expression and E/P ratio, and an inverse correlation between *Ascl2*expression and E/P ratio. Furthermore, *Mest* expression is predicted to increase with loss of methylation at the *Mest* DMD. In mouse placentas, *Mest* expression is limited to the fetal derived extraembryonic mesoderm and is most prominent in mid to late gestation placentas in the capillary endothelial cells of the LZ. Investigations suggest that *Mest* is involved in angiogenic sprouting in the LZ (*275*). An insertional disruption of the *Mest* gene when paternally inherited is associated with fetal and placental growth restriction at E18.5 of 86.4% and 87.5% respectively compared to wild-type (*274*). However, *Mest* null mutant placentas contained congruent LZ and JZ layers indicative a general growth defect rather than a disruption of a single layer. Loss of *Mest* DMD methylation, and concomitant upregulation of *Mest*, may thus be expected to increase the size of both embryo and placenta, and perhaps increase the placenta efficiency resulting an out-sized effect on fetal growth. It is also possible that *Mest* expression is a proxy for loss of imprinting of other genes within the *Mest* cluster (e.g. *Cpa4*, *Copg2* or *Klf14*) that are actually the main effectors. Although expression of *Klf14* is reduced in E17.5 placentas, no association was made between its expression and E/P ratio.

The inverse association between *Ascl2* expression and E/P ratio is more difficult to rationalize due to the unexpected increase in average methylation levels. Taken at face value the expected decrease in *Ascl2* expression associated with loss of *Kcnq1* DMD methylation should

be most similar to either the *Ascl2* null or hypomorphic alleles depending on how uniform the loss of imprinting is (*222*, *311*). Maternally inherited deletion of *Ascl2* is embryonic lethal at E10.5 due to excessive TGC accumulation and a lack of LZ and JZ layer development (*222*). *Ascl2* hypomorphs on the other hand are viable but growth restricted with a lesser degree TGC expansion and diminished LZ (*311*). One explanation for the inverse relationship would be that decreased *Ascl2* expression restricts placental growth disproportionately compared to fetal growth. However, there is also a cluster of four placental samples that have elevated *Ascl2* expression and lower E/P ratio, suggesting elevated *Ascl2* expression may be indicative or causative of inefficient placentas. It is also plausible that *Ascl2* expression is a proxy for other maternally expressed genes in the *Kcnq1* cluster (e.g. *Cdkn1c* and *Phlda2*). Although expression of *Phlda2* was also unexpectedly upregulated, its expression did not associate with E/P ratio. Intriguingly, transgenic over-expression of either *Phlda2* or *Cdkn1c* results in placental growth restriction (*314-316*). Lastly, it is important to note that the unexpected increase in the maternally expressed members of the *Kcnq1* cluster at E17.5 may be due to compensatory changes in placental layer contribution, selection for particular epigenotypes or gene expression patterns.

3.5.3 *Peg10* viability and labyrinth phenotypes

A strong association was observed between loss of *Peg10* DMD methylation and decreased fetal viability and LZ volume at E12.5 (Tables 3 and 4; Figure 36). Most placentas with loss of *Peg10* DMD methylation and decreased LZ volume were unable to support fetal development. I interpret these associations, and the gradual trend toward normal *Peg10* DMD methylation levels from E12.5 to E17.5 (Figure 32K), as a progressive requirement for *Peg10* methylation to sustain fetal viability during later gestation. The decreasing *Peg10* DMD methylation variability and

lack of phenotypic association at E15.5 and E17.5 could be explained by selection against certain low *Peg10* DMD methylation epigenotypes. The DMD methylation epigenotype of placentas with low *Peg10* methylation at E12.5 is different than the epigenotype of placentas with low *Peg10* DMD methylation recovered at E15.5 and E17.5 (Figures 33, 34 and 35). The combination of low *Peg10* DMD methylation (<50% wild-type level) plus low *Dlk1*, *Kcnq1*, *Nespas* or *Snrpn* DMD methylation (<50% wild-type) was observed at E12.5 (samples A5, A7, B1 and C3; Fig 33) but does not occur in any DNMT1o-deficient placentas at either E15.5 or E17.5 (Figures 34 and 35). In summary, my analysis of DNMT1o-deficient placentas reveals a novel link between placentas with low *Peg10* DMD methylation, poor LZ development and the inability to sustain fetal development.

A strong linkage between *Mest* and *Peg10* DMD methylation was found at E12.5 and E17.5 (Figures 34 and 35). This was expected given the proximity of the two DMDs on mouse chromosome 6, however *Mest* did not show significant associations with early placental phenotypes in this study. This observation does not preclude a role for *Mest* later in gestation, and in fact several associations were made between *Mest* DMD methylation and placental phenotypes at E15.5 and E17.5. An inverse association between *Mest* DMD methylation and spongiotrophoblast volume at E15.5, and a positive association between *Mest* and fetal weight at E17.5 were uncovered (Tables 6 and 7). Furthermore, regression analysis revealed a link between loss of *Mest* DMD methylation and placental lipid accumulation at E17.5 (Figure 39). I suggest that *Mest* and *Peg10* DMDs may exert their influence on placental development in a serial manner; loss of *Peg10* DMD methylation impairs LZ development early in gestation, which predisposes these placentas to metabolic abnormalities associated with lost *Mest* DMD methylation later in gestation.

The lethality and labyrinth failure in DNMT1o-deficient placentas with low *Peg10* DMD methylation is similar to the phenotype observed in chromosome 6 translocations and *Peg10* null mice (*10, 261, 262, 293*). Although the expected result of loss of *Peg10* DMD methylation is increased *Peg10* expression, I failed to detect significant changes in *Peg10* expression in DNMT1o-deficient placentas at any time point between E9.5 and E17.5 (Figure 28). However, I did observe a significant increase in *Sgce* and *Pon2* expression in late gestation DNMT1o-deficient placentas (Figure 28). It is difficult to correlate DMD methylation with imprinted gene expression in *Dnmt1*$^{\Delta 1o}$ maternal effect placentas because of the confounding factors of a mosaic model, cell-type expression biases and differential effects of loss of DMD methylation. Based on my direct observation that partial loss of a maternally methylated *Peg10* imprint is detrimental to placental development, I suggest that strict monoallelic dosage of *Peg10*, and/or other imprinted genes within the *Peg10* imprinted cluster is critical for placental development.

3.5.4 Loss of *Kcnq1* DMD methylation and TGC expansion

Mouse chromosome arm 7q contains three maternally methylated DMDs from proximal to distal: *Kcnq1*, *Snrpn* and *Peg3*. Not surprisingly, we found that the methylation status of the *Kcnq1* and *Snrpn* DMDs was linked at E12.5 in DNMT1o-deficient placentas (Figure 33). However, Peg3 is situated closer to *Snrpn* than *Kcnq1* but does not show linkage to the other two at any time point and is hypomethylated in DNMT1o-deficient placentas (Figure 32H and Figures 33-35). I found A strong association between DNA methylation at both the *Kcnq1* and *Snrpn* DMDs and accumulation of TGCs (Tables 4; Figure 36). Based on our forward step-wise regression model the combination of DMD methylation levels of *Kcnq1* and *Snrpn* is the best predictor of TGC abundance (Table 5). I speculate that the association between *Snrpn* methylation and TGC

accumulation is a passive effect due to close linkage with the *Kcnq1* cluster and consistent with lack of known placental function for *Snrpn* (*422*), although the possibility of the involvement of the *Snrpn* in TGC development cannot be ruled out completely. The *in situ* staining of TGCs for *Prl3d1* in DNMT1o-deficient placentas, an early TGC marker, indicates that not only is proliferation altered but also TGC differentiation (Figure 38). The morphology of DNMT1o-deficient placentas with low *Kcnq1* DMD methylation is similar to those described in null and hypomorphic *Ascl2* mouse models in which expansion of TGCs was observed (*222, 311*). These findings are substantiated by the diminished expression of *Ascl2* in DNMT1o-deficient E9.5 and E12.5 placentas (Figure 28).

The accumulation of TGCs observed in the $Dnmt1^{\Delta 1o}$ maternal effect model shown herein is remarkably similar to placentas derived from *Dnmt3L* null mothers, which lack all maternal imprinted DMD methylation (*97, 99*). One mechanistic explanation of the TGC expansion that is common between the $Dnmt1^{\Delta 1o}$, *Dnmt3L* and *Ascl2* models is that a decrease in *Ascl2* expression (by gene deletion or loss of *Kcnq1* DMD methylation) results in derepression of *Hand1*, a transcription factor that promotes differentiation of the EPC, and terminal differentiation of SpT into TGCs (*188, 231, 234*). Loss of *Kcnq1* DMD methylation in DNMT1o-deficient placentas has a distinct phenotype from paternal deletion of the *Kcnq1* ICR, which mimics a maternal (methylated) state with resulting increased maternal expression of *Ascl2*, *Phlda2*, and *Cdkn1c*, and growth restriction (*310*). Regression analysis did not reveal meaningful associations between loss of *Kcnq1* DMD methylation and placental overgrowth at E15.5 or E17.5 that might be expected based on targeted deletion mouse models of *Phlda2* and *Cdkn1c*, which exhibit pronounced placental overgrowth (*312, 313*). These findings taken together with prior research suggest that the imprinted gene *Ascl2* is a focal point for early placental development.

3.5.5 Loss of *Nespas* and *H19* DMD and junctional zone development

In addition to the effects of reduced *Peg10* and *Kcnq1* DMD methylation discussed above, regression analysis revealed weaker, but nonetheless significant, associations between loss of imprinted *Nespas* and *H19* DMD methylation and increased JZ volume (Table 4, and Figures 36B-D). Although both *Nespas* DMD amplicons assayed associated significantly with JZ expansion at E12.5 (Table 4 and Figures 36B and C), an association was not observed at E15.5 (Table 6), indicating this phenotype may resolve to a more normal one during development.

The observed association between loss of *H19* DMD methylation and JZ expansion bordered the significant cutoff (P=0.048, Table 4 and Figure 36D). *H19* DMD methylation gradually increased from E12.5 to E17.5 in DNMT1o-deficient placentas, indicating selection against loss of imprinting at this cluster (Figure 32D). Loss of methylation at the *H19* DMD is expected to depress transcription of the growth factor *Igf2*. Expression of of *Igf2* in DNMT1o-deficient placentas was reduced at E9.5 and E12.5 but was near normal levels at E15.5 and E17.5 (Figure 28). It is known that *Igf2* is paternally expressed throughout the placenta, and that the placenta specific isoform (*Igf2P0*) is expressed exclusively in SynT (*302, 303*). Paternal inheritance of either the *Igf2* null or *Igf2P0* null allele results in placenta with reduced JZ volume (*303*) . Based on this knowledge one explanation for the observed trend is that spongiotrophoblast is less dependent on IGF2 signaling than labyrinthine cell types, and may increase as an early compensatory mechanism to low placental *Igf2* expression. The association between *H19* DMD methylation and JZ volume is not found at E15.5 reflecting the resolving of both *H19* methylation levels and JZ volume toward normal levels.

3.5.6 No placental phenotypes associated with *Dlk1*, *Igf2r* or *Grb10* DMD methylation

At the onset of performing regression analysis I expected to find associations between imprinted DNA methylation at the *Dlk1* DMD and LZ development, and between both the *Grb10* and *Igf2r* DMDs and placental growth based on evidence from genetic models (*347, 374, 393*). In DNMT1o-deficient placentas imprinted DNA methylation at the *Dlk1* DMD did not significantly differ from wild-type although it did increase across gestation (Figures 32O and 32P). This pattern is perhaps indicative of early selection against cellular epigenotypes with loss of *Dlk1* DMD methylation during trophoblast differentiation and proliferation. No associations were found between *Dlk1* DMD methylation and placental phenotypes at E12.5 nor at E15.5, but a positive association between *Dlk1*.A methylation and placental weight at E17.5 was revealed (Table 7), indicating loss of *Dlk1* methylation restricts placental growth. Although there was substantial variation in DMD methylation at the *Igf2r* and *Grb10* DMDs (Figures 36B and 36E), associations between placental weight and DMD methylation were not significant for either loci at E12.5 and at E15. However, I discovered a positive relationship between *Igf2r* DMD methylation and fetal weight at E17.5 (Table 7), a counter intuitive finding given that loss of *Igf2r* methylation should repress expression of this growth suppressor. Regression analysis failed to identify DMDs responsible for the overgrowth of late gestation placentas and embryos but rather identified ones that promoted growth restriction. I interpret these results as evidence that in the context of the *Dnmt1*$^{\Delta 1o}$ mosaic loss of imprinting model, the mid to late gestation growth effects of the *Grb10* and *Igf2r* DMDs may be obscured by epigenetic epistatic interactions with loss of imprinting at other prominent DMDs within both placental and embryonic compartments. The clinically relevant dysregulation of placental and fetal growth associated with loss of imprinting previously highlighted in Chapter 2 is likely due to these complex interactions

between imprinted regions. In contrast, the stronger associations between both *Peg10* and *Kcnq1* and E12.5 placental phenotypes were not occluded by confounding epistatic effects.

3.5.7 *Zrsr1* is an imprinted DMD in placenta but *Nnat* and *Nap1l5* are not

We measured the DNA methylation levels of three additional imprinted DMDs (*Zrsr1*, *Nnat* and *Nap1l5*) in wild-type and DNMT1o-deficient E12.5 and E15.5 placentas (Figures 36Q-S). The mouse genomic coordinates for these three DMDs were previously established (*26*), but were not examined in placenta. EpiTYPER analysis showed that the *Commd1* DMD was methylated at a level consistent with imprinting in wild-type placenta, which was then lost in DNMT1o-deficient placentas (Figure 36Q). Both the *Nnat* and *Nap1l5* DMDs showed a methylation pattern that was not indicative of imprinted DMDs (Figure 36R and 36S). Both DMDs also had higher methylation levels in wild-type placentas than other imprinted DMDs tested (DMD methylation fraction >0.7), and furthermore, neither DMD lost methylation in DNMT1o-deficient placentas. We conclude that *Nnat* and *Nap1l5* are not imprinted DMDs that are perpetuated from gametes to mature trophoblast lineages, and that although the *Zrsr1* DMD is imprinted in the placenta, loss of imprinting at this locus is tolerated. Recent genome methylation studies have provided evidence that the *Nap1l5* but not the *Nnat* DMD retains its imprinted status in the human placenta (*423, 424*).

3.5.8 Conclusion

In summary, I have validated the placental epigenetic variability inherent in the *Dnmt1*$^{\Delta 1o}$ maternal effect model using a broad survey of imprinted gene expression and DMD methylation.

I revealed that the expression of both *Mest* and *Ascl2* are associated with E/P ratio at E17.5. I also discovered a novel association between loss of imprinting at the *Peg10* loci with fetal viability and placental labyrinth maldevelopment. In addition, I found a strong association between loss of imprinting at the *Kcnq1* cluster and TGC accumulation, validating prior genetic models. I conclude from the lack of *Dlk1* DMD methylation variability at E12.5 that *Dlk1* has an essential early trophoblast function. This chapter highlights the direct epigenetic effects of loss of imprinting on placenta development. My findings provide additional rationale to further dissect the *Peg10* and *Kcnq1* imprinting clusters for their roles in placental development.

4.0 *KLF14* IS AN IMPRINTED GENE REGULATING PLACENTAL GROWTH

4.1 SUMMARY

In the previous chapter I revealed two strong associations between loss of imprinting at the *Mest* locus and placental phenotypes. First, *Mest* gene expression was directly associated with the placental efficiency metric E/P ratio. Second, *Mest* DMD methylation was inversely associated with placental triacylglyceride levels. In this chapter I sought to interrogate the role of *Klf14*, a maternally expressed gene within the *Mest* imprinted locus, to determine if loss of its expression recapitulated any of the phenotypes observed in DNMT1o-deficient placentas. To these ends I generated a novel targeted deletion of *Klf14* in mouse. Using this new model, I confirmed that *Klf14* is an imprinted gene expressed from the maternal allele in the placenta. Although the *Klf14* gene appears to be non-essential based on the near Mendelian inheritance observed in heterozygous intercrosses, there were some placental differences. Homozygous late gestation placentas were larger than either heterozygous or wild-type littermates, suggesting a role for *Klf14* in limiting placental growth. No differences in placental layer fractions were observed between genotypes indicating that the placental overgrowth was symmetrical and not localized to one trophoblast cell type. In addition, placental triacylglyceride levels were unchanged in heterozygous intercrosses, however increased levels were observed in maternal null offspring

from homozygous null mothers fed a high fat diet. These results leave open the possibility that *Klf14* may regulate placental growth and mediate metabolic responses to dietary inputs.

4.2 INTRODUCTION

4.2.1 *Klf14* is part of the *Mest* imprinting cluster

Klf14 is a paternally imprinted (maternally expressed) gene within the *Mest* imprinted loci at mouse 6qB3 (See section 1.5.3; Figure 2C). Bimaternal inheritance of Robertsonian 6q translocations is embryonic lethal [10]. The proximal 6q *Peg10* imprinting cluster is most likely responsible for the lethal phenotype, whereas a more subtle intrauterine growth retardation phenotype is observed in embryos with bimaternal inheritance of the subproximal 6q region encompassing *Mest* (*262*). Epigenetic analysis of this region revealed that the *Mest* DMD is located in the promoter and exon 1 of the *Mest* gene and is maternally methylated (*265*). Multiple imprinted genes are regulated by the *Mest* DMD within a 400MB region including the paternally expressed *Mest* and the maternally expressed *Copg2*, *Cpa4*, and *Klf14* (*265*-*270*).

The *Klf14* gene was first identified as a maternally expressed transcript in mouse using RT-PCR restriction length polymorphism analysis of offspring of JF1 and BL6 inter-strain crosses (*270*). Maternal-specific expression of *Klf14* is dependent on *Mest* DMD methylation and is lost in *Dnmt3a*$^{null/+}$ maternal effect offspring (*270*). Additionally, I have shown that DNMT1o-deficient placentas show a strong reduction in *Klf14* expression throughout mid-gestation (Figure 28). Maternal-specific *Klf14* expression has also been confirmed in humans by sequence

comparison of maternal and fetal *Klf14* cDNAs using informative SNPs (*270*). Furthermore, human monochromosomal somatic cell hybrids with exclusively maternal or paternal chromosome 7 show either elevated or absent *Klf14* expression respectively (*270*). These results in mouse and man demonstrate a conserved *Klf14* imprinting mechanism and maternal-specific expression.

4.2.2 The *Mest* imprinting cluster is implicated in placenta development

In the previous chapter I uncovered four novel associations between loss of imprinting at the *Mest* locus and mid to late gestation placental phenotypes. At E15.5 *Mest* DMD methylation inversely associated with JZ central volume (Table 6). At E17.5 the placental efficiency metric E/P ratio is directly associated with *Mest* expression (Figure 29B). In addition, I found that methylation at the *Mest* DMD is positively associated with fetal weight (Table 7) and inversely associated with placental triacylglyceride levels (Figure 39). These results, using the *Dnmt1*$^{\Delta 1o}$ maternal effect model, suggest a role of the *Mest* imprinting cluster in regulating placental growth and metabolism.

The *Mest* gene itself is well studied, although the exact function of its encoded putative α/β hydrolase protein is unclear (*119, 266, 268, 271-276*). The *Mest* gene is expressed in the vascular endothelium of the mouse placenta where it is thought to influence branching morphogenesis (*275*). When paternally inherited insertional mutagenesis of the *Mest* gene completely eliminates its expression, and results in placental growth restriction (*274*). These prior studies, corroborated by my findings in Chapter 3, suggest that *Mest* expression modulates placental and fetal growth. However, they do not fully explain the phenotypic findings of fetal growth restriction and labyrinth lipid accumulation in DNMT1o-deficient offspring.

4.2.3 KLF14 is a transcription factor regulating metabolism

There are 17 mammalian genes encoding Krüppel-like factors (KLFs) including *Klf14* (*425, 426*). KLF proteins share a conserved C-terminal DNA-binding domain composed of a triad of C2H2 type zinc fingers. Each zinc finger is a 23-25 amino acid ββα peptide fold that coordinates a Zn^{2+} cation in a tetrahedral arrangement. The conserved KLF DNA-binding domains recognize similar CACCC or CGCCC sequence motifs and therefore may compete for DNA-binding sites (*425*). KLFs are classified into clades based on their N-terminal peptide sequences. The N-termini of KLFs are known sites of interactions with DNA-binding cofactors. Many KLFs harbor CTBP binding sites whereas others have SIN3A binding motifs. KLF14 falls into the latter group with a putative, although biochemically unconfirmed SIN3A interacting motif (an α-helical AA/VXXL peptide). SIN3a is a histone deacetylase repressive cofactor that acts as a multi-domain scaffold for HDAC1/2, NCOR, SMRT, IKAROS MAD, UMA6 and other chromatin modulators. KLF14 is most closely related to KLF16 based on peptide sequence homology. Moreover, because *Klf14* is an intronless gene, it has been suggested that it arose through retrotransposition during early protoeutherian evolution, whereby it acquired imprinting due to proximity of the *Mest* DMD (*270*). Intriguingly, *Klf14* has undergone recent human specific evolution with genetic changes predominantly in the N-terminal coding region (*270*).

The *Cabut* KLF-like family member in *Drosophila* is a TGFβ responsive developmental regulator and evolutionary precursor to mammalian KLFs 9, 10, 11, 13, 14 and 16 (*427, 428*). These proteins contain a similar tripartite transcriptional regulatory domain (TRD) (*428*). The N-terminal TRD1 contains SIN3A and HDAC interacting motifs whereas the more centrally located C-terminal contains proline rich domains that interact with WW and WD40 domain

containing proteins including numerous GTPases (*428*). The zinc finger domain, or TRD2, interacts with both DNA and histone acetyltransferases (*428*). The mammalian Cabut-related genes are divided into TGFβ inducible early growth response genes (*Klf10* and *Klf11*) and basic transcription factors (*Klf9, 13,14* and *16*) (*428*). The conservation of metabolic regulation is exemplified by *Klf11* which is mutated in maturity onset diabetes of the young (MODY7; OMIM 610508) and neonatal diabetes (*428*).

Although *Klf14* is not thought to be a TGFβ inducible early growth response gene it is involved in TGFβ signaling (*429*). In a human pancreatic epithelial cancer cell line (Panc-1) *KLF14* and *TGFβRII* are upregulated by exposure to TGFβ ligand (*429*). However, rapid upregulation of *TGFβRII* is tempered by the delayed transcription of *Klf14* in an inhibitory feedback circuit. Within TGFβ stimulated Panc-1 cells expression of a luciferase reporter driven by the *TGFβRII* promoter is repressed by KLF14 bound at CG rich sequences (*429*). Furthermore, TGFβ stimulation increases the presence of the repressive histone modification H3K20me3 and decreases the amount of the active histone marks H3K9ac and H4ac at the endogenous *TGFβRII* promoter (*429*). It was also revealed *in vitro* that FLAG-tagged KLF14 in Panc-1 cells pulls down SIN3A and HDAC2 (*429*). In addition, co-stimulation of Leydig cells with TGFβ and progesterone increases *Klf14* expression and leads to increased KLF14 based activation of the endoglin gene promoter (*430*). The endoglin protein is a co-receptor for ALK1 a component of larger TGFβ receptor complexes (*430*). These two studies show that KLF14 is a non-canonical (*i.e.* non-SMAD) protein effector of TGFβ that has both positive and negative feedback on TGFβ signaling. They also provide direct evidence that KLF14 can function as either an activator or repressor of transcription.

The mammalian family of KLFs have diverse physiological functions in cardiovascular, respiratory, digestive, hematological, and immune organ systems as well as in stem cells and tumor biology (*425*). It has recently been suggested that KLF14 is a master transcriptional regulator of adipose metabolism based on genome wide association studies (GWAS). Two separate microarray based studies utilizing human SNPs revealed strong parent-of-origin effects for allelic variants at rs4731702 and rs972283, roughly 14kb upstream of the *KLF14* promoter, that are associated with increased risk of type 2 diabetes when maternally inherited (*279, 281*). In the case of rs4731702, correlations were also made to elevated HDL levels (*280*). Furthermore, it has been shown that both of these risk alleles are associated with decreased adipose expression of *Klf14* in *cis*, when maternally, but not paternally inherited; thereby confirming that these alleles are parent-specific expressed quantitative trait loci (eQTLs) (*279, 281*).

To determine the mechanism by which the *KLF14* eQTL modulates metabolic activity researchers looked for *trans* changes in gene expressions associated with rs 4731702 (*282*, *283*). This approach was justified based on the assumption that *KLF14* encodes a transcription factor that modulates expression of a network of genes. This approach identified 10 genome wide significant trans (GWST) parent-of-origin specific associations between rs4731702 and the following genes in adipose tissue: *TPMT, ARSD, PRMT2, SLC7A10, C8ORF82, APH1B, NINJ2, KLF13, GNB1* and *MYL5*. Nearly all these genes were up-regulated (with SLC7A10 the lone exception) in conjunction with the rs4731702 risk allele suggesting that *KLF14* is in fact a repressor at this set of genomic loci. Furthermore, amongst these 10 genes, the majority were independently associated with other metabolic syndrome traits in GWAS studies. The promoters associated with a set of 50 GWST associations with a relaxed significance threshold, were enriched with CACCC KLF binding motifs. These results taken together suggest that *Klf14*

expression is imprinted and that it regulates a network of genes that modulate insulin response in adipose tissue. With this in mind it is important to understand whether *Klf14* has a similar role in regulating metabolism in mouse placental physiology given its high expression within the tissue, and to ascertain what targets it may regulate there.

4.2.4 Recombineering is a genetic engineering technique

I employed recombineering technology to construct a targeting vector and generate $Klf14^{flox}$ and $Klf14^{null}$ alleles. Recombineering utilizes bacterial strains that induce gap-repair recombination enzymes under certain conditions (*i.e*. temperature) that catalyze recombination between homologous 200-500bp sequences (homology boxes) enabling efficient exchange of sequences from one vector to another (for reviews see (*431, 432*)). A 13.5kb region encompassing *Klf14* and surrounding sequences was retrieved from a bacterial artificial chromosome (BAC) onto a plasmid adjacent to a diphtheria toxin gene (Dta). I then engineered plasmids to insert LNL and LFNTF cassettes to generate a targeting allele. Standard methods of ES cell transfection, homologous recombination, Flp electroporation, Neomycin/Ganciclovir positive/negative selection and blastocyst injection were used to generate a novel transgenic $Klf14^{flox}$ mouse line. This line was then crossed with *Sox2:CRE* transgenic females to generate a constitutive null allele ($Klf14^{null}$). I then used this novel mouse line to study the imprinted expression profile and functional role of *Klf14* in placental biology. Further details on the generation of this mouse line are provided in the material and methods (Sections 4.3.1-4.3.4).

4.2.5 Aims of chapter 4

I genetically engineered a novel targeted deletion to determine if *Klf14* downregulation was responsible for any of the placental phenotypes associated with loss of imprinting at the *Mest* loci in the *Dnmt1*$^{\Delta 1o}$ maternal effect model. This model was used to address important open questions including whether *Klf14* is an essential gene that when deleted results in a lethal phenotype. I also examined litters of heterozygous *Klf14*^{null} intercrosses at E16.5 for abnormal placental growth, layer development and lipid content. In addition, I attempted to provide absolute genetic proof of the maternal-specific expression of *Klf14* in mouse placentas. These efforts explored the functional role of *Klf14* in placenta biology.

4.3 MATERIALS AND METHODS

4.3.1 Recombineering

Primers were designed to amplify homology boxes (HB1-6) containing unique nonrepetitive DNA sequences with 5′ extensions that added restriction endonuclease sites and GCGC clamps (Appendix E). HB1-6 were amplified from a murine 129Sv BAC containing an 80KB genomic contig including *Klf14* (bMQ6044J02; Source Bioscience Lifesciences). HB1 primers were designed to amplify a region 5.5kb downstream of the *Klf14* 3′ UTR and added 3′ BglII and 5′ MluI restriction sites. HB2 primers amplified a region 4.4kb upstream of the *Klf14* TSS and added 5′ HindIII and 3′ MluI restriction sites. Both HB1 and HB2 were cloned into the HindIII and BglII restriction sites in place of the PGKneo and adjacent to the PGKdta cassettes in

plasmid vector PGKneolox2DTA.2 (addgene plasmid #13449). The resulting PGKHB2HB1DTA.2 was linearized with MluI and used as a *Klf14* retrieval plasmid. Prior to retrieval, *E.coli* strain SW106 was transfected with bMQ6044J02, selected for by chloramphenicol, and verified by PCR. On the day of retrieval, the bMQ6044J02 transfected SW106 was heat induced at 42°C and then directly co-transfected with the linearized *Klf14* retrieval plasmid. Positive recombinants were selected for by ampicillin resistance, then cloned and verified to contain PGKDta2 with a 13kb retrieved *Klf14* region by restriction digest and DNA sequencing.

Primers to generate HB3 and HB4 amplified adjacent 400bp regions centered less than 300bp downstream from the *Klf14* 3′UTR and incorporated 5′BamHI and 3′ internal KpnI and external BssHII restriction sites for HB3, and 5′SalI and 3′HindIII restriction sites for HB4. Restriction digested HB3 and HB4 amplicons, derived from PCRs using BMQ6044J02 as a substrate, were cloned together with a BssHII and XhoI digested loxp-frt-neomycin-TK-frt-loxp (LFNTF) cassette and a BamHI and HindIII linearized pBluscript backbone in a quadruple ligation reaction.

Primers to generate HB5 and HB6 amplified adjacent 400bp regions roughly 3kb from the TSS and incorporated 5′BglII and 3′KpnI restriction sites for HB3, and 5′SalI and 3′HindIII restriction sites for HB6. In addition, an endogenous BglII restriction site was removed from the 3′ end of HB3. Restriction digested HB3 and HB4 amplicons derived from PCRs using BMQ6044J02 as a substrate were cloned together with a loxp-neomycin-loxp (LNL) cassette (isolated by restriction digest of by KpnI and XhoI) and a BamHI and HindIII linearized pBluscript backbone in a quadruple ligation reaction.

HB5-LNL-HB6 was excised from pBS with NotI and XhoI, transfected into heat induced SW106 cultures pre-transfected with the *Klf14* positive retrieval plasmid and then plated onto Kanamycin/Ampicillin agar plates. Colony PCR was used to select clones with LNL inserted into the correct region upstream of *Klf14* within the retrieval plasmid, and then subcloned to ensure a single *Klf14* positive retrieval plasmid with a LNL cassette. The neomycin cassette of the LNL was then removed by transfection into SW106 arabinose inducible strain followed by ampicillin selection, and confirmation by HB5/6 PCR and lack of Kanamycin resistant colonies. Electrocompetent heat induced PGK-*Klf14*-5′loxp-Dta.2 transformed SW106 were transfected with linearized HB3LFNTFHB4 and plated onto dual Kanamycin/Ampicillin agar plates and subcloned to individual single vector colonies harboring the primary *Klf14* targeting plasmid. The *Klf14* targeting construct was confirmed by restriction digest analysis and full sequence coverage of the plasmid. To confirm the LFNTF cassette and *Klf14* gene could be deleted, the targeting construct was transfected into SW106 with arabinose induced FLP and SW105 with arabinose induced CRE respectively. Recombineering plasmid maps are shown in Figure 40 for clarity.

4.3.2 Transfection and selection of ESCs

30μg of the *Klf14* targeting construct was linearized by NotI and electroporated into murine J1 ESCs using standard protocols (30μg of pDNA) and plated at various dilutions on irradiated murine fibroblasts. After 48 hours, positive selection with G418 was initiated to eliminate non recombinant ESCs. Within 7-10 days individual colonies were picked and plated onto 96-well plates which were then passaged into 24-well plates after 70% confluence was observed. Homologous recombinants were screened by Southern blot using 5′ and 3′ probes external to the

targeting construct in conjunction with BglII and KpnI digests (Section 4.3.4). The LFNTF cassette was deleted (leaving behind a *loxp* site and ~100bp of additional sequence) by transient transfection of a Flp recombinase plasmid. Colonies surviving ganciclovir negative selection were determined to have deletion of the Neo-TK selectable marker, which was confirmed by PCR and ScaI based Southern digest.

4.3.3 Mouse colony establishment and CRE induced deletion

All mice were humanely cared for in adherence to IACUC guidelines at the University of Pittsburgh. Mouse 129Sv ESCs heterozygous for the *Klf14*^{flox} allele were injected into wild-type B6 blastocysts. Male chimeric offspring were bred with B6 females to test whether recombinant ESCs had been incorporated in the germline which manifests as yielding *agouti* offspring in those crosses. *Agouti* offspring were then genotyped for the presence of the *Klf14*^{flox} allele. Male heterozygous *Klf14*^{flox} offspring were mated to *Sox2:CRE* transgene positive females and the resultant offspring were screened for the combined presence of *Sox2:CRE* (via PCR), and null alleles (via ScaI Southern and PCR). The *Klf14*^{flox} and *Klf14*^{null} alleles, once established in mice, were backcrossed as heterozgotes for greater than five generations to both 129Sv and C57BL/6 (Taconic) strains prior to use in experiments.

4.3.4 *Klf14* genotyping

Southern blot genotyping was carried out using standard protocols utilizing agarose gel electrophoresis, capillary based transfer in alkaline buffer to charged nylon membranes, and hybridization with P^{32} radiolableled DNA probes. Primers that amplified probe templates from

bMQ6044J02 are shown in Appendix E. Probes were generated using random hexamer primed Klenow fragment polymerase based PCR with a nucleotide mixture with radiolableled cytosine (Perkin Elmer). Southern blots were exposed to X-ray film from 2hrs to 2 weeks depending on signal strength. Homologous recombinant ESCs were identified by Southern blotting using 10μg of genomic DNA digested with BglII, run on a 0.7%gel and probed with an external 5′ probe (*Klf14*^{wt} band of 4kb and *Klf14*^{targ} of 9.4kb). Incorporation of the 3′ targeting region was confirmed with a KpnI digest and 3′ external probe (*Klf14*^{wt} band of 16.1kb and *Klf14*^{targ} of 13.3kb). ScaI digestion and 5′ probes showed deletion of the PGKneo-tk marker (*Klf14*^{wt} and *Klf14*^{flox} bands of 11kb and *Klf14*^{targ} band of 12.7kb). Floxed alleles were confirmed by Southern blotting of BglII digests with an external 5′ probe (*Klf14*^{wt} band of 4kb and *Klf14*^{flox} band of 9.2kb). Null alleles were distinguished from wild-type and floxed alleles by Southern blot of ScaI digested genomic DNA with an external 5′ probe (*Klf14*^{wt} and *Klf14*^{flox} bands of 11kb and *Klf14*^{null} band of 6kb).

Klf14 floxed, null and wild-type alleles were also genotyped by semi-nested PCR using primers abutting homology boxes 4, 5 and 6 (Appendix A) using 350ng of genomic DNA and the following thermocycler program: 95°C 5′ denaturing followed by 35 cycles of 95°C 30″, 60°C 30″, 72°C 30″, a 72°C 7′ final extension and an indefinite hold at 4°C. PCR products were run on a 1.5% agarose gel to look for the presence of 200bp (*Klf14*^{wt}), 300bp (*Klf14*^{flox}) and 400bp (*Klf14*^{null}) alleles. *Sox2:CRE* transgenes were also genotyped by PCR using primers provided by the Barak lab (Appendix E)

4.3.5 RT-PCR

Nucleic acids were isolated using All-Prep or RNeasy kits (Qiagen) as previously described from fresh placental tissues, and other embryonic and adult tissues isolated by microdissection. Prior to reverse transcription, RNA was treated with RQ1 DNase for 1h to remove genomic contaminants. Reverse transcription was carried out using MMLV-RT (Promega) from approximately 500ng to 1ug of RNA template oligo dT primers. RT-PCR of *Klf14* was performed using Taq polymerase, approximately 200ng, and primers previously reporter by Parker-Katiraee et al. ((*270*) ; Appendix B)). The housekeeping gene *Gapdh* was used as a positive control. The RT-PCR themocycle was the following: 95°C 5′ denaturing followed by 30 cycles of 95°C 30″, 60°C 30″, 72°C 60″, a 72°C 7′ final extension and an indefinite hold at 4°C. PCR products were run on a 2.0% agarose gel and examined for the presence of an 800bp *Klf14* band.

4.3.6 *Mest* DMD methylation analysis

The methylation status of the *Mest* DMD in wild-type and null placentas was examined by BGS and COBRA assays. Bisulfite genome conversion and PCR of wild-type and $Klf14^{null}$ placental DNA was carried out as previously described using the same reagents and nested primers (Section 3.3.3, Appendix C). The BstBI restriction enzyme was utilized to cut 1ug of bisulfite converted *Mest* PCR amplicon in order to examine for the presence of unconverted (methylated) sites within the *Mest* DMD. Bisulfite genomic sequencing of the same populations of alleles and generation of dot-pot figures was performed as previously described (Section 3.3.3).

4.3.7 Embyronic and placental analysis

Placental analysis focused on the offspring of heterozygous *Klf14*^{null} intercrosses. Dissections were performed at E17.5 in the same manner as previously described (Section 2.3.3). Placenta and embryonic wet weights were recorded. Half of each placenta was preserved for cryo-histology as described earlier (Section 2.3.4). Portions of the remaining half were taken for genotyping, gene expression or lipid content analysis. Placental triacylglyeride content was measured as previously described (Section 2.3.6). Placental layer fractions were determined using the ratio of the average JZ and LZ area determined by random grid sampling of a single central placental section for each sample. Comparisons were made between wild-type, heterozygous and null placentas using students t-test and/or Rank-sum tests.

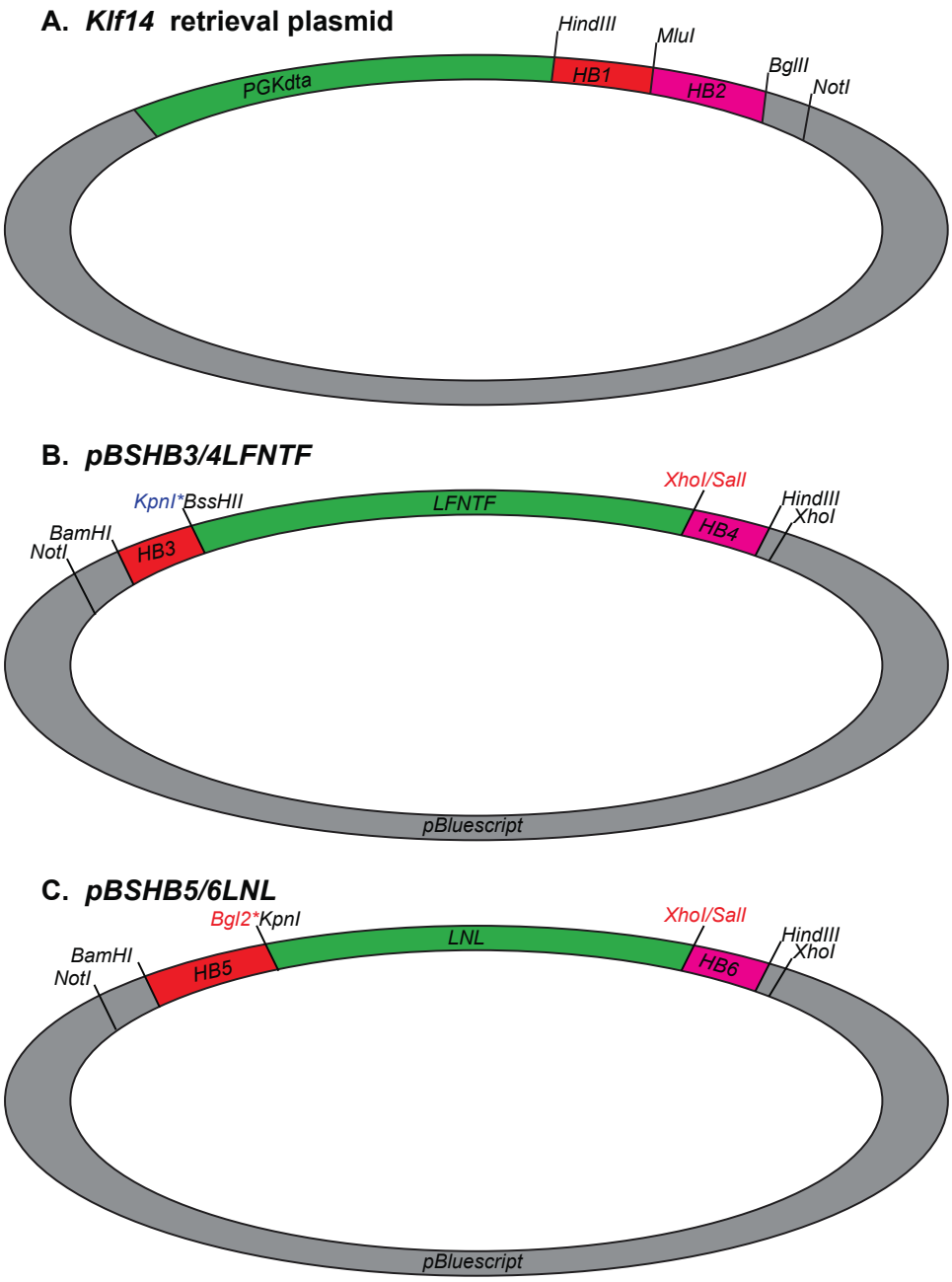


Figure 40. Recombineering plasmids. (A) Plasmid used to retrieve 13.5Kb region around *Klf14* from bMQ6044J02. (B) Plasmid used to place 3′ LFNTF selectable marker on the targeting construct. (C) Plasmid used to place a 5′ LNL selectable marker on the targeting construct.′

4.4 RESULTS

4.4.1 Confirmation of *Klf14* null allele

I engineered a novel targeting construct using recombineering to produce a targeted deletion of *Klf14* in mice (Figure 41A). Chimeric floxed allele mice were initially generated by blastocyst injection of 129Sv strain homologous recombinant ESCs into wild-type B6 blastocysts. Chimeric male offspring were crossed with B6 females and screened for passage of the *agouti* fur color trait indicating germline passage of the recombinant J1 ESCs. Offspring were then screened for the presence of a $Klf14^{flox}$ allele by Southern blot consisting of a BglII restriction digest and an external 5′ probe (Figure 41B). The wild-type BglII band is 4.9kb whereas the floxed allele is 9.2kb (Figure 41B). Full incorporation of the targeting construct was confirmed by Southern blot with KpnI digestion and an external 3′ probe to differentiate the 16.1kb wild-type allele and the13.2 kb floxed allele (Data not shown). Confirmed heterozygous $Klf14^{flox}$ males were mated to Sox2:CRE transgenic females to generate $Klf14^{null}$ heterozygous offspring (Figure 41A). The presence of a $Klf14^{null}$ allele was confirmed by Southern blot with ScaI restriction digest and the same external 5′ probe. The wild-type ScaI band is 11kb whereas the null allele is 6kb (Figure 41C). Semi-nested genomic PCR was used to assess genotyping of offspring as both the floxed and null alleles were backcrossed onto inbred 129Sv and B6 mouse strains. Using this strategy, the wild-type allele amplified a 200bp band from primer pairs *b* and c, whereas the floxed allele generated a 300bp doublet from primer pairs *b* and *c*, and the null allele generated a 400bp

fragment from primers *a* and *c* (Figure 41D). These genotyping results clearly show the generation and stable inheritance of floxed and null *Klf14* alleles.

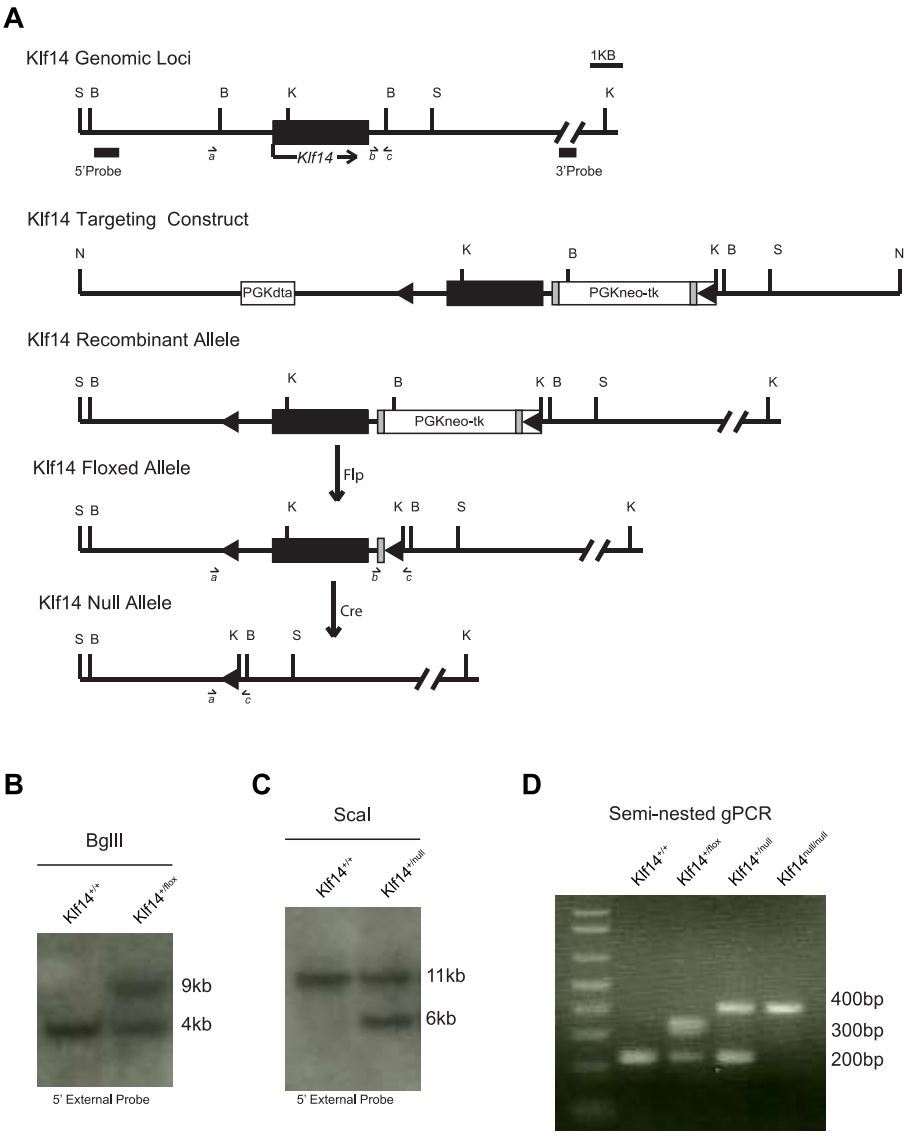


Figure 41. Targeted deletion of *Klf14* in mice. (A) Design and targeting of *Klf14*. (B) Southern blot confirming floxed allele in mice. (C) Southern blot confirming null allele in mice. (D) Genotyping PCR confirming flox and null alleles in mouse. Key: rectangles represent gene elements *Klf14* gene, PGK-neomycin-thymidine kinase cassette, PGK-diptheria toxin cassette; triangles represent loxp sites Abbreviations: S-ScalI, B-BglII, K-KpnI, N-NotI; a,b and c- semi-nested PCR primers. Scale bar is 1kb.

4.4.2 *Klf14* is an imprinted gene expressed in the placenta

I measured *Klf14* expression in an array of fetal and adult tissues by RT-PCR and found that expression levels were highest in yolk sac, fetal intestine and placenta and to a lesser degree in fetal brain, fetal liver, adult kidney and adult heart. However, *Klf14* expression was not detected in adult spleen, adult liver and homozygous null placenta (Figure 42A). Next, I sought to genetically confirm reports that *Klf14* expression is exclusively from the maternal allele in the mouse placenta. To these ends I compared expression of *Klf14* in wild-type ($Klf14^{+/+}$), maternal null ($Klf14^{null/+,}$), paternal null ($Klf14^{+/null}$), and homozygous null ($Klf14^{null/null}$) placentas using RT-PCR. Half of the offspring from heterozygous null dams crossed to wild-type males were $Klf14^{null/+}$ and had no detectable expression of *Kl1f14* after 35 RT-PCR cycles, and were queivalent in placental *Klf14* gene expression to that of $Klf14^{null/null}$ placentas derived from offspring of interbred heterozygotes (Figure 42A). Expression of *Klf14* in $Klf14^{+/null}$ placentas on the other hand were observed to be at or near $Klf14^{+/+}$ levels. These results provide definitive proof that *Klf14* is a maternally expressed gene within the mouse placenta.

To determine whether imprinting at the *Mest* DMD was altered in $Klf14^{null/null}$ placentas I assayed DNA methylation by COBRA and BGS. Bisulfite conversion and PCR amplification of a 550bp region of the *Mest* DMD yields two BstBI restriction sites, however if C→T conversion is blocked by DNA methylation these sites will not be generated. Approximately half of the bisulfite PCR product in $Klf14^{+/+}$ samples was undigested by BstBI, indicating roughly 50% methylation. Similar results were found in $Klf14^{null/null}$ placentas suggesting no changes in *Mest* DMD methylation in $Klf14^{null}$ transgenic placenta. However nearly all amplicons recovered from $Dnmt1^{c/c}$ ESCs were undigested by BstBI, indicative of the low methylation state of these cells.

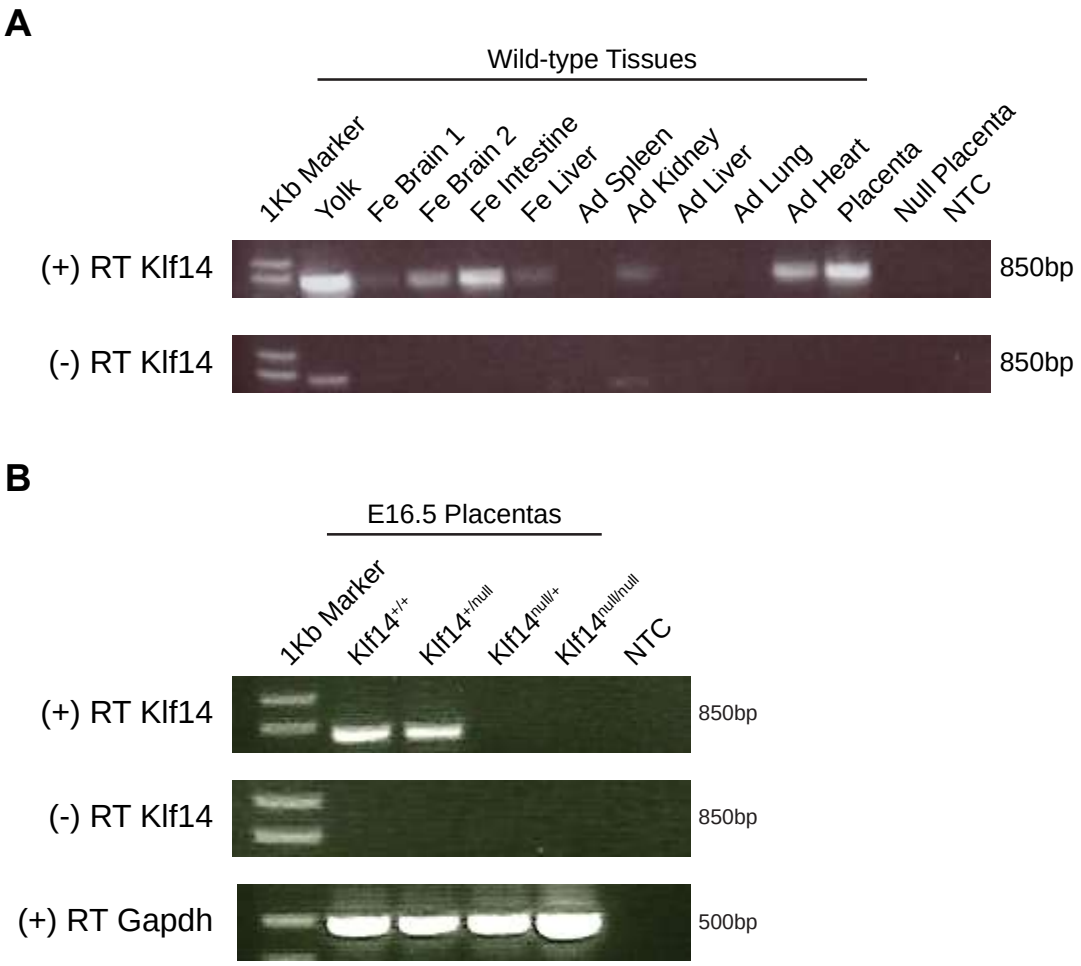


Figure 42. *Klf14* is expressed in the mouse placenta and is an imprinted gene. (A) RT-PCR based expression assay for *Klf14* in an array of fetal (Fe) and adult (Ad) tissues as well as extraembryonic tissue including homozygous null placenta. (B) Expression of *Klf14* in wild-type, heterozygous null and homozygous null E16.6 placentas. NTC-no template control.

I confirmed the COBRA assays by sequencing 16 cloned bisulfite converted PCR amplicons of the *Mest* DMD from *Klf14*$^{+/+}$ and *Klf14*$^{null/null}$ placentas (Figure 43A). Wild-type samples revealed the expected pattern of containing both fully methylated and fully unmethylated alleles. The average wild-type methylation value was 57.6%. Homozygous null placentas showed a similar pattern of *Mest* DMD methylation with both fully methylated and completely unmethylated alleles. The average *Mest* DMD CpG methylation in *Klf14*$^{null/null}$ placentas was 54.6%. These results further confirm that *Mest* DMD methylation is not altered by deletion of *Klf14*

Due to the tissue specific expression patterns of *Klf14* and the location of a CGI overlapping the *Klf14* transcriptional start site and 5′-end of the gene I hypothesized that there may be tissue specific methylation at this region. The EagI and MluI CpG methylation sensitive restriction enzymes have unique restriction sites within the *Klf14* coding region (Figure 43C). The EagI site falls within the CGI whereas the MluI site falls 3′ of it. Genomic DNA from an array of wild-type tissues was digested with ScaI in combination with either MluI or EagI, and probed with a 5′ internal radioprobe. The fetal liver was the only tissue in which partial digestion was observed in the ScaI-EagI double digest indicating partial methylation of the CGGCCG EagI CGI site (Figure 43C). Partial digestion was observed in genomic DNA derived from placental and adult liver samples suggesting partial methylation of the ACGCGT restriction sequence within the *Klf14* gene body (Figure 43C). I interpret these results as evidence that the *Klf14* CGI and gene body are generally hypomethylated but may have some tissue specific DNA methylation patterns

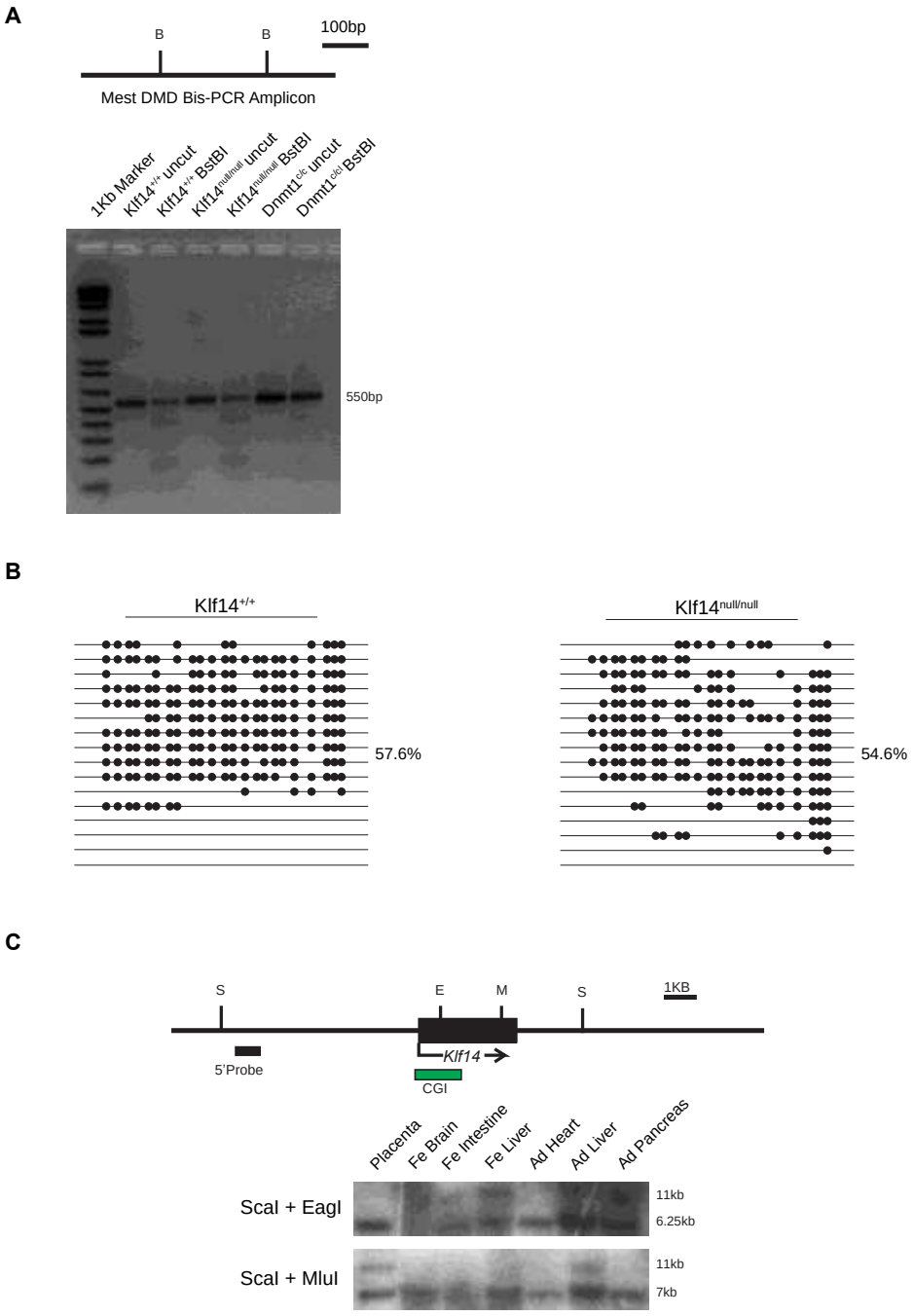


Figure 43. DNA methylation at the *Mest* DMD is normal in homozygous *Klf14* null placentas. (A) *Mest* DMD COBRA assay. (B) *Mest* DMD bisulfite genomice sequencing of 16 wild-type and *Klf14*$^{null/null}$ alleles. (C) Methylation-sensitive Southern blotting of the *Klf14* CGI and gene body. Abbreviations B-BstBI, S-ScaI, E-EagI, M-MluI.

4.4.3 *Klf14* null mice are viable and fertile

Heterozygous null mice were both viable and fertile regardless of sex, strain background or parent-of-origin of the *Klf14* null allele. The offspring of heterozygous intercrosses were found to contain wild-type, heterozygous null and homozygous null genotypes at near mendelian 1:2:1 frequency (Tables 8 and 9, Chi-square test P>0.05). Moreover, homozygous null mice of both sexes were viable and fertile on 129Sv and B6 strains. No differences were observed in the growth curves of wild-type, heterozygous and homozygous null littermates from weaning through postnatal day 90 (Data not shown). These results suggest that *Klf14* is not essential for murine development or reproduction, but do not rule out more sublte developmental or metabolic defects in null mice. Therefore, it was still important to determine if any abnormalities were present in developing *Klf14* null placentas.

Genotype	Obs	Exp		Genotype	Obs	Exp
Wild-type	37	27		Female Wild-type	18	13.5
Heterozygous Null	50	54		Female Heterozygous Null	26	27
Homozygous Null	21	27		Female Homozygous Null	11	13.5
Total	108	108		Male Wild-type	19	13.5
Chi-Square (df = 2)	5.33 (P>0.05)			Male Heterozygous Null	24	27
				Male Homozygous Null	10	13.5
				Total	108	108
				Chi-Square (df = 5)	5.48 (P>0.05)	

Table 8. Near Mendelian inheritance of the *Klf14* null allele in 129Sv strain heterozygous intercrosses. (Left) Chi-square analysis without gender influence. (Right) Chi-square analysis including gender. Abbreviations: Obs, Observed; Exp, Expected; df, degrees freedom; P, P-value.

Genotype	Obs	Exp		Genotype	Obs	Exp
Wild-type	20	16.5		Female Wild-type	8	8.25
Heterozygous Null	29	33		Female Heterozygous Null	18	16.5
Homozygous Null	18	16.5		Female Homozygous Null	9	8.25
Total	66	66		Male Wild-type	13	8.25
Chi-Square (df = 2)	1.36 (P>0.05)			Male Heterozygous Null	11	16.5
				Male Homozygous Null	8	8.25
				Total	66	66
				Chi-Square (df = 5)	4.79 (P>0.05)	

Table 9. Near Mendelian inheritance of the *Klf14* null allele in B6 strain heterozygous intercrosses. (Left) Chi-square analysis without gender influence. (Right) Chi-square analysis including gender. Abbreviations: Obs, Observed; Exp, Expected; df, degrees freedom; P, P-value.

4.4.4 Overgrowth in homozygous *Klf14* null placentas

Even though homozygous *Klf14* null animals were viable and fertile we examine E17.5 litters to determine if there were any growth effects on either heterozygous or homozygous null embryos and placentas. Average placental and fetal weights from wild-type and mutant offspring from litters of heterozygous *Klf14* null intercrosses are displayed in Figure 44. Homozygous null placentas were approximately 10% heavier than wild-type littermates ($P<0.05$; Figure 44A). The intermediate phenotype observed in heterozygous *Klf14* null placenta is most likely due to equal distributions of $Klf14^{+/null}$ and $Klf14^{null/+}$ that either had a functional or non-functional *Klf14*. Although the standard error of the mean (SEM) placenta weight is lower in heterozygotes than either wild-type or homozygous null littermates the standard error (uncorrected for sample size) is larger in heterozygotes. No difference in fetal weights was observed between genotypes from these crosses (Figure 44).

To determine whether there was an imprinting effect on placental development in heterozygous offspring I analyzed E17.5 litters of *Klf14*$^{null/+}$ mated to wild-type 129Sv males. Average placental and fetal weights from wild-type and *Klf14*$^{-/+}$ offspring are shown in Figure 45. The maternal null placentas were significantly heavier than wild-type littermates ($P<0.05$; Figure 45A). These results are interpreted as evidence rejecting a role for *Klf14* within the mother from influencing placental size. Rather the data suggest loss of active maternal *Klf14* in offspring autonomously enhances fetal growth. Similar to the offspring of heterozygous intercrosses no significant difference in fetal weights was detected in the offspring of *Klf14*$^{null/+}$ dams (Figure 45B).

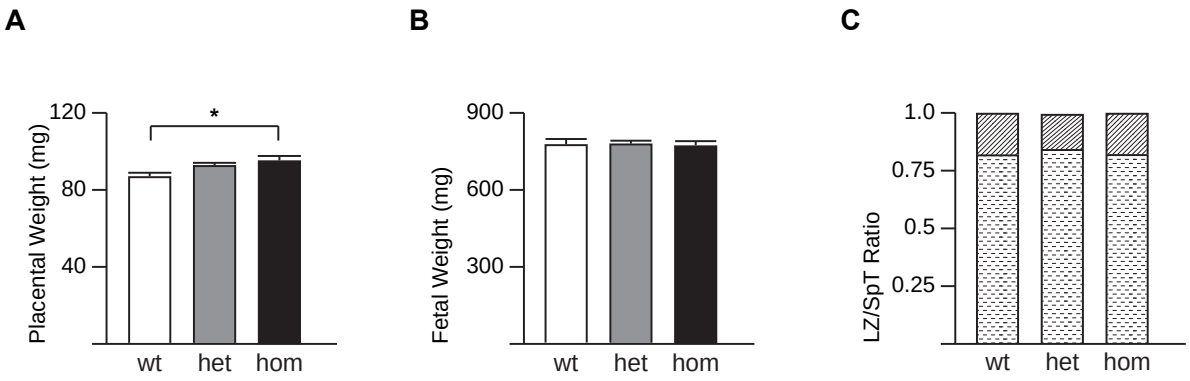


Figure 44. E17.5 Placental and fetal weights in offspring of heterozygous *Klf14* null intercrosses. (A) Wet placental weight of wild-type (wt, n=17), heterozygous (het, n=43) and homozygous null (hom, n=12) littermates (B) Corresponding fetal weights (C) Placental layer fractions *P<0.05 2-way Students T-Test.

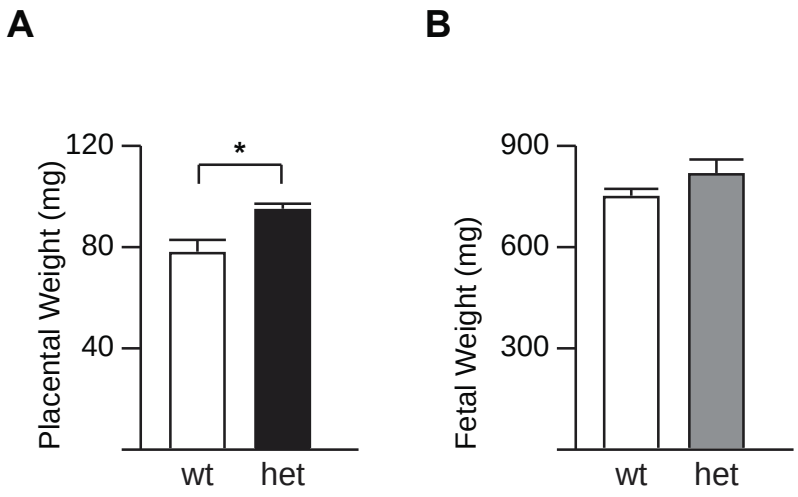


Figure 45. E17.5 Placental and fetal weights in offspring of maternal *Klf14* null dams. (A) Wet placental weight of wild-type (wt, n=9), and maternal null heterozygous (het, n=10) littermates (B) Corresponding fetal weights (C) Placental layer fractions *P<0.05 2-way Students T-Test.

4.4.5 Placenta layer structure in *Klf14* null placentas

I chose to further investigate the placental overgrowth phenotype using histological methods in order to determine if there were any structural abnormalities. The LZ and JZ layer fractions of homozygous and heterozygous *Klf14* null placentas from heterozygous intercrosses were not significantly different from wild-type littermates (44C). These results indicate that the observed placental overgrowth is evenly distributed between layers rather than attributable to expansion of either JZ or LZ.

4.4.6 Placental lipid content increased by high fat diet in *Klf14* null placentas

In the prior chapter loss of imprinting at the *Mest* cluster was determined to be associated with increased lipid content in the *Dnmt1*$^{\Delta 1o}$ maternal effect model. Therefore, it was important to determine if the increased placenta weight in the *Klf14* null model was due to lipid accumulation. However, I observed no difference in placental triacylglyceride concentrations in wild-type and *Klf14*$^{-/-}$ placentas (Figure 46). I challenged wild-type and *Klf14*$^{-/-}$ females with a high fat diet (HFD) for 6-weeks prior to and then during pregnancy to determine if placental lipid content was contingent on diet. Placentas recovered from pregnant *Klf14*$^{-/-}$ females challenged with HFD were significantly higher than those from wild-type dams fed with normal chow (Figure 46). However, no difference was found in wild-type litters fed with HFD nor was there a significant difference between offspring of *Klf14*$^{-/-}$ females compared with wild-type HFD offspring. These preliminary data suggest *Klf14* may regulate placental lipids in the context of HFD.

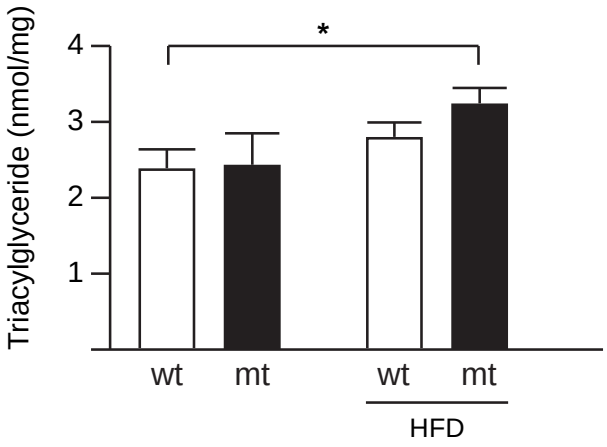


Figure 46. Lipid Accumulation in *Klf14* null offspring. Comparison of litters from wild-type and homozygous null females fed either normal or high fat diet (HFD) chow. *P<0.05 2-tailed Students T-Test

4.5 DISCUSSION

4.5.1 Opposing effects of *Klf14* and *Mest* on placental growth

The results presented in this section that show *Klf14* influences placental growth suggest that its loss of expression in the DNMT1o-deficient model may be in part responsible for the associations between loss of imprinting at the *Mest* cluster and placental phenotypes. In DNMT1o-deficient placentas the expression of *Klf14* is decreased more than 2-fold at E9.5, E15.5 and E17.5 whereas *Mest* expression is increased significantly only at the two later time points (Figure 28). Although the reciprocal changes in *Klf14* and *Mest* were concomitant, only *Mest* was identified by linear regression as associated with DNMT1o-deficient placental efficiency suggesting that *Mest* is either a marker of highly vascularized efficient placentas or that loss of imprinting of *Mest* influences the ratio of fetal and placental weight. Although fetal growth was unaffected in *Klf14* null mice, the E/P placental efficiency ratio was not significantly decreased despite the moderate placenta overgrowth (data not shown). Congruent placental and fetal growth restriction is observed in mice with a paternally inherited *Mest* null allele (*274*). Therefore, it is expected, although unconfirmed, that *Mest* overexpression would increase placental and fetal growth evenly. Based on my results regarding the *Klf14* null and the aforementioned *Mest* null model it is likely that the collective loss of imprinting at the *Mest* cluster integrates disparately towards fetal overgrowth. My results also show that *Klf14* and *Mest* have opposing influences on placental growth.

In Chapter 3 I revealed novel associations between loss of *Mest* DMD methylation and accumulation of spongiotrophoblast at E15.5, and accumulation of placental triacylglycerides at E17.5. The latter phenotype is particularly relevant given my findings in this section that placental lipid accumulation occurred in offspring of homozygous null *Klf14* females maintained on HFD during and six weeks prior to pregnancy. However, the lipid accumulation in DNMT1o-deficient placentas was more pronounced and occurred without HFD administration. I interpret these results to indicate that mosaic loss of DMD methylation in the context of *Mest* DMD hypomethylation and HFD in the context of maternal *Klf14null* development are insults to placental metabolism that result in excess lipid deposition. Based on my research herein, a further investigation into the role of genes within the *Mest* imprinting cluster with a focus on *Klf14* and *Mest* in fetal endothelial, syncytiotrophoblast and adipose lipid metabolism is warranted.

Klf14 null mice did not recapitulate the altered layer development observed in DNMT1o-deficient placentas. Despite the increase in spongiotrophoblast associated with loss of *Mest* DMD methylation in DNMT1o-deficient placentas I did not observe any changes in SpT layer fraction in E16.5 homozygous null placentas compared to littermates. Similarly, no difference in placental structure was observed in the growth restricted *Mest* null placentas. This suggests that either another gene within the *Mest* cluster was responsible for this phenotype or that epistatic interactions within the *Mest* loci or between the *Mest* loci and other clusters. Previously it has been pointed out that the components of the *H19, Grb10, Kcnq1* and *Plagl1* clusters are involved in SpT development and are part of a larger integrated network of imprinted genes to which *Klf14* and *Mest* may belong.

4.5.2 Placental and maternal influence on metabolism

Placental function and maternal environment during prenatal development has strong influences on the lifelong health of individuals. The association between adult metabolic disease and SNPs upstream of *Klf14* could be mediated by long lasting effects due to either maternal *Klf14* susceptibility alleles in either mother or placenta. Therefore, it was particularly of interest that homozygous null *Klf14* females when fed HFD had offspring with placentas enriched in triacylglycerides. These results show that *Klf14* may modulate lipid metabolism in pregnant females in a way that alters placental lipid deposition. This is exemplary of environmental influences being integrated through a genetic pathway. Further studies should be performed to validate my findings using a greater sample number of wild-type and homozygous *Klf14* null females. In addition, it would be interesting to determine if heterozygous maternal null or paternal null (*i.e.* $Klf14^{null/+}$ or $Klf14^{+/null}$) dams mated to wild-type males and fed HFD also yield increased placenta triacylglycerides in either wild-type or heterozygous offspring. Such a study would confirm that placental triacylglyceride accumulation is the result of HFD plus maternal *Klf14* deletion or provide evidence that the phenotype is caused by HFD plus placental *Klf14* deletion. Lastly, it might be interesting to perform a uterine transfer of wild-type embryos into homozygous *Klf14* null females to verify that lipid accumulation occurs independent of offspring genotype.

4.5.3 Comparison with other targeted *Klf14* models

During the time I was investigating the role of *Klf14* in placenta biology three different groups published reports on the effects of *Klf14* deletion in mouse (*433-435*). However, none of these

studies examined *in utero* fetal or placental development. These three groups utilized different targeted deletion models and examined diverse facets of mouse biology influenced by *Klf14* ranging from sphingosine-1-phosphate signaling in the liver, hepatic cholesterol metabolism, and centromere amplification (*433-435*). The fact that each of these groups published findings on homozygous *Klf14* null mice confirms my finding that *Klf14* is not an essential gene.

A group from the Mayo clinic utilized a neomycin insertion-deletion mouse available from the KOMP repository at UC Davis in their study of hepatic sphingosine kinase-1 (*Sk1*) transcriptional regulation (*435*). Using a luciferase assay this group observed that FGF2/FGFR1 induced *Sk1* promoter activity is dependent on GC rich sequences overlying KLF14 binding sites (*435*). They found that in HUVEC cells that *Klf14* siRNA reduces FGF2 stimulated *Sk1* expression, whereas *Klf14* overexpression increases basal and FGF2 stimulated *Sk1* expression (*435*). They also showed that KLF14 dependent FGF2 stimulation resulted in accumulation of activating histone marks (increased H4K8ac and H3K14ac) and a decrease in repressive marks (decreased H3k9me3 and H3K27me) at the *Sk1* promoter (*435*). Furthermore, they showed that epitope tagged KLF14 binds the endogenous *Sk1* promoter and interacts directly with the histone acetyltransferase p300 (*435*). This study provides firm genetic proof that KLF14 is a transcriptional regulator that can interact with more than just SIN3A, and is a mediator of FGF2 signaling.

Following up on the role of *Klf14* in liver a second group generated a liver-specific conditional null by mating a floxed *Klf14* allele with an *Albumin:CRE* mouse (*433*). They initially became interested in *Klf14* due to the GWAS studies linking it to T2D and HDL levels and their preliminary findings that *Klf14* expression is decreased in HFD fed wild-type mice, *Apoe* null coronary heart disease mouse model and in the leptin deficient (*ob*/*ob*) murine obesity

model (*433*). Overexpression of *Klf14* by adenoviral infection *in vivo* resulted in increased HDL-C and APO-A1 (*433*). Hepatocyte *in vitro* adenoviral induced *Klf14* overexpression showed a clear increase in *ApoA1* transcription that was dependent on two CACCC boxes bound by *Klf14* within the *ApoA1* promoter (*433*). Furthermore, *Klf14* expression was increased in perhexaline treated Apoe homozygous null mice and partially rescued the cardiac plaque phenotype in a manner independent of carnitine palmitoyltransferase1 inhibition by perhexaline (*433*). These results are particularly interesting given my findings that placentas derived from homozygous null *Klf14* dams are more susceptible to lipid accumulation. In addition, it was noted in DNMT1o-deficient placentas that mitochondrial carnitine efflux was highly distorted (*414*).

A third *Klf14* null model was developed using TALON zinc finger nucleases (*434*). This group became interested in *Klf14* when they discovered that it encodes a transcription factor that binds to and represses polo-like kinase-4 (*Plk4*) (*434*). It has previously been established that overexpression of *Plk4* induces centrosome amplification (*434*). They found that 13-14 month old homozygous *Klf14* null mice had more frequent tumors of the lung, lymph and spleen than wild-type (*434*). Homozygous *Klf14* null mice were also more susceptible to azoxymethane chemically induced colon tumors (*434*). They established homozygous *Klf14* null murine embryonic fibroblast cultures and found that roughly 12% of cells were polyploid. Additionally, they found that siRNA knockdown of *Klf14* in HeLa cells resulted in increased *Plk4* expression, centrosome amplification and misaligned metaphase plates, whereas *Klf14* overexpression provoked mitotic catastrophe (*434*). These results may in part explain why imprinting of *Klf14* is beneficial due to hazards of overexpression While I did not find any evidence of tumors in my *Klf14* null colony, I did not observe them beyond 9 months.

5.0 OVERALL DISCUSSION

5.1 SUMMARY AND SIGNIFICANCE

In Chapter 2 I described an array of abnormal placental phenotypes present in the *Dnmt1*$^{\Delta 1o}$ maternal effect offspring. Early in development trophoblast differentiation was severely disrupted leading to diminished LZ development and increased TGC abundance. Many of the abnormal placentas found at E12.5 could not support a viable fetus and lacked fetal vasculature in the placental labyrinth. The phenotypes in late gestation DNMT1o-placentas were very different. Although at E15.5 placental layers were not significantly different than wt, by E17.5 the fraction of JZ relative to LZ was increased. I observed extensions of SpT into the LZ in many E15.5 and E17.5 DNMT1o-deficient placentas. I also found that DNMT1o-deficient placentas recovered at E15.5 and E17.5 were enriched in glycogen deposits and lipid droplets. At E17.5 the ratio of fetal and placenta growth was highly dysregulated and revealed that both high and low E/P ratio placentas had unique gene expression profiles. Taken together these results demonstrate the strong effects on placenta development that occur in the the *Dnmt1*$^{\Delta 1o}$ maternal effect model.

In Chapter 3 I described the breadth of loss of imprinting in DNMT1o-deficient placentas and correlated these molecular changes with placental abnormalities. In DNMT1o-deficient placenta imprinted gene expression was disrupted across gestation. At later gestational ages the

directionality of imprinted gene expression changes was not always congruent with the changes expected with loss of DMD methylation. Novel associations between *Mest* and *Ascl2* expression levels and E/P ratio were revealed. A broad survey of 15 imprinted DMDs in DNMT1o-deficient placentas confirmed the epigenetic mosaicism previously postulated to occur in this model. DMD methylation was higher in the E17.5 than E12.5 placental cohorts suggesting selection against certain hypomethylated epigenotypes. In addition, methylation at some DMDs (*e.g. Dlk1* and *Nespas*) appeared less mutable than others. Ultimately, I used regression analysis to reveal novel associations between individual DMDs and placental phenotypes. Amongst the more notable associations were: a relationship between loss of *Peg10* DMD methylation and both reduced fetal viability and diminished labyrinth central volume at E12.5; a relationship between loss of *Kcnq1* DMD methylation and TGC accumulation at E12.5; and a relationship between loss of *Mest* DMD methylation and triacylglyceride accumulation at E17.5. These results provide an impetus to further dissect these three imprinting clusters for roles in placental development.

In my final results section, Chapter 4, I focused on the generation and study of a novel targeted deletion of the imprinted *Klf14* gene. I used this model to confirm previous assertions that *Klf14* is an imprinted gene expressed in the placenta. Although *Klf14* null mice were viable and fertile even in homozygous genotypes, I observed changes in the placenta. Both homozygous conceuptuses from heterozygous intercrosses, and heterozygous conceptuses from homozygous maternal null dams had significantly heavier placentas at E17.5 than wild-type littermates. In addition, placentas from homozygous null dams fed HFD were enriched in lipids. These results suggest a role for *Klf14* in regulating placental growth and metabolism and highlight the need for further investigation of this model.

The results presented within my dissertation significantly add to the scientific understanding of the role of genomic imprints in placental development. In characterizing the range of phenotypic and molecular abnormalities seen in DNMT1o-deficient placentas I have explicitly demonstrated that disruption of the inheritance of genomic imprinting has direct biological effects on trophoblast differentiation and metabolism and the balance of placental and fetal growth. Using this data, I was able to reveal novel associations between individual imprinted DMDs and specific placental phenotypes. This analysis revealed particular importance of the *Kcnq1*, *Peg10*, and *Mest* imprinting clusters for placental development. This study demonstrates the feasibility and importance of epigenotype-phenotype association studies. Lastly, my work developing and studying the imprinted *Klf14* gene revealed its role in regulating placental growth. My findings are summarized in Figure 47 demonstrate that mosaic loss of imprinting in the *Dnmt1*$^{\Delta 1o}$ maternal effect model lead to placental abnormalities, some of which can be replicated by deletion of *Klf14*.

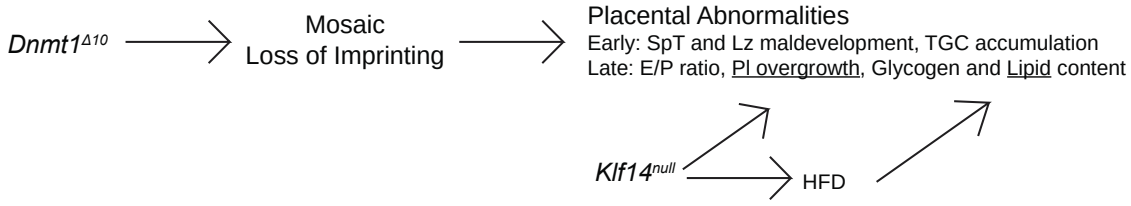


Figure 47. Dissertation summary model. The *Dnmt1*$^{\Delta 1o}$ maternal effect model results in partial and mosaic loss of genomic imprinting early in embryonic development. This influences abnormal placental development at early and late gestational time points resultin in specific phenotypes. Deletion of the *Klf14* gene also results in late gestation placenta overgrowth and when confronted with high fat diet increases lipid stores.

5.2 FUTURE DIRECTIONS

5.2.1 Coevolution of genomic imprinting and placentation

Genomic imprinting is a fascinating molecular phenomenon that has co-evolved with mammalian placentation. It is of note that prototheria (monotremes), metatheria (marsupials) and eutherian (placental mammals) have an increasing number of genomic imprints and reliance on *in utero* gestation (*436*). Furthermore, the boundaries of imprinted clusters are expanded to impose parent-of-origin specific expression to additional neighboring genes in extraembryonic tissues including the placenta and vitelline yolk sac (*307, 309*). For example, in extraembryonic tissues the *Igf2R* imprinted cluster includes *Slc22a1* and *Slc22a3*, the *Kcnq1* imprinted cluster includes *Th, Ascl2, Tspan32, Nap1l4,* and *Osbpl5*, the *Peg10* imprinted cluster includes *Tfpi2, Ppp1r9a, Pon2* and *Pon3*, the *H19* imprinted cluster includes *Ins2* and the *Grb10* cluster inclues *Cobl* (*307, 309*). The mechanisms by which this expansion occurs may have to do with unique nuclear architecture and chromatin topology in extraembryonic lineages that enable long distance interactions of ICs with expanded sets of imprinted genes and distant enhancers and repressors (*437*). Revealing the full scope of genomic imprinting within the placenta and the mechanisms that enable the expansion of imprinting boundaries will aide in the effort to ascribe placental functions to imprinted loci.

By understanding the evolutionary history of genomic imprinting we should be better equipped to understand the function of genomic imprints in mammalian biology. Therefore, I

think it is important to better grasp what placental physiological adaptations coincide with the acquisition of specific imprints. For example, the *H19* imprint (and paternal allele-specific *Igf2* expression) originated in the common ancestor of metatherian and eutherians possibly reflecting the changes in maternal/offspring resource allocation with the evolutionary acquisition of placentation (*438-440*). *Peg10* (but neither *Sgce* nor *Ppp1r9a*) and *Mest* are also imprinted in metatherians, but members of the *Snrpn, Kcnq1* and *Dlk1* cluster are not, suggesting the former are involved in early placentation while the latter are involved in eutherian-specific phenomena such as prolonged *in utero* development (*441, 442*). Furthermore, the *Impact* and *Zrsr1* imprints are specific to rodents (*442*). Intriguingly, strict *IGF2R* allele specific expression has been lost in primates, despite retention of imprinted *IGF2R* DMD methylation, perhaps indicative of changes in placental and fetal growth and maternal resource allocation in extended gestation of single conceptuses (*383, 439*). It is interesting to question whether the relaxation of *Igf2r* imprinting was due to a different adaptation to limit its expression, or changes in the threshold of IGF2 signaling, or to differences in the recognition of imprints in primates versus other mammals. The answers to these questions and more should help us understand our own genome and aid in the era of genomic medicine

The expansion of imprinted clusters to regulate additional genes in murine extraembryonic lineages is not conserved in humans. For example, in humans, the *Igf2r* DMD is maternally imprinted but expression of *IGF2R, SLC22A2* and *SLC22A3* is polymorphic and only imprinted in some but not all human term placentas (*383*). The expanded placenta-specific imprinting within the *Kcnq1* cluster is also lost in humans resulting in only the core *KCNQ1* cluster (*KCNQ1ot, KCNQ1, PHLDA2* and *CDKN1C*) and not the centromeric (*NAP1/4* and *OSBPL5*) nor telomeric (*CD81, TSPAN32* and ASCL2) adjacent genes retaining monoallelic

expression in trophoblast cells *in vivo* and *in vitro* (*383, 443*). Similarly, the extraembryonic paternally imprinted X chromosome inactivation phenomenon found in rodents is not conserved in humans, rather random inactivation analogous to the embryo proper occurs (*444*). The commonality of extraembryonic imprinted X-chromosome inactivation and expanded extraembryonic imprinted clusters processes suggests a co-evolution of an extraembryonic-specific imprinting mechanism that was lost during human evolution (*436*). The possibility of a mechanism involving ncRNA and repressive histone modifications that spread imprinting beyond core loci is particularly attractive (*436*).

There are three theories that explain how genomic imprinting arose during evolution (*440*). Each one postulates in its own way a selective advantage to the inheritance of parent-of-origin specific epigenetic information (*440*). The most prominent theory is the kinship theory in which a conflict of interest over maximizing reproductive fitness in maternal and paternal genomes exists (*411, 440*). In this paradigm the maternally inherited genome will be most successful if there are a large number of siblings across multiple litters from the same mother and favors an allocation of maternal resources that limits fetal growth so that it is not an impedance on future litters (*411, 440*). Paternally inherited genomes, particularly in non-monogamous mammals such as mice, maximize reproductive fitness by producing large vigorous offspring at the expense of future litters from the mother (*411, 440*). The kinship theory thus predicts and is validated by the fact that paternally expressed genes promote growth (*e.g. Igf2, Peg1*), whereas maternally expressed genes restrict growth (*e.g. Grb10*, *Phlda2*, *Cdkn1c*, *Igf2r*) (*440*). However, kinship theory is contradicted by the findings that many paternal UPDs result in growth restriction and/or embryonic lethality (*261*). Another argument against kinship theory is that it invokes a teleological argument that animates maternal and paternal genomes to selfishly direct

their own evolution towards different reproductive strategies. Theoretically, it is in the best interest of the species as a whole to have a balance of intra-litter and inter-litter fecundity to maximize species vitality.

The ovarian bomb theory is an alternative explanation to the evolution of genomic imprints (*445*). The central tenet to this theory is that genomic imprinting arose to prevent parthenogenetic oocyte activation from producing viable conceptuses or invasive trophoblastic diseases (*i.e.* ovarian teratomas) (*445*). This would limit trophoblastic disease to those derived from pregnancy (*i.e.* choriocarcinomas and molar pregnancies) (*445*). Like kinship theory, the ovarian bomb theory predicts that maternally expressed genes are growth limiting, and paternally expressed genes are growth promoting in order to overcome limitations the maternal genome has to promote growth (*445*). While this theory limits active selection to those genes involved in trophoblast development it does allow for new imprints to be acquired as passengers due to proximity or sequence similarity (*445*).

The authors of both the kinship and ovarian bomb theories focus entirely on the function of maternally and paternally expressed genes rather than the genomic imprints (*i.e.* DMD methylation) *per se* because the maternal imprints (*e.g. Kcnq1*, *Grb10*, and *Igf2r*) associate with expression of maternal genes and paternal imprints (e.g. *H19*) associate with expression of paternal genes often through indirect ncRNA or secondary epigenetic mechanisms. The evolution of maternal genomic imprints may have had as much to do with silencing of repressive ncRNAs (*e.g. Kcnq1ot*) than with the maternally expressed genes themselves. Similarly, the *H19* and *Dlk1* paternal imprints may have formed to silence the *H19* and *Meg3* ncRNAs rather than to promote the neighboring paternally expressed genes. I suggest that it may be more informative to ask how DMD methylation itself influences life history strategies to determine how genomic

imprints evolved. My results showed that loss of the maternal *Kcnq1* imprint was associated with TGC accumulation which in many ways looks like hydatidiform molar trophoblastic disease. This evidence fits the ovarian bomb theory better than the kinship theory. However, loss of the *Peg10* maternal imprint in DNMT1o-deficient mice results in diminished LZ development and fetal lethality, a result that neither the kinship nor ovarian bomb theory can adequately explain, suggesting that both theories may be too rigid.

A third theory on the evolution of genomic imprints suggests that haploid (monoallelic) expression enables rapid gene evolution (*446*). This theory is not mutually exclusive with the prior two. It is generally accepted among biologists that one of the benefits of diploid genomes is the robustness imparted by having two copies of each gene. However, when one allele is masked it can be mutated in compound fashion and bypass recessive lethal intermediates by not being expressed for multiple generations (*446*). In fact, it has been determined that many imprinted genes, including *Klf14*, are undergoing rapid evolution (*270*). It is also of note, although somewhat circumstantial, that most tissue-specific imprinted genes are either involved in placenta or the brain, the two fastest evolving organs in mammals (*447*).

Regardless of how genomic imprints initially evolved and what their selective fitness benefits are, they are clearly involved in reproductive isolation (*448*). In deer mice (*Peromyscus*) hybrids of *Peromyscus maniculatus* and *P. polionotus* have reciprocal parent-of origin effects (*448-451*). In crosses of *P.maniculatus* females with *P. polionotus* males offspring are viable but growth restricted and their JZ is reduced at E13 (*449-451*). In contrast, the reciprocal cross of *P. polionotus* females with *P.maniculatus* males are overgrown and dysmorphic with altered E/P ratio and disorganized hemorrhaging labyrinth (*449-451*). These overgrown mice have abnormal imprinting at the *Peg3*, *Mest*, *Snrpn*, *H19* and *Plagl1* imprinting clusters (*450, 451*). It has been

suggested due to the broad nature of loss of imprinting that there is a maternal effect locus (possibly *Dnmt1o* or *Dnmt3l*) responsible for the interspecific hybrid phenotypes (*450*). Furthermore, the placental dysplasia resulting from loss of imprinting is the main reason for the reproductive isolation of these two species (*448*). One explanation for these findings of incomplete loss of imprinting is that a differential expression difference in DNMT1o protein and interacting maternal/zygotic partners (*e.g.* DMAP1 or ZFP57) in the two species when interbred do not reach a sufficient threshold to maintain more sensitive imprints.

Many imprinted genes arose through retrotransposition. This mechanism generates new genes by reverse transcribing mRNA and integrating the resulting cDNA into the genome at a new site. This process is mediated by LTRs that flank transposable elements and are recognized by retroviral transposases. Essentially, these gene duplications events enabled the evolution of novel functions and/or or expression profiles. There are three classes of imprinted retrotransposed genes. The first class are transposed copies or endogenous retroviral genes (ERVs) that have been coopted to serve unique mammalian function (*e.g. Peg10, Rtl1*) (*289, 290, 373*). A second class of retrotransposed imprinted gene were derived from X-chromosome parent genes and inserted within introns of host genes (*e.g*. *Nap1l5* and *Zrsr1*) (*452*). A third class of retrotransposed imprinted gene as exemplified by Klf14 are intronless transposed copies of autosomal genes (*270*). Each of these types of imprinted genes acquired imprinting following transposition.

Both *Peg10* and *Rtl1* are copies of a Ty3/gypsy retrotransposon most similar to the Sushi-ichi retrotransposon family found in other vertebrates (*289, 290*). Of the 9 mammalian Sushi-like retrotransponons 5 are located on the X chromosome, 2 are imprinted and 2 are autosomal non-imprinted (*289*). It is likely that *Peg10* initiated the formation of an imprinted loci to block its

expression after transposition, although the Peg10 DMD sequence does not have homology to other Sushi-ichi family members (*289*). The *Peg10* locus is present in eutherian and marsupial mammals but not in monotremes further suggesting a link with placental evolution (*453*). *Rtl1* does contain a CGI, however there is no evidence of parent-specific methylation (*289*). *Rtl1* retrotransposition was an ancestral event to the acquisition of the intergenic DMD regulating the *Dlk1* cluster and insertion of miRNAs and snoRNAs in eutherians (*368*).

Retrotransposition of *Nap1l5* and *Zrsr1* resulted in novel micro imprinted domains that imposed parent-of-origin transcriptional effects on their host genes (*454*). The rodent-specific retrotransposition of *Zrsr1* led to direct imprinting of *Commd1*. In the case of *Nap1l5* retrotransposition resulted in imprinted allele-specific polyadenylation of *Herc3*. The *Nnat* microimprinted domain is similar in structure to *Nap1l5* and *Zrsr1,* and imprints its host gene *Bclap*. No ancestral gene has been identified, although a *Nnat* pseudogene exists on mouse chromosome 7 (*239, 454, 455*). It is perhaps not coincidental that alternative polyadenylation and transcriptional interference that occurs in these less complicated imprinted domains also occurs in the larger *Kcnq1, Mest*, and *Igf2r* domains. These findings on microimprinted domains have been interpreted as suggesting that such retrotranspositon events may have been the impetus for the earliest genomic imprints in eutherian development. My results showed that the *Nnat, Nap1l5*, and *Zrsr1* DMDs are approximately 40- 60% methylated in wild-type placentas which is in line with expected levels of a genomic imprint (Figures 32 Q-S). However, only the *Zrsr1* DMD had significant loss of methylation in DNMT1o-deficient placentas indicating that the *Nnat* and *Nap1l5* DMDs are either insensitive to loss of preimplantation maintenance methyltransferase activity or that they are absolutely required for TE development.

The final class of retrotransposed imprinted genes are copies of autosomal parent genes. For example, *Klf14* most likely acquired imprinting status upon its transposition in proximity of *Mest*, which may have been beneficial to limit the dosage of the ancestral gene product *Klf16* and enable its rapid evolution (*270*). Furthermore, retrotransposition of other genes have been vital to placental development in particular the syncytins *SynA* and *SynB* were coopted from viral envelope proteins to enable cell fusion of SynT progenitors within the labyrinth (*210, 211*). The syncytin 5′ LTR sequences are promoters dynamically regulated by DNA methylation in the human placenta (*456*).

It is also of note that species specific ERVs, or portions of their LTRs, are present in the promoters of many trophoblast expressed genes. These ERV promoters are enriched in binding sites for the placenta master transcriptional regulators ELF5, CDX2 and EOMES (*457*). These findings suggest a relationship between intergenic ERV transposition and adoption of trophoblast specific expression in mammalian genomes. It is clear from the above findings that retrotransposition had a strong impact on the evolution of both placentation and genomic imprinting. The placenta is a suitable target for live retroviruses and ERVs as it enables direct passage between generations as well as enabling horizontal transfer from heterozygous individuals to littermates *in utero* (*458*). In addition, the placenta may provide access to germline integration (*458*). ERVs once established would be maintained by positive selection if they were coopted to unique functions, efficiently silenced or genetically degraded (*458*). Retrotranspostition may be a key in the evolution of genomic imprinting because DNA methylation evolved as host genome defense mechanism used to silence transposable elements (*459, 460*). While not all imprinted loci contain transposed elements it is possible they are recognized by the same methylation machinery due to sequence similarities. It is still unclear

how imprinted DMDs, unlike the majority of the genome (including most ERVs), escape erasure and *de novo* DNA methylation during reprogramming.

5.2.2 Imprint-like sequences

Recently, imprint-like sequences that convey heritable epigenetic information from parental gametes have been identified. Methylation at these regions are similar to imprints in that they acquire parent-specific methylation patterns and are protected from DNA demethylation during preimplantation development. Much of the evidence of imprint-like sequences comes from the study of TET-off *Dnmt1* cell culture model of early perimplantation development (*110, 111*). In the TET-off system addition of tetracycline reduces genomic DNA methylation in ES cells within days of tetracycline application, however DNA methylation is also lost at imprinted DMDs (*110, 111*). After removal of tetracycline, genomic DNA methylation gradually recovers due to the combined activity of *de novo* and DNMT1 methyltransferase activity. However, genomic imprints and a larger group of imprint-like sequences do not recover DNA methylation to prior levels (*111*). Among 90 genomic loci that have greater than 80% reduction in DNA methylation following transient DNMT1 inactivation in ESCs as measured by RRBS 15 are imprinted loci and the remainder are imprint-like sequences (*111*). *Zfp787* is one imprint-like gene previously identified as a transient maternal DMD using the *Dnmt3l* maternal effect model (*98*). *Zfp787* is maternally imprinted in gametes and is protected from preimplantation demethylation, but acquires paternal methylation methylation at implantation such that embryonic livers are biallelically methylated by E9.5 (*98*). Unfortunately, the *Zfp787* DMD methylation status has not been determined in any extraembryonic lineage. A number of other

loci were co-identified in the TET-off and *Dnmt3l* maternal effect models but have not been further validated as imprinted DMDs or transient DMDs (*98, 111*).

DNA methylation at a limited set of these imprint-like sequence was measured in DNMT1o-deficient embryos and placentas to determine if the loci identified in the TET-off system are similarly effected by loss of maintenance methyltransferase activity during early reprogramming events (*111*). Many of these loci showed partial loss of methylation in both compartments (e.g. *Rnf216, Bbs9, Stk10* and *Zfp676*) while others showed loss of methylation only in the placenta (e.g. *Xlr4a, Xlr4b,* and *Prdm1*) or in the embryo (*1700018B08rik*) (*111*). This may indicate that the epigenetic status of some transient DMDs are inherited exclusively in embryonic or extraembryonic lineages while others are perpetuated in all lineages and are likely true DMDs. Intriguingly, *Prdm1* is a transcriptional repressor that is critical for differentiation of SpA-TGCs (*461*). I propose to identify the full set of imprinted DMDs and imprint-like sequences that is disrupted in *Dnmt1*$^{\Delta 1o}$ maternal effect offspring (both fetal and placenta tissues) using a global DNA methylation analysis method such as RRBS. This data may even be used to determine if any of the imprint-like sequences associate with placental phenotypes.

5.2.3 Role of placenta in imprinting disorders

It was my hope that some of my dissertation results would shed light on human imprinting disorders. Many imprinting disorders alter prenatal and/or postnatal growth rates. For example, BWS manifests as overgrowth and sometimes occurs with placentomegaly (*114*). This syndrome can have epigenetic etiologies based on loss of methylation of the *Kcnq1* DMD and gain of methylation of the *H19* DMD (*114*). My results showed a correlation with loss of *Ascl2* expression in DNMT1o-deficient placentas and increased E/P ratio suggesting loss of imprinting

within the *Kcnq1* cluster may increase placental efficiency in late gestation (Figure 29A). It was also intriguing that late gestation expression of both *Ascl2* and *Phlda2* was increased in DNMT1o-deficient placentas, as opposed to the decrease of expression expected with loss of *Kcnq1*DMD methylation, suggesting compensatory mechanisms that may alter late gestation loss of imprinting expression profiles and fetal and placental growth trajectories. I observed a strong correlation between loss of *Kcnq1* methylation and TGC accumulation during mid-gestation was made (Table 4 and 36G). and it would be interesting to determine if the abundance of the analogous extravillous cytotrophoblast in BWS cases is altered.

The SRS growth restriction clinical phenotype is due to loss of methylation at the *H19* DMD in the majority of cases and due to matUP7 or gain of *Mest* DMD methylation in a minority of cases (*125, 130*). I showed that loss of maintenance methyltransferase activity in DNMT1o-deficient conceptuses results in mosaic loss of *Igf2* expression in the placental compartment (Figures 4 and 28). However, I did not find an association between *H19* DMD methylation with either placental or fetal weight. I did however find an association between loss of *H19* DMD methylation and JZ layer development at E12.5 (Table 4). In addition, higher E/P ratio was associated with increased *Mest* expression and loss of *Mest* DMD methylation was associated with placental lipid accumulation (Figures 29B and 39). These results in combination with the placental overgrowth phenotype observed in the *Klf14* deletion (Figures 44A and 45A) suggest that the *Mest* cluster is involved in the growth phenotype of matUPD7 SRS cases where one would expect increased genetic dosage of *Klf14* and decreased *Mest* expression. It would certainly be worthwhile to examine SRS associated placentas for changes in cytotrophoblast histology and in sycytiotrophoblast lipid content.

The imprinting diseases PHPIa and Ib are caused by loss of imprinting at the *Nespas* and *Gnas* locus (*148*). I was intrigued to find that the *Nespas* DMD methylation was largely immutable in DNMT1o-deficient placenta, perhaps suggesting a developmental requirement (Figures 32M and 32N). While the placenta takes on some functions of thyroid and kidney during prenatal development it remains unclear what if any function the imprinted genes in this cluster do in the placenta. I revealed a positive association between loss of *Nespas* DMD methylation and spongiotrophoblast at E12.5 (Table 4).

Similarly, no known placental functions for the PWS/AS loci containing the *SNRPN* imprinting cluster have been described. My results using stepwise linear regression modeling suggest that both *Kcnq1* and *Snrpn* are informative to predicting TGC accumulation (Table 5). Although it is possible that the *Snrpn* association is due to close linkage with *Kcnq1* it may be informative to examine placentas associated with PWS/AS for abnormal extravillous trophoblast proliferation. Recently it has been shown that snoRNAs from the *Snrpn* cluster regulate alternative splicing of the serotonin receptor *5Htr2c*, and separately that the placenta is a transient source of serotonin during early forebrain development (*324, 462-464*). I conjecture based on those two lines of evidence that the *SNRPN* cluster may regulate serotonin levels during prenatal development and be altered in *PWS/AS* conceptuses. It would be interesting to examine fetal and placental serotonin receptor isoform expression and serotonin levels in DNMT1o-deficient offspring (with loss of *Snrpn* DMD methylation) and in *Snrpn* locus targeted mutation models (e.g. *Snrpn* DMD deletion, *Snord114/116* deletion). I also propose that some imprinting disorders cases may be caused by failure to maintain imprints during preimplantaion. The findings of mosaic loss of imprinting, particularly in BWS and SRS cases, suggests that the severity of phenotypes may be based on the degree of mosaicism and the tissue types (including

the placenta) in which loss of imprinting is found. Finally, I suggest that there may be distinct epi-alleles in the population that are more prone to loss of imprinting during preimplantation development due to environmental factors and maternal effects.

The separation of placental and fetal phenotypes in the DNMT1o-model is difficult due to the entanglement and codependence of their development. One tool to better ascertain the placenta autonomous phenotypes from those that are intertwined or the result of fetal maldevelopment is through tetrapoloid trophoblast complementation. It would be informative to determine if the partial fetal lethality observed at E12.5 in DNMT1o-deficient placentas could be rescued by tetrapolid complementation of *Dnmt1*$^{\Delta 1o}$ maternal effect blastocysts. If survival to late gestation and neonatal time points was observed at a greater frequency, placental phenotypes could firmly be attributed as the main antagonist of early fetal lethality associated with loss of imprinting. Similarly, the reciprocal experiment could be performed and normal ES cells injected into *Dnmt1*$^{\Delta 1o}$ maternal effect blastocysts and examined for developmental outcome. If abnormal wild-type fetal development was observed in such conceptuses it would be arguably caused by placental imprinting defects, however this system would likely result in at least partially chimeric fetuses with some cells having partial loss of imprinting. I also suggest the development of both ESC and TSC lines from DNMT1o-deficient conceptuses that have loss of imprinting at a small number of loci with clinical relevance (*e.g. Snrpn, H19, Kcnq1, Mest, Plagl1, Nespas*). These cell lines could be studied for their potential to generate normal chimeric fetal or trophoblast tissue after blastocyst injection. The experiments I have proposed above may yield results that are informative on the contribution of genomic imprinting in the placenta on fetal development.

5.2.4 Reproductive technologies

It has been reported that within the population of BWS patients there is enrichment for individuals conceived by assisted reproductive technologies (ART) (*465-468*). Although this suggests an increased incidence of imprinted diseases associated with ART, not all clinical research corroborates these findings (recently reviewed by (*469, 470*)). Nearly all cases of BWS associated with ART are concomitant with loss of methylation at IC1 (KCNQ1), whereas this is the case for only half of all BWS cases in the naturally conceived population (*114, 471*). Furthermore, loss of methylation in BWS cases from children born after ART is observed at a broad range of DMDs including *Mest*, *Snrpn* and *Plagl1* (*471, 472*). This multigenic, and sometimes mosaic effect suggests that loss of imprinting occurs post-fertilization within the embryo. It is important for future studies to calculate the ART associated risk of SRS and TNDM1, which are most commonly associated with loss of methylation at the *H19* and *PLAGL1* loci respectively (*469*). The gamete and embryo manipulation performed in ART procedures such as superovulation, *in vitro* fertilization and intra-cytoplasmic sperm injection directly expose both gametes and embryos during the window of time crucial for the maintenance of genomic imprints (*473, 474*). My results clearly show that the majority of DMDs, including *Kcnq1*, *H19*, *Mest* and *Plagl1* can be affected, either in isolation or in tandem, by loss of DNMT1o activity Therefore, it is rather intuitive to suggest that ART may be detrimental to DNMT1o maintenance methyltransferase activity.

Similarly, both ART and cloning in mouse alter imprinted DMD methylation. Mouse embryos generated from IVF and cultured during preimplantation development in Whittens medium have lower levels of *H19* methylation, and their placenta are afflicted with loss of imprinting to a greater degree than the fetus, showing biallelic expression of *H19*, *Snrpn*, *Ascl2*

and *Peg3* (*475, 476*). Likewise, aberrant imprinted gene expression is observed in preimplantation embryos cultured in M16+FBS (*477*). Blastocysts, mid-gestation embryos and placentas derived from superovulated oocytes developing *in vivo* have a modest partial loss of imprinting at the *Snrpn* and *H19* loci (*478, 479*). Both biallelic expression and loss of DMD methylation is greater in the placental compartment than in the embryo proper (*478*). Superovulated oocytes harbor normal imprinted DMD methylation ruling out superovulation induced effects on the establishment of imprints (*480*). Imprinted genes are overrepresented in those transcripts whose expression is increased or decreased more than 2-fold in placentas derived from *in vitro* fertilized superovulated oocytes (*481*). These results taken together suggest that environmental influences subject the early embryo to loss of imprinting during preimplantation development and that the maintenance of imprints is less robust in the TE than the ICM. It would certainly be of interest to determine the amount of DNMT1o present in superovulated oocytes and its activity in preimplantation *in vitro* culture.

Reconstitution of an enucleated oocyte with a donor nucleus (either ES or somatic cell) reformats the epigenetic state of the donor genome to one that emulates a nascent zygote in a process known as “reprograming” (*482, 483*). Cloning by nuclear transfer is an inefficient process (*484*). Only10-20% of ES cell nuclear donors and 50-70% of somatic cell nuclear donors mature into blastocysts (*484*). Of uterine transferred blastocysts 5-20% of ES nuclear donor and 1-3% of somatic nuclear donors survive to term and even fewer to adulthood (*484*). The majority of clones develop abnormally with fetal and placental overgrowth concomitant with altered imprinted gene expression and DMD methylation (*484-487*). Cloned placentas are overgrown almost 2-fold, have an expanded JZ enriched with GCs with SpT extensions into the LZ, have enlarged TGCs and a disorganized LZ (*488*). ESCs cultured from SCNT derived blastocysts are

epigentically equivalent to naturally fertilized blastocyst derived ESCs and can produce all fetal lineages when complemented with tetraploid TE further suggesting that extraembryonic development is the limiting factor in reproductive cloning (*489, 490*). The similarities between cloned mice and the JZ and GC expansions I observed in late gestation DNMT1o-deficient placentas is striking.

My results also showed a disruption of E/P ratio and a general overgrowth of DNMT1o-deficient placentas and fetuses surviving to late gestation which mirrors the findings in cloned animals and suggest that abnormal maintenance of imprints during preimplantation may be the central cause of cloning developmental failures. The work on reproductive cloning has implications for the large offspring syndrome observed in cloned livestock and in conservation efforts to restore native fauna and endangered species (*491, 492*). Interestingly, SCNT porcine embryos undergo replication dependent preimplantation partial demethylation of the *H19* DMD (*493*). Because this demethylation occurs during the first two cell cycles and is mimicked by *DNMT1* GV oocyte RNAi injection it implies that maternal DNMT1s activity is decreased in SCNT embryos (*493*). The above results taken together indicate that it is critical for reproductive biologists to better understand the details of maintenance methyltransferase activity in preimplantation development to improve the safety and efficacy of clinical ART and animal cloning. It would be insightful to determine if DNMT1o activity is altered in ART or nuclear transfer in mice, and whether ART or oocyte reconstitution can modulate the $Dnmt1^{\Delta 1o}$ maternal effect.

Reprogramming of somatic nuclei can also be carried out by transfection of the 4 Yamanaka pluripotency factors (*Oct3/4*, *c-Myc*, *Sox2* and *Klf4*) (*494*). A small percentage of these induced pluripotent stem (iPSCs) cells can generate high percentage chimerism when

injected into blastocysts and yield all iPSC pups from tetraploid TE complementation (*495, 496*). The most prominent transcriptional difference between iPSCs incapable of generating all iPSC pups via tetraploid complementation and genetically identical ESCs is found in the expression levels of maternally expressed *Meg3* and *Rian* transcripts within the *Dlk1* imprinting cluster (*380, 381*). Biallelic *Dlk1* DMD methylation represses maternal ncRNA transcription in these iPSCs (*380, 381*). However, the subset of iPSCs that are competent to generate chimeras and all iPSC tetraploid complemented offspring have normal monoallelic methylation and *Meg3* expression (*380, 381*). The incompetent iPSCs can be made to generate all iPSC offspring by the addition of the HDAC inhibitor valproic acid (*381*). Lastly, the addition of ascorbic acid (vitamin C), a potent histone methyltranferase and TET enzyme cofactor, during iPSC induction prevents *Dlk1* DMD hypermethylation and yields developmentally competent iPSCs (*497*). The fact that the *Dlk1* DMD becomes biallecially methylated during iPSC reprogramming suggests that there are molecular mechanisms during *in vivo* reprogramming that prevent *de novo* methylation at this locus. My results that *Dlk1* DMD methylation is invariable in DNMT1o-deficient placentas is in line with these findings and suggests an early selection against loss or gain of methylation at the *Dlk1* DMD (Figures 32O and 32P). Furthermore, parthenogenetic development can be induced with inheritance of one allele of Dlk1 and *H19* DMD deletion (both of which mimic the paternal imprinted state). I interpret these results to suggest that paternal imprints are required for embryonic development and as supporting evidence to the ovarian bomb theory.

The limited developmental potential of SCNT and iPSCs is perhaps not surprising given that ES cells themselves, particularly inbred and long term passaged lines, are restricted in their fetal chimera contribution when injected into host blastocysts or complemented with tetrapoloid TE (*484, 498, 499*). Mice derived from ES cells are often overgrown and riddled with epigenetic

defects including at imprinted loci (*498*, *499*). Clearly these results suggest that the maintenance of genomic imprints during reprogramming is a requirement for full embryonic developmental potential. It would be interesting to determine the extraembryonic developmental potential of ES, SCNT and iPSCs with imprinting defects that have been transformed via iRas to a TSC like state.

5.2.5 Mechanisms of imprinting

There are a number of unresolved issues regarding the molecular mechanisms involved in the maintenance of genomic imprints during preimplantation development. I have presented a summary of the factors present in the imprinting maintenance machinery including DNMT1, UHRF1, DNMT3, DMAP, ZFP57, TRIM28, and histone modifiers (Section 1.1.2). The maintenance of parent-of-origin specific monoallelic methylation during the dynamic genomic demethylation and remethylation events that occur with preimplantation reprogramming require that imprinted alleles be protected from demethylation and faithfully propagated, and the non-imprinted allele to be resistant to *de novo* methylation. Genetic ablation of the factors involved in these processes effects imprinted DNA methylation. Deletion of either *Zfp57* or *Trim28* results in partial loss of DMD methylation with certain loci more affected than others (*71, 72*, *74*). It is therefore reasonable to hypothesize that different imprinted loci have unique effectors and epigenetic readout, and that some are more robust than others. Furthermore, the maternal-zygotic lethality of compound *Dnmt1*^{v} and *Dmap* null alleles suggests that the interaction between DMAP and maternal DNMT1s is essential for early reprogramming either via maintenance of imprints or the formation of the TIP60 epigenetic complex (*107*). Detailed clarification of the

epigenetic components involved in preimplantation and their interactions although difficult to procure from early embryos will be essential to fully elucidate this process.

The role of ncRNA in the establishment, maintenance and expansion of genomic imprints is also unresolved (*500-502*). The macro long ncRNAs *Kcnq1ot, Airn,* and *Nespas* have been implicated to act *in cis* to silence neighboring genes directly by transcriptional interference and through recruitment of heterochromatin factors including EHMT2 (*500*). The expansion of the *Airn* and *Kcnq1ot* ncRNA spread across chromatin in extraembryonic tissue is one plausible explanation for larger imprinted domains at the *Igf2r* and *Kcnq1* clusters in placenta (*500*). In addition, the ncRNA may function in modulating nuclear architecture and long range chromatin interactions (*437*). Both the *Kcnq1ot* and *Airn* ncRNA are expressed in spermatagonia where the X-chromsome is imprinted via the *Xist* ncRNA and may share a common mechanism in regulation of their promoter (*436*).

Other imprinted ncRNAs like the snoRNAs and miRNAs in the *Snrpn* and *Dlk1* cluster likely function in trans to modulate alternative splicing, mRNA editing and overall gene expression levels of important developmental factors. For example, the miRNAs processed from the extended *Meg3* transcript have are thought to modulate PRC2 components that influence expression of numerous genes (*380*). The snoRNA genes *Snord114* and *Snord116* within the *Snrpn* cluster alter alternative splicing and mediate prominent PWS/AS phenotypes (*324, 325, 464*). The role of snoRNAs derived from *Meg8* within the *Dlk1* cluster nor the role of miRNAs from the same cluster are well defined. The snoRNAs from both the *Dlk1* and *Snrpn* clusters form unique imprinted chromatin architecture in which one allele is transcriptionally active and produces long transcripts that are found at nuclear foci near their respective imprinted domains, that are spliced into snoRNA products and distributed throughout the nucleus (*503*). The *H19*

ncRNA is processed into *Mir675*, a miRNA that influences placental development (*306*). Other ncRNA antisense transcripts including *Peg3as, Mestit,* and *Nespas* are even less studied. Furthermore, the large primate specific C19MC miRNA cluster is imprinted and is involved in placental exosome mediated fetal and maternal immunity (*504, 505*). I conjecture that future research will better define the functional roles of imprinted ncRNAs in health and disease.

5.2.6 Role of the *Kcnq1* cluster in TGC development

One of the major findings in my dissertation was an association between loss of methylation at the *Kcnq1* and *Snrpn* DMDs and accumulation of TGCs (Tables 4 and 5; Figures 36G and 36H). The TGC accumulation phenotype mimicked the *Dnmt3l* maternal effect and *Ascl2* targeted deletion models suggesting that loss of *Aslc2* expression in trophoblast is the underlying cause. In humans, *Ascl2* is expressed highly by intervillous and extravillous trophoblast but is not imprinted (*506, 507*). Complete hydatidiform molar pregnancies (which have a diploid paternally inherited genome) that are noninvasive do not express *ASCL2*, whereas invasive complete hydatidiform molar pregnancies do express *ASCL2* (*506*). Although the lack of *ASCL2* expression in the noninvasive molar pregnancies suggests paternal imprinting, it is more likely due to the predominance of microvillous-like cysts containing SynT and villous cytotrophoblast and the lack of extravillous cytotrophoblast which do not express *ASCL2*. The expression of *ASCL2* in intervillous cytotrophoblast is congruent with *Ascl2* expression in murine SpT, where it acts to repress TGC differentiation, but its expression in extravillous cytotrophoblst seems contradictory given their similar to TGCs. It would be interesting to determine if *ASCL2* is absent from more specialized endovascular extravillous cytotrophoblast subtypes.

Other members of the *KCNQ1* imprinted cluster that are imprinted in mouse extraembryonic lineages are not imprinted in humans including *CD81, TSSC4, NAP1/4* and *CARS* (*383*). However, *CDKN1C* and *PHLDA2* that are closer to the *KCNQ1* imprinting center are ubiquitously imprinted in human and are normally expressed in various cytotrophoblast lineages (*383, 508, 509*). In complete hydatidiform moles neither *CDKN1C* nor *PHLDA2* are not expressed (*508, 509*). Their expression is also limited in incomplete hydatidiform moles (triploid with diploid paternal and haploid maternal chromosome set) due to the single active maternal allele (*508, 509*). Although these correlative results suggest a possible role in molar pregnancies more profound evidence would be needed to rule out coincidental or passive associations. Many of the DNMT1o-deficient placentas collected at E9.5 presented hydatidiform molar-like cysts when viewed under the dissecting microscope (data not shown). I suspect that these may have been similar to the samples with expanded TGCs and flattened labyrinths, and diminish *Ascl2* and *Phlda2* expression (Figure 4). Based on the E9.5 DNMT1o-deficient phenotypes and the E12.5 associations between loss of *Kcnq1* DMD methylation and TGC accumulation along with the above hydatidiform molar molecular imprinting abnormalities I infer that loss of the *KCNQ1* imprint contributes to hydatidiform molar pregnancies.

The role of P-TGCs in mouse placenta development is to form a protective barrier between maternal endometrium and produce placental hormones (*231, 232*). Specifically, TGCs secrete choriogonadotropin hormone and the related family of prolactins (*225, 227*). In rodents this family of hormones has grown to include of 23 members in a large cluster and 3 additional members in a smaller cluster on mouse chromosome 13qA1 (*225, 227, 236*). While humans have a more limited set of prolactins their function in placenta development are poorly understood. The extravillous cytotrophoblast are similar to mouse TGCs and are polypoloid, but not to the

same extent as TGCs. Extravillous cytotrophoblast are however more invasive than their mouse counterparts extending into and taking up residence in the myometrium where they integrate into the maternal vascular system (*177*). Most studies on extravillous trophoblast have focused on their angiogenic and vascular remodeling properties in health and disease (*e.g*. PE). I think there may be crucial insights to be gained by studying their proliferation and secretion in loss of imprinting disorders. Given that placentomegaly is a common feature of BWS it would be pertinent to examine BWS associated placentas with loss of IC1 loss of methylation for extravillous cytotrophoblast numbers and examine whether *Ascl2* expression is disrupted.

5.2.7 Role of the *Peg10* cluster in labyrinth development and fetal viability

My regression studies revealed associations between loss of the maternal *Peg10* imprint and diminished labyrinth development and embryonic lethality at E12.5 (Table 4; Figure 36E). While deletion of *Peg10* results in similar neonatal lethality, loss of methylation is expected to lead to biallelic transcription of *Peg10* and *Sgce* and suppression of paternal *Pon2*, *Pon3* and *Ppp1r9a* transcription (*288*, *293*). In fact, I found that *Pon2* was significantly down regulated at E9.5 (Figure 28). The study of commercially available *Pon2, Pon3*, and *Ppp1r9a* targeted deletion mouse models for placental phenotypes may be informative. I also suggest the generation of a *Peg10* DMD deletion model which would likely replicate the imprinted allele due to the deletion of *Peg10* and *Sgce* promoter elements, and phenocopy embryonic lethality in the paternal *Peg10* null and 6q bimaternal translocation models. The *Peg10* DMD deletion may also reveal additional phenotypes from biallelic expression of maternally expressed genes in this cluster, particularly if crossed with a single copy *Peg10* transgene to rescue early lethality. Transgenic overexpression of *Peg10* and *Sgce* could mimic the loss of *Peg10* DMD methylation phenotypes

observed in DNMT1o-deficient placentas and would be worthwhile to pursue. Lastly it has been presented in the literature that the *Peg10* DMD regulates an expanded set of imprinted genes in extraembryonic tissues including *Ppp19ra,* and possibly other genes (*e.g. Dlx5*, *Calcr* and *Asb4*), therefore it is imperative to determine the exact boundaries of the *Peg10* imprinted cluster in mouse placenta (*263, 510*). This could be done by examining expression of genes in proximity of *Peg10* in DNMT1o-deficient E9.5 placentas with near complete loss of *Peg10* DMD methylation.

5.2.8 Identification of KLF14 transcriptional targets in mouse.

My results in Chapter 4 show that *Klf14* is an imprinted negative regulator of placental growth and that its deletion results in a significant increase in placental weight. It is important to determine what transcriptional targets are regulated by *Klf14* during placental development. To these ends I am currently working on comparing the genome-wide gene expression profiles of *Klf14* homozygous null and wild-type littermate placentas derived from heterozygous null intercrosses. Once significantly altered genes are identified I will test for enrichment of gene ontology pathways. I will also examine the promoters of up and down regulated genes for the presence of CACCC KLF14 DNA-binding sequence motifs. Transcriptional targets could be confirmed using luciferase reporter assays in trophoblast stem cells co-transfected with *Klf14* and luciferase reporters driven by the promoter of putative KLF14 targets. Additionally, it would be of interest to examine the presence of the KLF14 interacting partners SIN3A and p300 as well as various histone modification at target genes of interest in homozygous null and wild-type placentas using chromatin immunoprecipitation. These experiments should further reveal the functional role of *Klf14* in placental development.

During my thesis research I was unable to determine where in the placenta *Klf14* is expressed. It has been reported that *Klf14* is present in the placental labyrinth, which is an amalgam of trophoblast and fetal derived cells. I attempted to use commercially available polyclonal antibodies to identify KLF14 localization by immunofluorescence to no avail (data not shown). I had mixed results using these antibodies in western blotting as well (data not shown). Here I propose three different experiments to determine whether *Klf14* is expressed in trophoblast or fetal derived labyrinth cells. Heterozygous $Klf14^{flox}$ females could be crossed to male *Sox2:CRE* transgenics to generate epiblast specific *Klf14* maternal null conceptuses. If the fetal vascular endothelial cells in the labyrinth are the only cell type that express *Klf14* then fetuses that are heterozygous *Klf14* null would lack placental *Klf14* expression. Additionally, I could generate a trophoblast specific conditional deletion by crossing heterozygous $Klf14^{flox}$ females with *Cyp19*, *Tpbpa*, or *Gcm1* driven *CRE* transgenic males to generate ubiquitous trophoblast specific deletion or targeted deletion within SpT or SynT layer II respectively (*511*). A second method to generate trophoblast specific null mice is through the use of peripheral blastocyst lentiviral CRE infection (a new technique that specifically targets the mural and polar TE at the blastocyst stage) (*512*). Finally, I could attempt to isolate either SynT or fetal endothelial cells by using various cell capture methods (*ie.* antigen specific magnetic bead capture, or flow cytometry) and examine *Klf14* expression.

I also propose the construction of additional models to determine the role of the *Mest* imprinting cluster at large within the placenta. I think it would of interest to attempt to develop a *Klf14* transgenic overexpression model, although this may be difficult given the mitotic catastrophe revealed in HeLa cell *Klf14* overexpression (*434*). Engineering a targeted *Mest* DMD deletion model, while complicated due to the overlap with exon 1 and intron 1, would be

expected to generate an allele that replicates the methylated maternal allele. Paternal inheritance of a *Mest* DMD null might influence fetal and placental growth given the expected decrease in *Mest* expression and increase in *Klf14*. It is also important to define the function of MEST using biochemical *in vitro* experiments to determine putative hydrolase substrates. A *Mest* overexpression transgenic model may recapitulate the DNMT1o-deficient placental lipid accumulation phenotype and be insightful to determine causative mechanisms in a less heterogenous model. It would also be of particular interest to delete the placenta specific *Mest* promoter and/or exon and investigate whether placenta and/or fetal growth restriction still occurs similarly to the constitutive deletion. Each of these models suggested here could also be fed HFD to see whether metabolic phenotypes can be induced. Although I have focused on placental biology, it is likely that the *Mest* imprinting cluster has important roles in other organs.

5.3 CONCLUDING REMARKS

In conclusion, I have surveyed the placental morphological and epigenetic abnormalities in DNMT1o-deficient placentas and revealed unique roles for the *Kcnq1*, *Peg10* and *Mest* imprinting clusters. My work to generate and study a targeted deletion of *Klf14* has demonstrated a role for it in regulating placental growth. These findings add to the growing body of evidence that genomic imprints are vital for placental development. I hope that my dissertation research has added to our understanding of the mammalian genome. I look forward to further investigations by myself and others that resolve the functional roles and mechanisms involving the phenomenon known as genomic imprinting.

APPENDIX A

Primer Name	Primer Sequence
***Dnmt1o* Genotyping**	
Egg6	AGGAAAACAGTGGAGGAAC
Egg7	TACTTTGCACAGGGCTGTCCT
Sex Genotyping	
ZFYfwd	CCTATTGCATGGACAGCAGCTTATG
ZFYrev	GACTAGACATGTCTTAACATCTGTCC
***Sox2:CRE* Genotyping**	
SOX2fwd	AGGTGTAGAGAAGGCACTTAGC
SOX2rev	CTAATCGCCATCTTCCAGCAGG
***Klf14* Genotyping**	
HB3fwd	CTGCTCTTCCTTTCCTCACT
HB4rev	GAGAGACTTTTCTTCGCAGC
HB6rev	TGATTAGGAGGGGGAAAACAC

APPENDIX B

Primer Name	Primer Sequence	
qPCR		
Peg10	TGCTTGCACAGAGCTACAGTC	CTGAATCCAGCCATGTGGTAGA
Sgce	CGGATTCTTTGAAAAGCCGAGA	GTCTGTGTGCATGGGAGGTAT
Pon2	GCTCTGAGTTTGCTGGGCAT	GGCAGTTTGGAAGGTCTACAGAT
Dlk1	GGAACCATGGCAGTGCATCT	CGAACGTCTATTTCGCAGAATTT
Meg3	TCCTCACCTCCAATTTCCCCT	GAGCGAGAGCCGTTCGATG
Rtl1	CCTGTGCCAGGGGCTCAACG	CTTGGGCGCGACTCAGGTGG
Igf2	GTGCTGCATCGCTGCTTAC	ACGTCCCTCTCGGACTTGG
H19	GAACAGAAGCATTCTAGGCTGG	TTCTAAGTGAATTACGGTGGGTG
Igf2r	AGCCTCGGCAGATTTATTTT	CCCCATTGGTCCTCATGTCT
Slc22a2	GAACGCTGAGCTGTACCCTACA	GGGCAGAGCACACCATCAT
Mest	TCTCCAAAAGCTCCTCAAAG	ATGAATGGGGATGGACACAG
Klf14	CTCCGTGTGCCTCAACTAGC	CAGGCGCATCCAGGATAGC
Phlda2	CTCCGACGAGATCCTTTGCG	ACACGTACTTAGAGGTGTGCTC
Ascl2	AAGCACACCTTGACTGGTACG	AAGTGGACGTTTGCACCTTCA
Grb10	CCTGCCAAGCATGATGTCAAA	CCAGGCACCTCTCTAATCCCA
RT-PCR		
Klf14	TGGACACCCTCTCCAAAGTC	AAGCGACATCAGTGCTCCTT-

APPENDIX C

Primer Name	Primer Sequence	
Bisulfite Genomic Sequencing (nested)		
H19_R1	GAGTATTTAGGAGGTATAAGAATT	ATCAAAAACTAACATAAACCCT
H19_R2	GTAAGGAGATTATGTTTATTTTTG	CCTCATTAATCCCATAACTAT
Kcnq_R1	GGTTTAAAGGGTTTTAAGATTATTTTTG	CTTCTTTTCCCTCTATAsTAATTCTAC
Kcnq_R2	GTTTTTGTAAGTTTGGGTTATAAAG	AACTTTTCTATTCAACTTAATTCCC
Snrpn_R1	TATGTAATATGATATAGTTTAGAAATTAG	AATAAACCCAAATCTAAAATATTTTAATC
Snrpn_R2	AATTTGTGTGATGTTTGTAATTATTTGG	ATAAAATACACTTTCACTACTAAAATCC
Mest_R1	GATTTGGGATATAAAAGGTTAATGAG	TCATTAAAAACACAAACCTCCTTTAC
Mest_R2	TTTTAGATTTTGAGGGTTTTAGGTTG	AATCCCTTAAAAATCATCTTTCACAC

APPENDIX D

Primer Name	**Primer Sequence for EpiTYPER Mass Array**		
Primer 5′ tag			
	5' FWD Tag	AGGAAGAGAG	
	5' REV Tag (T7)	CAGTAATACGACTCACTATAGGGAGAAGGCT	
Imprinted gDMR		**Sequence (5' to 3')**	**Genomic Coordinates (GRCm38)**
Dlk1.A	FWD	ATAGTATTGGTTTGGTATATATGGATG	12:109527424-109527853
	REV	CCATAACATAAACATAAAAATCCACAA	
Dlk1.B	FWD	GATGTGTTGTGGATTTAGGTTGTAG	12:109528138-109528138
	REV	ATCCCCTATACTCAAAACATTCTCC	
Grb10	FWD	AGGAGTTGTTTATTATTTGGATTATTGT	11:12025702-12026046
	REV	CTCTAAACTCCAAAACCCTTTTTCT	
H19(*)	FWD	GTTGATGGTTTTAGAATTTTATAAGTTAG	7:142581609-142581931
	REV	CACAATACCACTAAAAAAACAAAACA	
Igf2r	FWD	GATAGGAGGATTTAGAGGGTTTTGT	17:12742752-12743024
	REV	AACCCCATTATCTACAACTCAAACA	
Impact.A	FWD	TTTGTATTAAGTAGGTTGTTTTAGGG	18:12972913-12973111
	REV	ACAACCAAACTAAAATTAACCAAACAA	
Impact.B	FWD	TTGTTTGGTTAATTTTAGTTTGGTT	18:12973084-12973497
	REV	TCATATAACAATACAACAAAACCTACTC	
Kcnq1	FWD	TGGAGAGTTTTTTTGTTTAGTTTGG	7:150481809 -150481430
	REV	CAAAACCACCCCTACTTCTATAAAC	
Nespas.A	FWD	TGGGGGTTTTTGATTTTTTTATTTTG	2:174295281-174295599
	REV	TAAATCTCAACCACTAACCCACTCC	
Nespas.B	FWD	TTTTTTTTAGGGTTTTGTAGGTTAGATTTG	2:174295985-174296393
	REV	CCCCTCCTCCTTCTATTATAAACACC	
Mest	FWD	ATATGTTGGGGAGGGATTTTTTTAG	6:30737763-30738178
	REV	CAACAAAAACAACAAACAACAACTC	
Peg3	FWD	GATTTTGTTTGGGGGTTTTTAATATTGAT	7:6683342 -6683054
	REV	CCACCAACCCAAAATAAACATCTCT	
Plagl1	FWD	TATTTTTGTGGGGATGGAGGAATTA	10:13090467-13090791

	REV	ATCCCAACCCAAACTAAATAACAAA	
Peg10	FWD	TTAGGATTTGGTTATTGAAGGTTTG	6:4697732 -4697319
	REV	CCCCTCCTAAAATCTCTCTATATAAAAC	
Snrpn	FWD	TGTGATGTTTGTAATTATTTGGGAG	7:67150146 -67149901
	REV	CTAAAATCCACAAACCCAACTAACC	
Nnat	FWD	TTTAGGTGGTAAGAGGGTATTTAAGG	2:157560062-157560273
	REV	AATACATACTCACCTACAACAACAC	
Nap1l5	FWD	AGTTTGGAATTTTTTGTTAAATTTGG	6:58906694-58907060
	REV	CAACTACAAAACCTCTCTAAACCAAC	
Zrsr1	FWD	GGTAAGGTAGATAATTATTGTTTTAGTTGT	11:22972131-22972559
	REV	CATAAACCTACCCATACAATTACCC	

(*) From McGraw et al (2013)

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