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Regulation of the Kcnq1ot1 Imprinting Domain in Mouse

Lauren SM Landschoot, *The University of Western Ontario*

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biochemistry

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REGULATION OF THE *KCNQ1OT1* IMPRINTING DOMAIN IN MOUSE

(Thesis format: Monograph)

by

Lauren Susanne Magri Landschoot

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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Abstract

Genomic imprinting is an epigenetic mechanism that controls gene expression based on parental-origin of an allele. The *Kcnq1ot1* imprinting cluster consists of an imprinting control region (ICR), the *Kcnq1ot1* ncRNA, and maternally expressed protein-coding genes. Truncation of the *Kcnq1ot1* ncRNA or deletion of the *Kcnq1ot1* ICR, including the *Kcnq1ot1* ncRNA promoter results in biallelic expression of normally paternally silent protein-coding genes in postimplantation, suggesting the *Kcnq1ot1* ICR/ncRNA are required for bidirectional silencing. However, *Kcnq1ot1* ncRNA regulation of imprinted genes during preimplantation is unknown. To address this, imprinted expression was investigated in preimplantation embryos with a paternally deleted *Kcnq1ot1* ICR or truncated *Kcnq1ot1* ncRNA. *Kcnq1ot1* mutant embryos were capable of silencing *Phlda2*, *Slc22a18* and *Cdkn1c* paternal alleles, suggesting the *Kcnq1ot1* ICR and ncRNA are dispensable for repression at this stage. Imprinted expression in early postimplantation embryos carrying a paternally deleted *Kcnq1ot1* ICR showed the ICR was necessary for maintenance of paternal repression at *Phlda2*, *Slc22a18*, *Cdkn1c*, *Kcnq1* and *Ascl2*. However, truncation of the *Kcnq1ot1* ncRNA resulted in paternal reactivation of distal genes, *Phlda2*, *Slc22a18* and *Ascl2*, while genes proximal to the ICR, *Kcnq1* and *Cdkn1c*, maintained maternal-specific or maternal-biased expression. This indicates the *Kcnq1ot1* ICR and ncRNA are dispensable in early development for paternal silencing but are required later for maintenance of imprinted expression. However, epigenetic modifiers maintaining paternal silencing of adjacent protein-coding genes in coordination with the *Kcnq1ot1* ICR or ncRNA are unknown. Therefore, epigenetic modifiers regulating imprinting at the *Kcnq1ot1* domain were identified in embryo-derived stem cells using a positive selection, loss-of-function RNA interference (RNAi) screen. Depletion of candidates *Ezh1*, *Smarca5* and *Smarcad1* resulted in loss of imprinted expression of *Cdkn1c* and *Kcnq1* but not *Osbp15* and *Slc22a18*, suggesting epigenetic modifiers identified here function at genes proximal to the *Kcnq1ot1* ICR and not domain-wide. *Kcnq1ot1* expression was reduced when *Smarca5* and *Smarcad1* but not when *Ezh1* were depleted, indicating loss of imprinting can occur independently of *Kcnq1ot1* ncRNA expression and epigenetic modifiers could be acting directly on imprinted genes. Further characterization of candidates will provide better understanding of imprinted gene regulation and the protein complexes responsible for maintaining repression.

Keywords

Genomic imprinting, non-coding RNA, imprinting control region, *Kenq1ot1*, preimplantation, stem cells, epigenetic modifiers

Co-Authorship Statement

1. Lauren S. M. Landschoot, Michelle M. Denomme, Michael J. Higgins and Mellissa R.W. Mann, *Kcnq1ot1* Imprinting Control Region and Non-coding RNA are Dispensable for Imprinted Domain Regulation During Early Development, PLoS Genetics, submitted.

All experimental work was carried out by myself except for:

Dr. Michelle Denomme harvested some wildtype, *Kcnq1ot1* ICR deletion and *Kcnq1ot1* truncated blastocysts.

Dr. Michael Higgins provided the *Kcnq1ot1* ICR deletion and *Kcnq1ot1* truncated mice.

Dr. Mellissa Mann and Lauren Landschoot conceived the project and experiments.

2. Lauren S. M. Landschoot, Saqib Sachani, Michael Golding, Mellissa R. W. Mann, Epigenetic Factors Regulating Paternal Allelic Repression at the *Kcnq1ot1* Domain, in preparation.

All experimental work was carried out by myself except for:

Dr. Michael Golding generated XEN stem cells, and shRNA libraries.

Saqib Sachani performed a transfection replicate and imprinted gene expression analysis for candidate-depletion in XEN cells.

Dr. Mellissa Mann, Dr. Golding and Lauren Landschoot conceived the project and experiments.

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Ethics Approval

Experiments were performed in compliance with guidelines set by the Canadian Council for Animal Care and policies and procedures approved by Western University Council on Animal Care.

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List of Abbreviations

%	Percent
<	Less than
>	Greater than
°C	Degree Celsius
A	Adenine
Ac	Acetyl group
<i>Airn</i>	Antisense <i>Igf2r</i> RNA
<i>Ascl2</i>	Achaete-scute complex homolog 2
B6	C57BL/6
B6(CAST7)	C57BL/6 (CAST chromosome 7)
BWS	Beckwith-Wiedemann Syndrome
CAST	Mus musculus castaneus
<i>Cd81</i>	CD81 antigen
<i>Cdkn1c</i>	Cyclin-dependent kinase inhibitor 1C, also known as p57
cDNA	Complementary deoxyribonucleic acid
CH3	Methyl Group
ChIP	Chromatin immunoprecipitation
CpG	Phosphorylated cytosine followed by guanine
CTCF	CCCTC-binding factor
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3a
DNMT3B	DNA methyltransferase 3b
DNMT3L	DNA methyltransferase 3-like
E	Enhancer
ES cells	Embryonic stem cells
Day 13.5	Embryonic day 13.5
Day 16.5	Embryonic day 16.5
Dpc	Days post coitum
EHMT2	Euchromatic histone lysine N-methyltransferase 2 (known as G9A)
ES cells	Embryonic stem cells
EZH1	Enhancer of zeste homolog 1
EZH2	Enhancer of zeste homolog 2
G	Guanine
H1	Histone 1
<i>H19</i>	Hepatocyte #19 fetal liver mRNA
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H2A.Z	Histone 2A, member Z
H2AK119Ub1	Histone 2A lysine 119 mono-ubiquitin
H2BK123Ub1	Histone 2B lysine 123 mono-ubiquitin

H3K27me3	Histone 3 lysine 27 tri-methylation
H3K36me3	Histone 3 lysine 36 tri-methylation
H3K4me3	Histone 3 lysine 4 tri-methylation
H3K9me2	Histone 3 lysine 9 di-methylation
H3K9me3	Histone 3 lysine 9 tri-methylation
H4	Histone 4
H4K20me3	Histone 4 lysine 20 tri-methylation
HDAC9	histone deacetylase 9
Hr	Hour
ICR	Imprinting control region
<i>Igf2</i>	Insulin-like growth factor 2
<i>Igf2r</i>	Insulin-like growth factor 2 receptor
JMJD2A	Lysine (K)-specific demethylase 4A
K	Lysine
<i>Kcnq1</i>	Potassium voltage-gated channel, KQT-like subfamily member 1
<i>Kcnq1ot1</i>	<i>Kcnq1</i> -overlapping transcript 1
M	Molar
Mat	Maternal
Me	Methyl group
mg/mL	Milligram per milliliter
Min	Minute
mM	Millimolar
mRNA	Messenger ribonucleic acid
<i>Mrpl</i>	Mitochondrial ribosomal protein L1
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ncRNA	Non-coding ribonucleic acid
<i>NeoR</i>	Neomycin Resistance gene
NTsi	Non-targeting siRNA
NUP107	Nucleoporin 107
<i>Osbpl5</i>	Oxysterol binding protein-like 5
Pat	Paternal
PCR	Polymerase chain reaction
<i>Phlda2</i>	Pleckstrin homology-like domain, family A, member 2
Pol II	RNA polymerase II
PRC2	Polycomb repressive complex 2
<i>Rasgrf1</i>	RAS protein-specific guanine nucleotide-releasing factor 1
RNA	Ribonucleic acid
RNase	Ribonuclease
RNF2	Ring finger protein 2
SATB1	Special AT-rich sequence binding protein 1
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
<i>Slc22a18</i>	Solute carrier family 22 (organic cation transporter), member 18
<i>Slc22a2</i>	Solute carrier family 22 (organic cation transporter), member 2
<i>Slc22a3</i>	Solute carrier family 22 (organic cation transporter), member 3

SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
SMARCAD1	SWI/SNF related, matrix associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1
SMARCC2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2
SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1
SUV420H1	Suppressor of variegation 4-20H1
T	Thymine
TAF6L	TAF6-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor
<i>Th</i>	Tyrosine hydroxylase
tRNA	Transfer ribonucleic acid
TS cells	Trophectoderm stem cells
<i>Tsix</i>	X (inactive)-specific transcript, opposite strand
<i>Tssc4</i>	Tumor-suppressing subchromosomal transferable fragment 4
Ub	Ubiquitin group
UPD	Uniparental disomy
WT	Wildtype
XCI	X-chromosome inactivation
XEN cells	Extra-embryonic endoderm stem cells
Xi	Inactive X chromosome
XIC	X inactivation centre
<i>Xist</i>	Inactive X specific transcript
Xm	Maternal X chromosome
Xp	Paternal X chromosome
µg	Microgram
µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer
µM	Micromolar

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Chapter 1

1 Introduction

1.1 Epigenetics

1.1.1 General Introduction

The term epigenetics was first coined by C.H. Waddington, to describe the complexities of developmental processes that cannot simply be explained by the connection between genotype and phenotype (Waddington, 2012). As an experimental embryologist, he observed the variability in sizes of structures and the differentiation of cells into various tissues despite all cells having the same genes. Therefore, the phenotype is guided by genetic content but influenced by “a set of organizers”, accounting for the variability between organs (Gilbert, 2012). These molecular and developmental pathways interact and converge on the genotype leading to the phenotype.

Evolving from Waddington’s description of the disconnect between genotype and phenotype in a developmental context, epigenetics had been used to explain previously unusual biological phenomena, such as position effect variegation (location and environment of genes influencing expression), genomic imprinting in mammals, and cellular differentiation (Goldberg et al., 2007). Modern day epigenetics provides a more specific definition: study of changes in gene expression by modifications that do not include alterations to the DNA sequence. Combining “genetics” and the prefix “epi” (meaning above), epigenetics influences gene expression by changing the way genes are recognized and read, affecting the accessibility of genes to transcriptional machinery. Epigenetic processes change the epigenetic “landscape” during germ cell specification and early embryonic development, generating cell types with a distinct gene expression profile and a unique cellular phenotype (Goldberg et al., 2007).

1.1.2 Mechanisms of Epigenetic Regulation

Epigenetic changes to the genome can occur by different complex molecular modifications that influence how genes are written, read and erased ultimately controlling the way genes are expressed. By changing the ionic microenvironment, macromolecular conformation of a gene locus and binding capabilities of regulatory factors, gene expression can either be activated or repressed (Goldberg et al., 2007; Kouzarides, 2007; Lee, 2012).

1.1.3 Histone Modifications

DNA exists as a string of nucleotides organized into a complex three-dimensional structure with proteins, known as chromatin, which condenses to form chromosomes. Chromatin is composed of DNA wrapped around an octamer of histone proteins (made of two molecules each of H2A, H2B, H3 and H4 histones) to form a nucleosome. Histone H1 acts as a linker between nucleosomes and condenses chromatin further into higher-order structure (Campos and Reinberg, 2009; Quina et al., 2006). The configuration of chromatin is important as it dictates the accessibility of regulatory factors to the incorporated DNA and modifications to this structure will influence the transcriptional state of nearby genes (Quina et al., 2006). Modifications to histones can occur along the length of the protein, including the N-terminal region, which protrudes from the DNA-histone particle. These histone “tails” allow for diverse post-translational modifications, influencing inter-nucleosomal interactions, DNA and histone interactions, as well as forming a hypothesized recognition code for specific regulatory proteins (Bannister and Kouzarides, 2011). Histone tails can be modified by acetylation, lysine and arginine methylation, phosphorylation and ubiquitination (Kouzarides, 2007). These modifications are typically associated with either increased gene activity or gene repression. Activation and repression are dependent on the amino acid that is modified and the type of modification applied (Figure 1). For methylation, lysine and arginine residues can be mono-, di- or tri-methylated. Tri-methylation at histone 3 lysine 4 (H3K4) and histone 3 lysine 36 (H3K36) are associated with active genes, while tri-methylation at histone 3 lysine 27 (H3K27), histone 4 lysine 20 (H4K20) and di- and tri-methylation of histone 3 lysine 9 (H3K9) are associated with repressed genes (Bannister and Kouzarides, 2011;

Campos and Reinberg, 2009). Addition of ubiquitin polypeptides to lysine residues has been characterized on histones H2A and H2B (Wang et al., 2004). Mono-ubiquitination of H2A at lysine 119 (H2AK119) is involved in gene silencing, while H2B mono-ubiquitination at K123 (H2BK123) is important for transcriptional initiation and elongation (Kim et al., 2009; Wang et al., 2004). Acetylation of lysine residues alters the charge of histones, allowing for a more open chromatin state (Campos and Reinberg, 2009), while lack of acetylation is associated with repressed chromatin. Multiple histone modifications may exist concurrently, adding an additional layer of complexity that is not fully understood.

Given the combinatorial variability of site and type of histone modifications, histone interactions with DNA and their mediation of higher-order chromatin structure is complex and not fully understood. However, evolutionary conservation of histones and the residues modified highlights their importance. Loss of histone modifiers causes lethality, abnormal chromatin structure and transcriptional output, imprinting defects and alterations in other epigenetic marks like DNA methylation (Ciccione et al., 2009; Terranova et al., 2008; Wagschal et al., 2008).

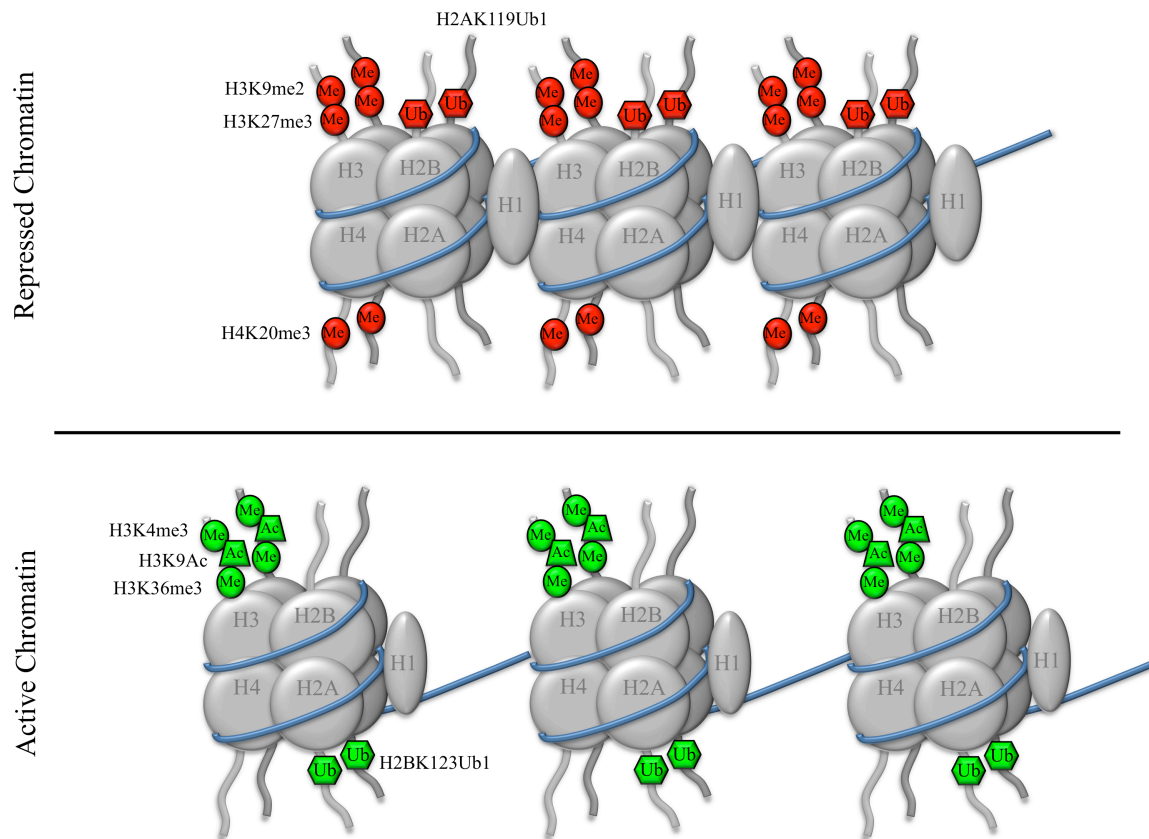


Figure 1: Histone Modifications

Chromatin is made of repeating units of nucleosomes. Nucleosomes are composed of DNA (blue) wrapped around an octamer of histones (gray): two dimers of H3/H4 and H2A/H2B. Nucleosomes are linked together by histone H1. Post-translational modifications to histone tails (wavy lines) can be activating (green) or repressive (red). Common modifications are depicted: acetylation (Ac), methylation (Me), ubiquitination (Ub). Repressed chromatin is more compact and closed off to gene expression. Active chromatin has a more open conformation and is accessible to transcriptional machinery.

1.1.4 DNA Methylation

Gene expression can be influenced by modifications to the DNA itself through the addition of a methyl group (CH₃) to cytosines in CpG dinucleotides (Figure 2). This modification is usually associated with gene repression by altering interactions of DNA with chromatin-binding proteins and transcription factors. DNA methylation is established at unmethylated DNA by *de novo* DNA methyltransferases DNMT3A, DNMT3B, and DNMT3L (Bourc'his et al., 2001; Hata et al., 2002; Okano et al., 1999). DNA methylation is subsequently maintained by the maintenance DNA methyltransferase DNMT1, which recognizes hemi-methylated DNA generated during DNA replication and adds methyl groups to the unmethylated (daughter) strand using the parental strand as a template.

DNA methylation controls gene expression, genomic stability, chromatin structure, X-chromosome inactivation, genomic imprinting and silencing of repetitive DNA elements (Robertson, 2002; Rodenhiser and Mann, 2006). Aberrant methylation state produces multiple defects including altered gene expression, genomic imprinting disorders, abnormal development, cancer and lethality (Biniszkiwicz et al., 2002; Bourc'his et al., 2001; Hata et al., 2002; Rodenhiser and Mann, 2006). Consequences of aberrant DNA methylation relating to genomic imprinting will be discussed later.

1.1.5 Non-coding RNAs

Once considered an intermediate during protein synthesis, RNA is now known to play important regulatory roles in gene expression. Only 1% of the genome encodes for proteins. However, 70-90% of the genome is transcribed, suggesting that the resultant RNA is functional (Lee, 2012). These noncoding RNAs (ncRNA) can vary in size from >100 nucleotides to 400,000 in length and can function in various biological processes, such as nuclear architecture, chromatin modification, RNA processing and genomic imprinting (Yan and Wang, 2012). ncRNAs are distributed throughout the genome and can be intragenic, intergenic, and intronic. Alterations in gene activity due to ncRNA-mediated regulation can occur at the level of individual genes, gene-clusters/domain, or over entire chromosomes. ncRNAs function by diverse and complex mechanisms,

working in *cis* or in *trans* to either activate or repress gene expression (Chaumeil et al., 2006; Fitzpatrick et al., 2002; Latos et al., 2012; Rinn et al., 2007; Tian et al., 2010). The ncRNA can act as a scaffold to mediate chromatin structure and chromatin-looping between distantly located regions (Zhang et al., 2014) (Figure 3). ncRNAs can also act as adaptors to recruit epigenetic modifiers that will alter the expression of nearby genes (Rinn and Chang, 2012; Wang et al., 2011; Zhao et al., 2010). Transcription of the ncRNA tethers the ncRNA to its genomic location, accounting for its ability to recruit epigenetic modifiers in *cis* (Lee, 2012). By this method, ncRNAs add specificity to chromatin modifiers, bringing them to specific sites in the genome, unlike transcription factors that recognize multiple sites in the genome (Lee, 2012).

An additional method of regulation excludes the RNA itself and proposes the act of transcription is functionally important, as depletion of two ncRNAs induced no changes in gene expression of nearby genes (Golding et al., 2011; Latos et al., 2012). How this is mediated is unclear but transcriptional interference has been shown to be important by preventing binding of RNA polymerase at repressed genes (Latos et al., 2012) (Figure 3). Overall, the complexity of ncRNA regulatory mechanisms and the potential for a RNA or transcriptional interference method makes studying ncRNAs difficult, as both mechanisms may be intrinsically linked.

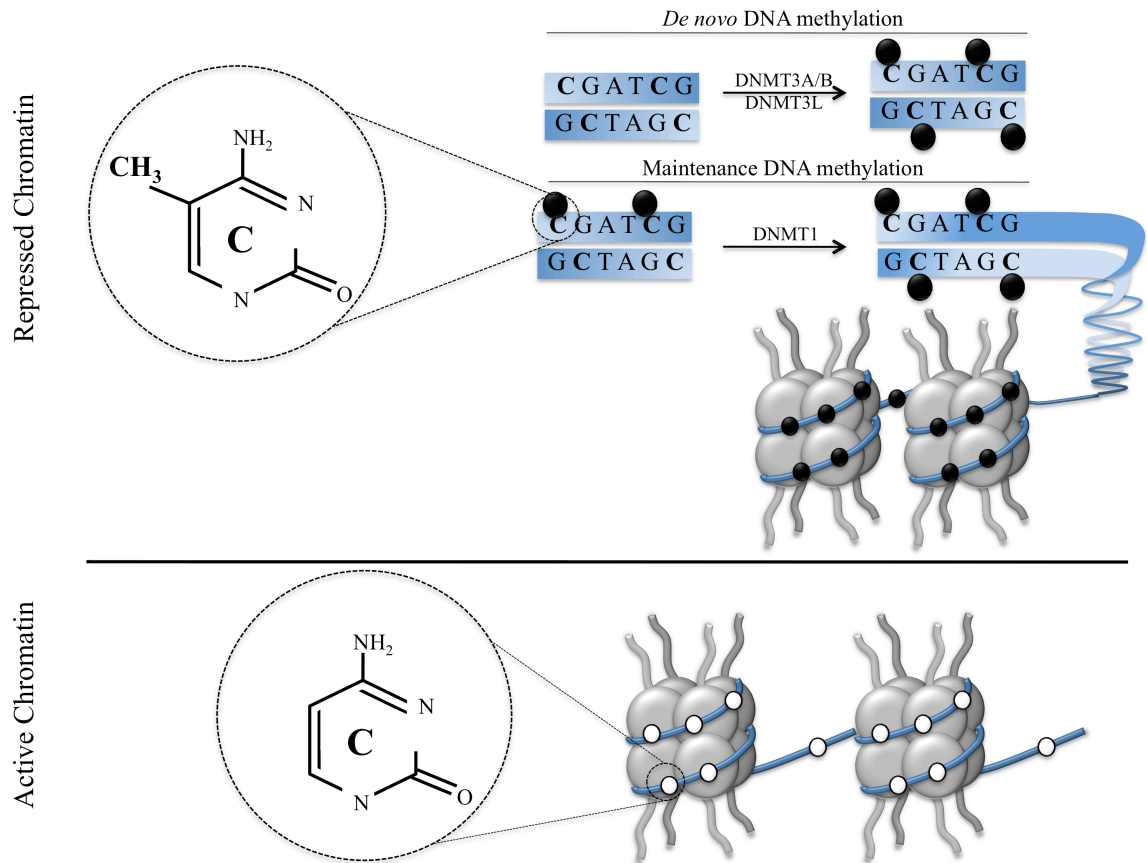


Figure 2: DNA Methylation

Modification to the DNA (blue) itself can influence chromatin and gene expression. Methyl groups (CH_3) are added to cytosines (C) within CpG dinucleotides. Methylated cytosines (black circles) are associated with repressed chromatin. DNA methylation is catalyzed by DNA methyltransferases (DNMT). *De novo* addition of methyl groups to unmethylated DNA is executed by DNMT3A/B and DNMT3L. Addition of methyl groups to hemi-methylated DNA is carried out by DNMT1. Active chromatin is not associated with DNA methylation, with cytosines being unmethylated (white circles).

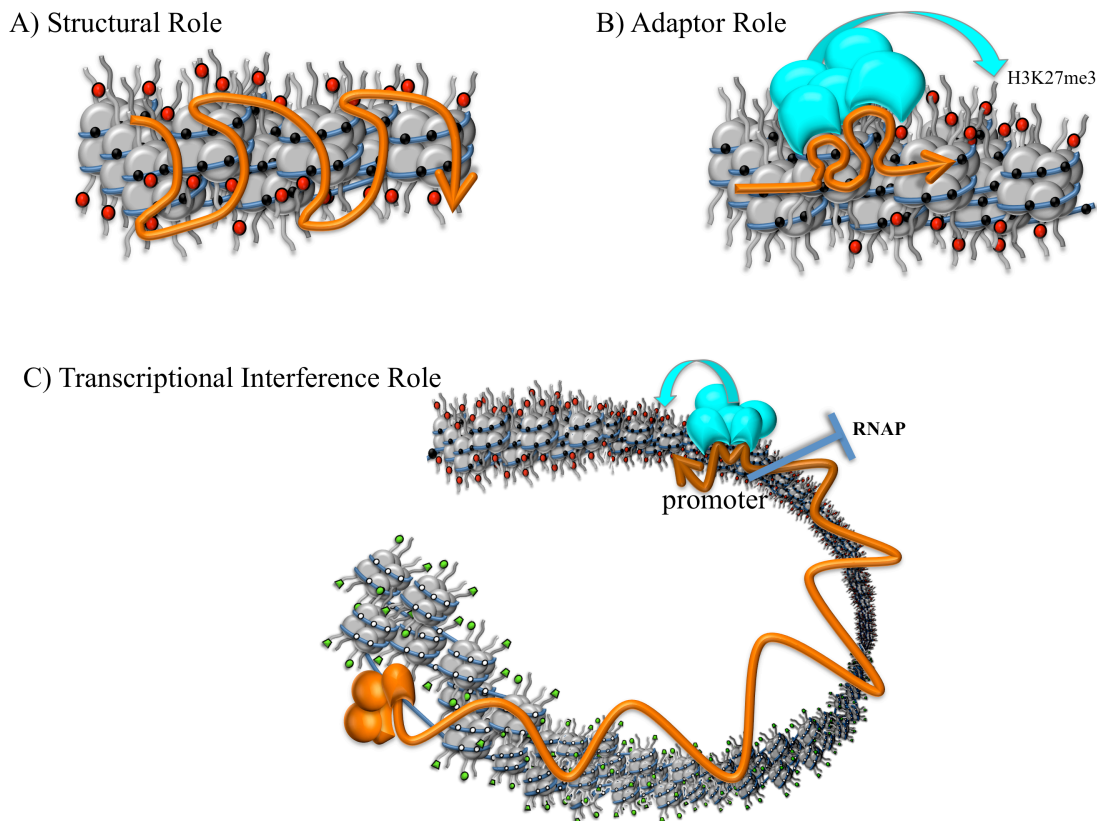


Figure 3: Long Non-coding RNAs

Long non-coding RNAs (orange lines) function beyond a protein-synthesis intermediate, controlling gene expression by various proposed methods. A) By acting structurally, ncRNAs function to maintain chromatin-looping and chromatin conformation. B) ncRNAs can also function as adaptors to protein-complexes, guiding and localizing chromatin-modifying enzymes (teal complex), like H3K27 histone methyltransferase to specific regions. C) During its transcription, the ncRNA is tethered by transcriptional machinery (orange complex), allowing recruitment of chromatin-modifying enzymes in *cis* (teal complex). Transcription *per se* of the ncRNA has also been shown to be important. Transcriptional interference preventing binding of RNA polymerase to downstream promoters, leading to their repression.

1.1.6 Nucleosome Remodelers

Epigenetic changes to histone tails or DNA occurs by enzymatic proteins like histone modifiers and DNA methyltransferases, respectively. Histone modifying enzymes add covalent marks (methyl, acetyl, ubiquitin groups) to histones. Non-covalent modifications are also important for controlling chromatin structure. Modifications to nucleosome structure and nucleosome placement can modify gene expression. Protein complexes called chromatin remodelers use ATP hydrolysis to disrupt DNA-histone contacts and alter DNA accessibility by modifying the position, spacing, presence of histones (eviction), and replacement of canonical histones with non-canonical histone variants (Quina et al., 2006; Wang et al., 2007) (Figure 4). Histone variants (H3.3, macroH2A, H2A.Z) can replace core histones (H3 and H2A) within nucleosomes via chromatin remodelers (Campos and Reinberg, 2009). Chromatin remodelers play roles in transcriptional regulation, functioning in both activation and repression, as well as regulating DNA replication and repair (Petty and Pillus, 2013; Wang et al., 2007). During transcription, nucleosomes are positioned to expose *cis*-regulatory regions and are removed during RNA polymerase passage and then are reassembled (Petty and Pillus, 2013).

Multiple families of chromatin remodelers exist in vertebrates, based on the shared structure of the ATPase subunit found between complexes. The vertebrate genome contains multiple ATPases with similarities to the yeast SWI2/SNF2 ATPase. Some of these include BRM/SMARCA4 or BRM/SMRCA2 ATPase within the SWI/SNF complex; Mi2- α or β ATPase in the NuRD complex; SNF2L/SMARCA1 or SNF2H/SMARCA5 ATPase in the ISWI complex (Hargreaves and Crabtree, 2011; Wu et al., 2009). The ATPases interact within a complex along with multiple accessory proteins, ranging from 4-12 subunits (Wu et al., 2009) (Table 1). Accessory subunits may alter the activity of the ATPase, facilitate binding to transcription factors, or target the complex to DNA-histones (Hargreaves and Crabtree, 2011).

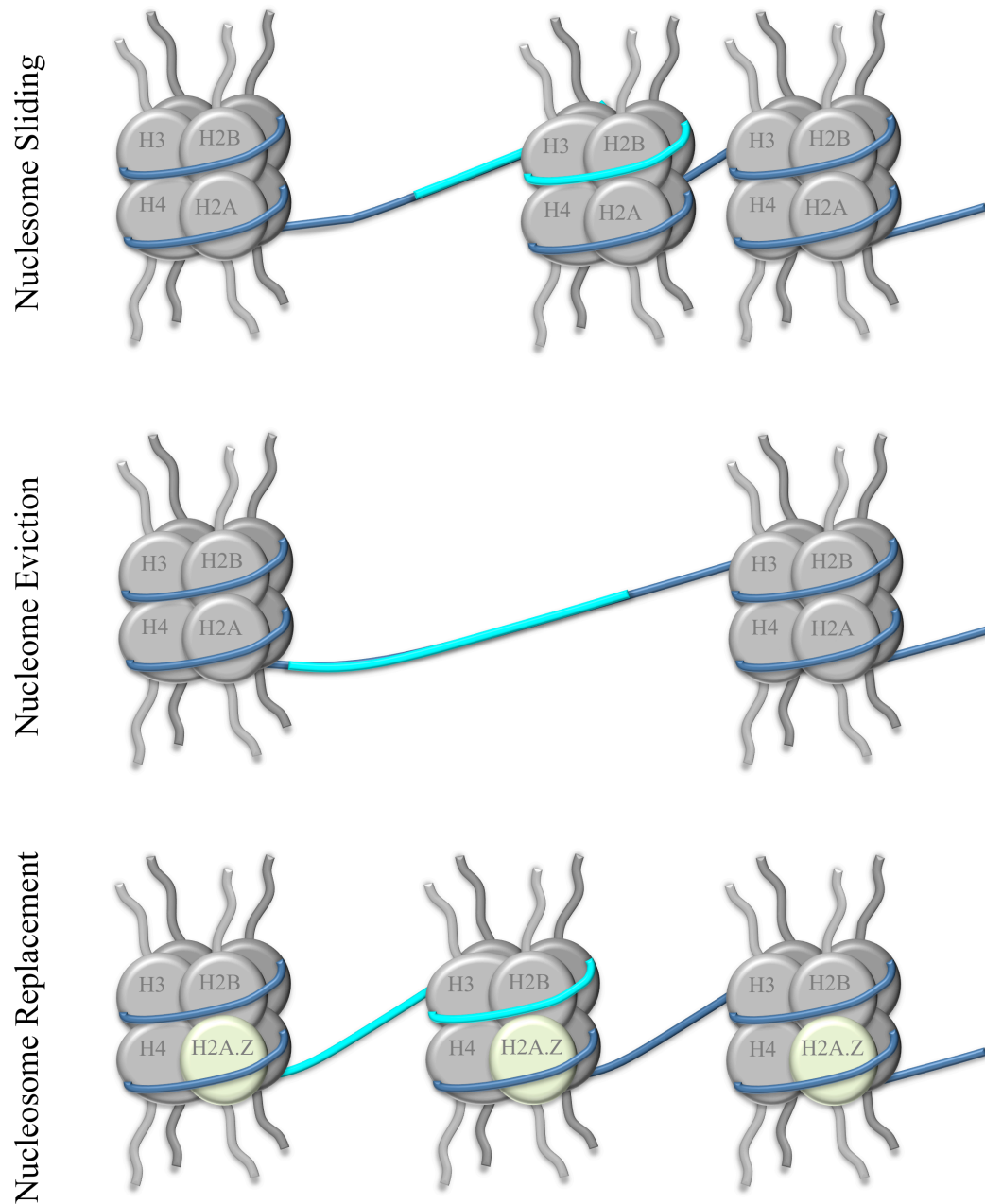


Figure 4: Chromatin Remodeling

Chromatin remodelers use ATP hydrolysis to alter chromatin accessibility (blue) of gene regulatory regions (teal) to the transcriptional machinery. Accessibility of gene regulatory elements can be occluded or exposed by chromatin remodelers when nucleosomes are repositioned (sliding), displaced (eviction) or replaced by a variant histone.

Table 1: ATP Chromatin Remodeling Complexes

Complex	Subunits
ISWI	NURF SMARCA1 BPTF RbAP46/ 48
	ACF/WCRF SMARCA5 ACF1/WCRF180
	CHRAC SMARCA5 ACF1/WCRF180 CHRAC-15/17
	WICH SMARCA5 WSTF
	RSF SMARCA5 RSF-1/p325
	NoRC SMARCA5 TIP5
	SNF2H/Cohesin SMARCA5 NuRD/Cohesin (RAD21, SMC1/2,SA1/2)
SWI/SNF	SMARCA4/ SMARCA2 SMARCC2 SMARCC1 SMARCE1 SMARCB1 SMARCD1/ SMARCD2/ SMARCD3
NuRD/Mi-2	Mi-2 α / β MTA1/ 2/ 3 HDAC1/ 2 RbAP46/ 48 MBD2/ MBD3 P66a/ P66b
SMARCA1	SMARCA1 KAP1 HDAC1/2 G9A

1.2 Genomic Imprinting

1.2.1 Overview of Genomic Imprinting

Genomic imprinting was first termed by Helen Crouse in 1960, who proposed that chromosomes can be marked outside of their genetic content and that this “imprint” is based on the sex from which the chromosome was inherited (Crouse, 1960). In the 1980s, parental-specific effects were observed in mammals using nuclear transplantation experiments. Mouse zygotes carrying two maternal genomes (gynogenotes) and zygotes carrying two paternal genomes (androgenotes) failed to develop to term, illustrating that parental contributions to the embryo are not equivalent (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984). Parental-specific effects were further established in mice and humans possessing uniparental disomy (chromosome or partial chromosome from a single parent). However, not all genomic regions contributed to parental-specific effects, indicating that imprinted regions are not found throughout the genome (Cattanach and Kirk, 1985; Ledbetter and Engel, 1995). These early studies demonstrated that parental contributions to offspring are distinct and critical for proper mammalian development.

Imprinted genes have a large influence on mammalian development and function in growth and development of the embryo and placenta, regulation of pre and postnatal resources, cell specification and differentiation, metabolism, neural function and behaviour (Barton et al., 1984; Constancia et al., 2004; McGrath and Solter, 1984; Plasschaert and Bartolomei, 2014). Imprinted genes in the prenatal embryo control tissue development and growth (Constancia et al., 2004). Imprinted genes in the postnatal embryo control energy homeostasis (hormonal regulation), neural function and behaviour (Constancia et al., 2004; Plasschaert and Bartolomei, 2014). Imprinted genes in extraembryonic lineages function to control morphogenesis, physiological processes and lineage specification in the placenta (Coan et al., 2005). Misregulation or mutations of imprinted genes cause human imprinting disorders (Constancia et al., 2004; Plasschaert and Bartolomei, 2014) (Table 2). Imprinted disorders are associated with growth restriction, overgrowth, neurological defects and behavioural abnormalities (Coan et al.,

2005; Constancia et al., 2004). Studying imprinted genes will aid in understanding imprinted gene regulation and the disorders they cause.

Genomic imprinting is currently defined as a specialized transcriptional mechanism that controls gene expression based on the parental-origin of an allele (Green et al., 2007; Rodenhiser and Mann, 2006; Terranova et al., 2008) (Figure 5). Imprinted genes frequently reside in clusters, allowing for coordinated regulation of imprinted expression. Within imprinting domains is a regulatory DNA element called the imprinting control region (ICR), which functions in *cis* to regulate imprinted genes within the cluster. ICRs are specific DNA regions with a high CpG content and are subject to differential DNA methylation, with one parental allele being methylated while the other parental copy is unmethylated. To be classified as an ICR in addition to a differentially methylated region, loss of imprinted expression of adjacent genes must occur when this element is deleted, demonstrating “control” over the domain (Spahn and Barlow, 2003). Parental-specific DNA methylation and histone modifications are established at ICRs during gametogenesis and maintained faithfully thereafter (Guseva et al., 2012).

Imprinting domains usually include a ncRNA. ncRNAs are monoallelically-expressed with the ncRNA promoter embedded or adjacent to the ICR. Like ICRs, ncRNAs may play a functional *cis*-acting role in regulating the domain. How imprinted ncRNAs mediate repression is unclear, although functions proposed include RNA interference, spread of heterochromatin, and inducing transcriptional interference or repressive effects (Koerner et al., 2009; Royo and Cavaille, 2008; Wan and Bartolomei, 2008).

Imprinting regulation begins in the gametes, with erasure of previous imprinting modifications to establish parental-specific marks at ICRs. Epigenetic marks found at ICRs include DNA methylation, activating H3K4me2/3 and repressive H3K9me2 (MacDonald and Mann, 2014). These parental-specific marks must be maintained or protected throughout development of the offspring. Failure to establish or maintain these marks will result in loss of imprinted expression and imprinting disorders.

Table 2: Imprinted Genes and Associated Human Disorders

	Gene/Domain	Human Imprinting Disorder	Effect
Intra-uterine growth	<i>IGF2</i> <i>CDKN1C</i>	Beckwith-Wiedemann Syndrome	Fetal Overgrowth; large organs, predisposition to tumors
Behavioural	Maternal UPD chromosome 7 (<i>H19</i> , <i>PEG1</i> / <i>MEST</i>)	Silver-Russell Syndrome	Intra-uterine growth restriction
	<i>SNRPN</i> paternal UPD	Angelman Syndrome	Motor and mental retardation, autistic-like behaviour, uncontrolled laughter
	<i>SNRPN</i> maternal UPD	Prader-Willi Syndrome	Mental retardation, obesity, short stature, suckling issues
	<i>Peg3</i> , <i>Peg1</i> / <i>Mest</i> (mouse)		Lack of maternal postnatal care
Physiological	<i>Grb10</i> (mouse)		Hyper-aggression and social dominance
	<i>GNAS</i>	Pseudohypoparathyroidism	Resistance to parathyroid hormone and other hormones
	<i>PLAG1</i>	Transient neonatal diabetes	Transient diabetes mellitus at birth

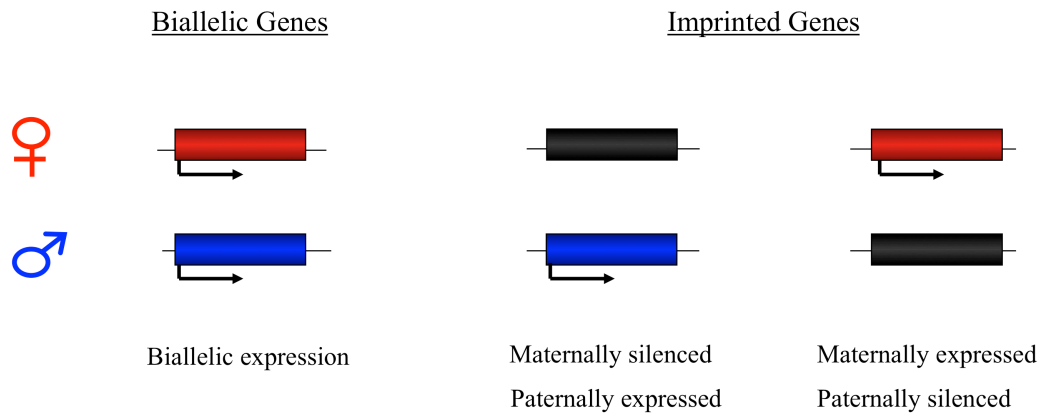


Figure 5: Genomic Imprinting

For most genes in the genome, expression occurs biallelically, that is from both parental alleles (red box, maternally expressed allele; blue box, paternally expressed allele). However, a sub-set of genes are expressed based on parental-origin. These imprinted genes are monoallelically expressed, either paternally expressed but maternally silent or maternally expressed but paternally silent (black box; silenced allele).

1.2.2 Epigenetic Reprogramming

Gametogenesis and early embryogenesis are dynamic periods of epigenetic changes. The first wave of epigenetic modifications begins in the gametes, with erasure of previous somatic DNA and histone modifications, including at imprinted genes. This is followed by acquisition of new epigenetic parental-specific marks (Hajkova et al., 2002; Kageyama et al., 2007). Once established in the gametes, these parental-specific marks are maintained or protected after fertilization and throughout development of the offspring.

A second wave of epigenetic programming occurs shortly after fertilization. The early embryo undergoes dynamic epigenetic changes to modify the genome from a haploid gametic-state to a diploid embryonic genome. Epigenetic changes occur globally through DNA methylation and histone modifications. Both parental genomes undergo global DNA demethylation (Santos et al., 2002).

On the paternal genome, DNA demethylation begins before the first cell division by an active mechanism (Oswald et al., 2000). The maternal genome loses methylation in a passive manner, after each cell division (Howell et al., 2001). However, ICRs at imprinted domains maintain DNA methylation during this demethylation wave. Oocyte and somatic forms of DNMT1 along with developmental pluripotency-associated 3 (DPPA3), zinc finger protein 57 (ZFP57) and tripartite motif-containing 28 (TRIM28) maintain DNA methylation at these important regulatory sites during preimplantation development (Cirio et al., 2008; Howell et al., 2001; Kurihara et al., 2008; Nakamura et al., 2007).

Asymmetry between parental-alleles is also seen with histone modifications shortly after fertilization. The maternal pronucleus maintains repressive H3K9me2/3 and H3K27me3 inherited from oocytes (Erhardt et al., 2003; Liu et al., 2004). The paternal pronucleus exchanges protamines (nuclear proteins that replace histones during spermatogenesis) for histones, which are hyperacetylated and hypomethylated (Santos et al., 2005). The presence of active marks and few repressive marks is hypothesized to allow for increased accessibility and remodeling of the condensed paternal genome. The

paternal allele gains repressive H3K9me2/3 and H3K27me3 marks by the four-cell stage (Santos et al., 2005). Protection of ICRs from these global histone changes is less well characterized than DNA methylation. In sperm, H3K9me2 is enriched at paternally-methylated ICRs of Hepatocyte #19 fetal liver mRNA (*H19*) and RAS protein-specific guanine nucleotide-releasing factor 1 (*Rasgrf1*), and H3K4me2/3 is enriched at maternally-methylated ICRs, suggesting that these marks could provide information to the preimplantation embryo (Guseva et al., 2012; Nakamura et al., 2012). Additionally, H3K9me3 and H3K27me3 are localized to the maternally-methylated KCNQ1 overlapping transcript 1 (*Kcnq1ot1* ICR) in 4-cell and morula embryos, suggesting that repressive histone marks from the gametes can be preserved during preimplantation (Kim and Ogura, 2009). Further studies are needed to examine how ICRs are regulated during preimplantation. Furthermore, less is known about transmission and protection of epigenetic information outside of the ICR during this dynamic epigenetic period, requiring studies to determine whether other regions within imprinted domains have regulatory information inherited from the gametes.

1.2.3 Imprinting Domains

Imprinted genes are found at specific regions in the genome. To date, 150 genes have been identified (<http://www.mousebook.org/catalog.php?catalog=imprinting>). These genes exist in clusters, most of which have an ICR/DMR and ncRNA. However, imprinting domains do have unique regulatory features that enable repression of one parental allele and activation of the other allele. Silencing can occur by ICR-mediated enhancer-blocking, transcriptional-interference and ncRNA-mediated repression. These three silencing mechanisms are discussed below, modeled by three imprinting domains/chromosomes: *H19* domain, *Airn* domain and imprinted X-chromosome inactivation, respectively.

1.2.3.1 *H19* Domain

The *H19* domain is a well-characterized example of an enhancer-blocking or insulator repressive mechanism. The *H19* domain contains an ICR, the *H19* ncRNA and the protein-coding gene Insulin-like growth factor 2 (*IGF2/Igf2*), and is located on

chromosome 11 and 7 in human and mouse, respectively. On the maternal allele, the ICR is unmethylated, *H19* is expressed and *Igf2* is silent. On the paternal allele, the ICR is methylated, *H19* is silent and *Igf2* is expressed (Figure 6). Deletion of the *H19* ncRNA has no effect on regulation of the domain (Jones et al., 1998). However, deletion of the ICR results in loss of imprinted expression, demonstrating ICR-mediated control of the domain (Thorvaldsen et al., 1998). By adopting a specific chromatin-looping conformation based on the parental origin of the ICR, *H19* and *Igf2* proximity to regulatory regions are altered. One structural protein responsible for this specific conformation is CCCTC-binding factor (CTCF), which functions to control higher-order chromatin structure and long-range interactions to “insulate” or block genes from regulatory elements like enhancers. CTCF binds to the unmethylated maternal *H19* ICR and induces a chromatin loop with regions upstream of *Igf2*, preventing *Igf2* from interacting with enhancer elements. The promoter of *H19* is unmethylated and interacts with available enhancers. On the paternal allele, CTCF cannot bind to the methylated ICR, and the domain adopts a conformation allowing *Igf2* to interact with enhancers. DNA methylation from the ICR spreads to the adjacent *H19* promoter, silencing its expression (Thorvaldsen et al., 1998).

In humans, defects in the *H19* domain are associated with the imprinting disorder Beckwith-Wiedemann syndrome (BWS) (Weksberg et al., 2010). This overgrowth disorder is characterized by macroglossia (enlarged tongue), visceromegaly (enlarged organs), abdominal wall defects and a predisposition to Wilm’s tumors (Zhang et al., 1997). In 5% of BWS patients, the maternal ICR is hypermethylated causing loss of *H19* and gain of *IGF2* expression (Weksberg et al., 2010).

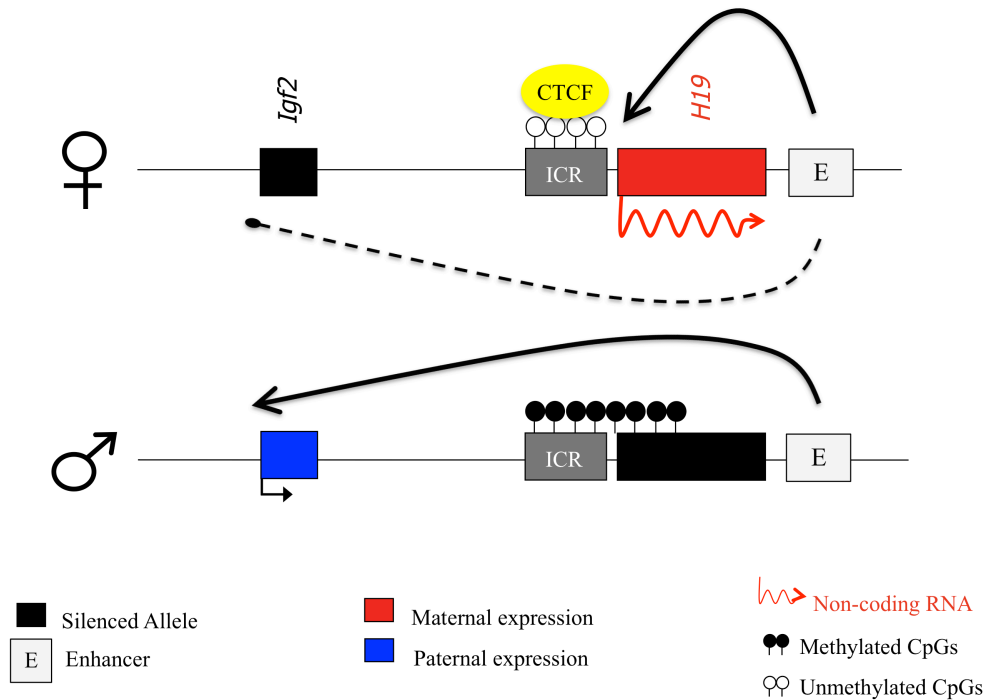


Figure 6: *H19* Domain

The *H19* domain contains an ICR, the *H19* ncRNA and protein-coding gene *Igf2*. This domain is an example of an insulator/enhancer regulatory mechanism. On the maternal allele, CTCF binds the unmethylated ICR and a conformation change is induced blocking *Igf2* from interacting with enhancers and allowing *H19* to associate with enhancers to become expressed. On the paternal allele, CTCF does not interact with the methylated ICR, instead adopting a conformation that allows *Igf2* to associate with enhancers. Methylation spreads from the paternal ICR to the *H19* promoter, silencing the gene.

1.2.3.2 *Airn* Domain

The *Airn/AIRN* domain is located on chromosome 17 and 6 in mouse and human, respectively, and is an example of the second type of allelic-silencing: transcriptional-interference. The *Airn* domain contains a maternally-methylated ICR, the paternally expressed Antisense of *Igf2r* RNA (*Airn*) ncRNA, and the maternally expressed protein-coding genes Insulin-like growth factor 2 receptor (*Igf2r*), Solute carrier family 22, member 2 (*Slc22a2*), and Solute carrier family 22, member 3 (*Slc22a3*) (Figure 7). The *Airn* ncRNA is paternally expressed from the unmethylated paternal ICR, and acts in *cis* to silence neighbouring imprinted genes. *Igf2r* is located downstream of *Airn*, and is silenced by transcriptional interference. Antisense transcription of the *Airn* ncRNA across the *Igf2r* promoter interferes with RNA polymerase II (Pol II), silencing *Igf2r* (Latos et al., 2012; Stricker et al., 2008). *Slc22a2* and *Slc22a3* are positioned upstream of *Airn* and are imprinted specifically in the placenta. Unlike *Igf2r*, they do not require transcriptional overlap to be silenced but are instead regulated by a ncRNA mechanism (Sleutels et al., 2003). The *Airn* RNA itself is important for repression by interacting with the *Slc22a3* promoter and recruiting the histone methyltransferase Euchromatic histone lysine N-methyltransferase 2 (EHMT2/ also known as G9A), which silences genes by catalyzing H3K9me2/3 (Nagano et al., 2008).

1.2.3.3 Imprinted X-chromosome Inactivation

Dosage compensation in mammalian females occurs by silencing one of two X chromosomes, equalizing X-linked expression with males. Imprinted X-chromosome inactivation (XCI) occurs during early development and silences the paternal X chromosome by the blastocyst stage (Huynh and Lee, 2003). This repressive process is a well-studied example of a ncRNA-mediated silencing mechanism, an example of the third type of allelic-specific repression. During XCI, silencing occurs at the level of an entire chromosome initiated in *cis* by the X inactive specific transcript (*Xist*) ncRNA. The 17 kb-*Xist* ncRNA is expressed from the future inactive paternal X chromosome, and is located within the X inactivation centre (XIC), a *cis*-acting element important for XCI (Leung and Panning, 2014). The paternal X chromosome is inherited from sperm in a partially silent state with repetitive elements already repressed (Namekawa et al., 2006;

Namekawa et al., 2010). Genes on the paternal X are initially active but become progressively silenced during preimplantation development, which may be facilitated by the repressed repetitive elements. The maternal X chromosome inherits markings from the oocyte, which protects it from inactivation during preimplantation (Goto and Takagi, 2000; Nesterova et al., 2001; Tada et al., 2000; Takagi and Abe, 1990). The X (inactive)-specific transcript, opposite strand (*Tsix*) is another ncRNA within the X inactivation center that is expressed solely from the maternal X chromosome during preimplantation development. *Tsix* represses *Xist* by antisense transcription (Sado et al., 2001).

Initiation of X-linked gene silencing begins with *Xist* coating and spreading across the future inactive X, eventually altering its chromosomal structure and forming a repressive compartment that represses most X-linked genes (Chaumeil et al., 2006). As *Xist* localizes along the inactivating X, epigenetic modifications accumulate, including H3K9me_{2/3} and H3K27me₃, H3K4 hypomethylation, loss of RNA polymerase II and eventually DNA methylation (Chaumeil et al., 2006; Okamoto et al., 2004; Reik and Lewis, 2005) (Figure 8). The importance of *Xist* RNA in mediating silencing is based on its ability to interact with and directly recruit to the X chromosome the Polycomb repressive complex (PRC2), which contains Enhancer of zeste homolog 2 (EZH2) histone methyltransferase that catalyzes H3K27 tri-methylation (Wutz et al., 2002; Zhao et al., 2008).

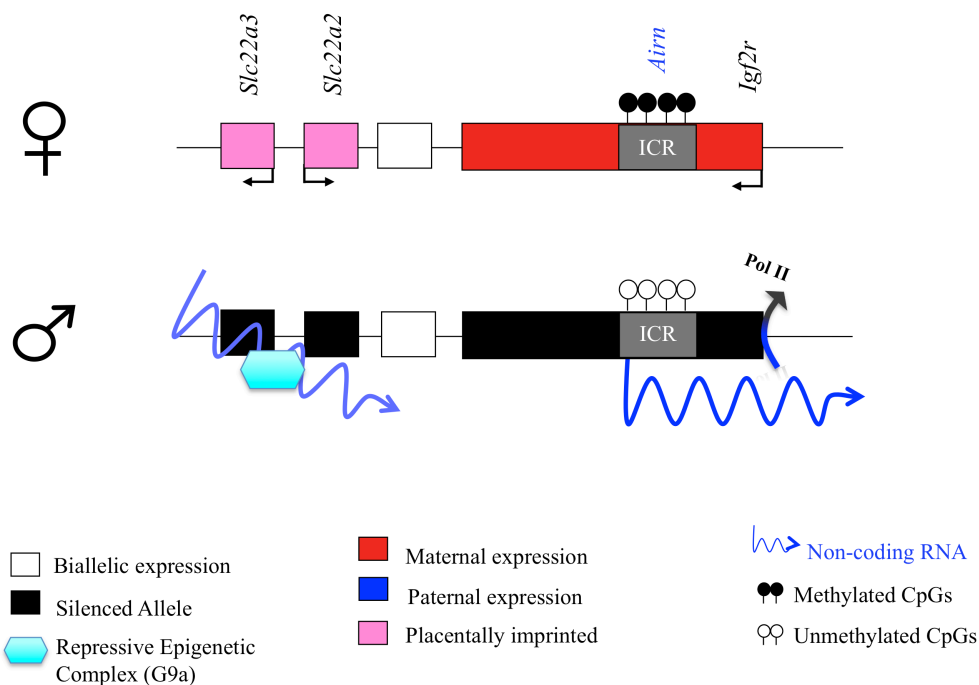


Figure 7: *Airn* Domain

The *Airn* domain contains an ICR, three protein-coding genes, *Igf2r*, *Slc22a2* and *Slc22a3*, and the *Airn* ncRNA (blue wavy arrow), whose promoter resides within the ICR. On the maternal allele, *Airn* is silenced by methylation at the ICR and *Igf2r*, *Slc22a2* and *Slc22a3* are expressed (red and pink boxes). *Igf2r* is maternally expressed in the embryo and placenta (red box). *Slc22a2* and *Slc22a3* are only maternally expressed in the placenta (pink boxes), and are biallelic expressed in the embryo. On the paternal allele, *Airn* is expressed from the unmethylated ICR and induces repression of adjacent imprinted genes in *cis* (black boxes). Transcription of *Airn* across the promoter of *Igf2r* impedes RNA polymerase II (Pol II) binding, thereby silencing *Igf2r*. The *Airn* ncRNA silences *Slc22a2* and *Slc22a3* by associating with gene promoters and recruits the histone methyltransferase G9A (teal hexagon), which catalyzes the repressive mark H3K9me3 to promoters of these genes.

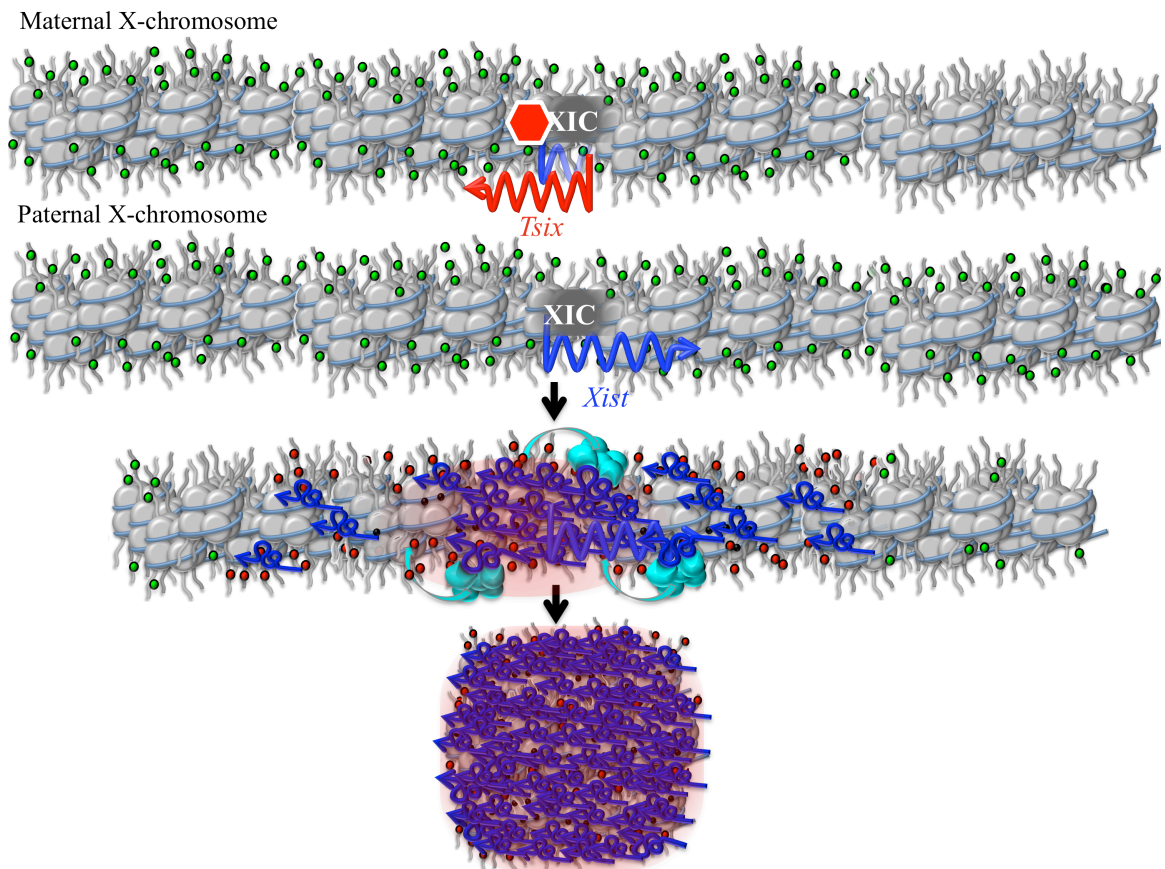


Figure 8: Imprinted X-Chromosome Inactivation

Imprinted X-chromosome inactivation equalizes X-linked expression between females and males by silencing the paternal X chromosome in females. The maternal X chromosome remains active while the paternal chromosome is silenced by the *Xist* ncRNA (blue wavy arrow). The promoter of *Xist* lies within an important DNA element, known as the X inactivation centre (XIC). Expression of *Xist* is blocked (red hexagon) from the maternal XIC by transcriptional interference from the maternally expressed *Tsix* RNA (red wavy arrow), leading to the maintenance of gene expression on the maternal chromosome. On the paternal chromosome, *Xist* is transcribed and associates with repressive complexes (teal complex), that are transferred to DNA where they confer repressive histone marks like H3K27me3 (red circle). *Xist* ncRNA begins to spread to adjacent regions, eventually coating and altering the structure of the entire chromosome, forming a repressive compartment (red shaded area) devoid of histone active marks (green circles) that leads to repression of X-linked genes by the blastocyst stage.

1.2.4 *Kcnq1ot1* Domain

The *Kcnq1ot1* imprinted cluster is located on syntenic regions of mouse chromosome 7 and human chromosome 11. Interestingly, investigation into the allelic silencing of the *Kcnq1ot1* domain supports multiple repressive models, including the three mechanisms outlined above. Description of three proposed regulatory models for the *Kcnq1ot1* domain will be discussed in detail in the following section.

The *Kcnq1ot1* domain contains a maternally methylated ICR, the paternally expressed *Kcnq1ot1* ncRNA, nine maternally expressed protein-coding genes and six biallelically expressed genes (Figure 9). The *Kcnq1ot1* ncRNA extends to 471 kb in length and originates from a promoter embedded in the ICR (Golding et al., 2011). On the maternal allele, the ICR is methylated, silencing *Kcnq1ot1* and permitting upstream and downstream protein-coding genes to be expressed. Genes monoallelically-expressed in both embryonic and placental-tissues genes in postimplantation are classified as ubiquitously imprinted: Pleckstrin homology-like domain, family A, member 2 (*Phlda2*); Solute carrier family 22, member 18 (*Slc22a18*); Cyclin-dependant kinase inhibitor 1C (*Cdkn1c*) and Potassium voltage-gated channel, subfamily Q, member 1 (*Kcnq1*). Genes imprinted in placental-tissue only are classified as placental-specific imprinted genes: Oxysterol binding protein-like 5 (*Osbpl5*); Tumor-suppressing subchromosomal transferable fragment 4 (*Tssc4*); CD81 antigen (*Cd81*); Achaete-scute complex homolog 2 (*Ascl2*) and Tyrosine-hydroxylase (*Th*). On the paternal allele, the *Kcnq1ot1* ncRNA is expressed due to an unmethylated ICR, and adjacent protein-coding genes are silent. The importance of *Kcnq1ot1* in silencing the paternal allele in *cis* was established through a paternally inherited deletion of the *Kcnq1ot1* ICR, including the *Kcnq1ot1* promoter, and truncation of the *Kcnq1ot1* ncRNA, which resulted in biallelic expression of the normally paternally silent imprinted genes within the domain (Fitzpatrick et al., 2002; Mancini-Dinardo et al., 2006; Shin et al., 2008).

Parental-specific epigenetic modifications are seen throughout the *Kcnq1ot1* domain in embryonic day 9.5 embryos and placentas, with active marks correlating with expression and repressive marks associating with repressed genes (Lewis et al., 2006; Lewis et al., 2004; Terranova et al., 2008; Umlauf et al., 2004). DNA methylation is low

at CpG-rich gene promoters throughout the domain, except for the maternal ICR and somatic DMR of *Cdkn1c* (Lewis et al., 2006; Lewis et al., 2004; Terranova et al., 2008; Umlauf et al., 2004). Active histone marks (H3K4me3) are present at the paternal ICR and maternally expressed genes while repressive histone marks (H3K27me3, H3K9me2/3) are found on the paternally silenced genes and maternal ICR (Lewis et al., 2006; Lewis et al., 2004; Terranova et al., 2008; Umlauf et al., 2004).

Misregulation of the *Kcnq1ot1* domain leads to the imprinting disorder Beckwith-Wiedemann Syndrome (BWS) (Weksberg et al., 2010). In 50% of BWS patients, loss of DNA methylation occurs on the maternal *KCNQ1OT1* ICR, causing biallelic expression of the *KCNQ1OT1* ncRNA and silencing of *CDKN1C* and *KCNQ1* on the maternal allele (Choufani et al., 2010; Weksberg et al., 2010). Mutations in *CDKN1C* occur in 5-10% of BWS patients (Choufani et al., 2010). The *H19* imprinted domain is also associated with BWS patients. In 5% of BWS patients, a gain of DNA methylation at the maternal ICR occurs causing increased *IGF2* expression (Choufani et al., 2010). In 20% of BWS patients, both the *KCNQ1OT1* and *H19* domains are altered due to inheritance of two (or partial) paternal chromosomes 11, causing lack of *CDKN1C* and increased *IGF2* expression (Weksberg et al., 2010).

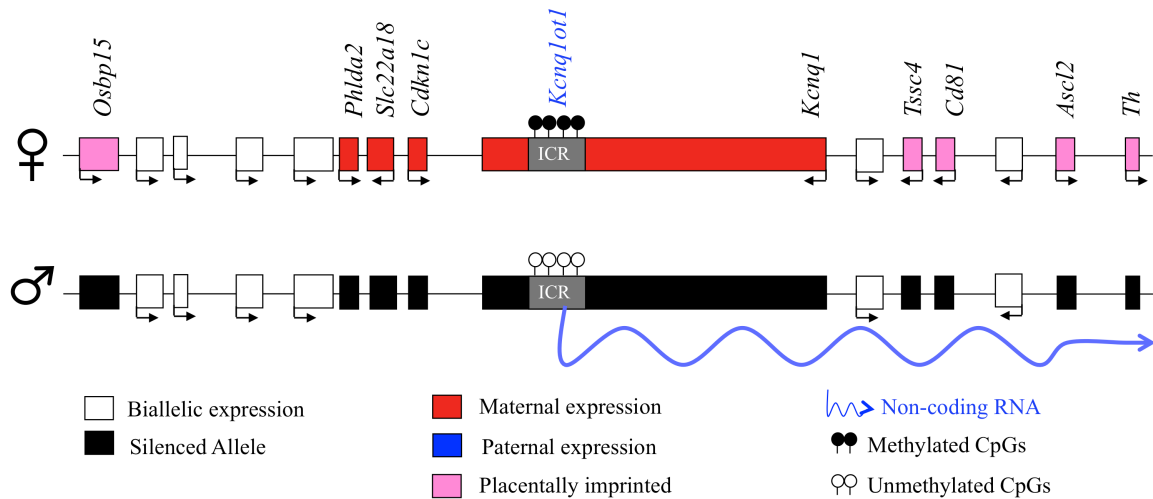


Figure 9: The *Kcnq1ot1* Imprinted Domain

The *Kcnq1ot1* domain consists of an ICR, which contains the promoter of the paternally expressed *Kcnq1ot1* ncRNA (blue wavy arrow), nine protein-coding with maternal-specific expression (red and pink boxes) and six biallelically-expressed genes (white boxes). On the maternal allele, the ICR is methylated and the *Kcnq1ot1* ncRNA is silent. Ubiquitously imprinted genes, *Phlda2*, *Slc22a18*, *Cdkn1c* and *Kcnq1* (red boxes), have maternal-specific expression in both embryonic and extraembryonic tissues. Placentally-specific imprinted genes, *Osbp15*, *Tssc4*, *Cd81*, *Ascl2* and *Th* (pink boxes), are maternally expressed in placental tissues but biallelic in the embryo proper. On the paternal allele, *Kcnq1ot1* is expressed from the unmethylated ICR and neighbouring protein-coding genes are silenced (black boxes).

1.2.5 Models of *Kcnq1ot1* Domain Regulation

The importance of the *Kcnq1ot1* ncRNA in silencing the paternal allele was established through paternal-inheritance of deletion and truncation mutations, where deletion of the ICR, including the *Kcnq1ot1* ncRNA promoter, and truncation of the *Kcnq1ot1* ncRNA resulted in biallelic expression of imprinted genes within the domain when analyzed at midgestation (day 13.5-16.5) (Fitzpatrick et al., 2002; Mancini-Dinardo et al., 2006; Shin et al., 2008). *Kcnq1ot1* is paternally expressed prior to the 2-cell stage and is credited with silencing the domain into adulthood (Lewis et al., 2006; Terranova et al., 2008). Several models have been proposed to regulate imprinting across the *Kcnq1ot1* domain: *Kcnq1ot1* ICR enhancer-blocking, *Kcnq1ot1* ncRNA-mediated silencing and *Kcnq1ot1* transcriptional interference models.

The first model is an enhancer-blocking mechanism similar to the *H19* domain. This model is based on the *Kcnq1ot1* ICR functioning as an insulator when placed between an enhancer and a gene of interest in an episomal system (Kanduri et al., 2002; Thakur et al., 2003). The ICR insulator model is supported by binding of CTCF insulator protein to the unmethylated paternal ICR, suggested to mediate a repressive ICR function by restricting access to the enhancer required for gene transcription (Fitzpatrick et al., 2007; Shin et al., 2008) (Figure 10). However, other studies have found that CTCF binding occurs at both the maternal and paternal *Kcnq1ot1* ICRs and is dispensable for enhancer-blocking mechanisms (Fitzpatrick et al., 2007; Lin et al., 2011). In addition, a region within the *Kcnq1ot1* ICR may have silencer activity, inducing repression of adjacent genes in an episomal system (Mancini-DiNardo et al., 2003; Thakur et al., 2003; Thakur et al., 2004). It is unclear how this silencer activity within the ICR is mediated.

The second model proposed to regulate domain imprinting is by *Kcnq1ot1* ncRNA-mediated paternal silencing, similar to X chromosome inactivation. The ncRNA model suggests the *Kcnq1ot1* ncRNA interacts with promoters of imprinted genes, directing repressive histone modifications to promoters (Mohammad et al., 2008; Pandey et al., 2008). The *Kcnq1ot1* ncRNA or its transcription can recruit histone methyltransferases, G9A and EZH2, to induce bidirectional repressive chromatin in *cis*. G9A is recruited to placental-imprinted genes to induce H3K9 di- and tri-methylation

(Umlauf et al., 2004; Wagschal et al., 2008) while EZH2/PRC2 catalyze H3K27me3 throughout the domain (Mager et al., 2003; Pandey et al., 2008; Terranova et al., 2008; Umlauf et al., 2004) (Figure 11). When there is decreased *Kcnq1ot1* ncRNA stability, paternal *Kcnq1ot1* ncRNA truncation or paternal ICR deletion, activation of the normally silent paternally imprinted genes within the domain occurs with concordant loss of repressive epigenetic marks (Fitzpatrick et al., 2002; Kanduri et al., 2006; Lewis et al., 2004; Mancini-Dinardo et al., 2006; Mohammad et al., 2008; Pandey et al., 2008; Shin et al., 2008; Thakur et al., 2003; Thakur et al., 2004). Additionally, the *Kcnq1ot1* ncRNA forms a nuclear domain for silent genes, which is suggested to subsequently target the domain to the nuclear periphery or perinucleolar regions (Mohammad et al., 2008; Redrup et al., 2009). This model necessitates additional epigenetic factors to allow for *Kcnq1ot1* ncRNA-promoter interactions and possibly the recruitment of other repressive complexes.

Debate exists as to whether it is the *Kcnq1ot1* RNA itself or its transcription that induces paternal silencing, leading to the third model of transcriptional interference. Similar to *Airn* transcriptional-silencing of *Igf2r*, the paternal *Kcnq1ot1* ncRNA is initiated from the ICR and is transcribed through downstream genes, *Kcnq1*, *Tssc4*, *Cd81*, *Ascl2* and *Th*. During antisense transcription, *Kcnq1ot1* may impede RNA polymerase binding, leading to paternal silencing of downstream genes. In support of this, our laboratory has reported that short-hairpin RNA (shRNA)-mediated depletion of the *Kcnq1ot1* ncRNA did not alter imprinted expression in embryo-derived stem cells, demonstrating that it is likely the act of transcription that is responsible for imprint maintenance rather than the RNA itself (Golding et al., 2011). However, antisense transcription cannot solely mediate paternal silencing at the *Kcnq1ot1* domain since overlapping transcription of *Kcnq1ot1* can only account for the repression of downstream and not upstream genes. Therefore, additional mechanisms must exist to silence upstream genes. Similar to *Airn* recruitment of G9A to silence *Slc22a2* and *Slc22a3*, *Kcnq1ot1* may recruit epigenetic factors to repress upstream genes within the domain (Figure 12). However, which factors are recruited is currently unclear and thus, requires further investigation.

In summary, silencing of the paternal allele within the *Kcnq1ot1* domain is complex and not fully understood. Several models have been proposed for the *Kcnq1ot1* domain paternal repression consisting of multiple regulatory features, such as the *Kcnq1ot1* ICR, *Kcnq1ot1* ncRNA and its transcription. The ICR contains the promoter of the *Kcnq1ot1* ncRNA and may also contain important regulatory roles outside the ncRNA, such as acting as an insulator or silencer to repress adjacent protein-coding genes while maintaining *Kcnq1ot1* ncRNA expression. The *Kcnq1ot1* ncRNA itself may recruit repressive epigenetic modifiers, targeting repressive epigenetic modifications like H3K27me3 and H3K9me2/3 to protein-coding genes. Transcription of the *Kcnq1ot1* ncRNA may also be important, and may silence the paternal allele by an unknown mechanism. Further investigation is required to better understand the mechanisms controlling paternal silencing at the *Kcnq1ot1* domain.

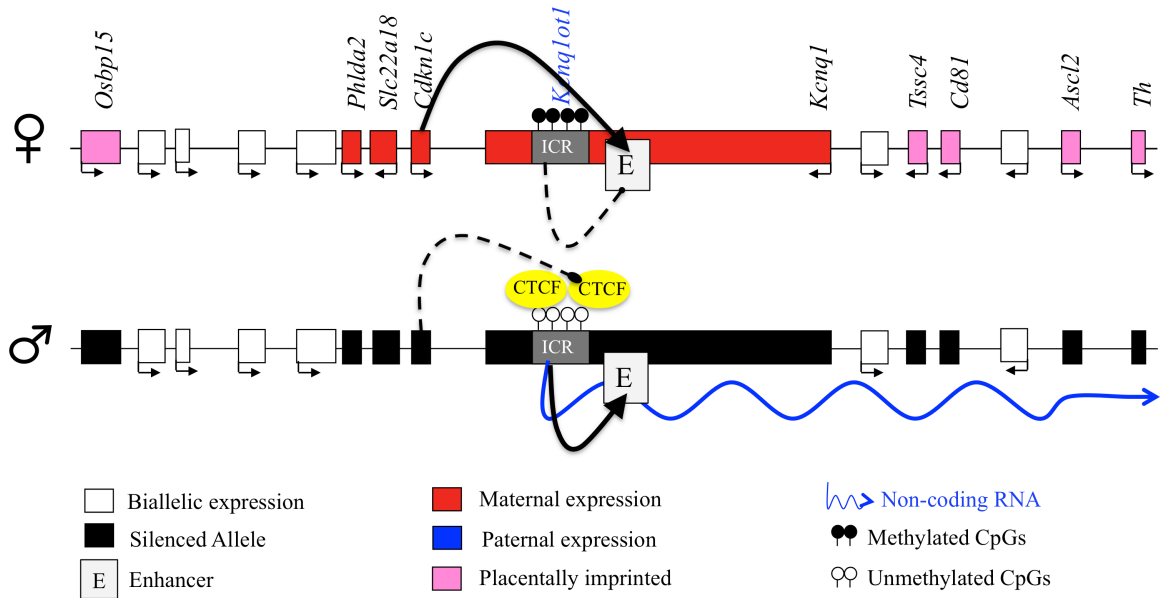


Figure 10: The *Kcnq1ot1* ICR Enhancer-Blocking Model

Kcnq1ot1 ICR can act as an insulator when placed between an enhancer (E) and a gene of interest and as a silencer to nearby genes in an episomal system, giving rise to an ICR-mediated silencing model. On the maternal allele, the methylated ICR cannot bind CTCF (yellow circle) and allows the interaction of imprinted genes (red and pink boxes), such as *Cdkn1c*, to interact with putative downstream enhancers, activating expression of these genes while blocking the interaction between the *Kcnq1ot1* promoter and enhancer. On the paternal allele, the unmethylated ICR binds CTCF, blocking the interaction of *Cdkn1c* and other protein-coding genes with downstream enhancers, inducing gene silencing while permitting *Kcnq1ot1* promoter-enhancer interactions.

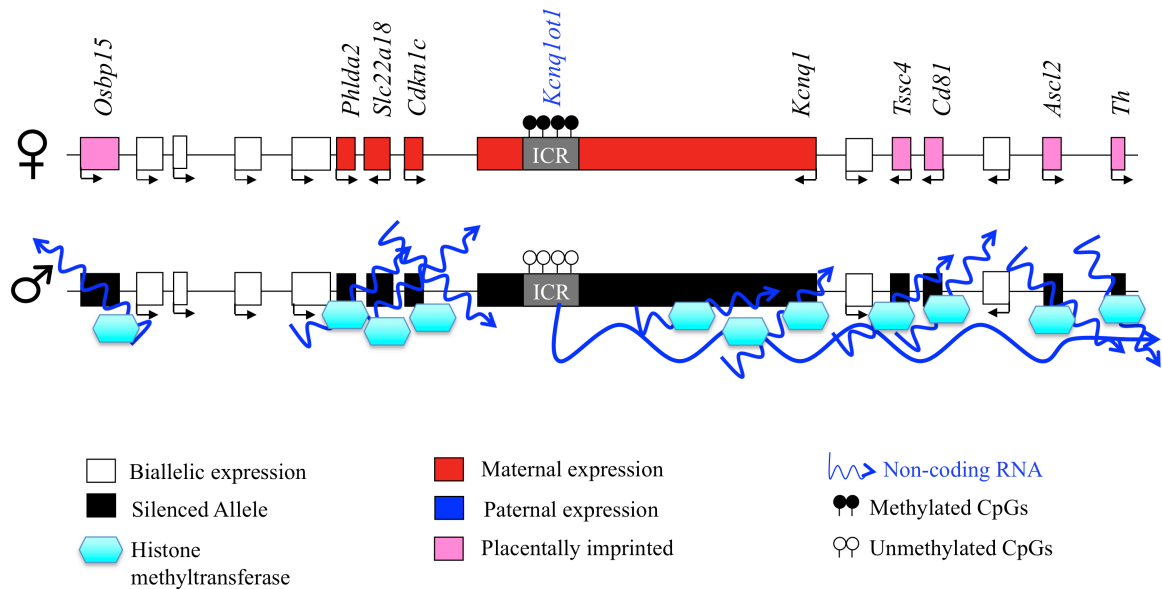


Figure 11: The *Kcnq1ot1* ncRNA-mediated Silencing Model

The ncRNA model of *Kcnq1ot1* imprinted regulation proposes the paternally expressed *Kcnq1ot1* ncRNA (blue wavy arrow) acts in *cis* to induce repressive chromatin modifications to silence the paternal alleles of adjacent imprinted genes (black boxes). The *Kcnq1ot1* ncRNA localizes to promoters of imprinted genes and recruits repressive histone methyltransferases (teal octagons) that induces paternal allelic silencing through repressive histone modifications. This model necessitates additional epigenetic factors to allow for *Kcnq1ot1* ncRNA-promoter interactions and possibly the recruitment of other repressive complexes.

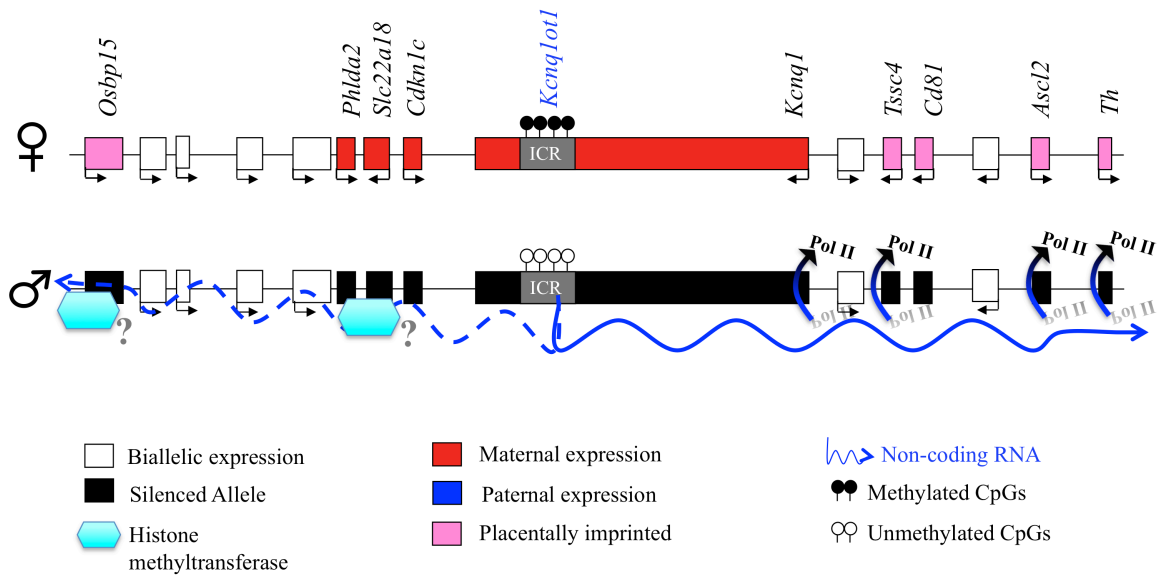


Figure 12: The *Kcnq1ot1* Transcriptional Interference Model

The ncRNA transcriptional interference model proposes that *Kcnq1ot1* transcription blocks transcription from other gene promoters. The *Kcnq1ot1* ncRNA (blue wavy arrow) is paternally expressed, initiating from the ICR and transcribes through downstream genes (*Kcnq1*, *Tssc4*, *Cd81*, *Ascl2* and *Th*). During antisense transcription, *Kcnq1ot1* impedes RNA polymerase II (Pol II) binding, leading to paternal silencing of downstream genes. However, silencing of upstream genes by transcriptional overlap must occur by another mechanism. Therefore, a hybrid model is proposed where both the *Kcnq1ot1* ncRNA and its transcription are required. The *Kcnq1ot1* ncRNA (blue dotted arrow) is tethered to the domain during transcription, and *Kcnq1ot1* ncRNA spreads recruiting repressive epigenetic modifiers (teal octagon) to silence the paternal alleles of upstream genes (as described in Figure 11). This hybrid model necessitates additional epigenetic factors for spreading function.

1.3 Rationale

The epigenetic silencing mechanisms of the paternal allele of imprinted genes within the *Kcnq1ot1* domain are poorly understood. While previous studies examining paternal *Kcnq1ot1* domain repression have expanded our understanding of *Kcnq1ot1* domain regulation, these studies are confounded by the fact that the majority of studies to date have investigated imprinted expression at stages when imprinting has already been established across the domain. For example, *Kcnq1ot1* ICR deletion and ncRNA truncation mutations showing loss of imprinting were analyzed at midgestation (day 13.6-16.5) (Fitzpatrick et al., 2002; Mancini-Dinardo et al., 2006; Shin et al., 2008). Since the *Kcnq1ot1* ncRNA is expressed at the zygote stage (Lewis et al., 2006; Terranova et al., 2008), studies are required to delineate the mechanisms regulating *Kcnq1ot1* domain imprinting during preimplantation development. Furthermore, it is currently unclear how *Kcnq1ot1* ICR and ncRNA induce and maintain paternal allelic silencing and through which epigenetic factors. The few epigenetic modifiers that do associate with the *Kcnq1ot1* domain are based on other imprinting models (Pandey et al., 2008). Thus, investigation is required to examine the epigenetic players acting on the domain.

My approach is to address when imprinting is acquired at the *Kcnq1ot1* domain and whether the *Kcnq1ot1* ICR or ncRNA are required for imprinted domain establishment. Additionally, I will use an approach independent of assumptions based on current proposed models to identify the epigenetic modifiers regulating paternal allelic repression at the *Kcnq1ot1* domain. Both approaches are imperative to understanding the regulatory mechanisms of the *Kcnq1ot1* domain.

1.4 Hypothesis

I hypothesize that the *Kcnq1ot1* ICR and ncRNA are required for paternal allelic repression of genes within the domain during preimplantation development. Furthermore, I hypothesize that factors identified independent of current models will act domain-wide to control paternal silencing of imprinted genes within the *Kcnq1ot1* domain.

1.5 Objectives

- (1) Determine the role of the *Kcnq1ot1* ICR or ncRNA in paternal allelic repression during preimplantation development.
 - a. Determine the stage during preimplantation development when paternal allelic silencing is established at protein-coding genes within the domain.
 - b. Determine whether the *Kcnq1ot1* ICR and/or ncRNA are required for the paternal allelic silencing of genes upstream or downstream of *Kcnq1ot1* using paternally inherited *Kcnq1ot1* ICR deletion and paternally inherited *Kcnq1ot1* truncation mutants.
 - c. Determine whether DNA methylation at the *Kcnq1ot1* ICR is altered during the establishment of paternal allelic silencing when the *Kcnq1ot1* ICR is paternally deleted and the paternal *Kcnq1ot1* ncRNA is truncated.
- (2) Determine the epigenetic modifiers associated with maintaining paternal allelic silencing.
 - a. Identify epigenetic modifiers involved in allelic silencing at the *Kcnq1ot1* domain using a positive selection, loss-of-function RNA interference screen.
 - b. Determine the effects on imprinted gene expression and *Kcnq1ot1* ncRNA levels when screen candidates are depleted in stem cells.
 - c. Determine the effects on imprinted DNA methylation at the *Kcnq1ot1* ICR when screen candidates are depleted in stem cells.

Chapter 2

2 Material and Methods

2.1 Embryo Collection

Female B6(CAST7) mice contained a *Mus musculus castaneus* chromosome 7 on a B6 background (Mann et al., 2004). Males were wildtype C57BL/6J (B6) (Charles River Laboratories, St. Constant, Quebec, Canada), congenic B6 heterozygous 2.8 kb *Kcnq1ot1* ICR deletion or congenic B6 heterozygous *Kcnq1ot1* ncRNA truncation (2.6 kb from the start site) mice (Fitzpatrick et al., 2002; Shin et al., 2008). B6(CAST7) females were mated to wild-type, deleted or truncated males to obtain F₁ 8-cell, blastocyst stage, and day 6.5 embryos (Figure 13). Wildtype 8-cell embryos were flushed from oviducts 2.5 dpc and frozen into pools of 5-7 embryos. In total, five 8-cell embryos pools were collected and analyzed for allelic-expression. Early blastocysts were flushed from uteri at 3.3 dpc (7am on 3 dpc) and late blastocysts at 3.5 dpc (noon on 3dpc). Embryos were washed in 20 µL drops of M2 media (Sigma). For each mutant (*Kcnq1ot1* ICR deletion and ncRNA truncation), 100 and 130 blastocysts were collected, of which 27 and 37 were analyzed for both DNA methylation and allelic expression, respectively. Wildtype blastocysts were collected from WT X WT and WT X Mutant heterozygotes crosses and 27 wildtype blastocysts were analyzed for allelic expression. Embryos were harvested from both WT X WT and WT X Mutant heterozygous crosses to rule out differences in developmental timing between control and mutant embryos. Day 6.5 embryos were dissected into embryonic, extraembryonic ectoderm and ectoplacental cone tissues with care taken to eliminate maternal tissue through successive washes. Experiments were performed in compliance with guidelines set by the Canadian Council for Animal Care, and the policies and procedures were approved by the University of Western Ontario Council on Animal Care.

2.2 Embryo Genotyping

Heterozygous *Kcnq1ot1* ICR deletion and ncRNA truncation males were mated with CAST7 females to collect F₁ blastocysts and day 6.5 embryos. Individual embryos

were genotyped for presence of the wildtype or mutant alleles. Deletion of the ICR was detected following bisulfite mutagenesis using first round primers BIS DEL OUTER F and R, and second round primers BIS DEL INNER F and R (Section 2.5). Lack of the B6 allele following bisulfite and sequencing of the BIS WT INNER amplicon also indicated deletion of the ICR. Truncation of the ncRNA was detected following bisulfite mutagenesis using first round primers BIS TRU OUTER F and R, and second round primers BIS TRU INNER F and R. Additionally, expression of the truncated *Kcnq1ot1* ncRNA was detected using TRU F and R following cDNA synthesis and first strand synthesis (Section 2.3-4).

2.3 RNA Isolation from Embryos and cDNA Synthesis

RNA isolation and cDNA library generation for preimplantation and early postimplantation embryos was performed as previously described (Korostowski et al., 2011). Second-strand synthesis was performed using forward primers and second-strand products were moved to PCR tubes containing Illustra Ready-to-Go PCR Beads (GE Healthcare Biosciences) containing the reverse (R) primer.

2.4 Allelic Expression Analysis

Primers were designed to span introns to avoid amplification of any remaining DNA. Primers were designed for *Phlda2*, *Slc22a18* and *Osbp15* or synthesized as previously reported for *Kcnq1*, *Cdkn1c*, and *Ascl2* (Golding et al., 2011) (Table 3). For *Phlda2* analysis, primers amplified a 389 bp fragment containing a polymorphism between B6 (A) and CAST (C). Restriction digestion with Hpy188III resulted in 282 bp and 107 bp bands in B6 and 231 bp, 107 bp and 51 bp fragments in CAST. For *Slc22a18* analysis, primers amplified a 227 bp fragment containing a polymorphism between B6 (C) and CAST (T). Restriction digestion with SacII resulted in 200 bp and 27 bp fragments in B6 and 231 bp, 107 bp and 51 bp bands in CAST. For *Osbp15* analysis, primers amplified a 415 bp fragment containing a polymorphism between B6 (T) and CAST (C). Restriction digestion with AluI resulted in 369 bp, 40 bp, 22 bp, 21 bp bands in B6 and 319 bp, 90 bp, 40 bp, 22bp, 21 bp fragments in CAST. Restriction enzymes that cleaved at B6:CAST polymorphisms were used to digest PCR products. Maternal

(red) and paternal (blue) transcript abundance was determined by densitometry of PCR amplicons following parental-specific digestion using the BioRad Gel Doc quantification system. Parental allele-specific expression was calculated as the percentage expression of the B6 or CAST allele relative to the total expression of both alleles. To control for primer bias, allelic expression analysis was also performed on cDNA from CAST and B6 adult kidney RNA, and on cDNA from equally mixed CAST and B6 RNA (C:B), shown on far left of allelic expression graphs (Figure 18).

2.5 Bisulfite mutagenesis and clonal sequencing assay

Bisulfite mutagenesis was performed as described (Golding et al., 2011; Golding et al., 2010; Market-Velker et al., 2010). Individual embryos or wildtype or siRNA-transfected XEN cells (15,000 cells) were lysed and embedded in 2% low melting point agarose (Sigma). Following bisulfite treatment, the agarose was divided into four independent PCR reactions, three containing the *Kcnq1ot1* ICR bisulfite (BIS WT OUTER) primers, and one containing either the BIS DEL OUTER or BIS TRU OUTER primers (Table 3). Following amplification, 5 μ l of the first round PCR product was added to the second round amplification reaction mix containing either the BIS WT INNER, BIS DEL INNER or BIS TRU INNER primers. PCR products were ligated into pGEM-Easy Vector according to the manufacturers' instructions (Promega) and sequenced at BioBasic Sequencing Facility (Markham, ON). Sequences with less than 90% conversion were excluded. Percent methylation was calculated as the number of methylated CpGs over the total number of CpGs.

2.6 Cell Culture

CASTXB6^{neoR} extraembryonic endoderm (XEN) stem cells were generated by crossing a *Mus musculus* (CAST) female to a congenic C57BL/6J (B6) male carrying a targeted mutation of the *Cdkn1c* gene, in which exons 1 and 2 were replaced by the PGK-Neomycin resistance cassette (*Cdkn1c*^{+/ Δ neo}) (Zhang et al., 1997) (Jackson Laboratory). Cells were genotyped for the paternal *Cdkn1c*^{+/ Δ neo} allele using primers p57neoF and p57neoR. B6XCAST wild-type and CASTXB6^{*Cdkn1c*/ Δ neo} XEN cells were generated and cultured as previously described (Golding et al., 2010) (Figure 14). Briefly, XEN cell

cultures were maintained in RPMI (Sigma) supplemented with 50 mg/ml penicillin/streptomycin (Sigma), 1 mM sodium pyruvate, 100 μ M β -mercaptoethanol (Sigma), 2 mM L-Glutamine (Sigma), and 15% Hyclone ESC grade fetal bovine serum (FBS) (Fisher Scientific). Cells were passaged on a layer of mitomycin C (Sigma) treated mouse embryonic fibroblasts.

2.7 Epigenetic shRNA Transduction of XEN Stem Cells and Screen Candidate Identification by PCR and DNA sequencing

A library of shRNAs targeting 250 different epigenetic factors used in this experiment was previously described (Golding et al., 2010). Transduction of shRNAs into XEN cells was modified from (Golding et al., 2010). shRNA vectors and lenti class plasmids encoding Vesicular stomatitis virus glycoprotein pseudotype (plasmid pMDG) and Psi (pPsi) packaging elements were transfected into 10 cm dish of HEK293 cells (ATCC) using Lipofectamine 2000 (Invitrogen). Media was changed 6 hr later. Viral-containing media was harvested and filtered (0.45 μ m pore) 72 hr posttransfection and added to freshly trypsinized XEN cells along with 1X polybrene (Sigma) into 1.5 X 6-well plates (Fisher). XEN cell media was changed 48 hr later. Low levels of infection were necessary to ensure single viral integration per cell and was verified using flow cytometry. XEN cells were selected for viral integration with 1 μ g/mL puromycin (Sigma). shRNA-containing XEN cells were moved to 8 X 10 cm dishes and selected for loss of silencing of *Cdkn1c*^{+/ Δ neo} allele with 50 μ g-200 μ g/mL neomycin (Sigma). While still under neomycin selection, XEN cells were plated at an even lower density into 15 cm dishes and incubated until individual colonies formed. Individual colonies were picked and moved into individual wells of 24-well plates and underwent a second round of neomycin selection. DNA of surviving colonies was isolated (Qiagen DNeasy Blood & Tissue Kit) and PCR amplification was performed for the hairpin region inside the shRNA construct with primers Lenti F and R. PCR amplicons were sequenced (BioBasic Inc, Markham ON) to determine identity of the shRNA and therefore the candidate epigenetic factor responsible for loss of neomycin silencing.

2.8 siRNA-transfection

XEN cells were plated at low density (5%) into 6-well plates and transfected 8 hr later with siRNAs (Dharmacon) targeting either *Ezh1* (L-058527-01-0005), *Smarca5* (L-041484-01-0005), *Smarcad1* (L-056219-01-0005) or Non-Targeting (D-001810-10-05) using PepMute siRNA Transfection Reagent (FroggaBio) according to manufacturers' instructions. Cells were incubated for 72 hr and then collected for DNA and RNA.

2.9 RNA Isolation from Cultured Cells and cDNA Synthesis

RNA from cultured cells was isolated using Trizol (Invitrogen) according to the manufacturers' instructions. Following removal of genomic DNA using DNaseI (Invitrogen), cDNA was synthesized from 2 µg of total RNA using Superscript II (Invitrogen) and oligo(dT) primers (Invitrogen). Depletion of siRNA-targeted mRNA and *Kcnq1ot1* abundance were determined using RT-PCR with *Mrpl* as the reference control (Table 3).

2.10 Statistical Analysis

For statistical difference of maternal-specific, maternal-bias, and biallelic expression of each imprinted gene between wildtype, *Kcnq1ot1* paternal deletion and truncation mutants was determined by Yate's Chi-squared test. P-value less than 0.05 was considered to be significant.

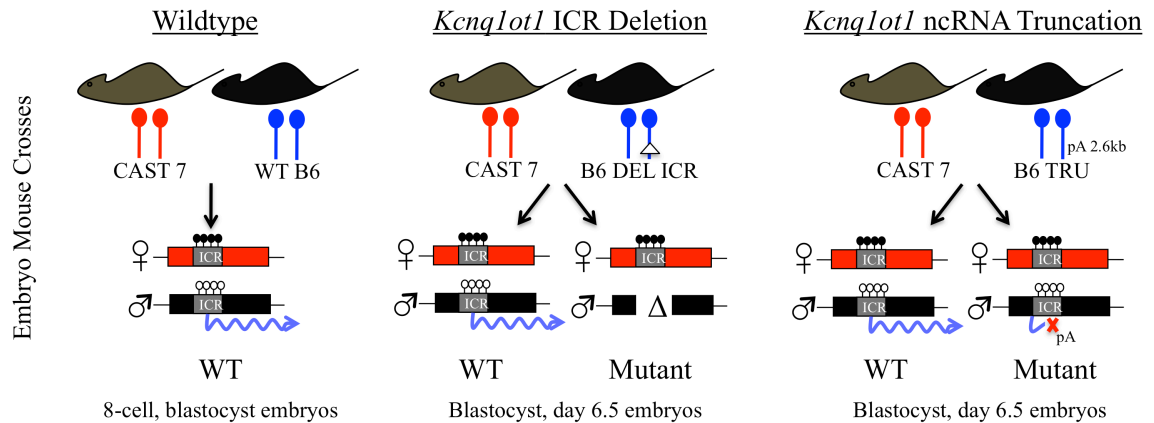


Figure 13: Preimplantation and Early Postimplantation Embryo Mouse Crosses

CAST7 (brown mouse; red chromosomes) females were mated with B6 (black mouse; blue chromosomes) males to generate wildtype embryos that were collected at the 8-cell and blastocyst embryo stages. *Kcnq1ot1* ncRNA (blue wavy line) is expressed from the paternally unmethylated imprinting control region (ICR). CAST7 females were mated with heterozygous congenic B6 males, carrying a 2.8 kb deletion (Δ) of the *Kcnq1ot1* ICR, to generate wildtype and mutant embryos lacking the *Kcnq1ot1* ICR and ncRNA. CAST7 females were mated with heterozygous congenic B6 males, carrying an insertion of a polyA cassette (pA) at 2.6 kb from the transcriptional start site, to generate wildtype and mutant embryos with a truncated *Kcnq1ot1* ncRNA. Blastocysts and day 6.5 embryos were collected from both mutant crosses.

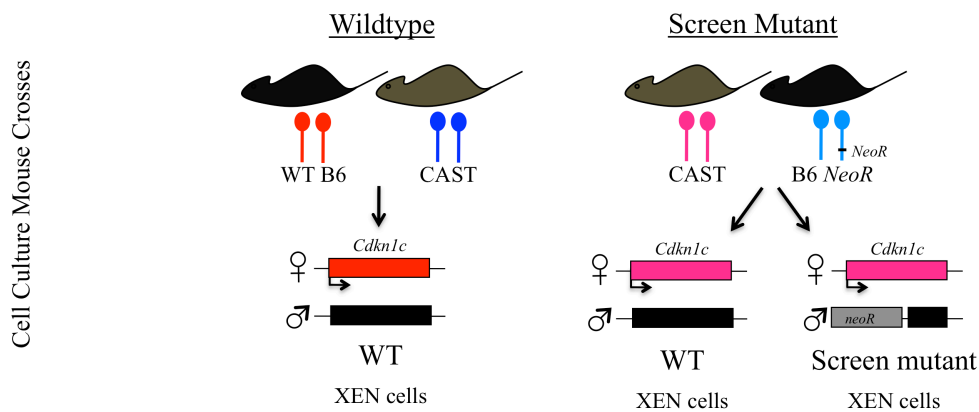


Figure 14: XEN Cell Mouse Crosses

Wildtype B6 (black mouse; red chromosomes) females were mated with CAST (brown mouse; blue chromosomes) males to collect embryos that were used to generate wildtype extra-embryonic endoderm (XEN) stem cells. *Cdkn1c* is maternally expressed (red box) and paternally silent (black box). Wildtype CAST (brown mouse; pink chromosomes) females were mated with heterozygous congenic B6 (black mouse; light blue chromosomes) males carrying the *Neomycin resistance* gene (*NeoR*) recombined at the *Cdkn1c* gene, removing the promoter and Exon 1 and 2. Wildtype and mutant embryos were collected and used to generate XEN cells. Wildtype XEN cells have maternally expressed *Cdkn1c* (pink box) and paternally silent *Cdkn1c* (black box). Mutant XEN cells have maternally expressed *Cdkn1c* (pink box) and paternally silent *neoR* (gray box) and were used to screen for epigenetic modifiers regulating paternal silencing of the *Kcnq1ot1* domain.

Table 3: PCR Primers

	Primer	Size (bp)	Enzyme	Reference
BIS DEL OUTER	F GGGATAGTTGAGAGGGTGGATTG R CCCAAAAAAAAATCCTACTTATC	539		
BIS DEL INNER	F GGAAGGGATGAGGAAGGTAGG R ACATACTCTAATTCACCAAACCTAC	270		
BIS TRU OUTER	F ATTTTTTTTTTAAGGTAATTAGGG R CCTTCTAATAAACCAACCTACACC	364		
BIS TRU INNER	F TAAATGATAAGGTAGAATATTTTTG R ATAAAACAACACAACAACCAACAC	240		
TRU	F TCTGTTTTGGGATTTGTGC R TGCCAAAATGATGAGACAGC	111		
BIS WT OUTER	F GTGTGATTTATTTGGAGAG R CCACTCACTACCTAATACTAACCAC	571		(Golding et al., 2011)
BIS WT INNER	F GGTTAGAAGTAGAGGTGATT R CAAAACCACCCCTACTTCTAT	207		(Golding et al., 2011)
<i>Cdkn1c</i>	F GCCAATGCGAACGACTTC R TACACCTTGGGACCAGCGTACTCC	364	TaqI	(Golding et al., 2011)
<i>Sle22a18</i>	F ATCAACAGGACTTTTGCCCC R AAAGGTTGTCTGTAGGTAGCC	257	SacII	
<i>Phlda2</i>	F ATCCTTTGCGAGGGCGAGC R GCGTTTCACGGACCCAGA	389	Hpy188III	
<i>Kcnq1</i>	F CATCGGTGCCCGTCTGAACAGG R TTGCTGGGTAGGAAGAGCTCAG	189	NlaIII	(Golding et al., 2011)
<i>Ascl2</i>	F TGAGCATCCCACCCCTA R CCAAACATCAGCGTCAGTATAG	474	SfeI	(Golding et al., 2011)
<i>Osbp15</i>	F CAGGGCAGCAGCAGAGGAT R GGAGGTTGTTCTTCTCACACTCG	415	AluI	
Lenti	F ACAACTACAACAGCCACAACG R GCAGCGTATCCACATAGCGT	834		(Golding et al., 2010)
P57neo	F GGGAACTTCCTGACTAGGGG R CCACGTTTGGAGAGGGACAC	284		
<i>Ezh1</i>	F TCCAGCCTGTTTCAGCCAATG R CTTCTCCTTCACCTCGTCACC	234		
<i>Smarca5</i>	F GCAATCATCCTTATCTCTTCG R CTTTCTTCCACAGTGTATCAG	478		
<i>Smarca4</i>	F GGAGTAAGATTGAGGAAGCAC R CTCCATCCATAGTGCTTGTTG	525		
<i>Kcnq1ot1</i>	F CAAGACCACCCCTGCTTCTG R GGGTTTCTTCTGAGTCAG	297		
<i>Mrpl</i>	F TTGGATATGCCAAGTGACCA R GCTTCTGCCGTTTGAGTTTC	212		(Golding et al., 2010)

Chapter 3

3 Results

3.1 Paternal Allelic Repression in Wildtype Blastocyst

Even though paternal *Kcnq1ot1* ncRNA begins to be transcribed at the 1-cell stage (Lewis et al., 2006; Terranova et al., 2008), *Cdkn1c* expression was observed previously to be biallelic in 4- and 8-cell embryos, with progressive silencing of the paternal allele by the late blastocyst stage (Market Velker et al., 2012). Imprinted expression of other genes within the *Kcnq1ot1* domain has not been examined until later stages (morula and blastocyst embryos) using pools of preimplantation embryos (Lewis et al., 2006; Terranova et al., 2008; Umlauf et al., 2004). To assess developmental expression of other imprinted genes within the domain, pools of 8-cell embryos, and individual blastocysts were examined for imprinted expression of genes upstream (*Phlda2*, *Slc22a18*, *Cdkn1c*) and downstream (*Kcnq1*, *Ascl2*) of the *Kcnq1ot1* ncRNA promoter. Here, I define maternal-specific expression as 90-100% expression from the maternal allele, maternal-biased expression as 65-89% expression from the maternal allele and biallelic expression as $\leq 64\%$ expression from the maternal allele. In our analysis, expression of both downstream genes, *Kcnq1* and *Ascl2*, was not detected in 8-cell or blastocyst stage embryos. The upstream genes, *Phlda2* and *Cdkn1c*, were biallelically expressed in 8-cell embryos (Figure 15). In blastocyst stage embryos, *Phlda2* and *Cdkn1c* underwent paternal repression. For *Phlda2*, 30%, 52% and 18% of wildtype (WT) blastocysts displayed maternal-specific, maternal-biased and biallelic expression, respectively (Figure 17). While for *Cdkn1c*, 11%, 50% and 39% of WT blastocysts displayed maternal-specific, maternal-biased and biallelic expression. While *Slc22a18* was not expressed at the 8-cell stage, embryonic expression was activated in 30% of WT blastocysts (Figure 15). Of these embryos, 75% had maternal-specific expression and 25% possessed biallelic expression (Figure 17). However, progressive maternal activation of imprinted genes in addition to paternal silencing cannot be ruled out. A comparison of imprinted expression across the three genes revealed that *Phlda2*, *Slc22a18* and *Cdkn1c* are not coordinately regulated (Figure 15). For example, embryo

12W had *Phlda2* and *Slc22a18* maternal-specific expression and *Cdkn1c* biallelic expression. Embryo 24W showed *Slc22a18* maternal-specific expression while *Phlda2* and *Cdkn1c* had maternal-biased expression. Embryo 26W had *Slc22a18* and *Cdkn1c* maternal-specific expression while *Phlda2* was biallelically expressed. Overall, this indicates that imprinted expression of the *Phlda2*, *Slc22a18* and *Cdkn1c* genes were differentially and developmentally regulated with the paternal alleles switching from an expressed to repressed state.

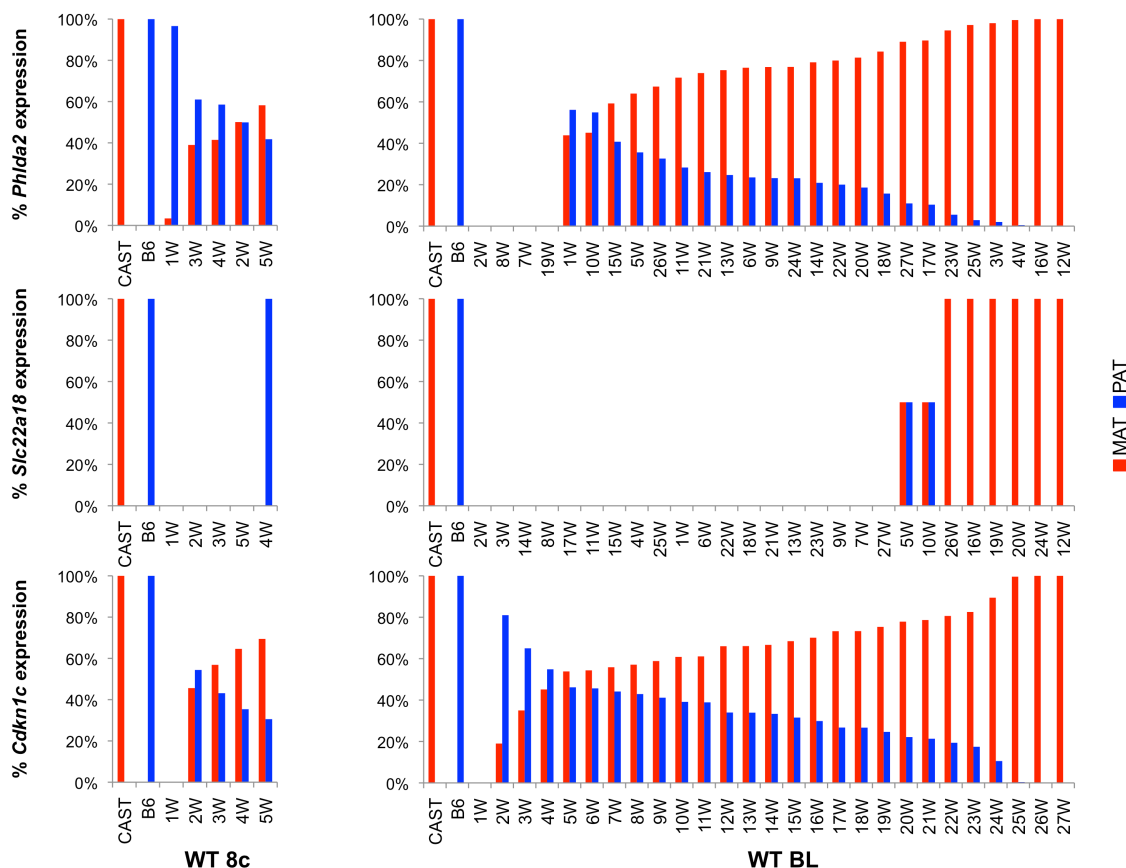


Figure 15: Developmentally Regulated *Cdkn1c*, *Slc22a18* and *Phlda2* Paternal Silencing During Preimplantation Development

Allelic expression of *Cdkn1c*, *Slc22a18* and *Phlda2* was examined in wildtype (WT), pools of 8-cells (5-7 per pool) and individual blastocysts (BL). *Cdkn1c* and *Phlda2* expression underwent paternal silencing beginning at the blastocyst stage. *Slc22a18* was expressed in few preimplantation embryos but displayed mostly maternal expression once activated in blastocysts. Red bar, maternal expression; blue bar, paternal expression; W, wildtype; Number, designation given to pools of embryos or blastocysts at the time of embryo collection.

3.2 Paternal Silencing in *Kcnq1ot1* ICR Deleted and *Kcnq1ot1* ncRNA Truncated Blastocysts

Imprinted expression of *Phlda2*, *Slc22a18* and *Cdkn1c* did not occur until the blastocyst stage, despite the presence of *Kcnq1ot1* ncRNA as early as the 1-cell stage (Lewis et al., 2006; Terranova et al., 2008). This led us to question whether the *Kcnq1ot1* ncRNA is required for the establishment of imprinted gene expression during preimplantation development. To delineate this, blastocyst stage embryos harboring the paternally inherited *Kcnq1ot1* ICR deletion or ncRNA truncation were analyzed for imprinted expression of *Phlda2*, *Slc22a18* and *Cdkn1c*. Mutant blastocysts with a paternal deletion or truncation of *Kcnq1ot1* were capable of silencing the paternal allele similar to WT blastocysts, with mutant embryos showing maternal-specific and maternal-biased expression (Figure 16). Importantly, for the *Kcnq1ot1* ICR deletion, maternal-specific and maternal-biased expression was observed in 44% of embryos for *Phlda2* (20% and 24%, respectively), 67% of embryos for *Slc22a18* (56% and 11%, respectively), and 32% of embryos for *Cdkn1c* (12% and 20%, respectively) (Figure 17). For *Kcnq1ot1* ncRNA truncation, maternal-specific and maternal-biased expression was found in 50% of embryos for *Phlda2* (15% and 35%, respectively), 67% of embryos for *Slc22a18* (42% and 25%, respectively), and 35% of embryos for *Cdkn1c* (6% and 29%, respectively) (Figure 17). Statistical analysis using the Yates Chi-squared test showed no significant difference between all three groups or between each pair-wise comparison ($p \geq 0.05$) (Preacher, 2001). Despite not being statistically different from controls, there appears to be an increased percentage of mutant embryos with biallelic expression. This may be due to developmental delay or an early effect of the *Kcnq1ot1* ICR or ncRNA. Since mutant embryos appeared to have slower developmental rates, the former explanation is favored. The important point is that mutant embryos had the ability to achieve paternal allelic silencing of *Phlda2*, *Slc22a18* and *Cdkn1c* in the absence of the *Kcnq1ot1* ICR or with truncation of the *Kcnq1ot1* ncRNA in blastocyst stage embryos, demonstrating that the *Kcnq1ot1* ICR and its ncRNA are dispensable for establishment of paternal allelic silencing during preimplantation development.

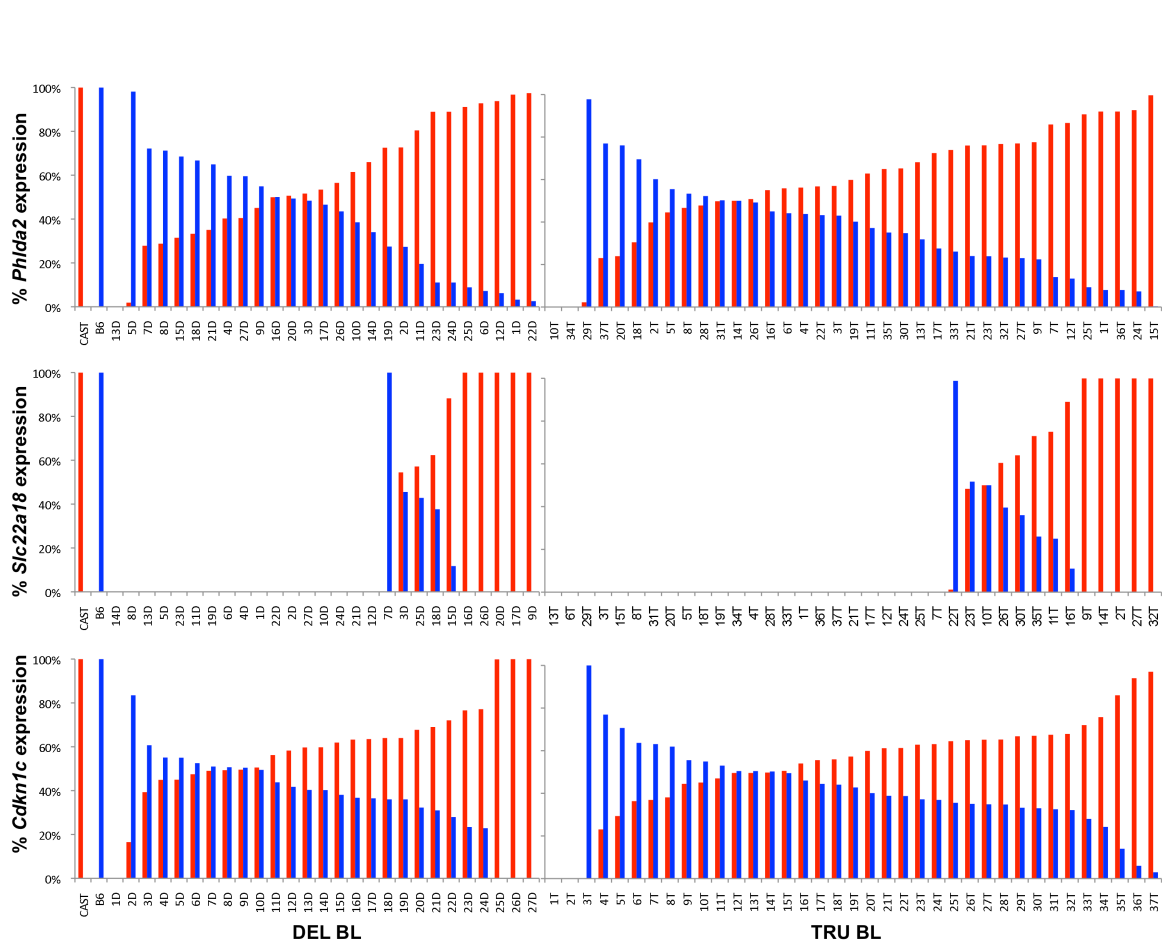


Figure 16: Paternal *Kcnq1ot1* ICR Deleted and ncRNA Truncated Blastocysts Possessed Developmentally Regulated Paternal Silencing

Allelic expression of *Cdkn1c*, *Slc22a18* and *Phlda2* was analyzed in individual blastocysts having either paternal *Kcnq1ot1* ICR deletion (DEL) or paternal *Kcnq1ot1* ncRNA truncated (TRU). *Cdkn1c* and *Phlda2* expression underwent paternal silencing beginning at the blastocyst stage. *Slc22a18* was expressed in few preimplantation embryos but displayed mostly maternal expression once activated. Red bar, maternal expression; blue bar, paternal expression; D, deletion; T, truncation; Number, designation given to blastocysts at the time of embryo collection.

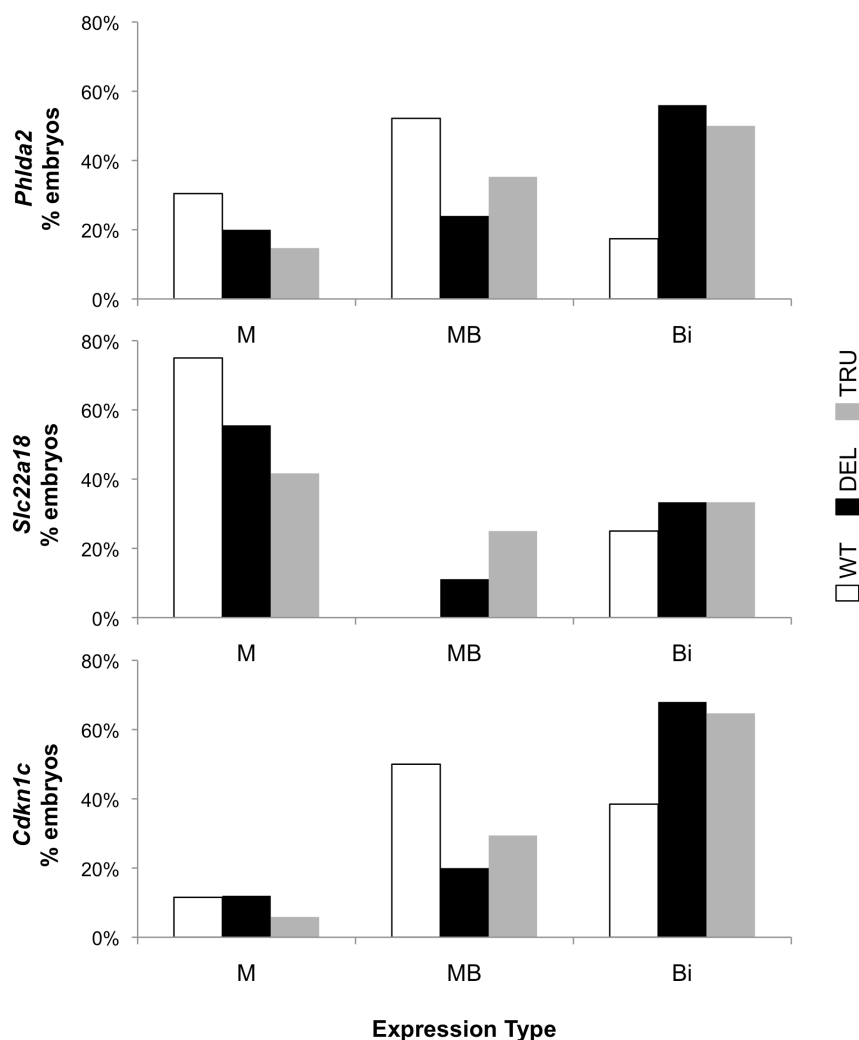


Figure 17: Paternal *Kcnq1ot1* Mutant Blastocysts Possessed a Similar Percentage of Embryos with Maternal-specific, Maternal-bias or Biallelic Expression

Statistical analysis based on expression patterns revealed no significant difference in the percentage of WT, paternal *Kcnq1ot1* ICR deleted (DEL) and ncRNA truncated (TRU) blastocysts having *Phlda2*, *Slc22a18* and *Cdkn1c* maternal-specific (M; >90%), maternal-bias (MB; 65%-89%) or biallelic (Bi; ≤64%) expression using the Yates Chi-squared test. Note that embryos with paternal only expression were not included in the analysis.

3.3 Gene-specific and Tissue-specific Requirement for *Kcnq1ot1* ICR and ncRNA at Day 6.5

To determine whether imprinted expression can be maintained in the absence of *Kcnq1ot1* ICR and ncRNA during early postimplantation development, imprinted expression of genes upstream (*Cdkn1c*, *Slc22a18*, *Phlda2*) and downstream (*Kcnq1*, *Ascl2*) of the *Kcnq1ot1* ICR/ncRNA promoter were examined in day 6.5 embryos that were dissected into three lineages, embryonic ectoderm (EMB), extra-embryonic ectoderm (EEE) and ectoplacental cone (EPC). WT embryos primarily displayed maternal-specific expression at all genes (mean maternal expression levels 91-100%), except for *Ascl2* where half of the tissue-samples had maternal-biased expression with mean maternal expression levels of 87%, 91% and 75% for EMB, EEE and EPC, respectively (Figure 18, Table 4). By comparison, day 6.5 embryos harbouring a paternally inherited *Kcnq1ot1* ICR deletion had activated the silent paternal alleles, resulting in biallelic expression for all genes (mean maternal expression levels of 40-64%), except for *Slc22a18* in EPC samples which exhibited maternal-biased expression (mean maternal expression 68%). Similarly, day 6.5 embryos inheriting a paternal *Kcnq1ot1* ncRNA truncated allele had activated the silent paternal alleles of *Phlda2*, *Slc22a18* and *Ascl2* genes (mean maternal expression levels of 50-63%), except for *Slc22a18* in EPC samples that exhibited maternal biased expression (mean maternal expression 71%). In contrast to deletion mutants, day 6.5 embryos with a paternal *Kcnq1ot1* truncation mutation maintained *Kcnq1* maternal-specific expression in EMB and EPC samples (mean maternal expression levels of 95%) and maternal-biased expression in EEE tissues (mean maternal expression levels of 88%). For *Cdkn1c*, paternal truncation mutants had maternal-biased expression with mean maternal expression levels of 68-73%. Thus, analysis of early postimplantation tissues revealed a gene-specific maintenance of imprinted expression in truncation mutants (*Kcnq1* and *Cdkn1c*) and tissue-specific maintenance of imprinted expression in truncation and deletion mutants (*Slc22a18* in EPC samples). A comparison of deletion and truncation mutants indicates that the *Kcnq1ot1* ICR and its ncRNA have distinct functions. In day 6.5 embryos, while the ICR was required to maintain paternal silencing of all genes in the domain, the *Kcnq1ot1* ncRNA was dispensable for imprint maintenance of genes closest

to the ICR (*Kcnq1*, *Cdkn1c*). In contrast, the *Kcnq1ot1* ncRNA was necessary for maintenance of imprinted expression of genes located at a distance (*Phlda2*, *Slc22a18*, *Ascl2*).

3.4 Preservation of DNA Methylation in Day 6.5 *Kcnq1ot1* ICR Deleted and *Kcnq1ot1* ncRNA Truncated Embryos

Even though there was no statistical difference in the number of embryos with maternal-specific, maternal-biased and biallelic expression of imprinted genes at the blastocyst stage, a larger number of embryos had biallelic expression when they inherited paternal *Kcnq1ot1* ICR deleted or *Kcnq1ot1* ncRNA truncated alleles. Although this may reflect developmental timing, I wanted to determine whether there was an aberrant gain of paternal DNA methylation at the *Kcnq1ot1* ICR in blastocysts exhibiting biallelic expression for one or more imprinted genes as well as in day 6.5 samples with biallelic and/or maternal-biased expression. Aberrant gain of paternal DNA methylation at the *Kcnq1ot1* ICR could potentially silence *Kcnq1ot1* in truncation mutants or alter the ICR function causing biallelic expression or maternal-biased expression. The bisulfite mutagenesis and clonal sequencing assay was performed on WT, *Kcnq1ot1* ICR deleted and *Kcnq1ot1* ncRNA truncated individual blastocysts and day 6.5 tissues. I observed that the maternal allele was hypermethylated as expected while the paternal allele was hypomethylated in all WT and *Kcnq1ot1* mutant embryos (Figure 19, Figure 20), consistent with previous results (Fitzpatrick et al., 2002; Shin et al., 2008).

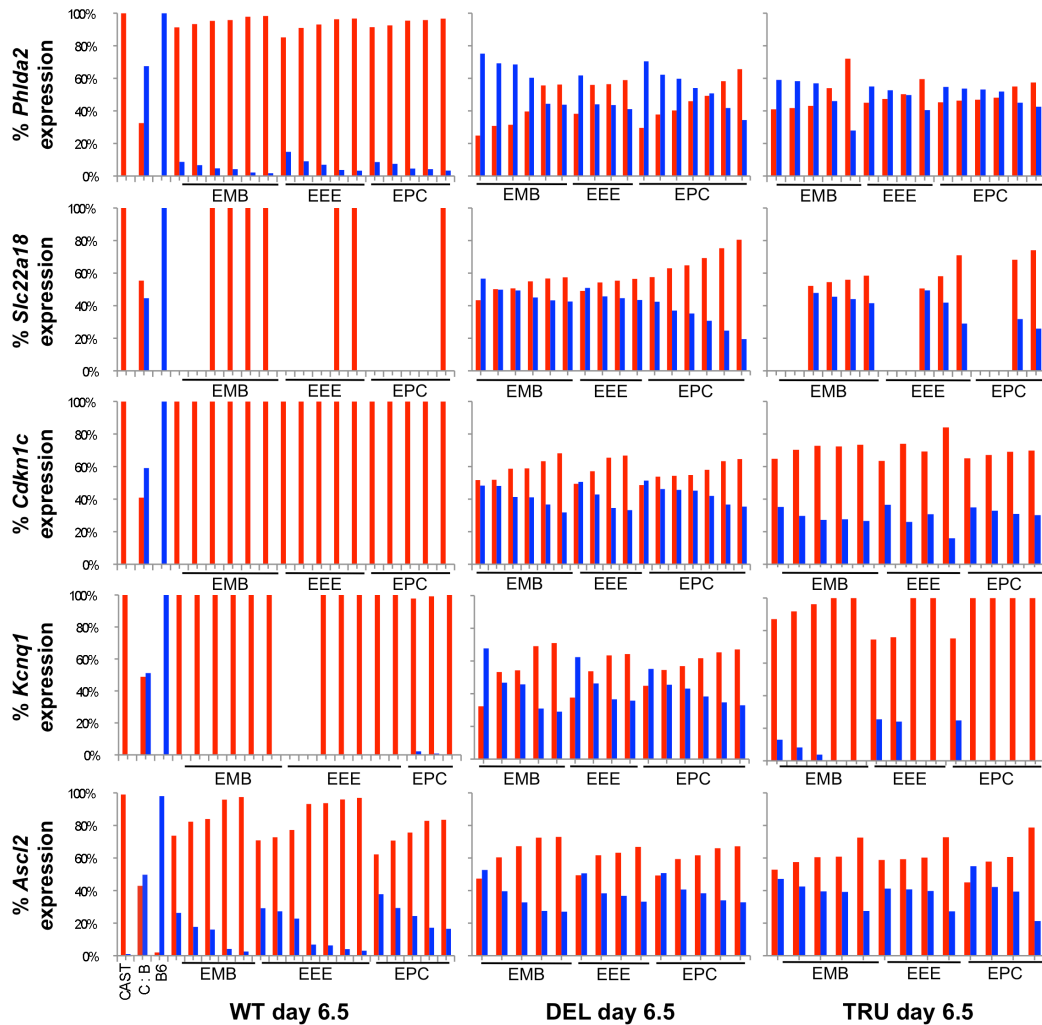


Figure 18: Gene-specific and Tissue-specific Reactivation of Paternal Alleles in Day 6.5 *Kcnq1ot1* Paternally ICR Deleted or ncRNA Truncated Embryos

Phlda2, *Slc22a18*, *Cdkn1c*, *Kcnq1* and *Ascl2* allelic expression was analyzed in individual day 6.5 embryonic ectoderm (EMB), extraembryonic ectoderm (EEE) and ectoplacental cone (EPC) having either a paternal wild-type (WT), *Kcnq1ot1* ICR deleted (DEL) or *Kcnq1ot1* ncRNA truncated (TRU) alleles.

Table 4: Maternal Expression in Day 6.5 WT, Paternal *Kcnq1ot1* ICR Deleted and *Kcnq1ot1* Truncated Embryos

Embryo	Tissue	<i>Phlda2</i>	<i>Slc22a18</i>	<i>Cdkn1c</i>	<i>Kcnq1</i>	<i>Ascl2</i>
WT	EMB	95%	100%	100%	100%	87%
	EEE	94%	100%	100%	100%	91%
	EPC	94%	100%	100%	99%	75%
<i>Kcnq1ot1</i> DEL	EMB	40%	51%	59%	56%	64%
	EEE	52%	54%	60%	55%	60%
	EPC	52%	68%	57%	58%	61%
<i>Kcnq1ot1</i> TRU	EMB	50%	55%	71%	95%	61%
	EEE	51%	60%	73%	88%	63%
	EPC	50%	71%	68%	95%	61%

Red, maternal-specific expression (>90%); light red, maternal-biased expression (65-89%); white, biallelic expression (<64%).

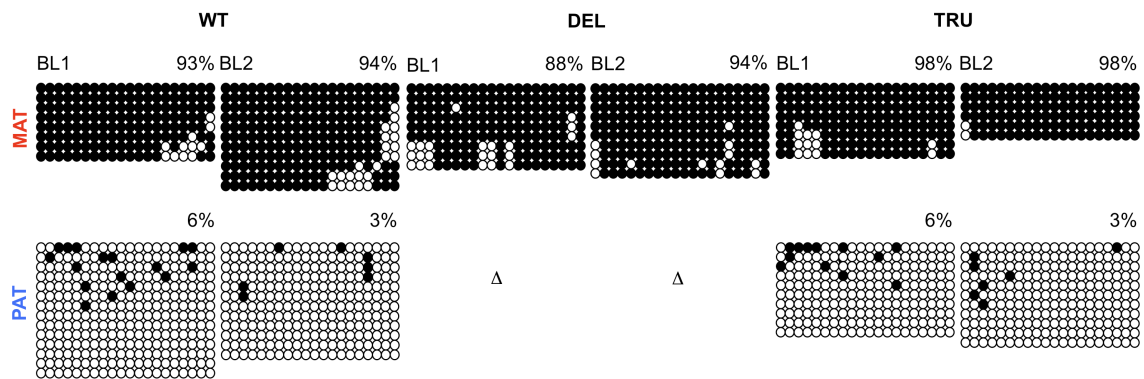


Figure 19: Maintenance of Maternal *Kcnq1ot1* ICR Methylation in *Kcnq1ot1* Paternally Deleted and Truncated Blastocysts

DNA methylation within the *Kcnq1ot1* ICR in blastocysts with paternal wildtype (WT), deleted (DEL) or truncated (TRU) *Kcnq1ot1* alleles. Two blastocysts (BL) were analyzed for each genotype. Each column represents an individual embryo. Each row represents a DNA strand with methylated CpGs (black circles) or unmethylated CpGs (white circles). Maternal allele (MAT) and paternal allele (PAT) with percent methylation shown at the top.

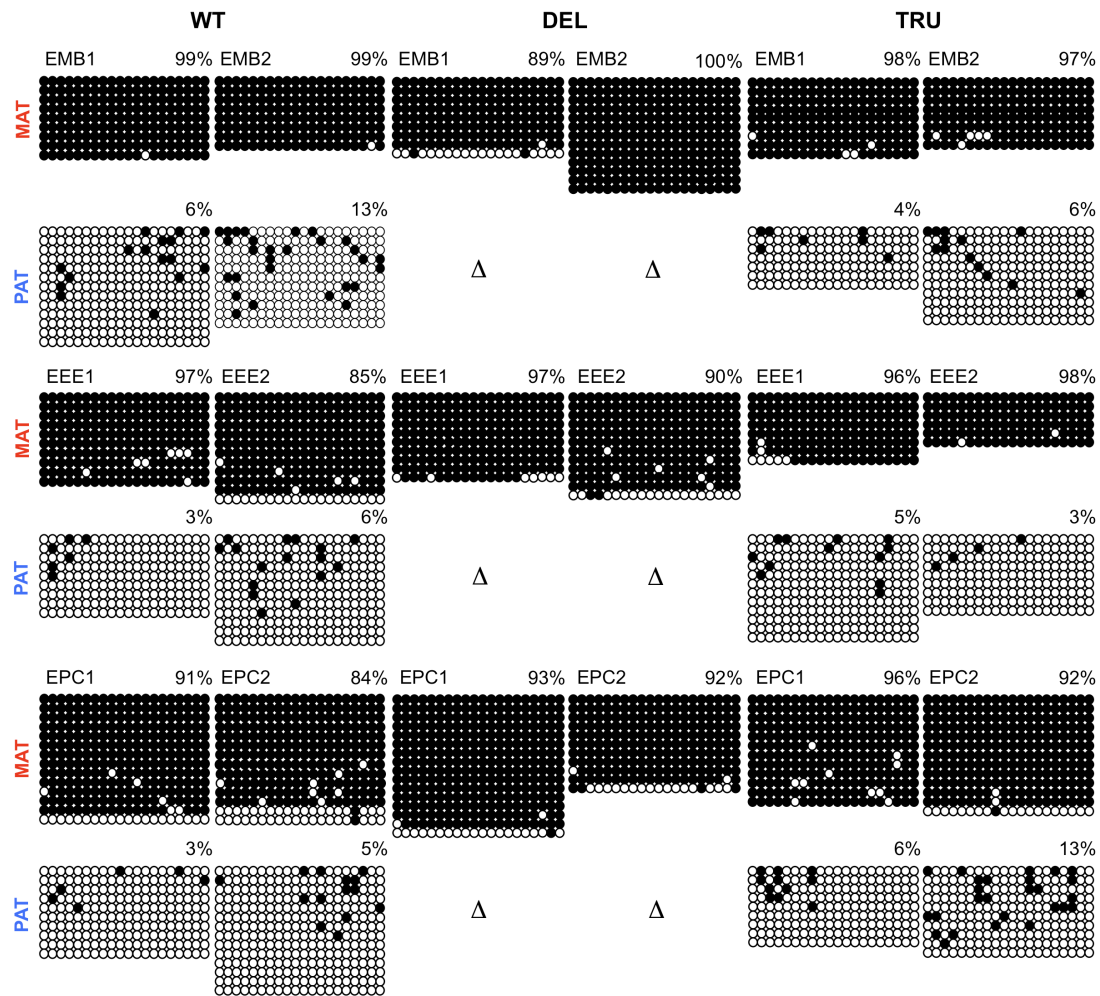


Figure 20: Maintenance of Maternal *Kcnq1ot1* ICR Methylation in *Kcnq1ot1* Paternally Deleted and Truncated in Day 6.5 Embryos

DNA methylation within the *Kcnq1ot1* ICR in day 6.5 embryonic ectoderm (EMB), extraembryonic ectoderm (EEE) and ectoplacental cone (EPC) with a paternal wild-type (WT), deleted (DEL) or truncated (TRU) *Kcnq1ot1* alleles. Two sets of tissue were analyzed per genotype. Each column represents a tissue from an individual embryo. Each line represents a DNA strand with methylated CpGs (black circles) or unmethylated CpGs (white circles). Maternal allele (MAT) and paternal allele (PAT) with percent methylation shown at the top.

3.5 Identification of Epigenetic Modifiers Controlling Paternal *Cdkn1c*^{+/ Δ neo} Allelic Silencing

As discussed in the introduction to this thesis, mechanisms controlling paternal allelic silencing of imprinted genes at the *Kcnq1ot1* domain remain unclear and several models to explain *Kcnq1ot1* domain regulation have been proposed. To date, only a few repressive factors have been linked to *Kcnq1ot1* domain silencing. Therefore, an independent characterization of the factors mediating paternal silencing is required. To determine the epigenetic modifiers involved in paternal allelic silencing at the *Kcnq1ot1* domain, a positive selection, loss of function screen was performed using RNA interference (RNAi) and a library of short hairpin RNAs (shRNAs) for 250 epigenetic factors, with ~three hairpins per factor. Extra-embryonic endoderm (XEN) cells carrying a paternal targeted mutation of the *Cdkn1c* gene where exons 1 and 2 were replaced by the PGK-Neomycin resistance cassette (*Cdkn1c*^{+/ Δ neo}) were used to select for loss of silencing (Figure 21). The initial plan had been to screen all three stem cell lineages from the early embryo. However, only XEN cells showed repression of paternally inherited *Cdkn1c*^{+/ Δ neo}, while *Cdkn1c*^{+/ Δ neo} silencing had not yet been achieved in embryonic stem and trophoctoderm stem cells (Figure 22). Reactivation of the silent *Cdkn1c*^{+/ Δ neo} allele following depletion of repressive mediators allowed for survival and selection of colonies in the presence of neomycin (Figure 23). The epigenetic library was divided into four pools, allowing for four independent rounds of the screen. In total, 584 individual colonies were picked for a second round of neomycin selection following which 192 individual colonies were selected and DNA was sequenced to identify shRNA targeted factors controlling paternal *Neo*^R repression. In total, 34 different epigenetic modifiers were identified. Ranking of top candidates was determined by: (1) the number of colonies recovered, (2) the number of different hairpins recovered, and (3) the number of pools from which the candidate was recovered (Table 5). The top 10 candidates were Enhancer of zeste homolog 1 (*Ezh1*), Histone deacetylase 9 (*Hdac9*), Lysine (K)-specific demethylase 4A (*Kdm4a*), Nucleoporin 107 (*Nup107*), SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1 (*Smarcad1*), SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, member 5 (*Smarca5*), SWI/SNF-related, matrix-associated actin-

dependent regulator of chromatin, subfamily e, member 1 (*Smarce1*), SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily c, member 2 (*Smarcc2*), Suppressor of variegation 4-20 homolog 1 (*Suv420h1*), TAF6-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor (*Taf6l*) (Table 6). Candidates *Smarcc2*, *Hdac9*, *Smarcad1* and *Smarce1* were isolated from multiple pools with reactivation of the paternal *Cdkn1c*^{+/ Δ neo} allele occurring with multiple shRNAs. *Ezh1*, *Nup107*, *Taf6l*, *Kdm4a* and *Suv420h1* were isolated from one pool and one shRNA elicited paternal *Cdkn1c*^{+/ Δ neo} allelic reactivation. Note that the shRNA for *Nup107* was not intended to be in the epigenetic shRNA library, but was inadvertently included when shRNAs were isolated from the whole genome shRNA library. The highest number of colonies recovered was for *Ezh1*. Interestingly, the list of candidates was composed of both repressive and activating epigenetic factors, which is a novel finding since the few factors (EZH2, RNF2, SUV420H1 and DNMT1) that were known to regulate imprinting act to repress chromatin (Mohammad et al., 2010; Pandey et al., 2008; Pannetier et al., 2008; Terranova et al., 2008). Some of these epigenetic factors were identified in this screen: *Ezh2*, *Rnf2*, *Suv420h1* and *Dnmt1*. However, they were recovered at a lower frequency than the other epigenetic candidates. This attests to the validity of this study's methods.

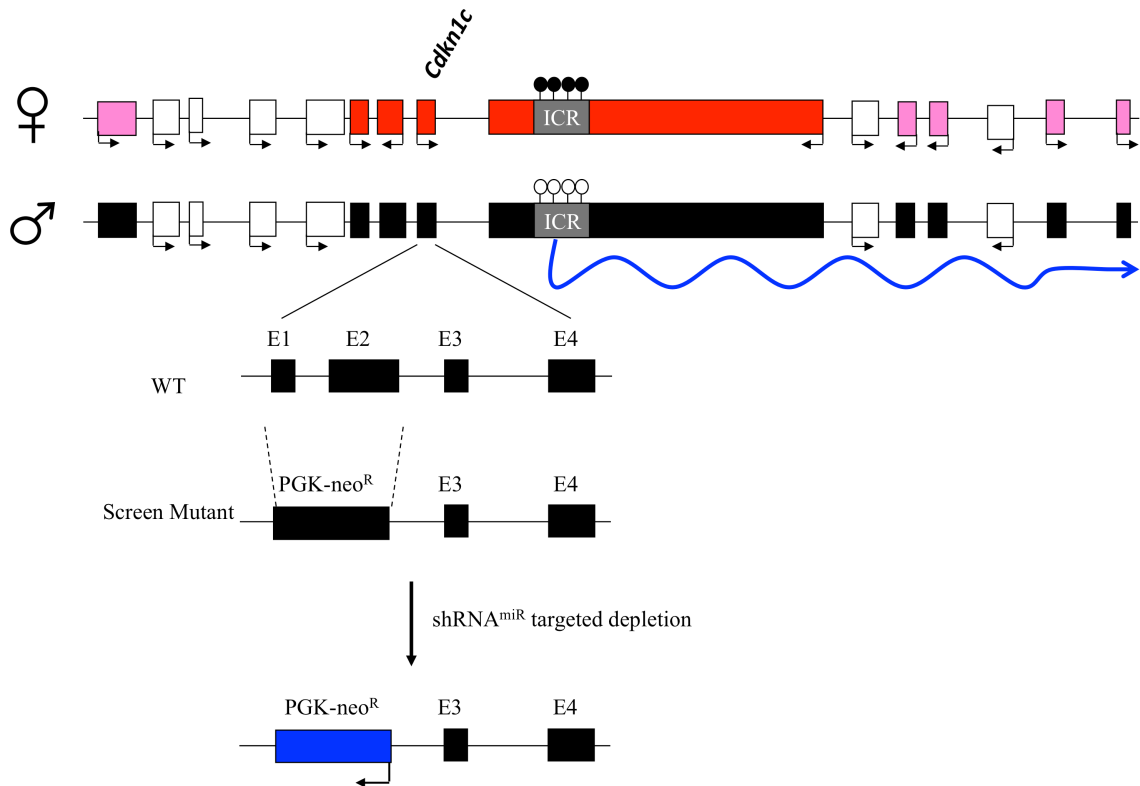


Figure 21: *Neo*^R Gene Recombined at *Cdkn1c* is Silent on the Paternal Allele at the *Kcnq1ot1* Domain

Gene targeting of *Cdkn1c* deleted the first two exons and replaced it with the PGK-driven *Neomycin Resistance* (*Cdkn1c*^{+/ Δ neo}) cassette. Like *Cdkn1c*, paternal inheritance of *Cdkn1c*^{+/ Δ neo} allele resulted in gene silencing. Targeted depletion of epigenetic regulators by shRNAs resulted in reactivation of *Cdkn1c*^{+/ Δ neo}, allowing for positive selection of colonies.

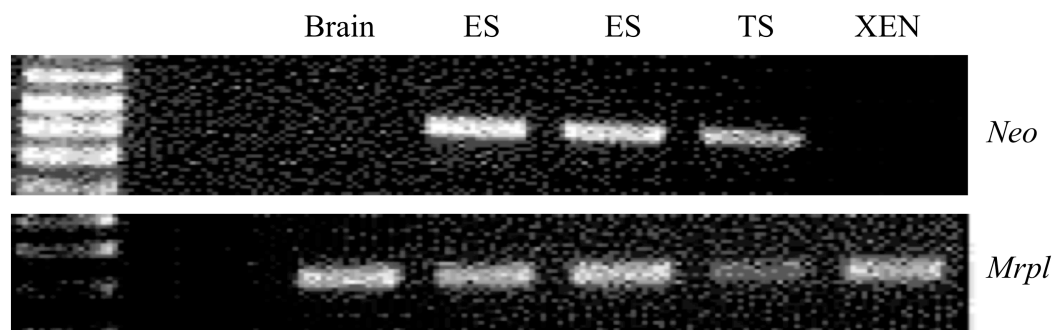


Figure 22: Neomycin Expression in WT Brain and in *Cdkn1c*^{+/ Δ neo} ES, TS and XEN cells.

Neomycin (*Neo*) expression was normalized to mitochondrial ribosomal protein L1 (*Mrpl*). Paternal silencing of *Cdkn1c*^{+/ Δ neo} was only observed in XEN cells. WT, wildtype; ES, embryonic stem cells; TS, trophoblast stem cells; XEN, extra-embryonic endoderm stem cells.

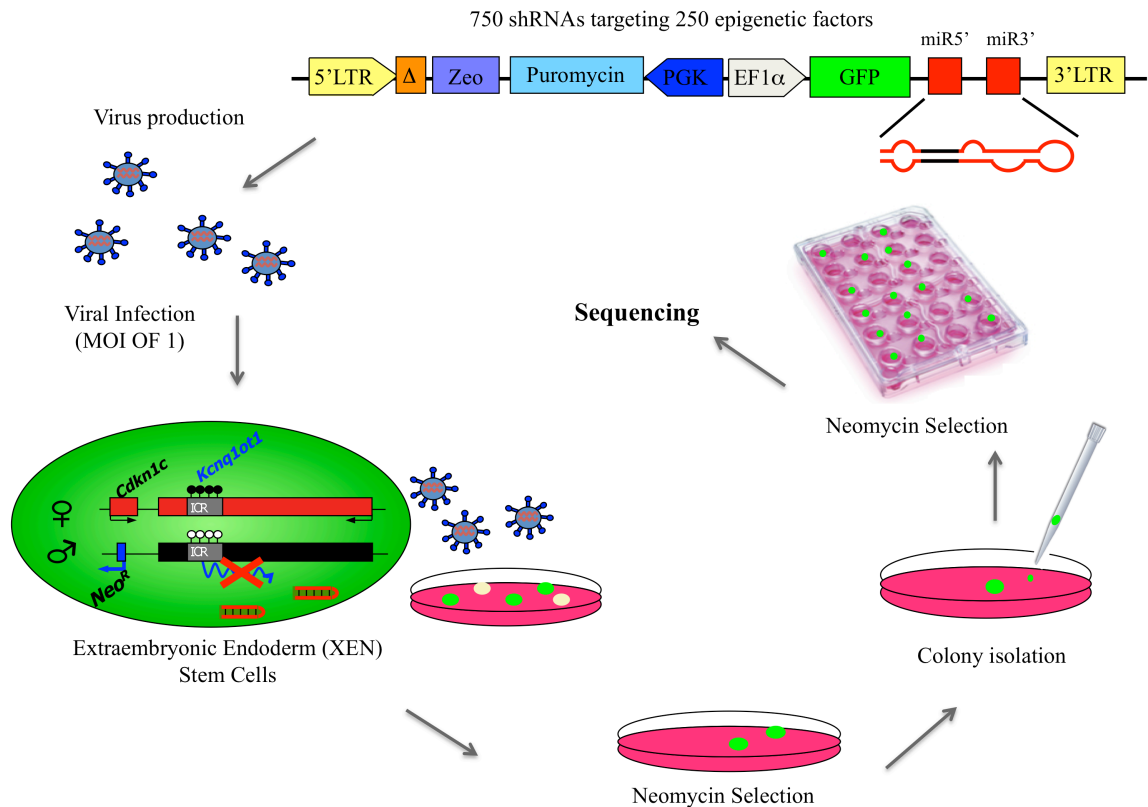


Figure 23: Screen for Epigenetic Modifiers of Genomic Imprinting at the *Kcnq1ot1* Domain

Lentivirus infection of extraembryonic endoderm (XEN) cells stably integrated the shRNA expressing construct in XEN cells with the paternal *Cdkn1c*^{+/ Δ neo} allele. RNA depletion of epigenetic regulators of imprinting caused expression of the paternally silenced *Cdkn1c*^{+/ Δ neo}, allowing selection of cells that had lost *Cdkn1c*^{+/ Δ neo} silencing by adding neomycin to the culture media. Following the first round of neomycin selection, colonies of cells were picked and expanded in a 24-well dish. Following a second-round of neomycin selection, candidate factors were identified by DNA sequencing.

Table 5: Candidate Epigenetic Modifiers of the *Kcnq1ot1* Domain

Candidate	Number of shRNAs	Total Colonies	Total Pools
<i>Ezh1</i>	1	21	1
<i>Nup107</i>	1	9	1
<i>Smarca5</i>	2	8	2
<i>Smarce1</i>	2	8	2
<i>Taf6l</i>	1	8	1
<i>Smarcc2</i>	4	7	4
<i>Kdm4</i>	1	7	1
<i>Smarcad1</i>	3	6	3
<i>Hdac9</i>	3	6	2
<i>Suv4-20h1*</i>	1	6	1
<i>Mbd4</i>	1	6	1
<i>Hdac1</i>	1	5	1
<i>Sin3a</i>	2	4	2
<i>Dnmt3a</i>	2	4	1
<i>Smyd3</i>	1	4	1
<i>Sirt1</i>	3	3	3
<i>Dnmt1*</i>	2	2	2
<i>Hdac2</i>	2	2	1
<i>Rnf2*</i>	2	2	1
<i>Smardc2</i>	2	2	1
<i>Gcn512</i>	1	2	1
<i>Hdac8</i>	1	2	1
<i>Rnf134</i>	1	2	1
<i>Taf5l</i>	1	1	1
<i>Phc3</i>	1	1	1
<i>Pcaf</i>	1	1	1
<i>Ep300</i>	1	1	1
<i>Ezh2*</i>	1	1	1
<i>Mill4</i>	1	1	1
<i>Myst2</i>	1	1	1
<i>Hdac5</i>	1	1	1
<i>Hdac7</i>	1	1	1
<i>Mbd3</i>	1	1	1
<i>Smarca1</i>	1	1	1
<i>Smarca3</i>	1	1	1

* Previous association with the *Kcnq1ot1* domain

Table 6: Top 10 Candidate Epigenetic Modifiers of the *Kcnq1ot1* Domain

Top Candidates		Number of shRNAs	Total Colonies	Total Pools	Putative Function	
<i>Ezh1</i>	Enhancer of zeste homolog 1	1	21	1	Repressive	
<i>Nup107</i>	Nucleoporin 107	1	9	1	Structural	
<i>Smarca5</i>	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	2	8	2	Activating	Repressive
<i>Smarca1</i>	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1	2	8	2	Activating	Repressive
<i>Taf6L</i>	TAF6-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor	1	8	1	Activating	
<i>Kdm4</i>	Lysine (K)-specific demethylase 4A	1	7	1	Activating	
<i>Smarca2</i>	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	4	7	4	Activating	Repressive
<i>Smarca1</i>	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1	3	6	3	Activating	Repressive
<i>Hdac9</i>	Histone deacetylase 9	3	6	2	Repressive	
<i>Suv4-20H1</i>	Suppressor of variegation 4-20 homolog 1	1	6	1	Repressive	

3.6 Loss of Imprinted Expression at Specific Genes Within the *Kcnq1ot1* Domain Following Candidate Depletion

Isolation of screen candidates was based on reactivation of the paternally inherited *Cdkn1c*^{+/ Δ neo} gene. Therefore, candidates may play a role in paternal allelic silencing, locally (based on DNA elements residing at *Cdkn1c*) or may act domain-wide. Three candidates were chosen for analysis based on the number of colonies recovered (*Ezh1*; 21 colonies), number of distinct shRNAs (*Smarcad1*, 3 shRNAs) and a combination of both (*Smarca5*, 8 colonies, 2 shRNAs). EZH1 is a noncanonical catalytic subunit of PRC2 (Shen et al., 2008). Its homologue EZH2 has been previously linked to the *Kcnq1ot1* domain, associating directly with the *Kcnq1ot1* RNA and catalyzes the H3K27me3 repressive mark to silent genes in ES cells (Pandey et al., 2008; Terranova et al., 2008). A large proportion of the top candidates were ATP-chromatin remodelers, albeit from different families of complexes. SMARCA5 is an ATPase in the SNF2-like family and SMARCAD1 is an ATPase of the SWR1-like family (Flaus et al., 2006).

To determine the candidates' role in paternal allelic silencing, wildtype B6 X CAST XEN cells were transfected with short-interfering (siRNAs) targeting candidates *Ezh1*, *Smarca5* and *Smarcad1*, as well as a non-targeting (NT) siRNA. Candidate depletion levels were measured by qRT-PCR (Figure 24). Compared to WT and NT siRNA controls, candidates were depleted to 62.4% for *Ezh1*; 9.4% for *Smarca5*; and 26.9% for *Smarcad1*.

To determine the role of the screen candidates in paternal *Cdkn1c* silencing, WT, NT siRNA, *Ezh1* siRNA, *Smarca5* siRNA, and *Smarcad1* siRNA-depleted XEN cells were assessed for *Cdkn1c* allelic expression. Maternal-specific expression was defined as 90-100% expression from the maternal allele, with less than 10% expression from the paternal allele. In contrast to WT and NT siRNA-transfected cells, which displayed paternal *Cdkn1c* silencing, the paternal *Cdkn1c* was reactivated when *Ezh1*, *Smarca5*, and *Smarcad1* were depleted, with 39%, 26% and 48% expression from the paternal allele, respectively (Figure 25). Thus, paternal reactivation of *Cdkn1c* in all candidate-depleted cells demonstrates that candidates identified here function in paternal allelic silencing.

To assess whether candidates play a role in domain-wide paternal allelic silencing or specifically at *Cdkn1c*, genes downstream (*Kcnq1*) and upstream (*Osbp15*, *Slc22a18*) of the *Kcnq1ot1* ncRNA promoter were examined for allelic expression. The downstream *Ascl2* gene and the upstream *Phlda2* gene possessed biallelic-expression in XEN cells and thus were not included in the analysis (Golding et al., 2011). For the downstream *Kcnq1* gene, paternal silencing was lost in *Ezh1*-, *Smarca5*- and *Smarcad1*-depleted XEN cells (41%, 38% and 36% paternal expression, respectively) (Figure 25). Upstream gene *Osbp15* had maternal-specific expression in wildtype XEN cells. Maternal-biased expression was observed in candidate-depleted samples and not different from non-targeting transfected cells, suggesting candidates are not acting to silence the paternal allele of this gene (Figure 25). For *Slc22a18*, NT cells maintained paternal allelic repression, as did *Smarcad1*-depleted cells (0% paternal expression), while the paternal *Slc22a18* allele was slightly reactivated in *Ezh1*- and *Smarca5*-depleted XEN cells (13% and 17% paternal expression, respectively) (Figure 25). All together, this indicates that candidate factors do not act domain-wide to control paternal allelic silencing, but instead function to regulate paternal silencing at proximal rather than distal genes.

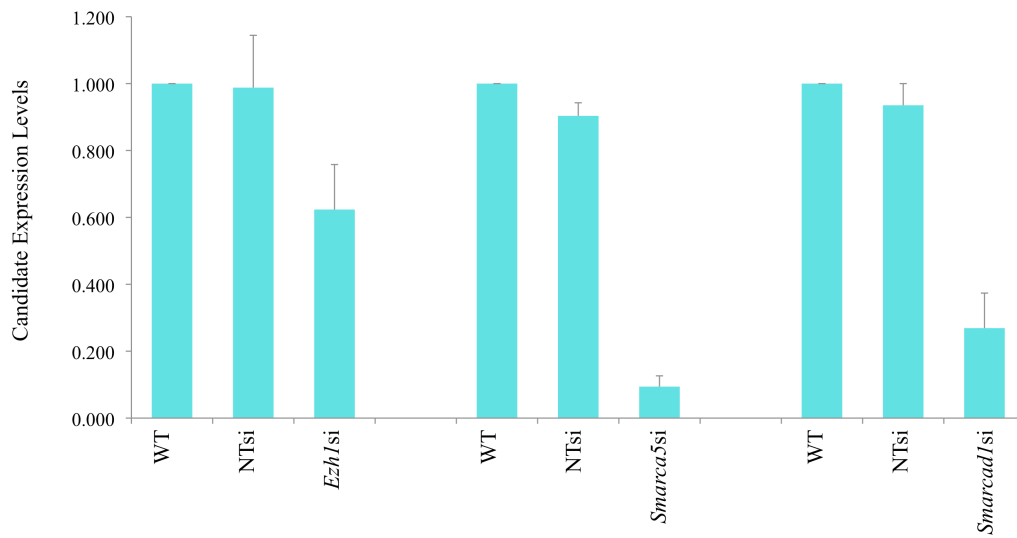


Figure 24: Candidate Depletion Levels in siRNA-transfected XEN Cells

Candidate expression was analyzed in wildtype (WT), non-targeting (NT), *Ezh1*, *Smarca5*, and *Smarcd1* siRNA-targeted XEN cells. Expression levels were compared to wildtype and averaged over multiple independent transfections (N=2 to 4) with each PCR done in duplicate replicates. Error bars indicate standard error of the mean.

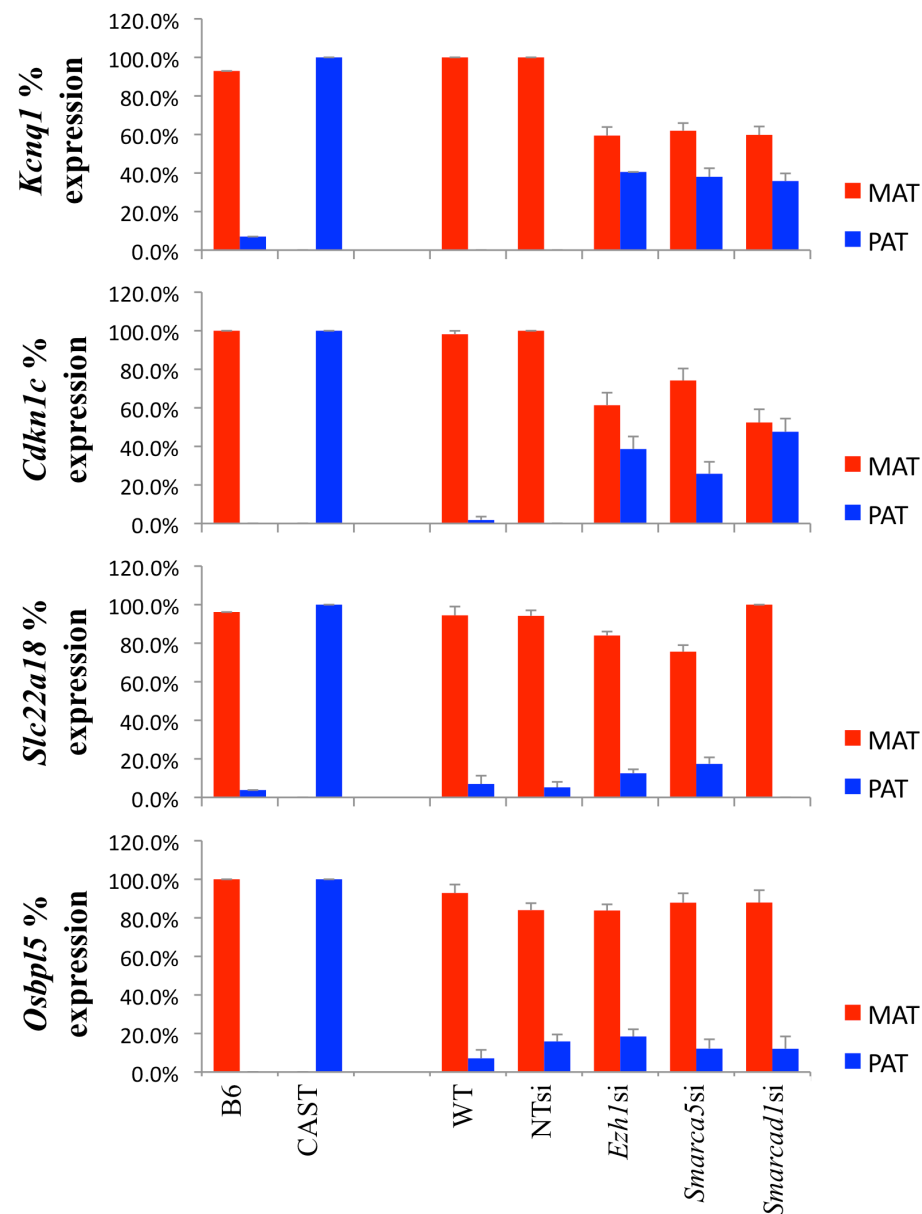


Figure 25: Imprinted Expression Analysis in Control and Candidate Deleted XEN Cells.

Allelic-expression of *Kcnq1*, *Cdkn1c*, *Slc22a18*, and *Osbp15* was analyzed in wildtype (WT), non-targeting (NT), *Ezh1*, *Smarca5* and *Smarcd1* siRNA-targeted cells. Percent maternal expression (red bar) and paternal expression (blue bar) was calculated from the total expression of both alleles averaged over multiple independent transfections (N=2 to 4). Error bars indicate standard error of the mean.

3.7 Reduced *Kcnq1ot1* Transcription Following Candidate Depletion

As described above, a novel observation for the RNA interference screen was that both repressive and active epigenetic factors were recovered. This includes SMARCA5 and SMARCA1 that have documented activating functions (Dluhosova et al., 2014; Hong et al., 2009). To determine whether the candidates play a role in maintaining *Kcnq1ot1* in an active chromatin configuration, total *Kcnq1ot1* transcript abundance was measured using quantitative real-time PCR in both control and siRNA-depleted XEN cells. Compared to wildtype and non-targeting siRNA-transfected XEN cells, *Kcnq1ot1* transcript levels were reduced when *Smarca5* (60.4%) and *Smarcad1* (55%) were depleted (Figure 26). Thus, loss of paternal allelic repression and reduced *Kcnq1ot1* expression suggests that SMARCA5 and SMARCA1 could be silencing the paternal allele by activating *Kcnq1ot1* transcription or maintaining the *Kcnq1ot1* ICR in an active configuration. Interestingly, *Kcnq1ot1* expression remained unchanged when *Ezh1* (103.4%) was depleted.

3.8 Maintenance of DNA Methylation Following Candidate Depletion

Reduced *Kcnq1ot1* expression in *Smarca5*- and *Smarcad1*-depleted XEN cells suggested that the *Kcnq1ot1* ncRNA is being silenced in the absence of the candidates. Aberrant gain of paternal ICR methylation could explain the reduced transcript abundance of the *Kcnq1ot1* ncRNA. To examine this, DNA methylation at the paternal ICR was analyzed in wildtype, non-targeting, *Ezh1*-, *Smarca5*- and *Smarcad1*-depleted cells using the bisulfite mutagenesis and clonal sequencing assay. In wildtype samples, the maternal ICR was hypermethylated while the paternal ICR was hypomethylated (Figure 27). Similarly, hypomethylation was observed on the paternal allele for depleted candidates, suggesting that changes in *Kcnq1ot1* expression or ICR function were not altered due to gain of paternal methylation.

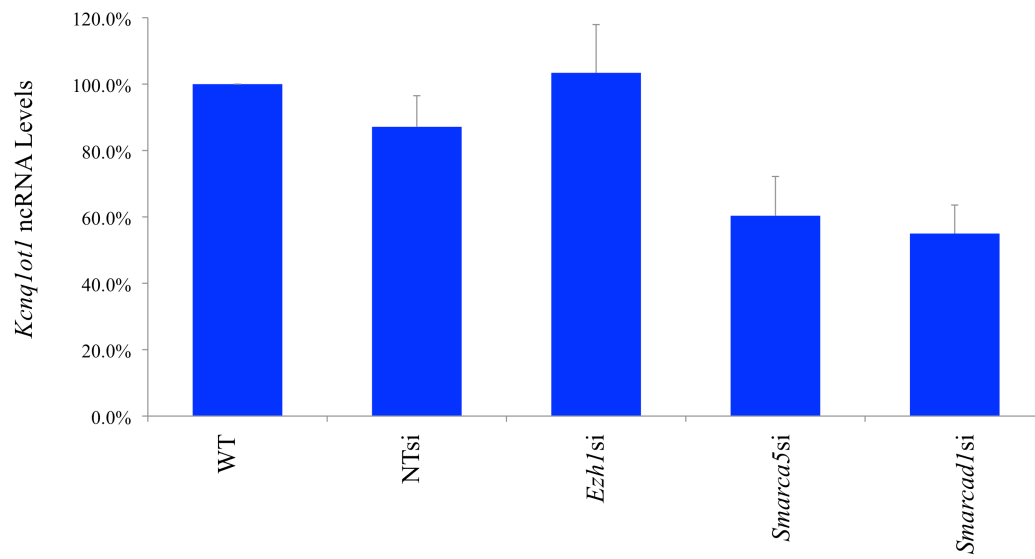


Figure 26: Reduced *Kcnq1ot1* Transcript Abundance in Specific Candidate-depleted XEN cells.

Kcnq1ot1 transcript abundance was analyzed in wildtype (WT), non-targeting (NT), *Ezh1*, *Smarca5* and *Smarcd1* siRNA-targeted XEN cells. Percent *Kcnq1ot1* transcript levels were averaged over multiple independent transfections (N=2 to 4) with each PCR done in duplicate replicates. Error bars indicate standard error of the mean.

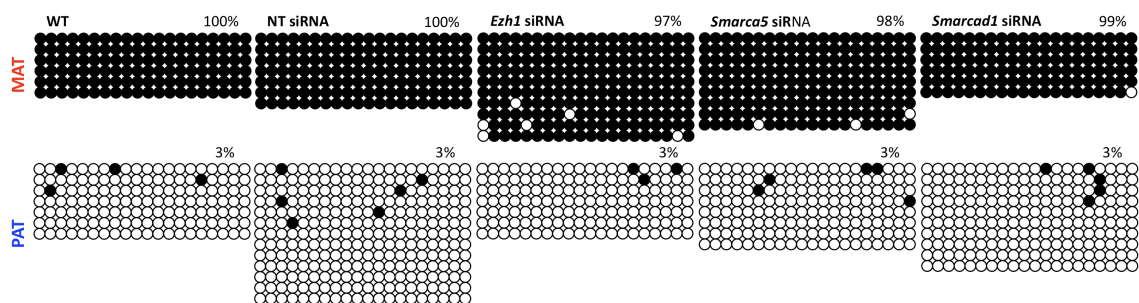


Figure 27: Maintenance of Maternal *Kcnq1ot1* ICR methylation in Candidate-depleted XEN Cells

DNA methylation was examined within the ICR of wildtype (WT), non-targeting (NT), *Ezh1*, *Smarca5* and *Smarca1* siRNA-targeted cells. Each line represents a DNA strand with methylated CpGs (black circles) or unmethylated CpGs (white circles). Maternal allele (MAT) and paternal allele (PAT) with percent methylation shown at the top.

Chapter 4

4 Discussion

4.1 *Kcnq1ot1* ICR and its ncRNA are Dispensable for Establishment of Paternal Silencing During Preimplantation Development

The *Kcnq1ot1* ICR possesses maternal DNA methylated and paternal unmethylated gametic marks. In the zygote, these gametic marks, together with the *Kcnq1ot1* ncRNA, were thought to function to establish *Kcnq1ot1* domain imprinting. The importance of *Kcnq1ot1* ICR and ncRNA in repressing adjacent protein-coding genes on the paternal allele was delineated from *Kcnq1ot1* ICR deletion or ncRNA truncation studies which resulted in loss of imprinting during postimplantation development (Fitzpatrick et al., 2002; Mancini-Dinardo et al., 2006; Shin et al., 2008). The caveat of those studies was that analysis of gene expression was performed during midgestation when domain imprinting had long been established and thus, would not be able to distinguish between failure to establish or failure to maintain *Kcnq1ot1* domain imprinting. Here, I set out to more precisely determine the timing of paternal allelic silencing of genes within the *Kcnq1ot1* domain and whether the *Kcnq1ot1* ICR and/or ncRNA were required for imprinted domain establishment or maintenance. In WT embryos, I observed that paternal *Phlda2*, *Slc22a18* and *Cdkn1c* alleles were becoming progressively silenced during preimplantation development. Significantly, I found that blastocyst-stage embryos with paternal *Kcnq1ot1* ICR deleted or *Kcnq1ot1* truncated allele underwent paternal silencing of the *Phlda2*, *Slc22a18* and *Cdkn1c* genes. In contrast, day 6.5 embryos carrying a paternally deleted *Kcnq1ot1* allele, the *Kcnq1ot1* ICR was necessary for maintenance of paternal repression at *Phlda2*, *Slc22a18*, *Cdkn1c*, *Kcnq1* and *Ascl2*. By comparison, truncation of the *Kcnq1ot1* ncRNA resulted in paternal reactivation of distal genes within the domain, *Phlda2*, *Slc22a18* and *Ascl2*, while genes more proximal to the ICR, *Kcnq1* and *Cdkn1c*, maintained maternal-specific or maternal-biased expression. The data indicates that the *Kcnq1ot1* ICR and ncRNA were dispensable for establishment of paternal allele silencing during preimplantation

development. During early postimplantation development, the *Kcnq1ot1* ICR and *Kcnq1ot1* ncRNA function differently, with the *Kcnq1ot1* ICR required for imprint maintenance at all imprinted genes while the *Kcnq1ot1* ncRNA was required for maintenance of paternal repression at only a subset of genes. Together, this indicates that the *Kcnq1ot1* ncRNA does not play a role in regulating genes domain-wide or genes specifically upstream and downstream of the *Kcnq1ot1* ICR/promoter. This maintenance of *Cdkn1c* and *Kcnq1* imprinted expression in truncation mutants can be considered transient since all genes show paternal reactivation by day 13.5-16.5 in a C57BL/6 background, while on a 129 background, *Cdkn1c* remains paternally repressed (Fitzpatrick et al., 2002; Mancini-Dinardo et al., 2006; Shin et al., 2008).

Maternal-biased expression of *Cdkn1c* in WT blastocyst was previously reported by our group as well as Terranova et al., who also reported maternal-biased expression of *Tssc4* and *Cd81* (Market Velker et al., 2012; Terranova et al., 2008). In contrast, Umlauf et al. and Lewis et al. observed maternal-specific *Kcnq1* and *Cdkn1c* expression (Lewis et al., 2006; Umlauf et al., 2004). This difference may relate to the use of a 100 pooled embryos or timing of embryo recovery (Lewis et al., 2006; Terranova et al., 2008). Analysis of imprinted-expression using individual preimplantation embryos rather than pooled embryos has been performed in previous studies for multiple imprinted genes, including *H19*, *Snrpn*, *Igf2r*, *Ascl2*, and *Cdkn1c* (Mann et al., 2003; Mann et al., 2004; Market Velker et al., 2012). I observed that *Cdkn1c* underwent completion of paternal silencing to be maternal-specific by day 6.5, consistent with previous data (Terranova et al., 2008). This switch to maternal-specific expression following implantation also occurred for *Cd81* and *Tssc4* by day 6.5 extra-embryonic ectoderm (Terranova et al., 2008). Developmental imprinted domain establishment also occurred at the *H19* domain. The neighbouring protein coding gene, *Igf2*, was biallelically expressed in blastocysts and was undergoing maternal allelic silencing at day 6.5 and possessed paternal-specific expression at day 9.5 (Thorvaldsen et al., 2006). Imprinted XCI also undergoes developmental silencing with paternal repression of protein-coding genes beginning in 8-16-cell stage embryos (Chaumeil et al., 2006; Kalantry et al., 2009). Interestingly, *Xist* is not required for imprinted XCI but is required by day 6.5 for maintenance of paternal silencing (Kalantry et al., 2009). Together, this suggests parental-specific expression of

imprinted genes is initiated at an early developmental stage and has been shown to occur in the absence of the *Kcnq1ot1* ICR and the *Kcnq1ot1* and *Xist* ncRNAs.

Since mutant embryos were capable of invoking paternal allelic silencing, indicating that the *Kcnq1ot1* ICR and ncRNA were dispensable for imprinted domain establishment, it invokes the question of what is directing the preimplantation silencing of paternal alleles of imprinted genes. One possibility is that gene-specific epigenetic marks, other than DNA methylation at the *Kcnq1ot1* ICR, are derived from the gametes. During spermatogenesis, histones are replaced by protamines with the exception of sperm-specific, developmental and imprinted genes as well as repetitive elements and unmethylated CpG-rich regions where histones are retained (Erkek et al., 2013; Hammoud et al., 2009). This includes the mouse/human *Kcnq1ot1/KCNQ1OT1* domain (Erkek et al., 2013; Hammoud et al., 2009). Mining of published data from mouse and human sperm revealed the presence of H3K27me3 (with or without H3K4me3) at *Phlda2/PHLDA2*, *Slc22a18/SLC22A18*, *Cdkn1c/CDKN1C*, *Kcnq1/KCNQ1* and *Ascl2/ASCL2*. However, these marks were absent at nearby non-imprinted genes, *Nap114/NAP1L4* and *Cars/CARS* (Erkek et al., 2013; Hammoud et al., 2009). Additionally, in the mouse, H3K27me3 foci were detected at the paternal *Kcnq1ot1* domain by immunofluorescence in zygotic through to blastocyst stage embryos, indicating retention of this repressive histone mark (Terranova et al., 2008). Repressive histone modifications retained at imprinted genes in sperm could explain why silencing occurs independently of the *Kcnq1ot1* ICR, suggesting that imprinted genes within a domain may harbour their own gametic mark contrary to current opinion that the ICR solely possesses gametic epigenetic modifications.

Intragenic regions within the *Kcnq1* gene, but outside of the deleted ICR, may also play a role in paternal allelic silencing of imprinted genes. Interestingly, mining of whole-genome bisulfite sequencing data revealed a germline, differentially methylated region within *Kcnq1* intron 2. In sperm, this CpG-rich region is unmethylated, in germinal vesicle oocytes it is 100% methylated, while in blastocysts its methylation level (24.5%) is similar to other imprinted control regions, indicating that differentiation methylation is likely preserved after fertilization (Kobayashi et al., 2012). This CpG-rich

region continues to maintain intermediate methylation levels (31%-61%) in day 14.5 placenta and adult tissues, which is suggestive of an important allelic role (Hon et al., 2013).

Another possibility is that paternal repression of protein-coding genes results from insulator or silencing elements with the domain. A cluster of matrix attachment regions (MARs) is situated just downstream of the *Kcnq1ot1* ICR outside of the deleted region (Purbowasito et al., 2004). MARs are AT-rich sequences involved in chromatin-looping, boundary formation and transcriptional regulation (Purbowasito et al., 2004; Tattermusch and Brockdorff, 2011). At the *H19* domain, on the maternal allele, a MAR associates with the differentially methylation region 1 upstream of *Igf2* together with the CTCF insulator protein, forming a chromatin loop around *Igf2*, that is suggested to contribute to its silencing (Kurukuti et al., 2006; Weber et al., 2003). Furthermore, MAR association within the *Gtl2* domain is altered when the maternal *Gtl2* ICR is deleted, resulting in loss of maternal *Gtl2* silencing (Braem et al., 2008). Similar to the *H19* and *Gtl2* domains, MARs within the *Kcnq1ot1* domain may mediate the generation of repressive chromatin loops that induce paternal allelic silencing of imprinted genes within the domain. Interestingly, ectopic expression of a MARs-binding protein Special AT-rich sequence binding protein 1 (SATB1) in lymphoma and embryonic stem cells, which do not normally undergo X-chromosome inactivation (even though *Xist* is expressed), initiates *Xist*-induced gene silencing and chromosome repression (Agrelo et al., 2009). Similarly, SATB1 or another MARs-binding protein may bind to MARs within the *Kcnq1ot1* domain, facilitating paternal allelic repression without the *Kcnq1ot1* ICR or ncRNA.

Numerous MARs and repetitive elements are dispersed throughout the *Kcnq1ot1* domain. The cluster of MARs closest to the *Kcnq1ot1* ICR (described above) contains some of the highest content of interspersed repeats, including LINEs, within the domain (Purbowasito et al., 2004). On the X chromosome, silencing of LINE1 elements occurs by the 2-cell stage, forming a silent compartment into which protein-coding genes are later recruited and repressed at the morula stage (Chow et al., 2010; Namekawa et al., 2010). If formation of a repressive compartment similarly takes place at the *Kcnq1ot1* domain, active marks would be excluded from the domain at the time of silencing.

Indeed, RNA polymerase II and H3K4me3 were absent at the paternal *Kcnq1ot1* domain in outer cells of the blastocyst (Terranova et al., 2008). Thus, MARs, repeat sequences and repressive modifications may act in concert to bring about the preimplantation paternal silencing of genes within the *Kcnq1ot1* domain.

My data suggests that the *Kcnq1ot1* ICR and *Kcnq1ot1* ncRNA function differently to regulate maintenance of imprinted expression at day 6.5. More specifically, the *Kcnq1ot1* ICR was required for maintenance of maternal-specific expression at imprinted genes while the *Kcnq1ot1* ncRNA was required for maintenance of imprinted expression at only a subset of genes. In truncation mutant embryos, the *Kcnq1ot1* ICR remained intact, leaving open the possibility that regulatory sequences contained within the ICR were sufficient to maintain repression at *Kcnq1* and *Cdkn1c* (Fitzpatrick et al., 2007; Thakur et al., 2004). In cell culture-based enhancer-blocking assays, the *Kcnq1ot1* ICR has been shown to function as an insulator when placed between an enhancer and a gene of interest. Since the unmethylated paternal *Kcnq1ot1* ICR remained intact in truncation mutants, it may prevent *Cdkn1c* and *Kcnq1* from accessing nearby enhancers on the paternal chromosome.

A region within the *Kcnq1ot1* ICR also possesses silencer activity by inducing repression of adjacent genes in an episomal system (Mancini-DiNardo et al., 2003; Thakur et al., 2003; Thakur et al., 2004). Paternal deletion of the silencer region reactivated the paternal *Kcnq1* and *Phlda2* alleles in midgestation placenta and the paternal *Kcnq1* allele in midgestation liver (Mohammad et al., 2010). Furthermore, paternal *Kcnq1* promoter association with the paternal *Kcnq1ot1* ICR was lost when the *Kcnq1ot1* ICR was deleted, resulting in lost paternal *Kcnq1* and *Cdkn1c* silencing (Zhang et al., 2014). Thus, it is possible that silencer activity at the *Kcnq1ot1* ICR may function to maintain paternal silencing of *Kcnq1* and *Cdkn1c* in day 6.5 ncRNA truncation mutants in this study.

A secondary differentially methylated region at *Cdkn1c* may also play a role in maintenance of paternal *Cdkn1c* silencing in truncation mutants. During embryonic development, *Cdkn1c* acquires *de novo* methylation at the paternal *Cdkn1c* differentially

methylation region beginning at day 6.5, which is complete by day 9.5 (Bhogal et al., 2004). This methylation acquisition is dependent on the *Kcnq1ot1* ICR, which when deleted abolishes *de novo* methylation acquisition. Thus, the *Kcnq1ot1* ICR-dependent *de novo* methylation initiated at *Cdkn1c* may account for the maternal-biased *Cdkn1c* expression that I observed when the *Kcnq1ot1* ncRNA was truncated.

Alternatively, the maintenance of paternal *Kcnq1* and *Cdkn1c* silencing in truncation mutants may be independent of the *Kcnq1ot1* ICR. In a previous study, it was reported that shRNA-mediated depletion of the *Kcnq1ot1* ncRNA did not alter imprinting in embryo-derived stem cells, demonstrating that it is likely the act of transcription that is responsible for imprint maintenance rather than the RNA itself (Golding et al., 2011). In this thesis, maintenance of *Kcnq1* and *Cdkn1c* imprinted expression in day 6.5 truncation mutants is most likely independent of the *Kcnq1ot1* ncRNA itself since most of the transcript has been deleted. Since truncation of *Kcnq1ot1* to 2.6 kb was sufficient to maintain paternal repression of *Kcnq1* and *Cdkn1c*, it indicates that if transcription through an important regulatory region exists, it most likely is localized within that non-truncated region. However, it is possible that sequences within the shortened RNA are capable of recruiting repressive factors to maintain paternal allelic silencing at some genes in the domain. During the process of X-chromosome inactivation, a small ncRNA embedded with the *Xist* gene, known as *RepA*, binds to EZH2 then deposits this protein to the *RepA* gene, where it initiates the deposition of H3K27me3 (Zhao et al., 2008). EZH2 also interacts directly with the *Kcnq1ot1* ncRNA and catalyzes the addition of H3K27me3 within the *Kcnq1ot1* domain (Pandey et al., 2008; Terranova et al., 2008). Despite this role of EZH2, it is unlikely to bind to the truncated *Kcnq1ot1* ncRNA since the interaction site occurs downstream of the truncation (Zhao et al., 2010). Having said this, an additional EZH2 binding site has been reported within the shortened *Kcnq1ot1* ncRNA (Pandey et al., 2008). In addition to EZH2, other unidentified epigenetic proteins may carry out a similar function with the truncated *Kcnq1ot1* ncRNA, allowing initial maintenance of *Kcnq1* and *Cdkn1c* imprinted expression at early postimplantation stages of development.

My preimplantation development model proposes that in wildtype embryos, imprinted protein-coding genes are recruited into a silent region and this repression is maintained during postimplantation (Figure 28). When the *Kcnq1ot1* ICR is deleted, imprinted genes are still recruited into this repressive compartment and undergo paternal silencing. However, paternal-repression is not maintained during postimplantation and genes become reactivated. When the *Kcnq1ot1* ncRNA is truncated, imprinted genes are still recruited into this repressive compartment and still undergo paternal silencing during preimplantation. However, paternal repression is only maintained for genes proximal to the ICR during postimplantation, with proximal genes remaining inside the repressive compartment.

My research shows that paternal allelic silencing is established later in preimplantation development and not with the onset of *Kcnq1ot1* ncRNA expression. The *Kcnq1ot1* ICR and ncRNA are dispensable for paternal repression at this stage. Imprinted expression in early postimplantation embryos carrying a paternally deleted or truncated *Kcnq1ot1* allele showed that the *Kcnq1ot1* ICR was necessary for maintenance of paternal repression at *Phlda2*, *Slc22a18*, *Cdkn1c*, *Kcnq1* and *Ascl2*, while truncation of the *Kcnq1ot1* ncRNA resulted in paternal reactivation of distal genes, *Phlda2*, *Slc22a18* and *Ascl2*, while genes more proximal to the ICR, *Kcnq1* and *Cdkn1c*, maintained maternal-specific or maternal-biased expression. Demonstrating for the first time that the *Kcnq1ot1* ICR and ncRNA are dispensable in early development for paternal silencing but are required later for maintenance of imprinted expression.

Further investigation is needed to understand the complex regulation of the *Kcnq1ot1* imprinted domain. Remaining questions include: through which mechanism(s) does paternal repression of genes within the domain occur, including whether repetitive elements facilitate repressive compartment formation; how does the *Kcnq1ot1* ICR mediate silencing of neighbouring genes; how does the *Kcnq1ot1* ncRNA or its transcription facilitate paternal allelic silencing of upstream and downstream genes; and which epigenetic modifiers facilitate imprinted domain establishment and maintenance. The analysis of preimplantation and early postimplantation embryos will be instrumental in addressing these questions.

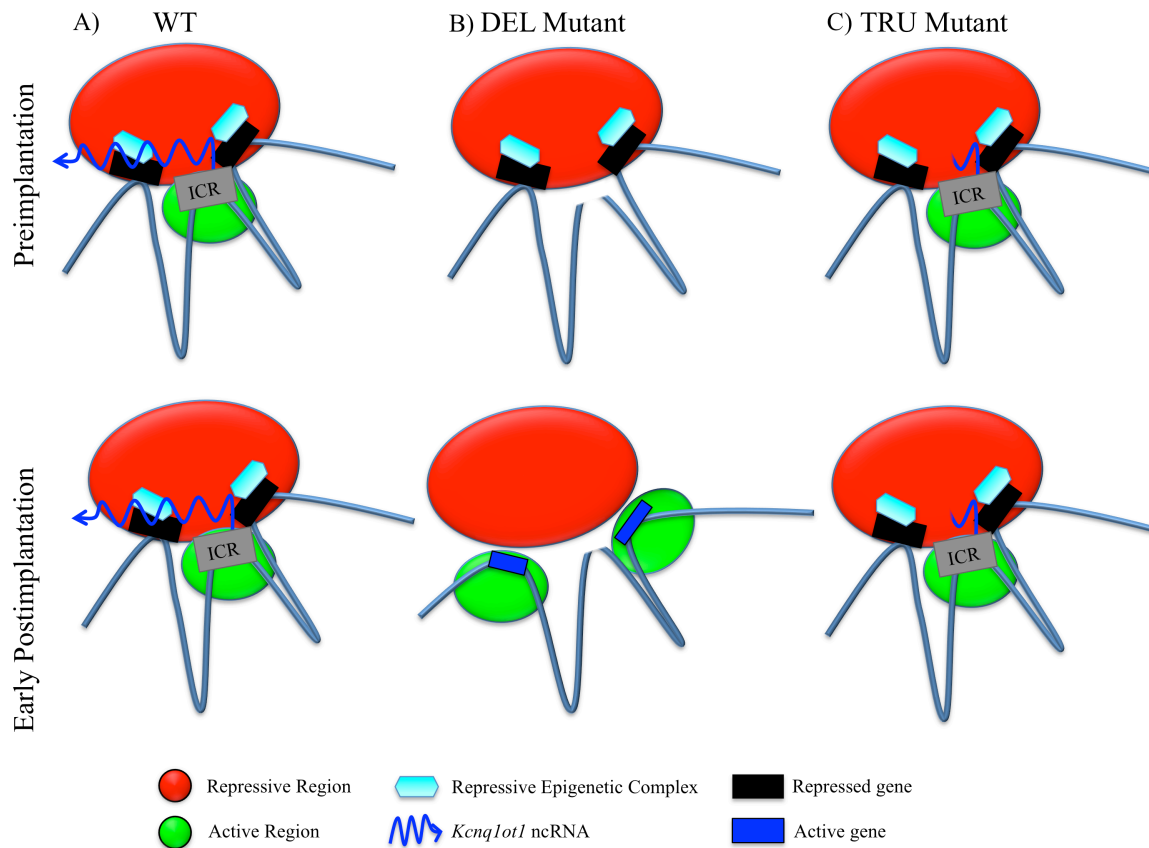


Figure 28: Model of *Kcnq1ot1* Paternal Silencing in Early Development

A) In both pre- and postimplantation, the wildtype paternal *Kcnq1ot1* domain consists of an ICR, which contains the promoter of the paternally expressed *Kcnq1ot1* ncRNA (blue wavy arrow) within an active region (green circle) and silent adjacent genes (black boxes) within a repressive compartment (red circle) along with repressive epigenetic complexes (teal octagon). B) Deletion of the paternal ICR results in no change in establishment of paternal repression (black boxes) during preimplantation. However, in early postimplantation embryos, maintenance of silencing is lost and expression of imprinted-genes occurs on the paternal allele (blue boxes). C) Truncation of *Kcnq1ot1* ncRNA results in establishment and maintenance of paternal silencing only at genes proximal to the ICR (distal genes not shown).

4.2 Epigenetic Factors Regulating Paternal Repression at the *Kcnq1ot1* Domain

To identify epigenetic factors that play a role in maintaining paternal allelic repression of imprinted genes in the *Kcnq1ot1* domain, I performed a loss-of-function RNA interference screen that was unbiased with respect to the proposed models for paternal silencing of imprinted genes within the *Kcnq1ot1* domain. The screen identified 35 epigenetic factors. Investigation of the top 3 candidates found that EZH1, SMARCA5, and SMARCAD1 regulated paternal allelic repression of imprinted genes at the *Kcnq1ot1* domain. More specifically, depletion of *Ezh1*, *Smarca5* and *Smarcad1* caused paternal reactivation of *Kcnq1* and *Cdkn1c* but not at *Osbpl5* and *Slc22a18*, indicating that the candidates identified here do not function domain-wide. Loss of paternal repression at the upstream gene, *Cdkn1c*, and downstream gene, *Kcnq1*, indicates that antisense transcription is not responsible for paternal repression across the entire domain. *Kcnq1ot1* transcript abundance was reduced in *Smarca5*- and *Smarcad1*-depleted cells, suggesting that nucleosome remodeling is important for *Kcnq1ot1* ncRNA transcription and/or *Kcnq1ot1* ICR function. Silencing of *Kcnq1ot1* expression was not due to aberrant paternal ICR DNA methylation. By comparison, EZH1 functioned to silence the proximal *Kcnq1* and *Cdkn1c* paternal alleles in a *Kcnq1ot1* ncRNA transcription independent manner, since *Kcnq1ot1* transcript levels were unchanged in *Ezh1*-depleted cells, suggesting that EZH1 acts through *Kcnq1ot1* ICR function or acts locally at specific genes. All together, the data suggest that multiple epigenetic modifiers and remodelers are required for paternal silencing in XEN cells

An interesting finding from my research is that depletion of *Ezh1*, *Smarca5* and *Smarcad1* reactivated the paternal allele of proximal genes *Cdkn1c* and *Kcnq1* but not the distal gene *Osbpl5* or *Slc22a18*, similar to the postimplantation *Kcnq1ot1* ncRNA truncation mutants maintenance of proximal genes *Cdkn1c* and *Kcnq1* paternal repression. Decreased *Kcnq1ot1* transcript abundance in *Smarca5*- and *Smarcad1*-depleted XEN cells indicates that the *Kcnq1ot1* ICR, the *Kcnq1ot1* ncRNA or its transcription are important for paternal silencing. Since the *Kcnq1ot1* ICR was intact in both the candidate-depleted cells and the *Kcnq1ot1* ncRNA truncated postimplantation

embryos, it indicates that the ICR could be important for maintaining paternal silencing at *Kcnq1* and *Cdkn1c*. For example, boundary/insulator elements on either side of the ICR could function to maintain the *Kcnq1ot1* ICR in an active state, requiring the activity of nucleosome remodelers. SMARCA5 was found to bind to the *H19* ICR in mouse erythroleukemia (MEL) cells and functioned to activate transcription of *H19* (Dluhosova et al., 2014). Therefore, SMARCA5 could function to maintain *Kcnq1ot1* transcription by allowing interaction with enhancers, similar to *H19*. As a result, loss of *Kcnq1ot1* expression in *Smarca5*-depleted cells could allow *Kcnq1* and *Cdkn1c* access to enhancers normally blocked by SMARCA5, which could lead to activation of *Kcnq1* and *Cdkn1c*.

The unmethylated paternal *Kcnq1ot1* ICR has been proposed to act as an insulator and binding site for the insulator protein CTCF, acting in a similar manner to the *H19* ICR to repress adjacent genes by restricting access to enhancers. Although my RNA interference screen was not exhaustive, the insulator protein CTCF was not isolated from our functional screen. Analysis of CTCF binding in online genomic databases in multiple tissues demonstrated that CTCF binds weakly to the *Kcnq1ot1* ICR relative to the strong peaks upstream of *Cdkn1c* and downstream of *Kcnq1*. Additionally, CTCF binds equally to the maternal and paternal *Kcnq1ot1* ICR (Lin et al., 2011). Together, these results suggest that CTCF function is limited at the *Kcnq1ot1* ICR, possibly explaining why it was not isolated from the functional screen. Consistent with this, imprinted expression of *Kcnq1*, *Kcnq1ot1* and *Cdkn1c* was maintained when CTCF was depleted in mouse embryonic fibroblasts (MEFs) (Lin et al., 2011). The function of boundary elements outside of CTCF could be mediated by SMARCA5, with an important role for nucleosome remodeling. Loss of *Kcnq1ot1* expression in *Smarca5*-depleted cells may result from an loss of nucleosome remodeling at the boundary/insulator region, no longer allowing the *Kcnq1ot1* ICR to maintain an active state, leading to activation of the paternal *Kcnq1* and *Cdkn1c* alleles.

Alternative to the *Kcnq1ot1* ICR having enhancer-blocking or insulator function, the ICR could act directly on promoters of proximal genes through a chromatin looping mechanism. At *Cdkn1c*, the ICR is required to methylate the paternal promoter in day 6.5 embryos, stabilizing paternal silencing (Bhogal et al., 2004). For *Kcnq1*, the paternal

promoter can associate with the ICR. However, control of paternal repression during this interaction seems to be tissue-specific: paternal *Kcnq1* promoter interaction with the ICR in MEFs correlated with silencing of *Kcnq1* (Zhang et al., 2014). Conversely, when the paternal *Kcnq1* allele lost paternal silencing in the developing heart, the *Kcnq1* promoter was able to bind to presumptive upstream heart-specific enhancers but not to the *Kcnq1ot1* ICR (Korostowski et al., 2011). Although the paternal ICR may stabilize proper looping conformation, it still does not explain how repression is lost when the ICR remains intact in screen candidate-depleted cells. Interestingly, interaction of the *Kcnq1* promoter with the paternal ICR is also influenced by *Kcnq1ot1* ncRNA transcription or the ncRNA itself (Korostowski et al., 2012; Zhang et al., 2014). Thus, reduced levels of *Kcnq1ot1* transcripts in *Smarca5*- and *Smarcad1*-and depleted cells may similarly interfere with *Kcnq1ot1* ICR-imprinted gene promoter interactions. Further investigation is required to understand the relationship between chromatin looping and *Kcnq1ot1* ncRNA transcription.

Previous research has shown that the SMARCA5 and SMARCAD1 can function in both gene activation and repression (Dluhosova et al., 2014; Hong et al., 2009; Rowbotham et al., 2011; Vargova et al., 2009). Thus, these remodeling proteins could mediate paternal allelic silencing by maintaining the ICR in an active conformation, activating *Kcnq1ot1* ncRNA expression or by repressing neighbouring protein-coding genes directly. However, the ACF1-ISWI chromatin remodeling complex containing SMARCA5 is required to replicate DNA within the densely packed chromatin of heterochromatin, presumably to move nucleosomes so the replication fork can progress (Collins et al., 2002). Furthermore, another SMARCA5 containing complex, NoRC, silences ribosomal RNA genes in yeast by re-positioning nucleosomes downstream of the transcriptional start site (Zhou et al., 2009). NoRC is recruited to promoters by RNA (pRNA), and this silencing occurs at the nucleolus during S-phase (Strohner et al., 2001; Zhou et al., 2009). Consistent with this, SMARCA5 associates with the inactive X chromosome at S-phase in MEFs, suggesting that it has a role in maintaining repressive marks during replication. Interestingly, *Kcnq1ot1* ncRNA also localizes near SMARCA5 foci and the nucleolus in XEN cells (Supplementary Figure 30) and near the nucleolus in JEG-3 cells (Mohammad et al., 2008). Interestingly, SMARCA5 mainly overlaps with

euchromatin by confocal microscopy in ES cells (Vargova et al., 2009). Therefore, it is possible that SMARCA5 interacts with the active paternal *Kcnq1ot1* gene and its ncRNA to re-establishing paternal allelic repression after replication. This is consistent with heterozygous SMARCA5 deletion which abolishes heterochromatin marks, suggesting that SMARCA5 regulates repressive chromatin structure (Vargova et al., 2009), perhaps at boundaries of active/repression chromatin.

Similar to SMARCA5, SMARCAD1 acts in a repressive complex along with KAP1, HDAC1, G9a and PCNA to re-establish repressive heterochromatin marks after DNA replication. Depletion of *SMARCAD1* in HeLa cells caused reduced global level of histone deacetylation and H3K9me3 (Rowbotham et al., 2011). If SMARCAD1 has a similar function in XEN cells, loss of paternal allelic repression at the *Kcnq1ot1* domain in *Smarcad1*-depleted cells could be the result of failure to re-establish heterochromatin after replication, following a similar loss of heterochromatin architecture that occurs on a global level. However, for both SMARCA5 and SMARCAD1, loss of global heterochromatin loss would imply that all genes on the paternal *Kcnq1ot1* domain would be activated, yet that is not the case, requiring other proteins within the repressive complex to provide specificity. Alternatively, there may be multiple boundary elements or chromatin looping regulatory regions within the *Kcnq1ot1* domain. Changes in nucleosome occupancy may lead to loss of *Kcnq1ot1* activation and activation of paternal alleles of proximal genes, changing active and repressive compartments when SMARCA5 and SMARCAD1 levels are reduced. Future studies are required to examine nucleosome occupancy in WT and *Smarca5*- and *Smarcad1*-depleted XEN cells.

Interestingly, EZH1, the homologue to EZH2, which is a member of the PRC2 complex, was identified in the functional screen. EZH1 can take the place of EZH2 within the PRC2 complex, interacting with SUZ12 and EED (Margueron et al., 2008). Loss of *Cdkn1c* paternal repression occurred in EZH2-deficient day 6.5 extra-embryonic ectoderm (*Kcnq1* was not examined) and *Kcnq1ot1* ncRNA expression was not altered in EZH2-deficient TS cells (Terranova et al., 2008), similar to *Ezh1*-depleted XEN cells. EZH2 has been proposed to interact with *Kcnq1ot1* ncRNA to recruit H3K27me3 to induce paternal allelic silencing at *Kcnq1*, *Cdkn1c* and *Ascl2* (Pandey et al., 2008;

Umlauf et al., 2004; Zhang et al., 2014). When EZH2 is deleted in ES cells, EZH1 was found to bind to *Osbp15*, *Phlda2*, *Cdkn1c*, *Kcnq1* and *Ascl2*, a subset of genes within the *Kcnq1ot1* domain (Shen et al., 2008), suggesting that EZH1 binding may be gene specific. To determine whether EZH1 replaced EZH2 in the PRC complex, EZH1 was depleted in EZH2-deficient ES cells (Margueron et al., 2008). However, little change in global H3K27me3 was observed. Therefore, it is possible that paternal allelic reactivation occurs without a change in H3K27me3, suggesting that an alternative repressive mechanism is mediated by EZH1. Consistent with this, EZH1 has little histone methyltransferase activity, but may instead function to induce chromatin compaction, altering chromatin structure resulting in repression of transcription (Margueron et al., 2008). This suggests that paternal activation of *Cdkn1c* and *Kcnq1* in *Ezh1*-depleted XEN cells could be result of chromatin decompaction. Future studies will need to investigate EZH1 binding to the *Kcnq1ot1* ncRNA, and whether EZH1 induces chromosome compaction.

My proposed model for candidate maintenance of paternal silencing suggests that on the paternal allele in wildtype XEN cells, *Kcnq1ot1* ncRNA is expressed and protein-coding genes are silent and contained within a silent region (Figure 29). When chromatin-remodelers are depleted, *Kcnq1ot1* ncRNA expression is reduced and adjacent protein-coding genes lose paternal allelic silencing, perhaps forming the basis for changes in active/repressive compartmentalization of proximal genes. When *Ezh1* is depleted, *Kcnq1ot1* ncRNA expression is unchanged and adjacent protein-coding genes lose paternal allelic silencing via decompaction of chromatin.

In summary, my research identified epigenetic modifiers regulating maintenance of imprinting at the *Kcnq1ot1* domain in embryo-derived stem cells using a positive selection, loss-of-function RNA interference (RNAi) screen. The RNAi screen was an excellent system to dissect the regulation of paternal allelic silencing as it does not rely on assumptions of previously proposed models. The screen represents a first in the field of genomic imprinting. Importantly, the identification of SMARCA5 and SMARCA1 as epigenetic regulators of genomic imprinting indicates that nucleosome occupancy plays a functional role in paternal allelic silencing at the *Kcnq1ot1* domain. Furthermore,

the identification of EZH1 as a regulator of paternal allelic silencing at the *Kcnq1ot1* domain suggests an important role for chromatin structure as EZH1 likely act via a non-enzymatic function. I had hypothesized that identified epigenetic factors would act locally or domain-wide. My data unexpectedly demonstrate for the first time that there is regional regulation of paternal allelic repression, identifying further complexity to imprinted domain regulation. The factors identified in the screen provide the foundation that will determine how paternal silencing is mediated. Further studies are required to identify where SMARCA5, SMARCAD1 and EZH1 bind within the domain; demarcate boundaries between active and repressed chromatin compartments; map nucleosome positioning particularly at boundary regions; and to further define chromatin structure, locally, regionally and domain-wide. These studies will provide the context to delineate the various models for *Kcnq1ot1* domain regulation.

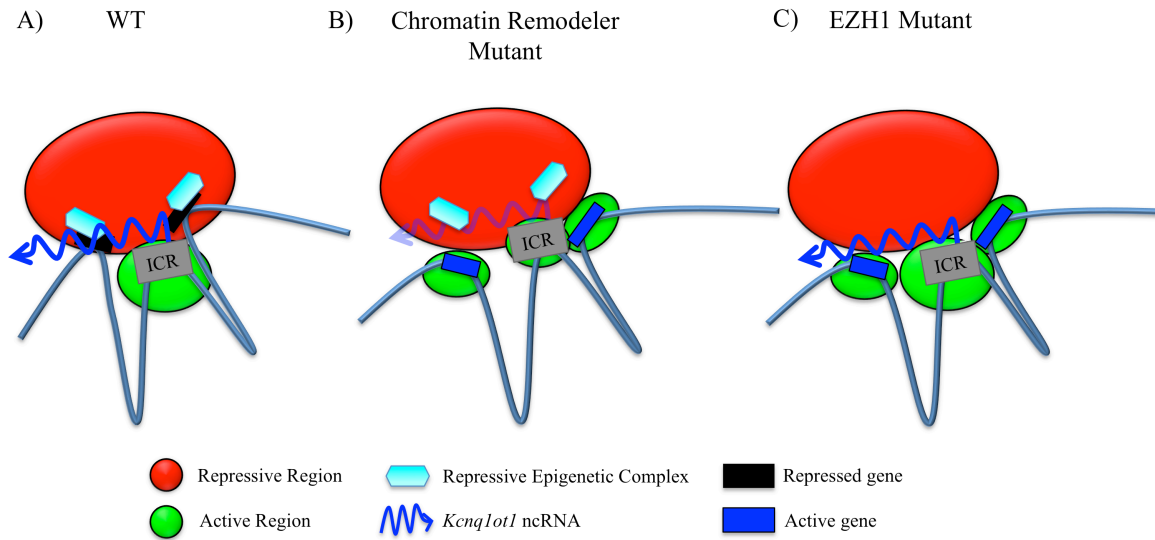


Figure 29: Model of *Kcnq1ot1* Paternal Silencing of Proximal Genes in XEN Cells

A) The wildtype paternal *Kcnq1ot1* domain consists of an ICR, which contains the promoter of the paternally expressed *Kcnq1ot1* ncRNA (blue wavy arrow) within an active region (green circle) and silent adjacent genes (black boxes) within a repressive compartment (red circle) with repressive epigenetic complexes (teal octagon). B) Depletion of chromatin remodelers results in reduced *Kcnq1ot1* levels and activation of the paternal alleles of genes proximal to the ICR (blue boxes), changing active/repressive compartmentalization of the *Kcnq1ot1* ICR and proximal genes. C) Depletion of *Ezh1* results in maintenance of *Kcnq1ot1* levels but activation of proximal genes (blue boxes), possibly through decompaction of proximal genes (distal genes not shown).

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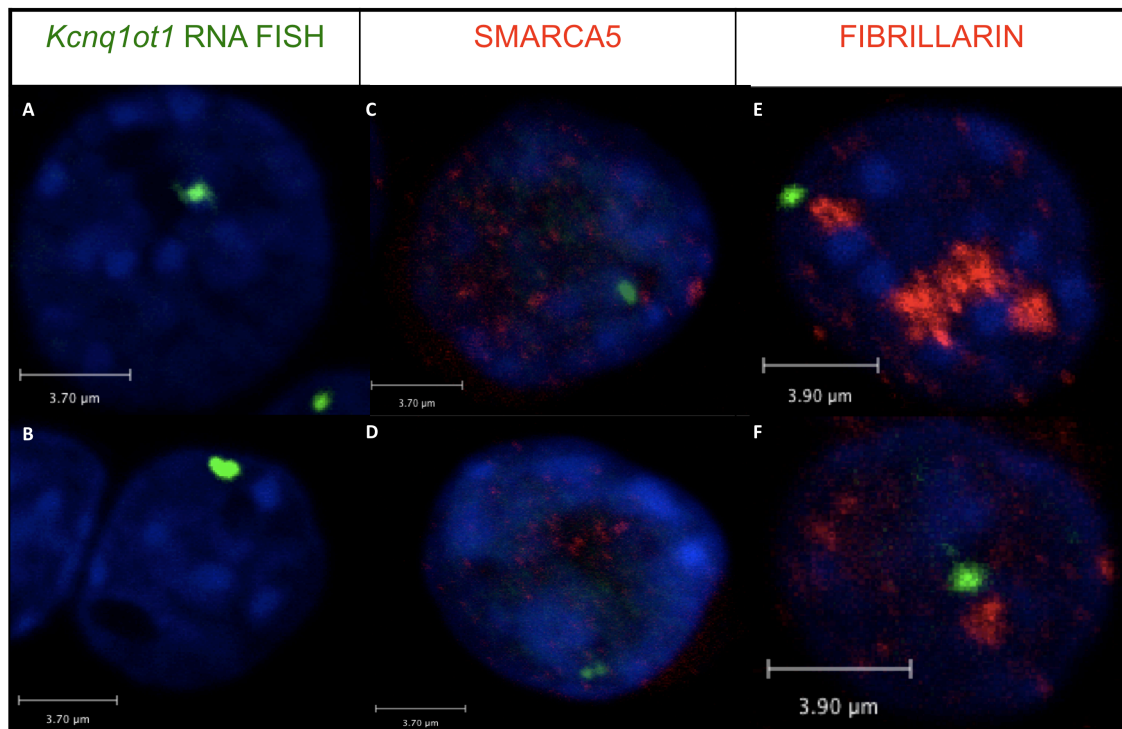
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Appendices

Appendix A:

Supplementary Figure 30: Colocalization of *Kcnq1ot1* ncRNA RNA with SMARCA5 and perinucleolar region in XEN cells.

A-B) RNA-FISH labeling of *Kcnq1ot1* (green) in XEN cells. RNA-FISH was performed on XEN cells with probes targeting *Kcnq1ot1* RNA, as previously described (Golding et al., 2011). (C-F) Sequential RNA-FISH and immunofluorescence in XEN cells was used to detect the co-localization of *Kcnq1ot1* (green) and proteins-of-interest (Red). RNA-FISH combined with immunofluorescence allows detection of *Kcnq1ot1* interactions with proteins of interest. *Kcnq1ot1* localizes near SMARCA5 foci and to the perinucleolar region in XEN cells. (C-D) SMARCA5 (Red) shows multiple foci with some adjacent to *Kcnq1ot1* (green). (E-F) Nucleolar marker FIBRILLARIN (Red) localizes adjacent to *Kcnq1ot1*, as shown in previous studies.



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CURRICULUM VITAE

EDUCATION

PhD, Biochemistry with Specialization in Developmental Biology 01/2009-Present

Western University, London, ON

Honours Bachelor of Science – Genetics Specialization Co-op 09/2004-12/2008

McMaster University, Hamilton, ON

HONOURS AND AWARDS

NSERC PhD	2011-present
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Epigenetic Eh! Conference Poster Presentation Award	2011
NSERC CGS Masters	2010-2011
Paul Harding Research Day Poster Presentation Award, 1 st Place	2009

WORK EXPERIENCE

Course Instructor 01/2014-04/2014
Fanshawe College, London ON

- Taught Immunology and Virology to senior Bachelor of Applied Technology students
- Discussed fundamentals of immunology, including innate and acquired immunity
- Taught basic virology, viral classification and epidemiology of viral human diseases
- Supervised and directed students through laboratory experiments
- Lead and delegated organization of laboratory experiments and materials
- Designed experiments to meet learning outcomes and curriculum standards when classical experiments were not feasible

Lentiviral Trainer 01/2009-Present

Children's Health Research Institute, London ON

- Supervised and trained multiple researchers in lentivirus techniques and viral biology

- Reinforced understanding of both practical and theoretical basis for virus work
- Placed utmost care and importance on training proper safety and handling procedures
- Assisted other researchers with performing and troubleshooting lentiviral techniques

Senior Co-op Student supervised by Dr. Juliet Daniel

McMaster University, Hamilton, ON

09/2008-12/2008

- Examined the role of Kaiso protein during epithelial-to-mesenchymal transition
- Troubleshoot and researched techniques when traditional assays of metastasis failed
- Strong knowledge of the WNT, TGF- β and RTK signalling pathways
- Wrote, presented and defended my fourth-year thesis

Co-op Student supervised by Dr. Mellissa Mann

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- Dissected and flushed mouse oviducts to isolate pre-implantation embryos
- Optimized PCR programs to examine specific genes from a single blastocyst
- Assisted in protocol development to analyze DNA methylation in single mouse blastocyst
- Investigated changes in DNA methylation following *in vitro* embryo culture

REFEREED PUBLICATIONS

1. **Landschoot LSM**, Denomme, MM., Higgins, MJ., and Mann, MRW. *Kcnq1ot1* Imprinting Control Region and Non-coding RNA are Dispensable for Imprinted Domain Regulation During Early Development, PLoS Genetics, submitted.
2. **Landschoot LSM**., Sachani, S., Golding, MC., Mann, MRW., Epigenetic Factors Regulating Paternal Allelic Repression at the *Kcnq1ot1* Domain, in preparation.
3. **Magri, LS** & Golding, MC., Lalone, SA, Zhang,L., Higgins, M., and Mann, MRW. (2011) Depletion of *Kcnq1ot1* non-coding RNA does not affect imprinting maintenance in stem cells. *Development*, 138(17):3667-78.
4. Market-Velker BA., Zhang, L., **Magri, LS.**, Bonvissuto, AC. and Mann, MRW. (2009) Dual Effects of Superovulation: Loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Human Molecular Genetics*, 19: 36-5.

NON-REFEREED CONTRIBUTIONS (presenter designated by*)

1. **Landschoot LSM***., Sachani, S., Golding, MC., Mann, MRW., Epigenetic Factors Regulating Paternal Allelic Repression at the *Kcnq1ot1* Domain. Epigenetics, Eh! Conference. London ON, June 24-27, 2014. Poster.

2. **LSM Landschoot***, MM Denomme, MJ Higgins, MRW Mann. The *Kcnq1ot1* Non-Coding RNA is Dispensable for Imprinted Genes Regulation During Early Development. Gordon Epigenetic Research Conference. Smithfield, RI. August 4-9, 2013. Poster.
3. **LSM Landschoot***, MM Denomme, MJ Higgins, MRW Mann. Regulation of the *Kcnq1ot1* Imprinting Domain. Department of Obstetrics and Gynaecology Paul Harding Research Day, London ON, May 1, 2013. Poster.
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5. **LS Magri***, MC Golding, SA Lalone, L Zhang, MRW Mann. *Kcnq1ot1* noncoding RNA depletion does not alter imprinting in stem cells. Great Lakes Developmental Biology Meeting, Toronto, ON, March 3-5, 2011.
6. **LS Magri***, MC Golding, SA Lalone, L Zhang, MRW Mann. *Kcnq1ot1* noncoding RNA depletion does not alter imprinting in stem cells. Paul Harding research day, London ON, April 27, 2011. Poster.
7. **LS Magri***, MC Golding, SA Lalone, L Zhang, MRW Mann. *Kcnq1ot1* noncoding RNA depletion does not alter imprinting in stem cells. Epigenetics Eh! Conference, London ON, May 4-7, 2011. Poster.
8. **LS Magri***, MC Golding, SA Lalone, L Zhang, MRW Mann. *Kcnq1ot1* noncoding RNA depletion does not alter imprinting in stem cells. Developmental Biology Research Symposium. London ON, May 4-7, 2011. Poster.
9. **LS Magri***, MC Golding, MRW Mann. Screen for Epigenetic Factors Controlling Genomic Imprinting During Early Mammalian Development. Department of Obstetrics and Gynaecology Paul Harding Research Day, London ON, May 19, 2010. Poster.
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12. BA Market-Velker, L Zhang, **LS Magri**, AC Bonvissuto, and MRW Mann*, Dual Effects of Superovulation: Loss of maternal and paternal imprinted methylation in a dose-dependent manner. Gordon Research Conference: Mammalian Gametogenesis and Embryogenesis, Waterville Valley, NH, August 2-7, 2009, Oral presentation
13. BA Market-Velker, L Zhang, **LS Magri**, AC Bonvissuto, and MRW Mann*, Dual Effects of Superovulation: Loss of maternal and paternal imprinted methylation in a dose-dependent manner. Gordon Research Conference: Epigenetics, Holderness, NH, August 9-14, 2009. Poster.
14. BA Market-Velker*, L Zhang, **LS Magri**, AC Bonvissuto, and MRW Mann, Dual Effects of Superovulation: Loss of maternal and paternal imprinted methylation in a dose-dependent manner. 42nd Society for the Study of Reproduction Meeting, Pittsburgh, PA, July 18-22, 2009. Poster.

15. **LS Magri***, MC Golding, MRW Mann. Screen for Epigenetic Factors Controlling Genomic Imprinting During Early Mammalian Development. Department of Obstetrics and Gynaecology Paul Harding Research Day, London ON, May 13, 2009. Poster.
16. BA Market-Velker*, L Zhang, **LS Magri**, AC Bonvissuto, and MRW Mann. Investigating the Molecular Effects of Assisted Reproductive Technologies on Genomic Imprinting. Paul Harding Research Day, London ON, May 13, 2009. Poster.
17. BA Market-Velker, L Zhang, **LS Magri**, AC Bonvissuto, and MRW Mann*. Susceptibility of Genomic Imprinting to Assisted Reproductive Technologies. Texas A&M University, College Station, TX, April 22, 2009, Oral Presentation.
18. **LS Magri***, MC Golding, MRW Mann. Screen for Epigenetic Factors Controlling Genomic Imprinting During Early Mammalian Development. Margaret Moffat Research Day, London ON, March 26, 2009, Poster.
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20. BA Market-Velker, L Zhang, **LS Magri**, AC Bonvissuto, and MRW Mann*. Susceptibility of Genomic Imprinting to Assisted Reproductive Technologies. Frontiers in Reproductive Biology and Regulation of Fertility, Keystone Symposia, Santa Fe, NM, Feb 4, 2009. Oral presentation.
21. BA Market-Velker*, L Zhang, **LS Magri**, AC Bonvissuto, and MRW Mann. Investigating the Molecular Effects of Assisted Reproductive Technologies on Genomic Imprinting. Canadian Society for Clinical Investigation Young Investigator's Forum, Toronto, ON, Sept 25, 2008. Oral presentation.
22. BA Market-Velker*, L Zhang, **LS Magri**, AC Bonvissuto, and MRW Mann. Investigating the Molecular Effects of Assisted Reproductive Technologies on Genomic Imprinting. Paul Harding Research Day, London, ON, May 14, 2008. Oral presentation.