REGULATION OF GENOMIC IMPRINTING DURING MOUSE EMBRYONIC DEVELOPMENT

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Katherine Ann Alexander

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REGULATION OF GENOMIC IMPRINTING DURING MOUSE EMBRYONIC DEVELOPMENT

Katherine Ann Alexander, Ph.D.

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Genomic imprinting is a process that results in allele-specific gene expression from either the maternally- or paternally-inherited chromosome. Proper genomic imprinting is essential for embryonic developmental, yet the mechanisms by which imprinted expression is achieved during embryogenesis are not fully understood. By investigating the regulation of genomic imprinting at different developmental stages and tissue types, we found that imprinted gene expression is regulated by different mechanisms that depend on developmental and tissue-specific contexts. First, we found that TRIM28, a transcriptional repressor that binds to all known imprinting control regions, has two distinct functions during embryogenesis. Shortly after fertilization, TRIM28 is essential for preserving DNA methyl marks at germline-inherited differentially methylated regions (gDMRs). In contrast, TRIM28 controls imprinting later in embryogenesis without altering gDMR methylation. Our experiments suggest that, at the *Dlk1-Dio3* imprinted cluster, TRIM28 binds the paternal germline intergenic DMR (IG-DMR) to prevent it from functioning as an enhancer for *Gtl2*. In addition, we found that TRIM28 also regulates imprinting through mechanisms other than IG-DMR binding. Interestingly, our investigations revealed a previously unappreciated requirement for the paternal IG-DMR to control imprinting in a tissue-specific manner. Consistent with previous reports, we

found that deletion of the paternal IG-DMR had minimal effects on imprinted gene expression in the E14.5 limb. However, qRT-PCR experiments showed that the paternal IG-DMR is essential in the yolk sac for cis repression of *Gtl2*, in the lung for cis activation of *Dlk1*, and in the E9.5 whole embryo for cis activation of both *Dlk1* and *Dio3*. Taken together, our studies provide evidence that imprinted gene expression is regulated through multiple mechanisms during development and that the regulatory elements and transcriptional regulators involved have different requirements depending on the developmental context.

BIOGRAPHICAL SKETCH

Katherine Alexander was born in Eugene, Oregon in 1988. She showed a great curiosity for exploring early in life, which at times necessitated rescue by her parents, James Alexander and Pamela Griffith, or her sisters, Margaret Alexander, Rebecca Alexander and Nancy Alexander. Family legend states that Katherine once climbed a basketball post and was found sitting inside the hoop, however Katherine claims to have no recollection of this incident. These energies were later focused on somewhat less daring athletics as Katherine became the captain of her high school swim team and cross-country team at Tualatin High School, which she attended 2002-2006. After high school, Katherine attended Carleton college with ambitions to double major in Studio Art and Physics. These ambitions were thwarted, however, after Katherine took her first biology class and became enthralled with the biological sciences. In 2010, this transformation led Katherine to Cornell University to pursue a Ph.D. in Biology.

To my family,

Donald Griffith, Constance Griffith, Pamela Griffith, Jim Alexander, Nancy Alexander,

Rebecca Alexander, and Margaret Alexander

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LIST OF ABBREVIATIONS

ΔIG^{MAT} - maternal deletion of the IG-DMR

ΔIG^{PAT} - paternal deletion of the IG-DMR

ChIP - Chromatin immunoprecipitation

DNA - deoxyribonucleic acid

DNMT1 - DNA methyltransferase 1

DNMT3A - DNA methylatransferase 3A

DNMT3B - DNA methyltransferase 3B

DPPA3 - development pluripotency-associated 3

ESC - embryonic stem cell

gDMR - germline differentially methylated region

ICR - imprinting control region

MEF - mouse embryonic fibroblast

PCR - polymerase chain reaction

PGC - primordial germ cell

RNA - ribonucleic acid

RNA-FISH - RNA fluorescent in situ hybridization

qRT-PCR - quantitative reverse transcriptase polymerase chain reaction

RRBS- reduced representation bisulfite sequencing

RT-PCR - reverse transcriptase polymerase chain reaction

sDMR - secondary differentially methylated region

SNP - single nucleotide polymorphism

TRIM28 - tripartate motif containing protein 28

WGBS - whole genome bisulfite sequencing

ZFP57 - zinc finger protein 57

CHAPTER 1

INTRODUCTION

A. Discovery of Genomic Imprinting

In 1869, Friedrich Miescher isolated a substance from the cell nuclei of pus-covered patient bandages that had chemical properties unlike any protein. Miescher termed this substance nuclein, which, as its chemical properties were further investigated, was renamed nucleic acid, then deoxyribonucleic acid (DNA). Given the relatively simple composition of DNA compared to protein, it was surprising when, by process of elimination, Alfred Avery and colleagues found that DNA, rather than protein or RNA, was essential for the pathogenicity of pneumococcal bacteria (Avery et al., 1944), providing the first evidence that DNA may be the heritable material. In 1952, Alfred Hersey and Martha Chase found that the T2 bacteriophage injects DNA, not protein, into the bacteria they infect (Hershey and Chase, 1952). Since T2 bacteriophage is capable of fully replicating from within bacteria, this finding demonstrated that the T2 bacteriophage protein was not the heritable material, but that the DNA “has some function.” DNA became more widely accepted as the heritable material later in the 1950s, after James Watson and Francis Crick proposed a structure of DNA that involved base pairing of the nucleic acids, and thus a potential mechanism by which DNA may be replicated (Watson and Crick, 1953).

That the DNA sequence is a critical component of inheritance is now undisputed. However, even as DNA became accepted as the heritable material, evidence existed suggesting that DNA sequence alone cannot fully explain inheritance. In sciarid flies, oocytes contribute one X chromosome, while sperm contribute two X chromosomes (Bois, 1933; Crouse, 1943; Metz, 1938). Sciarid somatic cells either eliminate one (in females) or both (in males) of the paternally-inherited X chromosomes. In 1960, Helen

Crouse mapped the location responsible for paternally-inherited X chromosome degradation to a heterochromatic control region within the X chromosome (Crouse, 1960). She astutely noted that the degradation of the paternal X chromosome(s) was dependent on their inheritance from the paternal germline, coining the term “imprint” to describe this phenomenon:

> “a chromosome which passes through the male germ line acquires an ‘imprint’ which will result in behavior exactly opposite to the ‘imprint’ conferred on the same chromosome by the female germ line. In other words, the ‘imprint’ a chromosome bears is unrelated to the genic constitution of the chromosome and is determined only by the sex of the germ line through which the chromosome has been inherited.”

In 1970, Jerry Kermicle observed another parent-of-origin specific effect at an autosomal gene in maize. Maize heterozygous for the color gene, *Rr*, were solidly colored when the *R* allele was inherited from the female gametophyte, but mottled when the *R* allele was inherited from the male gametophyte (Kermicle, 1970). Similar to Crouse, Kermicle concluded that the sex-dependent behavior of the *R* allele was a “paragenetic, rather than a conventional genetic” phenomenon, hypothesizing that passage through the germline affected the “expression but not the constitution of the *R* gene.”

These initial findings in sciarid flies and maize set the foundation for what we now know as the field of genomic imprinting. However, much of our current understanding of the factors that influence parent-of-origin inheritance has come from genetic manipulations in mice. In the 1970s, several publications observed different phenotypes

depending on whether a 3-5cM deletion on mouse chromosome 17 was inherited from the mother or from the father. Heterozygous animals were viable when the deletion was paternally inherited, but died in utero when the deletion was maternally inherited (Bennett, 1975; Johnson, 1974a; 1974b; Lyon and Glenister, 1977). While the evidence available could not yet explain this phenomenon, the authors speculated that the discrepancy in phenotypes could be due to differences in activity between the maternally and paternally-inherited chromosomes. However, they could not exclude the possibility that the deletion altered the content of the oocyte cytoplasm, leading to parent-of-origin specific phenotypes that were not due to imprinting of chromosome 17. In 1984, elegant nuclear transfer experiments distinguished between the effects of cytoplasmic and nuclear inheritance (McGrath and Solter, 1984; Surani et al., 1984). When mouse zygotes were reconstituted with a maternally-inherited pronucleus and a paternally-inherited pronucleus, embryos developed as normal. However, when embryos received two maternal or two paternal pronuclei, they failed to develop to term. These studies demonstrated that, despite contributing the same DNA sequence content, the maternally and paternally-inherited pronuclei are not functionally equivalent. Based on these findings, the authors concluded that the sperm and the oocyte genomes are imprinted during gametogenesis, acquiring heritable marks that distinguish between the pronuclei depending on whether they were inherited from the mother or from the father.

The finding that the maternally and paternally-inherited genomes are not functionally equivalent led to several fundamental questions that still shape the imprinting field. Which parts of genomes are imprinted? What is the mark that

differentiates between the maternally and paternally-inherited genomes? How is this mark set up in the sperm and the oocyte, how is it maintained after fertilization, and how does it have a functional effect capable of altering embryonic survival? In this Chapter, I review the progress toward understanding these questions and discuss the current gaps in our knowledge.

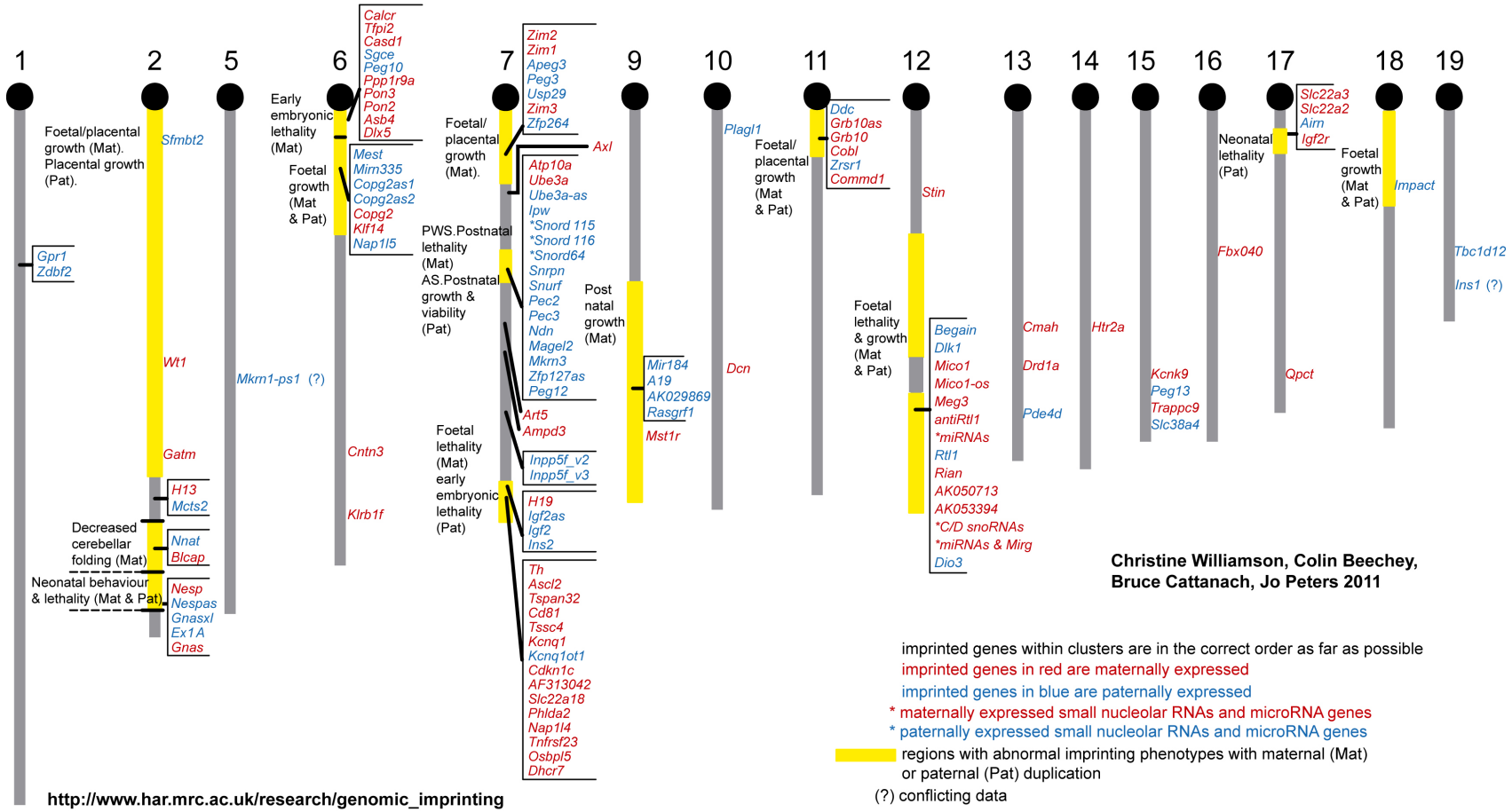
B. The Identification of Imprinted Regions

Phenotypic-based identification of imprinted regions. The first known imprinted region in mice was identified on chromosome 17 due to parent-of-origin specific phenotypes upon its deletion (Johnson, 1974a; 1974b). However, it was the study of mice that inherit both chromosomes from the mother or both from the father (maternal or paternal disomy), that allowed systematic identification of imprinted regions. These studies took advantage of the fact that Robertsonian translocations cause high frequencies of non-disjunction, allowing for chromosomal gain from one parent to be complemented with chromosomal loss from the other (Cattanach, 1986). In this manner, it was discovered that maternal or paternal disomy of 14 chromosomal regions causes severe phenotypes, including early embryonic lethality, disrupted embryonic and post-natal growth, and brain defects (Cattanach, 1986; Cattanach and Kirk, 1985; Lyon and Glenister, 1977; Searle and Beechey, 1985). This work provided a genomic map of developmentally-essential imprinted regions in the mouse (Figure 1.1).

As imprinted regions were systematically determined in mice, evidence began to emerge that certain human developmental syndromes were due to uniparental disomy or to parent-of-origin specific effects. Prader Willi syndrome, which is characterized by

Figure 1.1. Map of imprinted genes in mouse. Imprinted regions were identified based on the presence of phenotypes in mice with maternal or paternal disomy (yellow) (Cattanach, 1986; Cattanach and Kirk, 1985; Lyon and Glenister, 1977; Searle and Beechey, 1985). Figure from (Williamson et al., 2013).

Mouse Imprinted Genes, Regions and Phenotypes



genetic obesity coupled with mental retardation, was found in the 1980s to be associated specifically with paternal deletion of a region within human chromosome 15 (Butler et al., 1986; Nicholls et al., 1989b) or with maternal disomy of chromosome 15 (Nicholls et al., 1989a). On the other hand, maternal deletion or paternal disomy of the same region on chromosome 15 causes a clinically distinct developmental syndrome, Angelman syndrome (Knoll et al., 1989; Nicholls et al., 1992). These studies indicated that imprinting occurs in humans, identifying human chromosome 15 as a potential imprinted region. To date, several human diseases have been mapped to imprinted regions (Butler, 2009), and studies of particular human mutations can give us insight into the mechanisms controlling genomic imprinting as well as the consequences of its misregulation.

***Identification of individual imprinted genes*.** Subsequent to the identification of imprinted chromosomal regions, individual imprinted genes that are expressed from either the paternally- or maternally-inherited chromosomes began to be identified. Over 150 individual imprinted genes have now been found in mice (Morison et al., 2001; 2005; Wei et al., 2014), several of which are conserved in humans, and many of which reside in the aforementioned imprinted regions identified by uniparental disomy phenotypes (Figure 1.1). A few different methods have been employed to identify imprinted genes. *Igf2r,* an imprinted gene on chromosome 17, was determined to be paternally expressed because *Igf2r* expression was absent when a 3-5Mb chromosome 17 deletion was inherited paternally (Barlow et al., 1991). Similarly, *Igf2*, an imprinted gene on chromosome 7, showed proper expression when an *Igf2* deletion was

maternally-inherited, but was not expressed when the deletion was paternally-inherited (DeChiara et al., 1991), demonstrating that *Igf2* was a paternally-expressed imprinted gene. Another method used to detect imprinted genes utilized crosses between mouse genetic backgrounds that resulted in single nucleotide polymorphisms (SNPs) within the gene of interest. The non-coding RNA, *H19*, was identified as a maternally-expressed imprinted gene in this manner due to the presence of maternal-specific polymorphic patterns in RNase protection assays (Bartolomei et al., 1991). Many other imprinted genes have been identified based on allele-specific polymorphisms, although RNase protection assays have been replaced by other experimental methods such as restriction fragment polymorphism of reverse transcriptase PCR (RT-PCR) products, Sanger sequencing and quantitative pyrosequencing (Ge et al., 2005; Plass et al., 1996; Yang et al., 2013).

Genome-wide approaches to identify novel imprinted genes. The search for novel imprinted genes is ongoing. Genome-wide approaches for identifying imprinted genes began in the late 1990s and early 2000s with microarrays used to determine expression differences between uniparental disomy and wild type samples (Kaneko-Ishino et al., 1995; Mizuno et al., 2002; Piras et al., 2000). While analysis of uniparental disomy identified several imprinted genes, this approach was limited due to the recovery of many false positives, likely resulting from secondary effects of imprinting misregulation (Ruf et al., 2006). Computational predictions based on common features of imprinted genes have also been attempted, identifying a few more imprinted genes (Brideau et al., 2010; Ke et al., 2002; Luedi et al., 2007; 2005; Yang et al., 2003). More recently,

experimental genome-wide identification of imprinted genes has made use of RNA-seq in F1 hybrids that have SNPs distinguishing between the parental alleles (Gregg et al., 2010; Wang et al., 2008). One study found 400-500 novel imprinted genes in the E15 embryonic brain and 400-500 in the E17 embryonic brain, with only about 50 genes in common between the two datasets (Gregg et al., 2010). Although early RNA-seq studies suffered from high false discovery rates (DeVeale et al., 2012), recent technical and bioinformatic improvements have demonstrated the power of RNA-seq approaches for the discovery of novel imprinted genes (Wang and Clark, 2014). A recent study identified 160 imprinted transcripts in the brain. 41 of these were novel imprinted genes that were independently validated, with only 9 false positives (Perez et al., 2015). Thus, the appropriate application of RNA-seq should facilitate a more complete catalog of all the imprinted genes in mouse, humans, and in non-model organisms.

***Tissue-specific imprinting*.** One challenge to identifying all imprinted loci is that many imprinted genes only display parent-of-origin specific expression in certain tissues, and will thus only be detected if the correct tissue is examined. In 2012, a meta-analysis compiled the published imprinted gene expression patterns in different tissues. The authors found that 23 of 85 imprinted genes (28%) showed tissue-specific imprinting, the majority of which were imprinted only in the brain or in extraembryonic tissues (Prickett and Oakey, 2012). On the other hand, some imprinted genes are monoallelically expressed in most tissues and only bialllelic in a few specific cell types. For example, *Dlk1* is monoallelically expressed in most tissues analyzed, but biallelically expressed in niche astrocytes and neural stem cells of post-natal mice (Ferrón et al.,

2011). Intriguingly, RNA-seq based identification of imprinted genes has identified several genes that, rather than full expression from one parental allele, show expression bias towards one allele (ie. 70% paternal, 30% maternal)(Perez et al., 2015). Since typical RNA-seq studies are done in whole tissues, which represent a variety of cell types, it is tempting to speculate that the observed parental bias is due to full imprinting of the gene in some cell types and biallelic expression in other cell types. Methods for analyzing imprinted expression in single cell types are needed to test this prediction. Recent advances in high-throughput sequencing has made transcriptomics possible at a single cell level, and a couple of publications have detected monoallelic expression of non-imprinted genes in single cells (Borel et al., 2015; Deng et al., 2014). Although it is still unclear whether the low amounts of starting material in single cell transcriptomics can provide reliable identification of monoalllelic expression, future improvements to avoid experimental bias hold great potential for the use of this technique for imprinting studies (Zhang et al., 2015). Single molecule RNA fluorescence in situ hybridization (RNA-FISH) provides another promising method for detecting allele-specific expression in single cells. In a recent study, allele-specific RNA-FISH was able to detect allele-specific expression of *H19* in single cells (Ginart et al., 2016). Thus, the use of single molecule RNA-FISH can reveal whether known imprinted genes are mosaically imprinted within a population of cells. Together, these techniques have great potential for identifying genes that are imprinted only in specific tissues or cell types.

C. Mechanisms of Imprinting Control

Imprinted genes reside in clusters. Two of the first three imprinted genes identified, *Igf2* and *H19*, are neighboring genes on mouse chromosome 7 (Bartolomei et al., 1991; DeChiara et al., 1991). An additional two genes in the same region, *Igf2as* and *Ins2*, were later found to be imprinted (Giddings et al., 1994; Okutsu et al., 2000), constituting a cluster of imprinted genes (Figure 1.2 Ba). As more imprinted genes were discovered, it became clear that the majority of imprinted genes reside in clusters, with 95 imprinted genes found in 14 imprinted gene clusters (Figure 1.1-1.2). Intriguingly, deletion studies of particular regions within imprinted clusters revealed that imprinted genes within a cluster are regulated by a single control element. For example, expression of both *Igf2r* and *Airn*, two imprinted genes within an imprinted cluster on chromosome 17, was disrupted when an intronic CpG island was deleted (Figure 1.2 Aa)(Wutz et al., 1997). Likewise, deletion of a CpG island 5' to the *H19* gene resulted in misregulation of both *H19* and *Igf2* (Figure 1.2 Bb)(Thorvaldsen et al., 1998), and deletion of the *Snrpn* promoter caused disrupted expression of multiple genes within the *Snrpn* imprinted cluster (Figure 1.2 Ad)(Yang et al., 1998). Imprinting control regions (ICRs) have now been identified for many imprinted gene clusters (Table 1.1). Although the mechanisms by which ICRs coordinate imprinting are distinct between clusters, a common theme is the presence of differential DNA methylation between the sperm and oocyte inherited ICR (Table 1.1).

DNA methylation as the imprinting mark. In order to be expressed in a parent-of-origin specific manner, imprinted genes acquire heritable marks that are distinct between the sperm and the oocyte, that are maintained throughout embryogenesis, and

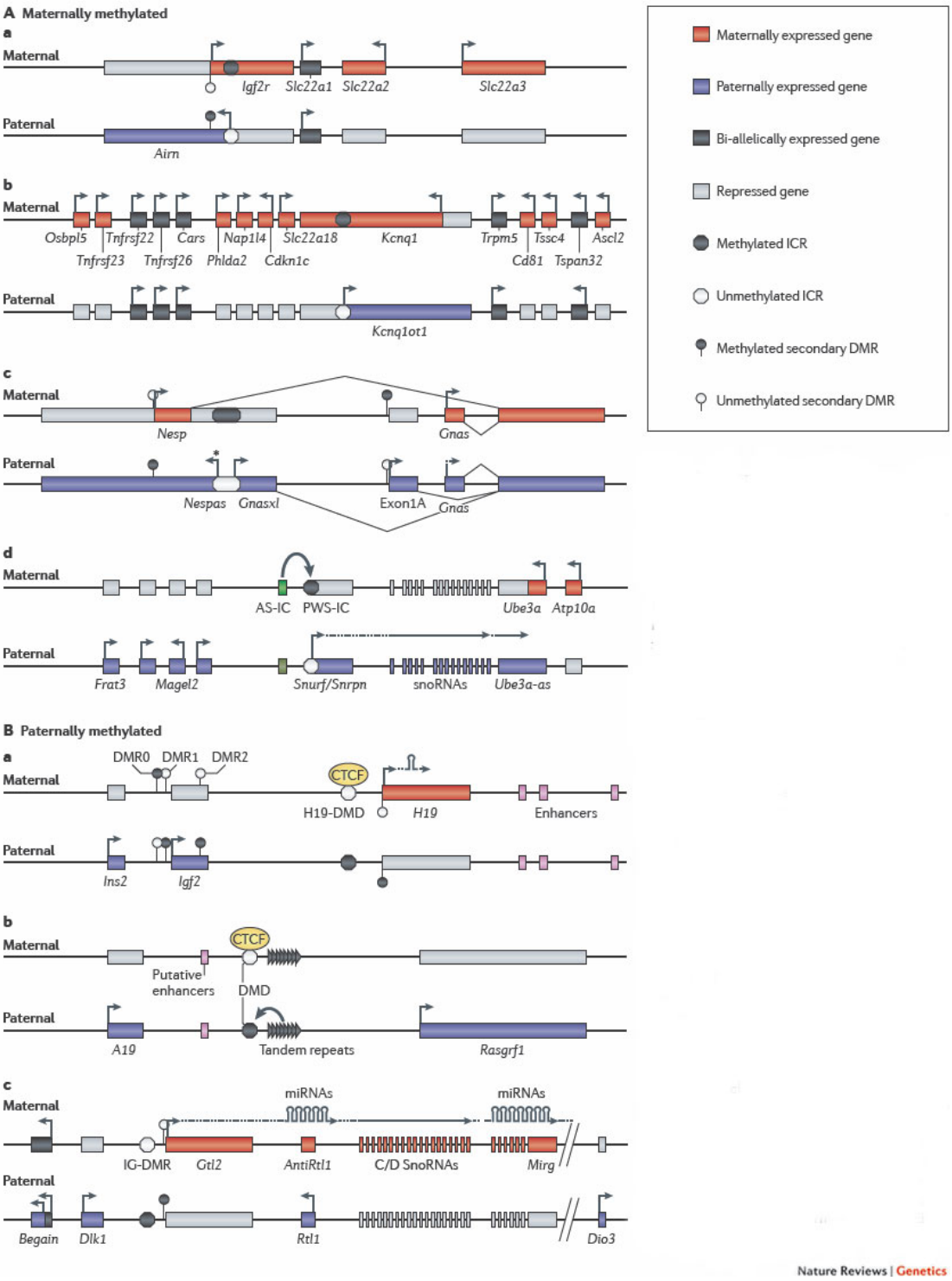


Figure 1.2. Examples of maternally (A) and paternally-methylated (B) imprinted gene clusters. Diagram shows the *Airn* (A.a), *Kcnq1ot1* (A.b), *Gnas* (A.c), *Snrpn* (A. d), *H19* (B.a), *Rasgrf1* (B.b), and *Gtl2* (B.c) imprinted clusters. From (Ferguson-Smith, 2011).

Table 1.1. List of imprinted gDMRs, their location, whether they are paternally or maternally methylated, whether they have been shown to be an imprinting control region (ICR), and whether they bind to ZFP57, as measured by ChIP-seq in ESCs (Quenneville et al., 2011). Table adapted from (Arnaud, 2010).

Table 1.1 (Continued)

Imprinted locus	Location of gDMR	Parental allele of methylation	ICR	Binds ZFP57 (TGCCGC motifs) Quennevile et al., 2011	Reference
Zbdf2	10kb upstream of *Zbdf2* exon 1 (not maintained after fertilization, but reestablished as an sDMR)	Paternal	nd	NO	Duffié et al., 2014 Kobayashi et al., 2012
Zdbf2	*Zdbf2* long isoform promoter	Maternal	nd	YES (3)	Duffié et al., 2014
Mcts2	*Mcts2* promoter/ exon1	Maternal	ICR	YES (3)	Wood et al., 2007
Nnat	*Nnat* promoter (identified in humans)	Maternal	nd	YES (2)	Evans et al., 2001
Gnas	*GnasXL/Nespas* promoter	Maternal	ICR	YES (2)	Coombes et al., 2003 Williamson et al., 2006
Gnas	*Gnas* 1A promoter	Maternal	nd	NO	Lui et al., 2000
Peg10	*Peg10/Scge* promoter	Maternal	nd	YES (1)	Ono et al., 2003
Mest	*Mest* promoter/ exon 1	Maternal	nd	YES (3)	Lucifero et al., 2004
Nap1l5	*Nap1l5* promoter/ exon1	Maternal	nd	YES (3)	Wood et al., 2007
Peg3	*Peg3* promoter/ exon1	Maternal	nd	YES (2)	Kim et al., 2003
Snrpn	*Snrpn* promoter/ exon1	Maternal	ICR	YES (3)	Shemer et al., 1997 Bielinska et al., 2000
Inpp5f	*Inpp5f-V2* promoter/exon1	Maternal	nd	YES (3)	Wood et al., 2007

Table 1.1 (Continued)

Imprinted locus	Location of gDMR	Parental allele of methylation	ICR	Binds ZFP57 (TGCCGC motifs) {Quenneville :2011do}	Reference
H19	2kb upstream of *H19*	Paternal	ICR	YES (2)	Tremblay et al., 1997 Thorvaldsen et al., 1998
Kcnq1	*Kcnq1ot1* promoter	Maternal	ICR	YES (3)	Fitzpatric et al., 2002 Yatsuki et al., 2002
Rasgrf1	30 kb upstream of *Rasgrf1* exon 1	Paternal	ICR	YES (3)	Shibata et al., 1998 Yoon et al., 2005
Zac1	*Zac1* promoter/ exon 1	Maternal	nd	YES (4)	Smith et al., 2002
Grb10	*Grb10* CpG island 2 (brain specific promoter)	Maternal	ICR	YES (1)	Arnaud et al., 2003 Shiura et al., 2009
Zrsr1	*Zrsr* promoter/exon 1	Maternal	nd	YES (2)	Wood et al., 2007
Gtl2	13 kb upstream of *Gtl2* (IG-DMR)	Paternal	ICR	YES (5)	Takada et al., 2000 Lin et al., 2003
Peg13	*Peg13* promoter	Maternal	nd	YES (3)	Ruf et al., 2007
Slc38a4	*Slc38a4* promoter	Maternal	nd	NO	Smith et al., 2003
Airn	*Airn* promoter	Maternal	ICR	YES (2)	Stoger et al., 1993 Wutz et al., 1997
Impact	*Impact* promoter/ exon1	Maternal	nd	YES (2)	Okamura et a., 2000

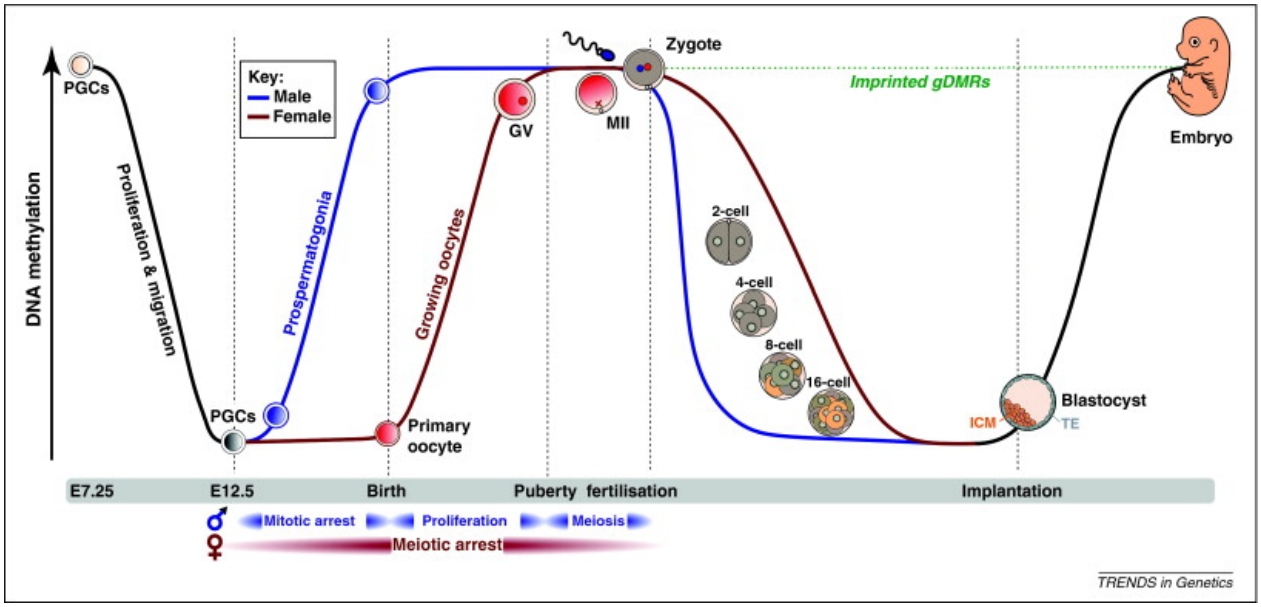


Figure 1.3. DNA methylation dynamics in germ cells and embryos. Over the course of development, the genome undergoes two DNA demethylation events: one during germ cell development as primordial germ cells (PGCs) migrate to the gonadal ridge (left) and one directly after fertilization (right). In germ cells, DNA methylation at imprinted DMRs is erased and then reset in a sperm or oocyte-specific manner. After fertilization, imprinted germline DMRs retain their methylation status. Figure from (Smallwood et al., 2012).

that are capable of regulating transcription. Thus far, DNA methylation is the most widely accepted modification that fits each of these criteria. Most ICRs are located at CpG dense regions that have differential DNA methylation between the sperm and the oocyte (Table 1.1). Furthermore, the mechanism by which DNA methylation can be inherited as DNA replicates is known: the DNA methyltransferase, *Dnmt1*, binds hemimethylated DNA to catalyze addition of DNA methylation to the newly synthesized DNA strand (reviewed in Dhe-Paganon et al., 2011). When *Dnmt1* is deleted, imprinted gene expression is disrupted (Li et al., 1993), supporting that DNA methylation status is capable of controlling imprinted gene expression. The reintroduction of transgenic *Dnmt1* in *Dnmt1*-deficient ESCs cannot restore imprinted gene expression. Rather, imprinting was only restored in these experiments by passage through the germline (Tucker et al., 1996), indicating that DNA methylation is reset during spermatogenesis and oogenesis.

Resetting DNA methylation in the germline. As germ cells develop, they undergo a massive DNA demethylation event where most genomic regions, including ICRs, lose DNA methylation (Figure 1.3). Germ cells then gain sperm- or oocyte-specific DNA methylation at ICRs (Hajkova et al., 2008; Lucifero et al., 2004), a process that depends on the *de novo* DNA methyltransferases, DNMT3A and DNMT3B, and their accessory protein DNMT3L (Bourc'his et al., 2001; Kaneda et al., 2010; 2004; Kato et al., 2007; Smallwood et al., 2011). Whether the DNA methyltransferases are directed to imprinted loci in a sequence-specific manner is unclear for most imprinted clusters. At *Snrpn*, the KRAB zinc finger protein, ZFP57, is required for DNA methylation in the oocyte (Li et al.,

2008). ZFP57 recruits DNA methyltransferases and binds directly to the DNA (Liu et al., 2012; Zuo et al., 2012), providing a mechanism by which *de novo* DNA methylation may be directed to ICRs. However, ZFP57 has been shown to bind specifically to methylated DNA (Liu et al., 2012; Quenneville et al., 2011), leaving it unclear whether ZFP57 directs *de novo* methylation to the unmethylated ICR or binds the already methylated ICR to stabilize DNA methylation. At the *Nnat, Peg3,* and *Mest* imprinted clusters, which also bind ZFP57 (Quenneville et al., 2011), ZFP57 is dispensable for oocyte DNA methylation, indicating that alternative mechanisms direct DNA methylation to these ICRs. In sperm, piRNAs play a critical role for *de novo* DNA methylation of the *Rasgrf1* ICR (Watanabe et al., 2011). The piRNA pathway is also required for DNA methylation at several other genomic locations in sperm (Aravin et al., 2007; Kuramochi-Miyagawa et al., 2008), but there is thus far no evidence that piRNAs mediate *de novo* methylation at the other paternally-methylated ICRs. Thus, with a couple of exceptions, whether specific factors recruit DNA methyltransferases to ICRs in the germline is not known.

Protection of DNA methylation at ICRs during post-fertilization reprogramming. After fertilization, there is a second wave of DNA demethylation, where the majority of DNA methylation inherited from the sperm and oocyte is erased (Kafri et al., 1993). In contrast to germ cell demethylation, DNA methylation at ICRs is maintained during post-fertilization methylation reprogramming (Smallwood et al., 2011). Prior to reprogramming, hundreds of germline differentially methylated regions (gDMRs) exist between the sperm and the oocyte genomes (Kobayashi et al., 2012; Smallwood et al., 2011; ZD et al., 2012). Many of these gDMRs are not associated with imprinted genes

and are erased during post-fertilization reprogramming. Therefore, the distinguishing feature of imprinted gDMRs is not necessarily that they are differentially methylated between the sperm and the oocyte, but that they retain their differential methylation during early embryonic development. A few proteins, such as DPPA3, DNMT1, and ZFP57, have been described to help protect imprinted gDMRs from loss of DNA methylation during post-fertilization reprogramming (Gu et al., 2011; Li et al., 2008; Nakamura et al., 2007; 2012). However, the exact mechanisms by which DNA methylation is maintained specifically at imprinted gDMRs is still not fully understood (discussed further in Chapter 2).

Establishment of secondary DMRs and protection from* de novo *DNA methylation. After the completion of post-fertilization reprogramming, there is a wave of *de novo* DNA methylation, which results in overall gain of genomic DNA methylation (Figure 1.3). During this time, secondary differentially methylated regions (sDMRs) acquire DNA methylation in an allele-specific manner. How DNA methylation is directed to sDMRs is not known. However, one theory is that DNA methylation at sDMRs occurs “by default” as the embryo is in an environment of methylation acquisition, and differential DNA methylation is achieved at sDMRs by protecting the unmethylated allele from *de novo* DNA methylation. A few lines of evidence support that unmethylated gDMRs and sDMRs may require protection from *de novo* DNA methylation. In embryonic stem cells (ESCs), the unmethylated maternal allele of the *Gtl2* gDMR depends on PRC2 to prevent *de novo* methylation (Das et al., 2015). Additionally, the unmethylated maternal *Gtl2* promoter sDMR requires the presence of the maternal gDMR to prevent sDMR

methylation (Lin et al., 2003). Whether DMRs at other genomic locations also require specific factors to maintain their unmethylated status is not clear. However, the *Zbdf2* imprinted locus contains a gDMR that does not resist *de novo* methylation under wild type conditions (Duffié et al., 2014). This gDMR is maintained throughout post-fertilization reprogramming, but during the *de novo* methylation phase gains methylation on the previously unmethylated allele, eliminating its differentially methylated status. Prior to methylation acquisition at the *Zdbf2* gDMR, a sDMR acquires allele-specific DNA methylation, which could contribute to the maintenance of imprinting at *Zbdf2*. Since the *Zbdf2* gDMR gains methylation during *de novo* methylation while other imprinted gDMRs and sDMRs retain their unmethylated status, comparing the factors that function at these different DMRs may give insights into the mechanisms that confer protection from *de novo* DNA methylation.

Mechanisms by which DNA methylation controls imprinted expression. Although DNA methylation is required on a global level to control the expression of imprinted genes (Li et al., 1993), the exact mechanisms by which DNA methylation regulates imprinted gene expression, or even the requirements of differential DNA methylation at particular genomic loci, has not been worked out at all imprinted DMRs. In general, the presence of DNA methylation is capable of changing which factors are recruited to the DNA (Tate and Bird, 1993). Many gDMRs and sDMRs are located at gene promoters (Table 1.1), where there is typically a negative correlation between DNA methylation and transcription (Holliday and Pugh, 1975; Jones and Takai, 2001; Riggs, 1975; Shen et al., 2007; Wolffe and Matzke, 1999). Whether DNA methylation is a cause or

consequence of transcriptional repression may differ depending on the sequence context and transcription factor binding landscape. It has been shown that the binding of transcription factors to DNA can be inhibited by the presence of DNA methylation (Choy et al., 2010; Perini et al., 2005; Tate and Bird, 1993), indicating an instructive role for DNA methylation in repressing transcription. However, a causal role of DNA methylation for repressing transcription at imprinted DMRs has only been suggested for a couple of imprinted gene promoters. For example, the *Peg3* gDMR binds the transcription factor YY1 specifically at the unmethylated paternal allele, but not at the methylated maternal allele (Kim et al., 2003). Mutations of YY1 binding sites at the *Peg3* gDMR disrupt gene expression (Kim et al., 2008), indicating that YY1 may interpret methylation status to control imprinted gene expression. However, binding of YY1 to the unmethylated allele is correlational and there is still no direct evidence that DNA methylation directly inhibits YY1 binding at the *Peg3* gDMR. Thus, while DNA methylation can prevent transcription factors from accessing DNA at some genomic contexts, whether DNA methylation has this function at all imprinted promoter DMRs is unclear.

DNA methylation can also recruit transcriptional repressors, such as MBD2, MeCP2 and ZFP57, that specifically bind to methylated CpGs (Hendrich and Bird, 1998; Nan et al., 1998; 1996; Wade et al., 1999; Quenneville et al., 2011; Liu et al., 2012). MeCP2 has been implicated in the regulation of the *Ube3a* and *Dlx5* imprinted genes, but its requirement for controlling imprinted expression of these genes is controversial (reviewed in LaSalle, 2007), and MeCP2 has not be found to control expression of other imprinted genes. ZFP57 binds the methylated allele of all known ICRs (Quenneville et al., 2011) and, through its interactions with TRIM28 (Li et al., 2008; Quenneville et al.,

2011), recruits a number of repressive chromatin modifying proteins (Frietze et al., 2010; Ivanov et al., 2007; Lechner et al., 2000; Nielsen et al., 1999; Ryan et al., 1999; Schultz et al., 2001; 2002; Sripathy et al., 2006). ZFP57 binds almost exclusively to gDMRs and a few dozen other genomic loci, but not to sDMRs (Quenneville et al., 2011), and therefore cannot account for the potential effects of DNA methylation on repression at sDMRs. Additionally, because ZFP57 disrupts DNA methylation at gDMRs (Li et al., 2008), it has not been determined whether ZFP57 also has important functions downstream of DNA methylation to control imprinting. Taken together, there is still no clear evidence that the recruitment of transcriptional repressors by methylated DNA is essential for the regulation of imprinted gene expression.

While DNA methylation at promoters is typically associated with repression, transcription can also occur independently of DNA methylation (Bird, 1984; Eckhardt et al., 2006; Weber et al., 2007). Methylated DNA can also sometimes recruit transcriptional activators (Rishi et al., 2010). Therefore, we cannot make the assumption that DNA methylation has repressive functions at all promoter DMRs. From deletion studies that reduce DNA methylation, it is clear that DNA methylation is critical for imprinted gene expression (Li et al., 1993). Yet, because of the global nature of DNA methylation loss in these mutants, it is not clear whether DNA methylation at every promoter DMR is critical for imprinting or may be a consequence of imprinting. With recent CRSPR/Cas9 genome targeting technologies, it may be possible to direct DNA methylation or DNA demethylation to specific DMRs, and thus isolate the requirements of DNA methylation.

There are a few DMRs that are located at intergenic regions (Table 1.1), where DNA methylation can have distinct roles compared to promoters. The *H19* gDMR is located 5kb upstream of *H19* and contains two regulatory elements: an insulator that blocks enhancer access to *Igf2* on the maternal allele and a repressor that inhibits *H19* transcription from the paternal allele (Drewell et al., 2000; Srivastava et al., 2000). The repressor element of the *H19* gDMR is capable of repressing a transgene reporter in *Drosophila*, which do not have extensive DNA methylation (Brenton et al., 1999; Lyko et al., 1997). Thus, the repressive activities of the methylated *H19* gDMR may be independent of DNA methylation. On the other hand, the insulator function of the unmethylated *H19* gDMR depends on binding of CTCF, which binds specifically to the unmethylated maternal allele and recognizes unmethylated, but not methylated, DNA binding sites *in vitro* (Bell and Felsenfeld, 2000; Hark et al., 2000; Kurukuti et al., 2006). Thus, CTCF binding to the *H19* gDMR is one clear example of a methylation-sensitive binding protein influencing imprinted gene expression. CTCF has similar functions at the *Rasgrf1* gDMR, where it binds the unmethlated maternal gDMR and blocks access of an enhancer to the *Rasgrf1* promoter (Yoon et al., 2005). However, CTCF-mediated enhancer blocking at intergenic DMRs is not universal. The *Gtl2* intergenic gDMR does not require CTCF for imprinted expression (Lin et al., 2011), and the exact mechanisms by which DNA methylation at the *Gtl2* gDMR controls imprinting are still unclear.

The unmethylated *Gtl2* gDMR has recently been shown to have characteristics of an active enhancer (Das et al., 2015). It has recently been shown that the protein AFF3 binds an enhancer element on the unmethylated maternal gDMR and is essential for activation of maternally-expressed imprinted genes (Luo et al., 2016). In ZFP57 null

ESCs, which have reduced DNA methylation and altered histone modifications (Quenneville et al., 2011), AFF3 binding to the enhancer region increases, suggesting that AFF3 may bind to both parental alleles in the absence of ZFP57 (Luo et al., 2016). These data point to a potential model whereby ZFP57 recognizes the methylated paternal *Gtl2* gDMR and inhibits enhancer activity by blocking AFF3 binding. However, because DNA methylation and histone modifications are both disrupted in ZFP57 null ESCs, it is unclear whether the observed changes in AFF3 binding were directly due to loss of ZFP57 or to the consequent chromatin changes.

Mechanisms other than DNA methylation control imprinted expression at some genes. While DNA methylation is the most well-supported imprinted mark, there are some cases where DNA methylation seems to have a reduced role. In mutants deficient in the DNMT1 catalytic domain (*Dnmt*$^{c/c}$), many imprinted genes are inappropriately expressed from both parental alleles. However, several genes within the *Kcnq1* cluster, *Osbpl5*, *Tssc4, Cd81*, and *Ascl2*, are properly imprinted in mutant *Dnmt*$^{c/c}$ placenta (Caspary et al., 1998; Green et al., 2007; Lewis et al., 2004; Tanaka et al., 1999). Intriguingly, imprinting of these genes is disrupted when the embryo fails to inherit DNA methylation from the oocyte (Green et al., 2007). Thus, at these genes, DNA methylation is required to establish imprinted expression, but other mechanisms are sufficient for imprinting maintenance.

Concluding Remarks

Over the course of development, imprinted loci overcome a number of challenges in order to maintain their imprinted status. Understanding the mechanisms by which imprinting is controlled, particularly in the context of dynamic changes that occur during embryogenesis, will inform us not only about how this critical class of genes is regulated, but will also provide general insights into how transcription is controlled and how transcriptional states can be inherited from the germline.

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CHAPTER 2

TRIM28 CONTROLS IMPRINTING THROUGH DISTINCT MECHANISMS DURING AND AFTER EARLY GENOME-WIDE REPROGRAMMING[1]

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Minor modifications have been made.
Contributions: Shibata M performed preliminary experiments showing that zygotic TRIM28 regulates imprinting expression. Wang X performed quantitative pyrosequencing.

ABSTRACT

Genomic imprinting depends on the establishment and maintenance of DNA methylation at imprinting control regions. However, the mechanisms by which these heritable marks influence allele-specific expression are not fully understood. By analyzing maternal, zygotic, maternal-zygotic, and conditional *Trim28* mutants, we found that the transcription factor TRIM28 controls genomic imprinting through distinct mechanisms at different developmental stages. During early genome-wide reprogramming, both maternal and zygotic TRIM28 are required to maintain methylation at germline imprints. However, in conditional *Trim28* mutants, *Gtl2* imprinted gene expression was lost despite normal methylation levels at the germline *IG-DMR*. These results provide evidence that TRIM28 controls imprinting after early embryonic reprogramming through a mechanism other than the maintenance of germline imprints. Additionally, our finding that secondary imprints were hypomethylated in TRIM28 mutants uncovers a requirement of TRIM28 after genome-wide reprogramming for interpreting germline imprints and regulating DNA methylation at imprinted gene promoters.

INTRODUCTION

Genomic imprinting is a process that regulates the allele-specific expression of certain genes depending on their maternal or paternal inheritance. To date, over a hundred genes have been described to be imprinted and defects in their expression have been associated with cancer and congenital disorders in humans (Lee and Bartolomei, 2013). Imprinted genes generally reside in gene clusters where their expression is controlled by regulatory sequences that are differentially methylated between the maternally and paternally-inherited chromosomes. Two types of these differentially methylated regions (DMRs), germline and secondary DMRs, have been identified in mammals (reviewed in Ferguson-Smith, 2011). DNA methylation at germline DMRs is established during gametogenesis and is later maintained in the zygote after fertilization (Ferguson-Smith, 2011). In contrast, allele-specific methylation at secondary DMRs is established only after fertilization (Bartolomei et al., 1993; Bhogal et al., 2004; Brandeis et al., 1993; Ferguson-Smith et al., 1993; Szabo and Mann, 1995; Tremblay et al., 1997; 1995) and is dependent on imprinting marks at germline DMRs (Lin et al., 2003; Lopes et al., 2003; Srivastava et al., 2003; 2000).

Once established, methylation at germline and secondary DMRs is maintained in somatic cells throughout embryonic development (reviewed in Messerschmidt et al., 2014). First, germline DMRs resist the genome-wide DNA demethylation event that reprograms the oocyte- and sperm-derived genomes shortly after fertilization (Kafri et al., 1993). During this process, germline DMRs are protected from enzymatic removal of DNA methylation that characterizes the early stages of genome-wide reprogramming. Additionally, both during and after genome-wide reprogramming, DNA methylation at

DMRs is maintained to prevent the replication-dependent dilution of methyl marks as the zygote starts to divide (Shen et al., 2014). Studies in mice have identified a few molecular mechanisms that prevent loss of DNA methylation at imprinted DMRs. The *developmental pluripotency-associated 3* gene (*Dppa3*, also known as *stella* or *PGC7*) encodes a DNA binding protein that is maternally required to protect imprinted loci from enzymatic demethylation during genome-wide reprogramming (Bian and Yu, 2014; Gu et al., 2011; Nakamura et al., 2007; 2012). Additionally, DNA methyltransferase 1 (DNMT1) is required both maternally and zygotically to prevent replication-dependent loss of methylation at DMRs (Hirasawa et al., 2008).

While many studies support a central role for DNA methylation in imprinting control, the mechanisms by which these heritable chromatin marks are interpreted to regulate allele-specific expression are still not entirely understood. The prevailing view is that differential methylation between the maternal and paternal germline DMRs influences the allele-specific recruitment of factors that function in cis to influence transcription. However, while a few proteins are known to bind specifically to either methylated DNA or unmethylated DMRs, some of these proteins are not required for imprinting (Filion et al., 2006; Hendrich et al., 2001; Monnier et al., 2013), and others only control imprinting at specific imprinted clusters (Balmer et al., 2002; Bell and Felsenfeld, 2000; Carr et al., 2007; Hark et al., 2000; Holmgren et al., 2001; Horike et al., 2005; Kanduri et al., 2000; Samaco et al., 2005; Szabo et al., 2004; Szabó et al., 2000). Therefore, the current data argues that rather than a universal mechanism to recognize methylated DNA, these epigenetic marks are interpreted in a locus-specific fashion to control transcription of nearby genes.

The transcriptional repressor TRIM28, also known as KAP1 and TIF1ß, is required for genomic imprinting (Lorthongpanich et al., 2013; Messerschmidt et al., 2012; Quenneville et al., 2011). TRIM28 has been described to bind to the methylated allele of all known imprinting control regions, a recruitment that is dependent on the KRüppel Associated Box (KRAB) domain zinc finger protein ZFP57 (Li et al., 2008; Quenneville et al., 2011). However, the molecular mechanisms by which TRIM28 controls imprinting are still unclear. TRIM28 interacts with a variety of effector proteins, including Heterochromatin protein 1 (Lechner et al., 2000; HP1; Nielsen et al., 1999; Ryan et al., 1999; Sripathy et al., 2006), histone deacetylases (Schultz et al., 2001), histone methyltransferases (Frietze et al., 2010; Ivanov et al., 2007; Schultz et al., 2002) and the DNA methyltransferases, DNMT1, DNMT3a and DNMT3b (Quenneville et al., 2011; Zuo et al., 2012). Whether one or more of these effectors is recruited by TRIM28 to control imprinting is not known. However, loss of function conditions for *Zfp57* or *Trim28* cause loss of DNA methylation and altered histone modifications at germline DMRs (Li et al., 2008; Lorthongpanich et al., 2013; Messerschmidt et al., 2012; Quenneville et al., 2011), indicating that these factors can, directly or indirectly, maintain epigenetic marks that preserve the imprinted status. Based on the facts that maternal depletion of TRIM28 causes loss of germline DNA methylation (Lorthongpanich et al., 2013; Messerschmidt et al., 2012) and that the zygote relies on maternally-deposited proteins during the early stages of genome-wide reprogramming, it has been proposed that TRIM28 functions by protecting imprinted loci from DNA demethylation during this early reprogramming event (Messerschmidt et al., 2012). However, despite the fact that TRIM28 binds to all known germline imprints, depletion of maternal TRIM28 is only

known to disrupt imprinting with variable penetrance at some imprinted clusters (Lorthongpanich et al., 2013; Messerschmidt et al., 2012). Variable effects on imprinting were also observed in *Zfp57* mutants, but the simultaneous loss of maternal and zygotic *Zfp57* caused more drastic effects than either mutant condition alone (Li et al., 2008), suggesting that effective maintenance of germline imprints requires both maternal and zygotic ZFP57.

To address the requirements of maternal and zygotic TRIM28 for genomic imprinting at different embryonic stages, we analyzed imprinted gene expression and DMR methylation in maternal, zygotic, maternal-zygotic and conditional *Trim28* mutants. Results from these studies showed that zygotic *Trim28* is required to control imprinting at many imprinted loci, including imprinted clusters that were not previously identified in embryos depleted of maternal *Trim28*. Consistent with previous studies, our results support a role for maternal and zygotic TRIM28 in the maintenance of DNA methylation at germline DMRs during early embryonic reprogramming. Surprisingly, we also found that loss of TRIM28 at later embryonic stages disrupted allele-specific gene expression without affecting germline DMR methylation, providing evidence that TRIM28 controls imprinting through a molecular mechanism that is distinct from its role to preserve germline imprints during genome-wide reprogramming. Our analysis of conditional *Trim28* mutants also revealed hypomethylation at the *H19* and *Gtl2* promoters. Together, our results provide insight into the *in vivo* requirements of TRIM28 and the mechanisms that govern allele-specific expression of imprinted genes at different stages of embryonic development.

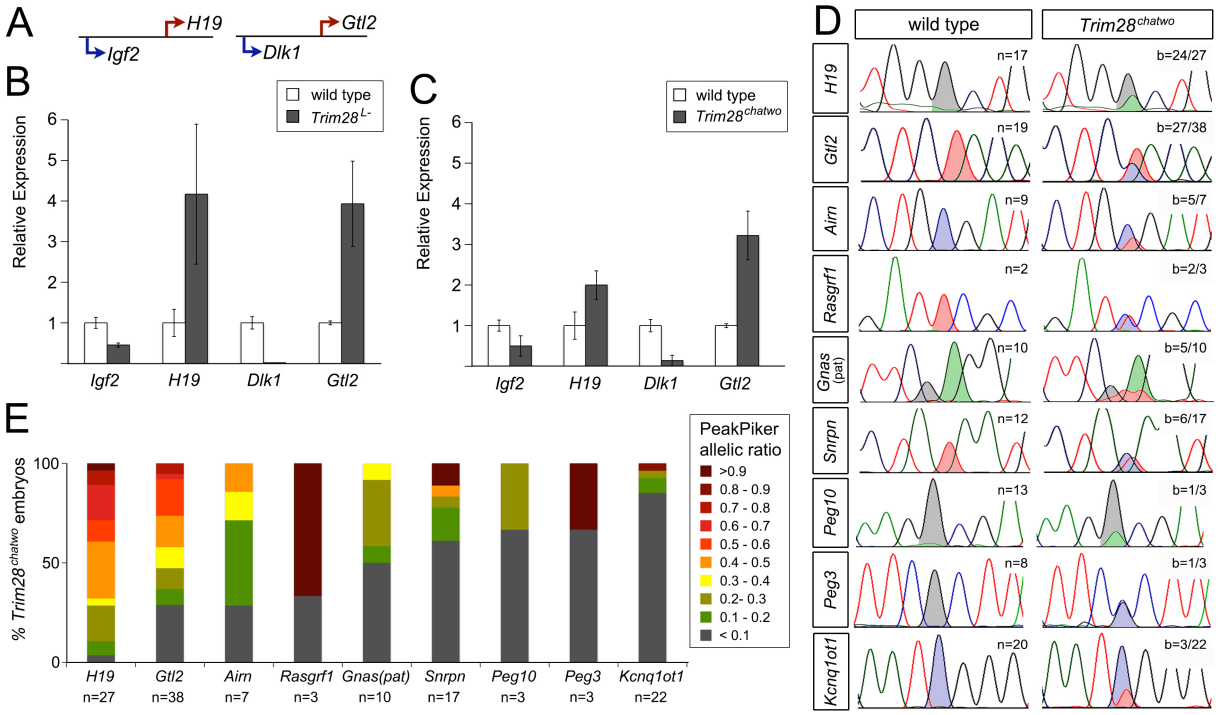


Figure 2.1. Imprinted gene expression in zygotic *Trim28* mutants. (**A**) Diagram of the *Igf2-H19* and *Dlk1-Gtl2* clusters, indicating maternally (red) and paternally (blue) expressed genes. (**B-C**) Expression of imprinted genes in the *Igf2-H19* and *Dlk1-Gtl2* clusters as determined by qRT-PCR in pools of 3-4 E7.5 *Trim28*$^{L-}$ (**B**) and E8.5 *Trim28*^{chatwo} (**C**) embryos. Data shown is normalized to *ß-actin* and relative to wild type controls. Error bars represent the standard deviation from two biological replicates. (**D**) Selected Sanger sequencing traces of cDNAs for *H19*, *Gtl2, Airn, Rasgrf1, Gnas* (paternal isoform), *Snrpn, Peg10, Peg3,* and *Kcnq1ot1* in individual E8.5 wild type and *Trim28*^{chatwo} embryos containing allele-specific SNPs (shaded peaks). All imprinted genes were analyzed in embryonic tissues, except for *Rasgrf1,* which is only imprinted in E8.5 extraembryonic tissues (Dockery et al., 2009). b = embryos with biallelic expression over the total number of embryos analyzed. (**E**) Percent of *Trim28*^{chatwo} embryos with biallelic expression of imprinted genes as analyzed by Sanger sequencing and quantified using PeakPicker. Wild type embryos showed PeakPicker allelic ratios between 0-0.1. Values higher than 0.1 were considered biallelic. A value of 1 corresponds to equal expression from both alleles. n = total number of embryos analyzed.

RESULTS

Zygotic TRIM28 is required for proper allelic expression of many imprinted genes.

To determine whether zygotic TRIM28 is required for genomic imprinting, we evaluated imprinted gene expression in *Trim28* null embryos (*Trim28*$^{L-}$; Cammas et al., 2000), and in homozygote mutants for *Trim28*^{chatwo}, a strong hypomorphic allele that causes developmental arrest at E8.5 and disrupts the protein stability and repressive activity of TRIM28 (Shibata et al., 2011). We first used quantitative RT-PCR (qRT-PCR) to test the levels of expression of imprinted genes in the *Igf2-H19* and *Dlk1-Gtl2* clusters (Figure 2.1 A). This analysis revealed that the maternally-expressed genes, *H19* and *Gtl2*, were upregulated in both *Trim28*$^{L-}$ and *Trim28*^{chatwo} mutants, while the respective paternally-expressed genes from these clusters, *Igf2* and *Dlk1*, were downregulated compared to wild type littermate controls (Figure 2.1 B-C).

To resolve whether abnormal *H19* and *Gtl2* expression levels in zygotic *Trim28* mutants were due to inappropriate biallelic expression, we sequenced cDNAs from embryos that contained single nucleotide polymorphisms (SNPs) distinguishing between the maternal and paternal alleles. While E8.5 wild type embryos expressed imprinted genes monoallelically, *Trim28*^{chatwo} embryos showed biallelic expression of *H19 and Gtl2*, as well as *Airn, Rasgfr1*, *Gnas* (paternal isoform), *Snrpn*, *Peg10, Peg3* and *Kcnq1ot1* (Figure 2.1 D). These results demonstrate that expression of *Trim28* from the zygotic genome is required for allele-specific expression at many imprinted loci. Notably, our results show that loss of zygotic TRIM28 disrupts imprinted expression of *Gtl2*, which was previously only found to be marginally affected by loss of maternal

TRIM28 (Messerschmidt et al., 2012). Thus, the analysis of *Trim28*^{chatwo} mutants shows that TRIM28 has widespread requirements for controlling imprinting.

Loss of imprinting is fully penetrant in maternal-zygotic *Trim28* mutants.

Our analysis of allele-specific imprinted gene expression in single embryos revealed that loss of imprinting was partially penetrant in zygotic *Trim28*^{chatwo} mutants (Figure 2.1 D-E; Figure 2.2 A-C, column 4). This partial penetrance was not due to the hypomorphic nature of the *chatwo* mutation, since *Trim28*$^{L-}$ mutants also showed partially penetrant loss of imprinting (Figure 2.2 A-C, column 5). We hypothesized that maternal TRIM28, which is present during the early development of zygotic *Trim28* mutants, may account for the partial penetrance of imprinting defects in *Trim28*$^{L-}$ and *Trim28*^{chatwo} embryos. To test this hypothesis, we generated embryos lacking both maternal and zygotic *Trim28* (*mzTrim28* mutants).

Maternal depletion of *Trim28* was accomplished by using a conditional allele of *Trim28* (*Trim28*^{L2}; Cammas et al., 2000) in combination with the *ZP3-Cre* transgene, which expresses *Cre*-recombinase from the oocyte-specific *Zona pellucida 3* promoter (de Vries et al., 2000). Mutants lacking both maternal and zygotic TRIM28 (*mzTrim28*$^{L-}$) arrested before implantation at the mid-blastocyst stage (Figure 2.2D). Some of these mutants formed a blastocele cavity similar to that of wild type blastocysts, but showed morphological abnormalities as compared with littermate controls, including slightly larger cells in the inner cell mass (n=5/16, Figure 2.2 F-F'). Other *mzTrim28*$^{L-}$ embryos failed to cavitate (n=11/16) and occasionally displayed fragmented nuclei characteristic

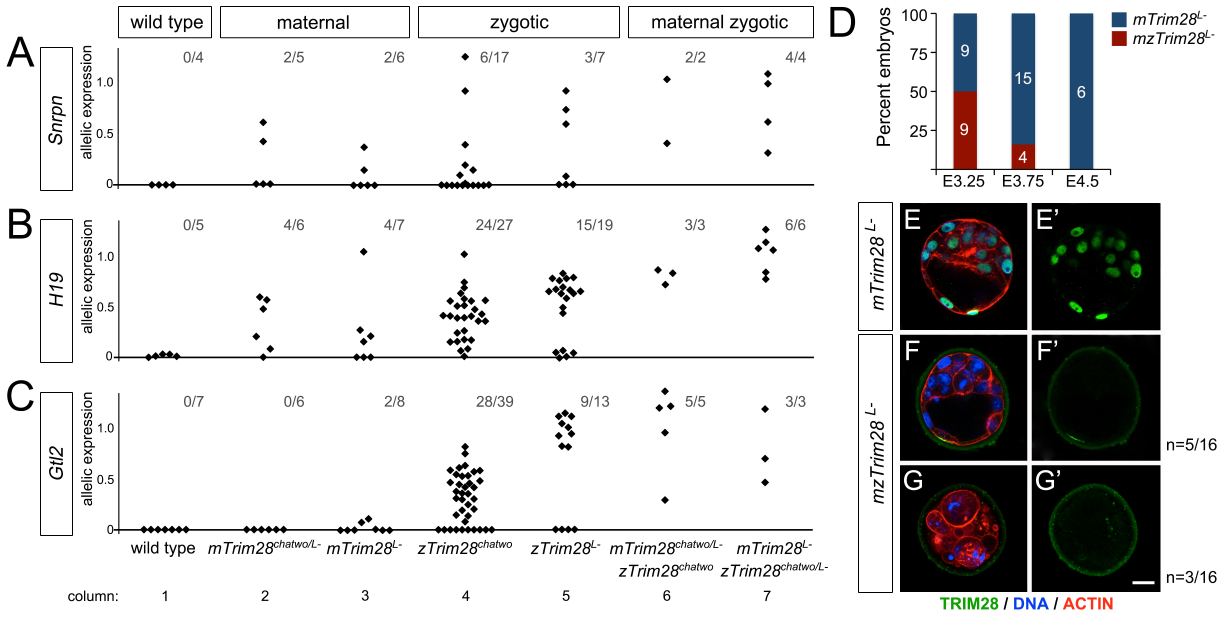


Figure 2.2. Analysis of maternal, zygotic, and maternal-zygotic *Trim28* mutants. Allelic expression in wild type, maternal (*mTrim28*$^{chatwo/L-}$ and *mTrim28*$^{L-}$), zygotic (*zTrim28*^{chatwo} and *zTrim28*$^{L-}$), and hypomorphic maternal-zygotic (*mTrim28*$^{L-}$ - *zTrim28*$^{chatwo/L-}$ and *mTrim28*$^{chatwo/L-}$ - *zTrim28*^{chatwo}) *Trim28* mutants was analyzed at *Snrpn* (**A**), *H19* (**B**), and *Gtl2* (**C**) by Sanger sequencing and quantified with PeakPicker. Each diamond represents a single embryo. The fractional numbers indicate the number of mutants with biallelic expression over the total number of embryos analyzed. All embryos were analyzed at E8.5 except for *zTrim28*$^{L-}$ and hypomorphic maternal-zygotic mutants, which were analyzed at E7.5. Analysis of wild type samples at E7.5 and E8.5 showed similar results (not shown). (**D**) Percentage of *mTrim28*$^{L-}$ (blue) and *mzTrim28*$^{L-}$ (red) mutants found in dissections at E3.25 (n=18), E3.75 (n=19), and E4.5 (n=6). (**E-G**) Fluorescence staining of TRIM28 (green), DNA (DAPI, blue), and ACTIN (phalloidin, red) in *mTrim28*$^{L-}$ (**E**) and *mzTrim28*$^{L-}$ (**F-G**) blastocysts. TRIM28 localization (green channel) is shown separately in E'-G'. Scale bar = 20μm.

of cell death (n=3/16, Figure 2.2 G-G'). While the early lethality of these embryos prevented the analysis of imprinted gene expression in *mzTrim28*$^{L-}$ mutants, we found that embryos completely lacking maternal *Trim28* and carrying the hypomorphic *Trim28*^{chatwo} allele zygotically (*mTrim28*$^{L-}$ - *zTrim28*$^{chatwo/L-}$ embryos), or embryos carrying the *Trim28*^{chatwo} allele maternally and zygotically (*mTrim28*$^{chatwo/L-}$ - *zTrim28*^{chatwo} embryos), survived past implantation and had a morphology and developmental arrest similar to that of zygotic *Trim28*$^{L-}$ mutants in dissections at E7.5. These two allelic combinations, from here onwards referred to as hypomorphic *mzTrim28* mutants, were used to analyze the effects of loss of maternal and zygotic *Trim28* on imprinted gene expression.

In contrast to the partially penetrant biallelic expression of *Snrpn*, *H19*, and *Gtl2* observed in maternal or zygotic *Trim28* mutants (Figure 2.2A-C, columns 2-5), we found that simultaneous depletion of maternal and zygotic *Trim28* in hypomorphic *mzTrim28* embryos disrupted imprinting in all embryos analyzed (Figure 2.2A-C, columns 6-7; *Snrpn* n=6; *H19* n=9; *Gtl2* n=8). One possible explanation for the complete penetrance of imprinting defects in hypomorphic *mzTrim28* mutants is that the amount of TRIM28 during the maternal to zygotic transition is critical for maintaining genomic imprinting during genome-wide reprogramming. In this scenario, loss of maternal TRIM28 could be partially compensated by zygotic TRIM28 and vice versa. However, it is also possible that the effects of lack of maternal and zygotic TRIM28 are additive, and represent separate requirements for TRIM28 during early embryonic reprogramming and during later stages of embryogenesis. To resolve the specific roles of TRIM28 at different

developmental stages, we analyzed the effects of *Trim28* depletion on DMR methylation in zygotic and conditional *Trim28* mutants.

DNA methylation at germline DMRs is disrupted in zygotic *Trim28* mutants.

Because maternal TRIM28 has been previously implicated in the protection of germline DMRs from demethylation during early genome-wide reprogramming (Messerschmidt et al., 2012), we sought to evaluate whether zygotic TRIM28 is also required to maintain DNA methylation at germline DMRs. To this end, we used combined restriction-bisulfite analysis (COBRA), bisulfite sequencing, and quantitative pyrosequencing on $Trim28^{L-}$ and $Trim28^{chatwo}$ embryos.

COBRA analysis showed loss of DNA methylation at the *H19, Snrpn* and *Gtl2* germline DMRs in null $Trim28^{L-}$ mutants (Figure 2.3 A-C) suggesting that, similar to maternal TRIM28, zygotic TRIM28 is also required to preserve DNA methylation at germline DMRs. Consistent with the variable penetrance of imprinting defects in $Trim28^{L-}$ mutants (Figure 2.2 A-C), our COBRA experiments showed that hypomethylation at the *H19, Snrpn* and *Gtl2* germline DMRs was also variable, with some $Trim28^{L-}$ embryos showing complete lack of methylation, while others only had partial or no effects (Figure 2.3 A-C). To further analyze the relationship between loss of germline DNA methylation and imprinted gene expression, we used pyrosequencing to quantify the allele expression ratio and germline DNA methylation level in an extensive collection of individual $Trim28^{chatwo}$ mutants.

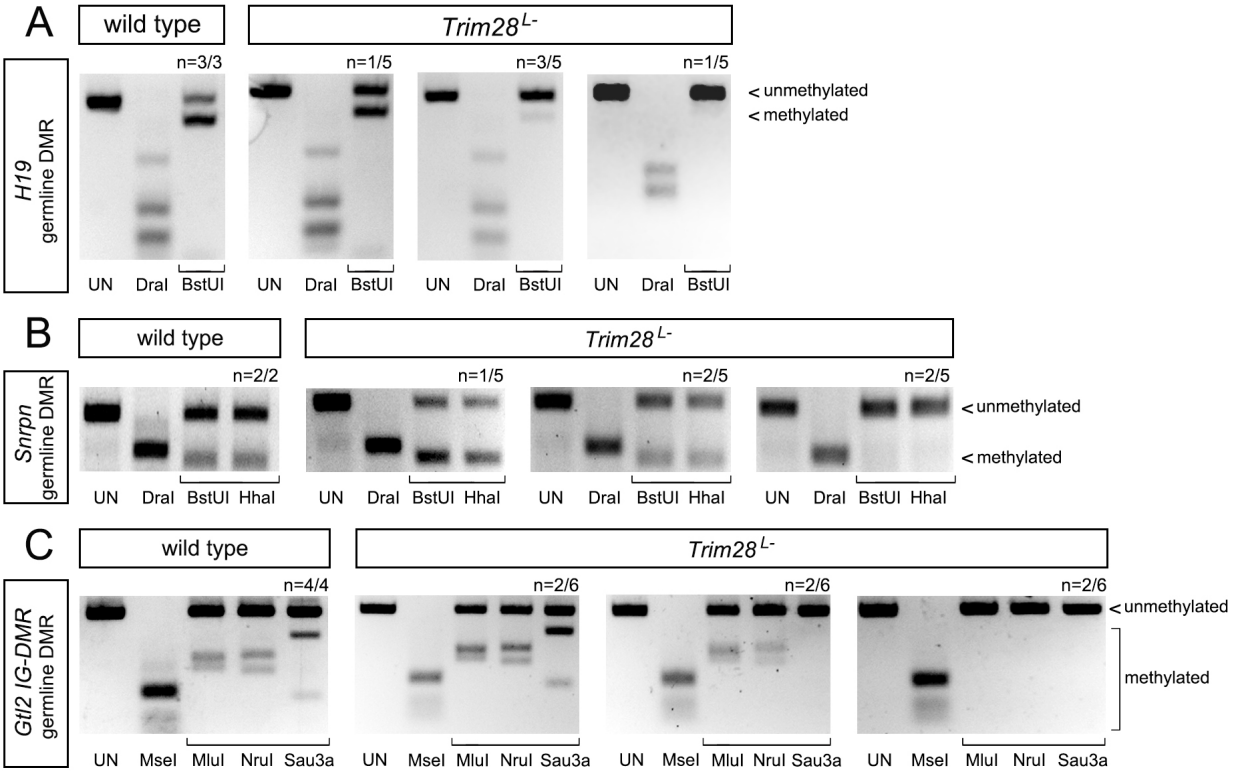


Figure 2.3. DNA methylation at the *H19* and *Gtl2* germline DMRs in *Trim28* null embryos. DNA methylation at the *H19* (**A**), *Snrpn* (**B**) and *Gtl2* (**C**) germline DMRs was detected by combined restriction-bisulfite analysis (COBRA) in single E7.5 wild type and *Trim28*$^{L-}$ embryos. n = number of embryos showing results similar to the one shown, relative to the total number of embryos analyzed. Restriction with DraI (A-B) and MseI (C) measured the efficiency of bisulfite conversion. All other restriction enzymes (lanes with brackets) only cut if the original sample was

Consistent with the hypomorphic nature of the *Trim28*^{chatwo} allele and with the variability of allele expression ratios in these mutants (Figure 2.2 A-C), we found that *Trim28*^{chatwo} embryos showed partial loss of methylation at the *H19* and *Snrpn* germline DMRs (Figure 2.4 A-B, Figure 2.5 A-B, Figure 2.6). The extent of hypomethylation correlated with the ratio of *H19* biallelic expression in a collection of twenty-six individual *Trim28*^{chatwo} embryos (Figure 2.5 A; $p<0.05$), supporting that abnormal *Igf2-H19* imprinting in *Trim28*^{chatwo} mutants was caused by loss of DNA methylation at the *H19* germline DMR. Similarly, *Snrpn* allelic expression ratios also correlated with loss of methylation at the germline DMR (Figure 2.5 B; $p=0.001$). In contrast, we found that the levels of *Gtl2* germline DMR (*IG-DMR*) methylation in hypomorphic *Trim28*^{chatwo} mutants were not significantly different from those of wild type littermates as analyzed with either COBRA (Figure 2.4 C-D) or bisulfite sequencing (Figure 2.5 C-D, Figure 2.6). Given that methylation of the *IG-DMR* was disrupted in null *Trim28*$^{L-}$ mutants (Figure 2.3 C), the lack of effects in *Trim28*^{chatwo} embryos indicates that this hypomorphic allele provides enough TRIM28 function to allow proper *IG-DMR* methylation maintenance. This result was nevertheless intriguing since the extent of *IG-DMR* methylation in *Trim28*^{chatwo} embryos did not correlate with the ratio of biallelic *Gtl2* expression observed in a collection of sixteen *Trim28*^{chatwo} mutants (Figure 2.5C, $p=0.56$). Therefore, these results provide evidence that, upon loss of TRIM28 function, germline DMR methylation is not sufficient to dictate imprinted expression of *Gtl2.* Consequently, our analysis of hypomorphic *Trim28*^{chatwo} embryos suggests that TRIM28 regulates imprinted expression at the *Dlk1-Gtl2* imprinted cluster through a molecular mechanism that is distinct from its function to preserve DNA methylation at germline DMRs.

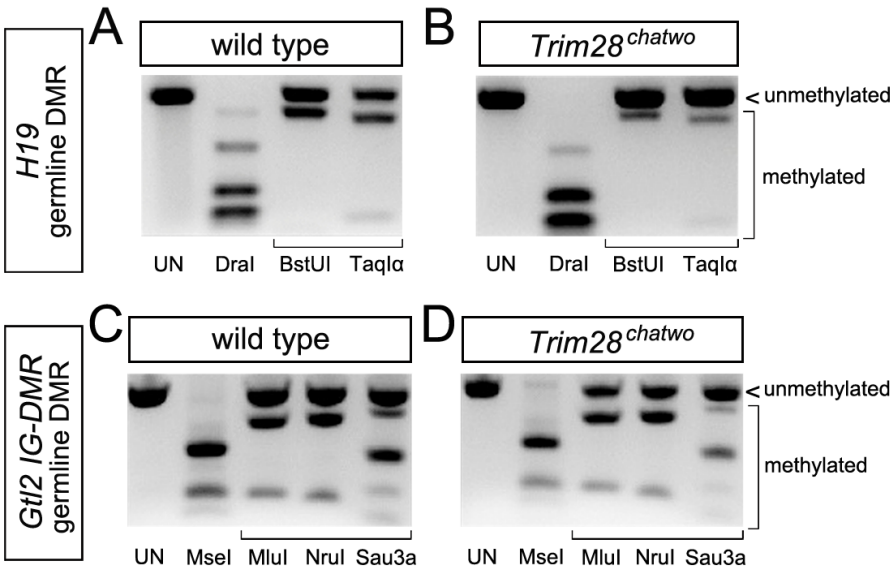


Figure 2.4. DNA methylation at the *H19* and *Gtl2* germline DMRs in *Trim28*^{chatwo} embryos. DNA methylation at the *H19* (A-B) and *Gtl2* (C-D) germline DMRs was detected by combined restriction-bisulfite analysis (COBRA) in wild type (A, C) and *Trim28*^{chatwo} embryos (B, D). Analysis was don on pools of 2-3 embryos collected at E8.5. Restriction with DraI (A-B) and MseI (C-D) measured the efficiency of bisulfite conversion. All other restriction enzymes (lanes with brackets) recognize the PCR product only if the original template contained methylated CpG dinucleotides.UN-undigested.

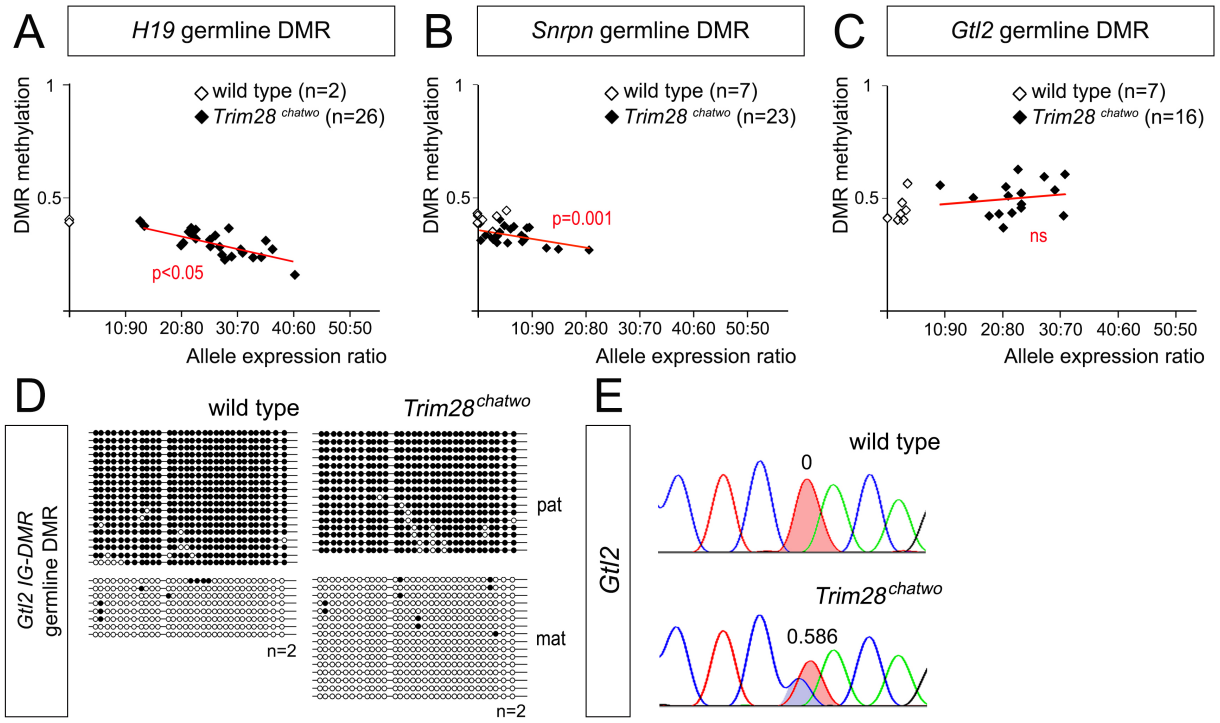


Figure 2.5. Correlation between DNA methylation and allelic expression at germline DMRs. DNA methylation at the *H19* (**A**), *Snrpn* (**B**) and *Gtl2* (**C-D**) germline DMRs was measured in single E8.5 wild type and *Trim28*^{chatwo} embryos through pyrosequencing (**A-C**) and bisulfite sequencing (**D**). Plots in **A-C** represent the allelic expression ratio versus DMR methylation as measured by pyrosequencing. Figure 2.7 illustrates the relationship between allelic expression ratios quantified by PeakPicker and pyrosequencing. Red lines show the linear regression model for *Trim28*^{chatwo} embryos. P-values (red) indicate the correlation between biallelic expression and DNA methylation. n = total embryos analyzed. (**D**) Representative bisulfite sequencing results for wild type and *Trim28*^{chatwo} embryos, additional results are shown in Figure 2.6. Filled circles, methylated DNA. Empty circles, unmethylated DNA. Maternal (mat) and paternal (pat) chromosomes were identified by allele-specific SNPs. (**E**) Sanger sequencing traces of cDNAs for *Gtl2* in the embryos analyzed in D. Numbers indicate the PeakPicker allelic expression ratio.

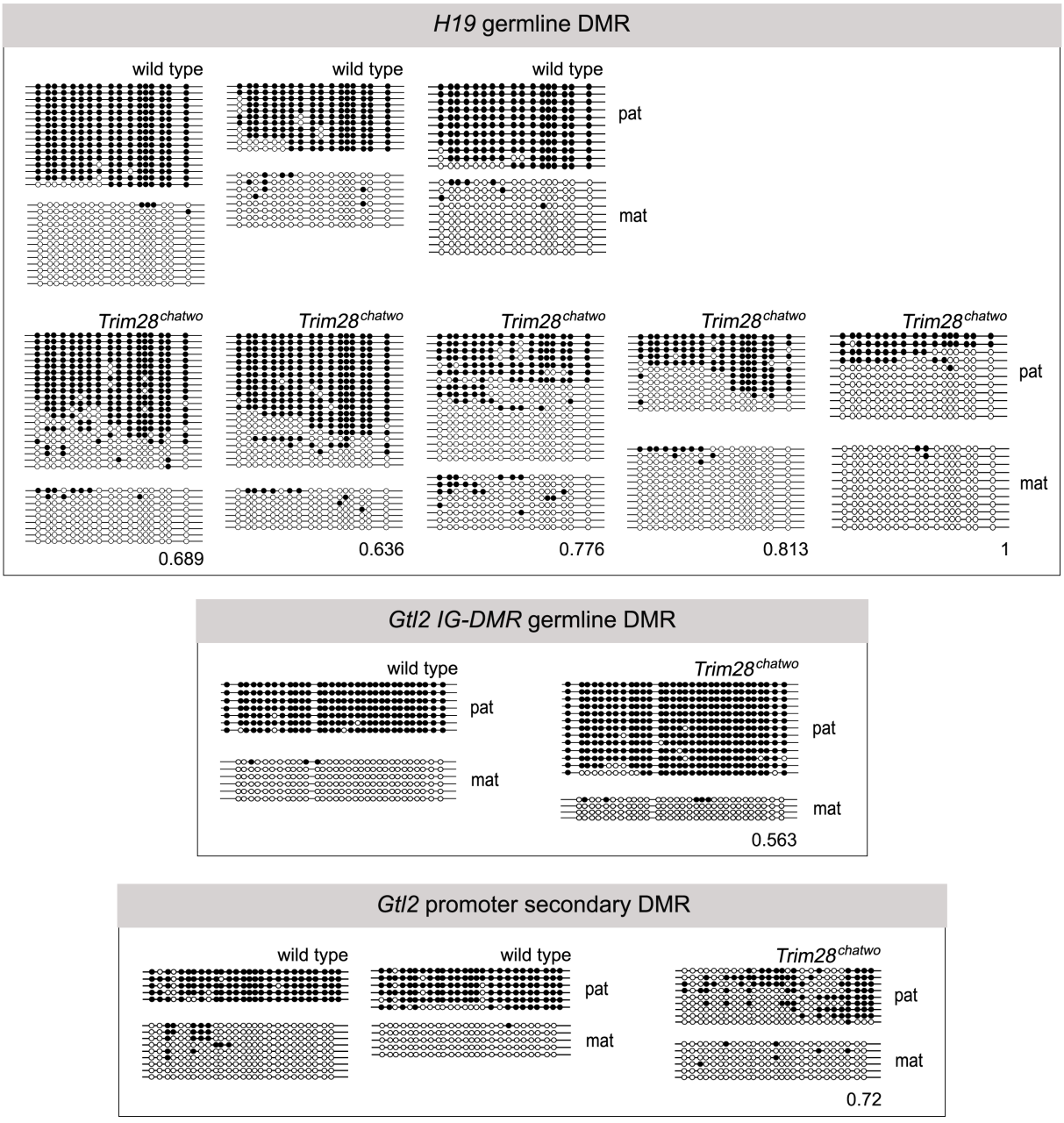


Figure 2.6. Bisulfite sequencing analysis of DNA methylation in *Trim28*^{chatwo} embryos. Results from the analysis of DNA methylation at the H19 germline DMR, the Gtl2 germline DMR, and the Gtl2 secondary DMR in additional wild type and *Trim28*^{chatwo} embryos to those shown in Figures 4 and 5. Methylated DNA is represented by filled circles. Unmethylated DNA is represented by empty circles. Maternal (mat) and paternal (pat) alelles were identified by allele-specific polymorphisms. The numbers at the bottom of each methylation plot indicate the PeakPicker allelic expression ratio for that particular embryo.

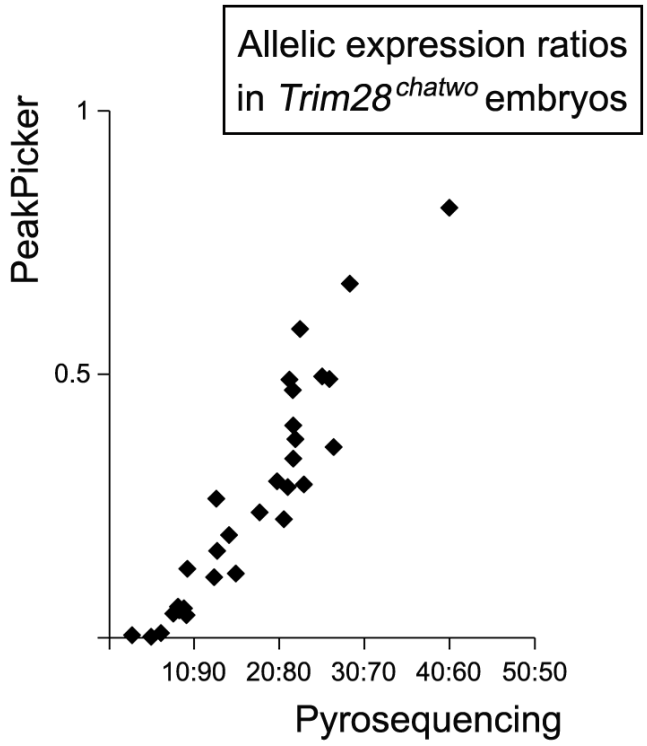


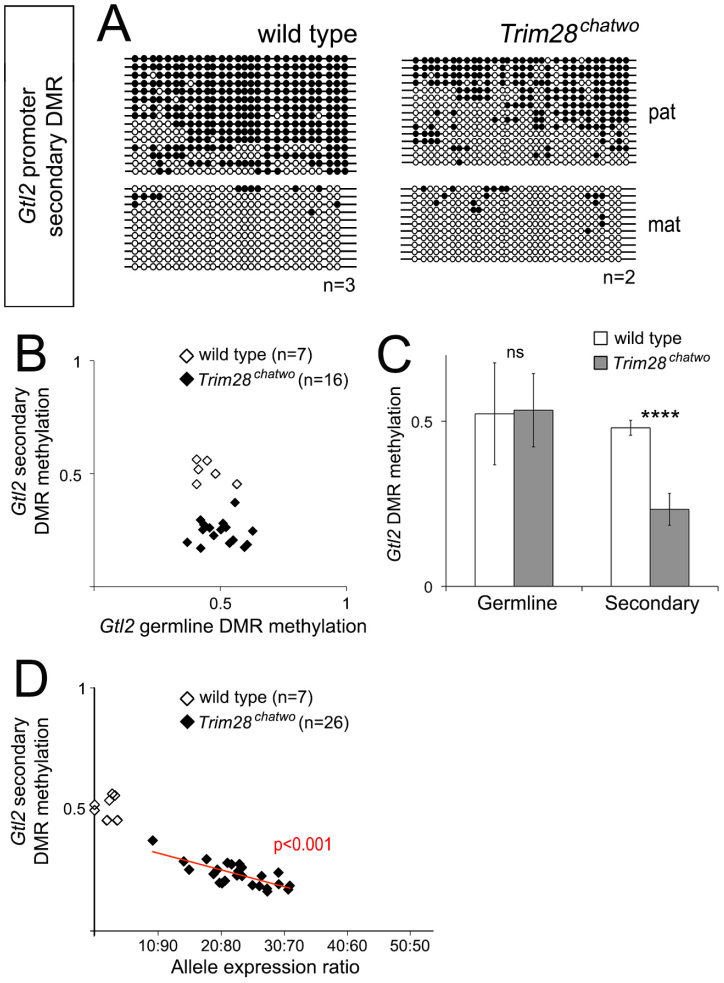
Figure 2.7. Relationship between PeakPicker and pyrosequencing quantification of allelic expression. Graph showing pyrosequencing versus PeakPicker quantification of allelic expression ratios from analysis of the *H19*, *Gtl2*, and *Snrpn* imprinted genes in *Trim28*^{chatwo} embryos. Note that PeakPicker uses Sanger sequencing chromatograms to calculate the relative SNP allele ratio normalized to control peaks and produces a value between 0 (monoallelic expression) and 1 (equal expression from both alleles). In contrast, pyrosequencing provides a ratio that represents the relative expression from each allele, with values that range from 0:100 (monoallelic expression) to 50:50 (equal expression from each allele). Data shows a strong correlation between the two methods ($p<0.0001$).

TRIM28 is required for DNA methylation at secondary DMRs.

Because allele-specific methylation at secondary DMRs has been proposed to maintain the imprinted status at some imprinted clusters (reviewed in John and Lefebvre, 2011), we hypothesized that biallelic expression of *Gtl2* in *Trim28*^{chatwo} embryos could be due to disrupted methylation of the secondary DMR located at the *Gtl2* promoter. To test this hypothesis, we used bisulfite sequencing and pyrosequencing to analyze DNA methylation at the *Gtl2* secondary DMR in hypomorphic *Trim28*^{chatwo} mutants. These experiments revealed that, despite normal levels of DNA methylation at the germline *IG-DMR* (Figure 2.5 C-D; Figure 2.8 B-C), *Trim28*^{chatwo} embryos had decreased levels of DNA methylation at the *Gtl2* secondary DMR as compared to wild type littermates (Figure 2.8 A-C). While loss of DNA methylation at the *Gtl2* secondary DMR was not complete in *Trim28*^{chatwo} mutants, the extent of hypomethylation was highly correlated with the ratio of biallelic expression of *Gtl2* ($p<0.001$, Figure 2.8 D). Therefore, these results suggest that biallelic expression of *Gtl2* in *Trim28*^{chatwo} mutants could be due to loss of secondary DMR methylation. Additionally, the analysis of *Trim28*^{chatwo} embryos demonstrates a requirement of TRIM28 for DNA methylation at the *Gtl2* secondary DMR.

TRIM28 has separate roles during and after genome-wide reprogramming.

Figure 2.8. DNA methylation at the *Gtl2* secondary DMR correlates with biallelic expression in *Trim28*^{chatwo} mutants. DNA methylation at the *Gtl2* secondary DMR was measured in single E8.5 wild type and *Trim28*^{chatwo} embryos by bisulfite sequencing (**A**) and pyrosequencing (**B-D**). (**A)** Representative bisulfite sequencing results for wild type and *Trim28*^{chatwo} embryos, additional results are shown in Figure 2.6. Bisulfite sequencing in A and Figure 2.6 shows results from *Trim28*^{chatwo} embryos that biallelically expressed *Gtl2*. Filled circles, methylated DNA. Empty circles, unmethylated DNA. Maternal (mat) and paternal (pat) chromosomes were identified by allele-specific SNPs. (**B**) DNA methylation at the germline *IG-DMR* versus the *Gtl2* secondary DMR as measured by pyrosequencing in single wild type and *Trim28*^{chatwo} embryos. (**C**) Average DMR methylation for all wild type and *Trim28*^{chatwo} embryos analyzed. (**D**) *Gtl2* allele expression ratios versus *Gtl2* secondary DMR methylation as measured by pyrosequencing in single wild type and *Trim28*^{chatwo} embryos. The red line in D shows the linear regression model for *Trim28*^{chatwo} embryos. The P-value (red) indicates the correlation between biallelic expression and DNA methylation. The data represented in **B-D** includes the same E8.5 wild type and *Trim28*^{chatwo} embryos as shown in Figure 4C. n = number of embryos analyzed. Error bars represent standard deviation. Statistical significance was measured using a paired student's t-test, ns-not significant, ****p<0.0001.


A
wild type
Trim28chatwo
Gtl2 promoter secondary DMR
pat
mat
n=3
n=2
B
wild type (n=7)
Trim28chatwo (n=16)
Gtl2 secondary DMR methylation
Gtl2 germline DMR methylation
C
wild type
Trim28chatwo
ns
****
Gtl2 DMR methylation
Germline
Secondary
D
wild type (n=7)
Trim28chatwo (n=26)
Gtl2 secondary DMR methylation
p<0.001
10:90
20:80
30:70
40:60
50:50
Allele expression ratio

Since methylation at secondary DMRs is established after implantation (Brandeis et al., 1993; Ferguson-Smith et al., 1993; Sato et al., 2011), our finding that zygotic TRIM28 is required for DNA methylation at the *Gtl2* secondary DMR supports the conclusion that zygotic TRIM28 controls imprinting beyond the stages of early embryonic reprogramming. To further explore the roles of TRIM28 after this genome-wide reprogramming event, we tested the effects of *Trim28* loss of function using a conditional allele of *Trim28* (*Trim28*^{L2}) and the *Sox2Cre* transgenic line, which expresses *Cre* recombinase in all embryonic cells starting at implantation (Hayashi et al., 2002).

Analysis of imprinted gene expression using allele-specific SNPs revealed that *Gtl2* was biallelically expressed in *Sox2Cre;Trim28*$^{L-/L2}$ embryos (Figure 2.9 A). Similar to our previous observations in *Trim28*^{chatwo} mutants, we found that *Sox2Cre;Trim28*$^{L-/L2}$ embryos had no significant differences in germline *IG-DMR* methylation (Figure 2.9 B-C, p=0.75), but showed a consistent decrease in DNA methylation levels at the *Gtl2* secondary DMR (Figure 2.9 B-C, p<0.01). Most significantly, biallelic *Glt2* expression in *Sox2Cre;Trim28*$^{L-/L2}$ embryos did not correlate with the small variations in DNA methylation at the *Gtl2* germline DMR (Figure 2.9 D; p=0.32; n=10), but was highly correlated with loss of DNA methylation at the *Gtl2* secondary DMR (Figure 2.9 E; p<0.001; n=10). Therefore, together with our previous analysis of *Trim28*^{chatwo} embryos, these results provide further support that the methylation at the *Gtl2* promoter is linked to the allele-specific silencing of *Gtl2.* Furthermore, since TRIM28 is only effectively depleted in *Sox2Cre;Trim28*$^{L-/L2}$ embryos after early embryonic genome-wide reprogramming (Shibata et al., 2011), our analysis of conditional *Trim28* mutants provides conclusive evidence that zygotic TRIM28 has separate roles to control

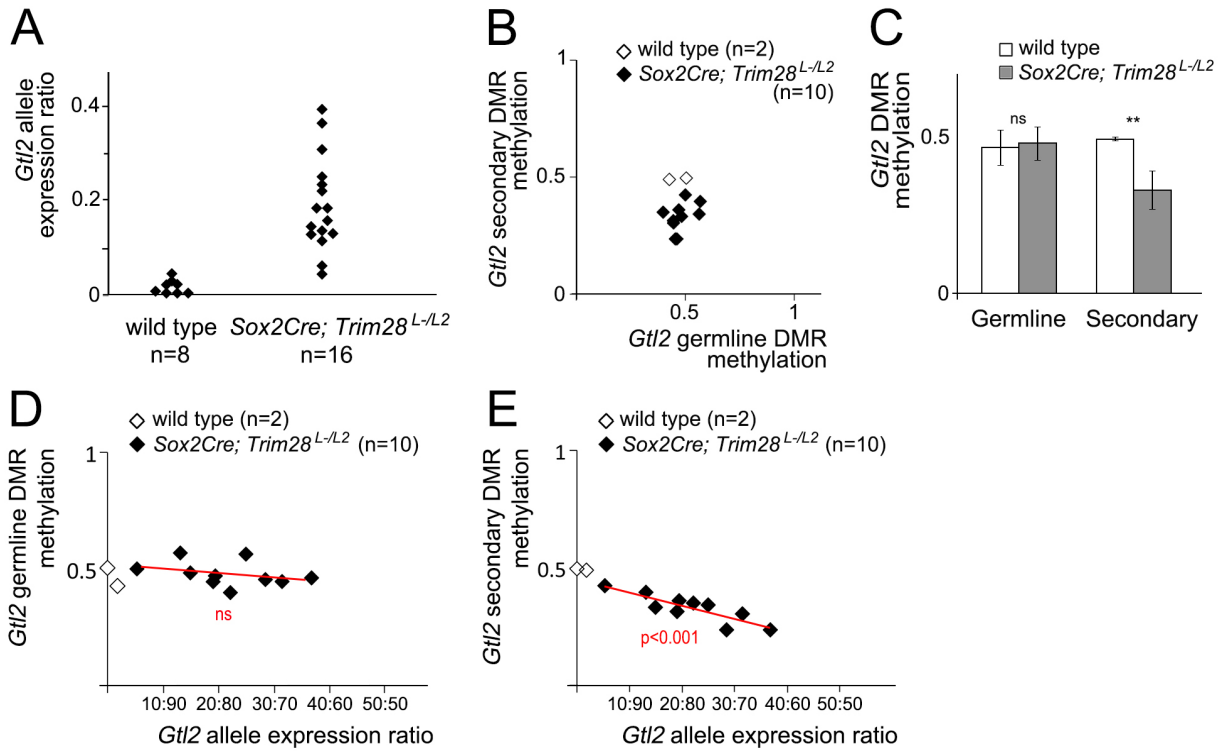


Figure 2.9. *Gtl2* DMR methylation and imprinted gene expression in *Sox2Cre;Trim28*$^{L-/L2}$ embryos. (**A**) PeakPicker allelic expression ratios for *Gtl2* in single E8.5 wild type and *Sox2Cre;Trim28*$^{L-/L2}$ embryonic tissues. (**B**) Germline *IG-DMR* methylation versus *Gtl2* secondary DMR methylation in E8.5 wild type and *Sox2Cre;Trim28*$^{L-/L2}$ embryonic tissues. (**C**) Average DMR methylation in wild type and *Sox2Cre;Trim28*$^{L-/L2}$ embryos. Error bars represent standard deviation. Statistical significance was measured using a paired student's t-test: ns-not significant, **p<0.01. (**D-E**) *Gtl2* allele expression ratio versus *Gtl2* germline DMR methylation (**D**) and *Gtl2* secondary DMR methylation (**E**) in wild type and *Sox2Cre;Trim28*$^{L-/L2}$ embryos. Red lines show the linear regression model for *Sox2Cre;Trim28*$^{L-/L2}$ embryos. P-value indicates the correlation between biallelic expression and DNA methylation, ns-not significant. n = number of embryos analyzed.

imprinting during and after early embryonic reprogramming.

To determine whether TRIM28 also has separate roles during and after early embryonic reprogramming at other imprinted clusters, we analyzed DMR methylation and allele-specific expression of *H19* and *Snrpn* in *Sox2Cre;Trim28*$^{L-/L2}$ embryos. Similar to our findings at *Gtl2*, we found that there were no significant changes in the level of DNA methylation at the *H19* and *Snrpn* germline DMRs in *Sox2Cre;Trim28*$^{L-/L2}$ embryos (Figure 2.10 A-B; Figure 2.11 A). Consequently, our data argues against a general role of TRIM28 to prevent DMR methylation during the replication-dependent dilution of DNA methyl marks that takes place as cells undergo mitosis. Also similar to *Gtl2*, we found that *Sox2Cre;Trim28*$^{L-/L2}$ embryos showed significant hypomethylation at the *H19* secondary DMR (Figure 2.10 A-B), demonstrating that the roles of TRIM28 to regulate the acquisition or maintenance of secondary DMR methylation are not exclusive to the *Gtl2* cluster. Interestingly, neither *H19* or *Snrpn* were biallelically expressed in *Sox2Cre;Trim28*$^{L-/L2}$ mutants (Figure 2.10 C and Figure 2.11 B). Therefore, these results suggest that either the roles of TRIM28 after genome-wide reprogramming differ amongst imprinted clusters or that secondary DMR methylation has distinct roles for imprinting control at different imprinted loci. While more experiments will be required to uncover the mechanisms by which TRIM28 and secondary DMRs control imprinting after genome-wide reprogramming, our experiments with *Trim28*^{chatwo} and conditional *Trim28* mutants provide conclusive evidence that zygotic TRIM28 is not required to maintain germline DMR methylation beyond the stages of early genome-wide reprogramming at both maternally and paternally imprinted loci.

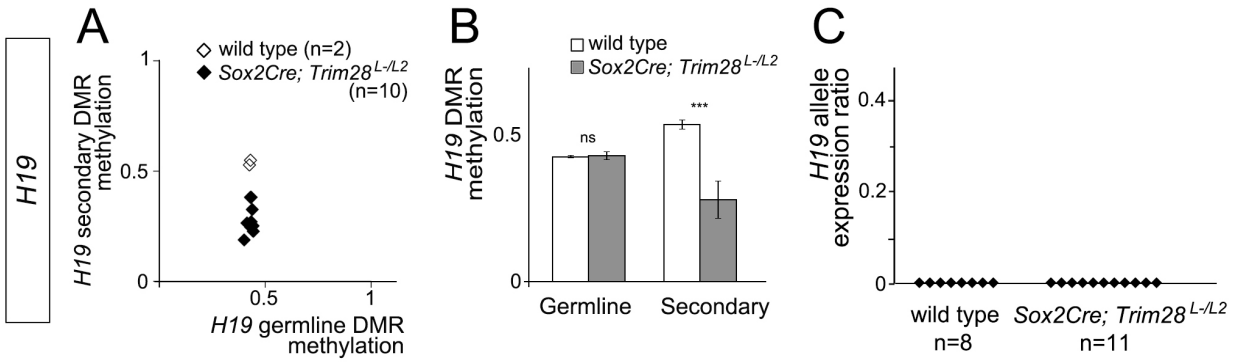


Figure 2.10. *H19* DMR methylation and imprinted gene expression in *Sox2Cre;Trim28$^{L-/L2}$* embryos. DNA methylation and allelic expression was measured at the *H19* imprinted clusters by pyrosequencing (**A-B**) and Sanger sequencing (**C**). (**A**) Germline DMR methylation versus secondary DMR methylation in E8.5 wild type and *Sox2Cre;Trim28$^{L-/L2}$* embryonic tissues. The same data is shown in (**B)** as the average DNA methylation levels at germline and secondary DMRs. Error bars represent standard deviation. Statistical significance was measured using a paired student's t-test: ns-not significant, ***p<0.001. (**C**) Allele expression ratios as quantified using PeakPicker.

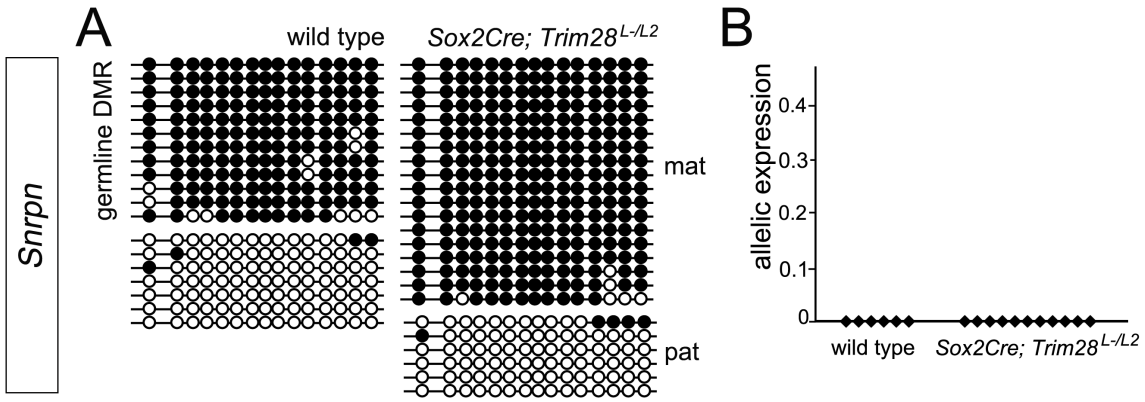


Figure 2.11, *Snrpn* DNA methylation and allelic expression in *Sox2Cre;Trim28*$^{L-/L2}$ embryos. (A) Allele-specific DNA methylation of the *Snrpn* germline DMR as measured by bisulfite sequencing in wild type and *Sox2Cre;Trim28*$^{L-/L2}$ embryos. (B) *Snrpn* allelic expression ratios as quantified using the PeakPicker program. n = number of embryos analyzed.

DISCUSSION

Our study provides insights into how genomic imprinting is regulated during mammalian embryogenesis by uncovering distinct requirements for TRIM28 at different embryonic stages. First, we found that both maternal and zygotic TRIM28 are required to maintain DNA methylation at germline DMRs and that this function is exclusive to the first stages of embryonic development, when genome-wide reprogramming takes place. Furthermore, our experiments revealed that TRIM28 controls genomic imprinting at later stages of embryogenesis through a different mechanism that is independent of its role in maintaining DNA methylation at germline DMRs. The implications of these findings and the molecular mechanisms by which TRIM28 might regulate imprinting at these different stages of embryonic development are discussed below.

TRIM28 has widespread requirements for imprinting control.

Based on the finding that TRIM28 binds all known imprinting control regions (Quenneville et al., 2011), TRIM28 has been lauded as a master regulator of genomic imprinting. However, loss of function studies in embryos lacking maternal TRIM28 showed abnormal imprinted gene expression only in a subset of imprinted clusters (Messerschmidt et al., 2012). We found that imprinted gene expression in zygotic *Trim28* mutants was disrupted in all the maternally and paternally imprinted clusters we tested, including some loci that were not previously described to be disrupted by

maternal *Trim28* depletion, such as *Airn*, *Rasgrf1*, *Gnas*, *Peg10*, *Peg3* and *Kcnq1ot1*. Therefore, the results described in this study provide genetic evidence that *Trim28* has widespread requirements for genomic imprinting.

The amount of TRIM28 is critical for genomic imprinting.

The maternal-to-zygotic transition in mouse embryos takes place at the 2-cell stage (reviewed in Li et al., 2013) and zygotic expression of TRIM28 is detectable as early as the 4-cell stage (Messerschmidt et al., 2012), when embryos are still undergoing genome-wide demethylation (Smith et al., 2012). Therefore, it is tempting to speculate that maternal and zygotic TRIM28 function redundantly to protect imprinted loci from demethylation during this early genome-wide reprogramming event. Consistent with this hypothesis, we found that either maternal or zygotic *Trim28* mutants showed a partially penetrant loss of imprinting, but that simultaneous removal of both maternal and zygotic TRIM28 resulted in loss of imprinting in all the embryos analyzed.

The sensitivity of genomic imprinting to the amount of TRIM28 was also remarkable in zygotic *Trim28* mutants. Specifically, we show that complete removal of zygotic TRIM28 in null $Trim28^{L-}$ mutants caused loss of germline DMR methylation at *H19*, *Snrpn* and *Gtl2*, but TRIM28 function in hypomorphic $Trim28^{chatwo}$ mutants was sufficient to maintain normal levels of DNA methylation at the *Gtl2* germline *IG-DMR*. Because our previous observations support that the hypomorphic nature of the *chatwo* allele is largely due to a drastic decrease in TRIM28 protein levels (Shibata et al., 2011), we suspect that the different effects of the $Trim28^{chatwo}$ allele on imprinting at the *H19,*

Snrpn and *Gtl2* might be a reflection of the specific dose-sensitive requirements for TRIM28 at these imprinted clusters. Together, these results indicate that genomic imprinting is particularly sensitive to the amount of TRIM28.

TRIM28 maintains germline DMR methylation exclusively during genome-wide reprogramming.

The ability of TRIM28 to interact with the maintenance DNA methyltransferase DNMT1 in embryonic stem cells, and the fact that loss of *Zfp57* in ESCs leads to loss of germline DMR methylation (Quenneville et al., 2011; Zuo et al., 2012) have led to propose that TRIM28 maintains germline imprints throughout embryonic development (Messerschmidt, 2014). However, our finding that germline DNA methylation was not disrupted in conditional *Sox2Cre;Trim28*$^{L-/L2}$ mutants at either the *H19*, *Snrpn* or *Gtl2* germline DMRs provides genetic evidence that TRIM28 is not required for replication-dependent maintenance of germline imprints after genome-wide reprogramming in vivo. Therefore, our results support the conclusion that TRIM28 maintains DNA methylation at germline imprints exclusively during the early stages of embryonic development.

DNA demethylation during genome-wide reprogramming is accomplished through both active and passive mechanisms (Shen et al., 2014). Active demethylation takes place through enzymatic oxidation of methylated cytosine residues by the Ten-eleven translocation-3 (TET3) methylcytosine dioxigenase (Gu et al., 2011; Iqbal et al., 2011). Additionally, DNA methylation is passively lost through replication-dependent dilution of methylated cytosines, which is facilitated by the exclusion of the maintenance

DNA methyltransferase DNMT1 from the nucleus during pre-implantation stages (Cardoso and Leonhardt, 1999; Doherty et al., 2002; Howell et al., 2001; Mertineit et al., 1998; Ratnam et al., 2002). Because TRIM28 has been shown to bind to the methylated allele of imprinting control regions (Quenneville et al., 2011), TRIM28 may interfere with active DNA demethylation by blocking the accessibility of TET3 to germline DMRs. However, it is also possible that TRIM28 interferes with passive mechanisms of DNA demethylation. In this respect, two studies have detected small amounts of DNMT1 in the nuclei of pre-implantation mouse embryos (Cirio et al., 2008; Kurihara et al., 2008). Given the ability of TRIM28 to interact with DNMT1 (Quenneville et al., 2011; Zuo et al., 2012), it is possible that TRIM28 might function by recruiting DNMT1 to germline DMRs during genome-wide reprogramming, ensuring that DNA methylation marks at these loci are perpetuated as the DNA replicates.

Separate roles for TRIM28 during and after early embryonic reprogramming.

Perhaps the most striking result from our analysis of zygotic *Trim28* mutants was the finding that imprinting at the *Gtl2* cluster was disrupted in *Trim28*^{chatwo} embryos despite normal levels of methylation at the *IG-DMR*. This result provides evidence for a role of TRIM28 in the regulation of genomic imprinting that is independent of DNA methylation maintenance at germline DMRs. Because this role of TRIM28 is also supported by our analysis of conditional *Trim28* mutants, our data argues that the imprinting defects in *Trim28*^{chatwo} embryos are not due to a neomorphic effect of the *chatwo* allele, but are rather caused by TRIM28 loss of function.

A role for TRIM28 interpreting germline imprints.

While multiple studies support an essential role for germline DMRs in imprinting control (Lin et al., 2003; Thorvaldsen et al., 1998), there are still large gaps in our understanding of how differential methylation at these regulatory regions controls allele-specific expression of imprinted genes. One of the best characterized imprinting control regions is the *H19* intergenic germline DMR (Ferguson-Smith, 2011). This imprinting control region is recognized in a methylation-specific manner by the chromatin insulator CCCTC-binding factor (CTCF), which is known to influence chromatin topology and favor the interaction of the *H19* promoter with downstream enhancers (Bell and Felsenfeld, 2000; Engel et al., 2004; Hark et al., 2000; Murrell et al., 2004; Szabó et al., 2000). The finding that CTCF also binds to other imprinted clusters (Fitzpatrick et al., 2007; Yoon et al., 2005) has brought some support towards an “insulator model” of imprinting regulation (Wan and Bartolomei, 2008). However, CTCF does not bind to all imprinting control regions (Carr et al., 2007). Consequently, the mechanisms by which germline DMRs function in cis to control allele-specific expression are likely specific of each imprinted locus. In this respect, the mechanisms that operate at the *Dlk1-Gtl2* germline *IG-DMR* to control imprinted gene expression are currently unknown (da Rocha et al., 2008). Chromatin immunoprecipitation experiments have failed to detect binding of CTCF, or methyl binding proteins MBD2 and MecP2, to the *Gtl2* germline *IG-DMR* (Carr et al., 2007). However, TRIM28 can bind the *Gtl2* germline *IG-DMR* in a methylation specific fashion (Quenneville et al., 2011). By identifying TRIM28 as a factor that controls allele-specific *Gtl2* expression without disrupting germline DMR

methylation, our studies provide genetic evidence supporting a role for TRIM28 in interpreting the epigenetic information inherited through the *Gtl2* germline *IG-DMR* to ultimately influence imprinted gene expression.

TRIM28 can recruit several histone-modifying enzymes (Schultz et al., 2001; 2002) and alterations in histone modifications are known to disrupt imprinted gene expression (Carr et al., 2007; Mager et al., 2003). TRIM28 is also known to mediate long-range transcriptional repression through heterochromatin spreading (Groner et al., 2010; Quenneville et al., 2012) Therefore, it is tempting to speculate that TRIM28 might regulate imprinting after early embryonic reprogramming by binding to the methylated *Gtl2* germline *IG-DMR* and spreading a repressive state through heterochromatin formation.

TRIM28 is required for secondary DMR methylation.

DNA methylation at secondary DMRs has been proposed to control imprinting, but its role in regulating allele-specific expression is still controversial (reviewed in John and Lefebvre, 2011). Several studies support an instructive role of certain secondary DMRs for allele-specific expression. Secondary DMR methylation at the *H19* and *Gtl2* promoters has been shown to correlate with allele-specific silencing (Lin et al., 2003; Srivastava et al., 2000; Steshina et al., 2006; Thorvaldsen et al., 1998). Additionally, a study that conditionally deleted the paternal *Igf2-H19* germline DMR late in embryogenesis suggested that, once established, secondary imprints can maintain the imprinted status in the absence of the germline DMR (Srivastava et al., 2000). Since

methylation at the *H19* and *Gtl2* promoters has been found to depend on the allele-specific methylation at germline DMRs (Lin et al., 2003; Srivastava et al., 2000; Thorvaldsen et al., 1998), a model has been put forward that secondary DMRs perpetuate the imprinted status inherited from germline DMRs. However, monoallelic expression of *H19* and *Gtl2* is established before DNA methylation is acquired at their secondary DMR promoters (Sasaki et al., 1995; Sato et al., 2011), arguing that secondary DMR methylation is a consequence of the imprinted status, rather than an instructive mechanism for allele-specific expression.

The fact that decreased levels of secondary DMR methylation in *Sox2Cre;Trim28*$^{L-/L2}$ embryos was highly correlated with biallelic expression of *Gtl2* provides additional data supporting the relationship between secondary DMR methylation and allele-specific expression. However, in *Sox2Cre;Trim28*$^{L-/L2}$ mutants, *H19* was not biallelically expressed despite significant loss of methylation at the *H19* promoter. These variable effects on different imprinted loci might be a reflection of the different mechanisms by which imprinting is regulated at specific clusters. For instance, it is possible that DNA methylation at the *H19* secondary DMR is dispensable for *H19* repression. Alternatively, it is possible that the roles of TRIM28 after genome-wide reprogramming differ at amongst imprinted loci. Although our experiments can not resolve whether methylation at secondary DMRs has an instructive role on imprinted gene expression or if is a secondary consequence of a previously established imprinted status, our results provide insight into the relationship between secondary DMR methylation and allele-specific expression of *Gtl2* and *H19*. The lymphoid specific helicase LSH/HELLS is required for methylation of somatic imprints (Fan, 2005).

However, LSH/HELLS seems to only be required at the *Cdkn1c* imprinted locus (Fan, 2005). Therefore, by identifying a requirement for TRIM28 in the regulation of DNA methylation at *Gtl2* and *H19* somatic imprints, our results provide an important contribution towards understanding the factors that control DNA methylation at secondary DMRs.

In conclusion, our analysis of maternal, zygotic and conditional *Trim28* mutants not only provides additional insight about how TRIM28 maintains methylation at germline DMRs, but also uncovers a requirement of TRIM28 after genome-wide reprogramming for deciphering germline imprints and influencing secondary DMR methylation.

EXPERIMENTAL PROCEDURES

Mice

Trim28^{chatwo}, *Trim28*$^{L-}$, maternal *Trim28* deletion (*ZP3-Cre; Trim28*$^{L-/L2}$), and *Sox2Cre; Trim28*$^{L-/L2}$ mutants (Cammas et al., 2000; Messerschmidt et al., 2012; Shibata et al., 2011). *Trim28*^{chatwo} mutants were analyzed in a mixed CAST/Ei background, where mutants show developmental arrest at E8.5 (Shibata et al., 2011). To generate maternal *Trim28*$^{chatwo/L-}$ embryos, *Zp3-Cre; Trim28*$^{chatwo/L2}$ females were mated to wild type males. For maternal-zygotic *Trim28* mutants, *Zp3-Cre;Trim28*$^{L-/L2}$ or *Zp3-Cre;Trim28*$^{chatwo/L2}$ females were mated to *Trim28*$^{L-/+}$ or *Trim28*$^{chatwo/+}$ males. For genetic backgrounds, SNPs and primers used see Supplemental Table 1. Experiments involving mice were done according to standard operating procedures approved by Cornell's Institutional Animal Care and Use Committee.

Embryo Collection

Embryos were dissected in phosphate buffered saline containing 4% bovine serum albumin. For post-implantation developmental stages (E6.5 and later), embryos were split into embryonic and extra-embryonic tissues that were processed separately for genotyping and imprinting analysis. In cases where allelic expression and DNA methylation were analyzed within the same embryo, the embryonic tissues were split and processed separately. At *Rasgrf1*, the embryonic tissues were used for genotyping and extra-embryonic tissues were used for analysis of imprinted gene expression. For pre-implantation developmental stages, embryos were flushed from the uterus and used directly for immunofluorescence.

Table 2.1. Primers used for genotyping, quantification of allele-specific expression, and DMR methylation

Gene expression

Quantification of imprinted gene expression was tested by qRT-PCR on RNA samples extracted from independent pools of 3-4 E7.5 *Trim28*$^{L-}$ or E8.5 *Trim28*^{chatwo} embryos and wild type littermates as previously described (Shibata et al., 2011). Allelic expression was detected by quantitative pyrosequencing and Sanger sequencing of RT-PCR products that amplified the SNP-containing region of the imprinted gene. Primer sequences and SNP positions are in Supplemental Table 1. Allele expression ratios were quantified with PeakPicker (Ge et al., 2005).

Immunofluorescence

Preimplantation embryos were fixed in 4% paraformaldehyde and used for staining with TRIM28 antibody (Santa Cruz, sc-33186), phalloidin, and 4',6-diamidino-2-phenylindole (DAPI).

DNA methylation

DNA was bisulfite converted using the EZ DNA methylation-direct kit (Zymo, D5020). For bisulfite sequencing analysis, PCR products of bisulfite-converted DNA were cloned using the TOPO TA cloning kit (Invitrogen, K450001) and individual clones were analyzed by Sanger sequencing. The efficiency of bisulfite conversion was >99%. Pyrosequencing was done as previously described (Wang et al., 2014). For primer sequences see Supplemental Table 1.

Allelic Expression (Sanger Sequencing)

	Primer1	Primer2	SNP position (mm10)	SNP	Background	Source
H19	actgaaggcgaggatgacag	gttcaaggtagggggaggag	7: 142576519	G/A	FVB/CAST	
Gtl2	gccaaagccatcatctggaatc	cacagatgtagactcaacagtgaag	12: 109571394	T/C	FVB/CAST	Schmidt et al., 2000
Snrpn	cccaccaggaattagaggc	gcagtaagaggggtcaaaagc	7: 59983201	C/T	FVB/CAST	Szabo and Mann, 1995; Doherty et al., 2000
Gnas (Paternal)	ctggcagtgagatcagtgga	aaaatcgctgggttgaagg	2: 174298128, 174298129	TT/GA	FVB/CAST	
Airn	cacaggcaaacaaccctaca	ctcggcagagaaagttttgg	17: 12816566	T/C	FVB/CAST	
Kcnq1ot1	ccaccagcctcagcatattt	gaaacgatccacacggaagt	7: 143296059	T/C	FVB/CAST	
Peg3	gggggagaatcctccattta	tgggaactgaaaacgtgaca	7: 6709700	G/C	FVB/BL6	
Peg10	atgattttgcacccagatcc	tcaggcagaatggtgacaaa	6: 4757990	C/T	FVB/BL6	
Rasgrf1	ggctcatgatgaatgccttt	tacagaagcttggcgttgtg	9: 89991554	T/C	FVB/BL6	

Allelic Expression (Quantitative pyrosequencing)

	Primer1	Primer2	Pyro Primer	SNP position (mm10)	SNP	Background
H19	TTTGGAGTCCCGGAGATAGCTTT	[Biotin-5]GCAAAGGATGAAGTAGGGCATGT	CCCAGTACCCACCTGT	7: 142575460	C/T	FVB/CAST
Gtl2	CCAGGACCCTCCAACTGTAAA	[Biotin-5]TCAGTCAGTAGGTGGGTCTCTCT	AGGCGTCCCCGTGGC	12: 109571145	T/C	FVB/CAST
Snrpn	CTTGCTACGTGGGGAGAACTT	[Biotin-5]TAGGTACACCTGCTGGCACTC	GGCCCTCCACAGTCA	7: 59986552	T/G	FVB/CAST

Quantitative Reverse Transcriptase PCR

	Primer1	Primer2	Source
H19	ttgcactaagtcgattgcact	ggaactgcttccagactaggc	
Igf2	ttgtgctgcatcgctgcttac	tagacacgtccctctcggact	
Gtl2	gggcgcccacagaagaa	ggtgtgagccgatgatgtca	
Dlk1	ttaccggggttccttagagc	tgcattaatagggaggaaggg	
Rian	tcgagacacaagaggactcc	attggaagtctgagccatgg	Sekita et al., 2005
Mirg	ccttcctggatctctcgctt	gtgggagttgaaacatgggt	Wu et al., 2006
Peg3	cacgaagacgacaccaacag	gtctcgaggctccacatctc	Shule et al., 2007
Mest	atcccggtgcttcttctca	aggcagcaagcagcaact	
Impact	aagaacgcgcagacttatcg	ttattttctccacccactggt	
Snprn	ttggttctgaggagtgatttgc	ccttgaattccaccaccttg	Tsai et al., 1999
Peg10	gtctctactgtggcaatgg	gggaccttattcgttctgg	Zaiton et al., 2010
Airn	gtggattcaggtttcatg	ggcccagatatagaatgt	
Igf2r	tagttgcagctctttgcacg	acagctcaaacctgaagcg	
IAP 5' UTR	cgggtcgcggtaataaaggt	actctcgttccccagctgaa	Rowe et al., 2010
Actin	aagtgacgttgacatccg	gatccacatctgctggaagg	

Bisulfite Sequencing and COBRA

	Primer1	Primer2	Background
H19 primary outer	gagtatttaggaggtataagaatt	atcaaaaactaacataaacccct	FVB/CAST
H19 primary inner	gtaaggagattatgtttatttttgg	cctcattaatcccataactat	FVB/CAST
H19 secondary outer	gactggtcagcccttgagtc	cctcaccacaactcccactt	FVB/CAST
H19 secondary inner	ggtgaggagtgcccaaatta	gagctagcccctcagtcctt	FVB/CAST
Gtl2 primary outer	agatgtgttgtggatttaggttgtag	ctaaactacaatctatataatcacaacac	FVB/CAST
Gtl2 primary inner	aagtgttgtggtttgttatgggtaag	ccattcccaatctataaaaatattttaacc	FVB/CAST
Gtl2 secondary outer	gaagaatttttttatttggtgagtg	caacactcaaatcaccccccc	FVB/CAST
Gtl2 secondary inner	gtttgaaaggatgtgtaaaaatg	ccccccccacatctattctacc	FVB/CAST

PyroMark

	Primer1	Primer2	Pyro Primer
H19 primary pyro	gattatgggattatagatggtgat	[Biotin-5]aatcaacaaaatccacttactctctacaa	aggggagaaaatttaattag
H19 secondary pyro	agttagggttttttttaattggagtg	[Biotin-5]aaaacaataccaaaccctatcta	tttgttaattaattagtatatggt
Gtl2 primary pyro	ggtttatagtggatagttagtgt	[Biotin-5]cttccctcactccaaaaat	gttatggattggtgttaag
Gtl2 secondary pyro	ttgtagtttgggtgaggttatagtaatttg	[Biotin-5]aaccactcaccaaataaaaaattcttcta	tttatttagttagtttttagtatag

Genotyping

	Primer1	Primer2	Source	Notes
Trim28 (chatwo)	atgtgttgtgtggcccagta	acctcttggcacctgcaac	Shibata et al., 2011	Restrict with BslI
Trim28 (L -)	ggaatggttgttcattggtg	gcgagcacgaatcaaggtcag	Cammas et al., 2000	
Trim28 (L2)	ggaatggttgttcattggtg	accttggcccatttattgataaag	Cammas et al., 2000	
Cre	ctaggccacagaattgaaagatct	gtaggtggaaattctagcatcatcc		
D7Mit361	tactaccaatgctaagcgctacc	accagagtttgtgcccattc	MIT marker, MGI	CAST versus FVB SSLP near *H19* and *Kcnq1ot1*
D7Mit259	cccctcctcctgacctctt	gtctccatgggaaccacact	MIT marker, MGI	CAST versus FVB SSLP near *H19* and *Kcnq1ot1*
D7Mit316	ccacacaaatgccatacacg	tgtgcattcctgtatatttgtatgc	MIT marker, MGI	CAST versus FVB SSLP near *Snrpn*
D12Mit80	caacccagatgtcccttaaca	ctggaaggtttcacctagttgg	MIT marker, MGI	CAST versus FVB SSLP near *Glt2*
D2Mit265	aataataatcaaggttgtcattgaacc	tagtcaaaattcttttgtgtgttgc	MIT marker, MGI	CAST versus FVB SSLP near *Gnas*
D17Mit114	ggatccttagggctcacaca	gcctattttccaatttggca	MIT marker, MGI	CAST versus FVB SSLP near *Airn*

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CHAPTER 3

DEVELOPMENTAL CONTEXT SPECIFIC REGULATION OF IMPRINTED GENE EXPRESSION AT THE *DLK1-DIO3* CLUSTER

ABSTRACT

Genes within the *Dlk1-Dio3* imprinted cluster are expressed in an allele-specific manner depending on an intergenic differentially methylated region (IG-DMR). The prevailing view is that the unmethylated maternal IG-DMR is essential for imprinting of the *Dlk1-Dio3* cluster, while the methylated paternal IG-DMR has minimal functions for imprinting control. By investigating the functions of the paternal IG-DMR in the context of different developmental stages and tissues, we challenge this view, finding diverse requirements for the paternal IG-DMR in different developmental contexts. Consistent with previous reports, we found that deletion of the paternal IG-DMR has minimal effects on imprinted gene expression in E14.5 limb. However, in E14.5 yolk sac, the paternal IG-DMR was required for cis repression of *Gtl2*. The paternal IG-DMR was also essential for activation of *Dlk1* in E14.5 lung, and for activation of *Dlk1* and *Dio3* in E9.5 whole embryos. These findings illuminate tissue-specific requirements of the paternal IG-DMR for controlling imprinted gene expression. Notably, these tissue-specific requirements were accompanied by tissue-specific marks of active enhancers at the paternal IG-DMR, suggesting that the ability of the paternal IG-DMR to activate paternally-expressed genes may be due to enhancer functions from the paternal IG-DMR. Overall, these findings reveal a new paradigm of imprinting control that is potentially tailored to the expression needs of individual tissue types with imprint gene expression being achieved through distinct mechanisms in different developmental contexts.

INTRODUCTION

While most genes are expressed from both parental alleles, imprinted genes are unique in that they are expressed selectively from either the maternally- or paternally-inherited chromosome. The importance of this monoallelic imprinted gene expression has been widely demonstrated by abnormal phenotypes caused by maternal or paternal disomy, conditions in which either two maternal or two paternal copies of certain genomic regions are inherited (Cattanach, 1986; Cattanach and Kirk, 1985; Searle and Beechey, 1978; 1990). Maternal or paternal disomy of mouse chromosome 12 leads to embryonic lethality due to defects in embryonic and extraembryonic tissues (Georgiades et al., 2001; 2000; Tevendale et al., 2006). In humans, similar uniparental disomy sometimes occurs at a homologous region on chromosome 14 (Liehr, 2010), and is also associated with developmental abnormalities (Falk et al., 2005; Temple et al., 1991). These parent-of-origin specific effects have been attributed to a particular group of imprinted genes on mouse chromosome 12, the *Dlk1-Dio3* imprinted gene cluster.

The *Dlk1-Dio3* imprinted cluster contains three protein-coding genes, *Dlk1*, *Rtl1* and *Dio3*, which are expressed from the paternal allele, and several non-coding RNAs (ncRNAs) that are expressed maternally (reviewed in da Rocha et al., 2008). Both protein-coding genes and ncRNAs within the *Dlk1-Dio3* gene cluster have critical functions for development. Paternally-expressed *Dlk1* and *Rtl1* are required for prenatal growth, with *Dlk1* deletion resulting in embryonic growth retardation (Moon et al., 2002), and loss of *Rtl1* resulting in placental growth retardation (Sekita et al., 2008). The other paternally-expressed gene, *Dio3,* is a regulator of thyroid hormone levels, and its deletion leads to neonatal thyrotoxis and postnatal growth retardation (Hernandez et al.,

2006). On the maternal chromosome, a long non-coding RNA, *Gtl2,* and several snoRNAs and microRNAs (found within *Rian, Mirg,* and *Rtl1as*) are expressed in tandem as a single transcription unit (Luo et al., 2016). Hence, deletion or activation of the *Gtl2* promoter affects expression of all maternally-expressed ncRNAs (Luo et al., 2016; Sekita et al., 2006; Takahashi et al., 2009; Tierling et al., 2006; Zhou et al., 2010). The maternal ncRNAs are essential for the full developmental potential of induced pluripotent stem cells (iPSCs) (Mo et al., 2015; Stadtfeld et al., 2010; 2012; Yu et al., 2015). In mice, mutations disrupting *Gtl2* result in lethality and have been linked to skeletal muscle, lung, and liver defects (Takahashi et al., 2009; Zhou et al., 2010).

Regulation of imprinted gene expression within the *Dlk1-Dio3* cluster depends on allele-specific DNA methylation at a regulatory region located between *Dlk1* and *Gtl2* (Takada et al., 2000). On the maternal chromosome, this intergenic differentially methylated region (IG-DMR) is unmethylated and it is essential for cis activation of maternal genes as well as cis repression of paternal genes in embryonic tissues (Lin et al., 2003). Recent studies in mouse embryonic stem cells (mESCs) have found enhancer-like properties at and near the maternal IG-DMR (Das et al., 2015; Kota et al., 2014; Luo et al., 2016). A possible role of the IG-DMR as an enhancer has also been supported by chromatin conformation capture experiments showing interactions between the IG-DMR and the *Gtl2* promoter (Das et al., 2015). Given that the maternal IG-DMR is required to activate *Gtl2* (Lin et al., 2003), these studies support that the maternal IG-DMR enhances transcription of the maternally-expressed genes at the *Dlk1-Dio3* imprinted cluster.

In contrast to the maternal IG-DMR, the paternal IG-DMR is hypermethylated and does not have enhancer-like properties in mESCs (Kota et al., 2014). The exact functions of the paternal IG-DMR are controversial. Mutants that lose DNA methylation at the paternal IG-DMR display maternalization of the paternal chromosome, with biallelic expression of *Gtl2* and biallelic repression of *Dlk1* (Alexander et al., 2015; Li et al., 2008; Schmidt et al., 2000). While these experiments suggest an important role of DNA methylation at the paternal IG-DMR, complete deletion of the methylated paternal IG-DMR has been reported not to disrupt imprinting in either whole embryos or placenta (Lin et al., 2003; 2007). Furthermore, paternal IG-DMR deletion mutants are viable and fertile, with no observed abnormal phenotypes.

A potential explanation for these seemingly contradictory results is that DNA methylation at the paternal IG-DMR could prevent it from functioning as an enhancer of the maternally-expressed genes. However, the mechanisms by which DNA methylation could prevent enhancer activity at the paternal IG-DMR are currently unclear. DNA methylation at the paternal IG-DMR recruits the methylation-specific DNA binding protein, ZFP57, which binds TRIM28, a corepressor that facilitates repressive chromatin (Li et al., 2008; Quenneville et al., 2011; Zuo et al., 2012). Deletion of either *Zfp57* or *Trim28* during early development results in inappropriate paternal activation of *Gtl2* with corresponding hypomethylation at the paternal IG-DMR (Alexander et al., 2015; Li et al., 2008). In addition to the roles of ZFP57 and TRIM28 during genome-wide reprogramming, we recently discovered that conditional and hypomorphic *Trim28* mutants inappropriately express *Gtl2* from the paternal allele, but have unaltered DNA methylation at the paternal IG-DMR (Alexander et al., 2015). These findings suggest

that TRIM28 controls imprinting at the *Gtl2* locus through mechanisms that are independent of its early roles during genome-wide reprogramming. To explore whether TRIM28 binds the methylated paternal IG-DMR to prevent aberrant enhancer activity, we analyzed the allele-specific IG-DMR enhancer properties in hypomorphic *Trim28* mutants. We found that *Trim28* mutants have increased enhancer activity from the paternal IG-DMR, indicating that TRIM28 prevents the paternal IG-DMR from enhancing transcription at maternally-expressed genes. However, the IG-DMR enhancer activity did not correlate with *Gtl2* biallelic expression, suggesting that TRIM28 also functions through other mechanisms to regulate imprinted expression.

As we investigated IG-DMR enhancer activity, we unexpectedly found that the paternal IG-DMR has characteristics of active enhancers in some embryonic tissues. Furthermore, the paternal IG-DMR has tissue-specific requirements for imprinting control. In the limb, the paternal IG-DMR is not required for imprinted gene expression. In the lung, the paternal IG-DMR is required for full activation of *Dlk1* from the paternal allele. In the yolk sac, the paternal IG-DMR is dispensable for paternal gene expression, but is essential for repression of *Gtl2.* Overall, these findings reveal distinct roles of the paternal IG-DMR depending on the tissue type, demonstrating that unique strategies of imprinting control exist in different tissues.

RESULTS AND DISCUSSION

The paternal IG-DMR shows enhancer activity in *Trim28* hypomorphic mutants. We recently discovered that mutants carrying the hypomorphic *chatwo* allele

(*Trim28*^{chatwo}) (Shibata et al., 2011) biallelically express *Gtl2* without altering the methylation status of the IG-DMR (Alexander et al., 2015). We therefore sought to determine whether biallelic expression of *Gtl2* in these *Trim28*^{chatwo} mutants could be due to aberrant enhancer activity from the paternal IG-DMR. One hallmark of active enhancers is the transcription of non-coding enhancer RNAs (Andersson et al., 2014; Hah et al., 2013; Melgar et al., 2011). In mESCs, bidirectional non-coding RNAs were found to be transcribed from the maternal IG-DMR (*IG-DMR RNA*), which was also marked with enhancer-specific histone modifications (Kota et al., 2014). We therefore used *IG-DMR RNA* expression as a proxy for enhancer activity. To determine whether the IG-DMR enhancer was active in *Trim28*^{chatwo} mutants (Shibata et al., 2011), we measured *IG-DMR RNA* expression by qRT-PCR. Embryos deleted for the maternal IG-DMR (*ΔIG*^{MAT})(Lin et al., 2003), were used as a negative control in these experiments, since *IG-DMR RNA* should not be expressed in these embryos. As previously reported in mESCs (Kota et al., 2014), *IG-DMR RNA* was expressed at very low levels in wild type E8.5 embryos (Figure 3.1 A). *IG-DMR RNA* expression was significantly reduced in *ΔIG*^{MAT} mutants, indicating that *IG-DMR RNA* is expressed in primarily from the maternal IG-DMR at E8.5. In *Trim28*^{chatwo} embryos, *IG-DMR RNA* expression was higher compared with wild type littermates (Figure 3.1 B), suggesting increased IG-DMR enhancer activity in *Trim28* mutants. To elucidate whether increased *IG-DMR RNA* expression in *Trim28* mutants was due to enhancer activity from the paternal IG-DMR, we measured allelic *IG-DMR RNA* expression in *Trim28*^{chatwo} mutants carrying subspecies-specific single nucleotide polymorphisms (SNPs) at the IG-DMR. While

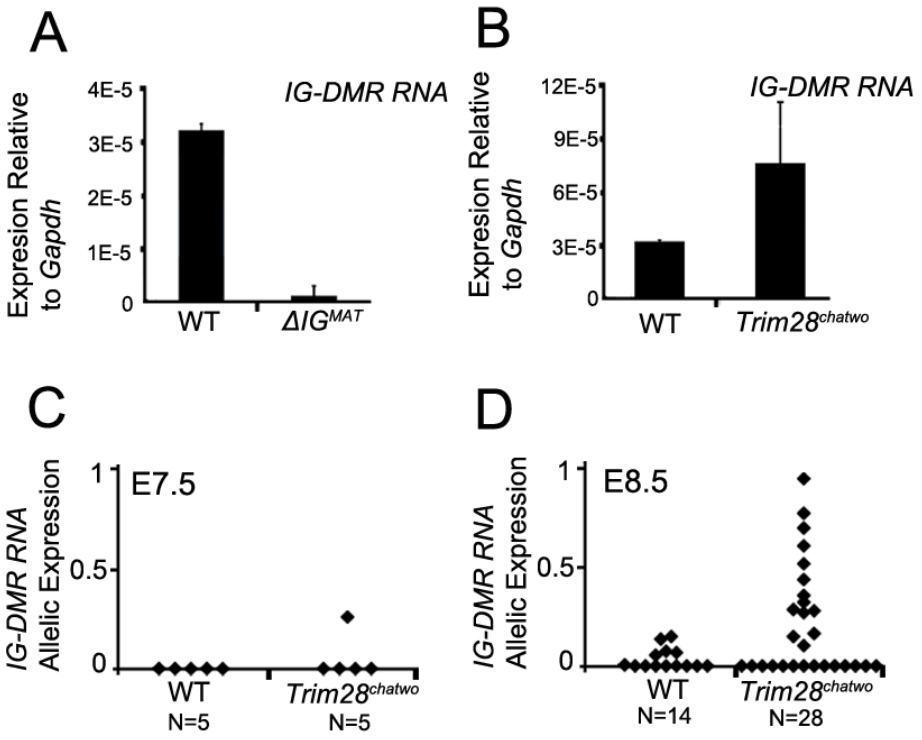

Figure 3.1. *IG-DMR RNA* is biallelically expressed in *Trim28^{chatwo}* mutants. (A-B) Average expression of *IG-DMR RNA* as measured by qRT-PCR normalized to *Gapdh* in wild type (WT), *ΔIG^{MAT}* mutants (A) and *Trim28^{chatwo}* embryos (B). Error bars represent standard deviation of three biological replicates. **(C-D)** PeakPicker quantification of allelic expression of *IG-DMR RNA* in E7.5 (C) and E8.5 (D) wild type and *Trim28^{chatwo}* embryos. Each diamond represents a single embryo. Sample sizes are indicated beneath labels. Allelic expression of zero indicates full expression from the maternal allele. Allelic expression of one indicates equal expression of *IG-DMR RNA* from both alleles.

E7.5 and E8.5 wild type embryos showed primarily maternal expression of *IG-DMR RNA*, many *Trim28*^{chatwo} embryos biallelically expressed *IG-DMR RNA*(Figure 3.1 C-D). These results suggest that TRIM28 is required to prevent enhancer activity from the paternal IG-DMR. Because the paternal IG-DMR is properly methylated in *Trim28*^{chatwo} mutants (Alexander et al., 2015), these findings also indicate that, despite retaining DNA methylation, the paternal IG-DMR is capable of having enhancer activity when TRIM28 is depleted.

Increased paternal IG-DMR enhancer activity cannot fully explain loss of imprinting in *Trim28*^{chatwo} mutants. The increased expression of *IG-DMR RNA* from the paternal IG-DMR was variable between individual mutant embryos, with several *Trim28*^{chatwo} embryos showing maternal-specific *IG-DMR RNA* expression similar to wild type embryos (Figure 3.1 C-D). This partially-penetrant biallelic expression was reminiscent of the partially penetrant loss of imprinting at *Gtl2* that we previously observed in *Trim28*^{chatwo} mutants (Alexander et al., 2015). To determine if biallelic expression of *Gtl2* could be explained by paternal IG-DMR enhancer activity, we measured *IG-DMR RNA* and *Gtl2* biallelic expression in single embryos. We found that every embryo that biallelically expressed *IG-DMR RNA* showed some level of *Gtl2* biallelic expression (Figure 3.2 A). However, many *Trim28*^{chatwo} embryos that biallelically expressed *Gtl2* did not biallelically express *IG-DMR RNA*, and the levels of biallelic expression of *Gtl2* and *IG-DMR RNA* were not correlated (Figure 3.2 A). Therefore, aberrant enhancer activation at the paternal IG-DMR cannot fully explain loss of imprinting in *Trim28*^{chatwo} mutants.

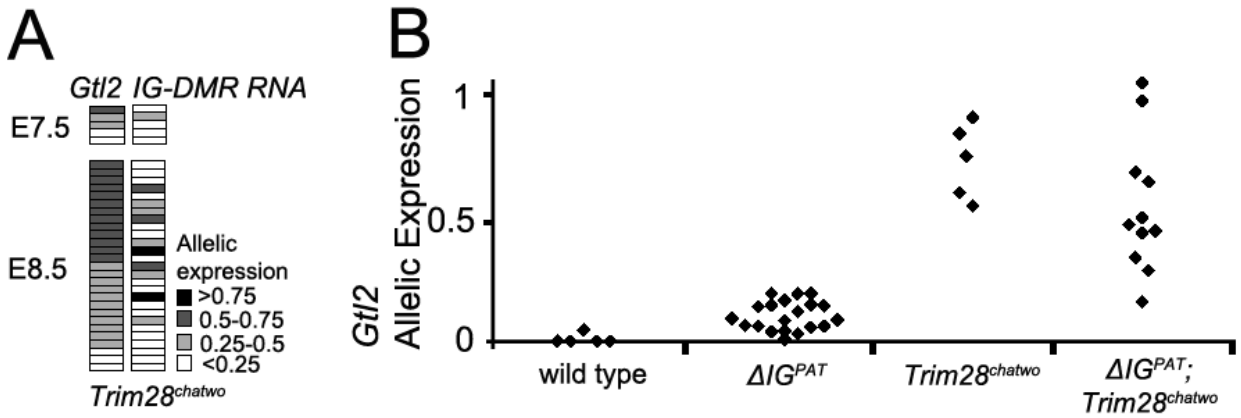

Figure 3.2. TRIM28 controls imprinting at *Gtl2* through mechanisms other than binding the paternal IG-DMR. (A) Comparison of allelic expression of *Gtl2* (left) and *IG-DMR RNA* (right) in individual E7.5 (top) and E8.5 (bottom) *Trim28*^{chatwo} embryos. Each row represents a single embryo. Rows are sorted by decreasing *Gtl2* biallelic expression. Analysis of Pearson's correlation coefficient showed that *Gtl2* and *IG-DMR RNA* allelic expression were not correlated in either E7.5 or E8.5 embryos **(B)** PeakPicker quantification of *Gtl2* allelic expression in wild type, *ΔIG*^{PAT}, *Trim28*^{chatwo}, *and ΔIG*^{PAT}*;Trim28*^{chatwo} E8.5 embryos. Each diamond represents allelic expression of a single embryo. A value of zero indicates full maternal expression. A value of one indicates equal expression from the maternal and paternal alleles.

TRIM28 controls imprinting of *Gtl2* through mechanisms other than binding the paternal IG-DMR. Given the inability of paternal IG-DMR enhancer activation to account for biallelic expression of *Gtl2* in *Trim28*^{chatwo} mutants, we hypothesized that TRIM28 may have additional functions outside of the IG-DMR for regulating of *Gtl2*. To test this, we performed epistasis experiments between *Trim28*^{chatwo} mutants and mutants deleted for the paternal IG-DMR (*ΔIG*^{PAT}). In these experiments, we would expect that if TRIM28 controls imprinting solely through its binding of the paternal IG-DMR, then deletion of the paternal IG-DMR should be epistatic over loss of *Trim28.* Consequently, given that deletion of the paternal IG-DMR in *ΔIG*^{PAT} embryos does not effect imprinting (Figure 3.2 B)(Lin et al., 2003), we predicted that *ΔIG*^{PAT}*;Trim28*^{chatwo} embryos would express *Gtl2* monoallelically. However, we observed that *ΔIG*^{PAT}*;Trim28*^{chatwo} mutants biallelically express *Gtl2* at similar levels to *Trim28*^{chatwo} embryos (Figure 3.2 B), demonstrating that TRIM28 is required outside of binding the paternal IG-DMR to repress paternal *Gtl2*.

Tissue-specific enhancer activity at the paternal IG-DMR. Interestingly, our analysis of *ΔIG*^{PAT} E8.5 embryos showed that, although these mutants expressed *Gtl2* primarily from the maternal allele, there was slight, but significant expression of paternal *Gtl2* compared to wild types (Figure 3.2 B, $p < 0.01$). This finding led us to hypothesize that the paternal IG-DMR may contribute to imprinting regulation in a subset of tissues. To further investigate this, we isolated individual tissues from E14.5 *ΔIG*^{PAT} embryos.

Analysis of expression levels of *IG-DMR RNA* in limb, yolk sac, liver, lung, forebrain, and heart showed that expression of *IG-DMR RNA* varied between tissues, with the highest expression in the limb, and nearly undetectable expression in heart (Figure 3.3 A). Expression of *IG-DMR RNA* was roughly correlated with expression of *Gtl2* (Figure 3.3 B). However, one exception was the yolk sac which, compared to the liver, showed similar expression of *Gtl2* but decreased *IG-DMR RNA* expression. Additionally, we found that *Dlk1* expression was not well correlated with *IG-DMR RNA* expression (Figure 3.3 C). Together, these results indicate that enhancer activity from the IG-DMR is variable amongst different embryonic tissues.

To determine whether the IG-DMR enhancer was active from one or both alleles, we measured allelic expression of *IG-DMR RNA* utilizing crosses that contained SNPs within the *IG-DMR RNA*. The limb and the yolk sac both showed maternal-specific expression of *IG-DMR RNA* as measured by Sanger sequencing in reciprocal crosses at two separate SNPs (Figure 3.4 A-B, left). To corroborate the maternal-specific expression of *IG-DMR RNA*, we measured *IG-DMR RNA* expression levels in ΔIG^{PAT} and ΔIG^{MAT} mutants. As expected, *IG-DMR RNA* expression in the limb and yolk sac was unchanged in ΔIG^{PAT} mutants, but decreased in ΔIG^{MAT} embryos (Figure 3.4 A-B, right), providing further support that the *IG-DMR RNA* is maternally expressed in limb and yolk sac. In contrast, *IG-DMR RNA* was biallelically expressed in both liver and lung (Figure 3.4 C-D, left). This biallelic *IG-DMR RNA* expression in liver and lung was corroborated by decreased levels of *IG-DMR RNA* when either the maternal or the paternal IG-DMR was deleted (Figure 3.4 C-D, right). Thus, contrary to mESC (Kota et

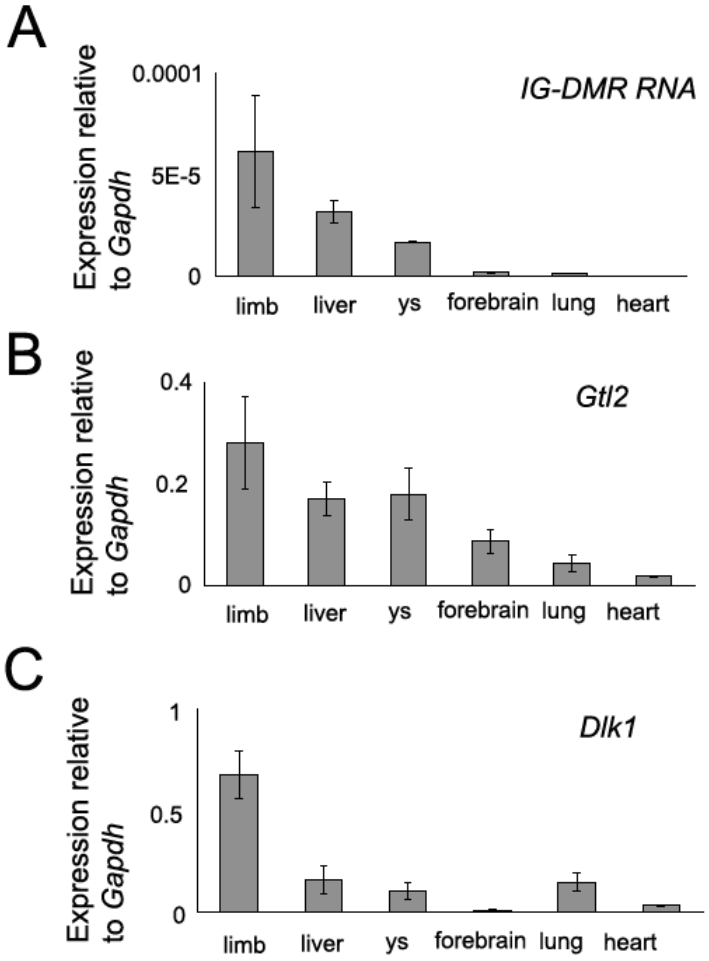

Figure 3.3. Expression levels of *IG-DMR RNA, Gtl2,* and *Dlk1* in different E14.5 tissues. Expression of *IG-DMR RNA* (A), *Gtl2* (B), and *Dlk1* (C) expression in limb, liver, yolk sac, forebrain, lung and heart as measured by qRT-PCR normalized to *Gapdh* expression levels. Error bars represent standard deviation of two biological replicates.

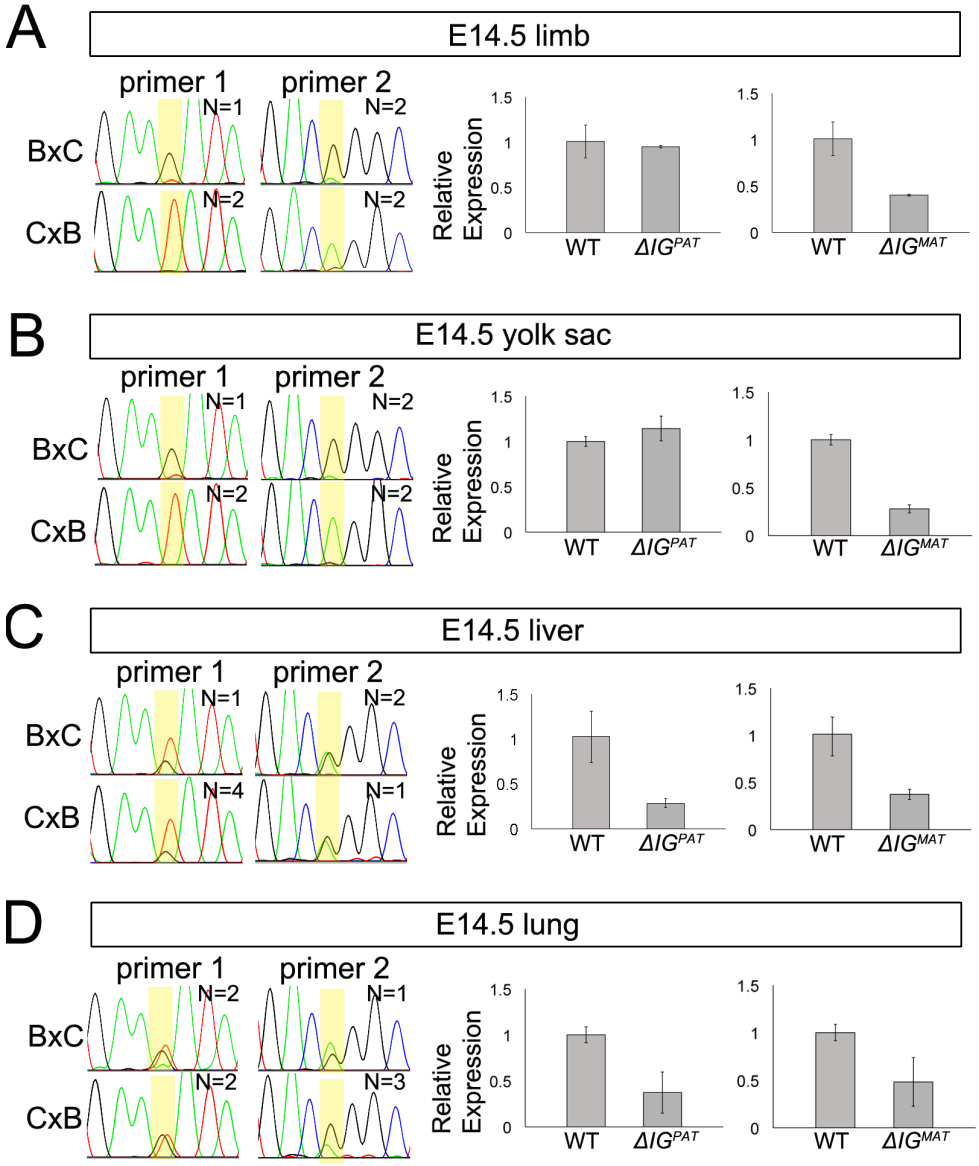

Figure 3.4. Tissue-specific biallelic expression of *IG-DMR RNA*. Sanger sequencing traces showing *IG-DMR RNA* allelic expression in wild type limb (A), yolk sac (B), liver (C), and lung (D) (left). Allelic expression was measured in reciprocal BL6 x CAST (BxC) and CAST x BL6 (CxB) crosses and measured at two different SNPs (primer 1 and primer 2). Relative expression of *IG-DMR RNA* was measured in wild type, *ΔIG*^{PAT}, and *ΔIG*^{MAT} embryos by qRT-PCR, normalized to *Gapdh*, and plotted relative to wild type expression levels (right). SNP positions are highlighted in yellow.

al., 2014) and early embryos (Figure 3.1 C), active transcription occurs at the paternal IG-DMR in liver and lung, indicating that the paternal IG-DMR contains an active enhancer in these tissues.

Tissue-specific allelic histone modifications. Active enhancers are nucleosome free, but the flanking areas tend to be associated with particular histone modifications (reviewed in Bell et al., 2011; Kouzarides, 2007). While no histone modification is a perfect predictor of enhancer activity (Arnold et al., 2013; Bonn et al., 2012), H3K27 acetylation (H3K27ac) and H3K4 methylation (H3K4me1, H3K4me2, and H3K4me3) tend to be associated with active enhancers (Arnold et al., 2013; Core et al., 2014; Pekowska et al., 2011; Rada-Iglesias et al., 2011). We therefore investigated whether H3K27ac and H3K4me2 showed tissue-specific binding patterns at the IG-DMR and flanking regions using ChIP-qPCR. Immunoprecipitated chromatin from these experiments was also analyzed by Sanger sequencing of ChIP PCR products to measure allele-specific histone occupancy. The levels of H3K27ac and H3K4me2 showed similar patterns between tissues, with the highest levels of H3K27ac downstream of the IG-DMR, high levels of H3K4me2 near the ZFP57 binding sites (Figure 3.5, yellow boxes) as well as some H3K4me2 binding downstream of the IG-DMR in yolk sac and lung(Figure 3.5). Consistent with the maternal-specific expression of *IG-DMR RNA* in yolk sac tissues, H3K27ac and H3K4me2 were enriched on the maternal allele throughout the IG-DMR and downstream regions in yolk sacs (Figure 3.5 A,D, pie charts). In liver and lung, where the *IG-DMR RNA* was biallelically expressed, the paternal IG-DMR showed maternal-specific binding of H3K27ac and H3K4me2 at

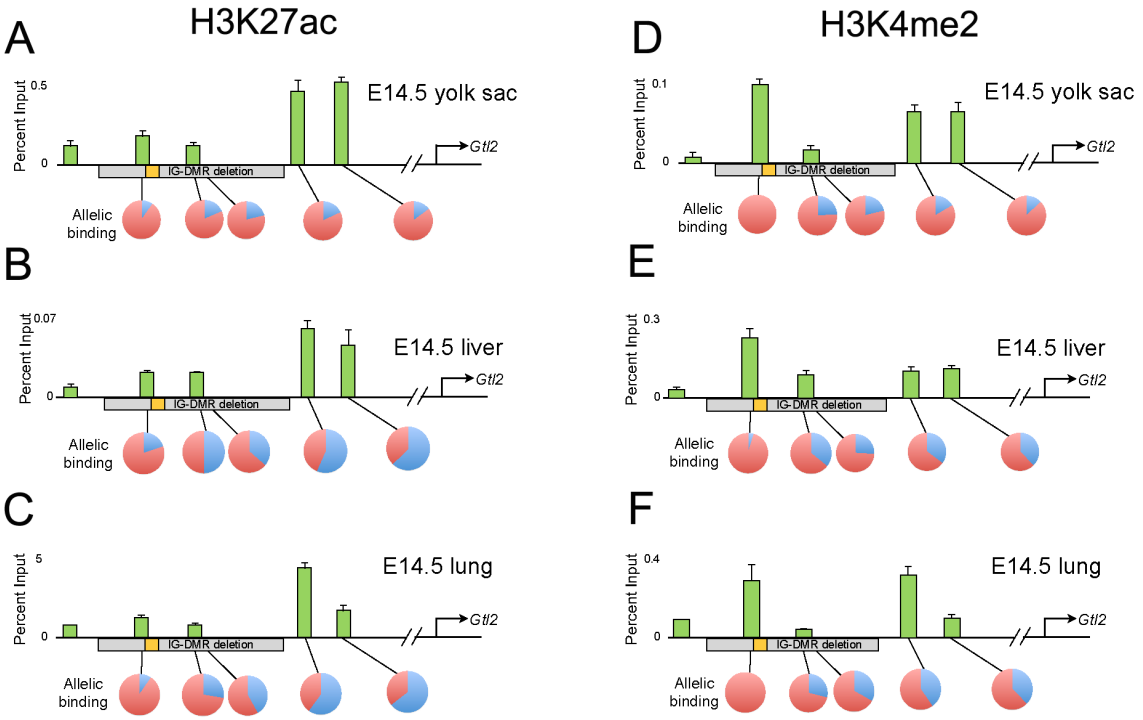

Figure 3.5. Allele-specific histone modifications at *Gtl2* intergenic region in the yolk sac, lung, and liver. ChIP qPCR (green bars) and allele-specific binding (pie charts) of H3K27ac (A-C) and H3K4me2 (D-F) at different locations at or near the IG-DMR (map diagramed below ChIP-qPCR, grey box indicates IG-DMR deletion, yellow box indicates location of ZFP57 binding) in E14.5 yolk sac (A, D), liver (B, E), and lung (C, F). Error bars of ChIP-qPCR represent the standard deviation of two technical ChIP-qPCR replicates. Allelic binding was quantified by PeakPicker of Sanger sequencing of ChIP-PCR products and represented in pie charts as the proportion maternally bound (red) and proportion paternally bound (blue).

the 5' end of the IG-DMR (Figure 3.5 B-F, leftmost pie chart), but biallelic histone binding at the 3' end and at downstream regions (Figure 3.5 B-F, right pie charts). Thus, as with RNA expression, the presence of allele-specific histone modifications at and near the IG-DMR differs between tissue types. Overall, these findings support the conclusion that the paternal IG-DMR has tissue-specific enhancer activity.

The paternal IG-DMR has tissue-specific requirements for paternal gene activation. Given our finding that the paternal IG-DMR has enhancer-like properties in the liver and the lung, we hypothesized that the paternal IG-DMR may have liver and lung specific functions for controlling expression of genes within the *Dlk1-Dio3* cluster. Since imprinted genes sometimes show tissue-specific loss of imprinting (reviewed in Prickett and Oakey, 2012), it is possible that the IG-DMR functions in cis in the liver and lung to promote biallelic expression of *Gtl2.* However, analysis of *Gtl2* allelic expression showed that *Gtl2* was expressed from only the maternal allele in wild type limb, yolk sac, liver, and lung (data not shown), demonstrating that the paternal IG-DMR does not activate paternal *Gtl2* in the liver and lung. Thus, to determine potential targets of the paternal IG-DMR enhancer, we investigated expression of the genes within the *Dlk1-Dio3* cluster in ΔIG^{PAT} mutants using qRT-PCR.

In the limb and yolk sac, the paternally-expressed genes *Dlk1, Rtl1* and *Dio3* were unaffected by paternal IG-DMR deletion(Figure 3.6 A-B), similar to what has been previously reported in whole embryos and placenta (Lin et al., 2003; 2007). These results are consistent with the absence of enhancer activity at the paternal IG-DMR in

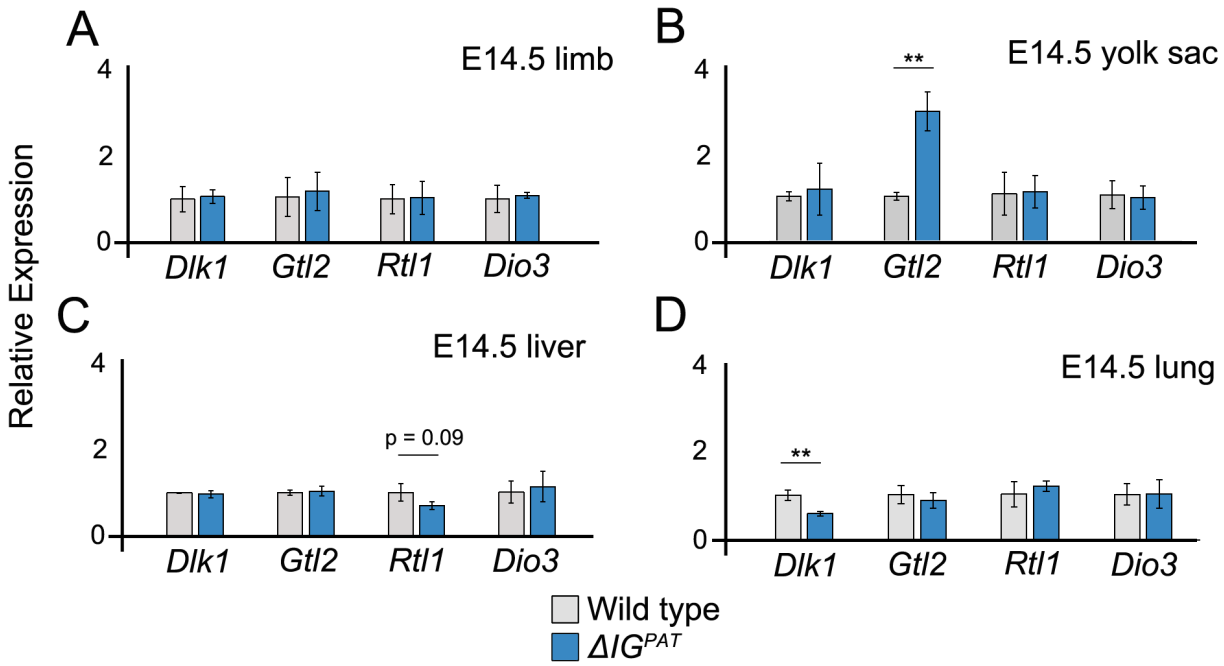

Figure 3.6. Expression of genes within *Dlk1-Dio3* cluster in different tissues of *ΔIG*^{PAT} deletion mutants. Relative expression of paternally expressed *Dlk1, Rtl1,* and *Dio3* and maternally-expressed *Gtl2* in *ΔIG*^{PAT} mutants as measured by qRT-PCR relative to wild type embryos, normalized to *Gapdh* expression levels in E14.5 limb (A), yolk sac (B), liver (normalized to *ßactin*)(C), and lung (D). Error bars represent standard deviation of three biological replicates. ** - $p < 0.05$.

limb and yolk sac. In the liver, despite the presence of active enhancer characteristics, *Dlk1, Dio3* and *Rtl1* were unaffected (Figure 3.6 C). Thus, if the paternal IG-DMR contributes to activation of paternally expressed genes in the liver, its role is not essential and there may be redundant mechanisms enhancing transcription. On the other hand, the lung of ΔIG^{PAT} mutants did not have decreased expression of *Rtl1* or *Dio3,* but showed a significant reduction in expression of *Dlk1* (Figure 3.6 D, $p < 0.05$), demonstrating a requirement of the paternal IG-DMR for *Dlk1* activation. Notably, *Dlk1* expression was not completely eliminated in ΔIG^{PAT} mutants, with expression in mutants about 50% of wild type expression levels. This is in contrast to the nearly complete loss of *Gtl2* expression in ΔIG^{MAT} whole embryos and in individual embryonic tissues (Lin et al., 2003)(Figure 3.7). Thus, while the maternal IG-DMR is absolutely essential for expression of maternal genes in embryonic tissues, the requirement of the paternal IG-DMR for expression of *Dlk1* in the lung is partial, indicating that the paternal IG-DMR is just one component that aids *Dlk1* transcription

One intriguing finding from these data is that deletion of the paternal IG-DMR differentially affects *Dlk1* expression in the liver and the lung, which both show enhancer activity at the paternal IG-DMR. Thus, it seems that the paternal IG-DMR contributes to activation of paternally-expressed genes depending on the developmental context. To further explore this, we measured the expression of *Dlk1* and *Dio3* in whole E9.5 embryos. As in E14.5 lung, E9.5 ΔIG^{PAT} embryos showed decreased expression of *Dlk1* (Figure 3.8, $p < 0.05$). Interestingly, E9.5 ΔIG^{PAT} embryos also showed a significant

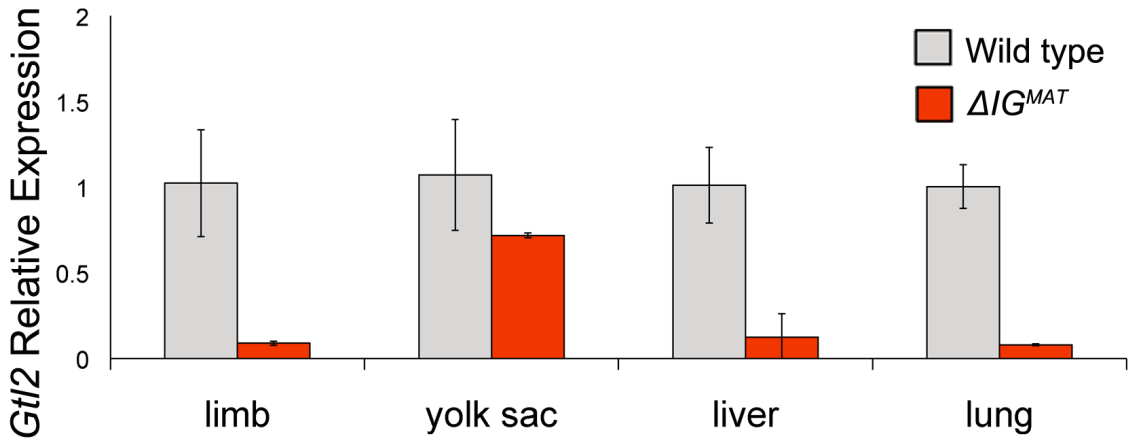

Figure 3.7. Expression of *Gtl2* in E14.5 limb, yolk sac, liver, and lung of *ΔIG*^{MAT} mutants compared to wild type litter mates. qRT-PCR in limb, yolk sac, liver, and lung in wild type and *ΔIG*^{MAT} embryos normalized to *Gapdh* and relative to wild type expression. Error bars represent standard deviation from two biological replicates.

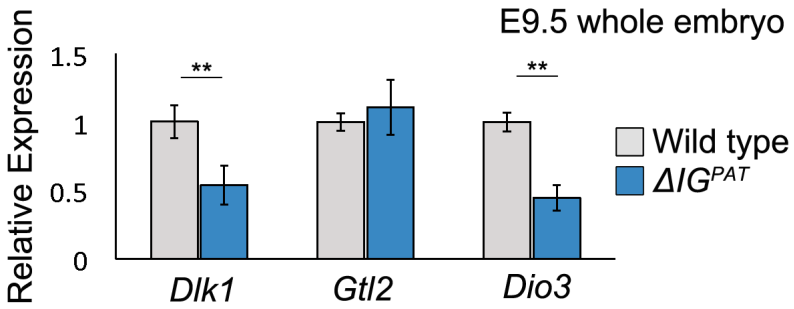

Figure 3.8. The paternal IG-DMR is required for activation of *Dlk1* and *Dio3* in E9.5 whole embryos. qRT-PCR analysis of expression levels normalized to *Gapdh* and shown relative to wild type embryos. Error bars represent standard deviation of two biological replicates. ** - $p < 0.05$.

reduction in levels of *Dio3* compared to littermate controls (Figure 3.8 $p < 0.05$). Based on our above finding that *Dio3* is not disrupted in the liver or the lung, the finding that *Dio3* is disrupted in E9.5 ΔIG^{PAT} mutants further support a context-specific requirement of the paternal IG-DMR for activating different paternally-expressed genes.

Over the course of embryonic development, genes within the *Dlk1-Dio3* cluster are dynamically expressed, fulfilling highly specific developmental functions (Falix and Tjon-A-Loi, 2013; Hernandez et al., 2007; Schuster-Gossler et al., 1998; Sekita et al., 2008; St Germain and Galton, 1997; Yevtodiyenko and Schmidt, 2006). Although the allele-specific expression at the *Dlk1-Dio3* cluster may be coordinately regulated by the IG-DMR, the overall expression levels of individual genes within the cluster are not highly correlated (da Rocha et al., 2007), suggesting that tissue-specific mechanisms control the level of imprinted gene expression. Our finding that the paternal IG-DMR is required for tissue-specific activation of paternally-expressed genes suggests a model whereby the need for cell type specific regulation of expression levels may be integrated with the control of allelic expression by the IG-DMR.

The paternal IG-DMR is required for repression of *Gtl2* in the yolk sac. As we measured expression levels of imprinted genes in different tissues, we noticed that the maternally-expressed gene, *Gtl2*, was significantly overexpressed in yolk sacs of ΔIG^{PAT} mutants (Figure 3.6 B, $p < 0.05$). Based on our previous finding that *Gtl2* was slightly biallelically expressed in E8.5 ΔIG^{PAT} embryonic tissues (Figure 3.2 B, $p < 0.05$), we hypothesized that the overexpression of *Gtl2* in E14.5 yolk sac may be due to a tissue-

specific requirement of the paternal IG-DMR for repressing paternal *Gtl2*. To investigate this, we measured allelic expression of *Gtl2* in different E14.5 and E8.5 tissues. In wild type tissues, *Gtl2* was consistently expressed from only the maternal allele (Figure 3.9 A-B). In ΔIG^{PAT} mutants, *Gtl2* was slightly, but significantly, biallelic in E14.5 limb and E8.5 embryos (Figure 3.9 A-B, E14.5 limb - $p < 0.005$, N =4; E8.5 embryo - $p < 0.01$ N = 20). More strikingly, *Gtl2* expression was fully biallelic in E14.5 yolk sacs of ΔIG^{PAT} mutants (Figure 3.9 A, $p < 0.005$, N = 7). In E8.5 ΔIG^{PAT} yolk sacs, *Gtl2* was also biallelically expressed, albeit to a lesser extent (Figure 3.9 B, $p < 0.01$, N = 5). Thus, the paternal IG-DMR plays a critical role in repressing paternal *Gtl2*, a function that is most evident in the yolk sac, but also is apparent in other tissue types.

Our finding that the paternal IG-DMR has an increased requirement for silencing *Gtl2* in the yolk sac extrembryonic tissue was intriguing based on a previous study that found that the maternal IG-DMR has a reduced requirement for activating *Gtl2* in placental extraembryonic tissue compared to embryonic tissues (Lin et al., 2007). The reduced role of the maternal IG-DMR for activating *Gtl2* extends to the E14.5 yolk sac tissue, which unlike embryonic-derived E14.5 tissues (limb, liver and lung), showed no significant reduction of *Gtl2* in ΔIG^{MAT} mutants (Figure 3.7). These data highlight reciprocal roles for the maternal and paternal IG-DMR in yolk sac compared to embryonic tissues. In embryonic tissues, the maternal IG-DMR facilitates activation of maternal *Gtl2*, and the paternal IG-DMR does not have strong requirements for repressing paternal *Gtl2.* In yolk sac, the maternal IG-DMR is not required to activate maternal *Gtl2,* but the paternal IG-DMR is essential for preventing activation at paternal

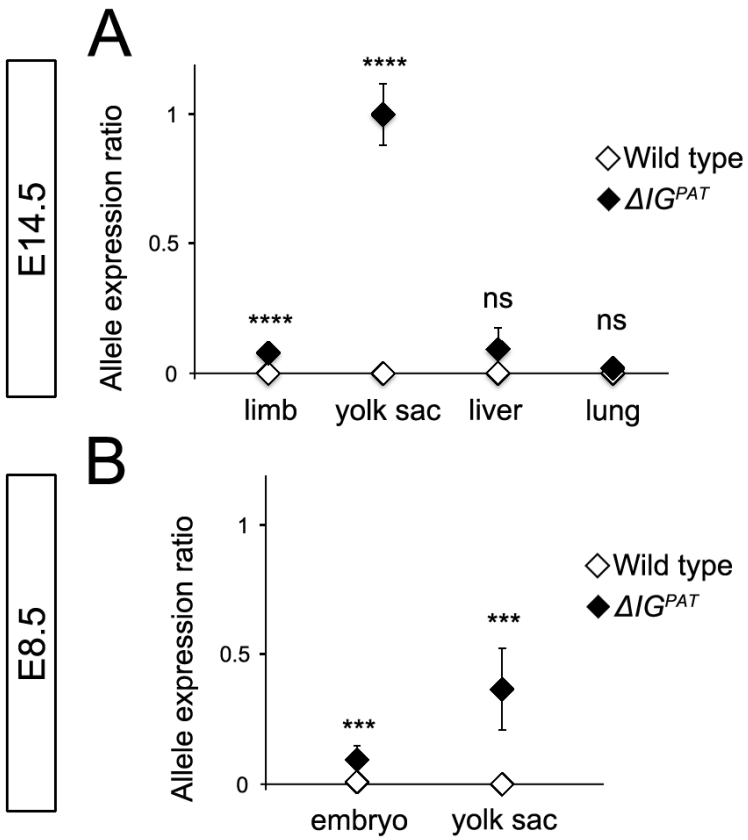

Figure 3.9. Requirements of the paternal IG-DMR for allelic expression of *Gtl2.* PeakPicker quantification of allelic expression in E14.5 (A) and E8.5 (B) tissues from wild type (white diamonds) and *ΔIG*^{PAT} (black diamonds) embryos. Error bars represent standard deviation of at least three biological replicates. Values of zero indicate full maternal expression of *Gtl2*. Value of one indicate equal expression from both alleles. *** - $p < 0.01$. **** $p < 0.005$. ns - not significant.

Gtl2. These data point to a model whereby alternative mechanisms, perhaps an alternative enhancer, activate *Gtl2* in the yolk sac and the paternal IG-DMR inhibits the ability of this alternative mechanism to function on the paternal allele. The reason for this differential regulation of imprinting in yolk sac compared to embryonic tissues is still unclear. However, given our other findings of tissue-specific requirements of the paternal IG-DMR, these data are consistent with our overall conclusion that the IG-DMR controls imprinting through distinct mechanisms in different tissue and developmental contexts.

The current understanding in the imprinting field is that the maternal IG-DMR functions as an imprinting control region, but that the paternal IG-DMR is relatively unimportant for imprinting control. Here we show that this is true only in a subset of tissue types, such as E14.5 limb, where deletion of the paternal IG-DMR has minimal consequences for imprinted gene expression. In other tissue types (E14.5 liver and lung), the paternal IG-DMR has enhancer activity similar to what has been described at the maternal IG-DMR (Kota et al., 2014). However, in contrast to the maternal IG-DMR, the paternal IG-DMR does not promote transcription of the maternally-expressed gene, *Gtl2*. Rather, the paternal IG-DMR is required for the full expression of specific paternally-expressed genes. The E14.5 yolk sac, similar to the limb, does not have paternal IG-DMR enhancer activity and the paternal IG-DMR is not required for activation of the paternally expressed genes. Yet deletion of the paternal IG-DMR in the yolk sac leads to overexpression and biallelic expression of *Gtl2*. Overall, these data demonstrate that the paternal IG-DMR has different functions in different tissues, acting

as an enhancer to paternally-expressed genes in some tissues and as a repressor to maternally-expressed genes in others.

EXPERIMENTAL PROCEDURES

Mice

Experiments involving allele-specific expression or allele-specific binding used crosses between mice of FVB or C57Bl6/NJ genetic backgrounds and a mixed CAST/eij and FVB genetic background that was CAST/CAST at mouse chromosome 12. *Trim28*^{chatwo} embryos derived from these crosses arrest at E8.5 (Shibata et al., 2011). Genotyping primers for *ΔIG, Trim28*^{chatwo} and for polymorphisms at chromosome 12 are shown in Table 3.1.

Embryo collection

Embryos were dissected in ice cold phosphate buffered saline with 4% bovine serum albumin.For analysis of individual tissue types, these whole tissues were dissected from the embryo and flash frozen in liquid nitrogen. For E8.5 embryos, the yolk sac tissues were dissected away from embryonic tissues and analyzed separately.

Expression analysis

RNA was isolated from mouse embryonic tissues using phenol-chloroform extraction. RNA was DNAse treated prior to First Strand cDNA synthesis using SuperScriptIII and

random hexamers (Invitrogen). Expression was quantified by qPCR of reverse transcriptase products (qRT-PCR) using KleenGreen (IBI). Expression was normalized to *Gapdh* or *ß-actin*. For *Rtl1*, which has an antisense transcript, expression levels were quantified using a primer specific to *Rtl1* and a poly-T primer. See Table 3.1 for primer sequences.

Chromatin Immunoprecipitation

Embryonic tissues were collected, flash frozen in liquid nitrogen, and stored in the -80 freezer. Tissues were broken up into a fine powder in liquid nitrogen using a tissue smasher and crosslinked for 10 minutes at room temperature in 1% formaldehyde. ChIP was performed according to the Abcam XChIP protocol (www.abcam.com/protocols/cross-linking-chromatin-immunoprecipitation-x-chip-protocol). Antibodies used: H3K4me2 (Abcam - ab32356), H3K27ac (Abcam ab4729).

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Table 3.1. Primers used in this study

Genotyping				
	Primer 1	Primer 2	Source	Notes
chatwo	atgtgttgtgtggcccagta	acctcttggcacctgcaac	Shibata et al., 2011	Use BslI RFLP to detect chatwo mutation
IG-DMR del	agtcttgacctggtgcttca	ggcaaccaacgctatagtgg		300 bp in delta IG-DMR, >2kb in wild type
IG-DMR WT	gccgctatgctatgctgttt	gcgtgatcccattcccaatc		136 bp in wild type, no product in delta IG-DMR
D12Mit80	caacccagatgtcccttaaca	ctggaaggtttcacctagttgg	MIT marker, MGI	CAST versus FVB SSLP near *Gtl2*

Allelic Expression and Allelic Binding						
	Primer 1	Primer 2	SNP possition (mm9, chr12)	SNP	Source	Notes
IG-1snp	tacggagatgtgctgtggac	ctagtccatggcgaaccttg				
IG-2snp	ccttggcatcctggctcaccg	cccgggggctcaggagtaggg	110767870	T/G	Kota et al., 2015	R7 in Kota et al., 2015
IG-3snp	tttgctaagttggggtttgc	aaagctgcactgggacagat	110768570	T/C	Kota et al., 2015	R9 in Kota et al., 2015, use Primer 2 for sequencing
Mid-1	caagagcgccagtttttcat	gccagcaggaaagctaactg	110,770,936 and 110,770,971	T/C, A/T		
Mid-2	tttgcagacacaaggagtgg	gacacggactcgaatgttca	110,771,615	A/T		
Gtl2-pA/B	gccaaagccatcatctggaatc	cacagatgtagactcaacagtgaag		T/C	Schmidt et al., 2000	

Quantitative PCR				
	Primer 1	Primer 2	Source	Notes
Dlk1-qRT	ttaccggggttccttagagc	tgcattaatagggaggaaggg		
IG upstream-qPCR	ctcccatgggacccagggttg	gtgtaccacatgtgggctgcc		
IG-1-qPCR	tgggtttaccgtaaaggatgattt	ctctggcacaaagcaatgataca	Quennevile et al., 2011	
IG-2-qPCR	agaagctgtggtgggattgct	agggccacttgcatcagaat	Kota et al., 2015	
Mid-1-qPCR	gcttggatctcttgggctgac	cctgtttgcagccctaactc		
Mid-2-qPCR	tggggaacaaaaggtttgag	tccgcagggttatacacaca		
Gtl2-qRT	gggcgcccacagaagaa	ggtgtgagccgatgatgtca		
Rtl1-qRT	ccagacccactgctcactgcc	ttttttttttttttttttt		Primer 2 poly T to avoid amplification of anti-Rtl1
Dio3-qRT	atgaacagttgcatggtactaggc	gtcacttgtcccttggttatttagaatc		

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CHAPTER 4

FUTURE DIRECTIONS

Our studies of TRIM28 and the mechanisms regulating the *Gtl2* imprinted cluster in the context of embryonic development have illuminated several potential future directions, outlined below:

To what extent does TRIM28 protect imprinted germline DMRs and other genomic regions from DNA demethylation during early embryonic reprogramming? In Chapter 2, we show that TRIM28 is essential for preserving DNA methyl marks at the *H19, Snrpn* and *Gtl2* germline DMRs during the genome-wide demethylation event that reprograms the genome shortly after fertilization. However, the mechanisms by which TRIM28 preserves DNA methylation and extent to which TRIM28 is required for protecting DNA methyl marks at other genomic loci are still unclear.

In mouse embryonic stem cells, TRIM28 co-immunoprecipitates with all known germline DMRs that resist pre-implantation DNA methylation reprogramming (Quenneville et al., 2011). Taken together with our finding that TRIM28 controls allelic expression of many different imprinted genes, this suggests that TRIM28 may broadly protect germline DMRs from DNA demethylation. However, we observed that in hypomorphic *Trim28* mutants, germline DMR methylation at the *H19* cluster is severely disrupted, while at the *Gtl2* cluster germline DMR methylation is not perturbed, suggesting that germline DMR methylation at some imprinted clusters is more sensitive to loss of *Trim28.* Since we have not directly tested DNA methylation levels across the genome, the extent to which TRIM28 protects DNA methylation at all the germline DMRs is still not known. Intriguingly, we found that IAPs, a non-imprinted class retroviral

elements that is protected from pre-implantation DNA demethylation, also require TRIM28 for DNA methylation, supporting that TRIM28 could have a general role for pre-implantation DNA methylation maintenance (see Appendix A). While it should be possible to assess the global requirements of TRIM28 for maintaining DNA methylation in pre-implantation embryos through genomic approaches, these experiments should take into account the following considerations: Since neither maternal nor zygotic *Trim28* deletion mutants show fully penetrant loss of imprinting, combined maternal-zygotic *Trim28* null embryos should be used for these experiments. However, the use of maternal-zygotic *Trim28* null mutants poses important challenges because these embryos developmentally arrest around the early blastocyst stage, when the embryos contain fewer than 200 cells. Whole genome bisulfite sequencing (WGBS) in single cells has been able to detect DNA methylation at nearly 50% of CpGs (Smallwood et al., 2014). Thus, WGBS should be possible for these small sample sizes. However, its interpretation can be complicated by lack of overlap in coverage between experimental and control groups. Some of these experimental issues can be partially alleviated by using sever sets of pooled maternal-zygotic *Trim28* mutants. Because maternal-zygotic *Trim28* mutant embryos are phenotypically indistinguishable from their littermates, embryos will need to be genotyped using immunofluorescence staining of TRIM28. This genotyping protocol should not be an impediment for WGBS since we were previously able to measure DNA methylation at IAPs in pools of morula stage embryos (see Appendix A). Finally, in order to accurately quantify methylation levels, at least 40x coverage is needed. For WGBS, this can be quite costly, with one sample occupying an entire sequencing lane. Reduced representation bisulfite sequencing (RRBS) is a less

costly alternative that enriches for CpG-rich genomic regions, thus eliminating sequencing reads from genomic regions that are unlikely to be methylated. We expect that high-throughput analysis will give us a more complete picture of the extent to which TRIM28 is required to maintain DNA methylation at both imprinted germline DMRs and non-imprinted genomic loci.

How does TRIM28 protect imprinted germline DMRs from DNA demethylation? Also unclear from our current studies are the mechanisms by which TRIM28 prevents DNA demethylation at germline DMRs. During pre-implantation development, DNA methylation is thought to be erased actively by enzymatic modification of methyl groups and passively as the DNA replicates in the absence of DNMT1 methylation maintenance activity (Messerschmidt et al., 2014). Theoretically, TRIM28 may help prevent both active and passive DNA demethylation at germline DMRs. Several experiments might shed light onto these potential mechanisms. One method by which DNA methylation marks are actively removed is by oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Recent technologies have been able to distinguish these different cytosine oxidation states at single nucleotide resolution (Booth et al., 2014; Wu et al., 2014; Yu et al., 2012). However, these methods require large amounts of starting material and will need to be optimized for examining cytosine modifications in pre-implantation *Trim28* mutant embryos.

TRIM28 could prevent passive DNA demethylation, one mechanism by recruiting DNMT1 to specific genomic loci. As discussed in Chapter 2, DNMT1 is largely excluded from the nucleus of preimplantation embryos, yet it is essential for maintaining DNA

methylation at particular genomic loci during this developmental stage. Therefore, one possibility is that TRIM28 recruits DNMT1 specifically to loci that do not lose DNA methylation. If this is the case, we would expect an overlap between the genomic binding sites for TRIM28 and DNMT1, a hypothesis that could be tested using ChIP-seq One challenge to these experiments is that ChIP-seq works most robustly when several million cells are used, a number that is not feasible to obtain for maternal-zygotic *Trim28* mutants. Low input ChIP protocols are emerging, lending promise that ChIP may be possible in preimplantation embryos in the near future (Schmidl et al., 2015).

To what extent is TRIM28 required for post-implantation acquisition of DNA methylation? By using *Sox2-Cre* to conditionally delete *Trim28* around the time of implantation, we found that TRIM28 is essential for DNA methylation at both the *Gtl2* and *H19* secondary DMRs (see Chapter 2). In these mutants, germline DMR methylation was not disrupted, indicating that TRIM28 is required for the establishment of DNA methylation at these secondary DMRs rather than the general DNA methylation maintenance. Thus far, we have only investigated the requirements of TRIM28 for DNA methylation at two secondary DMRs. Therefore, we do not yet know whether TRIM28 could have a universal role for methylating secondary DMRs or even a general role for the genome-wide DNA remethylation that occurs during implantation. To gain an initial indication of whether TRIM28 has a global function for post-implantation acquisition of DNA methylation, we can use immunofluorescence and Western blotting with an antibody against 5mC. Regardless of whether we observe a global decrease of DNA methylation in conditional *Trim28* mutants, it will still be informative to use WGBS or

RRBS to obtain specific information about which genomic loci require TRIM28 for methylation establishment.

Mechanisms by which TRIM28 facilitates secondary DMR methylation TRIM28 may directly recruit DNA methyltransferases to secondary DMRs. Alternatively, hypomethylation at secondary DMRs in conditional *Trim28* mutants may be a secondary consequence of TRIM28 loss of function effects on transcription or histone modifications. At the *Gtl2* secondary DMR, DNA methylation and transcription were both altered in *Trim28* mutants. Thus, it is possible that at this locus aberrant transcription from the paternal allele prevented DNA methylation acquisition at its promoter. The *H19* secondary DMR however, was also hypomethylated in *Trim28* conditional mutants, but in this case, loss of TRIM28 did not alter allele-specific transcription. Together, these results suggest that TRIM28 can have primary effects on secondary DMR methylation. Although it is unclear why hypomethylation at the *Gtl2* and *H19* promoters had different effects on transcription of these genes. To gain further insights into the mechanisms by which TRIM28 regulates DNA methylation and transcription post-implantation, it will be useful to measure changes in transcription, DNA methylation, and histone modifications in a time course after TRIM28 deletion. At the *Airn* imprinted locus, secondary DMR methylation has been successfully achieved when ESCs were differentiated in culture (Latos et al., 2009). Therefore, we could potentially measure the genome-wide effects of inducible *Trim28* deletion on secondary DMR methylation using this ESC differentiation system. One complication to these experiments is that TRIM28 is required for ESC pluripotency (Hu et al., 2009), which may affect the differentiation program of ESC upon *Trim28* deletion.

Dissecting specific functions of the *Dlk1-Dio3* IG-DMR. Our analysis of the functions of the IG-DMR in different embryonic tissues and embryonic stages indicates that the IG-DMR has diverse functions during development (see Chapter 3). Our data suggests that different regulatory elements within the IG-DMR regulate imprinting in a tissue-specific manner. Given our finding that the paternal IG-DMR has activating activity in some tissues, but repressive activity in others, it is likely that it contains both activating elements, such as enhancers, and repressive elements, that perhaps include TRIM28 binding sites. Our future goal will be to map all of the enhancers and repressor cis regulatory elements and query their individual importance for regulating transcription of imprinted genes in specific tissue types. This can be achieved by using genomic techniques, such as CHRO-seq and ChIP-seq. Subsequent chromosome conformation capture could help identify regulatory interactions of these elements with particular genes or other regulatory regions. Additionally, once specific regulatory sequences are identified, their individual functions can be determined by evaluating the effect of their mutation/deletion on imprinted gene expression.

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APPENDIX A

TRIM28 PROTECTS REPETITIVE IAP ELEMENTS FROM LOSS OF DNA METHYLATION DURING EARLY-EMBRYONIC REPROGRAMMING

INTRODUCTION

In addition to imprinted germline DMRs, a handful of other genomic loci are specifically protected from the global loss of DNA methylation that occurs during early embryogenesis (Smallwood et al., 2011). Key among these are the transposable repetitive elements, intracisternal A particles (IAPs), as well as genes nearby IAPs. Given that TRIM28 helps protect imprinted germline DMRs from loss of DNA methylation during early embryogenesis (Messerschmidt et al., 2012)(see Chapter 2), we hypothesized that TRIM28 may also play a role in preventing DNA demethylation at other genomic regions that resist reprogramming. Notably, while ZFP57 binds to imprinted germline DMRs, TRIM28 can also be directed to the genome by other members of KRAB zinc finger protein family, which number over 300 in mammals (Lupo et al., 2013). These include ZFP809, which directs TRIM28 to IAPs (Wolf and Goff, 2009). Zygotic deletion of TRIM28 causes a massive overexpression of IAPs, without altering DNA methylation at IAPs (Rowe et al., 2010), indicating that TRIM28 is required for repressing IAPs without altering DNA methylation status. However, because early embryonic reprogramming occurs during the maternal to zygotic transition, it is possible that maternal TRIM28 could help prevent loss of DNA methylation in zygotic *Trim28* mutants.

RESULTS AND DISCUSSION

To determine the contribution of maternal TRIM28 to preserving DNA methylation at IAPs, we measured DNA methylation in maternal *Trim28* null (*mTrim28*$^{L-}$) and maternal-

zygotic *Trim28* null morula (*mzTrim28*$^{L-}$) compared to wild type morula by bisulfite sequencing. Wild type and *mTrim28*$^{L-}$ morula showed similar levels of DNA methylation (Figure A.1). On the other hand, we observed reduction of DNA methylation in *mzTrim28*$^{L-}$ mutants (Figure A.1). These results demonstrate that, in addition to preserving methylation at imprinted DMRs, TRIM28 is also required to prevent loss of methyl marks at IAPs. These findings highlight a more diverse role of TRIM28 for preventing DNA demethylation at different genomic loci. In the future it will be exciting to determine the full extent to which TRIM28 protects DNA methylation during early embryonic reprogramming. The ability of TRIM28 to prevent demethylation would be particularly intriguing in situations where environmentally-induced changes in DNA methylation, as has been found to occur with certain exposures (Manikkam et al., 2012). Notably, DNA sequences that contain such epimutations are enriched for binding sites of ZNF219 and RREB1, two proteins that contain the conserved TRIM28-binding KRAB domain (Guerrero-Bosagna et al., 2014). Thus, it will be interesting to explore whether the scope of TRIM28 requirements during genome-wide reprogramming includes protecting environmentally-induced epimutations from loss of DNA methylation.

EXPERIMENTAL PROCEDURES

mTrim28^{L-} and *mzTrim28*$^{L-}$ were generated with crosses between *ZP3-Cre; Trim28*$^{L2/L-}$ females and *Trim28*$^{L-}$ males. These mutants arrest around the blastocyst stage (see Chapter 2; Figure 2.2D-F). *mTrim28*$^{L-}$ and *mzTrim28*$^{L-}$ were distinguished between one another based on TRIM28 immunofluorescent staining. 3-5 individual morula were pooled and their DNA was bisulfite converted using the EZ DNA Methylation-Direct kit

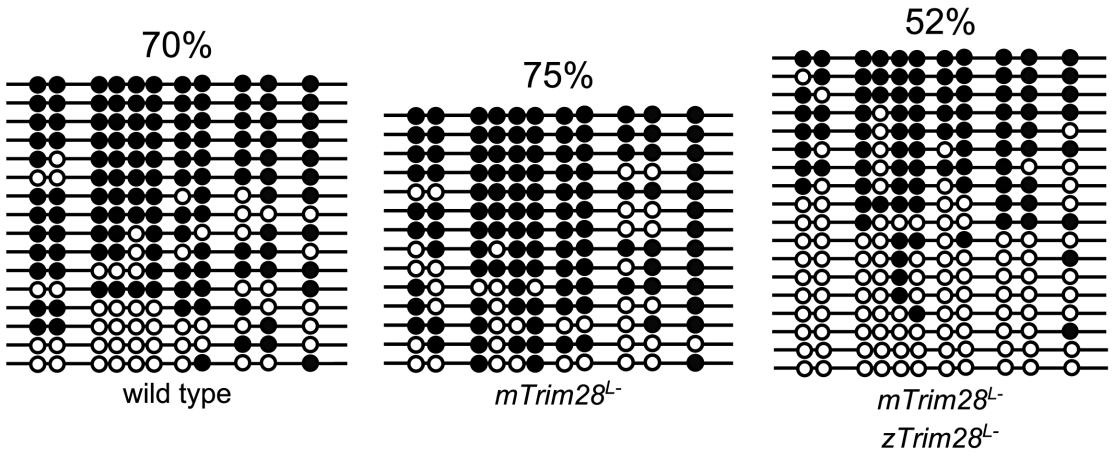

Figure A.1. DNA methylation at the 5‘UTR of IAPs in maternal and zygotic *Trim28* mutants. DNA methylation as measured by bisulfite sequencing at the 5’ UTR of IAPs in pools of 3-5 wild type, *mTrim28*$^{L-}$, and *mzTrim28*$^{L-}$ morula. *mTrim28*$^{:-}$ and *mzTrim28*$^{L-}$ morula were distinguished by the presence or absence of TRIM28 as determined by immunofluorescence. Filled circle represent methylated CpG dinucleotides. Empty circles represent unmethylated CpGs. Percentages (top) represent the percent of CpGs methylated in each sample.

(Zymo). Bisulfite converted DNA was PCR amplified using primers to IAPs as in (Quenneville et al., 2011). BS-PCR products were cloned into the TOPO-TA vector (Invitrogen), and individual clones were sequenced using Sanger sequencing.

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APPENDIX B

SEX SPECIFIC DISRUPTION OF GENOMIC IMPRINTING IN TRIM28 MUTANTS

INTRODUCTION

In Chapter 2, we noticed that imprinting was lost to varying degrees between individual *Trim28* mutant embryos. While one explanation for this variability between embryos could be differing contributions of maternal TRIM28, as described in Chapter 2, we also investigated the possibility that the variability was in part due to sex-specific responses to TRIM28 depletion.

RESULTS AND DISCUSSION

Sex-specific imprinted gene expression level changes in *Trim28^{chatwo}* mutants.

To determine if expression of imprinted genes was disrupted in a sex-specific manner, we measured expression levels of 13 different imprinted genes in 10 female and 10 male *Trim28^{chatwo}* embryos compared to 6 wild type females and males. The wild type embryos did not show variability between male and female embryos (data not shown). Therefore, we normalized imprinted gene expression in *Trim28^{chatwo}* embryos to the average of the combined male and female wild type embryos. As previously observed (Chapter 2), there was variability in imprinted gene expression between the 20 embryos (Figure B.1). Notably, hierarchical clustering that takes into account expression of all 13 imprinted genes divided the 20 embryos into two groups with the 10 male *Trim28^{chatwo}* embryos clustering together and the 10 female *Trim28^{chatwo}* embryos clustering in a separate group (Figure B.1, see top for clustering of embryos). Thus, there appears to be sex-specific changes in imprinted gene expression as a result of TRIM28 loss. Even within males and within females, imprinted gene expression was variable, indicating that

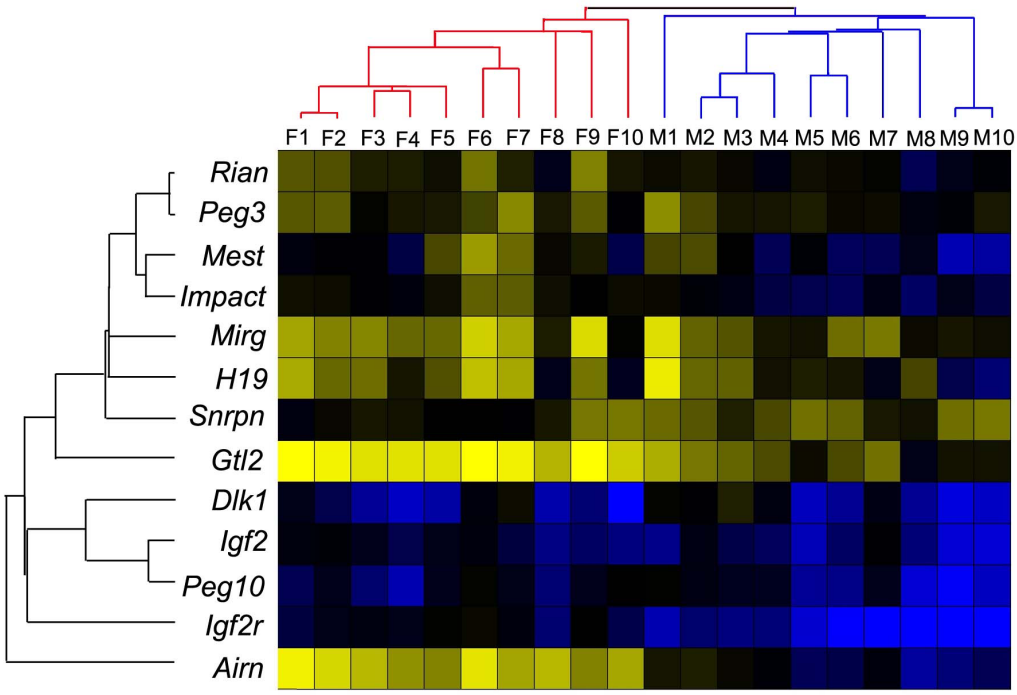

Figure B.1. Sex-specific clustering of imprinted gene expression *Trim28*^{chatwo} mutants. Heatmap representing the upregulation (yellow) and downregulation (blue) of *Rian, Peg3, Mest, Impact, Mirg, H19, Snrpn, Gtl2, Dlk1, Igf2, Peg10, Igf2r,* and *Airn* in individual female (F1-F10), and male (M1-M10) *Trim28*^{chatwo} mutants relative to the average expression of four wild type embryos as quantified by qRT-PCR and normalized to *ß-actin*. Wild type embryos showed little variability between different male and female individuals and were not included in graph. Imprinted genes and embryos were organized within the heatmap by hierarchical clustering.

the differences between males and females cannot fully explain variability between the embryos (variability between embryos is further discussed in Chapter 2).

Gtl2 is more severely biallelic in *Trim28^{chatwo}* females.

The expression differences between males and females in *Trim28^{chatwo}* mutants could be due directly to differences in allelic expression between males and females, or may be secondary effects to disruption of other genes in *Trim28* mutants. To directly measure allelic expression in males compared to females, we performed quantitative pyrosequencing in *Trim28^{chatwo}* embryos that contained SNPs within *H19, Snrpn,* and *Gtl2.* Intriguingly, there was no significant difference in the allelic expression between males and females at either *H19* or *Snrpn* (Figure B.2). On the other hand, *Gtl2* was significantly more biallelic in female *Trim28^{chatwo}* embryos compared to males ($p < 0.001$). Thus, some factor present in females causes more severe loss of imprinting in *Trim28* mutants at *Gtl2,* but not at *H19* or *Snrpn*. This result was interesting based on our previous finding that the mechanisms by which imprinting is disrupted differs between *Gtl2* versus *H19* and *Snrpn* in *Trim28^{chatwo}* mutants. At *H19* and *Snrpn*, loss of imprinting is correlated with loss of DNA methylation at the germline-inherited DMR, while *Gtl2* was biallelically expressed in the absence of changes in germline DMR methylation (see Chapter 2, Figure 2.5 A-C). Also in contrast to *H19* and *Snrpn*, *Gtl2* imprinting was disrupted in conditional *Sox2Cre* induced *Trim28* mutants that eliminate TRIM28 around the time of implantation (see Chapter 2, Figures 2.9-2.11). In *Sox2Cre; Trim28* mutants, females also showed significantly high biallelic expression of *Gtl2*

compared to males ($p < 0.05$). Together, these findings suggest that the sex-specific loss of imprinting may be a result of TRIM28 functions after the early embryonic genome-wide reprogramming event.

One puzzling result from these combined analyses was that only *Gtl2* of the three imprinted genes tested showed sex-specific disruption in allelic expression, but several imprinted genes showed sex-specific disruption in expression levels of imprinted genes. Over the past decade there has been increasing evidence for the existence of an imprinted gene network where imprinted genes regulate the expression levels of other imprinted genes, without affecting their allelic expression (Fauque et al., 2010; Gabory et al., 2009; Lui et al., 2008; Monnier et al., 2013; Varrault et al., 2006). Thus, expression differences between male and female *Trim28* mutants may be the result of sex-specific loss of imprinting at certain imprinted genes, such as *Gtl2*, affecting the expression levels of other imprinted genes.

Partially-penetrant overexpression of X-inactivated gene, *Pgk1*, in *Trim28*^{chatwo} females.

The reason for a more severe loss of imprinting of *Gtl2* in females compared to males is still unclear. Because these mutants arrest prior to primary sex-determination, it is unlikely that different responses to loss of TRIM28 are due to hormonal differences between males and females. At this time in development, one key difference between males and females is the presence of an inactive X chromosomes in females. In embryonic tissues, the X chromosome becomes randomly inactivated around the time

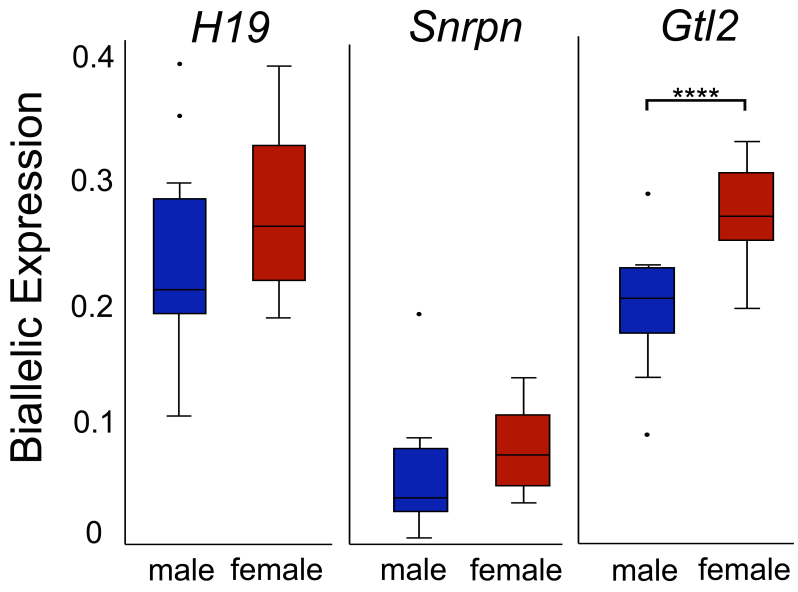

Figure B.2. Sex-specific effects of the *Trim28^{chatwo}* mutation on allelic expression of *H19, Snrpn,* and *Gtl2.* Box plots of pyrosequencing analysis of allelic imprinted gene expression in *Trim28^{chatwo}* males (blue, n = 13) and females (red n = 13). There was no significant difference between male and female allelic expression of *H19* and *Snrpn*. **** - $p < 0.001$.

of implantation. Coincidently, this is the same timing when *Gtl2* imprinting is disrupted in *Trim28*^{chatwo} and conditional *Trim28* mutants. Both *Trim28*^{chatwo} and conditional *Trim28* mutants may still have some functional *Trim28* present around the time of implantation, when the X chromosome becomes inactivated. Therefore, it is possible that the inactive X chromosome and *Gtl2* compete for the limited TRIM28 in these *Trim28* mutants, leading to an overall more severe loss of imprinting at *Gtl2* in females compared to males. This hypothesis still needs to be thoroughly tested. However to get an initial indication of whether TRIM28 may be involved in X inactivation, we measured expression levels of one X-inactivated gene, *Pgk1*, in single *Trim28*^{chatwo} males and females. The three males tested all showed similar expression levels of *Pgk1,* however, the three females showed variable expression of *Pgk1*, with *Pgk1* overexpression in two of three female mutants (Figure B.3). While more experiments are required to confirm the requirements of TRIM28 for X chromosome inactivation, this finding suggests that TRIM28 may have dual roles in imprinting control and X chromosome inactivation. In collaboration with Dr. Xu Wang, we are currently performing RNA-seq in *Trim28* mutants, which will give us a broader view of the extent to which TRIM28 affects X chromosome inactivation.

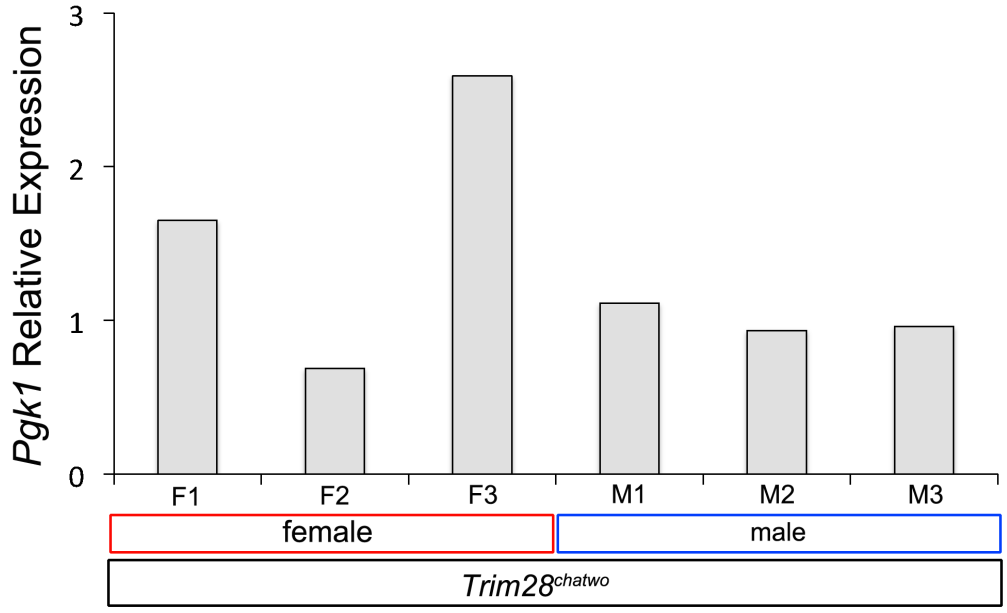

Figure B.3. Expression of the X-inactivated gene, *Pgk1*, in female and male *Trim28*^{chatwo} mutants. qRT-PCR of *Pgk1* expression in individual *Trim28*^{chatwo} females (F1-F3) and males (M1-M3), normalized to *ß-actin* and relative to expression in males.

EXPERIMENTAL PROCEDURES

qRT-PCR and quantitative pyrosequencing were performed as described in Chapter 2. *H19, Igf2, Gtl2,* and *Dlk1* qRT-PCR primers and pyrosequencing primers are in Table 2.1. Other qRT-PCR primers are as follows:

Peg10 - GTCTCTACTGTGGCAATGG, GGGACCTTATTCGTTCTGG

Rian - TCGAGACACAAGAGGACTGC, ATTGGAAGTCTGAGCCATGG

Mirg - CCTTCCTGGATCTCTCGCTT, GTGGGAGTTGAAACATGGGT

Mest - ATCCCGGTGCTTCTTCTCA, AGGCAGCAAGCAGCAACT

Snrpn - TTGGTTCTGAGGAGTGATTTGC, CCTTGAATTCCACCACCTTG

Zac1 - TTTTCTTTGCCTAGCTTAACCTACTACTT, CACAATCCTCTTGGGATACAAAAC

TAA

Peg3 - CACGAAGACGACACCAACAG, GTCTCGAGGCTCCACATCTC

Impact - AAGAACGCGCAGACTTATCG, TATTTTCTCCACCCACTGGT

Airn - GTGGATTCAGGTTTCATG, GGCCCAGATATAGAATGT

Igf2r - TAGTTGCAGCTCTTTGCACG, ACAGCTCAAACCTGAAGCG

Pgk1 - CAGCCTTGATCCTTTGGTTG, CTGACTTTGGACAAGCTGGA

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