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Comprehensive Review on the Existence of Genomic Imprinting in Aves

Derek Gygax
Virginia Commonwealth University

Comprehensive Review on the Existence of Genomic Imprinting in Aves

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

Derek Michael Gygax
Bachelor of Sciences in Biology, College of William and Mary, 2010

Director: Joseph W. Landry
Assistant Professor, Human and Molecular Genetics

Virginia Commonwealth University
Richmond, Virginia
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List of Abbreviations

AICDA	Activation-Induced Cytidine Deaminase
APOBEC	Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like
BORIS	Brother of Regulator of Imprinted Sites
CpG	Cytosine-Phosphate-Guanine
CTCF	CCCTC-Binding Factor
DMR	Differentially Methylated Region
DNMT1	DNA (Cytosine-5) Methyltransferase 1
DNMT3A	DNA (Cytosine-5) Methyltransferase 3A
DNMT3B	DNA (Cytosine-5) Methyltransferase 3B
DNMT3L	DNA (Cytosine-5) Methyltransferase 3-Like
ED	Embryonic Day
ES	Embryonic Stem Cells
gDRM	Germ Line Differentially Methylated Region
H3K4	Histone 3 Lysine 4
H3K9	Histone 3 Lysine 9
H3K9me2	Histone 3 Lysine 9 dimethylated
HP1	Heterochromatin Protein 1
ICR	Imprinting Control Region
ID	Incubation Day
KAP1	KRAB-Associated Protein 1
KDM1B	Lysine-Specific Histone Demethylase 1B
KRAB	Krüppel-Associated Box
MBD3	Methyl-CpG-Binding Domain Protein 3
MHM	Male Hypermethylated
NICE	Neighboring Imprinting Control Element
PGC	Primordial Germ Cell
PGC7	Primordial Germ Cell Protein 7
PHD	Plant Homeo Domain
PRMT7	Protein Arginine N-Methyltransferase 7
QTL	Quantitative Trait Loci

RING	Really Interesting New Gene
SET	Su(var)3-9 and ‘Enhancer of zeste’
SETDB1	SET domain, bifurcated 1
TDG	G/T Mistmatch-Specific Thymine DNA Glycosylase
TET3	Ten-Eleven Translocation-3
UHRF1	Ubiquitin-like, containing PHD and RING finger domains, 1
YY1	Yin Yang 1
ZFP42	Zinc Finger Protein 42
ZFP57	Zinc Finger Protein 57

Abstract

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By Derek Michael Gygax, B.S. in Biology

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Major Director: Joseph W. Landry
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Human and Molecular Genetics

Genomic imprinting results in monoallelic parent-of-origin gene expression. Therian mammals show conclusive evidence for imprinting, while the evidence in Aves is conflicting. It's unclear if Aves have the proteins necessary for establishment and maintenance of imprinting loci. Every examined avian orthologue to mammalian imprinted genes shows biallelic expression providing evidence for a lack of imprinting in Aves. While the known parent-of-origin quantitative trait loci in chicken do not overlap with differentiated methylated regions, further analysis with a larger sample size is required. No transcript in the chicken transcriptome at incubation day 4.5 shows parent-of-origin expression, providing strong evidence for a lack of imprinting at this stage of

development. Investigating expression of the chicken transcriptome at additional developmental time points, and the transcriptome of other Aves would provide decisive evidence on the presence or lack of imprinting in Aves. Based on current knowledge, Aves lack imprinting as observed in mammals.

Introduction

Diploid organisms generally express both alleles of a gene through biallelic expression (Otto and Goldstein 1992; Wilkins and Haig 2003). However, in the case of genomic imprinting, only a single allele is expressed, based upon the sex of the parent-of-origin of the allele (Wilkins and Haig 2003). The only vertebrate organisms that show indisputable evidence for genomic imprinting are the therian mammals. These mammals are viviparous and include the marsupials, such as opossums, and the eutherians, such as mice (Renfree et al. 2009).

Genomic imprinting was first observed in mice in 1984 (McGrath and Solter 1984). It was discovered that diploid mouse embryos would not fully mature when fertilization occurred with only maternal or paternal DNA. Therefore, it was concluded that DNA donated from the male and female germ cells each serve a separate purpose, and that one cannot be substituted for the other (McGrath and Solter 1984). Subsequent research has linked genomic imprinting effects to specific genes. These genes impact placental growth, maternal care, and affect social behavior (Fowden et al. 2011; Renfree et al. 2009b; Garfield et al. 2011).

The majority of imprinted genes are grouped together in imprinting clusters. To date 16 imprinting clusters have been identified in the eutherian genome. Genes within imprinting clusters can be maternally, paternally, or biallelically expressed (Dünzinger,

Haaf, and Zechner 2007a; Kelsey and Feil 2013; Lin et al. 2003). Many molecular mechanisms, including histone modification and DNA methylation are used to signify parental inheritance of a chromosome, and regulate parent-of-origin gene expression. Differentially Methylated Regions (DMRs) are defined as positions on the chromosome differentially methylated based upon parental inheritance. Each imprinting cluster contains an Imprinting Control Region (ICR), a germ line Differentially Methylated Region (gDMRs) set during gametogenesis to match the sex of the developing germ cell (Renfree et al. 2009b; Murdock and Walsh 2008). The ICR acts in cis to direct the establishment of secondary DMRs after fertilization and histone modifications important for regulating parent-of-origin expression in the imprinting cluster (Renfree et al. 2009b; Arnaud 2010a; Kelsey and Feil 2013; Ferguson-Smith 2011). Genomic imprinting has only been studied for 30 years, and researchers are actively searching vertebrates outside of therian mammals for genomic imprinting.

While the presence of imprinting in therian mammals is well documented, there is controversial evidence of whether imprinting occurs in birds (M Tuiskula-Haavisto and Vilkki 2007; Maria Tuiskula-Haavisto et al. 2004). Biological support for imprinting in Aves comes from their similarities with therian mammals, including child rearing after birth and social behavior, each of which have been linked to the function of imprinted genes (Collias 1952; Ruusila and Poysa 1998). On the other hand Aves are not viviparous, a biological consistency within all mammals that show imprinting (Renfree et al. 2009). In chicken, a good model organism for Aves, breeders using Quantitative Trait Loci (QTL) studies have observed loci that show different effects on traits such as growth and egg production when inherited from the mother compared to when inherited

from the father. These QTL studies provide evidence supporting parent-of-origin gene expression in Aves (Cañón, Herranz, & Manzanares, 2006; Renfree, Hore, Shaw, Graves, & Pask, 2009; M Tuiskula-Haavisto & Vilkki, 2007; T Yokomine, Hata, Tsudzuki, & Sasaki, 2006). However, it is also well documented that chickens lack some of the enzymes used for imprinting in mammals, leaving open to question the existence of imprinting in Aves (Cañón et al., 2006; Renfree et al., 2009; T Yokomine et al., 2006).

This review will investigate the biology of Aves for the conservation of regulatory mechanisms that have been established as important for imprinting in eutherian mammals, and summarize investigations on the expression of avian orthologues to mammalian imprinted genes. Towards this end, I will evaluate if chickens possess the molecular machinery needed for genomic imprinting as identified in mammals. I will then outline previous studies that have investigated chicken orthologues to mammalian imprinted genes in the search for parent-of-origin expression. Finally, chicken parent-of-origin QTLs will be cross-referenced with DMRs identified between male and female chicken primordial germ cells (PGCs) (Jang et al. 2013). Overlap between chicken DMRs and parent-of-origin QTLs would offer support for imprinting in Aves, as parent-of-origin gene expression in mammals is directly associated with DMRs (Arnaud 2010). This investigation will search for evidence of imprinting in Aves occurring as observed in mammals.

Life Cycle of Genomic Imprinting

During gametogenesis in therian mammals, genomic imprinting modifications are established to match the sex of the developing germ cell. After fertilization, the genome of the offspring contains both paternal and maternal imprinting modifications inherited from the sperm and ova respectively (Y. Li and Sasaki 2011). In the primordial germ cells (PGCs) of the offspring the parental specific modifications are erased, allowing the offspring to re-establish sex specific imprinting modification in their gametes. These steps form the imprinting life cycle, so that each generation passes on imprinting modifications to match the sex of the individual. In the somatic cells of the embryo the parental specific imprinting modification lead to additional epigenetic modifications causing parent-of-origin expression of imprinted genes (Y. Li and Sasaki 2011).

Epigenetic modifications are heritable genomic changes that influence gene activity without altering the underlying DNA sequence (Jaenisch and Bird 2003; Macdonald 2012). The epigenetic modification, DNA methylation of CpG residues is viewed as a defining factor for genomic imprinting, and is associated with almost every imprinted gene in mammals (Arnaud 2010). Mammals and chickens are distantly related, however many of the epigenetic regulatory mechanisms relevant to imprinting have been conserved (Figure 1). For example, hypermethylation of gene promoters is generally associated with reduced gene expression in both mammals and

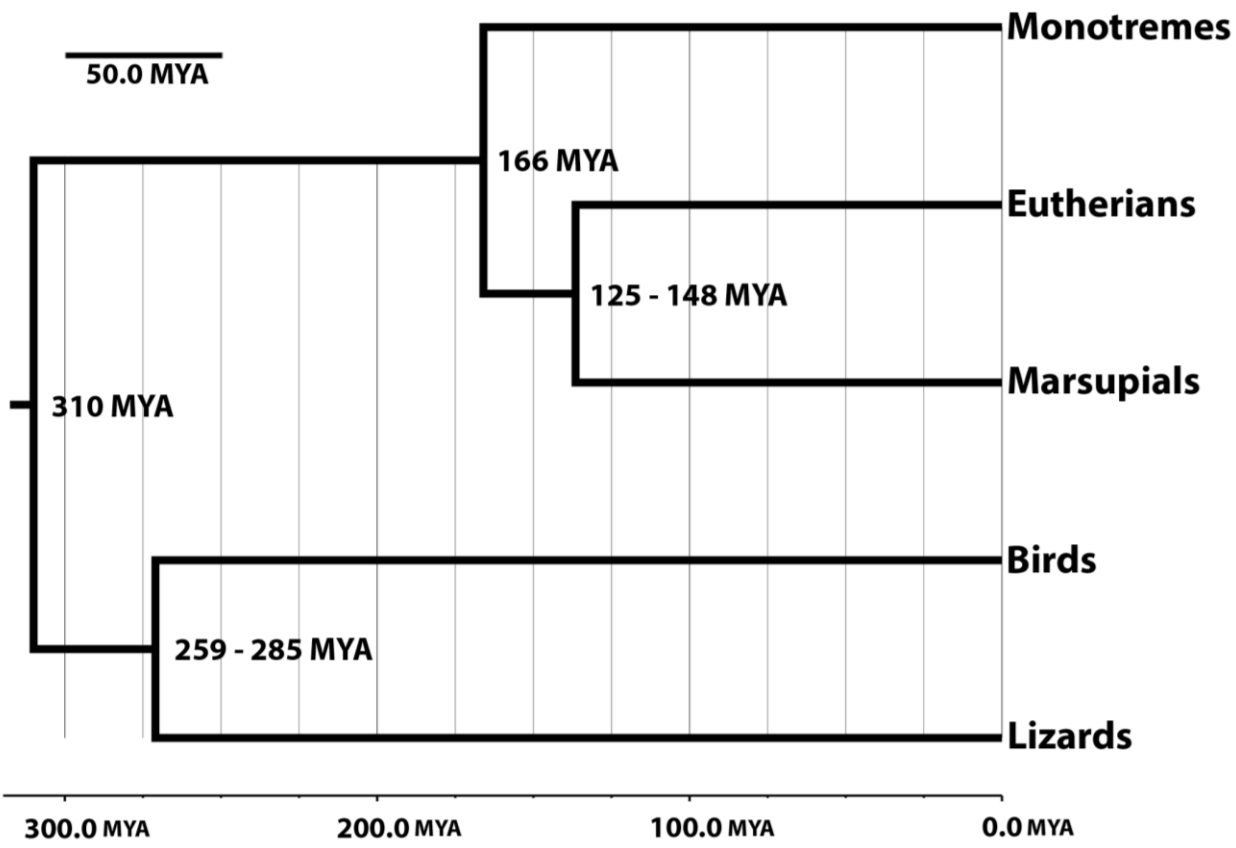

Figure 1. Divergence Time between Mammals and Chickens. Chronogram showing divergence times between mammals, birds, and lizards. Mammals and birds/lizards spilt 310 MYA. Within the mammals, monotremes and therian mammals split 166 MYA. The therian mammals split into eutherians and marsupials 125-148 MYA (Renfree et al. 2009). Birds and lizards split between 259 and 285 MYA (M. E. Jones et al. 2013). MYA = millions of years ago.

chickens (P. A. Jones and Takai 2001; Q. Li et al. 2011). Histone modifications are also important for imprinting (Kacem and Feil 2009). As with DNA methylation, histone modifications in chickens influence gene expression as observed in mammals (Schoenmakers et al. 2009; Schneider et al. 2004; Kisliouk and Meiri 2009; Kacem and Feil 2009). The association between DNA methylation and genomic imprinting is more clearly understood than the association between histone modifications and genomic imprinting. This review therefore, will concentrate on parent-of-origin DNA methylation and its influence on parent-of-origin gene expression.

gDMRs are differentially methylated regions established during gametogenesis to match the sex of the germ cell. Many gDMRs exist between male and female germ cells that are not associated with imprinted genes (Smallwood et al., 2011). On the other hand, ICRs are gDMRs with regulator mechanisms important for imprinting (Lees-murdock and Walsh 2008). This review therefore, will refer to ICRs in most cases. ICRs in therian mammals follow the lifecycle described above, with demethylation and re-methylation between generations (Figure 2). This ensures that the ICRs in the gametes of each generation are methylated to match the sex of the individual (Y. Li and Sasaki 2011). An investigation of the imprinting life cycle in mice and an examination of the similarities present in chicken development will provide evidence concerning the theoretical ability of Aves to establish and maintain ICRs.

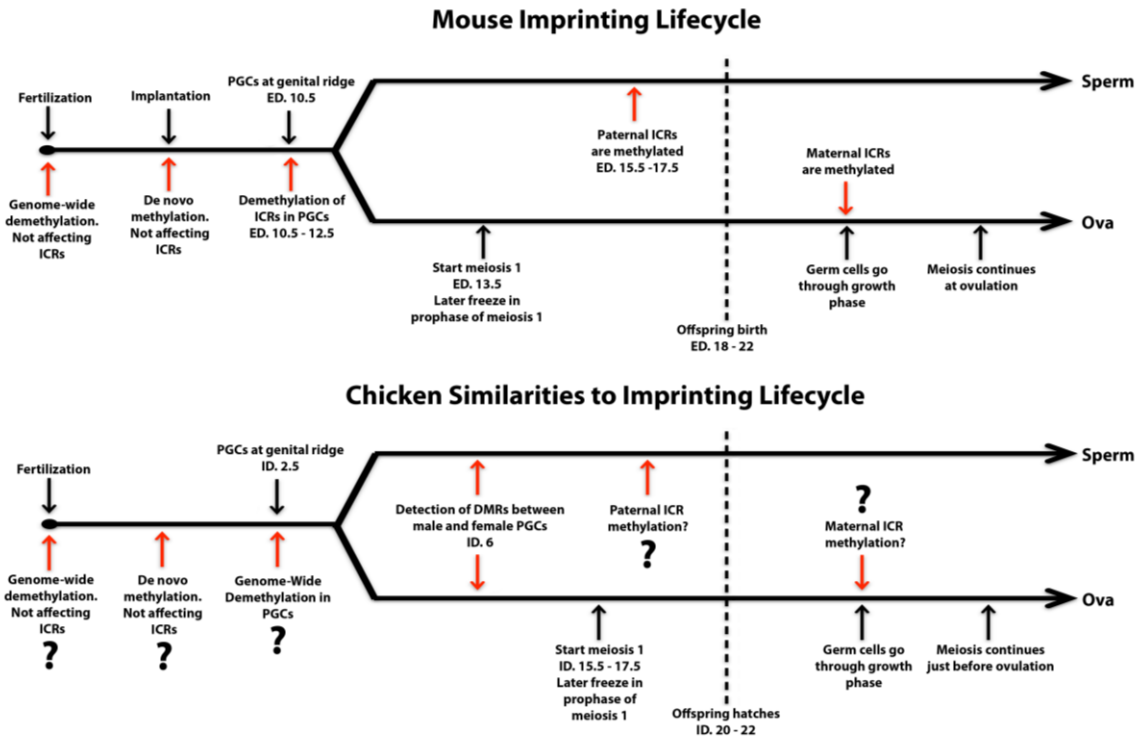

Fig 2. Mammalian Imprinting Life Cycle and Chicken Similarities. Representation of the life cycle of mouse ICRs and the similarities present in chickens. Black arrows show biological stages associated with imprinting, and red arrows point out stages in which epigenetic modifications occur that affect ICRs. (Mouse): ED = Embryonic day. After fertilization, the ICRs survive a wave of genome-wide demethylation affecting the rest of the genome (Y. Li and Sasaki 2011). After implantation a wave of de novo methylation occurs in the embryo that does not affect the ICRs (Proudhon et al. 2012). ICRs are erased in the PGCs between embryo day 10.5 and 12.5, after the PGCs have settled at the genital ridge (Seisenberger, Andrews, et al. 2012). Male PGCs set ICRs while in mitotic arrest between embryo day 15.5 and 17.5 (Henckel et al. 2012). Female germ cells set ICRs while arrested in meiosis 1 during the oocyte growth phase (Hiura et al. 2006). (Chicken): ID = Days of incubation. The methylation levels in the chicken genome during early embryogenesis and germ cell development have not been extensively studied. Chicken PGCs settle at the genital ridge at incubation day 2.5 (De Melo Bernardo et al. 2012). DMRs between chicken male and female PGCs on incubation day 6 have been identified (Jang et al. 2013). Female germ cells in chicken go through a growth phase while arrested in meiosis 1, as is seen in mice when maternally methylated ICRs are established (Mira 1998; Bujo et al. 1997; Y. Nakamura, Kagami, and Tagami 2013; Hiura et al. 2006).

Life Cycle of ICRs in Mammals

Ova and sperm have maternally and paternally methylated ICRs respectively. During fertilization the zygote inherits these parental specific modifications (Y. Li and Sasaki 2011). After fertilization the paternal and maternal genome undergo a wave of genome-wide demethylation (Figure 2). Demethylation of the maternal genome occurs passively, taking place during the first stage of DNA replication up to the morula. In passive demethylation, the newly synthesized DNA strand is not methylated to match the old strand; after many cell divisions the original methylation in the genome is lost (Morgan, Santos, Green, Dean, & Reik, 2005; Smallwood et al., 2011). In the paternal genome active demethylation takes place just after fertilization before the first cell division (Reik and Walter 2001). ICRs are protected from the wave of demethylation by the maintenance DNA methytransferase and DNA binding proteins, and maintain the parent-of-origin DNA methylation established during gametogenesis (X. Li et al., 2008; Y. Li & Sasaki, 2011; Reik & Walter, 2001).

In mice *de novo* methylation of the genome then takes place in the epiblast around the time of implantation (Seisenberger, Andrews, et al. 2012). ICRs are not affected by this *de novo* methylation (Figure 2)(Proudhon et al. 2012). Protection of the ICRs from this wave of *de novo* methylation by DNA binding proteins in the embryo allows ICRs to maintain their parental specific methylation in somatic cells, which is important for the regulation of imprinted genes (Reik and Walter 2001).

Mouse PGCs originate from the epiblast and are detected as a cluster of cells around embryo day 7.25 (Guibert, Forné, and Weber 2012; Hajkova et al. 2002). Evidence has been presented that the genome of PGCs is methylated as a result of the

de novo methylation in the epiblast (Seisenberger, Andrews, et al. 2012). The developing germ cells then go through a migration, and around embryo day 10.5 in mice are at the genital ridge (Figure 2)(Yamaguchi et al. 2013). A wave of genome-wide demethylation that exists in two separate stages occurs in PGCs. The first stage begins during PGC migration around embryo day 8 – 9 and affects most of the genome (Seisenberger, Andrews, et al. 2012; Guibert, Forné, and Weber 2012). The second stage affects ICRs and takes place after PGCs settle at the genital ridge. Demethylation of ICRs takes place around embryo day 10.5 – 12.5 (Figure 2)(Seisenberger, Andrews, et al. 2012; Guibert, Forné, and Weber 2012). This demethylation in PGCs erases the parental specific methylation at ICRs allowing the offspring to establish their own sex specific methylation at ICRs.

The establishment of gDMRs in male and female germ cells occurs at different time points. Between embryonic days 12.5 and 14.5 in mice, the male germ cells are arrested in mitosis (Western et al. 2008). Most of the methylation at paternally methylated ICRs is set when the germ cells are in mitotic arrest between embryonic days 15.5 and 17.5 (Figure 2)(Kelsey and Feil 2013; Henckel et al. 2012b; Bowles and Koopman 2007). During female gametogenesis, the female germ cells begin meiosis 1 on embryonic day 13.5. Mice are born between embryonic day 18 and 22, and around five days after birth the female germ cells are frozen at the diplotene stage of prophase (Bao et al. 2000; Silver and Laboratory 2008). While frozen in meiosis, the female germ cells enter a growth phase during which the maternally methylated ICRs are established (Figure 2)(Bao et al. 2000; Mira 1998). There is a direct association between an increase in size and an increase in the amount of methylation at ICRs during the oocyte

growth phase (Hiura et al. 2006). The female germ cells re-enter meiosis at ovulation, well after birth (Mira 1998). After fertilization the imprinting life cycle will start again.

Similarities to Life Cycle of ICRs in Chicken

Investigating embryogenesis and germ cell development in chickens will provide evidence if parental specific epigenetic modifications can occur in chickens, as they do in mammals. This review will discuss chicken development based on the length of incubation after egg laying. Chickens have the ability to demethylate DNA, however it is unknown if genome-wide demethylation occurs directly after fertilization (Figure 2). *In vitro* investigation has shown that nuclear extracts from advanced stage chicken embryos, incubation day 6 – 12, possess a strong ability to actively demethylate DNA (Jost 1993). Therefore the potential to actively demethylate DNA exists in chicken. In addition the male hypermethylated (MHM) region on the Z chromosome is demethylated in female embryos between fertilization and incubation day two (Teranishi et al. 2001). This shows the chicken embryo can demethylate DNA, but does not indicate if it affects gDMRs after fertilization. As will be elaborated on later, it is not clear if chickens have the DNA binding proteins necessary to protect specific gDMRs from demethylation after fertilization, which is an important component of the imprinting life cycle in mammals.

Similar to the *de novo* methylation in the epiblast of mice, evidence exists for *de novo* methylation in the early chicken embryo. The mRNA of a *de novo* DNA methyltransferase enzyme is highly expressed from incubation day 0 – 0.5 in the chicken embryo (Rengaraj et al. 2011). It will be described in detail later in this review, but it is only known how a few ICRs are protected from de novo methylation, and

therefore I cannot judge if chickens have the ability to protect specific gDMRs from de novo methylation (Kim et al. 2011; Engel, Thorvaldsen, and Bartolomei 2006).

Chicken PGCs are detected in the epiblast just after eggs have been laid (Motono et al. 2008; Eyal-Giladi and Kochav 1976; Y. Nakamura, Kagami, and Tagami 2013). The chicken PGCs then go through a migration and by incubation day 2.5 most are at the genital ridge (Figure 2)(De Melo Bernardo et al. 2012; Hamburger and Hamilton 1992). Similar to what is observed in mammals, genome-wide demethylation may occur in the migrating chicken PGCs. The DNA of some samples of migrating chicken PGCs is not methylated, offering support for a wave of genome-wide demethylation in migrating chicken PGCs (Rengaraj et al. 2011). Genome-wide demethylation at this point is critical for the existence of imprinting in Aves because the demethylation erases the parental specific methylation in gametes, allowing the gametes to re-establishment sex specific methylation at ICRs.

At incubation day 6, differentiation of the chicken gonads to testes and ovaries begins (Chue and Smith 2011). In mice, paternally methylated ICRs are established in the male germ cells during mitotic arrest, before the mice are born (Henckel et al. 2012). Similar to this, before the chicken egg hatches between incubation day 7 and 13 no cell division occurs in chicken male germ cells (Swift 1916). The transcript of a *de novo* DNA methyltransferase is highly expressed in chicken male germ cells from incubation day 8.5 to 12.5. This provides evidence that *de novo* methylation takes place in the chicken male germ cells while they are not dividing before the chicken egg hatches, just as the establishment of paternally methylated ICRs occurs in mice before they are born, while the male germ cells are in mitotic arrest (Rengaraj et al. 2011).

In mice, maternally methylated ICRs are established in the female germ cells after the mouse is born, when the female germ cells are arrested in meiosis and undergoing a growth phase (Bao et al. 2000; Mira 1998). In chickens, before the egg hatches, female germ cells initiate meiosis 1 between incubation days 15.5 and 17.5 (Smith et al. 2008). Chicken eggs hatch at incubation day 20 - 22 and within a week of hatching the female germ cells freeze in the diplotene stage of prophase (Figure 2). The germ cells will not re-enter meiosis until hours before ovulation (del Priore and Pigozzi 2012; Y. Nakamura, Kagami, and Tagami 2013; van de Ven et al. 2011). This freeze in meiosis in chicken female germ cells, parallels the freeze in meiosis in mouse female germ cells. The chicken female germ cells increase in size while frozen in meiosis, just as is seen in mice, especially during the seven days before ovulation (Figure 2)(Mira 1998; Bujo et al. 1997; Y. Nakamura, Kagami, and Tagami 2013; Hiura et al. 2006). The transcript of a *de novo* methyltransferase is highly expressed in the female germ cells post hatching up to at least 25 weeks. Because chickens begin laying eggs 20 - 22 weeks after hatching, the *de novo* methyltransferase is highly expressed in female germ cells while they are arrested in meiosis and going through a growth period after the chicken egg has hatched (Bujo et al. 1997; Rengaraj et al. 2011a; Berry). This time point of expression of the *de novo* methyltransferase in chicken female germ cells corresponds to how maternally methylated ICRs are established in mice after birth while the female germ cells are arrested in meiosis and going through a growth phase (Bao et al. 2000).

Molecular Machinery Involved in the Imprinting Life Cycle

A vast set of molecular machinery is important for the imprinting life cycle in mice. The presence or absence of homologues to this machinery in chickens will offer evidence supporting or refuting the presence of imprinting in Aves. It must be noted that many of the homologues that are described in chicken are predicted based upon DNA sequence and have not been studied to show if they share a similar function with the respective mouse protein. The evidence presented in this review, therefore, is preliminary, and further studies must be conducted to discern if these chicken homologues share functional similarities with the respective mouse proteins.

ICR Protection from Genome-Wide Demethylation after Fertilization

Proteins Proposed for Demethylation and ICR Protection after Fertilization in Mammals

Genome-wide demethylation after fertilization erases gDMRs that are not associated with imprinting loci (Kelsey & Feil, 2013; Smallwood et al., 2011). Passive demethylation of the maternal genome occurs when the maintenance DNA methyltransferase Dnmt1 does not methylate newly replicated DNA (Morgan et al. 2005). In contrast to the maternal genome, the paternal genome is actively demethylated, but how this occurs is uncertain (Seisenberger, Peat, et al. 2012).

Methods involving the base excision repair pathway, oxidation of methyl cytosine by TET3, and components of the elongator complex are thought to be involved (Table 1)(Seisenberger, Peat, et al. 2012).

ICRs are protected from demethylation after fertilization to ensure the parent-of-origin methylation established during gametogenesis is present in the zygote. This allows the ICRs to regulate imprinting in the embryo (Y. Li and Sasaki 2011). Many different factors have been proposed for this protection. For instance, DNMT1 works along with UHRF1 to methylate hemimethyated regions of newly replicated DNA (Sharif et al. 2007). This is essential, as it protects the genome from passive demethylation (Table 1)(Hirasawa et al. 2008). The KRAB zinc finger protein ZFP57, its cofactor KAP1, heterochromatin protein 1 (HP1), and the H3K9 methyltransferase SETDB1 bind the methylated allele at ICRs. This complex maintains DNA methylation at ICRs through association with UHRF1 and DNA methyltransferases (X. Li et al. 2008b; Messerschmidt et al. 2012; Quenneville et al. 2011). The ZFP57 complex maintains methylation at 4 ICRs (X. Li et al. 2008). Along with the ZFP57 complex, PGC7 (also called STELLA or DPPA3) protects ICRs from demethylation post fertilization. Maternal PGC7 binds ICRs that have the repressive histone modification H3K9me2, and prevents the conversion of methylated cytosine to hydroxymethylated cytosine by TET3 (T. Nakamura et al. 2012; T. Nakamura et al. 2007; Kelsey and Feil 2013). Protection by PGC7 has been documented as playing a role at 5 ICRs in the preimplantation embryo. Of these 5 ICRs protected from demethylation by PGC7, the ZFP57 complex is required at two of them as well, showing that PGC7 and ZFP57 have an overlapping role in maintaining methylation at ICRs in the preimplantation embryo (X. Li et al. 2008). MBD3

is important for maintaining methylation at the H19 ICR, but not the other ICRs examined. Further investigation on the function of MBD3 is required, as the protection of ICR from demethylation by this proteins has only been examined at a few ICRs (Reese et al. 2007; Ma et al. 2010). Collectively, all these proteins are important for maintaining methylation at the ICRs during demethylation after fertilization. Maintaining methylation at ICRs during this stage of demethylation is critical for imprinting, and therefore it is important to consider if these proteins are conserved in the chicken.

Homologues to Proteins for Demethylation and Protection of ICRs after Fertilization in Chickens

The observations that demethylation occurs in the MHM region on the Z chromosome between egg laying and incubation day two, and nuclear extracts of the chicken embryo are capable of active demethylation, shows that demethylation can occur in the chicken embryo (Teranishi et al. 2001; Jost 1993). Along with these observations, chickens have a homologue to TET3, many proteins in the base excision repair pathway, and components of the elongator complex that have been cited as important for demethylation in mammals (Table 1)(Flicek et al. 2013; Tatarinova and Kerton 2012; Zhu et al. 2000; Conticello et al. 2005; Heierhorst 2008). This shows chickens have homologues to many of the enzymes for demethylation after fertilization. Perhaps the chicken genome is demethylated at this stage, and therefore it is important to investigate if chickens have homologues to the proteins that protect ICRs from demethylation.

Proteins and Functions in Genomic Imprinting		Organisms					Mouse vs. Chicken protein sequence % Coverage %Identity %Positives E-value
Protein	Imprinting Function	Eutherian	Marsupial	Monotreme	Bird	Lizard	
DNMT1	DNA methyltransferase. Maintains ICR methylation during DNA replication	Yes	Yes	Yes	Yes	Yes	82% 78% 87% 0.0
DNMT3A	DNA methyltransferase. Establishes de novo methylation at ICRs	Yes	Yes	Yes	Yes	Yes	93% 87% 89% 0.0
DNMT3B	DNA methyltransferase. Establishes de novo methylation at ICRs	Yes	Yes	Yes	Yes	Yes	87% 61% 72% 0.0
DNMT3L	DNMT3A/B cofactor for establishing de novo methylation at ICRs	Yes	Yes	No	No	Yes	N/A
TET3	Involved in demethylation process in the zygote	Yes	Yes	Yes	Yes	Yes	72% 64% 71% 0.0
AICDA/AID	Demethylation process in PGCs	Yes	Yes	Yes	Yes	Yes	100% 88% 94% 1e-129
KDM1B	H3K4 demethylase. Involved in ICR establishment	Yes	Yes	Yes	Yes	Yes	100% 82% 90% 0.0
CTCF	Maintain H19 ICR during de novo methylation at implantation	Yes	Yes	Yes	Yes	Yes	100% 92% 94% 0.0
BORIS	Paralogue of CTCF. Aids in establishment of male ICRs	Yes	Yes	Yes	No	Yes	N/A
YY1	Zinc finger protein. Involved in establishment of a maternal ICR	Yes	Yes	Yes	Yes	Yes	100% 77% 80% 0.0
PGC7	Maintaining ICRs during post fertilization demethylation	Yes	No	No	No	No	N/A

Table 1. Proteins Important For Mammalian Imprinting Control Regions. The first column describes the function each enzymes serves in relation to genomic imprinting. The columns under organisms specify the existence of an enzyme within each organism. Literature searches, the ensembl database, and BLASTP were used to identify the presence of enzymes in each organism. The last column compares the mouse sequence of each protein to the chicken sequence for corresponding protein in the chicken database using BLASTP (Altschul et al., 1997; Arnaud, 2010; Cañón et al., 2006; Ciccone et al., 2009; UniProt Consortium 2014; Conticello, Thomas, Petersen-Mahrt, & Neuberger, 2005; Flicek et al., 2013; Hore, Deakin, & Marshall Graves, 2008; Kelsey & Feil, 2013; Kim et al., 2006; Renfree et al., 2009; T Yokomine, Hata, Tsudzuki, & Sasaki, 2006).

Chickens have homologues to DNMT1 and UHRF1, two proteins necessary for the protection of ICRs from passive demethylation (Biotechnology 2002; Altschul et al. 1997; Rengaraj et al. 2011). Chicken also have an homologue to MBD3, important for protecting the H19 ICR from demethylation in mammals (Flicek et al. 2013; Reese et al. 2007). Chickens do not possess a homologue to PGC7, important for preventing the conversion from methyl cytosine to hydroxymethyl cytosine by TET3 at ICRs (Cañón, Herranz, and Manzanares 2006; T. Nakamura et al. 2012). An orthologue to ZFP57 has not been identified in chickens. Because chickens, like all higher vertebrates have an enormous number of KRAB-ZFP proteins, a chicken orthologue to mouse *Zfp57* is difficult to identify (Quenneville et al. 2011). Further investigation is required to determine if chickens have an orthologue to mouse ZFP57. As chickens may lack two of the proteins important for the protection of ICRs from demethylation in mice, chickens may not have the ability to protect specific gDMRs from demethylation as observed in mice, a crucial part of the imprinting life cycle in mammals.

ICR Protection from De Novo Methylation at Implantation

Proteins Important for Protecting ICRs from De Novo Methylation at Implantation in Mammals

In mice, *de novo* methylation of the genome by DNMT3B occurs at the time of implantation (Borgel et al. 2010). ICRs are protected from DNMT3B and maintain parent-of-origin specific methylation (Proudhon et al. 2012). CTCF binding to the unmethylated allele at the H19 ICR ensures that the *de novo* methylation does not affect the H19 ICR (Engel, Thorvaldsen, and Bartolomei 2006). Along with CTCF,

ZFP42 protects ICRs from de novo methylation (Kim et al. 2011). ZFP42, resulting from a eutherian specific gene duplication of YY1, protects the Peg3 ICR and an ICR in the Gnas imprinting cluster from *de novo* methylation in mice, thus maintaining parent-of-origin specific methylation. Studies show that ZFP42 is specific to the Peg3 ICR and the Gnas ICR and does not protect other ICRs (Kim et al. 2011). Along with the DNA binding proteins CTCF and ZFP42, it is speculated that transcription factor binding and the active histone modification H3K4 methylation influence the protection of ICRs from de novo methylation. Further research is required to truly elucidate if transcription factor binding and H3K4 methylation really protect ICRs from de novo methylation (Proudhon et al. 2012).

Homologues to Proteins that Protect ICRs from De Novo Methylation at Implantation in Chickens

De novo methylation in the chicken embryo has not been directly investigated. However, indirect evidence for *de novo* methylation in the chicken embryo comes from the observation that chickens have homologues to DNMT3A and DNMT3B, and the detection of high levels of these homologues in the whole embryo between the time of egg laying and incubation day 0.5 (Rengaraj et al. 2011). This evidence offers support for *de novo* methylation in the early chicken embryo. As *de novo* methylation likely occurs in the chicken embryo, it is important to investigate if chickens have homologues to the proteins that protect ICRs from de novo methylation. Chickens have a functional homologue to CTCF, important for protecting the unmethylated H19 ICR allele from de novo methylation in mice (Bell, West, and Felsenfeld 1999; Valdes-Quezada et al.

2013). As ZFP42 is specific to eutherian mammals, chickens do not have a copy to ZFP42 (Kim et al. 2011).

Genome-Wide Demethylation in Primordial Germ Cells

Enzymes for Demethylation of Primordial Germ Cells in Mammals

Erasing parental methylation at ICRs in PGCs is required for the establishment of sex specific methylation at the ICRs in the gametes of offspring. As demethylation in PGCs occurs over a few days, between embryonic days 8 and 12.5, it has been speculated that both passive and active demethylation are involved (Guibert, Forné, and Weber 2012; Seisenberger, Andrews, et al. 2012). While empirical evidence shows that the Activation-Induced Cytidine Deaminase, AICDA, plays an important role in this process, knockout of AICDA in mouse PGCs does not completely prevent demethylation. Other enzymes in the AICDA/APOBEC family, the glycosylase enzyme TDG, or enzymes in the TET family have been proposed to play a role in the demethylation of PGCs as well (Seisenberger, Peat, et al. 2012; Popp et al. 2010).

Homologues to the Enzymes for Demethylation of the Primordial Germ Cells in Chicken

DNA methylation in early PGCs has not been thoroughly studied in chicken. As discussed earlier however, the study by Rengaraj (2011) provides evidence supporting a wave of demethylation in chicken PGCs (Rengaraj et al. 2011). This demethylation could occur by a novel lineage specific mechanism or by similar mechanisms as observed in mammals. Chickens have homologues to most of the proteins proposed to

play a role in PGCs demethylation in mammals including AICDA, TDG, the TET family, and APOBEC2 (Flicek et al. 2013; Tatarinova and Kerton 2012; Zhu et al. 2000; Conticello et al. 2005; Popp et al. 2010). This is strong observational evidence supporting genome-wide demethylation in chicken PGCs. Evidence supporting demethylation in the PGCs of chickens is crucial for imprinting, as this demethylation erases the parent-of-origin methylation of the previous generation, facilitating the establishment of new sex specific methylation in the gametes.

Establishing ICRs

Proteins Essential to the Establishment of ICRs in Mammals

Knowledge is incomplete on how methylation is established at ICRs during gametogenesis. Studies have shown that this is a complex process involving histone modification, active transcription, and DNA binding proteins (Kelsey and Feil 2013). Male and female ICR establishment occurs at differing time points during gametogenesis and is influenced by different proteins. Therefore, I will address the establishment of ICRs in male and female germ cells separately.

Maternal Establishment of ICRs

Sixteen maternally methylated imprinting gDMRs associated imprinting clusters are established during the oocyte growth phase (Hiura et al. 2006). DNMT3A and DNMT3L, but not DNMT3B, are important for the *de novo* methylation of maternally methylated imprinting gDMRs (Kaneda et al. 2004). While DNMT3L cannot methylate DNA on its own, it forms a complex with DNMT3A/DNMT3B to establish methylation (Y. Li and Sasaki 2011a; Suetake et al. 2004). DNA methylation of ICRs by the DNMTs is

inhibited by histone H3K4 methylation (Ciccone et al. 2009b; Ooi et al. 2007). This is because the DNMT3L-DNMT3A complex cannot interact with loci that are methylated at histone H3K4 (Ooi et al. 2007). In the male germ cells, ICRs that are maternally methylated have the histone modification H3K4 methylation, protecting them from becoming paternally methylated (Henckel et al. 2012). In the female germ cells, KDM1B demetylase removes methylation at histone H3K4, and facilitates the establishment of maternally methylated ICRs (Ciccone et al. 2009).

While DNMTs establish the *de novo* methylation at ICRs, other factors are important for directing the location of ICR establishment during gametogenesis. During the growth phase of the oocyte, active transcription occurs across many maternally methylated ICRs, which plays a fundamental role in the establishment of imprinting gDMRs in the oocyte (Chotalia et al. 2009). For example, transcription of the Neuroendocrine Secretory Protein gene, Nesp, overlapping with the maternally methylated imprinting gDMRs in the Gnas imprinting cluster is necessary for methylation at the gDMRs (Chotalia et al. 2009). When transcription of the Nesp gene is truncated and does not overlap the gDMRs, methylation at the gDMRs in the Gnas cluster is reduced (Chotalia et al. 2009).

Another factor essential for DNMTs to locate the loci for ICR establishment are DNA binding proteins, including YY1 and ZFP57. YY1 is required for establishment of the Peg3 ICR, but the ICRs in the Gnas cluster (Kim, Kang, and Kim 2009). Similar to the function of YY1 at the Peg3 ICR, ZFP57 must be present for the establishment of maternal methylation at the Snrpn ICR (X. Li et al. 2008). The successful establishment of maternally methylated ICRs during gametogenesis therefore, requires a variety of

factors including DNMTs, KDM1B, YY1, ZFP57, and active transcription. The required factors however are locus specific.

Paternal Establishment of ICRs

Three paternally methylated ICRs, in the H19, Dlk1-Dio3, and Rasgrf1 imprinting clusters, are established in the male germ cells of mice (Arnaud 2010). Dnmt3a, Dnmt3b, and Dnmt3l are each important for the establishment of these paternally methylated ICRs (Kato et al. 2007).

As described for the establishment of maternal ICRs, the DNMT3 enzymes are responsible for *de novo* methylation, while other factors work along with the DNMT3 enzymes to determine the location of ICR establishment. Similar to the requirement of active transcription across ICRs in the female germ cells, active transcription across paternally methylated ICRs is proposed to be necessary for their establishment (Henckel et al. 2012).

The protein BORIS is required for the establishment of the H19 ICR during male germ cell development. In therian mammals, BORIS, a paralogue of the transcription factor CTCF, is exclusively expressed in developing male germ cells (Jelinic, Stehle, & Shaw, 2006; Renfree et al., 2009). *In vitro* study shows BORIS and the protein arginine methyltransferase PRMT7 are essential for the DNMTs to fully methylate the H19 ICR (Jelinic, Stehle, and Shaw 2006). The presence of the proteins important for ICR establishment in chickens will offer some evidence of whether chickens can establish ICRs.

Homologues to the Proteins Essential for the Establishment of ICRs in Chickens

Chickens have homologues to DNMT3A and DNMT3B (Rengaraj et al. 2011). The chicken *DNMT3B* transcript shows low levels of expression in female germ cells during incubation, but is expressed at high levels 1 day, 12-weeks, and 25-weeks after hatching (Rengaraj et al. 2011). The heightened expression of *DNMT3B* occurs in chicken female germ cells while they are arrested in meiosis and going through a growth phase (Bujo et al. 1997; Y. Nakamura, Kagami, and Tagami 2013; Berry). In mice, maternal ICRs are established when the female germ cells are arrested in meiosis and in the growth phase (Hiura et al. 2006b; Bao et al. 2000). *DNMT3B* is thus expressed in chicken female germ cells at a time point corresponding to when maternal ICRs are established in mice. In chicken male germ cells, the *DNMT3B* transcript is expressed at high levels on incubation days 8.5, 10.5, and 12.5 (Rengaraj et al. 2011). The heightened expression of *DNMT3B* occurs before chicken eggs hatch, when the male germ cells are not going through cell division (Swift 1916). This is analogous to the stage when paternally methylated ICRs are established in mice (Bowles and Koopman 2007; Henckel et al. 2012). Similarity between the expression pattern of *DNMT3B* in chicken male and female germ cells and the time of ICR establishment in mice offers evidence supporting the chicken's ability to establish ICRs. While chickens have the *de novo* DNA methyltransferases DNMT3A and DNMT3B, chickens lack DNMT3L, which is crucial for the establishment of most ICRs in mammals (Arnaud et al., 2006; Kato et al., 2007; T Yokomine et al., 2006). Studies have shown that when DNMT3L is absent in mice, the majority of ICRs cannot be established. However, a minority of ICRs can still

be established even when DNMT3L not present during female gametogenesis, suggesting that DNMT3L is not essential for ICR establishment (Arnaud et al. 2006).

Chickens have homologues to many of the histone modifying enzymes and DNA binding proteins important for ICR establishment in mammals including *Kdm1b*, *Prmt7*, and *Yy1* (Kim et al. 2006; Flicek et al. 2013; Consortium 2014; Lefevre et al. 2008). Chickens do not however, have a functional orthologue to BORIS, which is important for the establishment of the H19 ICR in mice (Renfree et al., 2009). In addition it is not clear if chickens have an orthologue to ZFP57. The pattern of expression of *DNMT3B* in chicken germ cells, and the observation that some ICRs are established in the absence of DNMT3L, suggests that chickens could establish gDMRs as described in mammals.

Chicken Orthologous Regions to Mammalian Imprinting Clusters

Genomic imprinting affects over 100 genes in therian mammals (Table 2)(Jirtle 2012; Kelsey and Feil 2013). Examining the pattern of expression of orthologues to mammalian imprinted genes in Aves is a logical step in the attempt to identify genes subject to genomic imprinting in Aves. Within therian mammals, parent-of-origin expression of imprinted genes can be limited to certain tissues at specific development time points (T Yokomine et al. 2001). For example, *Tssc4* is maternally expressed in placental tissues, but biallelically expressed in all other tissues (Golding et al. 2011). When examining other organisms for imprinting therefore, it is important to inspect many tissues at various developmental time points (T Yokomine et al., 2001; Takaaki Yokomine et al., 2005).

H19 Imprinting Cluster

Genes and Regulatory Factors of the H19 Imprinting Cluster in Mice

In the H19 imprinting cluster the protein coding genes *Ins2* and *Igf2*, and the noncoding RNA *H19* are imprinted (Takaaki Yokomine et al., 2005). *H19* is maternally expressed, while *Igf2* is paternally expressed.

Gene	Expressed Allele	Gene	Expressed Allele	Gene	Expressed Allele
Znf264	Paternal	Peg12	Paternal	Zrsr1	Paternal
Gpr1	Paternal	Ube3a	Maternal	Commd1	Maternal
Zdbf2	Paternal	Mkrn3	Paternal	Mir380	Maternal
Epha4		Snrpn	Paternal	Mir134	Maternal
Mcts2	Paternal	Pwcr1	Paternal	AF357359	Maternal
Mir298	Paternal	Zfp127as	Maternal	B830012L14Rik	Maternal
Mir296	Paternal	Ampd3	Maternal	Mir431	Maternal
Sfmbt2	Paternal	H19	Maternal	Mir411	Maternal
Gatm	Maternal	Igf2as	Paternal	Mir410	Maternal
H13	Maternal	Igf2	Paternal	Mir376b	Maternal
Blcap	Isoform Dependent	Ins2	Paternal	AF357355	Maternal
Nnat	Paternal	Th	Maternal	Mir154	Maternal
Gnas	Isoform Dependent	Kcnq1ot1	Paternal	Mir136	Maternal
Nespas	Paternal	Ascl2	Maternal	Mir370	Maternal
Lin28a		Tssc4	Maternal	Mir127	Maternal
Magi2	Paternal	Kcnq1	Maternal	Mir337	Maternal
Il6		Cd81	Maternal	Gtl2	Maternal
Mkrn1-ps1	Paternal	Phlda2	Maternal	Dlk1	Paternal
Mir335		AF313042	Maternal	AF357428	Maternal
Peg10	Paternal	Slc22a18	Maternal	Rian	Maternal
Ppp1r9a	Maternal	Cdkn1c	Maternal	AF357341	Maternal
Asb4	Maternal	Nap1l4	Maternal	AF357426	Maternal
Tfpi2	Maternal	Tnfrsf23	Maternal	AF357425	Maternal
Sgce	Paternal	AK155734	Maternal	Dio3	Paternal
Calcr	Maternal	Tnfrsf22	Maternal	Rtl1	Paternal
Mest	Paternal	Tnfrsf26	Maternal	Mirg	Maternal
Klf14	Maternal	Nctc1	Paternal	Begain	Isoform Dependent
Copg2	Maternal	Zim2	Maternal	Htr2a	Maternal
Nap1l5	Paternal	Snurf	Paternal	Peg13	Paternal
Dhcr7	Maternal	Inpp5f V2	Paternal	Kcnk9	Maternal
LOC101055709		Gab1	Paternal	Slc38a4	Paternal
Ano1	Maternal	Ntm		Slc22a3	Maternal
Usp29	Paternal	Mir184	Paternal	Slc22a2	Maternal
Zim1	Maternal	Musd2		Igf2r	Maternal
Peg3	Paternal	Rasgrf1	Paternal	Air	Paternal
Zfp264	Paternal	Hymai	Paternal	Impact	Paternal
Zim3	Maternal	Plagl1	Paternal	Rhox5	Isoform Dependent
Ndn	Paternal	Dcn	Maternal	Tsix	Maternal
Magel2	Paternal	Ccdc40		Jpx	Paternal
U2af1-rs1	Paternal	Ddc	Paternal	Ftx	Paternal
Grb10	Isoform Dependent	Zcchc13	Maternal	Xist	Paternal

Table 2. List of Imprinted Genes in the Mouse. The column labeled Gene indicates the name of the imprinted genes. The column directly to the right labeled Expressed Allele indicates if the gene is paternally or maternally expressed. This list was directly extracted from the gene imprint website on April 24, 2014. (http://www.geneimprint.com/site/genes-by-species.Mus+musculus)

Ins2 is also paternally expressed in the yolk sac, but shows biallelic expression in other tissues (Takaaki Yokomine et al., 2005). The H19 ICR is paternally methylated, acts as an insulator, and exists in the intergenic region between *Igf2* and *H19* (Wan and Bartolomei 2008).

Two models have been proposed to explain how ICRs regulate imprinted genes, one the insulator model, and the other the noncoding RNA model (Wan and Bartolomei 2008). Regulation of *H19* and *Igf2* expression by the H19 ICR is a good representative of the insulator model. The H19 locus shares an enhancer that has the potential to regulate both *H19* and *Igf2*. When the H19 ICR is umethylated (maternally inherited allele) it is bound by CTCF, preventing the interaction between the enhancer and the *Igf2* promoter (Wan and Bartolomei 2008). This silences *Igf2* expression, while allowing for *H19* to be expressed. When methylated (paternally inherited allele), the H19 ICR is not bound by CTCF, allowing the enhancer and *Igf2* promoter to interact. Under this chromatin conformation *Igf2* is expressed, while *H19* is silenced (Wan and Bartolomei 2008).

Chicken orthologous region to the H19 Imprinting Clusters

Clustered together on chromosome 5 chickens have orthologues to the protein coding genes *Ins2* and *Igf2* (Dünzinger, Haaf, & Zechner, 2007; Takaaki Yokomine et al., 2005). In contrast, no chicken orthologue to the noncoding RNA *H19* has been identified in the region orthologous to where *H19* is located in mice (Takaaki Yokomine et al., 2005).

Conflicting evidence has been presented for parent-of-origin monoallelic expression of *IGF2* in chicken. One study found that the chicken *IGF2* gene is monoallelically expressed. However, these findings are in question because only blood samples were examined, and parent-of-origin expression was only observed in some embryos while other embryos showed biallelic expression (Koski et al. 2000). Other, more thorough studies present contradicting results. Biallelic expression of *IGF2* in chickens was always observed when examining multiple tissues at various developmental time points (Nolan, Killian, Petitte, & Jirtle, 2001; O'Neill, Ingram, Vrana, & Tilghman, 2000; T Yokomine et al., 2001). Biallelic expression of chicken *INS* was also observed in embryos and embryonic membrane tissues (Takaaki Yokomine et al., 2005). Because a majority of publications document biallelic expression for chicken *IGF2*, it is likely imprinting does not exist in the chicken orthologue to the mammalian H19 imprinting cluster.

Supporting a lack of imprinting in the chicken H19 cluster the regulatory sequences necessary for imprinting in mice are not well conserved in chickens. The chicken orthologous region to the H19 ICR lacks CTCF binding sites (Takaaki Yokomine et al., 2005). This is an important observation because CTCF binding to the H19 ICR in mice is a defining factor for imprinting in this cluster as described by the insulator model (Han, Lee, and Szabó 2008). Chickens have a functional homologue to CTCF, that can regulate gene expression (Bell, West, and Felsenfeld 1999; Valdes-Quezada et al. 2013). However, as CTCF cannot bind the chicken H19 ICR, it cannot regulate expression of the chicken H19 cluster. Based on this examination, chickens lack the regulatory sequences necessary for imprinting in mice.

Kcnq1 Imprinting Cluster

Genes and Regulatory Factors of the Kcnq1 Imprinting Cluster in Mice

A second imprinting cluster in close vicinity to the H19 imprinting cluster is the Kcnq1 imprinting cluster. The imprinted genes in this cluster are the protein coding genes *Osbpl5*, *Phlda2*, *Slc22a18*, *Cdkn1c*, *Kcnq1*, *Tssc4*, *Cd81*, *Ascl2*, and *Th,* and the long noncoding RNA *Kcnq1ot1*(Golding et al., 2011). *Kcnq1ot1* is paternally expressed, while *Kcnq1*, *Cdkn1c*, *Slc22a18*, and *Phlda2* are maternally expressed. *Th*, *Ascl2*, *Cd81*, *Tssc4*, and *Osbpl5* are also maternally expressed, but only in placental tissues, while they show biallelic expression in other tissues (Golding et al., 2011; Kanduri, 2011). The transcription start site of *Kcnq1ot1* is located in intron 10 of *Kcnq1*. The 471kb long noncoding RNA transcript *Kcnq1or1* runs antisense to Kcnq1, and overlaps with the transcription start sites of *Kcnq1*, *Tssc4*, *Cd81*, *Ascl2*, and *Th* (Golding et al., 2011). The Kcnq1 ICR (also called KvDMR1) is also located in intron 10 of *Kcnq1*. This ICR has two maternally methylated CpG islands, one at the promoter of *Kcnq1ot1* and the other in a silencing domain (Kanduri 2011).

In contrast to the H19 imprinting cluster, which is regulated by the insulator model, recent studies propose many of the imprinted genes in the Kcnq1 imprinting cluster are regulated via the noncoding RNA model. When the Kcnq1 ICR is unmethylated (paternally inherited allele) the long noncoding RNA *Kcnq1ot1* is transcribed. Transcription of *Kcnq1ot1* overlaps with the *Kcnq1*, *Tssc4*, *Cd81*, *Ascl2*, and *Th* promoters, causing the paternal allele of these genes to be silenced (Golding et al., 2011; Streets, Genetics, Health, & Kingdom, 1999). A different explanation for how

the Kcnq1 ICR regulates expression proposes that the *Kcnq1ot1* transcript interacts with histone modifying complexes, causing repressive histone modifications to be placed on paternally silenced genes in the Kcnq1 imprinting cluster (Kanduri 2011a; Pauler, Barlow, and Hudson 2012; Consortium 2014).

Chicken orthologous region to the Kcnq1 Imprinting Cluster

Chickens have an orthologue to *Osbpl5*, *Phlda1*, *Slc22a18*, *Kcnq1*, *Tssc4*, *Cd81*, *Ascl2*, and *Th*, associated with the Kcnq1 imprinting cluster in mammals. An orthologues to *Cdkn1c* has not been identified in chicken, and an orthologue to *Kcnq1ot1* has not been searched for (Dünzinger et al., 2007; Flicek et al., 2013; Takaaki Yokomine et al., 2005).

Chicken *ASCL2* and *TH* in the orthologous Kcnq1 cluster were investigated for imprinting and found to be biallelicaly expressed. However, few tissues were examined when drawing this conclusion, and further investigation would be merited in order to conclusively determine if these genes are imprinted in chicken (Takaaki Yokomine et al., 2005). Studies remain to be conducted to examine the expression of the other genes in the Kcnq1 cluster.

In mice, the Kcnq1 ICR is located in intron 10 of *Kcnq1* and serves as the promoter to the noncoding RNA *Kcnq1ot1*. It is proposed that expression of this noncoding RNA is required to imprint the Kcnq1 cluster in mice (Golding et al., 2011; Kanduri, 2011). The mouse *Kcnq1* intron 10 sequence is not highly conserved when compared to the orthologous region in chickens. The chicken *KCNQ1* intron 10 sequence contains a CpG island, however, the CpG island is 15kb downstream of

where the CpG island is in mice, and therefore it is unknown if this CpG island is orthologous to the ICR in mice (Ager et al. 2008) Also, the *KCNQ1* intron 10 sequence in chickens does not contain any sequences that show a high degree of similarity with the *Kcnq1ot1* transcription start site in mice (Ager et al. 2008). The *KCNQ1* intron 10 sequence in the tammar wallaby, a marsupial, however, does not contain any sequences that are similar to the *Kcnq1ot1* transcription start sites in mice either, yet still transcribe an orthologue to *Kcnq1ot1* (Ager et al. 2008). Further investigation for the transcription of an orthologue to *Kcnq1ot1* in the chicken therefore is merited. As chickens most likely lack an orthologue to the Kcnq1 ICR used by mice, further investigation searching for a Kcnq1ot1 orthologue in chicken is necessary to determine if chickens have any of the imprinting regulatory mechanisms important for imprinting the Kcnq1 cluster in eutherian mammals.

Igf2r Imprinting Cluster

Genes and Regulatory Factors of the Igf2r Imprinting Cluster in Mice

The Igf2r imprinting cluster contains the protein coding genes *Igf2r*, *Slc22a2*, and *Slc22a3*, and the long noncoding RNA *Airn*. (Latos et al. 2009; Monk et al. 2006). *Airn* is paternally expressed, while *Igf2r* is maternally expressed. In the placenta, *Slc22a2* and *Slc22a3* are also maternally expressed (Latos et al. 2009; Zwart et al. 2001; Yamasaki et al. 2005). The promoter on the noncoding RNA *Airn* lies in the second intron of *Igf2r.* The Airn transcript runs antisense to *Igf2r*, and overlaps with the *Igf2r* promoter (Latos et al. 2012). The Igf2r ICR is maternally methylated and serves as the *Airn* promoter (Ferguson-Smith 2011; Gibney and Nolan 2010).

Parent-of-origin regulation of *Igf2r* follows the noncoding RNA model, as described for the Kcnq1 imprinting cluster. When the Igf2r ICR is unmethylated (paternally inherited allele), transcription of *Airn* overlaps the *Igf2r* promoter, silencing *Igf2r* expression (Latos et al. 2012). On the maternally inherited allele the Igf2r ICR is methylated, which in turn silences *Airn* expression allowing *Igf2r* to be expressed. *Airn* transcription does not overlap with the *Slc22a2* or *Slc22a3* promoters, but still is required for paternal silencing (Wagschal et al. 2008). Similar to the *Kcnq1ot1* transcript, the *Airn* transcript is proposed to associate with histone modifying complexes directing repressive histone modifications to the *Slc22a2* and *Slc22a3* promoter on the paternally inherited alleles (Nagano et al. 2008; Pauler, Barlow, and Hudson 2012).

Chicken orthologous region to the Igf2r Imprinting Cluster

Chickens have an orthologue to the protein coding genes *Slc22a2*, *Slc22a3*, and *Igf2r*. The chicken orthologues are clustered together and in the same syntentic order as seen in mouse Igf2r imprinting cluster (Dünzinger, Haaf, and Zechner 2007). An orthologue to the noncoding RNA transcript *Airn* has not been searched for in chickens.

Two independent studies both observe biallelic expression of the chicken *IGF2R* gene. Both investigations conducted exhaustive studies, examining many different tissues at various development time points, extracted from many different chicken breeds (T Yokomine et al. 2001; Nolan et al. 2001). This offers conclusive evidence that chickens do not show imprinting at the *IGF2R* gene. Imprinting status of the other two genes in this cluster, *SLC22A2* and *SLC22A3*, has not been investigated in chickens.

Airn transcription is necessary for parent-of-origin expression of all the imprinted genes in the mouse Igf2r imprinting cluster (Wan and Bartolomei 2008). Chickens do not have a CpG island in the region orthologous to the Igf2r ICR in eutherians (Nolan et al. 2001). Opossum lack a CpG island in this region as well, and do not have an orthologue *Airn*, yet still somehow imprint the *IGF2R* gene (Weidman et al. 2006). It cannot be concluded therefore, that a lack of imprinting at chicken *IGF2R* is due to an absence of the imprinting regulatory mechanisms used by eutherian mammals. This observation points out the limitation that I can only conclude that imprinting in Aves does not occur as seen in the eutherian mammals, and shows that imprinting is not limited to the known imprinting regulatory mechanisms in mammals but can occur due to other unknown mechanisms.

Dlk1-Dio3 Imprinting Cluster

Genes and Regulatory Factors for the Dlk1-Dio3 Imprinting Cluster in Mice

The imprinted genes in the Dlk1-Dio3 imprinting cluster are the protein coding genes *Begain*, *Dlk1*, *Rtl1*, and *Dio3*, and the noncoding RNAs *Gtl2,* an *Anti-Rtl1, Meg8*, *Irm*, AK050713, AK053394, and *Meg9,* which also code for many micro RNAs and/or snoRNAs (Ferguson-Smith 2011; Hagan et al. 2009; Tierling et al. 2009). The *Begain* gene contains two transcripts, the shorter transcript is imprinted, while the longer transcript is biallelically expressed (Tierling et al. 2009). The syntenic order of the genes is *Begain*, *Dlk1*, *Gtl2*, *Anti-Rtl1*, *Rtl1*, *Meg8*, *Irm*, AK050713, AK053394, *Meg9*, and *Dio3. Dio3*, *Rtl1*, *Dlk1*, and the shorter transcript of *Begain* are paternally expressed, while the noncoding RNAs *Gtl2, Anti-Rtl1, Meg8*, *Irm*, AK050713, AK053394, *Meg9* are

maternally expressed (Hagan et al. 2009; Tierling et al. 2009). The Dlk1-Dio3 ICR is paternally methylated and is located in an intergenic region between *Gtl2* and *Dlk1* (Lin et al. 2007; Ferguson-Smith 2011).

The molecular mechanism regulating parent-of-origin expression in the Dlk1-Dio3 imprinting cluster is currently unknown. However, research has clearly shown that when the Dlk1-Dio3 ICR is unmethylated (maternally inherited allele), it regulates expression of all the imprinted genes in the cluster (Lin et al. 2003). It has also been proposed that many of the noncoding RNAs and micro RNAs in the cluster play a part in gene regulation (Hagan et al. 2009).

Chicken orthologous region to the Dlk1-Dio3 Imprinting Cluster

Chickens have an orthologue to *Begain*, *Dlk1* and *Dio3,* which are clustered together in chicken and in the same syntenic order as in mice (Flicek et al. 2013; Shin, Han, and Lee 2010). Chickens do not have an orthologue to *RTL1*, or the noncoding RNAs *GTL2* or *MEG8* (Shin, Han, and Lee 2010). It has not been examined if chickens have orthologues to the other noncoding RNAs in the Dlk1-Dio3 imprinting cluster.

DLK1 was tested for genomic imprinting by examining allelic gene expression in muscle and adipose tissue from both quail and chicken. Shin et al. observed biallelic expression of the *DLK1* gene in every sample from both organisms examined (Shin, Han, and Lee 2010). The other genes in the cluster were not examined for imprinting.

The molecular mechanism regulating this imprinting cluster in mice is unknown, however it has been shown the ICR is necessary for parent-of-origin expression (Lin et al. 2003). When comparing the ICR sequence in eutherians to the respective

orthologous region in chicken, no evolutionary conserved regions were identified (Edwards et al. 2008). Chickens do not have the regulatory sequence necessary for imprinting in the Dlk1-Dio3 imprinting cluster in eutherians, and therefore likely do not imprint any orthologues of the genes in the Dlk1-Dio3 cluster.

SNRPN Imprinting Cluster

Genes and Regulatory Factors for the SNRPN Imprinting Cluster in Humans

Mutations in the human SNRPN imprinting cluster cause Prader-Willi and Angelman syndrome leading to defects in brain development and eating habits. To learn how to treat these syndromes, the SNRPN imprinting cluster has been extensively studied in humans (Horsthemke and Wagstaff 2008). The human SNRPN cluster will be described in detail rather than the mouse SNRPN cluster. The imprinted protein coding genes in the SNRPN imprinting cluster in syntenic order include *MKRN3*, *MAGEL2*, *NDN*, *C15orf2*, *SNRPN*, *UBE3A* and *ATP10A* (Horsthemke and Wagstaff 2008; Cassidy et al. 2012). *MKRN3*, *MAGEL2*, *NDN*, *C15orf2*, and *SNRPN* are paternally expressed. While the majority of these genes show paternal expression in all tissues examined, paternal expression of *C15orf2* has only been identified in fetal brain tissue (Horsthemke and Wagstaff 2008). *UBE3A* and *ATP10A* show maternal expression, but only in brain tissue. The SNRPN ICR is maternally methylated and located on the *SNRPN* promoter (Horsthemke and Wagstaff 2008).

The paternally and maternally imprinted genes in the SNRPN cluster are regulated by different mechanisms. On the paternally inherited chromosome, the unmethylated ICR directly interacts with and activates the paternally expressed genes

(Rabinovitz et al. 2012). In mice, a long noncoding RNA transcript of *Snrpn* is exclusively expressed in neural tissue and overlaps with *Ube3a* (Plagge 2012). Although addition research is required, it has been proposed that the act of transcription of a long noncoding RNA *SNRPN* transcript over *UBE3A* inhibits paternal expression of *UBE3A* in human brain tissue. Contrarily, on the maternally inherited chromosome, the *SNRPN* noncoding RNA is silenced, which in turn allows *UBE3A* to be maternally expressed (Plagge 2012; Meng, Person, and Beaudet 2012; Horsthemke and Wagstaff 2008).

Chickens orthologous region to the SNRPN Imprinting Cluster

Chickens do not have an orthologue to many of the genes in the human SNRPN imprinting cluster (Rapkins et al. 2006). The only chicken orthologues are *UBE3A*, and *ATP10A*, and are clustered together on the same chromosome. Paralogues to *MKRN3* and *SNRPN* exist in chicken, however are not located on the same chromosomes as *UBE3A* and *ATP10A* (Rapkins et al. 2006).

Chicken UBE3A is biallelically expressed in tissues from the brain and hind limb (Colosi et al. 2006). These tissues are relevant because *UBE3A* is imprinted exclusively in brain tissue. Using brain tissue is important as monoallelic expression of the UBE3A gene in humans is limited to brain tissue (Horsthemke and Wagstaff 2008). The chicken tissue samples were collected on incubation day 11, giving the embryos time to age and ensuring that their central nervous system had developed (Colosi et al. 2006). This offers conclusive evidence that chickens do not imprint *UBE3A*, as humans do.

In mammals, it is proposed that transcription of the *SNRPN* noncoding RNA over *UBE3A* on the paternally inherited allele causes UBE3A to be silenced (Plagge 2012; Meng, Person, and Beaudet 2012; Horsthemke and Wagstaff 2008). Chickens do not present evidence of antisense transcription over *UBE3A* (Colosi et al. 2006). Lacking antisense transcription of UBE3A shows that chickens would not be able to imprint *UBE3A* as proposed for in humans. As chickens lack orthologues to most of the other imprinted genes in the human SNRPN imprinting cluster no further examination on the regulatory elements necessary for imprinting in humans was conducted. Based on biallelic expression of chicken *UBE3A* and the lack of orthologues to most of the other genes in the human SNRPN imprinting cluster, the chicken orthologue to the SNRPN cluster is most likely not imprinted.

Chicken DMRs vs. Parent-of-Origin QTLs

Chicken breeders have often observed differences affecting traits such as age at first egg, and feed intake between reciprocal crosses. Many factors have been proposed to account for these differences, one being genomic imprinting (Maria Tuiskula-Haavisto et al. 2004; M Tuiskula-Haavisto and Vilkki 2007). Quantitative trait loci (QTL) studies have been used to investigate the cause of these reciprocal cross differences (M Tuiskula-Haavisto and Vilkki 2007). Using statistical measurements, QTL studies allow scientists to identify genetic loci associated with specific phenotypic measurements by leveraging patterns of linkage (Miles and Wayne 2008).

To successfully conduct a QTLs study on chickens researchers require multiple chicken breeds that have separate genotypes affecting the phenotype being studied. These breeds also must have different genotypes at many genetic markers. These markers preferably do not directly affect the phenotype being studied (Miles and Wayne 2008). The separate breeds are treated as the parental generation and are crossed to form the F1 generation. Then many different crosses including backcrosses, intercrosses, or advanced intercrosses can be done to produce a final population (Darvasi 1998; Miles and Wayne 2008). The final population is then genotyped at the genetic markers and the phenotype being studied is scored. Makers that associate with the phenotypic trait being examined are linked with QTLs that influence that phenotype.

This type of study allows researchers to identify genetic loci that affect a trait influenced by many genes (Miles and Wayne 2008).

Many QTL studies have identified loci with parent-of-origin effects in the chicken genome, offering support for genomic imprinting in chickens. Many different traits were found to be influenced by parent-of-origin QTLs including age at first egg, total blood cell count, and components of bone structure like tibia weight (Maria Tuiskula-Haavisto et al. 2004; Sharman et al. 2007; Navarro et al. 2005; M Tuiskula-Haavisto and Vilkki 2007). In mammals, parent-of-origin gene expression is directly associated with DMRs (Arnaud 2010). Therefore, I looked for evidence of genomic imprinting in chickens by testing how often chicken QTLs with parent-of-origin effects overlap with chicken DMRs. For this study I used 289 DMRs identified from a comparison between male and female chicken PGCs at incubation day 6 (Figure 2)(Jang et al. 2013). Parent-of-origin QTLs were extracted from five separately published studies (Maria Tuiskula-Haavisto et al. 2004; Navarro et al. 2005; McElroy et al. 2006; Rowe et al. 2009a; Sharman et al. 2007). Some QTLs were excluded (see methods section), resulting in 20 non-redundant parent-of-origin QTLs. Of the 20 non-redundant parent-of-origin QTLs, 12 of them overlapped with chicken DMRs, while 8 did not (Figure 3). In a random control study, a dataset of equal size and number to the QTLs was compared to the 289 DMRs (see methods section). From this comparison 9 of the randomly produced QTLs overlapped with chicken DMRs, while 11 did not. A one-tailed Fisher's exact test returned a *P*-value of 0.2636, showing the overlap between the observed parent-of-origin QTLs and DMRs was not significantly greater than the overlap between the random QTL control dataset and DMRs. The power of the Fisher's exact test was found to be 0.1398, signifying that

even if the overlap between observed parent-of-origin QTLs and DMRs was significantly greater than the overlap between randomly produced parent-of-origin QTLs and DMRs, there was only a 0.14 probability of detecting it (Taylor and Gerrodette 1993; Calhoun 2013; Hornik 2013). Therefore, my analysis most likely would not have detected a significant overlap between observed parent-of-origin QTLs and DMRs, even if it did exist. To properly conduct this study a much larger dataset of QTLs and DMRs would be necessary to gain sufficient power. Based on the low power in this analysis I cannot conclude if chicken parent-of-origin QTLs significantly overlap with DMRs.

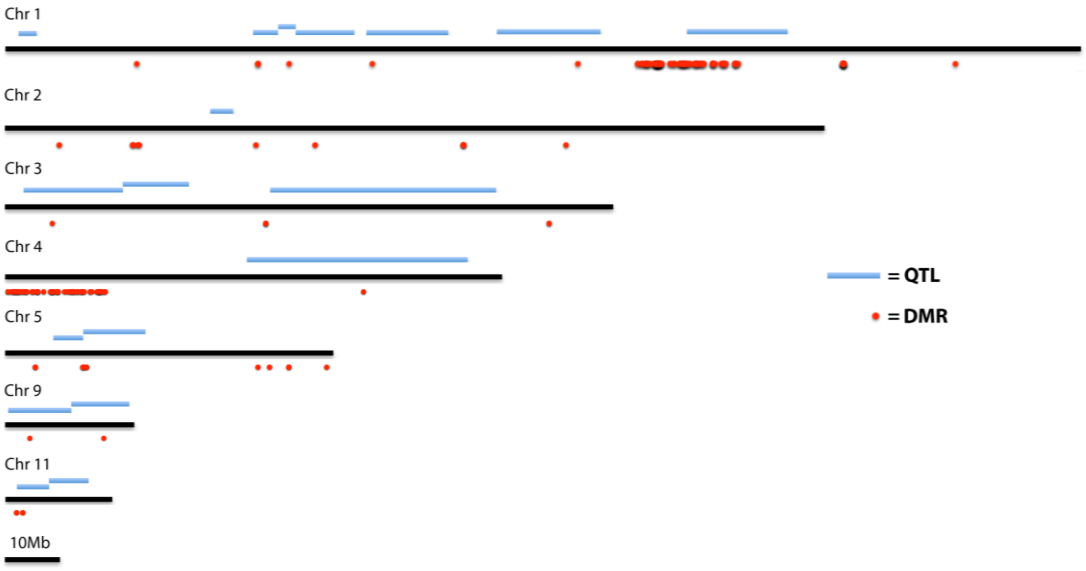

Fig 3. **Overlap Between Chicken DMRs and QTLs with Parent-of-Origin Effects.** Representation of chicken chromosomes 1, 2, 3, 4, 5, 9, 11. The blue bars above each chromosome represent QTLs with parent-of-origin effects, and the red dots below the chromosomes represent DMRs between male and female chicken PGCs on incubation day 6 (Jang et al. 2013). Only chromosomes that have both DMRs and QTLs with parent-of-origin effects are shown; therefore only 18 QLTs with parent-of-origin effects are shown. A one-tailed Fisher's exact test was used to compare the overlap between chicken parent-of-origin QTLs and DMRs. The *P*-value = 0.2636 showing that based on this analysis that the overlap between observed parent-of-origin QTLs and DMRs is not significantly greater than the overlap between random parent-of-origin QTLs and DMRs. Power = 0.1398 in this study showing that even if an overlap between observed QTLs and DMRs is significant greater than the overlap between random QTLs and DMRs there is only a 0.14 chance of detecting it. A conclusion concerning the overlap between observed parent-of-origin QTLS and DMRs thusly cannot be made based on this analysis.

Discussion

My objective with this thesis was to determine if there is evidence in the literature for imprinting in Aves. To achieve this objective, I determined the conservation of the imprinting life cycle and regulatory mechanism important for imprinting as determined in mammals to those in Aves, the pattern of expression of chicken orthologues to mammalian imprinted genes, and the overlap between parent-of-origin QTLs and DMRs as defined in chickens. From these comparisons, most evidence points toward a lack of imprinting in Aves. Chickens have homologues to the proteins important for establishing and demethylating ICRs in mammals, however it is unsure if they have the proteins that protect ICRs from genome-wide changes in DNA methylation after fertilization and at implantation. It has been shown that protecting ICRs from these changes in methylation is fundamental for imprinting in mammals, leaving open to question if Aves have the theoretical ability to establish and maintain ICRs as in mammals (Kelsey & Feil, 2013; Smallwood et al., 2011). Evidence for a lack of imprinting in birds comes from the observation that every examined orthologue to mammalian imprinted genes in Aves shows biallelic expression. Although it is important to recognize that Aves may imprint a unique set of genes (Colosi, Martin, Moré, & Lalande, 2006; Shin et al., 2010; T Yokomine et al., 2001; Takaaki Yokomine et al., 2005). I also addressed the overlap between chicken parent-of-origin QTLs and chicken DMRs because imprinted genes in

mammals are directly associated with DMRs. Consistent with a lack of imprinting in Aves, the parent-of-origin QTLs did not overlap with DMRs, strongly suggesting the QTLs are not associated with imprinted genes. This finding supports the proposal from other studies that most of these parent-of-origin QTLs can be explained by study bias rather than imprinting (Frésard et al. 2014). These conclusions however are limited by the assumption that imprinting in Aves occurs as described in mammals. This renders them inconclusive, as studies have shown that the mechanisms causing imprinting, and the genes subject to imprinting can be very diverse even between species and tissues in mammals (Das et al. 2012; Frésard et al. 2014; Maria Tuiskula-Haavisto et al. 2004). A recent study however, has overlooked most of these biases and examined the expression level of genes in most of the chicken transcriptome at one developmental time point, and found that no genes are expressed based on parent-of-origin (Frésard et al. 2014). While, further study using methodology not limited by the assumption that imprinting in Aves occurs as documented in mammals is required to attain an exhaustive conclusion on the presence of imprinting in Aves, current knowledge points to a lack of imprinting in Aves occurring as is observed in mammals.

There are four pivotal stages in the mammalian imprinting life cycle. At fertilization the zygote inherits maternally and paternally methylated ICRs from the oocyte and sperm, respectively. In the first stage of the imprinting life cycle the ICRs are protected from a genome-wide wave of demethylation in the early developing embryo (Y. Li and Sasaki 2011). At the second stage, the ICRs are again protected from *de novo* methylation in the epiblast. Protection from these epigenetic changes lets ICRs maintain their parental specific methylation, which is crucial for the regulation of

imprinting clusters (Proudhon et al. 2012; Borgel et al. 2010). At the third stage in the imprinting life cycle, the PGC undergo another wave of genome-wide demethylation that erases the parental methylation at ICRs. This is a vital part of the imprinting life cycle as erasing the parental methylation at ICRs allows for sex specific ICRs to be re-established in the gametes of the offspring. In the final stage of the imprinting life cycle the male or female gametes establish paternally or maternally methylated ICRs respectively (Y. Li and Sasaki 2011). Conservation of the stages in the imprinting life cycle when examining the embryonic and germ cell development of Aves will offer evidence supporting the ability to establish and maintain ICRs as established for mammals in Aves.

The first stage of the imprinting life cycle occurs after fertilization when the ICRs are protected from a genome-wide wave of demethylation (Y. Li and Sasaki 2011). In mammals, this demethylation occurs actively on the paternal genome before the first cell division, and passively on the maternal genome (Morgan et al. 2005; Y. Li and Sasaki 2011). While it is unknown if the chicken genome is demethylated after fertilization, studies show that nuclear extracts from the chicken embryo are capable of active demethylation (Jost 1993; Teranishi et al. 2001). This observation combined with the fact that chickens have homologues to most of the enzymes proposed to actively demethylate the mouse genome after fertilization strongly supports active demethylation in the chicken (Conticello et al. 2005; Tatarinova and Kerton 2012; Zhu et al. 2000; Flicek et al. 2013). While it is likely demethylation occurs, it is unknown how demethylation influences gDMRs and, more importantly, ICRs in chickens.

Protection of the inherited ICRs from demethylation after fertilization, allows the ICRs to regulate imprinting clusters in the offspring (Y. Li and Sasaki 2011). The protection of ICRs from demethylation at this stage is pivotal for the imprinting life cycle. Recent studies speculate that when methylation is first placed on ICRs during gametogenesis, the ICRs are not targeted for establishment, but rather ICRs are the gDMRs that retain parent-of-origin DNA methylation during epigenetic modifications in the embryo (Kelsey & Feil, 2013; Proudhon et al., 2012; Smallwood et al., 2011). Support comes from the observation that many CpG islands are methylated during female gametogenesis in mammals, but only around 15% of these CpG islands, including the maternally methylated ICRs, are maintained in the embryo. It is imperative, therefore, that chickens have homologues to the proteins necessary for the protection of ICRs from demethylation post fertilization.

The ICRs in mammals are protected from demethylation by pathways involving PGC7, ZFP57, and MBD3 (Y. Li and Sasaki 2011). Chickens have a homologue to *Mbd3* but the effect of MBD3 on the protection of ICRs from demethylation has not been extensively studied (Reese et al. 2007). We cannot speculate, therefore, on the importance of MBD3 when determining if chickens protect specific gDMRs from demethylation after fertilization. While it is clear chickens have a homologue to MBD3, it is less clear if chickens have an orthologue to ZFP57. The supposed transcript of many chicken genes share a high degree of sequence similarity with the mouse ZFP57 protein sequence, however without further investigation it cannot be determined if any of these sequences is an orthologue to mouse *Zfp57* (Quenneville et al. 2011). While it is unsure if chickens have an orthologue to ZFP57, it is clear that chickens do not have a

homologue to PGC7 (Cañón, Herranz, and Manzanares 2006). However the opossum, a marsupial, does not have a homologue to PGC7, yet still shows imprinting (Cañón, Herranz, and Manzanares 2006; Das et al. 2012). Therefore the lack of PGC7 in chickens does not necessarily present evidence dismissing the existence of imprinting in Aves. Therefore, because PGC7 in not necessary for imprinting and the importance of MBD3 is uncertain, further research investigating for a homologue with similar function to mouse ZFP57 in chicken is required to determine if chickens have the ability to protect specific gDMRs from demethylation, as seen for ICRs in mice.

At the second stage of the imprinting life cycle in mammals, ICRs are protected from the wave of *de novo* methylation that takes place at the time of implantation place in mice (Y. Li and Sasaki 2011). It is established that de novo methylation takes place in chickens as it does in mice, and the transcripts of the de novo DNA methyltransferases *DNMT3A* and *DNMT3B* are highly expressed in the chicken embryo from the time of laying until the end of incubation day 0.5 (Singal and VanWert 2001; Rengaraj et al. 2011). I hypothesize, therefore, that de novo methylation takes place in the early embryo of chicken as it does in mice.

Protection of ICRs from *de novo* methylation at the time of implantation is pivotal for imprinting because maintaining the parental specific methylation at ICRs is important for the regulation of imprinted genes (Borgel et al. 2010b; Proudhon et al. 2012). While the protection of ICRs from *de novo* methylation has not been extensively studied, it is clear in mice that CTCF is imperative for the protection of the H19 ICR, and that ZFP42 is crucial for the protection of the Peg3 ICR and an ICR in the Gnas imprinting cluster (Kim et al. 2011; Engel, Thorvaldsen, and Bartolomei 2006). Because *de novo*

methylation likely occurs in the chicken embryo, it is important to consider if chickens have homologues to these proteins responsible for protecting ICRs from *de novo* methylation. Chickens do not have an orthologue to ZFP42. However, because opossums do not have an orthologue to ZFP42 either, yet have imprinted genes, the lack of ZFP42 in chickens does not provide evidence for a lack of imprinting in Aves (Kim et al. 2011). Because ZFP42 is not necessary for imprinting, I can only reflect upon the function of CTCF when addressing if chickens have proteins that protect ICRs from *de novo* methylation. Chickens have a functional homologue to CTCF, however do not have a CTCF binding site at the DNA site orthologous to the H19 ICR, and therefore cannot protect the H19 ICR from de novo methylation as described for mammals (Bell et al., 1999; Valdes-Quezada et al., 2013; Takaaki Yokomine et al., 2005). However, because this is due to specifics at the H19 ICR, and not due to a lack of the protein, this observation does not offer strong support for a lack of the ability to protect ICRs from *de novo* methylation in chickens. Of the 19 gDMRs associated with imprinting clusters in mammals, it is only known how 3 are protected from *de novo* methylation (Kelsey and Feil 2013). It is essential to investigate how the other 16 imprinting gDMRs are protected from *de novo* methylation to gain a further understanding of the proteins necessary for ICR protection. This would allow us to further examine the conservation of proteins important for ICR protection from *de novo* methylation in chicken and clearly establish if chickens have the ability to protect ICRs from *de novo* methylation in the embryo.

In the third stage of the mammalian imprinting life cycle the ICRs are erased during a genome-wide wave of demethylation in the PGCs. This demethylation is a

fundamental stage for the imprinting life cycle because it erases the parental specific methylation at the ICRs allowing each generation to re-establish sex specific ICRs in their gametes (Y. Li and Sasaki 2011). In mice, demethylation of the ICRs occurs after PGCs have migrated from the epiblast to the genital ridge (Guibert, Forné, and Weber 2012; Seisenberger, Andrews, et al. 2012). A recent study provides evidence that a wave of genome-wide demethylation takes place in the chicken PGCs as well. This comes from the observation that while chicken PGC are migrating from the epiblast to the genital ridge their genome is not methylated. This suggests that demethylation in chicken PGCs occurs during migration (Rengaraj et al. 2011). Additional support comes from the fact that chickens have homologues to most of the enzymes proposed for PGC demethylation in mammals (Flicek et al. 2013; Conticello et al. 2005; Tatarinova and Kerton 2012; Zhu et al. 2000). This proposed timing of demethyation in chicken PGCs does not coincide with when ICRs are demethylated in mice. However, in the pig, a eutherian mammal that shows imprinting, at least one ICR is erased before PGCs arrive at the genital ridge (Hyldig et al. 2011). Demethylation of PGCs before they arrive at the genital ridge, therefore, is not unique to chickens when compared to the demethylation of ICRs in eutherian mammals. From these observations I conclude that chicken PGCs likely go through a wave of genome-wide demethylation. As this stage is required for imprinting in mammals, support for the demethylation of PGCs in chickens was crucial for the existence of imprinting in Aves.

At the final stage of the imprinting life cycle in mammals, ICRs are re-established during gametogenesis to match the sex of the germ cell (Y. Li and Sasaki 2011). This re-establishment allows mammals to pass ICRs on to their offspring, which will then

regulate imprinting clusters in the next generation. In mice, DNMT3A and DNMT3B form a complex with the cofactor DNMT3L to place *de novo* methylation at both maternally and paternally methylated ICRs (Hata, Kusumi, Yokomine, Li, & Sasaki, 2006; Jia, Jurkowska, Zhang, Jeltsch, & Cheng, 2007; Kaneda et al., 2004; Suetake et al., 2004). Chickens have DNMT3A and DNMT3B; however do not have DNMT3L (Rengaraj et al., 2011; T Yokomine et al., 2006). Past studies have speculated that the lack of an orthologue to DNMT3L is a strong indication of the lack of imprinting in organisms (T Yokomine et al., 2006). The function of DNMT3L is not specific to imprinting loci however, and while it is important for the establishment of ICRs in mice, studies have shown that when it is knocked out some maternally methylated ICRs can still be established (Arnaud et al., 2006; Hata et al., 2006; Smallwood et al., 2011). The lack of DNMT3L in chicken, therefore, does not lead to the conclusion that chickens cannot methylate ICRs.

Maternally and paternally methylated ICRs are established in mice at set time points during gametogenesis. The pattern of expression of the chicken *DNMT3B* transcript in male and female germ cells mirrors the time points when ICRs are established in the male and female germ cells of mice. Paternally methylated ICRs in mice are established before birth, while the male germ cells are in mitotic arrest (Henckel et al. 2012c; Western et al. 2008; Bowles and Koopman 2007). Similarly, DNMT3B is highly expressed in the chicken male germ cells before the chicken egg hatches while they are not dividing (Rengaraj et al. 2011b; Swift 1916). Maternally methylated ICRs are established after the mouse is born, while the female germ cells are arrested in meiosis 1 and undergoing the growth phase (Hiura et al. 2006b; Bao et

al. 2000). Mimicking this, DNMT3B shows high expression in the chicken female germ cells after the chicken eggs hatch, while the germ cells are arrested in meiosis 1 and going through a growth phase (Bujo et al. 1997; Y. Nakamura, Kagami, and Tagami 2013; Rengaraj et al. 2011). The observation that the pattern of expression of DNMT3B in male and female chicken germ cells corresponds to when ICRs are established in mice, provides support that *de novo* methylation might occur in chicken PGCs at similar time points when ICRs are established in mice. It is worth noting, however, that previous studies using sequence analysis have conflicting views on whether chicken DNMT3B is capable of catalytic activity (T Yokomine et al. 2006; Rengaraj et al. 2011). Future study investigating the potential activity of chicken DNMT3B is therefore merited.

While the DNMTs are responsible for establishing *de novo* methylation at the ICRs, the histone modification H3K4 methylation and its demethylase KDM1B, and the proteins YY1, ZFP57, and BORIS play a key role in determining the location of ICR establishment in mammals (Kim, Kang, and Kim 2009; X. Li et al. 2008). H3K4 methylation at ICRs blocks the DNMT3A DNMT3L complex from placing de novo methylation at ICRs (Ooi et al. 2007). The enzyme KDM1B removes this methylation at H3K4, and is important in the oocyte so H3K4 methylation is removed from maternally methylated ICRs allowing for their establishment (Ciccone et al., 2009; Smallwood et al., 2011). Chickens have an homologue to KDM1B, and just as observed in mice DNA methylation in chickens is generally associated with gene inactivation, while H3K4 methylation is usually present at active genes (Q. Li et al. 2011; Schneider et al. 2004; Flicek et al. 2013). Therefore, it would be interesting to see if H3K4 methylation at loci in chicken directly inhibits DNA methylation. If this is true KDM1B in chicken may be

responsible for ensuring de novo methylation is established at specific gDMRs during gametogenesis, just as has been described for the establishment of maternal ICRs in mice. Chickens have YY1, but do not have a functional orthologue to BORIS (Flicek et al., 2013; Renfree et al., 2009). BORIS is only expressed in the male germ cells of therian mammals however, while most ICRs are methylated in female germ cells (Kelsey and Feil 2013; Jelinic, Stehle, and Shaw 2006). Therefore, the lack of BORIS in chickens does not significantly impair the chicken's ability to establish the ICRs observed in therian mammals. As previously explained further investigation is necessary to determine if chickens have a homologue with similar function to mouse ZFP57 (Quenneville et al. 2011; Flicek et al. 2013). While chickens have homologues to many of the proteins important for the establishment of ICRs in mice, as previously discussed the de novo methylation during gametogenesis is most likely not directly linked the determination of ICRs (Kelsey & Feil, 2013; Proudhon et al., 2012; Smallwood et al., 2011). While the similarity between the pattern of *DNMT3B* expression in chicken germ cells and the timing of ICR establishment in mice therefore presents supporting evidence for the chicken's ability to establish gDMRs during gametogenesis, this likely does not provide evidence regarding the existence of imprinting in Aves.

Following the mammalian imprinting life cycle, there is support for the chicken's ability to demethylate and re-establish ICRs between generations. It is unclear, however, if chickens have the ability to protect ICRs from waves of genome-wide demethylation and de novo methylation in the embryo. This protection of ICRs is essential for imprinting, and therefore I cannot make a conclusion if chickens have the

ability to establish and maintain ICRs as described in mammals (Borgel et al., 2010; Kelsey & Feil, 2013; Smallwood et al., 2011).

Over 100 genes are imprinted in mammals, and investigating the pattern of expression of the orthologues to these genes in chicken is a logical step to identify imprinting (T Yokomine et al. 2001; Kelsey and Feil 2013). All chicken orthologues to mammalian imprinted genes show biallelic expression, providing strong evidence for a lack of imprinting in Aves (Colosi et al., 2006; Shin et al., 2010; T Yokomine et al., 2001; Takaaki Yokomine et al., 2005). Additionally, these studies found that the chicken orthologues to these imprinted genes are biallelically expressed in many tissues at many developmental time points. This is important, as the parent-of-origin expression of imprinted genes can be limited to specific tissues at certain developmental time points in mammals (Colosi et al., 2006; Shin et al., 2010; T Yokomine et al., 2001; Takaaki Yokomine et al., 2005). These studies present conclusive evidence that chickens do not show monoallelic gene expression at any of the genes studied. However, the genes subject to imprinting can vary even between closely related organisms. For example, the gene *Asb4* is imprinted in mice but not in humans (Jirtle 2012). As chickens and therian mammals are evolutionarily distantly related, it is much more likely for them to show differences between the genes subject to imprinting, compared to closely related organisms like humans and mice (Figure 1). Finding biallelic expression at the 7 imprinted orthologues examined in chicken compared to over 100 genes that are imprinted in mammals, therefore, does not offer conclusive evidence for a lack of imprinting in chicken (Kelsey and Feil 2013). Also as seen in mammals, perhaps closely related avian species differ based on which loci show parent-of-origin expression.

Consequently, a conclusion on imprinting in the class Aves cannot be drawn based on studies that only examine chicken and quail (Jirtle 2012; Maria Tuiskula-Haavisto et al. 2004; Frésard et al. 2014). Other studies propose that some genes may be uniquely imprinted in Aves, and that searching for imprinting in Aves by examining the orthologues to mammalian imprinted genes may overlook the genes subject to imprinting in birds (Maria Tuiskula-Haavisto et al. 2004; Frésard et al. 2013). While the lack of parent-of-origin expression at the chicken and quail orthologues to mammalian imprinted genes provides strong evidence for a lack of imprinting in Aves; it does not allow me to draw a decisive conclusion on the presence of imprinting in Aves as carried out in therian mammals.

I also investigated for evidence of imprinting in Aves by examining if chicken parent-of-origin QTLs significantly overlap with DMRs identified in chicken PGCs on incubation day 6 (Maria Tuiskula-Haavisto et al. 2004; Navarro et al. 2005; McElroy et al. 2006; Rowe et al. 2009; Sharman et al. 2007; Jang et al. 2013). A significant overlap would provide evidence for imprinting in chickens because almost all imprinted genes in mammals are directly associated with DMRs (Arnaud 2010). From this comparison a *P*-value of 0.2636 was calculated using a one-tailed Fisher's exact test, showing that chicken QTLs with parent-of-origin effects do not significantly overlap with chicken DMRs. this does not lead, however, to the conclusion that Aves lack imprinting for a number of reasons. The power of my study was only 0.1398. Therefore, even if chicken parent-of-origin QTLs significantly overlap with chicken DMRs, we have less of a chance of detecting the significance than we do of overlooking it. One cause of the low power is my small sample size of QTLs (Seltman 2013). The small sample size is a

result of the limited number of studies investigating the chicken genome for parent-of-origin QTLs, and the rigorous methods I applied to extract only meaningful QTLs from all the parent-of-origin QTLs identified in the literature examined. Also, QTL studies only identify loci corresponding to the phenotype examined (Miles and Wayne 2008). Therefore, the number of parent-of-origin QTLs used in my study is limited only to those that affect the phenotypes investigated in the literature examined. Along with the QTLs being biased to the phenotypes examined, the DMRs identified by Jang et al. were limited to the microarray constructed in their study, and thus would miss any other DMRs in chicken genome (Jang et al. 2013). Therefore, with the biased identification of chicken DMRs and parent-of-origin QTLs, the overlap I identified might be a misrepresentation of the actual overlap between chicken parent-of-origin QTLs and chicken DMRs. Another complication in this study comes from the observation stated earlier that many DMRs exist between male and female germ cells in mammals that are not used for imprinting (Smallwood et al., 2011). If this is true for chickens as well, then many of the identified chicken DMRs would not be associated with parent-of-origin expression, leading to confounding results in my study. Based on my analysis, the lack of overlap between chicken parent-of-origin QTLs and chicken DMRs does not provide evidence supporting or refuting the existence of imprinting in Aves.

The identification of parent-of-origin QTLs in chicken, the only positive support for imprinting in Aves, has been called into question. Rather than being the result of imprinting, studies show that parent-of-origin QTLs can often be falsely detected due to study bias (Sandor and Georges 2008; de Koning, Bovenhuis, and van Arendonk 2002). One assumption in QTL studies is that the breeds used as the parental generation have

different genotypes at QTL alleles (Miles and Wayne 2008). This is not extremely likely in livestock and can result in the false detection of QTLs with parent-of-origin effects (Sandor and Georges 2008; de Koning, Bovenhuis, and van Arendonk 2002). Also, linkage disequilibrium can lead to the false detection of parent-of-origin QTLs (Sandor and Georges 2008). When QTL alleles are not fixed based on breed and are in linkage disequilibrium with the DNA markers use in the QTL study, the false detection of QTLs with parent-of-origin effects greatly increase. As linkage disequilibrium in livestock can occur over large regions of the chromosome, it is likely that linkage disequilibrium lead to the spurious detection of parent-of-origin QTLs in chicken (Sandor and Georges 2008). However, Rowe et al. conducted a QTL study taking care to avoid these biases, yet still detected a QTL with maternal effects. Added support for the parent-of-origin QTL identified by Rowe et al. comes from the fact that a QTL with maternal effects had also previously been identified in this region by other studies (Rowe et al. 2009a; McElroy et al. 2006; Maria Tuiskula-Haavisto et al. 2004). However, Rowe et al. goes on to say that because only a two generation pedigree was used in this study, they cannot concluded that the maternal effects are a result of imprinting (Rowe et al. 2009a; Frésard et al. 2014). Therefore, the parent-of-origin QTLs identified in chickens most likely result from study bias and are not a result of imprinting.

A lot of the previous analysis outlined above returns questionable results on the existence of imprinting in Aves. Adding to this, even though imprinting exists in both marsupials and eutherians, most knowledge on genomic imprinting comes from studies addressing eutherians. Therefore, as my study is based on imprinting as described in mammals, my analysis on the existence of imprinting in Aves is limited to imprinting in

eutherians and does not address imprinting as it occurs in marsupials. This is detrimental as the few investigations on marsupials show there is a vast difference between imprinting in eutherians and imprinting in marsupials (Das et al. 2012). For example, opossum lack the imprinting regulatory elements used by eutherian mammals in the Igf2r cluster, but still imprint *IGF2R* (Weidman et al. 2006). Also, many of the imprinted genes in marsupials lack DMRs, the crucial imprinting regulatory element in eutherians (Das et al. 2012; Suzuki, Shaw, and Renfree 2013). The presence of imprinting and lack of eutherian regulatory elements in marsupials shows that imprinting mechanisms can be different between closely related organisms. On the other hand, imprinting also exists in some insects and some plants, which are very distantly related to mice and yet still share similar imprinting regulatory elements (Macdonald 2012). It is important to recognize however that the imprinted expression of the gene MEA in *Arabidopsis thaliana* does not rely upon parent-of-origin DNA methylation established during gametogenesis (Jullien et al. 2006). This observation that imprinting of at least one gene in *Arabidopsis thaliana* does not require DNA methylation along with the lack of DMRs at many imprinted genes in marsupials shows that the regulation of imprinting genes is not limited to parental specific DNA methylation, and can vary between species. I therefore determine that my previous analysis on the existence of imprinting in Aves is biased only to imprinting as described in this review for eutherians, and thus cannot be used to make a final determination on the existence of imprinting in Aves. A decisive conclusion on the presence of imprinting in Aves would only be reached by studies that are not biases toward orthologues of genes imprinted in eutherians, or are

conditional based upon parental specific DNA methylation being the imprinting regulatory element.

Recently a study questioning the existence of imprinting in Aves avoided most of the biases outlined above by using RNA-seq to investigate the expression levels of the transcriptome of chickens produced using reciprocal crosses in the search of any gene that shows parent-of-origin expression (Frésard et al. 2014). Using this methodology avoids most of the biases outlined above by not being limited to the imprinted genes identified in eutherian, and overlooking any regulatory mechanism involved in imprinting. Consistent with a lack of imprinting in Aves this study did not detect any loci that show parent-of-origin expression (Frésard et al. 2014). However, as the author explains this was an initial study, and only addressed the whole chicken embryo collectively at incubation day 4.5. Again, as discussed earlier, genes subject to imprinting at can vary based on tissue, developmental time point, and between closely related organisms (Frésard et al. 2014). Therefore, even though this study offers strong evidence for a lack of imprinting in the collective chicken embryo at incubation day 4.5, it does not provide evidence for the presence or lack of imprinted genes at other time points, or in Aves outside of chickens. As proposed by Frésard et al. future research using this methodology examining many chicken tissues at many developmental time points, and in avian species other than chickens is required for a decisive conclusion on imprinting in Aves (Frésard et al. 2014).

Based on the evidence presented in this review, I conclude that imprinting does not exist in Aves. Chickens have homologues to proteins necessary for the demethylation and re-establishment of ICRs, however it is questionable if they have

proteins to protect ICRs from demethylation and do novo methylation in the embryo (Kelsey & Feil, 2013; Smallwood et al., 2011). As protecting ICRs from these epigenetic modifications is essential for imprinting in mammals I cannot conclude that chickens have the ability to establish and maintain ICRs as in mammals, refuting the presence of imprinting in Aves. Additionally every chicken orthologue to mammalian imprinted gene examined show biallelic expression (Colosi et al., 2006; Shin et al., 2010; T Yokomine et al., 2001; Takaaki Yokomine et al., 2005). The only support for imprinting in Aves comes from the many QTLs with parent-of-origin effects identified in chicken. However, most of these findings can be explained by biases in the study, rather than imprinting. Also, I identified that these parent-of-origin QTLs do not overlap with chicken DMRs, implying they are not associated with imprinting. While all of the previously outlined components of my conclusion are biased to imprinting as it occurs in eutherian mammals, the completely objective study by Frésard et al. presents strong evidence that parent-of-origin monoallelic gene expression does not occur in the full chicken embryo at incubation day 4.5 (Frésard et al. 2014). While research at additional time points using the methodology outlined in the Fresard et al. study needs to be conducted for an exhaustive conclusion on imprinting in Aves, current knowledge provides substantial evidence for a lack of imprinting in Aves.

Methods

Chicken Homologues to Imprinting Enzymes

To fill table1, mice and humans were used as references for eutherians, opossums were used as references for marsupials, platypus were used as a reference for monotremes, and chickens were used as a reference for birds, and anoles were used as a reference for lizards. First, the table was filled out as completely as possible using previously published literature (Arnaud, 2010; Cañón et al., 2006; Ciccone et al., 2009; Conticello et al., 2005; Flicek et al., 2013; Hore et al., 2008; Kelsey & Feil, 2013; Kim et al., 2006; Renfree et al., 2009; T Yokomine et al., 2006). Ensembl was then searched to examine the existence of the left over enzymes in their respective organisms (Flicek et al. 2013). When Ensembl returned a negative result, the human and mouse protein sequence for each enzyme/protein were tested against the protein sequence databases on NCBI for opossum (taxid: 9265), platypus (taxid: 9258), chicken (taxid: 9031), or green anole (taxid: 28377) (Biotechnology 2002). If the best BLASTP result had an E-value greater than 0.05 using the BLOSUM62 matrix then it was concluded that the organism does not have a homologue to that protein that serves a similar function. A returned E-value of 0.05 using BLAST means that in database size you are searching you expect to see a specific match by chance 0.05 times (Altschul et al. 1997; Altschul et al. 1990; Altschul). Proteins that share a similar function are usually

highly conserved, and observing that conserved sequence by chance would not be very likely. The expected value of 0.05 is rather low, and I felt that only identifying a protein as a functional homologue if a value lower than 0.05 was returned was a good cutoff. A chicken homologue to ELP1 was identified in ensemble, the gene had the name IKBKAP, with nucleotide sequence labeled ENSGALG00000001947, and the protein sequence labeled ENSGALP00000003009 (Flicek et al. 2013). Using BLASTP this protein sequence was measured against the mouse protein sequence database on NCBI (taxid: 10090). The elongator complex protein 1, with the accession number NP_080355.2, and the GI number 158937298 was returned (Biotechnology 2002; Altschul et al. 1997).The chicken protein sequence from ensemble ENSGALP00000003009 covered 100% of the mouse elongator complex protein 1 sequence and shared 63% sequence identity (Biotechnology 2002; Altschul et al. 1997). The mouse protein sequence of PGC7/Stella/DPPA3, sequence accession number BAB86304.1 and GI number was 19570421, along with the human protein sequence of PGC7/Stella/DPPA3, accession number was AAQ84110.1 and GI number 35186894, were measure against the platypus (taxid: 9258), and green anole (taxid: 28377) protein sequence databases using BLASTP (Biotechnology 2002). No matches returned an E-value of 0.05 or lower when searching the platypus or green anole protein database using the mouse or human PGC7/Stella/DPPA3 protein sequence. The protein sequence for Uhrf1 isoform A in the mouse, accession number NP_035061.3 and GI number 161621269, and the protein sequence for Uhrf1 isoform 1 in humans, accession number NP_001041666.1 and GI number 115430235, were also measured for matches against the chicken genome using BLASTP. The best sequence returned for both

searches was UHRF1 isoform X6 for *Gallus gallus*, accession number XP_004949015.1 and GI number 513227877. The chicken UHRF1 isoform X6 predicted protein sequence covered 95% of the human UHRF1 protein sequence and shared 73% identity, and also covered 95% of the mouse UHRF1 protein sequence and shared 65% identity (Biotechnology 2002; Altschul et al. 1997). I concluded therefore that chickens have a homologue to UHRF1. The mouse protein sequence for DNMT1, accession number AAH53047.1 and GI number 31419356, and the human protein sequence for DNMT1, accession number AAH92517.1 and GI number 62204780 were tested against the platypus database (Biotechnology 2002; Altschul et al. 1997). Most of the identified results covered less than 10% the human and mouse protein sequence. However, the UniProt database contains a sequence called cytosine-specific methyltransferase in the platypus genome, identification: F7F8U9_ORNAN, with a sequence closely resembling the DNMT1 sequence in mice and human. The sequence contains 83% identity covering 100% of the human DNMT1 protein sequence, accession number AAH92517.1 and GI number 62204780; and 78% identity covering 99% of the mouse DNMT1 protein sequence, accession number AAH53047.1 and GI number 31419356, and therefore it was concluded that platypus contain a homologue to DNMT1 that shares a great deal of similarity with eutherian mammals (Consortium 2014; Altschul et al. 1997). To fill the final column in Table 1 the following sequences in the mouse were used as the query and compared to the chicken protein database on NCBI (taxid: 9031) using BLATP, word size 3 and the BLOSUM62 matrix. The E-values results come from a comparison to the whole chicken protein database. The mouse DNMT1 sequence, accession number NP_034196.5 and GI number 327180743, returned the chicken

DNMT1 sequence, accession number NP996835.1 and GI number 46048774. The mouse DNMT3A sequence, accession number NP_031898.1 and GI number 6681209, returned the chicken DNMT3A sequence, accession number NP_001020003 and GI number 67514539. The mouse DNMT3B sequence, accession number NP_001003961.2 and GI number 172088099, returned the chicken DNMT3B sequence, accession number NP_001019999.1 and GI number 67514587. The mouse TET sequence, accession number NP_898961.2 and GI number 256773243, returned the predicted chicken TET3 sequence, accession number XP_003642602.2 and GI number 513220788. The mouse AICDA sequence, accession number EDK99688.1 and GI number 148667272, returned the chicken AICDA sequence, accession number NP_001230151.1 and GI number 342366802. The mouse KDM1B sequence, accession number NP_758466.1 and GI number 26986559, returned the predicted chicken KDM1B isoform X3 sequence, accession number XP_418920.3 and GI number 363730386. The mouse CTCF sequence, accession number NP_851839.1 and GI number 31044459, returned the chicken CTCF sequence, accession number NP_990663.1 and GI number 45384498. The mouse YY1 sequence, accession number NP033563.2 and GI number 31982421, returned the chicken YY1 sequence, accession number NP_001026381.1 and GI number 71894941.

Chicken gDMRs

Chicken DMRs were identified between male and female PGC in White Leghorn chickens on incubation day 6 by a recent study conducted Jang et al (Jang et al. 2013). This study did not examine the methylation of the full chicken genome but only focused

on 5kb regions upstream of chicken genes that were orthologous to mammalian imprinted genes, orthologous to mammalian X-linked genes, or genes that Jang et al. identified as showing differential expression between chicken embryonic fibroblasts and chicken PGCs (Jang et al. 2013). For an extended outline of the methods to obtain chicken DMRs see the Jang et al. paper (Jang et al. 2013). We received a comprehensive spreadsheet of all the DMRs labeled with their chromosome and base pair (bp) location by Jang et al. maternally vs. paternally methylated DMRs were not differentiated in this study and were treated together as a single group.

Real Chicken Parent-Of-Origin QTLs

Chicken QTLs with parent-of-origin effects were extracted from five papers (Navarro et al. 2005; Rowe et al. 2009a; Sharman et al. 2007; McElroy et al. 2006; Maria Tuiskula-Haavisto et al. 2004). Parent-of-origin QTLs that show maternal effects were not differentiated from QTLs that show paternal effects in this study. The paper by Sharman et al. showed discrepancies about the number of QTLs with parent-of-origin effects identified; in the text Sharman et al. states there are 12 parent-of-origin QTLs but only shows 11 in the tables. This study only address the 11 parent-of-origin QTLs described as showing imprinting effects in Table 5 from the Sharman et al. paper (Sharman et al. 2007). QTL locations were given based on cM position. The DNA flanking markers surrounding each QTL were used to identify the bp and chromosome location of each QTL. The QTLs with parent-of-origin effects from the McElroy et al. paper are provided in Table 3 with estimated cM position. These positions were then used to infer the surrounding markers based on the estimated cM position of DNA

markers provided in Table 1 (McElroy et al. 2006). A similar method of inference was used between Table2 and Fig. 1 in the paper by Tuiskula-Haavisto et al. (Maria Tuiskula-Haavisto et al. 2004). The DNA markers surrounding the QTLs with parent-of-origin effects were directly provided by the other papers (Rowe et al. 2009b; Navarro et al. 2005). The chromosome and bp position of each of the DNA markers was obtained using NCBI Mapviewer for Gallus gallus, Annotation Release 102 Statistics on December 2013 (Dombrowski and Maglott 2002; Wheeler et al. 2002). The window size of each QTL ranged from the chromosome bp position of one DNA flanking marker to the chromosome bp position of the other DNA flanking marker. If a QTL existed directly on top of a DNA marker then the surrounding markers identified by the paper were used. Each QTL with a DNA marker that did not have an identified chromosome and bp location was not used in this study. There was one exception to this rule. A QTL from the Tuiskula-Haavisto et al. paper located on chromosome 9, existed directly on top of the DNA marker MCW0135 (Maria Tuiskula-Haavisto et al. 2004). The higher surrounding marker based on cM position, MCW0134, provided a bp position, while the lower marker ADL0191 did not. A QTL from the Navarro et al. paper goes from the DNA marker MCW0135, to another DNA marker, ROS0078, which does provide a bp position. As the DNA markers ADL0191, located around 44 cM, and the marker ROS0078, located at 0 cM are both in a lower orientation to the marker MCW0135, which is located at 61 cM based on cM position in the chicken consensus linkage map, it can be concluded that most likely the lower bp position of chromosome 9 is already covered by the Navarro et al. QTL, and that only considering the top half of the Tuiskula-Haavisto et al. QTL from chromosome 9 in this study will not cause problems

(Dombrowski and Maglott 2002; Wheeler et al. 2002). Any QTL that did not cover a novel DNA region but instead only existed within previously identified QTLs was not used in the study. QTLs that overlapped but also covered novel regions of DNA were still used in the study and treated as separate QTLs. The average size of the observed parent-of-origin QTLs was calculated.

Randomly Produced Chicken Parent-Of-Origin QTLs

Random chicken parent-of-origin QTLs for each chromosome were made using the Integer Generator on the website Random.org (Haahr 2013).The number of random parent-of-origin QTLs produced for each chromosome was equal to the number of real parent-of-origin QTLs observed for each chromosome. The length of each chromosome was input into Integer Generator so the random numbers produced would be between 0 and the bp size of the chromosome. This provided an equal likelihood of the random parent-of-origin QTLs existing anywhere on the chromosome. The numbers produced by Random.org were treated as the starting positions of the random parent-of-origin QTLs. The average size of real parent-of-origin QTLs was then added to the random QTL start positions to calculate the window size of the random parent-of-origin QTLs.

Fisher Exact Test

A 2x2 contingency table was created to compare the number of times real parent-of-origin QTLs overlapped with chicken DMRs and the number of times random parent-of-origin QTLs overlapped with chicken DMRs. One column was for real parent-of-origin QTLs and showed the number of times they overlapped with DMRs and the

number of times they did not overlap with DMRs. The other column was for randomly produced parent-of-origin QTLs and showed their overlap with DMRs. Using the GraphPad QuickCalcs website http://graphpad.com/quickcalcs/contingency1/ (accessed Dec 2013) a one tailed Fisher's exact test was performed.

Power of Fisher Test

The power of the Fisher's exact test was computed using the package 'Exact' in the programming language R (Calhoun, 2013; Hornik, 2013; R Development CoreTeam, 2008) . The function power.exact.test(p1, p2, n1, n2, alpha, alternative, method, simulation) was used. "p1" represents the probability of getting a success in group 1, represented by the fraction of real QTLs overlapping with DMRs. "p2" represents the same calculation but for the group of randomly produced QTLs (Calhoun 2013). "n1" and "n2" equal the group sizes for real QTLs and random QTLs respectively. "alpha" equals the level of significance, which was 0.05 for this study (Calhoun 2013). "alternative" was set as "greater" because a one-tailed Fisher's exact test was used to measure if the overlap between real chicken parent-of-origin QTLs and DMRs was significantly greater than the overlap between random parent-of-origin QTLs and DMRs. Method equals "Fisher" because a Fisher's exact test was used. Simulation equals FALSE since real data was used and not simulated data (Calhoun 2013).

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Vita

Derek Michael Gygax is an American citizen and was born in the Cheverly, Maryland on February 23, 1988. He graduated from Kent Island High School as Valedictorian in 2006. In 2010 he graduated from the College of William and Mary with a Bachelor of Science in Biology.