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OCT4 AND SOX2 REGULATE METHYLATION AT THE IGF2/H19 IMPRINTING CONTROL REGION

BY

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DISSERTATION

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ABSTRACT

Mono-allelic expression of H19 and Igf2 depends on parental-specific methylation of an imprinting control region (ICR). Although CTCF sites maintain the absence of DNA methylation on maternal ICRs after fertilization, some Beckwith-Wiedemann patients showed maternal ICR methylation that correlated with mutations in one of the pair of octamer binding sites within the ICR. Using a tissue culture system, we found that both octamers were required for transgene hypomethylation in F9 cells and that Oct4 was present at the endogenous ICR. In addition, each of the octamers is flanked by a sequence that allowed cooperative binding of Oct4 and Sox2 in vitro. To determine at which developmental stage the octamers regulate ICR methylation, we created a knock-in mouse with mutations in the ICR that eliminate Oct4 binding. Maternal transmission of this mutant allele resulted in partial methylation of the maternal ICR in somatic tissues but had little effect on imprinted expression of H19 and Igf2. Although a subset of oocytes showed partial methylation of the mutant ICR, maternally inherited mutant alleles were unmethylated in blastocysts, indicating that the methylation acquired during oogenesis was not a stable imprint. Absence of mutant ICR methylation in female primordial germ cells regardless of parental origin demonstrated that the octamers are not required for imprint erasure but protect the ICR from de novo methylation during oogenesis. These findings provide the first direct evidence that octamers are required for hypomethylation of an endogenous ICR.

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CHAPTER 1: INTRODUCTION

GENOMIC IMPRINTING

Development of multicellular organisms depends on the transmission and inheritance of stable gene expression profiles that result from differential DNA methylation and histone modifications. These reversible "epigenetic" marks have a profound effect on the chromatin architecture and accessibility of genes to cis- and trans-acting regulatory elements without changing the underlying genetic sequence [6,10]. Therefore, while the individual cells comprising an organism generally share a common genome, they acquire unique epigenomes as a means of establishing and maintaining the differential expression of genes associated with disparate cell structures and functions (reviewed in [7,47]). During mammalian gametogenesis divergent pathways of cellular differentiation for sperm and oocyte result in epigenomic heterogeneity that renders maternal and paternal genomes functionally non-equivalent [79,119,120]. Parental specific epigenetic marks or 'imprints' established in the different germ lines result in unequal expression of autosomal alleles that is essential for normal organism development. The requirements for establishing and maintaining these imprints and the mechanism by which epigenetic modification regulate imprinted gene expression has been an active area of research for more than two decades.

To date 144 imprinted mouse genes have been identified from uniparental disomies of whole chromosomes and uniparental duplications of selected chromosome regions [139]. However, this number is likely to increase as researchers begin validating some of the more than 1300 loci showing parent-of –origin effects in RNA-seq experiments performed on embryonic and adult brain [34]. More than 80% of genes whose imprinted expression has been independently validated are clustered in genomic regions containing two or more imprinted genes and more than 40 of these genes are distributed between 7 well characterized genomic clusters (*Gnas* on chr:2; *Kcnq1*, *Pws/As* and *Igf2* on chr7; *Grb10* on chr11, *Dlk1* on chr12; *Igf2r* on chr17) [3]. Imprinted genes within a given cluster can be spread over as much as 2-3 Mb, as is the case for the *Pws/As*

locus, but are regulated by a common imprinting control region (ICR) capable of influencing gene expression *in cis* over long distances [77]. At many of these loci, parental-specific epigenetic modifications to the ICR results in both activation and repression of different neighboring genes. Genes regulated in this way are said to be reciprocally imprinted as lack of the same imprint on the opposite parental allele reverses the expression profile. All 7 major clusters contain reciprocally imprinted genes, and except for *Grb10*, the imprints at their respective ICRs are associated with activate protein coding genes and an inactive noncoding RNA gene (ncRNA) [3].

Differential DNA methylation is a hallmark of the genetic imprints. Each ICR contains a differentially methylated region (DMR) that acquires methylation in only one of the germ lines. Mammalian DNA methylation involves a reversible covalent modification at the C-5 position of cytosine residues in CpG dinucleotides that is catalyzed by two distinct families of DNA methyltransferases (DNMTs) [8]. The DNMT3 family, which includes DNMT3a, DNMT3b and DNMT3L, is responsible for *de novo* methylation of CpGs, and the DNMT1 family, which includes DNMT1 and (an oocyte specific isoform) DNMTo, is responsible for maintaining CpG methylation [9]. Establishment of maternal germline specific methylation imprints requires both DNMT3a and the non-catalytically active DNMT3L [12,52,53]. Establishment of paternal imprints depends predominantly on DNMT3a and to a lesser extent on DNMT3L [9,52]. Maintenance of parental imprints following fertilization depends on oocyte derived DNMT1 and DNMT1o for the earliest cleavage stages and conceptus derived DNMT1 for the rest of development [20,21]. Once established, the majority of methylation imprints are stably maintained in adult somatic cell lineages irrespective of the imprinted gene's tissue-specific expression [141]. However, the mechanism by which ICRs recruit or inhibit DNMT and other epigenetic regulators as a means of establishing and maintaining parental imprints is not entirely clear.

ESTABLISHING IMPRINTS

During gametogenesis, establishment of identical sex-specific methylation patterns on homologous chromosomes is preceded by genome wide erasure of methylation including imprints. By embryonic (E) day 13.5, CpG methylation in male and female primordial germ cells (PGCs) has been reduced to 16.3% and 7.8% respectively compared to 73.2% for the whole embryo [98]. This process of demethylation takes place from E7.5-12.5 and likely requires both active and passive mechanisms [103]. Transcription of DNMT3a, DNMT3b, DNMT1 recruitment factor UHRF1/NP45 and histone H3K9me2 methyltransferase GLP are repressed in PGCs shortly after lineage commitment (~E7.5) [61,108,109]. A second H3K9 methyltransferase, G9a, is also repressed at ~E9.5 [108]. Consequently, PGCs show reduced genome wide methylation, including 50% demethylation of the H19 and Peg10 imprinted genes, and significant loss of H3K9me2 by the time the cells have finished migrating to the genital ridge (~E10.5) [66,109]. Other imprinted genes, including *Snrpn*, *Peg3* and *Kcnq1ot1*, are almost completely methylated at E11.5 but actively lose most of their methylation by E12.5. Activation-induced cytosine deaminase (AID) mediated deamination of cytosine to thymine followed by base excision repair (BER) is one possible mechanism for active demethylation in the germline [38,98]. However, AID-deficient PGCs still have low levels of genomic methylation (~21%) possibly due the presence of compensatory deaminases or 5-methylcytosine (5mC) hydroxylases [103].

Sex-specific differential methylation at ICRs may result from different temporal expression profiles of *de novo* methyltransferases [14,115]. In the male germ line DNMT3a and DNMT3L expression resumes in diploid prospermatogonia at E14 and results in gradual CpG methylation until shortly after birth [63,104]. Both DNMT3s are required for complete methylation of retrotransposons and paternal ICRs and for spermatogonia to successfully complete meiosis [15,54]. DNMT3a and DNMT3L are also essential for maternal ICR methylation, but its expression in oocytes begins postnatally after which time meiotic recombination has already taken place [63,73]. The concomitant lack of cell divisions and prolonged high levels of DNMT3 in growing

oocytes likely contributes to dimorphic methylation patterns including the greater number of maternally methylated ICRs and the presence of non-CpG methylation in oocytes and not sperm [129].

Differences in pre- and post-meiotic chromatin architecture influence the accessibility of ICRs to DNMTs [14]. During meiosis there are global changes in histone composition and modifications including changes in H3K4 methylation that are required for DNA methylation at some imprinted loci [1,74]. DNMT3L has been shown to interact with H3 amino terminus and its binding is strongly inhibited by H3K4me [91]. The presence of this histone modification in male PGCs may therefore prevent ICR methylation of maternally imprinted genes. Moreover, the maternally expressed H3K4 demethylase KDM1B is necessary for establishment of DNA methylation imprints at a subset of loci in oocytes [19]. In addition to H3K4 hypomethylation, repressive histone marks such H3K9 di/trimethylation, H4K20 trimethylation and heterochromatin protein 1 (HP1) may be sufficient to initiating *de novo* ICR methylation by recruiting DNMTs through the KRAB box zinc-finger protein Zfp57 and other mediators[115,149] . The maternally methylated *Snrpn* ICR is unmethylated in oocytes deficient in Zfp57, but it reacquires methylation via a Zfp57 dependent mechanism in the zygote suggesting the existence of a chromatin specific initiator of methylation [70].

Differential expression of male and female specific trans-acting factors have been found essential for establishing imprints at only a few loci. In addition to the maternally expressed *KDM1B*, paternal expression of MILI and MIWI2 are required for piRNA dependent methylation of *Rasgrf1* ICR in spermatogonia [136]. A paternally expressed paralog of CTCF, CTCFL/BORIS, has also been proposed to facilitate methylation at the *H19/Igf2* ICR through the recruitment of a complex involving the H4 methyltransferase PRMT7 and DNMT3s [48]. However, the establishment of normal methylation patterns in male PGCs with ICRs containing mutant CTCFL binding sites indicates this mechanism is not essential [106].

Parental-specific protection of ICRs from *de novo* methylation in PGCs is equally important for inheritance of imprinted traits but its mechanics are even less well understood. Unmethylated DMRs at ICRs can function as CpG islands (CGIs) which utilize cis-regulatory elements to promote a euchromatic state that is not conducive to DNA methylation. Five such elements, including two Oct and one Sox binding site, have been identified at the paternally imprinted Angelman syndrome ICR (AS-IC) which are required for ICR hypomethylation in oocytes [55]. A more universal mechanism of maintaining ICR hypomethylation may be through the CxxC finger protein, Cfp1, which is required for H3K4me enrichment at unmethylated CGIs [127]. Cfp1 binds specifically to unmethylated CpGs and associates with the H3K4 methyltransferase Setd1 [67,133].

MAINTAINING IMPRINTS

The genome undergoes dynamic epigenetic modifications as the totipotent state of the zygote gives way to the progressively differentiated cells that will comprise either the extra-embryonic tissue or the adult organism. This process includes a wave of genome-wide demethylation during cleavage followed by extensive *de novo* methylation during gastrulation [83,84,103]. Both of these events challenge allelic imprinting differences. DNA demethylation is initiated in the male pronuclei prior to the first cell division by TET dependent iterative oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and finally 5-carboxylcytosine (5caC) [35,44]. Demethylation of both male and female chromosomes then proceeds through a passive replication-dependent dilution of 5-cytosine moieties [45]. However, the maternal pronuclei and at least two paternal imprints at the *Igf2/H19* and *Rasgrf1* loci are protected from 5mC oxidation by the DNA binding protein PGC7/Dppa3/Stella [86,142]. PGC7/Stella also contributes to transcriptional silencing and chromatin condensation in late stage oogenesis, but how it is targeted specifically to methylation imprints and selected repeats and not the rest of the male pronucleus, which is ~ 80% methylated at CpGs, is unknown [72,103,135].

Imprints are also preserved by direct binding of Zfp57 to specific methylated DNA sequences post-fertilization and concomitant recruitment of KAP1 [70,99]. KAP1 can in turn form complexes with UHRF1, DNMTs, repressive histone deacetylases (HDACs) and the histone methyltransferase SETDB1 all of which have been shown to localize at methylated ICRs [140,149]. At the *H19* ICR, four of six Zfp57 consensus sequences, TGCCGC, partially overlap three CTCF binding sites [99]. The *H19* ICR has been thoroughly investigated and a retrospective assessment of previous work is consistent with a possible role for Zfp57 in maintaining *H19* ICR methylation. Deletions of CTCF and Zfp57 sites in mice reduced methylation on the paternally imprinted allele in somatic tissue while mutations inhibiting only CTCF binding had no effect on the paternal imprint [29,106]. Disruption of Zfp57 and CTCF sites in stably expressed *H19* ICR transgenes also indicated these sequences were required for methylation-dependent repression in specific cell types [17].

The stability of many imprints in the preimplantation embryo likely depends on positive feedback loops that exist between DNA methylation and histone modifications [16,18,47]. Although most nucleosomes are replaced by protamines in sperm, the majority of methylation imprints are maternally derived and can be inherited with repressive chromatin [27]. H3K9 di/trimethylation, H4K20 trimethylation and sometimes heterochromatin protein 1 (HP1) show enrichment on alleles bearing methylation imprints while euchromatic histone modification such as H3K4 di/trimethylation and H3 acetylation are absent [51,81,100,144]. The presence of H3K9me and H4K20me enrichment at most ICRs is methylation dependent [41]. Methylated DNA maintains these differential histone modifications through the activity of Zfp57, MeCP2, and other related methyl-CPG binding domain proteins (MBDs) which can recruit co-repressor complexes containing HDACs [32,50,87,149]. UHRF1 and DNMT1, which bind preferentially to hemimethylated DNA and are required for methylating newly synthesized strands, also recruit the H3K9 methyltransferases G9a and GLP [11,110,146]. Methylated H3K9 can then reinforce CpG methylation through UHRF1 or HP1 - Kap1 complex mediated recruitment of DNMTs [101,111,146].

Mechanism required for preserving differential methylation at ICRs during genome wide remethylation likely depends on which parent the hypomethylated ICR was inherited. Rapid exchange of protamines with maternal histones during male pronuclear decondensation combined with histone acetylation and lack of repressive histone modifications following genome wide demethylation may create an unmethylated default state for most paternal ICRs [132]. Alternatively, the unmethylated paternal ICR at the *Kcnq1* cluster has been shown to retain nucleosomes with H3K4me in sperm [36]. Protection from de novo methylation may also depend on many of the same cis-regulatory elements that insure hypomethylation of ICR in the germline. The H19/Igf2 ICR has demonstrated the capacity to promote hypomethylation of transgenes in tissue culture in an Oct dependent manner similar to the AS-IC [42,55]. However, fundamental differences in chromatin architecture between gametes and the differentiating cells of the conceptus likely explain the requirements for different trans-regulatory factors. One such protein is CTCF which is required for hypomethylation of the maternal *H19/Igf2* ICR in somatic tissue but not in oocytes [29,106].

REGULATING TRANSCRIPTION

ICRs regulate mono-allelic expression of neighboring genes through two primary mechanisms which function in a tissue- and developmental–specific manner.[59]. The most common of which involves the establishment and maintenance of repressive heterochromatin either at a proximal promoter or at multiple promoters over long distances through a mechanism involving long ncRNAs [64]. ICRs are frequently located at promoters for ncRNAs and when methylated are capable of directly inhibiting their expression [13]. ICRs at the maternally imprinted *Kcnq1* and *Igf2r* clusters are located at promoters for the respective ncRNAs *Kcnqot1* and *Airn* which are only expressed from the paternally unmethylated alleles [75,76,114]. These ncRNAs functions in *cis* to repress paternal genes via transcription-mediated silencing or RNA-mediated silencing [94]. In the placenta *Kcnqot1* silencing of imprinted genes largely depends on an RNA-dependent recruitment of both polycomb repressive complex 1 (PRC1) and G9a for respective methylation of H3K27 and HK9 and polycomb repressive complex 2 (PRC2) for

repressive H2AK119 monoubiquitination [125,131,134]. The placental-specific imprinted expression of *Slc22a3* in the *Igf2r* cluster also requires G9a [82]. However, other genes at these loci which are ubiquitously imprinted in both the placenta and the embryo demonstrate different tissue specific requirements for PRC1 and 2 or G9a indicating the existence of alternative mechanisms of repression. Both ubiquitously imprinted *Igf2r* and *Kcnq1* genes are partially overlapped by the respective ncRNA at their locus which is transcribed in an antisense direction and therefore potentially capable of direct interference. Alternatively, *Airn* may disrupt placental enhancers at the *Igf2r* locus thereby interfering with transcription of *Slc22a3* [58].

The other primary mechanism by which imprinted genes are regulated involves establishment and maintenance of differential insulators via the zinc finger DNA binding protein CTCF [85]. CTCF has the capacity to mediate long range intra-chromosomal interaction by binding to distant sites and recruiting the cohesin complex which can unite the sites to form chromosomal loops [80,138]. This partitioning in turn facilitates or blocks the interaction of promoters with distant enhancers and can separate regions of active or repressed chromatin. Both the *H19/Igf2* ICR, described in detail below, and the *Rasgrf1* ICR functions as CTCF dependent enhancer blockers [5,40,145].

THE *H19/IGF2* LOCUS

Insulin like growth factor 2 (*Igf2*) and *H19* were among the first imprinted genes discovered and their reciprocal expression has been extensively studied [2,4,24]. They are part of a cluster of four imprinted genes, including the insulin 2 (*Ins2*) gene and *Igf2* anti-sense (*Igf2as*) gene, located on mouse distal chromosome 7 and in shared synteny on human chromosome 11p15 [68,90]. *Igf2* is one of only two paternally expressed imprinted genes that are required for viability of parthenogenetic embryos [60,143]. H19 is a maternally expressed non-coding RNA that is not essential for development but may serve to modulate the expression of *Igf2* and other imprinted genes *in trans* [33]. Increased *Igf2*expression resulting from paternal duplications, uniparental disomy (UPD) or maternal loss of imprinting results in a fetal overgrowth phenotype in mouse or a

pediatric overgrowth disorder in humans known as Beckwith-Wiedemann syndrome (BWS), which is also associated with a predisposition for tumor development [68,137]. In contrast, loss of paternal imprinting results in diminished or absent *Igf2* expression and is frequently responsible for the intrauterine and postnatal growth retardation associated with Siver-Russel syndrome in humans [102].

The mouse *Igf2* cluster is spread over ~100 kb with a 72 kb intergenic region separating *Igf2* and *H19* (Fig.1a). An ICR located with the intergenic region ~2 kb upstream of *H19* contains four CTCF binding sites that are required for imprinted expression at the locus [106]. CTCF binds to the unmethylated ICR and mediates cohesin complex dependent interactions with DMR 1 at *Igf2* and the matrix attachment region 3 (MAR3) on the maternal allele to form intra-chromosomal loops (Fig. 1b) [62,88,138]. These loops prevent shared enhancers from associating with *Igf2* and create a boundary that separates an active chromatin region containing *H19* from a repressive chromatin region containing Igf2 [113]. In addition to the physical boundary, CTCF contributes to *Igf2* repression by interacting with Suz12 and recruiting PRC 2 to direct H3K27 methylation at the *Igf2* promoters [69]. In contrast, hypermethylation at the paternal ICR inhibits CTCF binding and prevents the formation of chromatin loops that block enhancer access to *Igf2* [5,62]. The methylated ICR also represses H19 transcription both by facilitating H19 promoter methylation and through poorly defined *cis*-regulatory elements [43,128].

PROJECT SUMMARY

Differential ICR methylation is essential for imprinted *H19/Igf2* expression. The focus of this project was to investigate the role of the conserved octamers identified by Hori et al. in establishing and/or maintaining the hypomethylated state of the ICR *in vivo*. In order to answer that question we first utilized a tissue culture based system to compare the effect of octamer and CTCF site mutations on acquisition and erasure of DNA methylation of stably incorporated ICR transgenes. Contrary to the requirements for CTCF in protecting the ICR from *de novo* methylation in mouse somatic cells, we found

that the octamers were the primary regulator of ICR transgene methylation [106]. Moreover, we determined that octamer mediated demethylation was dependent on flanking Sox binding sites and specific to F9 cells which express both Oct4 and Sox2 pluripotency factors. Further characterization of the ICR Sox/Oct motifs using electromobility shift assay (EMSA) demonstrated that they cooperatively bind Oct4 and Sox2. Using a luciferase reporter assay we also discovered that the ICR Sox/Oct motifs can function as an Oct4/Sox2 dependent transcriptional activator. As a definitive test of octamer dependent regulation of ICR methylation at the endogenous locus, we created a knock-in mouse with mutant octamers. Analysis of ICR tissue-specific methylation and *H19* and *Igf2* allelic expression demonstrated that the octamers are required for protecting the maternal allele from *de novo* methylation in oocytes and somatic tissue but not for imprinted expression.

FIGURES

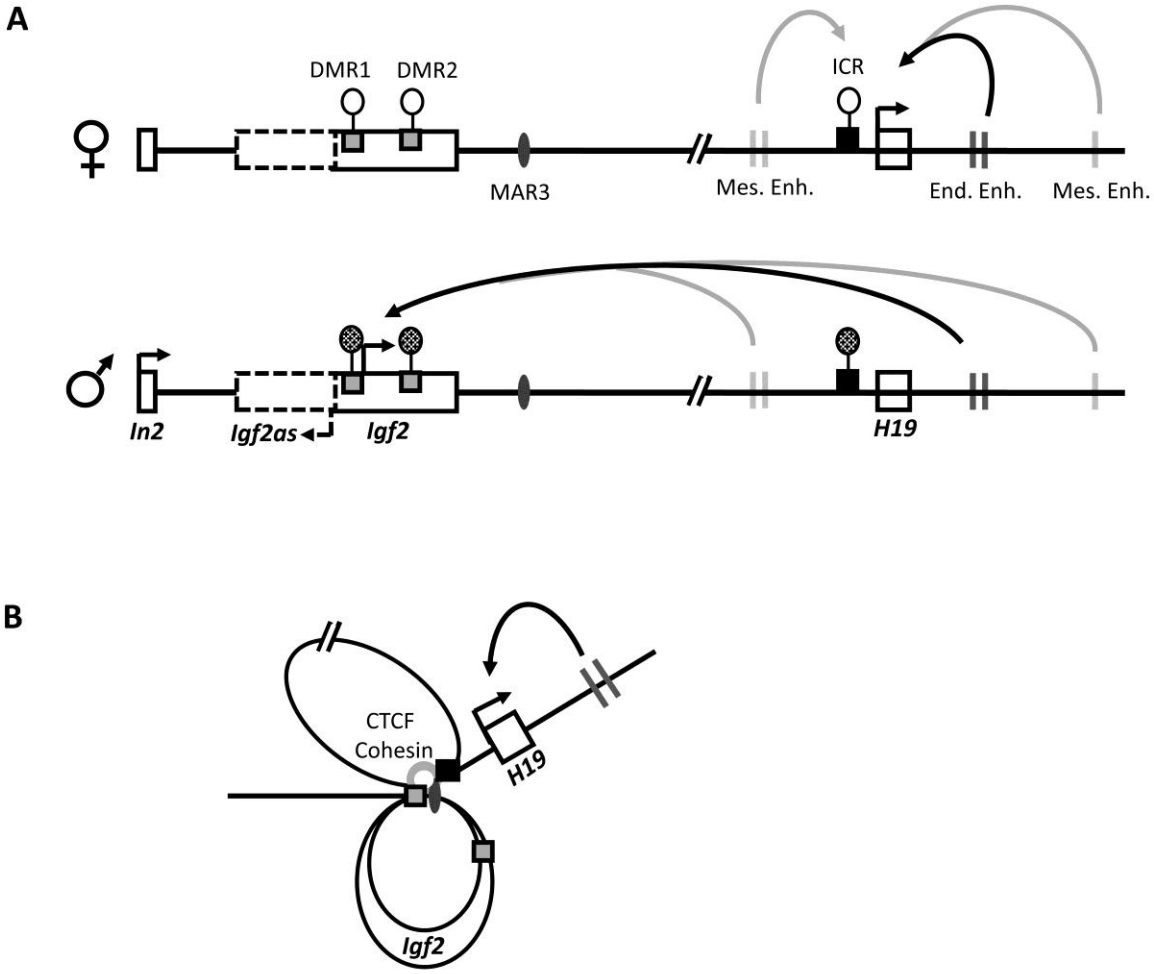

Figure 1. **ICR dependent regulation of *H19* and *Igf2* imprinted expression in the mouse.** (**A**) Maternal (top) and paternal (bottom) alleles are shown with their respective unmethylated (open lollipop) or methylated (closed lollipop) ICR and secondary DMRs. The unmethylated maternal ICR binds CTCF and establishes cohesin dependent chromatin loops that restrict localization of shared enhancers to the *H19* promoter (**B**). Methylation at the paternal ICR prevents CTCF dependent looping and provides downstream enhancers access to the *Igf2* promoter. Mesodermal and endodermal enhancers are represented by light and dark hash mars respectively. The matrix attachment region, MAR3, is represented by a closed oval. The looping model is based on chromosome conformation capture (3C) analysis of neonatal liver [62].

CHAPTER 2: OCT4 AND SOX2 REGULATE METHYLATION AT THE IGF2/H19 IMPRINT CONTROL REGION

INTRODUCTION

Genomic imprinting is an epigenetic phenomenon that results in unequal expression of genes based on their parent of origin. Maternal and paternal alleles are distinguished by differentially methylated regions (DMRs) which acquire unique gamete specific DNA methylation and chromatin modifications. Once established, these parental -specific imprints are maintained in somatic cell linages where they direct expression and repression of neighboring imprinted genes either directly through cis-regulatory elements or indirectly by driving the expression of anti-sense regulatory RNAs and/or mediating long range chromatin interaction between promoters and distal enhancers.

A DMR at the imprint control region (ICR) upstream of the *H19* gene on mouse chromosome 7 and in shared synteny on human chromosome 11P15 (IC1) coordinates the reciprocal expression of *H19* and *Igf2* by controlling access to shared enhancers. The maternal ICR is hypomethylated and functions as a CTCF dependent chromatin boundary that facilitates interaction of the H19 promoter with downstream enhancers through the formation of Cohesin dependent intra-chromosomal loops [62,88,95]. Hypermethylation of the paternal ICR represses *H19* expression and blocks CTCF binding which results in an alternative looping arrangement that facilitates interaction of the enhancers with the *Igf2* promoter. Deletion of the ICR in mice results in a loss of parental-specific partitioning of promoters and enhancers and biallelic expression of *H19* and *Igf2* [30]. In humans, inheritance of maternally methylated IC1 accounts for 10% of cases of Beckwith-Wiedemann Syndrome (BWS) – a fetal overgrowth syndrome that also includes an increased risk of childhood tumors[137]. Analysis of maternally methylated alleles from BWS cell lines detected a loss of CTCF-Cohesin binding at the ICR and chromatin architecture similar to the paternally imprinted allele.[89]

In addition to its insulator function, CTCF has been shown to protect the maternal ICR in somatic cells from *de novo* methylation during embryogenesis [31,106,123]. Loss of CTCF binding at one or more of the four binding sites in the mouse ICR results in methylation of the mutant maternal allele and biallelic expression of Igf2[29,93,106]. Mutations that allow CTCF to bind methylated paternal ICRs have also shown CTCF capable of facilitating demethylation of the paternal allele in mouse somatic tissue [28]. However, the mechanism of demethylation and the involvement of other cis-regulatory elements within the ICR are unknown. CTCF binding is not required for erasure of paternal imprints in mice primordial germ cells (PGCs) or protection of the ICR from de novo methyltransferases during postnatal methylation imprint establishment in oocytes[29,106,123].

A conserved pair of Sox-Oct motifs located between CTCF sites 2 and 3 in mice and in both A repeats of the human IC1 have been suggested as possible regulators of ICR methylation. N. Hori, et al. demonstrated that Oct4 binding sites were required for demethylation of partially methylated ICR transgenes in a mouse embryonic carcinoma cell line [42]. Analysis of genomic ChIP-seq data for Oct4 and Sox2 has also found the pluripotency factors enriched at the endogenous ICR in mouse embryonic stem (ES) cells implying that they may play a role in establishment and or maintenance of maternal imprints [78]. Furthermore, all reported IC1 micro-deletions and point mutations associated with abnormal maternal ICR methylation in BWS patients have included the loss or disruption of Oct- and/or Sox2-binding sites[23,25,97,116,117]. Genetic alterations that only affect CTCF sites at the IC1 have not been reported for BWS.

In this study we investigated the cooperative binding characteristics of Oct4 and Sox2 at the ICR and determined that cooperative binding is required at both Sox-Oct motifs for robust ICR transgene hypomethylation and octamer induced transcriptional activation in a murine embryonic carcinoma cell line. In contrast to Sox-Oct motifs, CTCF binding contributed little to ICR transgene demethylation. We also analyzed the effect of octamer mutations on establishment and maintenance of the maternal hypomethylation imprint in a knock-in mouse model. Oct motifs were required for

protection of the maternal ICR from de novo methylation in somatic tissue and oocytes but are not essential for imprint establishment or mono-allelic expression of *H19* and *Igf2*.

RESULTS

CTCF and octamer sites regulated ICR DNA methylation in F9 cells

The mouse *Igf2/H19* ICR has four CTCF sites and a closely spaced pair of octamers (Oct sites Intro) that lie about 200 bases from CTCF site 2 (Fig. 1 A). Results from mouse and cell culture experiments indicated that CTCF and octamer sites separately regulate CpG methylation of the ICR. Given the importance of the octamer pair, we searched for similar sequences in the ICR and found an additional consensus octamer site 450bp upstream of CTCF site 1. To determine whether these three sites cooperate in regulating ICR methylation, we mutated the elements and assessed the methylation status of stably transfected ICR-containing plasmids. The plasmids were stably integrated into F9 embryonic carcinoma cells, as these cells have demonstrated both de novo methylation and demethylation of transgenes. Using methylation sensitive Southern analysis, we found that integrated transgenes containing the wild type ICR and *H19* promoter did not acquire methylation, while mutation of both octamers resulted in a small amount of de novo ICR methylation (Fig. 2 A and B). In contrast to results seen in mice, mutation of the four CTCF sites did not lead to increased methylation of the ICR. The protective activity of CTCF was apparent only when both the CTCF and octamer sites were mutated, as the double mutant ICR showed less digestion by HhaI and HpaII than the octamer mutant alone.

In addition to protection from *de novo* methylation, binding by certain transcription factors also can drive demethylation of partially methylated sequences. We tested the demethylation activity of the CTCF and octamer sites using stably integrated plasmids that had been treated with HhaI and HpaII methyltransferases and found that both WT and CTCF mutant ICRs were substantially demethylated (Fig. 2 C), By contrast, ICR transgenes with mutant octamers retained most of their partial methylation.

Morever, ICR constructs with single mutations in either of the centrally located octamers retained methylation to the same degree as the double octamer mutant. Combining CTCF and octamer mutations resulted in a modest decrease in demethylation activity (Fig. 2 C). Transgenes containing a mutation in only this third octamer showed wild type levels of demethylation (Fig. 3 A).

Octamer mediated demethylation of the ICR was both specific to embryonic carcinoma cells and limited to ICR constructs with low density methylation. Partially methylated WT and mutant ICR transgenes stably integrated into either mouse C2C12 or 3T3 remained highly methylated (Fig. 3 B). Likewise, WT ICR transgenes fully methylated in vitro with SssI methyltransferase also remained highly methylated in F9 cells (Fig. 3 C).

Sox-Oct motifs regulated ICR methylation in F9 cells

Octamers often are found adjacent to Sox binding sites, forming a functional Sox-Oct motif. We compared the ICR octamers to known Sox-Oct motifs and found potential Sox sites flanking each octamer (Fig. 2 A). We tested the activity of these elements in the F9 demethylation assay using partially methylated ICR constructs containing separate mutations in the two putative Sox sites (Fig. 2 E). Southern analysis demonstrated a moderate reduction in ICR demethylation activity when the Sox site upstream of the promoter distal octamer was mutated. Mutation of the Sox site upstream of the proximal octamer exhibited a greater loss in demethylation activity, which was comparable to the single octamer mutations. We also identified a sequence 22bp downstream from the proximal octamer that bound Sox2 in vitro (Fig. 3 A). Mutation of this element, however, did not effect ICR demethylation alone nor in combination with mutation of the first Sox site (Fig. 2 E and Fig. 3 D).

Oct4 and Sox2 bound cooperatively to the ICR Sox-Oct motifs

F9 cells express Oct4 and Sox2, suggesting that they bind to the Oct-Sox motifs in the ICR and mediate demethylation. Consistent with this idea, EMSAs performed with F9 nuclear extract showed that both motifs formed heterodimeric complexes with Oct4

and Sox2 (Fig. 4 A). Moreover, the same octamer mutations that disrupted ICR demethylation also prevented Oct4 binding in vitro (Fig. 4 B). In addition, EMSAs with recombinant Oct4 and Sox2 demonstrated that mutations made to the putative Sox sites blocked Sox2 binding but did not prevent Oct 4 binding (Fig. 4 C). Because Sox-Oct mediated ICR demethylation in F9 cells was limited to partially methylated transgenes, we performed EMSAs with ICR probes methylated at CpGs and found that the methylation did not inhibit formation of Sox2 and Oct4 heterodimeric complexes (Fig. 3 C). [The lone Sox binding site identified downstream of the promoter proximal octamer also bound to endogenous and recombinant Sox2.] In vivo binding by Oct4 and Sox2 was suggested by chromatin immunoprecipitations (ChIP) performed on F9 cell lines that showed enrichment for Oct4 and Sox2 at both the endogenous and wild type transgenic ICRs, but not at the octamer mutant ICR (Fig. 4 E).

Oct4 and Sox2 often bind DNA cooperatively, and we determined if this occurred on the two ICR Sox-Oct elements (Fig. 4 D). In EMSAs with recombinant proteins, we found that Oct4 bound to both elements equally well, but Sox2 alone showed only modest binding to the distal element and almost no binding to proximal. However, in the presence of Oct4, Sox2-Oct4 complexes formed with the distal element that predominated over either single protein complex, consistent with a cooperative Sox2-Oct4 interaction. Similarly, the second (proximal) element also bound Sox2 in the presence of Oct4 as demonstrated by the formation of Sox2-Oct4 and the near absence of Sox2 complex regardless of the Sox2 concentration. Conserved Sox-Oct motifs in the Human ICR A2 repeat (HD1, Hori et al.) also demonstrated cooperative binding of recombinant Sox2 and Oct4 in EMSAs (Fig. 5 A).

Given the proximity of the two Sox-Oct sites to one another, we performed EMSAs with probes that included both sites to assess whether the dual motifs could simultaneously bind two Sox2-Oct4 complexes. We found that recombinant Oct4 and Sox2 were capable of forming two heterodimers on the same WT mouse probe. However, this heterotetramer represented a small fraction of observed complexes, and the exact binding site of Sox2 in the more abundant heterotrimer was unclear. The formation an

Oct4-Sox2-Oct4 specific heterotrimeric complex was observed using a Human ICR A2 probe with a mutant Sox binding site upstream of the octamer 1 homolog (Fig 5 B).

The ICR Sox-Oct motifs functioned as a transcriptional activator

In many cases, the demethylation activity of DNA sequences has been shown to correlate with their ability to activate transcription. To determine whether the ICR Sox-Oct motifs activate transcription, we placed ICR fragments containing the elements upstream of a luciferase gene driven by the H19 promoter (Fig. 6 A). In transient transfections of F9 cells, the Sox-Oct regions activated the reporters more than five-fold compared to the H19 promoter alone. Consistent with their ICR demethylation activity, mutation of one or both octamer motifs abolished transactivation and produced a modest repression. Mutations disrupting the cooperative Sox binding sites also abolished transcriptional activation, while the lone 3' Sox motif mutant showed a modest reduction in luciferase expression. To determine if Oct4 was required for transactivation, we transfected F9 cells that had been depleted of Oct4 by shRNAs and found that relative luciferase expression dropped at least 90% compared to control shRNA expressing cells (Fig. 6 B). As an alternative to Oct 4 knock-down, mouse 3T3 cells lacking endogenous Oct4 and Sox2 expression were also transiently transfected with ICR-reporter constructs and found to have luciferase levels equal to or less than that of H19 promoter controls (data not shown).

Octamers were required for maintenance of the unmethylated maternal ICR in somatic cells

To determine if the octamers participated in establishing or maintaining the ICR's differentially methylated state in vivo, we used homologous recombination and ES cells to create mice with mutations in both ICR octamers (Fig. 7 A and B). Southern analysis on DNA from neonatal liver and muscle (Fig. 7 C) showed that the loss of octamer binding had no effect on the methylation of paternally transmitted mutant ICR's. However, methylation of maternally transmitted mutant ICRs increased significantly, as indicated by reduced HpaII and HhaI digestion compared to wild-type maternal ICR DNA. The asymmetric distribution of methyl sensitive restriction sites in the probed

region also provided a means for assessing the relative location of methylated CpGs. Analysis of the banding patterns from 5' HpaII and 3' HhaI digests suggests that the majority of mutant allele methylation was concentrated near the octamers while the region including the promoter proximal CTCF sites was largely unmethylated. To more accurately characterize the increased methylation, we performed bisulfite sequencing of the region including CTCF site 2 and the octamers from neonatal liver. Previously published bisulfite analysis of maternal alleles from somatic tissue had indicated that CpGs located near the octamers were more likely to acquire methylation than upstream sequences including CTCF site 2 [130]. Consistent with those results we found that the 3 CpGs within 7bp of the WT maternal octamers were 41% methylated, despite a less than 15% total methylation for all CpGs assayed (Fig.8 A). In contrast, the maternally inherited mutant alleles were 78-93% methylated at all CpGs within 35bps of the octamers and more than 53% for all CpGs assayed. Two CpGs in particular, located 35bps upstream and 5bp downstream of the first octamer, had 78% and 72% respective increases in methylation. The three CpGs within CTCF site 2 showed a modest 28-35% methylation for octamer mutant alleles compared to maternal wild-type alleles (12-23%). A comparison of WT and mutant maternal alleles from neonatal muscle yielded similar results (Fig. 9). Additional bisulfite analysis of the ICR region proximal to the *H19* promoter from neonatal liver showed vertically no methylation of maternal mutant alleles - consistent with the Southern results (Fig. 8 A). Paternal methylation imprints were like wise unaffected by the octamer mutations (Fig. 8 A. and data not shown).

Octamer mutations increased methylation in oocytes but not blastocysts

Methylation of the mutant maternal ICR appeared stable and was relatively unchanged from methylation patterns present in somatic tissue from 12dpc embryos (Fig. 8 B). In order to determine if this methylation pattern was the result of stable inheritance from maternal gametes or occurred during embryogenesis, we conducted additional bisulfite analysis of maternally derived mutant oocytes and blastocyst (Fig.8 C and D). Half of the mutant oocytes sequenced exhibited methylation patterns similar to that of somatic tissue while the other half remained unmethylated. Surprisingly, we found the ICR of mutant blastocysts to be unmethylated despite the presence of methylation at the

ICR of some mutant oocytes indicating the maternal ICR methylation was not to stably inherited.

The octamers were not required for establishing an unmethylated ICR in the primordial germ line

We assessed mutant ICR methylation in germ cells during different stages of embryogenesis in order to determine if the mutant ICR methylation in oocytes resulted from a failure to erase paternal methylation imprints and/or inadequate protection of the ICR from de novo methylation during normal gamete imprint establishment. Primordial germ cells (PGCs) were isolated from 14, 16 and 18dpc ovaries and testes by crossing ICR mutants with a Pou5f1-EGFP strain and FACS sorting the respective dissociated tissues. [94-99% of GFP+ FACS isolated cells tested also stained positive for alkaline phosphatase (data not shown)]. Bisulfite analysis of PGCs from 14.5 ovary and testes showed normal hypomethylation of maternally inherited mutant ICRs (Fig. 10). The ICR octamers were not required for erasure of paternal methylation in female PGCs as evidenced by the absence of methylation on paternally derived mutant alleles from16dpc ovaries. Nor were they required for preventing de novo methylation of the female germ line up to and including 18dpc development. In testes, mutant alleles acquired paternal specific methylation imprints irrespective of parental origin (Fig. 10).

The ICR octamers had little influence on mono-allelic expression of H19 and Igf2

We performed a single nucleotide primer extension assay (SNuPE) in order to determine if the methylation observed on the maternal mutant ICR was sufficient to alter the normal allelic expression ratios of *H19* and *Igf2* in liver, muscle, tongue, kidney, and brain from neonatal wild type and heterozygous mutant mice. Despite the methylation of mutant ICRs in liver and muscle, no significant difference in the ratio of maternal/paternal *H19* allelic expression was observed for the maternally transmitted mutant alleles (Fig. 11 A). The ratio of paternal/maternal *Igf2* expression was similarly unchanged in all tissues assayed except for kidney where we observed a 2.5 fold decrease ($p=0.014$) (Fig. 8 B). Consistent with normal repression of the maternal *Igf2*, we

observed no difference in size between neonatal mice that inherited the mutant maternal allele and their WT litter mates (data not shown).

DISCUSSION

Investigations of the mechanics responsible for mono-allelic expression of *H19/IGF2* have predominantly focused on methyl sensitive binding of CTCF to the ICR and its effect on chromatin architecture. However, the characterization of CTCF as a ubiquitous enhancer blocker responsible for modulating chromatin boundaries at hundreds if not thousands of diverse loci, means that its binding is unlikely to be sufficient for establishing and maintaining the unique differential methylation pattern that is a functional requirement of the ICR. Attempts to identify sequence outside of the CTCF sites capable of influencing ICR methylation *in vivo* have been largely unsuccessful. In this study we demonstrated that conserved sox-oct motifs within the ICR were more influential than CTCF sites in insuring the hypomethylation of transgenes in F9 cells. Moreover, the extent of ICR methylation maintained by octamer mutants indicated a low degree of functional redundancy between the octamers and CTCF sites with respect to sequence-directed demethylation. We also showed that robust octamer-dependent ICR demethylation required two sox-oct motifs capable of cooperatively binding Sox2 and Oct4. This unique double sox-oct motif formed a single functional unit capable of driving demethylation or transcriptional activation in cells expressing Oct4 and Sox2. Oct1, which was ubiquitously expressed in all cells assayed, was insufficient to induce ICR transgene demethylation in non-embryonic carcinoma cells or octamer dependent transcriptional activation in F9 cells following knock-down of Oct4 by shRNA.

These findings in conjunction with the expression profile of Oct4 and Sox2 during early embryogenesis and germ cell maturation suggested the octamer sequences could play an essential role in establishing and maintaining the maternal ICR hypomethylation imprint *in vivo*. To test this hypothesis, we created a mouse with mutations at the three know octamer sites associated with the ICR and observed the effect of these changes on

ICR methylation and allelic expression profiles of *Igf2* and *H19*. In somatic tissue we found the region centered at the mutant octamer pair to be hypermethylated on the maternally transmitted alleles. This methylation extended to CTCF site 2 on 33% of sequenced mutant alleles but did not extend as far as CTCF site 4. In contrast, maternal transmission of an ICR with mutant CTCF sites resulted in methylation of nearly 64% of CpGs at CTCF site 2 in addition to significant methylation of the 3' region of the ICR including CTCF site 4 [106]. Ectopic methylation for both octamer and CTCF mutants was absent from maternal alleles during the blastocyst stage indicating that both motifs are required, but insufficient, to prevent *de novo* methylation of the maternal ICR during post-implantation associated cellular differentiation. Lack of methylation at regions of the ICR distal to the mutant octamers could be the result of CTCF forming boundaries that block the spread of methylation and/or actively facilitating a euchromatic architecture either through direct interaction with chromatin remodelers such as CHD8 or indirectly by mediating interaction with distal enhancers [46,57]. The demethylation of mutant paternal ICRs that exhibit methylation independent binding of CTCF during pre- and post-implantation embryogenesis supports a more active role for CTCF in regulating ICR methylation [28].

With the exception of WT ICR methylation in oocytes depleted of CTCF [31], CTCF binding has proven unnecessary for both erasure of paternal methylation imprints and protection of the ICR from methylation in female germ cells [106,123] However, Lee and Sing et al. have implicated CTCF binding at the maternal ICR to delayed methylation imprint establishment in male PGCs [65]. Here we showed that octamers are also nonessential for germ cell erasure of imprints but are necessary for preventing *de novo* methylation of the ICR during post-natal oocyte maturation. Although mutant ICR methylation in oocytes did not represent a stable imprint, its acquisition coincided with increased Dnmt3L methyltransferase expression and normal maternal methylation imprint establishment [14]. In addition, the absence of a significant difference in ICR methylation between maternally inherited mutant alleles and WT paternal alleles in 16dpc testicular PGCs, suggests the ability of octamers to prevent methylation may also contribute to a delayed paternal imprint establishment.

Protecting maternal hypomethylation imprints may be a common function of Oct4 and Sox2 at imprinted loci. Similar to the mutant H19/Igf2 ICR, the normally hypomethylated human Angelman Syndrome – imprinting center (AS-IC) acquires methylation in mouse oocytes when a mutation is made to one of two Oct binding sites or an independent Sox site [55]. At both loci oocyte methylation occurs on only a fraction of mutant alleles and is lost during fertilization. Although the individual Oct and Sox sites are distributed over a 370bp region of AS-IC and separated by two additional trans-factor binding sites, the observation that all three sites are required for *in vivo* regulation of methylation is consistent with our *in vitro* functional analysis of the two ICR Sox-Oct motifs.

Methylation of the mutant maternal ICR may depend on an additional *cis*-regulatory element located within 100-200bps of the mutant octamer pair. Surprisingly, mice possessing a ~0.9kb deletion of the ICR between CTCF sites 2 and 3, which includes the octamer pair, showed increased expression of H19 from the paternally inherited mutant allele but no acquisition of methylation at maternally inherited mutant ICR [43]. This discrepancy in maternal ICR methylation between mice with octamer point mutations verses an octamer inclusive deletion could be explained by the existence of an as yet unidentified repressive element located between the octamer and neighboring CTCF site(s). Therefore, the function of WT octamers would be, at least in part, the inhibition of putative repressor(s) at the maternal ICR. Consistent with this possibility, we observed that luciferase reporters containing an octamer mutation in the context of a 352bp ICR fragment averaged a near 2 fold reduction in luciferase expression compared to the H19 promoter alone (Fig. 6 A, standard dev. = 0.5).

Maternal transmission of either a single point mutation or a deletion effecting one of the octamers in repeat A2 of the human ICR have been implicated in five different cases of Beckwith-Wiedemann Syndrome (BWS)[25,97]. The mouse model presented here provides the first direct evidence that maternal transmission of octamer mutations alone can result in loss of differential methylation at the ICR. In contrast to BWS cases, we observed that mutant octamer induced methylation was mostly absent from the

CTCF-sites of maternally inherited alleles and consequently imprinted expression of H19 and Igf2 was unaffected. These results suggest that the extent to which conserved octamers control ICR methylation may depend on non-conserved aspects of ICR architecture and/ or species specific developmental modalities. The existence of unique species requirements for maintenance of differential methylation at the H19 and Igf2 locus was previously reflected in the inability of human ICR transgenes to maintain methylation acquired in the male germ line throughout mouse embryogenesis [49]. Further studies are needed to determine whether conserved octamers regulate mouse and human ICR methylation through a common mechanism including the potential modulation of neighboring repressive elements.

METHODS

Plasmid construction

Octamer and Sox site mutations were inserted into the WT and CTCF mutant ICR using fragments created by overlapping PCR with mutant primers. Substitutions at octamers 2 and 3 (-3248 and -4338 with respect to the H19 start site) created novel *BspH*I sites. The 1.5kb mutant PCR products were ligated into the *Xho*I and *BspE*I sites of a pcDNA3-luciferase vector containing the mouse H19 upstream region (from -5547 to +695) with either WT or mutant CTCF sites. WT and mutant Sox constructs were prepared by replacing the octamer region from -3305 to -3209 in the same pcDNA vector containing the mouse H19 upstream region with a unique *Spe*I site via overlapping PCR. WT and mutant 83bp sequences corresponding to the deleted region and possessing 5'-CTAG overhangs were constructed by ligating three double-strand oligonucleotides containing the appropriate mutations were then ligated to a *Spe*I linearized vector. (See EMSA probes in Table 1 for WT and mutant sequences.)

Luciferase reporter constructs were prepared by inserting either a 352bp WT or mutant octamer PCR product (-3396 to -3044) or the 83bp WT or mutant Sox sequence into the *Nhe*I site of the pH19-Luc vector [57]. All PCR-derived constructs were sequenced to ensure fidelity.

Oct4 and Sox2 recombinant protein expression vectors were prepared by cloning *BamH*I – *Sal*I fragments containing the respective cDNAs from pMXS-Oct3/4 and pMXS-Sox2 [Addgene plasmids 13366 and 13367[124]] into the *BamH*I site of pET-15b (Novagen).

We prepared the mutant octamer knock-in vector by ligating the mutant ICR contained within an *Xba*I fragment from -5.5 to -0.8kb between the *lox*511 and *lox*P sites of a previously described targeting vector[106].

Cell culture, transfection, luciferase assays, and Oct4 knock-down

F9, 3T3, and C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. F9 cells were cultured on 0.1% gelatin-coated dishes. For ICR methylation analysis, partially methylated constructs were treatment with *Hha*I and *Hpa*II methyltransferases. The fully methylated WT ICR construct was *in vitro* methylated with SssI methyl transferase. Complete methylation was verified by HhaI and *Hpa*II restriction digest followed by agarose gel electrophoresis and ethidium bromide staining. Only DNA showing no digestion was used in transfections. Cells were co-transfected with test constructs and either a pcDNA3.1 or pBS-puromycin selection plasmid at a ratio of 5:1 using either FuGENE 6 (Roche) or MirusTransIT-LT1 at a lipofectant to plasmid ratio of 3:1. Cells underwent 10 days of selection with either G418 or puromycin. Colonies were passaged onto 60mm plates and harvested when confluent.

Transient transfections of F9 cells were performed in six well plates using 3 μL MirusTransIT-LT1 and 0.5 μg each of test construct and pSV-β-gal (Promega). After 48 hours, cells were harvested in Reporter Lysis Buffer (Promega), and β-Galactosidase and luciferase activity were assayed as previously described[17]. Relative luciferase expression was calculated after normalizing for β-galactosidase activity. Student's t-test was used to determine statistical significance of at least three independent transfections.

Knock-down of Oct4 in F9 cells was achieved using lentiviral particles derived from Sigma-Aldrich MISSION plasmids pLKO.1, (S1= Clone ID: NM_013633.1-659s1c1, and S3= Clone ID: NM_013633.1-544s1c1). A pLKO.1 vector with a non-targeting shRNA (SCR= Addgene plasmid 1864[105]) served as a negative control. Lentivirus was produced in 293T cells and used to infect F9 cells on a 6 well plate according to the Addgene pLKO.1 Protocol version 1.0 (December 2006). F9 cells underwent selection with puromycin from 25-48 hrs post-infection and then were passaged onto a 6 well plate. At 72 hrs post-infection, cells were transfected with luciferase vectors and pSV-β-gal and then collected for analysis after 48 hrs. Oct4 knock-down was verified by Western blotting extracts from cells infected in parallel and collected 5 days post-infection using rabbit α-Oct4 [Santa Cruz sc-9081 (1:500 dilution)] and mouse α-nucleophosmin (NPM) [LabVision ms-1849 (1:10,000)]. HRP-conjugated human α-rabbit IgG [Sigma-Aldrich A0545 (1:5,000)] and goat α-mouse IgG [Jackson ImmunoResearch 63656 (1:8,000)] secondary antibodies. Blots were visualized using Peirce SuperSignal West Pico Chemiluminescent Substrate.

Southern Blot

To assess ICR methylation, genomic DNA was purified as previously described and digested with *Bgl*II followed by *Hha*I or *Hpa*II methyl-sensitive restriction enzymes [106]. *Msp*I, an isoschizomer of *Hpa*II not sensitive to methyl-cytosine, was used as a positive control for complete digestion. Digested DNA was separated by agarose gel electrophoresis followed by blotting to Hybond-XL (Amersham Biosciences). Probes were radiolabeled with the Takara Random Primer DNA Labeling Kit and hybridized to membranes in either Rapid-Hybe Buffer (GE Healthcare) supplemented with 25 μg/ml salmon sperm (SS)DNA at 65 °C for 2hrs or 3% Poly(acrylic acid)pH8.0, 6x SSPE, 1% SDS, 0.1% BSA, 25ug/ml SS DNA at 65 °C overnight. After hybridization membranes were washed in 2x SSC, 0.1% SDS at room temperature for 2 min, twice in 1x SSC, 0.1% SDS at 65 °C for 15 min, and once in 0.1x SSC, 0.1% SDS at 65 °C for 30 min.

Electrophoretic mobility shift assays

F9 nuclear extract (NE) was prepared as previously described [107]. Recombinant Oct4 and Sox 2 were purified from IPTG induced Rosetta (DE3) pLys competent cells (Novagen) transformed with pET-15b-Oct4 or pET-15b-Sox2 expression vectors and cultured in LB supplemented with ampicillin and chloramphenicol. After lysis with BugBuster Protein Extraction Reagent (Novagen), Oct4 and Sox2 were purified from inclusion bodies by preparative SDS-polyacrylamide gel electrophoresis. Isolation and renaturation of the gel-isolated proteins were performed according to Hager and Burgess [37].

Oligonucleotide probes and competitors used in EMSA are listed in the Supplementary material, Table S1. For each reaction 25 fmoles of labeled double-stranded probe was incubated for 10 min at room temperature with either F9 NE or recombinant Oct4 and/or Sox2 proteins in a 16 μl reaction buffer containing 10mM Tris pH8.0, 2 mM MgCl, 100mM KCl, 1.25 mM DTT, 5% glycerol, 0.0625% BSA, and 19 μg/ml dGdC. Supershifts included an additional 10 min RT incubation with 1 μg of anti-Oct4 (Santa Cruz sc-9081), anti-Sox2 (Santa Cruz sc-20088), or IgG (Santa Cruz sc-2028) as a negative control. Reactions were separated on a 4% 0.25X TBE polyacrylamide gel at room temperature, which was dried before exposing to film.

Chromatin immunoprecipitations (ChIP)

Chromatin from F9 and F9 mutant ICR stable transfectants was prepared and immunoprecipitated using the ChIP-IT Express kit (Active Motif). Chromatin was cross-linked at room temperature with either 1% formaldehyde for 10 min or 2 mM disuccinimidyl glutarate (DSG) 45min followed by formaldehyde for 10 min (Sox2 endogenous ICR precipitations only) and sonicated using a Vibra Cell (VC 375) ultrasonic processor. Each ChIP reaction contained 25 μg of sheared chromatin precipitated with 6ug anti-Oct4 (Santa Cruz sc-9081), 6 μg anti-Sox2 (3 μg each of Santa Cruz sc-20088 and sc-17320) or 6 μg IgG (Santa Cruz sc-2028 and sc-2027) as a negative control. Precipitated DNA was analyzed using a two-step qRT-PCR reaction in SsoFast EvaGreen Supermix (Bio Rad) on a Bio Rad iCycler. The octamer region of the

ICR was amplified using the following primer set: 5'-ATGCAGACCCCACTAAGCAT-3' AND 5'-CGGAGATCATTAGCATCTGA-3'.

Generation of transgenic mice

ES cell lines and chimeric mice were created by the University of Illinois Transgenic Mouse Facility. CJ7 cells were electroporated with *Sal*I linearized knock-in vector and colonies were selected for G418-resistance. ES clones demonstrating proper targeting by Southern analysis were injected into C57BL/6J (B6) derived blastocysts. Genotyping and confirmation of Cre-mediated deletion of the neo cassette were performed by Southern analysis and PCR of tail DNA (MOPrimer, 5'-ATCATTGGGGCGTTCAGATAATC-3' AND OCR3, 5'-GACAGTGCAAAA-CAGGTGAA-3'). All animal experiments in this study followed protocols approved by the Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

Germ cell isolation

Embryos for PGC isolation were obtained from matings between mutant ICR mice and B6;CBA-Tg (Pou5f1-EGFP) [122]. Embryos were sexed and staged according to morphology [126]. Embryonic gonads were separated from the mesonephros, digested with 150 μl of trypsin-EDTA [0.2% trypsin, 1 mM EDTA, 1× phosphate-buffered saline (PBS)] for 15min at 37°C, washed with DMEM supplemented with 10% fetal bovine serum and manually triturated to a single cell suspension using a 1 ml syringe and 30G needle. GFP positive cells were isolated by flow cytometry on a BD Biosciences LSR II. PGC purity was verified by assaying for alkaline phosphatase activity using the Vector Blue Phosphatase Substrate Kit III (Vector Laboratories).

Bisulfite sequencing

Genomic DNA from PGCs, oocytes, and blasts was prepared and bisulfite treated using the EZ DNA Methylation-Direct Kit (Zymo Research) according to the manufacturer's instructions. Genomic DNA from neonatal liver and muscle was prepared separately and added directly to the conversion reagent [106]. Primers (Bi3000F and Bi3455R) and PCR conditions for amplification of CTCF site 4 were as previously

described [106]. Amplification of CTCF site 2 and the octamer region were performed using the following primer set: Bi1825F, 5'-TTGTAAA-GAATTTTTTGTGTGTAAAG-3' and Bi2310R, 5'-ATACAATTTCAAAATTATTTACAA-CCC-3' and 35-40 cycles of PCR under the following conditions: 95 °C, 30 s; 52 °C 40 s; 72 °C, 100 s. For oocytes a secondary PCR with 1-5 μl of the first reaction was performed for 35 cycles with the same parameters. PCR products were gel-purified and cloned following the manufacture's protocol (pGEM-T, Promega).

RNA isolation and SNuPE analysis

Tissue was dissected from WT and mutant 4 day old pups obtained from mating female mice heterozygous for the mutant ICR with males that were homozygous for the *M. casteneus* Igf2/H19 locus. RNA was purified using the RNeasy Mini Kit (Qiagen), and all samples were subjected to on-column DNase digestion (RNase-Free DNase Set, Qiagen). Tissue was homogenized for 3 min in RLT buffer using 0.1mm Zirconia/Silica beads and a Mini-BeadBeater (Biospecs Products). Igf2 and H19 transcripts were amplified by using the SuperScript First Strand Synthesis System (Invitrogen), the following primer sets: Igf2-168F/U, 5'- ACGTGTCTACCTCTCAGGCCGTACT-3' and Igf2-166R, 5'-GGGTTGTTTAGAGCCAA-TCAA-3' [121]; H19-172F, 5'-GCACTAA-GTCGATTGCACTGG-3'and H19-172R, 5'-GCCTCAAGCACACGGCCACA-3' [118], and 35 cycles of PCR under the following conditions: (Igf2) 95 °C, 20 s; 53 °C 20 s; 72 °C, 30 s; and (H19) 95 °C, 20 s; 56 °C 20 s; 72 °C, 20 s. Control reactions lacking RT did not yield a PCR product. SNuPE was performed as previously described [112] using the following primers: Igf2-169R/S, 5'-TCAAATTTGGTTT-TTTAGAA-3' [121]; H19-173R/S, 5'-GGCAGCATTGCCAAAGAGG-3'[118]. Quantification of SNuPE reactions following electrophoresis was performed using a Storm 860 Phosphoimager and ImageQuant 5.2 software.

FIGURES AND TABLES

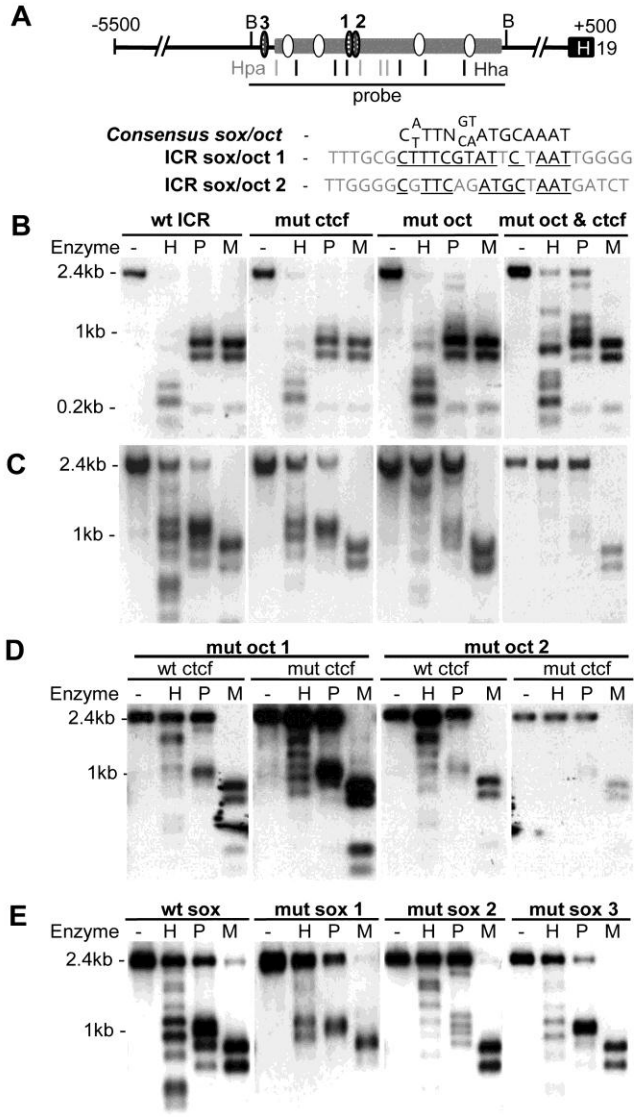

Figure 2. Analysis of ICR transgene methylation in F9 cells. (**A**) Structural map of ICR construct depicting CTCF (white oval), octamers (shaded oval), restriction enzymes *Bgl*II (B), *Hha*I, (dark grey bar), *Hpa*II (light grey bar). Octamers 1 and 2, (ICR sox/oct 1 and 2 respectively), are compared to the consensus sox2-oct4 motif. Southern-blots are shown for stably incorporated WT (wt) or mutant ICR constructs which were either *in vitro* unmodified (**B**) or *in vitro* partially methylated with *Hha*I and *Hpa*II methyltransferases prior to transfection(**C-E**). Mutations to the ICR transgenes include: disruption of the 4 CTCF sites (mut ctcf), the three octamer sites (mut oct), both octamer and CTCF sites (mut oct & ctcf); either the first or second octamer site (mut oct 1 and mut oct 2, respectively) alone (wt ctcf) or in combination with CTCF mutations; and the replacement of an 83 bp region including the octamer pair with a either a modified WT sequence (wt sox), or mutations to the 5' flanking sequence of the first octamer (mut sox 1), the sequence between the octamer pair (mut sox 2), or the 3' flanking sequence of the second octamer. All genomic DNA was digested with *Bgl*II alone or in combination with *Hha*I (H), *Hpa*II (P), or *Msp*I (M) as a positive control for *Hpa*II. For Southern hybridization, DNA was probed with the 2.4kb *Bgl*II fragment.

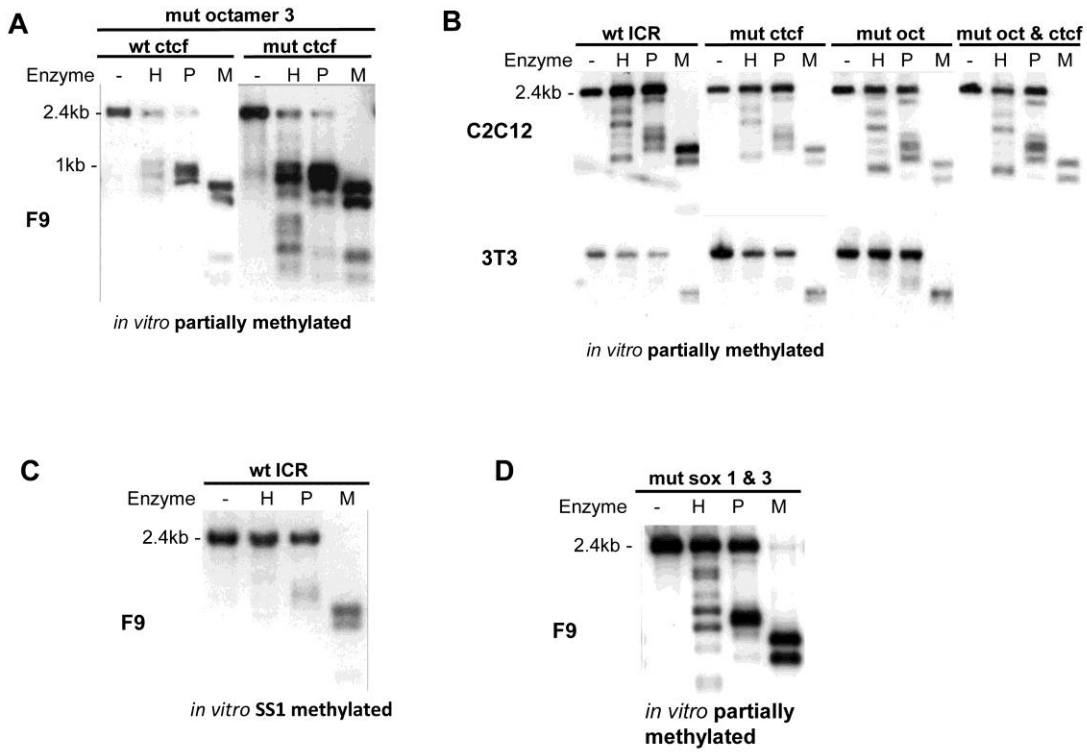

Figure 3. Analysis of WT and mutant ICR transgene demethylation in F9, C2C12, and 3T3 mouse cell lines via methylation sensitive Southern-blot. ICR constructs as described in figure 1 or with mutations to octamer 3 or sox sites 1 and 3 were either *in vitro* partially methylated and stably incorporated into F9 cells **(A and D)**, C2C12 cells, or 3T3 cells **(B)** or fully methylated and stably incorporated into F9 cells **(C)**.

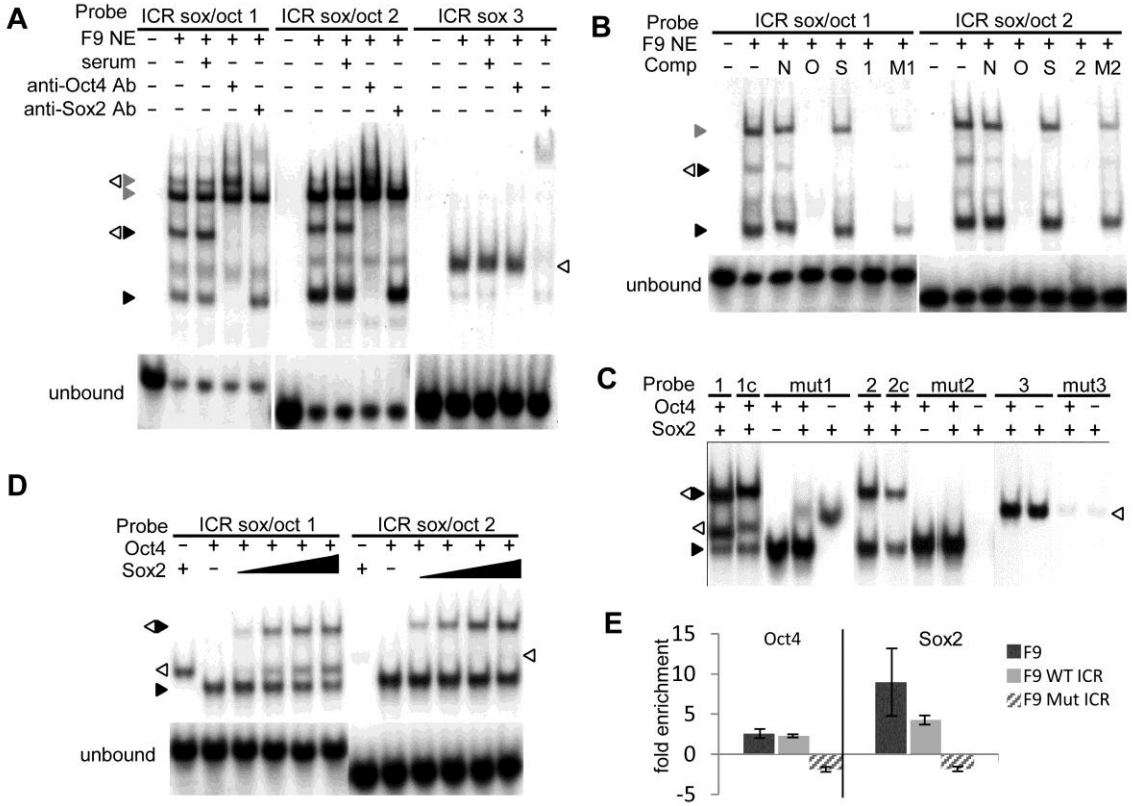

Figure 4. Sox2 and Oct4 binding at the ICR. EMSAs were performed using labeled WT or mutant ICR sox-oct motifs (1 and 2) or a 3' sox site down-stream of the second octamer (3). Oct 4 and Sox 2 specific binding was assessed using F9 NE (**A,B**) or recombinant Oct4 and Sox2 (**C,D**). (**A**) Sox2 and Oct4 protein-DNA complexes (white and black triangles respectively) were identified by the addition of antibodies (Ab). Oct 1 complexes are indicated by a grey triangle. (**B**)The effect of octamer specific point mutations on Oct4 and Sox2 binding was assessed by addition of unlabeled nonspecific (N), Oct 4 specific (O), Sox2 specific (S), wild type ICR sox-oct 1 and 2, or ICR octamer 1 and 2 mutant (M1 and M2) competitors. (**C**) ICR probes with mutations at the respective upstream and downstream octamer flanking sequences (mut1, mut2, and mut3) or CpG methylation (1c and 2c) were used to validate disruption of Sox 2 binding. (**D**) Cooperative binding characteristics of Oct 4 and Sox 2 at the ICR were assessed by comparison of Sox2 binary and Oct4-Sox2 ternary protein-DNA complexes formed using a fixed amount of Oct 4 and increasing amounts of Sox2. See supplemental table I for a complete list of EMSA oligonucleotides. (**E**) Enrichment of endogenous F9 or wt and mut transgenic ICR octamers by Oct 4 and Sox2 ChIP was determined by QRT-PCR and normalized to serum controls. Statistical significance of enrichments for Oct 4 and Sox 2 at the endogenous locus according to the t.test (n=3) are p=0.05 and p=0.047 respectively. Error bars represent standard deviation of the mean.

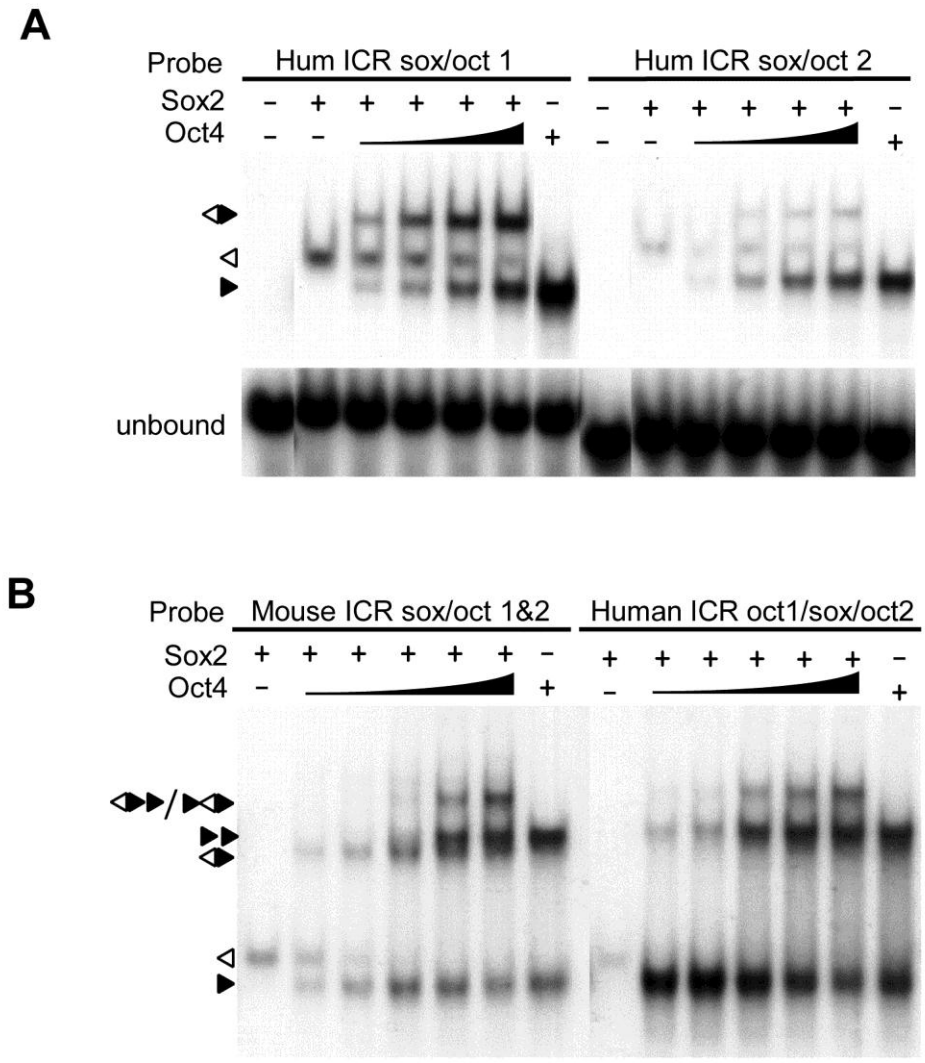

Figure 5. EMSAs with WT octamer probes for the human and mouse ICR conducted with recombinant Oct4 and Sox2. (A) Probes containing conserved sox/oct motifs from the Human ICR A2 repeat were incubated with Sox2 and increasing amounts of Oct4. **(B)** Probes containing the complete mouse ICR double sox/oct motif or the first octamer and the second sox/oct motif of the Human A2 repeat were incubated with Sox2 and increasing amounts of Oct4. Probe sequences are located in supplemental table I.

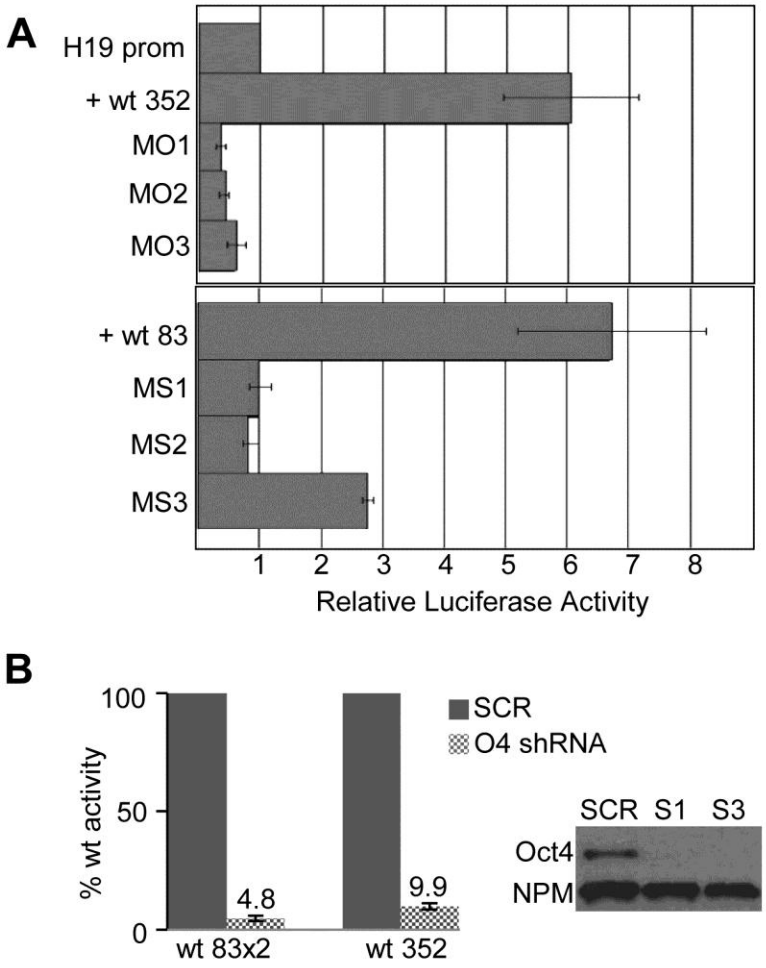

Figure 6. ICR sox-oct motifs function as Oct-4 and Sox-2 dependent transcriptional activators. F9 cells were co-transfected with a β-gal. reporter and constructs containing a luciferase report driven by the H19 promoter alone or in combination with either a 352bp or an 83bp upstream ICR fragment. (**A**) Transcriptional activation is shown as the total luciferase activity normalized to b-gal and H19 promoter luciferase expression. Mutations disrupting Oct 4 binding to either octamer 1 (MO1), octamer 2(MO2) or both octamers (MO3) were part of a 352bp ICR fragment. Mutations disrupting Sox 2 binding upstream of octamer 1 (MS1), upstream and downstream of octamer 2 (MS2 and MS3 respectively) were part of an 83bp ICR fragment. (**B**) The effect of Oct 4 shRNA knock-down on relative luciferase activity is shown for constructs containing either two copies of the wt 83bp ICR fragment (wt 83x2) or the wt 352bp fragment. Average relative luciferase activities for F9 cells infected with the shRNA negative control (SCR) and transiently transfected with wt 83x2 or wt 352 were 39.7 and 6.9 (standard dev. = 10.3 and 3.1) respectively. 'O4 shRNA' includes both S1 and S3 shRNA sample populations. Western blot with F9 CE from Oct4 #1, Oct4 #3, or SCR treated cells probed with α-Oct-4 and α-NPM antibodies.

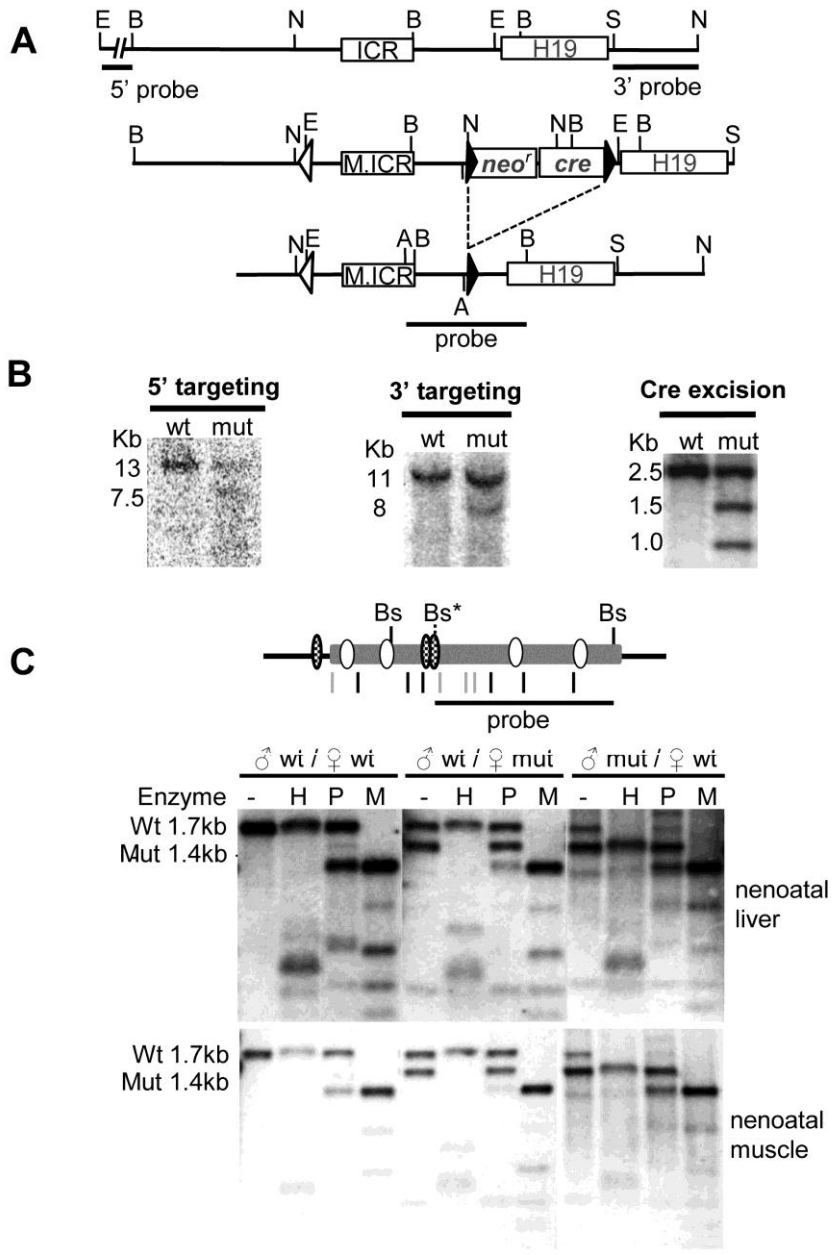

Figure 7. Targeting of the three octamer mutations in ES cells and methyl sensitive Southern analysis of somatic tissue from knock-in mice. **(A)** In top-down order are the H19/Igf2 locus, the targeting vector, and the targeted locus. The neomycin resistance (*neo*^{r}) and Cre recombinase (*cre*) genes were removed in the germ line of male chimeras by testes-specific expression of Cre. Restriction sites are *Ase*I (A), *BamH*I (B), *Ecor*V (E), *Nde*I (N), and *Sal*I (S). **(B)** Southern-blot analysis to confirm targeting of the mutant ICR (M.ICR) was carried out on *Ecor*V –digested WT and targeted ES cell DNA hybridized to a probe that lies 5' of the targeting arm or on *Nde*I-digested DNA hybridized to a probe 3' of the targeting vector. Correct Cre-mediated excision of the selection cassette was confirmed by *BamH*I and *Ase*I-digested tail DNA from F1 mice. **(C)** Southern analysis of maternally and paternally inherited WT and mutant ICR neonatal liver and muscle. DNA were digested with *Bsph*I (Bs) and then *Hha*I (H), *Hpa*II (P), or *Msp*I (M). The mutation in the promoter proximal octamer creates an additional *Bsph*I (Bs*) restriction site. The 1.4kb mutant ICR fragment was used for the probe.

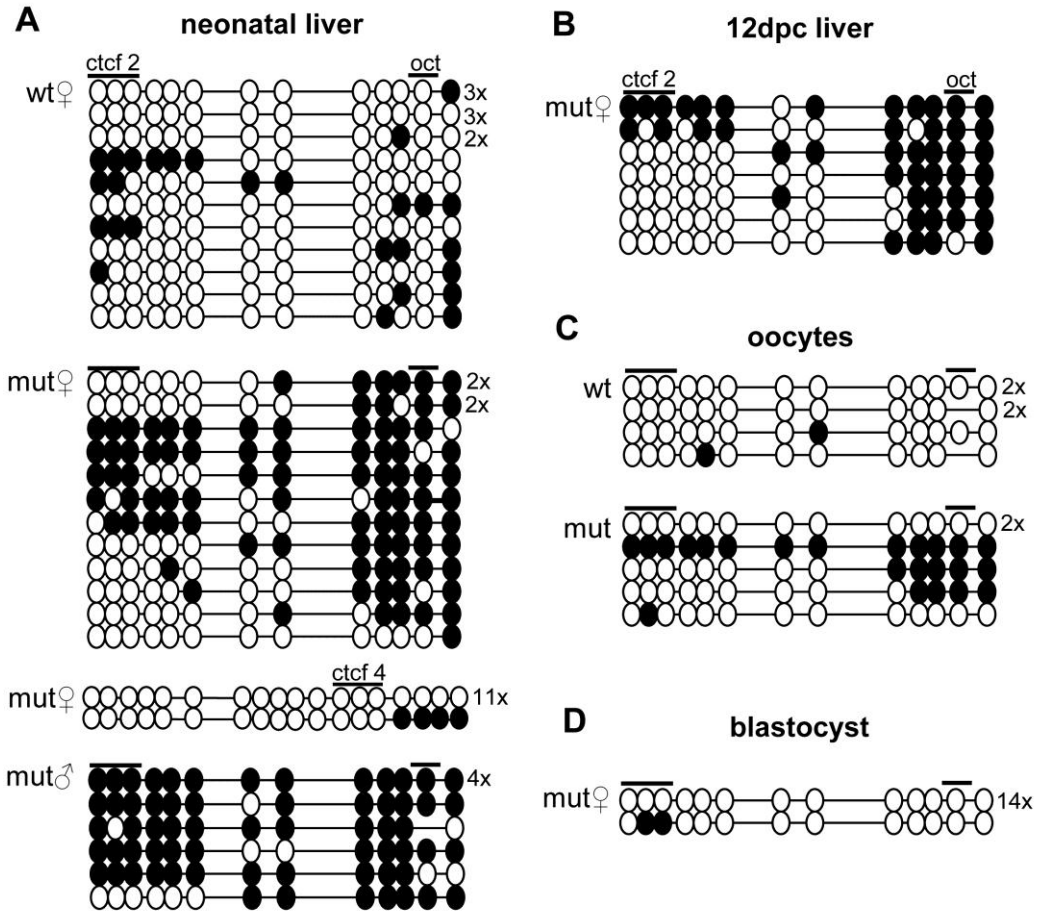

Figure 8. **Bisulfite sequencing of WT and mutant ICRs**. Each horizontal line represents an independent sequence. Unmethylated and methylated CpGs are presented as open and closed circles, respectively. Multiple occurrences of the same pattern are indicated to the right of each sequence. Lines above the circles indicate relative location of CTCF and Oct sites. The mutant status and the allelic parent of origin are indicated to the upper left of each block of sequences. PCR products were obtained from **(A)** the neonatal liver DNA of heterozygous 4 day old mice with maternally WT, maternally mutant, or paternally mutant transmitted ICRs and **(B)** maternally transmitted mutant alleles from 12dpc embryonic liver. **(C)** WT and mutant bisulfite products from oocytes were the result of separate PCR reactions each using the DNA from 10-20 oocytes. Each reaction product was cloned separately, and 3-6 clones of each product were sequenced. Identical sequences from each reaction were counted only once. **(D)** For blastocysts, two separate PCR reactions were performed each using the DNA from 6-10 bastocysts. Each product was cloned separately and considered to be unique.

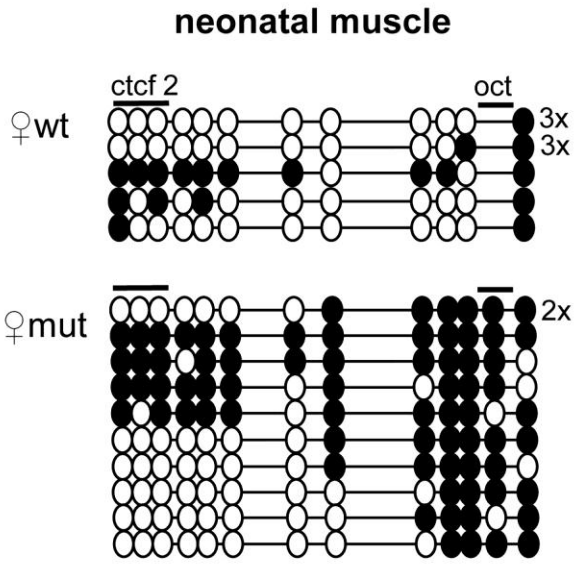

Figure 9. Bisulfite analysis of WT and mutant ICRs from transgenic mice. **(A)**Bisulfite sequencing of muscle from 4 dpn pups generated from the cross of a female with a mutant ICR and a father with a WT *castaneous* ICR **(B)** Bisulfite sequences for PGCs from 18 dpc testes with paternally inherited mutant allele and a maternally inherited WT allele.

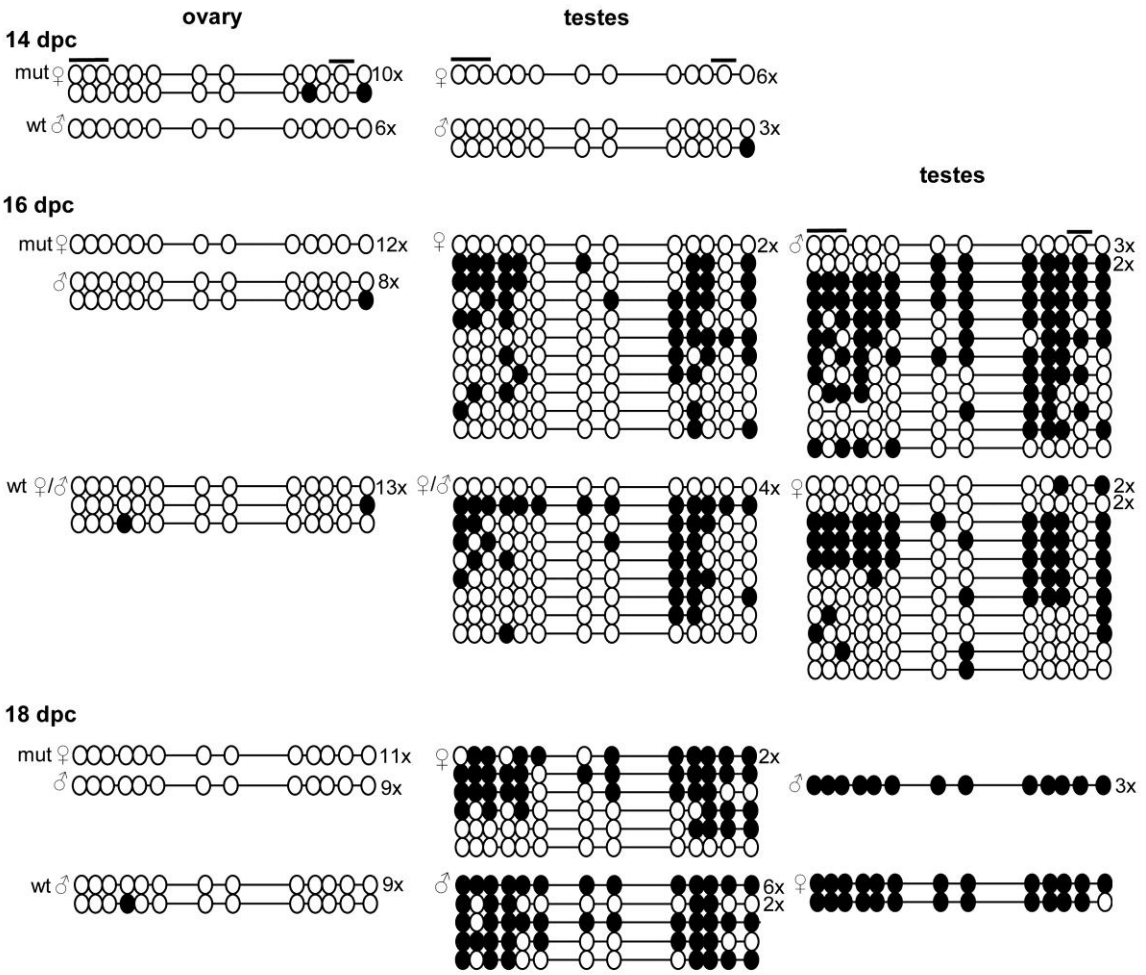

Figure 10. **Methylation profile of WT and mutant ICRs from female and male germ cells**. Bisulfite sequencing of the CTCF 2 and octamer region are shown for germ cells isolated from 14, 16, and 18dpc ovaries and testes. Each block of bisulfite sequences was the result of 1-2 separate PCR reactions performed with DNA from $1x10^3$-$1x10^4$ pooled germ cells. All products were cloned separately and considered to be unique. See figure 5 for other details.

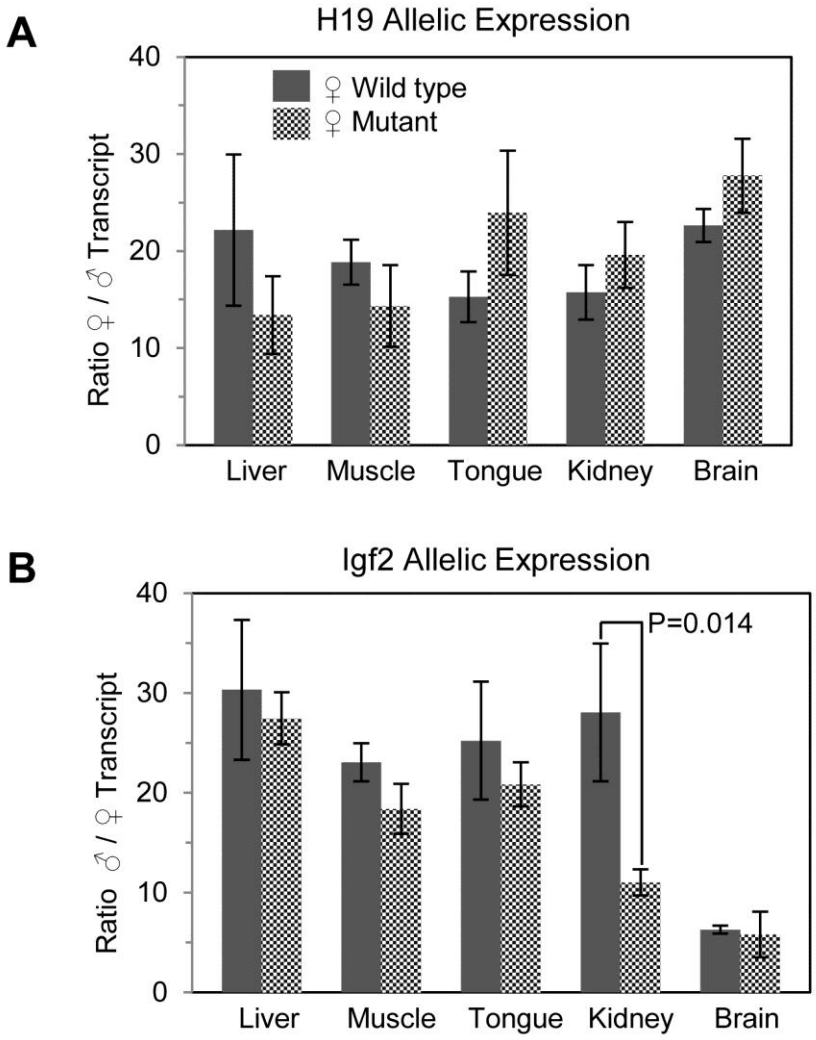

Figure 11. **Allelic H19 and Igf2 mRNA expression ratios in neonatal mice.** SNuPE analysis was carried out on **(A)** H19 and **(B)** Igf2 cDNA from 4dpn heterozygous mice having a paternally inherited WT *castanious* allele and a maternally inherited WT or mutant B6 allele. The ratio of transcript for each tissue was determined by averaging the individual allelic ratios for 3-5 different mice. Error bars represent the standard deviation of the mean. The p value for the kidney Igf2 transcript ratio was generated using the t.test (n=8).

NAME	Sequence
Mouse Ref.	*(2223) 5'-CTGGTGATTTGCGCTTTCGTATTCTAATTGGGGCGTTCAGATGCTAATGATCTCCGGCTCAGGGCTGCAAACAATTCTGAAACTGCATTCTC-3' (2314)*
Mouse ICR sox/oct1&2	TTTCGTATTCTAATTGGGGCGTTCAGATGCTAATGATCTC
ICR sox/oct 1	CTGGTGATTTGCGCTTTCGTATTCTAATTGGGGAC
(mut oct) **M1**	CTGGTGATTTGCGCTTTCGTATAATCATTGGGGAC
(mut sox) **mut1**	CTAAGCGCTACGTCCTAGGTATTCTAATTGGGGAC
[methyl-C (M)] **1c**	MTGGTGATTTGMGMTTTMGTATTMTAATTGGGGAM
ICR sox/oct 2	TTGGGGCGTTCAGATGCTAATGATCTCCGGCT
(mut oct) **M2**	GCGTTCAGATAATCATGATCTCCGGCT
(mut sox) **M2**	TTGGGGAACGAAGATGCTAATGATCTCCGGCT
[methyl-C (M)] **2c**	TTGGGGMGTTMAGATGMTAATGATMTMMGGMT
ICR sox 3	GCTCAGGGCTGCAAACAATTCTGAAACTGCATTCTC
(sox) **3**	atgaTCTCCGGCTCAGGGCTGCAAACAATTCTGAAGctag
(mut sox) **mut3**	atgaCTAGTGTAGTGTCATACCGGTAAGAAGCTGAGctag
Human Ref.	*(6173) 5'- CATTCAGTGCAGGTTTGAGATGCTAATTTAGGAGCTTGAGATGCTAAAGAGCTG-3' (6120)*
Human ICR oct1/sox/oct2	CGCGGAGATGCTAATTTAGGAGCTTGAGATGCTAAAGAGCTG
Human ICR sox/oct 1	CATTCAGTGCAGGTTTGAGATGCTAATTTAGG
Human ICR sox/oct 2	AGGAGCTTGAGATGCTAAAGAGCTG
(oct) **O**	GAGCCCATATGCAAATCCAAGAGG
(sox) **S**	GTATCCCGGGTTACCCCAAACAAAGAGTTTTC
(ap1) **N**	CGCTTGATGAGTCAGCCGGAA

Table 1. EMSA oligonucleotides. Only the top strand of each dsDNA probe or competitor is provided. Mouse and human sequence positions correspond to AF049091 and AF125183 respectively. Changes made to the reference sequences are underlined. Lowercase sequence was added for cloning.

CHAPTER 3: CONCLUSION

At the time this project was begun in 2002, little was known about the cis-regulatory elements governing ICR methylation. At the *H19/Igf2* locus only CTCF binding sites had been proven necessary for preventing *de novo* methylation of the maternal ICR during embryogenesis, and no *cis*-elements were known to be required for establishment of differential methylation patterns in PGCs [106]. In August of 2002 conserved octamer sites at the ICR were found to be necessary for both preventing *de novo* methylation and promoting demethylation of ICR transgenes in tissue culture [42]. Despite the implications for octamers in regulating imprint establishment and maintenance and the relative lack of alternative candidates, a direct assessment of octamer involvement in imprinting was not published for more than seven years. In 2009 Oct and Sox sites at the human *PWs/AS* locus were found to be required for protecting the human ICR on an exogenous transgene from *de novo* methylation in mouse oocytes [55] Furthermore, methylation of the mutant transgenes correlated with loss of imprinting. More recently maternal inheritance of mutations disrupting octamers at the human *H19/Igf2* ICR has been associated with maternal ICR methylation and loss of imprinting in BWS patients [25,97].

Given the importance of CTCF in regulating the mouse ICR, this project began by comparing the effect of octamer and CTCF site mutation on ICR methylation in order to determine if they have synergistic or redundant functions. Utilizing a murine tissue culture based system we discovered that octamers were the primary regulator of ICR transgene methylation and that CTCF had only a modest additive effect on protecting and removing methylation from stably incorporated plasmids. These results are particularly important in light of recent findings that Oct4 contributes to X-chromosome pairing and counting in part through the formation of a complex with CTCF and other proteins [26]. From their position 350bp downstream of CTCF site 2 and 600bp upstream of CTCF site 3 the two ICR octamers could potentially influence ICR methylation by regulating the binding of CTCF. However, our results indicate octamer and CTCF sites function independently.

Because the adjacent sequences and unique relative spacing of the two octamers was highly conserved between human and mouse, we tested additional constructs with separate mutations at either a single octamer or its respective 3' or 5' flanking sequences. We discovered that mutation of only one octamers or the sequence that links them resulted in a loss of demethylation activity equivalent to that of a double octamer mutant indicating that these three regions function as a single regulatory element. EMSA confirmed the presence of Sox2 binding sites upstream of each octamer and the cooperative binding of Sox2 and Oct4 to each respective dual motif. This double Sox/Oct motif also functioned as a single element capable of inducing transcriptional activation. Mutation of any one Sox or Oct biding site resulted in a complete loss of luciferase reporter activation. Moreover, both DNA demethylation and transcriptional activation were specific to F9 cells expressing Oct4 and Sox2.

The mechanism by which Oct4 and Sox2 influence ICR methylation is unclear, but the F9 tissue culture system used here could continue to serve as a valuable tool for future studies. Using shRNA we were able to successfully knock-down Oct4 for short periods in order to test its necessity for transcriptional activation. A modified approach could be used to test the necessity of trans-factors associated with active demethylation such as the TET family of proteins, AID, or members of the BER pathway [35,38,98]. Alternatively, one could complete the same demethylation experiments but utilize an assay sensitive to 5hmC. We also did not address the possibility that the Sox-Oct motifs may function through transcriptional activation of an unidentified local promoter. This possibility could be explored by searching for enrichment of ICR ncRNAs in F9 stable transfectants.

A similar system could also be used to assess requirements for maintaining ICR methylation. In our studies only partially methylated constructs were demethylated while fully methylated constructs remained methylated despite the methylation independent binding characteristics of Oct4 and Sox2. This result is consistent with the observation that paternal methylation imprints in somatic cells generally remain stable even during Oct4- and Sox2- mediated genome wide reprogramming associated with creation of

induced pluripotent stem (IPS) cells [96]. Mutation of putative Zfp57 binding sites on methylated ICR constructs or knock-down of ZfP57 and/or its associated partners, KAP1, UHRF1 and SETDB1, and other MBDs could be used to investigate their respective contribution to maintaining ICR methylation [99,149].

To ascertain if the octamers are directly involved in establishing and maintaining *H19/Igf2* imprints we created a knock-in mouse possessing octamer mutations at the endogenous ICR. These mice demonstrated normal paternal methylation imprints but abnormal acquisition of methylation on the maternal allele in somatic tissue and oocytes. Maternal ICR methylation was concentrated at the region nearest the octamers and extended as far as the nearest upstream CTCF site. However, the methylation had little effect on maternal imprinted expression of *H19* and *Igf2* and the mice were phenotypically normal. Moreover, the methylation observed in oocytes was erased in the blastocyst stage and therefore did not constitute a stable imprint.

This work documents the first instance of endogenous Sox-Oct motifs being required for differential methylation of a mouse ICR. The pattern and temporal distribution of methylation observed provides important insights into the state of the ICR during gametogenesis and early development. Oct4 is expressed in PGCs during imprint erasure but we show here that it is not required for erasing methylation at the paternal *H19* ICR. However, Oct4 present during the establishment of methylation imprints in male PGCs may contribute to the characteristically slower rate of methylation observed on the maternal allele [22,65]. We observed a modest increase in the amount of mutant maternal ICR methylation in 16 dpc male PGCs compared to the WT alleles from the same embryo population indicating that Oct4 may have a protective effect at the ICR that must be overcome in the male germ line. This result was similar to what has been observed for PGCs with CTCF site mutations [65].

The *de novo* methylation of the region surrounding the mutant octamers could be explained by the active recruitment of methyltransferases. Modest bi-allelic expression of H19 in mice that have inherited a paternal deletion of the sequence between CTCF site 2

and 3 suggests a repressive *cis*-regulatory element may exist in the octamer region [43]. However, deletion of the region did not effect ICR methylation so the mechanism responsible for *H19* repression is unclear. Because the octamers were also removed, a modal in which Sox-Oct motifs regulate the activity of a putative repressive element capable of initiating *de novo* methylation is consistent with both our findings.

The mechanism by which CTCF regulates overall ICR methylation and contributes to the localized methylation pattern of octamer mutants is unknown. The binding of CTCF to the unmethylated mutant ICR in both late stage oocytes and the developing embryo may be capable of establishing a defined chromatin boundary that restricts the spread of methylation to the rest of the ICR. Alternatively, CTCF may actively block *de novo* methylation by promoting euchromatic histone modifications [39]. Whether histone modifying complexes are recruited directly by interacting CTCF activation domains or through the activity of enhancers tethered to the ICR by CTCF dependent intra-chromosomal loops is unclear [57]. The latter hypothesis is enticing because it places the centrally located octamers at the putative transition point between a repressive loop containing *Igf2* and an active loop containing *H19*. In this model the ICR would be split into two CTCF binding domains, each with two CTCF binding sites, joined by a 1 KB linker region containing the octamer sites. Interactions between the two ICR domains, DMR1 and MAR3 would result in a small loop or partition of the linker region and its methylated state would be governed by its own cis-regulatory elements (Fig 12). Mutations to either CTCF site 1-3 or only site 4 results in loss of maternal imprinting and biallelic expression of *Igf2* suggesting independent functions for CTCF sites in forming the higher-order chromatin structure normally associated with the maternal allele [92,93]. Additional single or double CTCF site mutants are needed to ascertain the extent to which sites 1 and 2 and sites 3 and 4 contribute to distinct elements. If either CTCF site 2 or 3 were shown to be expendable for maternal imprinting, a combination of mutations to both octamer and CTCF sites 2 and 3 could shed light on the extent to which these CTCF sites restrict the spread of mutant octamer induced methylation.

The different degrees to which mutant ICRs were methylated in oocytes and somatic tissue suggests disparate mechanisms exist for preventing *de novo* ICR methylation. In somatic tissue CpGs surrounding the mutant octamers were methylated on all alleles indicating a unique role for octamers in maintaining ICR hypomethylation. However, in oocytes octamer mutations resulted in methylation on only a fraction of alleles suggesting a redundant mechanism may be in place to insure proper imprint establishment. One possibility is that CTCF may provide a redundant function in protecting the ICR from *de novo* methylation during oocyte maturation. The complete absence of methylation at ICRs lacking CTCF binding in oocytes however, suggests an alternative mechanism. Despite elevated Oct4 in oocytes during the time of imprint establishment, it is unclear whether octamer sites would be sufficient to insure hypomethylation as they are not sufficient for preventing *de novo* methylation of the maternally inherited CTCF mutant ICR during embryogenesis [106,147,148]. A second hypothesis is that Oct4 may regulate CTCF mediated chromatin looping in the oocyte by antagonizing its interaction with cohesin similar to what has been observed at the *HOXA* locus [56]. CTCF has been suggested to mediate both intra- and inter-chromosomal interactions at the *H19* ICR [71]. Similar interactions involving the octamer mutant ICR in oocytes could lead to sporadic methylation of alleles if the other loci contained cis-acting repressive elements. Both hypotheses could be tested by creating a mouse with ICR mutations at all CTCF and octamer sites as the former would lead to more pervasive methylation while the latter should reduce methylation.

FIGURE

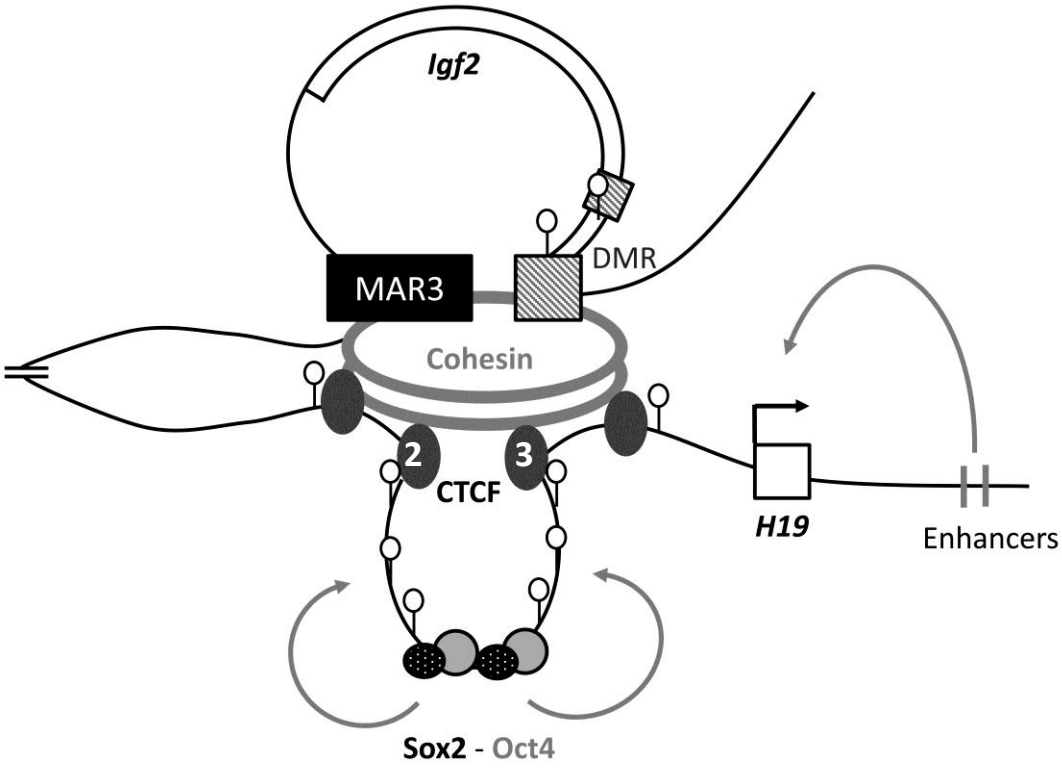

Figure 12. Sox2-Oct4 mediated hypomethylation of the maternal ICR. An expanded view of the unmethylated (open lollipops) ICR is shown depicting the role of Sox2 and Oct4 in protecting the sequences linking CTCF sites 2 and 3.

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