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Identification of Imprinting Regulators at the *Meg3* Differentially Methylated Region

Erin N. McMurray and Jennifer V. Schmidt

Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL 60607

Abstract

Genomic imprinting at the *Delta-like 1 (Dlk1) - Maternally expressed gene 3 (Meg3)* locus is regulated by the *Meg3* differentially methylated region (DMR), but the mechanism by which this DMR acts is unknown. The goal of this study was to analyze the *Meg3* DMR during imprinting establishment and maintenance for the presence of histone modifications and trans-acting DNA binding proteins using chromatin immunoprecipitation. In embryonic stem (ES) cells, where *Meg3* is biallelically expressed, the DMR showed variable DNA methylation, with biallelic methylation at one region but paternal allele-specific methylation at another. All histone modifications detected at the *Meg3* DMR of ES cells were biallelic. In embryonic day 12.5 (e12.5) embryos, where *Meg3* is maternally expressed, the paternal *Meg3* DMR was methylated, and activating histone modifications were specific to the maternal DMR. DNA-binding proteins that represent potential regulatory factors were identified in both ES cells and embryos.

Keywords

Dlk1; *Meg3*; genomic imprinting; differentially methylated region; epigenetics; histone modifications; chromatin immunoprecipitation

1.0 Introduction

Genomic imprinting is the differential expression of the two alleles of a gene based on their parent of origin. For a gene to be imprinted, each chromosome must be marked such that the transcriptional machinery is able to distinguish the parental alleles. Examples of such imprinting marks are DNA (CpG) methylation and covalent modifications to histone proteins. At imprinted genes, DNA methylation is applied in a parent-of-origin specific manner at CpG islands within differentially methylated regions (DMRs). DMRs have proven to be critical for maintenance of imprinting, as deletion or mutation of these regions often results in loss of imprinting [1–5]. Core histone proteins have N-terminal tails to which acetyl, methyl, SUMO, ubiquitin, and phosphate groups can be added to influence expression of associated genes.

The imprinted *Dlk1-Meg3* locus is located on distal mouse chromosome 12 (human 14q32) and contains the paternally expressed *Delta-like 1 (Dlk1)* gene and the maternally expressed

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Corresponding Author: Jennifer V. Schmidt, Ph.D., Department of Biological Sciences, University of Illinois at Chicago, 900 S. Ashland Avenue, MC 567, Chicago, IL 60607, Phone 312-996-5655, Fax 312-413-2691, jvs@uic.edu.

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Maternally expressed gene 3 (Meg3) gene, along with several additional maternally-expressed noncoding genes (Figure 1A and data not shown) [6–8]. *Dlk1* encodes a transmembrane protein belonging to the Notch signaling family [9]. *Meg3*, previously known as *Gene trap locus 2 (Gtl2)*, was discovered by gene trap insertion, and produces a multiply spliced, untranslated RNA of unknown function [10, 11]. Within the *Dlk1-Meg3* region lie three DMRs that are methylated solely on the paternal chromosome: the *Dlk1* DMR located at the 3' end of the *Dlk1* gene, the intergenic (IG)-DMR located 13 kb upstream of *Meg3*, and the *Meg3* DMR, which begins approximately 1.5 kb upstream of the *Meg3* transcriptional initiation site and ends within the first intron of *Meg3* (Figure 1A) [6, 7, 12]. Deletion of the IG-DMR has demonstrated that it is required for maternal, but not paternal, imprinting at this locus [3]. Previous studies suggest that the *Meg3* DMR is necessary for proper *Dlk1-Meg3* imprinting on both parental chromosomes, but the mechanism by which the *Meg3* DMR controls imprinting is not well understood [5, 13, 14].

Steshina *et al.* [5] generated *Gtl2Δ5'Neo* mice by replacing a 2.8 kb region upstream of *Meg3* with the *Neomycin (Neo)* gene. One hundred base pairs of the *Meg3* DMR were deleted by this mutation, but the remainder of the *Meg3* DMR and the *Meg3* promoter were left undisturbed. Altered expression of *Dlk1* and *Meg3* was detected when the transgene was passed either maternally or paternally, and these abnormal expression levels were due to loss of imprinting (LOI) of the normally silent allele on the chromosome carrying the mutation. LOI upon paternal transmission of the *Gtl2Δ5'Neo* allele, and the associated biallelic expression of *Meg3*, appeared to result from the loss of paternal *Meg3* DMR methylation. No methylation change was seen after maternal transmission of the *Gtl2Δ5'Neo* allele, where *Dlk1* is biallelically expressed, so the molecular mechanism of maternal LOI is unknown. Similar LOI and methylation changes were observed in *Gtl2lacZ* mice, which carry a *lacZ/β-actin-Neo* transgene insertion 2.3 kb upstream of *Meg3*, in a position analogous to that of *Neo* in the *Gtl2Δ5'Neo* mice [10, 13]. These data suggest that the *Meg3* upstream region plays a regulatory role in *Dlk1-Meg3* imprinting on both chromosomes, with perturbation of this region resulting in LOI.

Carr *et al.* used chromatin immunoprecipitation (ChIP) experiments on midgestation mouse embryos to search for imprinting regulators at five sites spanning the IG and *Meg3* DMRs [15]. One of the sites tested was located in the *Meg3* DMR, and while this region displayed high levels of maternal-specific acetylation of histones H3 and H4, little evidence was found for other allele-specific histone modifications. The goal of the current study was a more detailed characterization of the allele-specific epigenetic profile of the *Meg3* DMR, incorporating chromosomal regions and epigenetic modifications not previously examined. ChIP was first conducted using chromatin from embryonic stem (ES) cells to determine the epigenetic status of this region at the time imprinted expression is being established. ChIP was then performed using embryonic day 12.5 (e12.5) midgestation embryos, a developmental time point when *Dlk1* and *Meg3* display fully imprinted expression. The resulting data provide a more complete picture of how imprinting is regulated at the *Dlk1-Meg3* region.

2.0 Results

2.1 Methylation status of the *Meg3* DMR and imprinting analysis of *Meg3* in ES cells and e12.5 embryos

To conduct an in-depth analysis of histone modifications and DNA-binding proteins present at the *Meg3* DMR, primers were designed to amplify nine overlapping 300-base pair regions of the DMR (Figure 1B, Supplementary Table 1). In e12.5 embryos, the *Meg3* DMR is methylated on the paternal allele, and the *Meg3* gene is maternally expressed [6]. To our knowledge, neither the methylation profile of the *Meg3* DMR, nor the expression status of

the *Meg3* gene, have been determined in ES cells. To determine the allele-specific methylation status of the *Meg3* DMR in ES cells and e12.5 embryos, the DMR was first analyzed for single nucleotide polymorphisms (SNPs) between two mouse subspecies: *Mus musculus domesticus* (strains C57BL/6 and 129X1/Sv, abbreviated D) and *Mus musculus castaneus* (strain Cast/Ei, abbreviated C). Sequencing the nine regions of the *Meg3* DMR identified SNPs that alter a restriction enzyme recognition site in regions 3 and 9, allowing allele-specific analysis at these two regions. When the region 3 PCR product is digested with the restriction enzyme *MboI*, D DNA is cut into two fragments, whereas C DNA is not cut. When the region 9 PCR product is digested with the restriction enzyme *HinA*, D DNA is cut into two fragments of 258 and 41 base pairs, and C DNA is cut into three fragments of 192, 67, and 41 base pairs. Only the 258 and 192 base pair fragments are shown for region 9 (Figure 1C).

For *Meg3* DMR methylation analysis, 129X1/Sv (D) x Cast/Ei (C) F₁ ES cell DNA and C57BL/6 (D) x B6.CAST-D12Mit30-D12Mit263 (Cg12) F₁ e12.5 embryo DNA was used. Cg12 mice are a congenic strain carrying a C-derived *Dlk1-Meg3* interval on a D background [16]. After digestion with the methylation sensitive enzyme *HpaII*, DNA was amplified by PCR using region 3 and 9 primers (Supplementary Table 1), and the resulting products were digested with the appropriate restriction enzyme. The percent of maternal allele in the digested sample was calculated to determine allele-specificity. DNA methylation was considered allele-specific if the sample contained more than 80% of one parental allele. The data showed that in ES cells, the *Meg3* DMR is biallelically methylated at region 3, but is methylated only on the paternal allele at region 9. This variable DNA methylation may reflect an intermediate state as imprinting is in the process of being established. As expected, methylation is paternal-specific at both regions 3 and 9 in e12.5 embryos (Figure 1C).

To determine the imprinting status of *Meg3* in D x C ES cells, expression analysis of *Meg3* was carried out as described [6], using cDNA from D x C ES cells. Parental D and C cDNAs, as well as those from D x C and C x D e12.5 embryo, were used as controls. PCR amplification with *Meg3*-specific primers was followed by digestion with *SfiI*, which cuts only the D allele. *Meg3* was found to be expressed from both parental alleles in ES cells, but was fully imprinted in e12.5 embryos, with expression only from the maternal allele (Figure 1D).

2.2 Histone modifications at the *Meg3* DMR in ES cells

Acetylation of histones is commonly associated with transcriptional activity; acetylation of histone H3 (H3ac), histone H4 (H4ac), and histone H4 lysine 91 (H4K91ac) all promote the transcription of associated genes [17]. Phosphorylation of histone H3 serine 10 (H3S10phos), mono-, di-, and tri-methylation of histone H3 lysine 4 (H3K4me, H3K4me₂, H3K4me₃), and mono-methylation of lysine 79 of histone H3 (H3K79me) have also been associated with transcriptional activation [17]. Many different histone modifications have been identified at various imprinted loci, often applied in an allele-specific manner that correlates with imprinted expression. In mouse embryonic fibroblast cells (MEFs), H3ac, H4ac, and H3K4me₃ have been detected at the maternal DMR of the active *H19* allele, the promoter of the active paternal *Snrpn* allele, and the maternal allele of the IG-DMR [18]. H3K4me₃ was present at the maternal allele of the IG-DMR in ES cells [19]. H3K79me and H4K91ac have been observed at DMR1, DMR2, and the promoter of the active paternal *Igf2* allele in MEFs [17].

Histone methylation may also be linked to transcriptional repression. For example, di- and tri-methylation of histone H3 lysine 9 (H3K9me₂, H3K9me₃), tri-methylation of histone H3 lysine 27 (H3K27me₃), tri-methylation of histone H4 lysine 20 (H4K20me₃), and tri-

methylation of histone H3 lysine 79 (H3K79me3) are associated with silent chromatin [17]. In MEFs, ES cells, and the murine brain, H3K9me3 has been detected at the inactive maternal promoter of *Snrpn* [18, 20]. This inactive *Snrpn* allele also carries H3K27me3 in ES cells, and H4K20me3 in the mouse brain [18, 20]. In ES cells, H3K9me2 and H3K27me3 were present at the promoter of the inactive paternal *Kcnq1* allele [21]. In MEFs, H3K9me3 was found at the paternal allele of the IG-DMR, and H3K79me3 has been detected at the imprinting control region and promoter of the inactive *H19* allele [17, 18].

To determine the pattern of histone modifications at the *Meg3* DMR of the early mouse embryo, ChIP was conducted using D x C ES cells. As ES cells are derived from the inner cell mass of the e3.5 mouse blastocyst, they approximate the early mouse embryo. Following immunoprecipitation with an antibody of interest, the immunoprecipitated DNA was amplified using primers corresponding to each region of the *Meg3* DMR (Supplementary Table 1). The percent precipitation of a given histone modification was determined by dividing the signal of a particular modification by the input signal and subtracting the normalized IgG signal. In many ChIP analyses, the convention has been to report modifications as simply present or absent. To introduce greater quantitative rigor to the data, and therefore make determinations about imprinting status, we established cutoffs for reporting a modification as positive. These cutoffs were determined by the signal-to-noise observed in our raw data, and the ability to confidently call a modification as present above background. For this study, a histone modification was considered to be present at the *Meg3* DMR if it had a calculated percent precipitation of 5% or greater.

The activating histone modifications H3ac, H4ac, H3K4me, H3K4me2, H3K4me3, H3S10phos, H3K79me and H4K91ac, and the repressive modifications H3K9me3, H3K27me3, H4K20me3 and H3K79me3, were present at all regions of the *Meg3* DMR tested. Percent precipitations across the DMR ranged from 10% to 365%. While many biological and technical variables contribute to percent precipitation, this range suggests there may be differences in abundance of the various modifications (Figure 2, Supplementary Table 3). H3K9me2 was present across the entire *Meg3* DMR except at region 7 where the percent precipitation of this modification was 4%, just below the cutoff for a positive result (Figure 2B, Supplementary Table 3).

2.3 Allele-specificity of histone modifications at the *Meg3* DMR in ES cells

To determine the allele-specificity of histone modifications detected in D x C ES cells, immunoprecipitated DNA was amplified with region 3 and region 9 primers (the two regions with informative SNPs), and the resulting PCR products were digested with *MboI* and *HinI* respectively, as described for the DNA methylation analysis. The percent of maternal allele in the digested sample was calculated, and modifications that exhibited parental bias of 80% or greater were considered to be allele-specific. This analysis revealed that at regions 3 and 9 of the *Meg3* DMR, all histone modifications were present in a biallelic distribution in ES cells (Figure 3, Supplementary Table 4). The presence of both activating and repressing histone modifications at the *Meg3* DMR corresponds to the biallelic expression of *Meg3* in ES cells. Similarly to the variable DNA methylation across this region in ES cells, histone modifications may also be in a state of flux as the imprinting process is ongoing.

2.4 Histone modifications at the *Meg3* DMR in e12.5 embryos

ChIP was then used to analyze the *Meg3* DMR of D x C e12.5 embryos for the histone modifications studied in ES cells. ChIP analysis revealed that the activating modifications H3ac, H4ac, H3K4me2, H3K4me3, and H3K79me were present across the *Meg3* DMR (Figure 4, A and C, Supplementary Table 5), while H3K4me, H3S10phos, and H4K91ac

were absent, with none exceeding 4% precipitation (Figure 4, Supplementary Table 5). For each of the active modifications detected, enrichment was highest at region 6, which overlies *Meg3* exon 1 and the beginning of the first intron (Figure 1B, 4, Supplementary Table 5).

The repressive histone modifications H3K9me2, H3K9me3, H3K27me3, H4K20me3, and H3K79me3 were largely absent from the *Meg3* DMR, but a few were found at specific regions. H3K27me3 was present at region 6 of the *Meg3* DMR at 7% precipitation, and H3K79me3 was present at regions 6 and 7 at 10% precipitation (Figure 4, B and C, Supplementary Table 5). H3K9me2, H3K9me3, and H4K20me3 were not detected at any DMR region (Figure 4B). In general, histone modifications were found at a lower level at the *Meg3* DMR of e12.5 embryos than in ES cells (Figure 2 and Figure 4). The absence of most of the repressive histone modifications tested at the *Meg3* DMR of e12.5 embryos, where imprinting is fully established, suggests that they are not required for the maintenance of imprinted expression of *Meg3* at this stage.

2.5 Allele-specificity of histone modifications at the *Meg3* DMR in e12.5 embryos

Histone modifications present at the *Meg3* DMR of D x Cg12 e12.5 embryos were analyzed for allele-specific localization as described for ES cells. This analysis revealed that H3Ac, H4Ac, H3K4me2, and H3K4me3 localized to the maternal allele at regions 3 and 9 of the *Meg3* DMR. The average amount of maternal allele in the immunoprecipitated DNA was 82% or higher for each of these modifications (Figure 5A and B, Supplementary Table 6). The presence of the maternal allele-specific activating modifications H3Ac, H4Ac, H3K4me2, and H3K4me3 suggests that these histone modifications may play a role in the maternal expression of *Meg3*. H3K79me was biallelically distributed at these regions, with 70% maternal allele in immunoprecipitated samples (Figure 5A and B, Supplementary Table 6). H3K4me, H3K9me2, H3K9me3, H3K27me3, H4K20me3, H3S10phos, H3K79me3, and H4K91ac were not subjected to allele-specific analysis as these modifications are not present at regions 3 and 9 which carry informative SNPs (Figure 4).

2.6 Trans-acting DNA binding proteins at the *Meg3* DMR of e12.5 embryos and ES cells

In addition to histone modifications, trans-acting DNA binding proteins play a role in the regulation of imprinted genes. Methyl-CpG binding proteins (MBPs), including methyl-CpG binding protein 2 (MeCp2) and the methyl-CpG binding domain proteins 1–4 (MBD1, 2, 3, and 4), are capable of binding DNA at methylated CpG dinucleotides through a methyl-binding domain [22, 23]. MBPs have the ability to recruit histone deacetylase complexes (HDACs) resulting in transcriptional repression [24–27]. MeCp2 has been detected at the maternal allele of the paternally expressed *U2af-rs1* gene and at the *Meg3* DMR in the brains of 8 week old mice. In these experiments, MeCp2 localized approximately 700 base pairs downstream of the *Meg3* DMR region tested by Carr *et al.* [15, 28, 29]. MBD1 and MBD3 localized to the maternal *U2af-rs1* allele, and MBD3 bound the DMR of the imprinted *H19* gene and was required for maintenance of *H19* imprinting in mouse blastocysts [29, 30].

We tested MeCp2, MBD1, and MBD3 for localization to the *Meg3* DMR of ES cells and e12.5 embryos using ChIP. For this study, proteins with percent precipitations above 2% were considered to be present at the *Meg3* DMR. As with the histone modification analysis, this cutoff was determined by the signal-to-noise observed in our raw data, and the ability to confidently call a modification as present above background. ChIP analysis demonstrated that MeCp2 was present at DMR regions 1, 3, 4, 5, and 6 in ES cells, and at all regions in e12.5 embryos, identifying new sites of MeCp2 binding in this region (Figure 6). MBD1 and MBD3 were not present at any region of the *Meg3* DMR in ES cells (Figure 6A), while in e12.5 embryos MBD3 was detected at region 8 and MBD1 was detected at region 3 (Figure

6B). The presence of one or more MBD proteins is not surprising, as this correlates with the fully methylated paternal DMR in e12.5 embryos. Like MBPs, the protein Kaiso also binds methylated DNA, but does so through a zinc finger domain [31]. In addition to binding methylated CpGs, Kaiso can directly bind DNA, and a putative Kaiso binding site is located in region 6 of the *Meg3* DMR. Kaiso has not yet been found at any imprinted domains, but we detected it binding to *Meg3* DMR region 4 in ES cells. Kaiso was not present at any region of the *Meg3* DMR in e12.5 embryos (Figure 6A and B).

Polycomb group proteins are typically associated with transcriptional repression and play a role in the maintenance of imprinting and X-inactivation [21, 32]. EED belongs to this protein family, and mice lacking the *Eed* gene display biallelic expression of several imprinted genes including *Meg3* [33, 34]. EED interacts with HDACs and has been suggested to interact with DNA through YY1, a multifunctional zinc finger protein that can act as either a transcriptional activator or repressor [34–37]. Our previous studies showed that YY1 does not recognize a putative YY1 binding site approximately 5 kb upstream of the *Meg3* transcriptional initiation site [15]. In the current study, EED and YY1 were tested for binding to the *Meg3* DMR. Two putative YY1 binding sites are present at the *Meg3* DMR, one at region 3 and another at region 8. The current analysis showed that EED was not present at the *Meg3* DMR in ES cells, while YY1 was present at regions 3, 4, and 6 (Figure 6A). In e12.5 embryos, EED was not detected at any *Meg3* DMR region, and YY1 was present at all nine DMR regions (Figure 6B).

The atypical histone macroH2A1 is incorporated into the silent X chromosome in female mammals, suggesting it plays a role in transcriptional repression [38]. Recently, macroH2A1 was found to localize to the paternal allele of the *Meg3* DMR in the brain and liver of neonatal mice [28]. We show here that in both ES cells and e12.5 embryos, macroH2A1 is present at all nine regions of the *Meg3* DMR (Figure 6).

The protein CTCF localizes to several imprinted domains [39–44]. At the *Igf2/H19* locus, CTCF binds the maternal imprinting control region, silencing the *Igf2* gene by preventing its interaction with downstream enhancers [41]. CTCF plays a role in chromatin looping, generating chromosomal domains permissive and restrictive for transcription that are required for proper imprinted gene expression at *H19* and other loci [45, 46]. Previous studies showed that CTCF does not bind to a putative site in the first intron of *Meg3* in e12.5 embryos, but it has been detected at the *Meg3* DMR in MEFs and human fibroblast cells [15, 47, 48]. In the current study, CTCF was detected at DMR region 9 in ES cells, but was not detected at any region in e12.5 embryos (Figure 6). All ChIP results from this work are summarized in Table 1.

3.0 Discussion

3.1 Expression and methylation analysis

Previous studies have demonstrated that the *Meg3* DMR is required for proper imprinting of *Dlk1* and *Meg3*, but the factors necessary for this regulation remain elusive [5, 13–15]. The goal of the present study was to analyze the *Meg3* DMR in ES cells and midgestation embryos for the presence of DNA methylation, histone modifications and DNA binding proteins that may play a role in regulation of *Dlk1-Meg3* imprinting.

Methylation sensitive PCR revealed that in ES cells, DNA methylation is biallelic at DMR region 3, but localized to the paternal allele at region 9, the two regions that carry SNPs for allele-specific analysis (Figure 1C). These data suggest a pattern of partial methylation across the paternal DMR in early mouse embryos. Despite the presence of some paternal-specific methylation at the ES cell DMR, allele-specific analysis showed that the *Meg3* gene

is biallelically expressed in these cells (Figure 1D). In e12.5 embryos, the *Meg3* DMR has acquired the appropriate paternal methylation at both regions 3 and 9 (Figure 1C), suggesting fully established allele-specific methylation across the paternal DMR. This pattern correlates with the paternal silencing of the *Meg3* gene in midgestation embryos (Figure 1D). Our data suggest that the epigenetic status of the *Meg3* DMR in ES cells represents a window into the establishment phase of the *Dlk1-Meg3* imprint, with key paternal silencing marks still being applied, while the epigenetic status of e12.5 embryos represents a fully mature imprint. The region 9 paternal-specific methylation suggests that certain CpG dinucleotides within the DMR are methylated earlier than others, and may serve to nucleate methylation spreading across the DMR.

3.2 Chromatin regulatory factors in embryonic stem cells

In ES cells, both activating and repressive histone modifications were found at the *Meg3* DMR, and allele-specific analysis revealed that all modifications present were biallelically distributed at regions 3 and 9 (Figure 2, 3). These data mirror the biallelic expression of the *Meg3* gene at this stage, and contrast with the finding of partial allele-specific DNA methylation in ES cells. Several trans-acting DNA binding proteins were also detected at the *Meg3* DMR of ES cells; the histone variant macroH2A1 was present at all DMR regions, while the transcription factors MeCp2, YY1, Kaiso, and CTCF were detected only at specific regions (Figure 6A). Putative YY1 binding sites are located in regions 3 and 8, and YY1 was detected at regions 3, 4, and 6. A putative Kaiso binding site is located in region 6, but Kaiso was detected at region 4. No consensus CTCF binding sites are contained within the *Meg3* DMR, but CTCF was found to localize to region 9. These data illustrate the difficulty in predicting transcription factor binding by sequence analysis alone. Each of these proteins has a highly variable consensus sequence, and as we demonstrate, is capable of binding regions of DNA without a recognizable binding site. The presence of MeCp2, YY1, Kaiso, and CTCF at specific regions of the *Meg3* DMR suggest that they may play a yet unknown role in the establishment of *Meg3* imprinted expression (Figure 1D).

3.3 Chromatin regulatory factors in midgestation embryos

In midgestation mouse embryos, the activating modifications H3ac, H4ac, H3K4me2, H3K4me3, and H3K79me, were detected at all regions of the *Meg3* DMR (Figure 4, A and C). With the exception of H3K79me, all modifications were maternal-specific at DMR regions 3 and 9 (Figure 5). These maternal-specific modifications likely act to specify the transcriptionally active state of the maternal *Meg3* allele. Repressive histone modifications were largely absent from the *Meg3* DMR in e12.5 embryos, with H3K27me3 and H3K79me3 showing slight enrichment at specific DMR regions. Therefore, it is unlikely that repressive histone modifications play a role in the maintenance of *Meg3* imprinting in midgestation mouse embryos, perhaps because continued silencing of the paternal allele can be achieved through DNA methylation alone.

A number of trans-acting DNA binding proteins were also detected at the *Meg3* DMR of e12.5 embryos, and differences between ES cells and midgestation embryos suggest a dynamic pattern of trans-acting protein activity. Kaiso and CTCF, proteins that are found at the *Meg3* DMR of ES cells, are no longer present in e12.5 embryos, suggesting they may be involved in establishment of imprinting but are unnecessary beyond this stage. On the other hand, MeCp2 and YY1, which are found at some DMR regions in ES cells, are found across all regions of the DMR in midgestation embryos. The proteins MBD3 and MBD1 which are not present at the *Meg3* DMR of ES cells, localize to specific regions of the e12.5 *Meg3* DMR. The protein macroH2A1 is present at all regions of the *Meg3* DMR in both ES cells and midgestation embryos. One role for the factors MeCp2, YY1, MBD3, and/or MBD1 may be the removal of the activating H3ac and H4ac modifications from the paternal *Meg3*

DMR, as all of these proteins are capable of recruiting HDACs. Since HDACs interact with DNA methyltransferases (DNMTs), removal of the activating modifications may reinforce the methylation of the paternal *Meg3* DMR and therefore also reinforce the silencing of the paternal *Meg3* gene [49, 50]. The interaction between DNA binding proteins, HDACs, and DNMTs would result in the stable silencing of the paternal *Meg3* allele, while leaving the maternal *Meg3* allele active.

3.4 Conclusion

This study provides a window into the temporal progression of the epigenetic modifications and DNA binding proteins associated with imprinting establishment at the *Dlk1-Meg3* DMR. These data suggest that multiple chromatin regulatory pathways must interact to confer proper imprinting at this locus, and that those regulators responsible for imprinting establishment are likely distinct from the regulators necessary for imprinting maintenance. These data also suggest that epigenetic modifications may work in concert with DNA binding proteins to ensure stable silencing of the paternal *Meg3* allele independently of the maternal allele. Future work will determine the role each histone modification and/or DNA binding protein plays in the establishment and maintenance of *Dlk1-Meg3* imprinting.

4.0 Materials & Methods

4.1 Propagation of ES cells

129X1/Sv x Cast/Ei (D x C) ES cells were obtained from Stemgent Inc. and were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (4.5 g/L) with 15% ES cell qualified fetal bovine serum, 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin, 1 µl/100 ml β-mercaptoethanol, and 10 µl/100 ml LIF. Cells were grown on mouse embryonic fibroblast feeder cells and were maintained in a humidified incubator at 37°C with 5% CO₂. The cells were frozen or split 1:5 when they reached 80% confluence. For ChIP, cells were grown to 80% confluency on 10 cm plates, trypsinized, resuspended, and plated for 1 hour to remove feeder cells. The ES-enriched supernatant was removed from the plate and used for ChIP analysis.

4.2 Mouse maintenance and breeding

Embryos were analyzed at midgestation (e12.5) and were the offspring from a cross between the *M. m. domesticus* strain C57BL/6 and the congenic mouse strain B6.CAST-D12Mit30-D12Mit263 (Cg12) [16]. Animals were maintained in microisolator cages, on a standard diet, with a 14:10 light:dark cycle. All animals used in these experiments were maintained in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and The University of Illinois at Chicago Animal Care Committee guidelines.

4.3 Identification of SNPs

Regions 1–9 were PCR amplified from C57BL/6, 129X1/Sv and Cg12 genomic DNA and sequenced to identify SNPs. The strains C57BL/6, 129X1/Sv belong to the D subspecies, and Cg12 is a congenic line carrying a C-derived distal chromosome 12 on a D background [16]. A SNP in region 3 was identified that abolishes an *MboI* restriction site in Cg12 DNA, and a SNP in region 9 alters a *HinfI* site. *MboI* digestion of region 3 gives 190 and 90 base pair fragments from D DNA, while C or Cg12 DNA is not cut. *HinfI* digestion of region 9 DNA give 258 and 41 base pair fragments from D DNA, and 192, 67, and 41 base pair fragments from C or Cg12 DNA. Only the 258 and 192 base pair fragments are shown in the figures for region 9 analysis.

4.4 Determination of Meg3 DMR methylation status in ES cells and e12.5 embryos

Two hundred ng of genomic DNA from D x Cg12 e12.5 embryos and D x C ES cells was digested with 20 units of *HpaII* overnight. Digested DNA was amplified with region 3 and region 9 primers, with conditions 95°C for 30 seconds, 60°C for 30 seconds, and 70°C for 30 seconds. After 25 cycles, 0.3 μ Ci [α -³²P]dCTP (PerkinElmer) was added, and amplification resumed. Region 3 reactions were carried out for a total of 32 cycles, while region 9 reactions were carried out for 34 cycles. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The region 3 product was cut overnight at 37°C with *MboI* and region 9 was cut for 1 hour at 37°C with *HinfI*. Digested PCR products were separated on a 7.5% acrylamide gel, and the gel was dried and exposed to film, and then to a phosphorimaging screen. Phosphorimaging screens were scanned using a Molecular Dynamics Storm 860 Phosphorimager, and the maternal and paternal alleles were quantified using ImageQuant Software.

4.5 Determination of Meg3 imprinting status in ES cells and e12.5 embryos

Total RNA was extracted from D, C, D x C, and C x D e12.5 embryos and from approximately 3×10^6 D x C ES cells with Trizol (Invitrogen) according to manufacturer's instructions. RNA was treated with TURBO DNA-free DNase (Ambion). Oligo-dT-primed reverse transcription reactions were carried out using 2 μ g total RNA and Superscript III (Invitrogen). Imprinting analysis of the *Meg3* gene was performed as described using 2 μ l of a 1:10 dilution of the RT reactions [6]. After 25 cycles, 0.3 μ Ci [α -³²P]dCTP (PerkinElmer) was added to each reaction. PCR products were run on a 7.5% acrylamide gel, dried, and exposed to film. Control reactions carried out in the absence of reverse transcriptase were negative.

4.6 Preparation of nuclei for chromatin immunoprecipitation

Nuclei were prepared using the Millipore ChIP Assay Kit according to the manufacturer's instructions with the following modifications. Midgestation embryos were minced with a razor blade and crosslinked with 1% formaldehyde in DMEM. When testing for the presence of histone modifications, cells were crosslinked for 10 minutes. When testing for the presence of a DNA binding protein, cells were crosslinked for 20 minutes. The crosslinking reaction was stopped by the addition of glycine to a concentration of 0.125M for 5 minutes. The cells were spun at 2000 rpm for 5 minutes, washed 2 times with wash buffer (1X PBS containing 1mM PMSF, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin A) and homogenized in a dounce homogenizer with a tight pestle for 3 cycles of 10 strokes with a 2 minute rest between cycles. The nuclei were washed twice with wash buffer, split into two equal fractions, pelleted, and the pellets were snap frozen.

To prepare ES cell nuclei, 1×10^6 ES cells were crosslinked and treated as described above with the following changes. ES cells were homogenized in a dounce homogenizer with a tight pestle for 2 cycles of 10 strokes with a 2 minute rest between cycles. ES cell nuclei were not split into two fractions before snap freezing.

4.7 Chromatin immunoprecipitation

ChIP was carried out on nuclei from e12.5 embryos and ES cells using the Millipore ChIP Assay Kit according to the manufacturer's instructions with the following modifications. One tube of previously prepared nuclei was thawed on ice, the pellet was resuspended in 200 μ l of SDS lysis buffer, and was incubated on ice for 10 minutes. The nuclei were sonicated using a Branson Model 450 sonicator with a double-step tip for 150 sec (10 cycles of 15 seconds with a 2 minute rest between cycles) at 30% power. Chromatin was diluted in ChIP dilution buffer and divided into two fractions. One fraction was treated with the

antibody against the histone modification or protein of interest, and the other was treated with a preimmune antibody to serve as a negative control. Chromatin was pre-cleared with 75 μ l Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) for 1 hour at 4°C. For immunoprecipitation, 10 μ g of antibody against a histone modification or 20 μ g of antibody against a protein was added to the chromatin and was incubated overnight at 4°C.

4.8 Collection of immune complexes and DNA purification

Antibody/protein/DNA complexes were collected by the addition of 60 μ l Protein A/G PLUS-Agarose that had been blocked overnight at 4°C in 5% BSA in ChIP Dilution Buffer. The immune complex was washed, the DNA/protein complex was eluted from the beads, DNA/protein crosslinks were reversed, and the protein was digested as described in the Millipore ChIP Assay Kit instructions. DNA was purified using the MinElute PCR Purification Kit (Qiagen) according to the manufacturer's instructions and was resuspended in 20 μ l dH₂O.

4.9 PCR amplification

PCR was conducted using region 1–9 primers (Supplementary Table 1) and 1 μ l of DNA from DNA binding protein immunoprecipitations or 1 μ l of a 1:2 dilution of DNA from histone modification immunoprecipitations. The conditions for all PCR reactions were 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. After 25 cycles, 0.3 μ Ci [α -³²P]dCTP (PerkinElmer) was added, and the PCR was resumed for the necessary number of cycles. For regions 1–5 and 7–10, DNA from ES cell histone modification immunoprecipitations, e12.5 histone modification immunoprecipitations, ES cell protein immunoprecipitations, and e12.5 protein immunoprecipitations was amplified for 33, 31, 30, and 28 total cycles respectively. Regions 6 and 11 were amplified for 35, 33, 32, and 30 total cycles respectively. All PCR reactions carried out without template DNA were negative. Ten microliters of the PCR reaction was separated on a 7.5% acrylamide gel. The gel was dried and exposed to film and to a phosphorimaging screen. Phosphorimaging screens were scanned using a Molecular Dynamics Storm 860 Phosphorimager, and the intensity of each band was quantified using ImageQuant Software. The IP signal was first normalized by dividing the intensity of the signal by the intensity of the corresponding input signal. Percent precipitation was then determined by multiplying the normalized IP signal by 100. To determine the allele-specificity of a histone modification, regions 3 and 9 were cut with the appropriate restriction enzyme as described above and separated on a 7.5% acrylamide gel that was subsequently dried and exposed to a phosphorimaging screen. Phosphorimaging screens were scanned using a Molecular Dynamics Storm 860 Phosphorimager, and the bands were quantified using ImageQuant Software. The signal from each allele was normalized to the input DNA as described, and the percentage of maternal allele present in the sample was calculated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

We analyze epigenetic modifications at the mouse *Meg3* DMR imprinting regulatory element.

In ES cells, *Meg3* is biallelically expressed and the DMR shows biallelic epigenetic modifications.

In midgestation embryos, *Meg3* is imprinted, and the DMR shows allele-specific epigenetic modifications.

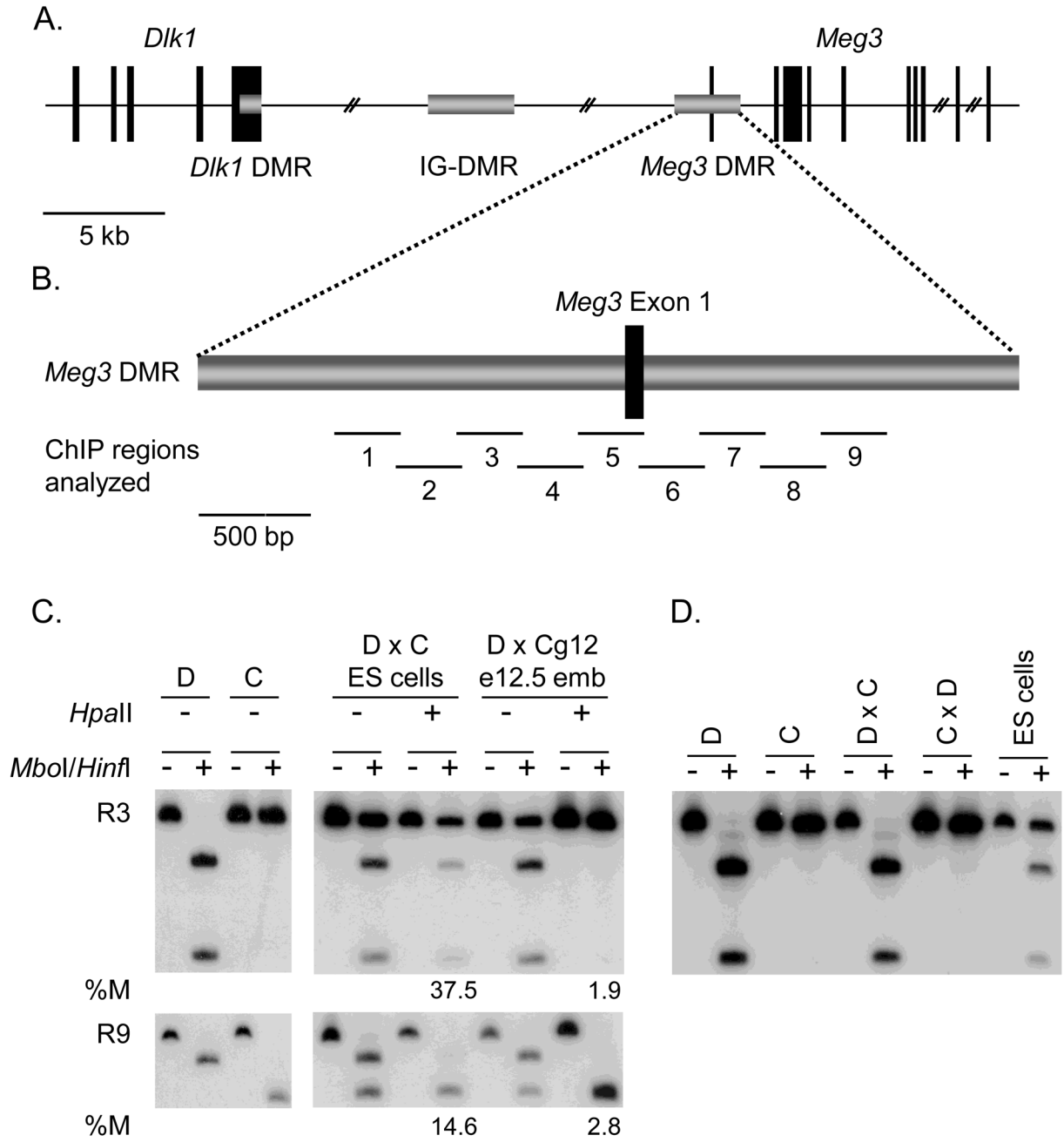


Figure 1. Regions of the *Meg3* DMR analyzed for epigenetic status

(A) Genomic organization of the *Dlk1-Meg3* domain. Black boxes represent exons and gray boxes represent DMRs. (B) Depiction of the *Meg3* DMR. The gray box represents the *Meg3* DMR, and the black box represents *Meg3* exon 1. Regions of the *Meg3* DMR analyzed by ChIP are indicated by horizontal lines below the DMR. (C) *Meg3* DMR methylation analysis in ES cells and e12.5 embryos. D, C, D x C ES cell, and D x Cg12 e12.5 embryo genomic DNA was digested with *HpaII*, amplified with region 3 (R3) and region 9 (R9) primers, and cut with the appropriate restriction enzyme to determine DNA methylation allele specificity. The uncut DNA is represented by “-”, while “+” denotes DNA cut with the appropriate restriction enzyme; %M indicates the amount of methylation on the maternal

allele relative to the total methylation. (D) *Meg3* expression analysis in e12.5 embryos and ES cells, using D, C, D x C, and C x D e12.5 embryo cDNA and D x C ES cell cDNA for analysis. Uncut DNA is represented by “-”, while “+” denotes *Sfi*I cut DNA, %M indicates the amount of methylation on the maternal allele relative to the total level of methylation.

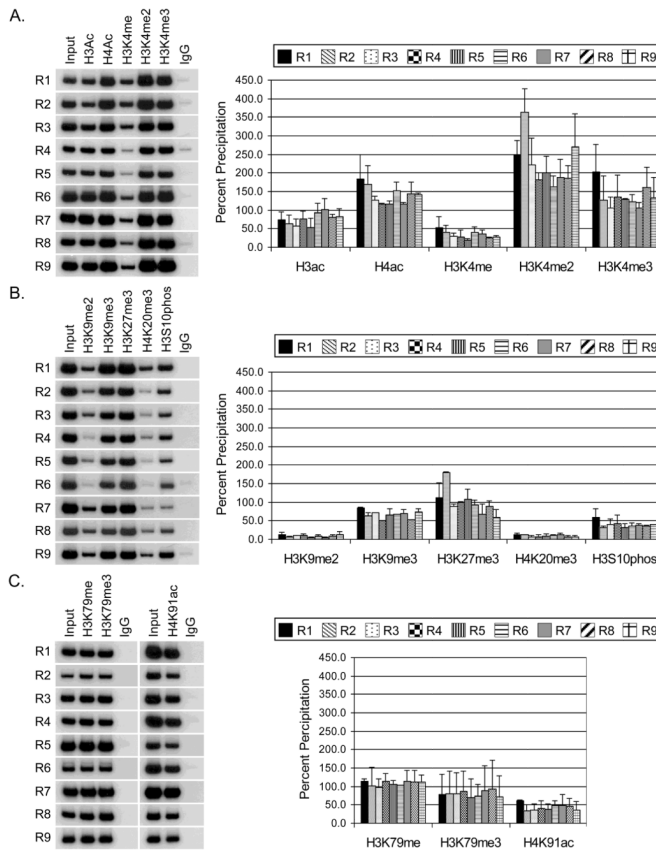


Figure 2. Analysis of histone modifications at *Meg3* DMR regions R1 through R9 in ES cells ChIP analysis of (A) H3ac, H4ac, H3K4me, H3K4me2, H3K4me3, (B) H3K9me2, H3K9me3, H3K27me3, H4K20me3, H3S10phos, (C) H3K79me, H3K79me3, and H4K91ac. Left panels display a representative ChIP assay for each modification at each region of the DMR. Right panels depict the average percent precipitation for each modification at each region of the DMR. Average percent precipitation was calculated from at least two independent ChIP experiments. Error bars represent standard error of the mean (SEM).

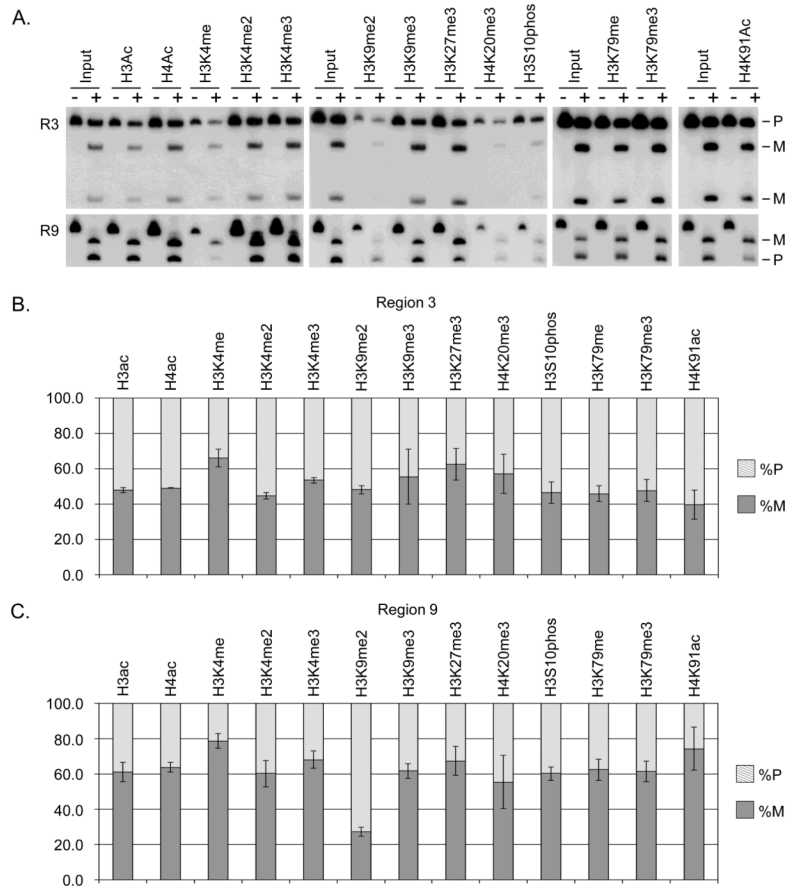


Figure 3. Allele-specific analysis of histone modifications at *Meg3* DMR regions R3 and R9 in ES cells

(A) The region 3 product was cut with *MboI* and the region 9 product was cut with *HinfI* to distinguish the maternal and paternal alleles of the DMR. Paternal and maternal bands are indicated by “P” and “M” respectively. The uncut PCR product is represented by “–”, while “+” denotes the PCR product cut with the appropriate restriction enzyme. Average percent of maternal and paternal allele (%M and %P, respectively) of each histone modification present at region 3 (B) and region 9 (C) was calculated using data collected from at least two independent ChIP experiments. Error bars represent SEM.

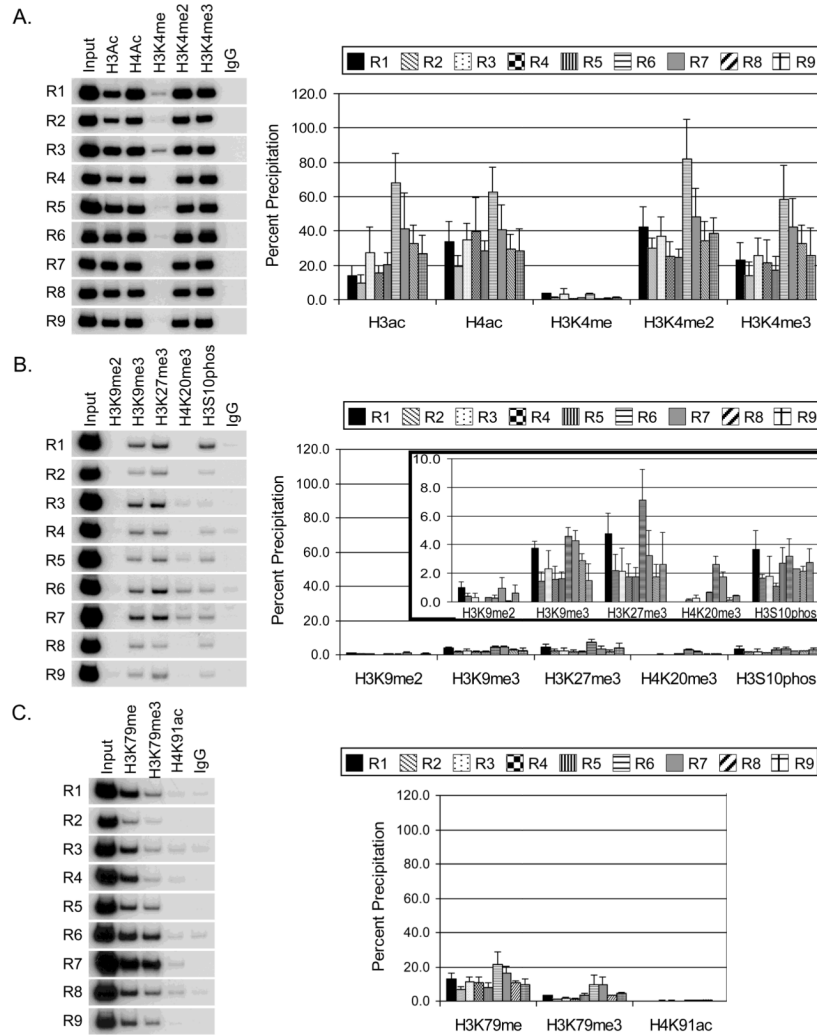


Figure 4. Analysis of histone modifications at *Meg3* DMR regions R1 through R9 in e12.5 embryos
 ChIP analysis of (A) H3ac, H4ac, H3K4me, H3K4me2, H3K4me3, (B) H3K9me2, H3K9me3, H3K27me3, H4K20me3, H3S10phos, (C) H3K79me, H3K79me3, and H4K91ac. Left panels show a representative ChIP assay for each modification at each region of the DMR. Right panels depict the average percent precipitation of each modification at each region. Average percent precipitation was calculated from at least two independent ChIP experiments. Error bars represent SEM.

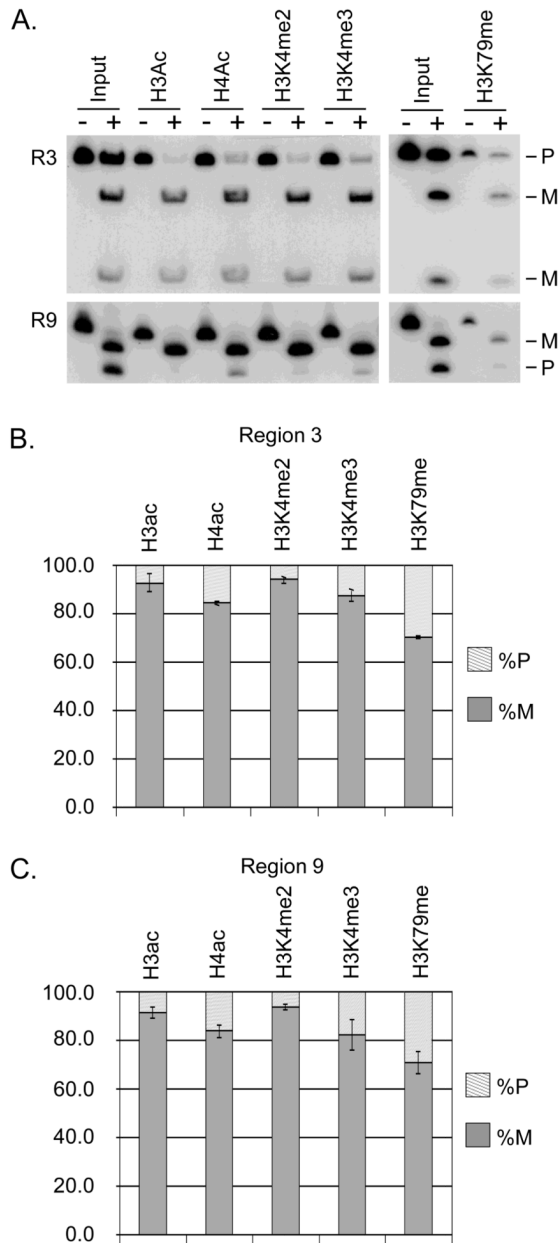


Figure 5. Allele-specific analysis of histone modifications at *Meg3* DMR regions R3 and R9 in e12.5 embryos

(A) The region 3 product was cut with *MboI* and the region 9 product was cut with *HinfI* to distinguish the maternal and paternal alleles of the DMR. Paternal and maternal bands are indicated by “P” and “M” respectively. The uncut PCR product is represented by “-”, while “+” denotes the PCR product cut with the appropriate restriction enzyme. Average percent of maternal and paternal allele (%M and %P, respectively) of each histone modification present at region 3 (B) and region 9 (C) was calculated using data collected from at least two independent ChIP experiments. Error bars represent SEM.

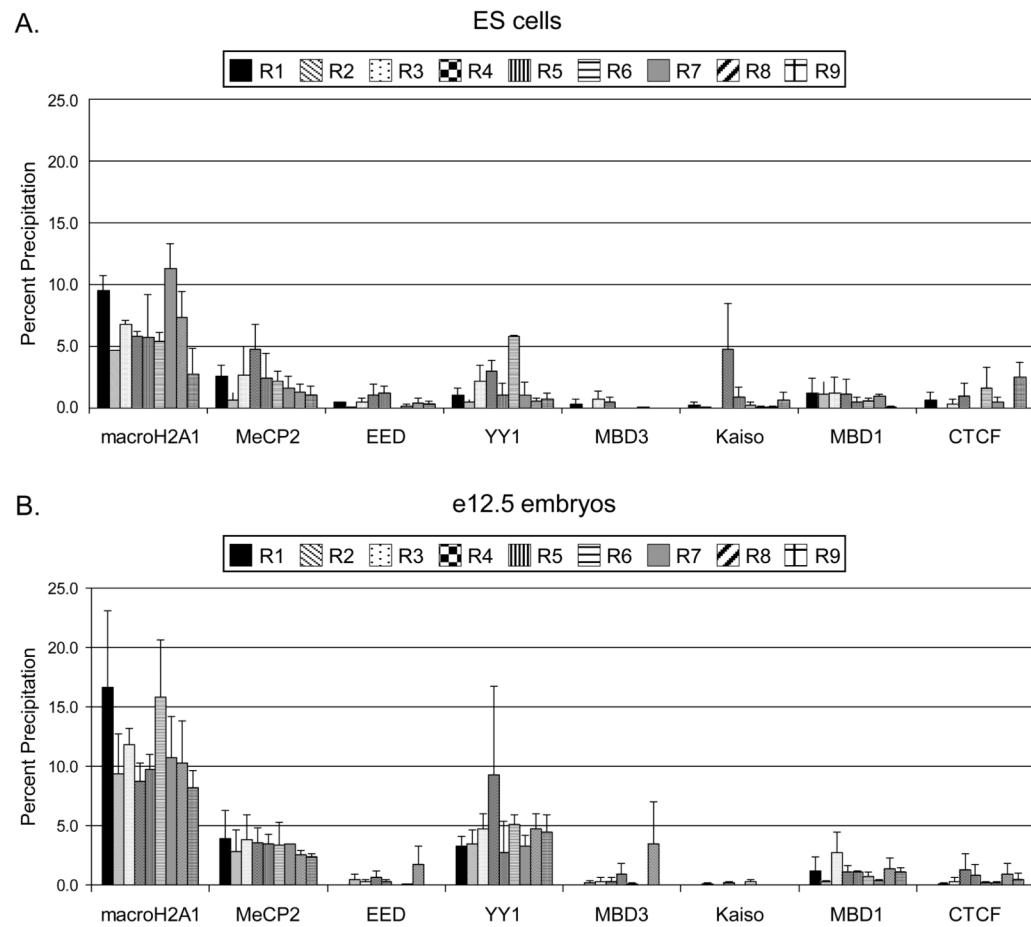


Figure 6. Analysis of transcription factors at *Meg3* DMR regions R1 through R9 in ES cells and e12.5 embryos
ChIP analysis of macroH2A1, MeCP2, EED, YY1, MBD3, Kaiso, MBD1, and CTCF in (A) D x C ES cells and (B) D x Cg e12.5 embryos. Each panel depicts the average percent precipitation of a given protein at each region of the *Meg3* DMR. Average percent precipitation was calculated from at least two independent ChIP experiments. Error bars represent SEM.

Table 1

Summary of ES cell and e12.5 embryo ChIP results at regions 1–9 of the *Meg3* DMR

	ES cells									e12.5 embryos								
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R1	R2	R3	R4	R5	R6	R7	R8	R9
H3ac	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H4ac	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H3K4me	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
H3K4me2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H3K4me3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H3K9me2	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-
H3K9me3	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
H3K27me3	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
H4K20me3	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
H3S10phos	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
H3K79me	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H3K79me3	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
H4K91ac	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
macroH2A1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MeCp2	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
BED	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YY1	-	-	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+
MBD3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kaiso	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MBD1	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
CTCF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Shown is the presence or absence of a histone modification or DNA binding protein at a particular *Meg3* DMR region in ES cells and e12.5 embryos. "+" denotes the presence and "-" denotes the absence of a given histone modification or protein. "ES" stands for ES cells, while "12.5" stands for e12.5 embryos.