Domain-Specific Response of Imprinted Genes to Reduced DNMT1[▽]

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Imprinted genes are expressed in a monoallelic, parent-of-origin-specific manner. Clusters of imprinted genes are regulated by imprinting control regions (ICRs) characterized by DNA methylation of one allele. This methylation is critical for imprinting; a reduction in the DNA methyltransferase DNMT1 causes a widespread loss of imprinting. To better understand the role of DNA methylation in the regulation of imprinting, we characterized the effects of *Dnmt1* mutations on the expression of a panel of imprinted genes in the embryo and placenta. We found striking differences among imprinted domains. The *Igf2* and *Peg3* domains showed imprinting perturbations with both null and partial loss-of-function mutations, and both domains had pairs of coordinately regulated genes with opposite responses to loss of DNMT1 function, suggesting these domains employ similar regulatory mechanisms. Genes in the *Kcnq1* domain were less sensitive to the absence of DNMT1. *Cdkn1c* exhibited imprinting perturbations only in null mutants, while *Kcnq1* and *Ascl2* were largely unaffected by a loss of DNMT1 function. These results emphasize the critical role for DNA methylation in imprinting and reveal the different ways it controls gene expression.

In the mammalian genome, a small group of genes known as imprinted genes is monoallelically expressed in a parent-of-origin-specific manner (1). Genomic imprinting is regulated by epigenetic modifications that distinguish the two alleles and mark their parental identity. While many chromatin characteristics are associated with imprinted genes in somatic tissues, the only gamete-established and preimplantation-maintained epigenetic modification that differs between the two alleles and is definitively linked to imprinted gene regulation is DNA methylation. DNA methylation is the covalent addition of a methyl group to cytosines within CpG dinucleotides and is generally associated with repressive chromatin and gene silencing.

Imprinted genes are found in clusters (see Fig. 1A), where an imprinting control region (ICR) controls their expression (13, 30, 41, 48, 49, 51). For all known ICRs, parental allele-specific methylation patterns are established in the germ line and subsequently maintained throughout the life of the organism (10, 44). This methylation is critical to the function of the ICR. Mutations in the DNA methyltransferase (*Dnmt*) genes result in demethylation and widespread loss of imprinting (LOI). Deposition of methylation at ICRs is performed by the *de novo* methyltransferase DNMT3 family (4, 5, 17, 22, 47). The maintenance methyltransferase DNMT1 replicates DNA methylation patterns during S phase by recognizing hemimethylated CpG dinucleotides and catalyzing the addition of a methyl group to the newly synthesized

daughter strand (26). In the preimplantation embryo, maternal stores of an oocyte-specific isoform, DNMT1o, are required in addition to the somatic form (DNMT1s or DNMT1) to maintain methylation (7, 19, 20, 23). Embryonic loss of *Dnmt1* function causes a global loss of methylation, LOI, and embryonic lethality (28, 29).

The role of somatic DNMT1 in imprinting has been studied using three mutant alleles. The *Dnmt1^c* allele harbors a deletion of the PC and ENV motifs of the catalytic domain and is presumed to be a null allele (24). The moderately severe *Dnmt1^s* allele contains an insertion that disrupts a region important for targeting DNMT1 to replication forks (24, 28) and that may be involved in dimerization (12). The least-severe allele, *Dnmt1ⁿ*, replaces most of exon 4 and exon 5 with a neomycin resistance cassette (29). This region codes for about 60 amino acids near the N-terminal end of the somatic form of DNMT1 and also includes the translation start site for DNMT1o. *Dnmt1^{n/n}* embryonic stem cells (ESCs) harbor about one-third the cytosine methylation of wild-type ESCs, and *Dnmt1^{n/n}* embryos have a 70% reduction in global DNA methylation (29). A small amount of a lower-molecular-weight protein was detected in these embryos, but the exact nature of this protein is unknown. *Dnmt1^{s/s}* and *Dnmt1^{c/c}* ESCs had no detectable DNMT1 protein and a more severe loss of methylation (24). *Dnmt1^{n/n}* embryos die between embryonic day 10.5 (E10.5) and E12.5, at which point they are developmentally delayed (29). *Dnmt1^s* and *Dnmt1^c* cause more-severe developmental delay than *Dnmt1ⁿ*. At E9.5, homozygous embryos have distorted, open neural tubes, no or reduced numbers of somites, and a more severe loss of methylation (24).

All *Dnmt1* mutants exhibit perturbations in the regulation of imprinted genes with respect to both allelic expression and expression levels (2, 3, 6, 27, 28, 36, 39, 40). However, there are important exceptions. Genomic imprinting is more widely deregulated in embryos homozygous for the *Dnmt1^s* and *Dnmt1^c* alleles than for the partial loss-of-function *Dnmt1ⁿ* allele. For

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example, *Igf2r* loses imprinting in *Dnmt1^{c/c}* but not *Dnmt1^{n/n}* embryos (24, 28). Furthermore, analysis of *Dnmt1* mutant embryos has suggested that some genes may utilize different mechanisms to regulate imprinting in embryonic and extraembryonic tissues. In the *Kcnq1* cluster, expression of genes that are imprinted only in the placenta, including *Osbpl5*, *Tssc4*, *Cd81*, and *Ascl2*, is unaffected in *Dnmt1^{c/c}* placentas (6, 15, 27, 40). These genes are differentially marked by histone modifications in the placenta, with active histone modifications on the maternal, expressed allele and repressive marks on the silent paternal allele (27, 43). These observations have led to the proposal that somatic DNMT1 is not required for maintenance of imprinting of these genes, which are instead regulated by histone modifications (27, 43, 46). However, placentas derived from *Dnmt1^{Δ10/Δ10}* oocytes, which lack maternal DNMT1, do have biallelic expression of these genes, suggesting DNMT1 is required earlier to establish imprinting and is dispensable only later in development (15).

Many analyses of the effects of *Dnmt1* mutations on imprinting have utilized only one mutation or focused on genes in a single domain. More specifically, *H19* and *Igf2* expression has been examined in *Dnmt1ⁿ* and *Dnmt1^s* mutants, expression of genes in the *Kcnq1* domain has been assayed in *Dnmt1^c* mutants, *Snrpn* expression has been tested in *Dnmt1^s* mutants, and genes in the *Peg3* domain have not yet been examined in any *Dnmt1* mutant. Thus, a more comprehensive analysis of the effects of multiple *Dnmt1* alleles on genes in many domains would provide insight into the ways different genes respond to loss of methylation, their relative sensitivities to the effects of different mutations, and the tissue-specific responses to loss of methylation in the embryo and the placenta. A comparison of the various clusters would also provide insight into the specific regulatory mechanisms each domain employs. To this end, we undertook a systematic characterization of the effects of the *Dnmt1ⁿ* and *Dnmt1^c* mutant alleles on the expression of a panel of imprinted genes in four domains on chromosome 7 in the embryo and placenta at E9.5. We found substantial differences among imprinted domains in response to *Dnmt1* mutations. The *Igf2* and *Peg3* domains exhibited many similarities. Genes in these domains consistently exhibited LOI in *Dnmt1* mutants. Furthermore, paternally expressed *Peg3* and maternally expressed *Zim1* had opposite responses to loss of methylation, suggesting that they may share enhancers, in similarity to *H19* and *Igf2*. In the *Kcnq1* domain, genes tended to be less sensitive to a loss of DNMT1 function. *Cdkn1c* retained imprinting in *Dnmt1^{n/n}* tissues, similarly to *Igf2r* (28). *Kcnq1* was not as consistently imprinted as other genes studied and was largely unaffected by *Dnmt1* mutations. These results provide a comprehensive view of the ways in which DNA methylation affects the expression of imprinted genes and demonstrate that it plays diverse roles in domains that utilize different regulatory mechanisms.

MATERIALS AND METHODS

***Dnmt1^{n/n}* and *Dnmt1^{c/c}* mice.** For the purpose of allele-specific expression studies, heterozygous *Dnmt1^{+/-}* (The Jackson Laboratory) and *Dnmt1^{+/-}* (generously provided by En Li) mice were crossed with either C57BL/6 (B6) or C57BL/6(CAST7) (C7) mice to obtain animals with the following genetic combinations: (i) B6 *Dnmt1^{+/-}*, (ii) C7 *Dnmt1^{+/-}*, (iii) B6 *Dnmt1^{+/-}*, and (iv) C7 *Dnmt1^{+/-}*. C7 mice bear *Mus musculus castaneus* (CAST) (the Jackson Labora-

tory) chromosome 7s on a B6 background. These mice served as a source of CAST alleles (33, 34). For the C7 crosses, progeny were genotyped with MIT markers for chromosome 7 (D7Mit305, D7Mit57, D7Mit112, D7Mit52, D7Mit27, D7Mit211, D7Mit200, D7Mit163, D7Mit148, D7Mit222, D7Mit285, D7Mit207, D7Mit140, and D7Mit362; Invitrogen) approximately every 5 centimorgans (cM) as described previously (33, 34).

Genotyping for the *Dnmt1ⁿ* allele was performed using multiplex PCR amplification with the following primers: Dm2 (5'GGCTACCTGGCTAAAGTCAA GTCC3'; final concentration, 0.375 μM), Dm4 (5'CAAAAAGGGTGTCACTG TCCGAC3'; final concentration, 0.375 μM), and Pgl1 (5'GGGAACCTCCTG ACTAGGGG3'; final concentration, 0.75 μM) for 1 cycle at 95°C for 2 min followed by 40 cycles at 95°C for 15 s, 58°C for 10 s, and 72°C for 20 s. Dm2 acts as the 5' primer for both the wild-type and *Dnmt1ⁿ* mutant alleles. Primers Dm2 and Dm4 amplify a 211-bp fragment from the wild-type allele, while primers Dm2 and Pgl1 produce a fragment of 302 bp from the *Dnmt1ⁿ* mutant allele.

For the *Dnmt1^c* allele, multiplex PCR was conducted at 95°C for 2 min followed by 40 cycles at 95°C for 15 s, 58°C for 10 s, and 72°C for 20 s using the primers Dm7 (5'GCACCACAGTGTTCACAGAGG3'; final concentration, 0.375 μM), Dm6 (5'TAAGTCGGGGAGTTGAAGCG3'; final concentration, 0.375 μM), and Pgl1 (final concentration, 0.75 μM). Dm7 acts as the 5' primer for both the wild-type and *Dnmt1^c* mutant alleles. The primers Dm7 and Dm6 amplify a fragment of 171 bp from the wild-type allele, while the primers Dm7 and Pgl1 amplify a 256-bp fragment from the *Dnmt1^c* mutant allele.

Matings and embryo collection. F₁ embryos were obtained from reciprocal crosses with B6 *Dnmt1^{+/-}* and C7 *Dnmt1^{+/-}* mice and with B6 *Dnmt1^{+/-}* and C7 *Dnmt1^{+/-}* mice (see Fig. 1B). Embryos, placentas, and yolk sacs were recovered at E9.5 of pregnancy. E6.5 ectoplacental cone, extraembryonic ectoderm, and epiblast and E7.5, E8.5, and E9.5 embryos and placentas were collected from matings of B6 females and C7 or P12X9/11 (which are *Mus musculus castaneus* on portions of chromosomes 9, 11, 12, and X in addition to chromosome 7) males. Yolk sacs were collected for genotyping. DNA was extracted by incubation at 55°C overnight in lysis buffer, followed by heat inactivation at 95°C for 10 min (37), and cell lysate (1 to 4 μl) was used to genotype embryos as described above. RNA was isolated from embryos and placentas using the HighPure RNA tissue kit (Roche Molecular Biochemicals), with minor modifications to the manufacturer's recommendations (33). RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and 100 ng RNA was used for cDNA synthesis, performed as described previously (37).

All animal work was conducted with the approval of the Institutional Animal Care and Use Committee.

Western blot. Individual *Dnmt1^{+/-}*, *Dnmt1^{+/-}*, and *Dnmt1^{n/n}* E9.5 embryos and placentas were lysed in radioimmunoprecipitation assay (RIPA) buffer. Lysates were mixed with 5× loading buffer, denatured by heating at 95°C for 10 min, and fractionated on an 8% SDS-PAGE gel. The protein was transferred to a nitrocellulose membrane (Bio-Rad), blocked in 5% nonfat milk (Bio-Rad) in PBST (0.1% Tween 20 in 1× phosphate-buffered saline [PBS]) and probed with antibodies against alpha-tubulin (mouse monoclonal, 1:2,000; Sigma) and DNMT1 (mouse monoclonal, 1:650; AbCam). After washing with PBST, the blot was incubated with secondary antibody (horseradish peroxidase [HRP]-conjugated anti-mouse antibody, 1:2,000 for alpha-tubulin, 1:5,000 for DNMT1; GE). The blot was visualized using chemiluminescence (ECL Plus; GE). Quantification was performed using the ImageJ software program.

Total RNA quantification. Quantification of RNA levels was conducted on E9.5 embryonic and placental cDNA using the LightCycler real-time PCR system (Roche). cDNA was added to a Ready-To-Go PCR bead (General Electric) along with sterile H₂O, 0.10 to 0.45 μM primers, 0.38 μl TaqStart antibody (Clontech), 2 to 4.5 mM MgCl₂ (Roche), and 1× EvaGreen (Biotium, Inc.). PCR conditions were as described previously (38) with the following modifications. For *Cdkn1c*, the forward primer was 5'GCCAATGCGAACGGTGGCG3'; the reverse primer and PCR product size were unchanged. For *Igf2*, the forward primer was 5'CGCTTCGTTTGTCTGTTCG3'; the reverse primer was 5'GCA GCACTCTCCACGATG3', and the product size was 94 bp. For *Peg3*, the forward primer was 5'ATGCCCCACTCCGTCACGCG3'; the reverse primer was 5'GCTCATCCTTGTGAACCTTG3', and the product size was 487 bp. For *Zim1*, the forward primer was 5'CTCAAGCAGAGCACAAAGC3'; the reverse primer was 5'GTGGCACACGAAAGGTTTCTC3', and the product size was 490 bp. The housekeeping genes used were *Arpp0* (acidic phosphoprotein P0 subunit) (45) and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) (38). Samples were set up in triplicate and analyzed with the LightCycler 4.0 software. Concentrations of imprinted genes were calculated relative to the geometric mean of the concentrations of *Arpp0* and *Gapdh*. To control for variability between runs, quantification was performed on a standard cDNA sample included in each run; all samples were normalized to this control. Because

EvaGreen binds nonspecifically to double-stranded DNA, a dissociation curve step was included to determine the specificity of PCR amplification and only samples with a single peak were included in analysis. For primer sets that did not cross exon boundaries, a minus reverse transcriptase (RT) control was included to ensure there was no genomic DNA contamination.

Allele-specific expression analysis. Allele-specific *H19* and *Snrpn* expression assays were conducted on E9.5 embryonic and placental cDNA using the Light-Cycler real-time PCR System (33, 34). For *Ascl2*, 0.3 μ M (each) primer (34) and α -³²P-labeled dCTP (1 μ Ci) were added to a Ready-To-Go PCR bead (General Electric). PCR amplification was performed for 30 cycles, and the PCR product was digested with *Sfi*I and then resolved on a 7% polyacrylamide gel as described previously (33, 34). After exposure (approximately 15 h), the relative band intensities were quantified using ImageQuant (Molecular Dynamics). For *Igf2*, PCR amplification and restriction digestion were performed as previously described (42), except that α -³²P-labeled dCTP was not included and ethidium bromide-stained polyacrylamide gels were imaged and quantified using a digital gel documentation system (Bio-Rad). For *Cdkn1c*, *Kcnq1*, *Kcnqlot1*, *Peg3*, and *Zim1*, primers and LightCycler PCR amplification conditions were as described above, but amplification was terminated in the linear range of the plot of fluorescence versus cycle number. The PCR product was restriction digested, resolved on a polyacrylamide gel, and imaged and quantified using a digital gel documentation system (Bio-Rad) as described previously (38) with the modifications described above for relative quantification, as well as the following. *Kcnq1* PCR product was digested with *Bsm*FI. The B6 allele was undigested (189 bp), and the CAST allele produced 113-bp and 76-bp fragments. The *Peg3* PCR product was digested with *Mn*II. The B6 allele produced 377-bp and 110-bp fragments, and the CAST allele was undigested (487 bp). The *Zim1* PCR product was digested with *Fau*I. The B6 allele was undigested (490 bp), and the CAST allele produced 254-bp and 236-bp fragments. Samples that had 10% or more expression from the repressed allele were considered biallelic.

Statistical analysis. Statistical analysis was performed using the PRISM software program. To test for differences in the mean of relative expression between samples, one-way analysis of variance (ANOVA) and Tukey's posttest were used. To test for differences in allelic expression between genotypes, samples were designated as having either monoallelic (0) or biallelic (1) expression of a gene. Because percent data are not normally distributed, an arcsine transformation was applied to these values. One-way analysis of variance and Tukey's posttest were then used.

RESULTS

Expression analysis of imprinted genes in *Dnmt1* mutant embryos and placentas. To study the effects of loss of DNMT1 function on genomic imprinting, we made use of the null *Dnmt1*^c allele (24) and the less-severe *Dnmt1*ⁿ allele (29). These mutations were bred into two strain backgrounds: C57BL/6 [B6] and C57BL/6(CAST7) [C7], which contained chromosome 7s from the *Mus musculus castaneus* strain in a B6 background (34). Crosses between heterozygous mice from each strain produced F₁ hybrid embryos and placentas that were collected at E9.5 (Fig. 1B). Reciprocal crosses were performed to control for the effects of strain differences in expression. The few strain-specific effects observed are discussed below. Homozygosity for *Dnmt1*ⁿ and *Dnmt1*^c causes embryonic lethality prior to E12.5 (24, 29). In similarity to previous studies, we observed that at E9.5, *Dnmt1*^{n/n} embryos were smaller than their littermates. *Dnmt1*^{n/n} embryos from the B6 $\times$ C7 cross (note that the female parent is written first) were phenotypically E8.5 in appearance and were unturned, while those from the C7 $\times$ B6 cross were phenotypically E8.5 with the majority of embryos half-turned. To characterize the severity of the defect in our *Dnmt1*^{n/n} embryos, we performed Western blot analysis (Fig. 1C). We found that the *Dnmt1*^{n/n} embryos and placentas analyzed had 24% and 35% DNMT1 protein compared to their wild-type counterparts, respectively (Fig. 1C). This is in contrast to previous articles, which reported a more substantial reduction in protein in ESCs (24,

29). *Dnmt1*^{c/c} embryos from the B6 $\times$ C7 cross were phenotypically late head-fold stage to E8.0, with no somites. Additional embryos were in the process of resorbing. *Dnmt1*^{c/c} embryos from the C7 $\times$ B6 cross were phenotypically E8.0 to E8.5. None of the analyzed conceptuses were necrotic.

Embryos and placentas were analyzed for the expression of imprinted genes on chromosome 7. This chromosome contains many imprinted genes, most of which are organized into four large clusters (Fig. 1A). Maternally and paternally expressed genes from these domains were selected for analysis. Relative RNA levels were measured by quantitative real-time PCR. To control for any effects of the *Dnmt1* mutations on housekeeping gene expression, expression of imprinted genes was normalized to the geometric mean of the concentrations of two housekeeping genes, *acidic phosphoprotein P0 subunit (Arpp0)* and *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)*. Allele-specific expression was measured by RT-PCR amplification followed by digestion at restriction enzyme sites that are polymorphic between the two mouse strains or by melting-curve analysis using probes that bind with different affinities to the two alleles. Samples that had 10% or more expression from the repressed allele were considered biallelic. As anticipated, we found substantial expression changes in *Dnmt1* mutants. To ensure that changes in imprinted gene expression were not the result of developmental delay, wild-type embryos and extraembryonic tissues were collected at E6.5, E7.5, E8.5, and E9.5 and included in the allele-specific expression analyses described.

Loss of imprinting in the *Igf2* domain in *Dnmt1* mutants. The *Igf2* domain includes the maternally expressed *H19* RNA and the paternally expressed growth factor *Igf2*, which are jointly regulated by a paternally methylated ICR and shared enhancers (Fig. 1A). *H19* mRNA was previously reported to exhibit increased abundance in *Dnmt1*^{n/n} (28) and *Dnmt1*^{s/s} embryos (18), with biallelic expression demonstrated in the former (28). We observed biallelic *H19* expression in *Dnmt1*^{n/n} embryos and in *Dnmt1*^{c/c} embryos, confirming these earlier reports (Fig. 2 and Tables 1 to 4). Furthermore, C7 $\times$ B6 *Dnmt1*^{n/n}, B6 $\times$ C7 *Dnmt1*^{c/c}, and C7 $\times$ B6 *Dnmt1*^{c/c} placentas demonstrated a loss of imprinting (Fig. 2 and Tables 1 to 4). *H19* was monoallelically expressed in wild-type embryos between E6.5 and E9.5 and in wild-type extraembryonic tissues between E7.5 and E9.5 (data not shown), so biallelic expression in mutants was not due to developmental delay. It was previously reported that *Dnmt1*^{+/-} embryos occasionally displayed biallelic *H19* expression (8). In this study, we also observed biallelic *H19* expression in a substantial number of B6 $\times$ C7 *Dnmt1*^{+/-} embryos (4/9) and placentas (7/9) (Table 1). Heterozygotes from the reciprocal cross retained monoallelic expression of *H19*, suggesting that the paternal allele is more easily reactivated when it is derived from the *Mus musculus castaneus* strain than when it is derived from B6. Paradoxically, *Dnmt1*^{+/-} conceptuses had monoallelic *H19* expression, suggesting that the LOI in the former is related to the deletion within the N-terminal domain of the protein in the *Dnmt1*ⁿ mutation.

Conversely, *Igf2* was shown to be silenced in *Dnmt1*^{n/n} and *Dnmt1*^{s/s} embryos (18, 28). Likewise, our analysis of relative mRNA levels demonstrated a loss of *Igf2* expression in B6 $\times$ C7 *Dnmt1*^{n/n} embryos and placentas, B6 $\times$ C7 *Dnmt1*^{c/c} pla-

FIG. 1. Experimental design and characterization of *Dnmt1*ⁿ. (A) Imprinted gene domains on chromosome 7. Genes that were analyzed are boxed. Imprinted genes are shaded. The transcriptional and methylation statuses of the maternal and paternal chromosomes are represented on the top and bottom of the DNA strand, respectively. (B) Breeding scheme and embryo and placenta collection. Note that the female parent is written first in crosses. (C) Analysis of the DNMT1 protein in *Dnmt1*ⁿ mutants. Top, Western blot of individual embryos and placentas with antibodies against DNMT1 and alpha-tubulin. Bottom: quantification of Western blot. DNMT1 levels are normalized to that of alpha-tubulin. DNMT1 levels in *Dnmt1*^{+/-} and *Dnmt1*^{n/n} embryos and placentas are expressed relative to those in *Dnmt1*^{+/+} embryos and placentas, respectively.

centas, and C7 × B6 *Dnmt1*^{c/c} embryos relative to their wild-type counterparts (Fig. 3A). Allele-specific analysis demonstrated that the little remaining *Igf2* mRNA in mutant samples was often biallelic (Fig. 2 and Tables 1 to 4), likely reflecting low-level basal transcription from both alleles. These results confirm that imprinted expression of *H19* and *Igf2* is dependent on DNMT1 in the embryo and demonstrate that the same is true in the placenta.

FIG. 2. Changes in allele-specific expression of imprinted genes in *Dnmt1* mutants. Results of statistical analysis of data in Tables 1 to 4. Black, a significant difference in the proportion of samples of the two genotypes with biallelic expression based on ANOVA ($P < 0.05$) and Tukey's posttest. White, no significant difference in the proportion of samples of the two genotypes with biallelic expression based on ANOVA ($P > 0.05$). M/B, all samples of one genotype had

Coordinate regulation of genes in the *Peg3* domain. The *Peg3* domain, which is located at the proximal end of mouse chromosome 7 and harbors at least six imprinted genes over a 320-kb region (Fig. 1A), has not been previously examined in *Dnmt1* mutants. Allele-specific expression analysis revealed that the two genes assayed in this domain, *Peg3* and *Zim1*, experienced LOI in *Dnmt1* mutant tissues. *Peg3* was expressed primarily from the paternal allele in all wild-type and heterozygous embryos and placentas. In contrast, all *Dnmt1^{n/n}* and *Dnmt1^{c/c}* embryos and placentas exhibited biallelic *Peg3* expression (Fig. 2 and Tables 1 to 4). This biallelic expression cannot be explained by developmental delay, since *Peg3* was monoallelically expressed between E7.5 and E9.5 (data not shown). LOI was not always accompanied by a change in the total amount of *Peg3* mRNA, but when the total amount of mRNA changed, it increased (Fig. 3B). There was a significant increase in *Peg3* expression in B6 $\times$ C7 *Dnmt1^{n/n}* placentas and C7 $\times$ B6 *Dnmt1^{c/c}* embryos compared to that for their wild-type and heterozygous counterparts. Possible explanations for why so few crosses produced a significant difference in *Peg3* expression between genotypes include developmental delay in mutants, which may have altered *Peg3* expression independently of LOI and obscured the effect of DNMT1 mutation on *Peg3* expression levels, or a tissue distribution in mutants different from that in wild-type samples. A smaller proportion of *Peg3*-expressing cells in mutants would counteract any increase due to biallelic expression. Nevertheless, these results demonstrate that imprinting of *Peg3* is dependent on DNMT1 and that DNA methylation primarily serves to prevent expression of the maternal allele, which is derepressed in mutants.

Analysis of the maternally expressed *Zim1* gene revealed decreased expression in mutant samples. B6 $\times$ C7 *Dnmt1^{n/n}* embryos, C7 $\times$ B6 *Dnmt1^{n/n}* embryos, B6 $\times$ C7 *Dnmt1^{c/c}* placentas, and C7 $\times$ B6 *Dnmt1^{c/c}* placentas exhibited significantly reduced *Zim1* mRNA levels relative to those of their wild-type counterparts (Fig. 3C). Furthermore, while *Zim1* was imprinted in wild-type and heterozygous samples, the little *Zim1* mRNA remaining in *Dnmt1* mutant samples was transcribed from both parental alleles (Fig. 2 and Tables 1 to 4). As with *Igf2*, we explain this finding as the result of basal expression of the two alleles. *Zim1* was monoallelically expressed in all E6.5 to E9.5 wild-type samples (data not shown). These results demonstrate that expression of the maternal allele of *Zim1* is dependent on the function of DNMT1. The coordinate expression of these two genes in the *Peg3* domain is analogous to what was observed for *H19* and *Igf2* above, strongly suggesting that *Peg3* and *Zim1* are also coregulated.

Incomplete loss of imprinting in the *Kcnq1* domain in *Dnmt1* mutants. The *Kcnq1* cluster is adjacent to the *Igf2* domain and contains 11 imprinted genes over an approximately 1-Mb region (Fig. 1A). This cluster has been the most extensively

biallelic expression, while all samples of the other genotype had monoallelic expression. However, due to a lack of variability, we were unable to perform a statistical analysis. M/M, all samples of both genotypes had monoallelic expression. However, due to a lack of variability, we were unable to perform a statistic analysis. X, not expressed in this tissue at this time.

TABLE 1. Allele-specific expression of imprinted genes in B6 *Dnmt1*^{+/n} × C7 *Dnmt1*^{+/n} samples

Sample	% of RNA derived from normally repressed allele ^c								
	<i>Zim1</i> ^a	<i>Peg3</i> ^b	<i>Snrpn</i> ^b	<i>H19</i> ^a	<i>Igf2</i> ^b	<i>Ascl2</i> ^a	<i>Kcnq1</i> ^a	<i>Kcnq1ot1</i> ^b	<i>Cdkn1c</i> ^a
Embryos									
<i>Dnmt1</i> ^{+/+}									
2439 E7	0.0	0.0	0.0	0.0	2.9	NE	0.0	4.1	0.0
E1	0.0	0.0	0.0	0.0	1.0	NE	0.0	0.0	0.0
E2	0.0	0.0	0.0	0.0	3.1	NE	0.0	0.0	0.0
E8	0.0	0.0	0.0	0.0	4.8	NE	<u>23.4</u>	0.0	0.0
<i>Dnmt1</i> ^{+/n}									
2439 E1	0.0	0.0	0.0	<u>43.5</u>	5.0	NE	0.0	3.1	0.0
2459 E2	0.0	0.0	0.0	<u>29.0</u>	2.7	NE	0.0	6.1	0.0
2603 E5	0.0	0.0	—	—	3.7	NE	0.0	2.7	ND
2439 E2	0.0	0.0	—	—	4.8	NE	0.0	1.7	0.0
2459 E3	—	—	0.0	<u>15.3</u>	—	NE	—	—	—
E3	—	—	—	0.0	—	NE	—	—	—
E4	—	—	—	0.0	—	NE	—	—	—
E5	—	—	—	0.0	—	NE	—	—	—
E6	—	—	—	0.0	—	NE	—	—	—
E7	—	—	—	0.0	—	NE	—	—	—
E8	—	—	—	<u>13.6</u>	—	NE	—	—	—
<i>Dnmt1</i> ^{n/n}									
2439 E6	<u>28.0</u>	<u>41.8</u>	<u>27.9</u>	<u>47.2</u>	1.1	NE	<u>59.6</u>	<u>50.8</u>	7.2
2459 E7	<u>17.7</u>	<u>42.0</u>	<u>39.8</u>	<u>47.3</u>	0.6	NE	<u>32.5</u>	<u>47.2</u>	<u>19.1</u>
2459 E8	<u>14.7</u>	<u>38.7</u>	<u>21.4</u>	<u>46.5</u>	<u>24.5</u>	NE	<u>34.8</u>	<u>57.0</u>	<u>23.7</u>
2603 E6	<u>12.8</u>	<u>43.6</u>	—	—	<u>40.2</u>	NE	<u>72.0</u>	<u>41.7</u>	0.0
Placentas									
<i>Dnmt1</i> ^{+/+}									
2439 P7	9.7	0.0	0.0	0.0	9.4	1.4	0.0	4.0	0.0
P1	4.0	0.0	0.0	0.0	6.3	0.0	<u>14.6</u>	3.4	0.0
P2	4.0	0.0	0.0	0.0	4.5	0.0	0.0	4.1	0.0
P8	3.3	0.0	0.0	<u>28.2</u>	6.0	0.0	<u>12.4</u>	2.8	0.0
<i>Dnmt1</i> ^{+/n}									
2439 P1	6.4	<u>10.1</u>	0.0	0.0	0.0	5.4	<u>20.5</u>	1.9	0.0
2459 P2	8.5	0.0	0.0	0.0	7.2	5.9	0.0	6.3	2.7
2603 P5	1.2	5.1	0.0	—	9.8	0.0	0.0	3.8	5.7
2439 P2	2.6	0.0	0.0	—	4.8	—	<u>13.1</u>	2.6	0.0
2459 P3	—	—	—	<u>17.3</u>	—	4.5	—	—	—
P3	—	—	—	<u>21.9</u>	—	—	—	—	—
P4	—	—	—	<u>31.1</u>	—	—	—	—	—
P5	—	—	—	<u>30.3</u>	—	—	—	—	—
P6	—	—	—	<u>34.3</u>	—	—	—	—	—
P7	—	—	—	<u>30.9</u>	—	—	—	—	—
P8	—	—	—	<u>30.3</u>	—	—	—	—	—
<i>Dnmt1</i> ^{n/n}									
2439 P6	<u>23.9</u>	<u>41.3</u>	<u>39.8</u>	<u>26.8</u>	<u>10.9</u>	<u>79.3</u>	0.0	<u>31.4</u>	7.5
2459 P7	<u>29.2</u>	<u>37.0</u>	<u>45.5</u>	<u>40.2</u>	<u>12.6</u>	6.4	0.0	<u>21.5</u>	5.1
2459 P8	<u>18.7</u>	<u>39.6</u>	<u>36.3</u>	<u>40.2</u>	<u>18.1</u>	<u>13.0</u>	0.0	<u>25.9</u>	<u>11.2</u>
2603 P6	<u>26.1</u>	<u>42.8</u>	<u>41.6</u>	—	<u>10.6</u>	0.0	<u>16.4</u>	<u>27.2</u>	<u>10.1</u>

^a Percentage of total RNA derived from the repressed paternal allele.^b Percentage of total RNA derived from the repressed maternal allele.^c Underlining indicates biallelic expression. ND, no expression detected for this gene in this sample; NE, not expressed at this stage; —, not assayed.

studied in *Dnmt1* mutants and contains the placentally imprinted genes that are postulated to be regulated independently of somatic DNMT1 (6, 27, 46). Our goals in analyzing genes in this domain were to validate this observation in our mice and to expand these studies to include the *Dnmt1*ⁿ allele. We found that the genes in this domain had much more variable responses to the loss of DNMT1 function than other genes we had studied.

In previous studies of *Dnmt1*^{c/c} conceptuses, *Kcnq1ot1* expression increased in embryos and became biallelic in embryos and placentas (15, 27). In this study, we found that *Kcnq1ot1* expression was significantly increased only in C7 × B6

Dnmt1^{c/c} embryos relative to that of their wild-type counterparts (Fig. 3D). As with *Peg3*, this could be due to differences in age or tissue distribution between genotypes. In contrast, the allele-specific results were dramatic. All wild-type samples and all but two heterozygous samples were monoallelically expressed, while all *Dnmt1*^{n/n} and *Dnmt1*^{c/c} samples exhibited LOI (Fig. 2 and Tables 1 to 4). Our analysis of wild-type conceptuses between E7.5 and E9.5 revealed mainly monoallelic *Kcnq1ot1* expression, although a proportion of samples had baseline levels of expression of slightly greater than 10% from the maternal allele (10 to 16%; data not shown). However, this cannot account for the consistent, nearly equal ex-

TABLE 2. Allele-specific expression of imprinted genes in C7 *Dnmt1*^{+/n} × B6 *Dnmt1*^{+/n} samples

Sample	% of RNA derived from normally repressed allele ^c								
	<i>Zim1</i> ^a	<i>Peg3</i> ^b	<i>Snrpn</i> ^b	<i>H19</i> ^a	<i>Igf2</i> ^b	<i>Ascl2</i> ^a	<i>Kcnq1</i> ^a	<i>Kcnq1ot1</i> ^b	<i>Cdkn1c</i> ^a
Embryos									
<i>Dnmt1</i> ^{+/+}									
1206 E3	8.5	0.0	0.0	0.0	0.0	NE	0.0	0.0	1.7
5124 E1	0.0	0.0	0.0	0.0	0.0	NE	0.0	0.0	0.0
865 E2	0.0	0.0	0.0	2.8	0.0	NE	0.0	0.0	0.0
865 E3	0.0	0.0	0.0	0.0	0.0	NE	0.0	0.0	0.0
<i>Dnmt1</i> ^{+/<i>n</i>}									
1206 E1	0.0	0.0	0.0	0.0	0.0	NE	0.0	0.0	0.8
1206 E2	1.7	0.0	0.0	0.0	0.0	NE	0.0	0.0	0.0
1434 E5	8.6	0.0	—	—	0.0	NE	0.0	0.0	0.0
1434 E9	5.7	0.0	—	—	0.0	NE	0.0	0.0	0.0
1434 E7	—	—	0.0	0.0	—	NE	—	—	—
<i>Dnmt1</i> ^{<i>n</i>/<i>n</i>}									
1206 E7	—	68.8	53.3	41.6	20.0	NE	0.0	53.1	2.2
1206 E8	54.0	65.8	49.2	41.4	20.0	NE	25.4	71.0	4.6
1440 E3	13.2	66.0	—	—	18.4	NE	14.7	36.3	8.3
1265 E4	34.6	68.6	—	—	13.9	NE	0.0	46.2	9.8
1434 E6	—	—	27.5	44.8	—	NE	—	—	—
Placentas									
<i>Dnmt1</i> ^{+/+}									
1206 P3	7.0	0.0	0.0	0.0	—	2.9	5.5	0.0	2.2
5124 P1	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0	0.0
865 P2	0.0	0.0	0.0	0.0	0.0	26.2	0.0	0.0	0.0
865 P3	0.0	0.0	0.0	8.8	0.0	9.9	0.0	0.0	0.0
<i>Dnmt1</i> ^{+/<i>n</i>}									
1206 P1	5.0	0.0	0.0	0.0	0.0	4.9	0.0	0.0	1.6
1206 P2	7.8	0.0	0.0	0.0	0.0	10.7	0.0	18.0	4.8
1434 P5	4.7	0.0	0.0	—	0.0	—	0.0	0.0	4.5
1434 P9	8.0	0.0	0.0	—	0.0	3.4	0.0	0.0	0.0
865 P2	—	—	—	—	0.0	—	—	—	—
1434 P7	—	—	—	0.0	—	6.6	—	—	—
<i>Dnmt1</i> ^{<i>n</i>/<i>n</i>}									
1206 P7	25.0	46.1	0.0	28.2	0.0	15.6	0.0	20.7	8.2
1206 P8	29.9	57.5	46.6	39.0	ND	21.6	0.0	18.1	5.4
1440 P3	37.8	51.0	48.3	—	0.0	6.9	0.0	20.0	0.0
1265 P4	56.3	52.4	40.5	—	ND	—	0.0	39.4	7.2
1434 P6	—	—	—	37.3	—	4.1	—	—	—

^a Percentage of total RNA derived from the repressed paternal allele.^b Percentage of total RNA derived from the repressed maternal allele.^c Underlining indicates biallelic expression. ND, no expression detected for this gene in this sample; NE, not expressed at this stage; —, not assayed.

pression of the two alleles seen in *Dnmt1* mutants. In addition, there was a difference in the response to the two mutations between the embryo and the placenta. *Dnmt1*^{c/c} and *Dnmt1*^{n/n} embryos displayed similar LOI with roughly equal expression of the two parental alleles. In contrast, *Dnmt1*^{n/n} placentas showed less activation from the silent allele than *Dnmt1*^{c/c} placentas (compare Tables 1 and 2 to Tables 3 and 4), suggesting that the placenta is less sensitive than the embryo to a loss of DNMT1 function. These results demonstrate that imprinting of *Kcnq1ot1* is dependent on DNMT1, which primarily represses expression of the maternal allele, and that the embryo is more sensitive than the placenta to a loss of DNMT1 function.

Ascl2, the first gene identified that remained imprinted in the absence of somatic DNMT1 function (6), is maternally transcribed in the placenta but not expressed in the embryo at E9.5 (6, 16, 40). Caspary et al. reported no change in its expression in *Dnmt1*^{s/s} embryos (6), while Tanaka et al. reported a slight increase in the number of cells with an active paternal allele in *Dnmt1*^{n/n} and *Dnmt1*^{s/s} embryos but not

enough to eliminate the maternal bias of transcription (40). We found that *Ascl2* allelic expression was heterogeneous. In B6 × C7 placentas, wild-type and heterozygous samples had monoallelic expression. All B6 × C7 *Dnmt1*^{c/c} placentas likewise had monoallelic expression. Two *Dnmt1*^{n/n} placentas had biallelic expression of *Ascl2*, but this did not produce a statistical difference in the number of samples with biallelic expression between genotypes (Fig. 2). It was possible that the few samples that did have biallelic expression were due to developmental delay, since it has been observed that *Ascl2* is biallelically expressed earlier in development and the paternal allele is not fully repressed until around E9.5 (6, 16, 40). The exact timing could vary by strain, so we analyzed *Ascl2* expression in B6 × C7 wild-type placentas between E6.5 and E9.5. We found that *Ascl2* was monoallelically expressed throughout this time period (data not shown), suggesting that delay was not the cause of biallelic expression in these samples, which may have been true LOI. In C7 × B6 placentas, in contrast, a number of wild-type and heterozygous E9.5 samples displayed biallelic *Ascl2* expression. One possible explanation for these

TABLE 3. Allele-specific expression of imprinted genes in B6 *Dnmt1*^{+/c} × C7 *Dnmt1*^{+/c} samples

Sample	% of RNA derived from normally repressed allele ^c								
	<i>Zim1</i> ^a	<i>Peg3</i> ^b	<i>Snrpn</i> ^b	<i>H19</i> ^a	<i>Igf2</i> ^b	<i>Ascl2</i> ^a	<i>Kcnq1</i> ^a	<i>Kcnq1ot1</i> ^b	<i>Cdkn1c</i> ^a
Embryos									
<i>Dnmt1</i> ^{+/+}									
2020 E3	0.0	0.0	0.0	0.0	0.0	NE	<u>11.1</u>	0.0	0.0
2017 E3	0.0	0.0	0.0	0.0	2.5	NE	0.0	5.6	0.0
2146 E2	0.0	0.0	0.0	0.0	1.1	NE	0.0	5.6	0.0
1 E1	0.0	0.0	0.0	0.0	1.1	NE	<u>43.3</u>	6.9	0.0
<i>Dnmt1</i> ^{+/c}									
2017 E1	0.0	0.0	0.0	0.0	0.2	NE	0.0	3.7	0.0
2148 E2	<u>19.0</u>	0.0	0.0	0.0	1.3	NE	0.0	3.6	0.0
2148 E9	1.9	0.0	0.0	—	2.6	NE	0.0	4.6	0.0
2148 E7	0.0	0.0	0.0	—	0.0	NE	0.0	3.1	0.0
1734 E5	—	—	—	0.0	—	NE	—	—	—
1734 E6	—	—	—	0.0	—	NE	—	—	—
<i>Dnmt1</i> ^{c/c}									
1734 E7	<u>70.1</u>	<u>36.0</u>	—	—	0.0	NE	<u>34.5</u>	<u>50.0</u>	<u>52.7</u>
2017 E5	<u>51.0</u>	<u>19.2</u>	—	—	15.6	NE	<u>44.9</u>	<u>28.6</u>	—
2017 E4	<u>59.9</u>	<u>36.1</u>	<u>52.5</u>	<u>55.2</u>	<u>39.1</u>	NE	<u>41.5</u>	<u>39.6</u>	<u>75.4</u>
2148 E4	<u>28.6</u>	<u>35.8</u>	<u>53.6</u>	<u>53.1</u>	<u>32.5</u>	NE	0.0	<u>38.8</u>	<u>37.0</u>
2148 E5	0.0	<u>43.1</u>	—	<u>54.6</u>	<u>74.3</u>	NE	—	<u>35.8</u>	<u>38.4</u>
1215 E5	—	—	<u>47.0</u>	—	—	NE	—	—	—
Placentas									
<i>Dnmt1</i> ^{+/+}									
2020 P3	0.0	0.0	—	0.0	0.0	0.5	0.0	3.8	0.0
2017 P3	2.0	0.0	0.0	<u>18.1</u>	7.4	0.0	<u>25.3</u>	5.1	0.0
2146 P2	3.2	0.0	0.0	0.0	7.3	0.0	0.0	2.5	2.5
1 P1	1.5	0.0	0.0	2.0	8.3	0.0	0.0	5.0	0.8
<i>Dnmt1</i> ^{+/c}									
2017 P1	3.2	0.0	0.0	0.0	8.2	2.0	<u>43.1</u>	<u>56.5</u>	0.0
2148 P2	0.0	0.0	0.0	0.0	0.0	7.0	0.0	4.3	5.4
2148 P9	0.0	0.0	0.0	—	4.4	0.0	0.0	3.0	1.2
1734 P5	—	—	—	0.0	—	3.0	—	—	—
1734 P6	—	—	—	0.0	—	4.1	—	—	—
<i>Dnmt1</i> ^{c/c}									
2017 P4	<u>100.0</u>	<u>40.3</u>	<u>32.8</u>	<u>56.7</u>	<u>69.5</u>	8.9	0.0	<u>53.6</u>	<u>45.8</u>
2148 P4	ND	<u>43.2</u>	<u>38.8</u>	<u>48.7</u>	0.0	0.4	0.0	<u>71.6</u>	<u>46.0</u>
2148 P5	<u>100.0</u>	<u>29.8</u>	—	<u>37.6</u>	<u>10.4</u>	4.0	0.0	<u>44.1</u>	<u>42.3</u>
1215 P5	—	—	<u>44.6</u>	—	—	—	—	—	—

^a Percentage of total RNA derived from the repressed paternal allele.^b Percentage of total RNA derived from the repressed maternal allele.^c Underlining indicates biallelic expression. ND, no expression detected for this gene in this sample. NE, not expressed at this stage. —, not assayed.

strain differences is that the B6 allele is more highly expressed than the *Mus musculus castaneus* allele, so that it is more difficult or takes longer to fully silence the paternal allele when it is B6. Only some *Dnmt1*^{n/n} and *Dnmt1*^{c/c} placentas had biallelic *Ascl2*, and there was no statistical difference in the fraction of placentas with biallelic *Ascl2* between genotypes in either case (Fig. 2). Our results, therefore, are consistent with the hypothesis that *Dnmt1* mutations do not affect *Ascl2* imprinting.

The maternally expressed *Kcnq1* gene was reported to have reduced, biallelic expression in *Dnmt1*^{c/c} embryos, while placentas had no change in total expression and only one in three had LOI (15, 27). *Kcnq1* was also shown to be silenced in *Dnmt1*^{s/s} embryos and ectoplacental cones (6). We found that *Kcnq1* expression levels were unchanged in most mutant tissues, with the exception of C7 × B6 *Dnmt1*^{c/c} placentas, where *Kcnq1* mRNA was significantly reduced compared to that of the wild type (Fig. 3E). Our results from the allele-specific assay revealed variability in imprinted expression (Tables 1 to 4). In many cases, wild-type and heterozygous samples had

biallelic *Kcnq1* expression, while many mutant samples remained imprinted. The variability in imprinting status among wild-type embryos can likely be attributed to the changes in expression *Kcnq1* undergoes during development. *Kcnq1* has been reported to cease being imprinted around E9.5 (6, 14, 21, 27). In wild-type B6 × C7 samples collected for our time course analysis, we found that *Kcnq1* was monoallelically expressed in wild-type embryos and placentas at E7.5, E8.5, and E9.5 (data not shown). It is likely that the transition from monoallelic to biallelic *Kcnq1* expression occurs around E9.5 and the wild-type and heterozygous samples we found to have biallelic expression were slightly older than those with monoallelic expression or else transitioned to biallelic expression slightly earlier. *Kcnq1* was biallelically expressed in the majority of *Dnmt1*^{n/n} and *Dnmt1*^{c/c} embryos (Tables 1 to 4), though there was a significant difference in the proportion of biallelic embryos only when comparing B6 × C7 *Dnmt1*^{n/n} samples to their wild-type and heterozygous counterparts (Fig. 2). Only one B6 × C7 *Dnmt1*^{n/n} and one C7 × B6 *Dnmt1*^{c/c} placenta had

TABLE 4. Allele-specific expression of imprinted genes in C7 *Dnmt1*^{+/c} × B6 *Dnmt1*^{+/c} samples

Sample	% of RNA derived from normally repressed allele ^c								
	<i>Zim1</i> ^a	<i>Peg3</i> ^b	<i>Snrpn</i> ^b	<i>H19</i> ^a	<i>Igf2</i> ^b	<i>Ascl2</i> ^a	<i>Kcnq1</i> ^a	<i>Kcnq1ot1</i> ^b	<i>Cdkn1c</i> ^a
Embryos									
<i>Dnmt1</i> ^{+/+}									
1899 E7	0.0	3.2	0.0	0.0	0.0	NE	4.3	0.0	0.0
1801 E7	0.0	4.7	0.0	0.0	0.0	NE	0.0	0.0	0.0
1801 E1	0.0	3.4	0.0	0.0	0.0	NE	<u>27.5</u>	0.0	0.0
1801 E5	0.0	6.8	0.0	0.0	0.0	NE	<u>0.0</u>	0.0	0.0
1899 E6	—	—	0.0	0.0	—	—	—	—	—
<i>Dnmt1</i> ^{+/c}									
1581 E3	0.0	4.6	0.0	0.0	0.0	NE	0.0	0.0	0.0
1899 E2	0.0	7.1	0.0	0.0	0.0	NE	4.9	0.0	0.0
1899 E4	0.0	5.8	—	—	0.0	NE	<u>25.0</u>	0.0	0.0
1899 E3	0.0	<u>10.9</u>	0.0	0.0	0.0	NE	0.0	0.0	0.0
<i>Dnmt1</i> ^{c/c}									
1595 E5	<u>34.8</u>	<u>64.0</u>	—	<u>36.6</u>	<u>51.2</u>	NE	<u>31.4</u>	<u>55.3</u>	<u>45.5</u>
1899 E1	—	—	<u>43.6</u>	<u>44.3</u>	—	NE	—	—	<u>82.0</u>
1581 E2	—	—	<u>37.6</u>	<u>43.1</u>	—	NE	—	—	<u>41.2</u>
1581 E8	—	—	<u>50.9</u>	<u>44.3</u>	—	NE	—	—	0.0
978 E4	3.6	<u>70.5</u>	—	—	<u>61.0</u>	NE	<u>59.3</u>	<u>40.4</u>	—
978 E5	<u>57.8</u>	<u>71.3</u>	—	—	<u>46.2</u>	NE	<u>66.6</u>	<u>43.0</u>	—
Placentas									
<i>Dnmt1</i> ^{+/+}									
1899 P7	0.0	0.0	0.0	0.0	1.0	—	8.9	0.0	0.0
1801 P7	0.0	0.0	0.0	0.0	1.6	<u>13.1</u>	0.0	0.0	0.0
1801 P1	0.0	0.0	0.0	0.0	2.9	0.0	0.0	0.0	0.0
1801 P5	0.0	0.0	—	0.0	0.0	9.4	0.0	0.0	0.0
1899 P6	—	—	—	0.0	—	3.3	—	—	—
<i>Dnmt1</i> ^{+/c}									
1581 P3	0.0	0.0	0.0	0.0	1.5	<u>18.6</u>	9.9	0.0	0.0
1899 P2	0.0	0.0	0.0	<u>24.8</u>	1.3	0.0	5.2	8.6	0.0
1899 P4	0.0	0.0	0.0	—	0.0	0.0	0.0	0.0	0.0
1899 P3	0.0	0.0	0.0	0.0	0.0	<u>14.4</u>	0.0	4.7	0.0
<i>Dnmt1</i> ^{c/c}									
1595 P5	<u>58.8</u>	<u>59.0</u>	<u>52.7</u>	—	<u>35.9</u>	<u>18.5</u>	0.0	<u>57.6</u>	<u>44.5</u>
1899 P1	<u>61.3</u>	<u>65.4</u>	<u>47.7</u>	<u>39.7</u>	ND	<u>35.7</u>	0.0	<u>52.0</u>	<u>40.2</u>
1581 P2	<u>78.1</u>	<u>58.0</u>	<u>51.2</u>	<u>56.0</u>	<u>34.0</u>	<u>41.6</u>	<u>38.2</u>	<u>58.1</u>	<u>37.0</u>
1581 P8	<u>67.8</u>	<u>60.7</u>	<u>56.3</u>	<u>50.8</u>	<u>38.2</u>	8.3	0.0	<u>58.7</u>	<u>35.0</u>

^a Percentage of total RNA derived from the repressed paternal allele.^b Percentage of total RNA derived from the repressed maternal allele.^c Underlining indicates biallelic expression. ND, no expression detected for this gene in this sample. NE, not expressed at this stage. —, not assayed.

biallelic *Kcnq1* expression (Table 1–4). As a result, there was no significant difference between genotypes in the fraction of placentas with biallelic *Kcnq1* resulting from any of the four crosses (Fig. 2). Our results, therefore, suggest that maintenance of imprinting of *Kcnq1* does not require DNA methylation in the placenta and that methylation is possibly dispensable in the embryo as well. Given the variability of the imprinting status of this gene in wild-type samples at this stage, however, a more extensive analysis with more samples will be needed to resolve the question.

Cdkn1c was previously reported to be biallelically expressed in *Dnmt1*^{+/s} embryos and ectoplacental cones (6) and to have reduced (15) but biallelic (3, 27) expression in *Dnmt1*^{c/c} embryos and placentas. We found that *Cdkn1c* mRNA was significantly reduced in B6 × C7 *Dnmt1*^{n/n} embryos, C7 × B6 *Dnmt1*^{n/n} embryos, B6 × C7 *Dnmt1*^{c/c} embryos and placentas, and C7 × B6 *Dnmt1*^{c/c} embryos and placentas (Fig. 3F) relative to their wild-type counterparts. Allele-specific analysis of *Dnmt1* mutant samples also revealed LOI of *Cdkn1c* (Fig. 2 and Tables 1 to 4). Interest-

ingly, comparison of the *Dnmt1*ⁿ and *Dnmt1*^c alleles revealed a difference in the effects of the two mutations. Nearly all *Dnmt1*^{c/c} embryos and placentas lost imprinting and had roughly equal expression of the two parental alleles (Fig. 2 and Tables 3 and 4). In contrast, there was no significant difference in the fraction of *Dnmt1*^{n/n} embryos or placentas with biallelic *Cdkn1c* expression compared to their wild-type and heterozygous counterparts (Fig. 2 and Tables 1 and 2). These results demonstrate that imprinting of *Cdkn1c* is more resistant to the effects of reduced DNMT1 than that of other genes in the domain. Interestingly, *Cdkn1c* is regulated by a paternally methylated differentially methylated region (DMR) at its promoter (3). This DMR acquires methylation during the postimplantation wave of *de novo* methylation between E6.5 and E9.5 (3). The action of *de novo* methyltransferases in this region may compensate for some loss of DNMT1 function. Alternatively, the *Dnmt1*ⁿ mutation may affect the specificity of the protein in such a way that it is still targeted to *Cdkn1c* but not to other genes. The difference is unlikely to be related to

FIG. 3. Changes in relative expression of imprinted genes in *Dnmt1* mutants. Mean *Igf2* (A), *Peg3* (B), *Zim1* (C), *Kcnq1ot1* (D), *Kcnq1* (E), or *Cdkn1c* (F) mRNA levels by genotype. Imprinted gene concentrations were normalized to a standard sample included in all runs and to the geometric mean of the concentrations of the housekeeping genes *Arpp0* and *Gapdh*. Finally, values were normalized to wild-type values within each cross and tissue. For each genotype, $n = 2$ to 5. +/+, *Dnmt1*^{+/+}; +/-, *Dnmt1*^{+/n} or *Dnmt1*^{+/c}; -/-, *Dnmt1*^{n/n} or *Dnmt1*^{c/c}. Error bars represent standard errors of the means. *, significant difference in the mean between genotypes based on one-way ANOVA ($P < 0.05$) and Tukey's posttest.

the greater developmental delay in *Dnmt1*^{c/c} conceptuses, since *Cdkn1c* is monoallelically expressed as early as E6.5 through to E9.5 (3) (data not shown). In sum, imprinting of *Cdkn1c* is dependent on DNMT1 but is largely resistant to

the effects of the *Dnmt1*ⁿ mutation. Overall, we found that genes in the *Kcnq1ot1* domain had much more variable responses to the loss of DNMT1 function than other genes we had studied.

Loss of imprinting of *Snrpn* in *Dnmt1* mutants. The *Snrpn* domain (Fig. 1A) contains a group of paternally expressed genes, including a putative long polycistronic transcript encoding *Snrpn*, a cluster of snoRNAs, and two maternally expressed genes (44). While previous studies had shown that *Snrpn* was monoallelically expressed in *Dnmt1^{c/c}* ESCs (50) and that expression of *Snrpn* was increased in *Dnmt1^{s/s}* embryos at E9.5 (39), the effects of *Dnmt1* mutations on *Snrpn* allelic expression have not been examined in E9.5 embryos or placentas. We found that *Snrpn* was biallelically expressed in *Dnmt1^{n/n}* and *Dnmt1^{c/c}* embryos and placentas (Fig. 2 and Tables 1 to 4). This biallelic expression was not due to developmental delay, since *Snrpn* was monoallelically expressed in wild-type conceptuses from E6.5 to E9.5 (data not shown). These results demonstrate that DNMT1 is necessary for monoallelic expression of at least one gene in this domain. When combined with the previous finding, these results suggest that DNA methylation serves primarily to repress expression of the maternal allele of *Snrpn*.

DISCUSSION

In this study, we undertook a systematic characterization of the effects of mutations in *Dnmt1* on the expression of imprinted genes on chromosome 7. With this approach, we compared the effects of two *Dnmt1* alleles on expression of imprinted genes, the effects of *Dnmt1* mutations on imprinted gene expression in the embryo versus the placenta, and the role of DNMT1 in imprinted domain regulation. Our results provide insight into the role of DNA methylation in the regulation of imprinting and how this role varies among genes, imprinted domains, and tissues.

Comparison of *Dnmt1* alleles reveals degrees of dependence on DNA methylation. Comparison of the two mutant alleles of *Dnmt1* revealed that the majority of imprinted genes are dependent on DNA methylation for their regulation. However, in accordance with the greater reduction in global DNA methylation reported in *Dnmt1^{c/c}* ESCs than in *Dnmt1^{n/n}* ESCs and the more severe phenotype produced in *Dnmt1^{c/c}* embryos than in *Dnmt1^{n/n}* embryos (24), *Dnmt1^{c/c}* samples had a more substantial change in the total expression of imprinted genes and/or more equivalent expression of the two parental alleles. For example, *Kcnq1* and *Kcnq1ot1* expression was significantly altered only in *Dnmt1^{c/c}* samples. More dramatically, *Dnmt1^{c/c}* samples had LOI of *Cdkn1c*, while *Dnmt1^{n/n}* embryos and placentas retained monoallelic *Cdkn1c* expression. Genes that displayed this differential response to *Dnmt1* mutations were less dependent on DNA methylation than those that were more severely affected by either mutation.

There are a few possible explanations for this differential response. One explanation is that these genes depend on a threshold level of methylation for proper expression and that only the *Dnmt1^c* allele causes methylation to drop below this level. This could depend on the number of methylated CpGs. Other epigenetic mechanisms may be able to compensate for a limited loss of methylation in *Dnmt1^{n/n}* samples but not in *Dnmt1^{c/c}* samples. *Cdkn1c* is notable since this gene was particularly resistant to the loss of DNMT1 function. The *Cdkn1c* promoter is the primary site of allele-specific DNA methylation in this domain outside of the *Kcnq1ot1* promoter and

acquires methylation between E6.5 and E9.5. This DMR may possess a distinct epigenetic signature that attracts *de novo* methylation and also allows the monoallelic expression of *Cdkn1c* in *Dnmt1^{n/n}* conceptuses. This could be through the action of the *de novo* methyltransferases keeping the DMR methylated despite a partial loss of DNMT1 function or because other chromatin factors, such as histone modifications, maintain proper expression. Support for the latter hypothesis comes from mutation studies of two chromatin remodelers, LSH and EED, both of which are required for imprinting of *Cdkn1c* but not *Kcnq1ot1* (11, 31, 35, 52). Despite these alternate mechanisms, our results indicate imprinted expression of *Cdkn1c* is dependent on DNA methylation, as indicated by LOI in *Dnmt1^{c/c}* samples.

Comparison of tissues reveals widespread dependence on DNMT1. Comparison of the effects of *Dnmt1* mutations on the embryo and placenta suggests that DNMT1-dependent regulation of imprinting in embryonic and extraembryonic tissues is generally the rule rather than the exception. Although embryonic and extraembryonic tissues typically responded similarly to mutations in *Dnmt1*, there were a few notable exceptions. One potential exception was *Kcnq1*, which did not exhibit LOI in mutant placentas; however, it is unclear whether imprinting was maintained in mutant embryos. A second exception was *Kcnq1ot1*, which was biallelically expressed in embryos with either mutation, while in the placenta only the null *Dnmt1^c* allele caused complete LOI. These results suggest that other regulatory mechanisms, present in the placenta but not in the embryo, may compensate for reduced DNMT1 function in *Dnmt1^{n/n}* placentas. For example, the *Kcnq1ot1* promoter is differentially marked by histone modifications on the two alleles (9, 15, 43, 45). Histone modifications have been proposed to maintain monoallelic expression of genes imprinted only in the placenta (15, 46). Although these modifications are not sufficient for imprinting maintenance of *Kcnq1ot1* in *Dnmt1^{c/c}* placentas, they may be able to maintain a paternal bias of expression in *Dnmt1^{n/n}* placentas.

Comparison of imprinted domains reveals different roles for DNA methylation. A major goal of this study was to compare the effects of *Dnmt1* mutations on the different domains of imprinted genes to gain insight into the regulatory mechanisms that maintain monoallelic gene expression. Similarities and differences in responses to loss of DNMT1 function were found among genes in different domains. We observed many similarities between the *Igf2* and *Peg3* domains. Generally, genes in these domains were expressed monoallelically in wild-type and heterozygous samples. This imprinting was entirely dependent on DNA methylation, as demonstrated by the consistent LOI of these genes in *Dnmt1^{n/n}* and *Dnmt1^{c/c}* samples. Another similarity between these two domains is the presence of inversely regulated pairs of genes. It was previously shown and has been confirmed here that *Igf2* and *H19*, which are expressed from opposite parental alleles, have the opposite responses to a loss of DNMT1 function (28). For *H19* and *Igf2*, methylation of the ICR determines which gene is able to access shared enhancers located downstream of *H19* (25). On the maternal allele, where *H19* is expressed, the insulator protein CTCF binds to the unmethylated ICR and prevents *Igf2* from accessing the enhancers, silencing it. On the paternal allele, methylation of the ICR prevents CTCF binding, allowing *Igf2*

exclusive access to the enhancers. In the absence of DNA methylation, the paternal allele acquires a maternal epigenotype; *H19* is biallelically expressed, and *Igf2* is silenced (28). Similar effects are noted in the *Peg3* domain. *Peg3* is upregulated and biallelically expressed, while *Zim1* is silenced. Little is known about how imprinting of the *Peg3* domain is regulated, but the similarities to the *Igf2* domain suggest that they may employ similar regulatory mechanisms. It will be interesting to determine whether any shared enhancers can be found in the *Peg3* domain.

The genes in the *Kcnq1* domain, in contrast, had more-complex responses to the *Dnmt1* mutations. Previous experiments suggested that maternal methylation of the ICR represses transcription of *Kcnq1ot1* and paternal transcription of this noncoding RNA (ncRNA) prevents paternal expression of the linked, maternally expressed genes (32). Therefore, loss of methylation should result in biallelic *Kcnq1ot1* expression and silencing of the other genes in the domain. Moreover, *Ascl2* would remain maternally expressed if its regulation were independent of DNA methylation. Many of these expectations were confirmed. *Kcnq1ot1* was biallelically expressed in mutants, indicating derepression of the maternal allele. *Kcnq1* and *Cdkn1c* mRNA levels were reduced. Nevertheless, all three genes produced results that suggested some independence from DNMT1 at this stage of development. *Cdkn1c* remained largely monoallelic in *Dnmt1^{mut}* samples. *Kcnq1ot1*, although biallelic in all mutant samples, retained some paternal bias of expression in *Dnmt1^{mut}* placentas, indicating that this tissue was less susceptible to the effects of reduced DNMT1 function. *Kcnq1* and *Ascl2* were largely unaffected by *Dnmt1* mutations. Furthermore, many of the genes in this domain undergo changes in imprinted expression at this time: *Kcnq1* loses imprinting in the embryo, and *Ascl2* becomes imprinted in the placenta. A simple model of maternal ICR methylation regulating imprinting throughout the domain is not sufficient to explain the differences observed between tissues and developmental time points, warranting further mechanistic studies.

Conclusions. In this study, we revealed both the central role for DNMT1 in the regulation of genomic imprinting and many instances of genes whose expression is not wholly dependent on DNA methylation. Some of these, such as the resistance of *Cdkn1c* to the effects of a less-severe *Dnmt1* mutation and the continued imprinting of *Kcnq1* in mutant conceptuses, were not fully appreciated previously. In the future, it will be crucial to determine the DNMT1-independent mechanisms that regulate the expression of these genes. Finally, we described the first effects of *Dnmt1* mutations on the *Peg3* domain; much remains to be learned about the regulatory mechanism in this domain.

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