The Dnmt1 Intrinsically Disordered Domain Regulates Genomic Methylation During Development

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ABSTRACT The DNMT1 cytosine methyltransferase enzyme contains a large ~300-aa intrinsically disordered domain (IDD) that we previously showed regulated DNA methylation patterns in mouse ES cells. Here we generated seven mouse lines with different mutations in the IDD. Homozygous mutant mice of five lines developed normally, with normal levels of methylation on both imprinted and nonimprinted DNA sequences. The other two lines, however, had alterations in imprinted and/or nonimprinted (global) DNA methylation appearing during embryonic development. Embryos of one line expressing a DNMT1 variant containing a 6-aa rat orthologous sequence in the IDD maintained imprinted methylation, showed very reduced levels of global methylation and occasionally completed fetal development. These *in vivo* studies demonstrate that at least two DNMT1-dependent methylation processes can be distinguished during fetal development. One process maintains the bulk of genomic methylation on nonimprinted sequences. The other process maintains methylation on a much smaller class of sequences including but not limited to gametic differentially methylated domains (gDMDs) that transmit essential imprinted parent-specific methylation for embryonic development.

KEYWORDS DNA methylation; Dnmt1; genomic imprinting; intrinsically disordered protein; methyltransferase

PROFOUND increases and decreases in genomic methylation are seen during the reproductive cycle (Li 2002). The genomes of female and male germ cells gain enormous methylation, including maternal and paternal gDMD methylation as they differentiate from unmethylated primordial germ cells (<1% of CpG dinucleotides methylated) into mature gametes (~70% sperm and ~30% egg CpG methylation) (Li 2002; Peters 2014). During preimplantation, the parental genomes lose most of their global methylation yet retain gDMD methylation, resulting in blastocysts with <10% of their CpGs methylated. After implantation, genomic methylation climbs rapidly as cells differentiate, rising to ~60% methylated CpGs in early postimplantation embryos and peaking at ~75% methylated CpGs in adult somatic cells.

Changes in methylation are uneven across the genome; for example, during preimplantation, gDMD methylation is retained while global methylation falls sharply depending on the type of sequence element (Smith and Meissner 2013). Thus, at different times in development there are very different methylomes, or unique patterns of DNA methylation across the genome. The coincidence of shifts in the methylome and key developmental events suggest that methylome changes promote development.

The importance of DNA methylation for normal development was shown in genetic studies. Targeted mutations of the *Dnmt1* maintenance methyltransferase gene in mice have detrimental effects on development; homozygous *Dnmt1*-null embryos die soon after implantation with major structural abnormalities, complete loss of gametic differentially methylated domain (gDMD) methylation and ~85% loss of global methylation (Li *et al.* 1992). Lesser phenotypic effects and methylation losses are seen in embryonic knockouts of *de novo* methyltransferase genes *Dnmt3a* and *Dnmt3b*. Notably, embryos with knockouts of both *Dnmt3a* and *Dnmt3b* (*3a*^{-/-}, *3b*^{-/-}) show partial loss of repetitive-sequence

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methylation and sporadic loss of gDMD methylation, with some $3a^{-/-}, 3b^{-/-}$ embryos retaining gDMD methylation and others losing methylation at some gDMDs (Okano *et al.* 1999). From these genetic experiments, we conclude that DNA methylation is an essential developmental process. At present, it is not known how much of the complete methylome is required for normal development.

The role of inherited gDMD methylation in development was illustrated in knockouts of two maternal-effect genes. The *Dnmt3l* gene encodes an accessory protein for DNMT3a and is required for establishment of maternal imprints at gDMD sequences in oocytes (Bourc'his *et al.* 2001). Embryos derived from *Dnmt3l*^{-/-} oocytes lack maternal imprints, retain near-normal levels of global methylation, and die soon after implantation. Similarly, embryos derived from *Dnmt1* ^{$\Delta 10/\Delta 10$} oocytes, which lack the maternal-effect DNMT1o enzyme, lose on average 50% gDMD methylation due to failure to maintain gDMD methylation in eight-cell embryos (Howell *et al.* 2001). Because global methylation is approximately normal, they likely die in the latter half of gestation because of the partial loss of gDMD methylation. Thus, gDMD methylation is essential for normal and complete embryogenesis, but it is not known if gDMD methylation alone is sufficient for mammalian development.

Putting aside active demethylation, which appears to require many genes (Wu and Zhang 2014), methylation during embryo development is controlled by the collective actions of *Dnmt1*, *Dnmt3a*, and *Dnmt3b*. In principle, a logical genetic approach to determining the developmental purposes of DNA methylation would be to study the consequences of noninactivating mutations in *Dnmt1*, *Dnmt3a*, and/or *Dnmt3b* on genomic methylation. DNMT1 is involved in both maintenance methylation and passive demethylation (Li 2002; Wu and Zhang 2014), actions that can occur simultaneously in a cell but on different sequences (Reinhart and Chaillet 2005; Reinhart *et al.* 2006). This suggests that DNMT1 activity is regulated and that noninactivating mutations in *Dnmt1* that generate informative mutant methylomes can be found. Based on this notion, we studied the effects on genomic methylation of expressing mutant DNMT1 proteins in ES cells using a *Dnmt1*^{tet/tet} line in which the endogenous DNMT1 enzyme is extinguished by the administration of doxycycline (Borowczyk *et al.* 2009). We found that methylome changes were induced by expressing DNMT1 proteins with mutations in its large 300-aa intrinsically disordered domain (IDD)—some IDD mutants produced methylomes specifically lacking gDMD methylation, whereas other mutants produced methylomes retaining normal gDMD methylation but with much reduced global methylation. These findings and the knowledge that IDDs of many proteins regulate their catalytic functions prompted us to explore the *in vivo* purposes of DNA methylation during development through the study of mice with DNMT1 IDD mutations.

Materials and Methods

ES-cell lines

Heterozygous ES-cell lines were generated from wild-type R1 ES cells using targeting plasmids and screening for

homologous recombinant clones. Mutations in exon 12 and exon 13 of the *Dnmt1* gene were produced by site-directed mutagenesis using the QuickChange kit (Stratagene), followed by insertion of the LTNL (loxP-HSVtk-neo^r-loxP) selection cassette into an *EcoRI* restriction site in the intron between exons 12 and 13 (Supporting Information, Figure S1). Oligonucleotides for site-directed mutagenesis of codons are listed in Table S1. Identification of homologous recombinant clones was performed by screening DNA from neo-resistant ES-cell clones using *KpnI* digestion followed by Southern hybridization with a genomic fragment located 3' of the targeting plasmid (Figure S1). Accurate homologous recombination was confirmed by Southern hybridization with a genomic fragment located 5' of the targeting plasmid. The LTNL selection cassette was then removed by expression of Cre recombinase and selection in presence of 1 μ M gancyclovir. An identical process was then performed to generate homozygous mutant ES clones from heterozygous clones. ES-cell lines were generated from blastocysts of the mutant *Dnmt1*^P mouse line using techniques previously described (Cirio *et al.* 2008a).

Mouse lines

Heterozygous *Dnmt1* mutant ES-cell lines were generated from R1 ES cells using a similar scheme as described above, except that a floxed pgk-neo^R selection cassette instead of the LTNL cassette was placed in the intronic *EcoRI* site. Chimeric mice were produced by blastocyst injection of heterozygous ES cells. Following germ-line transmission of the mutant allele, the selection cassette was removed by crossing heterozygous mice to a *Sox2-Cre* transgenic mouse (Hayashi *et al.* 2002). Each line was serially backcrossed to wild-type inbred 129/Sv mice for at least eight generations. Genotypes of mice were determined by PCR using oligonucleotides flanking exon 12 or exon 13 followed by an appropriate restriction enzyme digestion of the PCR product that distinguishes wild type from mutant allele (Figure S1). To easily assess imprinted methylation in single early stage embryos, the normally imprinted high-copy Igf2r-Ig/myc transgene (Mohan *et al.* 2011) was introduced into some of the *Dnmt1* mutant strains through mating. All experiments were performed in compliance with guidelines established by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Immunoblotting

Detection of DNMT1 proteins was performed using an immunoblot assay as previously described (Cirio *et al.* 2008b). Affinity-purified rabbit anti-DNMT1 antibody UPT82 was used at a 1:1000 dilution. The anti-rabbit secondary antibody was used at a 1:50,000 dilution.

Determinations of DNA methylation

Global levels of DNA methylation were qualitatively assessed by *HpaII* digestion of genomic DNA followed by Southern blot hybridization with an intracisternal A particle long terminal repeat (IAP LTR) probe (Howell *et al.* 2001) and the LUMA (LUMinometric Methylation Assay) assay (Karimi *et al.* 2006;

Pilsner *et al.* 2010). DNA methylation standards generated from mixtures of wild-type (WT) and *Dnmt1*^{c/c} ES-cell DNA were used to determine global methylation in the LUMA assay.

Methylation at the imprinted *Snurp/Snrpn* gDMD was determined by traditional bisulfite genomic sequencing and Southern blotting (Cirio *et al.* 2008a). Methylation in the normally imprinted Igf2r-Ig/myc transgene was determined using Southern blots as previously described (Reinhart *et al.* 2006; Mohan *et al.* 2011). For more widespread quantitative methylation analysis of gDMD methylation, the EpiTYPER method (Sequenom) was used as previously described (Ehrich *et al.* 2005). EpiTyper absolute methylation levels were calculated as the unweighted average CpG methylation fraction across each individual imprinted gDMD amplicon, and the overall imprinted gDMD methylation was displayed as the sum of 12 nonredundant gDMD amplicon methylation levels. Primers for the EpiTYPER experiments are included in Table S2. DNA methylation standards (0, 33, 67, and 100%) were generated from mixtures of WT and *Dnmt1*^{c/c} ES-cell DNA.

Genome-wide methylation was assessed using the reduced representation bisulfite sequencing (RRBS) technique (Boyle *et al.* 2012) and analyzed according to the pipeline established at the McGill Epigenomics Mapping and Data Coordinating Centres in the McGill University and Génome Québec Innovation Centre (Magnus *et al.* 2014). Sequencing was performed at the Tufts University Core Facility (TUCF Genomics) using an Illumina HiSeq2500 rapid flow cell.

Results

IDD mutant mouse lines

Our previous studies using mouse ES cells strongly suggested that a portion of DNMT1's IDD that is unique to mammals (aa 280–350) is needed to maintain DNA methylation in a sequence-specific manner (Borowczyk *et al.* 2009). As shown in Figure 1A, within this region of interest “variable,” “conserved,” and “mouse–rat” regions can be defined. Within the mouse–rat region, the mouse amino-acid sequence LESHTV is aligned to the rat PEPLSI sequence; the alignment is “forced” by the flanking sequence identities in the two species. We approached the study of this region by first generating mouse lines with mutations in these different regions. We then studied these mouse lines to determine the effects of the mutations on genomic methylation and development.

The *Dnmt1*^{IDel12} allele was generated by a nucleotide insertion in the 5' end of exon 12 and an accompanying deletion of a nucleotide in the 3' end of exon 12. The altered exon 12 codons produce a mutant DNMT1 protein with amino-acid changes across the variable region. Homozygous *Dnmt1*^{IDel12/IDel12} mice developed normally, and WT, *Dnmt1*^{IDel12/+}, and *Dnmt1*^{IDel12/IDel12} mice were obtained at the time of weaning (Figure 1B). Both homozygous *Dnmt1*^{IDel12/IDel12} female and male mice were fertile and the obligate heterozygotes from crosses between *Dnmt1*^{IDel12/IDel12} females and WT males were viable and

fertile, indicating an absence of any maternal-effect abnormalities. Moreover, the global level of genomic methylation was normal in both *Dnmt1*^{IDel12/IDel12} ES cells and embryos, as assessed on a Southern blot of *HpaII*-digested DNA hybridized with an IAP LTR probe (Figure 2A). A homozygous *Dnmt1*^{IDel12/IDel12} ES-cell line expressed a level of mutant protein comparable to that expressed in wild-type ES cells (Figure S4A) and had levels of imprinted and nonimprinted methylation indistinguishable from wild-type mice (Figure 2C). We conclude that there is no apparent effect of the *Dnmt1*^{IDel12} mutation on genomic methylation.

The *Dnmt1*^{IDel13} mutation was produced by a nucleotide deletion in the 5' end of exon 13 and a nucleotide insertion in the 3' end of the same exon. It alters codons spanning the conserved and mouse–rat regions (Figure 1A). Homozygous *Dnmt1*^{IDel13/IDel13} mice died during the early stages of embryogenesis (Figure 1B). E8.5 *Dnmt1*^{IDel13/IDel13} homozygous embryos were recovered from crosses between heterozygous parents, but no normal-appearing E9.5 homozygous mutants were recovered. Global methylation was much reduced in the E8.5 *Dnmt1*^{IDel13/IDel13} embryos, as assessed by Southern blotting (Figure 2A). Moreover, imprinting of an incorporated Igf2r-Ig/myc transgene was lost in the homozygous *Dnmt1*^{IDel13/IDel13} mutants (Figure 2B). Normal levels of the mutant protein were present in homozygous *Dnmt1*^{IDel13/IDel13} E8.5 embryos and three *Dnmt1*^{IDel13/IDel13} mutant ES-cell lines generated from the same heterozygous *Dnmt1*^{IDel13/+} cell line (Figure S4A). Surprisingly, in contrast to its expression and demonstrated maintenance methyltransferase activity in ES cells (Figure 2C and Figure S2), the IDel13 protein was ineffective in maintaining methylation in mouse embryos, as assessed by the level of genomic methylation (Figure 2, A and B). We infer from these studies that the maintenance methyltransferase activity of the wild-type DNMT1 protein can be regulated in the developing embryo and this regulation requires DNMT1's IDD.

Additional exon 13 mutant mouse lines

In an attempt to identify an amino-acid motif or motifs governing the developmental regulation of DNMT1, we generated additional mouse lines with mutations in exon 13 codons. The *Dnmt1*^{IDel13} mutation described above spans two distinct regions of exon 13, the 5' portion encoding amino acids highly conserved among mammalian species (conserved region) and the 3' portion encoding amino acids present in rat and mouse (mouse–rat region), but not in other Eutherian mammals for which *Dnmt1* sequence is available, including humans (Figure 1A). Interestingly, the mouse–rat region encodes a divergent 6-aa sequence and an adjacent 4-aa sequence identity (PVQS). Homozygous *Dnmt1*^{TT-AA/TT-AA} and *Dnmt1*^{KK-AA/KK-AA} female and male mice with mutations in the conserved region developed normally. In addition, homozygous *Dnmt1*^{EEE/EEE} mice in which the amino-acid sequence VPV of the mouse–rat region was replaced by EEE also developed normally (Figure 1B). We conclude from this analysis that small amino-acid substitutions within the IDel13 region are well tolerated, with no measureable effects on the normal regulation of methylation during development.

Figure 1 Effects of mutations in the DNMT1 intrinsically disordered domain (IDD) on embryonic development. (A) Positions of seven *Dnmt1* mutations in the IDD. Top: Alignments of mouse, rat, and human amino-acid sequences in carboxyterminal portion of the IDD, amino acids 290–344. Bottom: Seven IDD mutations in “variable,” “conserved,” and “mouse-rat” IDD regions. Embryonic phenotypes of homozygous mutants are summarized at right. (B) Genotypes of live embryos at different developmental stages for six of the seven *Dnmt1* mutant mouse lines.

Alignment of the two disparate amino-acid sequences in the mouse-rat region, mouse LESHTV, and rat PEPLSI, is intriguing. The alignment is “forced” by flanking sequence identity (Figure 1A). Would PEPLSI provide the same or different function as LESHTV to the mouse DNMT1 protein? To address this, a mutant mouse line (designated *Dnmt1^P*) in which the LESHTV codons were replaced by PEPLSI codons was generated. Heterozygous *Dnmt1^{P/+}* mice were phenotypically normal, whereas *Dnmt1^{P/P}* homozygotes died in the late stages of gestation or the perinatal period (Figure 3C). Homozygous *Dnmt1^{P/P}* pups were usually distinguished from *Dnmt1^{P/+}* and WT littermates by their small size (Figure 3A). Even in cases in which WT and *Dnmt1^{P/P}* embryos in the same litter were of similar size, the placentas of the *Dnmt1^{P/P}* embryos were much smaller than those of the WT and *Dnmt1^{P/+}* heterozygous embryos (Figure 3B). Despite the extended survival of homozygous *Dnmt1^{P/P}* embryos, in one observed instance to 2 days following birth (Figure 3C), they exhibited profound alterations in genomic methylation. Global methylation at E13.5 was significantly reduced to approximately one-third of the level present in wild-type E13.5 embryos, but above

the very low level found in *Dnmt1*–null mouse ES cells (Figure 4, A and B). (*Dnmt1*–null ES cells retain ~15% of WT ES-cell methylation; this residual methylation is due to DNMT3 enzyme activities, as mouse ES cells in which the *Dnmt1*, *Dnmt3a*, and *Dnmt3b* genes were knocked out have no genomic methylation (Tsumura *et al.* 2006)). Imprinted gDMD methylation was assessed in three ways. Fully methylated *Snurp/Snrpn* gDMD alleles, consistent with normally methylated maternal *Snurp/Snrpn* alleles, were identified in *Dnmt1^{P/P}* embryos and ES cells by traditional bisulfite genomic sequencing (Figure 4C). When analyzed by Southern blotting, very similar ratios of methylated to unmethylated alleles were present in *Dnmt1^{P/P}*, *Dnmt1^{P/+}*, and WT embryos, findings consistent with normal *Snurp/Snrpn* gDMD methylation in homozygous *Dnmt1^{P/P}* embryos (Figure S3). EpiTyper analysis showed a small decrease in average gDMD methylation across 12 gDMDs in E15.5 *Dnmt1^{P/P}* embryos compared to wild-type age-matched controls (Figure 4D). We conclude that the mutant P protein is a partial-loss-of-function enzyme, with ability to maintain only a small fraction of normal methylation. Among the retained *Dnmt1^{P/P}*

Figure 2 Methylation analysis of Dnmt1 ES lines and mutant mice and ES-cell lines. (A) Southern blot of *HpaII*-digested DNA of *Dnmt1*^{IDel12/IDel12} and *Dnmt1*^{IDel13/IDel13} embryos, hybridized with an IAP LTR probe. (B) Southern blot of *HpaII*-digested DNA from E8.5 Igf2r-Ig/myc transgenic embryos in either homozygous *Dnmt1*^{IDel13/IDel13} (IDel13/IDel13) or wild-type (WT) genetic background. The Igf2r-Ig/myc transgene was inherited from either the maternal or the paternal parent. Control *MspI*-digested Igf2r-Ig/myc transgenic embryo in a WT background is shown on the right. DNA in each lane is from a single embryo. The Southern blots were hybridized with the C α fragment of the Igf2r-Ig/myc transgene. (C) Southern blot analysis of DNA methylation from ES-cell lines R1, *Dnmt1*^{c/c}, *Dnmt1*^{IDel12/IDel12}, and *Dnmt1*^{IDel13/IDel13}. DNA samples are from either pluripotent ES cells (Undiff) or following 2 days of growth as unattached embryoid bodies in the absence of LIF (EB(2d)). ES-cell DNA was digested with *HpaII* and hybridized to an IAP probe. c/c, *Dnmt1*^{c/c}. H, *HpaII*. —, no restriction endonuclease.

methylation is gDMD methylation, which is known to be required for normal embryonic development.

Full-length protein from the mutant *Dnmt1*^P allele was detected in homozygous *Dnmt1*^{P/P} ES cells and embryos from E8.5 to E13.5 (Figure S4B). The reduced level of mutant protein compared to the wild-type level in ES cells is associated with wild-type levels of IAP methylation (Figure S5), whereas low levels in embryos are associated with preferential loss of nonimprinted DNA methylation in embryos (Figure 4, A and B). We infer from these studies that the maintenance methyltransferase activity of the wild-type DNMT1 protein can be regulated in the developing embryo and this regulation requires DNMT1's IDD. We derived the same conclusion from analysis of the overlapping *Dnmt1*^{IDel13} mutation (see above).

To better quantify the alterations in methylation in *Dnmt1*^{P/P} embryos, RRBS libraries were constructed from DNA of six E15.5 embryos (two female WT embryos, two female *Dnmt1*^{P/P} embryos, one male WT embryo, one male *Dnmt1*^{P/P} embryo) and two E15.5 cerebra (from one WT and one *Dnmt1*^{P/P} male embryo) and paired-end sequencing performed. RRBS libraries are subgenomic, derived from small *MspI* fragments representing ~1% of the mouse genome and 1–2 million CpG dinucleotides (Boyle *et al.* 2012). *Dnmt1*^{P/P} embryos showed a substantial loss of highly methylated tiles (>80%) (Figure 5A) coupled with an increase in tiles exhibiting lower methylation. All three *Dnmt1*^{P/P} embryos showed very consistent and significant methylation

alterations in intergenic as well as genic regions compared to WT, as seen in 74,296 of ~190,000 100-nt sequenced tiles (Figure 5, A and B, and Figure S6). Interestingly, 74,288 tiles showed loss of methylation (>20%) in *Dnmt1*^{P/P} compared to WT embryos, whereas only 8 tiles (~0.01% of altered tiles) showed hypermethylation, a remarkably low percentage for any genome methylation comparison. Approximately 80% of the remaining ~115,000 tiles are unmethylated or have <5% methylation in both WT and *Dnmt1*^{P/P} embryos, and only 2705 tiles (~3%) with >40% methylation in WT remained equally methylated in *Dnmt1*^{P/P} embryos ($\pm 10\%$) (Figure 5D). The retained methylation in *Dnmt1*^{P/P} embryos was mostly observed in intergenic regions and gene bodies; similar observations were made for the E15.5 *Dnmt1*^{P/P} cerebrum (3026 tiles). A total of 668 tiles overlapped between *Dnmt1*^{P/P} embryos and brain, with similar genomic distribution to that observed for all retained methylation.

To test the specificity of the P mutant protein in preventing the maintenance of most genomic methylation in the face of maintenance of inherited gDMD methylation, an allele with two alanine codon substitutions in the mouse–rat region was generated. The *Dnmt1*^{ASA} allele expresses a mutant DNMT1 protein in which LESHTV is replaced by LASATV. Two homozygous *Dnmt1*^{ASA/ASA} offspring out of a total of nine were obtained from crosses between heterozygous *Dnmt1*^{ASA/+} mice; these mice survived to 4 months of age with no visible abnormalities and normal global levels of genomic methylation. We

Figure 3 Phenotypes of *Dnmt1^P* embryos. (A) Genotyped E15.5 litter from mating between *Dnmt1^{P/+}* parents. WT or +, wild type; P, *Dnmt1^P* allele. (B) Mid-sagittal (embryo) and mid-transverse (placenta) sections of WT and *Dnmt1^{P/P}* conceptuses. Sections stained with hematoxylin and eosin. (C) Genotypes of live embryos from crosses between *Dnmt1^{P/+}* parents. E8.5–E19.5 are days 8.5–19.5 of embryonic development. P0 is day of birth. P21 is 21 days after birth. The live *Dnmt1^{P/P}* embryo identified at P0 survived for 2 days following its birth.

conclude that small changes in the mouse LESHTV sequence preserve DNMT1-dependent methylation during development, whereas the complete change to the rat orthologous sequence produces a mutant DNMT1 protein (the P protein) that clearly fails to regulate genomic methylation properly during development.

Discussion

Function of the IDD in regulating embryonic methylation

Targeted mutations in a small portion of DNMT1's IDD produced mutant mouse lines with altered methylomes appearing in the course of embryonic development. We infer from this that the IDD is involved in regulating genomic methylation during development. Because homozygous *Dnmt1^{P/P}* embryos retain gDMD methylation, but not the majority of global methylation, we can further infer that the mouse LESHTV motif in the IDD is required to maintain global methylation,

but not methylation on gDMD and possibly related sequences. The aligned mouse LESHTV and rat PEPLSI motifs may have evolved to maintain perpetuated DNA methylation on species-specific sequences during development, most notably on very abundant interspersed retroelements (Bohne *et al.* 2008). Other Eutherian mammals for which *Dnmt1* sequence is available do not have the mouse–rat region yet have conserved gDMD methylation and equally highly methylated genomes as the mouse and rat. Comparable mouse–rat region function for these other species presumably resides at other locations in their DNMT1 proteins.

A striking difference between the aligned rat PEPLSI and mouse LESHTV motifs is the presence of two prolines in the rat sequence and no prolines in the mouse sequence. Proline richness is a distinctive feature of many IDDs, and prolines are considered to be disorder promoting (Dyson 2011; Oldfield and Dunker 2014). This may seem paradoxical given the restricted rotation of a peptide chain and hence localized “structure” imposed by the imino acid proline. Nevertheless, PEPLSI and LESHTV would be expected to contribute

Figure 4 Methylation in *Dnmt1^P* embryos. (A) Southern blot of *HpaII*-digested DNA of E18.5 embryos and *Dnmt1*-null ES cells (c/c) hybridized with IAP probe. (B) Global methylation (percentage of CpG dinucleotides methylated) estimated from LUMA assays. (C) Methylation of *Snurp/Snrpn* gDMD in an E15.5 *Dnmt1^{P/P}* embryo by bisulfite sequencing showing a normal mixture of mostly methylated (solid circles) and mostly unmethylated (no circles) alleles. (D) Methylation of 12 gDMD sequences in E15.5 *Dnmt1^{P/P}*, *Dnmt1^{P/+}*, and WT embryos determined by EpiTYPER method. Two *Dnmt1^{P/P}* embryos (1 and 2) were studied. The values are averages of two independent methylation determinations. The average methylation across all 12 gDMDs was significantly different between the *Dnmt1^{P/P}* embryos and the two other genotypes ($P < 0.01$; Kruskal–Wallis equality-of-population rank test).

Figure 5 Genome-wide DNA methylation dynamics in E15.5 *Dnmt1^{P/P}* embryos. (A) Fraction of 100-bp tiles with <20% (pink), 20–40% (light blue), 40–60% (dark blue), 60–80% (green), and >80% (red) methylation in WT and *Dnmt1^{P/P}* (P/P) embryos. (B) Heat map of methylation levels for 74,296 tiles with methylation alterations in *Dnmt1^{P/P}* compared to WT embryos (methylation variance >20% and $P < 0.05$). Paired-end sequenced libraries of WT ($N = 3$) and P/P ($N = 3$) embryos. 100-nt tiles with >10-fold coverage. (C) Methylation mean surrounding the transcription start site (TSS) for all tiles in each E15.5 embryo group. (D) Genomic distribution for tiles with retained methylation in *Dnmt1^{P/P}* (>40%) and equally methylated in WT ($\pm 10\%$). E15.5 WT and *Dnmt1^{P/P}* embryos and cerebra were analyzed.

differently to the disorderness of DNMT1's IDD. In this regard, exon 8 of *Dnmt1* encodes two similar 6-aa motifs, also with conserved flanking aa's; PERVRP is the rat ortholog of the mouse sequence LEEVTA (amino acids 216–221), and CQEEQG is the rat ortholog of the mouse sequence GPPEPC (amino acids 226–231). The three 6-aa motifs may cooperate in each species to ensure maintenance of the majority of nonimprinted DNA methylation. Based on this model we speculate that substituting the orthologous rat sequence for the mouse sequence at either one or both exon 8-encoded motifs would interfere with the normal IDD maintenance methylation function and achieve a similar dramatic genomic methylation change to that seen in *Dnmt1^{P/P}* homozygous embryos.

The *Dnmt1^{IDel13}* and *Dnmt1^P* mutations share an important feature. Most importantly, both maintain normal levels of gDMD and global methylation in homozygous mutant ES cells (assessed by level of IAP methylation; Figure 2C and Figure S5), yet homozygous mutant embryos show very significant loss of methylation (Figure 2A, Figure 4, A and B, and Figure 5). Although analysis of deletion mutants in ES cells pointed to a role for the IDD in maintenance methylation (Borowczyk *et al.* 2009), the contrast between effects in ES cells and embryos for the *Dnmt1^{IDel13}* and *Dnmt1^P* mutations suggest that it is unlikely that important details of IDD function can be obtained from ES-cell studies alone.

We can speculate on the cause of the low level of DNMT1 protein and its possible relationship to genomic methylation in *Dnmt1^{P/P}* ES cells and embryos. A proposed function of intrinsically disordered proteins is binding to a large number of interacting molecules such as other proteins and small

chemicals (Dyson 2011). Because the *Dnmt1^P* mutation resides in DNMT1's IDD, it is possible that the main effect of the mutation is disruption of very specific interactions between DNMT1 and key function cellular components such as nuclear proteins, cytoplasmic proteins, and DNA. We speculate that failure to establish some of these interactions leads to loss or degradation of the noninteracting mutant P protein and reduced levels of DNMT1, proportionate to the extent of remaining interactions. We further propose that during development there is a change in the composition of DNMT1 interacting molecules leading to additional losses of key interactions, further reduction of steady-state DNMT1 levels, and loss of non-imprinted methylation.

Categories of genomic methylation

Dnmt1^{P/P} embryos uncouple at least two categories of genomic methylation, that residing on a small number of gDMD sequences and the majority of methylation on nonimprinted sequences. Based on different assays, the level of methylation on different gDMD sequences in inbred *Dnmt1^{P/P}* embryos is normal or very nearly normal (Figure 4). More accurate assessments of parental allele-specific gDMD methylation in *Dnmt1^{P/P}* embryos must await incorporation, through breeding, of the *Dnmt1^P* allele into the CAST/Ei strain such that maternal and paternal gDMD alleles can be easily distinguished by sequence differences between 129/Sv and CAST/Ei strains in F1 (129/Sv $\times$ CAST/Ei) embryos. gDMD sequences are unique in their ability to maintain two distinct, heritable epigenetic states—the unmethylated and methylated parental alleles of each gDMD reside in the same nucleus and both are faithfully

inherited with each cell division. Moreover, when a methylated gDMD allele is converted to the unmethylated state by transient loss of DNMT1 activity, it remains unmethylated, immune to remethylation outside of gametogenesis. This may distinguish gDMD sequences from most methylated sequences, whose methylation depends on DNMT1, but do not retain an unmethylated state following a loss of DNMT1 activity—upon reexpression of DNMT1, they rapidly regain their initial methylation levels (Tucker *et al.* 1996; Damelin and Bestor 2007; Borowczyk *et al.* 2009). This distinction between gDMDs and the majority of genomic methylation is interesting in light of observations in ES cells demonstrating that DNMT3 activity is required for maintenance of DNA methylation (Liang *et al.* 2002; Chen *et al.* 2003). This requirement is most obvious at repetitive DNA sequences and may not apply *in vivo* to gDMDs. This raises the intriguing possibility that the genome is divided into two fundamental types of CpG methylation. One type, exemplified by methylated alleles of gDMDs, is strictly inherited with each cell division by the action of DNMT1 (Howell *et al.* 2001; Borowczyk *et al.* 2009). The other type, exemplified by methylated mouse IAPs, is perpetuated by combined DNMT3 and DNMT1 activities (Liang *et al.* 2002; Damelin and Bestor 2007; Jeltsch and Jurkowska 2014). Importantly, both types require DNMT1 maintenance methylation and are affected differently in homozygous *Dnmt1*^{P/P} embryos, raising the possibility that the DNMT1 P protein fails to engage DNMT3 proteins during embryonic development.

What methylation is required for embryonic development? Inherited DNA methylation at gDMDs is essential for normal development (Bourc'his *et al.* 2001), and we conclude that the survival of *Dnmt1*^{P/P} conceptuses to a very late stage of gestation is due, at least in part, to maintained gDMD methylation. Apart from gDMD methylation, some or all of the methylation retained in *Dnmt1*^{P/P} embryos may be required for their extensive fetal development. If so, this nonimprinted essential methylation would likely be maintained by the same DNMT1-dependent mechanism that maintains gDMD methylation. Further studies are needed to understand the relationship between the changes in different categories of genomic methylation and effects on embryonic development.

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GENETICS

Supporting Information

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The Dnmt1 Intrinsically Disordered Domain Regulates Genomic Methylation During Development

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Figure S1 Generation and identification of *Dnmt1* alleles. A. Strategy of generating targeted mutations in exons 12 and 13 of the *Dnmt1* gene. Grey rectangle is location of 3' hybridization probe used to identify homologous recombinant clones on Southern blots. B. PCR-based assays for genotyping the seven different *Dnmt1* mutant alleles. Sizes of DNA fragments after restriction digestion indicated. Primer pairs for genotyping: Exon12genF CTA CCA TCT ATG GCG GTG CT; Exon12genR GCT GGA AAA CCA CAG GAC AT; Exon13genF AGT GGG GTT AGG GCA GAC TT; Exon13genR GGG CAC TTT GGT GAG TTG AT. Restriction enzymes for genotyping: *Dnmt1*^{IDel12} *Ava*I; *Dnmt1*^{IDel13} *Bmr*I; *Dnmt1*^{TT-AA} *Kpn*I; *Dnmt1*^{KK-AA} *Pml*I; *Dnmt1*^P *Ava*I; *Dnmt1*^{ASA} *Ple*I; *Dnmt1*^{EEE} *Hpy*188I.

	Peg3	Sgce	Plagl1	Dlk1	Nespa5	Snrpn	Igf2r	Peg1	Kcnq1	Grb10	Impact	H19
<i>IDel12/IDel12</i>	0.38	0.02	0.38	0.24	0.47	0.07	0.33	0.38	0.37	0.70	0.24	0.74
<i>IDel13/IDel13(A)</i>	0.37	0.07	0.43	0.30	0.36	0.01	0.33	0.77	0.05	0.62	0.38	0.60
<i>IDel13/IDel13(A)</i>	0.35	0.02	0.38	0.28	0.41	0.03	0.36	0.78	0.07	0.45	0.20	0.69
<i>IDel13/IDel13(B)</i>	0.32	0.01	0.25	0.29	0.41	0.01	0.35	0.81	0.05	0.40	0.45	0.63
<i>IDel13/IDel13(C)</i>	0.27	0.09	0.41	0.29	0.43	0.01	0.34	0.81	0.05	0.43	0.20	0.74
R1	0.33	0.35	0.39	0.27	0.40	0.02	0.35	0.37	0.30	N/A	0.26	0.65

Figure S2 Methylation in wild-type and homozygous mutant ES cells. EpiTYPER results of methylation at 12 gDMDs in homozygous *Dnmt1*^{IDel12/IDel12}, *Dnmt1*^{IDel13/IDel13} and WT R1 pluripotent ES cells. Values are averages of two independent methylation determinations on same DNA sample. Two separate DNA collections were analyzed for ES line IDel13/IDel13 (A).

Figure S3 Analysis of *Snurp/Snrpn* gDMD methylation in the *Dnmt1^P* mouse line at E15.5 and E18.5. DNA samples were obtained from entire embryos, digested with *HhaI* and *PstI* and hybridized with a fragment of the *Snurp/Snrpn* gDMD according to Cirio et al. (2008a). P = *Dnmt1^P* allele. R1 = wild-type ES cell line. c/c = *Dnmt1*-null ES cell line. The 1.9- and 0.9-Kb fragments are methylated and therefore resistant to *HhaI* digestion.

Figure S4 A. Immunoblots of DNMT1 protein expression in homozygous mutant Dnmt1IDel12 and Dnmt1IDel13 ES cell lines and embryos. B. Immunoblots of mutant DNMT1 protein expressed from the Dnmt1P allele in ES cells, E8.5 (pool of 5 embryos), and individual E10.5, E13.5 and E15.5 embryos. “P/P” indicates homozygous Dnmt1P/P genotype. “P/+” indicates heterozygous Dnmt1P/+ genotype and “+” indicates a wild type genotype. R1 is an additional wild-type ES cell line.

Figure S5 Southern blot of *HpaII*-digested genomic DNA samples from four different ES cell lines. P = *Dnmt1^P* allele. c/c = *Dnmt1*-null ES cell line. M = *MspI*. H = *HpaII*. “-” is undigested DNA. Panels A and B are the same Southern blot exposed overnight without (panel A) or with (panel B) an intensifying screen.

Figure S6 Distribution of annotated sequence features presented in tiles with methylation alterations in *Dnmt1^{P/P}* (white box) compared to WT (grey box) embryos. *** $p < 0.001$ by Wilcoxon test.

File S1

RRBS methylation data sets

Available for download as an Excel file at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.173609/-/DC1>

Table S1 Sequences of oligonucleotides for site-directed mutagenesis

<u>Dnmt1 allele</u>	<u>Exon sequence</u>
Exon 12 WT	A GCT GCC AAA CGG AGA CCC AAG GAA GCA GAG CCA GAG CAG GTA GCT CCA GAG ACT CCC GAG GAC AGA GAC GAG GAT GAG AGG
<i>IDel12</i>	A GCT GCC AAA CGG AGA CCC AAG GAA GTC AGA GCC AGA GCA GGT AGC TCC AGA GAC TCC GAG GAC AGA GAC GAG GAT GAG AGG
Exon 13 WT	GAG GAG AAG AGA CGA AAA ACG ACA CGT AAA AAA CTG GAG TCA CAC ACC GTT CCC GTT CAG AGC AG
<i>IDel13</i>	GAG GAG AAG GAC GAA AAA CGA CAC GTA AAA AAC TGG AGT CAC ACA CCG TTC CCA GTT CAG AGC AG
<i>TT-AA</i>	GAG GAG AAG AGA CGA AAA GCG GCA CGT AAA AAA CTG GAG TCA CAC ACC GTT CCC GTT CAG AGC AG
<i>P</i>	GAG GAG AAG AGA CGA AAA ACG ACA CGT AAA AAA CCC GAG CCC CTC TCC ATC CCC GTT CAG AGC AG
<i>ASA</i>	GAG GAG AAG AGA CGA AAA ACG ACA CGT AAA AAA CTG GCC TCA GCC ACC GTT CCC GTT CAG AGC AG
<i>EEE</i>	GAG GAG AAG AGA CGA AAA ACG ACA CGT AAA AAA CTG GAG TCA CAC ACC GAG GAG GAG CAG AGC AG
<i>KK-AA</i>	GAG GAG AAG AGA CGA AAA ACG ACA CGT GCA GCA CTG GAG TCA CAC ACC GTT CCC GTT CAG AGC AG

Table S2 Oligonucleotide pairs for EpiTYPER assay of gDMD methylation

	<u>Forward primer</u>	<u>Reverse primer</u>
Peg3	aggaagagagGATTTTGTGGGGGTTTTTAATATTGAT	cagtaatacgactcactatagggagaaggctCCACCAACCCAAAATAAACATCTCT
Sgce	aggaagagagTTAGGATTTGGTTATTGAAGGTTTG	cagtaatacgactcactatagggagaaggctCCCCTCCTAAAATCTCTCTATATAAAAC
Plagl1	aggaagagagTATTTTTGTGGGGATGGAGGAATTA	cagtaatacgactcactatagggagaaggctATCCCAACCCAACTAAATAACAAA
Dlk1	aggaagagagGATGTGTTGTGGATTTAGGTTGTAG	cagtaatacgactcactatagggagaaggctATCCCCTATACTCAAAACATTCTCC
Nespas	aggaagagagTTTTTTTTAGGGTTTTGTAGGTTAGATTTG	cagtaatacgactcactatagggagaaggctCCCCTCCTCTTCTATTATAAACACC
Snrpn	aggaagagagTGTGATGTTTGTAATTATTTGGGAG	cagtaatacgactcactatagggagaaggctCTAAAATCCACAAACCCAACTAACC
Igf2r	aggaagagagGATAGGAGGATTTAGAGGGTTTTGT	cagtaatacgactcactatagggagaaggctAACCCATTATCTACAACCTCAAACA
Peg1	aggaagagagATATGTTGGGGAGGGATTTTTTTAG	cagtaatacgactcactatagggagaaggctCAACAAAACAACAACAACAACCTC
Kcnq1	aggaagagagTGGAGAGTTTTTTTGTTTAGTTTGG	cagtaatacgactcactatagggagaaggctCAAAACCACCCCTACTTCTATAAAC
Grb10	aggaagagagAGGAGTTGTTTATTATTTGGATTATTGT	cagtaatacgactcactatagggagaaggctCTCTAAACTCCAAAACCCTTTTTTCT
Impact	aggaagagagTTGTTTGGTTAATTTTAGTTTGGTT	cagtaatacgactcactatagggagaaggctTCATATAACAATACAACAAAACCTACTC
H19	aggaagagagGTTGATGGTTTAGAATTTTATAAGTTAG	cagtaatacgactcactatagggagaaggctCACAATACCACTAAAAAACAAAACA