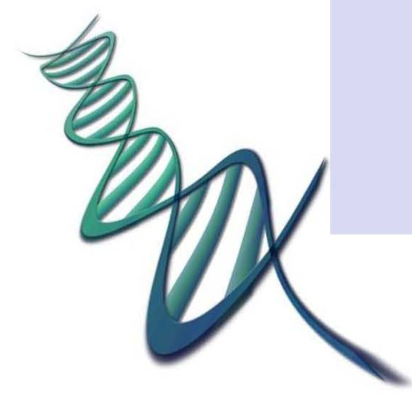

The role of DNA methyltransferase 1 in maintaining methylation at differentially methylated regions associated with imprinted genes

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Introduction to Genomic Imprinting

Some mammalian genes have monoallelic expression where either only the paternal allele or only the maternal allele is expressed (Figure 1). This mammalian-specific phenomenon is known as genomic imprinting. Differential methylation of DNA on the cytosine of a CpG dinucleotide determines the expression of imprinted genes where the methylated allele is typically silenced and the unmethylated allele is expressed. These differentially methylated regions (DMRs) are known to be maintained by DNA methyltransferase 1 (Dnmt1). Primary (1˚) DMRs are established in the germ cells and are consistently maintained throughout growth and development while secondary (2˚) DMRs are acquired during embryogenesis and are more variable.

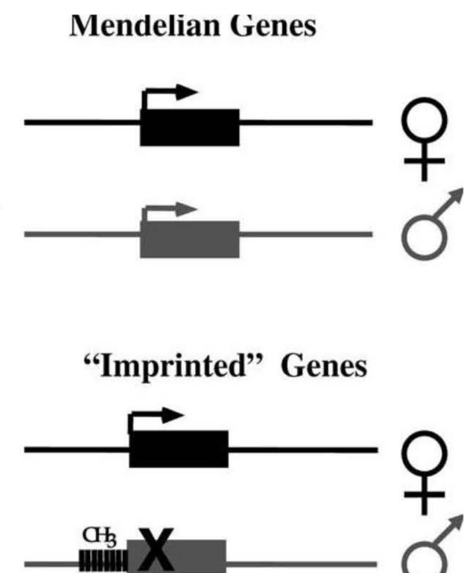

Figure 1. Mendelian genes vs Imprinted genes. Arrows represent expression, X represents lack of expression and CH_3 represents methylation. Source: Vrana (2007) Journal of Mammalogy, 88(1):5–23.

The Role of Dnmt1 in maintaining methylation at secondary DMRs

Maintaining differential methylation is essential for proper expression of imprinted genes. Failure to maintain methylation can result in imprinting disorders such as Silver-Russell and Beckwith-Wiedemann syndromes, which affect growth and development. We hypothesize that the variable methylation at secondary DMRs is not well maintained and hence may need to be re-acquired, involving multiple DNA methyltransferases. To test this hypothesis, we studied the role of Dnmt1 by exploring the methylation patterns of primary and secondary DMRs at imprinted genes in mice bearing a loss of function mutation in *Dnmt1,* resulting in compromised methyltransferase activity (P allele). The P mutation is categorized by a substitution of the mouse-specific sequence with the rat-specific sequence in the intrinsically disordered domain (IDD) of *Dnmt1* (Figure 2). When homozygous, the mutant P allele results in late embryonic lethality, likely as a result of a dramatic reduction in global methylation. In contrast, primary DMRs showed a less drastic reduction in methylation when compared to WT and P/+ embryos, suggesting that *Dnmt1* may function differently at different sequences (Shaffer *et al.*, 2015).

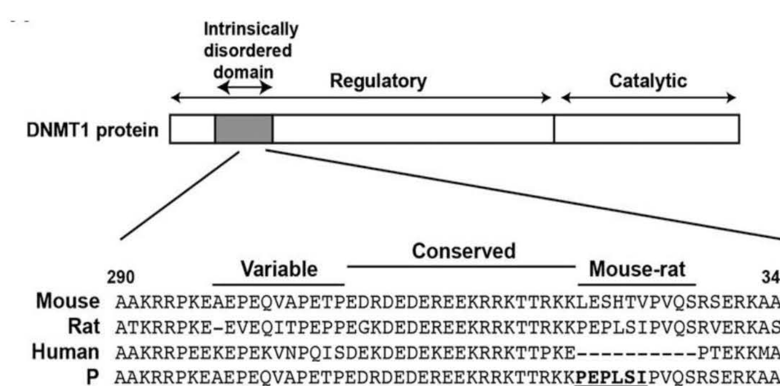

Figure 2. A section of the IDD of Dnmt1 in different mammals and the position of the P allele mutation. Modified from Shaffer *et al.* (2015), Genetics Vol.199, 533-541.

Figure 3. 15.5 day old embryos with placentae from a cross between two mice heterozygous for the P mutation. P/P embryos and placentae are smaller in size as compared to their WT and P/+ litter mates. Source: Shaffer *et al.* (2015), Genetics Vol.199, 533-541.

A comparison of methylation patterns in mutant embryos with wild-type or heterozygous embryos can help us understand the role Dnmt1 plays in maintaining DMRs and can further our understanding into the regulation of imprinted genes, especially how different epigenetic domains interact to regulate expression at clusters of imprinted genes. We studied homozygous mutant embryos using bisulfite mutagenesis to determine how methylation patterns change over time at DMRs. We investigated the methylation patterns at a total of 16 DMRs – 7 primary DMRs and 9 secondary DMRs (summarized in Table 1). For 7 DMRs, we analyzed P/P embryos at 12.5 days post coitum (dpc) as well as WT and P/+ controls using bisulfite mutagenesis and sequencing of individual subclones. We further analyzed 15 DMRs in 15.5 dpc WT and P/P embryos using bisulfite mutagenesis and Next Generation Amplicon Sequencing.

Table 1. Summary of DMRs analyzed

Locus/Gene Name	DMR and Methylation Type	Stage (dpc) Studied
Airn Region B	1˚ Maternal	15.5
Lit1	1˚ Maternal	12.5 & 15.5
Snrpn	1˚ Maternal	15.5
Grb10	1˚ Maternal	12.5
H19 ICR	1˚ Paternal	15.5
IG-DMR	1˚ Paternal	12.5 & 15.5
Rasgrf1	1˚ Paternal	12.5 & 15.5
Magel2	2˚ Maternal	12.5 & 15.5
Mkrn3	2˚ Maternal	15.5
Ndn	2˚ Maternal	15.5
Peg12	2˚ Maternal	12.5 & 15.5
Dlk1	2˚ Paternal	15.5
Gtl2	2˚ Paternal	12.5 & 15.5
H19-pp	2˚ Paternal	15.5
Igf2r-DMR1	2˚ Paternal	15.5
Cdkn1c	2˚ Paternal	15.5

Methodology

Studying methylation pattern using bisulfite mutagenesis

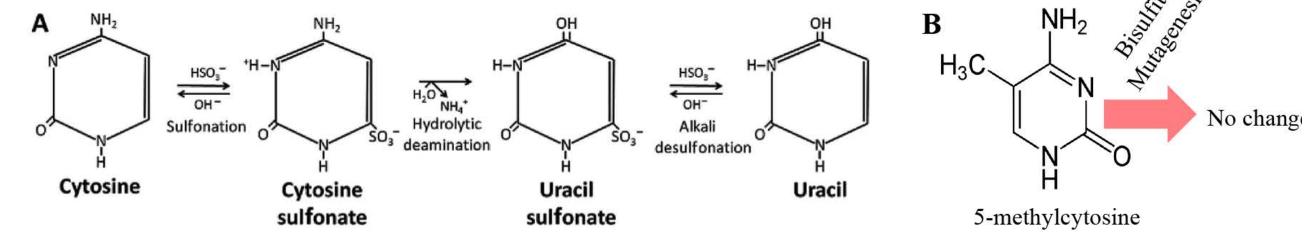

Figure 4. The chemistry of bisulfite mutagenesis. (A) Any unmethylated cytosine is converted into uracil. When amplified by PCR, the uracil is replaced by thymine. Source: Kristensen and Hansen (2009), Clinical Chemistry 55:8 1471–1483. (B) Methylcytosine remains as cytosine after PCR.

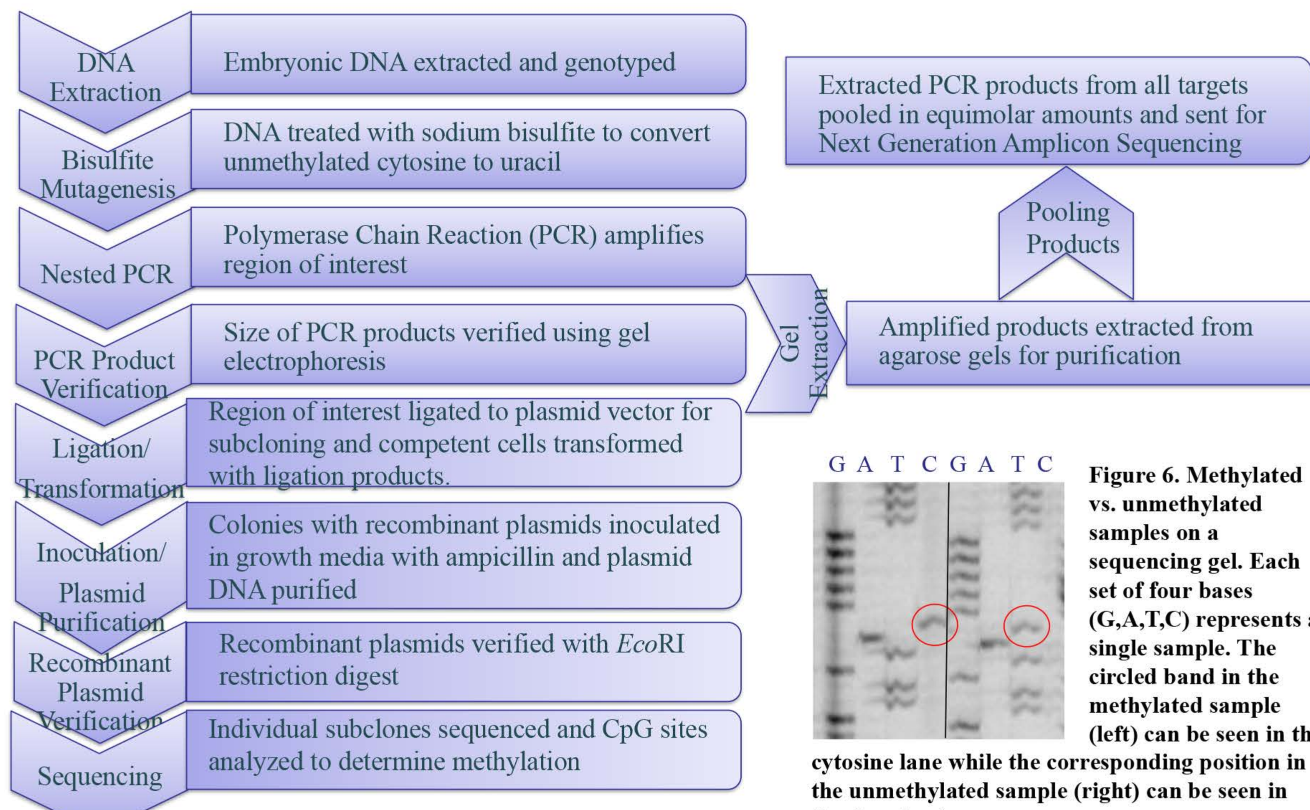

Figure 5. Flowchart showing the procedures followed in the experiment.

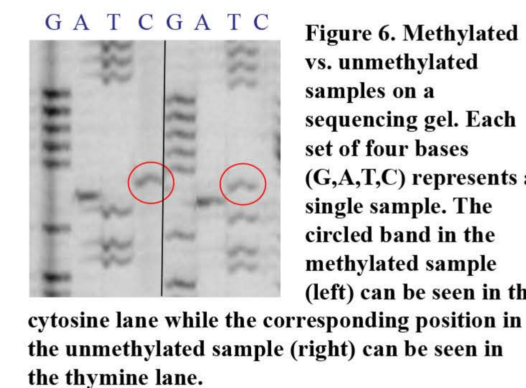

Figure 6. Methylated vs. unmethylated samples on a sequencing gel. Each set of four bases (G,A,T,C) represents a single sample. The circled band in the methylated sample (left) can be seen in the cytosine lane while the corresponding position in the unmethylated sample (right) can be seen in the thymine lane.

Methylation at 15.5 dpc in WT vs. P/P showed a greater decrease in 2˚ DMRs compared to 1˚ DMRs with some exceptions

Since the results from analyzing individual subclones were limited by sample size, methylation data at 15.5 dpc was collected following Next Generation Sequencing (NGS). A total of 15 DMRs were analyzed. A summary of the results is shown in Figure 9.

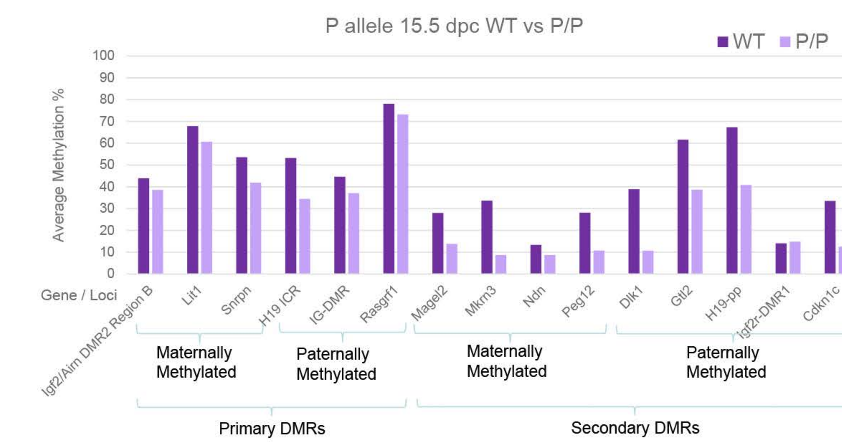

Figure 9. Average methylation at the 15 analyzed DMRs in WT vs. P/P 15.5 dpc embryos.

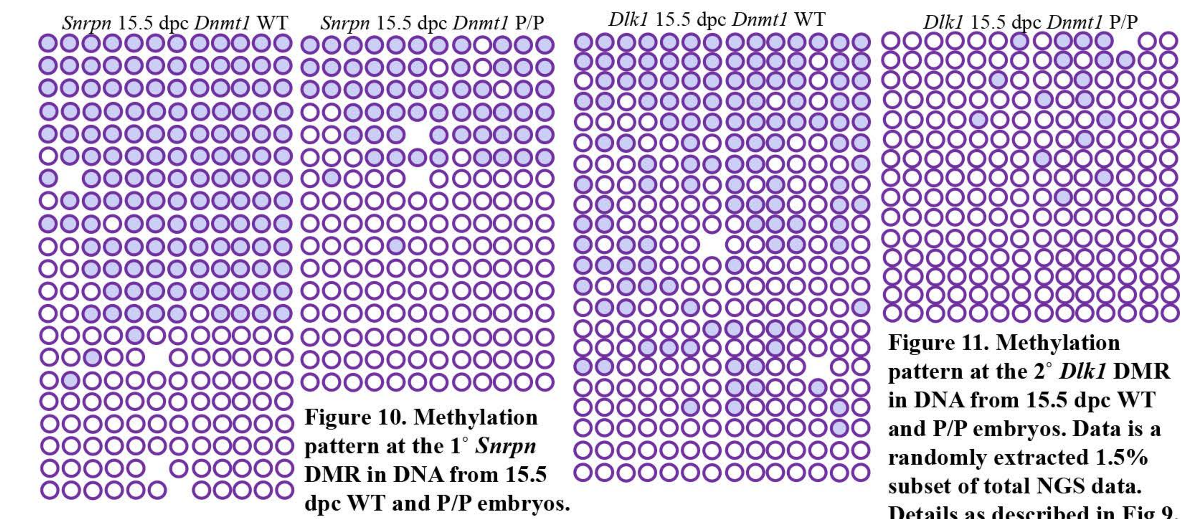

Figure 10. Methylation pattern at the 1˚ *Snrpn* DMR in DNA from 15.5 dpc WT and P/P embryos. Data is a randomly extracted 2% subset of total NGS data. Filled circles represent methylated sites, unfilled ones represent unmethylated sites, while absence of circles represents anomalous data.

Figure 11. Methylation pattern at the 2˚ *Dlk1* DMR in DNA from 15.5 dpc WT and P/P embryos. Data is a randomly extracted 1.5% subset of total NGS data. Details as described in Fig 9.

In general, the data from 15.5 dpc embryos showed that most 2˚ DMRs have a drastic reduction in methylation in P/P as compared to WT while most 1˚ DMRs have a small reduction (Figure 9). The exceptions are the 1˚ DMR *H19*-ICR which showed a drastic reduction, and the 2˚ DMRs *Ndn* which showed a small reduction and *Igf2r*-DMR1 which showed a slight increase. A deeper analysis with individual sequences is needed to see what kind of methylation patterns these DMRs have. The data in Figures 10 & 11 represent a small random subset of the total sequences analyzed of the 1˚ DMR *Snrpn* and the 2˚ DMR *Dlk1*. This random subset showed the same general trend as we saw with the larger dataset with *Snrpn* illustrating a less drastic reduction in methylation between WT and P/P as compared to *Dlk1*. It is also noteworthy that some of the 1˚ DMRs such as *Rasgrf1* have shown a bias towards the methylated allele (average methlylation significantly over the expected 50% in the WT) even with much larger sample size compared to the 12.5 dpc dataset.

Methylation at 12.5 dpc in P/P vs. WT, P/+ varied by DMR

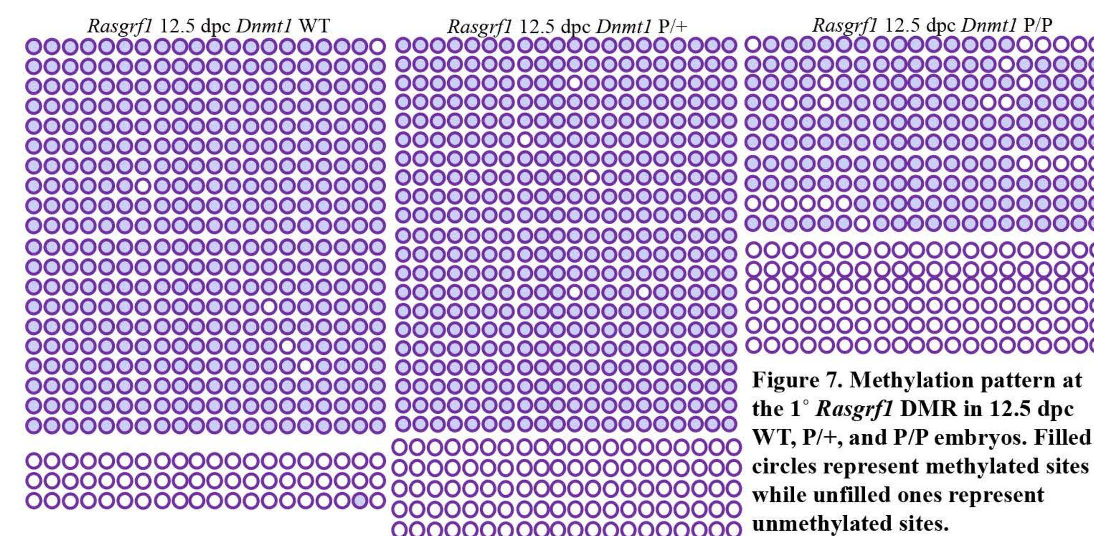

Figure 7. Methylation pattern at the 1˚ *Rasgrf1* DMR in 12.5 dpc WT, P/+, and P/P embryos. Filled circles represent methylated sites while unfilled ones represent unmethylated sites.

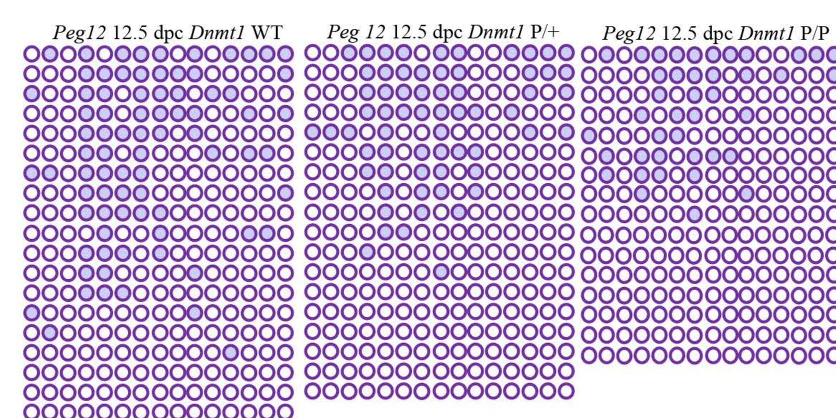

Figure 8. Methylation pattern at the 2˚ *Peg12* DMR in 12.5 dpc WT,P/+, P/P embryos. Details as described in Figure 7.

We analyzed individual subclones from 1˚ and 2˚ DMRs to see if methylation varied in P/P vs. WT, P/+. As expected of a 1˚ DMR, we saw 90-100% methylation on the methylated *Rasgrf1* allele and almost none on the unmethylated one (Figure 7). There was no significant difference between methylation in WT vs. P/+, but the differences between WT vs. P/P and P/+ vs. P/P were significant (Table 2). We did not observe the expected allele distribution of roughly 50-50 methylated-unmethylated alleles, which could be attributed to the sample size. As expected of a 2˚ DMR, variable methylation was observed at *Peg12* with methylation of individual sequences ranging from 0-73% (Figure 8). The differences in methylation among all the groups were not significant (Table 3) but this could be attributed to the small sample size. The data in Figures 7 & 8 are representative, and Tables 2 & 3 show average methylation for the seven loci analyzed in each genetic background as well as whether or not the methylation between genotypes is significantly different. In general, most DMRs showed a reduction in average methylation between WT & P/+ as compared to P/P (except IG-DMR), although whether the difference was significant varied according to genotypes and DMRs.

Table 2. Comparison of average methylation % and P-values at primary DMRs among the various genotypes at 12.5 dpc

Primary DMRs	Average Methylation			Conclusion about Difference (P-value)		
	WT	P/+	P/P	WT vs P/+	WT vs P/P	P/+ vs P/P
Rasgrf1	0.861	0.798	0.544	Not Significant (0.8337)	Significant (0.001)	Significant (0.0045)
Grb10	0.867	0.986	0.759	Not Significant (0.2225)	Significant (0.0124)	Significant (0.0001)
Lit1	0.855	0.966	0.921	Not Significant (0.5419)	Not Significant (0.1336)	Significant (0.0063)
IG-DMR*	0.174	0.483	0.667	Significant (0.0375)	Significant (0.002)	Not Significant (0.6312)

Table 3. Comparison of average methylation % and P-values at secondary DMRs among the various genotypes at 12.5 dpc

Secondary DMRs	Average Methylation			Conclusion about Difference (P-value)		
	WT	P/+	P/P	WT vs P/+	WT vs P/P	P/+ vs P/P
Peg12	0.281	0.237	0.171	Not Significant (0.4839)	Not Significant (0.0854)	Not Significant (0.4295)
Gtl2*	0.745	0.634	0.349	Not Significant (0.865)	Significant (0.0271)	Significant (0.0257)
Magel2*	0.237	0.271	0.090	Not Significant (0.7795)	Not Significant (0.2585)	Significant (0.03)

*WT and P/+ data collected and analyzed by fellow student researcher Christine Siebels-Lindquist.

Discussion and Future Directions

We want to understand how methylation is maintained at secondary DMRs given their high level of variability, and whether methylation is truly maintained vs. lost and reacquired. Analyzing methylation across embryonic development in P/P mice might help us understand how methylation is being maintained and/or reacquired and how these processes are being hindered in the P/P mutants. Our future goal is to examine how the methylation patterns at secondary DMRs change over time which will be done by collecting embryos at different stages of growth. We plan to conduct a more thorough analysis at the genome-wide level to see how this mutation is affecting non-DMR methylation using Reduced Representation Bisulfite Sequencing (RRBS). As a long-term goal, we plan to introduce the P allele mutation in BL6/*castaneus* hybrid mice so that we can analyze SNPs to definitively categorize alleles as maternal and paternal. This will help us differentiate sequences derived from the unmethylated parental allele vs. sequences from the methylated parental allele that have lost methylation in the P/P background. It will also give us better insight on the skew we are seeing in some of the data like the 12.5 dpc and 15.5 dpc *Rasgrf1* data. It is important to understand the reason behind this skew because it might compromise our ability to study methylation pattern over time especially if methylation is being lost and reacquired.

The mouse-rat region where the P mutation is located is absent in the human *Dnmt1* sequence. This could suggest a species-specific role for this portion of the Dnmt1 protein (Shaffer *et al.*, 2015). An example of species-specific expression can be seen at *Rasgrf1* which has paternal allele-specific expression in mice and rat but is non-imprinted in deer mice (Arnaud *et al.*, 2003, Shorter *et al.*, 2012). Since methylation is an important epigenetic factor that regulates gene expression, this study could help us understand species-specific expression of imprinted genes better. Further, as the results showed that many 1˚ DMRs can maintain most methylation in the P/P mutants, Dnmt1 might not be the only methyltransferase playing a role in the maintenance hinting to possible roles of other methyltransferases like Dnmt3a/b which are primarily known to be de-novo methyltransferases.

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