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DNA methylation at differentially methylated regions of imprinted genes is resistant to developmental programming by maternal nutrition

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Abbreviations: ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphate; *Cdkn1c,* cyclin-dependent kinase inhibitor 1c gene; CpG, dinucleotide CG; DMR, differentially methylated region; Dnmt1, DNA methyltransferase 1; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase gene; GLP, maternal gestational low protein diet; *Gnas*, guanine nucleotide binding protein, alpha stimulating, complex locus; *Grb10*, growth factor receptor-bound protein 10 gene; *H19*, H19 gene; H3K4me1, histone H3 monomethylated at lysine 4; H3K4me3, histone H3 trimethylated at lysine 4; H3K9me2, histone H3 dimethylated at lysine 9; *Hnf4a*, hepatic nuclear factor 4 alpha gene; ICR, imprinting control region; *Igf2*, insulin-like growth factor II gene; *Kcnq1ot1*, *Kcnq1* opposite strand/antisense transcript 1 (non-protein coding) gene; KvDMR, DMR in the *Kcnq1ot1* locus; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; *Nespas/Gnasxl*, neuroendocrine secretory protein antisense transcript/Gnas extra large DMR; PCR, polymerase chain reaction; *Pdx1*, pancreatic and duodenal homeobox 1 gene; *Peg3*, paternally expressed gene-3; *Phlda2*, pleckstrin homology-like domain, family A, member 2 gene; PLP, maternal postnatal low protein diet; Pparα, peroxisome proliferator-activated receptor α; RT-qPCR, reverse transcriptase-quantitative PCR; Tet, ten-eleven translocation protein

The nutritional environment in which the mammalian fetus or infant develops is recognized as influencing the risk of chronic diseases, such as type 2 diabetes and hypertension, in a phenomenon that has become known as developmental programming. The late onset of such diseases in response to earlier transient experiences has led to the suggestion that developmental programming may have an epigenetic component, because epigenetic marks such as DNA methylation or histone tail modifications could provide a persistent memory of earlier nutritional states. One class of genes that has been considered a potential target or mediator of programming events is imprinted genes, because these genes critically depend upon epigenetic modifications for correct expression and because many imprinted genes have roles in controlling fetal growth as well as neonatal and adult metabolism. In this study, we have used an established model of developmental programming—isocaloric protein restriction to female mice during gestation or lactation—to examine whether there are effects on expression and DNA methylation of imprinted genes in the offspring. We find that although expression of some imprinted genes in liver of offspring is robustly and sustainably changed, methylation of the differentially methylated regions (DMRs) that control their monoallelic expression remains largely unaltered. We conclude that deregulation of imprinting through a general effect on DMR methylation is unlikely to be a common factor in developmental programming.

Introduction

Epidemiological and animal studies have shown that adverse environments or suboptimal nutritional conditions during pregnancy can alter the physiology of offspring and increase their predisposition to many diseases in adult life. This phenomena is referred to as developmental programming.[1,2] One explanation advanced to account for long-term or later onset outcomes of early life experiences is epigenetic: that interventions, through adaptive or maladaptive responses, result in a permanent resetting of gene expression programs that is mediated by altered epigenetic marking (DNA methylation or posttranslational modifications

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of histones), which can persist long after the duration of the initiating factor or condition.[3,4] This takes account of the fact that patterns of DNA methylation and histone modifications experience wholescale changes during embryogenesis, lineage specification and differentiation, and that there may be time-points particularly in pre- and early post-implantation development and lineage segregation during which epigenetic modifications could be especially vulnerable to abnormal nutritional states. Some epigenetic modifications, in particular DNA methylation, can be stably propagated through numerous cell divisions as a cellular memory of earlier events.

Nutritional and other environmental challenges could affect epigenetic marks in a variety of ways. They could change the availability of the common methyl donor, *S*-adenosylmethionine (SAM), used in DNA and histone methylation reactions, or alter the expression or activity of epigenetic modifiers. Accordingly, DNA methylation patterns have been reported to be sensitive to methyl deficient diets[5-7] or to variations in other components of one-carbon metabolism, such as folate.[8] Histone demethylases require flavin adenine dinucleotide or α-ketoglutarate,[9] so that their activities are also potentially responsive to cellular energy status. In addition the Ten-eleven translocation (Tet) proteins involved in the active removal of cytosine methylation by oxidation depend upon oxoglutarate. It is possible, therefore, that nutritional status could have global, non-specific effects on histone modifications and DNA methylation, or locus-specific effects that could be more adaptive in nature.

A set of genes that has received a great deal of interest as possible mediators of developmental programming effects are imprinted genes.[10-12] This is because these genes are entirely dependent on long-term epigenetic marks for their normal expression. Imprinted genes are unusual in that the two alleles are expressed to different degrees depending on their parental origin; in many cases, there is complete silencing of one parental allele.[13] Imprinting is determined by the establishment of different states of epigenetic modification in male and female gametes at imprinting control regions (ICRs), typified by differences in DNA methylation (giving rise to the term germline differentially methylated region or gDMR). Imprinting also depends on the faithful maintenance of these parental allele-specific marks throughout the lifetime of the individual. We have some understanding about how these gametic marks are created and then maintained in embryos,[14,15] but the degree to which they can be eroded during the lifetime is less clear, although some studies have shown individual variation in DMR methylation levels in human populations.[16-18] Most imprinted genes are clustered, with monoallelic expression of multiple imprinted genes in a domain being controlled in cis by a single ICR.[19,20] The action of the ICR in the embryo results in elaboration of further epigenetic differences between the two alleles across the imprinted domain, including the establishment of additional DMRs (secondary or somatic DMRs) and allelic differences in histone modifications. A particular appeal of imprinted genes as possible targets of developmental programming is that they play important roles in controlling offspring growth, both pre- and post-natally, in early post-natal adaptations and in adult energy homeostasis.[21,22] Owing to their clustered nature and shared dependence on single ICRs, changes in DNA methylation of an ICR would result in altered expression of multiple imprinted genes. Because of their monoallelic expression, imprinted genes are considered to be particularly dosage-sensitive,[23,24] such that even modest deregulation of imprinting at single loci could have phenotypic consequences. Moreover, there could be additive effects from deregulation of several imprinted domains if their imprinting was similarly vulnerable to nutritional or other interventions. In addition, once perturbed, there is thought to be no mechanism to restore normal DNA methylation at ICRs during embryogenesis or later life, because methylation of these elements is specified primarily by mechanisms acting in gametogenesis.[25] There is evidence for altered methylation of DMRs of imprinted genes in some developmental programming phenomena, such as the Dutch Hunger Winter,[26,27] and methylation of some DMRs has been shown to be affected by pre- and peri-conceptional micronutrient supplementation.[28] Nevertheless, the magnitude of reported effects is small and the analysis has necessarily been conducted in accessible tissues in which the physiological relevance of observed changes cannot easily be inferred.

In this study, we have specifically addressed whether expression at imprinted genes is altered in a paradigm of developmental programming and whether this is associated with altered methylation of their DMRs. Using an established model of developmental programming that employs protein restriction of maternal diets during gestation or lactation,[29,30] we find that although changes in expression level of imprinted genes can be detected in liver of offspring from dietary-restricted female mice, DMR methylation appears to be robust.

Results

Expression of imprinted genes in offspring from maternal dietary restriction. Quantitative reverse transcriptase PCR (RT-qPCR) assays were performed to ascertain if imprinted genes exhibited altered expression in liver of offspring of female mice fed an isocaloric low-protein diet during gestation (GLP) or lactation (PLP) and whether any observed gene expression changes were sustained beyond weaning. Five imprinted genes from four imprinted domains—*Gnas*, *Grb10*, *Igf2*, *Cdkn1c* and *Phlda2*—were chosen for analysis owing to their established or possible roles in fetal growth, liver development or physiology. Expression was determined first in liver at the end of postnatal week 3. Significant changes in transcript levels were detected for *Gnas* (reduced in GLP pups; $p = 0.036$) and *Grb10* (elevated in PLP pups; $p = 0.025$) (**Fig. 1A**). Both *Cdkn1c* and *Phlda2* showed a tendency toward reduced expression in the GLP group, but not significantly (*Phlda2* expression levels were particularly low in general; **Table S1**); these genes were not assessed further. *Igf2* expression was not changed in either of the groups compared with controls at this age. In addition, we examined expression of the peroxisome proliferator-activated receptor α gene (*Pparα*), which has been described to be modulated in other developmental programming paradigms, and found that it was upregulated

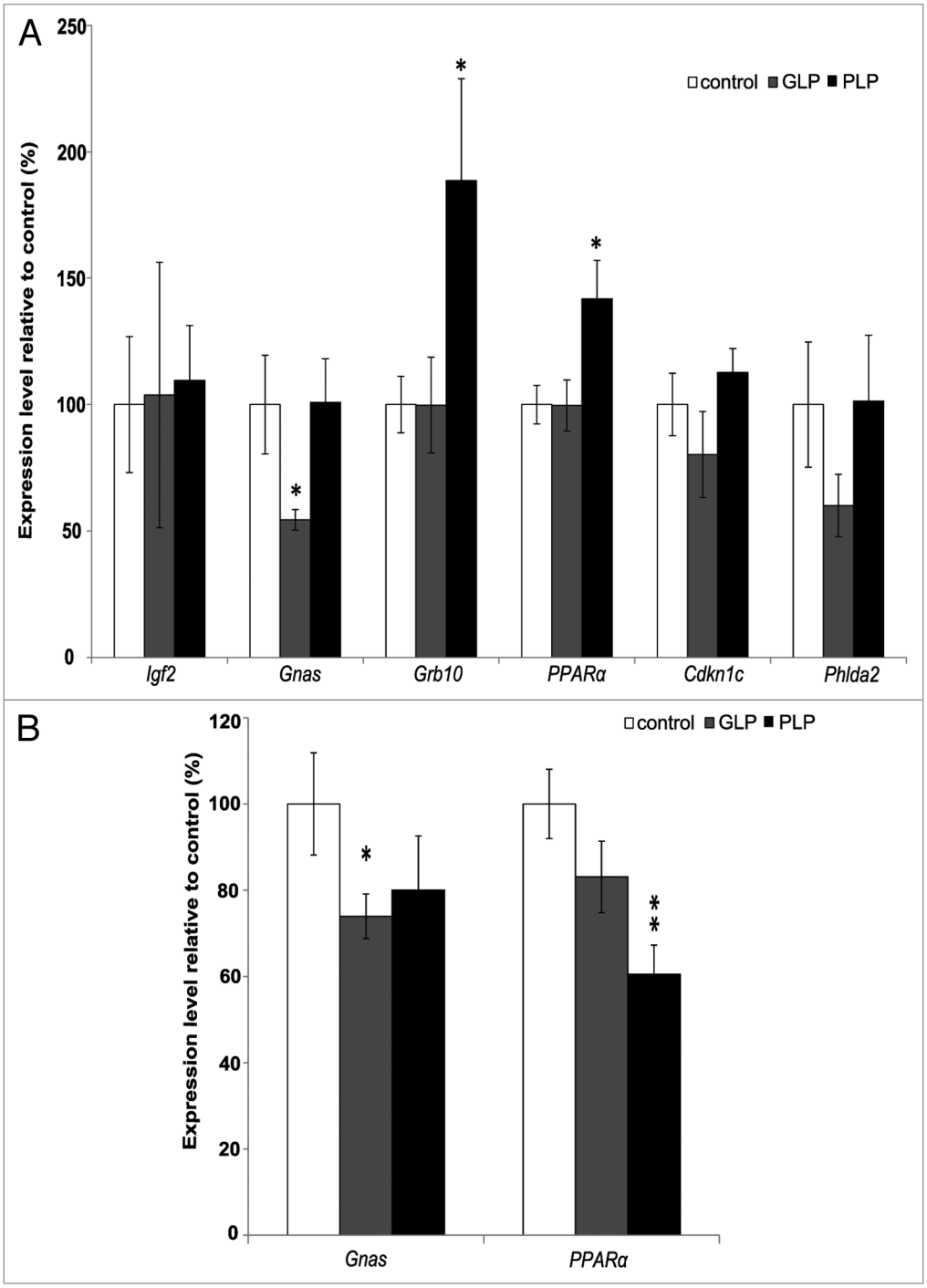


Figure 1. Expression analysis of imprinted and other genes in offspring from dams experiencing dietary protein restriction during gestation (GLP) or lactation (PLP). (**A**) RT-qPCR analysis for the indicated genes at week 3 after birth. (**B**) RT-qPCR analysis for the indicated genes at week 12 after birth. mRNA levels are expressed as the percentage of the means of the control groups (set at 100%) following normalization for each gene against three housekeeping genes. Bars represent standard errors; *p < 0.05, **p < 0.01 compared with control.

in PLP pups (p = 0.014). To assess whether any of these expression changes was sustained into adult life, RT-qPCR was also performed on liver of mice sacrificed at the end of postnatal week 12 (**Fig. 1B**). The significant downregulation of *Gnas* in the GLP group was maintained at week 12 (p = 0.039), although the magnitude of the effect was reduced as compared with week 3. It was not possible to assess expression of *Igf2* or *Grb10*, as these genes are known to be downregulated postnatally and expression was no longer detectable by RT-qPCR (**Table S2**). Surprisingly, the effect on hepatic *Pparα* expression was reversed at week 12, with transcript levels in the PLP group reduced in comparison with the control group (p = 0.002), suggesting dynamic regulation of

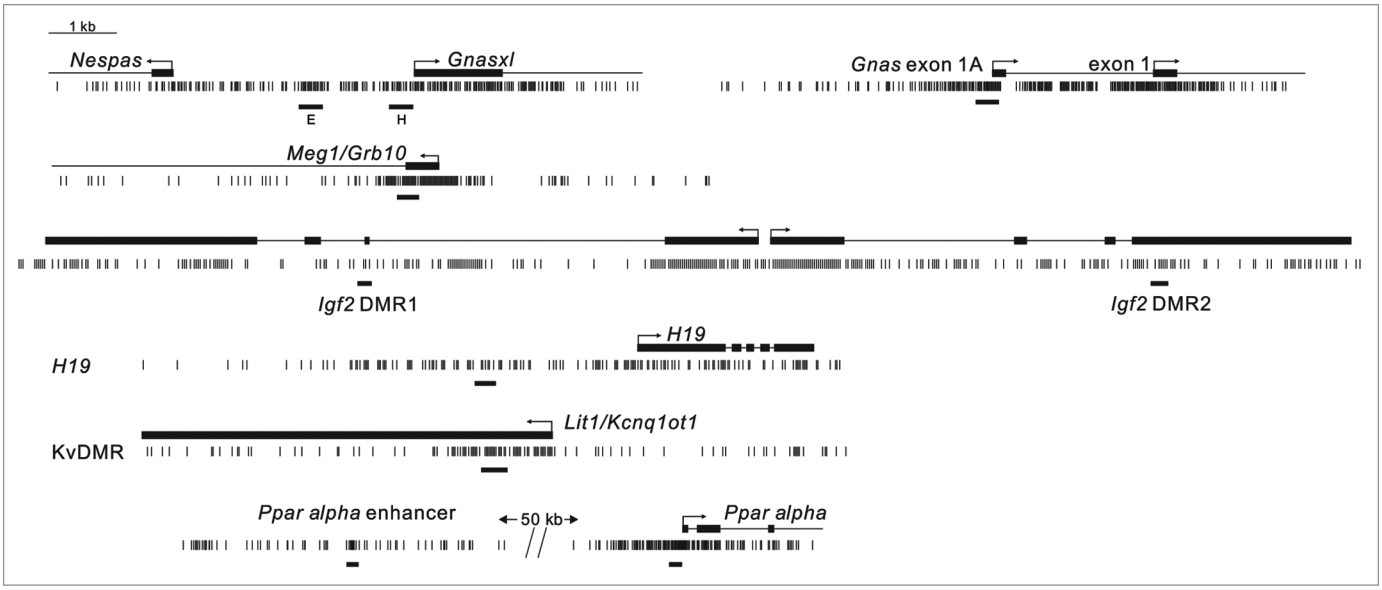
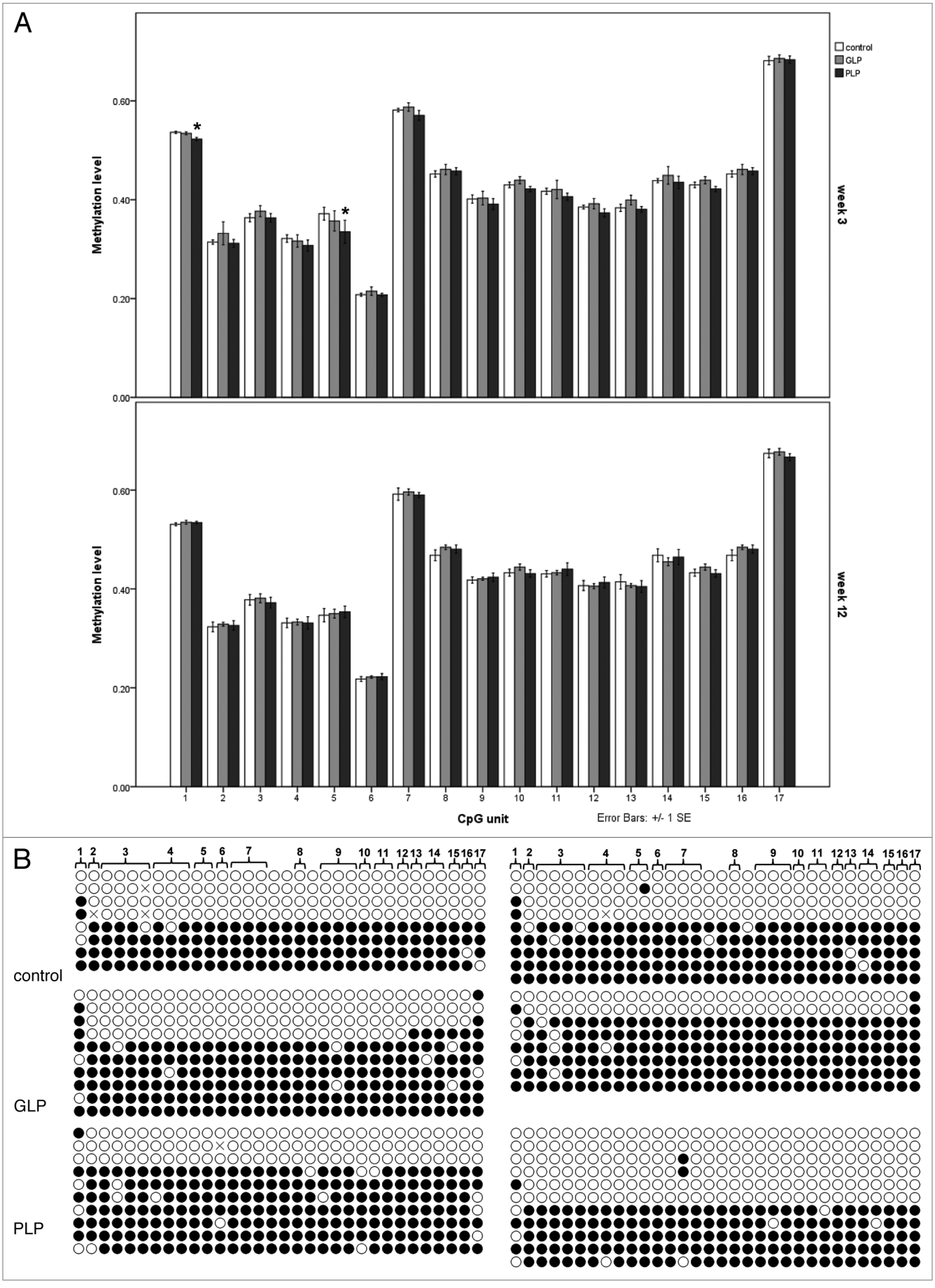


Figure 2. Representation of the location of the amplicons tested in relation to the DMRs within each imprinted domain. Each vertical line represents an individual CpG site. Thick bars above the CpG track represent exons of the indicated transcripts, with transcription start sites and direction of transcription indicated by arrows. The locations of the amplicons used for methylation analysis is given by the short bars under each DMR. In addition, the locations of the amplicons to assay methylation at the *Ppar*α promoter and upstream enhancer are shown similarly.

this gene in pups nursed by dams with dietary protein restriction specifically during lactation.

DNA methylation analysis of imprinted genes in offspring from maternal dietary restriction. For the quantitative assessment of DMR methylation, we used the EpiTYPER system on the Sequenom platform. This method depends upon bisulphite conversion of DNA in which, as in conventional bisulphite sequencing, unmethylated cytosines (C) are converted to uracil (U) but methylated cytosines are resistant to conversion. Bisulphite treated DNA is subject to PCR amplification using primers that include T7 RNA promoter sequences. Amplification products are used as templates for in vitro transcription by T7 RNA polymerase, followed by digestion with RNase A, which cleaves at U and C corresponding to adenosine and guanine in the originating DNA strand. The methylation status of the input DNA will determine the presence of U or C in the RNase A cleaved fragments, affecting their mass, which is revealed with precision by mass spectrometry (MALDI-TOF). In addition, fragment peak height is a direct function of the absolute quantity in the sample, such that the relative heights of the corresponding "methylated" and "unmethylated" peaks provide a quantitative score of methylation of the corresponding "CpG unit." The frequency of RNase A cleavage sites within the region analyzed is such that multiple CpG units can be scored; amplicons are typically designed to cover 300–500 bp. It should be noted, however, that this method does not necessarily provide information on all CpGs at single base resolution: some CpG units comprise multiple CpGs and some CpG units are outside of the effective size range for mass analysis. In addition, the assay provides a quantitative score for CpG unit methylation of the amplicon as a whole, not CpG methylation profiles of individual DNA strands, so it is not possible to discriminate methylation on maternal or paternal alleles by this method. Assays were designed for seven DMRs in four imprinted domains (**Fig. 2**). This included the maternal germline DMRs in the *Gnas* locus (the *1A* DMR and two amplicons in the *Nespas/Gnasxl* DMR), in *Grb10* and at *Kcnq1ot1* (the KvDMR, which also regulates monoallelic expression of the *Cdkn1c* and *Phlda2* genes), the paternal germline DMR at *H19* (responsible for monoallelic expression of *Igf2*) and the paternally methylated, somatic DMRs in *Igf2* (DMR1 and DMR2). Between 9 and 17 CpG units were scored for each amplicon, apart from the two, less CG-rich DMRs at *Igf2*, for which only three CpG units were assessed (**Table S3**). In addition, CpG islands at the *Ppar*α promoter and an upstream enhancer[31] were assayed, as well as B1 elements to provide a measure of global DNA methylation.

We found that the EpiTYPER method was quantitative and reproducible, but exhibited some idiosyncrasies that suggested that the methylation score does not necessarily correspond directly to the percentage methylation of given CpGs (or CpG units). This was apparent, for example, in analysis of the *Gnas* exon 1A DMR (**Fig. 3**). Although the mean methylation score across the amplicon as a whole was consistently between 42.1% and 44.2% (**Table 1**), close to the expected 50% for a faithfully maintained germline DMR, the individual methylation scores of the 17 CpG units analyzed ranged between 20% and 65% (**Fig. 3A**). However, the methylation profile was highly reproducible between samples (**Fig. S1**). Although it might be anticipated that the maternal allele is fully methylated and the paternal allele is fully unmethylated at this DMR, the pattern from EpiTYPER analysis could be interpreted to suggest that the CpG comprising CpG unit 6, for example, is substantially unmethylated also on the maternal allele and, conversely, the three CpGs corresponding to CpG unit 7 have a degree of methylation on the normally


A
control
GLP
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Methylation level
0.00
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CpG unit
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Figure 3 (See opposite page). Methylation analysis of the *Gnas* exon 1A DMR. (**A**) Methylation analysis by EpiTYPER of the DMR in control, GLP and PLP liver samples at week 3 (above) and week 12 (below) after birth. Methylation values on a scale 0.0 to 1.0 for each CpG unit that can be resolved by EpiTYPER are given. Values expressed are means and bars represent standard errors; n = 7–9. Where individual CpG units exhibited a significant difference in methylation level in treated group compared with control, * $p < 0.05$. (**B**) Bisulphite sequencing analysis of two representative samples from each of the control, GLP and PLP groups (week 3). Each row of circles represents a single, non-redundant bisulphite sequence clone. Individual CpGs are represented by circles, with open circles indicating unmethylated and filled circles methylated CpGs. The numbering at the top refers to the CpG units identified by EpITYPER analysis; note that some CpG units contain multiple CpGs (bracketed), while other CpGs are not scored by EpiTYPER analysis.

unmethylated paternal allele, because the methylation indices at these positions depart substantially and reproducibly from 50%. To test whether this was indeed the case, we cloned the corresponding PCR products for sequencing of individual clones. This revealed the expected distribution of near fully methylated and near fully unmethylated sequences that would be consistent with strict parental-allele-specific methylation levels (**Fig. 3B**), with no evidence for the skewed methylation level of the adjacent CpGs corresponding to CpG units 6 and 7 predicted by EpiTYPER. A similar analysis was conducted for the *Grb10* DMR (data not shown) and here, again, a strong methylation peak predicted by EpiTYPER (CpG unit 2) was not found as a site of skewed methylation in cloned bisulphite products. Aside from the fact that the methylation profile reported by EpiTYPER does not appear to correspond to the absolute percentage methylation of individual CpGs, the strong sample-to-sample reproducibility (**Fig. S1**) suggests that it provides a reliable method to quantify methylation differences between samples.

To quantify methylation across the DMRs and at the other loci tested using EpiTYPER, in the first place methylation levels for each CpG unit were averaged per group and the total methylation level of the DMR obtained as the mean of the means. The data are summarized in **Table 1**. Considering methylation status at weaning (week 3), most of the DMRs were not significantly altered in either GLP or PLP groups compared with controls. This applies to maternal germline DMRs (*Gnas* exon 1A and the KvDMR), a paternal germline DMR (*H19* DMD) as well as to somatically acquired DMRs (*Igf2* DMR1 and DMR2). In the GLP group (offspring from dams protein restricted during gestation) we observed an increase in methylation in the *Grb10* maternal germline DMR (34.05 ± 1.11 in control compared with 37.97 ± 1.46 in the PLP group; $p = 0.014$; **Table 1**). However, at the CpG-unit level, the difference was significant, or only reached borderline significance, at six of the 15 CpG units scored (**Fig. 4**). Similarly, there was an increase in methylation in the maternal germline DMR at *Nespas/Gnasxl* amplicon E (control = 68.7 ± 1.60, PLP = 72.98 ± 1.86; $p = 0.041$; **Table 1**). However, only three of 16 CpG units in this amplicon had a significant difference (data not shown), and the overall methylation level of a second amplicon tested in the *Nespas/Gnasxl* DMR, amplicon H, showed no significant difference between groups. When tested at week 12, no significant differences between the groups were detected at any of the DMRs (**Table 1**). There was no evidence for greater variation in DMR methylation levels in the GLP or PLP groups; nor was there a general increase in variation of DMR methylation between week 3 and week 12 (data not shown). We also assessed methylation of the promoter and upstream enhancer of *Ppar*α, in view of the significantly altered expression detected in the PLP group both at week 3 and 12. Methylation of the promoter was low and not significantly altered in either experimental group at either time point (**Table 1**). On the other hand, there was an overall increase in methylation of the upstream enhancer region of *Ppar*α at week 3 in the PLP group (from 60.3 ± 0.86 to 64.0 ± 1.6; $p = 0.048$). The overall genome methylation level was not found to be affected in offspring by maternal diet as assessed by methylation of B1 repetitive elements (**Table 1**).

Discussion

In the current study, we investigated germline and somatic DMR methylation and imprinted gene transcript levels in offspring of female mice that had experienced a low protein diet during gestation (GLP) or the early postnatal period. Early nutritional deficits can potentially affect DNA methylation of the developing organism through one-carbon mechanism, which provides the methyl groups for all biological methylation reactions and depends on dietary methyl donors. Epigenetic marks could be particularly vulnerable during early stages of embryonic development, which is a critical period for their establishment and maintenance.[32] Despite a number of reports concerning the effects of parental or early life dietary status on DNA methylation,[26,27,33-39] it is still unclear the extent to which manipulating maternal diet can lead to substantial and sustained methylation changes in offspring and what the underlying mechanisms might be.

We investigated DMR methylation at five imprinted loci and found that dietary protein deficiency either during gestation or the early postnatal period did not have a substantial impact on the methylation levels of imprinted gene DMRs in the liver of offspring. It is possible that there are tissue-specific effects on the methylation of one or more of the DMRs, but our results indicate that there are no constitutive effects in response to major maternal nutritional deprivation. Therefore, DMRs do not appear to be especially sensitive to early dietary manipulations, and it might be that mechanisms that faithfully maintain methylation at DMRs have evolved to help ensure that imprinted genes are normally regulated within quite narrow boundaries and that imprinted genes are not especially plastic to nutritional or environmental states. We did observe slight gain of methylation in the *Grb10* and *Nespas/Gnasxl* DMRs in 3-week old offspring from females protein-restricted during gestation, but the changes affected a limited number of CpGs, were not present throughout the DMR in the case of *Nespas/Gnasxl*, and were not sustained into adulthood. Such transient changes in methylation could be explained in a variety of ways. If gain of methylation on the normally unmethylated allele was mosaic, it might be of insufficient density to ensure maintenance by DNA methyltransferase 1 (Dnmt1) at DNA replication, or insufficient to recruit repressive

Table 1. Effect of maternal low protein diet on DMR methylation in offspring

DMR	DMR status	Week	Group				
			Control	GLP-control	p value	PLP-control	p value
***Gnas* (Exon 1A)**	Maternal germline	Week 3	42.2 ± 0.99	1.62 ± 1.39	0.399	-0.13 ± 1.34	0.993
		Week 12	44.2 ± 0.88	-0.71 ± 1.32	0.812	-0.15 ± 0.99	0.990
Grb10	Maternal germline	Week 3	34.05 ± 1.11	**3.92 ± 1.46**	**0.014**	-0.54 ± 1.39	0.895
		Week 12	47.6 ± 1.15	-2.91 ± 1.49	0.094	-1.98 ± 1.43	0.285
H19	Paternal germline	Week 3	31.6 ± 1.56	-0.17 ± 2.58	0.997	0.85 ± 2.43	0.914
		Week 12	35.9 ± 1.70	-1.29 ± 2.63	0.844	-0.29 ± 2.4	0.990
***Igf2* (DMR1)**	Paternal somatic	Week 3	65.7 ± 0.55	0.22 ± 1.06	0.971	-2.21 ± 1.14	0.106
		Week 12	57.4 ± 0.74	-0.41 ± 0.86	0.849	0.52 ± 0.84	0.763
***Igf 2* (DMR2)**	Paternal somatic	Week 3	43.3 ± 1.47	0.66 ± 2.08	0.933	1.09 ± 2.34	0.862
		Week 12	36.7 ± 1.33	1.48 ± 1.6	0.541	0.32 ± 1.54	0. 966
***Kcnq1ot1* (KvDMR)**	Maternal germline	Week 3	36.1 ± 0.98	-1.35 ± 1.5	0.574	1.71 ± 0.46	0.458
		Week 12	41.0 ± 0.74	1.66 ± 1.16	0.262	2.19 ± 1.08	0.081
***Gnas* Nespas/Gnasxl (Amplicon E)**	Maternal germline	Week 3	**68.7 ± 1.6**	**4.28 ± 1.86**	**0.041**	0.69 ± 1.75	0.890
		Week 12	64.4 ± 1.5	2.77 ± 2.3	0.388	3.63 ± 2.26	0.197
***Gnas* Nespas/Gnasxl (Amplicon H)**	Maternal germline	Week 3	61.7 ± 0.86	-1.86 ± 1.39	0.310	1.51 ± 1.44	0.478
		Week 12	42.3 ± 0.56	1.20 ± 0.76	0.200	-1.56 ± 0.72	0.055
***Pparα* promoter**	Not applicable	Week 3	6.08 ± 1.07	0.22 ± 1.86	0.990	0.55 ± 1.9	0.938
		Week 12	6.38 ± 1.27	-0.17 ± 1.71	0.993	-0.57 ± 1.7	0.916
***Pparα* enhancer**	Not applicable	Week 3	60.3 ± 0.86	0.4 ± 1.2	0.633	**3.74 ± 1.6**	**0.048**
		Week 12	45.3 ± 1.95	-3.05 ± 2.1	0.243	1.18 ± 2.3	0.799
B1 repetitive elements	Not applicable	Week 3	40.8 ± 6.27	-1.02 ± 9.18	0.991	4.19 ± 9.34	0.867
		Week 12	40.4 ± 7.95	-1.52 ± 12.4	0.989	-5.11 ± 11.5	0.868

Overall DMR methylation levels in mice at week 3 and 12 of age born to dams fed a low protein diet during gestation (GLP) or lactation (PLP). The columns GLP-control and PLP-control give the difference in absolute methylation percentage between the treated groups and control group. n = 4–10 per group; data are expressed as means ± standard error. P values were obtained from Dunnett's post-hoc tests following 1-way ANOVAs.

complexes that in turn recruit DNA methyltransferase activities. Alternatively, as the liver experiences continual replacement of cell populations, the cell populations with altered methylation might have been replaced by week 12 from progenitor cell pools in which DMR methylation was normal. The magnitude of the effects we observe are similar to the findings of Tobi et al.[27] in blood samples from individuals from the Dutch Hunger Winter who were exposed to malnutrition in utero, as well as those from Cooper et al.[28] who studied an African cohort receiving periconceptional micronutrient supplementation, or Kovacheva et al.,[38] who report changes in *Igf2* DMR2 methylation in response to altered maternal dietary choline. In each case, as with the current study, only isolated DMRs demonstrated altered methylation and the magnitude of change is small.

In contrast to the relatively small changes in DMR methylation, we detected more robust changes in expression of some imprinted genes as a consequence of maternal dietary protein restriction. Substantial upregulation of *Igf2* mRNA in liver of pups on the day of birth without corresponding changes in DMR methylation have also been reported in a rat maternal low protein model model.[39] We found a significant increase in *Grb10* expression in liver of 3-week-old offspring from females experiencing protein restriction during lactation, but not in the GLP group in which offspring were assessed three weeks after the end of the maternal dietary restriction. Radford et al.[40] also reported upregulation of *Grb10* transcripts in liver of e16.5-old fetuses of dams experiencing caloric restriction late in gestation, suggesting that upregulation of *Grb10* expression is a consistent, but transient, response to nutritional insufficiency. Grb10 is an adaptor protein that interacts with a number of receptor tyrosine kinases that regulate cell metabolism, development and growth.[41] Analysis of mice deficient in Grb10 has demonstrated a role of Grb10 in attenuating insulin receptor signaling and in glucose metabolism in the liver around birth,[42,43] so the effect we observe could be physiologically relevant, at least at that time, although *Grb10* expression in liver is declining in the immediate postnatal period and does not persist to adulthood.[43] If increased expression reflected a degree of loss of imprinting of *Grb10* caused by altered methylation of its DMR, one would anticipate it to be accompanied by gain in methylation, as methylation is normally present on the expressed maternal allele and the unmethylated copy of the DMR is proposed to operate as a silencer.[44,45] However, we did not

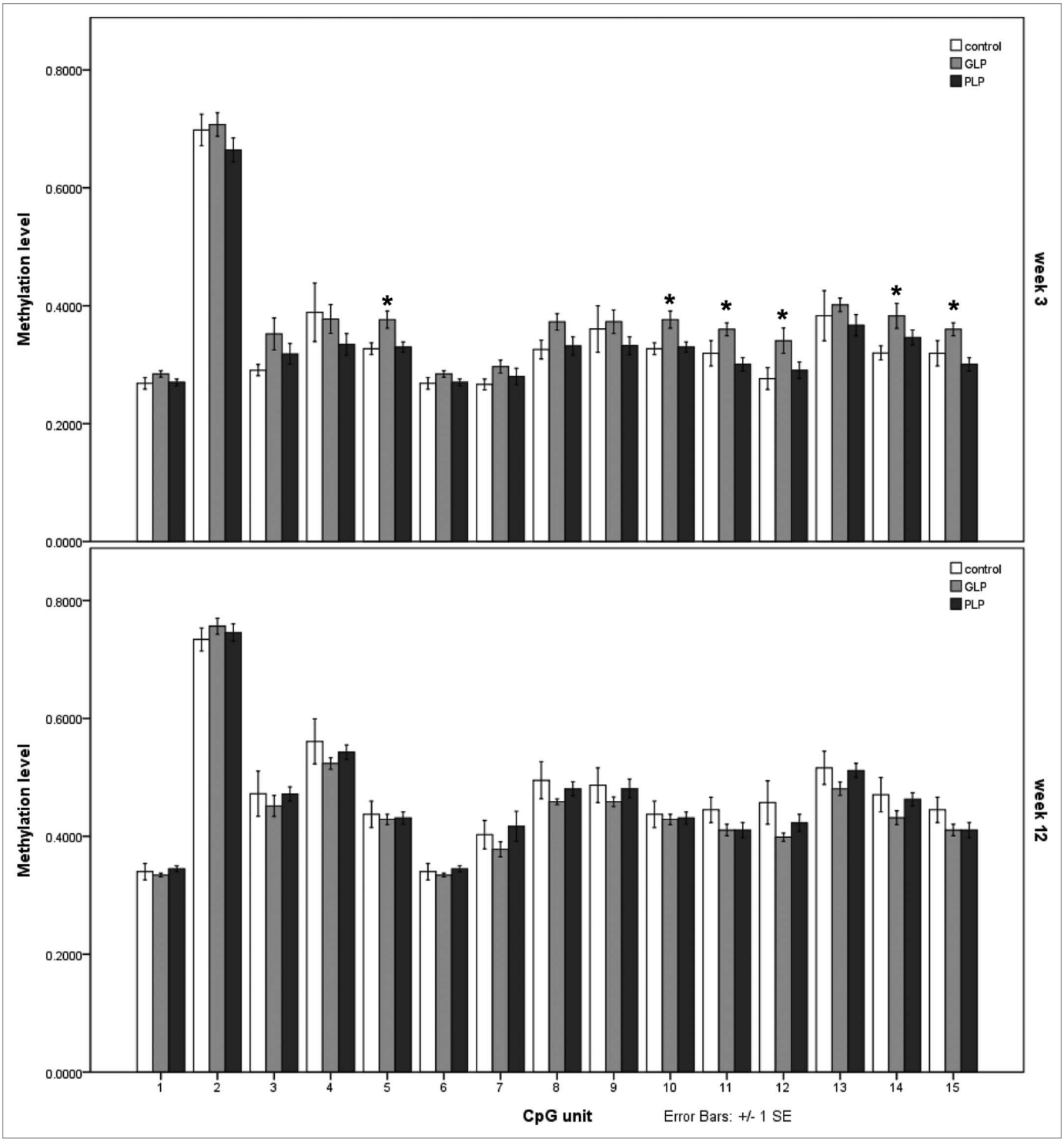

Figure 4. Methylation analysis of the *Grb10* DMR. EpiTYPER analysis of the DMR in control, GLP and PLP liver samples at week 3 (above) and week 12 (below) after birth. Methylation values for each CpG unit that can be resolved by EpiTYPER are given. Values expressed are means and bars represent standard errors. n = 7–9; *p ≤ 0.055 compared with control.

detect significantly altered *Grb10* DMR methylation in the PLP group, rather in the GLP group, showing that adaptive changes in imprinted gene expression can occur independently of changes in DMR methylation. For *Gnas*, we detected downregulation of expression in liver of 3-week as well as 12-week-old offspring from females experiencing gestational protein restriction. The stimulatory G-protein subunit Gsα, encoded by *Gnas*, mediates responses to glucagon and catecholamines in the liver, via the cAMP-potein kinase A pathway, including transcriptional activation of genes encoding gluconeogenetic enzymes. Liver-specific

ablation of Gsα results in increased insulin sensitivity, increased hepatic glycogen storage and blunted induction of gluconeogenic enzymes but, paradoxically, little impairment of gluconeogenesis on fasting, probably owing to a compensatory increase in extrahepatic sites of gluconeogenesis.[46] Considering its role in glucose mobilization in fasting, the reduction in *Gnas* expression in livers of the GLP group might reflect a rebound effect in offspring that have come out of gestational deprivation and have experienced normal maternal diet during lactation. *Gnas* expression is normally biallelic in liver, so changes in expression would presumably be immune to altered methylation of the 1A DMR, which has major importance in regulating allelic expression of *Gnas* in tissues with imprinted expression.[47,48] In this case, therefore, there are adaptive and sustained changes in imprinted gene expression independent of changes in DNA methylation. The notion that transcription factor dependent changes in imprinted gene expression rather than epigenetic deregulation occur in developmental programming paradigms was also noted by Radford et al.,[40] in their study of offspring from calorie-restricted dams, in which they found altered expression of the imprinted gene *Peg3* in liver of offspring with no detectable effect on the methylation level of the *Peg3* DMR. Similarly, the upregulation of *Igf2* mRNA detected in liver at birth[39] without corresponding effects on DMR methylation noted above could reflect a transcriptional response to altered maternal physiology at a time that gene expression patterns in the liver are being substantially reprogrammed as the pup adapts to the transition from placental supply of nutrients to gastric supply of milk, rather than a specific deregulation of *Igf2* imprinting.

Offspring of female rats fed a protein-deficient diet have been reported as having decreased methylation at the *Ppar*α promoter. Pparα is a transcription factor involved in the regulation of numerous metabolic processes and abundantly expressed in the liver. The absolute difference in methylation reported was relatively small, but was associated with a large change in *Ppar*α expression,[33] and the observed reduction in methylation was transmitted from F1 to F2 generations.[49] In our study, we did not detect any significant changes in *Ppar*α promoter methylation at week 3 or 12 of postnatal development in either GLP or PLP groups compared with controls; however, *Ppar*α gene expression was affected. Postnatal maternal protein restriction was associated with upregulation of *Ppar*α transcripts at week 3 in offspring. This could be explained by the physiological state of mice rather than changes in promoter methylation, as it is known that *Ppar*α expression is activated by fasting.[50] Conversely, *Ppar*α expression was found to be downregulated at postnatal week 12 in the same group but, again, this was not associated with any changes in promoter methylation. In addition to effects at the promoter, altered methylation of an enhancer element for *Ppar*α in liver of offspring of male mice subjected to a protein-restricted diet has been reported.[31] However, although we detected increased methylation of this element at weaning in the PLP group, the gain in methylation was small, correlated unexpectedly with increased transcript levels and was not sustained to the later time point when *Ppar*α expression is downregulated, again indicating a disconnect between methylation and expression.

Altered gene expression, including imprinted genes, in the offspring of diet manipulated females could be brought about and perpetuated by epigenetic mechanisms other than DNA methylation. For example, maternal protein restriction results in reduced expression of the *Hnf4a* gene in pancreatic islets of adult rat offspring. This change is accompanied by altered levels of histone modifications, e.g., a reduction in histone H3 lysine-4 monomethylation (H3K4me1) and elevation of the repressive modification H3K9me2 at an enhancer element, and by a reduced interaction of this enhancer with the pancreatic-specific promoter.[36] In another model of developmental programming, intrauterine growth restriction induced by uterine artery ligation in pregnant rats is associated with reduced expression of the *Pdx1* gene in islets, also accompanied by altered histone modification state.[37] In the growth restricted fetuses, there is increased binding of histone deacetylases and reductions in H3 and H4 acetylation at the *Pdx1* promoter, effects on H3K4me3 and H3K9me2 are detected after birth, and only in adults does the promoter become DNA methylated. Despite these important observations, the mechanisms by which early life experiences program longer-term changes in gene expression, and whether the observed epigenetic modifications by themselves are able to sustain altered expression of the associated genes in the absence of ongoing, perhaps unidentified, physiological signals, are still unclear. Our study, together with other recent results,[40] does not single out imprinted genes and relaxation of imprinting as particular purveyors of developmental programming. The challenges for future work are to identify the biochemical signals linking nutritional deficiency and epigenetic changes and the degree to which epigenetic outcomes are selected or global.

Materials and Methods

Animals and tissues. The animal model used in this study has been described previously.[51] Mice (C57BL/6J) were bred at a designated animal unit (University or Cambridge) following approval from the Local Ethics Committee and in accordance with the Home Office Animals (Scientific Procedures) Act 1986. Adult females were individually housed and maintained at 22°C on a 12h:12h light:dark cycle. At six weeks of age mice were mated and pregnancy established by visual appearance of a vaginal plug. Pregnant females were divided into two groups with the first fed ad libitum (20% protein) and the second maintained on isocaloric low protein diet (8%) during gestation. Both diets had identical contents of methyl donor folic acid and choline. To establish the final experimental groups, pups were cross-fostered at three days of age, as follows. The first experimental group comprised pups born and nursed by normal fed dams (control group); the second group were pups born from ad libitum mothers and nursed by females fed low protein diet during lactation (postnatal low protein group, PLP); the third group were pups born from protein restricted dams and nursed by control dams (recuperated or gestational low protein group, GLP). Effects in offspring of prenatal or postnatal maternal dietary protein restriction were studied at 21 d after birth (week 3, weaning) and 12 weeks after birth. Prior to tissue collection, food was withdrawn overnight

from mice. Liver samples were snap frozen in liquid nitrogen and stored at -80°C before processing. Experimental groups for all analysis reported here were 8–9 animals from separate litters.

Real-time quantitative RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen). Total RNA, 2 μg, was reverse-transcribed using SuperScript II (Invitrogen). Real-time quantitative PCR (qPCR) was performed using a Biorad CFX-96 system, according to standard protocols, using SYBR Green Master Mix (Applied Biosystems). PCR reactions (12 μl) contained 2 μl of 1:20 diluted cDNA and each sample was assessed in triplicate. Expression levels were calculated by the standard curve method[52] using three housekeeping genes (β-actin, β-microglobulin and *Gapdh*) for normalization. Primer sequences for *Gnas*, *Igf2*, *Grb10*, *Cdkn1c*, *Phlda2* and *Ppar*α transcripts, as well as the housekeeping genes, are listed in **Table S4**.

DNA methylation analysis. Genomic DNA was isolated from liver using All Prep DNA/RNA kits (Invitrogen). DNA, 1 μg, was bisulphite converted using the EZ-DNA Methylation Gold kit (Zymo Research). DNA methylation at the indicated loci was measured using a mass-spectrometry based method (EpiTYPER, Sequenom). Pre-analysis sample treatment was performed according to the manufacturer's specifications. After conversion, DNA was eluted in 50 μl and PCR was performed on 20 ng of bisulphite treated DNA. PCR primers specific for bisulphite converted DNA for the DMRs in the imprinted loci *Gnas* (*Nespas/Gnasxl* and *Exon 1A* DMRs), *Igf2* (DMR1 and 2), *Grb10, H19* and *Kcnq1ot1* (KvDMR), as well as regions of the *Ppar*α promoter and enhancer, and B1 repetitive elements are listed in **Table S3**. All samples were analyzed in duplicate, and all experimental and control samples for a given time point were analyzed on a single plate. Data quality filtering included removal of CpG units from analysis for which the measurement success rate was below 90%, which was typically caused by low or high masses of fragments. For methylation analysis the difference in methylation score for each CpG unit between technical replicates was expressed in percentages, the mean technical variability (difference) and confidence intervals were calculated for each amplicon, and replicates between which the difference was above the upper boundary were removed from the analysis. Selected amplicons were also analyzed by conventional bisulphite sequencing and cloning, as described in.[53] Amplicons were cloned into the vector pGEM-T Easy (Promega) and 12 clones per sample were sequenced. Analysis of sequences was done using BiQ Analyzer.[54] Sequences with < 90% conversion based on conversion rate of non-CpGs and duplicate sequences based on identical patterns of non-CpG non-conversion events were excluded.

Statistical analysis. All data are expressed as means ± standard error. The effects of maternal diet manipulation on gene expression and DNA methylation were assessed by one-way ANOVA or two-way ANOVA followed by Dunnet's post-hoc tests, performed with PASW Statistics v.18.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/22141

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