A mono-allelic bivalent chromatin domain controls tissue-specific imprinting at *Grb10*

Lionel A Sanz¹, Stormy Chamberlain^{2,4},
Jean-Charles Sabourin^{3,4}, Amandine
Henckel¹, Terry Magnuson²,
Jean-Philippe Hugnot³, Robert Feil^{1,*}
and Philippe Arnaud^{1,*}

¹Institute of Molecular Genetics, CNRS UMR-5535 and University of Montpellier II, Montpellier, France, ²Department of Genetics, Carolina Center for the Genome Sciences, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA and ³INSERM U583, Physiopathologie et Thérapie des Déficiences Sensorielles et Moteurs, Institut des Neurosciences de Montpellier, Hôpital St Eloi, Montpellier, France

Genomic imprinting is a developmental mechanism that mediates parent-of-origin-specific expression in a subset of genes. How the tissue specificity of imprinted gene expression is controlled remains poorly understood. As a model to address this question, we studied *Grb10*, a gene that displays brain-specific expression from the paternal chromosome. Here, we show in the mouse that the paternal promoter region is marked by allelic bivalent chromatin enriched in both H3K4me2 and H3K27me3, from early embryonic stages onwards. This is maintained in all somatic tissues, but brain. The bivalent domain is resolved upon neural commitment, during the developmental window in which paternal expression is activated. Our data indicate that bivalent chromatin, in combination with neuronal factors, controls the paternal expression of *Grb10* in brain. This finding highlights a novel mechanism to control tissue-specific imprinting.

The EMBO Journal (2008) 27, 2523–2532. doi:10.1038/emboj.2008.142; Published online 24 July 2008

Subject Categories: chromatin & transcription

Keywords: bivalent chromatin; Eed; *Grb10*; imprinting; tissue-specific imprinting

Introduction

Genomic imprinting is a form of non-Mendelian inheritance in mammals, whereby some genes are expressed from a single allele, depending on whether it is inherited from the mother or the father. Many of the 80 imprinted genes discovered so far in humans and mice are involved in the regulation of cellular proliferation and growth, whereas others have key functions in neurological processes and behaviour (Smith *et al*, 2006; Wilkinson *et al*, 2007). Not surprisingly, therefore, deregulation of imprinting gives rise

to abnormal development and is causally involved in a number of growth and behavioural syndromes in humans (Arnaud and Feil, 2005).

To achieve allele-specific expression, imprinted loci are regulated by epigenetic modifications that differentially mark the parental alleles as either active or repressed. This is manifest at key control elements known as imprinting control regions (ICRs), which mediate imprinted gene expression. All known ICRs are marked by DNA methylation on either their maternal or their paternal allele. This allelic methylation is established in the female or male germ line and is subsequently maintained throughout development (Delaval and Feil, 2004). In addition to DNA methylation, ICRs are also marked by differential histone modifications in somatic cells. The allele harbouring DNA methylation is associated with 'repressive' histone marks such as trimethylation of lysine 9 of histone H3 (H3K9me3), whereas the unmethylated allele is marked by 'permissive' marks such as H3K4me2 (Fournier *et al*, 2002; Yang *et al*, 2003; Delaval *et al*, 2007). DNA methylation at ICRs is essential for orchestrating mono-allelic gene expression. However, the precise role of histone tail modifications in this process remains poorly understood.

One possibility is that histone modifications could be important for the ICR-mediated repression of imprinted domains. It is well documented that these modifications can influence gene expression (Margueron *et al*, 2005; Li *et al*, 2007). Thus, their allelic distribution at imprinted loci could be involved in regulating mono-allelic expression. Recent studies of tissue-specific imprinting at the *Igf2r*, *NDN* and *Gnas* loci (Yang *et al*, 2003; Lau *et al*, 2004; Li *et al*, 2004) have correlated imprinted expression with tissue-specific differences in histone modifications. A more direct relationship between histone modifications and tissue-specific imprinting exists at the imprinted *Kcnq1* domain in the mice, where a maternally methylated ICR controls a number of paternally repressed genes, some of which are imprinted in the placenta only. Across the *Kcnq1* domain, the repressed paternal allele is enriched in repressive marks (H3K9me2 and H3K27me3), especially in the placenta (Umlauf *et al*, 2004). A functional role for these marks is suggested by studies showing that placenta-specific imprinting at *Kcnq1* domain is perturbed in mouse conceptuses deficient in G9A, a H3K9me2 methyltransferase (Wagschal *et al*, 2008), or in EED (embryonic ectoderm development), a component of the PRC2 complex that mediates H3K27 methylation (Mager *et al*, 2003).

The growth factor receptor-binding protein 10, GRB10, is a potent growth inhibitor (Charalambous *et al*, 2003). We and others have shown that the mouse *Grb10* gene displays a tissue-specific reciprocal imprinting pattern based on the use of tissue-specific promoters (Arnaud *et al*, 2003; Hikichi *et al*, 2003). In most tissues, *Grb10* is expressed from the maternal allele, whereas in brain, its expression is mainly from the paternal allele. Strikingly, the maternal and paternal expressions initiate from different promoter regions.

*Corresponding authors. P Arnaud or R Feil, Institute of Molecular Genetics, CNRS UMR-5535 and University of Montpellier II, 1919 route de Mende, Montpellier 34293, France. Tel.: +33 4 6761 3661; Fax: +33 4 6704 0231; E-mails: philippe.arnaud@igmm.cnrs.fr or robert.feil@igmm.cnrs.fr

⁴These authors contributed equally to this work

Received: 4 March 2008; accepted: 27 June 2008; published online: 24 July 2008

DNA methylation has been shown to be important in the imprinting of *Grb10* (Arnaud *et al*, 2006). This agrees with the current model to explain the maternal *Grb10* expression that proposes the existence of a methylation-sensitive boundary mechanism that prevents promoter–enhancer interactions (Arnaud *et al*, 2003; Hikichi *et al*, 2003). However, this model does not account for the brain-specific paternal expression where other epigenetic modifications are likely to be involved. To investigate whether histone modifications could be involved, we compared their distribution across key chromosomal regions of *Grb10* in brain and several other tissues. We focused our analysis on the CpG island (CGI) region that corresponds to the putative ICR and comprises brain-specific paternal promoters. We identified a mono-allelic bivalent chromatin domain that carries both active (H3K4me2) and repressive (H3K27me3) histone marks and that controls paternal expression of *Grb10*. The loss of this bivalent chromatin, together with neuronal-specific factors, is important to de-repress *Grb10* expression in brain.

Bi-allelic bivalent chromatin has been observed at the promoters of many genes in embryonic stem (ES) cells as well as in differentiated cells (Azuara *et al*, 2006; Bernstein *et al*, 2006; Roh *et al*, 2006; Pan *et al*, 2007; Mikkelsen *et al*, 2007; Zhao *et al*, 2007), and has been considered to be involved in maintaining key developmental genes in a ‘permissive’ chromatin configuration, while being repressed by H3K27me3 (Azuara *et al*, 2006; Bernstein *et al*, 2006). Our work shows that bivalent chromatin is also important in the control of imprinted gene expression. Furthermore, our data provide one of the first examples of the implication of bivalent chromatin in the control of gene expression in a differentiated tissue.

Results

Paternal enrichment for H3K27me3 on the brain-specific paternal promoters is lost in brain

We previously established that in neonatal tissues, the three CGIs located in the 5′ untranslated region (UTR) of *Grb10* display reciprocally imprinted and tissue-specific promoter activities (Arnaud *et al*, 2003). Here, we extended and confirmed this analysis by using the material obtained from reciprocal crosses between C57BL/6J (B6) and *Mus musculus molossinus* JF1 mice. Maternal expression was detected in all tissues and developmental stages analysed, except in brain, and initiates from exon 1A located in CGI1. By contrast, the brain-specific paternal expression arises from three independent regions: exon 1C in CGI3 and exons 1B1 and 1B2, immediately upstream and at the 3′ end of CGI2 (Figure 1A; Supplementary Figures 1 and 2A). Further analysis of RNA from glial cells and a neuron-enriched fraction of cells derived from embryonic cerebral cortices showed that the paternal expression from exons 1B2 (Yamasaki-Ishizaki *et al*, 2007) and 1B1 (this study) is restricted to neuronal cells (Supplementary Figure 1D).

We previously observed brain-specific hypomethylation of CGI3 on the paternal allele (Arnaud *et al*, 2003), suggesting that DNA methylation is involved in controlling the expression from promoter 1C. However, the regulation of *Grb10* paternal expression cannot be explained solely by DNA methylation. Indeed, DNA methylation pattern does not vary at the brain-specific paternal promoters 1B1 and 1B2

between brain and other tissues. The putative ICR CGI2, which comprises 1B1 and 1B2, maintains its maternal methylation in all tissues and developmental stages analysed (Arnaud *et al*, 2003; Hikichi *et al*, 2003; Yamasaki-Ishizaki *et al*, 2007). Therefore, we focused our analysis on histone tail modifications at CGI2. Particularly, we wished to determine whether allelic histone modification patterns could vary between brain and other tissues. To this purpose, we performed chromatin immunoprecipitation (ChIP), followed by electrophoretic detection of single strand conformational polymorphism (SSCP) to evaluate for each antibody-bound and unbound fractions the relative abundance of the maternal and paternal alleles. ChIP was performed with antisera against permissive (H3K9ac and H3K4me2) and repressive (H3K27me3, H3K9me3 and H4K20me3) marks. In all tissues and developmental stages analysed, we observed constitutive allelic enrichment of specific histone modifications. H3K4me2 was always associated with the unmethylated paternal allele of CGI2, whereas H3K9me3 and H4K20me3 were consistently associated with the DNA-methylated maternal allele (Figure 1B, not shown). By contrast H3K9ac, which marks active chromatin (Jenuwein and Allis, 2001), was found to be specifically associated with the paternal allele in brain and, to a lesser extent, in 12.5 days post-coitum (d.p.c.) embryos in which paternal expression arising from the developing brain is detected (not shown) (Figure 1B and C). A similar distribution was observed for H3K27ac, with no allelic enrichment in adult liver and kidney and a clear paternal enrichment in brain (Supplementary Figure 3A).

Unexpectedly, we observed that the repressive mark H3K27me3 was enriched on the unmethylated paternal allele of CGI2 (Figure 1B). This observation suggested that both the repressive H3K27me3 and the permissive H3K4me2 coexisted on the paternal allele of CGI2. Moreover, paternal H3K27me3 enrichment was specifically lost in brain (Figure 1B and C, not shown). Indeed, relative H3K27me3 paternal enrichment in placenta and kidney (3.29 ± 0.56 and 3.20 ± 0.35 , respectively) was much higher than in adult (1.03 ± 0.05) and neonatal brain (1.26 ± 0.04) (Figure 1C). These observations indicated that at CGI2, the paternal enrichment in H3K27me3 correlates with repression of this promoter region, suggesting a role for this modification in the regulation of the paternal *Grb10* expression.

Allele-specific bivalent ‘H3K4me2/H3K27me3’ chromatin marks CGI2

The finding that the permissive H3K4me2 and the repressive H3K27me3 marks are both enriched on the paternal allele of CGI2 is reminiscent of the bivalent chromatin domains recently described in ES cells (Azuara *et al*, 2006; Bernstein *et al*, 2006). Accordingly, we established that the allelic chromatin pattern across CGI2 in undifferentiated ES cells (line SF1-G) was also characterized by paternal co-enrichment of H3K4me2 and H3K27me3 (Figure 2A). This chromatin pattern was also observed in undifferentiated trophoblast stem (TS) cells (Supplementary Figure 3B). To firmly establish that CGI2 is mono-allelically marked by bivalent chromatin in differentiated cells, we performed sequential ChIP on mouse embryonic fibroblasts (MEFs). In this approach, a first round of ChIP precipitated chromatin fragments enriched in H3K4me2. These fragments were subsequently precipitated with antibodies against H3K27me3. This sequential

Figure 1 Paternal enrichment of H3K27me3 at CGI2 is lost in brain. (A) Overview of tissue-specific imprinted expression of *Grb10*. The upper panel gives a schematic representation of the *Grb10* gene. Untranslated and coding exons are shown in grey and black, respectively. The 5' UTR region encompassing the three CpG islands (CGI1, CGI3 and CGI2) is drawn to scale. CGI1 is located roughly 70 kb upstream of the first coding exon; CGI2 10 kb downstream of CGI1 and CGI3 8.5 kb downstream of CGI1. Maternal expression arises from exon 1A within CGI1, whereas brain-specific paternal expression arises from exons 1C, 1B1 and 1B2 located in CGI3 and CGI2. (B) Representative results of allele-specific analysis of histone marks at CGI2. Native ChIP followed by SSCP-mediated allelic discrimination in the antibody-bound (B) and unbound (U) fractions was performed on the material from (B6 × JF1)F1 mice (indicated on the left). Maternal (mat.) and paternal (pat.) alleles are indicated. The relative position of the SSCP polymorphism at CGI2 is shown (black star). (C) Relative enrichment of the paternal versus maternal allele in chromatin precipitated with anti-H3K27me3 and anti-H3K9ac antisera, respectively. For each tissue, the value is reported as the mean of the results obtained from at least two independent ChIP experiments (n) normalized to the allelic results obtained in the corresponding input chromatin. Brain (n=3); neonatal brain (n=3); 12.5 d.p.c. embryo (n=4); 12.5 d.p.c. placenta (n=2); liver (n=4); kidney (n=5).

round of immunoprecipitation retains only chromatin fragments that carry both the histone modifications. To validate this approach, we included the *Irx2* and *Tcf4* genes as controls. The promoter region of *Irx2* has a H3K4me3/H3K27me3 bivalent chromatin in ES cells (Bernstein *et al*, 2006; Azuara *et al*, 2006) that is maintained in MEFs (Mikkelsen *et al*, 2007; http://www.broad.mit.edu/seq_platform/chip/). The detection of the *Irx2* promoter in the final bound fractions was consistent with these observations (Figure 2B). Conversely, the *Tcf4* promoter is known to be enriched in H3K4me3 only in MEFs (Mikkelsen *et al*, 2007; http://www.broad.mit.edu/seq_platform/chip/), and could be PCR amplified only after the first round of ChIP with the anti-H3K4me2 antibody (Figure 2B). Analysis performed on the CGI2 region demonstrated that both the modifications were present on the same

chromatin fragments (Figure 2B), indicating the presence of a bivalent chromatin in this region in somatic cells.

To evaluate the extent of this bivalent chromatin domain, we analysed chromatin at CGI1, CGI3 and at a third region upstream of the transcriptional unit, which we called UR, for upstream region (Figure 2C). At all three regions, in 'non-brain-tissues' H3K27me3 was associated with the paternal allele. However, H3K4me2 was enriched only on the maternal CGI1 allele and displayed no allelic enrichment at CGI3. This finding shows that the bivalent domain observed in non-brain tissues does not extend beyond the CGI2 region. We completed these observations by consulting an online ChIP database (Mikkelsen *et al*, 2007; http://www.broad.mit.edu/seq_platform/chip/). Chromatin patterns obtained from ES and MEF cells indicate that the entire *Grb10* 5' UTR region is

Figure 2 CGI2 is specifically marked by an H3K4me2/H3K27me3 bivalent domain. (A) H3K4me2 and H3K27me3 are enriched on the paternal allele of CGI2 in undifferentiated ES cells. Analysis was performed on (C57BL/6J $\times$ *M. spretus*) F1 undifferentiated ES cells. (B) H3K4me2 and H3K27me3 are enriched on the same chromatin fragments at the CGI2 region. Sequential ChIP was performed on MEFs to detect chromatin fragments enriched for both H3K4me2 and H3K27me3. *Irx2* was used as a positive and *Tcf4* as a negative control. (C) The H3K4me2/H3K27me3 bivalent domain is restricted to CGI2. Allelic analysis of histone modifications in kidney and brain at CGI1, CGI3 and a non-CpG-rich region located upstream of the transcriptional unit (region 'UR'). Kidney data are shown as an example of representative results obtained with 'non-brain tissues'. At the three regions, H3K27me3, but not H3K4me2, was enriched on the paternal allele. This paternal enrichment is lost in brain tissue. Relative positions of polymorphisms used for the SSCP-based allelic discrimination are shown (black stars). TSS: transcriptional start site.

enriched in H3K27me3, whereas H3K4me3 is more specifically localized in CGI2 and CGI1 (Supplementary Figure 4). This information together with our allelic analysis establish that the paternal allele of the *Grb10* 5' UTR region is enriched in H3K27me3 and that at CGI2, this mark is specifically associated with H3K4me2/me3, thus forming a bivalent chromatin domain.

During neural precursor cell differentiation the CGI2 bivalent chromatin domain is resolved and *Grb10* paternal expression is induced

To evaluate how the CGI2 bivalent chromatin evolves during nervous system development and to explore its effects on *Grb10* imprinted expression, we used cultures of highly enriched embryonic neural precursor cells called neurospheres (Reynolds and Weiss, 1992). Neurospheres are generated in suspension by clonal expansion of neural stem cells and are composed of a mixture of neural stem-progenitor cells with little or no differentiated cells (Deleyrolle *et al*, 2006; Supplementary Figure 5A). Upon growth factor withdrawal in adherent culture conditions, neurospheres generated about 70% astrocytic cells (GFAP⁺), 10–20% neurons (β -3-tubulin⁺) and approximately 1% oligodendrocytes (O4⁺) (Supplementary Figure 5B; Deleyrolle *et al*, 2006).

By exon-specific RT-PCR amplification coupled with sequencing, we revealed that in undifferentiated embryonic neurospheres, *Grb10* was maternally expressed from exon 1A (Figure 3A; Supplementary Figure 2B). This expression pattern correlated with the presence of bivalent H3K4me2/H3K27me3 chromatin on the paternal CGI2 allele (Figure 3B, upper panel). Upon differentiation, we detected both maternal expression from exon 1A and paternal expression

from exon 1B1 (Figure 3A; Supplementary Figure 2B). The bivalent chromatin was almost resolved, as the H3K27me3 enrichment on the paternal allele was largely reduced, whereas H3K4me2 remained unchanged (Figure 3B). To evaluate whether this relative decrease in paternal H3K27me3 was due to the loss of H3K27me3, we determined its abundance by real-time PCR amplification after ChIP. A decrease of nearly 75% was detected at CGI2 upon neurosphere differentiation (Figure 3C). A similar decrease was also observed at CGI1 (Supplementary Figure 5D). Paternal enrichment of H3K9ac was detected in both undifferentiated and differentiated neuronal cells. However, the amount of this modification at CGI2 clearly increased upon differentiation (Figure 3C), suggesting that the allelic enrichment observed in undifferentiated neurospheres arises from a limited subset of cells. We observed a similar pattern for H3K27ac (Supplementary Figure 5C). Thus, during neural stem/progenitor cell differentiation, the CGI2 bivalent domain is almost completely resolved through a decrease of H3K27me3 whereas acetylation increases. This chromatin change is concomitant with the induction of *Grb10* paternal expression from exon 1B1 (Figure 3A).

Ectopic *Grb10* paternal expression in *Eed*^{−/−} ES cells and embryos

In ES cells, bivalent chromatin is thought to maintain key developmental genes in a 'permissive' chromatin configuration through H3K4me2, while repressing them by means of H3K27me3 (Azuara *et al*, 2006; Bernstein *et al*, 2006; [35]Mikkelsen *et al*, 2007). This idea is supported by the finding that some of these genes are inappropriately upregulated in ES cells deficient for EED (Azuara *et al*, 2006), a component of the PRC2 complex that mediates H3K27

Figure 3 Resolution of the bivalent domain upon neural differentiation correlates with paternal *Grb10* expression from CGI2. (A) The promoter usage in undifferentiated (undiff.) and differentiated (diff.) embryonic neurospheres was determined by exon-specific RT-PCR amplification. Relative positions of the polymorphism (black star) and primers used are indicated. Paternal expression from the CGI2 region was detected only after differentiation. De-repression appeared to be limited to exon1B1, as we failed to detect transcripts from exon1B2. (B) Representative allele-specific analysis of histone modifications at CGI2 in undifferentiated (undiff.) and differentiated (diff.) neurospheres. The upper panel shows the results of the SSCP analysis. The lower panel shows the relative enrichment of the paternal versus the maternal allele in H3K27me3 precipitations. (C) The level of H3K27me3 decreases at CGI2 during neurosphere differentiation, whereas H3K9ac increases. Following ChIP, the abundance of H3K27me3 and H3K9ac was determined by real-time PCR quantification of bound fractions and reported as a percentage of the value obtained with input chromatin.

tri-methylation. Therefore, to investigate the functional significance of the developmental loss of H3K27me3, we analysed *Grb10* imprinted expression in two *Eed*^{-/-} ES cell lines (i.e. G8.1 and B1.3) (Azuara *et al*, 2006). As controls, undifferentiated wild-type ES cell lines were analysed. Expression from the 1B1 promoter was detected in both mutant lines, but not in the wild-type cells (Figure 4A). However, the absence of allelic polymorphisms in G8.1 and B1.3 cells did not allow us to draw formal conclusions about the parental origin of this transcript. Nevertheless, bisulphite treatment of DNA followed by sequencing suggested that the maternal DNA methylation at CGI2 was unaltered in the two mutant cell lines (data not shown). The detected transcript was thus likely to originate from the unmethylated paternal allele. These results indicate that, in ES cells, *EED* deficiency and the resulting loss of H3K27me3 leads to a partial de-repression of brain-specific promoters at CGI2.

To extend our analysis, we studied mice bearing a novel gene-trap insertion in intron 7 of *Grb10*, referred to as *Grb10*^{XC302}. In these mice, LacZ staining can be used to visualize *Grb10* expression. RNA *in situ* hybridization confirmed that LacZ staining faithfully recapitulated endogenous *Grb10* expression (data not shown). Through paternal and maternal transmission of *Grb10*^{XC302}, *Grb10* imprinted expression was monitored in wild-type and *Eed*^{-/-} embryos. As expected, *Grb10* was widely expressed from the maternal

allele in both cases, as indicated by the ubiquitous blue staining (Supplementary Figure 6). Conversely, following paternal transmission, *Grb10* expression was restricted to specific cell populations in wild-type 6.5 d.p.c. embryos (Figure 4B). Particularly, *Grb10* was expressed in the anterior visceral endoderm (AVE), an extra-embryonic tissue required for specifying early anterior patterning in the mouse embryo. In *Grb10*^{XC302}/*Eed*^{-/-} conceptuses, ectopic paternal expression of *Grb10* was not found to be ubiquitous, but rather, was limited to one part of the extra-embryonic ectoderm (Figure 4B). This ectopic expression is likely to be a direct consequence of the absence of *EED* rather than an expansion of the AVE region in the mutant embryo. Previous lineage analysis showed that the AVE does not expand into the extra-embryonic region (Faust *et al*, 1998; Rivera-Pérez *et al*, 2003). Together these observations suggest that absence of *EED* (and the associated H3K27me3 mark), although involved in, is not sufficient on its own to promote the paternal expression of *Grb10*.

Discussion

In this study, we unravelled how the paternal expression of *Grb10* in brain is controlled. Our main finding is that this process is regulated at least in part by a mono-allelic bivalent chromatin domain that marks the brain-specific paternal promoters of *Grb10*.

Figure 4 Effects of EED deficiency on *Grb10* imprinted expression. (A) EED is required for repressing the CGI2 promoter 1B1 in ES cells. *Eed*-deficient and wild-type ES cells were studied by RT-PCR amplification; *Gapdh* is used as a semiquantitative reference. We failed to detect transcripts initiating from exons 1C and 1B2 in both wild-type and *Eed*-deficient ES cell lines. (B) EED deficiency has a limited impact on *Grb10* paternal expression in 6.5 d.p.c. *Grb10*^{XC302} conceptuses. Embryos from a cross between an *Eed*^{-/+} female and an *Eed*^{-/+}; *Grb10*^{XC302} male were collected at 6.5 d.p.c., genotyped and stained with X-Gal.

We confirmed and extended previous *Grb10* imprinted expression studies (Miyoshi *et al*, 1998; Arnaud *et al*, 2003; Hikichi *et al*, 2003). *Grb10* is likely to be imprinted at pre-implantation stages as maternal expression is detected in both undifferentiated ES and trophoblast cells. Maternal expression is subsequently maintained in the developing embryo, placenta and in all adult tissues, except brain. The reciprocally imprinted, paternal expression observed in brain relies on three different promoters and is restricted to neuronal cells (this study; Yamasaki-Ishizaki *et al*, 2007). This is consistent with the expression pattern we observed in neurospheres, where paternal expression from the promoter in exon 1B1 is initiated upon differentiation. Hence, the paternal expression is activated in the neural lineage, when the neural stem/progenitor cells are committed towards the neuronal fate. Nonetheless, the three promoters are not activated simultaneously, and paternal expression from exon 1C is detected only after birth (data not shown). Therefore, the discrete paternal expression we observed in 6.5 d.p.c. conceptuses is intriguing. This paternal expression could result from a 'leakage' of imprinting at this stage, or could somehow pre-empt the forthcoming brain-specific paternal expression.

The CGI2 region corresponds to the putative ICR of the *Grb10* locus and displays chromatin features found at other ICR regions (Fournier *et al*, 2002; Yang *et al*, 2003;

Delaval *et al*, 2007). Specifically, the DNA-methylated maternal allele is associated with repressive marks (H3K9me3 and H4K20me3), whereas the unmethylated paternal allele is enriched for a permissive modification (namely H3K4me2). This constitutive chromatin pattern could be involved in the maintenance of the allelic DNA methylation observed at ICRs (Fournier *et al*, 2002; Ooi *et al*, 2007; Jia *et al*, 2007). Unlike these constitutive marks, H3K27me3 is enriched on the paternal unmethylated allele of the CGI2 region in all tissues but brain, suggesting that it could be involved in the control of the brain-specific paternal expression of *Grb10*. This paternal enrichment was not restricted to CGI2 but was detected along the whole 5' UTR. Interestingly, we observed a reciprocal enrichment for H3K27me1 that we detected on the maternal allele of the three CGI in different adult tissues, but brain (Supplementary Figure 3C, data not shown). H3K27me1 was shown in human T cells to be enriched downstream of active transcriptional start sites (Barski *et al*, 2007), and could thus be linked to the maternal expression of *Grb10*.

The most striking finding of our study is the colocalization of H3K27me3, a repressive mark, with the permissive mark H3K4me2 on the paternal allele of the putative ICR, constituting an allele-specific bivalent chromatin domain. Bi-allelic bivalent chromatin domains have been characterized for numerous genes in human and mouse ES cells, as well as in differentiated cells (Azuara *et al*, 2006; Bernstein *et al*, 2006; Roh *et al*, 2006; Pan *et al*, 2007; Mikkelsen *et al*, 2007; Zhao *et al*, 2007), but so far they have not been linked to imprinted gene expression. The *Grb10* mono-allelic bivalent chromatin domain is established at an early developmental stage and is maintained in all somatic tissues, but is absent in the brain. The mechanism involved in establishing and maintaining this peculiar chromatin configuration remains elusive. MLL and PRC2 histone methyltransferase complexes, involved in H3K4 and H3K27 methylation, respectively, could localize to the same nucleosomes or on different nucleosomes in close proximity to each other. A direct interaction or colocalization of homologues of these protein complexes has been described in plants and flies (Cavalli and Paro, 1998; Orlando *et al*, 1998; Klymenko and Müller, 2004; Saleh *et al*, 2007) and was suggested in mice (Xia *et al*, 2003). Such close proximity could account for the formation and maintenance of bivalent chromatin. Important information on the mechanisms involved could be obtained by investigating the allelic distribution of the components of these complexes at the putative ICR of *Grb10*.

Bivalent domains have been proposed to mark genes that are 'poised' for expression to occur in response to appropriate developmental cues (Azuara *et al*, 2006; Bernstein *et al*, 2006) and whose transcription is temporarily repressed by H3K27me3 (Boyer *et al*, 2006; Lee *et al*, 2006). At the *Grb10* locus, the presence of bivalent chromatin is strictly correlated with paternal *Grb10* repression. Furthermore, in neurospheres the bivalent domain is resolved upon neural commitment, in the same developmental window during which the paternal promoters become de-repressed. Moreover, one of the paternal-specific promoters is de-repressed in ES cells deficient for EED, a component of the PRC2 complex. Nonetheless, removal of H3K27me3 on its own is probably not sufficient to trigger de-repression. Indeed, analysis of *Grb10*^{XC302} conceptuses indicates that the absence of EED and the resulting loss of H3K27me3 fail to activate *Grb10*

paternal expression in the whole embryo. Similar results have been reported for *Gata1*, *Eomes* and *Hoxa1*, genes with bivalent domains that do not become de-repressed in *Eed*^{-/-} ES cells or following loss of H3K27me3 (Azuara *et al*, 2006; Jørgensen *et al*, 2006; Lee *et al*, 2007a). Analysis of *Hoxa1* suggested that in addition to the loss of H3K27me3, increased histone acetylation could be important for gene reactivation (Lee *et al*, 2007a). Upon removal of H3K27me3, histone acetyltransferases could be recruited by specific transcription factors, or indirectly through H3K4 methylation (Pray-Grant *et al*, 2005). If this pathway relies on tissue-specific factors, de-repression will be observed only in cells or tissues where such factors are expressed.

Our data lead to a model for the brain-specific paternal expression of *Grb10* from the CGI2 region (Figure 5). In non-neural lineages, paternal expression is poised but silenced through the bivalent chromatin domain, whereas DNA methylation associated with repressive histone marks locks promoters on the maternal allele. In the developing neural lineage, the bivalent chromatin on the paternal allele is resolved, through the loss of H3K27me3. This leads to de-repression of the promoters at CGI2. To account for the neuron-specific paternal expression, the loss of the bivalent chromatin should occur only upon neural stem cell commitment towards neurons. However, the large decrease in H3K27me3 in neurospheres, when they are induced to differentiate, suggested that removal of this mark occurs in both glial and neuronal cells. Therefore, neuronal-specific factors are probably required to translate the loss of H3K27me3 into active gene expression. Possibly, such factors could be involved in recruiting histone acetyltransferases, through H3K4me2 and/or by interacting with a putative neuronal-specific enhancer.

An intriguing aspect of our model is the removal of H3K27me3 from *Grb10* specifically during lineage commitment in the developing brain. The loss of H3K27me3 in brain could be actively carried out by JMJD3 and the homologous UTX protein, which are functional H3K27me3 demethylases (Agger *et al*, 2007; De Santa *et al*, 2007; Lan *et al*, 2007; Lee

et al, 2007b). A recent report by Jepsen *et al* (2007) suggests that JMJD3 has a key function in the differentiation of neural stem cells towards neurons. Removal of H3K27me3 from the *Grb10* promoter upon commitment to the neural lineage could be part of a more general epigenetic programme that exerts an effect on several genes involved in neural development. Furthermore, it is tempting to speculate that the mechanism described here for *Grb10* could apply to other imprinted genes as well. There is indeed increasing evidence that genomic imprinting influences brain function through effects on neuro-developmental processes (Wilkinson *et al*, 2007). Among the roughly 80 imprinted genes characterized so far, many are expressed in the brain and some, such as *Ube3a* and *Commd1*, show a brain-specific imprinting pattern (Yamasaki *et al*, 2003; Wang *et al*, 2004; Kishino, 2006). The recently developed allelic large-scale mapping of histone methylation (Mikkelsen *et al*, 2007) offers a promising tool to investigate whether bivalent chromatin could be a feature of other imprinted genes as well. If so, the biological significance of mono-allelic bivalent chromatin in imprinted gene regulation, particularly in brain, could be assessed by analysis of cells and tissues deficient for Polycomb group proteins and specific histone demethylases. Beyond imprinting, our study represents one of the first examples of the implication of bivalent chromatin in tissue-specific gene expression. We demonstrate for the *Grb10* bivalent chromatin domain that it is faithfully maintained in somatic tissues and becomes resolved in a lineage-specific manner, underscoring the potential role of bivalent chromatin in the developmental control of gene expression.

Materials and methods

Material collection

Material was obtained from reciprocal crosses between C57BL/6J (B6) with *M. musculus molossinus* JF1 mice, (B6xJF1) F1 and (JF1xB6)F1. Whole embryos, placentae and isolated tissues (brain, liver and kidney) were recovered at various developmental stages.

Figure 5 Model for the brain-specific paternal expression of *Grb10*. From early developmental stages onwards, in all the non-neural tissues, promoters at CGI2 are poised but silenced through the paternal allele-specific bivalent chromatin. Upon neural lineage formation, the bivalent chromatin is resolved through the loss of H3K27me3. As a consequence, in neurons, the maintained H3K4me2 in association with neuronal-specific factors allows transcriptional activation, possibly through recruitment of histone acetyltransferases. Repressive marks are in bold letters, permissive in italics. DNA methylation is represented by filled lollipops.

Directly following dissection, tissues were frozen in liquid nitrogen and stored at -80°C .

ES cell lines

Allelic chromatin and expression analyses were performed on a (C57BL/6J $\times$ M. spretus)F1 ES cell line (SF1-G). Cells were grown and maintained in an undifferentiated state as previously described (Dean *et al*, 1998). DNAs and RNAs from OS25 (WT) and G8.1 and B1.3 (*Eed*^{-/-}) ES lines (Azuara *et al*, 2006) were a kind gift from Drs Helle Jorgensen, Veronique Azuara and Amanda Fisher (Lymphocyte Development Group, MRC, London, UK).

RNA extraction and allelic expression analysis

Total RNA extraction and first-strand cDNA synthesis were performed as previously described (Arnaud *et al*, 2006). RT-PCR products from maternal and paternal alleles were distinguished by SSCPs, or by direct sequencing. Expression analyses were performed in ES and TS cells and in brain, liver and kidney obtained from 1- to 3-day-old and, 2-, 4-week, and 4- and 8-month-old animals. Similar results were obtained in (B6 $\times$ JF1)F1 and (JF1 $\times$ B6)F1 reciprocal crosses (not shown). Primers used and details on polymorphism are given in Supplementary Table 1. RNAs from glial and neuronal cells were a kind gift from Drs Takahiro Yamada, Layla Parker-Katirae, and Stephen W Scherer (The Hospital for Sick Children, Toronto, Ontario, Canada). RNAs were prepared by Dr Takahiro Yamada from the cerebral cortex of 15.5–16.5 d.p.c. (B6 $\times$ JF1)F1 embryos as described in Mnatzakanian *et al* (2004).

ChIP

ChIP on native chromatin was carried out as described in our protocol at <http://www.epigenome-noe.net/researchtools/protocol.php?protid=22>. We used antisera against H3K9ac (06–942), H3K4me2 (07–030), H3K9me3 (07–442), H4K20me3 (07–463) and H3K27me1 (07–448) (Upstate Biotechnology) and H3K27ac (ab4729) (Abcam). For H3K27me3, we obtained similar results with two different antisera: 07–449 from Upstate Biotechnology and Ab6002 from Abcam. As a negative control (mock precipitation), we used a rabbit antiserum against chicken IgG (Sigma; C2288). For each tissue, at least two independent chromatin immunoprecipitations were performed. The same allelic enrichments were observed in adult tissues obtained from (B6 $\times$ JF1)F1 and (JF1 $\times$ B6)F1 crosses (not shown). Sequential ChIP was conducted as described by Bernstein *et al* (2006), on cross-linked chromatin obtained from semiconfluent MEF cells.

Analysis of immunoprecipitated chromatin

For each antiserum used, in the antibody-bound and unbound fractions the parental alleles were distinguished by radioactive PCR amplification, performed in the presence of [$\alpha^{32}\text{P}$]dCTP (1% of total dCTP), followed by electrophoretic detection of SSCP polymorphisms. Relative intensities of the maternal and paternal bands were determined using the Aida 1D quantification software (Fujifilm). Primer sequences used and details on polymorphism are given in Supplementary Table 2. Precipitation levels were determined by real-time PCR amplification, using a SYBR Green PCR Kit (Qiagen). Each PCR was run in triplicate and results are presented as a percentage of immunoprecipitation, calculated by dividing the average value of immunoprecipitated DNA by the average value of the corresponding input chromatin. For H3K27me3, precipitation levels were similar between two different antisera, 07–449 from Upstate Biotechnology and Ab6002 from Abcam.

Neurosphere culture

Neurospheres were derived from spinal cord of 13.5 d.p.c. (B6 $\times$ JF1)F1 embryos and cultured with EGF and FGF2 growth

factors as described (Deleyrolle *et al*, 2006). Neurospheres were passaged 4–5 times and then allowed to differentiate for 3 days. This was achieved by plating undissociated neurospheres at 300 000 cells/cm² on poly-D-lysine-coated (20 $\mu\text{g}/\text{ml}$) dishes in differentiation medium (neurobasal-B27; Invitrogen) supplemented with 0.5% fetal calf serum, 10 ng/ml neurotrophin 3 (Peprotech) and 10 $\mu\text{g}/\text{ml}$ gentamicin. Neurosphere differentiation into neurons and glial cells was checked by immunofluorescence using cell-specific markers (GFAP, β -3-tubulin and O4) as described in Deleyrolle *et al* (2006). Accurate quantification of the number of astrocytic (GFAP), neuronal (β -3-tubulin) and oligodendrocytic (O4) cells was achieved in a separate experiment in which neurospheres were first dissociated with trypsin before differentiation and plated for 4 days without growth factor at a density of 250 000 cells/cm² onto 20 $\mu\text{g}/\text{ml}$ poly-D-lysine (Sigma)-coated coverslips, as described (Deleyrolle *et al*, 2006). Around 200 cells were individually observed and counted at high magnification ($\times$ 400) for expression of differentiated cell markers.

Grb10^{XC302} expression in WT and *Eed*^{-/-} conceptuses

We used *Eed*^{17Rn5-3354SB}-null 6.5 d.p.c. embryos, in which *Eed* deficiency results from a proline substitution (L196P) that leads to a null mutation (Rinchik and Carpenter, 1993). To assess allelic expression of *Grb10*, female mice heterozygous for the *Eed*^{17Rn5-3354SB} allele were mated with heterozygous males that also carried a gene trap insertion of a β -geo cassette in intron 7 of the *Grb10* gene. This *Grb10gtTMag1* strain was generated from the XC302 ES cell line from BayGenomics, and is herein referred to as *Grb10*^{XC302}. Gravid females were killed at 6.5 d.p.c. Embryos were dissected from the maternal uterus and decidual tissue, and a small tissue sample was used for genotyping. Embryos were fixed in a mixture of glutaraldehyde and formaldehyde and stained with X-Gal, a substrate for galactosidase, as previously described (Morin-Kensicki *et al*, 2001). Stained embryos were post-fixed in 4% paraformaldehyde and cleared through a glycerol gradient before photographing. Blue staining indicates cells expressing the β -geo gene trap. *Eed* genotyping was carried out as previously described (Kalantry and Magnuson, 2006). *Grb10*^{XC302} genotyping was performed using specific primers for the neomycin gene within the gene trap insertion. Primer sequences are available upon request.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank T Forné and E Julien for valuable advice and critical reading of the paper, A Fisher, H Jorgensen and V Azuara (for the generous gift of DNA and RNA from *Eed*^{-/-} ES cell lines, and T Yamada, L Parker-Katirae and SW Scherer for the kind gift of RNA from glial and neuronal cells. We are grateful to M Pannetier for her precious help with 'cross-link' ChIP and to K Chebli, C Jacquet and the Animal Facility for expert animal husbandry. This project was supported mainly by the ESF EUROCORES programme EuroSTELLS, the ARC programme ARECA and the Agence Nationale de la Recherche (ANR, 'EMPREINTE') awarded to RF. Additional funding was derived from an NIH grant to TM, and European FP6 Rescue project and the Association Française contre les myopathies (AFM) grants awarded to J-PH. We thank the European Framework-6 network EPIGENOME for encouragement and support. SC is a recipient of an NIH fellowship. LAS, J-CS and AH are recipients of a PhD fellowship from the French ministry of Education and Science.

References

- Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, Issaeva I, Canaani E, Salcini AE, Helin K (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature* **449**: 731–734
- Arnaud P, Feil R (2005) Epigenetic deregulation of genomic imprinting in human disorders and following assisted reproduction. *Birth Defects Res C Embryo Today* **75**: 81–97
- Arnaud P, Hata K, Kaneda M, Li E, Sasaki H, Feil R, Kelsey G (2006) Stochastic imprinting in the progeny of *Dnmt3L*^{-/-} females. *Hum Mol Genet* **15**: 589–598
- Arnaud P, Monk D, Hitchins M, Gordon E, Dean W, Beechey CV, Peters J, Craigen W, Preece M, Stanier P, Moore GE, Kelsey G (2003) Conserved methylation imprints in the human and mouse GRB10 genes with divergent allelic expression suggests

- differential reading of the same mark. *Hum Mol Genet* **12**: 1005–1019
- Azuara V, Perry P, Sauer S, Spivakov M, Jørgensen HF, John RM, Gouti M, Casanova M, Warnes G, Merckenschlager M, Fisher AG (2006) Chromatin signatures of pluripotent cell lines. *Nat Cell Biol* **8**: 532–538
- Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. *Cell* **129**: 823–837
- Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**: 315–326
- Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441**: 349–353
- Cavalli G, Paro R (1998) The *Drosophila* Fab-7 chromosomal element conveys epigenetic inheritance during mitosis and meiosis. *Cell* **93**: 505–518
- Charalambous M, Smith FM, Bennett WR, Crew TE, Mackenzie F, Ward A (2003) Disruption of the imprinted *Grb10* gene leads to disproportionate overgrowth by an Igf2-independent mechanism. *Proc Natl Acad Sci USA* **100**: 8292–8297
- Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses JJ, Reik W, Feil R (1998) Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development* **125**: 2273–2282
- De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G (2007) The histone H3 lysine-27 demethylase *Jmjd3* links inflammation to inhibition of Polycomb-mediated gene silencing. *Cell* **130**: 1083–1094
- Delaval K, Feil R (2004) Epigenetic regulation of mammalian genomic imprinting. *Curr Opin Genet Dev* **14**: 188–195
- Delaval K, Govin J, Cerqueira F, Rousseaux S, Khochbin S, Feil R (2007) Differential histone modifications mark mouse imprinting control regions during spermatogenesis. *EMBO J* **26**: 720–729
- Deleyrolle L, Marchal-Victorion S, Dromard C, Fritz V, Saunier M, Sabourin JC, Tran Van Ba C, Privat A, Hugnot JP (2006) Exogenous and fibroblast growth factor 2/epidermal growth factor-regulated endogenous cytokines regulate neural precursor cell growth and differentiation. *Stem Cells* **24**: 748–762
- Faust C, Lawson KA, Schork NJ, Thiel B, Magnuson T (1998) The Polycomb-group gene *ee* is required for normal morphogenetic movements during gastrulation in the mouse embryo. *Development* **125**: 4495–4506
- Fournier C, Goto Y, Ballestar E, Delaval K, Hever AM, Esteller M, Feil R (2002) Allele-specific histone lysine methylation marks regulatory regions at imprinted mouse genes. *EMBO J* **21**: 6560–6570
- Hikichi T, Kohda T, Kaneko-Ishino T, Ishino F (2003) Imprinting regulation of the murine *Meg1/Grb10* and human *GRB10* genes; roles of brain-specific promoters and mouse-specific CTCF-binding sites. *Nucleic Acids Res* **31**: 1398–1406
- Jenuwein T, Allis CD (2001) Translating the histone code. *Science* **293**: 1074–1080
- Jepsen K, Solum D, Zhou T, McEvilly RJ, Kim HJ, Glass CK, Hermanson O, Rosenfeld MG (2007) SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. *Nature* **450**: 415–419
- Jia D, Jurkowska RZ, Zhang X, Jeltsch A, Cheng X (2007) Structure of *Dnmt3a* bound to *Dnmt3L* suggests a model for *de novo* DNA methylation. *Nature* **449**: 248–251
- Jørgensen HF, Giadrossi S, Casanova M, Endoh M, Koseki H, Brockdorff N, Fisher AG (2006) Stem cells primed for action: Polycomb repressive complexes restrain the expression of lineage-specific regulators in embryonic stem cells. *Cell Cycle* **5**: 1411–1414
- Kalanitry S, Magnuson T (2006) The Polycomb group protein *EED* is dispensable for the initiation of random X-chromosome inactivation. *PLoS Genet* **2**: e66
- Kishino T (2006) Imprinting in neurons. *Cytogenet Genome Res* **113**: 209–214
- Klymenko T, Müller J (2004) The histone methyltransferases trithorax and Ash1 prevent transcriptional silencing by Polycomb group proteins. *EMBO Rep* **5**: 373–377
- Lan F, Bayliss PE, Rinn JL, Whetstone JR, Wang JK, Chen S, Iwase S, Alpatov R, Issaeva I, Canaan E, Roberts TM, Chang HY, Shi Y (2007) A histone H3 lysine 27 demethylase regulates animal posterior development. *Nature* **449**: 689–694
- Lau JC, Hanel ML, Wevrick R (2004) Tissue-specific and imprinted epigenetic modifications of the human *NDN* gene. *Nucleic Acids Res* **32**: 3376–3382
- Lee ER, Murdoch FE, Fritsch MK (2007a) High histone acetylation and decreased Polycomb repressive complex 2 member levels regulate gene specific transcriptional changes during early embryonic stem cell differentiation induced by retinoic acid. *Stem Cells* **25**: 2191–2199
- Lee MG, Villa R, Trojer P, Norman J, Yan KP, Reinberg D, Di Croce L, Shiekhkhattar R (2007b) Demethylation of H3K27 regulates Polycomb recruitment and H2A ubiquitination. *Science* **318**: 447–450
- Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K, Koseki H, Fuchikami T, Abe K, Murray HL, Zucker JP, Yuan B, Bell GW, Herbolsheimer E, Hannett NM, Sun K *et al* (2006) Control developmental regulators by Polycomb in human embryonic stem cells. *Cell* **125**: 301–313
- Li B, Carey M, Workman JL (2007) The role of chromatin during transcription. *Cell* **128**: 707–719
- Li T, Vu TH, Ulaner GA, Yang Y, Hu JF, Hoffman AR (2004) Activating and silencing histone modifications form independent allelic switch regions in the imprinted *Gnas* gene. *Hum Mol Genet* **13**: 741–750
- Mager J, Montgomery ND, de Villena FP, Magnuson T (2003) Genome imprinting regulated by the mouse Polycomb group protein *Eed*. *Nat Genet* **33**: 502–507
- Margueron R, Trojer P, Reinberg D (2005) The key to development: interpreting the histone code. *Curr Opin Genet Dev* **15**: 163–176
- Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C *et al* (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**: 553–560
- Miyoshi N, Kuroiwa Y, Kohda T, Shitara H, Yonekawa H, Kawabe T, Hasegawa H, Barton SC, Surani MA, Kaneko-Ishino T, Ishino F (1998) Identification of the *Meg1/Grb10* imprinted gene on mouse proximal chromosome 11, a candidate for the Silver-Russell syndrome gene. *Proc Natl Acad Sci USA* **95**: 1102–1107
- Mnatzakanian GN, Lohi H, Munteanu I, Alfred SE, Yamada T, MacLeod PJ, Jones JR, Scherer SW, Schanen NC, Friez MJ, Vincent JB, Minassian BA (2004) A previously unidentified *MECP2* open reading frame defines a new protein isoform relevant to Rett syndrome. *Nat Genet* **36**: 339–341
- Morin-Kensicki EM, Faust C, LaMantia C, Magnuson T (2001) Cell and tissue requirements for the gene *Eed* during mouse gastrulation and organogenesis. *Genesis* **31**: 142–146
- Ooi SK, Qiu C, Bernstein E, Yang Z, Erdjument-Bromage H, Tempst P, Lin SP, Allis CD, Cheng X, Bestor TH (2007) DNMT3L connects unmethylated lysine 4 of histone H3 to *de novo* methylation of DNA. *Nature* **448**: 714–717
- Orlando V, Jane EP, Chinwalla V, Harte PJ, Paro R (1998) Binding of trithorax and Polycomb proteins to the bithorax complex: dynamic changes during early *Drosophila* embryogenesis. *EMBO J* **17**: 5141–5150
- Pan G, Tian S, Nie J, Yang C, Ruotti V, Wei H, Jonsdottir GA, Stewart R, Thomson JA (2007) Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. *Cell Stem Cell* **1**: 299–312
- Pray-Grant MG, Daniel JA, Schieltz D, Yates III JR, Grant PA (2005) Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. *Nature* **433**: 434–438
- Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**: 1707–1710
- Rinchik EM, Carpenter DA (1993) *N*-Ethyl-*N*-nitrosourea-induced prenatally lethal mutations define at least two complementation groups within the embryonic ectoderm development (*eed*) locus in mouse chromosome 7. *Mamm Genome* **4**: 349–353

- Rivera-Pérez JA, Mager J, Magnuson T (2003) Dynamic morphogenetic events characterize the mouse visceral endoderm. *Dev Biol* **261**: 470–487
- Roh TY, Cuddapah S, Cui K, Zhao K (2006) The genomic landscape of histone modifications in human T cells. *Proc Natl Acad Sci USA* **103**: 15782–15787
- Saleh A, Al-Abdallat A, Ndamukong I, Alvarez-Venegas R, Avramova Z (2007) The *Arabidopsis* homologs of trithorax (ATX1) and enhancer of zeste (CLF) establish 'bivalent chromatin marks' at the silent AGAMOUS locus. *Nucleic Acids Res* **35**: 6290–6296
- Smith FM, Garfield AS, Ward A (2006) Regulation of growth and metabolism by imprinted genes. *Cytogenet Genome Res* **113**: 279–291
- Umlauf D, Goto Y, Cao R, Cerqueira F, Wagschal A, Zhang Y, Feil R (2004) Imprinting along the *Kcnq1* domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes. *Nat Genet* **36**: 1296–1300
- Wagschal A, Sutherland HG, Woodfine K, Henckel A, Chebli K, Schulz R, Oakey RJ, Bickmore WA, Feil R (2008) G9a histone methyltransferase contributes to imprinting in the mouse placenta. *Mol Cell Biol* **28**: 1104–1113
- Wang Y, Joh K, Masuko S, Yatsuki H, Soejima H, Nabetani A, Beechey CV, Okinami S, Mukai T (2004) The mouse *Murr1* gene is imprinted in the adult brain, presumably due to transcriptional interference by the antisense-oriented *U2af1-rs1* gene. *Mol Cell Biol* **24**: 270–279
- Wilkinson LS, Davies W, Isles AR (2007) Genomic imprinting effects on brain development and function. *Nat Rev Neurosci* **8**: 832–843
- Xia ZB, Anderson M, Diaz MO, Zeleznik-Le NJ (2003) MLL repression domain interacts with histone deacetylases, the Polycomb group proteins HPC2 and BMI-1, and the corepressor C-terminal-binding protein. *Proc Natl Acad Sci USA* **100**: 8342–8347
- Yamasaki K, Joh K, Ohta T, Masuzaki H, Ishimaru T, Mukai T, Niikawa N, Ogawa M, Wagstaff J, Kishino T (2003) Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of Ube3a. *Hum Mol Genet* **12**: 837–847
- Yamasaki-Ishizaki Y, Kayashima T, Mapendano CK, Soejima H, Ohta T, Masuzaki H, Kinoshita A, Urano T, Yoshiura K, Matsumoto N, Ishimaru T, Mukai T, Niikawa N, Kishino T (2007) Role of DNA methylation and histone H3 lysine 27 methylation in tissue-specific imprinting of mouse *Grb10*. *Mol Cell Biol* **27**: 732–742
- Yang Y, Li T, Vu TH, Ulaner GA, Hu JF, Hoffman AR (2003) The histone code regulating expression of the imprinted mouse *Igf2r* gene. *Endocrinology* **144**: 5658–5670
- Zhao XD, Han X, Chew JL, Liu J, Chiu KP, Choo A, Orlov YL, Sung WK, Shahab A, Kuznetsov VA, Bourque G, Oh S, Ruan Y, Ng HH, Wei CL (2007) Whole-genome mapping of histone H3 lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. *Cell Stem Cell* **1**: 286–298