# GENOMIC IMPRINTING: PARENTAL INFLUENCE ON THE GENOME

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Genomic imprinting affects several dozen mammalian genes and results in the expression of those genes from only one of the two parental chromosomes. This is brought about by epigenetic instructions — imprints — that are laid down in the parental germ cells. Imprinting is a particularly important genetic mechanism in mammals, and is thought to influence the transfer of nutrients to the fetus and the newborn from the mother. Consistent with this view is the fact that imprinted genes tend to affect growth in the womb and behaviour after birth. Aberrant imprinting disturbs development and is the cause of various disease syndromes. The study of imprinting also provides new insights into epigenetic gene modification during development.

EUTHERIANS
Mammals that give birth to live offspring (viviparous) and possess an allantoic placenta.

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Genomic imprinting in mammals was discovered in the early 1980s as a result of two types of mouse experiment. Nuclear transplantation was used to make embryos that had only one of the two sets of parental chromosomes (uniparental embryos) and other sophisticated genetic techniques were used to make embryos that inherited specific chromosomes from one parent only (uniparental disomy). In both cases, the surprising finding was that mammalian genes could function differently depending on whether they came from the mother or the father[1–6]. The early 1990s then saw the discovery of the first imprinted genes, which were indeed expressed differently on maternal and paternal chromosomes[7–9], and the realization that imprinting had a substantial effect on human genetic disease[10,11]. It was also found that DNA methylation was a key molecular mechanism of imprinting; methylation marks the imprinted genes differently in egg and sperm, and inheritance of these epigenetic marks leads to differential gene expression[12–17].

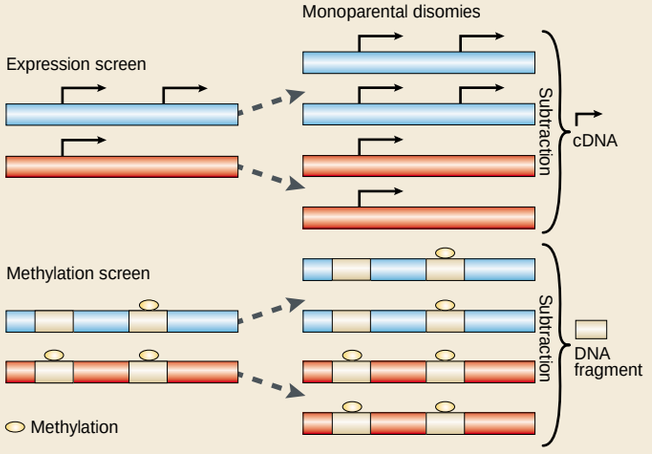
Substantial progress has been made in our understanding of imprinting in the past few years: important phenotypic effects of imprinted genes have been discovered, particularly in the control of fetal growth and behaviour after birth; a number of *cis*-acting sequences are being defined that are important for the control of imprinted gene expression; the evolutionary understanding of imprinting and its likely biological purposes is increasing[18,19]; and the study of imprinting is providing general insights into the importance of epigenetic mechanisms in development.

Here we review these recent developments. We begin with a brief summary of imprinted genes, then look at what is known about establishment and maintenance of imprints, and the important role of the germ line. We review the various 'reading mechanisms' that convert the imprint into differential gene expression. We discuss the evolution of imprinting, and its main phenotypic effects, in healthy and diseased states. Finally, we consider the effect of imprinting on important general issues in epigenetics, such as cloning and genome reprogramming.

## Imprinted genes

Using several approaches (BOX 1), around 45 imprinted genes have so far been identified in the mouse (see the Harwell imprinting web site for up-to-date statistics on imprinted gene numbers and characteristics). Some of these genes have been tested in other mammals and for many (but not all), the imprinting status is conserved in humans, in some other EUTHERIAN mammals and in a marsupial[20–22] (but only a few genes have been tested). What are the genetic and epigenetic features that characterize imprinted genes?

Box 1 | Finding imprinted genes




Imprinted genes have been identified in various ways: by chance (usually knockouts that then showed parent-specific expression); based on position (next to other imprinted genes or in a chromosome region associated with an imprinting phenotype); or by using two types of systematic screen. In both screens, embryos are used that have a duplication of one of the parental chromosomes or genomes, together with embryos that have the opposite parental chromosome duplicated. This results in gene expression or methylation in one type of embryo but not the other if the gene is imprinted. The first screen is based on subtraction of cDNAs between such uniparental embryos[115]. The second is based on methylation differences. One approach using restriction landmark genome scanning (by two-dimensional electrophoresis of DNA) has estimated that there are roughly 100 imprinted genes in the mouse genome[116]. Another methylation screen uses representational difference analysis[117](RDA). The estimate of 100 imprinted genes in the genome is likely to be an underestimate but, in any event, imprinted genes constitute a minority of all the genes in the genome.

One remarkable and characteristic feature of imprinted genes is that they are rarely found on their own: around 80% are physically linked in clusters with other imprinted genes (FIG. 1). The clustered organization of imprinted genes is thought to reflect coordinated regulation of the genes in a chromosomal domain. By analogy to X-chromosome inactivation in which an X-inactivation centre controls the inactivation of the entire chromosome, imprinting centres or imprinting control elements (ICs) have been discovered in some clusters. These ICs are needed for the regional control of imprinting or imprinted expression.

No common features are recognizable when comparing the protein sequences encoded by imprinted genes, although there are functional relationships between some proteins with roles in fetal growth and development. Furthermore, two general features of the DNA sequence environment of imprinted genes have been noted. First, they are unusually rich in CPG ISLANDS[23]: around 88% of mouse imprinted genes have CpG islands, compared with the average figure of 47%. Second, clustered, direct repeats are common near to or within the CpG islands. The repeats might or might not belong to one of the known repeat families and they have been proposed to be involved in conferring or maintaining differential methylation[24]. Neither the repeats nor the CpG islands are unique to imprinted genes, so these features cannot be used in a systematic search for new imprinted genes.

The great majority of imprinted genes examined so far show differences in DNA methylation between the parental alleles (FIG. 2), but the differentially methylated regions (DMRs) can have different properties. For example, the differential DNA methylation in some DMRs is introduced in parental germ cells and maintained in all developmental stages and tissues[25–28]. Others show considerable changes in methylation during development and acquire tissue-specific methylation patterns[29], which can be associated with tissue-specific imprinted expression. Some DMRs are methylated in the inactive gene copy, whereas others are methylated in the active one. Imprinted genes can also differ with respect to bulk chromatin structure, as well as with respect to more specific modifications, such as histone acetylation[30–36] (R. Feil and R. Gregory, personal communication).

Two other epigenetic features have been discovered that might reflect the larger-scale organization of imprinted genes into clusters or domains. First, it has been shown that the DNA in imprinted regions replicates asynchronously in the S phase of the cell cycle; for most imprinted regions, the paternal copy replicates earlier than the maternal one[37,38]. Because maternally and paternally expressed genes are interspersed in some regions, this is not likely to be a gene-specific property and its molecular basis is not understood. Second, different frequencies of meiotic recombination are found in or near to imprinted clusters, with an elevated recombination rate during male meiosis[39,40]. How these regional epigenetic features are linked with methylation and chromatin structure is not known.

The precise nature of the primary imprint and its fate during development is still a mystery, but it is likely that all the above epigenetic modifications are relevant to imprinting. However, at present there is no direct evidence that histone or other chromatin modifications have roles in imprinting that are independent of DNA methylation. Indeed, the importance of DNA methylation, at least in the maintenance of imprints, has been clearly established genetically[17]. For the most part, we therefore equate 'imprints' with 'methylation imprints' or 'differential methylation' to simplify the discussion. Imprinted expression is then a result of the 'reading' of the imprint in somatic tissues.

## The life cycle of imprints

Genomic imprints change in characteristic ways during the life cycle of the organism (FIG. 3). Imprints are 'established' during the development of germ cells into sperm or eggs. After fertilization, they are 'maintained' as chromosomes duplicate and segregate in the developing organism. In the germ cells of the new organism, imprints are 'erased' at an early stage. This is followed by establishment again at a later stage of germ-cell development, thus completing the imprinting cycle. In somatic cells, imprints are maintained and are modified during development. For example,

CPG ISLAND
DNA region of >500 bp that has a high CpG density and is usually unmethylated. CpG islands are found upstream of many mammalian genes.

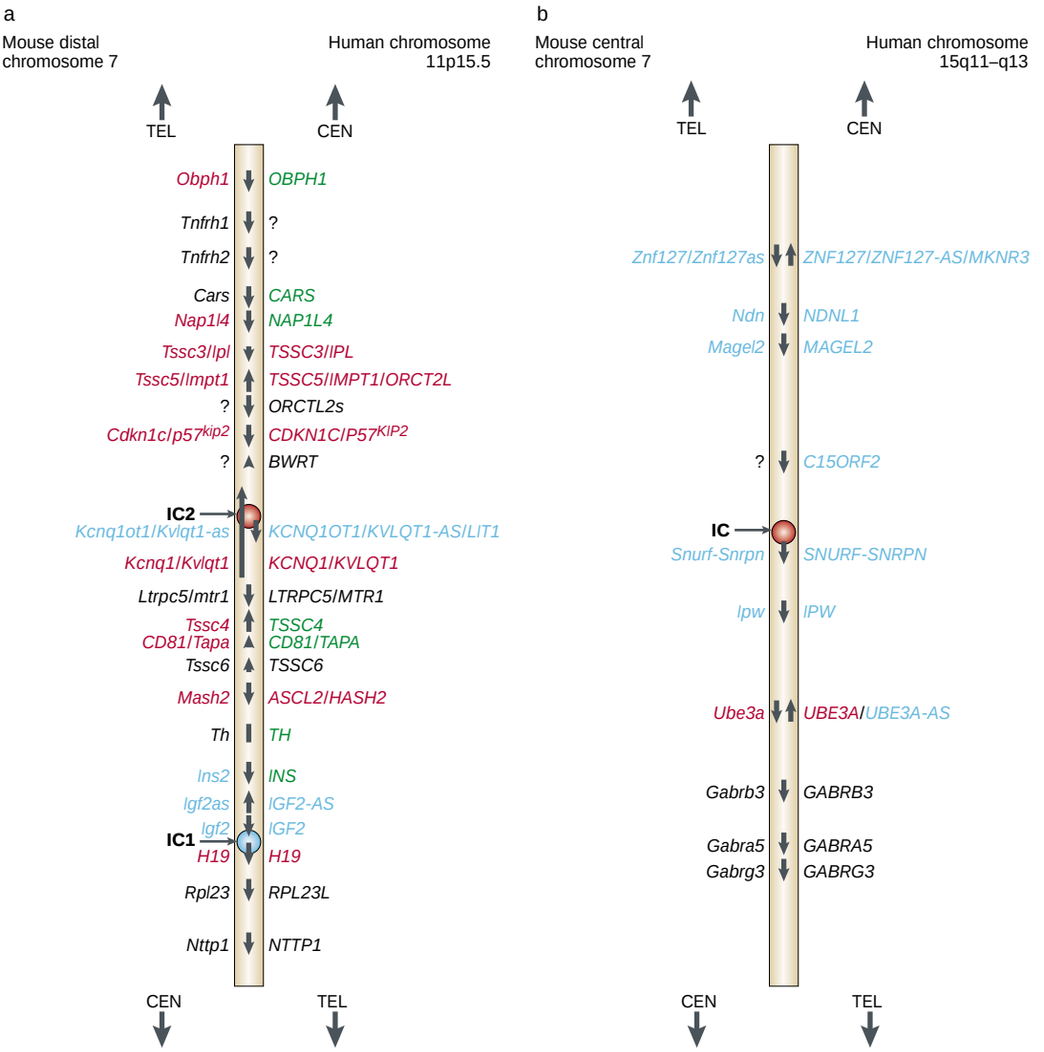



Figure 1 | **Imprinting clusters in human and mouse genomes.** Human chromosomes **a** | 11p15.5 and **b** | 15q11–-q13 and orthologous clusters on **a** | mouse chromosome distal 7 and **b** | central 7. The relative location and transcriptional orientation of genes are indicated by arrows. The imprinting status is shown in red (maternally expressed), blue (paternally expressed), black (biallelic expression) and green (imprinted expression not known or not yet precisely defined). Question marks (?) indicate that the orthologues of the mouse or human genes, respectively, are not known. The drawings are not to scale. The Beckwith–Wiedemann (BWS) cluster (**a**) comprises about 1 Mb, and the Prader–Willi syndrome/Angelman syndrome (PWS/AS) cluster (**b**) roughly 2 Mb. Imprinting centres (IC) are marked by circles coloured according to the parental origin of the imprint.

methylation may spread from an IC into the promoter. The imprints are eventually read, resulting in parent-specific gene expression.

*Erasure.* The germ line has the role of resetting imprints such that in mature gametes they reflect the sex of that germ line. For most imprints, current evidence indicates that there might be two stages for this resetting process — the first one is erasure. This is followed later by establishment. During erasure, there is marked and apparently genome-wide demethylation in germ cells, which is completed by embryonic day 12–13 (E12–13) in both sexes[41,42] (FIG. 4). Indeed, germ cells cultured from these stages (EG cells) have a dominant demethylating activity when fused with somatic cells[43]; whether this demethylation is active or passive is not known. The evidence so far indicates that all methylation imprints probably become erased at this stage[41,42,44,45]. This is important because it implies that imprints inherited from a parent with the same sex as the developing embryo are erased and are unlikely to persist unchanged.

There is preliminary evidence that methylation imprints are still present and may be functionally intact before the erasure stage[46]. After erasure, functional evidence from nuclear transplantation experiments with both male and female germ-cell nuclei indicates that imprints have indeed been substantially altered[47,48]; expression of imprinted genes in these reconstituted embryos reflects their lack of methylation (for example, *H19* is expressed and *Igf2* is not expressed). In some instances, this has led to interest-

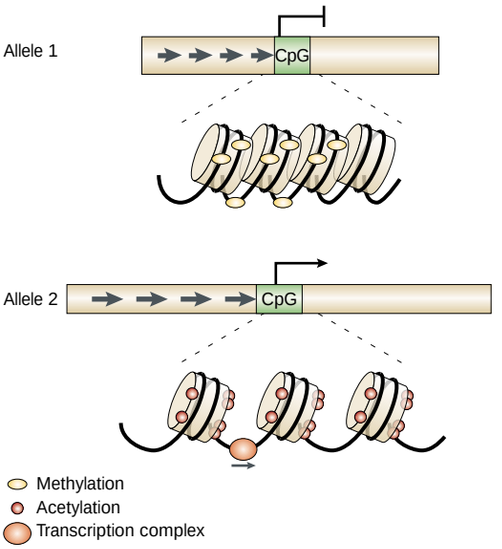


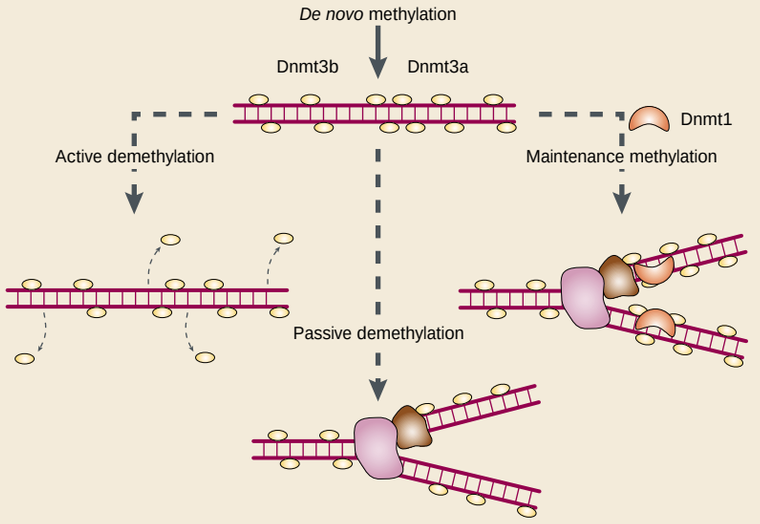

Figure 2 | **Characteristics of imprinted genes.** The figure shows a schematic pair of imprinted alleles. Hallmarks of imprinted genes such as CpG islands and repeats (arrows) are indicated. The enlarged region below the chromosomes highlights the allele-specific epigenetic changes, such as nucleosomal condensation through deacetylation, and methylation (allele 1) and opening of the chromatin by acetylation and demethylation (allele 2). The transcriptional competence of allele 2 is indicated by the binding of a transcription complex.

ing new insights about the role of methylation imprints (for example, *Cdkn1c* requires a maternal methylation imprint to be expressed; see below). In addition to methylation imprints, differential replication of DNA is also apparently erased in both germ lines; in the female germ line this coincides with demethylation, but in the male germline it occurs substantially later, after birth[49].

*Establishment.* After erasure, *de novo* methylation begins in both germ lines at late fetal stages, and continues after birth[41,50] (FIGS 3,4). Oocytes are in meiotic arrest and methylation occurs during their growth[47], whereas during spermatogenesis, methylation occurs before meiosis[44,45]. Nuclear transplantation experiments indicate that this DNA methylation coincides roughly with the acquisition of functional imprints both for autosomal genes and for X chromosome imprinting, at least in oocytes[47,51]. It is not yet clear which enzymes are responsible for *de novo* methylation in germ cells (BOX 2). Dnmt1 (DNA methyltransferase 1) and its germ-cell-specific isoforms are candidates[52], but it is also possible that Dnmt3a or Dnmt3b, which are required for *de novo* methylation in postimplantation embryos[53], carry out this function in germ cells. It is also unclear how Dnmts specifically target DMRs in either female or male germ cells. DMRs in imprinted genes might be specifically targeted for *de novo* methylation in one of the germ lines. It is equally possible that there is general *de novo* methylation in both germ lines and that DMRs are specifically protected from methylation in one germ line but not in the other. In either case, this would require factors that recognize DMRs and that are germline-specific. The existence of such factors is supported by the observation that deficiency of Dnmt1 causes loss of imprints postzygotically, and the imprints cannot be restored by Dnmt1, Dnmt3a or Dnmt3b[54].

DMRs are generally CpG rich and often fulfil the criteria for CpG islands (see below). However, autosomal CpG islands do not become methylated *de novo*. So it is likely that imprinted DMRs are genetically or epigenetically modified so that *de novo* methylation can occur. Genetic modification has been previously postulated to be due to stretches of unique direct repeats that often flank DMRs[24]. More recent work has shown that the repeats are not necessarily unique to DMRs but that clusters of known repeat families, such

## Box 2 | DNA methylation and demethylation




DNA methylation in mammals occurs in the dinucleotide CpG. Methyl groups can be introduced into unmethylated DNA by the *de novo* methylation enzymes Dnmt3a and Dnmt3b (and perhaps others). When DNA is replicated, the methyl group on the template strand is recognized and a new one is introduced on the opposite (daughter) strand by the enzyme Dnmt1, which can be associated with the replication machinery. In the presence of Dnmt1, hemi-methylated DNA becomes fully methylated and so DNA methylation patterns tend to be maintained (maintenance methylation). Demethylation can occur in the absence of Dnmt1 with continued rounds of DNA replication (passive demethylation), as well as actively (without DNA replication). The nature of demethylases is unknown.

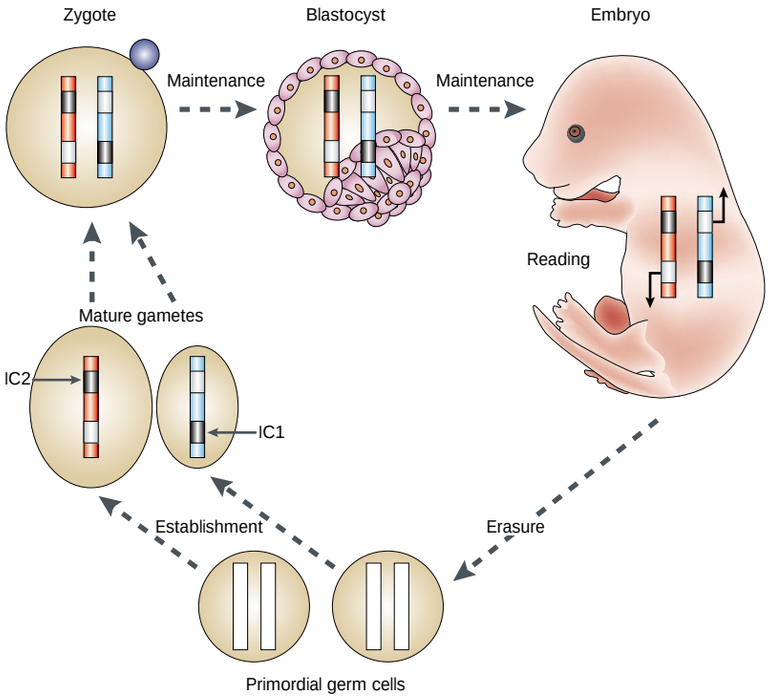



Figure 3 | **Life cycle of methylation imprints.** Erasure, establishment and maintenance of methylation imprints at imprinting centres during germ cell and embryonic development. Imprinting control elements 1 (IC1) and IC2 are shown as examples (see chromosome 11p15.5 in FIG. 1). Grey indicates modification and white indicates no modification at the corresponding alleles. Parental chromosomes are marked according to their sex in blue (male) or red (female). The reading (transcriptional interpretation of the primary imprints) in the developing embryo is indicated by arrows.

as LINE-1 ELEMENTS, can also be found next to DMRs[55]. Intriguingly, in various organisms there are epigenetic-targeting systems including methylation, that lead to heterochromatization and inactivation of tandemly repeated gene arrays[56]. Local heterochromatization could therefore lead to methylation of nearby CpG-rich DMRs.

CpG-rich DMRs could also be modified epigenetically so that they can become methylated. A hallmark of imprinting clusters is their different timing of DNA replication in S phase; recent work indicates that this property might be already acquired in the germ cells and, at least in oocytes, might precede acquisition of the methylation imprint [49]; it is thus possible that different timing of replication results in different accessibility of DNA to the *de novo* or maintenance methylation machinery. Indeed, regulation of methyltransferases can be cell-cycle specific[57].

It is also possible that some imprints are established not in the germline, but rather by immediate demethylation or *de novo* methylation, after fertilization, of only one of the alleles. Parent-specific demethylation or *de novo* methylation immediately after fertilization has indeed been documented (see below).

*Maintenance.* It might be imagined that maintenance of methylation imprints after fertilization is trivial and that, once DMRs are differentially methylated in the parental germ cells, these patterns are simply maintained after fertilization by Dnmt1. The problem is that there is genome-wide demethylation after fertilization and a wave of *de novo* methylation after implantation[58], both of which have to be resisted by DMRs (FIG. 4).

How is demethylation resisted by DMRs? First, demethylation occurs both by active and by passive mechanisms (BOX 2). Whereas the paternal genome is largely demethylated by an active mechanism only hours after fertilization when the parental genomes are still separate in the pronuclei[59,60], the maternal genome is largely demethylated passively by failure to maintain methylation during DNA replication[61,62]. The maternal genome presumably needs a protection mechanism against active demethylation at fertilization; because demethylation might be triggered by chromatin remodelling of the sperm genome, the maternal genome might be protected by its nucleosomal chromatin structure. Thus it would be interesting to know whether paternally methylated DMRs adopt a specialized chromatin structure in the sperm that does not undergo substantial remodelling after fertilization. Passive demethylation is thought to come about by exclusion of Dnmt1 from the nucleus during cleavage divisions[63]. So it remains a mystery how methylation in DMRs is maintained during cleavage and which enzymes might be involved. The different requirements that the parental genomes have for their protection from demethylation might explain why there seem to be more maternal germline methylation imprints than paternal ones (W.R. and J.W., unpublished).

Resistance to *de novo* methylation after implantation might be conferred by the specialized chromatin features of unmethylated alleles, or their earlier replication during S phase. Indeed, CpG islands on the inactive X chromosome become methylated *de novo* owing to the action of Dnmt3a/Dnmt3b after implantation, when the inactive chromosome becomes late-replicating[64].

## Reading mechanisms

Once the imprints are introduced in the parental germlines, maintained in the early embryo and fully matured during differentiation, they need to be read. Reading means the conversion of methylation or chromatin imprints into differential gene expression. Differential gene expression is thought to be largely at the level of transcription, although there might be the possibility of post-transcriptional mechanisms as well[65].

A general feature of the reading mechanisms of imprinted genes is that they seem to be complex. The complexity is likely to arise from the fact that many imprinted genes are clustered, and that clustering involves interactions between neighbouring genes and their control sequences. The clustering and interactions between neighbouring genes might be explained by extension of the conflict theory to mechanistic interactions between genes that oppose each other's function[66]. So far, imprinted genes have been shown to be regulated by epigenetic

LINE-1 ELEMENT
A class of repetitive transposable element interspersed between and within genes throughout the genome. Most have degenerated and lost transposition activity.

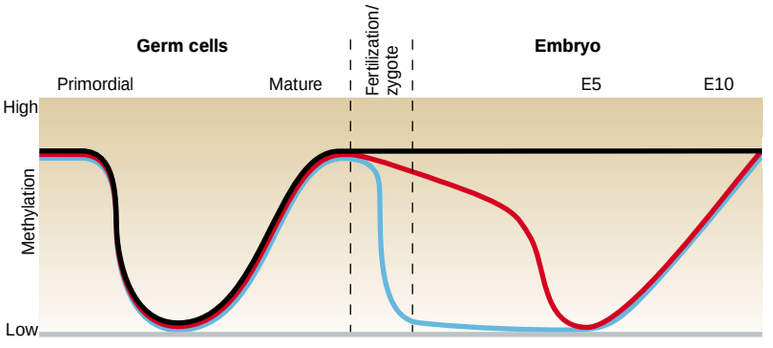



Figure 4 | **Methylation reprogramming in the germ line and embryo.** The figure shows the level of methylation in methylated (black) and non-methylated (grey) imprinted genes and non-imprinted sequences (red, maternal; blue, paternal) during germ-cell and early embryonic development. The horizontal time axis and the vertical axis indicating the relative methylation levels are not to scale. (E, embryonic day.)

modifications of promoter sequences, of SILENCERS, of BOUNDARY ELEMENTS, and possibly of overlapping antisense transcripts. So imprinted genes use the normal arsenal of transcriptional control mechanisms, but some of these are controlled by differential epigenetic modifications on parental chromosomes.

*Promoter methylation.* A fairly common way to achieve transcriptional silencing of one allele is by promoter methylation (FIG. 5a). Here, the promoter region, which is often CpG rich, is heavily methylated on one allele. Note that the primary germline imprint region (the region in which epigenetic differences occur between egg and sperm) is often distinct from the promoter, but is necessary for promoter methylation during development[67]. In the cases tested, DNASEI HYPERSENSITIVE SITES are absent from a paternally methylated promoter[30,31] and a maternally methylated promoter[32]. The details of how methylated promoters are transcriptionally repressed are not known yet, but it is clear that several methyl-CpG-binding proteins (MBDs), as well as the maintenance methyltransferase itself (Dnmt1), form a complex with histone deacetylases. This presumably leads to a closed chromatin conformation in which transcription factors cannot gain access to the promoter[68]. Indeed, differences in specific patterns of histone acetylation consistent with this model have been observed between alleles of imprinted genes (R. Feil and R. Gregory, personal communication).

*Antisense transcripts.* A considerable proportion of imprinted genes are associated with antisense transcripts (at present 15%; FIG. 5b). Surprisingly, all antisense transcripts discovered so far in imprinted genes are themselves imprinted and are paternally expressed (with the exception of *Tsix*, the antisense transcript to *Xist*[69]), regardless of whether they occur in sense genes that are paternally or maternally expressed (W.R. and J.W., unpublished). Most antisense transcripts are non-coding and may have regulatory functions. Among the best studied are those overlapping the maternally expressed *Igf2r* and *Kcnq1* genes (called *Air* and *Kcnq1ot1*, respectively)[70–72]. Both of these antisense RNAs originate in introns of the sense genes and are co-linear with DNA. The *Air* gene overlaps the promoter of the sense gene[70], but this is not the case for *Kcnq1ot1* (REF. 55). The promoter regions of the antisense transcripts are CpG-rich and are methylated on the inactive maternal allele; for *Air* and *Kcnq1ot1*, these CpG islands or sequences near them carry the primary germline imprint[25,55]. Deletions of both of these DMRs leads to loss of expression of the antisense transcripts and loss of imprinting of the sense genes[73,74] (*Igf2r* or *KCNQ1*). It is not known whether expression of the antisense transcript itself interferes with transcription of the sense gene. The antisense transcript could lead to alterations of chromatin structure and DNA methylation, to promoter exclusion or work by RNA-directed mechanisms, such as RNA interference. Alternatively, it is possible that the antisense transcripts have no role by themselves, but simply reflect the activity of other regulatory elements such as silencers or boundaries (see below). Indeed, deletion of the *KCNQ1OT1* DMR leads to loss of imprinted repression not only of *KCNQ1*, but also of *CDKN1C*, which does not overlap with the antisense transcript[74]. It is important to note that this deletion was made in somatic cells, so that any effects of germline transmission could not be assessed. Finally, in a marsupial, the opossum, the *Igf2r* gene is imprinted without having a differentially methylated antisense promoter or an antisense transcript[22]. This may also indicate that imprinting mechanisms can evolve rapidly and that the primitive (or primordial) imprinting mechanism of *Igf2r* worked differently from the one now seen in the mouse (W.R. and J.W., unpublished).

*Boundaries.* The observation that endoderm-specific enhancers can be shared between the paternally expressed *Igf2* and the maternally expressed *H19* (a non-coding RNA) genes suggested the possibility that chromatin boundaries might be involved (FIG. 5c). The region upstream of *H19* carries the paternal germline methylation imprint; when this was deleted, the maternal *Igf2* gene was expressed, albeit not at its full level in all tissues[67]. This led to the model that the *H19* DMR is a chromatin boundary that is 'closed' when unmethylated, and 'open' when methylated. A specific chromatin structure with several DNaseI hypersensitive sites exists on the unmethylated maternal allele[33,34], and the previously characterized repressor factor CTCF (CCCTC-binding factor), which is important for the function of a chick globin boundary element, binds to this maternal allele but not to the paternal methylated one[75–78]. This region has a boundary function in transfection assays and deletion of CTCF-binding motifs abolishes this function[75–78]. It is not known how CTCF and perhaps other factors prevent the *H19* endoderm enhancers from activating the *Igf2* promoters. However, it is remarkable that the maternal *Igf2* promoters are nevertheless DNaseI hypersensitive[79], indicating factor binding despite transcriptional silence. Enhancers for lineages other than endoderm have so far not been discovered, although there are candidates for muscle-specific enhancers[80,81].

SILENCER
DNA sequence at which repressor factors bind and mediate silencing of promoters through interaction with the basal transcriptional machinery or the enhancer.

BOUNDARY ELEMENT
DNA sequence that lies between two gene-controlling elements, such as a promoter and enhancer, preventing their communication or interaction. Boundary element function is usually mediated by the binding of specific factors.

DNASEI HYPERSENSITIVE SITE
Chromosomal region highly accessible to cleavage by deoxyribonuclease I (DNAseI). Such sites are associated with open chromatin conformations and transcriptional activity.

EPIGENOTYPE
Methylation and chromatin pattern of a gene or allele.

In the previous section, we suggested that the *Kcnq1ot1* DMR could also have boundary functions at least for *Cdkn1c*, in addition to being a promoter region for the antisense transcript, and this could be true of other DMRs as well. A new imprinted gene cluster on mouse chromosome 12 and human chromosome 14 has been isolated recently that consists of the paternally expressed *Dlk* gene flanked by a maternally expressed gene, *Gtl2*, which is a non-coding RNA[82–84]. This arrangement is intriguingly similar to that of *Igf2* and *H19*, including a paternally methylated DMR upstream of *Gtl2* and a paternally methylated DMR in *Dlk*[82–84]. It is puzzling how such a markedly similar arrangement could have evolved in different genes.

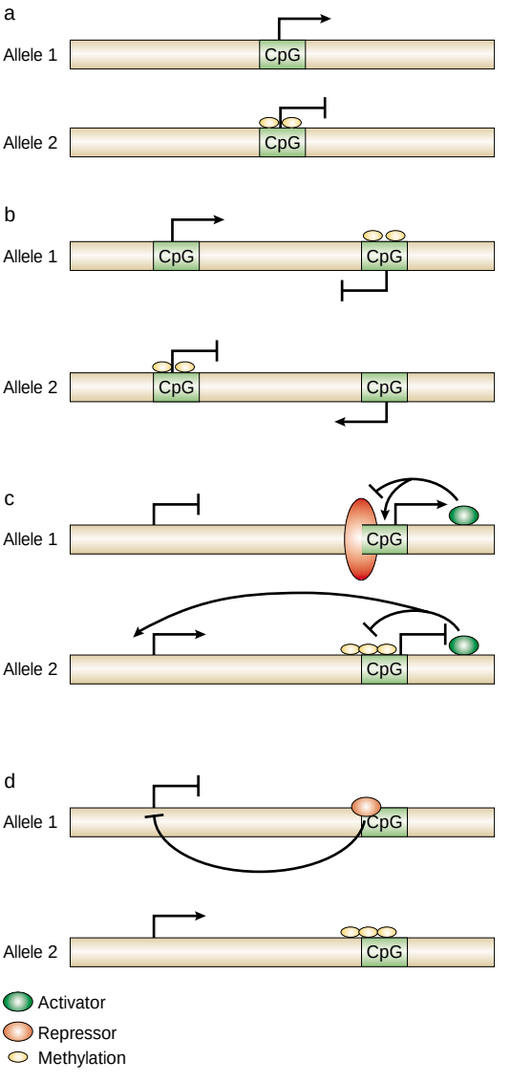



Figure 5 | **Reading mechanisms in imprinted genes. a** | Differential silencing by CpG island or promoter methylation. **b** | Regulation by antisense transcripts in conjunction with CpG island or promoter methylation; **c** | Allele-specific regulation of neighbouring genes by differential methylation of boundary elements within a CpG island. Factors such as CCCTC-binding factor (CTCF) (red disc) bind to the unmethylated allele and block the access of upstream promoters to downstream enhancers (green), leading to transcriptional repression of the upstream gene. **d** | Differential methylation results in differential binding of silencing factors (red, in this case methylation-sensitive), which repress the promoter *in cis*.

*Silencers.* Several imprinted genes have DMRs that are methylated on the active allele. This has led to the proposal that these sequences contain silencers that are inactivated by methylation, perhaps by excluding repressor factors[25,29,41,79] (FIG. 5d). This model has now been corroborated in the case of DMR1 in *Igf2*. *Igf2* is paternally expressed in various fetal tissues and DMR1 functions as a maternal silencer in a subset of those tissues. DMR1 is paternally methylated in mesodermal tissues (heart, kidney and lung), in which *Igf2* is expressed. In a DMR1 knockout, the maternal allele of *Igf2* is derepressed in these tissues[85]. In addition, *Igf2* continues to be expressed postnatally in the same tissues, when it is normally silenced soon after birth[85]. These experiments reveal that DMR1 is a mesodermal silencer; *in vitro* transfection experiments confirm this and show that the silencer is methylation sensitive (H. Cedar, personal communication).

Another tissue-specific silencer has been detected between *Igf2* and *H19* in a conserved region that shows DNaseI-hypersensitive sites but no differential methylation. Deletion of this sequence from a yeast artificial chromosome (YAC) transgene results in expression of the maternal *Igf2* allele specifically in skeletal muscle and tongue[86]. So it is possible that for specific sets of enhancers (acting in different tissues) there are specific silencers that might or might not be epigenetically controlled. Indeed, the *H19* DMR also contains a silencer (in addition to the boundary) that is endoderm-specific[87].

The findings on antisense transcripts, boundaries and silencers reinforce the idea that various elements, some of which are under epigenetic control, interact to regulate expression of imprinted genes in clusters.

## ICs and epigenetic spreading in clusters

The existence of ICs was first proposed from the molecular- genetic analysis of imprinting disorders, and from knockouts in the mouse[88–91]. In the imprinted region on human chromosome 15 (FIG. 1), small deletions were found in patients with Prader–Willi syndrome (PWS) in the promoter region of *SNURF–SNRPN*, and, a few kilobases upstream of this, deletions were found in patients with Angelman syndrome (AS). PWS requires paternal transmission of the deletion, whereas AS requires maternal transmission. The intriguing feature of these deletions is that they lead to altered expression and altered methylation patterns of many of the imprinted genes in the region, even if the genes are separated from the deletions by several megabases[88]. This is defined as 'EPIGENOTYPE spreading'. In the PWS deletions, when paternally transmitted, otherwise paternally expressed genes are silenced and methylated. In the AS deletions with maternal transmission, genes that are otherwise repressed are now demethylated and

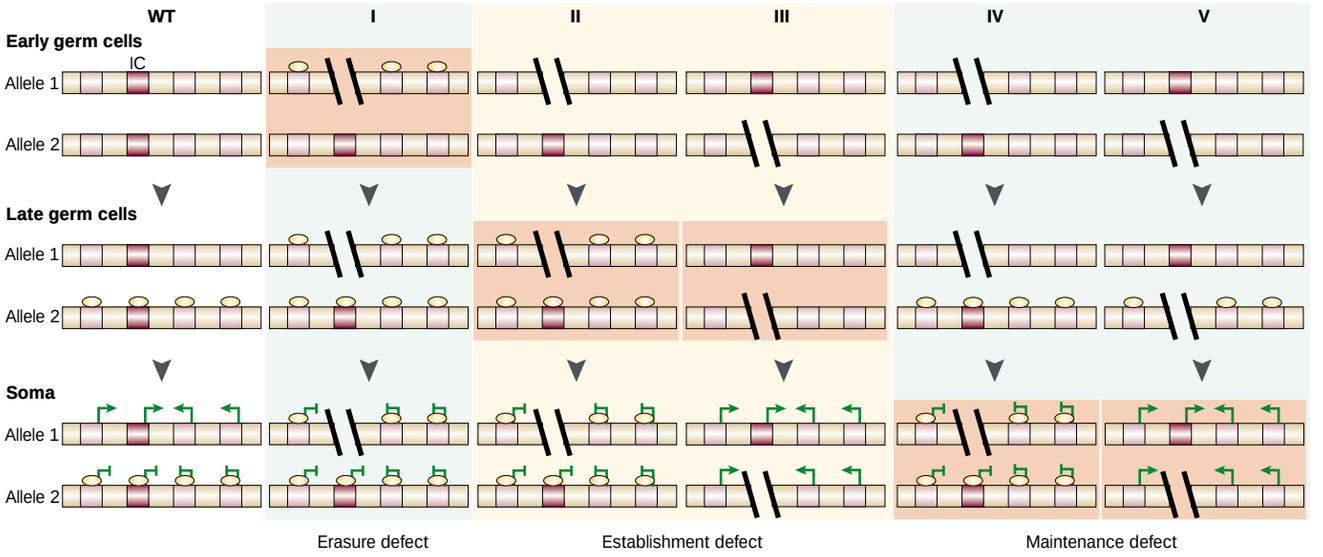



Figure 6 | **Control of imprinting clusters by imprinting centres.** An imprinting centre (IC) with neighbouring imprinted genes is shown. Imprinting centre deletions have been shown to lead to altered regional imprinting (and altered 'epigenotype spreading') but the developmental stage at which this occurs is not known. The figure shows the three developmental stages at which defects might occur. In the 'erasure defect', IC deletion leads to the inability to remove the previous methylation imprints in early germ cells (box I). In the 'establishment defect', an IC deleted chromosome is aberrantly methylated (box II) or wrongly demethylated (box III) in late germ cells. In the 'maintenance defect', an IC deletion leads to postzygotic methylation (box IV) or loss of methylation (box V). (WT, wild type.)

expressed. The same is true in a mouse knockout model of the PWS deletion[91]. A model has been proposed whereby deletion of these ICs makes the switching of the regional epigenotype in the germ line impossible[88] (FIG. 6). Thus, in the paternal germ line, it is suggested that the incoming deleted chromosome from the grandmother cannot be demethylated, and remains in a 'maternal' epigenotype despite passing through a male germ line. However, no direct proof of this model has been obtained. In fact, recent evidence has shown that a deleted chromosome switched its epigenotype (from unmethylated to methylated) after fertilization, indicating that an IC might be important for maintenance of the epigenotype[92]. Methylation analysis of the germ cells from parents of patients with PWS and AS, and of the appropriate mouse models, is urgently needed to resolve this important issue.

There is functional evidence for ICs in other clusters as well. Deletion of the maternal DMR upstream of *H19* leads to expression of the maternal *Igf2* and *Ins2* genes, and to methylation of DMR1 and DMR2 in *Igf2* (REFS 67,90,93); and deletion of the DMR in *KCNQ1OT1* alters imprinting of *KCNQ1* and *CDKN1C*[74] (see above). It is remarkable that for the imprinting cluster on distal mouse chromosome 7/human chromosome 11p15.5, there seem to be two ICs[71,72,94,95](*H19* DMR, *Kcnq1ot1* DMR, FIG. 1) that are largely independent from each other. However, the possibility cannot be excluded that there are other mechanisms that coordinate the two ICs, perhaps involving cluster-wide epigenetic modifications, such as altered replication timing of DNA. Indeed, some patients with Beckwith–Wiedemann syndrome (BWS) have imprinting alterations in both subdomains of the cluster[96].

It is still unknown whether epigenotype spreading occurs in the germ line or postzygotically (or perhaps both) (FIG. 6). But more mysterious still is how methylation spreading works mechanistically. It could be imagined that non-transcribed promoters become methylated, or that promoters that are overlapped by antisense transcripts become methylated. Alternatively, methylation spreading may be independent of transcription. It is possible that DMRs in a cluster adopt specific spatial arrangements that bring them in close contact with each other. They could thus protect each other from becoming methylated, or conversely attract methylation to their points of contact. Indeed, a special nuclear arrangement (possibly involving matrix-attachment sites) has been described for the maternal copy of the PWS/AS cluster[97].

## Evolution — conflicts and arms races

Imprinting has been found in eutherian mammals, marsupials and flowering plants. However, in MONOTREMES; and all other vertebrates and invertebrates, analysis of uniparental embryos or of genes imprinted in other organisms indicates that imprinting might not be present. But some of these assays are crude and certainly not comprehensive. More subtle effects of imprinting in other organisms cannot therefore be ruled out.

This phylogenetic distribution of imprinting, together with the observation that a sizeable proportion of imprinted genes affect fetal growth in a potentially antagonistic manner (paternally expressed genes enhance fetal growth and maternally expressed ones suppress fetal growth) led to the proposal that genetic conflict over maternal resources was the driving force in the evolution of imprinting[18]. Paternally expressed genes are therefore selected to extract more resources

MONOTREMES
Non-eutherian, egg-laying mammals.

SPONGIOTROPHOBLAST
Junctional zone between the labyrinth and the maternal side of the placenta.

LABYRINTHINE TROPHOBLAST
Placental zone where fetal and maternal exchange takes place.

HYPERKINETIC, HYPOKINETIC
Exceeding (hyper) or reduced (hypo) movement of the body or extremeties.

from the mother to benefit offspring fitness, whereas maternally expressed genes tend to conserve resources, to divide them among more offspring and to maximize reproductive performance of the female[18].

Accordingly, some imprinted genes could be in 'arms races' with others and this might result in accelerated rates of evolution of imprinted genes[18]. However, the rate of evolution of proteins encoded by imprinted genes has not been found to be elevated over that of 'normal' genes[98]. Instead, the imprinting patterns themselves seem more variable. Although the imprinting status of a considerable number of imprinted genes is conserved between mouse and human, there are now several notable exceptions where imprinting status is markedly different. For example, the human *IGF2R* gene only shows imprinted expression during early development, or imprinted expression is polymorphic, whereas the mouse gene shows stable imprinted expression in most fetal tissues and developmental stages (Harwell imprinting web site). Moreover, disruptions of imprinting of various genes were observed in interspecific hybrids of the deermouse *Peromyscus maniculatus*[99]. So, it may be that regulation of imprinted genes rather than the encoded proteins evolves rapidly, a phenomenon that could also contribute to mammalian speciation mechanisms[99]. Such changes might take place at the level of the multiple regulatory sequences involved in reading imprints (enhancers, silencers or boundary elements), as well as at the level of epigenetic modification.

One possible evolutionary territory for imprinting arms races is in the zygote. Here, the observed paternal-specific active demethylation might make it difficult to maintain a paternal germline methylation imprint. In other words, the capacity of the oocyte to demethylate paternal genomes might have evolved as a means to counteract paternal growth-enhancing strategies (W.R. and J.W., unpublished). It is interesting to note in this regard that genome-wide demethylation does not occur in the zebrafish[100] (which does not have imprinting), but is present in mammalian species with imprinting.

**Box 3 | Outstanding questions**

- How and precisely when during germline develoment are old imprints removed and new ones introduced? Which Dnmts, demethylating activities and chromatin factors are involved?
- How does the spreading of epigenetic information in clusters work, and is this a germline-specific phenomenon, a postzygotic phenomenon or both?
- How are imprints maintained when there is genome-wide active and passive demethylation in the early embryo?
- How many fundamentally different arrangements of imprinted genes and imprinting control elements are there in the genome?
- How conserved is imprinting between mammalian species?
- How precisely do imprinted genes affect extraembryonic and embryonic development, and the nutritional exchange with the mother?
- Are there interactions of imprinted genes (particularly antagonistic ones) in known, or in novel, physiological pathways?
- In addition to growth and behaviour, are there other developmental processes and mechanisms in which imprinted genes have a decisive role, and how will these fit with evolutionary theories?

## Phenotypic consequences

A substantial proportion of imprinted genes are implicated in the control of fetal growth by transgenic studies, by their location in chromosome regions that (in uniparental disomies) affect growth, or because the gene product indicates a role in growth or cell proliferation (Harwell imprinting web site). Paternally expressed genes generally enhance fetal growth and maternally expressed genes suppress fetal growth. Although the numbers are still small, this pattern is consistent with the conflict theory[18]. Regardless of imprinting, only one system of genes has been discovered so far that has an important role in growth of the fetus as a whole. This is the insulin and insulin-like growth factor system together with its receptors, binding proteins and associated signal-transduction pathways[101]. So far, three members of this system (*Igf2*, *Igf2r*, *Ins*) are known to be imprinted. Imprinted genes that affect growth by an unknown mechanism could either encode new members of the Igf system (Grb10 might well be involved in transducing an Igf/Ins signal) or belong to an as yet unknown system of growth control.

Fetal growth depends on the availability of nutrients provided by the mother. An indirect way of regulating fetal growth is therefore to restrict nutrient transfer through altered placental growth or function. Remarkably, most imprinted genes are expressed in the placenta (Harwell imprinting web site). Furthermore, placental growth is generally affected in those transgenic studies that also showed an effect on fetal growth. Two imprinted genes have so far been discovered that might have a role specifically in placental development or growth. *Mash2* regulates the development of the SPONGIOTROPHOBLAST[102], whereas an *Igf2* transcript is expressed specifically in the LABYRINTHINE TROPHOBLAST[85]. Altered growth and development of these important tissues could conceivably lead to effects on nutrient transfer to the fetus, but functional studies are needed to clarify this. Effects of imprinting might also be expected in immediate postnatal growth (from birth to weaning) because resources (milk) continue to be provided by the mother[18].

The first indication that postnatal behaviour might be influenced by imprinted genes was obtained from studies on mouse pups disomic for distal chromosome 2. Just after birth, the mice with paternal disomy showed HYPERKINETIC behaviours whereas those with maternal disomy were HYPOKINETIC[6]. Other evidence that imprinted genes have a role in brain development or function is provided by the surprisingly large number of neurological and psychiatric disorders in which parent-of-origin effects are observed[103]. In PWS and AS (classical imprinting diseases), as well as in autism, bipolar affective disorder, epilepsy, schizophrenia, Tourette syndrome and Turner syndrome, the occurrence or severity of symptoms depends on which parent transmits the disease susceptibility. With the exception of PWS and AS, however, no specific imprinted genes have yet been found that have a role in these diseases. In AS, the only maternally expressed gene found

in the cluster so far, *UBE3A*, is probably solely responsible for the disease symptoms, whereas in PWS, several otherwise paternally expressed genes (FIG. 1) are deleted or silent. The individual contributions that these deficiencies make to the disease are not yet clear.

Some more specific insights have been gained from knockout experiments in the mouse. Knockouts of *Grf1* (REF. 104) and *Ube3a*[105] (the mouse homologue of the human AS gene) have defects in contextual learning and memory (among others). *Peg1* and *Peg3*, by contrast, have a role in maternal behaviour such that mothers that lack these molecules neglect and do not feed their offspring[106,107]. How these phenotypes could be interpreted in terms of the genetic conflict theory or other theories is not clear. Because several imprinted genes that affect behaviour also have a role in fetal growth, these effects need to be genetically separated (for example by conditional knockouts), particularly because intra-uterine growth retardation can have long-term effects on cognitive functions.

## Epimutations, reprogramming and cloning

Just as mutations alter DNA, epimutations alter DNA methylation or chromatin patterns. Epimutations in imprinted genes can lead either to biallelic expression (loss of imprinting) or to biallelic silencing. How frequent these alterations are either in the germ line or during somatic development is not known. Epimutations that are not likely to have been caused by underlying DNA mutations have been observed in several disease situations, including Wilms tumour[108,109] (*H19* methylation), BWS[71,72,89] (*H19* methylation, *KvDMR1* demethylation), and PWS/AS[110] (*SNURF-SNRPN* methylation/demethylation).

BWS and PWS epimutations are likely to occur in the germ line presumably by failing to erase the grandparental imprint (or by establishing the wrong imprint), or in the early embryo. By contrast, aberrant *H19* methylation in Wilms tumour arises somatically[108,109]. The possibility that epimutations can arise during development has been explored in embryonic stem cells. Indeed, with prolonged culture of embryonic stem cells, a high frequency of epimutations arises in *H19*, *Igf2*, *Igf2r* and *U2af-rs1*, which persist during fetal development[111] (and are associated with developmental abnormalities).

Cloning of various mammalian organisms has been achieved recently using donor nuclei from differentiated cells[112]. Gene expression and, presumably, epigenetic modifications need to be reprogrammed when the somatic nuclei are introduced into the enucleated oocyte. Whereas the introduced genome might undergo passive or active demethylation, methylation in imprinted DMRs again needs to be protected from this reprogramming so that imprints are maintained intact in the cloned organism. The fact that cloning is still very inefficient and the large majority of clones die during development might indicate that the reprogramming process is inefficient. In addition, cloned animals frequently show abnormalities (placental and fetal overgrowth, and perinatal death)[113] that are typical of deregulation of imprinted genes, perhaps indicating that somatic donor cells might have had aberrant imprint patterns, or that reprogramming might interfere with proper imprint maintenance. Finally, the striking observation that ageing might be reversible by cloning[114] prompts the speculation that an important component of the ageing process, as well as of diseases, could be the somatic acquisition of epigenetic modifications.

## Perspectives

It is instructive to view mammalian genomic imprinting from many viewpoints, including mechanisms, phenotypic consequences and evolutionary significance. In the next few years, exciting developments will occur in all these areas, making this synthetic view ever more productive and enjoyable. Particular advances will come from the comparison of the sequences of imprinted regions in different mammalian species, including perhaps marsupials and monotremes, and with other vertebrates that do not have imprinting. This will provide important insights into the evolution of arrangements and clusters of imprinted genes, and will pinpoint conserved regions with possible regulatory roles. Their function can then be examined in precisely timed, and tissue-specific, gene-targeting experiments. The functions of regulatory sequences will depend on complexes that involve the chromatin factors and methyltransferases, and such complexes can be analysed using proteomic approaches. Once all the imprinted genes have been isolated, targeting experiments will provide crucial insights, particularly into interactions between their products in physiological pathways. Many questions about the biology of imprinting remain (BOX 3), but using the array of approaches summarized above, some fascinating answers will surely follow.

## Update — added in proof

A recent mouse transgenic study[118] reports that the Angelman syndrome deletion region contains a sequence that can protect the maternal *Snrpn* promoter from becoming demethylated after fertilization, and the paternal *Snrpn* promoter from becoming *de novo* methylated in sperm and in the postimplantation embryo. This is the first report of a sequence that can protect from *de novo* methylation as well as maintain a methylation imprint.

## Links

DATABASE LINKS H19 | Igf2 | Cdkn1c | Dnmt1 | Dnmt3a | Dnmt3b | Tsix | Xist | Igf2r | Kcnq1 | Air | Kcnq1ot1 | CTCF | Dlk | Gtl2 | Prader–Willi syndrome | SNURF–SNRPN | Angelmann syndrome | Ins2 | Beckwith–Wiedemann syndrome | Ins | Grb10 | autism | bipolar affective disorder | schizophrenia | Tourette syndrome | Turner syndrome | UBE3A | Grf1 | Ube3a | Peg1 | Peg3 | Wilms tumour

FURTHER INFORMATION Harwell imprinting web site

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