Stability and flexibility of epigenetic gene regulation in mammalian development

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During development, cells start in a pluripotent state, from which they can differentiate into many cell types, and progressively develop a narrower potential. Their gene-expression programmes become more defined, restricted and, potentially, 'locked in'. Pluripotent stem cells express genes that encode a set of core transcription factors, while genes that are required later in development are repressed by histone marks, which confer short-term, and therefore flexible, epigenetic silencing. By contrast, the methylation of DNA confers long-term epigenetic silencing of particular sequences — transposons, imprinted genes and pluripotency-associated genes — in somatic cells. Long-term silencing can be reprogrammed by demethylation of DNA, and this process might involve DNA repair. It is not known whether any of the epigenetic marks has a primary role in determining cell and lineage commitment during development.

Development is, by definition, epigenetic. Differences in the programmes of gene expression that result in the development of different organs and tissues occur without changes to the sequence of our DNA (with one or two exceptions). There is nothing mysterious in this concept; subsets of the ~30,000 genes in our genome are active in different tissues and organs, depending on their regulation by different sets or combinations of transcription factors. This implies that if we were to take all of the transcription factors that activate genes in a liver cell and transfer them to a brain cell (while inactivating all brain-specific transcription factors), then the brain cell would turn into a liver cell.

A recent study provides tantalizing insight into this concept of epigenetic control of development. Takahashi and Yamanaka identified four transcriptional regulators that when expressed in fibroblasts, resulted in these cells being reprogrammed to become embryonic stem (ES)-like cells[1]. Extending this concept a little further, in somatic-cell nuclear transfer, the nucleus of a somatic cell from an adult individual is transplanted into an oocyte from which the nucleus has been removed, resulting in reprogramming of the adult nucleus and therefore successful development of the cloned animal.

Cloning, however, is inefficient, because most (if not all) cloned animals have epigenetic defects, particularly in DNA methylation. Therefore, our lack of understanding of how epigenetic marks are reprogrammed is a key obstacle to cloning[2]. Similarly, the reprogramming of fibroblasts to become ES-like cells is a rare event *in vitro*, and epigenetic defects such as lack of demethylation of the *Oct4* (also known as *Pou5f1*) promoter, affecting expression of the encoded transcription factor, have been noted in these ES-like cells[1].

These observations highlight that, in addition to transcription factors, changes in gene expression during development are accompanied or caused by epigenetic modifications[2–7], such as methylation of DNA at CpG sequences (in vertebrates[4,5]), modification of histone tails[6] and the presence of non-nucleosomal chromatin-associated proteins[7]. Therefore, as development and differentiation proceed, differentiated cells accumulate epigenetic marks that differ from those of pluripotent cells, and differentiated cells of different lineages also accumulate different marks.

In this review, I focus on the role of epigenetic regulation in development, particularly comparing the short-term flexibility of certain epigenetic marks (which can be removed before a cell divides or within very few cell divisions) with the long-term stability and heritability of other marks (which can be maintained for many divisions) (Fig. 1). During the early stages of development, genes that are required later in development are transiently held in a repressed state by histone modifications, which are highly flexible and easily reversed when expression of these genes is needed. During differentiation, genes that are crucial for pluripotency are silenced by histone modifications, as well as by DNA methylation. Some of these genes are also silent in mature germ cells, meaning that epigenetic marks probably need to be reversed rapidly after fertilization to allow re-expression of pluripotency-associated genes in the next generation. By contrast, long-term silencing of transposons and imprinted genes — which is based on DNA methylation — needs to be stably maintained from the gametes into the early embryo and the adult organism. Methylation of imprinted genes can only be erased in primordial germ cells (PGCs), the cells that ultimately give rise to the germ line. Probably because there is a requirement for both removing epigenetic marks and retaining epigenetic marks between generations, epigenetic information can sometimes be inherited across multiple generations. In this review, I address how the fascinating interplay between transcription factors and epigenetic factors is beginning to provide an explanation for how pluripotency and development are regulated.

Flexibility for developmental gene regulation

In this section, three issues are addressed. First, are differentiation-specific genes held in an epigenetically silenced manner in pluripotent cell types, in order to be activated later? And is the removal of epigenetic marks from these genes needed for their activation? Second, are pluripotency-associated genes epigenetically inactivated in differentiated cell types? This inactivation could, in principle, be irreversible, because somatic cell types are not required to give rise to pluripotent cells. One exception is the germ line, where reactivation of pluripotency-associated genes is needed at the initial stages of development; however, later, the silencing of these genes is essential for the differentiation of mature germ cells. And therefore, third, is the removal of 'permanent' silencing marks from the gametic genomes after fertilization crucial to activate essential genes, such as pluripotency-associated genes, early in development?

There is recent evidence for the first type of epigenetic regulation: that is, the temporary inactivation of differentiation-specific genes in pluripotent cell types (Fig. 2a). Genes that are required during development and differentiation — for example, those in the homeobox (*Hox*), distal-less homeobox (*Dlx*), paired box (*Pax*) and sine-oculis-related homeobox (*Six*) gene families — are held repressed in pluripotent ES cells by the Polycomb group (PcG)-protein repressive system in mice and humans. This system marks the histones associated with these genes by inducing methylation of the lysine residue at position 27 of histone H3 (H3K27)[8–10]. ES cells that lack EED (embryonic ectoderm development), a component of the PcG-protein repressive complex (PRC), have partly derepressed developmental genes and are prone to spontaneous differentiation[8,10]. Interestingly, some developmental genes are present within 'bivalent' chromatin regions, which contain both inactivating marks (methylated H3K27) and activating marks (methylated H3K4)[9,11]. This could indicate that after the repressive marks have been removed (when expression of the components of PRCs are downregulated during differentiation), these genes are automatically poised for transcriptional activation through the H3K4 methylation mark. It is important to note that epigenetic silencing by PRCs might be mitotically heritable (through an unknown mechanism)[7], but these marks could presumably be rapidly removed by enzymatic demethylation of H3K27 (by an uni-

dentified demethylase)[12]. The H3K27 methylation mark occurs mostly outside the context of DNA methylation. In contrast to the terminal silencing achieved by DNA methylation (discussed later), developmental genes that are silenced by PRCs in pluripotent tissues require repressive marks to be rapidly and flexibly removed when differentiation begins. Strikingly, in cancer cells, the genes targeted by PRCs often become DNA methylated, which might result in a more permanent locking-in of a 'pluripotent' state in cancer stem cells[13].

The second type of epigenetic regulation to be considered is whether pluripotency-associated genes are epigenetically inactivated in differentiated cell types. Several genes that are required for early development or for germ-cell development only — for example, those that encode pluripotency-sustaining transcription factors (such as OCT4 and NANOG) — are known to be expressed by ES cells but silenced on the differentiation of these cells, with a defined kinetics of acquiring repressive histone modifications and DNA methylation[14] (Fig. 2b). Silencing by both histone modifications and DNA methylation in somatic tissues seems to be typical of this group of genes and of those that encode cancer–testis antigens, which are expressed during spermatogenesis[15]. It is probable that this permanent type of epigenetic silencing safeguards against accidental expression of these genes in differentiated cells, because that might lead to dedifferentiation and, perhaps, to a predisposition to
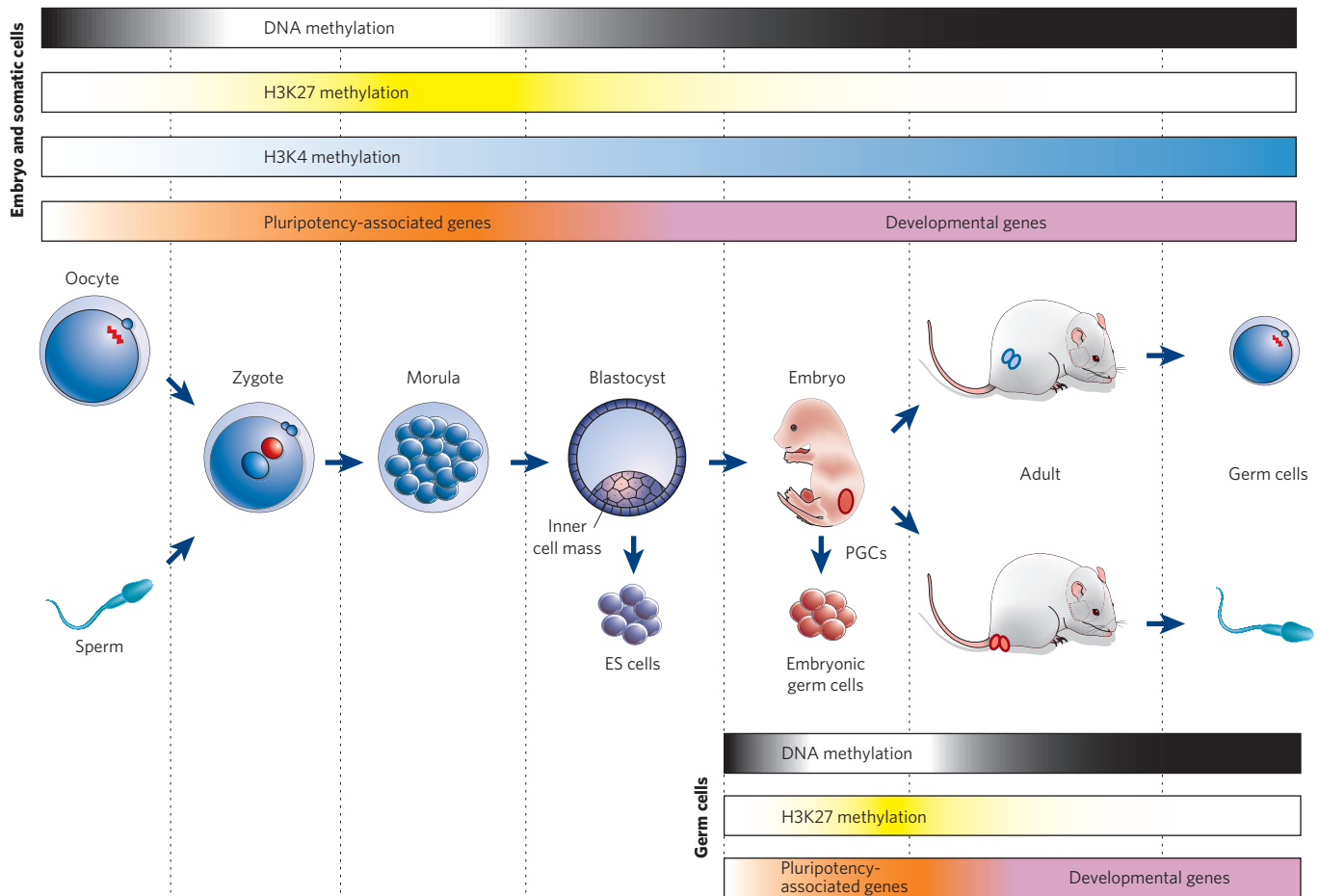

Figure 1 | Epigenetic gene regulation during mammalian development.
Key developmental events are shown together with global epigenetic modifications and gene-expression patterns. Very early in development, DNA methylation is erased. In addition, pluripotency-associated genes begin to be expressed, and developmental genes are repressed by the PcG protein system and H3K27 methylation. During the differentiation of pluripotent cells such as ES cells, pluripotency-associated genes are repressed, potentially permanently, as a result of DNA methylation. At the same time, developmental genes begin to be expressed, and there is an increase in H3K4

methylation. During the early development of PGCs, DNA methylation and repressive histone modifications (such as H3K9 methylation) are also erased. Pluripotency-associated genes are re-expressed during a time window that allows embryonic germ cells to be derived in culture. Imprinted genes are demethylated during this period, and developmental genes are expressed afterwards. Flexible histone marks such as H3K27 methylation enable developmental genes to be silenced for a short time in pluripotent cells. By contrast, DNA methylation enables the stable silencing of imprinted genes, transposons and some pluripotency-associated genes.

cancer[16]. Consequently, these genes are difficult to reactivate in cloned embryos because of inefficient reprogramming of repressive marks, particularly of DNA methylation[17].

Special epigenetic regulation needs to occur in PGCs developing in the early post-implantation embryo[18]. Because these cells emerge from cell types in the egg cylinder that are already on the way to lineage commitment and differentiation, the somatic gene-expression programme needs to be suppressed. One of the key regulators of this process is BLIMP1 (B-lymphocyte-induced maturation protein 1), which associates with the arginine methyltransferase PRMT5. PRMT5 might partly repress *Hox*-family genes and other somatic genes in PGCs[19] (Fig. 2c). Pluripotency-associated genes and genes that have later roles in germ-cell development can also be repressed by DNA methylation (Fig. 2b). So genes such as *Mvh* (also known as *Ddx4*), *Dazl* (deleted in azoospermia-like) and *Sycp3* (synaptonemal complex protein 3) are methylated in early PGCs and begin to be expressed after the erasure of DNA methylation[20], which occurs between embryonic day (E) 8.0 and E12.5 in PGCs. Interestingly, pluripotency-associated genes such as *Nanog* also begin to be reactivated at these stages, but it is not known whether this involves demethylation of DNA. PGCs at these stages have similar properties to pluripotent cells, including the ability to form embryonic germ cells in culture[21]. These studies are important because they are the first to show that in some developmental situations, removal of epigenetic marks (H3K27 methylation in the ES-cell study, and DNA methylation in the PGC study) could be crucial for the activation of developmental genes. Whether DNA methylation in PGCs is erased by an active or a passive mechanism is unclear (discussed later). The promoters of the genes that undergo 'developmental' demethylation (for example, *Mvh*, *Dazl* and *Sycp3*) contain CpG islands, as do the differentially methylated regions (DMRs) of imprinted genes, which also undergo demethylation at these stages of PGC development. I am not aware of any reports of demethylation of CpG islands during development other than in PGCs or in the zygote and pre-implantation embryo (discussed later). Methylation of CpG islands might only be removable under exceptional circumstances.

Some key pluripotency-associated genes (such as *Oct4* and *Nanog*) are epigenetically inactivated at later stages of gametogenesis and in the mature gametes, including by DNA methylation. Therefore, after

a   Temporary repression of developmental genes by the PcG protein system
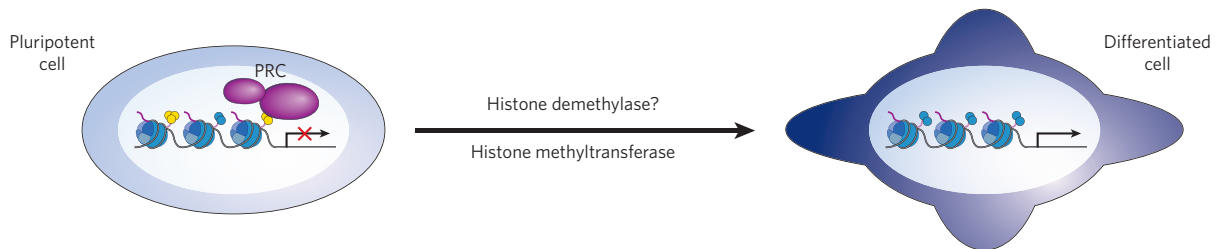
b   Repression of pluripotency-associated genes by histone methylation and DNA methylation
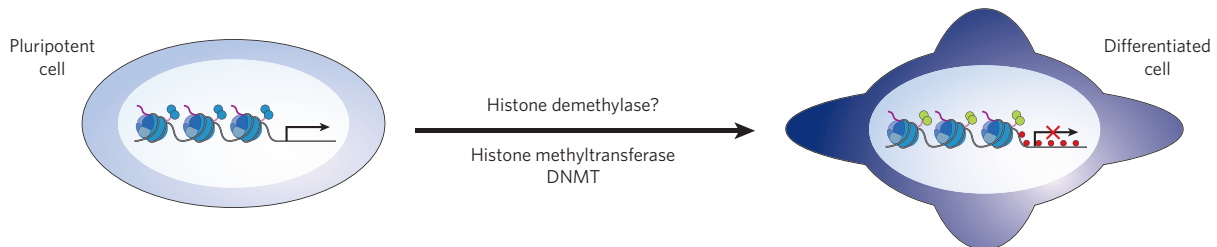
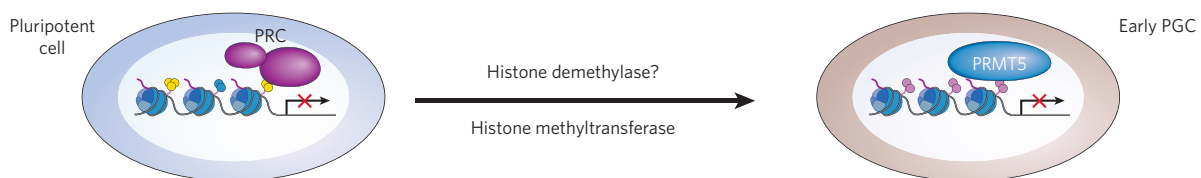**c**   Maintenance of silencing of somatic genes in early germ cells

Figure 2 | Epigenetic regulation of pluripotency-associated genes and developmental genes during the differentiation of somatic cells and germ cells. The expression or repression of pluripotency-associated genes and developmental genes is indicated, and the associated modifications of the histone tails and/or DNA are represented by different colours. **a,** In pluripotent cells, the repression of genes that are needed later in development is flexible and can involve the PcG-protein repressive system. Silent developmental genes can be marked by both H3K27 methylation (yellow) and H3K4 methylation (blue), possibly allowing rapid gene activation after loss of repression by PcG-protein-containing repressive complexes (PRCs). Whether the loss of H3K27 methylation involves a histone demethylase is unknown. Further increases in H3K4 methylation might be required for proper developmental gene expression.

b, Pluripotency-associated genes are stably silenced during differentiation, through histone methylation and DNA methylation. For example, genes such as *Oct4* and *Nanog* are silenced during ES-cell differentiation, and this process can involve both histone methylation (such as methylation of H3K9 mediated by G9A; also known as EHMT2) (green) and DNA methylation (red). Whether a histone demethylase is required for the removal of H3K4 methylation is unknown. **c,** For germ-cell development, the repression of somatic genes needs to be maintained in early germ cells, and this process might involve histone arginine methylation (pink). *Hox*-family genes and other developmental genes remain silent in early germ cells; some of this silencing might require histone arginine methylation brought about by PRMT5.

fertilization, the repressive epigenetic marks might need to be removed for transcriptional activation of these genes and correct early lineage development to take place (discussed later).

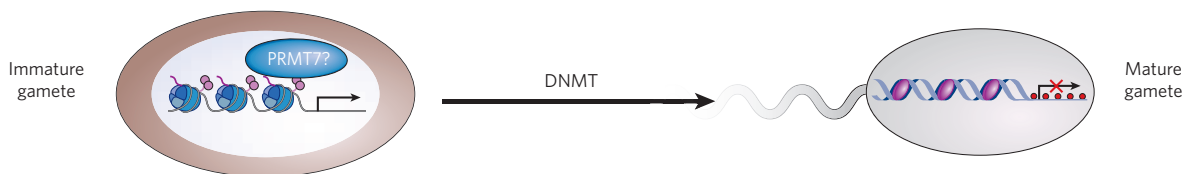## Stability for transposon silencing and imprinting

In contrast to developmental genes, which need to be epigenetically regulated with flexibility, transposons (if possible) need to be silenced completely and stably (at least from the perspective of the host) to prevent them from moving around in the genome and potentially causing mutations[22]. Therefore, many transposon families are both methylated themselves and marked by repressive histone modifications (such as H3K9 methylation), and these marks are important for the heritable silencing of transposons. Some transposon families (such as intracisternal A particles; IAPs) are also resistant to the erasure of DNA methylation in the zygote and in PGCs, possibly resulting in epigenetic inheritance across generations (discussed later).

Imprinted genes are a class of mammalian genes with possible mechanistic relationships to transposons[23], in that CpG islands in their promoters become methylated and in that silencing relies on long-term epigenetic stability. In imprinted genes (and transposons), DNA methylation is introduced during either oogenesis or spermatogenesis, by the *de novo* methyltransferase DNA methyltransferase 3A (DNMT3A) and its cofactor DNMT3-like (DNMT3L)[24,25] (Fig. 3a). How particular imprinted genes are selected for *de novo* methylation during oogenesis or spermatogenesis is not understood, although this targeting could

involve pre-existing histone marks[26]. After fertilization, the methylation of imprinted-gene DMRs is maintained by DNMT1o (the oocyte form of DNMT1) for one division cycle during very early pre-implantation development[27] and then by DNMT1s (the somatic form of DNMT1) in embryonic and adult tissues[28].

Imprinted genes can be directly silenced by methylation of DMRs (which often contain CpG islands) that overlap the promoter. More frequently, however, imprinted genes occur in clusters, and there is usually a single DMR that is methylated in the germ line and is responsible for regulating gene silencing in the rest of the cluster. So far, there are two distinct models for how, after fertilization, imprinted genes are silenced through the action of nearby unmethylated DMRs. First, the DMR overlaps the promoter of a long, non-coding, unspliced, nuclear RNA[29,30]. The presence of the unmethylated and expressed copy of the non-coding RNA results in the silencing of linked genes, a process that involves repressive histone modifications[31,32]. It is unclear how the presence of the non-coding RNA leads to gene silencing *in cis*. In one model, repressive complexes (for example, PRCs) might be targeted during transcription[33]. Alternatively, the RNA might 'coat' the region to be inactivated, similarly to how *Xist* RNA (inactive X-specific transcripts) coats the inactive X chromosome[31,34]. This might establish a physical structure from which RNA polymerase II (Pol II) is excluded, resulting in transcriptional silencing[35] (Fig. 3b). In one case of silencing mediated by an imprinted non-coding RNA, the developmental kinetics of inactivation are markedly similar to those of imprinted X-chromosome inactivation. Both

a Acquisition of DNA methylation in germ cells

Immature gamete — PRMT7? — DNMT → Mature gamete

b Silencing of the X chromosome and imprinted genes

Pluripotent cell — Pol II

DNMT
Histone methyltransferase? → Embryonic lineage — Pol II

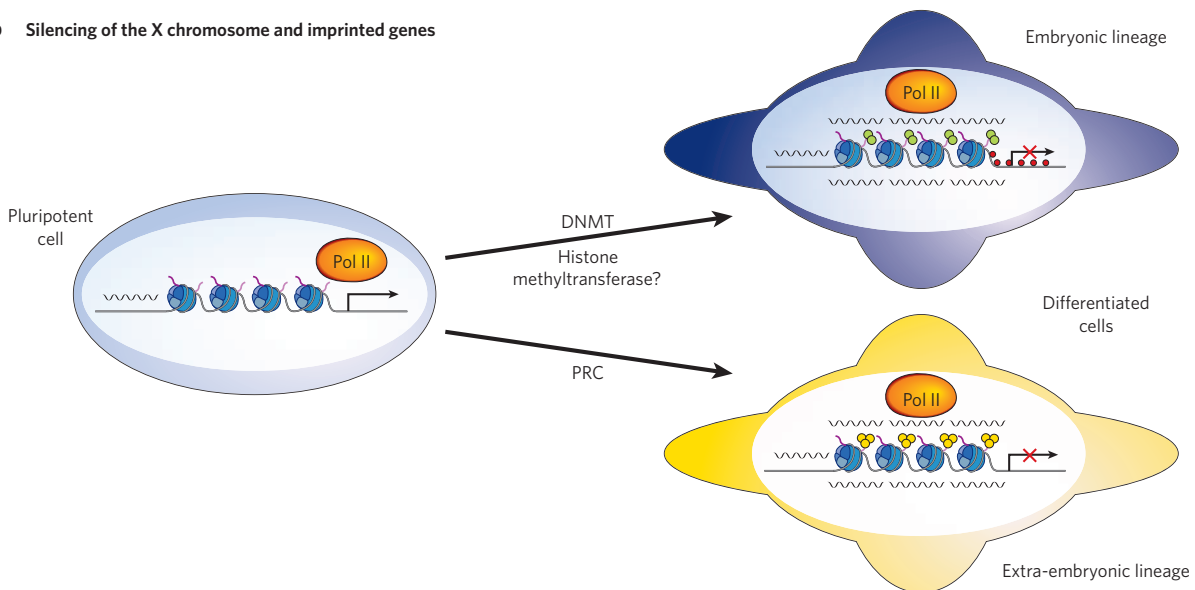PRC → Differentiated cells — Pol II — Extra-embryonic lineage

Figure 3 | Developmental regulation of imprinting and X-chromosome inactivation. a, During germ-cell development, selected imprinted genes and transposons become methylated. This process depends on *de novo* methyltransferases such as DNMT3A and its cofactor DNMT3L. It is possible that the targeting of DNA methylation requires arginine methylation of histones, carried out by PRMT7. Mature male germ cells have chromatin that is largely based on non-histone proteins known as protamines (dark pink); this alters the packaging of the DNA. **b,** Expression of non-coding RNAs (wavy black line) *in cis* can result in the silencing of

adjacent genes as a consequence of the physical exclusion of Pol II and the acquisition of histone modifications and/or DNA methylation, depending on the embryonic lineage. DNA methylation stabilizes gene silencing in embryonic tissues but is less important in extra-embryonic tissues, where PRC-mediated silencing might predominate. This mechanism of postzygotic gene silencing occurs in X-chromosome inactivation and in some forms of autosomal gene imprinting. H3K9 methylation is shown in green, H3K27 methylation in yellow, histone arginine methylation in pink and DNA methylation in red.

non-coding RNAs (*Kcnq1ot1* and *Xist*) begin to be expressed from the paternal allele in the two-cell embryo, and gene silencing *in cis* and the acquisition of histone modifications follow during the next few cleavage divisions and are largely complete by the blastocyst stage[34] (Fig. 3b).

The second model of how imprinted genes are silenced involves an epigenetically regulated chromatin insulator. In this model, tissue-specific enhancers are located on one side of the DMR overlapping with the insulator, whereas the silenced genes are on the other side[36]. Silencing occurs when the DMR is unmethylated and binds chromatin-organizing proteins such as CTCF (CCCTC-binding factor), resulting in a higher-order chromatin structure that prevents interactions between remote enhancers and promoters[37].

X-chromosome inactivation is another example of a relatively stable epigenetic silencing event; in this case, large regions of a whole chromosome are involved. In mice, imprinted X-chromosome inactivation is probably largely initiated by expression of *Xist* from the paternal chromosome at the two-cell stage[38]. (The nature of the imprinting leading to paternal expression is still unknown, but it is unlikely to be DNA methylation.) Imprinted X-chromosome inactivation is then stable (even in the absence of DNA methylation[39]) in the extra-embryonic tissues. Although the PcG protein system (which confers H3K27 methylation marks) has some influence on gene silencing, these modifications do not seem to confer heritable silencing[40]. Random X-chromosome inactivation is initiated in the epiblast after reprogramming of imprinted inactivation[41,42]. This reprogramming might be initiated by the silencing of *Xist* expression, and if this is the case, it is possible that the mitotic 'memory' for inactivation simply resides in the expression of *Xist*. The subsequent upregulation of *Xist* expression during the differentiation of epiblast cells is again followed by coating, gene silencing and acquisition of histone marks[43]. However, in contrast to imprinted X-chromosome inactivation, CpG islands in inactivated genes on the X chromosome become methylated and, although it has not been tested genetically, this might constitute long-term memory for inactivation during embryonic and adult life[43] (Fig. 3b). It is important to note that this methylation of CpG islands seems to be a dead end in that it does not need to be reprogrammed during the normal life cycle. (In the germ line, the inactivated X chromosome does not become methylated.)

Breaking stability by epigenetic reprogramming

DNA-methylation patterns that have been acquired during development are stable in somatic cells and during adult life. DNA-methylation patterns are somatically heritable essentially through the action of DNMT1, the maintenance methyltransferase[44]. At most CpG sites, the error rate of maintaining methylation (~1% per division) is low in relation to the number of cell divisions that are needed to produce a mammalian organism (44 for humans). Indeed, methylation of CpG islands is never erased during normal development. By contrast, methylation of CpG islands in imprinted-gene DMRs needs to be erased in the germ line so that gender-specific methylation can be imposed subsequently, during germ-cell development. This erasure takes place in a defined period — from E10.5 to E12.5 in PGCs — in all imprinted genes that have been tested[45,46], and it could occur by active demethylation of DNA by an unknown mechanism, possibly involving DNA repair (discussed later). This mechanism for erasure might also underlie the demethylation and activation of non-imprinted genes such as *Mvh*, *Dazl* and *Sycp3,* which takes place at about the same stage[20] (Fig. 4a).

Epigenetic reprogramming in PGCs entails widespread loss of DNA methylation, as well as H3K9 methylation[47]. In addition to the erasure of genomic imprints, this epigenetic reprogramming might also help to return PGCs to a pluripotent state (because at these stages of PGC development, pluripotent embryonic germ cells can be established in culture), through the reactivation of genes such as *Nanog*. Not all genomic methylation is lost, however, at these stages; some transposons such as IAPs remain fairly highly methylated[48]. Later in oogenesis and spermatogenesis, *de novo* methylation occurs not only sex-specifically in imprinted genes but also in transposons and in single-copy gene sequences. For example, the *Nanog* promoter becomes highly methylated in mature sperm[49].

Distinct genome-wide reprogramming events also occur immediately after fertilization and during early pre-implantation development (Fig. 4b). Many sequences in the paternal genome become suddenly demethylated shortly after fertilization[50–53]. This demethylation occurs after the removal of protamines (basic proteins that are associated with DNA in sperm) and the acquisition of histones by the paternal genome during the long G1 phase, before DNA replication. Methylation can be observed by staining cells with an immunofluorescently labelled antibody specific for 5-methylcytosine. Judged by the substantial loss of immunofluorescence signal, together with the considerable loss of methylation of *Line1* elements as determined by bisulphite sequencing[48], the paternal genome loses a significant amount of methylation, although more precise measurements and more information about which sequences are affected and unaffected would be valuable. Sequences that are known not to be affected include IAPs and paternally methylated DMRs in imprinted genes (Fig. 4c). A recent study provides intriguing insight into a protein that might protect the genome from demethylation. The protein stella (also known as DPPA3) binds to DNA and was originally identified because expression of the encoding gene is upregulated during early PGC development. Stella is present in large amounts in oocytes and, after fertilization, translocates to both pronuclei. Deletion of the gene from the oocyte (and therefore removal of the protein from the zygote) results in early pre-implantation lethality of embryos, as well as loss of methylation of the following sequences: the maternally methylated genes *Peg1* (also known as *Mest*), *Peg5* (also known as *Nnat*) and *Peg10*; the paternally methylated genes *H19* and *Rasgrf1* (Ras protein-specific guanine-nucleotide-releasing factor 1); and IAPs[54]. So stella might, either directly or indirectly, protect specific sequences from demethylation in the zygote, but it is unknown how other sequences are protected (Fig. 4c).

The mechanism of active demethylation in the zygote is still unknown. However, the DNA deaminases AID and APOBEC1 have been shown *in vitro* to deaminate 5-methylcytosine in DNA to thymine[55]; this results in T•G mismatches, which can be repaired by the base-excision repair pathway. Interestingly, *Aid* and *Apobec1* are located in a cluster of genes with *Stella*, growth differentiation factor 3 (*Gdf3*) and *Nanog*. *Stella*, *Gdf3* and *Nanog* are all expressed in pluripotent tissues, and *Gdf3* and *Nanog* have important roles in conferring stem-cell identity on ES cells. Indeed, *Aid* and *Apobec1* are also expressed by oocytes, stem cells and germ cells[55], and recent work shows that *in vivo* targeting of AID to the methylated *H19* DMR in the zygote results in efficient and substantial demethylation of this region (C. F. Chan, H. Morgan, F. Santos, D. Lucifero, S. Petersen-Mahrt, W. Dean and W.R., unpublished observations). Although it is unclear whether AID and/or APOBEC1 are responsible for the demethylation of the paternal genome in the zygote, the evidence suggests that base-excision or mismatch repair might have a role in this process. I think that this suggestion is supported by the recent identification of a DNA glycosylase–lyase — DEMETER — that preferentially excises 5-methylcytosine from DNA in *Arabidopsis thaliana*[56,57]. DEMETER is required for the demethylation and activation of the imprinted gene *MEDEA* (see page 418). Another DNA-damage-responsive gene, the mouse gene *Gadd45* (growth arrest and DNA-damage-inducible 45), might also have a role in demethylation[58].

Although there have been suggestions that the methyl group could be directly removed from DNA by hydrolytic attack or by oxidation, these mechanisms have not been substantiated[2]. The relative flexibility of histone methylation might be brought about by the attachment of the methyl group through a carbon–nitrogen bond, together with the existence of enzymes that can directly remove the methyl group, leaving the rest of the histone molecule intact[12]. By contrast, the current evidence suggests that methyl groups attach through a carbon–carbon bond to the cytosine base and therefore might not be able to be directly removed, so demethylation inevitably has to proceed by pathways that involve base-excision or mismatch repair[55–57].

This active demethylation of the paternal genome is followed by passive demethylation of both maternal and paternal genomes, presumably brought about by exclusion of DNMT1o (the main form of DNMT1 present in the oocyte) from the nuclei of pre-implantation embryos[27]. Although DNMT1s can maintain the methylation of imprinted-gene DMRs during this period, total genome methylation decreases, reaching an overall low at the blastocyst stage. The purpose of active and passive demethylation during early embryogenesis is unknown. Demethylation of the paternal genome has been proposed to account for the paucity of paternal imprints[59] or to be a consequence of DNA-repair processes that are potentially involved in the protamine-to-histone transition[53]. General demethylation during this period could also have a role in returning the gametic genomes to pluripotency. For example, early expression of genes such as *Oct4* or *Nanog* is required for the establishment and maintenance of the inner-cell-mass lineage in the blastocyst[60]. Because the *Nanog* and *Oct4* promoters are methylated in sperm, and because methylation of these promoters is repressive, they need to be demethylated for proper expression to occur (Fig. 4b).

Epigenetic spillover across generations

Many of the epigenetic marks that are inherited and acquired by germ cells are therefore erased in PGCs and in early embryos, making way for new generations to develop and grow into adults purely on the basis of their genetic make-up. However, it also seems that epigenetic information can spill over to the next generation. The ability of somatic cells in the offspring to inherit the methylation of imprinted genes from parental germ cells is a mechanistic example of this (Fig. 4c). Another important example of spillover is inheritance of the epigenetic states conferred on some genes by adjacent insertion of IAPs. This can alter the expression of the endogenous genes; however, more importantly, the epigenetic state of the IAP (that is, methylated or unmethylated)

regulates the expression of the nearby gene[61]. Because IAPs seem generally resistant to reprogramming during PGC and pre-implantation development, the state of expression of the genes that are regulated by IAP insertion can be inherited across several generations. It is interesting to note that there is an example of epigenetic inheritance being maternally transmitted but not paternally transmitted (the agouti viable yellow epiallele in mice), and the methylation of the IAP in the sperm is, unusually, erased in the zygote in this case[62]. So epigenetic inheritance is 'broken' by erasure of methylation of the paternal genome after fertilization.

There are other possible spillovers across generations. In *Caenorhabditis elegans*, the X chromosomes are epigenetically marked (by histone modifications) during gametogenesis, and some of these marks are maintained for several cell divisions in the new embryo (for an unknown reason)[63]. In mammalian embryos, some of the histone modifications acquired during the silencing of X-linked genes in spermatogenesis might be carried over into the zygote, leading to early silencing of some genes on the paternal X chromosome without the action of *Xist*[64].

One other area that is unique to mammalian biology deserves consideration with regard to epigenetic spillovers from the previous generation. At present, we have no understanding of how molecular decisions are taken to set up the first two cell lineages in the embryo: the trophectoderm and the inner cell mass[65]. However, a recent study suggests that differential histone arginine methylation of individual blastomeres, as early as the four-cell stage, could be one of the earliest marks for this lineage commitment[66]. There is much work to be done in this area, but it is an exciting possibility that the spillover of epigenetic marks from the gametes of parents might be responsible for setting up some of the earliest developmental decisions in the newly developing embryo.
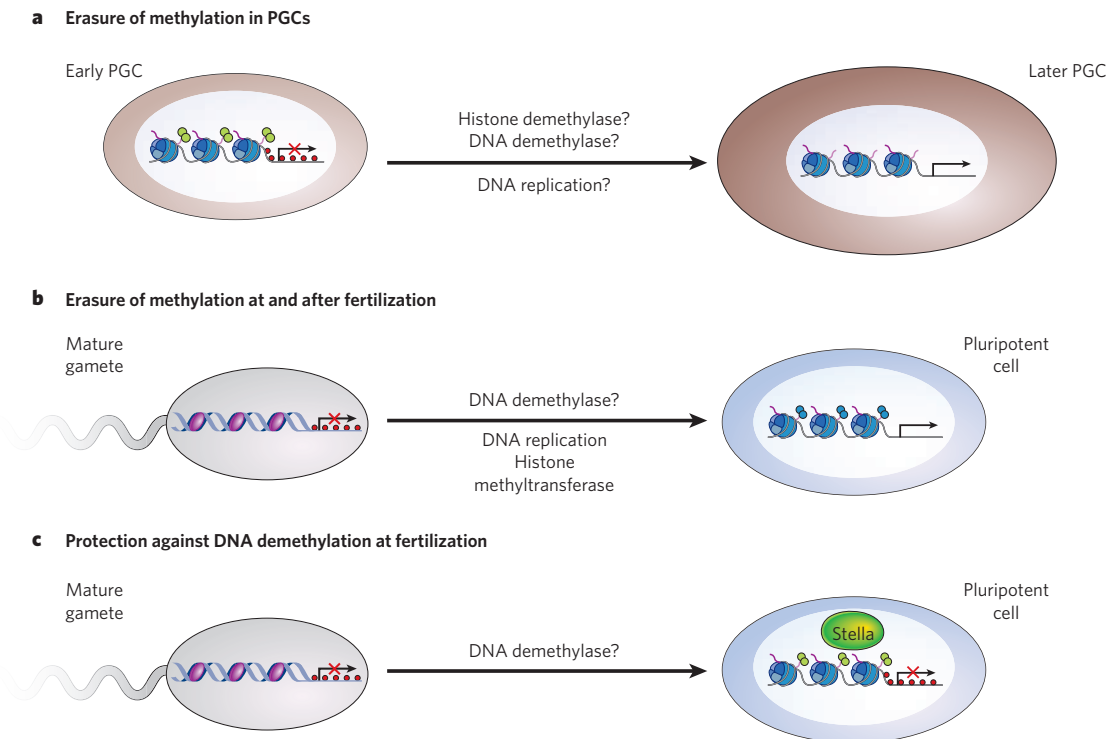

Figure 4 | Reprogramming of epigenetic marks in the germ line and the early embryo. a, During the development of PGCs, methylation of CpG islands in imprinted genes and other genes can be erased. This is a rapid process and might involve a demethylase or might occur by DNA replication without methylation being maintained. **b,** Many gene sequences that are methylated in mature gametes become demethylated in the early embryo. Some of this demethylation occurs in the absence of DNA replication and is therefore likely to be mediated by a demethylase. Demethylation might be important for the expression of pluripotency-associated genes. Active histone marks are also likely to be important for the expression of pluripotency-associated genes. **c,** In mature gametes, some DNA sequences that are methylated are protected from demethylation at or after fertilization. These sequences include imprinted genes and some transposons. The protein stella has recently been implicated in protection against demethylation at fertilization. H3K9 methylation is shown in green, H3K4 methylation in blue and DNA methylation in red.

Conclusions and outlook

Development might be a one-way street because of the somatic inheritance of epigenetic marks. Whether there is a linear relationship between acquisition of epigenetic marks and developmental progression is doubtful; some key restrictions in developmental potential that are brought about by epigenetic regulation might occur very early in development. Judging from somatic-cell nuclear-transfer experiments, it is far from clear whether more-differentiated cells have more epigenetic marks or have marks that are more difficult for the oocyte to reprogramme[67].

Natural epigenetic reprogramming might be needed to ensure that development can start afresh in every new generation. Although various mechanisms for the rapid erasure of histone modifications have recently been identified, the mechanism of DNA demethylation still needs to be determined. Recent work on the erasure of DNA methylation from imprinted plant genes shows that base-excision repair has an important role, and it is possible that this is also the case in mammals. Because of the generally accurate heritability of DNA methylation and because of its chemical stability, erasure of DNA methylation might only be possible either by replicating DNA in the absence of DNMT1 or by breaking DNA.

It is fascinating to see that both transcription-factor interactions and epigenetic programming and reprogramming seem to be needed to maintain pluripotency in early embryos and ES cells. Indeed, experimental reprogramming of differentiated nuclei without using somatic-cell nuclear transfer or cell fusion has been achieved recently, using a mix of pluripotency factors[1]. It could be expected that forcing the expression of pluripotency transcription-factor networks would also activate epigenetic reprogramming factors, but whether this occurs is unclear. Perhaps combinations of transcription factors and epigenetic reprogramming factors are needed for more complete reprogramming of somatic cells to a pluripotent state, and this would be of great fundamental scientific and medical interest.

In the animal kingdom, some epigenetic systems, such as imprinting, have evolved only in mammals. Many of the basic molecular building blocks for epigenetics, such as the enzymes for DNA methylation and histone modifications, are highly conserved in vertebrates, but the regulation of epigenetic modifiers might evolve more rapidly together with specific developmental strategies. Therefore, evolutionary epigenetics and epigenomics will have an important role in discovering links between developmental adaptations and epigenetic regulators.

There is probably a conflict between the requirement for erasing epigenetic marks between generations and the requirement for maintaining others, such as those in imprinted genes and in some transposons. This conflict most probably underlies the observation that some epigenetic marks are not erased between generations, thereby leading to multigenerational influences on inheritance and phenotype (see page 396). Epigenetic inheritance across generations is relatively common in plants, but it is still unclear how widespread this phenomenon is in mammals or whether it has any role in shaping evolution[61].

An exciting question for future work is whether segregation of epigenetic marks in early development has any primary role in determining cell and lineage commitment. For example, the mechanism by which the first two cell lineages are allocated in mammalian pre-implantation embryos, although a matter of hot debate, is not really understood. An epigenetic hypothesis might allow us to take a fresh look at a long-standing fundamental problem in developmental biology. ∎

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