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Competing interests statement

The authors declare no competing financial interests.

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OPINION

Silence of the genes — mechanisms of long-term repression

Laura Lande-Diner and Howard Cedar

Abstract | A large fraction of genes in the mammalian genome is repressed in every cell throughout development. Here, we propose that this long-term silencing is carried out by distinct molecular mechanisms that operate in a global manner and, once established, can be maintained autonomously through DNA replication. Both individually and in combination these mechanisms bring about repression, mainly by lowering gene accessibility through closed chromatin structures.

In unicellular organisms, most of the genes in the genome are in a perpetual state of activity, with only a small number being specifically recognized as targets for repression. By contrast, repression is a dominant theme in the regulation of gene expression in animal cells, with more than 50% of the genome being silenced in any particular cell type. From a biological point of view, there seem to be two principal categories of long-term repression. On the one hand, genes that are specifically turned on during early embryogenesis are subsequently silenced and then remain in this state throughout development in almost all somatic cell types[1]. On the other hand, most tissue-specific genes are set up to be repressed at an early stage of development and they too are maintained in this form in almost all cell types, only undergoing reactivation in their tissues of expression.

Although much is known about the dynamic regulation of gene expression, there have been few efforts aimed at understanding long-term repression. The fact that gene silencing is highly stable throughout development and seems to work in a global manner indicates that the mechanisms that bring it about must be able to autonomously preserve the correct repression pattern even though the genome is copied at every cell division. Here, we propose that although repression itself is mediated mainly through effects on the local transcription machinery or chromatin structure, the maintenance of long-term silencing is carried out by three fundamental mechanisms that involve sequence-dependent repression factors, DNA methylation and late replication timing. These mechanisms operate either individually or in combination to generate layers of repression that not only ensure correct developmental expression patterns but might also lower transcriptional noise[2].

Layers of long-term silencing

Sequence-dependent repression. The simplest mechanism of gene silencing is mediated by

repression factors. In this strategy, the genes themselves are marked by specific regulatory sequences that can bind factors that can bring about repression (BOX 1). Although this is usually carried out by proteins, recent studies have shown that it can also be mediated by RNA sequences that recognize specific sites in the DNA[3]. Assuming that these protein or RNA effectors are constitutively expressed, this mechanism will maintain the specific silencing of individual genes, even after DNA replication. This simple mode of action can be said to operate as a 'genetic' mechanism, as the actual blueprint for repression is inscribed in the local DNA sequence itself.

Although gene-specific, this process can also operate in a global manner, as some repressors recognize docking sequences that are present in many gene promoters. One example of this is the neural restrictive silencer element (NRSE) motif that functions as a mark for many neuron-specific genes[4]. This sequence is recognized by the RE1-silencing transcription factor (REST), which is constitutively expressed in all cell types except neurons[5,6]. In this way, widely dispersed functionally similar genes are stably inactivated in a coordinated manner. In a similar way, Polycomb proteins function as long-term repressors for embryonically expressed genes that are turned off during later development[7].

DNA methylation. In animal cells, DNA methylation at CpG residues functions as an important mechanism for maintaining gene repression during development. Genes controlled by promoters that contain methyl cytosines are transcribed at much lower levels than unmethylated genes[2]. This inhibition is carried out by several molecular strategies that can either affect the transcription machinery itself or operate by altering the local chromatin structure (BOX 2).

DNA methylation is organized into a bimodal pattern in the animal genome[8]. In every cell, almost all the DNA is methylated; CpG ISLANDS, located mainly in the promoters of housekeeping genes, are an exception.

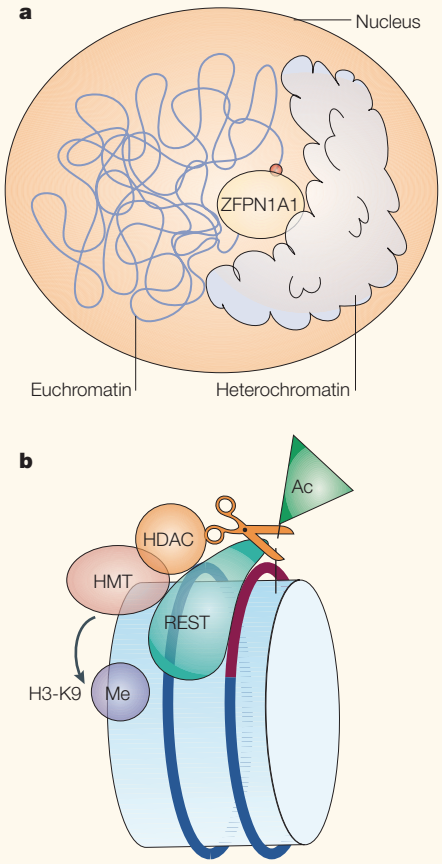
The profile of DNA methylation is re-established in each individual through a simple two-step process. First, almost all DNA methylation is erased from the genome in the early MORULA[8,9], followed by a general wave of *de novo* methylation at about the time of implantation[8,10]. Although this methylation occurs globally, CpG islands seem to be protected by specific *cis*-acting sequences that are inherent to the island regions[11–14], and in this way, the basic pattern of bimodal methylation is restored. Once established, this pattern is maintained through replication by a semi-conservative mechanism for copying the methylation residues from parental strands onto the newly synthesized nascent DNA[15].

In this system, tissue-specific genes that lack CpG-island promoters are automatically methylated, which brings about global repression that is maintained without the need to recognize specific regulatory elements. In parallel, this mechanism also ensures the constitutive expression of housekeeping genes. In contrast to factor-mediated repression, which requires local sequence information, DNA-methylation-mediated repression is based on epigenetic signals that modify the DNA.

DNA-replication timing. The entire genome is divided into large replication time zones, each of which is programmed to replicate in a particular window of time during S phase and it is this division that gives the chromosome its distinctive banding pattern[16,17]. There is a strong correlation between late replication and gene repression at the level of individual genes[18]. Housekeeping genes seem to be arranged in clusters[19,20] that replicate early, whereas many tissue-specific genes comprise miniature bands that replicate late in most tissues, but become early-replicating in cells in which they are expressed[21]. These regional replication-timing patterns are controlled by long-range *cis*-acting sequences[22,23] that can instruct several replication origins to initiate in a coordinated manner[24] at a particular time during S phase. Although little is known about the mechanism that underlies this process, it is clear that it must involve *trans*-acting factors that can be regulated during development.

Although the cause-and-effect relationship between replication and expression has not yet been established, recent evidence indicates that late replication timing has a role in maintaining repression. This is based on the idea that chromatin structure is repeatedly disrupted at each round of DNA

Box 1 | **Sequence-dependent repression**



One of the simplest mechanisms for maintaining developmentally regulated gene repression involves protein factors that recognize and bind to local *cis*-acting sequences. These repressors can operate in several ways. Some, such as the TATA-box binding protein (TBP)[84], work by directly inhibiting key components of the transcription machinery[53]. Others, such as the lymphoid-specific zinc-finger protein, subfamily 1A, 1 (ZFPN1A1; also known as IKAROS), bring about the relocation of a gene region from its euchromatic position within the nucleus to specialized regions of heterochromatin. ZFPN1A1 (see part a in the figure) is thought to bind to specific recognition signals near its target genes (red), and in this way function as a 'flag' for directing their recruitment to heterochromatin where they are exposed to a repressive environment[85,86].

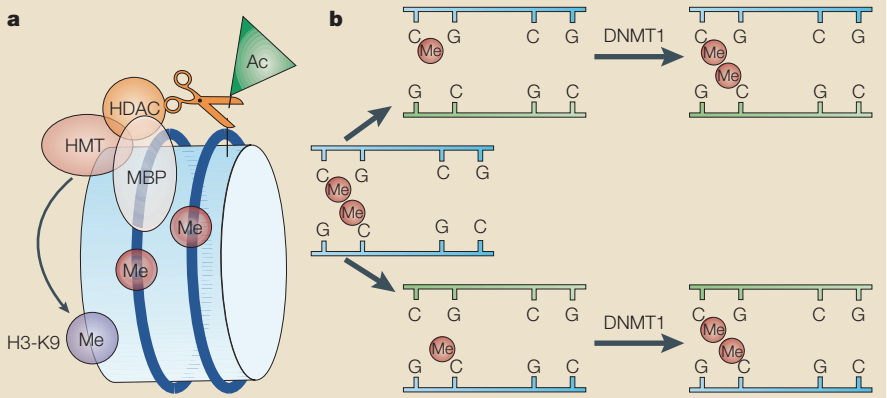
Repression factors (such as RE1-silencing transcription factor; REST) that recognize specific sequence elements might also influence the local nucleosome structure (see part b in the figure). They achieve this either by recruiting enzymes that deacetylate[29,87] (histone deacetylases; HDACs) or methylate[39] (histone methyl transferases; HMTs) specific residues in histones H3 and H4 by causing the nucleosomes to slide into positions that are unfavourable for the initiation of transcription[52], or by bringing about nucleosome compaction[88]. Indeed, in the immune system, elements within the recombination signal sequences guide the nucleosomes themselves to positions that inhibit gene rearrangement[89]. Ac, acetylation; H3-K9, lysine 9 on histone 3; Me, methylation.

Box 2 | **DNA methylation**

DNA methylation of promoters inhibits transcription by several molecular strategies. One simple model is that DNA methylation of specific promoter sites blocks the binding of factors that are necessary for transcription[54]. A good example of this is the abnormal methylation that occurs on the fragile X mental retardation 1 (*FMR1*) promoter that prevents binding of nuclear respiratory factor (NRF1; also known as α-PAL) (REF. 90) and inhibits transcription of this gene[91–93], causing fragile X syndrome[94].

More commonly, methylated CpGs are recognized by methyl-binding domain proteins (MBP)[95] that can recruit histone-modification enzymes such as histone deacetylase (HDAC) and histone methyl transferase (HMT)[38], and in this way bring about histone deacetylation and H3-K9 (lysine 9 on histone 3) methylation that alters the local chromatin environment[96] (see part **a** in the figure). Methyl groups might also have a ubiquitous role in the positioning of nucleosomes[97]. As well as these more local effects, DNA methylation brings about a general decrease in DNaseI sensitivity and alterations in higher order structure[56] that make these regions less accessible to the transcription machinery.

All these effects can be propagated through cell division because of the unique way of maintaining DNA-methylation patterns in the genome. In higher eukaryotes, methyl moieties are always located on cytosines within palindromic sequences (CNG, CG)[98], and in animal cells, methylation is restricted exclusively to CpG residues[99]. Because these sites have identical dinucleotide sequences on both strands (blue), they are always methylated symmetrically. During replication (see part **b** in the figure), the newly made DNA strand (green) is generated using unmodified cytosine. DNA methyltransferase (cytosine 5) 1 (DNMT1) then progressively modifies only those sites that already have a methyl moiety on the parental DNA strand[15]. In this way, the pattern of methylation is faithfully copied to the daughter cells, where it then contributes to reconstructing the chromatin structure[55] and factor composition at the promoter. Ac, acetylation; Me, methylation.



synthesis, and must be reconstructed in the wake of the replication fork[25,26]. Regions that replicate early during S phase would be exposed to a nuclear environment that is conducive to reassembly into open chromatin. By contrast, packaging during late S phase results in a closed chromatin conformation[27] (BOX 3). Because the genomic replication-timing pattern itself is extremely stable and seems to be autonomously preserved through cell division, it provides a cycling, time-based mechanism for global maintenance of repressed chromatin structure. It should be noted that in this case, the basis for the maintenance of repression is not 'engraved' in either the local DNA sequence or its modification pattern, but seems to depend on distant genetic and epigenetic signals that drive the replication-timing programme.

Histone modification

Histone modification is one of the main ways of mediating gene repression through chromatin structure. Acetylation of lysine residues on histones H3 or H4 induces a more open DNA–protein structure, whereas methylation of specific lysines (for example, H3-K9 or H3-K27) can bring about an opposite effect[28]. Altering histone-modification patterns is a common strategy of all three basic repression mechanisms. For example, many repression factors have been shown to operate by recruiting histone deacetylases that alter the local chromatin structure[29]. In a similar manner, DNA methylation functions as a binding cue for factors that recruit deacetylases[30,31], and late replication timing is also associated with the repackaging of DNA using nucleosomes that specifically contain deacetylated histones[27].

Experiments in animal cells indicate that although transcription of many genes is activated by treatment with the histone-deacetylase inhibitor, TSA, transcription returns to its repressed state once the drug is removed[32]. This indicates that the histone-acetylation state is determined by underlying basic mechanisms and is not simply copied autonomously following replication. One exception might be H3-K9 methylation, which is characteristic of heterochromatin[33]. This type of methylation can be carried out by one of several methyl transferases[34,35]. Once modified, lysine 9 functions as a docking site for heterochromatin protein 1 (HP1) (REFS 36,37); it is perhaps this marking mechanism that gives the DNA its heterochromatin-like properties. Interestingly, HP1 can recruit methyl transferases[37]. On this basis it has been proposed that, by analogy to chromatin spreading[29], nascent nucleosomes that contain trimethylated H3-K9 can bring about methylation of H3 on newly assembled nucleosomes at the replication fork, and in this way produce an 'epi-copy' of the original histone-modification pattern. Although attractive, this putative mechanism has never been rigorously tested. It is equally likely that continued methylation at H3-K9 is propagated by underlying mechanisms such as those caused by DNA methyl-binding proteins[38] or sequence-specific *trans*-acting factors that themselves recruit the histone-methylation enzymes[39].

Layers of repression

All three basic repression mechanisms are probably controlled independently of each other. For example, the presence of DNA methylation does not seem to influence local replication-timing patterns[40], and neither of these parameters has any substantial effect on repression by sequence-dependent factor binding. At any particular gene region, repression by sequence-dependent factors might be mediated by a single mode of action or a combination of several effectors. To use a previous example, the repression of neuron-specific genes by REST is mediated through the NRSE sequence motif. Because these consensus sequences[5,6] are normally located in CpG-island promoters (L.L.-D., unpublished observations), most of these target genes are unmethylated[41,42] and probably replicate early during S phase[32]. It therefore seems that these genes might be subject to repression exclusively by a factor-mediated process. In support of this idea, it has been shown that interference with the ability of REST to bind its target motifs is, in itself, sufficient to reactivate these genes[5]. To use another example, microarray expression

analysis on cells that lack DNA methyltransferase (cytosine 5) 1 (DNMT1), the maintenance methylase, reveals that expression of a considerable number of repressed genes can be induced simply by removing local DNA methylation[43]. These genes therefore seem to be maintained in their repressed state almost exclusively by DNA methylation.

In contrast to these examples of single-mode regulation, many well-known tissue-specific genes have methylated promoter sequences and lie in late-replicating regions[32], which indicates that these genes might be regulated by at least two independent mechanisms.

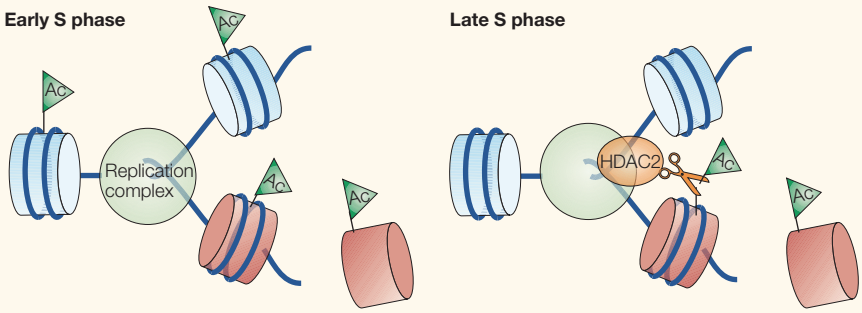
Embryonically expressed POU domain, class 5, transcription factor 1 (*Pou5f1*; also known as *Oct4*) provides another example of multiple-mechanism repression. It is inactivated after implantation[1] and then maintained in this state both by DNA methylation at its promoter[44] and the binding of a Polycomb-repression complex (N. Feldman, personal communication). Both X-chromosome inactivation[45] and heterochromatin silencing[46] are also characterized by the use of several basic mechanisms that include DNA methylation and late-replication timing[47]. In comparison with genes that are subject to a single layer of inhibition, multi-layer repression genes such as β-globin, pepck and α-crystallin[43] seem to be expressed at extremely low levels, as detected by microarray analysis, and are not readily reactivated by demethylation (see the supplementary information in REF. 43) or by treatment with TSA[32]. This is in keeping with the idea that these independent layers of repression work together. Consequently, the removal of a single mechanism alone is not sufficient for reactivation (FIG. 1).

Repression in animal cells can be highly efficient. In some cases, the difference between the high tissue-specific levels of expression and the minimal transcription observed in the non-expressing phenotype can be many orders of magnitude[48]. Considering that factor-mediated repression, as seen in bacteria, can only generate 100–1,000-fold differences in expression, it is likely that repression of many genes in animal cells is carried out by multiple mechanisms. This is presumably accomplished at two levels. Each basic mechanism can mediate repression by several independent pathways. For example, repression factors can 'recruit' genes to heterochromatin[49], alter histone modification[50] or nucleosome positioning[51,52], but might also work by directly inhibiting the transcription machinery[53]. By the same rationale, DNA methylation can prevent *trans*-acting factor binding[54], alter histone-modification patterns[55] and cause key changes in chromatin structure[56]. As well as these multiple effects, each gene might be subject to repression by two or more basic mechanisms. Taken together, this might help to explain the strong levels of repression that are observed in animal cells. As only a small fraction of the genome is transcribed in any given cell, these mechanisms might reduce what could represent a large amount of background noise[2].

Although some degree of repression might be brought about by factors that prevent the initiation of RNA polymerase, the principal cause of silencing seems to be driven by nuclear structural components that ultimately limit the accessibility of transcription factors to the gene locus, thereby preventing its transcription. This insight into gene structure was originally demonstrated in simple experiments showing that large molecular probes such as RNA polymerase[57,58] or DNaseI[59] have limited access to the genome as a whole, and preferentially choose as substrates only those genes that are expressed in that particular cell type[60]. Perhaps the most elegant proof of this idea comes from studies showing that when the recombination activating genes *RAG1* and *RAG2* are inserted into a B-cell nucleus, their products can cleave recombination signal sequences in the immune-receptor loci. By contrast, when introduced into non-lymphoid cells, the RAG proteins have no access to these loci and rearrangement is reduced by many orders of magnitude[61]. This *in vivo* experiment clearly highlights the importance of chromatin structure in gene accessibility.

X-chromosome inactivation in female mammals represents a good example of multi-layer repression, as it involves chromosome-specific binding of the X-specific inactive transcript (*Xist*) RNA, DNA methylation and late-replication timing, all of which are used to maintain a closed heterochromatin-type structure. Once silencing is established randomly in the early embryo, the choice of which allele — the paternal or maternal — undergoes inactivation is clonally preserved through all cell divisions. This implies that the X-chromosome repression pattern is not constantly generated in each cell, but rather is maintained during development. In this case, it is clear that multiple mechanisms are required to ensure complete gene inactivation[45]. In mammalian cells, the removal

Box 3 | **Replication timing**



There is a strong correlation between late-replication timing and gene repression, although the cause-and-effect relationship between them is not yet clear. Recent findings indicate that the time of replication might have a role in fashioning chromatin structure after each round of DNA synthesis[22]. According to this scheme, replication timing itself is set up in a regional manner through interactions between *trans*-acting factors and long-range *cis*-acting sequences that control origin firing.

Because replication involves opening the DNA helix, the existing nucleosome structure becomes disrupted during this process and must therefore be regenerated after the polymerase machinery has passed. Microinjection experiments show that replicating DNA is packaged into nucleosomes that contain acetylated histones during early S phase, but it is assembled with deacetylated histones during late S phase[27] (see the figure). In this way, replication-timing control provides a time-based system for maintaining nucleosome structure and gene-repression patterns. One factor that might have a role in this differential packaging is the histone deacetylase HDAC2, which is an integral part of the replication complex, but only during late S phase[100]. During early S phase, newly added nucleosomes would carry pre-acetylated histone H4 (REF.100), but these acetyl groups would be removed from DNA that replicates during late S phase.

As well as its effect on nucleosome modification, late-replication timing is correlated with a closed, DNaseI-resistant chromatin structure[101], and might also influence nuclear localization by recruiting gene sequences to heterochromatin[78]. Ac, acetylation.

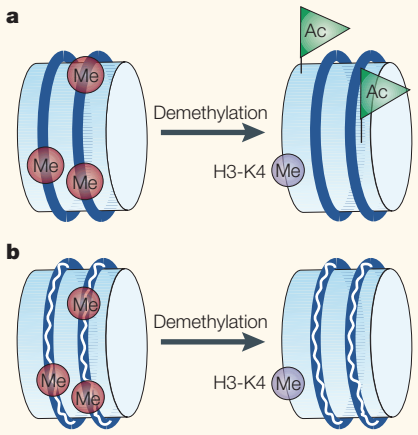


Figure 1 | **Several repression mechanisms.** The model shows the nucleosome-level structure of genes that are subject to DNA methylation (red) either as a single layer of repression (**a**) or in conjunction with another repression mechanism (**b**). **a** | The presence of DNA methylation brings about histone deacetylation and prevents methylation of H3-K4 (lysine 4 on histone 3). When DNA methylation is removed, H3-K4 becomes methylated (purple), and because there is no longer any underlying repression mechanism, local histones undergo acetylation. **b** | A gene can be repressed both by DNA methylation and a second mechanism, such as late-replication timing (indicated by a white wavy line on the DNA). Removal of DNA methylation from this gene region does not result in a change in acetylation because another mechanism that brings about histone deacetylation is still present. It is likely that histone H3 still undergoes methylation at K4, as this modification might be affected primarily by DNA methylation regardless of gene activity. Ac, acetylation; Me, methylation.

of methylation has little effect on repression[62], presumably because there are further mechanisms that can maintain inactivation even in the absence of DNA modification. Furthermore, X-chromosome inactivation seems to be stable over time and does not allow significant expression of genes even in long-lived somatic cells[63]. By contrast, X-chromosome inactivation in marsupials does not involve DNA methylation, and consequently many genes become reactivated as a function of age[64].

Relieving repression

Molecular studies of gene repression indicate that many tissue-specific genes are packaged in a hierarchical manner[65] into higher order chromatin structures that prevent access to the transcription machinery. As a consequence, multiple layers of repression must be peeled off, probably in a step-wise manner, to activate these genes during cell-type-specific differentiation.

The gene that encodes the κ-light chain is carefully regulated to undergo rearrangement at a particular stage of B-cell development[66]. Epigenetic studies now show that this gene locus becomes accessible to the recombination machinery through a programmed series of carefully regulated molecular events[67,68]. In the earliest stages of B-cell development, this locus is packaged in a closed chromatin conformation, with the DNA being methylated and late-replicating. It is also positioned on the periphery of the nucleus[69], a site that is known to be associated with heterochromatin.

During the pro-B-cell stage, the locus becomes relocated to the centre of the nucleus, and undergoes increased histone acetylation that might signal a slight opening of the chromatin as reflected by the onset of germline transcription[70]. As development progresses into the pre-B-cell stage, there is a marked shift in replication timing towards early S phase[71], which is accompanied by a further increase in histone acetylation. Finally, the κ-locus probably undergoes demethylation[72] and nucleosome remodelling at the recombination signal sequences[73]. It is these later events that finally allow rearrangement[74,75]. Studies in mice in which the κ-locus has been targeted and in transfected cells show that neither forced demethylation nor histone acetylation are sufficient by themselves to activate recombination[76,77], indicating that rearrangement can only occur if all the layers of repression are removed.

A similar series of events seems to be associated with activation of the human β-globin locus in erythroid cells. Here too, differentiation is associated with an early change in nuclear localization[78,79], a shift to early replication of the entire domain[23,80], an increase in DNaseI sensitivity[81,82] and finally, local demethylation that is specific for individual genes at this locus[83]. In the case of the globin genes, histone acetylation seems to occur after demethylation (A. Goren *et al.*, personal communication) and it is this event that 'opens' the correct gene within the β-globin locus for full transcription.

Conclusions and future directions

In many multicellular organisms a large fraction of the genome is programmed to be repressed. This silencing pattern seems to be set up in a global manner in the embryo and is then stably maintained for a prolonged period of time through many cell divisions that take place during development. At each gene locus, repression is mediated both by factors that affect the transcription machinery and by a closed chromatin structure that limits accessibility. As these components are probably disrupted at the time of replication, there must be molecular mechanisms to restore gene-repression structures at each cell division and in this way maintain the epigenetic repression pattern.

We have suggested that there are at least three fundamental mechanisms for autonomously maintaining gene-repression structure: they are based on the local DNA sequence, DNA modification and replication timing. An important feature of this process is that these mechanisms can be used individually or in combination to generate different levels of gene repression.

Very little is known about gene repression in the early embryo. It seems that each embryo initially inherits the DNA-methylation pattern from the gametes, but this pattern is then almost completely erased in the early morula. A new methylation pattern is then established around the time of implantation. Although the full biological significance of these events is not clear, it seems that this erasure has a crucial part in reprogramming somatic nuclei during animal cloning. It is likely that during development, the erasure of DNA methylation functions to reactivate genes that underwent repression during the normal process of gametogenesis, and in this way brings about a resetting of the epigenome before GASTRULATION.

In light of the model we have presented, one might expect that the pattern of expression for many genes in the blastula is affected by the lack of DNA methylation. It would therefore be interesting to analyse the chromatin structure at this early stage of embryogenesis where one of the fundamental maintenance mechanisms is apparently missing. These cells might mimic the epigenetic programme characteristic of most other eukaryotic organisms that completely lack DNA methylation.

Many studies have focused on sequence-dependent repression and DNA methylation,

Glossary

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

GASTRULATION
A morphogenetic process in vertebrate embryogenesis during which the endoderm, mesoderm and ectoderm germ layers are formed.

MORULA
A pre-implantation embryo that consists of a solid cluster of cells.

but little is known about the effects of replication timing on chromatin structure and gene repression. This is an especially fascinating mechanism that seems to control regional expression. Indeed, replication time zones define the chromosome banding pattern itself. Deciphering how replication timing is set up and how it influences chromatin structure should therefore help us to understand this basic subunit structure of the genome.

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Competing interests statement

The authors declare no competing financial interests.

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