⊚ **MODES OF TRANSCRIPTIONAL REGULATION**

Silencing chromatin: comparing modes and mechanisms

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Abstract | Recent transcriptome analyses show that substantial proportions of eukaryotic genomes can be copied into RNAs, many of which do not encode protein sequences. However, cells have developed mechanisms to control and counteract the high transcriptional activity of RNA polymerases in order to achieve cell-specific gene activity or to prevent the expression of deleterious sequences. Here we compare how two silencing modes — the Polycomb system and heterochromatin — are targeted, established and maintained at different chromosomal locations and how DNA-binding proteins and non-coding RNAs connect these epigenetically stable and heritable structures to the sequence information of the DNA.

Heterochromatin
The portion of the genome that stays highly condensed throughout the cell cycle. Compared with euchromatin, it replicates late in S phase and is relatively gene-poor. Molecularly, heterochromatin is characterized by DNA methylation, histone hypoacetylation, methylation of histone H3 at lysine 9 and the presence of heterochromatin protein 1 (HP1).

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Genomes provide many opportunities to initiate transcription. Indeed, recent analyses indicate that substantial proportions of eukaryotic genomes are copied into RNAs, including many that do not encode proteins. However, it has long been known that cells have sophisticated mechanisms to keep RNA polymerase activity in check. The classical example of this is heterochromatin, a large, microscopically distinguishable section of the genome that is considered to have evolved its highly repressive structures to prevent the ectopic expression of, and thereby activation of, transposable elements and other deleterious sequences. As always, nature does not operate in black and white patterns and, fascinatingly, heterochromatic sequences can also become transcribed as part of the silencing system.

There must also be control of the expression of protein-coding genes. In particular, genes encoding regulatory factors, such as those that control patterning or the cell cycle, need to be kept strictly silent in cells in which, or at times at which, their presence would distort developmental decisions. As we discuss here, long-term maintenance of silencing is common to gene and heterochromatin silencing; in fact, components that shape chromatin silencing are typically not part of the initial transient decision process of transcriptional control, but are required for the heritable transmission of the choice through cell division.

Some 20 years ago, the discovery of a motif (the chromo domain) that is conserved between a heterochromatin-associated protein (heterochromatin protein 1 (HP1)) and a protein involved in maintaining homeotic genes silenced during development (Polycomb)

suggested that the processes of transcriptional repression that involve these proteins might have shared molecular grounds. Since then, we have learned that there are many shared features between the heterochromatin and Polycomb systems, such as the requirement to interact with specific histone modifications (methylation) to produce the stable and long-lasting silencing, or that histone methyl marks are set by methyltransferases that are intrinsic to the systems. Mutual roots of the two systems can be traced back to the ancient unicellular ciliate *Tetrahymena thermophila* (BOX 1).

Chromatin-based silencing has three main steps: a decision-making process that targets specific silencing complexes to the DNA sequences to be inactivated; a chromatin structuring process that results in efficient inhibition of RNA polymerases or other nuclear enzymes; and, most enigmatically, the epigenetic part, the propagation of the silent chromatin through DNA replication and mitosis to the daughter cells. In this Review, we introduce the molecular factors involved in the control of the three steps and discuss possible underlying molecular mechanisms. DNA sequence-specific binding proteins seem to have a major role in the first step, as may many non-coding RNAs (ncRNAs). The crosstalk of silencing complexes with nucleosomes is fundamental to the stable anchoring of repressive functions and could explain the heritability of silencing; ncRNAs might also be involved in this still mysterious epigenetic step.

Forms of chromatin-based silencing
The transcriptional activity of a gene is dependent on the local composition and organization of its chromatin

Box 1 | Evolutionary origins

The heterochromatin and Polycomb group (PcG) protein epigenetic silencing systems have a unified role in the ancient unicellular ciliate *Tetrahymena thermophila*. *T. thermophila* possesses a transcriptionally inactive germline micronucleus and a transcriptionally active somatic macronucleus. Macronuclei originate from micronuclei during the sexual phase of the life cycle (known as conjugation). This process is accompanied by major genome reorganizations in the macronucleus that lead, through heterochromatin formation, to the elimination of 15% of the micronuclear genome. Similarly to the formation of centromeric heterochromatin in fission yeast (*Schizosaccharomyces pombe*), the formation of heterochromatin in *T. thermophila* is dependent on the RNAi machinery. In contrast to fission yeast and higher eukaryotes, the heterochromatin in *T. thermophila* is marked by histone H3 lysine 27 trimethylation (H3K27me3), which is laid down by EZL1, the homologue of *Drosophila* Enhancer of zeste[106]. In order to initiate heterochromatin formation, short non-coding RNAs are involved in targeting EZL1 to genomic loci. EZL1 methylates H3K27. This is followed by H3K9 methylation (which may also be mediated by EZL1). Subsequently, Pdd1p — a protein containing two chromo domains — binds to both methylation marks, leading to heterochromatin formation and ensuing DNA elimination. Therefore, in *T. thermophila*, the PcG system is essential for heterochromatin formation, whereas during later evolutionary steps these epigenetic silencing systems seem to have separated and act independently.

Histone
A family of small, highly conserved basic proteins, found in the chromatin of all eukaryotic cells. The core histones (H2A, H2B, H3 and H4) associate with DNA to form a nucleosome. The histone proteins are subject to various chemical modifications, including acetylation, methylation and phosphorylation.

Constitutive heterochromatin
A subtype of heterochromatin that is present at the highly repetitive DNA sequences found at the centromeres and telomeres of chromosomes, where it hinders transposable elements from becoming activated and thereby ensures genome stability and integrity.

Facultative heterochromatin
A subtype of heterochromatin that is formed in the euchromatic environment, where heterochromatin proteins are used to stably repress the activity of certain target genes.
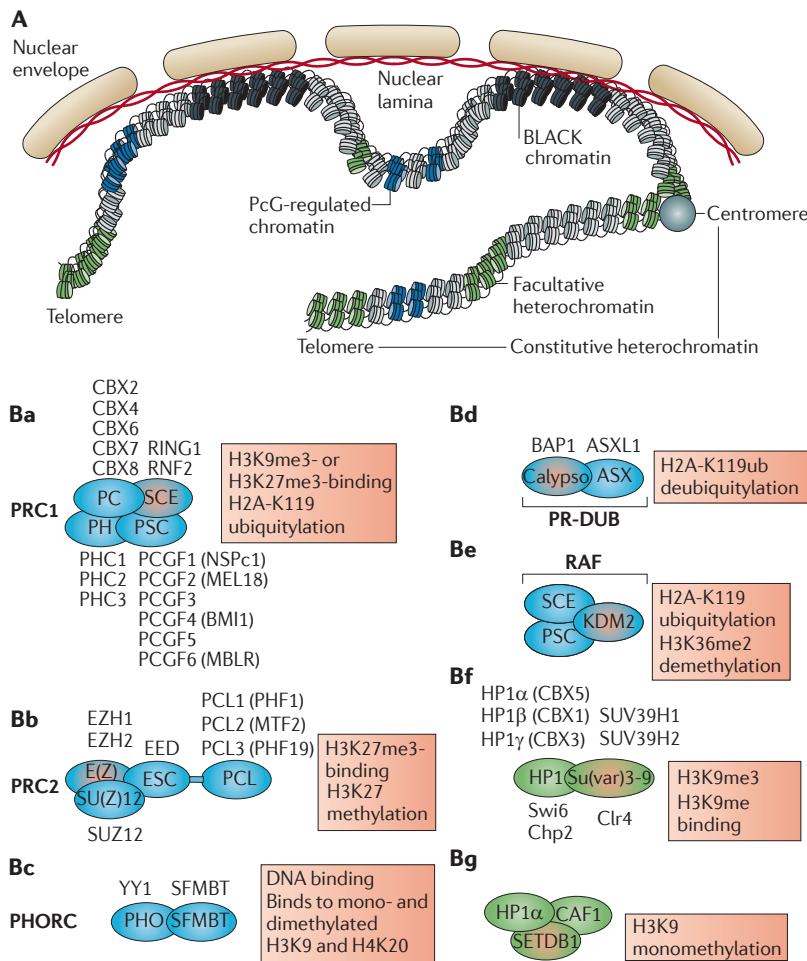
Polycomb group
(PcG). A class of proteins — originally described in *Drosophila melanogaster* — that maintain the stable and heritable repression of several genes, including the homeotic genes.

environment. Chromatin-based silencing mechanisms (FIG. 1A) form specialized higher-order chromatin structures to ensure well-timed and spatially restricted gene-expression patterns. Heterochromatin remains highly condensed throughout the cell cycle. Compared with euchromatin, it replicates late in S phase and is relatively gene-poor. Molecularly, heterochromatin is characterized by histone hypoacetylation, methylation of histone H3 at lysine 9 (H3K9me) and the presence of HP1 (the proteins involved are shown in FIG. 1B) (in this Review we use 'heterochromatin' to refer to HP1-associated repressive chromatin). Heterochromatin can be classified into two subtypes: constitutive heterochromatin and facultative heterochromatin. Constitutive heterochromatin is typically present at the highly repetitive DNA sequences found at chromosome centromeres and telomeres, where — besides its function in chromosome mechanics and structure — it represses transposable-element activity and thereby ensures genome stability and integrity. By contrast, facultative heterochromatin forms in the euchromatic environment at sites where heterochromatin proteins stably target genes. The best-studied example of facultative heterochromatin is the inactive X chromosome in the somatic cells of female mammals[1].

The stable and heritable maintenance of specific gene-expression patterns is important to achieve and sustain cell-lineage identity. The heterogeneous class of Polycomb group (PcG) proteins ensures the long-term controlled repression of specific target genes. PcG protein complexes are shown in FIG. 1B. PcG-mediated gene repression is characterized by H3K27me and seems to primarily control genes involved in developmental decisions. Silencing by facultative heterochromatin and the PcG proteins acts on unique gene sequences, which facilitates the search for DNA elements bound by these silencing complexes (discussed further below). Heterochromatin and PcG-mediated repression are tightly linked to DNA methylation (BOX 2).

Figure 1 | **Types of repressed chromatin and the protein complexes involved. A** | Schematic of types of repressed chromatin in the context of the nucleus. Constitutive and facultative heterochromatin (green) and Polycomb group (PcG) protein-regulated chromatin (blue) are discussed in the main text. Recently, another type of repressive chromatin — termed BLACK chromatin — has been identified in embryonic *Drosophila melanogaster* cells (its presence in other organisms has yet to be demonstrated)[118]. It covers 48% of the genome and forms large domains (many of them >100 kb) that harbour many developmentally regulated genes[118]. It is depleted of heterochromatin protein 1 (HP1) and PcG proteins, and is enriched in histone H1, the AT-Hook protein D1, Ipl1-aurora-like kinase (IAL), Suppressor of underreplication (SUUR), Effete (EFF) and Lamin (LAM)[118]. These proteins have been implicated in transcriptional repression, higher-order chromatin structure formation and replication control[119–122]. LAM is a component of the nuclear lamina (red), which might explain the localization of BLACK chromatin to the nuclear periphery. Previous work has also linked heterochromatin and PcG-repressed chromatin to the nuclear periphery (not shown here). **B** | PcG- and HP1-containing core complexes. Five PcG complexes (**a**–**e**) have been identified in *D. melanogaster*[77,78,123–126]; only the core components of the PcG are shown (except in RING-associated factors complex (RAF), where KDM2, which is not a *bona fide* PcG member, is included). *D. melanogaster* proteins are in the shapes; the human homologues are listed adjacent to these. Known biochemical activities and the enzymatically active proteins are indicated in red. Polycomb repressive complex 1 (PRC1) and PRC2 have been identified in mammals; Polycomb repressive deubiquitinase (PR-DUB) has been reconstituted with the human proteins[125]; Pleiohomeotic repressive complex (PHORC) and RAF have only been investigated in *D. melanogaster*[123,124]. Mammalian chromobox (CBX) protein homologues might mediate differential binding specificities of PRC1 complexes to histone H3 that is methylated at lysine 9 (K9) or K27 (REF. 64). Polycomb-like (PCL) seems to be required for high-level H3K27 trimethylation (H3K27me3) at target genes[127]. For an in-depth discussion on the biochemical activities of these complexes, see recent reviews[83,84]. HP1-containing protein complexes (**f**, **g**) are less clearly defined than PcG complexes. In **f**, *D. melanogaster* proteins are in the shapes, human homologues are above and *Schizosaccharomyces pombe* homologues are below. In *S. pombe*, Swi6 and Chp2 form complexes with different molecular functions[128]. The HP1α–CAF1–SETDB1 complex (**g**) has been found in humans. It monomethylates H3K9 in constitutive heterochromatin; SUV39 then catalyses trimethylation[128]. ASX, Additional sex combs; BAP1, BRCA1-associated protein 1; CAF1, chromatin assembly factor; Clr4, histone H3 methyltransferase; EED, embryonic ectoderm development; ESC, Extra sex combs; E(Z), Enhancer of zeste; EZH, Enhancer of zeste homologue; MTF2, Metal response element-binding transcription factor 2; PC, Polycomb; PCGF, Polycomb group ring finger; PH, Polyhomeotic; PHC, Polyhomeotic-like; PHF, PHD-finger protein; PHO, Pleiohomeotic; PSC, Posterior sex combs; RNF2, RING finger protein 2; SCE, Sex combs extra; SETDB1, SET domain, bifurcated 1; SFMBT, SCM-related gene containing four MBT domains; SU(Z), Supressor of zeste; YY1, yin-yang 1.

A

Nuclear envelope

Nuclear lamina

BLACK chromatin

PcG-regulated chromatin

Centromere

Telomere

Facultative heterochromatin

Telomere — Constitutive heterochromatin

Ba

CBX2
CBX4
CBX6
CBX7 RING1
CBX8 RNF2

PRC1

PC SCE
PH PSC

H3K9me3- or H3K27me3-binding H2A-K119 ubiquitylation

PHC1 PCGF1 (NSPc1)
PHC2 PCGF2 (MEL18)
PHC3 PCGF3
       PCGF4 (BMI1)
       PCGF5
       PCGF6 (MBLR)

Bb

EZH1
EZH2 EED
PCL1 (PHF1)
PCL2 (MTF2)
PCL3 (PHF19)

PRC2

E(Z) ESC PCL
SU(Z)12

SUZ12

H3K27me3-binding H3K27 methylation

Bc

YY1 SFMBT

PHORC

PHO SFMBT

DNA binding
Binds to mono- and dimethylated H3K9 and H4K20

Bd

BAP1 ASXL1

Calypso ASX

PR-DUB

H2A-K119ub deubiquitylation

Be

RAF

SCE KDM2
PSC

H2A-K119 ubiquitylation
H3K36me2 demethylation

Bf

HP1α (CBX5)
HP1β (CBX1) SUV39H1
HP1γ (CBX3) SUV39H2

HP1(Su(var)3-9

Swi6 Clr4
Chp2

H3K9me3
H3K9me binding

Bg

HP1α CAF1
SETDB1

H3K9 monomethylation

Targeting silencing to specific DNA sequences

PcG targeting in Drosophila melanogaster. In flies, the major PcG core-protein complexes, Polycomb repressive complex 1 (PRC1) and PRC2 (FIG. 1B), were found to bind defined elements, termed PcG response elements (PREs)[2–4]. These are switch-like *cis*-regulatory elements that can act as enhancers or silencers, depending on the transcriptional history of their associated gene. PREs are composite DNA elements characterized by a complex pattern of different motifs recognized by various sequence-specific DNA-binding proteins. Factors such as GAGA factor (GAF), Zeste, Dorsal switch protein 1 (DSP1), Pipsqueak, Grainyhead and Specificity factor 1 (SP1; also known as KLF) have been implicated in PcG protein targeting[5–12] (FIG. 2). However, all of these also participate in processes other than PcG silencing, and are involved in general gene repression and activation. It is still unclear which part of their range of activities is involved in targeting PREs. For more detailed discussion of PREs and DNA-binding proteins in *D. melanogaster*, see recent reviews[3,4,13].

Sequences involved in mammalian PcG targeting. Promising advances have recently been made towards the identification of DNA elements required for the targeting of PcG proteins to specific genes in mammalian genomes. Several chromatin-profiling studies have mapped PcG protein distributions genome-wide[14–19]. Interestingly, a large proportion of binding sites coincide with promoter regions of target genes. However, computational analyses of the target DNA sequences have failed to reveal distinguishing motifs that could be involved in the global targeting of PcG proteins. However, binding of the PRC2 PcG-silencing complex in embryonic stem (ES) cells is entirely restricted to sequences with high CpG content[17]. This raises the possibility that proteins that have a zinc finger CXXC (zf-CXXC) domain with high affinity for unmethylated CpGs are involved in the process. Such specific targeting factors for PcG proteins have yet to be identified. However, it is interesting that mixed lineage leukaemia 1 (MLL1) — a homologue of *D. melanogaster* Trithorax (TRX) and a PcG antagonist — contains a zf-CXXC domain, so it is conceivable that PcG-counteracting activities might be recruited through the same DNA sequence determinants[20].

Two recent studies report the identification of specific mammalian PREs[21,22]. Sing *et al.* identified a 3-kb element termed PRE-kr that regulates the expression of the mouse *MafB* gene[21]. Endogenous PRE-kr is bound by the PRC1 and PRC2 subunits BMI1 and SUZ12, respectively, and regulates reporter gene expression in a PcG-dependent manner. Protein binding occurs in a 450-bp segment of PRE-kr that is highly conserved between humans, mice and chickens. This minimal PRE, called hcPRE, contains GAGA-binding and double palindromic yin-yang 1 (YY1)-binding motifs, reflecting striking similarities with *D. melanogaster* PREs (YY1 is the homologue of the *D. melanogaster* PcG protein Pleiohomeotic (PHO) and GAGA motifs are bound by *D. melanogaster* GAF; a potential mammalian homologue of GAF has recently been identified[5,12,23]). Ectopically inserted hcPRE seems to have a preference for PRC1, because it recruits BMI1 but not SUZ12. PRC2 may require additional sequence motifs outside of hcPRE and be targeted independently of PRC1.

The second mammalian PRE that has been identified is a 1.8-kb region between the human homeotic genes *HOXD11* and *HOXD12*, termed D11.12 (REF. 22). It contains a highly conserved 237-bp region and a cluster of four YY1-binding sites that are essential for the repressive activity of D11.12. Repression by D11.12 is dependent on PcG proteins and is maintained through cell differentiation — a hallmark of PRE function in flies. This functional feature — yet to be demonstrated for PRE-kr — makes a convincing case that D11.12 is a mammalian PRE.

Sequence-specific DNA-targeting factors that nucleate PcG silencing in mammals. As indicated above, factors similar to those used in *D. melanogaster* for PcG targeting have also been highlighted by recent mammalian studies, for example: YY1, adipocyte enhancer-binding protein 2 (AEBP2, which is a core member of PRC2) and Krüppel-related zinc-finger protein/T-helper-inducing POZ/Krüppel-like factor (cKrox/Th-POK, which is the vertebrate orthologue of *Drosophila* GAF)[12,23,24]. In addition, there seem to be a number

Box 2 | DNA methylation

Repetitive sequences   Exon 1   Exon 2
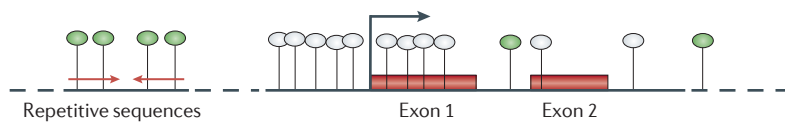
The best-characterized epigenetic system is DNA methylation at CpG dinucleotides, which is commonly associated with gene silencing[107–109]. During replication, DNA methyltransferase 1 (DNMT1) maintains the DNA methylation pattern of the newly synthesized daughter strands. The repressive activity of methylated DNA is mediated by methyl-CpG-binding domain proteins (MBDs), which recruit further chromatin modifiers such as histone deacetylases or methyltransferases[110]. In addition, transcriptional activators containing zinc-finger CXXC domains can be selectively targeted to unmethylated CpGs[20,111].

The majority of CpG dinucleotides throughout mammalian genomes are methylated, with more than 90% of all methylated cytosines residing within repetitive elements of constitutive heterochromatin regions (see the figure, green circles). In this way, DNA methylation has a major role in maintaining genome stability by preventing the reactivation of transposable elements[112].

CpG sites are not randomly distributed in the genome. Instead, there are CpG-rich regions known as CpG islands (centre of figure), which span the 5′ regions of 60% of all genes[113]. CpG sites in CpG islands are usually unmethylated (see the figure, open circles), which correlates with their potential for active gene transcription. Nevertheless, promoters can be de novo methylated and thereby repressed. For example, in embryonic stem cells CpG-rich promoters are mostly free of methylation, but after cell-fate commitment hundreds of promoters involved in regulating pluripotency and controlling other cell fates become methylated and silenced[114]. Interestingly, promoters with histone H3 lysine 27 methylation, which is associated with Polycomb group (PcG) proteins, are more frequently de novo methylated than other promoters[114]. Furthermore, DNA methyltransferases have been found to interact with Polycomb repressive complex 2 components and to be recruited to sites of PcG-mediated repression in cancer cells[115]. Intriguingly, PcG target genes undergo aberrant DNA methylation in human cancers, which suggests that the PcG-repressed state that is established during development may predispose these genes to later de novo methylation[116,117].

In summary, DNA methylation is intricately interconnected with heterochromatin and PcG-mediated silencing and is involved in genome stability, developmental gene regulation, genomic imprinting and X-chromosome inactivation.

Genomic imprinting
The epigenetic marking of a gene on the basis of parental origin, which results in monoallelic expression.
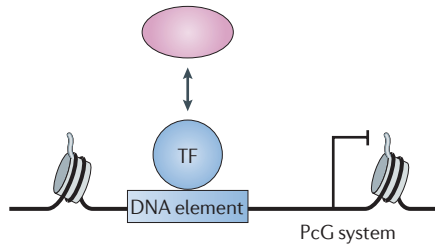
Poised promoter state
A promoter bound by the transcription-initiating form of RNA polymerase II while the gene is not being actively transcribed. Transcriptionally poised genes are suggested to be rapidly upregulated in, for example, developmental processes.

Mating type locus
A well-studied chromosome region that forms a model for epigenetic gene silencing in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The mating type locus controls the sexual identities of both haploid and diploid cells.

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of context-specific DNA-binding proteins that target subsets of genes in specific tissues (FIG. 2).

Another factor was recently discovered independently by five laboratories that were reanalysing the composition of PRC2 with the aim of finding new interaction partners[25–29]. Using mammalian cells, all five groups identified JARID2, a protein previously recognized as being required for early embryonic development and a target of the pluripotency transcription regulatory network[30–32]. JARID2 colocalizes with PRC2 components to most PcG target genes in ES cells[25–29]. The AT-rich interactive domain (ARID) of JARID2 binds DNA and is required for recruitment of PRC2 (REFS 29,33). Surprisingly, the effect of JARID2 depletion on global H3K27 methylation is mild, supporting the idea that a combinatorial and potentially redundant set of factors, not just a single protein, targets PcG proteins to repressed genes. In contrast to the observed ectopic activation of PcG target genes in the absence of other PRC1 or PRC2 components, the withdrawal of JARID2 leads to a decrease in the expression of these genes to below the basal level detected in wild-type ES cells[14,16,27].

A possible explanation for this divergent behaviour has been contributed by Landeira et al.[28]; they demonstrated that JARID2 is required to establish a poised promoter state at PcG targets in ES cells. This state is defined by the presence of RNA polymerase II (Pol II) phosphorylated at serine 5 and PRC1 and/or PRC2. If JARID2 is withdrawn, S5-phosphorylated RNA Pol II is missing from these promoters. As a consequence, transcriptionally poised bivalent genes are not upregulated in a timely way and differentiation fails. Because JARID2 is apparently an integral part of the PRC2-silencing complex, it is surprising that it seems to be required for the recruitment of a component of the transcription apparatus, S5-phosphorylated RNA Pol II.

Sequence-specific DNA-targeting factors that nucleate heterochromatin. In fission yeast (*Schizosaccharomyces pombe*), constitutive heterochromatin is formed at the telomeres and centromeres, and also at the mating type loci (mat loci)[34]. The RNAi machinery has been recognized as a major driver of the targeting and maintenance of heterochromatin at these loci (discussed below)[35]. In budding yeast (*Saccharomyces cerevisiae*), no clear homologues of the well-defined RNAi machinery of *S. pombe* have been identified and the assembly of heterochromatin-like structures at telomeres relies on the targeting function of DNA-binding proteins (for a review, see REF. 36). Similarly, in *S. pombe*, in addition to the RNAi pathway, distinct DNA-binding factors can bring heterochromatin proteins to specific sites. At the mat loci, the transcription factors Atf1 and Pcr1, as well as the telomere-binding proteins Taz1 and Ccq1, can nucleate heterochromatin formation by recruitment of the histone deacetylase Clr3 (REFS 36–39). This subsequently attracts the Su(var)3-9 homologue Clr4 and Swi6, leading to establishment and spreading of heterochromatic structures.

Site-specific deposition of HP1, which is mediated by transcription factors, also establishes microenvironments of heterochromatin for the repression of gene transcription[40]. The RNAi machinery seems not to be involved in this type of repression. HP1 is crucial for the transcriptional regulation of Krüppel-associated box domain zinc-finger proteins (KRAB-ZFPs), which constitute the largest group of transcriptional regulators encoded by the genomes of higher organisms, with more than 350 members in mice and humans[40,41]. This class of genes is transcriptionally auto-regulated by specific KRAB-ZFPs that bind to the 3′ end of KRAB-ZFP genes that are to be repressed[42]. They recruit KAP1 (KRAB-associated protein 1), which acts as a scaffold for various heterochromatin-inducing factors, such as HP1 and the H3K9-specific histone methyltransferase SETDB1 (REFS 42–44). Recruitment of KAP1 leads to the spread of heterochromatin, with binding of HP1 and H3K9me3 through the gene bodies, thus mediating long-range repression of the promoters (which can be up to several tens of kilobases upstream of the 3′-end nucleation site)[43]. The extended family of KRAB-ZFP genes originates from iterative gene-duplication events, so there are large sequence homologies among the genes,

a DNA-binding factors

Heterochromatin	PcG system
General targeting factors Atf1 (Sp) Pcr1 (Sp) Taz1 (Sp) Ccq1 (Sp)	**General targeting factors** GAF (Dm), GAF (Dr), cKrox/Th-POK (Mm, Hs) Zeste (Dm) DSP1 (Dm) Pipsqueak (Dm) Grainyhead (Dm) SP1 (Dm) PHO/PHOL (Dm), YY1 (Mm, Hs) AEBP2 (Mm, Hs) JARID2/Jumonji (Mm, Hs) OCT4 (Mm,Hs)
Gene-specific targeting factors RB (Mm, Hs) KRAB-ZNF (Mm, Hs)	**Gene-specific targeting factors** E2F6 (Mm, Hs) BCL-6 (Mm, Hs) SNAIL1 (Mm, Hs) PML-RARα (Hs) PLZF-RARα (Hs)
DNA elements REIII/mat locus (Sp) Telomeric repeats (Sp)	**DNA elements** PREs (Dm) CpG islands (Mm, Hs) PRE-kr (Mm) D11.12 (Mm)

b Histone modifications
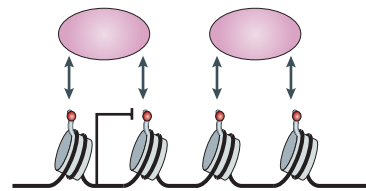
Heterochromatin	PcG system
Modification H3K9me	**Modification** H3K27me
Reader HP1 (Dm, Mm, Hs), Swi6 (Sp)	**Reader** Polycomb (Dm), CBX2/7 (Mm, Hs) ESC (Dm), EED (Mm, Hs)

Writer
Clr4 (Sp), SU(VAR)3-9 (Dm),
  SUV39H1 (Mm,Hs)
Eggless (Dm), SETDB1 (Mm,Hs)
	Writer
	E(Z) (Dm), EZH1 (Mm, Hs),
	  EZH2 (Mm, Hs)

c Non-coding RNA
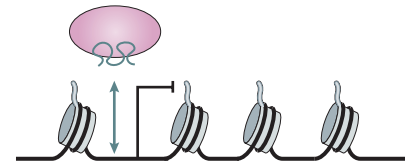
Heterochromatin	PcG system
RNAi (Sp, At, Ce, Dm)	*HOTAIR* (Mm, Hs) *Xist-RepA* (Mm, Hs) *Kcnqlot1* (Mm) *ANRIL* (Mm, Hs) Promoter proximal short ncRNAs (Mm, Hs)

Figure 2 | **Targeting mechanisms for heterochromatin and Polycomb group proteins.** In principle, the heterochromatin and Polycomb group (PcG) protein silencing systems deploy similar factors and mechanisms for recruitment and anchoring to target genes: DNA-binding proteins (transcription factors (TFs)) and their corresponding DNA elements (**a**); histone modifications (**b**); and non-coding RNAs (ncRNAs) (**c**). A schematic of each process is shown (the silent gene is represented by a T bar; proteins are shown as coloured ovals). Specific factors involved in the targeting of heterochromatin and PcG proteins are listed. In heterochromatin formation, the DNA element REIII is located in the mating type locus (mat locus) of *Schizosaccharomyces pombe* (Sp) and is bound by Atf1 and Pcr1, whereas Taz1 and Ccq1 bind to telomeric repeat regions. In *Drosophila melanogaster* (Dm) all DNA-binding factors have been implicated in the targeting of PcG proteins to PcG response elements (PREs). For the histone modifications, the corresponding histone methyltransferases (writers) and binding proteins (readers) are indicated. RNA-mediated recruitment mechanisms are also depicted in more detail in FIG. 3. AEBP2, adipocyte enhancer-binding protein 2; *ANRIL*, long ncRNA; At, *Arabidopsis thaliana*; BCL-6, B-cell lymphoma 6 protein; cKrox/Th-POK, Krüppel-related zinc finger protein/T-helper-inducing POZ/Krüppel-like factor; CBX, chromobox protein homologue; Ce, *Caenorhabditis elegans*; D11.12, a PRE; Dr, *Danio rerio*; DSP1, dorsal switch protein 1; E2F6, E2F transcription factor 6; EED, embryonic ectoderm development; ESC, Extra sex combs; E(Z), Enhancer of zeste; EZH, enhancer of zeste homologue; GAF, GAGA factor (also known as Trl); H3K9me, methylation of histone H3 at lysine 9; HP1, heterochromatin protein 1; *HOTAIR*, *trans*-acting long intergenic ncRNA; Hs, *Homo sapiens*; JARID2, Jumonji AT-rich interactive domain; *Kcnq1ot1*, *Kcnq1*-overlapping transcript 1; KRAB-ZNF, Krüppel-associated box domain zinc finger proteins; Mm, *Mus musculus*; OCT4, octamer binding transcription factor 1 (also known as POU5F1); PHO, Pleiohomeotic; PHOL, PHO-like; PML–RARα, promyelocytic leukaemia–retinoic acid receptor-α; PLZF–RARα, zinc-finger protein– retinoic acid receptor-α; RB, Retinoblastoma; SETDB1, SET domain, bifurcated 1; SP1, Specificity factor 1 (also known as KLF); SU(VAR)3-9, Suppressor of variegation 3–9; SUV39H1, SU(VAR)3-9 homologue 1; *Xist*, X-inactive specific transcript; YY1, Yin-yang 1.

RNAi
(RNA interference). Cellular mechanism involved in gene silencing and 'protection' against retroviral and transposable element invasion. Regulated by proteins such as Dicer and Argonaute, which are responsible for the production of small interfering RNAs that target messenger RNAs for cleavage and that localize silencing factors to heterochromatic regions.

which are often found in multi-gene clusters. Therefore, the large heterochromatin domains containing HP1β and H3K9me3 at KRAB-ZFP genes might also protect against deleterious recombination events among the extended homologous regions[44]. KAP1 has also been shown to control endogenous retroviruses in ES cells; it is probably recruited by KRAB-ZFPs to the 5′ UTR of the retroviral genome and then deposits HP1 (REF. 45). Therefore, targeting of HP1 by the vast and still ill-defined class of sequence-specific KRAB-ZFPs, in combination with KAP1, might mediate sequence-specific heterochromatin formation.

Because initiation of PcG silencing and heterochromatin formation seem to rely on DNA-binding proteins, it seems that specificity is generated by underlying DNA sequences rather than by certain protein features of the chromatin environment. In the case of the PcG system, there may be more general factors such as JARID2 that bind to a large proportion of target genes, in addition to transcription factors that target only context-specific subsets. Moreover, although transcription factors recruit heterochromatin-specific proteins to their target loci in fission yeast and in mammalian facultative heterochromatin, such a process has not been demonstrated for mammalian constitutive heterochromatin domains.

An RNA bridge over silencing

In addition to sequence-specific DNA-binding proteins, various forms of ncRNA have been implicated in the targeting of heterochromatin and PcG proteins. In both fission yeast and higher eukaryotes, the RNAi machinery nucleates the formation of constitutive heterochromatin. As yet, there is no strong evidence for a contribution of the RNAi pathway to PcG-mediated gene silencing. By contrast, several studies have implicated long ncRNAs (lncRNAs) and a new class of short ncRNAs as factors that interact with PcG complexes (FIG. 3).

Establishing heterochromatin using RNAi. In fission yeast, transcription of repeat regions within heterochromatin domains triggers the RNAi machinery, which generates small interfering RNAs (siRNAs), which are about 21 nucleotides in length. The siRNA molecules associate with the Argonaute protein (Ago1) and guide the Ago1-containing RNA-induced initiation of transcriptional gene-silencing complex (RITS complex) to homologous sequences of nascent chromatin-associated transcripts. The chromatin-associated RNAs serve as assembly platforms for the RITS complex and additional protein components. This leads to the generation of more double-stranded RNAs, which are processed into more siRNAs, thus enabling the spread of heterochromatin[46]. The RITS complex also recruits Clr4, a homologue of *D. melanogaster* SU(VAR)3-9, which methylates H3K9 and results in Swi6 targeting and subsequent heterochromatin propagation. (For further molecular details on RNAi-dependent heterochromatin formation, see REF. 47.) The role of siRNAs seems to be restricted to the targeting function, because tethering Clr4 to euchromatic sites artificially results in the formation of 'synthetic' heterochromatin even without active RNAi machinery[48,49].

Figure 3 | **Current working models for RNA-mediated recruitment mechanisms. a** | RNAi-related processes are involved in the initiation of constitutive heterochromatin in *Schizosaccharomyces pombe*. Transcription of heterochromatin leads to the formation of RNA duplexes processed by Dcr1 into small interfering RNAs (siRNAs, short grey lines). Argonaute protein 1 (Ago1) binds these siRNAs and is subsequently targeted to nascent transcripts, leading to the recruitment of the histone H3 lysine 9 (H3K9)-specific histone methyltransferase Clr4 through Stc1 and the spreading of heterochromatin[48,129–131]. **b** | *Xist* and *Tsix* are large non-coding RNA elements located in the mammalian X-inactivation centre. The 17 kb *Xist* RNA is expressed exclusively by the inactive X chromosome (Xi) and is antagonized by the 40 kb antisense transcript *Tsix*, which needs to be expressed from the active X chromosome[59,132–134] (inactive X is shown). *Xist* contains *RepA*, a repeat element that can also be expressed as separate 1.6 kb RNA and that is necessary for the recruitment of Polycomb repressive complex 2 (PRC2)[61]. The arrows indicate the spreading of *Xist* and PRC2 across Xi. **c** | *Kcnq1*-overlapping transcript 1 (*Kcnq1ot1*) has been discovered in mice as 91 kb antisense transcript in the imprinted *Kcnq1* domain[63]. It interacts with PRC2 and with the H3K9-specific methyltransferase G9a (not shown), and its expression coincides with the assembly of repressive chromatin defined by H3K27me3 and H3K9me3 (REF. 63). Imprinted repressed genes are depicted in red, biallelically expressed genes are depicted in green. **d** | *ANRIL* is a non-coding antisense transcript overlapping the INK4a/ARF tumour-suppressor locus[135]. It binds the PRC1 subunit chromobox protein homologue 7 (CBX7) which is required for the repression of genes in *cis*[65]. **e** | Expression of promoter-proximal short non-coding RNAs (ncRNAs) of 50–200 nucleotides coincides with the repressed state of Polycomb group (PcG) protein target genes in embryonic stem cells and are proposed to be involved in the anchoring of PRC2 complexes[68]. **f** | *HOTAIR* is a 2.1 kb RNA transcribed antisense to HOXC genes. Its expression is correlated with the repression of genes in the HOXD locus, supposedly by targeting of PRC2 (REF. 69).

Fission yeast is the best understood model system for heterochromatin formation and provides valuable hints regarding higher eukaryotes. Similar RNAi-dependent targeting mechanisms for heterochromatin can be found in *Arabidopsis thaliana*, *Caenorhabditis elegans* and *D. melanogaster*; for mammalian genomes the situation remains largely unresolved[50–52].

Non-coding RNAs that interact with PcG proteins. In *D. melanogaster*, many ncRNAs have been identified in the homeotic gene clusters and it has been realized that the transcription through a PRE is accompanied by derepression of its associated gene[53–55]. In this case, it is suggested that, rather than RNA-recruitment of protein complexes, the active transcription through the *cis*-acting element leads to loss of PcG protein binding, which results in an open chromatin conformation[56]. In another case, a study suggested that transcription of an *Ultrabithorax* (*Ubx*) PRE (known as *bxd*-PRE) during a specific stage of development produces ncRNA

Small interfering RNA
(siRNA). A short, non-coding RNA (∼22-nucleotides long) that is processed from longer double-stranded RNA by the RNAi machinery. Such non-coding RNAs confer target specificity to the silencing complexes in which they reside.

RNA-induced initiation of transcriptional gene silencing complex
(RITS complex). An RNAi effector complex required for heterochromatin assembly in fission yeast. It targets centromeric transcripts to induce both H3K9 methylation and small interfering RNA amplification.

a RNAi

b Xist/RepA

c Kcnq1ot1

d ANRIL

e Short ncRNAs

f HOTAIR
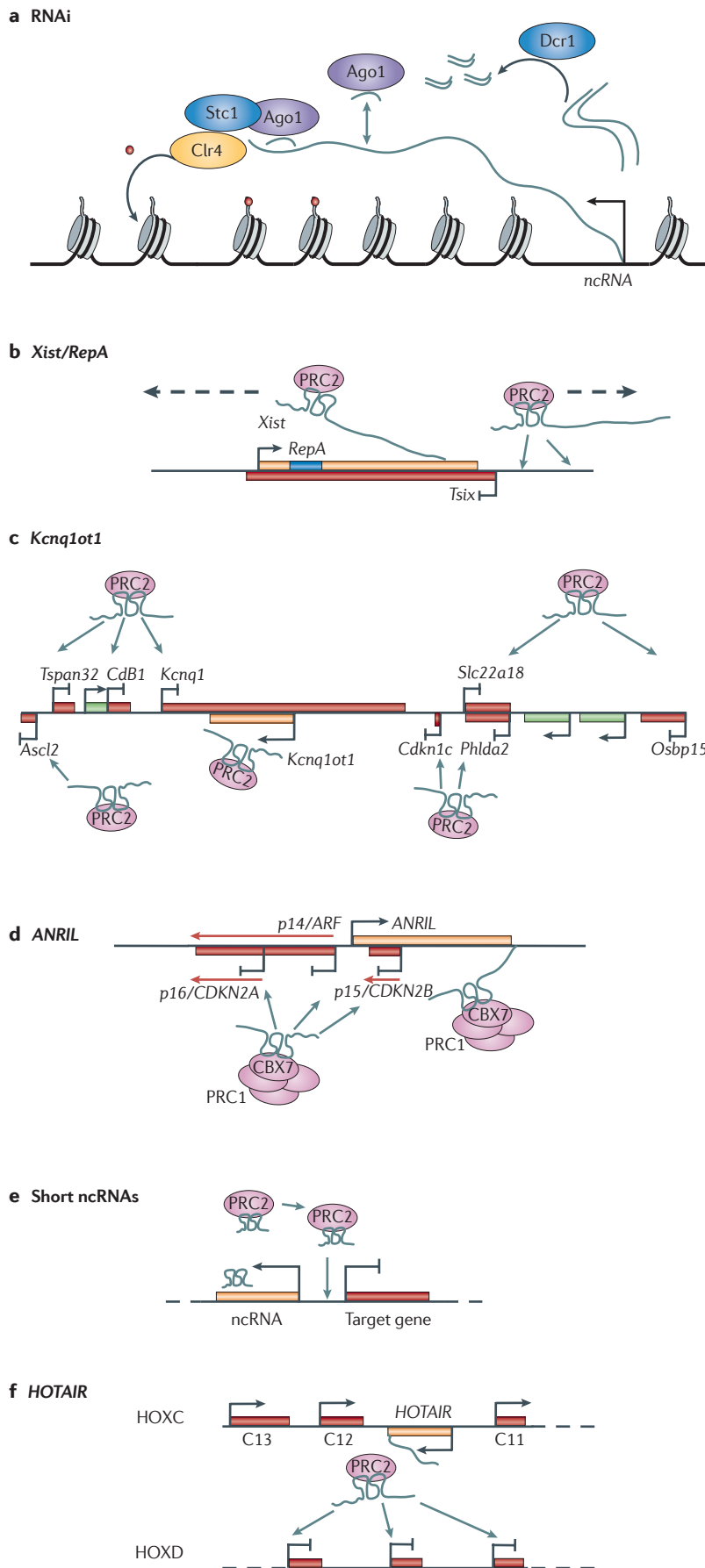
transcripts that mediate the recruitment of absent, small or homeotic discs 1 (ASH1), a member of the anti-silencing Trithorax group (TRXG) of chromatin proteins[57]. However, these findings are inconsistent with a demonstration of transcriptional interference between *Ubx* and *bxd* lncRNAs[58]. Indeed, there is currently no strong evidence that ncRNAs are involved in the targeting of PcG proteins in *Drosophila*. The situation in mammals seems to be different.

Targeting of PcG proteins by lncRNAs in **cis.** In mammals, X inactivation has long been known to be dependent on the transcription of the lncRNA *Xist* on the X chromosome that is inactivated[59,60]. *Xist* can be immunoprecipitated with PRC2 components and also binds to recombinant EZH2 (enhancer of zeste homologue 2)[59–61]. A short motif, termed *RepA*, folds into two conserved stem–loop structures, which mediate the protein–RNA interaction *in vitro*[61,62]. Similarly, *Kcnq1*–overlapping transcript 1 (*Kcnq1ot1*) — a paternally transcribed lncRNA from the imprinted *Kcnq1* gene cluster — immunoprecipitates with PRC2 and the G9a histone methyltransferase. This suggests that *Kcnq1ot1* recruits these histone methyltransferases in *cis* to set the repressive H3K27 trimethylation (H3K27me3) and H3K9me3 marks, respectively[63]. In addition, PRC1 may be directly targeted to the inactive X by chromobox protein homologue 7 (CBX7) in an RNA-dependent manner[64]; the chromo domain of CBX7 was found to bind single-stranded RNA, as well as H3K27me3, and to localize the protein to the inactive X chromosome[64].

A recent study in human cells adds a new candidate to the list of lncRNAs and corroborates RNA-dependent targeting of CBX7 (REF. 65). *ANRIL* is an lncRNA that is transcribed from the INK4a/ARF tumour-suppressor locus and regulates the expression of *INK4b*. Using NMR spectroscopy and fluorescence anisotropy, Yap *et al.* provide evidence for a CBX7–RNA interaction on the basis of structural and kinetics measurements[65]. The CBX7 chromo domain binds *ANRIL* and H3 peptides methylated at K9 or K27 with similar affinities. Most intriguingly, two different point mutations in the chromo domain independently disrupt the binding either to methylated H3K27 or to RNA and lead to derepression of the INK4a/ARF tumour-suppressor genes. The binding of CBX7 to the INK4a/ARF locus is also dependent on MOV10, an RNA helicase, which suggests that MOV10 may facilitate the CBX7–*ANRIL* interaction[66].

In summary, these studies suggest a mechanism by which nascent lncRNAs, together with the methylated H3K27 moieties, provide multiple binding sites that may increase the local concentration of PRC1 in *cis*. Although both types of interaction are of relatively low affinity, the multitude of binding sites may generate a local high-affinity environment that acts as a molecular cage to dynamically maintain PRC1 complexes in place at the target genes (see below). At the same time, this configuration can be regulated so that the equilibrium shifts to gene activation — for example, by recruitment of histone demethylases and components of the TRXG, and/or by switching off the transcription of the *cis*-acting lncRNA.

Targeting of PcG proteins by short ncRNAs in **cis.**
PRC2-repressed promoters are often associated with the 'active' histone mark H3K4me3, a condition termed 'bivalent'[67]. In human CD4+ T cells and mouse ES cells, Kanhere *et al.* have identified a class of short ncRNAs (in the size range of 50–200 nucleotides) that are derived from loci within approximately 700 bp of the transcription start site (TSS) of these PcG-repressed target genes[68]. The transcription of these short RNAs at bivalent domains might explain why marks associated with transcription, such as H3K4me3, are found at apparently repressed genes. Although bivalent domains have not been identified in *D. melanogaster*, concomitant occurrence of PcG and TRXG proteins is often seen at the PREs of repressed genes, which might suggest a similar condition of 'transcription underlying repression'. In mammals, the short transcripts could interact with PRC2, most likely through SUZ12, by means of a stem–loop structure — similar to the interaction with the *RepA* motif of *Xist* — and this could cause gene repression in *cis*. After differentiation of ES cells and activation of PcG-repressed genes, the associated short RNAs are depleted. These ideas support a role for the short TSS-derived ncRNAs in targeting PRC2 in *cis* to initiate the repressive state. To corroborate this model, nucleotide exchanges could be made at endogenous loci that abolish stem–loop formation in the ncRNAs and thereby impair targeting of PRC2. As such, it would be interesting to examine SNP databases for mutations affecting such stem–loops, as this would serve as additional confirmation for a general molecular mechanism and, moreover, could be a new entry point by which to explain SNP-associated disease states.

PcG proteins and **trans**-*acting lncRNAs.* More than 3,000 large intergenic ncRNAs (lincRNAs) have been identified in various human cell types[69,70]. Approximately 20% of them associate with PRC2, as determined by immunoprecipitation with subsequent microarray analysis[70]. One of these transcripts, called *HOTAIR*, is a 2.2 kb ncRNA expressed by the HOXC locus[69]. Depletion of *HOTAIR* results in the derepression of HOXD genes. This has been explained by the association of *HOTAIR* with PRC2, which would normally stimulate H3K27 methylation at the HOXD locus in *trans*. *HOTAIR* also seems to interact with PRC2 and the H3K4-specific demethylase complex LSD1 simultaneously, with the two protein complexes associating with different sites of the RNA[71]. Thus, *HOTAIR* has been suggested to be a 'scaffold' that organizes the concerted action of various chromatin modifiers[71]. The interaction of *HOTAIR* with PRC2 has not been investigated in detail and the molecular mechanism underlying the *trans*-targeting remains elusive.

Overexpression of *HOTAIR* in human breast cancer cells induces the mislocalization of PRC2 to more than 850 new targets, suggesting that *HOTAIR* has a genome-wide role in targeting PRC2 (REF. 72). How can a single RNA be responsible for the targeting of a protein complex to such a large number of binding sites? An attractive mechanism would involve the formation of sequence-specific hybrids between corresponding homologous parts of the ncRNA and the target DNA or nascent transcripts of the target gene. Another explanation would be that *HOTAIR*, rather than being required as targeting factor, has a structural role in a specific PRC2 variant that is responsible for the regulation of the 850 genes. Hybrid formation, as well as structural incorporation of the RNA into PRC2 complexes, would predict the presence of *HOTAIR* at PcG target sites, a detail that has not yet been experimentally shown. In addition, demonstration of a defined *HOTAIR*–PRC2 complex and the definition of the RNA–protein interaction surface (or surfaces) will yield important insight towards a better understanding of the suggested *trans*-targeting mechanism.

Anchoring silencing to the nucleosomal backbone

It is commonly recognized that H3K9- and H3K27-methylated moieties are important for the formation of heterochromatin and PcG-mediated silencing, respectively. However, their relevance and contribution to the silencing process are still under debate, because it is not yet clear whether histone modifications are the cause or consequence of silencing.

Methylation of H3K9 is a mark of pericentric heterochromatin and is catalysed by SU(VAR)3-9 in *D. melanogaster* (SUV39H1 in humans)[73,74]. SUV39H1 binds to HP1, and the amino-terminal chromo domain of HP1 can bind to di- and trimethylated H3K9 (REFS 74,75). Similarly for the PcG system, Enhancer of zeste (E(Z)) is a H3K27-specific histone methyltransferase that binds, in the context of PRC2, to methylated H3K27 (REFS 76–80). These interdependencies have been suggested to constitute self-reinforcing loops, which are required for the spreading and maintenance of pericentric heterochromatin and PcG-repressed chromatin domains (discussed further in the next section).

Polycomb, a core subunit of PRC1 (FIG. 1B), also binds methylated H3K27 through its chromo domain[76–78]. A hierarchical recruitment model was proposed in which the DNA-binding protein PHO recruits PRC2, which sets the histone mark that leads to PRC1 binding and repression of the target gene[81]. This model is challenged by an experiment performed in the mid-1990s by Platero *et al.*[82] that argues against histone methylation being the predominant targeting determinant. A chimeric *D. melanogaster* HP1–Polycomb protein, consisting of the chromo domain of Polycomb joined to the backbone of HP1, localizes to heterochromatin, as well as to Polycomb-binding sites in polytene chromosomes. Endogenous HP1 is misdirected to Polycomb binding sites and endogenous Polycomb is misdirected to pericentric heterochromatin, which suggests that both proteins are targeted primarily through protein–protein interactions rather than by binding only to a certain histone methylation mark through the chromo domain. Furthermore, in *D. melanogaster*, H3K27me3-marked regions extend over many kilobases and can encompass several repressed genes, such as the entire silenced homeotic clusters. By contrast, PRC1 proteins are restricted to distinct sites at promoters and PREs[83–86].

Trithorax group
(TRXG). A class of proteins — originally identified as suppressors for mutations in PcG genes in *Drosophila melanogaster* — that maintain the stable and heritable active state of several genes, including the homeotic genes.

Many additional studies have discussed further functional aspects of the role of H3K27 methylation in PcG-mediated silencing; two recent reviews summarize these findings[83,84].

In addition, the binding affinity of chromo domains is in the micromolar range, which speaks against a mechanism in which PcG and HP1 protein complexes are initially recruited by histone modifications[87]; the targeting functions seem to be primarily sustained by transcription factors binding directly to specific DNA sequences with affinities in the nanomolar range. Instead, the increased local concentration of histone modifications may be required to build up large repressive chromatin domains, whereby the low binding affinities are overcome by increasing the number of binding epitopes (avidity). Moreover, the affinity of protein complexes for histones in specific chromatin domains may be fine-tuned by post-translational modifications of certain PcG proteins and HP1 (REFS 88–90).

Signal propagation

The key question for a better understanding of epigenetic gene regulation is how the repressed gene-expression state is transmitted through cell division, in particular through DNA replication during which the specific epigenetic signal has to be faithfully duplicated and distributed into the daughter chromosomes in a sequence-specific manner.

Copying silencing during the process of DNA replication. A recent study indicated that PcG proteins themselves could 'survive' the process of replication and stay bound at replicating DNA[91]. Furthermore, two other studies have revealed that not only PRC1 but also PRC2 can recognize and bind H3K27me3 (REFS 79,80). The binding activates the methyltransferase activity of PRC2 and is mediated by the seven-bladed β-propeller domain of embryonic ectoderm development (EED)[80]. Similarly to the SU(VAR)3-9–HP1–H3K9me3 interdependency in heterochromatin formation, the concomitant presence of H3K27 methylation and H3K27me3 binding activities in PRC2 suggests a self-reinforcing loop that might ensure the propagation through replication of an epigenetic mark characteristic of PcG-mediated gene silencing. In this model, nucleosomes carrying the H3K27me3 mark are equally distributed between the daughter strands and target PRC2 to these chromatin domains after the replication machinery has passed (FIG. 4a). The methyltransferase activity of PRC2 subsequently fills the blank histone H3 tails of the newly placed nucleosomes with three methyl groups at lysine 27, thereby maintaining the repressed state of the underlying gene (or genes).

Can histones act as carriers of stable epigenetic information at all? This question was raised in a recent study investigating the kinetics of nucleosome turnover in *D. melanogaster* cells[92]. The work demonstrates that histones within active genes, at promoters and, most intriguingly, at binding sites of PcG and TrxG proteins are exchanged multiple times during the cell cycle. This means that histone modifications are also highly dynamic at these sites, and might be erased and re-established multiple times within the lifetime of a cell, thus raising the question of whether such a highly dynamic structure can be used to carry information to daughter cells. However, if the enzymes setting the histone marks act faster than nucleosome-turnover rates, histone modifications could still be stably maintained in a highly dynamic way. In the case of PcG and hetero-chromatin silencing, this might have been resolved by the combination of readers and writers in one protein complex (EED–EZH2 in PRC2 and HP1–SUV39H). Together with the fact that the corresponding histone methylation marks are not localized to a single nucleosome but are spread across entire repressed chromatin domains, this still leaves open the possibility that histone marks are epigenetically transmissible signals.

An alternative model, independent of histone modifications at the outset, has been suggested for the propagation of centromeric heterochromatin in fission yeast, which seems to be mediated by the activation of the RNAi machinery[93,94] (FIG. 4b). In mitosis, Swi6 dissociates from the condensed chromosomes, leading to the derepression of the transcription of centromeric repeats in early S phase. This triggers the RNAi machinery, which generates siRNAs that subsequently target the RITS complex and Clr4. Methylation of H3K9 leads to the recruitment and spreading of Swi6, leading to the re-establishment of heterochromatin and its propagation through replication.

Propagation of silencing through mitosis. The condensation of chromosomes during mitosis is associated with global gene silencing and the dissociation of transcription factors that are normally bound to promoter regions in interphase chromatin[95]. In addition, PcG proteins, HP1 and SUV39 dissociate, while a small fraction of proteins may stay at mitotic chromosomes to serve as 'seeds' for re-targeting the other components after mitosis[96–98].

In contrast to what has been observed for PcG proteins, a recent study demonstrated that MLL1 (the mammalian orthologue of *Drosophila* TRX) stays bound at mitotic chromosomes[99]. MLL1 is required for the maintenance of mammalian HOX gene expression and binds to several thousand promoters of active genes[100–102]. Chromatin immunoprecipitation analysis of interphase and mitotically arrested HeLa cells demonstrated a cell-cycle dependent global reorganization of the MLL1 binding pattern[99]. Interestingly, genes that are occupied by MLL1 in mitosis are highly expressed in interphase, with MLL1 promoting the rapid transcriptional reactivation on mitotic exit, whereas those genes bound by MLL1 only during interphase show a moderate expression level. If one assumes that PcG-mediated repression is the default state, MLL1 could act as an anti-silencing factor counteracting PcG recruitment after mitosis[103].

Conclusions and future directions

The list of potential targeting mechanisms for chromatin modifiers that mediate epigenetic silencing has expanded substantially during recent years. It is an open question whether the various mechanisms are context- or gene-dependent, or whether they act generally and
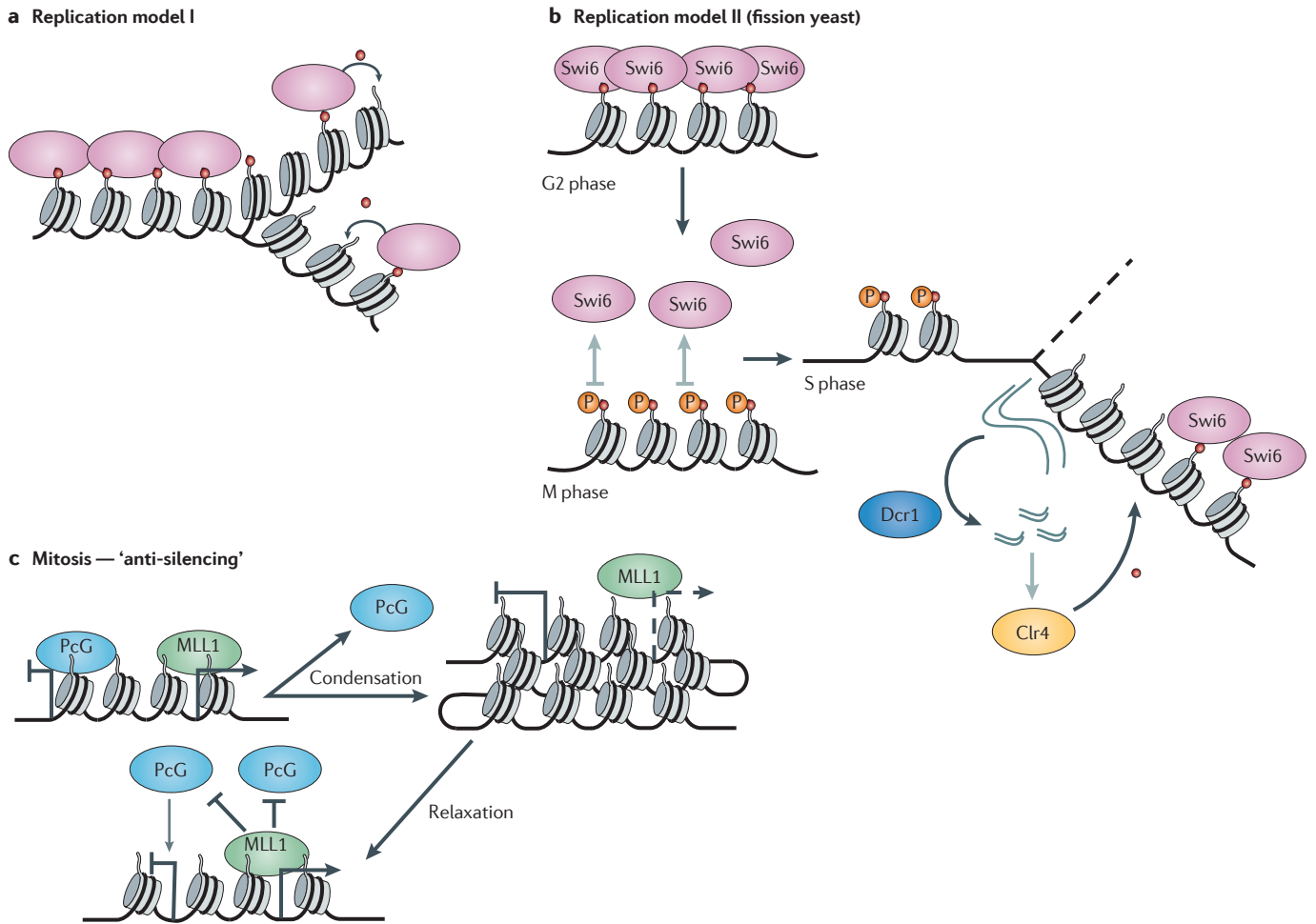
a Replication model I

b Replication model II (fission yeast)

c Mitosis — 'anti-silencing'

Figure 4 | **Current working models for the propagation of silent chromatin through replication and mitosis.** **a** | Replication model I. Histone modifications are evenly distributed to the daughter strands during the replication process. Silencing complexes (heterochromatin protein 1–SUV39 and Polycomb repressive complexes 1 and 2), indicated in pink, are recruited by diluted histone marks (red) and label newly inserted nucleosomes to re-establish the repressed chromatin domains. Alternatively, Polycomb group (PcG) proteins could stay bound at replicating DNA as suggested in REF. 91. **b** | Replication model II (fission yeast). Cells in G2 phase have high levels of methylated histone H3 lysine 9 (H3K9) and Swi6 associated with centromeric heterochromatin. Phosphorylation of H3 serine 10 (H3S10, indicated by an orange P) in mitosis leads to loss of Swi6 (methyl/phosphate switch[97,136]) and transcription of centromeric repeats in early S phase[93,94]. H3K9me2 levels are diluted during replication but are restored by the RNAi machinery (depicted as Dcr1), which guides Clr4 for H3K9 methylation and subsequent Swi6 recruitment. **c** | At the onset of mitosis, most chromatin regulators, including PcG proteins, dissociate from condensing chromosomes. Mixed lineage leukaemia 1 (MLL1) acts as an anti-silencer and stays bound at genes, which need to be rapidly activated after mitosis, and prevents the binding of PcG proteins after mitosis and chromosome relaxation[99,103]. See text for details. Histone modifications and residual heterochromatin or PcG proteins that do remain at mitotic chromosomes could serve as seeds to re-establish chromatin silencing after mitosis.

in a combinatorial manner. In *S. pombe*, constitutive heterochromatin targeting by RNAi and by sequence-specific transcription factors occur as independent, parallel pathways[36,38]. Both pathways converge on recruitment of Clr4, which recruits Swi6 and results in the establishment and propagation of mitotically stable heterochromatin. On the other hand, similarly to PcG-mediated silencing, gene-specific targeting of facultative heterochromatin in the euchromatic environment relies on sequence-specific transcription factors. RNAi-related recruitment seems not to be involved.

In mammals, several DNA-binding proteins have also been implicated in the targeting of PcG proteins. What role in this process could be attributed to the other possible targeting mechanisms discussed above? In analogy to the nucleation of constitutive heterochromatin in *S. pombe*, alternative targeting pathways that act in parallel could ensure proper silencing of important developmental regulators. Considering the importance of epigenetic silencing mechanisms for diverse cellular functions such as gene control, genome stability and chromosome segregation, it is not surprising that
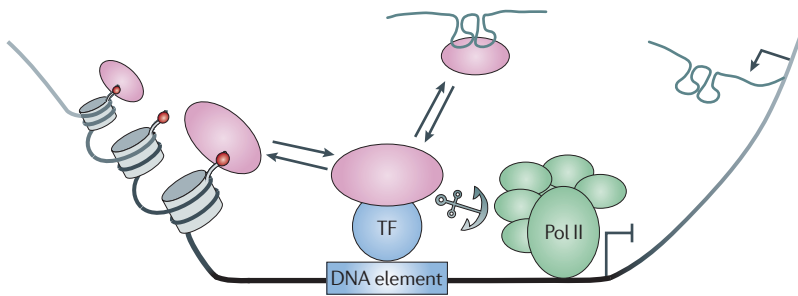
Figure 5 | A model for the targeting and anchoring of silencing complexes.
The primary event for the nucleation of heterochromatin formation and Polycomb repression is targeting of the silencing complex to specific DNA elements, mediated by DNA-binding proteins (transcription factors, TFs; other proteins are represented by pink ovals). The silencing complex can then prevent transcriptional activity by anchoring the RNA polymerase II (Pol II) complex at the promoter[137]. In order to maintain transcriptional repression through subsequent cell divisions, additional mechanisms are deployed. A multitude of low-affinity binding sites provided by histone modifications (red dots) and *cis*-acting non-coding RNAs (grey lines) perpetuate a local repressive environment that retains the silencing complexes. This environment can also be dynamically regulated by enzymes that erase histone marks and by the repression of non-coding RNA transcription to switch the gene from a repressed to an active state.

cells have evolved multiple pathways for assembling PcG-dependent repressive chromatin domains and heterochromatin. Alternatively, some pathways could act separately on specific sets of target genes, whereas others could act in a combinatorial way.

In recent years, a diverse set of ncRNAs have been identified and linked to gene-regulatory events, although their specific molecular roles remain largely unresolved. Small RNAs generated by the RNAi machinery in fission yeast constitute the best-defined system, as they have been functionally validated to target and establish constitutive heterochromatin. By contrast, the targeting functions of the various ncRNAs that potentially initiate PcG silencing have not yet been clearly demonstrated. It remains open how the RNA molecules could mediate the recruitment of PcG proteins to specific sites; for example, which PcG proteins bind RNA? (In the case of

PRC2, is it SUZ12, EZH2 or the complete complex that is required?) And which genomic sites are targeted by ncRNAs? As noted above, at centromeres in *S. pombe* the siRNA pathway directs selective silencing of repeat sequences that can generate bidirectional transcripts. One possibility is that a similar principle operates in PcG silencing if ncRNA transcription could act as sensor for open chromatin that needs to be re-silenced. ncRNAs transcribed in the vicinity of PcG target loci could perhaps serve as bait for PcG proteins that dissociate from chromatin, thereby keeping them close to their target sites.

Photobleaching experiments have demonstrated that the binding of HP1 and PcG proteins is surprisingly dynamic[104,105]. In the case of the PcG system, this feature may be reflected by the combination of a diverse set of targeting mechanisms that might fine-tune the dynamic behaviour of PcG complex binding rather than irreversibly fix repressed chromatin states. As a consequence, low-affinity anchor points, such as histone modifications or RNA-binding motifs, need to be assembled to allow a local trapping of silencing complexes and thereby ensure an uninterrupted supply of repressing factors at the target gene (FIG. 5). In this respect, ncRNAs would act in the maintenance of PcG silencing rather than to initiate it. Potentially, the sequence specificity provided by the ncRNAs could sustain the heritable propagation of PcG silencing.

Further studies are required to systematically dissect the various epigenetic silencing targeting mechanisms and their interdependencies. However, recent findings have paved the way for rapid development of this field. Research on global gene-expression profiles in different model organisms, and in disease and cell-differentiation states has revealed a well-interconnected and highly dynamic transcriptional network underlying cellular function. Understanding the initial steps of epigenetic gene silencing — the targeting of PcG and HP1 complexes as global regulators of transcription — is crucial to deciphering these networks and enabling predictions for applied research approaches.

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