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Ten principles of heterochromatin formation and function

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Abstract

Heterochromatin is a key architectural feature of eukaryotic chromosomes, which endows particular genomic domains with specific functional properties. The capacity of heterochromatin to restrain the activity of mobile elements, isolate DNA repair in repetitive regions and ensure accurate chromosome segregation is crucial for maintaining genomic stability. Nucleosomes at heterochromatin regions display histone post-translational modifications that contribute to developmental regulation by restricting lineage-specific gene expression. The mechanisms of heterochromatin establishment and of heterochromatin maintenance are separable and involve the ability of sequence-specific factors bound to nascent transcripts to recruit chromatin-modifying enzymes. Heterochromatin can spread along the chromatin from nucleation sites. The propensity of heterochromatin to promote its own spreading and inheritance is counteracted by inhibitory factors. Because of its importance for chromosome function, heterochromatin has key roles in the pathogenesis of various human diseases. In this Review, we discuss conserved principles of heterochromatin formation and function using selected examples from studies of a range of eukaryotes, from yeast to human, with an emphasis on insights obtained from unicellular model organisms.

Heterochromatin is a fundamental architectural feature of eukaryotic chromosomes that endows particular genomic regions with specific functional properties. The term ‘heterochromatin’ was coined based on the differential staining of chromosomal regions but now generally refers to molecular subtypes of transcriptionally repressed chromatin domains that extend beyond a single gene or regulatory element (BOX 1). Different varieties of heterochromatin are distinguished by their combination of histone post-translational modifications (PTMs). These affect the recruitment of proteins to, and the folding of, chromatin. Sequences embedded in heterochromatin often contain repetitive elements, such as satellite repeats and transposable elements. A crucial function of heterochromatin, which

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is generally more compact than euchromatin, is to prevent such selfish sequences from producing genetic instability. Additional heterochromatin roles include asserting cell-type-specific transcription and centromere function.

Histone PTMs, particularly on lysine residues of the unstructured histone tails that protrude from nucleosomes, are often referred to as ‘epigenetic marks’ because they can confer gene expression properties that are not strictly dependent on DNA sequence (BOX 2). Histone PTMs regulate the propensity of the underlying DNA to participate in the processes of transcription, replication, repair and recombination. Specific PTMs control binding of particular proteins to nucleosomes via specific protein domains known as ‘reader’ domains or modules (FIG. 1). Reader domains can be joined in the same protein with domains that modify chromatin, or reader proteins can be part of complexes that contain or recruit chromatin-modifying enzymes, which modify histones by the addition of PTMs (‘writers’) or the removal of PTMs (‘erasers’). Other enzymes recruited by histone PTMs are chromatin remodellers, which alter contacts between the histone octamer core and DNA to accomplish a variety of tasks[1].

The best-studied types of heterochromatin are marked by the addition of one (me1), two (me2) or three (me3) methyl groups to histone H3 lysine 9 (H3K9me) or lysine 27 (H3K27me). In this Review, we focus mostly on H3K9 methylation-dependent heterochromatin, which forms the major blocks of heterochromatin in cells and represents the defining molecular feature of constitutive heterochromatin in many eukaryotes. We also touch on other paradigms, namely, the silent information regulator (SIR) and Polycomb silencing systems, to highlight specific concepts. Histone H3K9 methylation on ε-amine groups of lysine residues is catalysed by suppressor of variegation 3-9 (Su(var)3-9), enhancer of zeste and trithorax (SET) domains of the histone–lysine *N*-methyltransferase (KMT) orthologues of the *Drosophila melanogaster* Su(var)3-9 proteins (SUV39H1 and SUV39H2 in mammals and abbreviated here as Suv39 when referring to both fruit fly and mammalian proteins) and *Schizosaccharomyces pombe* cryptic loci regulator 4 (Clr4)[2–4] (FIG. 1a). The H3K9me readers, heterochromatin protein la (HP1a) and HP1b in *D. melanogaster* (FIG. 1b) and their *S. pombe* orthologues Swi6 and chromo domain-containing protein 2 (Chp2), selectively bind methylated H3K9 through their chromodomains[2–13]. Additional factors and epigenetic modifications contribute to the formation and maintenance of heterochromatin; for example, the gene-repressive histone PTM H3K9me can be coupled with the repressive DNA 5-methylcytosine (5meC) in some systems[14–17].

In this Review, we discuss the key principles of heterochromatin formation and function. We illustrate these with examples taken mainly from unicellular yeasts, but include selected studies from a variety of model organisms. It is not our intention to be comprehensive; therefore, we have included only limited discussion of system-specific details and caveats. We focus on studies and experimental approaches that illuminate how histone modifications recruit heterochromatin factors, the role of RNA as a recruiting platform, the differences between heterochromatin establishment and maintenance, the processes of heterochromatin spreading and inheritance and the contributions of heterochromatin to genome stability, development and disease.

One: coupling of readers and modifiers

The SIR proteins of the budding yeast *Saccharomyces cerevisiae* (reviewed in REFS 18,19) comprise the first system in which the molecular mechanism of chromatin silencing was molecularly defined, although the silenced chromatin is distinct from canonical, H3K9me-dependent heterochromatin. In the SIR system, silencer elements are recognized by sequence-specific DNA-binding proteins that then recruit four proteins: Sir1, Sir2, Sir3 and Sir4. Sir2 is a NAD^+-dependent histone deacetylase (HDAC) that acts on acetylated histone H4 lysine 16, thereby enabling the Bromo-associated homology domain of Sir3 (a component of the Sir3–Sir4 complex) to bind nucleosomes[20]. Deacetylation by Sir2 thus promotes Sir3 binding, allowing further cycles of Sir protein recruitment to form silent domains. The SIR system illustrates the principle of reader–modifier coupling (FIG. 1c), in this case between the Sir3 reader and the Sir2 eraser. It also illustrates the principle of gene-silencing initiation by the recruitment of sequence-specific DNA-binding proteins (FIG. 1d). Although paradigmatic, the SIR system is restricted to *S. cerevisiae* and its relatives and thus evolved quite recently during evolution[21].

Reader–modifier coupling is also a key feature of the more canonical H3K9me-dependent heterochromatin[5,6,22–24]. Both fruitfly and mammalian Suv39 and *S. pombe* Clr4 H3K9 methyltransferases have a similar organization with an *N*-terminal chromodomain and C-terminal SET domain (FIG. 1a), thereby coupling writer and reader modules in the same protein. Methylation of H3K9 by the SET domain enables recruitment of Suv39 or Clr4 through their respective chromodomains. HP1 proteins contain not only a chromodomain reader module but also a more C-terminal chromoshadow domain (CSD; FIG. 1b). CSD dimerization forms a binding platform for other effector proteins[25,26]. Thus, the reading of H3K9me by HP1 proteins provides another route to reader–modifier coupling through CSD dimerization. For example, in *S. pombe*, recruitment of HDAC (eraser) complexes such as SHREC (Snf2/Hdac-containing repressor complex and Clr6 complex) by Swi6 and Chp2 removes acetylation, thereby allowing H3K9 methylation[27,28]. The recruitment of SHREC, which harbours the Mit1 (Mi2-like protein interacting with Clr3) chromatin remodeller subunit, also has a role in the elimination of nucleosome-free regions — nucleosome absence is a hallmark of heterochromatin in *S. pombe*[29,30]. As part of protein dimers, the reader domains of HP1 are also coupled as pairs with ensuing functions: two dimers of Swi6 bind a single H3K9me-modified nucleosome and provide ‘sticky ends’ that enable Swi6 to bridge two nucleosomes[31]. In some systems, H3K9 readers can be coupled with DNA modification. In mammals and plants, 5meC DNA methyltransferases can be recruited with H3K9 methyl-transferases so that the two reciprocally bolster each other to ensure that the DNA is rendered inaccessible[15–17].

Reader–modifier coupling is also a feature of the more dynamic silencing complexes recruited by H3K27me. Methylation of H3K27 by the Polycomb repressive complex 2 (PRC2) catalytic subunit enhancer of zeste (E(z)) in *D. melanogaster* or enhancer of zeste homologue 2 (EZH2) in mammals promotes binding of the Polycomb subunit of PRC1 to chromatin through its chromodomain[32–36]. In addition, the PRC2 subunits extra sex combs (ESC) in *D. melanogaster* and embryonic ectoderm development (EED) in mammals recognize the H3K27me mark and allosterically activate E(z) or EZH2, respectively[37].

Two: noncoding RNAs recruit modifiers

Noncoding RNAs (ncRNAs) that may be processed into small RNAs are transcribed from heterochromatin[16,38]. This may seem surprising because heterochromatin induces transcription silencing. Nonetheless, a low level of transcription occurs in heterochromatin, and this is important for heterochromatin formation in several organisms. Heterochromatin transcription can be cell-cycle regulated, occurring only during DNA replication, when heterochromatin becomes accessible[39–41]. One function for this transcription appears to be recruitment of silencing factors (FIG. 1d) through association with nascent transcripts, for example, in *S. pombe*[42–50]. The ncRNAs also provide a substrate for the generation of small RNAs, which promote the recruitment of silencing factors through base pairing, likely with nascent transcripts.

In *S. pombe*, RNA polymerase II (Pol II) transcribes heterochromatin repeats. The Argonaute protein (Ago1) binds single-stranded siRNAs and uses them to target homologous nascent repeat transcripts that emerge from chromatin-associated Pol II and thus to recruit silencing factors[47,51,52]. Ago1 is part of the three-subunit complex RNA-induced transcriptional silencing (RITS)[46], which associates with both the RNA-dependent RNA polymerase complex (RDRC)[43] and the histone Clr4 complex (CLRC)[45,53–55]. RDRC uses primary transcripts as templates for synthesis of double-stranded RNA (dsRNA), which is subsequently processed into siRNAs, thereby increasing siRNA production[43–56]. In a positive feedback loop, CLRC is required for all H3K9 methylation in *S. pombe,* and H3K9me promotes efficient siRNA production. Such feedback is, in part, mediated through the recruitment of RITS to H3K9-methylated chromatin through its chromodomain protein Chp1 (REFS 24,57,58). Two bridging factors connect the different heterochromatin-promoting complexes: Stc1 (siRNA to chromatin) recruits CLRC through RITS[44], whereas Ers1 (essential for RNA silencing) couples RDRC, RITS and Swi6 (REFS 59–61).

In plants, a similar feedback loop promotes H3K9 methylation. Most details come from studies in *Arabidopsis thaliana* where, similarly to *S. pombe*, nascent transcripts provide the platform for the recruitment of ARGONAUTE–siRNA complexes. Pol V, which is a specialized plant Pol II paralogue, produces transcripts that are targeted by siRNA-guided AGO4 (REF. 62). AGO4 recruits the *de novo* DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2)[63], which in turn recruits the KMTs and H3K9 methyltransferases SUVH4, SUVH6 and SUVH9 through the DDR complex (DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) and RNA-DIRECTED DNA METHYLATION 1 (RDM1))[64]. Another Pol II paralogue, Pol IV, produces dsRNA from template transcripts in association with RNA-dependent RNA polymerase. The processing of the resulting dsRNA by Dicer generates siRNAs that are loaded into AGO4 and targeted at the complementary, Pol V-generated transcripts[65–67]. Pol IV is commonly recruited by an H3K9me reader, SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1)[68,69]. Thus, as in *S. pombe*, nascent transcripts have two functions in *A. thaliana:* transcripts produced by Pol V recruit chromatin-modifying enzymes through base pairing with siRNAs, which in turn are produced from homologous, Pol IV-generated transcripts.

Another function of nascent heterochromatin transcripts is to recruit silencing-promoting proteins without the intermediary of small RNAs. *S. pombe* possesses an RNAi-independent pathway that promotes H3K9 methylation and functions to maintain pericentromeric heterochromatin[70]. One component of this pathway is seven binding 1 (Seb1), which contains an RNA recognition motif that recognizes GUA trinucleotides in nascent transcripts and a Pol II C-terminal-domain interaction domain[71,72]. Seb1 acts upstream of the SHREC complex[73], which participates in an RNAi-independent silencing pathway[74]. The Seb1–SHREC pathway is partially redundant with RNAi because H3K9 methylation is eliminated only in mutants where both pathways are inactivated[74]. As GUA trinucleotides occur frequently, how Seb1 promotes H3K9 methylation selectively at pericentromeric regions is not fully understood, but the selectivity could be facilitated by the relative depletion of GUA sequences from *S. pombe* protein-coding genes[72]. Furthermore, recent findings show that Suv39 KMTs are stabilized on heterochromatin by their nonspecific affinity for nascent RNA produced from mammalian centromere repeat arrays[75–77].

Similar transcript-driven processes mediate X chromosome inactivation in female mammals, a process that produces a condensed, silenced X chromosome marked by H3K27me3. Although the inactive X is not considered constitutive heterochromatin, this form of silent chromatin serves to illustrate related important principles. The A-repeat region of the long noncoding RNA X-inactive specific transcript (XIST) recruits SPEN (split ends; also known as SMART/HDAC1-associated repressor protein and Msx2-interacting protein), a protein that contains RNA recognition motifs[78–81] and that in turn recruits HDAC3 through the adaptor protein SMRT (silencing mediator of retinoid and thyroid hormone receptor; also known as NCOR2)[31,82,83]. Ensuing histone deacetylation probably triggers the recruitment of at least two redundant silencing machineries: one comprising PRC2 and PRC1, and the other remains to be identified (reviewed in REF. 83). As with Seb1, it is unclear whether SPEN alone has sufficient specificity to target XIST and the X chromosome for inactivation.

Three: establishment versus maintenance

Some signals and factors required to initiate *de novo* assembly of heterochromatin (that is, to convert euchromatin into heterochromatin) differ from those required for heterochromatin maintenance. This distinction between establishment and maintenance is crucial for understanding how heterochromatin formation occurs.

Testing whether a nonessential factor is required to establish heterochromatin (an establishment factor) is performed in *S. pombe* as follows (FIG. 2). Heterochromatin is first erased by removing the gene encoding a key chromatin modifier (for example, a KMT or HDAC). The re-introduction of that gene into otherwise wild-type cells allows heterochromatin re-establishment; however, cells lacking a heterochromatin establishment factor are unable to assemble heterochromatin[24]. Another approach compares the outcome of introducing naive DNA that can serve as a heterochromatin template (for example, centromere repeats) into wild-type versus mutant cells[24,84,85]. A third way is to erase heterochromatin by exposure to inhibitors (for example, the HDAC inhibitor Trichostatin A) and determine whether mutant cells recover heterochromatin after the removal of the inhibitor[86,87]. Such assays revealed that in *S. pombe* RNAi has an essential role in

establishing heterochromatin. For instance, in the absence of RNAi factors, no H3K9me can be established at centromere repeats or related sequences when the KMT Clr4 is re-introduced into cells lacking Clr4. Likewise, H3K9me is established on repeats transformed into wild-type cells but not cells lacking RNAi. This stands in contrast to the partially redundant role of RNAi with Seb1 or with the HDACs Clr3 or Sir2 in the maintenance of H3K9me at peri-centromeric regions, in which double mutants of RNAi and Seb1 or RNAi and a HDAC are required to eliminate H3K9me2 (REFS 74,85).

Establishment of heterochromatin on *S. pombe* centromeric outer repeats requires RNAi, but it remains unclear how the initiating source of dsRNA is generated. Possibilities include dsRNA produced by convergent, overlapping transcripts[38], RNA secondary structures[88] and degradation products[89]. Another possibility is that the RDRC synthesizes the initiating dsRNA from centromere repeat transcripts[43,56], as in plants (see above). In the latter case, specific features must exist that distinguish repeat-element transcripts from mRNAs to ensure the specific recruitment of RDRC to the former.

In *S. pombe*, dsRNA induced by expression of an artificial, hairpin-encoding DNA is sufficient to generate synthetic siRNAs and direct H3K9me–heterochromatin formation in *cis* at the locus producing the dsRNA[90]. Here, no inherent special features are required to trigger heterochromatin formation once dsRNA is synthesized. Surprisingly, siRNAs produced from such artificial dsRNAs only weakly induce heterochromatin assembly in *trans* at transcribed homologous loci in euchromatin[91]. Such synthetic siRNAs trigger more efficient H3K9me–heterochromatin formation in *trans* in cells harbouring mutations in the Pol II-associated polymerase associated factor complex[92–94]. Defective canonical polyadenylation signals at the transcribed target locus also enhance silencing[95]. Thus, nascent transcripts that are held at native heterochromatin loci owing to inefficient transcription elongation and/or termination could bolster RNAi-mediated H3K9 methylation.

RNAi-independent mechanisms of heterochromatin establishment also exist in *S. pombe* as RNAi is not required for establishment of heterochromatin adjacent to telomeres. Clr4 is recruited to telomere repeats through the telomere-binding protein complex shelterin[96]. However, RNAi contributes to subtelomeric silencing in *S. pombe* at centromere-related, telomere-adjacent repeats[97,98].

Heterochromatin establishment-specific factors that function through H3K9 methylation have also been identified in the *Caenorhabditis elegans* germ line, where small Piwi-associated RNAs (piRNAs) trigger an siRNA–H3K9 methylation feedback loop, much like those in *S. pombe* and plants[99]. However, piRNAs are dispensable for the maintenance of the feedback loop. This was revealed through genetic crosses that removed the two Piwi-related genes, *prg-1* and *prg-2*, after triggering heterochromatin formation[100]. Piwi also has a role in the establishment of HP1a-bound heterochromatin during *D. melanogaster* development[101].

In *A. thaliana,* where DNA methylation and H3K9 methylation are connected, most loci controlled by RNAi can re-establish silencing following transient disruption of DNA

methylation[102]. However, at a small subset of these loci, DNA methylation cannot be rescued by the re-introduction of the maintenance DNA (CYTOSINE 5)-METHYTRANSFERASE (MET1) to *MET1* mutants[102]. This suggests that once DNA methylation has been erased from these particular loci, they lack the cues required for its re-establishment.

Finally, during X chromosome inactivation in murine epiblasts, the XIST ncRNA is required to establish silencing on one of the X chromosomes (see above). However, removal of XIST later in development has no effect on the maintenance of silencing[103,104]. Furthermore, analyses in embryonic stem cell models showed that although SPEN and other factors are required to establish XIST-mediated gene silencing, once DNA methylation has been installed on the inactive X, the silent state is inherited in the absence of SPEN or other initiating factors[78–81].

Four: heterochromatin can spread

Once nucleated at a particular location, the biochemical properties of heterochromatin components enable the expansion of the domain in a manner that is largely independent of DNA sequence. The classic example of this is position-effect variegation in *D. melanogaster,* where specific chromosome rearrangements can juxtapose heterochromatin with euchromatin; in such cases, heterochromatin spreads over large distances into euchromatin (reviewed in REF. 105). In *D. melanogaster,* the presence of additional heterochromatin elsewhere titrates limiting factors away from, and consequently weakens, heterochromatin, thereby alleviating repression at different loci[106–108]. Thus, spreading requires a surplus of unassembled heterochromatin components and can be driven by their overexpression[109–111].

Heterochromatin spreading requires reader–writer coupling. Nucleosomes bearing H3K9me are bound by the chromodomains of H3K9me writers such as Suv39 and Clr4, and mutations in the Clr4 chromodomain impede spreading in *S. pombe*[45,112]. However, spreading also requires the HP1-dependent recruitment of HDACs[28,43,50,113]. Thus, interconnections among reader, writer and eraser modules form positive feedback loops that extend heterochromatin domains.

Single-cell reporter analysis in *S. pombe* showed that nucleation of heterochromatin at the mating-type locus can take several cell divisions and that the expansion of the domain to its full size requires even longer time[114]. This indicates that feedback mechanisms act both locally, on adjacent nucleosomes, and more broadly over greater distances to mediate this two-step process[114]. Thus, the spreading of silent chromatin does not necessarily occur in a linear fashion; random collisions between a heterochromatin domain and chromatin that is spatially located nearby may allow the key modification to be deposited discontinuously through ‘hops’ that decline in frequency with distance from the nucleation site or domain. Subsequently, gaps between the original domain and the new heterochromatin patch could be filled by a pincer-like movement, although exceptions to this scenario have been observed in *D. melanogaster*[108]. Modelling of available data suggests that reader–writer-driven feedback coupled with collisions between modified and unmodified sites optimally describes the dynamics of heterochromatin domains[115].

Heterochromatin-spreading models may be influenced by recent findings of a role for HP1-induced liquid–liquid phase separation in heterochromatin assembly[116,117]. Purified *D. melanogaster* HP1a can form proteinaceous liquid droplets that undergo liquid–liquid de-mixing *in vitro* in particular conditions[117]. In *D. melanogaster* and mammalian cells, heterochromatin domains display properties of phase-separated liquids[117]. *In vitro* de-mixing has also been reported for the human HP1α protein. Phosphorylated HP1α de-mixes more efficiently than unphosphorylated HP1α, suggesting potential for regulation *in vivo*[116]. Indeed, a mutant that cannot be phosphorylated forms smaller heterochromatic foci when introduced into cells. Nucleosomes and DNA preferentially partition into these phosphor-HP1α droplets *in vitro,* suggesting that the HP1α 'solvent' controls entry of molecules into heterochromatin[116]. We anticipate that future work will reveal further the functions of phase separation in heterochromatin assembly, spreading and/or function.

Mammalian X chromosome inactivation is initiated by XIST expression from the X inactivation centre. XIST spreads discontinuously along the X chromosome and may first affect noncontiguous chromosomal regions that contact the X inactivation centre in three-dimensional space[118,119]. XIST spreading, accompanied by gene silencing, is not limited to X chromosomes. Specifically, rearrangements that fuse autosomes to an inactive X result in spreading of silencing into the autosome, albeit with limited efficiency[120–122]. Likewise, ectopic expression of XIST from autosomes results in reduced gene expression over large adjacent domains[83,103,123–127].

Five: spreading is restrained

Because heterochromatin can spread, mechanisms to restrict its expansion are necessary to avoid erroneous and potentially deleterious gene silencing (FIG. 3). Mechanisms to create such barriers and interrupt lateral heterochromatin spreading include the following: generation of nucleosome-depleted regions by binding of proteins such as transcription factors; processes that promote nucleosome turnover; recruitment of antisilencing factors by ongoing transcription and associated regulatory elements; recruitment of readers with antisilencing activity; and restricting silencing factors to their sites of prior action.

tRNA genes are a class of heterochromatin-spreading barrier conserved from yeast to man[128–130]. Binding sites for the Pol III-associated transcription factor complex TFIIIC appear to be crucial for the barrier function as clusters of these sites alone, independent of tRNA genes, function as heterochromatin barriers. One example derives from the boundaries of the silent mating-type region in *S. pombe*[131]. These regions have large nucleosome-free regions, which may prevent heterochromatin spreading by forming a 'gap' in chromatin over which some reader–writer machineries cannot cross[29] (FIG. 3b). tRNA genes, such as the TFIIIC sites at the mating-type locus, are themselves accessible and essentially free of nucleosomes[132–134]. Turnover of nucleosomes assembled in heterochromatin is low[92,135], and factors such as the polymerase associated factor complex, which promote their turnover, are required for barrier function[92,94,135] (FIG. 3c). Myriad other types of boundary elements and factors have been described suggesting that there are many mechanisms for interrupting heterochromatin assembly and spreading.

Euchromatin is marked by a variety of chromatin modifications that antagonize heterochromatin assembly. These include the histone variant H2A.Z, which is deposited in response to the formation of nucleosome-free regions at the first nucleosome (+1) downstream of transcription initiation sites[136–138], and histone PTMs triggered by active transcription (for example, methylation at H3K4, H3K36 and H3K79 and general histone acetylation). In *S. cerevisiae*, such PTMs have an antisilencing role at SIR-dependent heterochromatin (see above and FIG. 3d)[139–144]. Thus, competition between the two opposing mechanisms of heterochromatin spreading and transcription likely explains the classic bistability of heterochromatic (repressed) versus euchromatic (expressed) states implied by the phenomenon of position-effect variegation described above.

Heterochromatin can itself recruit the inhibitors that limit its own spreading through reader–eraser coupling (FIG. 3e). An example in *S. pombe* is the Epe1 (enhancement of position effect 1; also known as Jmjc domain chromatin-associated protein) protein, which is a putative H3K9 demethylase that is recruited by the reader Swi6 (REFS 145–148). Epe1 is degraded through the action of a ubiquitin ligase, the activity of which is limited to the interior of heterochromatin domains and absent from their edges, thereby providing a mechanism by which heterochromatin can recruit an antisilencing factor in a restricted manner[149]. Epe1 acts in parallel with boundary elements, as loss of both Epe1 and TFIIIC sites that flank the mating-type locus result in extensive heterochromatin spreading and slow cell growth[150]. Likewise, cells lacking both Epe1 and the globally acting histone acetyltransferase Mst2 display widespread ectopic heterochromatin assembly and slow growth, again emphasizing the importance of redundancy of antisilencing mechanisms[151]. Ectopic heterochromatin formation in such double mutants suggests that the processes that trigger heterochromatin assembly at the primary genomic locations can act globally, albeit normally less effectively. The detection of low levels of H3K9me at several euchromatic loci in wild-type cells, in specific conditions or in mutants, may be a manifestation of pathways that are important for gene regulation in response to various cues[92,151–155].

Tethering silencing machinery to its sites of prior action provides another mechanism to restrict heterochromatin to particular loci. Numerous chromatin-modifying complexes have domains that recognize the products of their enzymatic activity. In the budding yeast *Cryptococcus neoformans,* the H3K27-specific methyl-transferase PRC2 contains a subunit with a chromodomain, Ccc1 (chromodomain and coiled coil 1), which recognizes the H3K27me modification. In this yeast, H3K27me3 is selectively generated over sub-telomeric regions[156]. Mutations that prevent Ccc1 from recognizing H3K27me3 cause ectopic H3K27 methylation at centromeres. This ectopic methylation requires prior H3K9 methylation at centromeres, indicating that tethering of PRC2 to its sites of prior action (subtelomeres) through reader–writer coupling suppresses a latent attraction of PRC2 to H3K9-methylated domains, perhaps through the methyl-lysine binding activity of Eed.

Six: heterochromatin can be inherited

During DNA replication, the H3–H4 tetramers of old, parental nucleosomes are randomly distributed to sister chromatids during their synthesis (reviewed in REF. 157). Nucleosome occupancy on the newly synthesized DNA molecules is fully restored with new nucleosomes

that are assembled from free histones. The recruitment of KMTs by the H3K9me modification that they catalyse (reader–writer coupling) may allow the modification of such newly assembled neighbouring nucleosomes and suggests that heterochromatin self-propagates in a manner that is independent of the underlying DNA sequence (FIG. 4a). This process would enable the preservation of silent chromatin through DNA replication into progeny cells. Such inheritance is termed *'cis* inheritance of a chromatin state'.

Epigenetic inheritance is well known to be mediated by DNA methylation in some organisms, where the maintenance DNA (cytosine-5)-methyltransferase 1 is associated with the replisome, recognizes 5meC in CG dinucleotides and adds a methyl group to cytosine in the CG of the complementary strand (reviewed in REF. 158). In the filamentous fungus *Neurospora crassa*, H3K9 methylation and 5meC can reinforce each other; H3K9me nucleosomes can recruit the DIM2 (defective in methylation 2) DNA methyltransferase through HP1, and DNA methylation recruits the H3K9 methyltransferase DIM5 (REFS 14,159). Connections between H3K9 methylation and DNA methylation are also well established in other organisms (reviewed in REF. 160). Because 5meC on CG dinucleotides is heritable through DNA replication, its influence on H3K9 methylation could mask the *cis* inheritance of chromatin states mediated by H3K9me read–write systems themselves.

Thus, a strong test of H3K9me-dependent heterochromatin heritability would be enabled by a system lacking DNA methylation. In fission yeast, DNA methylation is undetectable, and stable *cis* inheritance of heterochromatin occurs at the silent mating-type locus[87,161]. Domains of synthetic heterochromatin formed when the SET domain of the KMT Clr4 was fused to a DNA-binding domain and through it recruited to cognate binding sites placed at loci in euchromatin, resulting in gene silencing[162]. A DNA-binding domain controlled by a small molecule allowed conditional use of such an artificial heterochromatin nucleator to test whether endogenous wild-type Clr4, along with other effector proteins, could maintain heterochromatin and gene silencing through cell division[163,164] (FIG. 4b). Release of the tethered Clr4 resulted in rapid loss of H3K9me, even when the cell cycle was arrested, suggesting that rather than being passively diluted through rounds of replication, H3K9me is actively removed. The histone demethylase Epe1 was found to be responsible for the rapid removal of this ectopic H3K9 methylation. Cells lacking Epe1 can transmit H3K9me at the manipulated locus into progeny through multiple cell divisions and even through meiosis (FIG. 4c). Thus, H3K9 methylation can be heritable and affect phenotype. Nonetheless, even in the absence of Epe1, such engineered H3K9-methylated heterochromatin and associated gene silencing eventually dissipates, presumably because of imperfect maintenance during replication and/or transcription-coupled loss of H3K9me nucleosomes.

Analogous transient targeting experiments in mammalian cells suggest that H3K9me-mediated repression is reversible, whereas DNA methylation allows the silent state to persist for many cell divisions[165,166]. Thus, mammalian cells also appear to restrict the heritability of H3K9me-mediated repression after the initial recruiting mechanism is disabled. By contrast, H3K9me-dependent heterochromatin formed by tethering HP1 persisted for many cell divisions following HP1 release from an engineered murine locus, although a potential role for DNA methylation in its maintenance at this locus seems difficult to rule out[167].

There is now strong evidence that silencing by the Polycomb proteins can mediate the *cis* inheritance of a chromatin state[168]. Interestingly, in *D. melanogaster*, specific sequences that mediate PRC recruitment are required for this inheritance, suggesting again that the heritability of silent chromatin is tightly regulated[169,170]. Thus, Polycomb-mediated silencing exhibits similarity to heterochromatin assembly at the *S. pombe* mating-type locus, which also involves sequence-specific binding proteins[161,171,172]. In the latter case, heterochromatin maintenance is further promoted by chromatin remodelling enzymes that curb nucleosome turnover, limit euchromatin assembly and affect the spatial positioning of chromatin in the nucleus[173–175].

Seven: defending the genome

Repetitive sequences are a threat to genome stability and organism viability. Mechanisms of genome destabilization by DNA repeats include mutations produced by the integration or excision of transposable elements and recombination between repeats. Heterochromatin has a pivotal role in suppressing these deleterious events through diverse mechanisms.

Studies in plants have revealed increased transposon copy numbers in mutants defective in the RNA-dependent DNA methylation (RdDM) silencing pathway described above[176]. Surprisingly, the copy number of only a single *copia*-type retrotransposon, EVD (evadé), increases when this pathway is mutated. Additional analyses confirm this observation[177], which has several potential implications. First, it suggests that it is the latent activity of this single transposon that drives maintenance of RdDM in *A. thaliana*. Theoretical work shows that a single transposable element can spread through a sexually reproducing population despite a negative impact on fitness[178]. Second, the lack of effect of RdDM pathway loss on the copy number of other transposons, despite an increase in their transcript levels, suggests that these other elements are not active or that other mechanisms limit their transposition. Nevertheless, their silencing could be important for genetic stability, as discussed below.

In *C. elegans*, loss of Piwi proteins, which control a nuclear RNAi pathway that is coupled to H3K9me, has been shown to increase the transposition of the Tc3 family of transposable elements[100,179]. Worms lacking the H3K9 methyltransferases *met-2* and *set-25* show widespread upregulation of Tc3 transcripts in both germline and somatic tissues. Strikingly, this resulted in the formation of R-loops, replication stress and increased mutation frequency within repetitive elements[179]. Thus, transcribed transposons can be mutagenic even without undergoing transposition *per se.*

A less-appreciated characteristic of heterochromatin is that it can control transposon activity by promoting the biogenesis of specialized small RNAs, rather than by transcriptional silencing. In the *D. melanogaster* female gonad, mutations in the HP1 paralogue Rhino result in defective piRNA biogenesis from clustered elements[180]. This is highly reminiscent of the role of H3K9 methylation in siRNA biogenesis in *S. pombe*. These genetic clusters are heterochromatin islands that produce transposon-homologous piRNAs[181,182], which act transcriptionally and post-transcriptionally to silence transposable elements[181]. Insertion of a transposon into a piRNA cluster in female gonads activates a mechanism that monitors and

silences the transposon. The piRNA system also operates in mammalian testes to silence transposons through DNA methylation[183–185].

An important mechanism of genome defence is the suppression of chromosomal rearrangements in repetitive elements following DNA damage. Homologous recombination between repeats such as dispersed transposable elements can result in chromosomal deletions, inversions and translocations. Intrachromosomal homologous recombination within repeat arrays often results in array expansion and contraction that may cause little harm (an exception being recombination within ribosomal DNA repeats[186]). By contrast, homologous recombination between repeats on nonhomologous chromosomes can cause translocations and result in the formation of dicentric and acentric chromosomes. Studies in *D. melanogaster* and mammalian cells demonstrated that breaks within heterochromatin are sequestered to the periphery of heterochromatin compartments[187–189]. This is thought to favour repair by homologous recombination within cognate heterochromatin repeats and thereby prevent illegitimate recombination with similar repeats on nonhomologous chromosomes[190,191].

Eight: influencing centromere function

Centromeres are the chromosomal loci where kinetochores assemble. Most eukaryotic centromeres are composed of repetitive arrays of DNA; the majority of these repeats are embedded in H3K9me-dependent heterochromatin and their DNA is heavily methylated in mammalian somatic cells. However, patches of repeats assemble unusual nucleosomes, in which histone H3 is replaced by its variant centromere protein A (CENP-A). These centromere-specific nucleosomes form the physical foundation for the kinetochore (reviewed in REF. 192). Heterochromatin has two important roles in centromere and kinetochore function.

First, heterochromatin influences the assembly of CENP-A into nucleosomes. In *S. pombe*, CENP-A-containing chromatin and functional kinetochores cannot be established on centromere DNA lacking flanking pericentromeric heterochromatin. Heterochromatin provides a crucial, but unknown, function to ensure CENP-A assembly into adjacent chromatin. Heterochromatin-directed histone modifications and/or nuclear–periphery association may promote CENP-A incorporation. Heterochromatin may also act to limit the size of the CENP-A–kinetochore domain[130,193]. Conversely, inadvertent or forced heterochromatin formation in fission yeast[194] or mammalian cell centromeres prevents CENP-A and kinetochore assembly[195,196].

A second role for heterochromatin at centromeres involves sister-chromatin cohesion, which is mediated by cohesin[197]. In most metazoans, at metaphase sister chromatids remain associated through cohesion only at their centromeres. This is because centromeric cohesin, which embraces both sister chromatids, is protected from degradation until anaphase. In *S. pombe*, centromeric heterochromatin is required to mediate tight physical sister-centromere cohesion by trapping high levels of centromeric cohesin. This occurs through physical association of the cohesin complex with $Swi6^{HP1}$ (REFS 198,199). In cells lacking heterochromatin, single kinetochores are disorganized and display aberrant attachment to

spindles, and sister centromeres prematurely dissociate, leading to chromosome loss and gain[200–202]. This explains the elevated frequency of chromosome loss in *S. pombe* cells with defective heterochromatin[8,202]. Sister-centromere cohesion may also be weaker in human cells with reduced levels of centromeric H3K9me heterochromatin[203].

Nine: controlling cell differentiation

Gene silencing by heterochromatin provides the capacity to control cell-type specification. An example in *S. pombe* is the silencing of two gene cassettes, *mat2P* and *mat3M*, that encode transcription factors that programme the cell mating type, either Plus (P) or Minus (M). The heterochromatin domain that silences these cassettes is called the *mat2–mat3* region. Lineage-regulated recombination places copies of these transcription-factor-encoding genes into the expression site (*mat1*), thereby producing a switch in mating type, P to M or M to P[204,205]. In addition to the silencing of *mat2* and *mat3,* H3K9me-heterochromatin has a role in regulating the directionality of this recombination and therefore the pattern of mating-type switching so that P-to-P and M-to-M are disfavoured[205].

In mammals, megabase-sized islands of H3K9me-dependent heterochromatin are formed in a cell-type-specific manner[206]. One function of these islands is to form a barrier to transcription-factor-mediated cell-type reprogramming; hence, they are termed ‘differentially bound’ or ‘reprogramming-resistant regions’ (FIG. 5a). This type of heterochromatin is important for preserving the cell-type identity of differentiated cells, as depletion of proteins involved in maintenance of this heterochromatin — chromatin assembly factor 1 (CAF1), SET domain bifurcated 1 (SETDB1), KRAB-associated protein 1 (KAP1; also known as transcription intermediary factor 1β and TRIM28) — enables efficient reprogramming of differentiated cells to induced pluripotent stem cells[206–210] or of somatic nuclei transferred into oocytes[211]. The determinants required to establish these large heterochromatin islands in *cis* remain unknown, but heterochromatin nucleation may be linked to mechanisms that silence endogenous retroelements (EREs), including endogenous retroviruses (ERVs), and neighbouring genes in somatic cells (FIG. 5b). A family of Krüppel-associated box zinc-finger (KRAB-ZFP) proteins recruits the H3K9 methyltransferase SETDB1 to EREs through the adaptor KAP1, where they elicit the formation of repressive heterochromatin[212–215]. Thus, ancient transposable elements appear to have been co-opted for the regulation of adjacent chromatin and genes.

Ten: medical relevance

Heterochromatin function is involved in various aspects of human health. We focus here on a handful of examples that illustrate and extend some of the principles introduced above.

Viral dormancy.

Heterochromatin protects genomes from pathogenic viruses. A fraction of genomic integrations of HIV-1 can occur in heterochromatin regions[216]. Retroviral reporters in lymphocyte cell lines are subject to silencing by H3K9me-dependent heterochromatin through the human silencing hub (HUSH) complex, which includes the proteins M-phase phosphoprotein 8 (MPP8), periphilin 1 (PPHLN1), TASOR (transgene activation suppressor

protein; also known as FAM208A) and SETDB1. HUSH spreads across the viral genome from neighbouring heterochromatin[217] (FIG. 5c). Although speculative, silencing of integrated HIV-1 viruses may allow dormant viral genomes to persist in T cells long after therapeutic clearance of circulating virus is achieved. Sporadic reactivation of these proviruses could allow the later reappearance of viruses. Interestingly, a distinct chromodomain protein, MPP8, and not HP1, binds HUSH-installed H3K9me3. Other human viruses may also be rendered dormant by HUSH-mediated heterochromatin spreading[216]. HUSH-directed silencing is distinct from that mediated by KRAB-ZFPs, which target heterochromatin formation to ERVs and EREs (see above)[212–216,218].

Obesity.

The increasing frequency of obesity and its associated health risks in humans have a heritable component. Intriguingly, KAP1 haploinsufficiency in mice results in stochastic production of either normal or obese offspring from genetically identical parents. Analyses of human lean and obese cohorts indicate that KAP1 expression levels correlate with expression patterns of key obesity-associated genes and with body mass index[219].

Premature ageing.

The progeroid (premature ageing) Werner syndrome is caused by mutations in the *WRN* gene, which encodes a helicase. *WRN*-null human mesenchymal stem cells display disrupted heterochromatin with loss of H3K9me3 from heterochromatin islands[220]. The WRN protein is targeted to centromeric repeats and associates with the H3K9 methyltransferase SUV39H1 and HP1α. This WRN complex may stabilize repeat arrays within heterochromatin, thereby preventing DNA damage. Comparison of primary human mesenchymal stem cells from young and old individuals revealed reduced levels of WRN protein and heterochromatin loss in the cells of old individuals. This implies that WRN protects heterochromatin and thereby prevents the irreversible genome instability associated with ageing. Alternatively, DNA damage associated with defective WRN might induce the loss of heterochromatin.

Metabolism.

DNA and histone methyltransferases and demethylases require metabolites for their function (reviewed in REFS 221,222). *S*-Adenosyl-methionine is the methyl donor used by nucleic acid and histone methyltransferases. Many demethylases require α-ketoglutarate, which is a metabolic intermediate of the Krebs cycle, as a co-substrate, whereas other demethylases utilize flavin adenine dinucleotide. Acetyl-CoA is the acetyl donor used by histone acetyltransferases, and the sirtuin family of histone deacetylases requires NAD^+ as a cofactor. Consequently, nutritional changes or mutations that affect levels of metabolites can cause the accumulation of inhibitors of writers and erasers, which can alter chromatin.

Mutations in the genes encoding the Krebs cycle enzymes isocitrate dehydrogenase, fumarate hydratase and succinate dehydrogenase cause the accumulation of their substrates 2-hydroxyglutarate, fumarate and succinate, respectively. These metabolites are competitive inhibitors of α-ketoglutarate-dependent histone and DNA demethylases[223,224]. Consequently, such mutations promote tumorigenesis. Accumulation of 2-hydroxyglutarate

results in elevated H3K9me levels and blocks cellular differentiation[223]; conversely, provision of α-ketoglutarate to embryonic stem cells reduces histone and DNA methylation and promotes pluripotency whereas succinate has the opposite effect. Histone methylation in embryonic stem cells is sensitive to glutamate and thus to fluctuations in α-ketoglutarate levels[225]. Poor nutrient availability is a feature of many solid tumours, the interiors of which are deprived of glutamine and hence of α-ketoglutarate, leading to elevated histone methylation and cellular dedifferentiation within such tumours[226]. In *S. cerevisiae,* equivalent mutations to those that cause 2-hydroxyglutarate accumulation were found to enhance SIR-mediated silencing by inhibiting H3K36 methyltransferases[227].

Concluding remarks

We have discussed fundamental principles that have emerged from the study of heterochromatin in a broad range of organisms. Among many unanswered questions in the field, several stand out. What are the signals that initially trigger heterochromatin at specific sites? What determines the heritability or lack of herit ability of heterochromatin? What is the role of liquid-liquid phase separation in maintaining heterochromatin integrity? What enables transcription at heterochromatin regions? How is heterochromatin regulated during stress and development? Addressing these outstanding questions will require new model organisms and technologies as well as ingenious experimental strategies.

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Glossary

Post-translational modifications (PTMs)
Chemical groups (such as methyl or acetyl) on amino acid side chains that are enzymatically added by ‘writer’, removed by ‘eraser’ and recognized by ‘reader’ protein modules.

Satellite repeats
Short repetitive sequences that exhibit a distinct satellite peak on buoyant density gradients ‘owing ho their skewed base composition.

Constitutive heterochromatin
In most eukaryotes, heterochromatin that is consistently termed throughout the cell cycle and in many cell types, for example, centrome-reassociated heterochromatin.

Facultative heterochromatin
Locus-specific and cell-type-specific heterochromatin, for example, the inactive X chromosome in mammals.

Chromoshadow domain (CSD)

Dimerization domain in heterochromatin protein 1 -related proteins that forms a peptide-binding groove at the dimer interface that can recruit additional heterochromatin proteins.

Argonaute
Proteins with PAZ and Piwi domains that are loaded with small RNAs, which target them and their associated proteins to long RNAs that bear homology to the small RNA.

Pericentromeric heterochromatin
Large blocks of heterochromatin formed on the tandem repeats that surround the centromere–kinetochore region.

X chromosome inactivation
Mechanism of dosage compensation in female mammals in which one of the two X chromosomes is inactivated by the formation of facultative heterochromatin.

X-inactive specific transcript (XIST)
Long noncoding RNA that designates the X chromosome from which it is expressed for X chromosome inactivation.

Piwi-associated RNAs (piRNAs)
Small RNAs associated with Piwi members of the Argonaute protein superfamily, which promotes repression of transposable elements in animal gonads.

R-loops
Nascent RNA that remains associated with its DNA template through hybridization, thereby dislodging the opposite, nontemplate DNA strand.

Heterochromatin islands
Extensive domains of heterochromatin on chromosome arms, which are distinct from the main centromeric and telomeric heterochromatin domains.

Reprogramming-resistant regions
Large lineage-specific chromosomal regions that are assembled into heterochromatin and thus resist binding by reprogramming factors.

Endogenous retroelements
Mobile elements that replicate through reverse transcription followed by genomic integration. The term also includes degenerate, immobile elements.

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Box 1 |

Heterochromatin history

The term heterochromatin was first coined in 1928 by Emil Heitz, who developed chromatin staining methods and found that chromosomes are composed of regions that are not stained after telophase (euchromatin) and regions that stained throughout the cell cycle (heterochromatin). He noted that staining patterns are chromosome-specific and later suggested that genes are found in euchromatin, whereas heterochromatin is genetically inert. He also noted that heterochromatin is often associated with sex chromosomes (reviewed in REF. 228). Finally, Heitz recognized that some regions are stained only in certain cells; these were later termed facultative heterochromatin to distinguish them from constitutively stained regions, which were dubbed ‘constitutive heterochromatin’.

In the early 1930s, Hermann Muller isolated the experimentally induced white mottled mutations in *Drosophila melanogaster,* which exhibited a mosaic or variegated pattern of red (wild-type) or white (mutant) eye facets as a result of chromosome rearrangements that displaced the white gene from its original position[229]. In 1936, the examination of *D. melanogaster* polytene chromosomes revealed that variegating mutants were freguently associated with rearrangement breakpoints in heterochromatic regions[230]. Thus, the vague cytological entity ‘heterochromatin’ became intertwined with a phenomenon that was termed ‘position-effect variegation’, which refers to phenotypic variegation due to variable inactivation of a gene triggered by its placement in or near heterochromatin. Extra copies of heterochromatic chromosomes were found to alleviate position-effect variegation, perhaps because they compete for binding by limiting factors[231,232]. Later, mutations were isolated in single genes that increased or decreased the variegated eye colour phenotype[233–235].

In the 1960s, reassociation kinetics of sheared denatured DNA revealed that a substantial fraction of eukaryotic genomes is repetitive[236]. These rapidly annealing fractions were found to correspond to genomic sequences that exhibited distinct buoyant density on CsCl gradients because of their skewed base composition relative to the rest of the genome[237–238]. Because these sequences formed an ancillary peak in the density profile, they were termed ‘satellites’. As satellite peaks form with both sheared, low-molecular-weight DNA and with high-molecular-weight DNA, it was concluded that the constituent repeats are organized in long, tandem arrays[239–241]. Because of its repetitive nature, satellite DNA was the first eukaryotic DNA to be sequenced by early methods[242,243].

The use of purified satellite DNA as labelled probes for *in situ* hybridization to metaphase chromosomes revealed that these satellites are located in the pericentromeric heterochromatin regions of metaphase chromosomes[244,245] and colocalize with dense chromatin at the nuclear periphery in interphase cells[246]. Thus, it became apparent that large blocks of constitutive pericentromeric heterochromatin contain arrays of repetitive sequences and that artificial juxtaposition of genes with such regions by a chromosomal rearrangement led to their inactivation.

The above findings, coupled with the inability to detect satellite-complementary RNA, suggested that heterochromatin is transcriptionally inactive[247]. Moreover, the differential centrifugal sedimentation of chromatin containing satellite DNA was consistent with heterochromatin being more compact[248]. In addition, satellite DNA replicated late during S phase[249] and under-replicated in polytene nuclei[250], suggesting that heterochromatin also affects DNA replication.

Box 2 |

The use of the terms ‘epigenetic’ and ‘epigenetics’

Conrad Waddington originally coined the term ‘epigenetics’ to refer to the mechanisms of acguisition of stable cell fates during development, but subseguently this definition was repeatedly modified (reviewed in REF. 251). Robin Holliday defined epigenetics as the inheritance of changes in gene expression patterns and, more generally, the inheritance of any change in gene function that does not involve a change in DNA sequence. Arthur Riggs defined epigenetics as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence. Mark Ptashne defined the phrase ‘epigenetic change’ as a heritable change in the expression of a gene that does not involve a change in its sequence and persists in the absence of the initiating signal. Conversely, Adrian Bird questioned whether heritability should be a compulsory component of a modern definition of epigenetics because it does not specify how many generations of inheritance might be required to satisfy the definition. Instead, Bird suggested as an all-encompassing definition of epigenetics “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states” (REF. 252). This chromosome-based definition excludes any number of other feedback mechanisms that can mediate heritable change without a change in DNA sequence, such as post-transcriptional positive feedback loops that occur in *Drosophila melanogaster* sex determination and in prions.

Despite these foundational differences in definition, the use of the noun ‘epigenetics’ and the adjective ‘epigenetic’ has been essentially redefined by many to refer to chemical modifications of histones and DNA because, in some cases, these are required for or contribute to a heritable change in gene expression. The adjective ‘epigenetic’ has thus been used in the context of phrases such as ‘epigenetic mark’ or ‘epigenetic modification’ in a manner synonymous with chemical modification of nucleic acids or associated proteins, or more generally as synonymous with ‘chromatin modification’. The ensemble of such modifications has been referred to as the ‘epigenome’. Such extensions, although entrenched, may be misconstrued or imply an untruth (depending on the definition being applied)—namely, that any chemical modification of a nucleic acid or associated protein mediates a heritable change in the expression of a gene.

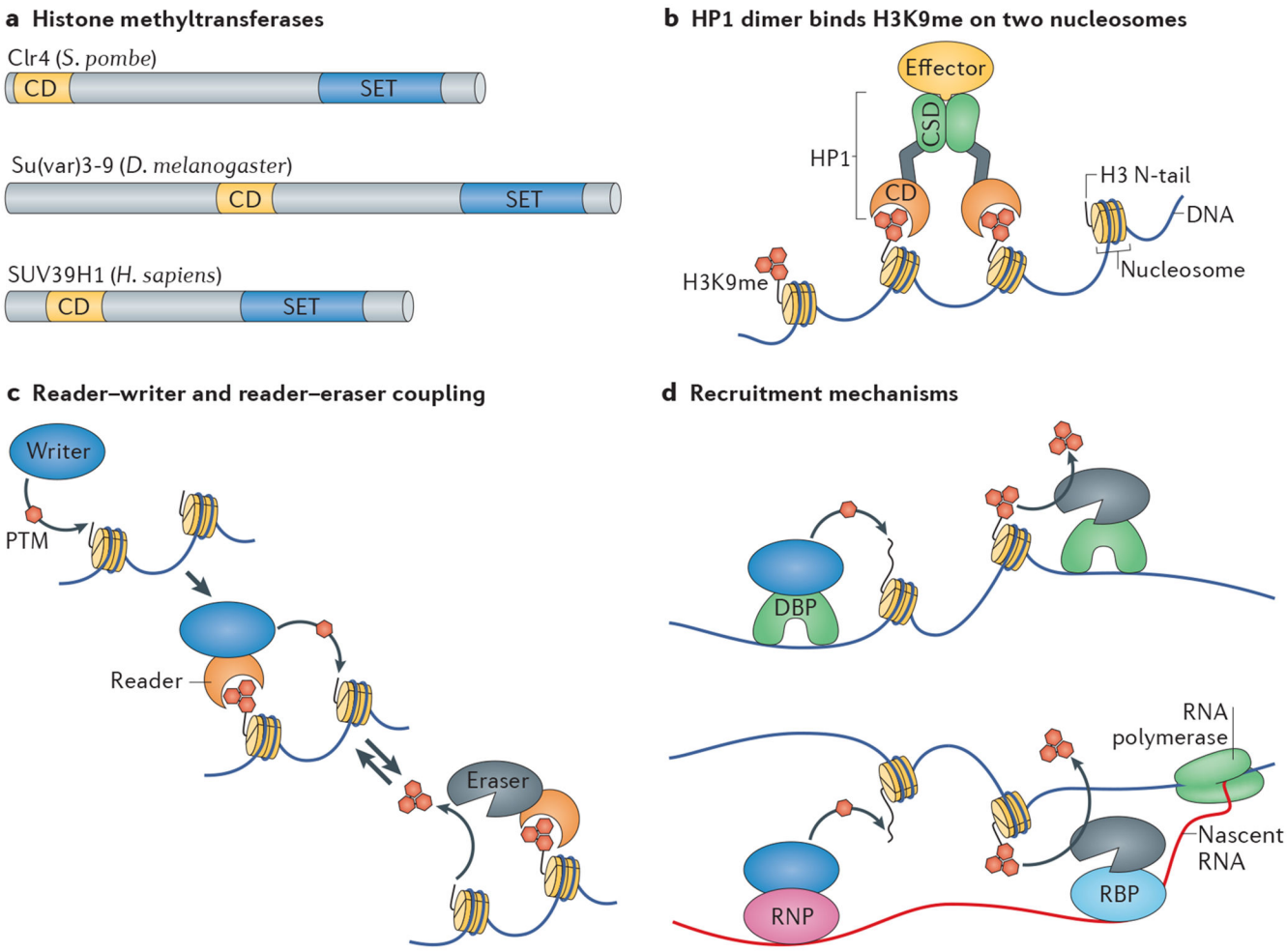

Figure 1 |. Core heterochromatin components and mechanisms.

a | The protein domain organization of the histone–lysine *N*-methyltransferases (KMTs) cryptic loci regulator 4 (Clr4) of *Schizosaccharomyces pombe*, suppressor of variegation 3-9 (Su(var)3-9) of *Drosophila melanogaster* and SUV39H1 (SU(VAR)3-9 homologue 1) of *Homo sapiens*. The SET (Su(var)3-9, enhancer of zeste and trithorax) domain isthe KMT catalytic domain and uses S-adenosyl-methionine as a methyl donor to methylate histone H3 lysine 9. The chromodomain (CD) specifically recognizes methylated histone H3 lysine 9 (H3K9me). **b** | Depiction of a heterochromatin protein 1 (HP1) dimer bound to nucleosomes modified with H3K9me (red hexagons). The chromodomain and the chromoshadow domain (CSD), which is a dimerization domain, of HP1 are shown. The platform produced by the CSD dimer enables binding of effector proteins. For simplicity, only one of the two H3 tails that protrude from the octamer core is shown on each nucleosome. **c** | Heterochromatin assembly and disassembly by reader-modifier coupling. Different ‘writer’ enzymes catalyse the addition of a post-translational modification (PTM) to a histone within a nucleosome, whereas ‘eraser’ enzymes catalyse the removal of PTMs. ‘Reader’ proteins or protein domains recognize and bind PTMs and are often coupled with writer or eraser proteins or protein domains in the same protein, protein complex or via reversible protein-protein interactions, **d** | Recruitment mechanisms. DNA-binding proteins (DBPs) can recruit writers

or erasers to chromatin (top). Alternatively, a nascent transcript associated with the RNA polymerase can harbour recognition signals for a sequence-specific and/or structure-specific ribonucleoprotein (RNP) or RNA binding protein (RBP) (bottom). The latter include the Argonaute family proteins (not shown), which recognize and bind RNA by incorporating cognate small RNAs such as siRNAs or Piwi-associated RNAs (reviewed in REF. 253). In turn the RNP or RBP can recruit writers or erasers that modify chromatin.

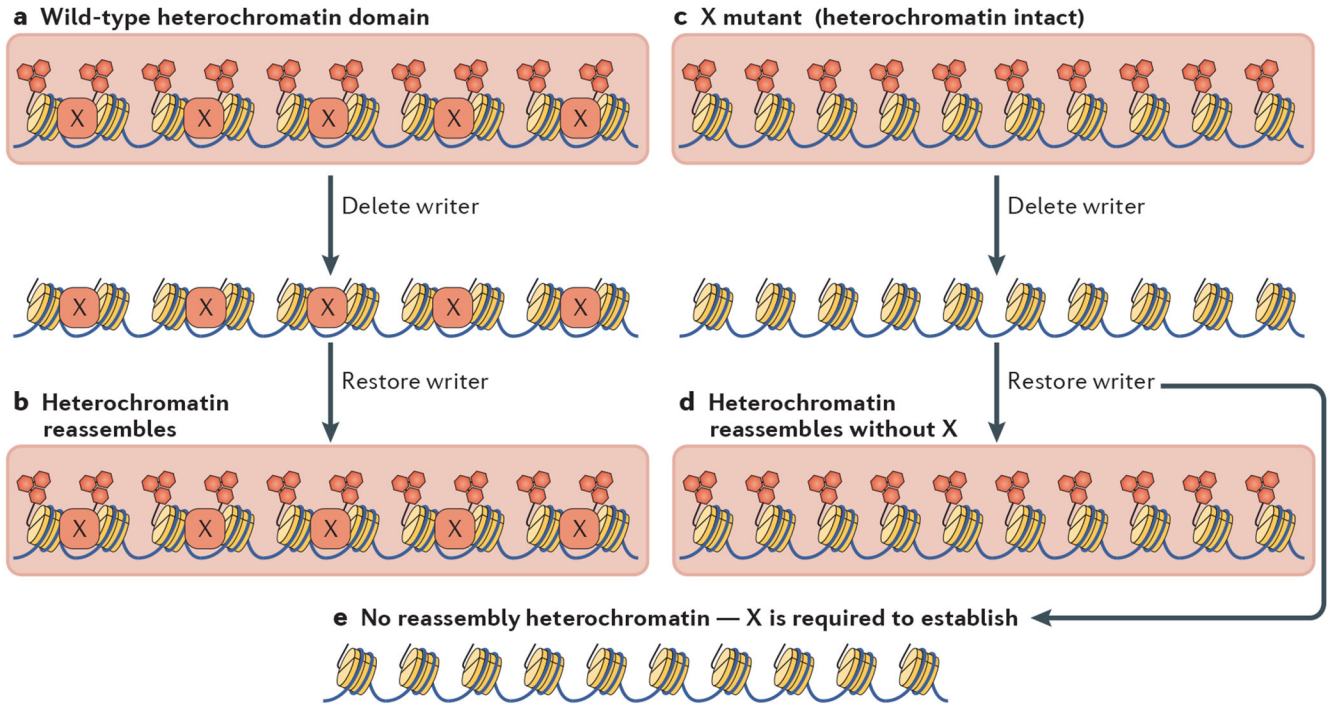

Figure 2 |. Determining whether a factor is required for the establishment, but not maintenance, of heterochromatin.

Identifying a factor that is required to maintain repressive heterochromatin is straightforward because deletion of the gene encoding that factor will disrupt heterochromatin formation and associated phenotypes such as gene silencing. Determining whether a factor has a role in heterochromatin establishment requires additional experiments. **a** | The gene for an endogenous pivotal writer is inactivated, resulting in the loss of a heterochromatin domain (large red rectangles) such as that mediated by histone H3 lysine 9 methylation (red hexagons) in these cells. A heterochromatin-associated factor (protein, RNA or post-translational modification) is marked with 'X'. **b** | Restoration of the writer to these cells allows re-establishment of a full heterochromatin domain, indicating that all factors required for heterochromatin nucleation, spreading and maintenance are present, including factor X. **c** | Cells lacking the heterochromatin-associated factor X are similarly tested. Note that X may be required for heterochromatin establishment but not strictly required for maintenance. **d** | The full assembly of a silent heterochromatin domain upon restoration of the writer indicates that X is not required for nucleating heterochromatin formation. **e** | The inability to re-establish a full heterochromatin domain indicates that X is required to trigger heterochromatin assembly but is not required for its maintenance. RNAi in *Schizosaccharomyces pombe* and the long noncoding RNA X-inactive specific transcript in mammals are examples of such heterochromatin establishment factors.

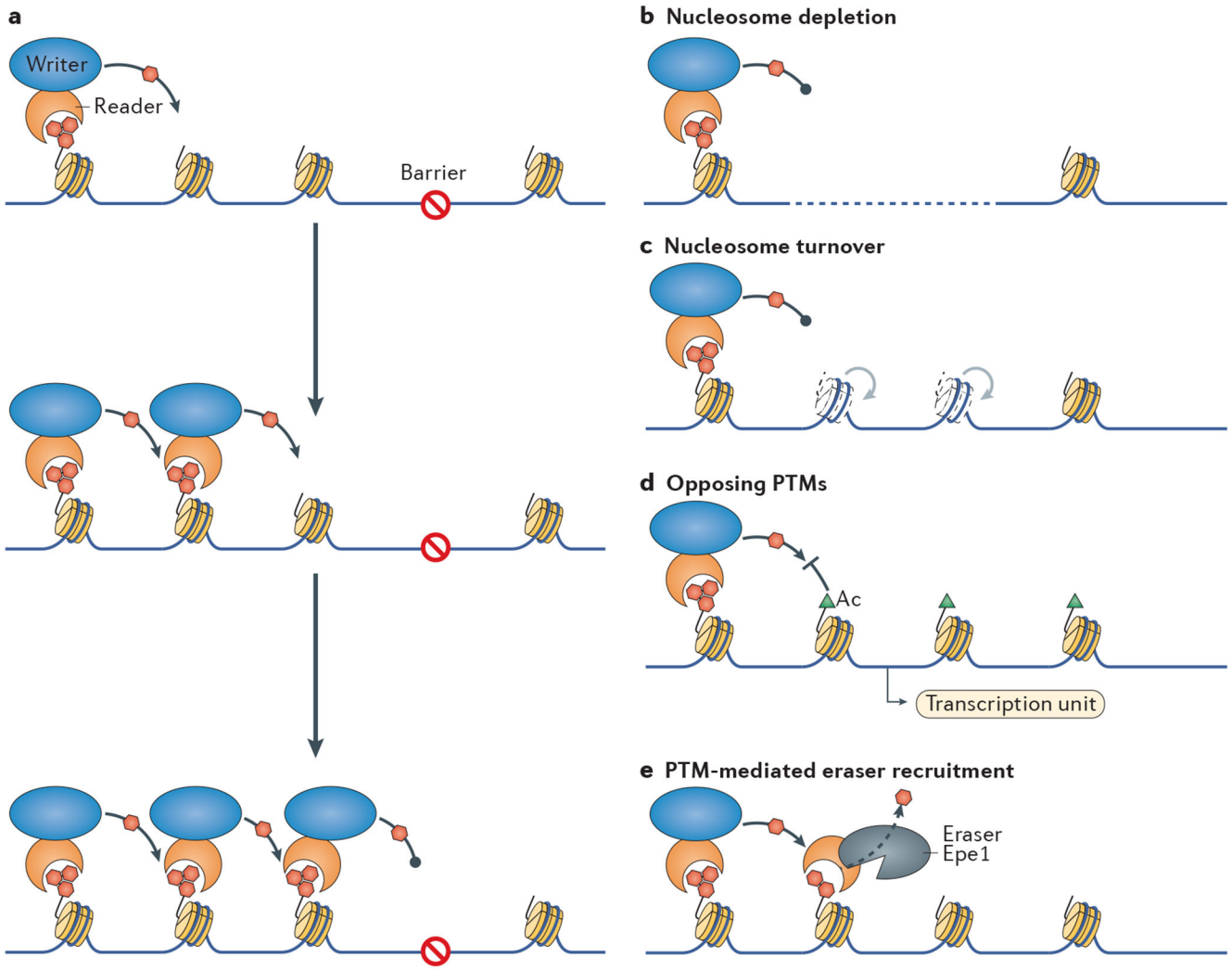

Figure 3 |. The regulation of heterochromatin spreading.

a | A model for the expansion of a heterochromatin domain, in which a 'reader' is associated with a 'writer', thereby enhancing the formation of repressive histone post-translational modifications(PTMs; red hexagons) in adjacent nucleosomes. Iterative cycles result in the formation of extensive heterochromatin domains. The barrier represents a series of mechanisms that restrict such spreading, which are shown in parts b–e. **b** | Sequencesthat are bound by factors that disfavour nucleosome assembly create extensive gaps (dashed line) that prevent heterochromatin from spreading. **c** | Factors that promote nucleosome turnover through disassembly and reassembly and/or through cycles of histone exchange (light nucleosomes and arrows) effectively block heterochromatin domain expansion. **d** | Adjacently expressed transcription units mediate the addition of active PTMs (green triangles) to histones, which prevent the intrusion of repressive PTMs and heterochromatin. **e** | Erasers such as the *Schizosaccharomyces pombe* demethylase enhancer of position effect 1 (Epe1) are recruited by readers of repressive PTMs at the edge of heterochromatin and prevent heterochromatin expansion. Ac, acetylation.

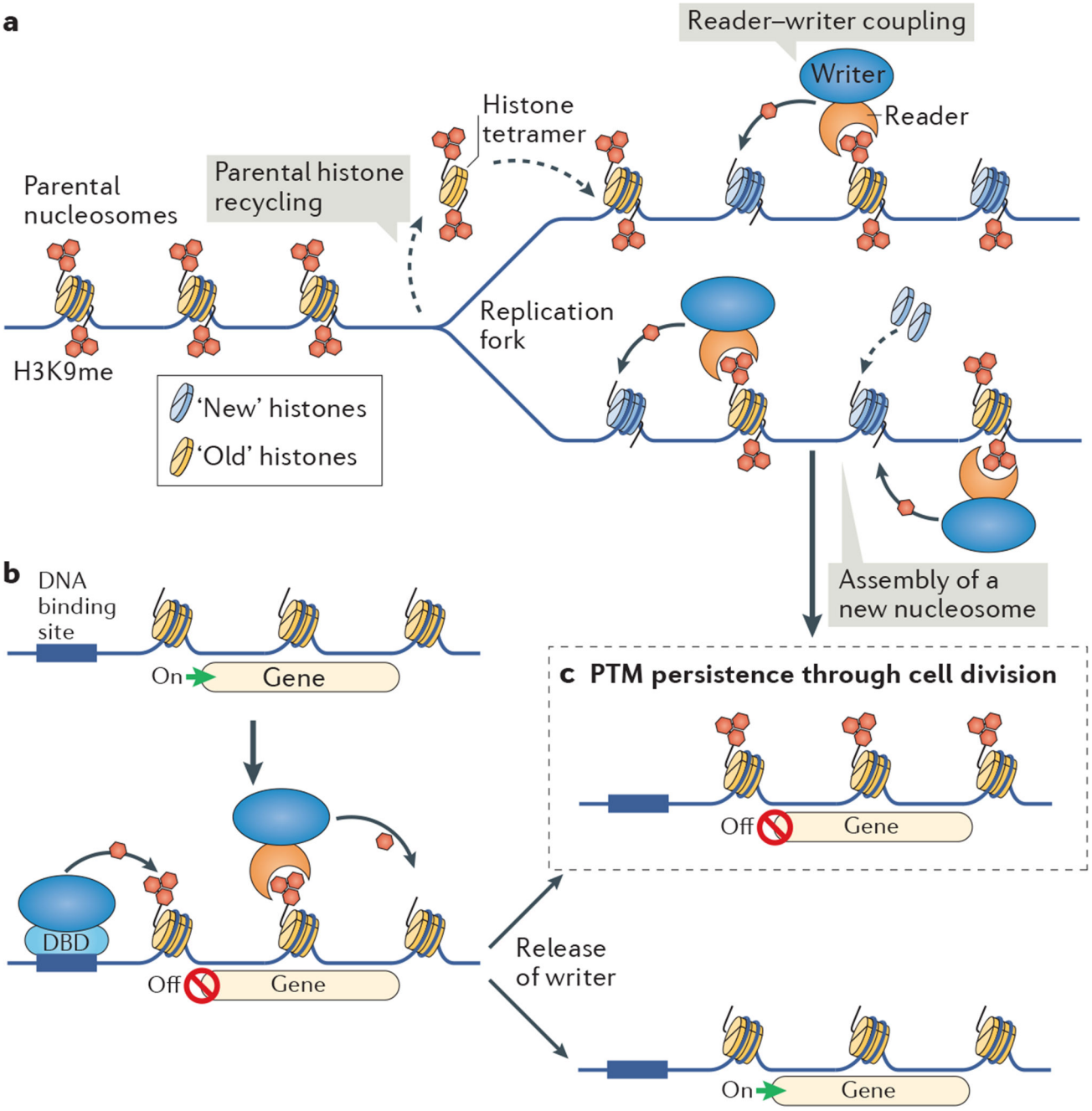

Figure 4 |. Reader–writer coupling allows the maintenance of repressive chromatin modifications through DNA replication and their transmission through cell division.

a | The maintenance of repressive histone post-translational modifications (PTMs) through DNA replication by reader–writer coupling. During replication, H3–H4 tetramers from pre-existing parental 'old' nucleosomes are randomly recycled to either of the two newly synthesized DNA molecules. Consequently, the number of H3 histones bearing a PTM, such as methylation of H3 Lys 9 (H3K9me), on the two new DNA molecules will be reduced by half compared with the parental DNA. Reader–writer coupling should enable propagation of

the PTM from old nucleosomes that retained the PTM to newly assembled nucleosomes, thereby replenishing PTM levels and reinstating the full chromatin domain on both sister chromatids, ultimately allowing its transmission to progeny cells, **b** | A writer module such as the SET (Su(var)3-9, enhancer of zeste and trithorax) domain of an H3K9 methyltransferase, can be artificially recruited to DNA by its fusion to a DNA-binding domain (DBD) whose binding site is inserted at a neutral genomic location. This generates a region with a specific, newly catalysed chromatin PTM such as H3K9me, which can recruit additional reader–writers that can spread the PTM over a nearby reporter gene, thereby silencing its expression. Release of the artificial writer from DNA by inhibition of its DBD enables assessment of the persistence and heritability of this heterochromatin. **c** | If heterochromatin and gene silencing persist through cell division (by the mechanism shown in part **a),** then the modification, in this case H3K9me, must be capable of mediating a heritable epigenetic change (BOX 2).

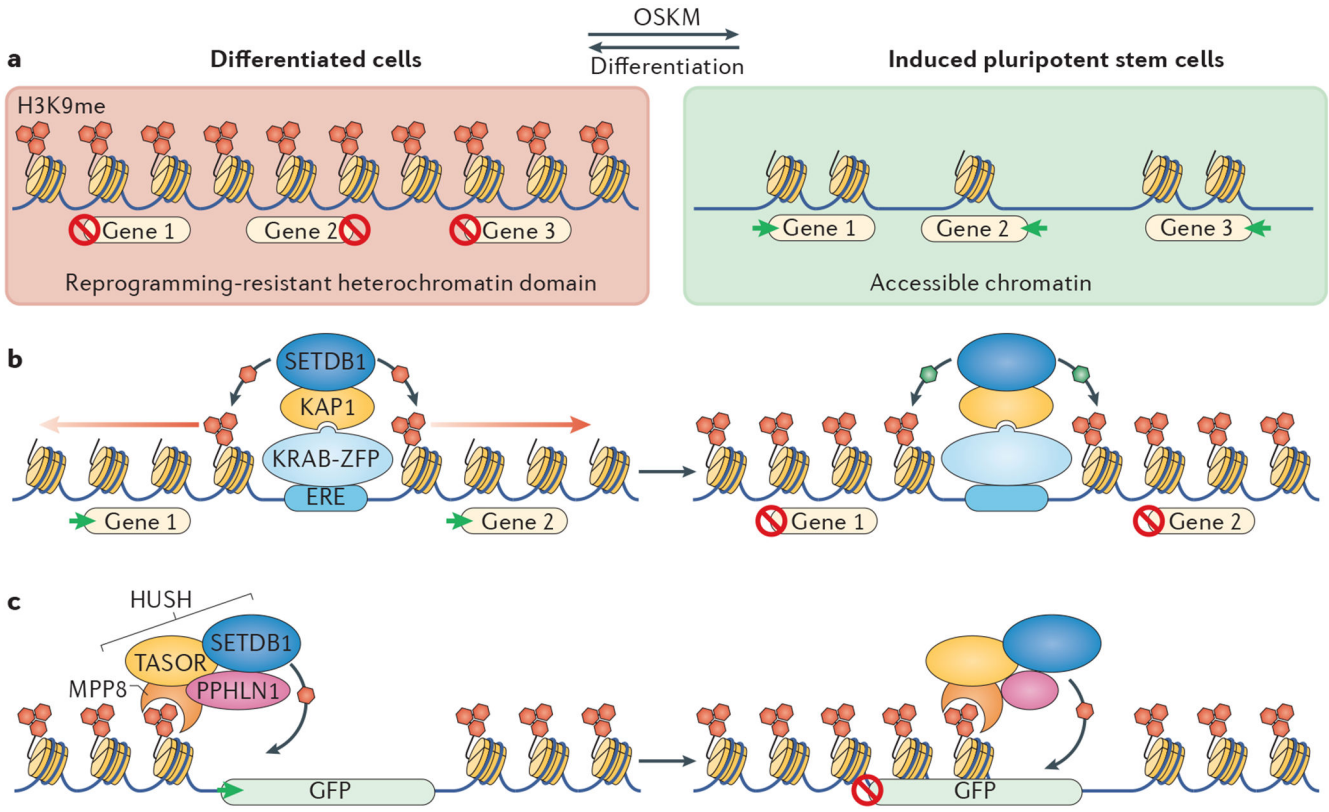

Figure 5 |. Heterochromatin functions in mammalian cells.

a | The forced expression of four transcription factors (OCT4, SRY-box 2, Krüppel-like factor 4 and MYC, collectively known as OSKM) induces dedifferentiation of somatic cells into induced pluripotent stem cells. Such cell-type reprogramming is inefficient because large heterochromatin domains (depicted in the large red rectangle) present a barrier to the activation of key genes that are reguired for pluripotency. Reprogramming efficiency can be increased by depletion of proteins that are reguired for heterochromatin maintenance, thereby allowing activation (large green rectangle) of reprogramming pathways. **b** | In mammalian cells, histone H3 Lys 9 methylation (H3K9me)-dependent heterochromatin formation can be nucleated by transposable elements such as endogenous retroelements (EREs). EREs are bound by members of the large family of Krüppel-associated box zinc-finger proteins (KRAB-ZFPs), which recruit the H3K9me writer methyltransferase SET domain bifurcated 1 (SETDB1) through the adaptor protein KRAB-associated protein 1 (KAP1). This in turn allows the recruitment of H3K9me readers (such as heterochromatin protein 1) and writers to expand the heterochromatin domain. Heterochromatin spreading can silence adjacent genes, suggesting that remnants of transposable elements have been co-opted for defining and regulating heterochromatin domain formation. **c** | Retroviral GFP reporter constructs can be silenced by heterochromatin spreading mediated by the human silencing hub (HUSH) complex, which comprises the proteins M-phase phosphoprotein 8 (MPP8), periphilin 1 (PPHLN1), transgene activation suppressor protein (TASOR) and SETDB1. MPP8 binds flanking H3K9me and recruits SETDB1 through the adaptor protein TASOR. This silencing mechanism may be used to render pathogenic viruses latent. HUSH

might also promote the formation of heterochromatin islands by mediating spreading from dispersed repeats, transposable elements or EREs.