Title

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Journal

Open Biology, 13(11)

Authors

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Publication Date

2023-11-01

DOI

10.1098/rsob.230271

OPEN BIOLOGY

royalsocietypublishing.org/journal/rsob

Review

Cite this article: Hamali B, Amine AAA, Al-Sady B. 2023 Regulation of the heterochromatin spreading reaction by *trans*-acting factors. *Open Biol.* **13**: 230271. https://doi.org/10.1098/rsob.230271

Received: 10 August 2023
Accepted: 3 October 2023

Subject Area:
genetics/molecular biology/biochemistry

Keywords:
heterochromatin spreading, gene silencing, *trans*-acting factors, polycomb, Suv39, Silent Information Regulator

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THE ROYAL SOCIETY PUBLISHING

Regulation of the heterochromatin spreading reaction by *trans*-acting factors

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Heterochromatin is a gene-repressive protein–nucleic acid ultrastructure that is initially nucleated by DNA sequences. However, following nucleation, heterochromatin can then propagate along the chromatin template in a sequence-independent manner in a reaction termed spreading. At the heart of this process are enzymes that deposit chemical information on chromatin, which attracts the factors that execute chromatin compaction and transcriptional or co/post-transcriptional gene silencing. Given that these enzymes deposit guiding chemical information on chromatin they are commonly termed 'writers'. While the processes of nucleation and central actions of writers have been extensively studied and reviewed, less is understood about how the spreading process is regulated. We discuss how the chromatin substrate is prepared for heterochromatic spreading, and how *trans*-acting factors beyond writer enzymes regulate it. We examine mechanisms by which *trans*-acting factors in Suv39, PRC2, SETDB1 and SIR writer systems regulate spreading of the respective heterochromatic marks across chromatin. While these systems are in some cases evolutionarily and mechanistically quite distant, common mechanisms emerge which these *trans*-acting factors exploit to tune the spreading reaction.

1. Introduction

Heterochromatin is a gene-repressive chromatin structure that has been visualized cytologically for over a century. The name was coined by Emil Heitz and describes chromosomal domains that remain condensed throughout the cell cycle [1]. We have learned much about heterochromatin over the century, yet its behaviours remain important to explore, given its central role in the eukaryotic cell: constitutive heterochromatin shapes the normal functioning of the genome, while facultative heterochromatin, which can change across lineages, directs normal development in multicellular organisms. In some ways, both types of heterochromatin, but especially facultative heterochromatin, are formed by a process similar to the activation of transcription: DNA sequences dictate the local recruitment of repressive factors [2,3]. The field terms those 'nucleation sites' rather than promoters and enhancers. What has remained intriguing about heterochromatin is its ability to propagate itself outwards from such DNA-sequence encoded signals for significant distances along the chromosome. This sequence-independent extension of heterochromatin is a process referred to as 'spreading' [4] and encompasses both the chromosomal extension of function and structure (i.e. gene expression and the associated changed chromatin state and protein composition). This process is highly dosage-sensitive to key regulators, which was for example observed for subtelomeric silencing by the Silent Information Regulator (SIR) proteins in *S. cerevisiae* [5–7], and position effect variegation in *D. melanogaster* [8]. At the heart of nucleation and spreading is the action of the central enzymes (writers) that deposit repressive chromatin marks that signal the assembly of the gene repressive heterochromatic structure. Much has been written about the properties of the writer enzymes, here we want to explore how *trans*-acting factors enable

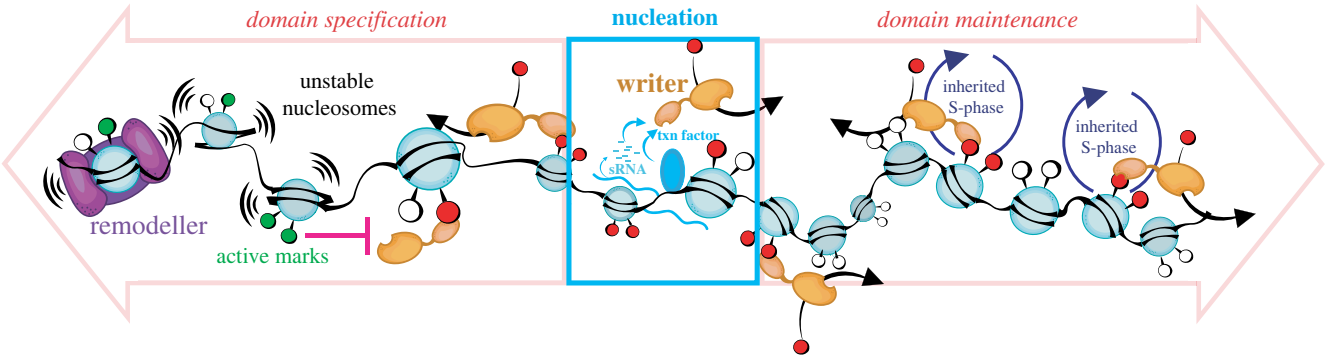

Figure 1. Heterochromatin spreading occurs in different chromatin environments and on substrates of different histories. 'Writers' are nucleated directly via transcription factors or indirectly via small RNA processes (centre). The writer then can spread on chromatin that was previously heterochromatic (domain maintenance, right), thus inheriting nucleosomes through S-phase, enabling positive feedback. The region also may be diminished in spreading-antagonizing transcriptional activities, such as in repetitive regions. The writer may also spread into a region de novo (domain specification, left), where it has to contend with multiple antagonizing activities, including nucleosome destabilization and inhibitory chromatin marks.

and control this process by writers across systems and examine some unique and shared characteristics.

2. General substrate requirements for heterochromatin spreading

While the heterochromatin systems we will discuss below do have some differences in their requirements for spreading away from DNA-encoded nucleation sites, there are some universal features of the substrate, chromatin, that either encourage spreading or hinder it. On a first level, it is useful to think of heterochromatin spreading as a reaction that can occur to re-establish the initial stage (domain maintenance), or, to establish a repressed domain for the first time (domain specification, we avoid the term establishment as this typically refers to the first nucleation event; figure 1). The chromatin environments in these two contexts are rather different: in the case of maintenance, this occurs in regions that have already been repressed and may be additionally gene-poor, such as constitutive heterochromatin. Here, RNA polymerase is typically less active (although, for co-transcriptional gene silencing, some transcription does occur). Nucleosomes bearing repressive marks, such as methylation at H3 lysine 9 or 27, are partially inherited (figure 1), which directly facilitates the re-establishment of the initial state by the writer enzymes. This is because writer enzymes exploit positive feedback encoded in those inherited methylated nucleosomes: As 'read-write' enzymes, the writers recognize their reaction product in the modifying subunit or other complex subunit, which facilitates further modification via a variety of mechanisms [9–13]. This type of positive feedback has long been predicted by theoretical approaches to be required for the formation of a stably repressed domain (e.g. [14]).

By contrast, a newly specified heterochromatin domain cannot inherit pre-modified nucleosomes, therefore, the initial specification by spreading does not have the opportunity to exploit this positive feedback. Moreover, the chromatin template is more hostile to heterochromatin: genes are active, and transcription can directly or indirectly abrogate spreading. It does so largely in two ways: first, destabilizing nucleosomes, or even creating nucleosome-free regions (figure 1). Just as nucleosome-free regions 'poison' spreading [15], unstable nucleosomes can inhibit spreading as well, especially in systems that need to reach a fully methylated state for repression and spreading (PRC2, Suv39, SETDB1; figure 2). This trimethyl state is often critical for gene silencing, for example, H3K9me2 can be permissive to transcription [16], and the trimethyl state can be instructive for positive feedback [9]. Methyl writers are thought to require stable nucleosomes to reach the fully methylated state as they are not processive for trimethylation on the nucleosome substrate, e.g. Suv39h1 [17]. The *in vivo* appearance kinetics of H3K27me3 also suggest that PRC2 is not primarily processive for the terminal state [18,19]. In a distributive mode and relatively slow kinetics, continuous residence of the target is essential for reaching trimethylation. Hence, these heterochromatin systems are sensitive to nucleosome turnover: factors that mobilize or stabilize nucleosomes antagonize and promote spreading, respectively. Second, active genes can contain chromatin marks that directly antagonize the enzyme itself. For example, H3K4 or K36 trimethylation, which mark active genes, can inhibit heterochromatic enzymes such as G9a/GLP and Suv39 [20], as well as PRC2 [21,22]. The SIR3 protein which spreads SIR heterochromatin is directly inhibited in its ability to bind nucleosomes by chromatin marks associated with gene activity [23,24] (figure 1). This antagonism can be critical to rejecting ectopic nucleation and spreading into active genes.

Another broad level of regulation is the nuclear compartment. It has been known for a long time that hetero- and euchromatin segregate into different nuclear compartments, for example via microscopy approaches, a general finding that was re-emphasized by chromosome contact mapping [25,26]. Some of the heterochromatic compartments may be in a different biophysical state than euchromatin, i.e. in a phase condensate [27,28] (see below). Whether or not such condensates represent a requirement for heterochromatin spreading, for example by providing a more permissive environment, remains less understood. However, newer theoretical efforts by the Mirny and Jost/Vaillant groups have emphasized the requirement for self-attraction in three-dimensional space and compaction of the repressed domain in space for accurate reformation by spreading. In this way, modified nucleosomes are brought into close proximity with those yet to be modified [29,30]. On the more local scale, three-dimensional contacts appear necessary for efficient spreading [31,32]. Together these theoretical advances may provide a rationale for *trans*-acting factors promoting local looping, long-range contacts, spatial and/or biophysical segregation to enable heterochromatin spreading.

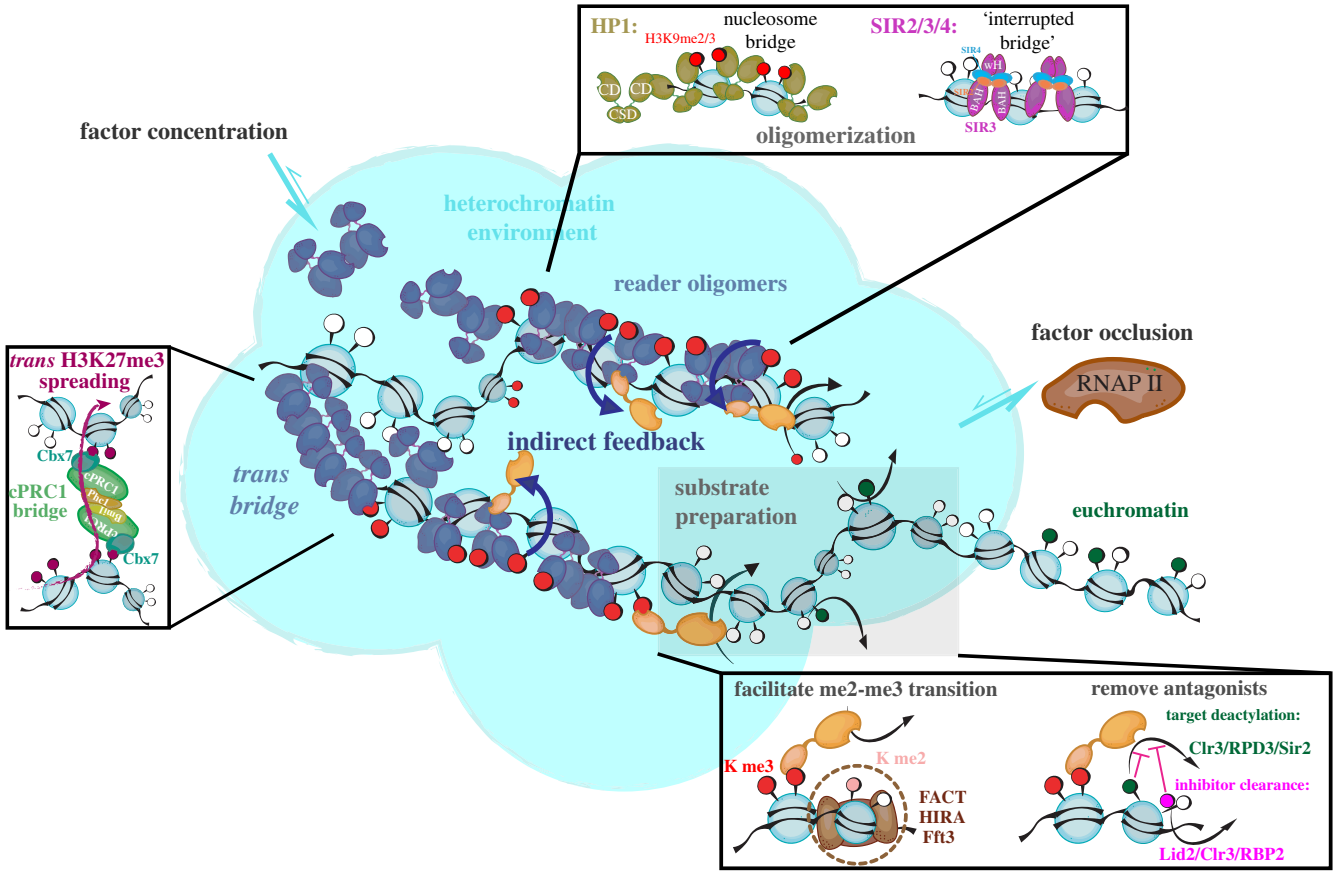

Figure 2. Mechanisms by which *trans*-acting factors promote spreading. Central *trans*-acting factors produce a heterochromatin niche, either via condensates, or subnuclear localization, where heterochromatin factors are enriched, and transcription-activating factors are de-enriched. These central *trans*-acting factors direct feed into the positive feedback of the writer and stabilize the chromatin substrate via oligomerization, which also promotes spreading via the positive feedback. TOP BOX: HP1 and Sir3 are examples of two proteins that cross-bridge nucleosomes via their oligomeric properties. HP1 domains highlighted: chromodomain (CD, me2/3 binding and oligomerization), chromo shadow domain (CSD, dimerization). Sir3 domains highlighted: winged helix (wH, di- and oligomerization), bromo-adjacent homology (BAH, nucleosome binding). LEFT BOX: Long-range spreading (trans-spreading is enabled by canonical PRC1 (cPRC1) via its H3K27me binding and oligomeric properties. RIGHT BOX: The chromatin substrate is prepared for productive spreading by factors that stabilize nucleosomes, such as FACT, Fft3 or HIRA. This allows productive methylation to the trimethylated state, which is required for spreading and silencing in the case of Suv39 and PRC2 'writers'. *Trans*-acting factors also remove occluding (on the substrate lysine) and antagonizing (inhibit writer activity on substrate lysine) marks.

3. Factors promoting the spreading of H3K9me3 via Suv39 'writers'

3.1. Position effect variegation and the early identification of *trans*-acting regulators

A great deal of our understanding of heterochromatin formation in the last few decades derives from studies with *Drosophila* and the position-effect variegation (PEV) phenomenon. PEV occurs when a normally expressed gene becomes silenced in some cells. Silencing results from a change in the gene position, for example due to recombination (i.e. when the gene becomes juxtaposed to heterochromatin), hence the name 'position effect' [8,33–35]. Subsequently, PEV has been observed in a variety of organisms including yeasts and mammals [36,37]; but it primarily has been used in *Drosophila* as a tool to study heterochromatin formation [38,39]. A fly line with a PEV phenotype was used to screen for mutations that are either suppressors or enhancers of the phenotype. Approximately 150 genetic loci have been identified from such screens including suppressors of variegation Su(var) as well as enhancers of variegation E(var) [8,38], with a smaller fraction cloned and described. The screens revealed that the *su(var)3–9* mutant has a dominant effect over the majority of PEV modifier mutations. Later, mammalian SU(VAR)3–9 homologous (human SUV39H1 and murine Suv39h1) were shown to be histone methyltransferases (HMTs) that selectively methylate lysine 9 of the histone 3 (H3K9me) tail through their SET domains [40]. The fact that the mutant identifier, deriving from the chromosome number and linkage group, ended up matching the lysine target was a happy coincidence.

3.2. The central role of HP1 in positive feedback

Another important modifier identified from the screens is SU(VAR)2–5, which encodes a heterochromatin-associated protein (now called HP1a) [41,42]. HP1a interacts with many other chromosomal proteins and contains two conserved domains, an amino-terminal chromo (CD) and a carboxy-terminal chromo-shadow domain (CSD) along with a variable hinge region. HP1a belongs to a highly conserved family of chromatin proteins, with homologous present from fission yeast (Swi6, Chp2) to humans (HP1α-γ) [2,43]. The CD of HP1 binds the product of SU(VAR)3–9, H3K9me. The combination of H3K9me recognition and HP1's ability to dimerize (via the CSD) and multimerize (via the CD) makes HP1 a central spreading regulator [44–46]. HP1 binds to both the H3K9me mark on one nucleosome and the neighbouring nucleosome via a bridging

interaction [47]. This nucleosome-bridging by HP1 in turn promotes H3K9me spreading [48,49]. It does so primarily via the recruitment of SU(VAR)3–9, which in turn produces more H3K9me. In *Drosophila* for instance, the N-terminus of SU(VAR)3–9 was found to interact with the HP1 CSD both *in vitro* and *in vivo* [50]. HP1 thus produces a positive feedback loop of H3K9 methylation across the chromatin fibre, bringing in more SU(VAR)3–9 at the edge of the spreading heterochromatin domain. In addition, oligomerization and bridging itself are central to the spreading process, stabilizing the nascent heterochromatin domain [2,9,47]. HP1s role in spreading has been studied in quite some detail in *S. pombe*: The cryptic loci regulator 4 (Clr4, the fission yeast SU(VAR)3–9 homologue), initiates H3K9 methylation independently of Swi6 (the main *S. pombe* HP1 homologue), but then the subsequent spreading of H3K9 methylation across the domain is Swi6-dependent [51]. Whether this occurs in mammals primarily via direct Suv39 recruitment, nucleosome bridging, or downstream interactions with other proteins (see below) is not fully clear.

3.3. Recruitment of additional spreading regulators by HP1

Beyond this central positive feedback and signal amplification role of HP1 it further contributes to spreading in two ways: first, HP1 recruits to H3K9me marked chromatin a diverse set of factors (more than 100 putative interacting proteins were identified by mass spectrometry) including chromatin remodellers and modifiers, such as histone deacetylases (HDACs) [52–57]. These factors, besides executing the actual gene silencing actions, produce an environment more favourable to H3K9 methylation, via removal of antagonistic activities, such as acetylation and transcription, or direct promotion of the stability of the heterochromatic state. For instance, Swi6 recruits Clr3, a fission yeast homologue of mammalian class II HDACs, which promotes spreading and maintains heterochromatin through the stabilization of H3K9me3 [58–60]. The trimethylated state is required for the transcriptionally silent heterochromatin in *S. pombe*, but also for feedback by Clr4 itself, as the CD of Clr4 is quite specific for H3K9me3 [9,16,61]. The HDAC function of Clr3 is also important for preventing histone modifications associated with active transcription and limiting RNA polymerase II accessibility at the repressed site (transcriptional gene silencing) [62,63]. As another example, Swi6 attracts the chaperone FACT, which is required for spreading in constitutive heterochromatin, probably via nucleosome stabilization [64–66].

3.4. HP1 as a regulator of heterochromatin position and biophysical state

Second, HP1 may be required to promote an environment inside the nucleus conducive to spreading. Swi6 connects heterochromatin to the nuclear periphery via the nuclear rim protein Amo1, which associates with Swi6-interacting FACT (see above) and RIXC complexes [64]. Localization of H3K9me heterochromatin to the periphery is commonly observed across systems, for example via the CEC-4 protein in worms and PRR14 to the nuclear lamina in mammals [67,68], and in some cases is critical for heterochromatin formation. How this environment promotes heterochromatic spreading and silencing remains mechanistically opaque; however, one mechanism is likely the concentration of pro-spreading factors into this niche. The ability to form a specialized heterochromatin compartment or biophysical environment is likely linked to HP1s ability to oligomerize, which is considered central to its potential to bridge nucleosomes in H3K9me spreading. Oligomerization also underlies HP1's propensity to undergo liquid–liquid phase separation (LLPS), a process where biomolecules separate into distinct liquid-like compartments within the cytoplasm or nucleus [27,28,69]. Phase separation is typically driven by weak and multivalent interactions between biomolecules. These interactions involve both folded regions, such as the CD or CSD of HP1 as well as intrinsically disordered regions, such as the N-terminal extension of HP1 or its hinge region. These associations can lead to phase separation in the cell into condensates, which can have apparent LLPS characteristics, though it remains notoriously difficult to test *in vivo* [70]. These condensates can sequester other proteins and RNAs, thereby regulating their availability for biological processes [71]. HP1 has been shown to undergo LLPS on its own *in vitro* upon phosphorylation [27,28] and induce it in chromatin in part via nucleosome distortions [72]. It has been proposed that this behaviour, and/or its ability to induce phase separation of chromatin, may underlie the formation of heterochromatin domains *in vivo*. Whether these condensates represent the cytologically observed dense domains of heterochromatin, where gene expression is typically repressed, is not fully clear. It is possible that in this context, this formed liquid droplet encloses heterochromatic sequences and helps to exclude the transcription machinery, triggering gene silencing by forming a 'boundary' that separates heterochromatin from the surrounding chromatin [73,74]. Whether HP1-induced phase separation applies to all H3K9me-marked heterochromatin territories is not known. HP1 containing chromocenters in the nucleoplasm that are made of alpha-satellite repeats show properties of phase-separated bodies [27,28]. By contrast, whether phase separation is involved in heterochromatin found at the nuclear periphery is unclear. It is possible that HP1 uses phase-separation to package and insulate distinct heterochromatin types.

3.5. Beyond HP1

Additional chromatin modifications regulate H3K9me spreading, for example by regulating chromatin structure. One such mark is trimethylation at lysine 20 of H4 (H4K20me3). This mark is produced from H4K20me1 by the SUV4-20H1 and H2 enzymes (reviewed in [75] and on its own, can compact chromatin fibres [76]. This by itself may support H3K9me spreading. In addition, SUV4-20H1 in a non-enzymatic role changes nucleosome structure when bound, promotes phase condensation of chromatin, and alters the HP1-formed chromatin condensates [77]. Both these activities of SUV4-20H might promote either a spreading compatible chromatin structure and/or biophysical environment. Further, it appears that ubiquitination (Ub) of H3K14 may be critically required to stimulate Suv39 enzymes *in situ* on the chromatin substrate and enable spreading. In *S. pombe*, the Clr4 complex contains an E3 ligase [78–80], which we now understand ubiquitinates H3K14 which binds to a partially conserved Ub-binding pocket in Clr4 [81]. Stimulation by H3K14-Ub appears conserved with mammalian Suv39 enzymes [81]. Separately, in

a screen for spreading versus nucleation regulators, Greenstein *et al.* identified a complex of the Clr6 HDAC with the Fkh2 transcription factor as specifically required for heterochromatin spreading at constitutive sites. Fkh2 recruits Clr6 to nucleation-distal chromatin sites in such contexts [82]. This points to the fact that regions outside the active nucleation zone require additional manipulation of the chromatin substrate to make it compatible with heterochromatin assembly and H3K9 methylation.

4. Factors promoting the spreading of H3K27me3 by polycomb

4.1. PRC2 and PRC1 in H3K27me spreading

Heterochromatin marked by H3K27 methylation is critically involved in the control of animal and plant development, as was demonstrated via an elegant series of genetic studies in *Drosophila melanogaster* by Ed Lewis [83] and others. The central polycomb H3K27 methylase is PRC2, which consists of the evolutionary conserved Enhancer of zeste (Ezh) SET domain methylase, the H3K27me binding Embryonic ectoderm development (Eed), Suppressor of zeste 12 (Suz12) proteins, plus Rpab46/48. Repression by the polycomb system requires another enzymatic complex called PRC1, which catalyses H2AK119 ubiquitination [84]. In *Drosophila* and mammals, polycomb proteins are antagonized by trithorax group activator proteins, both systems establish a balance of activation and repression over the developmentally regulated loci such that only genes appropriate to the body segment are expressed [85]. In flies, it has become recognized that the PRC2 protein complex is recruited to specific polycomb response elements (PREs) [86,87], which have not been identified in this form in mammals. However, specific sequence contexts have been identified in mammals that appear to initially attract PRC2. These sequences contain CpG islands, and are unmethylated, along with other features [88]. Experiments that fully deplete the essential Eed protein and reintroduce it following the full loss of H3K27me have further solidified the location of these elements, which likely represent nucleation sites [18]. These sites also attract variant PRC1 complexes, triggering H2A ubiquitination and subsequent PRC2 recruitment [89]. Beyond these nucleation sites, other regions repressed by PRC2 are subject to H3K27me3 spreading from those nucleation sites. Elegant experiments tracking H3K27me3 domains in the cell cycle also indicate a continuous need for PRC2-nucleation, and later, Eed-dependent spreading, for domain reformation [90].

4.2. H3k27me2 versus me3 spreading

Spreading appears to be divided into short and more longer-range spreading, and here, there is a critical distinction concerning the methylation state: At first, the spreading of H3K27me2 appears relatively wide-ranging from the nucleation centre [18,91], yet the functionally critical H3K27me3 is initially more restricted [18]. Allosteric activation of Ezh2 via the Eed subunit binding to H3K27me3 [11,92], is likely required to enable further spreading of trimethylation. PRC2's activity in trimethylating H3K27 is also strongly regulated by activating PRC2 auto-methylation [93]. Oncogenic PRC2 antagonists, like H3K27M and Ezhip [94], which traps allosterically activated PRC2, instead abrogate spreading. Insertion of a regulatory step between me2 and me3 is reminiscent of Suv39 enzymes discussed above and appears to be a key gate in regulating spreading of the repressive state.

4.3. Regulation of H3K27me3 spreading by the chromatin substrate

Tied into this regulation of the methylation state is the regulation of these transitions by the chromatin substrate itself. Beyond the influence of histone modifications other than H3K27, which are not further discussed here, a central concept already touched on is regulation of nucleosome density and stability. In the case of PRC2, it was shown that the nucleosome spacing influences nucleosome methylation activity by PRC2, with 40 bp being the ideal spacing for methylation on dinucleosomes and arrays [95]. This is consistent with the results from structural biology that show PRC2 directly reaching from a methylated to a unmethylated substrate nucleosome with 30–35 bp spacing [96]. Another report showed that a high density of nucleosome *in cis* is required for stimulation [97]. Recent evidence indicates one key factor that regulates the optimal nucleosome arrangement for spreading may be the linker histone H1. *in vitro* and *in vivo* evidence [98] seems to support a role for H1 creating a chromatin structure that stimulates spreading by PRC2. The details of the precise nucleosome arrangement produced by H1 and favoured by PRC2, and how it relates to prior work on nucleosome spacing and density remains to be determined.

4.4. Trans-H3K27me3 spreading

As alluded to above, H3K27me3 after this initial local spreading is capable of long-range spreading apparently both *in cis* and *in trans*. How is this long-range spreading facilitated? The answer may reside in the biophysical nature of the polycomb domains, which appear to form 'polycomb bodies' (PBs) in the nucleus [99]. There, PBs bring together relatively distal sequences in a manner that is not only independent of the general looping and architecture regulatory pair CTCF and cohesion, but rather appears to be antagonized by it [100,101]. Nucleation sites appear distally contacted through canonical PRC1 complexes. These canonical PRC1 complexes, unlike its variant PRC1 complex cousins, are less capable of the central enzymatic activity of PRC1, H2A ubiquitination, but instead are more prone to oligomerize [89,102–106]. Such capacity to oligomerize has long been associated with PRC1 proteins such as the *Drosophila* Ph [107] as well as with the mammalian homologue Phc1/2 [103]. Similarly, the *Drosophila* PSC oligomerizes [108] and the *Drosophila* protein has been shown to mediate nucleosome compaction [102]. Further, hetero-oligomerization between the PRC1 component is thought to be essential in phase separation and likely PB formation [109–111]. It is likely that the Phc1 and the PSC homologue Bmi-1 (PCGF4) in canonical PRC1, in addition to CBX2, could mediate clustering and phase separation [106,109,111]. These clustered nucleation sites then allow PRC2 to spread H3K27me3 across distal regions, enabling PRC2 to exit the local nucleation environment [18], a model that is also consistent with theoretical modelling of heterochromatin spreading in three dimensions via self-attraction [29,30]. Such nucleation site clustering, presumably in PBs, also apparently ensures

redundancy in targeting spreading sites from several spatially adjacent nucleation sites, which may be at various distances in genomic space. To what degree the biophysical environment inside the PBs is important for the spreading process itself remains to be determined.

4.5. Potential roles of PRC2 oligomerization

While PRC1 oligomerization appears to allow the connection of distal sites, this type of oligomer formation is different from the coupling of oligomerization to writer product reading we saw for the Suv39 system. There is no apparent evidence that PRC1 oligomerization is connected to H3K27me reading. However, recent evidence implies that different PRC2 types can form dimeric complexes. For example, EZH1 containing PRC2 can dimerize on the nucleosome [112]. The structure of that complex, with two 'reading' Eed domains facing outward, may directly couple 'writing' to cross-nucleosome spreading. How this is related to structures of EZH2 PRC2 [96,113,114] and when dimerization is active *in vivo* remains to be determined. Finally, whether a linked oligomerization/product recognition cycle is operational for this system is not clear.

4.6. Lessons from plants: coupling nucleation and spreading

Interestingly, in plants, H3K27me3 spreading shares some features with Suv39 H3K9me3 spreading, as uniquely in plants, an HP1 protein LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) appears to act downstream or in parallel to PRC2 [115]. LHP1 binds H3K27me3 [116,117] that is deposited by PRC2 over flowering time loci and appears to aid in the spreading of this mark [118,119]. Intriguingly, spreading of the mark beyond nucleation sites is required for the epigenetic stability of the domain [119], highlighting the unique role of spreading in intergenerational maintenance.

5. Spreading by SETDB1

5.1. KAP1, KRAB-zfps and SETDB1s initial recruitment

SET domain bifurcated 1 (SETDB1) is a specific H3K9 methyltransferase that primarily acts in euchromatin sequences to silence retroelements or developmental regulators. An extensive amount of research has been conducted on the recruitment of SETDB1 to retroelements. Nucleation requires recognition of target sites by KRAB zinc finger proteins (KRAB-zfps), which then recruit SETDB1 via the critical co-repressor KAP1 (also known as TRIM28) [120,121]. KAP1 forms a complex with SETDB1 [122], which requires the intramolecular SUMOylation of KAP1 [123–125]. When KAP1 is depleted, SETDB1 enrichment in class I/II ERVs is decreased [120], indicating that the formation of the KAP-KRAB-zfps complex is a central step in SETDB1 recruitment to ERVs.

5.2. Pathways of H3K9me3 spreading by SETDB1

A first indication of H3K9me3 spreading by SETDB1 is that KRAB/KAP1 binding sites may be found only in one region of the retroelement, while H3K9me3 enrichment occurs throughout the entire retroelement [126,127]. Separately, SETDB1-generated H3K9me3 can repress the transcription of genes located distally to KRAB-zfp binding sites, in some cases up to tens of kilobases away [128–131]. This H3K9me3 spreading via SETDB1 may be facilitated by product recognition feedback. There are two potential pathways for this, outside the SETDB1 enzyme subunit, which remain underexplored:

1. HP1 may perform 'double duty' as a key spreading regulator for both Suv39 and SETDB1. Targeting of HP1α, HP1β and HP1γ to heterologous loci is sufficient to induce the recruitment of SETDB1 and deposition of H3K9me3 [132,133]. In mESCs, ERVs are enriched in HP1α, HP1β and HP1γ and this occurs in part due to SETDB1-deposited H3K9me3 [120]. There is a slight derepression in the expression of ERVs, as well as a partial reduction in H3K9me3 around the ERVs in HP1β KO mESCs [133,134]. The HP1 protein is therefore thought to be partially implicated in SETDB1-mediated H3K9me3 spreading.
2. Beyond HP1, a specialized complex has been identified that may be required for SETDB1 to mark target domains with H3K9me3. Using a similar strategy to find the PEV elements in drosophila cells, a non-lethal forward genetic screen conducted in haploid human KBM7 cells identified the human silencing hub (HUSH), which consists of the proteins TASOR, MPP8 and periphilin [130]. TASOR appears to be the hub of HUSH, providing a platform for assembling the other subunits, and has been identified as a pseudo-Poly (ADP-ribose) polymerase essential for HUSH complex assembly [135]. One central activity of HUSH is the recruitment of both SETDB1, as well as another factor MORC2 to sites of initial H3K9me3 in retroelements. MORC2 appears to have ATP-dependent remodelling activity key to the compaction of the underlying chromatin [130,136,137]. Whether HUSH is directly involved in H3K9me3 spreading is still contentious. The mechanisms by which HUSH may be involved in spreading comes back to product recognition. The CD of MPP8 binds H3K9me2 and H3K9me3 [138]. Hence MPP8 could 'read' the product of SETBD1 on nucleosomes. However, MPP8 also recognizes the methylation of an H3-like mimic sequence found in other proteins, such as ATF7IP, the nuclear chaperone of SETDB1 [139]. This methylation on ATF7IP is thought to be partially required for HUSH-dependent silencing [140]. Another critical piece of information is that the CD of MPP8 can form dimers [141]. Since ATF7IP is required for SETDB1 stability [139], a model could be envisioned where following recruitment of SETDB1 to a transgene, initial methylation occurs on H3K9 and AT7IP. MPP8 dimers could bridge chromatin and ATF7IP, recruiting the active form of SETDB1, which would constitute a read-write cycle for outward spreading over HUSH targets. This would nicely mirror parts of the Suv39 model where stabilization of the H3K9me substrate, multimerization and writer recruitment enable spreading. Yet, a H3K9me3 read-write mechanism involving MPP8 alone and ATF7IP/SETDB1 is probably too simplistic: HUSH-dependent lentiviral reporter repression requires both TASOR and Periphilin, and the MPP8 chromodomain is not required for the maintenance of repression [130]. However, the establishment of repression, so possibly the initial domain specification, is dependent on the MPP8 chromo domain [130].

It is also noteworthy that HUSH targets are enriched within transcriptionally active chromatin, as opposed to

classical heterochromatin regulators [142,143], which is akin to de novo spreading, for example, by the polycomb system in cell fate decisions. Together spreading by the SETDB1 system is still a new field, especially with the relatively recent discovery of HUSH. Whether it follows a 'read–write' type of mechanism and to what degree this interfaces with HP1 remains to be determined.

6. Spreading via SIR proteins

6.1. The SIR2/3/4 system

Yeast *Saccharomyces cerevisiae* SIR genes encode a family of nuclear proteins that are targeted to specific genomic sequences and targeted for silencing. SIR proteins are associated with three classes of genomic sequences: subtelomeres (which serve as the principal storage sites for the SIR proteins), silenced mating-type loci (i.e. HMR and HML) and rDNA sequences. To efficiently silence the HM loci, Sir1, Sir2, Sir3 and Sir4 are required, while Sir2, Sir3 and Sir4 are required to silence subtelomeres effectively. The central writer of the SIR complement is Sir2 which is a NAD-dependent deacetylase that targets histone H3 and H4.

Silent chromatin assembles in two stages at the molecular level: nucleation and spreading. Nucleation occurs when the Sir2/3/4 complex is recruited to silencers for the first time. The spreading step occurs following the assembling of the extended domain of silent chromatin by the complex. As a result of the intrinsic properties of the SIR proteins, the nucleation and spreading steps are closely linked. Nucleation without spreading and spreading without nucleation can be studied through mutations and other experimental manipulations (e.g. [144]).

The process of nucleation relatively well understood. The Sir2/3/4 complex is recruited to proteins bound at silencers by a network of interactions. Both Sir3 and Sir4 associate with the transcription factor Repressor-activator protein 1 (Rap1), while Sir4 also associates with Origin Recognition Complex (ORC)-bound Sir1 [145,146]. The transcription factor ARS-binding factor (Abf1) also cooperates in this process at HMR and HML [147]. Mutants of Sir2 that exhibit a catalytic defect restrict the Sir2/3/4 complex to silencers [148–150]. This suggests that the deacetylation of histones by Sir2 triggers the transition from nucleation to spreading.

6.2. SIR spreading: coupling of deacetylation and Sir3 oligomeric engagement

According to the original sequential model of spreading, Sir2 first deacetylates the nucleosomes adjacent to silencers, creating additional recruitment sites for Sir2/3/4 complexes [151]. Sir3 prefers to bind to deacetylated H4 tails, specifically amino acid H4K16 [6,152]. In an alternative view, based on the observed affinity of the Sir2/3/4 complex for acetylated H4K16, the complex is thought to bind acetylated nucleosomes first, and then acquire additional stability via deacetylation of H4K16 and docking of Sir3 to the deacetylated tails [153]. As a result of successive spreading, Sir2/3/4 binding, histone deacetylation and interactions between Sir2/3/4 complexes expand the silent chromatin domain until either a barrier is reached, or the pool of free SIR proteins falls below a threshold that facilitates efficient binding. In this view, the sequential spreading of Sir2/3/4 complexes is analogous to a linear polymerization reaction.

A more detailed mechanism has been proposed recently, involving Sir3 and Sir4 propagation along the fibre. The domain architecture of Sir3 has some conceptual similarities to HP1 in that it contains a nucleosome binding and dimerization domain. The N-terminus of Sir3 contains a bromo-adjacent homology (BAH) domain (amino acids 11–196). Many chromatin-associated proteins, including Orc1, Dnmt1, Rsc1, Rsc2 and Mta1 [154,155], contain the BAH domain, which is involved in nucleosome binding [156,157], and in Sir3's case specifically, nucleosomes that are the products of Sir2 (see below). Separately, Sir3's winged helix-turn-helix domain mediates self-interaction [158] and dimerization. This dimer/multimerization of Sir3 is likely at the heart of spreading. A model driven by biophysical measurements proposes that Sir3 spreads along the chromatin fibre using an 'interrupted bridges' [159] mechanism, where a Sir3 dimer bridges from one face of the nucleosome to the adjacent nucleosome face (figure 2). Further functional crosstalk with the enzymatic step via Sir2 also may feed directly into the oligomerization process.

6.3. Possible modulation by the Sir2 deacetylation by-product O-acetyl-ADP-ribose

But unlike the case of HP1, there is another interesting wrinkle to the SIR system, and that is regulation by one of the reaction products: Sir2 and other NAD-dependent protein Sir2/Class III HDAC uses NAD as a cofactor for deacetylation. Two products derive from NAD: nicotinamide and O-acetyl-ADP-ribose (AAR) [160,161]. AAR is associated with silent heterochromatin domains and demonstrates a similar pattern to that of Sir2 [162]. Intriguingly, the *in vitro* association of SIR2-3-4 complex and Sir3 alone with recombinant trinucleosomes is enhanced by AAR [163]. Similar modulation of binding to purified yeast nucleosomes was also reported [157,164]. The effect of AAR on chromatin epigenetic gene silencing has been demonstrated *in vivo* [165]. Moreover, previous observations regarding the role of AAR in the assembly of the SIR complex, as discussed above, strongly suggest that AAR binds to at least one of the SIR proteins [152]. For example, AAR might associate with the AAA ATPase-like domain within SIR3's C-terminus [158,166,167]. However, there is no strong direct evidence that AAR binds Sir3, instead, evidence supports binding to Sir2 [162], leaving the mechanism of action unclear. Even if AAR enhances the efficiency of Sir3-nucleosome complex formation, it does not appear that AAR is necessary for SIR silencing. This is because silent chromatin can be assembled *in vivo* using Hos3 (where Hos3 is targeted by a SIR3-Hos3 fusion), a deacetylase of the Rpd3 family that does not consume NAD nor produce AAR [168]. Despite the absence of all NAD-dependent deacetylases, the chimera produced robust transcriptional silencing. Therefore, if AAR is involved in the spreading of silencing, it may act to modulate, rather than drive the process.

6.4. Antagonism to SIR spreading

Spreading via Sir3 is also downregulated or limited in several ways. A prominent example is the acetylation of H4K16, which has been shown to impact Sir3 chromatin association by mutational analyses, ChIP and co-immunoprecipitation studies,

as well as biochemical studies [149–151,153,157,169–171]. The co-crystal structure of the nucleosome and the Sir3 BAH domain [10] visualized how this antagonism by H4K16 acetylation, but also H3K79 methylation would regulate Sir3 association with the nucleosome surface. A majority of the BAH domain's electrostatic contacts are with histone residues K16 and H18 in the H4 N terminal tail. A significant decrease in the affinity of Sir3 for the nucleosome is expected to occur as a result of the acetylation of K16. This is consistent with previous studies, which have indicated that acetylation has a 1000-fold impact [157]. Thus, Sir3/4 spreading, like that of HP1, requires recognition of the writer enzyme product on chromatin, in this case, a deacetylated H4 tail.

7. Conclusion

In the above, we have attempted to summarize what is known about how *trans*-acting factors regulate of heterochromatin spreading by writers in four systems. Some common principles emerge about how *trans*-acting factors promote the spreading of heterochromatin by writer enzymes. These are also summarized in figure 2.

First, *trans*-acting factors directly promote the positive feedback inherent in most heterochromatic writers. They do so by acting as a second, redundant feedback layer. The redundant layer, obvious in Suv39, SIR, and likely SETDB1 systems, consists of 'reader' proteins binding the writer product on chromatin and recruiting more writers via direct physical interactions.

Second, through processes of oligomerization on the chromatin substrate, writers like HP1 and Sir3 stabilize the structure and ensure that opportunities for redundant feedback are present across the forming heterochromatin domain. How the writers however do not end up getting 'trapped' in the core of the domain remains unresolved. A hint at a possible mechanism is differential preferences for methylation states of the chromatin mark in systems where methylation is the instructive chemical change (hence, not in the case of Sir2). Swi6 and Clr4 have differential preferences for H3K9me2 and me3, with Clr4 strongly preferring H3K9me3 [9,61]. Since H3K9me2 is more abundant than H3K9me3, one can consider one mark the assembly and the other the spreading and silencing mark [16].

Third, *trans*-acting factors likely promote spreading by shaping a microenvironment conducive to spreading, in part via nucleosome stabilization and possibly by producing an altered biophysical environment. Spatial segregation and self-attraction of heterochromatin has been predicted to be required for efficient spreading and inheritance by modelling approaches [29,30]. HP1 appears to be involved in anchoring some but not all H3K9me domains to the nuclear periphery, for example, those that fall into lamin-associated domains (controlled largely by G9a/GLP which we did not discuss in this review). The nuclear periphery in the case of fission yeast may be enriched for factors that stabilize nucleosomes [64]. Nucleosome stabilization, in turn, is central for efficient spreading, and especially for production of the trimethylated state in the case of H3K9me and K27me systems, which drives positive feedback. This state is also favoured by deacetylases that are recruited to the spreading zone. Finally, at least HP1 and PRC1 appear to be involved in forming condensates or condensate-like domains *in vivo* that may be required to promote the stability of heterochromatin and its spreading. In principle, condensate formation may promote writer enzyme activity directly, for example by increasing local concentration and altering the chromatin structure, or by rejecting antagonistic factors, such as excluding acetylases, transcription factors, or RNA polymerase (figure 2). In either case, resolving how condensates influence heterochromatin spreading remains a very active area of research.

However, there are also unique aspects not shared across the different heterochromatin systems. Firstly, the polycomb system's ability to perform long-distance spreading via PRC1 clusters in PBs does not have an exact parallel for other systems, and may be required for the reliable silencing of large developmental loci via redundant of spreading from dispersed nucleation sites [18]. SIR proteins may be regulated in their chromatin interactions via the Sir2 writer NAD deacetylation reaction by-product AAR, achieving a potential level of feedback lacking in the S-adenosyl methionine-dependent methyltransferase writers Suv39, PRC2 and SETDB1. The by-product in this case, S-adenosyl homocysteine is largely inhibitory via product inhibition. The interactions of HP1/Swi6 appear particularly diverse and contain inbuilt autoregulation not observed to the same extent elsewhere. In a facet not reviewed above, HP1/Swi6 even directly recruits negative heterochromatin spreading regulators such as Epe1 [172,173] in a manner tightly coordinated with H3K9 methylation. This restrains propagation of heterochromatin beyond the natural borders in fission yeast.

More work remains on unravelling how some of these unique mechanisms shape the spreading reaction and how they interface with the common operating principles discussed above.

Data accessibility. This article has no additional data.

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. B.H.: conceptualization, writing—original draft, writing—review and editing; A.A.A.A.: conceptualization, writing—original draft, writing—review and editing; B.A.-S.: conceptualization, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. B.A.-S. was supported by a grant from the National Institutes of Health R35GM141888 and a grant no. 2113319 from the National Science Foundation.

Acknowledgements. We like to thank Geeta J Narlikar, Sigurd Braun and Karim-Jean Amache for very valuable feedback and discussion of this review. We also thank Can Goksal for comments and feedback. We apologize to the community for the literature we could not cite due to space limitations.

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