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Review Article

Storing memories: the distinct phases of Polycomb-mediated silencing of *Arabidopsis FLC*

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Polycomb-mediated epigenetic silencing is central to correct growth and development in higher eukaryotes. The evolutionarily conserved Polycomb repressive complex 2 (PRC2) transcriptionally silences target genes through a mechanism requiring the histone modification H3K27me3. However, we still do not fully understand what defines Polycomb targets, how their expression state is switched from epigenetically ON to OFF and how silencing is subsequently maintained through many cell divisions. An excellent system in which to dissect the sequence of events underlying an epigenetic switch is the *Arabidopsis FLC* locus. Exposure to cold temperatures progressively induces a PRC2-dependent switch in an increasing proportion of cells, through a mechanism that is driven by the local chromatin environment. Temporally distinct phases of this silencing mechanism have been identified. First, the locus is transcriptionally silenced in a process involving cold-induced antisense transcripts; second, nucleation at the first exon/intron boundary of a Polycomb complex containing cold-induced accessory proteins induces a metastable epigenetically silenced state; third, a Polycomb complex with a distinct composition spreads across the locus in a process requiring DNA replication to deliver long-term epigenetic silencing. Detailed understanding from this system is likely to provide mechanistic insights important for epigenetic silencing in eukaryotes generally.

Introduction

The Polycomb silencing mechanism was originally identified using molecular genetic approaches in *Drosophila*. Distinct complexes of Polycomb proteins, known as Polycomb repressive complex 2 (PRC2) and Polycomb repressive complex 1 (PRC1), were shown to impart a *cis*-acting transcriptional silencing to the local chromatin [1–6]. The conservation in mechanisms of Polycomb silencing across mammals and plants has spurred an investigation into how epigenetic states are established and their role in response to environmental stimuli and developmental signals. Polycomb targets appear to exist in bistable states—they are either epigenetically ON, marked by H3K36me3 or other active histone modifications, or OFF and marked by H3K27me3. The switch from one state to another occurs via distinct phases [6–10]. However, the mechanisms underlying epigenetic switching and inheritance of the different states through cell division are still not well understood.

A Polycomb target that has received considerable attention is *Arabidopsis FLC*. This encodes a MADS box transcriptional repressor that delays flowering through repression of a set of genes involved in the transition from vegetative to reproductive development [11,12]. *FLC* expression is epigenetically silenced by exposure to cold. This process, known as vernalisation, is quantitative and at the whole plant level, *FLC* silencing is progressive and gradual. However, at the gene level, it is an infrequent ON/OFF epigenetic switch, with more cold leading to more silenced loci [9,13–15]. For plants in the field, this slow overall quantitative silencing response ensures plants wait until the end of winter to flower, aligning their transition to reproductive development with favourable spring conditions [11].

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The long timescale over which the *FLC* silencing occurs has enabled its distinct phases to be elaborated: first, transcription is down-regulated, and similarly to other Polycomb targets, this involves non-coding transcripts. Once the transcription is down-regulated, there is a switch to epigenetic silencing mediated by PRC2 and accessory proteins, independently of DNA methylation [16–19]. Spreading of Polycomb silencing across the locus, in a process requiring DNA replication, then locks in the silenced state, giving stable long-term epigenetic silencing through multiple rounds of cell division [9,20]. The silencing is reversed in a process requiring histone demethylation [21], ensuring each generation overwinters before flowering. In this review, we summarise and discuss the current mechanistic understanding of the distinct phases of the Polycomb-mediated switching mechanism at *FLC*.

Setting the stage: down-regulation of transcription

FLC transcription is rapidly down-regulated in response to cold exposure, and this occurs independently of the Polycomb accessory proteins [22]. This is consistent with the original view that PRC2 functions to maintain rather than initiate transcriptional repression [23–25]. What drives the cold-induced transcriptional down-regulation at *FLC* is still being elucidated, but it is linked to the up-regulation of expression of *COOLAIR*, a set of long, antisense, non-coding RNAs (lncRNA) transcribed from the opposite strand of *FLC* starting in the proximity of the poly(A) site of the *FLC* sense transcript (Figure 1a) [22]. This early involvement of non-coding transcription shows some parallels with the up-regulation of Xist, the ncRNA central to X inactivation in mammalian cells [26]. For example, single molecule RNA FISH analysis in root cells shows that *COOLAIR* antisense transcription is mutually exclusive to the sense *FLC* transcription—only one or other FISH signal is seen at each locus [27]. *COOLAIR* transcription occurs independently at each diploid copy suggesting that cold induction is via changes of the local chromatin, i.e. the *trans*-factors operate in a context-dependent manner. The antisense signal strongly increases with one to two weeks of cold exposure and *COOLAIR* forms large 'clouds' over the *FLC* locus, rather like Xist over the inactive X chromosome [27]. The mutually exclusive transcription is interesting given that *FLC* and *COOLAIR* expressions are positively correlated in RNA from populations of cells. The local chromatin environment appears to affect both *FLC* and *COOLAIR* transcription, with an additional mechanism temporally allowing transcription of only one strand at a time. We now have new mutants identified with high *COOLAIR* expression in the warm and these show very low *FLC* sense expression. These should help elucidate how transcription occurs on only one strand at a time and how cold induction of *COOLAIR* results in *FLC* down-regulation (Zhao and Dean, unpublished).

Two other published non-coding RNAs at *FLC* are *COLDAIR*, a sense transcript originating from within *FLC* intron 1, and *COLDWRAP*, another sense transcript originating instead from the promoter region of *FLC* [28,29]. These are reported to be induced by cold and to immunoprecipitate with the core PRC2 machinery [28,29]. There are now many examples of non-coding RNAs associating with PRC2 [30]. Current thinking is that PRC2 binds to RNA in a non-sequence specific manner and influences the methyltransferase activity of PRC2 and/or prevents the interaction of the complex with chromatin [31–36].

Connecting the two: linking transcriptional down-regulation to Polycomb silencing

Once *FLC* transcription is down-regulated, the nucleation of Polycomb silencing can occur [37]. Transcription needs to be down-regulated as it opposes H3K27me3 silencing through the delivery of H3K27me3 demethylases and general nucleosome disruption [10]. The *FLC* nucleation region covers ~3 nucleosomes over *FLC* exon 1 and the beginning of intron 1 and, with increasing cold, there is a progressive reduction in H3K36me3 over these nucleosomes, and a concomitant increase in H3K27me3 [25,38]. *COOLAIR* expression is required for this co-ordinated switch of histone modifications [25,39].

The nucleation region was also defined by mutational analysis, which showed that one intronic single nucleotide change at the 3′-end of the nucleation region, within intron 1, could attenuate *FLC* silencing [40,41]. VAL1 was identified as the protein factor binding to this genomic region, with *in vitro* and *in vivo* association with a tandem RY *cis* motif reduced by the mutation; VAL1 acts redundantly with VAL2 [40,41]. VAL1 and VAL2 are B3 DNA-binding proteins with PHD, B3, CW-ZF and EAR domains, and broad functions across the *Arabidopsis* genome in transcriptional repression [42]. VAL1 interacts with the histone deacetylase HDA19, with components of the apoptosis and spliceosome (ASAP) complex involved in co-transcriptional regulation [43,44], and with the PRC1 [40]. The ASAP components physically link to the PRC2 accessory proteins VIN3

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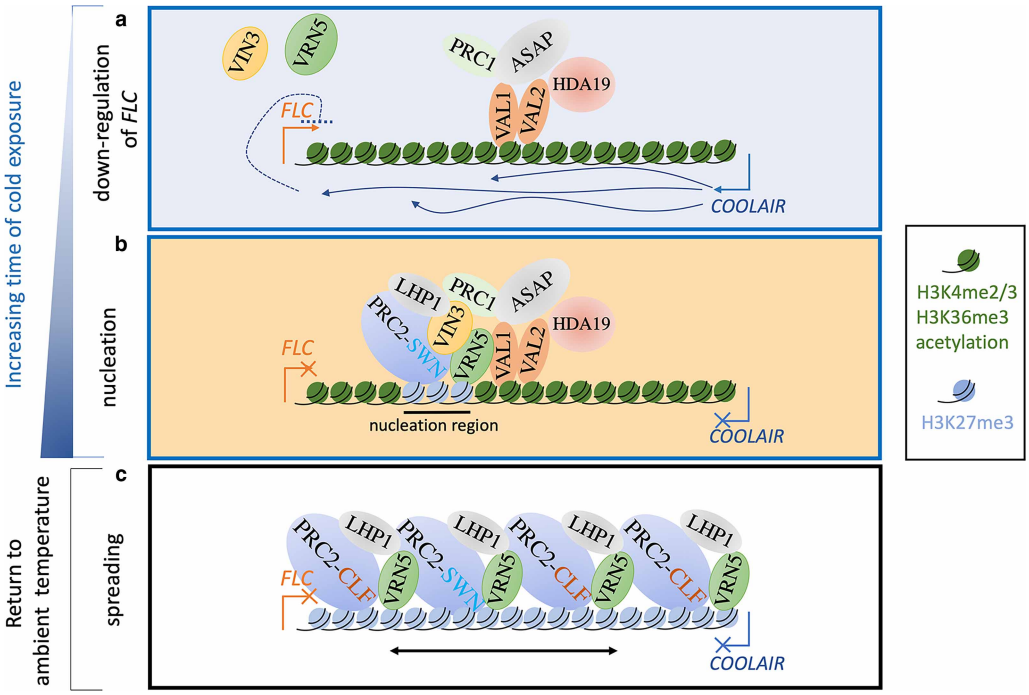

Figure 1. Model for the epigenetic switching mechanism at *FLC*.

(**a**) Prior to cold exposure, the *FLC* locus is expressed and enriched in H3K4me2, H3K4me3, H3K36me3 and histone acetylation. During the first weeks of cold exposure, *COOLAIR* transcripts are transiently expressed at a high level causing the down-regulation of *FLC* transcription. The binding of VAL1 (and possibly its homologue VAL2) at the nucleation region and their interactions with the histone deacetylase HDA19, the ASAP complex, involved in transcriptional regulation, and the PRC1, link the down-regulation of transcription to Polycomb silencing. VIN3 is induced by cold. (**b**) The ASAP complex associates with the PRC2 accessory proteins VIN3 and VRN5 and with the PRC2-SWN complex at the nucleation region establishing the metastably silenced state at *FLC*. This state is characterised by *FLC* not being transcribed and accumulation of H3K27me3 at the nucleation region. (**c**) Upon return to warm temperatures, H3K27me3 spreads across the locus, a process requiring the formation and spreading of the PRC2–CLF complex, LHP1 and the incorporation of the histone variant H3.1. This last phase establishes a long-term epigenetically silenced state that is maintained across multiple cell divisions until the state is reset during reproductive development.

and VRN5 [40], providing a mechanism through which PRC2-induced silencing could be targeted to specific sequences (Figure 1b). How all these factors link the transcription state to the ability to nucleate Polycomb activity is under investigation. The demonstration that Polycomb nucleation is a *cis*-mediated event, driven by local chromatin and thus involving co-transcriptional regulators [9,14], argues that the VAL1 sequence-specific DNA-binding protein is required for, but does not drive the epigenetic silencing.

Slow and steady: Polycomb nucleation

Forward genetic screens for mutants defective in vernalisation identified a key role for VIN3 and VRN5 in triggering the epigenetic silencing of *FLC*. VIN3 and VRN5 are two homologues in a four-member gene family in *Arabidopsis*, which all share PHD and FNIII domains and a VEL protein interaction domain at their C-termini [18,45]. VIN3 and VRN5 co-immunoprecipitate with both ASAP components and core PRC2 subunits SWN (the EZH1/2 histone methyltransferase homologue), FIE (the EED homologue), MSI (the RBAP46/48 homologue) and VRN2 (the SUZ12 homologue) (Table 1) [19,46]. Once *FLC* is transcriptionally down-regulated the VIN3–VRN5–PRC2 complex deposits H3K27me3 at the nucleation site in *FLC*. At the whole plant level, the H3K27me3 deposition looks gradual, but at each locus, there is a switch from H3K36me3 to H3K27me3. This appears to be a cell-autonomous, stochastic and infrequent epigenetic switch, so the longer the exposure to cold, the higher number of cells contain nucleated *FLC* [9,13–15]. Each *FLC* parental allele nucleates

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Table 1 Equivalent PRC2 core subunits proteins in different systems

Mammals	Flies	Plants	Characteristic domain
EZH1/2	E(z)	SWN CLF MEA	SET
SUZ12	Su(z)12	VRN2 FIS2 EMF2	Zinc finger VEFS box
EED	ESC ESC-like	FIE	WD-40
RBAP48 (synonym RBBP4) RBAP46 (synonym RBAP7)	p55 (synonym Nurf55)	MSI1	WD-40

independently, showing the nucleation event is driven by mechanisms acting through the local chromatin environment, rather than limited by *trans*-factors (Figure 2) [9,14].

An interesting aspect of nucleation is its intragenic location. Many features point to the *FLC* nucleation region being equivalent to a Polycomb Response Element (PRE) [47], well characterised in *Drosophila*. PREs bind the Polycomb factors either through sequence specificity or a structural feature, e.g. non-methylated CpG islands act as PREs in mammalian genomes [48]. VIN3 associates specifically at the nucleation region during cold [9] and together with VRN5 shares many functional domains (zinc finger—PHD and winged-helix domains, Nielson, Fiedler and Dean, unpublished) with PRC2 accessory proteins characterised from mammals: PHF1, PHF19 and MTF2. These PRC2 accessory proteins appear to regulate the recruitment and activity of the core PRC2 complex, composed of EED, EZH1/2, SUZ12, RBAP46/48 [49]. The PHF1 winged-helix domain binds non-sequence specifically to DNA and prolongs the residence time of the PHF1–PRC2 on chromatin, making it a more efficient H3K27 methyltransferase than PRC2 alone [50,51]. Modulation of PRC2 activity appears a common theme: the mammalian PRC2 accessory proteins AEBP2 and JARID2 are substrates for PRC2 methylation, and this feeds back to stimulate the methyltransferase activity of the core PRC2 complex [52–56]; SUZ12 enhances PRC2 association with DNA through the N-terminal part of its VEFS domain [57]. However, when in complex with the other core subunits, SUZ12 mediates the inhibition that the active histone marks H3K4me3 and H3K36me3 exert on the PRC2 methyltransferase activity [52,58]. Interestingly, plants can relieve the inhibitory effect of the active histone marks by exchanging the SUZ12 homologue EMF2 with the SUZ12 homologue VRN2, as VRN2 does not inhibit the methyltransferase activity of the complex in their presence [58]. Thus, the modulation of the activity of PRC2 complex via accessory proteins or by swapping between SUZ12 homologues might provide switch-like properties to the system facilitating the initial establishment of PRC2-dependent repression when H3K27me3 is absent or its density is low.

During the cold, there is the inheritance of deposited H3K27me3 through cell division, but little or no spreading of the K27me3 marks along the locus. Yet spreading readily occurs once plants are returned to the warm [9,20]. During replication, the redistribution of the histones carrying the H3K27me3 modification onto the newly synthesised strands has been proposed to help to copy the mark from the modified nucleosomes to the unmodified neighbouring nucleosomes giving rise to self-maintenance of methylation patterns [6,7,13,59–61]. This hypothesis is supported by the observation that the core PRC2 subunit EED can bind H3K27 and that this binding also allosterically stimulates the methyltransferase activity of the complex [8,62–64]. Why then does the nucleation region remain restricted to three nucleosomes in the cold and does not spread? The structural analysis of the PRC2 complex (reviewed in [65,66]) has given some clues. The catalytic centre of the core PRC2 complex resides in the SET-domain histone methyltransferase (EZH2) [3,67], but the stability and catalytic activity of the complex depend on the contacts that the zinc-finger protein SUZ12 establishes with the WD-40 protein EED and EZH2 [53,63,68,69]. The fourth subunit, the WD-40 protein RBAP48, folds together with the N-terminal part of SUZ12 to form the module that binds to the nucleosome [53,56,63]. The mammalian PRC2 accessory proteins affect this structural arrangement [52,56], and since, as mentioned above, the plant accessory proteins VIN3 and VRN5 share many functional domains with PRC2 accessory proteins characterised from mammals, a possibility is that they may do the same and during cold hold PRC2 in a conformation with minimal catalytic activity to prevent the spreading. Upon return to warm temperatures, the

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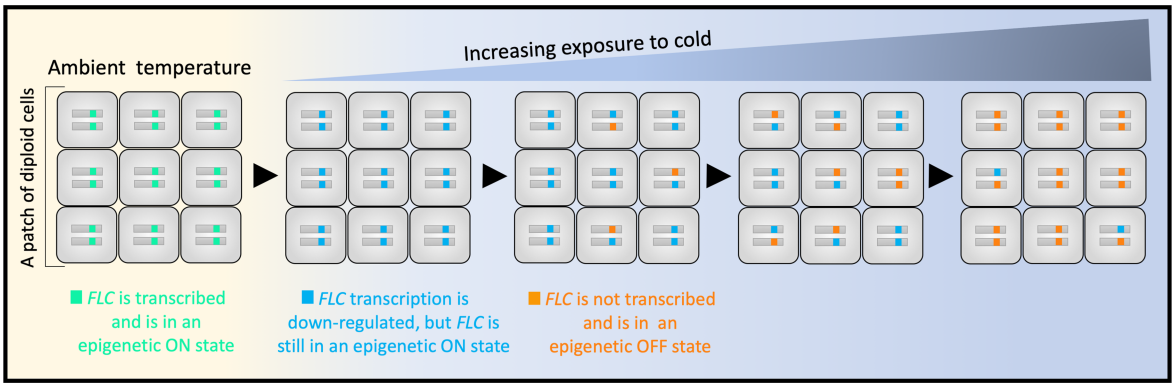

Figure 2. Cell-autonomous and *cis*-based silencing of *FLC* expression.

FLC can exist in ON or OFF epigenetic states. At ambient temperatures, *FLC* is transcribed and epigenetically ON in every cell. Upon exposure to cold, *FLC* transcription is shut down rapidly and epigenetic switching to an OFF state occurs cell-autonomously, i.e. independently in each cell and in *cis*, i.e. separately at each *FLC* copy (two alleles in each cell of a diploid plant). Because the switching events are infrequent, it takes many weeks of cold exposure before all *FLC* copies are epigenetically OFF (so unable to reactivate *FLC* transcription upon return to the warm). The molecular events occurring at each *FLC* copy during exposure to cold are described in Figure 1a and b.

conformation and/or the composition of the complex could change increasing PRC2 allosteric activation and promoting spreading of the H3K27me3. Experimental observations are consistent with this notion: VIN3 is rapidly lost from the locus; VRN5 slowly decreases at the nucleation region over 10 days, but also it redistributes along the locus [9]. Thus, both proteins could be important for docking and keeping the PRC2 at the nucleation region, with VIN3 potentially keeping the methyltransferase activity of PRC2 'in check'.

Take me for a ride: H3K27me3 spreading

After cold, upon return to warm temperatures, H3K27me3 spreads from the nucleation region to cover the *FLC* locus and this spreading is required for the long-term stability of the epigenetic silencing (Figure 1c) [9,20]. This memory state is propagated in *cis*, i.e. by local chromatin, as demonstrated by the independent behaviour of two *FLC* gene fusions in the same nucleus, which can be inherited in different transcriptional states (Figure 2) [14].

Analysis of different mutants revealed that the nucleation and spreading phases could be genetically uncoupled [9,20]. *lhp1*, *clf* and *h3.1kd* mutants could nucleate H3K27me3 but not enable H3K27me3 spreading across the locus. LHP1 is the homologue of HP1 and associates with PRC2 [70]; CLF functions in a partially redundant manner with SWN, they are the homologues of EZH1 and EZH2 [71], and H3.1 is an histone variant deposited during DNA replication that facilitates the restoration of H3K27me3 [20]. The spreading process is also blocked by the CDK inhibitors that halt cell cycle progression [9,20] and by mutations in the DNA replication primase Pol α [72]. Proteomic studies in *Arabidopsis* show that several PRC2 components, including CLF, immunoprecipitate with DNA Pol ε, the DNA polymerase responsible for replicating the leading strand [73]. CLF, SWN and LHP1 also interact with the helicase complex at the replication forks via a protein related to the yeast replication factor Ctf4 [74]. Thus, a possibility is that the PRC2 complex associates with the replication machinery and by progressing along with the replication fork, it is able to efficiently methylate newly deposited histones just behind the replication fork.

A real puzzle is the functional distinction between the two closely homologous H3K27 methyltransferases, CLF and SWN at the *FLC* locus, where SWN predominantly mediates nucleation, whilst CLF is required for spreading [9]. Interestingly, the two mammalian paralogues EZH1 and EZH2 also have complementary roles [75,76]. EZH2 is associated with proliferative tissue, whilst EZH1 is not [77]. However, PRC2-EZH1 differs from PRC2-EZH2 in that it has low methyltransferase activity [55,77,78]. A possibility therefore is that SWN function is similar to EZH1 with a minimal catalytic activity, which would be sufficient to establish the nucleation region and to restrict it to three nucleosomes during cold. CLF, may be more like EZH2, able to be allosterically activated and thus promote efficient spreading concomitant with DNA replication upon return to

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warm temperatures. Like CLF, LHP1 is required for H3K27me3 spreading along the *FLC* locus [9] and for maintaining the *FLC* silenced state [79,80]. LHP1 associates with CLF and together they co-ordinate spreading of H3K27me3 at many genomic regions [81]. LHP1 function requires an RNA-binding domain and LHP1 forms distinct and RNA-dependent heterochromatic-like foci in *Arabidopsis* nuclei [82]. However, whilst *FLC* loci cluster within the nucleus as Polycomb nucleation occurs, this process is not dependent on LHP1 [83]. LHP1 function is thus downstream of H3K27me3 deposition, maintaining the spread H3K27me3 state, ensuring long-term epigenetic silencing through many mitotic cycles.

FLC expression needs to be epigenetically reset every embryogenesis [84,85] because in plants, the germline is not laid down separately like in animals, but it arises from somatic tissue during reproductive development. A mutant defective in resetting, *elf6*, was found to disrupt an H3K27 demethylase activity, and this led to trans-generational inheritance of the silenced H3K27me3 state [21]. Resetting mechanisms during seed development thus play crucial roles in ensuring plants can align their development with the seasons.

Conclusions

The analysis of what is a specific plant process—the developmental transition to reproduction in response to temperature changes—has produced a detailed mechanistic understanding of Polycomb-mediated epigenetic switching. The long timescales involved in the epigenetic silencing of *FLC* provide an excellent system to dissect the distinct Polycomb complexes that operate in sequence to give transcriptional repression, metastable *cis*-based nucleation and then long-term epigenetic silencing. Similar stochastic epigenetic switching systems underlie other developmental switches: in mating type switching in fission yeast [86], X chromosome inactivation in mammals [26] and the switch to haematopoietic T-cell development in mammals [87]. In all of these, the local chromatin environment is central to the switching and memory mechanisms [14,87,88]. These local chromatin-driven (*cis*-based) epigenetic mechanisms are the outcome of integrated and interdependent functions of *trans*-factors, non-coding transcription and histone modifications. Thus, dissection of the silencing mechanism at *FLC* with respect to non-coding transcription, allosteric interactions between *trans*-factors and histone modification dynamics will undoubtedly provide information important for understanding quantitative gene regulation and epigenetic switching generally.

Perspectives

- Importance of the field: Polycomb-mediated epigenetic silencing is central to correct growth and development in higher eukaryotes. However, we still do not fully understand what defines Polycomb targets, how their expression state is switched from epigenetically ON to OFF and how silencing is subsequently maintained through many cell divisions.
- Current thinking: the long timescales involved in the epigenetic silencing of *Arabidopsis FLC* have provided an excellent system to dissect the distinct Polycomb complexes that function over different timescales in transcriptional repression, metastable *cis*-based nucleation and long-term epigenetic silencing.
- Future directions: future work will focus on the interaction of non-coding transcription, allosteric interactions between *trans*-factors and histone modification dynamics in the mechanisms underpinning quantitative gene regulation and epigenetic switching.

Abbreviations

ASAP, apoptosis and spliceosome; PRC1, Polycomb repressive complex 1; PRC2, Polycomb repressive complex 2; PRE, Polycomb response element.

Author Contribution

S.C. and C.D. both contributed to the writing of the manuscript.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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