Dissection of PRC1 and PRC2 recruitment in Arabidopsis connects EAR repressome to PRC2 anchoring

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Short title: The EAR acts as a docking point for PRC2 and HDACs

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Abstract

PcG complexes ensure that every cell in an organism expresses the genes needed at a particular stage, time or condition. However, it is still not fully understood how PRC1 and PRC2 are recruited to target genes in plants. Recent results in Arabidopsis support that PRC2 recruitment is mediated by different TFs. However, it is unclear how all these TFs interact with PRC2 and whether they can also recruit PRC1 activity. Here, by using a system to *in vivo* bind selected factors to a synthetic promoter lacking the complexity of PcG target promoters, we show that while VAL1 binding recapitulates PRC1 and PRC2 marking, the binding of other TFs only render PRC2 marking. Interestingly, all these TFs contain an EAR domain that acts as docking point for PRC2 and HDACs, connecting two different repressive mechanisms. Furthermore, we show that different TFs act synergistically in PRC2 anchoring to maintain a long-term repression.

34

35 **Introduction**

36 The evolutionary conserved Polycomb group (PcG) factors are required to
 37 maintain gene repression (Ringrose and Paro, 2004; Merini and Calonje, 2015).
 38 These factors form multiprotein complexes with different histone modifying
 39 activities, including PcG repressive complex 1 (PRC1), which has H2A E3
 40 ubiquitin ligase activity towards lysine 119, 120 or 121 in *Drosophila*, mammals
 41 or *Arabidopsis*, respectively (Wang et al., 2004; Cao et al., 2005; Bratzel et al.,
 42 2010; Yang et al., 2013), and PRC2, which has H3 lysine 27 (H3K27)
 43 trimethyltransferase activity (Müller et al., 2002; Makarevich et al., 2006;
 44 Mozgova and Hennig, 2015). Despite the conserved activity of these
 45 complexes, several data indicate that distinct rules operate for PcG recruitment
 46 in the different organisms (Müller and Kassis, 2006; Mendenhall et al., 2010;
 47 Xiao et al., 2017).

48 In *Arabidopsis*, PRC2 core subunits are well conserved to their animal
 49 counterpart (Mozgova et al., 2015); however, PRC1 composition is less
 50 conserved (Merini and Calonje, 2015). Although a H2A E3 ubiquitin ligase
 51 module containing one AtBMI1 (A, B or C) and one AtRING1 (A or B) protein
 52 has been identified (Bratzel et al., 2010; Sanchez-Pulido et al., 2008), homologs
 53 for other PRC1 components are missing and instead several plant-specific
 54 proteins seem to play PcG functions (Merini and Calonje, 2015; Calonje, 2014).
 55 Distribution analysis of H2AK121ub and H3K27me3 peaks in *Arabidopsis*
 56 showed that both marks are generally targeted to gene regions, although
 57 H3K27me3 peaks are longer than H2AK121ub peaks. In addition, this analysis
 58 revealed that despite H2AK121ub marks frequently co-localize with H3K27me3,
 59 there are also genes only marked with H3K27me3 or H2AK121ub (Zhou et al.,
 60 2017).

61 Concerning PcG recruitment to target genes in *Arabidopsis*, a high number of
 62 transcription factors (TFs) has been related to PRC2 tethering. Among these
 63 factors are the GAGA motif binding proteins BASIC PENTACYSTEINE (BPC) 1-
 64 6 (Hecker et al., 2015a; Xiao et al., 2017), the *TELOBOX* motif binding proteins
 65 *ARABIDOPSIS* ZINC FINGER 1 (AZF1), ZINC FINGER OF *ARABIDOPSIS*

66 THALIANA 6 (ZAT6) (Xiao et al., 2017) and TELOMERE-REPEAT-BINDING
67 FACTOR (TRB)1/2/3 (Zhou et al., 2018), the MYB TF ASYMMETRIC LEAVES
68 1 (AS1) (Lodha et al., 2013), the C2H2 TFs SUPERMAN (SUP) (Xu et al.,
69 2018) and KNUCKLES (KNU) (Sun et al., 2019), and the MADS-box TFs
70 FLOWERING LOCUS C (FLC) and SHORT VEGETATIVE PHASE (SVP)
71 (Wang et al., 2014; Richter et al., 2019). Furthermore, it has been recently
72 shown that certain genomic fragments located at several PcG targets, which
73 contain binding sites for a wide variety of TF families, can recruit PRC2, thus,
74 functioning as *Drosophila* Polycomb Recruiting Elements (PREs) (Xiao et al.,
75 2017). In addition, localization analyses of H2AK121ub and H3K27me3 marks
76 in WT and PcG mutants showed that levels of H3K27me3 are substantially
77 reduced in the PRC1 mutant *atbmi1abc* (Zhou et al., 2017), indicating that
78 PRC1 also plays a role in PRC2 recruitment.

79 Unlike PRC2, the recruitment of PRC1 H2A E3 ubiquitin ligase module has so
80 far only been associated to VIVIPAROUS1/ABI3-LIKE (VAL)1/2 proteins (Yang
81 et al., 2013; Qüesta et al., 2016), which is surprising given the number of TFs
82 involved in PRC2 recruitment and the apparent dependence of PRC1 for
83 H3K27me3 marking. Thus, despite recent advances in understanding PcG
84 recruitment in plants, there are still many unknowns. For instance, it is still far
85 from clear whether the recruitment of one complex is required for the
86 recruitment of the other, how PRC2 can interact with such a diversity of TFs,
87 and whether these interactions take place independently or in parallel. In
88 addition, as there are genes marked with H2AK121ub/H3K27me3, H2AK121ub
89 or H3K27me3 (Zhou et al., 2017), it is unknown whether this differential marking
90 depends on different recruiting factors and, in that case, if these factors can
91 function synergistically at some target genes.

92 To address all these questions, we developed a system to *in vivo* mediate the
93 binding of selected factors to a synthetic promoter lacking the *cis* regulatory
94 elements involved in PcG recruitment, allowing us to investigate their role under
95 controlled conditions. Our results show that VAL1 can recapitulate PRC1 and
96 PRC2 marking. However, while PRC1 recruitment is directly mediated by
97 interaction with VAL1, PRC2 tethering involves both VAL1 and PRC1.
98 Interestingly, we also found that PRC2 can be recruited independently of PRC1

99 by TFs from different families that contain an EAR domain as a common
 100 feature. We show that the EAR domain, through its interaction with TOPLESS
 101 (TPL)/TPL-RELATED (TPR)1-4 corepressors or the SIN3-associated protein 18
 102 (SAP18), acts as a docking point for both PRC2 and HISTONE
 103 DEACETHYLASE COMPLEXES (HDACs). Furthermore, we found that different
 104 TFs could act synergistically in PRC2 recruitment, leading to increased levels of
 105 H3K27me3 at target genes. Our results not only unveil how the different PcG
 106 complexes are recruited to target genes, but also how different histone
 107 modifying activities are coupled to promote gene repression in Arabidopsis.

108

109

110 Results

111 VAL1 acts as a platform for simultaneous recruitment of PRC1, PRC2 and 112 HDACs

113 VAL1/2 TFs have been involved in both PRC1- and PRC2-mediated repression
114 (Yang et al., 2013; Qüesta et al., 2016; Chen et al., 2018; Jing et al., 2019;
115 Zeng et al., 2020). VAL factors contain a B3 DNA-binding domain that
116 specifically recognizes RY elements (CATGCA) (Suzuki et al., 2007).
117 Accordingly, when analyzing the 6-mer DNA motifs present at the proximal
118 promoter (500 bp upstream the start codon) of the genes marked with
119 H2AK121ub/H3K27me3 in WT and upregulated in the PRC1 mutant *atbmi1abc*
120 (Zhou et al., 2017) (n=1030), we found an enrichment of these elements over
121 other motifs (**Figure 1A; Supplementary Dataset 1**). In addition, VAL1/2
122 interact with HISTONE DEACETYLASES (HDAs)(Zeng et al., 2020; Zhou et al.,
123 2013). Besides the B3 domain, VAL1/2 contain a Plant homeodomain like
124 (PHD-L), a CW and an EAR domain(Suzuki and McCarty, 2008). While the
125 PHD-L and the CW domains act as readers of H3 methylation states (Yuan et
126 al., 2016; Hoppmann et al., 2011), the EAR domain is involved in the interaction
127 with TPL/TRP or SAP18, which in turn recruit HDA activities (Kagale and
128 Rozwadowski, 2011). Nevertheless, despite VAL1/2 can interact with these
129 different repressive complexes, it is not clear whether these interactions take
130 place simultaneously or within different contexts.

131 To investigate this, we developed a system to direct VAL1 recruitment to a
132 constitutive promoter that lacks any of the *cis* regulatory element proposed to
133 recruit PcG activity, including RY elements. For this, we built a synthetic target
134 promoter, consisting on a *cauliflower mosaic virus* (*CaMV35S*) promoter in
135 which the bacterial LexA *operator* (*LexO*) was inserted. This promoter was
136 placed upstream of the *beta-glucuronidase* (*GUS*) reporter gene, obtaining the
137 *pLexO::GUS* construct (**Figure 1B**). In parallel, we generated a construct to
138 express a translational fusion between LexA DNA-binding domain (BD) and
139 VAL1, and another to express the BD alone as control (**Figure 1B**). The three
140 constructs were independently transformed into Wild type Col-0 Arabidopsis
141 plants (WT) and, after selecting the appropriate lines (**Supplementary Figure**
142 **1; Supplementary Figure 2**), they were crossed to obtain

143 WT/*pLexO::GUS/BD-VAL1* and WT/*pLexO::GUS/BD* plants. To verified the
 144 functionality of the system, we confirmed the binding of the BD fusion proteins
 145 to the synthetic promoter by Chromatin Immunoprecipitation (ChIP) using anti-
 146 LexA BD antibody (**Figure 1C,D**). Next, we investigated whether H2AK121ub
 147 and H3K27me3 marks were incorporated at the reporter *locus* in the different
 148 plants. ChIP results using anti-H2AK121ub and anti-H3K27me3 antibodies
 149 showed that the incorporation of these marks was only observed in
 150 WT/*pLexO::GUS/BD-VAL1* (**Figure 2A,B**), indicating that the binding of VAL1 is
 151 able to recapitulate PRC1 and PRC2 marking. We also checked the levels of
 152 H3ac marks at the reporter *locus* in the different lines (**Figure 2C**), finding that
 153 they were considerable decreased in WT/*pLexO::GUS/BD-VAL1* compared to
 154 WT/*pLexO::GUS/BD* plants. In addition, we examined the effect of these
 155 proteins on gene expression by measuring GUS activity in the different
 156 transgenic plants (**Figure 2D**). While the binding of lexA BD did not affect the
 157 levels of GUS activity compared to control plants, the binding of BD-VAL1 led to
 158 a significant reduction of GUS activity. All together, these results indicate that
 159 VAL1 acts as a platform for the simultaneous recruitment of different histone
 160 modifying complexes involved in gene repression.

161 Previous reports have shown that VAL1 and AtBMI1 proteins directly interact
 162 (Yang et al., 2013; Qüesta et al., 2016) and that the levels of H3K27me3 were
 163 reduced in both *val1val2* and *atbmi1abc* mutants at seed maturation genes
 164 (Yang et al., 2013). Furthermore, genome wide analyses showed that
 165 H3K27me3 levels were reduced to some extent at most of
 166 H2AK121ub/H3K27me3 marked genes in *atbmi1abc* (Zhou et al., 2017); thus,
 167 we wondered whether VAL1 directly recruit PRC2 or if this is mediated by PRC1
 168 interaction. To investigate this, we introduced the *pLexO::GUS* and *BD-VAL1*
 169 transgenes into *atbmi1abc* mutant (**Figure 2E**, left panel) and analyzed the
 170 levels of H3K27me3 at the reporter *locus* (**Figure 2E**, right panel). We found
 171 that despite the levels of H3K27me3 marks were considerably reduced in
 172 *atbmi1abc* mutant, they were not completely eliminated, indicating that PRC2
 173 recruitment is mediated by both PRC1 and VAL1.

174

175 **PRC2-independent recruitment is mediated by TFs other than the VAL** 176 **factors**

177 A broad diversity of TFs belonging to different gene families are able to bind to
178 PRE-like sequences in Arabidopsis (Xiao et al., 2017). Among the most
179 enriched ones are the C2H2 and AP2-ERF families (Xiao et al., 2017) (**Figure**
180 **3A**). Accordingly, several evidence showed that the C2H2 factors SUP, KNU
181 and AZF1 are able to recruit PRC2 activity (Xiao et al., 2017; Xu et al., 2018;
182 Sun et al., 2019). Besides, the MADS-box TFs FLC and SVP have been
183 connected to PRC2 repression (Wang et al., 2014; Richter et al., 2019) (**Figure**
184 **3A**). Therefore, we wondered whether all these TFs were able to work as
185 recruiting platforms for PRC1, PRC2 and possibly other histone modifying
186 complexes as VAL1 did. To test this, we generated BD-KNU, BD-FLC and BD-
187 ERF10 fusions and analyzed their effects on our synthetic target *locus*. We
188 found that the three fusion proteins were able to repress gene expression
189 (**Figure 3B**) and led to the incorporation of H3K27me3 and the removal of H3ac
190 marks (**Figure C,D**). However, we did not detect incorporation of H2AK121ub
191 marks (**Figure 3E**). Accordingly, the *cis* regulatory motifs recognized by these
192 TFs were enriched at the proximal promoter of the genes marked only with
193 H3K27me3 (Zhou et al., 2017), whereas the RY elements were not enriched in
194 this subset of genes (**Supplementary Figure 3; Supplementary Dataset 1**).
195 These results indicate that PRC2 activity can be recruited independently of
196 PRC1 through TFs other than VAL factors.

197 Nevertheless, since the promoters of H2AK121ub/H3K27me3 marked genes in
198 addition to RY elements showed enrichment in other *cis* regulatory motifs
199 (**Figure 1A; Supplementary Dataset 1**), we wondered whether different
200 recruiting factors could collaborate in H3K27me3 marking at these genes. To
201 test this, we inserted into the synthetic promoter a DNA fragment containing one
202 GAGA and two *TELOBOX* motifs to generate the WT/*p(G+2T)LexO::GUS* line,
203 as these motifs have been extensively related to PRC2 recruitment in
204 Arabidopsis (Xiao et al., 2017; Zhou et al., 2018; Hecker et al., 2015b) (**Figure**
205 **3F; Supplementary Figures 1 and 4**). We first analyzed the levels of
206 H3K27me3 at *GUS* reporter *locus* in WT/*p(G+2T)LexO::GUS* and
207 WT/*pLexO::GUS* plants in absence of any of the BD fusion proteins. We

208 detected some levels of H3K27me3 marks at the reporter *locus* when these
209 motifs were present (**Figure 3G**), supporting that the TFs recognizing these
210 motifs can mediate PRC2 recruitment. Then, we checked the levels of
211 H3K27me3 at these reporter *loci* after the binding of BD-VAL1 (**Figure 3G**). We
212 found higher levels of H3K27me3 in WT/*p(G+2T)LexO::GUS/BD-VAL1* than in
213 WT/*p(G+2T)LexO::GUS*; moreover, the levels in WT/*p(G+2T)LexO::GUS/BD-*
214 *VAL1* were higher than in WT/*pLexO::GUS/BD-VAL1*. Consistent with this, we
215 found that GUS activity was lower in *p(G+2T)LexO::GUS/BD-VAL1* than in
216 *pLexO::GUS/BD-VAL1* plants (**Figure 3H**), indicating that the levels of
217 H3K27me3 are important to maintain gene repression. All together, these
218 results support that different TFs can act synergistically in PRC2 recruiting.

219

220 **EAR repressome connects histone deacetylation and PRC2 marking**

221 Since all the TFs tested, including VAL1, were able to recruit PRC2 activity, we
222 examined if they display some common feature. Interestingly, despite the lack
223 of sequence homology among them, these TFs contain an EAR domain.
224 Furthermore, except for the case of TRB factors, all the TFs that have been
225 related to PRC2 recruitment before contain an EAR domain (**Figure 4A**). The
226 EAR domain is defined as LxLxL, DLNxP, and DLNxxP. This domain has been
227 found in a high number of TFs of different gene families with repressive activity,
228 constituting what has been named the EAR repressome (Kagale et al., 2010).
229 The EAR domain mediates interaction with TPL/TPR corepressors or SAP18
230 protein (Kagale and Rozwadowski, 2011; Causier et al., 2012; Song and
231 Galbraith, 2006). TPL/TPR in addition interact with the HDAs HDA6 and
232 HDA19(Liu et al., 2014), and, importantly, with the PcG proteins EMBRYONIC
233 FLOWER1 (EMF1) and VERNALIZATION 5 (VRN5) (Causier et al., 2012; Ke et
234 al., 2015; Collins et al., 2019). On the other hand, SAP18 is both a component
235 of the SIN3-HDAC (Zhang et al., 1997) and the APOPTOSIS AND SPLICING-
236 ASSOCIATED PROTEIN (ASAP) complex (Deka and Singh, 2017). The SIN3-
237 HDAC in Arabidopsis includes a SIN3-like protein (SNL1-6), SAP18, SAP30,
238 one HDA activity (HDA19, HDA9, HDA7 or HDA6) and MULTICOPY
239 SUPPRESSOR OF IRA1 (MSI1)(Liu et al., 2014). Interestingly, MSI1 is also a
240 PRC2 core component (Derkacheva et al., 2013; Mehdi et al., 2016; Ning et al.,

2019). Moreover, it has been shown that SAP18 co-purifies with PRC2 core components and HDA19 (Qüesta et al., 2016). All together, these data strongly suggest a direct connection between EAR factors, TPL/TPR-HDAC or SAP18-HDAC and PRC2, which so far has not been deeply investigated. Therefore, to explore whether the EAR domain can serve as a docking point for both PRC2 and HDAC recruitment via TPL/TPR or SAP18 interaction, we compared the levels of H3K27me3 marks and H3ac at the reporter *locus* after the binding of BD-KNU or a mutated BD-KNU version in which the EAR domain was removed (BD-KNU(-EAR)) (**Figure 4B,C**). We found that the levels of H3K27me3 were significantly reduced in *pLexO::GUS/BD-KNU(-EAR)* plants compared to *WT/pLexO::GUS/BD-KNU* plants, while the opposite effect was observed for the case of H3ac. Furthermore, the levels of GUS activity in *pLexO::GUS/BD-KNU(-EAR)* were as in control plants (**Figure 4D**). To further verify these results, we checked if the LexA BD fused to an EAR domain (BD-EAR) was able to reduce H3ac, increase H3K27me3 levels and repress gene expression when recruited to the synthetic promoter. Indeed, we found that the EAR domain by itself was able to cause all these effects (**Figure 4E,F,G**); however, it was unable to recruit PRC1 (**Figure 4H**), which connects the EAR repressome to PRC2 recruitment.

261 **EMF1-TPL interaction couples H3K27me3 marking to H3 deacetylation**

We also used our system to investigate the exact role of the plant-specific PcG associated factor EMF1 (Calonje et al., 2008). EMF1 has been proposed to be a PRC1 component due to its ability to *in vitro* perform similar functions to those of *Drosophila* Psc and to interact with AtBMI1 proteins (Bratzel et al., 2010; Calonje et al., 2008; Beh et al., 2012). However, several data indicate that EMF1 is required for H3K27me3 marking at some PcG target genes (Calonje et al., 2008; Kim et al., 2012; Li et al., 2018). Accordingly, EMF1 interacts with MSI1 (Calonje et al., 2008) and co-purifies with PRC2 components (Liang et al., 2015). In addition, EMF1 interacts with FLC and the HISTONE DEMETHYLASE JMJ14 to mediate *FT* repression (Wang et al., 2014). However, it is not clear whether EMF1 is also required for PRC1 marking. Thus, we analyzed the levels of H3K27me3 and H2AK121ub at the reporter *locus* after the binding of BD-

274 EMF1 to the synthetic promoter (**Figure 5A**). We found that the levels of
 275 H3K27me3 were increased in WT/*pLexO::GUS/BD-EMF1* compared to
 276 WT/*pLexO::GUS/BD* plants but we did not find considerable changes in
 277 H2AK121ub levels, indicating that the recruitment of EMF1 leads to H3K27me3
 278 marking but not to H2AK121 monoubiquitination. Since EMF1 directly interact
 279 with JM14 (Wang et al., 2014), we also analyzed H3K4me3 levels at the
 280 reporter *locus* (**Figure 5B**). Accordingly, we found reduced levels of these
 281 marks after BD-EMF1 binding. On the other hand, EMF1 has been shown to
 282 interact with TPL in yeast two hybrid assay (Causier et al., 2012). In support of
 283 this, we found TPL among the proteins that co-immunoprecipitate with EMF1
 284 (Bloomer et al., 2020) (**Supplementary Dataset 2**). Furthermore, the levels of
 285 H3ac at the reporter *locus* were reduced after EMF1 binding (**Figure 5B**),
 286 confirming an EMF1-TPL-HDA interaction. We then analyzed the levels of GUS
 287 activity in WT/*pLexO::GUS/BD-EMF1* (**Figure 5C**), finding decreased levels
 288 compared to control plants; however, the levels were not as low as after the
 289 binding of the TFs, indicating that factor/s acting upstream EMF1 may be
 290 required for proper repression.

291

292

293 Discussion

294 PcG complexes ensure that each cell in an organism expresses the genes that
295 are needed at a particular stage, time or condition. However, as PcG proteins
296 do not have the ability to recognize DNA sequences, how PRC1 and PRC2 are
297 recruited to the appropriate target gene is still not fully understood. Recent data
298 support that PRC2 is recruited via interaction with different TFs; however, it is
299 not known whether these TFs display a common feature to do so, whether the
300 same TF can recruit PRC2 and PRC1 and how PcG-mediated differential
301 marking is achieved. In this work, we were able to dissect how PRC1 and PRC2
302 recruitment take places in Arabidopsis.

303 We found that the binding of VAL1 TF was able to recapitulate PRC1 and PRC2
304 marking and to assemble HDAC activities, acting as a recruiting platform for
305 different repressive complexes (**Figure 5D**, left panel). While PRC1 recruitment
306 is mediated by AtBMI1 direct interaction with VAL1 (Yang et al., 2013; Qüesta
307 et al., 2016), PRC2 recruitment involves both PRC1 and VAL1. Interestingly,
308 our data showed that while TFs like KNU, FLC or ERF10 can mediate PRC2
309 and HDACs recruitment, they cannot attract PRC1 for H2A monoubiquitination
310 (**Figure 5D**, right panel), indicating that PRC2 recruitment relies on a more
311 general mechanism.

312 When comparing the protein domains present in VAL1, KNU, FLC, ERF10 and
313 other TFs related to PRC2 recruitment before, we found that, except for the
314 case of TRB1/2/3 factors that seem to be stable PRC2-accessory proteins as
315 they co-purify with PRC2 (Bloomer et al., 2020), all of these TFs contain an
316 EAR domain. The EAR domain interacts with TPL/TPR or SAP18, which in turn
317 recruit HDA activities (Kagale and Rozwadowski, 2011; Kagale et al., 2010;
318 Causier et al., 2012; Song and Galbraith, 2006). Interestingly, TPL and SAP18
319 also interact with PcG proteins. In fact, TPL co-purify with EMF1 (Bloomer et al.,
320 2020) and SAP18 with MSI1 (Mehdi et al., 2016), suggesting that they serve as
321 scaffolds for HDACs and PRC2 assembly. In support of this, we found that the
322 binding of three EAR-containing TFs led to the incorporation of H3K27me3 and
323 removal of H3ac marks at the reporter *locus*, and that this depends on the EAR
324 domain. Moreover, the recruitment of EMF1 leads to H3K27me3 incorporation

325 and H3ac removal. Therefore, we propose that the EAR repressome acts as
326 anchoring point for PRC2 and HDACs recruitment (**Figure 5**).

327 It is unknown whether the interaction of the EAR factors with TPL/TRP or
328 SAP18 depends on the type of EAR domain or on adjacent sequences, or
329 whether they functionally overlap, as some of the EAR factors have been
330 reported to interact with both (Kagale and Rozwadowski, 2011; Kagale et al.,
331 2010; Causier et al., 2012). In any case, since TPL/TRP and SAP18 are
332 expressed in most plant tissues (Kagale and Rozwadowski, 2011), the ability of
333 the PcG machinery to maintain specific transcriptional states in different cell
334 types, at different times or under different conditions, may rely on the EAR-
335 containing recruiting factors, which expression is tightly regulated in response to
336 internal and external signals.

337 We also found that different TFs can act synergistically in PRC2 recruitment at
338 the same target gene, leading to increased levels of H3K27me3. PcG proteins
339 in plants seems to be involved in both transient and long-term repression that
340 persist through multiple cell divisions. Long-term repression has been reported
341 to require spreading and maintenance of high levels of H3K27me3 marks
342 across the target genes (Costa and Dean, 2019). Interestingly, *FLC* initial
343 repression requires the RY elements for PcG nucleation (Costa and Dean,
344 2019), but its long-term repression involves other *cis* regulatory sequences
345 located along *FLC locus* (Qüesta et al., 2020). Similarly, a PcG long-term
346 repression in *Drosophila* requires sequence-specific targeting of PRC2 (Laprell
347 et al., 2017). Thus, it might be possible that the combined action of different
348 recruiting factors propagates and maintains appropriate H3K27me3 levels to
349 mediate long-term repression in *Arabidopsis*.

350 Nevertheless, in *Arabidopsis* there is also a high number of only-H2AK121ub
351 marked genes¹⁶. The promoters of these genes are highly enriched in G-box
352 motifs (**Supplementary Figure 3; Supplementary Dataset 1**). This motif is
353 recognized by two large families of TFs in *Arabidopsis*, the basic helix-loop-helix
354 (bHLH) and Leu zipper (bZIP) families (Ezer et al., 2017), raising the possibility
355 that TFs from these families may be involved in PRC1-independent recruitment

356 and suggesting that PcG differential marking depends on different recruiting
357 factors.

358

359

360 **Methods**

361 **Plant material and culture conditions**

362 *Arabidopsis thaliana* Col-0 wild type (WT), *atbmi1abc* (Yang et al., 2013) and
363 transgenic plants harboring the different constructs were grown under long-day
364 conditions (16 h light and 8 h dark) at 21 °C on MS agar plates containing 1.5%
365 sucrose and 0.8% agar for 7 days. MS-agar plates were appropriately
366 supplemented with Kanamycin (50 µg.ml⁻¹) and/or hygromycin (10 µg.ml⁻¹) for
367 selection of transgenic plants.

368 **Synthetic system constructs and transgenic plants**

369 To generate the synthetic target gene constructs, we used as backbone the
370 pCambia 1305.1 binary vector that contains the *GUS reported gene* under the
371 control of the *cauliflower mosaic virus* (CaMV35S) promoter. We replaced the
372 CaMV35S promoter by a CaMV35S in which the LexA DNA binding element
373 (Lex A operator (LexO), amplified from pER8 vector (Zuo et al., 2000), was
374 inserted upstream of the TATA box, resulting in the *pLexO::GUS* construct. To
375 generate *p(G+2T)LexO::GUS* construct, a DNA fragment of 100 bp from the
376 regulatory region of *ABSCISIC ACID INSENSITIVE 3 (ABI3)* gene (see
377 **Supplementary Figure 4**), which contains one GAGA and two TELOBOX
378 motifs, was introduced into the synthetic promoter upstream of the LexO. These
379 constructs were transformed into WT Col-0 plants to generate WT/*pLexO::GUS*
380 and WT/*p(G+2T)LexO::GUS* transgenic plants. To build the BD translational
381 fusion constructs, we inserted into the pPZP211 vector the G10-90 promoter,
382 the LexA BD (252 bp N-terminal region of LexA protein amplified from pER8
383 vector (Zuo et al., 2000)), the TF cDNA and the OCTOPINE SYNTHASE (OCS)
384 terminator . To construct the BD-EAR fusion, we used the C-terminal region of
385 VAL1 cDNA that contains a predicted Nuclear Localization Signal (NLS) and the
386 EAR domain (region from 2041 bp to stop codon of VAL1 cDNA; See
387 **Supplementary Figure 5**). To ensure that the BD when expressed alone was
388 transported to the nucleus, the sequence corresponding to VAL1 predicted NLS
389 (region from 2041 to 2183 bp of VAL1 cDNA; See **Supplementary Figure 5**)
390 was fused to the C-terminal region of the BD. The different BD fusion constructs
391 were transformed into WT Col-0 plants. The expression of the protein in the
392 different transgenic lines was verified by Western blot using anti-LexA BD

antibody. One line from each BD fusion was crossed to the same WT/*pLexO::GUS* or WT/*p(G+2T)LexO::GUS* line. Primers used are listed in **Supplementary Dataset 3**.

Western blot assay

Total protein extract from the different plants was separated on 10% SDS-PAGE gel and transferred to a PVDF membrane (Immobilon-P Transfer membrane, Millipore) by semi-dry blotting in 25 mM Tris-HCl, 192 mM glycine, and 10% methanol. To detect the fusion proteins, anti-LexA BD antibody (Millipore 06-719; 1:2000) was used as primary antibody and Horseradish peroxidase-conjugated goat anti-rabbit (Sigma-Aldrich, A0545; 1:10,000) as secondary. Chemiluminescence detection was performed with ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) following the manufacturer's instructions.

Fluorometric assay of beta-glucuronidase (GUS) activity

The activity of beta-glucuronidase (GUS) was determined on whole seedlings using 4-methylumbelliferyl β -D-glucuronide (4-MUG) as a substrate (Halder and Kombrink, 2015). One-Single 7-day-old seedlings were placed in 96-well microplates and incubated with 150 μ L lysis buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100) containing 1mM 4-MUG at 37°C for 90 min. At the end of the incubation period, 50 μ L of 1M Na₂CO₃ (stop solution) was added to each well and 4-MU fluorescence was directly determined in a microplate reader (excitation/emission wavelength of 365/455 nm). Activity is expressed as relative fluorescence units (RFU).

Chromatin immunoprecipitation (ChIP) and ChIP-qPCR

ChIP experiments were performed on one gr of 7-day-old whole seedlings fixed in 1% Formaldehyde. Chromatin was extracted from fixed tissue and fragmented using a Bioruptor® Pico (Diagenode) in fragments of 200-500 bp. The sheared chromatin was immunoprecipitated overnight using the following antibodies and dilutions: anti-LexA BD (Millipore 06-719, 1:300) Anti-H3K27me3 (Millipore, 07-449, 1:300), anti-H2AK121ub (Cell Signaling, 8240S; 1:100) Anti-Histone H3 (acetyl K9 + K14 + K18 + K23 + K27) (Abcam ab47915, 1:300), or anti-H3K4me3 (Diagenode, C15410003-50; 1:300). Immunocomplexes were

captures using Protein-A Sepharose beads CL-4B (GE Healthcare). After washing the Protein-A beads, chromatin was eluted and the cross-linking was reversed overnight at 65°C. The DNA from the immunoprecipitated chromatin was treated with RNase and proteinase K and purified by phenol-chloroform extraction followed by ethanol precipitation. For ChIP-qPCR, amplification was performed using SensiFAST™ SYBR® & Fluorescein Kit (Bioline) and iQ5 Biorad system. Results are given as percentage of input or as relative level to *FLOWERING LOCUS C (FLC)*, *ACTIN 7 (ACT7)* or *AGAMOUS (AG)*, depending on the histone mark and the genotype. qPCR data are shown as the means of two to four biological replicates as indicated. Primers used for ChIP-qPCR are listed in **Supplementary Dataset 3**.

436

437 **Author Contributions and Acknowledgements**

438 FB and WM performed the experiments with the help of IH. MC designed the study, interpreted the results and wrote the manuscript.

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444

445 **Competing Interests statement**

446 The authors declare that they have no competing interests.

447

448 **Figure legends**

449 **Figure 1. LexA BD fusion proteins *in vivo* bind to the synthetic promoter.**

450 **(A)** Bar chart showing RY element as the most significantly enriched cis-regulatory motif found at the proximal promoter of the H2AK121ub/H3K27me3 marked genes in WT that become upregulated in *atbmi1abc* mutant (n=1030 genes; see Supplementary Dataset 1). Analysis was carried out using Tair Motif finder tool (<https://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp>). Other significantly enriched 6-mer motifs are also shown. **(B)** Schematic representation of the synthetic *GUS* reporter locus. The LexO element

457 recognized by LexA binding domain (BD) is indicated. Numbered arrows
458 indicate the position of the primer pairs used to examine the binding of the
459 fusion proteins to the synthetic *locus*. **(C,D)** Bar charts showing BD (in blue) and
460 DB-VAL1 (in orange) enrichment at *GUS* reporter *locus* determined by ChIP
461 using anti-LexA DB antibody. WT/*pLexO::GUS* plants (in grey) were used as
462 negative control. Results are indicated as percentage of input. Error bars
463 represent standard deviation of n=2-3 biological replicates. Significant
464 differences as determined by Student's t-test are indicated (**P < 0.001).

465 **Figure 2. VAL1 acts as a platform for PRC1, PRC2 and HDACs recruitment.**

466 **(A,B,C)** H2AK121ub, H3K27me3 and H3ac levels at *GUS* reporter *locus* in
467 WT/*pLexO::GUS/BD* and WT/*pLexO::GUS/BD-VAL1* plants. Numbers at x-axis
468 indicate the position of amplified regions as indicated in Figure 1b. H2AK121ub
469 and H3K27me3 levels were normalized to *FLC* and H3ac levels to *ACT7*. Error
470 bars indicate standard deviation of n=2-4 biological replicates. Significant
471 differences at position 4 are indicated as determined by Student's t-test (**P <
472 0.001; *P < 0.05). **(D)** Box plots showing GUS activity levels in
473 WT/*pLexO::GUS*, WT/*pLexO::GUS/BD* and WT/*pLexO::GUS/BD-VAL1*
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475 tested in independent seedlings (N≥17). In each case, the median (segment
476 inside rectangle), the mean (cross inside the rectangle), upper and lower
477 quartiles (boxes), and minimum and maximum values (whiskers) are indicated.
478 Significant differences as determined by Student's t-test are indicated (**P <
479 0.001). “ns” indicates not significant. **(E)** Left panel, schematic representation of
480 the experiment showed at the right panel in which the levels of H3K27me3 at
481 the reporter *locus* were compared between WT/*pLexO::GUS/BD-VAL1* and
482 *atbmi1abc/pLexO::GUS/BD-VAL1* plants. H3K27me3 levels were normalized to
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485 (**P < 0.01).

486 **Figure 3. BD-KNU, BD-FLC and BD-ERF10 are able to recruit PRC2 and**

487 **HDACs but not PRC1. (A)** Sequence logos of the cis regulatory motifs
488 recognized by C2H2, MADS and AP2ERF TFs. **(B)** Box plots showing GUS
489 activity levels in WT/*pLexO::GUS*, WT/*pLexO::GUS/BD*, WT/*pLexO::GUS/BD-*

490 *KNU*, WT/*pLexO::GUS/BD-FLC* and WT/*pLexO::GUS/BD-ERF10* seedlings at 7
 491 DAG indicated as relative fluorescence units (RFU). Activity was tested in
 492 independent seedlings (N≥18). In each case, the median (segment inside
 493 rectangle), the mean (cross inside the rectangle), upper and lower quartiles
 494 (boxes), and minimum and maximum values (whiskers) are indicated.
 495 Significant differences as determined by Student's t-test are indicated (**P <
 496 0.001). **(C,D,E)** H3K27me3, H3ac and H2AK121ub levels at *GUS* reporter *locus*
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 500 deviation of n=2 biological replicates. Significant differences compared to
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 503 WT/*pLexO::GUS/BD-VAL1* was included as additional control. **(F)** Schematic
 504 representation of *p(G+2T)LexO::GUS* construct in which one GAGA and two
 505 *TELOBOX* motifs were inserted upstream of the *LexO*. **(G)** H3K27me3 levels at
 506 *GUS* reporter *locus* in WT/*pLexO::GUS* and WT/*p(G+2T)LexO::GUS* plants
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 511 GUS activity levels in the same plants. Activity was tested in N=14 independent
 512 seedlings. Significant differences as determined by Student's t-test are indicated
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514 **Figure 4. EAR domain acts as an anchoring point for PRC2 and HDACs.**

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 516 recruitment. The TFs analyzed in this work are indicated. **(B,C)** Comparison of
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Figure 5. EMF1 recruitment leads to the incorporation of H3K27me3 and removal of H3K4me3 and H3ac. **(A)** H3K27me3 and H2AK121ub levels at *GUS* reporter locus in WT/*pLexO::GUS*, WT/*pLexO::GUS/BD* and WT/*pLexO::GUS/BD-EMF1* plants. Numbers at x-axis indicate the position of amplified regions as indicated in Figure 1b. H2AK121ub and H3K27me3 levels were normalized to *FLC*. Error bars indicate standard deviation of n=2 biological replicates. Significant differences compared to WT/*pLexO::GUS/BD* are indicated as determined by Student's t-test (***P < 0.001). “ns” indicates not significant. **(B)** H3K4me3 and H3ac levels at *GUS* reporter locus in the different plants. The levels were normalized to *ACT7*. Error bars indicate standard deviation of n=2 biological replicates. Significant differences between BD and BD-EMF1 are indicated as determined by Student's t-test (**P < 0.01). **(C)** Box plots showing GUS activity levels in WT/*pLexO::GUS*, WT/*pLexO::GUS/BD* and WT/*pLexO::GUS/BD-EMF1* seedlings at 7 DAG. RFU indicates relative

556 fluorescence units. Activity was tested in independent seedlings ($N \geq 20$). In each
 557 case, the median (segment inside rectangle), the mean (cross inside the
 558 rectangle), upper and lower quartiles (boxes), and minimum and maximum
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 560 Student's t-test are indicated ($***P < 0.001$). **(D)** Drawing summarizing the
 561 histone modifying complexes recruited by VAL1 or by other EAR-containing TFs
 562 to promote transcriptional repression.

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