The EMBO Journal (2013) 32, 2073–2085
www.embojournal.org

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THE EMBO JOURNAL

Arabidopsis MSI1 connects LHP1 to PRC2 complexes

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Polycomb group (PcG) proteins form essential epigenetic memory systems for controlling gene expression during development in plants and animals. However, the mechanism of plant PcG protein functions remains poorly understood. Here, we probed the composition and function of plant Polycomb repressive complex 2 (PRC2). This work established the fact that all known plant PRC2 complexes contain MSI1, a homologue of *Drosophila* p55. While p55 is not essential for the *in vitro* enzymatic activity of PRC2, plant MSI1 was required for the functions of the EMBRYONIC FLOWER and the VERNALIZATION PRC2 complexes including trimethylation of histone H3 Lys27 (H3K27) at the target chromatin, as well as gene repression and establishment of competence to flower. We found that MSI1 serves to link PRC2 to LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), a protein that binds H3K27me3 *in vitro* and *in vivo* and is required for a functional plant PcG system. The LHP1–MSI1 interaction forms a positive feedback loop to recruit PRC2 to chromatin that carries H3K27me3. Consequently, this can provide a mechanism for the faithful inheritance of local epigenetic information through replication.

The EMBO Journal (2013) **32,** 2073–2085. doi:10.1038/emboj.2013.145; Published online 18 June 2013
Subject Categories: chromatin & transcription; plant biology
Keywords: *arabidopsis*; chromatin; epigenetics; MSI1; polycomb group proteins

Introduction

Most developmental decisions are based on tight regulation of transcription to establish and maintain specific gene expression patterns, and polycomb group (PcG) proteins are among the master regulators of different developmental programmes. PcG proteins were first identified in *Drosophila* as regulators of *Hox* gene expression (Lewis, 1978) and were subsequently found to represent an ancient and evolutionarily conserved mechanism of gene silencing (for reviews see Hennig and Derkacheva, 2009; Butenko and Ohad, 2011; Margueron and Reinberg, 2011). Animal and plant PcG proteins function by forming multi-subunit protein complexes such as Polycomb repressive complex 1 (PRC1) and PRC2. PRC2 is recruited to target genes and catalyses the trimethylation of lysine 27 of histone H3 (H3K27me3). Animal PRC1 binds to H3K27me3 and establishes monoubiquitylation of H2AK119. H3K27me3 is, however, not always required for PRC1 recruitment to target genes. Eventually, animal PcG proteins repress transcription by means of mechanisms that are not fully understood and that probably involve compaction of nucleosomes and interference with transcription elongation. In *Drosophila* and *Arabidopsis*, silencing by PcG proteins involves local restriction of DNA accessibility (Shu *et al*, 2012).

The PRC1 complex was originally characterized in *Drosophila*, where it consists of four main subunits: polycomb (Pc), polyhomeotic (PH), posterior sex combs (Psc) and RING (Francis *et al*, 2001; Mohd-Sarip *et al*, 2002). Pc binds to H3K27me3 (Fischle *et al*, 2003), and RING catalyses H2AK119 monoubiquitylation (Wang *et al*, 2004; de Napoles *et al*, 2004). Similar to animals, plant PcG function seems to involve RING proteins that can monoubiquitylate H2A (Sanchez-Pulido *et al*, 2008; Xu and Shen, 2008; Bratzel *et al*, 2010; Li *et al*, 2011). Although plants lack Pc homologues, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), also known as TERMINAL FLOWER 2, is considered to fulfil the role of Pc in plants based on its ability to bind to H3K27me3 *in vitro* and its genome-wide co-localization with H3K27me3 *in vivo* (Turck *et al*, 2007; Zhang *et al*, 2007). LHP1 binding to H3K27me3 is required for its function (Exner *et al*, 2009), and LHP1 is required for repression of several PcG protein targets such as *FLOWERING LOCUS C* (*FLC*), *FLOWERING TIME* (*FT*) and *AGAMOUS* (*AG*) (Kotake *et al*, 2003; Libault *et al*, 2005). However, it remains unknown whether LHP1 has additional functions independent of the plant PcG system.

In contrast to PRC1, homologues of all four core subunits of animal PRC2 exist in plants. The *Arabidopsis* genome encodes three homologues of the histone methyltransferase enhancer of zeste (E(z)): CURLY LEAF (CLF), SWINGER (SWN) and MEDEA (MEA); three homologues of the suppressor of zeste: EMBRYONIC FLOWER 2 (EMF2), FERTILIZATION INDEPENDENT SEED 2 (FIS2) and VERNALIZATION 2 (VRN2); a single extra sex comb homologue: FERTILIZATION INDEPENDENT ENDOSPERM (FIE); and five homologues of p55: MULTICOPY SUPRESSOR OF IRA 1–5 (MSI1–5). The diverse PRC2 subunit homologues in *Arabidopsis* probably form at least three different PRC2-like complexes with distinct functions. The VERNALIZATION (VRN) complex comprises VRN2, FIE, CLF or SWN and MSI1, and accelerates flowering in response to prolonged

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Received: 19 December 2012; accepted: 17 May 2013; published online: 18 June 2013

exposure to cold (Wood *et al*, 2006; De Lucia *et al*, 2008). The EMBRYONIC FLOWER (EMF) complex was proposed to control vegetative development and the transition to flowering and to comprise EMF2, FIE, CLF or SWN and one p55 homologue. An interaction of EMF2 with CLF was shown *in vitro* and in yeast two-hybrid assays (Chanvivattana *et al*, 2004), but the *in vivo* composition of the EMF complex awaits confirmation. Both EMF2 and VRN2 contribute to repression of the *FLC* (Gendall *et al*, 2001; Jiang *et al*, 2008). The FERTILIZATION INDEPENDENT SEED (FIS) complex has specific functions in the female gametophyte and the endosperm and comprises FIS2, FIE, MEA and MSI1 (Köhler *et al*, 2003; Spillane *et al*, 2000).

MSI1–5 proteins belong to a subfamily of WD-40 repeat proteins, which are subunits of several chromatin-remodelling complexes in animals, plants and yeast. They do not have enzymatic activity but can bind to histones and serve as protein scaffolds (for a review, see Hennig *et al*, 2005). Although MSI1-like proteins were usually found among the core subunits of animal PRC2, they are not required for enzymatic activity *in vitro* (Cao and Zhang, 2004; Ketel *et al*, 2005; Schmitges *et al*, 2011). Similarly, the role of plant MSI1-like proteins in PcG gene silencing has been under debate. *Arabidopsis* MSI1 was shown to be part of the FIS complex and is essential for gametophyte and seed development (Köhler *et al*, 2003; Guitton *et al*, 2004; Guitton and Berger, 2005; Leroy *et al*, 2007). MSI1 co-purified with VRN2 (De Lucia *et al*, 2008), but it is not known whether MSI1 is required for VRN complex function and the vernalization response. Finally, which of the five MSI1-like proteins function in the EMF complex has not been established yet. Deficiency of MSI1 affects shoot apical meristems and floral meristems and primordia, suggesting a role in vegetative plant development and transition to flowering (Hennig *et al*, 2003; Bouveret *et al*, 2006; Schönrock *et al*, 2006), possibly as part of the EMF complex. Similar to MSI1, MSI4 and MSI5 regulate the transition to flowering (Kim *et al*, 2004; Ausin *et al*, 2004; Gu *et al*, 2011). Recently, co-immunoprecipitation of MSI4 with CLF was shown, suggesting that MSI4 instead of MSI1 could be part of the EMF complex (Pazhouhandeh *et al*, 2011).

In this study, we have analysed the function of MSI1 in sporophytic PRC2 complexes in *Arabidopsis*. Purification of the EMF complex established MSI1 but not MSI4 as a core subunit. Similarly, MSI1 but not MSI4 interacts with EMF2. MSI1 is recruited to the chromatin of EMF target genes, where it is required for transcriptional silencing. Further, we find that MSI1 is recruited to the *FLC* locus where it is required for stable repression by cold and for a normal vernalization response. Our data indicate that MSI1 is an indispensable subunit of all PRC2 complexes in *Arabidopsis*. MSI1 was found to interact with LHP1, a major protein for PRC1-like functions in plants. We suggest that a physical link between plant PRC2-like and PRC1-like complexes contributes to the inheritance of H3K27me3 during DNA replication and to the maintenance of H3K27me3 levels during interphase.

Results

MSI1 is a core subunit of the EMF complex

EMF2 is essential for vegetative plant development (Yang *et al*, 1995; Yoshida *et al*, 2001), but the proposed EMF complex has not been isolated yet. To uncover the composition of the EMF complex *in vivo*, we expressed a FLAG-tagged EMF2 in *Arabidopsis* and immunoaffinity-purified the FLAG–EMF2 complex from inflorescences. Wild-type plants served as controls. The purified fractions from four independent experiments were analysed by mass spectrometry. Measured spectra were searched with Mascot against the *Arabidopsis* TAIR9 protein database using a concatenated decoy database and imported into Scaffold. Cutoffs of 90% minimal confidence for protein identification and of 95% minimal confidence for peptide identification were applied. These criteria resulted in a spectrum false-discovery rate below 1%. Only proteins identified with at least two peptides in at least two replicates but not in control samples were taken into account. Three plant PcG proteins were found to co-purify with EMF2: FIE, SWN and MSI1 (Table I and Supplementary Table S1). This is the first demonstration of the composition of the plant EMF complex *in vivo*, showing that the core EMF complex consists of the four main subunits EMF2, MSI1, FIE and SWN. MSI2, 3, 4 and 5 were not found in any experiment, suggesting that these MSI1 homologues are not part of the core EMF complex in inflorescences.

To verify the presence of MSI1 in the EMF complex, we tested the interaction of MSI1 and EMF2 *in vivo*. YFP-tagged EMF2 (YFP–EMF2) and HA-tagged MSI1 (HA–MSI1) or MSI4 (HA–MSI4) were transiently co-expressed in *Nicotiana benthamiana* leaves. YFP–EMF2 was immunoaffinity-purified, and the presence of the co-precipitating proteins was analysed on protein immunoblots. HA–MSI1 but not HA–MSI4 was co-precipitated with YFP–EMF2 (Figure 1A). This result confirms that MSI1 and EMF2 associate into a common complex *in vivo*. MSI4 did not interact with EMF2 *in vivo* in this assay. This finding not only establishes the specificity of the assay but also strengthens the notion that MSI1 but not MSI4 is a core EMF complex subunit *in vivo*.

To provide independent confirmation for the presence of MSI1 in the EMF complex, we performed reciprocal immunoaffinity purification experiments using an *Arabidopsis* line expressing GFP-tagged MSI1 (MSI1–GFP) (Alexandre *et al*, 2009) and a GFP control line. Purified fractions were analysed by mass spectrometry in order to identify proteins co-precipitating with MSI1–GFP. Four independent experiments firmly established the presence of MSI1, EMF2, FIE and SWN in the complex (Table II). PcG proteins EMF2, FIE

Table I EMF2 co-purifies with PcG proteins

Protein	Number of unique peptides/ probability of identification 95% IP1-IP2-IP3-IP4	Sequence coverage (%) IP1-IP2-IP3-IP4	Protein identification probability (%) IP1-IP2-IP3-IP4
EMF2	12-13-12-12	15-21-16-16	100-100-100-100
MSI1	10-7-7-10	34-23-23-38	100-100-100-100
FIE	3-5-5-5	9.8-19-17-14	100-100-100-100
SWN	3-5-3-4	5-7.4-5-5.5	100-100-100-100

FLAG–EMF2 was expressed in *Arabidopsis* under the control of the *35S* promoter. Proteins were identified by immunoaffinity purification of FLAG–EMF2 and mass spectrometry. The experiment was performed with four biological replicates (IP1-4) using inflorescences. Shown are all identified plant PcG proteins.

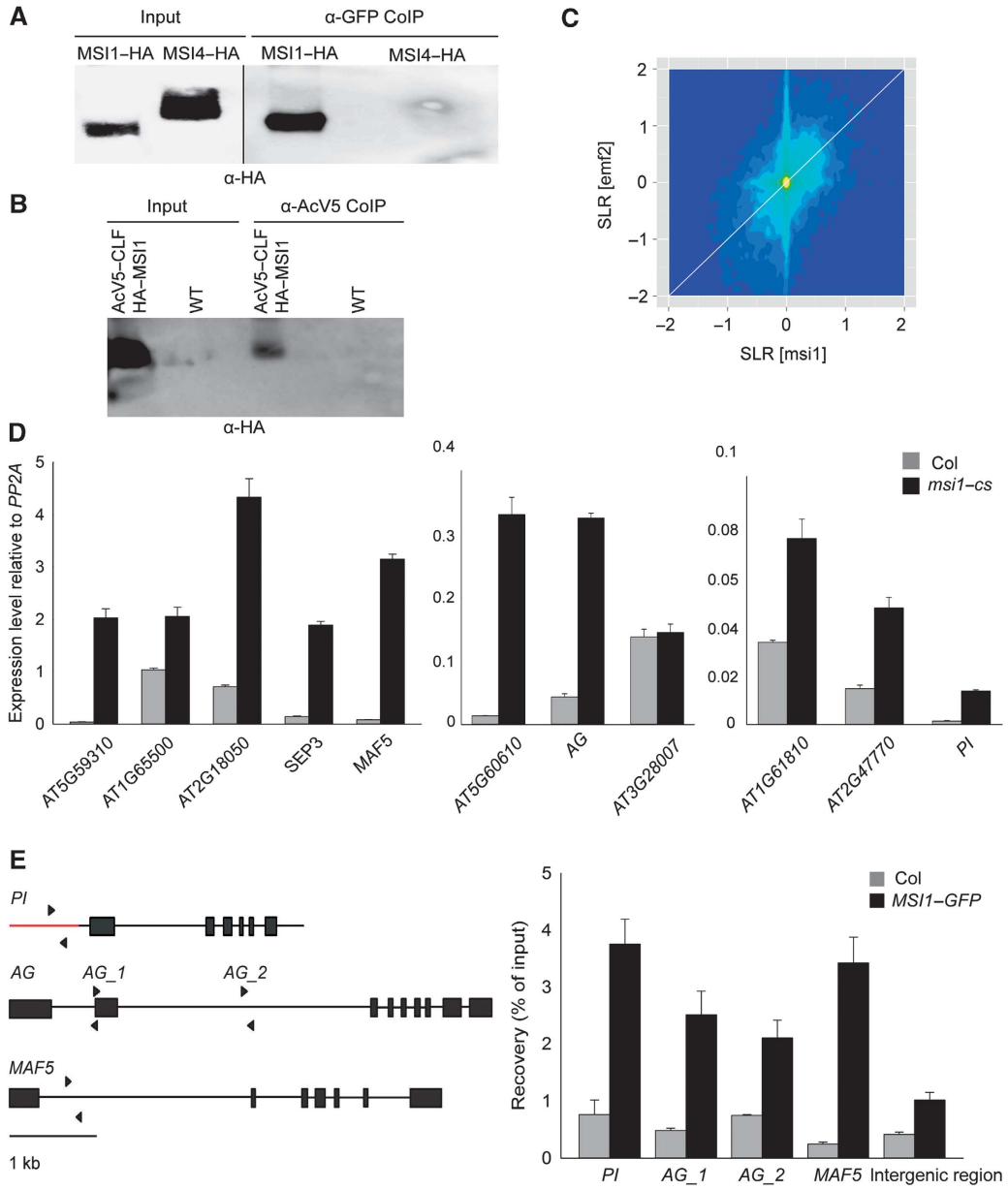

Figure 1 MSI1 is a key subunit of the EMF complex *in vivo*. (**A**) MSI1 co-purifies with EMF2. HA–MSI1 and YFP–EMF2 or HA–MSI4 and YFP–EMF2 were expressed in *N. benthamiana* leaves under the control of *35 S* promoter. YFP–EMF2 was immunoprecipitated, and precipitates were analysed by immunoblotting using anti-HA antibodies. (**B**) MSI1 and CLF are present in the same complex *in vivo*. AcV5–CLF and HA–MSI1 were expressed in *N. benthamiana* leaves under the control of *35 S* promoter. AcV5–CLF was immunoprecipitated, and the precipitates were analysed by immunoblotting using anti-HA antibodies. Wild-type *N. benthamiana* leaves were used as a control. (**C**) Lack of MSI1 and lack of EMF2 cause similar changes in the transcriptome. Transcript signal log ratios (SLR) for an MSI1 co-suppression line (*msi1–cs*) and an *emf2* mutant were plotted. The colour gradient (dark blue to yellow) represents local data point density. The white diagonal line represents identical changes in *msi1–cs* and *emf2*. (**D**) MSI1 is needed for repression of EMF target genes. Quantitative RT–PCR was performed on cDNA from rosette leaves of 6-week-old plants. Relative expression values are shown as mean ± s.e. ($n = 3$). Values were normalized to a *PP2A* gene (*At1g13320*). (**E**) MSI1 is recruited to the chromatin of the EMF target genes. Left: Genomic structure of *PI, AG and MAF5*. Black lines, introns; red line, promoter region; wide bars, exons. Arrows represent the position of primers used for qPCR. The intergenic control region is on chromosome 1 from 8383019 to 8383083 between *At1G23700* and *At1G23710*. Values are recovery as percent of input; shown are mean ± s.d. ($n = 3$). Source data for this figure is available on the online supplementary information page.

and SWN consistently co-purified with MSI1–GFP, confirming that MSI1 is a core subunit of the EMF complex *in vivo*. Consistent with earlier observations (De Lucia *et al*, 2008), the VRN2, VRN5 and VEL1 subunits of the VRN PRC2 complex were also found to associate with MSI1 *in vivo* (Table II). Several non-PcG proteins co-purified with MSI1, including homologues of yeast Rpd3 histone deacetylase complexes (Supplementary Table S2). To confirm these results, we performed additional immunoaffinity purification experiments using a modified protocol involving protein–protein cross-linking prior to protein extraction. These experiments confirmed the presence of the initially identified MSI1 interactors, except for VRN5, and revealed additional candidate interactions (Table III and Supplementary Table S2). Notably, the plant PcG protein LHP1 was found with high confidence in both additional experiments.

Table II MSI1 co-purifies with PcG proteins

Protein	Number of unique peptides/probability of identification 95% IP1-IP2-IP3-IP4	Sequence coverage (%) IP1-IP2-IP3-IP4	Protein identification probability (%) IP1-IP2-IP3-IP4
MSI1	19-34-20-27	52-77-60-71	100-100-100-100
EMF2	10-11-8-6	21-23-15-12	100-100-100-100
FIE	9-0-9-0	29-0-27-0	100-0-100-0
SWN	15-8-14-3	19-9.5-20-4.1	100-100-100-100
VRN2	3-3-2-0	7-8.2-4.8-0	100-100-100-0
VRN5	3-2-0-0	5.1-4.7-0-0	100-100-0-0
VEL1	6-7-2-3	10-11-3.8-5.3	100-100-100-100

MSI1–GFP was expressed in *Arabidopsis* under the control of the *MSI1* promoter. Proteins were identified by immunoaffinity purification of MSI1–GFP and mass spectrometry. The experiment was performed with four biological replicates (IP1-4) using inflorescences. Shown are all identified plant PcG proteins.

Table III Co-purification of MSI1 with PcG proteins from cross-linked protein extracts

Protein	Number of unique peptides/probability of identification 95% IP5c-IP6c	Sequence coverage (%) IP5c-IP6c	Protein identification probability (%) IP5c-IP6c
MSI1	26-23	67-55	100-100
EMF2	11-7	20-11	100-100
FIE	10-4	36-15	100-100
SWN	15-5	19-6.1	100-100
VEL1	16-4	23-6.9	100-100
LHP1	7-3	14-10	100-100

MSI1–GFP was expressed in *Arabidopsis* under the control of the *MSI1* promoter. Proteins were identified by immunoaffinity purification of MSI1–GFP and mass spectrometry. The experiment was performed with two biological replicates (IP5c-6c) using inflorescences. Shown are all identified plant PcG proteins.

Unexpectedly, the well-characterized *Arabidopsis* PcG protein CLF (Goodrich *et al*, 1997) was not found among the MSI1-binding partners. CLF plays a major role during sporophytic plant development (Goodrich *et al*, 1997; Chanvivattana *et al*, 2004; Katz *et al*, 2004; Wood *et al*, 2006; Jiang *et al*, 2008; Doyle and Amasino, 2009) and interacts with EMF2 *in vitro* and in yeast two-hybrid assays (Chanvivattana *et al*, 2004), suggesting that CLF is part of the EMF complex. Identification of proteins by mass spectrometry is affected by many protein-specific factors including protein abundance (Lubec and Afjehi-Sadat, 2007), and it is possible that CLF interacts with MSI1 but failed to be detected under our experimental conditions. This notion was supported by the considerably weaker expression of CLF compared with SWN at both transcript and protein levels (Zimmermann *et al*, 2004; Baerenfaller *et al*, 2011). Therefore, we tested whether MSI1 interacts with CLF *in vivo* using an alternative approach. AcV5-tagged CLF (AcV5–CLF) and HA–MSI1 were transiently co-expressed in tobacco leaves, AcV5–CLF was immunoaffinity-purified, and the presence of the co-precipitating proteins was analysed on protein immunoblots. HA–MSI1 was co-precipitated with AcV5–CLF (Figure 1B). This result demonstrates that MSI1 and CLF can associate into a common complex *in vivo*.

Together, these experiments establish that MSI1, EMF2 and FIE, together with SWN or CLF, constitute the EMF complex.

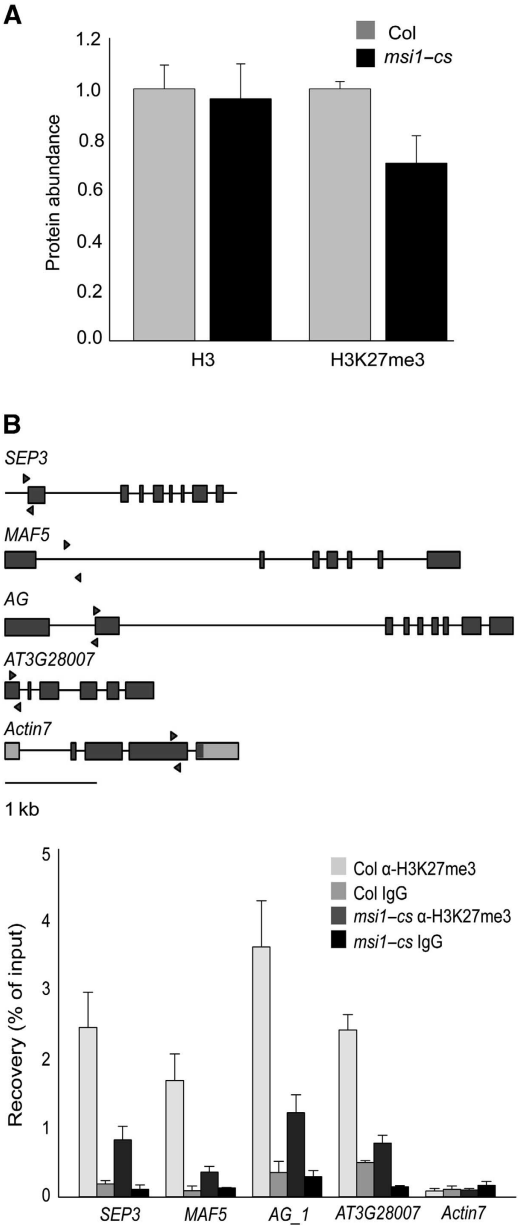

Figure 2 MSI1 is needed for trimethylation of H3K27. (**A**) Global H3K27me3 levels are reduced in *msi1–cs* plants. Total protein levels were analysed by quantitative immunoblotting using anti-H3K27me3 and anti-H3 antibodies in Col and *msi1–cs* plants. Shown are mean ± s.d. ($n = 3$). (**B**) H3K27me3 is reduced at the chromatin of EMF target genes in *msi1–cs* plants. Top: genomic structure of *SEP3*, *MAF5*, *AG*, *AT3G28007* and *ACTIN7*. Black lines, introns; wide bars, exons. Arrows represent the position of primers used for qPCR. Values are recovery as percent of input; shown are mean ± s.d. ($n = 3$).

In contrast, there is no strong evidence for functions of MSI2–5 in the EMF complex.

MSI1 is essential for the function of the EMF complex

To establish whether MSI1 is required for the function of the EMF complex, we determined the expression levels of EMF target genes in an MSI1 co-suppression line (*msi1–cs*) in which the MSI1 protein level is reduced to less than 10% (Hennig *et al*, 2003). We compared the transcriptional profiles of *msi1–cs* (Alexandre *et al*, 2009) and *emf2* plants

(Liu *et al*, 2012) and found that transcriptional changes were strongly and significantly correlated between plants of the two genotypes (Pearson correlation = 0.44, $P < 2.2e-16$) (Figure 1C). Note that this strong correlation was observed despite considerable differences in experimental conditions (rosette leaves of 23-day-old *msi1–cs* plants that retain ~5% MSI1 protein and 7-day-old *emf2*-null mutant seedlings). The global similarity of transcriptional changes caused by reduced MSI1 or EMF2 loss of function strongly suggests that the biochemical interaction of MSI1 and EMF2 is of functional relevance. The data also confirm that redundancy among MSI1 homologues is limited and that MSI2–5 can only partially, if at all, substitute MSI1 in the EMF complex.

To confirm the microarray data on deregulation of EMF target genes in *msi1–cs* plants, we tested the expression of some known PcG target genes in leaves (Lafos *et al*, 2011) by RT–qPCR using independent samples (Figure 1D). Ten of 11 tested PcG target genes were upregulated in *msi1–cs* plants, demonstrating that the presence of MSI1 in the EMF complex is necessary for the repression of many EMF target genes. Next, we used ChIP to test whether MSI1 binds to EMF target genes. The results show an enrichment of MSI1 at the previously described EMF target genes *PISTILLATA (PI)*, *AG* and *MADS AFFECTING FLOWERING 5(MAF5)* (Figure 1E), demonstrating that MSI1 is recruited to at least some EMF target genes. Because PRC2 complexes trimethylate H3K27 in target chromatin, we tested whether MSI1 is needed for this PRC2 function. We found that global H3K27me3 levels were reduced to 70% in *msi1–cs* plants (Figure 2A, Supplementary Figure S1). Similarly, ChIP results also showed that H3K27me3 is highly reduced in EMF target genes in *msi1–cs* plants (Figure 2B). Notably, *At3g28007* has no increase in expression in *msi1–cs* but has reduced H3K27me3 demonstrating that loss of H3K27me3 is not a consequence of increased transcription. Together, these results demonstrate that MSI1 is required for full PRC2 function and normal H3K27me3 levels *in vivo*. Because the MSI1-like subunit was found to be dispensable for PRC2 catalytic activity *in vitro* (Schmitges *et al*, 2011), our findings suggest that MSI1 functions in PRC2 regulation or targeting *in vivo*.

MSI1 regulates *FLC* expression and the vernalization response

MSI1 is a subunit of the VRN–PHD complex (Table II and De Lucia *et al*, 2008), which represses *FLC* after vernalization, but the function of MSI1 in this complex has not been addressed so far. The *msi1–cs* line showed MSI1 protein reduction and developmental alterations only at the rosette stage (Hennig *et al*, 2003) and thus did not appear suitable for testing MSI1 function in seedling vernalization. In contrast, *MSI1* anti-sense (*msi1–as*) lines contain about 30–50% of wild-type MSI1 levels in seedlings and exhibit developmental alterations at seedling and rosette stages (Exner *et al*, 2006). To test whether MSI1 also functions in the vernalization response, we analysed flowering time and *FLC* expression with and without vernalization in *msi1–as* and wild-type plants. Vernalized wild-type plants flowered earlier than non-vernalized plants, forming only about half the number of rosette leaves (Figure 3A). Consistent with the phenotype, *FLC* transcript levels were strongly reduced in vernalized wild-type plants compared with non-vernalized controls

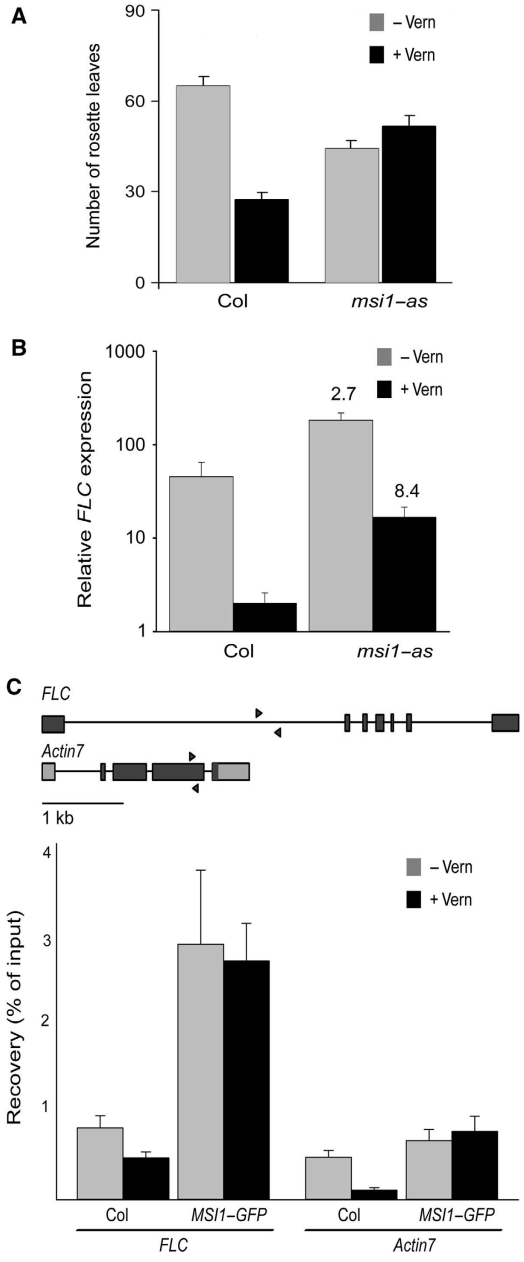

Figure 3 MSI1 functions in the vernalization response via regulation of *FLC* expression. (**A**) The vernalization response is strongly impaired in *MSI1* anti-sense plants (*msi1–as*). Plants were vernalized for 6 weeks followed by cultivation in SD. Flowering time was measured as the number of rosette leaves produced before bolting. Shown are means ± SE ($n \geqslant 14$). (**B**) *FLC* is only partially repressed by vernalization in *msi1–as* plants. Quantitative RT–PCR was performed on cDNA from vernalized (6 weeks at 4°C and 10 days at 23°C) and non-vernalized (10 days at 23°C) plants grown in SD. Relative expression values are shown as mean ± SE ($n = 3$). Values were normalized to a *PP2A* gene. Values shown above bars represent fold change relative to the wild-type control. (**C**) MSI1 is recruited to the *FLC* locus. Top: Genomic structure of *FLC* and *ACTIN7*. Black lines, introns; wide bars, exons. Arrows represent the position of primers used for qPCR. Values are recovery as percent of input; shown are mean ± s.d. ($n = 3$).

(Figure 3B). In contrast, vernalized *msi1–as* plants flowered similarly to non-vernalized *msi1–as* plants (Figure 3A), revealing that a normal vernalization response requires MSI1. Non-vernalized *msi1–as* plants flowered earlier than

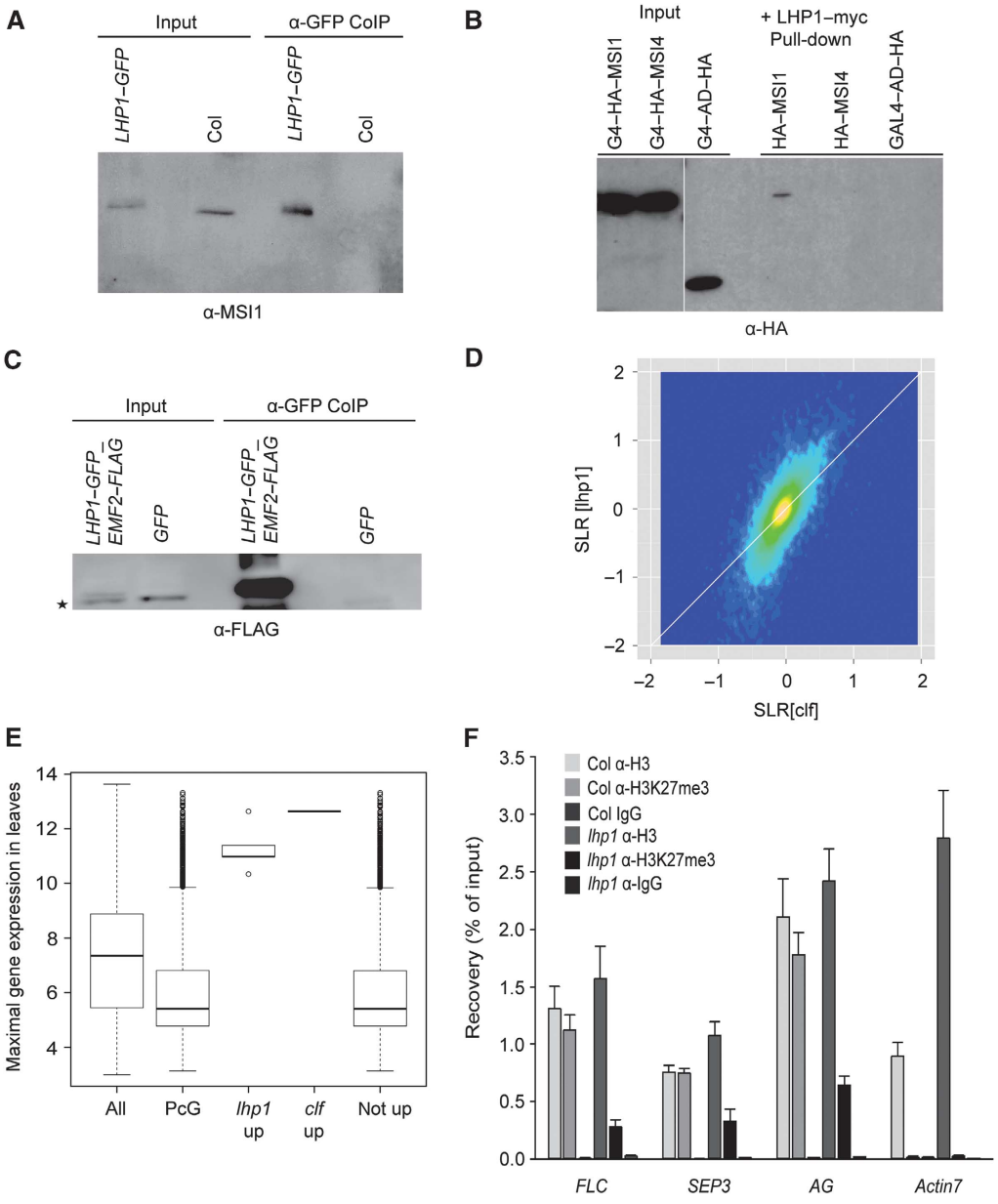

Figure 4 MSI1 connects LHP1 to PRC2. (**A**) MSI1 co-purifies with LHP1. LHP1–GFP was immunoprecipitated from inflorescences of *35 S::LHP1–GFP* plants, and precipitates were analysed by immunoblotting using anti-MSI1 antibodies. Col wild-type plants served as control. (**B**) MSI1 interacts directly with LHP1. LHP1–myc was immunoprecipitated from extracts of yeast expressing either HA–GAL4–AD–MSI1 and LHP1–myc or HA–GAL4–AD-–MSI4 and LHP1–myc or HA–GAL4–AD and LHP1–myc. Precipitates were analysed by immunoblotting using anti-HA antibodies. (**C**) LHP1 co-purifies with EMF2. GFP was immunoprecipitated from inflorescences of plants expressing LHP1–GFP and EMF2–FLAG or GFP, respectively, under the control of the *35S* promoter and analysed by immunoblotting using anti-FLAG antibodies. The asterisk marks an unspecific, cross-reacting band. (**D**) LHP1 function in gene silencing is restricted to the PcG system. Lack of LHP1 and lack of CLF cause similar changes in the transcriptome. Signal log ratios (SLR) for a *clf* and an *lhp1* mutant were plotted. The colour gradient (dark blue to yellow) represents local data point density. The white diagonal line represents identical changes in *clf* and *lhp1*. (**E**) Only PcG target genes with the potential to be expressed in leaves are upregulated in *lhp1* and *clf* mutants. A gene's potential to be expressed in wild-type leaves was estimated as its maximal expression in wild-type leaves according to the developmental series of AtGenExpress transcriptome data (Schmid *et al*, 2003). (All) all genes; (PcG) all PcG target genes in leaves (Lafos *et al*, 2011); (*lhp1* up) PcG target genes from leaves that are upregulated in *lhp1*; (*clf* up) PcG target genes from leaves that are upregulated in *clf*; (not up) PcG target genes from leaves that are not upregulated in *lhp1* or *clf*. While most PcG genes have very low leaf expression potentials and are thus inactive throughout wild-type leaf development, PcG target genes that were upregulated in *lhp1* or *clf* had very high expression potentials and thus are active at certain stages of wild-type leaf development. **(F)** H3K27me3 at PcG target genes is reduced in *lhp1* mutants. ChIP was done using roots enriched for dividing cells by 2,4-D treatment. Values are recovery as percent of input; shown are mean ± s.d. ($n = 3$).

non-vernalized wild type, possibly because of a partial loss of repression of floral activators that are under PcG protein control. Without vernalization, *FLC* levels were increased in *msi1–as* (Figure 3B). Under such conditions, *FLC* is controlled by the EMF complex (Jiang *et al*, 2008), and the increased *FLC* expression in *msi1–as* is consistent with the requirement for MSI1 in EMF complex function. More importantly, vernalization was less effective in reducing *FLC* transcript levels in *msi1–as* than in wild-type plants (11-fold versus 22-fold reduction) (Figure 3B). The reduced efficiency of

vernalization treatments to repress *FLC* and accelerate flowering in *msi1–as* demonstrates that MSI1 is required for a normal vernalization response.

Next, we tested whether regulation of *FLC* by MSI1 is direct. In ChIP experiments, MSI1 was enriched at *FLC* both without and after vernalization (Figure 3C), demonstrating that MSI1 is indeed recruited to *FLC*. The core VRN complex is present at the *FLC* locus already without vernalization (De Lucia *et al*, 2008) and EMF2 also regulates *FLC* (Jiang *et al*, 2008), suggesting that MSI1 can bind to *FLC* as part of the EMF complex and as part of the VRN complex. Together, these results demonstrate that MSI1 is needed for VRN complex function and a normal vernalization response.

MSI1 connects LHP1 to PRC2

The cross-linked immunoaffinity purification of MSI1–GFP identified LHP1 among the interacting proteins (Table III). To confirm the interaction between MSI1 and LHP1 *in vivo*, we performed a co-immunoprecipitation (CoIP) assay. Immunoblot analyses revealed co-immunoprecipitation of MSI1 with LHP1, demonstrating that LHP1 and MSI1 indeed coexist in shared complex(es) *in vivo* (Figure 4A).

To determine whether MSI1 interacts directly with LHP1, we carried out an *in vitro* pull-down assay. HA–GAL4–AD-tagged MSI1, HA–GAL4–AD-tagged MSI4 and myc-tagged LHP1 were expressed in yeast, and extracts were used for immunoprecipitation with anti-myc antibodies. Immunoblot analyses revealed the presence of MSI1 but not of MSI4 or the negative control in the bound fraction (Figure 4B). The binding of MSI1 and not of MSI4 to LHP1 in the absence of any other plant proteins strongly suggests that the MSI1–LHP1 interaction is specific and direct.

LHP1 fulfils PRC1-like functions in plants (Turck *et al*, 2007; Zhang *et al*, 2007; Exner *et al*, 2009), and we found that LHP1 interacts with MSI1. Thus, it appeared possible that LHP1 interacts with plant PRC2 complexes via MSI1. To test whether LHP1 also interacts with EMF2 *in vivo*, we performed a CoIP assay using *EMF2–FLAG LHP1–GFP* double-transgenic plants. Immunoblot analyses clearly showed that EMF2 co-precipitated with LHP1, demonstrating that both proteins coexist in shared complex(es) *in vivo* (Figure 4C). Because LHP1 interacted with both MSI1 and EMF2, which are present together in the EMF complex, we conclude that LHP1 interacts with the EMF complex *in vivo*. This suggests that in plants PRC1- and PRC2-like functions are closely integrated.

LHP1 function in gene silencing is restricted to the PcG system

Our finding that MSI1 connects LHP1 to plant PRC2 complexes extends previous findings of LHP1 functions in the plant PcG system (Kotake *et al*, 2003; Libault *et al*, 2005; Mylne *et al*, 2006; Sung *et al*, 2006; Turck *et al*, 2007; Zhang *et al*, 2007; Xu and Shen, 2008; Exner *et al*, 2009; Bratzel *et al*, 2010; Latrasse *et al*, 2011). At the same time, our results raise the question regarding the extent to which LHP1 may function independently of the PcG system. LHP1 is a homologue of HP1 and SWI6, which in metazoa and fission yeast, respectively, function in heterochromatic gene silencing (Zeng *et al*, 2010) and can bind to heterochromatic H3K9me2 *in vitro*. To search for potential PcG-unrelated functions of LHP1, we profiled transcriptional changes in *lhp1* and *clf* mutants. One concern with transcript profiling experiments in *lhp1*, *clf* and other mutants with pleiotropic phenotypes is the confounding effect of secondary transcriptional changes. Because the pleiotropic phenotype of *clf* is mostly suppressed under short-day photoperiods (SD) (Schatlowski *et al*, 2010), the experiment was carried out in SD. The pleiotropic phenotype of *lhp1* is less repressed by SD but greatly depends on *FT* (Kotake *et al*, 2003). Therefore, we used a *lhp1 ft* double mutant. Together, we expect that these conditions will considerably reduce secondary transcriptional changes. The transcriptional changes between *lhp1* and *clf* were strongly and significantly correlated (Pearson correlation $= 0.725$, $P = 2.2e-16$) (Figure 4D). There was no considerable subpopulation of genes that was miss-expressed in *lhp1* and not changed in *clf*. The amplitude of changes, however, was frequently higher in *lhp1* than in *clf* (cf. the deviation from the diagonal in Figure 4D). Linear regression suggested that fold changes were on average two-fold larger in *lhp1* than in *clf*, which was probably caused by partial redundancy between CLF and SWN. A Venn diagram representation of the most strongly upregulated genes in *emf2*, *clf*, *msi1–cs* and *lhp1* plants shows considerable overlap (Supplementary Figure S2). Differences between gene sets are probably caused by false negatives, differences in plant material and assay conditions and by partial redundancy of some of the genes. It is interesting to note that only a subset of PcG target genes lost repression in the mutants. It was possible that only genes that have the potential to be expressed in leaves were detected as upregulated in *lhp1* or *clf* rosette leaves. We tested this hypothesis using gene-specific leaf expression potentials that were based on all wild-type leaf samples in the developmental AtGenExpress data resource, including cotyledons, rosette and cauline leaves of diverse age or harvesting time (Schmid *et al*, 2003). The *leaf expression potential* for a gene is the maximal expression of this gene observed in any of the wild-type leaf samples. Genes with low leaf expression potentials are inactive throughout wild-type leaf development, while genes with high leaf expression potentials are active at certain stages of leaf development. Leaf expression potentials of PcG target genes were considerably smaller than the genome average, demonstrating that many PcG target genes were not expressed in the leaf samples (Figure 4E). Similarly, the PcG target genes that were not upregulated in *lhp1* or *clf* had generally low expression potentials, demonstrating that most of them were not expressed in any leaf sample. In contrast, the PcG target genes that were strongly upregulated in *lhp1* or *clf* had a very high expression potential, demonstrating that they were highly expressed in some leaf samples (Figure 4E). This result is in agreement with the proposal that PcG targets become upregulated in PcG mutants only in tissues in which they have a potential to be expressed (Farrona *et al*, 2011). Together, our data establish that the main function of LHP1 in gene regulation is related to the PcG system and that there is no evidence of a PcG-independent function of LHP1.

LHP1 is needed to establish full H3K27me3 levels

During the S-phase, new histones are incorporated into replicating chromatin, and existing histone modifications are transiently diluted. Cells have various mechanisms for

re-establishing local histone modifications during replication. We sought to determine whether LHP1 could recruit plant PRC2 complexes to PcG target genes and contribute to the re-establishment of H3K27me3 in dividing cells. In order to establish the high number of dividing cells needed to test this hypothesis, lateral root outgrowth was induced by the synthetic auxin 2,4-Dichlorophenoxyacetic acid in wild-type and *lhp1* seedlings (Supplementary Figure S3). ChIP with anti-H3 and anti-H3K27me3 antibodies was performed using roots. In wild type, H3K27me3 signals were detected at known PcG protein target genes including *SEP3* and *FLC* but not at the negative control gene *ACT7*. Consistent with our hypothesis of a contribution of LHP1 to the establishment of H3K27me3 in dividing cells, H3K27me3 of the PcG target genes was significantly lower in *lhp1* than in wild type (Figure 4F). Thus, the discovered physical link between LHP1 and PRC2 is highly relevant for the function of the plant PcG protein system.

Discussion

MSI1-like proteins form an evolutionarily conserved family of proteins that is present in all organisms except prokaryotes. Some organisms such as *Drosophila* have only one MSI1 homologue, while others such as *Arabidopsis* have several (for review, see Hennig *et al*, 2005). It has been suggested that MSI1-like proteins function via their histone H3 and H4 binding pockets as it has been demonstrated in detail for the *Drosophila* MSI1 homologue p55 (Song *et al*, 2008; Nowak *et al*, 2011; Schmitges *et al*, 2011). *Drosophila* p55 is involved in many chromatin-remodelling complexes: CHROMATIN ASSEMBLY FACTOR1 (CAF-1), histone deacetylase complexes, histone acetyl–transferase complex, the nucleosome-remodelling factor NURF complex and PRC2 (Tyler *et al*, 1996; Martinez-Balbas *et al*, 1998; Tie *et al*, 2001; Czermin *et al*, 2002; Müller *et al*, 2002; Nekrasov *et al*, 2005). Notably, conflicting results have been published about the role of p55 in *Drosophila* PRC2: although p55 is not required for PRC2 enzymatic activity *in vitro* (Cao and Zhang, 2004; Ketel *et al*, 2005) and complete loss of the p55 gene did not affect global H3K27me3 levels in fly larvae (Wen *et al*, 2012), sectorial loss of p55 caused reduced H3K27me3 in eye discs (Anderson *et al*, 2011). In organisms that have multiple *MSI1*-like genes, it is not clear how much functional redundancy exists between them. In *Arabidopsis,* there are three main clades of *MSI1*-like genes, MSI1, MSI2/MSI3 and MSI4/MSI5, which evolved before the divergence of monocots and dicots (Hennig *et al*, 2005). The existence of five MSI1-like proteins in *Arabidopsis* raises the question of whether only one or different MSI1-like proteins are subunits of the various PRC2 complexes and whether they are functionally redundant. Earlier work had revealed that MSI1 has an essential function in the FIS–PRC2 complex during seed development that is not redundant with MSI2–5 (Köhler *et al*, 2003; Guitton *et al*, 2004; Guitton and Berger, 2005; Leroy *et al*, 2007). However, knowledge about the predicted MSI1-like subunit in the sporophytic PRC2 complexes in *Arabidopsis* has remained fragmented. Because MSI1-like proteins are not needed for *in vitro* PRC2 enzymatic activity in animals, it was even possible that trimeric PRC2 complexes lacking an MSI1-like subunit exist.

MSI1 functions in the EMF and VRN complexes

On the basis of genetic and *in vitro* protein–protein interaction data, the EMF complex is considered to be the major sporophytic plant PRC2 complex (Yoshida *et al*, 2001; Chanvivattana *et al*, 2004; Katz *et al*, 2004; Schönrock *et al*, 2006; Jiang *et al*, 2008). Here, we provide biochemical *in vivo* evidence for the presence and composition of the EMF complex. We found that this complex comprises MSI1, EMF2, FIE and SWN. MSI1 and FIE are also known to be subunits of the FIS (Köhler *et al*, 2003) and VRN complexes (De Lucia *et al*, 2008) and are thus conserved among all known *Arabidopsis* PRC2 complexes. Interestingly, the histone methyltransferase SWN was well represented in the EMF and VRN complexes, while its homologue CLF was not or was only weakly represented (this work and De Lucia *et al*, 2008). The role of CLF as a key PcG protein in sporophyte development has been well established (Goodrich *et al*, 1997; Chanvivattana *et al*, 2004; Katz *et al*, 2004; Wood *et al*, 2006; Jiang *et al*, 2008; Doyle and Amasino, 2009), and we confirmed that CLF can form a complex with MSI1 *in vivo*. Several explanations exist for the low representation of CLF in PRC2 complexes analysed by MS/MS: first, identification of CLF and SWN in MS/MS assays could differ, as it has been observed for other proteins (for review, see Lubec and Afjehi-Sadat, 2007). Second, SWN could associate with other PRC2 subunits stronger than CLF, resulting in a preferential loss of CLF during purification. Third, stronger expression of SWN as evident from transcript and protein abundance compendia (Zimmermann *et al*, 2004; Baerenfaller *et al*, 2011) could lead to higher levels of SWN- than CLF-containing PRC2 complexes. Genetic analysis showed that *CLF* is partially redundant with *SWN* (Chanvivattana *et al*, 2004) but that only *clf* and not *swn* mutants have obvious developmental defects. The strong developmental alterations upon loss of the less abundant CLF may be caused by a subset of CLF-specific PcG protein target genes not shared with SWN-containing PRC2 complexes. Notably, most of the strongest developmental alterations in *clf* depend on misexpression of a few genes, including *AG* and *FT* (Goodrich *et al*, 1997; Lopez-Vernaza *et al*, 2012), and it is possible that these genes specifically depend on a CLF complex. Future experiments will have to establish the differential roles of CLF and SWN in plant PRC2 complexes; here, we conclude from our own and other data (De Lucia *et al*, 2008) that SWN is a major histone methyltransferase subunit in the EMF and VRN complexes *in vivo*.

Unlike the p55 function in *Drosophila* PRC2 that remains controversial (Anderson *et al*, 2011; Schmitges *et al*, 2011; Wen *et al*, 2012), we found that *Arabidopsis* MSI1 is required for EMF complex function *in vivo* and that it is recruited to the chromatin of at least some EMF target genes. MSI1 has four homologues in *Arabidopsis*, of which MSI4 and MSI5 are known to act redundantly in the repression of *FLC* and its homologues *MAF4* and *MAF5*, promoting floral transition (Ausin *et al*, 2004; Kim *et al*, 2004; Gu *et al*, 2011). Both proteins associate with HISTONE DEACETYLASE 6 (HDA6) and are recruited to the *FLC* locus, leading to de-acetylation of histones and silencing of *FLC* (Gu *et al*, 2011). MSI4 was also implicated in the silencing of *FLC* and *FT* through its association with the CLF–PRC2 complex and the cullin-RING ubiquitin ligase (CUL4 DDB) (Pazhouhandeh *et al*, 2011). Here, we show that MSI1 is recruited to the *FLC*

locus and that it is required for *FLC* repression, demonstrating that MSI4 and MSI5 cannot substitute for MSI1 function in *FLC* silencing. MSI4 and MSI5 were also not found in the EMF or VRN complexes *in vivo* (this work; De Lucia *et al*, 2008) and MSI4 failed to interact with EMF2 *in vitro*. Together, we suggest that MSI1 functions as the subunit of the EMF and VRN complexes that is homologous to p55 in *Drosophila* PRC2, while MSI4 interacts with PRC2 as part of histone deacetylase and/or CUL4 DDB complexes.

MSI1 functions in the VRN complex and is required for the vernalization response

A vernalization response is the increase in capacity to flower after long exposure to cold. It ensures that plants start flowering only in the spring when conditions are optimal. The main effect of vernalization at the molecular level is a cold-induced epigenetic silencing of *FLC* by the VRN PRC2 complex, which is maintained through further plant development (Michaels and Amasino, 1999; Sheldon *et al*, 1999; Gendall *et al*, 2001; Bastow *et al*, 2004). The core VRN complex is already associated with the *FLC* locus before cold treatment. After exposure to cold, the VRN–PHD complex, which contains the additional three PHD-finger proteins VRN5, VIL2 and VIN3, promotes spreading of H3K27me3 over *FLC* (De Lucia *et al*, 2008). MSI1 co-purified with the VRN–PHD complex *in vivo* (this work and De Lucia *et al*, 2008), but it was not known whether this reflects a function of MSI1 in the vernalization response. Here, we report that MSI1 is present at the *FLC* locus and is required for normal repression of *FLC* and for accelerated flowering after vernalization. Thus, MSI1 is essential for the function of the core VRN complex and of the VRN–PHD complex *in vivo* and is needed for vernalization response in plants.

MSI1 bridges LHP1 to PRC2

Parts of the PcG system are conserved between animals and plants, but it has remained unclear how far the functional similarities extend (Hennig and Derkacheva, 2009). In particular, repression by PcG proteins in animals requires the coordinated function of PRC2 and PRC1, but the identity of PRC1 complexes in plants has not yet been fully established. In *Arabidopsis*, LHP1 is considered to be one of the main proteins with a PRC1-like function (Turck *et al*, 2007; Zhang *et al*, 2007; Exner *et al*, 2009). We found that LHP1 can directly bind MSI1 *in vitro* and can be co-immunoprecipated with MSI1 and EMF2 *in vivo*, establishing that LHP1 and the EMF–PRC2 complex interact. Although the function of LHP1 in the plant PcG system has been established before, it remained possible that LHP1 had not only PcG-related but also PcG-independent functions. This idea was supported by the homology of LHP1 to animal HP1 and fission yeast SWI6, by some reports of LHP1 targeted to heterochromatin (Zemach *et al*, 2006) and by the finding that LHP1 binds methylated H3K9 (Turck *et al*, 2007; Zhang *et al*, 2007). Our genome-wide transcript profiling established that LHP1 and CLF have very similar effects in the transcriptome. Thus, we conclude that the main function of LHP1 in gene regulation is restricted to the PcG system.

Possible functions of an LHP1–PRC2 interaction

It is possible that the LHP1–PRC2 interaction facilitates recruitment of PRC2 to target genes. In animals and plants, targeting of PcG proteins is poorly understood. It has been suggested for *Drosophila* PcG proteins that they are recruited by DNA-binding proteins and also by non-coding RNAs (ncRNAs) (for review, see Sawarkar and Paro, 2010). At least in some cases, ncRNAs contribute to PcG targeting in

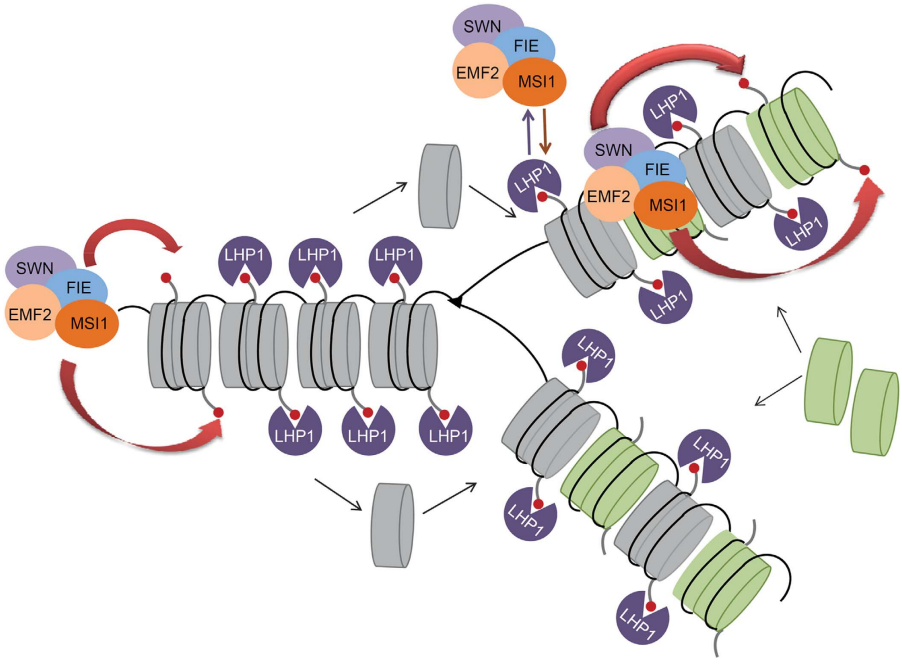

Figure 5 Model of LHP1 function in semi-conservative inheritance of H3K27me3. During DNA replication, new histones are incorporated into chromatin diluting epigenetic marks. We propose that LHP1 binds to nucleosomes with old histones that carry H3K27me3 and via binding to MSI1 recruits the EMF complex, which trimethylates H3K27 of newly incorporated histones. H3K27me3 is symbolized by red circles, old and new nucleosomes are grey and green, respectively.

Arabidopsis as well, such as during *FLC* repression by the VRN complex (Heo and Sung, 2011). Because LHP1 is recruited by the transcription factors SCARECROW and SHORT VEGETATIVE PHASE to the *MAGPIE* and *SEP3* loci, respectively (Cui and Benfey, 2009; Liu *et al*, 2009), it is possible that *Arabidopsis* PRC2 recruitment to some target genes depends on transcription factor—LHP1 interactions.

Histone demethylation, as well as incorporation of newly synthesized, non-methylated histones during DNA replication and histone exchange in interphase, causes a continuous loss of H3K27me3 from target chromatin, and stable PcG silencing requires re-establishment of full H3K27me3 levels at target loci. In mammals, the ESC subunit of PRC2 can bind to H3K27me3, suggesting a self-recruiting mechanism to counteract loss of H3K27me3 during DNA replication, histone exchange or demethylation (Hansen *et al*, 2007; Margueron *et al*, 2009), while *Drosophila* PcG proteins remain associated with DNA during replication independent of H3K27me3 (Petruk *et al*, 2012). For plants, our findings suggest a model in which LHP1 assists in the recruitment of PRC2 to target sites for re-establishing reduced H3K27me3 levels. This model is consistent with the high LHP1 expression in proliferating cells (Kotake *et al*, 2003; Baerenfaller *et al*, 2011) and the interaction of LHP1 with the POL2a subunit of DNA polymerase epsilon (DNA Pol ε) (del Olmo *et al*, 2010). Because LHP1 interacts with plant POL2a, it is possible that LHP1 functions during S-phase in the re-establishment of full H3K27me3 levels after replication (Figure 5). This model is strongly supported by the H3K27me3 ChIP experiment in *lhp1* mutants. The model we propose for inheritance of the repressed state of PcG target genes in plants has striking similarity to a model of inheritance of heterochromatic states in yeast and animals (Bannister *et al*, 2001). In the latter, the LHP1 homologues SWI6 and HP1 bind to H3K9me2 containing chromatin and recruit the H3K9 methyltransferase CLR4/SUV39H1 to re-establish H3K9me2 after replication. Similar to plant LHP1, which interacts with DNA Pol ε, SWI6 and HP1 are recruited to DNA replication forks (Lewis, 1978; Murzina *et al*, 1999). Thus, it is possible that a common function of HP1 homologues is based on their association with DNA-replication forks to recruit effector proteins such as histone methyltransferases to target chromatin.

Together, we propose that MSI1 functions in *Arabidopsis* PRC2 complexes to link PRC2 to LHP1, which then serves to tether PRC2 to target chromatin and maintain full H3K27me3 levels after DNA replication, histone exchange or demethylation.

Materials and methods

Plant material and growth conditions

Wild-type plants were *Arabidopsis thaliana* accession Columbia (Col). Transgenic plants were generated by floral dip with *Agrobacterium tumefaciens* (strain GV 3101) (Logemann *et al*, 2006). To generate constructs for tagged EMF2, CLF, MSI1 and MSI4 proteins, cDNAs were cloned into vectors pEarleyGate 201, 202 and 204 (Earley *et al*, 2006), which were transformed into Col plants or infiltrated into leaves of *N. benthamiana* as previously described (Goodin *et al*, 2002). The *MSI1–GFP*, *LHP1–GFP*, MSI1 co-suppression (*msi1–cs*) and *MSI1* anti-sense (*msi1–as*) plant lines have been described earlier (Hennig *et al*, 2003; Exner *et al*, 2006; Alexandre *et al*, 2009; Exner *et al*, 2009). The *lhp1-6*, *clf1-29* and *ft-10* alleles were used (Yoo *et al*, 2005; Schönrock *et al*, 2006; Exner *et al*, 2009). *EMF2–FLAG*, *LHP1–GFP* and *lhp1-6 ft-10* plants were obtained by crossing. Plant growth conditions were as described previously (Exner *et al*, 2009). Vernalization treatments and measuring of flowering time were carried out as described earlier (Bouveret *et al*, 2006; Möller-Steinbach *et al*, 2010). For induction of root cell division, seedlings were grown vertically on ½ MS, 1% sucrose and 0.8% agar plates under constant light conditions. After 5 days, seedlings were transferred to plates supplemented with 750 nM 2,4-dichlorophenoxyacetic acid (2,4-D) and grown for an additional 3 days. Roots were separated from the shoots and used for ChIP and gene expression analyses.

Immunoprecipitation and protein immunoblot analyses

For immunoprecipitation (IP) followed by mass spectrometry 10 g of plant material was ground in a mortar with liquid nitrogen; for co-immunoprecipitation (CoIP) 2–4 g of plant material was used. Soluble proteins were extracted in 2 volumes of extraction buffer (10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% Igepal, 1% Triton -00x and protease inhibitors (Roche, Basel, Switzerland)) at 4°C for 30 min with gentle rocking. For protein cross-linking 2 mM DTSSP was added to the extraction buffer, in which Tris was replaced with 20 mM HEPES, and the extracts were incubated at 4°C for 2 h. To stop cross-linking, 50 mM Tris–HCl (pH 7.5) was added, followed by incubation at 4°C for 20 min. To extract non-soluble proteins, NaCl was added to the final concentration of 2.5 M, followed by incubation at 4°C for 1 h. The centrifuged supernatant (4500 *g*) was filtered through four layers of Miracloth (Calbiochem, San Diego, CA, USA) and desalted using PD-10 desalting columns (GE Healthcare, Little Chalfont, UK). The centrifuged (4500 *g*) supernatant was pre-cleared with 30 µl of pre-washed protein A sepharose beads (GE Healthcare, Little Chalfont, UK) at 4°C for 20 min with gentle rocking. An input aliquot was taken from the pre-cleared centrifuged ($2000 \times g$) supernatant before the rest of the supernatant was subjected to IP with 50 µl of bead-coupled antibodies at 4°C for 2 h with gentle rocking. The precipitate was washed six times in extraction buffer and eluted in 2 × Laemmli buffer. The following antibodies were used for IP: anti-FLAG magnetic beads (Sigma, #M8823), anti-GFP Trap_A (Chromotek, Planegg-Martinsried, Germany), anti-HA antibodies (Sigma, #H3663) and anti-AcV5 antibodies (Sigma, #A2980) coupled to protein A agarose beads and anti-myc beads (Sigma, #A7470). For protein immunoblots, proteins were separated by 12% SDS–PAGE and transferred to a PVDF membrane (Roth, Karlsruhe, Germany) by semi-dry blotting in 25 mM Tris–HCl (pH 8.3), 150 mM Glycin and 10% methanol for 1 h at 15 V. Enhanced chemiluminescence detection was performed as recommended by the manufacturer (GE Healthcare). The following antibodies and dilutions were used for immunoblotting: anti-HA (Sigma, #H3663), 1:1000; anti-V5 (Sigma, #V8012), 1:1000; anti-MSI1 (Hennig *et al*, 2003), 1:1000; anti-FLAG (Sigma, #A8592), 1:1000; anti-H3K27me3 (Millipore, Billerica, Massachusetts, USA, #07-449), 1:1000; and anti-H3 (Abcam, Cambridge, USA, # ab24834), 1:1000.

Histone extraction and quantitative immunoblotting

Approximately 2 g of frozen rosette leaves were ground to a fine powder and homogenized for 15 min in histone extraction buffer (0.25 M sucrose, 1 mM $CaCl_2$, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 15 mM PIPES, pH 7, 0.5% Triton X-100 including protease inhibitors (Roche) and 10 mM sodium butyrate). Extracts were cleared by centrifugation and pellets were dissolved in 0.2 N H_2SO_4. Total histones were precipitated with 33% Trichloroacetic acid, washed twice with acetone containing 0.1% HCl and once with acetone, briefly air-dried and dissolved in 1 x Laemmli buffer. The histone extract was run on 15% SDS–PAGE gels and transferred onto PVDF membranes (Roth). Proteins were probed with rabbit anti-H3K27me3 mixed with mouse anti-H3 (Abcam) antibodies. Goat anti-mouse IgG-IRDye 800CW (LI-COR, #926-32210) and goat anti-rabbit IgG-IRDye 680LT (LI-COR, #926-68021) were used as secondary antibodies. Membranes were scanned using an Odyssey Fc Imager (LI-COR Biosciences, Bad Homburg, Germany), and band intensities were quantified using Odyssey quantification software.

Tandem mass spectrometry analyses

After IP, the proteins were separated by 12% SDS–PAGE, and in-gel digestion was performed (Shevchenko *et al*, 1996). Mass spectrometry measurements were recorded on an LTQ Orbitrap-XL (Thermo Finnigan, Vernon Hills, IL, USA). MS/MS spectra were searched with MASCOT (Matrix Science, London, UK) against

the *Arabidopsis* TAIR9 protein database with a concatenated decoy database (download on 19 June 2009) supplemented with contaminants. The search parameters were as follows: requirement for tryptic ends, one missed cleavage allowed, peptide tolerance ± 5 p.p.m., MS/MS tolerance ± 0.6 Da. Carbamidomethylation of cysteine was set as fixed modification, and oxidation of methionine was set as a variable modification. The processed data were imported into Scaffold (Proteome Software). The cutoff for data analyses was set to a minimum confidence of 90% for protein identification and to a minimum confidence of 95% for peptide identification. The spectrum false-discovery rate was calculated by dividing the number of decoy database spectrum assignments by the number of spectrum assignments. The false-positive rate was below 1% in all measured experiments. Proteins identified with at least two unique peptides in at least two replicates but never in control samples were taken into account.

Protein expression in Saccharomyces cerevisiae and immunoprecipitation assays

The *MSI1* and *MSI4* cDNAs were cloned into vector pGADT7 (Clonetech, Mountain View, CA); the *LHP1* cDNA was cloned into vector pFLAG–attR (Stanyon *et al*, 2003). Proteins were expressed in *S. cerevisiae* strain BY4741 (Brachmann, 1998 #11297). If a constitutive promoter was used (pGADT7), cells were grown until $OD_{600} = 1.1$; they were then harvested and frozen in liquid nitrogen. If an inducible promoter was used (pFLAG–attR, pYES2), protein expression was induced at $OD_{600} = 1.1$ by 2% galactose; the cells were then harvested after 6 h and frozen in liquid nitrogen. After re-suspension in extraction buffer (20 mM HEPES pH 7.6, 10% glycerol, 200 mM potassium acetate, 1 mM EDTA, 1 mM DTT, protease inhibitors (Roche), 1 mM PMSF), cells were disrupted using a French Press (20 K, 1200 p.s.i., three times). The input sample was taken from the centrifuged supernatant (10 min at 4000 *g* followed by 10 min at 14.000 *g*); the rest of the supernatant was used for immunoprecipitation as described above.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described before (Exner *et al*, 2009) using the LowCell# ChIP kit (Diagenode, Liège, Belgium) according to the manufacturer's instructions. Antibodies used in ChIP were anti-GFP (Molecular Probes Invitrogen, #A11122), IgG (Sigma-Aldrich, #I5006), anti-histone H3 (Millipore, #07690) and anti-H3K27me3 (Millipore, #07690). qPCR with gene-specific primers (Supplementary Table. S3) was performed using a MyiQ system (BIO-RAD, Hercules, CA, USA) and Sybr Green master mix (Fermentas) according to the manufacturer's instructions.

RNA isolation and RT–qPCR

RNA extraction and RT–qPCR were performed as described previously (Leroy *et al*, 2007; Alexandre *et al*, 2009) with some modifications: qPCR with gene-specific primers (Supplementary Table S4) was performed using a MyiQ system and either the Sybr Green master mix (Fermentas) or the Fast Start Universal Probe Master (Rox) reagent and the Universal Probe Library set (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions.

Microarray analysis

Plants were grown for 48 days in short-day photoperiods. Leaf number 6 was harvested at ZT (*zeitgeber* time) = 7 h, and RNA was isolated, labelled using the GeneChip WT Sense Target Labeling Assay and hybridized to Affymetrix AGRONOMICS1 *Arabidopsis* tiling arrays as described (Rehrauer *et al*, 2010; Müller *et al*, 2012). Data were normalized and analysed as described (Rehrauer *et al*, 2010; Müller *et al*, 2012), based on TAIR10 annotations (http://www.arabidopsis.org). Leaf-specific expression potentials were estimated as the maximum expression measured in any of the leaf samples from the AtGenExpress reference set for development (Schmid *et al*, 2005). PcG targets in leaves were taken from Lafos *et al* (2011). Gene expression data for *msi1–cs* and *emf2* plants were taken from Alexandre *et al* (2009) and Liu *et al* (2012).

Data availability

The microarray raw data from this publication were submitted to the ArrayExpress (www.ebi.ac.uk/arrayexpress) database (accession number E-MTAB-1412). The protein interactions from this publication have been submitted to the IMEx (Orchard *et al*, 2012) consortium through IntAct (Aranda *et al*, 2010) and assigned the identifier IM-18782. Processed microarray data are enclosed as Supplementary Table S5.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Drs. Gilles Beck and Nicole Schatlowski for critical reading of the manuscript. We thank Dr. R. L. Finley Jr (Wayne State University) for providing plasmid pFLAG–attR. This work was supported by grants from the Swiss National Research Foundation, ETH Zurich, the Swedish Research Council and the Sixth Framework Program of the European Commission through the AGRON-OMICS Integrated Project (grant no. LSHG–CT–2006–037704).

Author contributions: MD, YMS, TW, IM and WM performed the experiments; MD, TW, PN and SB performed the MS/MS analyses; MD, YMS, TW, WM, WG and LH planned the experiments; and MD, LH and WG wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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