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# The Arabidopsis CUL4–DDB1 complex interacts with MSI1 and is required to maintain *MEDEA* parental imprinting

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**Protein ubiquitylation regulates a broad variety of biological processes in all eukaryotes. Recent work identified a novel class of cullin-containing ubiquitin ligases (E3s) composed of CUL4, DDB1, and one WD40 protein, believed to act as a substrate receptor. Strikingly, CUL4-based E3 ligases (CRL4s) have important functions at the chromatin level, including responses to DNA damage in metazoans and plants and, in fission yeast, in heterochromatin silencing. Among putative CRL4 receptors we identified MULTICOPY SUPPRESSOR OF IRA1 (MSI1), which belongs to an evolutionary conserved protein family. MSI1-like proteins contribute to different protein complexes, including the epigenetic regulatory *Polycomb* repressive complex 2 (PRC2). Here, we provide evidence that Arabidopsis MSI1 physically interacts with DDB1A and is part of a multimeric protein complex including CUL4. CUL4 and DDB1 loss-of-function lead to embryo lethality. Interestingly, as in *fis* class mutants, *cul4* mutants exhibit autonomous endosperm initiation and loss of parental imprinting of *MEDEA*, a target gene of the Arabidopsis PRC2 complex. In addition, after pollination both *MEDEA* transcript and protein accumulate in a *cul4* mutant background. Overall, our work provides the first evidence of a physical and functional link between a CRL4 E3 ligase and a PRC2 complex, thus indicating a novel role of ubiquitylation in the repression of gene expression.**

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## Introduction

Regulation of protein stability by the ubiquitin/proteasome system participates in a broad variety of physiologically and developmentally controlled processes in all eukaryotes (Ciechanover *et al*, 2000; Smalle and Vierstra, 2004). In this pathway, a critical step involves ubiquitin ligases (E3s), which facilitate the transfer of ubiquitin moieties to a substrate protein, the preparative step for degradation via the 26S proteasome. Among the different E3 enzymes, the composition of CUL4-based E3 ligases (CRL4s) was only recently identified (Higa and Zhang, 2007). CUL4 binds RBX1 to recruit a specific E2 ubiquitin-conjugating enzyme, and also binds DDB1, an adaptor protein, which itself associates with a substrate receptor. Affinity purification of CLR4s from mammalian cells identified various WD40 proteins as possible substrate receptors (Angers *et al*, 2006; He *et al*, 2006; Higa *et al*, 2006; Jin *et al*, 2006). Many of these proteins, also called DDB1 and CUL4-associated factors (DCAFs), contain WDxR motifs that are required for efficient DDB1 binding. However, for most of them, their roles and substrates remain unknown. In humans, about 90 different DCAFs have been predicted (He *et al*, 2006), suggesting the existence of a large number of CRL4s. A similar number of WD40 repeat proteins harbouring at least one WDxR motif have been identified in the model plant *Arabidopsis thaliana* (Lee *et al*, 2008). One of the predicted Arabidopsis DCAFs is MULTICOPY SUPPRESSOR OF IRA1 (MSI1), which belongs to an evolutionary conserved protein family (reviewed in Hennig *et al*, 2005), whose founding member is MSI1 from yeast (Ruggieri *et al*, 1989). In both metazoans and plants, MSI1-like proteins are part of several protein complexes involved in diverse chromatin functions (reviewed in Hennig *et al*, 2005). In particular, MSI1 has been proposed to maintain epigenetic memory during development by targeting silencing complexes to chromatin.

In Arabidopsis, MSI1 is essential for plant reproductive development (Köhler *et al*, 2003; Guitton *et al*, 2004). In *msi1* mutants, seeds abort when the mutant allele is inherited from the mother regardless of the paternal contribution. In such seeds, the endosperm (an embryo nourishing tissue) does not cellularize, whereas the embryo exhibits cell-cycle and developmental defects. *msi1* mutants have a strong penetrance of autonomous endosperm development in the absence of fertilization and form rare parthenogenetic embryos (Köhler *et al*, 2003; Guitton and Berger, 2005). MSI1 is part of the FIS–PRC2 complex together with at least three other proteins, MEDEA (MEA), FERTILIZATION-INDEPENDENT SEED2 (FIS2) and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE), which is required for normal seed development (Köhler *et al*, 2003). *MEA* encodes a SET-domain-containing histone methyltransferase homologous to *Drosophila* Enhancer of Zeste (Grossniklaus *et al*, 1998) and regulates the imprinted expression of itself, as well as of its target gene *PHERES1*

(*PHE1*), encoding a MADS-domain transcription factor (Köhler *et al*, 2005). Imprinting regulation by FIS–PRC2 involves the silencing of the paternal allele of *MEA* and the maternal allele of *PHE1*, respectively (Köhler *et al*, 2005; Baroux *et al*, 2006; Gehring *et al*, 2006; Jullien *et al*, 2006). In contrast, auto-repression of the maternal *MEA* allele is FIS–PRC2 independent (Baroux *et al*, 2006).

Here, we report that all WD40 repeat MSI1-like proteins from various organisms carry at least one conserved WDxR motif, a signature of DCAFs. Arabidopsis MSI1 physically interacts with DDB1A and is part of a CUL4–DDB1A–MSI1 protein complex. Functional analysis revealed that *CUL4*, as well as the Arabidopsis *DDB1* homologs, are essential for seed production. Importantly, the *cul4* mutation leads to autonomous endosperm development and loss of parental *MEA* imprinting, that is reactivation of the paternal *MEA* allele, supporting a functional link of this E3 ligase and the FIS–PRC2 complex.

## Results

### *MSI-like proteins are evolutionary conserved WD40 proteins that carry WDxR motifs*

Recent work identified DDB1 and DCAFs as possible substrate receptors of CRL4 E3 ligases (reviewed in Lee and Zhou, 2007). The largest class of DCAFs are WD40 repeat proteins, which interact with DDB1 via one or several conserved WDxR motifs. The Arabidopsis genome encodes 237 WD40 repeat proteins; however, only a subset of them (~80 proteins) carry one or more WDxR motif(s) (Lee *et al*, 2008 and our unpublished data). Among these proteins we identified MSI1 and four other Arabidopsis MSI1-related proteins, named MSI2–MSI5 (reviewed in Hennig *et al*, 2005). When all MSI1-like proteins from plant and non-plant organisms were compared, it appeared that most of them share a highly conserved WDxR motif (Figure 1). In metazoans, MSI1-like proteins exhibit also a second WDxR motif, which is less conserved in plants, but is also present in fungi. Therefore, most if not all MSI1-like proteins are structurally related to DCAFs.

### *MSI1 associates with DDB1A and CUL4 in Arabidopsis*

We first investigated whether MSI1 interacts with DDB1A in a yeast two-hybrid assay. Similarly to DDB2 (Molinier *et al*, 2008), which served as a positive control, MSI1 and DDB1A interacted, although the interaction was weak as yeast growth was only detected on (-LWH) medium (Figure 2A). We further confirmed this interaction by an *in vitro* pull-down assay. In this experiment, a fusion protein between glutathione-S-transferase (GST) and DDB1A, GST-DDB1A, was incubated with *in vitro* translated, $^{35}$S-methionine-labelled MSI1 or DDB2. Consistently, MSI1 and DDB2 co-precipitated with GST-DDB1A, but not with GST alone (Figure 2B). To provide evidence for a physical interaction between both proteins in plant cells, we carried out bimolecular fluorescence complementation (BiFC) experiments. Plasmids YC-MSI1 and YN-DDB1A were co-bombarded into etiolated mustard hypocotyls. A strong YFP signal was observed in the nucleus of 81% examined cells (35/43; Figure 2C). These data are similar to those obtained with cells transformed with the positive control YN-DDB1A + YC-DDB2 (43/46). Only a weak fluorescence signal was observed after bombardment with the following plasmid combinations YN-DDB1A + YC-BPM3 (9/35) and YN-BPM3 + YC-MSI1 (2/27), where BPM3 (BTB/POZ-MATH3 protein encoded by *At2g39760*) is a nuclear cullin-ring ubiquitin ligase3 (CLR3) receptor, used here as a negative control. Taken together, our data clearly demonstrate a physical interaction between DDB1A and MSI1.

Next, we tested whether MSI1 is also part of a protein complex containing Arabidopsis CUL4. Thus, we immunoprecipitated Arabidopsis CUL4 from plants expressing the MSI1–RFP fusion protein under the control of its own promoter (Chen *et al*, 2008). Hence, MSI1 was successfully co-immunoprecipitated in this assay (Figure 2D). Since CUL4 interacts with DDB1A (Bernhardt *et al*, 2006) our results, collectively, support the existence of a CUL4–DDB1A–MSI1 protein complex in Arabidopsis.

### *CUL4 and its adaptors DDB1A and DDB1B are required for embryogenesis*

In Arabidopsis, loss-of-function of *MSI1* causes maternal effect embryo lethality leading to seed abortion early in development (Köhler *et al*, 2003). We have previously isolated a T-DNA mutant, *cul4-1* (Bernhardt *et al*, 2006), in which *CUL4* expression was severely downregulated. Although viable *cul4-1* homozygous mutants were obtained, these plants showed various developmental abnormalities (Bernhardt *et al*, 2006). When selfed, we noticed that *cul4-1* homozygous plants exhibited altered seed development leading eventually to seed abortion (Supplementary Figure S1). Thus, we examined *cul4-1* homozygous mutant seeds at different developmental stages (Figure 3). Already at the octant stage, we observed a lower proliferation of the endosperm (Figure 3B) while at later seed developmental stages we scored abnormally large endosperm nuclei and delayed embryo development (Figure 3D and F). Because of the pleiotropic and hypomorphic nature of the *cul4-1* allele, we aimed to identify amorphic *CUL4* loss-of-function mutants. As no such mutants were available in public collections, we screened a collection of Arabidopsis T-DNA insertion lines (Ríos *et al*, 2002). Two T-DNA insertions were identified within the coding region of *CUL4*, called *cul4-2* and *cul4-3* (Supplementary Figure S2A). Both *cul4-2* and *cul4*-3 mutants were backcrossed to the wild type and Southern blots confirmed single T-DNA insertions. Although we genotyped 137 and 72 progeny from selfed *cul4-2* and *cul4-3* mutant plants, respectively, we were unable to identify homozygous mutants, suggesting that *CUL4* is an essential gene in Arabidopsis.

As both lines contained single T-DNA insertions with integral hygromycin selection markers, we self-pollinated *cul4-2* and *cul4-3* heterozygous plants and analysed the segregation of this marker among their progeny (Table I). This genetic analysis revealed a segregation ratio close to 2:1 consistent with nearly fully penetrant zygotic embryo lethality. Because the segregation ratio of the marker was slightly below 2:1 for the *cul4-2* allele, suggesting a weak defect in gametophytic transmission, we performed reciprocal crosses with wild-type plants. The transmission efficiency of the marker was slightly reduced through both male and female gametophytes (Table I).

Next, we examined mature siliques for the presence of aborted seeds. The number of aborted seeds was consistent with zygotic embryo lethality, where a segregation

 

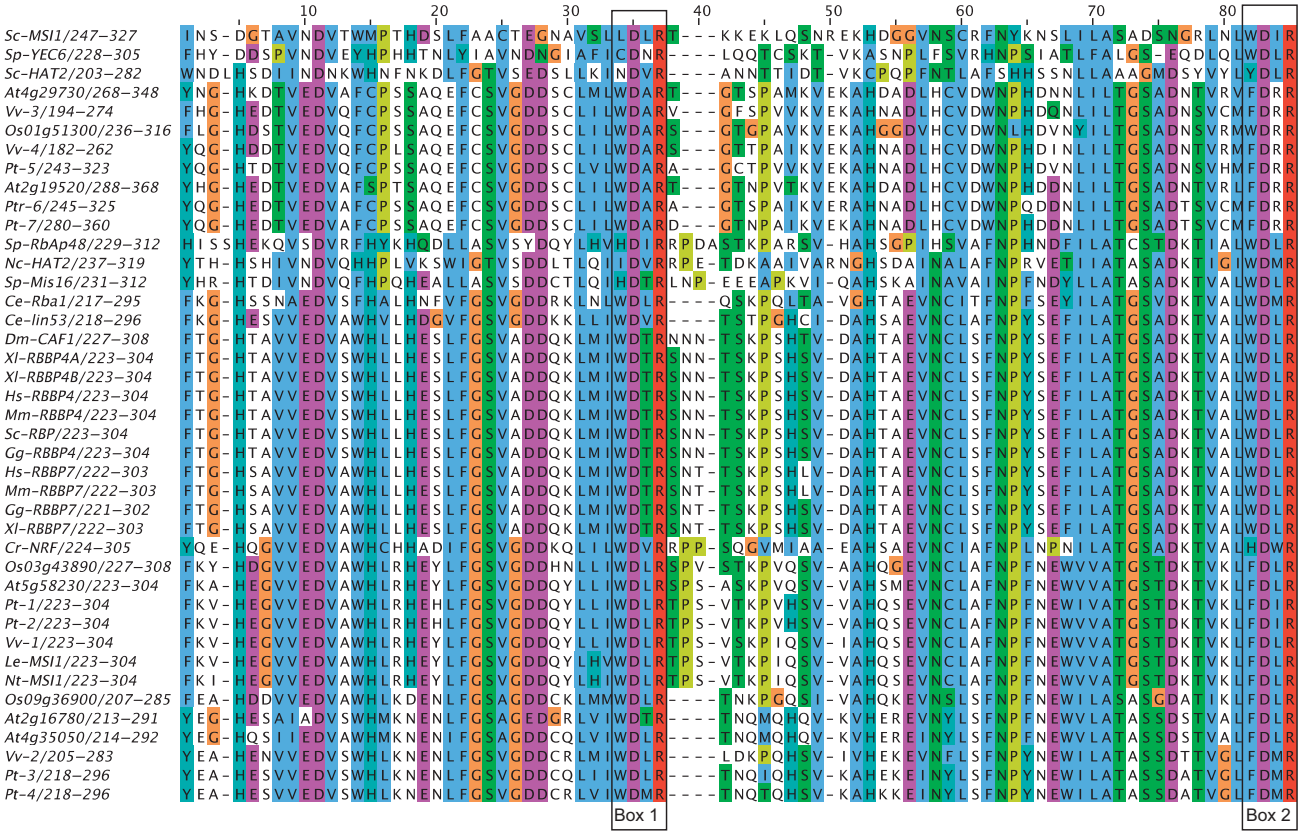


**Figure 1** Alignments of MSI1-like proteins and WDxR motifs. All five Arabidopsis MSI1-like protein sequences (MSI1, AT5G58230; MSI2, AT2G16780; MSI3, AT4G35050; MSI4, AT2G19520 and MSI5, AT4G29730) were used to identify MSI1-like proteins by BLAST (Altschul *et al*, 1990). We used the following databases: for *Oryza sativa* (http://rice.plantbiology.msu.edu/; Os03g43890; Os09g36900; Os01g51300); *Vitis vinifera* (http://www.genoscope.cns.fr/spip/Vitis-vinifera-whole-genome.html; Vv-1 GSVIVP00030810001; Vv-2 GSVIVP00036121001; Vv-3 GSVIVP00016560001; Vv-4 GSVIVP00034167001); *Lycopersicon esculentum* (NCBI; http://www.ncbi.nlm.nih.gov/; Le-MSI1 O22466.1); *Nicotiana tabacum* (NCBI; Nt-MSI1 ABY84675.1); *Homo sapiens* (NCBI; Hs-RBBP4 NP_005601.1; Hs-RBBP7 NP_002884.1); *Mus musculus* (NCBI; Mm-RBBP4 NP_033056.2; Mm-RBBP7 NP_033057.3); *Gallus gallus* (NCBI; Gg-RBBP4 Q9W7I5.3; Gg-RBBP7 Q9I8G9.1); *Drosophila melanogaster* (NCBI; Dm-CAF-1 NP_524354.1); *Caenorhabditis elegans* (NCBI; Ce-lin53 NP_492552.1; Ce-Rba1 NP_492551.1); *Xenopus laevis* (NCBI; Xl-RBBP4B Q6INH0.3; Xl-RBBP4A O93377.3; Xl-RBBP7 Q8AVH1.1); *Chlamydomonas reinhardtii* (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html; Cr-NRF XP_001696907.1); *Populus trichocarpa* (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html; Pt-1 estExt_fgenesh4_pg.C_LG_II1945; Pt-2 estExt_fgenesh4_pg.C_LG_XIV1179; Pt-3 gw1.IX.1159.1; Pt-4 estExt_fgenesh4_pg.C_LG_IV1464; Pt-5 eugene3.00440093; Ptr-6 gw1.145.113.1; Pt-7 eugene3.02850001); *Schizosaccharomyces pombe* (NCBI; Sp-RbAp48 O14021.1; Sp-YEC6 Q9Y825.1; Sp-Mis16 NP_587881.1); *Saccharomyces cerevisiae* (NCBI; Sc-HAT2 P39984.1; Sc-MSI1 P13712.1; Sc-RBP 1919423A), and *Neurospora crassa* (NCBI; Nc-HAT2 Q7S7N3.2). All proteins identified were aligned using the program Muscle v3.6 (Edgar, 2004). Non-conserved protein regions were removed by GBlocks v0.91b using the following settings: minimum number of sequences for a conserved position: 21; minimum number of sequences for a flanking position: 34; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half. The positions of two conserved WDxR motifs are indicated (Box1 and Box2).

of aborted:normal seeds of 1:3 is expected (Table II; Supplementary Figure S1). To further investigate at which developmental stage embryogenesis is arrested, we analysed cleared seed specimens from siliques of selfed *cul4-2* mutant plants at different developmental stages. At the octant stage, the mutant seeds exhibited a low number of large nuclei in the endosperm (Figure 3H). At later stages, embryos arrested their development at the globular stage with abnormal shapes and cell division defects in both the suspensor and the embryo proper (Figure 3J and L). Moreover, in *cul4-2* homozygous mutant seeds, the endosperm was always severely underdeveloped with a dozen fewer enlarged, abnormal nuclei. When siliques were analysed at later stages, harbouring bent-cotyledon stage or mature wild-type sibling embryos, the arrested seeds had degenerated (not shown), indicating a strict arrest and not only a delay in seed development. Similar results were obtained with the *cul4-3* mutant allele (Supplementary Figure S3B).

Because CUL4 interacts with DDB1 to form CRL4 E3 complexes, we also investigated whether DDB1 is required for embryogenesis. The Arabidopsis genome encodes two expressed DDB1-related proteins, named *DDB1A* (*At4g05420*) and *DDB1B* (*At4g21100*), exhibiting 89% sequence identity at the amino-acid level (Schroeder *et al*, 2002). DDB1A loss-of-function mutants are viable (Molinier *et al*, 2008). Therefore, we searched for T-DNA insertion mutants in the related *DDB1B* gene and identified one mutant, named *ddb1b-1*, from the SALK collection (SALK 061944) (Alonso *et al*, 2003). In the *dbb1b-1* allele, the T-DNA interrupts the coding sequence in the last exon (Supplementary Figure S2B). Homozygous *ddb1b-1* mutant plants developed normally and were fully fertile. To test whether *DDB1A* and *DDB1B* act redundantly during embryogenesis, the *ddb1a-2* mutant was used to pollinate a homozygous *ddb1b-1* mutant plant. Among the progeny of this cross, we selected F2 plants that were *DDB1A/ddb1a-2*

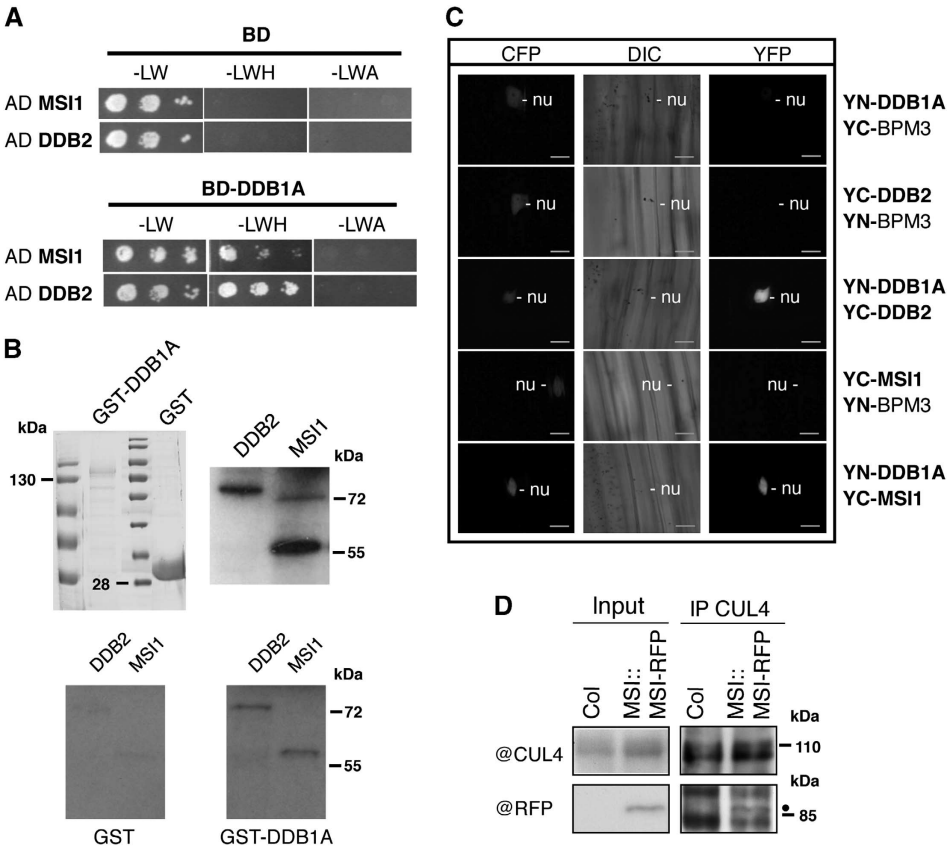


**Figure 2** MSI1 forms a complex with DDB1A and CUL4. (**A**) Yeast two-hybrid experiments showing MSI1 interaction with DDB1A. Dilution series of yeast cells co-expressing the indicated proteins were grown for 3 days at 28°C on LWH (low-stringency selection) and on LWA (high-stringency selection). As a positive control, we used DDB2. (**B**) The interactions from the Y2H assay were confirmed by using bacterially expressed GST or GST-DDB1A proteins in pull down *in vitro* assays. Upper panel shows GST-DDB1A protein (left) and 5 µl of *in vitro* translated $^{35}$S-Met-labelled MSI1 and DDB2 proteins (right) used for pull downs (lower panels). (**C**) BiFC of YN-DDB1A/YC-MSI1. Different combinations of plasmids expressing the indicated YN- and YC-fusion proteins were bombarded into hypocotyls of dark-grown mustard seedling. The nuclear-localized CUL3 receptor BPM3 protein was used here as a negative control. A transfection control CPRF2 expressing a fused CFP targeted to the nucleus (nu) was systematically included to identify transformed cells. Images were recorded 5 h after bombardment via CFP- (left panels) and YFP-specific filters (right panels). Differential interference contrast (DIC) images are shown (middle panels). Reconstitution of functional YFP as detected by YFP fluorescence occurs only in the nucleus with both MSI1 and DDB2. Scale bars = 20 µm. (**D**) *In vivo* pull down with CUL4 and MSI1. MSI1–RFP expressing and control wild-type plants were used for immunoprecipitation (IP) assays using anti-CUL4 antibody. Both CUL4 (upper right panel) and MSI1–RFP (lower right panel) were detected in the IPs, using anti-CUL4 and anti-RFP antibodies, respectively. An asterisk indicates the MSI1–RFP protein band. A full-colour version of this figure is available at *The EMBO Journal* Online.

*ddb1b-1/ddb1b-1* (referred as *DDB1A/ddb1a ddb1b*) and *ddb1a-2/ddb1a-2 DDB1B/ddb1b-1* (referred as *ddb1a DDB1B/ddb1b*). Because both *ddb1a-2* and *ddb1b-1* mutants carry the same selection marker, we used PCR-based genotyping for further genetic analyses. Among the progeny of self-pollinated *DDB1A/ddb1a ddb1b* and *ddb1a DDB1B/ddb1b* plants, no double mutant were identified, despite the analysis of ~60 plants for each genotype (Table III).

Next, we evaluated the effect of both *DDB1*-related genes on male and female gametophytic transmission (Table III). Reciprocal crosses between the different genotypes revealed that the two genes do not contribute equally to gametophyte development and/or function as indicated by unequal transmission defects: while in the absence of *DDB1B*, *DDB1A* is required for normal transmission through the male, the converse is true for the female gametophyte.

The number of aborted seeds was consistent with zygotic embryo lethality in self-pollinated *ddb1a DDB1B/ddb1b* plants (Table II). Light microscopic observations of cleared seeds revealed that double homozygous *ddb1a dbb1b* embryos derived from selfed *DDB1A/ddb1a ddb1b* (not shown) or *ddb1a DDB1B/ddb1b* (Supplementary Figure S3D)) mutants arrest at the globular stage, with a phenotype reminiscent of that of the *cul4* mutants. Thus, both *CUL4* and *DDB1A/B* functions are required for normal development of embryo and endosperm.

### *CUL4 is expressed during embryogenesis*

To determine the expression pattern of *CUL4* in reproductive tissues and during embryogenesis, we performed mRNA *in situ* hybridization experiments on sections of flower buds and developing siliques using *CUL4*-specific antisense and sense control probes. *CUL4* transcripts were detected in the tissues of young flower buds, that is in petals, stamens, and carpels (Supplementary Figure S4A). A distinctive signal was observed in emerging ovules (Supplementary Figure S4B), but not in the developing embryo sac (Supplementary Figure S4E). After fertilization, the expression level of *CUL4* was prominent in the developing embryo (Supplementary Figure S4C, D, G–L). The signal intensity decreased after the

 

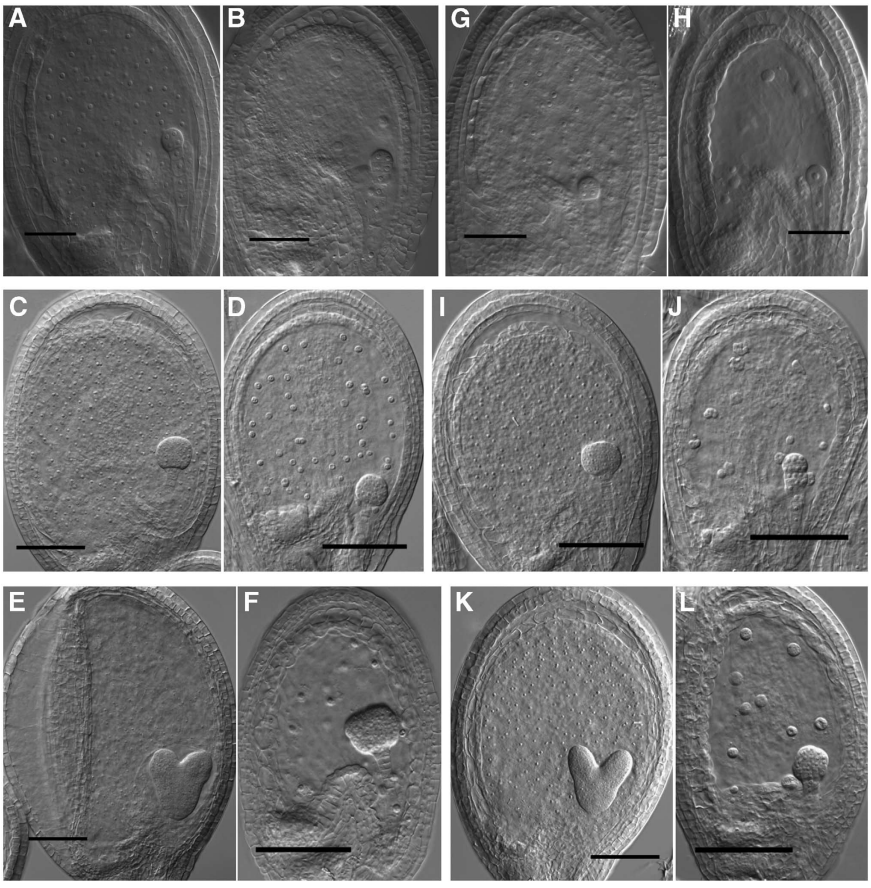


**Figure 3** Embryo and endosperm development is affected in *cul4* mutant seeds. (**A**) Cleared seed with an embryo at the octant stage from the same *cul4-1* homozygous mutant silique as the seed shown in (**B**). (**B**) Mutant embryo and endosperm with reduced proliferation and large nuclei. (**C**) Cleared seed with an embryo at the globular stage from the same *cul4-1* homozygous mutant silique as the seed shown in (**D**). (**D**) Delayed mutant with large endosperm nuclei. (**E**) Cleared seed with an embryo at the heart stage from the same *cul4-1* homozygous mutant silique as the seed shown in (**F**). (**F**) Delayed mutant with reduced proliferation and enlarged endosperm nuclei. (**G**) Cleared seed with an embryo at the octant stage from the same silique as the seed shown in (**H**). (**H**) *cul4-2* homozygous mutant with a reduced number of large endosperm nuclei. (**I**) Cleared seed with an embryo at the globular stage from the same silique as the seed shown in (**J**). (**J**) Delayed *cul4-2* homozygous mutant with enlarged and aggregated endosperm nuclei. (**K**) Cleared seed with an embryo at the heart stage from the same silique as the seed shown in (**L**). (**L**) Delayed *cul4-2* homozygous mutant with a reduced number of enlarged endosperm nuclei. Bars = 50 μm (**A–B**, **G–H**); 100 μm (**C–F**, **I–L**).

**Table I** Genetic analysis of *cul4* mutant plants

| Parental genotype (female × male) | $Hyg^R$ | $Hyg^s$ | *n* | *P*-value | $TE_F$ (%) | $TE_M$ (%) |
|---|---|---|---|---|---|---|
| cul4-2 (selfed) | 470 | 296 | 766 | 0.002 | NA | NA |
| cul4-3 (selfed) | 474 | 236 | 710 | 0.936 | NA | NA |
| Col-0 × cul4-2 | 213 | 251 | 464 | 0.077 | NA | 84.9% |
| cul4-2 × Col-0 | 198 | 236 | 434 | 0.068 | 83.9% | NA |
| Col-0 × cul4-3 | 229 | 256 | 485 | 0.220 | NA | 89.4% |
| cul4-3 × Col-0 | 221 | 254 | 475 | 0.130 | 87.0% | NA |

Resistance to Hygromycin ($Hyg^R$, Hygromycin resistant seedlings; $Hyg^S$, Hygromycin sensitive seedlings) was used as a marker for the *cul4-2* and *cul4-3* insertions. Transmission efficiencies were calculated according to Howden *et al.* (1998): $TE = Hyg^R/Hyg^S \times 100\%$. *P*-value, based on a 2:1 segregation ratio as expected for a zygotic embryo lethal mutation and 1:1 for the reciprocal crosses as expected for normal transmission; $TE_F$, female transmission efficiency; $TE_M$, male transmission efficiency; NA, not applicable. At a *P*-value of $<0.05$ the null hypothesis is rejected.

heart stage (Supplementary Figure S4I–G). This expression pattern correlates with the requirement of *CUL4* for embryo development. In endosperm cells, the hybridization signal was low but detectable at all stages of seed development (Supplementary Figure S4C, D, G–L). Overall, the *CUL4* expression pattern in developing seeds is consistent with the *CUL4* loss-of-function phenotype.

### *Imprinted expression of MEA is lost in cul4 mutants*

MSI1 is a component of the FIS–PRC2 complex, which is required for seed development (Köhler *et al*, 2003). However, MSI1 was also found in other protein complexes potentially involved in chromatin functions (Hennig *et al*, 2005). Thus, we wondered whether loss of *CUL4* and/or *DDB1* activity affects PRC2-like functions during plant reproduction.

**Table II** Analysis of mature siliques

| Parental genotype (female × male) | Normal seeds | Aborted seeds | Seeds scored | *P*-value |
|---|---|---|---|---|
| *Col-0* × *Col-0* | 512 | 8 (1.5%) | 520 | NA |
| cul4-2$^{+/-}$ *(selfed)* | 1129 | 444 (28.2%) | 1573 | 0.003 |
| cul4-3$^{+/-}$ *(selfed)* | 1030 | 383 (27.1%) | 1413 | 0.065 |
| ddb1a-2 DDB1B/ddb1b-1 *(selfed)* | 215 | 81 (27.4%) | 296 | 0.347 |

Mature siliques were analysed for the presence of aborted seeds. *P*-value, based on a 3:1 ratio as expected for zygotic embryo lethality. NA, not applicable. At a *P*-value of $<0.05$ the null hypothesis is rejected.

**Table III** Genetic analysis of *ddb1a ddb1b* mutant plants

| Genotyping | Doubly homozygous (−/− ; −/−) | Heterozygous for one allele (+/− ; −/−) | WT allele (+/+ ; −/−) | *n* | *P*-value | $TE_F$ (%) | $TE_M$ (%) |
|---|---|---|---|---|---|---|---|
| DDB1A/ddb1a-2 ddb1b-1 (selfed) | 0 | 36 | 20 | 56 | 0.567 | NA | NA |
| ddb1a-2 DDB1B/ddb1b-1 (selfed) | 0 | 38 | 25 | 63 | 0.285 | NA | NA |
| ddb1b-1 × DDB1A/ddb1a-2 ddb1b-1 | 0 | 26 | 68 | 94 | <0.0001 | NA | 38.2% |
| DDB1A/ddb1a-2 ddb1b-1 × ddb1b-1 | 0 | 44 | 45 | 89 | 0.9156 | 97.8% | NA |
| ddb1a-2 × ddb1a-2 DDB1B/ddb1b-1 | 0 | 43 | 41 | 84 | 0.8273 | NA | 104.8% |
| ddb1a-2 DDB1B/ddb1b-1 × ddb1a-2 | 0 | 20 | 66 | 86 | <0.0001 | 30.3% | NA |

$TE_F$, female transmission efficiency; $TE_M$, male transmission efficiency; NA, not applicable.
The progeny of *DDB1A/ddb1a ddb1b* and *ddb1a DDB1B/ddb1b* plants were genotyped. No double mutant was identified. *P*-value, based on a 2:1 segregation ratio as expected for a zygotic embryo lethal mutation and 1:1 for the reciprocal crosses as expected for normal transmission. At a *P*-value of $<0.05$ the null hypothesis is rejected.

In contrast to the *msi1* mutant, *cul4-1* siliques did not show a clear elongation after emasculation (Supplementary Figure S5A). Nevertheless, we observed 16% ($n=229$) and 3.6% ($n=224$) of autonomous endosperm division in homozygote *cul4-1* and heterozygote *cul4-2* mutants, respectively (Supplementary Figure S5B). As expected, no extra divisions were observed in wild-type ovules ($n=71$) (Supplementary Figure S5B). Thus, although at a lower penetrance, *cul4* mutants share the *fis* class phenotype of endosperm initiation in the absence of fertilization (Ohad *et al*, 1996; Chaudhury *et al*, 1997; Grossniklaus and Vielle-Calzada, 1998; Kiyosue *et al*, 1999; Köhler *et al*, 2003; Guitton *et al*, 2004). Next, we investigated whether mutations of *CUL4* affect parental imprinting of *MEA* and/or *PHE1*, two genes that are regulated by the FIS–PRC2 complex (Köhler *et al*, 2003, 2005; Baroux *et al*, 2006; Gehring *et al*, 2006; Jullien *et al*, 2006). We used sequence polymorphisms between different Arabidopsis accessions to distinguish parental alleles. Interestingly, we detected paternal *MEA* expression in the *cul4-1* mutant (Figure 4A). It is noteworthy that repression of the paternal *MEA* allele in the control experiment was not complete, as a weak but detectable expression was observed when Columbia (Col-0) pollen was used. Indeed, previous genetic analyses suggested that Col-0 carries a paternal modifier of *mea* seed abortion (Vielle-Calzada *et al*, 1999), and it is possible that this leads to a weak de-repression of the paternal *MEA* allele. However, full expression of paternal *MEA* allele was only observed 3 days after pollination (DAP) when *cul4-1* or *cul4-1 ddb1a* pollen was used.

To further investigate the loss of repression of the paternal *MEA* allele in the *cul4-1* or *cul4-1 ddb1a* mutants, we introgressed the *pMEA::MEA-YFP* reporter gene (Wang *et al*, 2006a) into the *cul4-1* and *cul4-2* mutant backgrounds. When *pMEA::MEA-YFP* line in a Col-0 background was used to pollinate Col-0 plants, we observed by confocal microscopy a detectable fluorescence signal in the endosperm of about half of the seeds (Figure 4B; Table IV). This result is inconsistent with a full repression of the paternal *MEA* allele in the Col-0 background, but is in agreement with our results using sequence polymorphisms (see above). A similar result was observed when the *cul4-1* hypomorphic mutant was used as a female and pollinated with wild-type pollen carrying the *pMEA::MEA-YFP* reporter. However, when we used the *cul4-2 pMEA::MEA-YFP* pollen to fertilize Col-0 plants, we observed a stronger fluorescence signal in some seeds (Figure 4B), supporting a reactivation of the paternal *pMEA::MEA-YFP* reporter gene if derived from *cul4* mutant pollen.

To better deplete *CUL4* activity in such experiments, we combined the weak *cul4-1* with the strong *cul4-2* alleles. When homozygous *cul4-1* plants were used as a female and pollinated with pollen from *cul4-2* heterozygote plants, we observed a category of seeds (~50%), which arrested at the late globular stage, thus corresponding to *cul4-1/cul4-2* homozygous embryos. In these seeds, the underdeveloped endosperm contained large coenocytic cells (Supplementary Figure S6) and degenerated at 4 DAP. Interestingly, ~70% of seeds were scored for a fluorescent signal when the *cul4-1* mutant was pollinated with pollen from *cul4-2 pMEA::MEA-YFP* plants (Table IV). The fluorescence signal was particularly strong in the aberrant endosperm of arrested *cul4-1/cul4-2* seeds at 2 and 3 DAP (Figure 4B). This finding was further supported by the accumulation of paternally expressed MEA-YFP protein in this cross, as detected by western blotting (Figure 4C). Thus, our results indicate that *CUL4* is necessary to maintain repression of the paternal *MEA* allele.

Since the PRC2 complex mediates trimethylation of histone H3 at the lysine residue 27 (H3K27me3) (Gehring *et al*, 2006; Jullien *et al*, 2006; Makarevich *et al*, 2006), we investigated whether *CUL4* knockdown affects histone methylation. Interestingly, chromatin immunoprecipitation (ChIP) analysis performed on young siliques revealed a decrease in H3K27 trimethylation at the *MEA* locus (Figure 5B). It is remarkable that a similar effect was not observed when ChIP assays were performed with young floral buds (Figure 5A), suggesting that CUL4 is involved in the maintenance rather than in the establishment of those histone repressive marks.

 

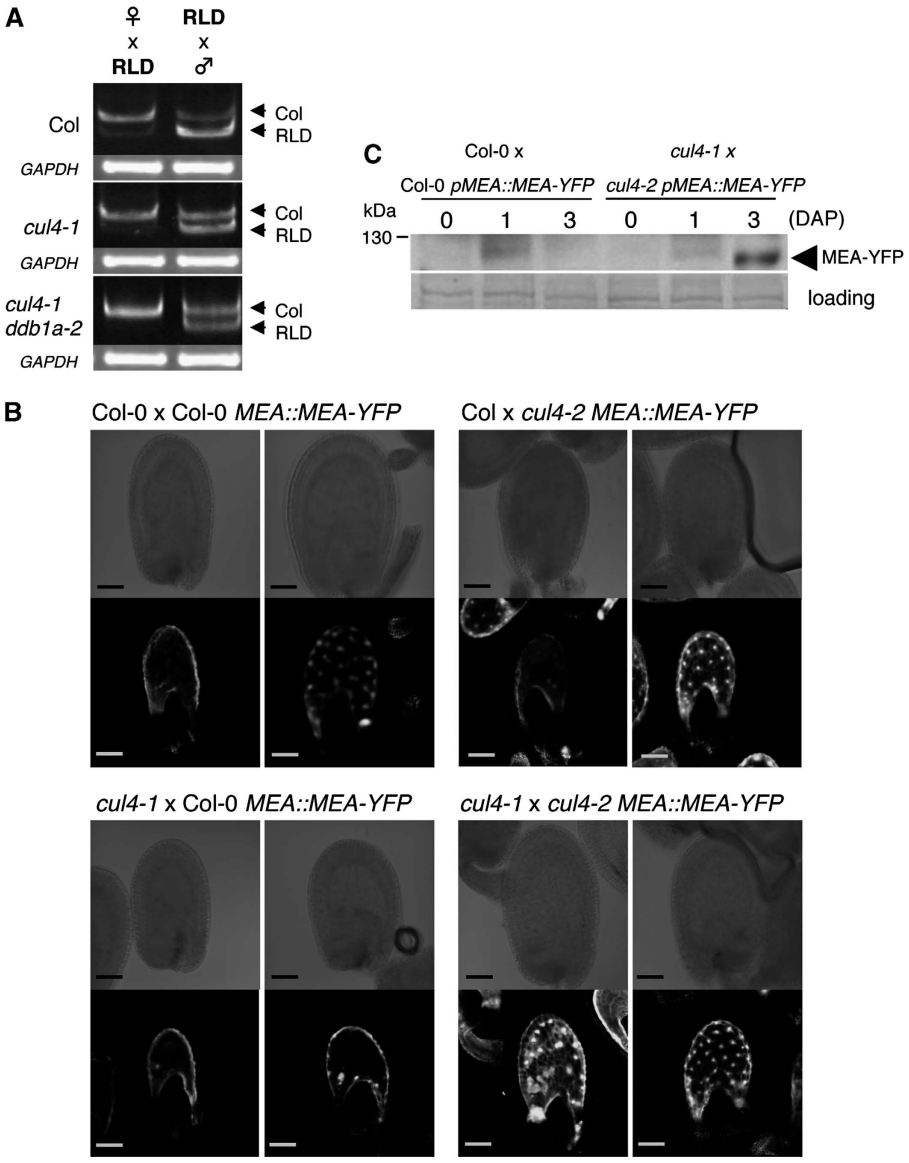


**Figure 4** *MEA* parental imprinting is lost in *cul4* mutants. (**A**) Parental allele-specific expression analysis of *MEA* in wild-type, *cul4-1* and *cul4-1 ddb1a-2* seeds at 3 DAP. For *MEA* expression, Col-0 was crossed with the RLD accession. (**B**) Representative confocal and corresponding DIC images showing *pMEA::MEA-YFP* detection in various crosses at 2 DAP. A strong fluorescence signal was detected in the abnormally large coenocytic endosperm cells resulting from the *cul4-1 cul4-2 pMEA::MEA-YFP* crosses. Bar = 50 μm. (**C**) Paternally expressed MEA-YFP protein accumulation in two different crosses, as indicated. Protein extracts were analysed by immunoblotting using the anti-GFP antibody. Coomassie blue staining used as a loading control. A full-colour version of this figure is available at *The EMBO Journal* Online.

**Table IV** Paternal MEA-YFP signal detection in wild-type and *cul4* mutants

| Parental genotype (female × male) | Negative | Positive (%) |
|---|---|---|
| Col-0 × Col-0 *MEA ::MEA-YFP* | 14 | 15 (51.7%) |
| Col-0 × *cul4-2 MEA ::MEA-YFP* | 13 | 15 (54.6%) |
| *cul4-1* × Col-0 *MEA ::MEA-YFP* | 13 | 14 (51.9%) |
| *cul4-1* × *cul4-2 MEA ::MEA-YFP* | 11 | 26 (70.2%) |

In vegetative tissues, both alleles of *MEA* are silenced by PRC2 complexes through the deposition of H3K27me3 marks on chromatin (Gehring *et al*, 2006; Jullien *et al*, 2006). Thus, we tested whether CUL4 is also required to repress *MEA* later during development. Indeed, *MEA* expression was detected in homozygous *cul4-1* knockdown plants, though to a lesser extend than in a mutant compromised in the SET-domain protein CURLY LEAF (CLF) used here as control (Supplementary Figure S7). Moreover, ChIP analysis revealed that *MEA* reactivation was correlated with a decrease in H3K27 trimethylation (Supplementary Figure S7C).

### *PHE1 3′ region maintains maternal PHE1 repression in cul4 mutants*

An intriguing observation was that in contrast to *MEA*, we did not observe the activation of the maternal *PHE1* allele in the *cul4* knockdown mutant when using the different Arabidopsis accessions (Figure 6A). Nevertheless, when examining H3K27 trimethylation at the *PHE1* locus (Figure 5), we observed a clear reduction in the repressive

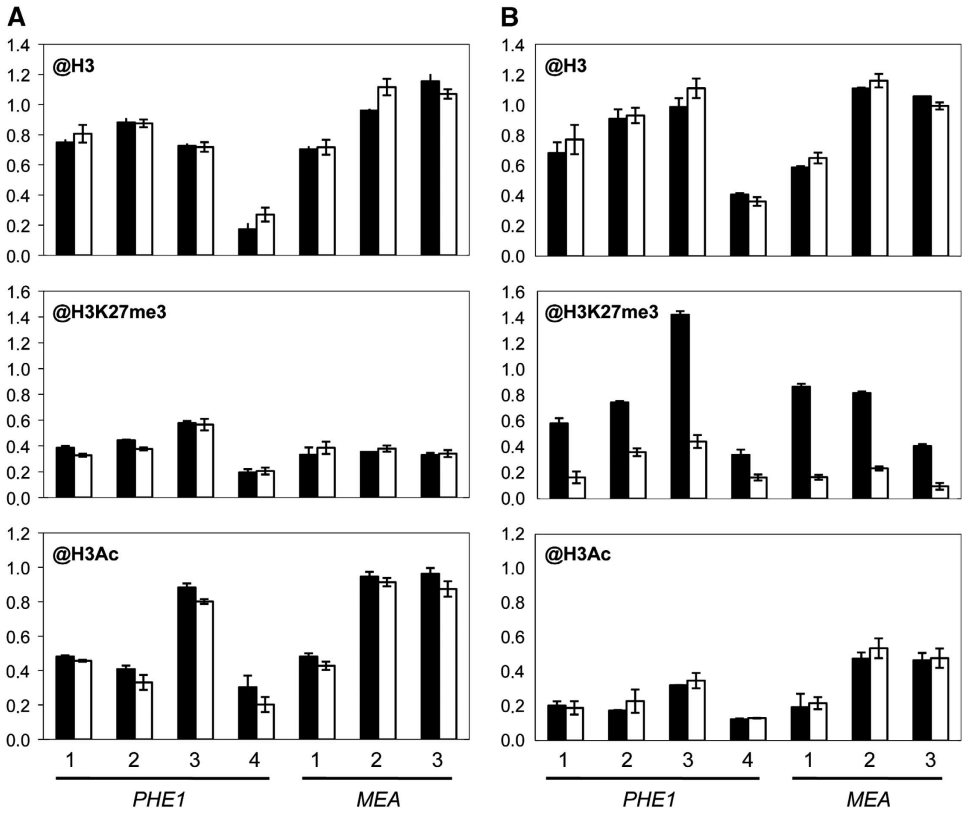


**Figure 5** *CUL4* knockdown induces loss of the repressive H3K27me3 mark on both *MEA* and *PHE1* loci in young siliques. Relative levels of histone modifications on *PHE1* and *MEA* chromatin examined after ChIP assays using anti-H3K27me3 and anti-H3ac antibodies. Chromatin of Col-0 (black bars) and homozygous *cul4-1* mutant (white bars) was prepared from either closed floral buds prior to fertilization (**A**), or young siliques at 3–4 DAP (**B**). DNA fragments after ChIP were quantified by real-time quantitative PCR and were subsequently normalized to internal controls. Data shown are means ± s.d. of three technical replicates. Similar results were obtained in three independent experiments.

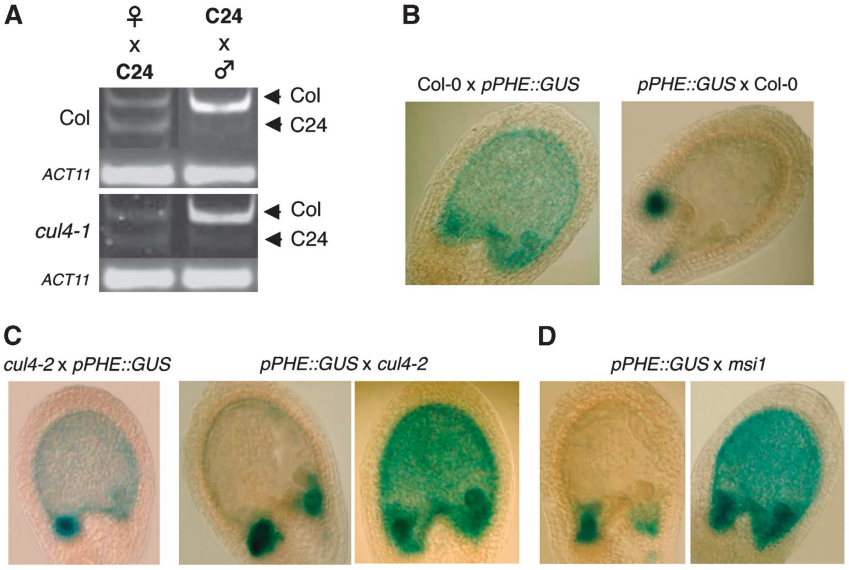


**Figure 6** Paternally expressed CUL4 and MSI1 are required to maintain maternal repression of the *PHE1-GUS* reporter. (**A**) Parental allele-specific expression analysis of *PHE1* in wild-type and *cul4-1* seeds at 3 DAP. For *PHE1* expression, Col-0 was crossed with C24. (**B**) Expression of paternal (left panel) and maternal (right panel) *PHE1-GUS* transgene in wild-type seeds 3 DAP. (**C**) Expression of maternal (middle and right panels) *PHE1-GUS* transgene 3 DAP using *cul4-2* mutant pollen. Seeds presenting a strong GUS staining (~50%) correspond to *cul4-2* heterozygote genotype. (**D**) Expression of maternal *PHE1-GUS* transgene 3 DAP using *msi1* mutant pollen. Seeds presenting a strong GUS staining (~50%) correspond to *msi1* heterozygotes.

histone marks, to a similar level as at the *MEA* locus. Although parental imprinting of both genes requires the FIS–PRC2, it was however recently found that *Polycomb*-dependent silencing is necessary but not sufficient to establish *PHE1* imprinting. Indeed, a distantly located region downstream of the *PHE1* locus, named DMR (differentially

 

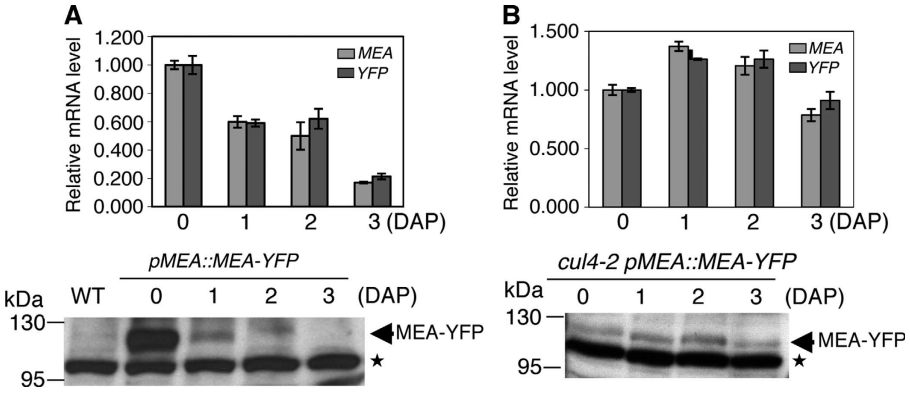


**Figure 7** MEA expression in developing fruits of wild-type and *cul4* mutant plants. (**A**, **B**) MEA-YFP mRNA and protein levels in developing fruits of self-fertilized Col-0 (**A**) and *cul4-2* heterozygote mutant plants (**B**). Relative levels of total *MEA* (blue bars) and *MEA-YFP* (red bars) transcripts determined by quantitative RT–PCR are shown on the upper panels. The transcript level of MEA-YFP was reduced by ~50% in *cul4-2* when compared with Col-0 at 0 DAP (not shown). Data are means ± s.d. MEA-YFP protein levels are shown in the lower part. A measure of 40 μg of total protein extracts was analysed by immunoblotting using an anti-GFP antibody. A full-colour version of this figure is available at *The EMBO Journal* Online.

methylated region), has also an important function in silencing the maternal *PHE1* allele (Makarevich *et al*, 2008). Thus, it is possible that the reduction in repressive histone methylation marks seen in *cul4-1* is not sufficient to impair maternal *PHE1* silencing. To examine this possibility, we took advantage of the *PHE1-GUS* reporter construct, which contains the ~3 kbp promoter sequence but no 3′ regulatory elements (Köhler *et al*, 2003). The paternally derived PHE1-GUS transgene was similarly expressed in wild-type ($n = 55$; Figure 6B) and *cul4-2* heterozygote mutant seeds ($n = 49$; Figure 6C). Expression of the maternally derived *PHE1-GUS* was mainly restricted to the chalazal endosperm when pollinated with wild-type pollen (Figure 6B). In contrast, 3 DAP with heterozygote *cul4*-2 pollen, the maternally derived *PHE-GUS* transgene was expressed at a higher level in about half of the seeds (56 out of 118 seeds exhibited GUS staining in the endosperm; Figure 6C). A similar result was observed with pollen from heterozygote *msi1* mutant plants (78 out of 148 seeds exhibited GUS staining in the endosperm; Figure 6D). Thus, paternally expressed CUL4 and MSI1 are both required to maintain the silencing of the maternal *PHE1-GUS* reporter, suggesting haplo-insufficiency. It is, therefore, likely that the silencing of endogenous maternal *PHE1* is maintained in *cul4* mutant seeds due to additional regulatory elements, most likely located in the 3′ region of the gene (Makarevich *et al*, 2008).

### *MEA transcript and protein accumulate in CUL4-deficient seeds*

Next, we investigated the kinetics of both *MEA* transcripts and protein accumulation. The Col-0 *pMEA::MEA-YFP* line was self-pollinated and MEA-YFP mRNA and protein levels were determined before and during the first 3 DAP. While the *MEA* mRNA level gradually decreased after pollination, MEA protein quickly disappeared and was hardly detectable at 2 DAP (Figure 7A). Thus, the MEA protein level is highly dynamic and seems under both transcriptional and post-transcriptional control. Therefore, we investigated MEA-YFP protein accumulation in *cul4* mutant plants. It is noteworthy that the *pMEA::MEA-YFP* transgene was partially silenced in the *cul4-2* background. This phenomenon, at least in part, could explain the lower MEA-YFP protein content detected in the mutant (Figure 7B). Since *MEA* transcript levels did not decay in the *cul4-2* mutant (Figure 7B), it seems that the MEA-YFP protein accumulation mainly reflects transcriptional regulation by CUL4, though a post-transcriptional regulation cannot be excluded. Nevertheless, our results clearly demonstrate that *CUL4* activity is necessary to restrict *MEA* expression during seed development.

## Discussion

MSI1 is a well-characterized WD40 protein with several functions in the control of chromatin dynamics and gene expression (Hennig *et al*, 2005). In particular, MSI1 was identified as a subunit of the FIS–PRC2 complex (Köhler *et al*, 2003), which regulates parental imprinting during seed development. Here, we provide evidence that CUL4–DDB1 physically associates with MSI1 and is involved in the regulation of the FIS–PRC2 in Arabidopsis. However, because the MSI1 protein associates with additional protein complexes such as chromatin assembling factor 1 (CAF-1) and a complex with the retinoblastoma-related protein (Exner *et al*, 2006; Jullien *et al*, 2008), we do not exclude the possibility that CUL4–DDB1 acts at more than one level.

In flowering plants, imprinting has been mostly studied in the endosperm, which is a terminal tissue developing after fertilization of the central cell (reviewed in Grossniklaus, 2005; Feil and Berger, 2007; Köhler and Weinhofer-Molisch, 2010). Thus far, several genes have been found to be maternally expressed but paternally silenced including *MEA*, *FIS2* and *FWA* (Vielle-Calzada *et al*, 1999; Kinoshita *et al*, 1999, 2004; Jullien *et al*, 2006). In particular, the paternal silencing of *MEA* requires the activity of the FIS–PRC2, which mediates trimethylation of histone H3 at the lysine residue 27 (H3K27me3) (Gehring *et al*, 2006; Jullien *et al*, 2006; Makarevich *et al*, 2006). In contrast, the MADS-box gene *PHE1* is predominantly expressed from the paternal allele while the maternal allele is downregulated (Köhler *et al*, 2005). The maternal *PHE1* allele is repressed through the combined action of the FIS–PRC2 containing MEA and the unmethylated DNA state of a DMR in the 3′ region of *PHE1* (Köhler *et al*, 2005; Makarevich *et al*, 2006). In sperm cells, the DMR is most likely methylated by the maintenance DNA

methyltransferase MET1, preventing silencing and leading to an active paternal *PHE1* allele (Makarevich *et al*, 2008).

In line with a role of CUL4–DDB1 in the regulation of the FIS–PRC2 complex, we could show that reduced *CUL4* activity leads to autonomous endosperm division, although at a lower penetrance than in some other *fis* class mutants (Ohad *et al*, 1996; Chaudhury *et al*, 1997; Köhler *et al*, 2003; Guitton *et al*, 2004). However, the percentage of seeds with a *fis* phenotype observed in *cul4* (3.6–16%) is rather similar to that of *mea* mutants ranging from 3 to 20% (Grossniklaus and Vielle-Calzada, 1998; Kiyosue *et al*, 1999). For *mea* this low penetrance can be explained due to functional redundancy with its paralogue *SWINGER* (Wang *et al*, 2006a). Moreover, we showed that paternal *MEA* silencing was lost when *CUL4* function was compromised. Consistently, H3K27me3 repressive marks were significantly reduced in the *cul4-1* knockdown mutant. A similar reduction in H3K27me3 marks was also observed at the *PHE1* locus, albeit this was not sufficient to abolish downregulation of the maternal *PHE1* allele, most likely because of the presence of additional regulatory mechanisms depending on DNA methylation (Makarevich *et al*, 2008).

Several observations suggest that CUL4–DDB1 is not required for the establishment of *MEA* paternal silencing, but rather for its maintenance. First, we never observed paternal *MEA* reactivation in the pollen of *cul4* knockdown or null mutants (data not shown). Second, we only visualized a strong paternal *MEA* expression 2–3 DAP, but not at 1 DAP. Third, ChIP experiments failed to reveal a loss of H3K27me3 marks at the *MEA* locus in the young floral buds, but only became evident after fertilization in young siliques. Finally, we also found that *CUL4* participates in the maintenance of *MEA* repression at a later developmental stage (e.g. in 17-day-old plants), which depends on another form of PRC2 containing CLF as the histone methyltransferase.

Whether CUL4 and DDB1 only associate transiently or are more stable components of the FIS–PRC2 complex will need further investigations. However, it is noteworthy that MSI1 together with FIE, MEA and FIS2 were found in a very large protein complex of about 650 kDa (Köhler *et al*, 2003), leading the authors to speculate that other proteins associate with FIS–PRC2. Another intriguing observation is that the PRC2 core component FIE, which is also part of various other forms of PRC2 in Arabidopsis (Pien and Grossniklaus, 2007), was predicted to interact with DDB1 based on its structure (Lee *et al*, 2008). Thus, not only one but even two PRC2 components may recruit CUL4 to the FIS–PRC2. Moreover, because metazoan homologs of Arabidopsis MSI1-like proteins, such as the retinoblastoma-binding proteins P55 in Drosophila and RbAp48 in mammals, have structural features of typical CUL4 substrate receptors, it is probable that our findings will extend to other organisms beyond plants.

Although CUL4–DDB1 is involved in FIS–PRC2 functions, *CUL4* loss-of-function mutations do not phenocopy all aspects of *fis* class mutants. In particular, we did not observe in *cul4* mutants as strong penetrance of autonomous endosperm development as in *msi1* mutants (Köhler *et al*, 2003; Guitton *et al*, 2004), nor the presence of parthenogenetic embryos (Guitton and Berger, 2005). This could be explained by at least two different features that distinguish *cul4* from the *fis* class mutants. First, recent work suggests that Arabidopsis *CUL4* is also involved in cell-cycle regulation (Marrocco *et al*, 2010; Roodbarkelari *et al*, 2010), as it has been shown in metazoans (Jin *et al*, 2006; Abbas *et al*, 2008; Havens and Walter, 2009). Thus, instead to promote cell proliferation in the endosperm as observed in the *fis* class mutants, the loss of *CUL4* restricts cell division in this tissue counteracting its *fis* phenotype. Second, the *cul4-1* knockdown does not affect parental imprinting of all FIS–PRC2 targets. In particular, we did not observe the de-repression of the maternal *PHE1* allele, although we found a decrease in H3K27 methylation at this locus. *PHE1* encodes a MADS-domain transcription factor (Köhler *et al*, 2003), while misexpression in *fis* class mutants, but not in *cul4-1*, could explain some phenotypic differences during seed development.

Ubiquitylation has already been linked to *Polycomb*-mediated repression (reviewed in Niessen *et al*, 2009). Indeed, the human PRC1 complex exhibits an E3 ligase activity for histone H2A (Wang *et al*, 2004), which is triggered by two of its subunits, RING1 and RNF2 (also referred to RING1B or RING2) (de Napoles *et al*, 2004; Buchwald *et al*, 2006). In the prevailing model, PRC1 binds to histone H3K27me3 to catalyse monoubiquitination of histone H2A, which in turn could interfere with the transcriptional machinery or chromatin remodelling proteins to repress transcription of target genes (Stock *et al*, 2007; Zhou *et al*, 2008). Moreover, it was recently shown that a tight balance between histone H2A ubiquitylation and deubiquitylation is important for *Polycomb*-mediated repression in Drosophila (Scheuermann *et al*, 2010). Arabidopsis also contains RING-domain proteins that, together with LHP1, may fulfil PRC1-like functions (Xu and Shen, 2008), most likely via histone H2A ubiquitylation (Bratzel *et al*, 2010).

Our finding that the CUL4–DDB1$^{MSI1}$ E3 ligase is required in the maintenance of FIS–PRC2-dependent parental imprinting in Arabidopsis raises the question which substrate(s) are targeted for ubiquitylation in this process. One possibility is that CUL4–DDB1$^{MSI1}$ ubiquitylates directly one of the FIS–PRC2 subunits.

We initially speculated that MSI1 could be either a substrate or a substrate receptor of this E3 ligase, or even both. Thus, we introgressed the *pMSI::MSI1–RFP* reporter gene (Chen *et al*, 2008) into the *cul4-1* mutant background and checked for MSI1 protein accumulation. However, when *cul4-1 pMSI::MSI1–RFP* plants were self-pollinated, the MSI1–RFP protein level was only slightly higher than in a wild-type background (Supplementary Figure S8), suggesting that MSI1 protein turnover is not controlled by CUL4.

In contrast, we noticed that MEA protein does not decay after pollination in *cul4* mutants. Thus, it is possible that ubiquitylation controls its stability, although we cannot exclude that this accumulation mainly results from the persistence of the *MEA* transcript. Nevertheless, unscheduled MEA protein accumulation may alter FIS–PRC2 activity by, for example, titering some of its components or associated proteins. This may also explain the paradox why in the presence of more MEA protein, repression of the paternal *MEA* allele is lost.

Finally, it is also possible that CUL4–DDB1$^{MSI1}$ acts at the FIS–PRC2 level by a mechanism that does not imply protein degradation. In this respect, it is well established that CRL4 E3 ligases trigger different kinds of non-proteolytic ubiquitylation reactions, including the assembly of K63-linked polyubiquitin chains and monoubiquitylation. Thus,

 

in the process of nucleotide excision repair after UV damage, a CUL4–DDB1$^{DDB2}$ E3 ligase (DDB2 being a WD40 substrate receptor) triggers non-proteolytic ubiquitylation of XPC (xeroderma pigmentosum complementation group C) to permit its binding to damaged DNA (Sugasawa *et al*, 2005) where it induces histone ubiquitylation, presumably to modify the chromatin structure at the sites of DNA lesions (Kapetanaki *et al*, 2006; Wang *et al*, 2006b). Interestingly, the fission yeast Cul4 associates with Clr4 histone methyltransferase and is required for RNAi-mediated heterochromatin formation (Hong *et al*, 2005; Jia *et al*, 2005), although the CUL4-mediated modifications, which are involved in this process, remain unknown. Future investigations will identify CUL4–DDB1$^{MSI1}$ E3 substrates and clarify its function(s) in PRC2-dependent epigenetic regulation.

## Materials and methods

### *Plant material and growth conditions*

The Arabidopsis *ddb1a-2* mutant is described in Molinier *et al* (2008). The *ddb1b-1* (SALK 061944) mutant was identified using the web-assisted program at http://signal.salk.edu/cgi-bin/tdnaexpress. The precise location of the T-DNA in *ddb1b-1* mutant was determined by sequencing, showing an insertion after nucleotide 6211 in the last exon of *DDB1B*. The *cul4-1* (GABI-KAT 600H03) mutant is described in Bernhardt *et al* (2006). The *cul4-2* (Koncz 42460) and *cul4-3* (Koncz 57891) mutants were identified by PCR screening of the Köln Arabidopsis T-DNA mutant collection (Ríos *et al*, 2002). The precise location of the T-DNA in *cul4-2* and *cul4-3* was determined by sequencing, showing an insertion after nucleotide 4478 in the 14th exon and after nucleotide 2718 in the 7th intron of *CUL4*, respectively.

For *in vitro* culture, seeds were surface sterilized using the ethanol method, plated on GM medium (MS salts (Duchefa, The Netherlands), 1% sucrose, 0.8% agar, pH 5.8) in the presence or absence of a selectable agent, stored 2 to 3 days at 4°C in the dark, and then transferred to a plant growth chamber under a 16-h/8-h photoperiod (22uC/20uC). For soil-cultured plants, seeds were sown (20/pot) and put at 4°C in the dark during 3 days. Two weeks later, single plants were transferred to pots in the greenhouse and kept under a regime of 16 h/8 h photoperiod (20uC/16uC; 70% humidity).

### *Yeast two-hybrid assays*

The DDB2 and MSI1 cDNAs were cloned as fusions to the GAL4 activation domain and the DBB1A cDNA fused to the GAL4-binding domain, respectively, in Gateway-compatible pGAD424gate and pGBT9gate (Ghent plasmids collection, http://bccm.belspo.be/index.php) yeast two-hybrid vectors. The yeast strain AH109 (Clontech) was transformed with the appropriate combinations of bait and prey vectors. Transformants were selected on synthetic (SD)/-Leu/-Trp (-LW) media and interactions were tested on SD/-Leu/-Trp/-His (-LWH) or SD/-Leu/-Trp/-Ade (-LWA) media, allowing growth for 4 days at 28°C.

### *GST pull-down assay*

The full-length DDB1A cDNA was cloned into Gateway vector pDEST15 (Invitrogen) by recombination for expression in *Escherichia coli* BL21AI (Invitrogen). In this construct GST is placed in frame at the N-terminus of DDB1A protein. After 4 h of 0.2% Arabinose induction at 16°C, the fusion proteins were purified on bulk gluthatione-sepharose following the manufacturer's instructions (GE Healthcare). For GST pull-down assays, DDB2 and MSI1 proteins were translated *in vitro*, using the TNT7-coupled wheat germ extract system (Promega) and radiolabelled with [$^{35}$S]-methionine. Purified GST-DDB1A or control GST proteins, immobilized on glutathione-sepharose beads, were incubated for 2 h at 4°C with equal amounts of $^{35}$S-methionine-labelled DDB2 and MSI1 protein following the manufacturer's instructions (GE Healthcare). Labelled DDB2 and MSI1 proteins were detected by autoradiography.

### *Bimolecular fluorescence complementation*

The DDB1A, DDB2 and MSI1 coding sequences were cloned into the split YFP destination vectors by recombination (Invitrogen) in order to obtain the YN-DDB1A, YC-DDB2 and YC-MSI1 constructs. The YN-, YC- and $^{35}$S-CPRF2-CFP vectors and split YFP experiments were performed as described in Stolpe *et al* (2005). Vectors bearing YN or YC either alone or fused to BPM3 (At2g39760) were used as negative controls. Images were recorded 20 h after bombardment with a Nikon fluorescent stereomicroscope E800 equipped with a 40× water immersion optic by using CFP- and YFP-specific filters.

### *Immunoprecipitation experiments*

Total soluble proteins were extracted from pMSI1::MSI1–RFP plants (Chen *et al*, 2008) using buffer A (100 mM $NaHPO_4$ pH 8.0, 1% Triton ×100, protease inhibitor mix (Complete; Roche Molecular Biochemical)). Immunoprecipitation assay was performed using anti-CUL4 polyclonal antibody coupled to ProteinA-sepharose beads. The MSI1–RFP protein was detected using anti-DsRed antibodies (Clontech Laboratories Cat#632496) diluted 1:1000 (v:v).

### *Histology and microscopy*

Developing seeds were prepared from siliques of different developmental stages and directly mounted on microscope slides in a clearing solution of 8:2:1 chloral hydrate:distilled water:glycerol as described in Grini *et al* (2002). Observations were performed with a Zeiss Axiophot or a Leica DMR microscope under differential interference contrast (DIC) ×20 and ×40 optics. For YFP marker analysis, seeds from dissected siliques at different DAP were mounted in a 1:10 glycerol:distilled water. Specimens were observed under a Zeiss confocal laser-scanning microscope. Histochemical assays to detect GUS activity were performed as described in Capron *et al* (2003).

### *In situ hybridization*

*In situ* hybridization was performed on the 10-μm Paraplast Plus (Sigma) sections as described in Brukhin *et al* (2005). For probe synthesis, the fragment spanning the region of the CUL4 (At5g46210) cDNA from 1501 to 2000 (500 bp) sequence was used. The fragment was inserted into the pGemT plasmid (Promega). Sense and antisense digoxygenin-UTP-labelled riboprobes were generated by run-off transcription using T7 and Sp6 RNA polymerases, respectively. The probes were hydrolyzed into 120 bp fragments in carbonate buffer, pH = 10.2 for 59 min at 60°C.

### *RNA extraction and RT–PCR analysis*

RNAs from siliques at 3 DAP and from 17-day-old plantlets were prepared with the Trizol reagent (Invitrogen). In all, 5 μg of total RNA was treated with Dnase1 kit (Fermentas) and reverse transcribed with Superscript III Reverse Transcriptase kit (Invitrogen). To detect *PHE1* and *MEA* mRNA, allele-specific RT–PCR was performed as described previously in Kinoshita *et al* (1999) and Köhler *et al* (2005). Primers used to amplify the control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RNA, were GAPDH3′ (5′-GTAGCCCCACTCGTTGTCGTA-3′) and GAPDH5′ (5′-AGGGTGGT GCCAAGAAGGTTG-3′). To detect DDB1B, specific primers DDB1FWD (5′GGAAAATGAACCAACTAAGGAAGG-3′) and DDB1bREV (5′AGAG CTTGGATTTGCTTCAGTG-3′) were used. To detect EF1α-specific primers EF1Fwd (5′-TTGCTCCACAGGATTGACCACTG-3′) and EF1Rev 5′-TCACTTCGCACCCTTCTTGACG-3′) were used.

### *Quantitative PCR*

Total RNA for quantitative PCR (qPCR) was extracted from inflorescences and siliques at different DAP of pMEA::MEA-YFP plants in Col-0 and *cul4-2* heterozygous mutant backgrounds, respectively, using the kit RNeasy MINI PLUS (Qiagen). In all, 2 μg of total RNA was reverse transcribed with Superscript III Reverse Transcriptase kit (Invitrogen). PCR was performed using gene-specific primers in a total volume of 10 μl SYBR Green Master mix (Roche) on a Lightcycler LC480 apparatus (Roche) according to the manufacturer's instructions.

The mean value of three replicates was normalized using the ACTIN2 (AT3G18780), GAPDH (AT3G26650) genes as internal controls.

Primer list:

MEA: GCAGGACTATGGTTTGGATG and CACCTTGAGGTAACA ATGCTC

YFP: ATATCATGGCCGACAAGCA and GAACTCCAGCAGGACCA TGT
ACTIN2: CTTGCACCAAGCAGCATGAA and CCGATCCAGACAC TGTACTTCCTT
GAPDH: TTGGTGACAACAGGTCAAGCA and AAACTTGTCGCTC AATGCAATC

#### *Chromatin immunoprecipitation*

ChIP experiments were performed as described by Jiang *et al* (2008) using young flowers before fertilization and young siliques around 3 DAP as well as 17-day-old plants. A measure of 5 ml mixed tissue powder was prepared after cross-linking DNA with proteins by formaldehyde. Preparation of chromatin, sonication, and immunoprecipitation using anti-H3 (05-499; Millipore), anti-trimethyl-histone H3K27 (07-449; Millipore) and anti-acetyl-H3 (06-599; Millipore) antibodies were carried out using Millipore ChIP kit according to the manufacturer's instructions. The immunoprecipitated DNA was analysed by real-time qPCR using *MEA* primers (region −700–1500; Baroux *et al*, 2006) and *PHE1* primers (Makarevich *et al*, 2006). *FUSCA* primers (Kwon *et al*, 2009) were used as internal standards for normalization. Data analysis was done as described in Mutskov and Felsenfeld (2004). Experiment was performed three times using independent biological samples.

#### *Protein extraction and immunoblotting*

Total proteins were extracted from Col-0 and *cul4* siliques at 1, 2 and 3 DAP using denaturating buffer as described in Büche *et al* (2000). A measure of 40 μg of total protein extracts were separated on SDS–PAGE gels and blotted onto Immobilon-P membrane (Millipore). The MSI1–RFP protein was detected by using the anti-DsRed antibody (Clontech Laboratories Cat#632496) diluted 1:1000 (v:v), whereas the MEA-YFP protein was identified by using a Rabbit anti-GFP polyclonal antibody diluted 1:10 000 (v/v).

#### *Supplementary data*

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

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## Conflict of interest

The authors declare that they have no conflict of interest.

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