Arabidopsis SWR1-associated protein methyl-CpG-binding domain 9 is required for histone H2A.Z deposition

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Deposition of the histone variant H2A.Z by the SWI2/SNF2-Related 1 chromatin remodeling complex (SWR1-C) is important for gene regulation in eukaryotes, but the composition of the Arabidopsis SWR1-C has not been thoroughly characterized. Here, we aim to identify interacting partners of a conserved Arabidopsis SWR1 subunit ACTIN-RELATED PROTEIN 6 (ARP6). We isolate nine predicted components and identify additional interactors implicated in histone acetylation and chromatin biology. One of the interacting partners, methyl-CpG-binding domain 9 (MBD9), also strongly interacts with the Imitation SWItch (ISWI) chromatin remodeling complex. MBD9 is required for deposition of H2A.Z at a distinct subset of ARP6-dependent loci. MBD9 is preferentially bound to nucleosome-depleted regions at the 5' ends of genes containing high levels of activating histone marks. These data suggest that MBD9 is a SWR1-C interacting protein required for H2A.Z deposition at a subset of actively transcribing genes.
Chromatin dynamics plays a central role in regulating gene expression and has important implications in various cellular processes. The basic unit of chromatin is the nucleosome, which consists of 147 base-pairs (bp) of DNA wrapped around a histone octamer composed of two of each of the histones H2A, H2B, H3, and H4 (ref. 1). Cells contain chromatin remodeling complexes, which modulate the packaging, spacing, and composition of nucleosomes3–5. The activity, targeting, and composition of remodeling complexes is tightly regulated to enable proper development of an organism6–9.

The most highly conserved histone variant in eukaryotes is H2A.Z4–6. H2A.Z is found at many genes, most often around transcription start sites (TSS), with a particular preference for the +1 nucleosome7–9. The relationship between H2A.Z accumulation and transcription is complex. When present in the active genes of some organisms, the levels of H2A.Z at the TSS positively correlate with gene expression, typically when found together with H3/H4 acetylation, marks of active transcription10–13. H2A.Z is nonetheless absent at the most highly expressed genes7 and is also localized around the TSS of repressed genes that are poised for activation in yeast and animals9,14–16. Although the function of gene body methylation is unclear, one study in Arabidopsis suggests that a function of gene body DNA methylation may be to prevent H2A.Z deposition over housekeeping genes17, while another study found no such evidence18. However, it is clear that an anti-correlation between H2A.Z and DNA methylation exists across diverse systems18,19–21. In Arabidopsis, genes bearing high levels of gene body DNA methylation tend to be long and highly expressed, and show H2A.Z enrichment at their 5′-ends overlapping with activating histone modifications17,22–24. By contrast, enrichment of H2A.Z over gene bodies (including high H2A.Z levels at the TSS) is typically found in shorter, weakly expressed genes involved in environmental/stress responses that are marked with H3K27me3 (refs. 17,22–25). Recently, levels of H3K27me3 were shown to be dependent on H2A.Z deposition in Arabidopsis23,26. Together, these studies demonstrate that H2A.Z can participate in both activation and repression of gene expression, likely in concert with other factors and chromatin marks.

H2A.Z is deposited by the SWI2/SNF2-Related 1 (SWR1) complex, which involves the replacement of H2A-H2B dimers by H2A.Z-H2B dimers27–29. In yeast, the SWR1 complex (SWR1-C) is composed of 14 subunits30. In mammals, the structurally and functionally equivalent homolog of the yeast SWR1 complex, Snf2-Related CREBBP Activator Protein (SRCAP), is composed of 11 subunits, which are also conserved in yeast31–33. Based on homology, Arabidopsis contains one or several orthologs of the 11 conserved subunits from the yeast and human SWR1/SRCAP complexes, respectively34. Based on genetic and protein–protein interaction studies, several components have been validated as components of the Arabidopsis SWR1 complex, including PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1), the ATPase/scaffold subunit; ACTIN-RELATED PROTEIN 6 (ARP6); SERRATED LEAVES AND EARLY FLOWERING (SEF), the homolog of the yeast SWC6; and SWR1 complex subunit 2 (SWC2)35–37. A recent pull-down study of the SWC6 subunit identified additional subunits of the Arabidopsis SWR1 complex in yeast and validated their interaction with SWR1 complex subunit 4 (SWC4)38. Mutations in the genes encoding the SWR1 subunits share various phenotypes, with null mutations in the core catalytic subunit PIE1 producing the most severe phenotypes, including reduced size, loss of apical dominance, reduced fertility, and early flowering35–37,39–41.

How the SWR1 complex is targeted across the genome remains unclear. Evidence suggests that H2A.Z localization around TSS in yeast is dependent on open chromatin and histone acetylation deposited by the Nucleosome acetyltransferase of H4 (NuA4) complex4,8,42–46. The SWR1-C and NuA4 complexes share four common members: SWC4, YEAST ALL1-FUSED GENE FROM CHROMOSOME 9 (YAF9), ACTIN-RELATED PROTEIN 4 (ARP4), and ACTIN 1 (ACT1)27–29,47–49. Homologs of these four proteins are present in the mammalian TIP60 complex, which possesses the dual activities of acetylating histones and depositing H2A.Z31,36,37. The Arabidopsis genome also contains homologs for many of the yeast NuA4 subunits; however, a functional link between histone acetylation and H2A.Z deposition is unclear50. YAF9 proteins are required for both the proper expression of FLOWERING LOCUS C (FLC) and H2A.Z acetylation at its regulatory regions, but not for H2A.Z deposition51. Much more needs to be learned about the potential NuA4 complex in Arabidopsis, including its composition and genome-wide effects on H2A.Z deposition and modification. Although genome-wide localization of the SWR1 components is lacking in Arabidopsis, it has been reported that ARP6 and SWC4 directly bind their target locus FLOWERING LOCUS T (FT)38; likewise, ARP6 and SWC6 bind directly to FLC39.

In this study, we used the conserved ARP6 subunit as bait to analyze the composition of the Arabidopsis SWR1 complex. We identify Arabidopsis homologs of most of the conserved SWR1 complex components in yeast, as well as several additional interacting partners: MBD9, four of the seven members of the ALFIN1-LIKE family (AL4, AL5, AL6, AL7), and two components of the yeast NuA4 and Arabidopsis SAGA histone acetyltransferase complexes, TRANSCRIPTION-ASSOCIATED PROTEIN 1A AND 1B (TRA1A and TRA1B). Our data are consistent with a related study where ARP6-interacting proteins were identified using ARP6-TAP-tag purification followed by mass spectrometry54. We focus on characterizing MBD9, a protein implicated in flowering time regulation55. Using biochemical, genetic, and transcriptome analyses, we show that MB9 interacts with the SWR1 complex in Arabidopsis, and is required for H2A.Z deposition at a subset of ARP6-dependent genes. Furthermore, immunoprecipitation of MB9 followed by mass spectrometry (IP–mass spec) reveals its association with the ISWI chromatin remodeling complex, suggesting additional functions for MB9. Genome-wide ChIP-seq analyses of MB9 show that it localizes to nucleosome-depleted regions of active genes but does not influence open chromatin itself. MB9 is thus an interacting partner of the Arabidopsis SWR1 complex required for H2A.Z deposition at a subset of actively transcribed genes.

**Results**

MBD9 controls flowering time via its interaction with SWR1 complex. To identify components of the SWR1 complex in Arabidopsis, we used the conserved ARP6 subunit of the SWR1 complex as bait. We generated the epitope-tagged ARP6 lines pARP6:ARP6-3xFLAG and pARP6:ARP6-9xMYC. Both lines complemented arp6-1 mutants (Supplementary Fig. 1), indicating that epitope-tagged ARP6 is functional in vivo. To detect as many potential protein interactions as possible, we performed IP–mass spec in both FLAG- and MYC-tagged ARP6 lines using highly proliferating inflorescence tissue where levels of proteins are high. In all replicates (three biological replicates with two technical replicates each), we observed enrichment of the Arabidopsis homologs of most of the conserved yeast SWR1 complex components—ARP6, PIE1, RuB-like protein 1 and 2 (RIN1, RIN2), ARP4, SWC4, SWC2, YAF9A, and SEF. This includes three of the four common subunits of the yeast NuA4 complex, ARP4, SWC4, and YAF9A (Table 1). Our experiment confirms previous well-characterized in vivo and in vitro interactions of ARP6 with PIE1 and SEF36–38. We additionally identified two homologs of the yeast Tra1 proteins, subunits of the yeast NuA4 complex, TRA1A

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and TRA1B. We also observed consistent enrichment for several interacting partners previously implicated in chromatin biology, MBD9 (refs. 35,36) and AL4, AL5, AL6, and AL7, which recognize di- and trimethyl H3K4 histone marks57. The interaction between ARP6 and MBD9 was further validated by co-immunoprecipitation (co-IP) in Arabidopsis flowers using an antibody raised against MBD9 (Fig. 1a). We then generated epitope-tagged MBD9 lines, pMBD9:MBD9-3xFLAG and pMBD9:MBD9-9xMYC. Both lines complemented mbd9-3 mutants (Supplementary Fig. 2), indicating that the epitope-tagged MBD9 is functional in vivo. We performed IP-mass spec using floral tissue from both FLAG- and MYC-tagged MBD9 lines. In both experiments we observed enrichment of the SWR1 complex components PIE1, RIN1, ARP4, RIN2, SWC2, ARP6, SWC4, and TRA1B. We also observed consistent enrichment for several proteins that interact with ARP6 and MBD9, including PIE1, RIN1, ARP4, RIN2, SWC2, ARP6, SWC4, YAF9A, and MAF4 (Supplementary Methods). We surveyed the gene annotations in each common interacting partners between ARP6 and MBD9, and the role of MBD9 in H2A.Z deposition, we analyzed H2A.Z levels in Arabidopsis seedlings before the major developmental transition to flowering in order to minimize secondary effects in mutants. We performed western blot on total extracts, and observed the levels to decline in the mutants: wild type > mbd9-3 > arp6-1 > arp6-1 mbd9-3 (Fig. 2a). To investigate the impact of H2A.Z loss on chromatin, we performed two replicates of H2A.Z ChIP-seq in wild type, mbd9-3, arp6-1, and arp6-1 mbd9-3, with H3 ChIP-seq as a control. Pearson correlation indicated a considerable overlap between the ChIP-seq replicates (Supplementary Fig. 3a). As with H2A.Z protein levels, we detected a sequential decrease in H2A.Z signal over H2A.Z peaks in wild type and over protein-coding genes as follows: wild type > mbd9-3 > arp6-1 > arp6-1 mbd9-3 (Fig. 2b). By contrast, H3 levels were unchanged over these regions (Supplementary Fig. 3b). Using the MACS2 peak caller for each replicate (q value <0.01), we identified common H2A.Z enrichment. We surveyed the gene annotations in each differential region and identified genes that lost H2A.Z in the mutants compared to wild type: 2594 for mbd9-3, 12,222 for arp6-1, and 15,226 for arp6-1 mbd9-3. GO term enrichment for mbd9-3, arp6-1, and arp6-1 mbd9-3 revealed a high degree of overlap (Fig. 2c). A meta-gene plot of MADS AFFECTING FLOWERING 4 and 5 (MAF4 and MAF5), indicated that both the single mutants and the double mutant had a similar reduction of expression of these genes (Fig. 1d). Together, these results indicate that MBD9 and ARP6 act together in the same genetic pathway controlling flowering time as part of the SWR1 complex.

### Table 1 IP-mass spectrometry of ARP6

| Protein       | Annotation | ARP6-9xMYC | R1 | R2 | R3 | R4 | ARP6-3xFLAG | Wild type |
|---------------|------------|------------|----|----|----|----|------------|-----------|
| AT3G33520     | ARP6       | 123        | 119| 138| 123|    |            |           |
| AT3G12810     | PIE1       | 175        | 208| 206| 202|    |            |           |
| AT5G22330     | RIN1       | 176        | 189| 224| 196|    |            |           |
| AT5G67630     | RIN2       | 115        | 136| 175| 146|    |            |           |
| ATIG18450     | ARP4       | 75         | 76 | 64 | 56 |    |            |           |
| AT2G62710     | SWC4       | 45         | 46 | 33 | 39 |    |            |           |
| AT2G36740     | SWC2       | 37         | 32 | 35 | 34 |    |            |           |
| AT5G45600     | YAF9A      | 25         | 19 | 20 | 21 |    |            |           |
| AT5G37055     | SEF        | 11         | 9  | 9  | 8  |    |            |           |
| AT3G01462     | MBD9       | 67         | 71 | 97 | 108|    |            |           |
| AT2G17930     | TRA1A      | 84         | 94 | 117| 117|    |            |           |
| AT4G36608     | TRA1B      | 72         | 78 | 91 | 91 |    |            |           |
| AT5G26210     | AL4        | 2          | 3  | 2  | 2  |    |            |           |
| AT5G20510     | AL5        | 12         | 16 | 15 | 14 |    |            |           |
| AT2G02470     | AL6        | 5          | 6  | 4  | 5  |    |            |           |
| AT1G14510     | AL7        | 6          | 3  | 5  | 6  |    |            |           |

Spectral counts for ARP6-9xMyc and ARP6-3xFlag lines representing enriched genes over wild type. Source data are provided as Source Data file.
mbd9-3 mutant vs arp6-1, q value <0.01). These genes were enriched for cell cycle progression functions (Supplementary Fig. 3c). The H2A.Z profiles over this set of genes (Fig. 2g) support the observation that these regions were dependent on both MBD9 and ARP6 for H2A.Z deposition. In Arabidopsis, housekeeping genes typically contain H2A.Z exclusively over the +1 nucleosome and lowly expressed/responsive genes contain H2A.Z over the entire gene bodies, with highest levels of H2A.Z at the TSS. To investigate which types of genes are most impacted by the loss of H2A.Z in our mutants, we divided genes (into nine classes) according to the levels of H2A.Z in wild type over gene bodies (low, medium, and high) and TSS (low, medium, and high) as described in ref. 17 (Supplementary Fig. 4, Supplementary Methods). We intersected these classes of gene with genes that lost H2A.Z in our mutants and plotted their distribution (Fig. 2h). This revealed that loss of H2A.Z in the mbd9-3 mutant tends to occur over genes possessing high levels of H2A.Z over the TSS and low levels of H2A.Z over gene bodies (HL class—High TSS, Low gene body), whereas loss of H2A.Z in the arp6-1 mutant tends to occur over genes containing high levels of H2A.Z over gene bodies (LH, MH, and HH classes) (Fig. 2h).

**MBD9/ARP6 partial redundancy in gene expression control.** To investigate the relationship between H2A.Z loss and transcription, we performed RNA-seq in seedlings in wild type, mbd9-3, arp6-1, and arp6-1 mbd9-3. We defined differentially
expressed genes (fold change >2, FDR <0.05, Supplementary Methods). We detected 277 misregulated genes in mbd9-3, 2042 in arp6-1, and 1620 in arp6-1 mbd9-3. Among the down-regulated genes we detected FLc, MAF4, and MAF5 as targets of both MBD9 and ARP6, confirming our RT-qPCR results (Supplementary Fig. 5a). Importantly, the expression of genes encoding the predicted subunits of the SWR1 complex as well as H2AZ genes were largely unaffected in the mutants (Supplementary Fig. 5b). We examined down-regulated and up-regulated genes (Fig. 3) and observed a high degree of overlap between the arp6-1 and arp6-1 mbd9-3 mutants, with 584 common up-regulated and 616 common down-regulated genes (Fig. 3a, b). These overlapping genes showed the same trend in expression change in mbd9-3 mutants, but to a lesser degree that did not meet our significance cutoffs (Fig. 3c, d). We also defined genes specifically up- and down-regulated in either mbd9-3 or arp6-1, implying independent functions at a subset of loci (Fig. 3a–d) as well as a set of 218 up-regulated genes that were dependent on both ARP6 and MBD9 for expression (Fig. 3a, c), suggesting a redundant role of MBD9 and ARP6 at this set of genes. By contrast, the unique down-regulated genes (n = 170) did not show a significant change between arp6-1 and arp6-1 mbd9-3 (Fig. 3b, d). GO term analysis indicated that up-regulated genes in mbd9-3 mutants are involved in post-embryonic development and response to stress, whereas for arp6-1 and arp6-1 mbd9-3 up-regulated genes are involved in lipid localization, biosynthetic processes, and response to drug (Supplementary Fig. 5c). By contrast, down-regulated genes are involved in response to stress, stimulus, and defense in all mutants (Supplementary Fig. 5c).

We compared differentially expressed genes in relation to H2A.Z changes in the mutants. First, to obtain general trends, we generated boxplots of expression and profiles of H2A.Z in the nine classes of genes divided according to their H2A.Z occupancy (Supplementary Fig. 4) for wild type, mbd9-3, arp6-1, and arp6-1 mbd9-3 (Supplementary Fig. 6a, b). One general trend stood out, genes containing high-medium H2A.Z levels over gene bodies and the highest H2A.Z levels over TSS showed increased expression upon loss of H2A.Z in arp6-1, which was even more pronounced in the arp6-1 mbd9-3 mutant (Supplementary Fig. 6a, b). Only a weak loss of H2A.Z was observed in the mbd9-3 mutant over these classes of genes where expression was not significantly increased (Supplementary Fig. 6a, b). Interestingly, looking at a typical H2A.Z profile of housekeeping genes (low H2A.Z at gene body with high levels of H2A.Z at TSS), H2A.Z deposition depended on both ARP6 and MBD9; however, this loss did not cause major transcriptional changes even in arp6-1 mbd9-3 where H2A.Z levels were nearly depleted (Supplementary Fig. 6a, b). This perhaps is not surprising since genes that typically change in expression (become up-regulated) upon H2A.Z misregulation are weakly expressed/environmentally responsive genes that contain highest levels of H2A.Z over the gene body and the TSS, most likely reflecting changes in chromatin accessibility. We next profiled H2A.Z changes in the significantly up- and down-regulated genes in our RNA-seq datasets (Fig. 3e). Both up- and down-regulated genes lost H2A.Z in arp6-1 and arp6-1 mbd9-3 mutants (Supplementary Fig. 5d). In agreement with the observed general trends, the up-regulated genes tend to belong to gene classes containing high levels of H2A.Z over the gene body (LH, MH, HH classes). More specifically, loss of H2A.Z in mbd9-3 was observed most over genes up-regulated in mbd9-3 (n = 84 and n = 33) and those specifically up-regulated in arp6-1 mbd9-3 (n = 218) (Supplementary Fig. 5d), suggesting that MBD9-dependent H2A.Z deposition may be directly involved in repression of this subset of genes. arp6-1 dependent up-regulated genes (n = 252 and n = 500) had minimal if any change in H2A.Z levels in mbd9-3 (Supplementary Fig. 5d). The down-regulated genes did not tend to belong to any particular class of H2A.Z-containing genes (Fig. 3e). Except for few well-studied genes (MADS-box proteins), the down-regulated genes in the SWR1-C mutants (including MBD9) most likely reflect indirect effects related to H2A.Z loss perhaps due to misregulated transcription factors in our mutants (Supplementary Fig. 7). Altogether, these data suggest that (1) mbd9-3 has a much weaker effect on transcription than arp6-1; (2) genes misregulated in the mbd9-3 background are a subset of genes misregulated in the arp6-1 background; (3) MBD9 and ARP6 also cooperate to control the expression of a subset of up-regulated genes; and (4) although loss of H2A.Z is observed genome-wide in arp6-1, and is even more pronounced in arp6-1 mbd9-3, genes that change in expression upon the loss of H2A.Z are generally those that contain high levels of H2A.Z at TSS and high-medium levels of H2A.Z over the gene body.

Table 2 IP-mass spectrometry of MBD9

| Protein  | Annotation | MBD9-9xMYC | MBD9-3xFLAG | Wild-type-9xMYC | Wild-type-3xFLAG |
|---------|------------|------------|-------------|----------------|------------------|
| AT3G01460 | MBD9       | 355        | 607         | 0              | 0                |
| AT3G12810 | PIE1       | 75         | 49          | 0              | 0                |
| AT5G22330 | RIN1       | 33         | 26          | 0              | 0                |
| AT1G18450 | ARP4       | 31         | 24          | 0              | 0                |
| AT5G67630 | RIN2       | 29         | 22          | 0              | 0                |
| AT2G36740 | SWC2       | 17         | 0           | 0              | 0                |
| AT3G33520 | ARP6       | 16         | 5           | 0              | 0                |
| AT2G47210 | SWC4       | 12         | 9           | 0              | 0                |
| AT5G45600 | YAF9A      | 5          | 6           | 0              | 0                |
| AT5G37055 | SEP        | 2          | 0           | 0              | 0                |
| AT2G17930 | TRA1A      | 128        | 76          | 0              | 0                |
| AT4G36080 | TRA1B      | 120        | 74          | 0              | 0                |
| AT2G02470 | AL6        | 2          | 0           | 0              | 0                |
| AT5G18620 | CHR17      | 122        | 190         | 0              | 0                |
| AT3G0640  | CHR11      | 118        | 177         | 0              | 0                |

*Significant expression differences in MBD9-9xMYc and MBD9-3xFlag lines.*
Fig. 2 Characterization of H2A.Z in wild type and mutant chromatin. a Western blot and quantification for H2A.Z and H3 levels in total extracts from wild type, mbd9−3, arp6−1, and arp6−1 mbd9−3. The upper band in the H2A.Z blot likely represents a modified form of H2A.Z. Only the lower band was used in the quantification. Average levels of three independent westerns ± standard deviations are shown. Paired two-tailed Student’s t-test was used to determine significance between wild type and mutants (black asterisks) or between mutants (red asterisks); ns p value >0.05, * p value ≤0.05, ** p value ≤0.01, *** p value ≤ 0.001. b Distribution of normalized H2A.Z ChIP-seq signal as read count kbp−1 million−1 mapped reads (RPKM) from merged replicates over H2A.Z common peaks in wild type and protein-coding genes. c Overlap of H2A.Z-depleted genes for mbd9−3, arp6−1, and arp6−1 mbd9−3 mutants. d-g Distribution of normalized H2A.Z ChIP-seq signal (RPKM) from merged replicates over H2A.Z-enriched genes for indicated mutants. h Intersection of H2A.Z-depleted genes from d to g with nine classes of genes characterized by their distribution of H2A.Z in wild type at TSS and gene body. Labels H—high, M—medium, L—low refer to levels of H2A.Z at TSS (first letter) and gene body (second letter). Source data for Figs. 2a and 2c are provided as Source Data file.

having a peak within 1 kb upstream of the TSS (Supplementary Methods). The distribution of all MBD9 peaks showed a preference for promoter and intergenic regions (68%) compared to a control set of peaks randomly selected from the genome based on length and number (Fig. 4a). Although MBD9 contains a methyl-CpG-binding domain, it is predicted not to bind methylated DNA since it lacks key conserved residues required for mCpG binding61. Consistently, the level of methylation over MBD9 peaks in wild-type plants was lower in all cytosine contexts than over a control set of peaks (Fig. 4b).

We plotted MBD9 peaks alongside open chromatin regions, as determined by ATAC-seq, given MBD9’s clear preference for promoter regions. This revealed a significant overlap between MBD9 peaks and open chromatin regions (Fig. 4c). At these regions, the open chromatin was largely unaffected in mbd9−3 (Fig. 4c). Metaplots of MBD9 ChIP-seq signal over
protein-coding genes revealed two major MBD9 peaks, one over nucleosome-depleted regions just upstream of the TSS and a smaller peak just 3’ of the transcription end site (TES) (Fig. 4d). At the 5’ end, the MBD9 peaks overlap with the ATAC-seq peaks and are just upstream of the H2A.Z peak (Fig. 4d, e).

We further investigated MBD9 occupancy in relation to H2A.Z distribution. When plotting H2A.Z over MBD9-occupied genes, we detected a loss of H2A.Z at the 5’-ends of genes in the mbd9-3 mutant (Fig. 4f). To investigate whether the loss of H2A.Z occurs over a specific subset of genes, we ordered MBD9-occupied genes based on H2A.Z occupancy in the wild type using k-means.
clustering (n = 5) to reflect typical patterns of H2A.Z occupancy in Arabidopsis17 (Fig. 4g). This analysis revealed that MBD9 occupies genes with various H2A.Z localization patterns, including genes with low H2A.Z throughout (cluster 2, Fig. 4g) and genes containing H2A.Z over gene bodies (clusters 1 and 5, Fig. 4g). However, the locations that were most sensitive to the loss of H2A.Z in the mbd9-3 mutants were those that contain H2A.Z predominately over their 5′-ends (clusters 3 and 4, Fig. 4g, ratio plots). These regions (clusters 3 and 4) contain low levels of H3K27me3. This is in contrast to the arp6-1 and the arp6-1 mbd9-3 mutants, where H2A.Z loss occurred throughout all MBD9-occupied genes, with the greatest loss observed in the double mutant (Fig. 4g).

MBD9-enriched genes contain specialized chromatin features. To determine if specific chromatin features mark the regions that are enriched with MBD9, we analyzed ATAC-seq data from wild-type plants and observed that MBD9-occupied genes tended to have more open chromatin than an equivalent set of genes with similar expression levels, or all protein-coding genes (Fig. 5a, b). We also investigated preferences for histone modifications using previously published and generated data62–64 from equivalent tissues and stages of development (Supplementary Note). MBD9-occupied genes contained higher levels of H3K4me3 when compared to all protein-coding genes and the H3K4me3 signal was broader when compared to a control set of genes (Fig. 5c). A similar relative enrichment was also true for histone acetylation levels (Fig. 5c), whereas MBD9-occupied genes were depleted of H3K27me3 when compared to gene averages (Fig. 5c). This general trend was also true for regions depleted of H2A.Z in mbd9-3, as depicted in the heatmap ordered by expression level at all protein-coding genes (Supplementary Fig. 8). Regions that lost H2A.Z most in mbd9-3 tended to occur predominantly at the 5′-ends of genes. These regions also contained high levels of activating histone modifications (H3K4me3, H3Ac) and low levels of H3K27me3, and coincided with MBD9-occupied peaks and open chromatin. In agreement, a related study also identified an enrichment of histone acetylation marks at MBD9-dependent H2A.Z sites54.

Overall, these results suggest that MBD9 preferentially localizes to the open chromatin regions of actively transcribing genes, which contain high levels of H3K4me3 and histone acetylation and are different from genes controlled by the Polycomb Group Complex.

Discussion
H2A.Z has been implicated in a wide variety of functions, including transcription, meiosis, and DNA damage52. How it is specifically targeted to the locations where these events occur is poorly understood. In this study we have characterized the interacting partners of Arabidopsis ARP6, a homolog of Arp6 found in the yeast SWR1-C involved in H2A.Z deposition. We identified nine Arabidopsis proteins that are homologous to subunits common to the yeast (SWR1) and mammalian (SRCAP) complexes, ARP6, PIE1, RIN1, RIN2, ARP4, SWC4, SWC2, YAF9A, and SEF. We further identified several additional ARP6-interacting partners, TRA1A, TRA1B, MBD9, and four members of the Alfin1-like family (AL4, AL5, AL6, and AL7). These Alfin1-like-containing proteins recognize H3K4me3 histones57, suggesting that they play a role in recruiting the SWR1 complex to H3K4me3 regions. The ARP6-interacting partners identified in our study are in agreement with a related study involving ARP6-TAP purification followed by mass spectrometry54. However, there were a few differences. Specifically, we did not enrich for H2A.Z or ACT1 in our mass spec data, perhaps reflecting different methods of isolation, while we did identify TRA1A and AL4 as ARP6-interacting partners that were not reported by Sijacic et al.54.

We focused on characterizing the interaction between ARP6 and MBD9. We identified interacting partners for MBD9 and showed that it interacted with most components of the SWR1 complex, including PIE1, RIN1, ARP4, RIN2, SWC2, ARP6, SWC4, YAF9A, and SEF. MBD9 also interacted with TRA1A and TRA1B, further implicating histone acetylation in H2A.Z deposition. Additionally, we detected high enrichment of the ISWI chromatin remodeling complex in our MBD9 IP, which was not seen in the ARP6 IP–mass spec. The ISWI remodeling complex interacts with other chromatin remodelers65, MADS proteins66, and DTT-containing proteins67. MBD9 was among the reported interacting partners of CHR17, independently validating our results65. However, only two other SWR1 complex components (ARP4 and RIN1) were detected in the same CHR17 IP–mass spec study. Since we did not detect preferential enrichment between CHR11/17 and ARP6 in our data, this suggests that MBD9 interacts with the two complexes separately. Our analysis of the genetic interactions between mbd9-3 and arp6-1 suggests that, despite the more severe developmental phenotype of the double mutant, ARP6 is epistatic to MBD9 regarding flowering time. These two observations are in agreement since flowering time is controlled by a particular set of genes, whereas H2A.Z deposition is globally affected in our mutants. The stronger developmental phenotype of the double mutant could have two plausible explanations: (1) multiple pathways are involved or (2) since the double mutant closely resembles pie1-2 (a mutation affecting the catalytic subunit of the SWR1 complex), the phenotype results from MBD9 and ARP6 being part of the SWR1 complex, where the complex may be partly functional without one of the components but not without both. Future work will discriminate between these possibilities. Our genomic observations, H2A.Z ChIP-seq data, and transcriptome analysis revealed that MBD9 and ARP6 share many common targets, but the mbd9-3 mutant shows a weaker phenotype than arp6-1. We observed that MBD9 is required for the deposition of H2A.Z at a subset of arp6-1 dependent loci, similar to what was reported by Sijacic et al.54. We also identified a subset of genes that require both ARP6 and MBD9 for both their expression and for H2A.Z deposition, suggesting the two proteins act redundantly in some cases. Additionally, we determined the genome-wide occupancy of MBD9 and showed that it...
Fig. 4 Characterization of MBD9 chromatin binding. 

a) Genomic distribution of MBD9-enriched peaks vs wild type (mac2 peak caller, q value <0.01).

b) Average distribution of DNA methylation levels in CG, CHG, and CHH contexts in wild type over MBD9-occupied peaks and MBD9 control peaks.

c) Heatmap of normalized MBD9 signal (log2, RPKM) over wild-type control and normalized ATAC-seq signal (RPKM) from merged replicates in wild type and mbd9-3 mutant over MBD9-occupied peaks.

d) Upper: Distribution of normalized signal (RPKM) for MBD9-3XFlag ChIP-seq, wild-type Flag ChIP-seq control, H2A.Z, and H3 in wild type over protein-coding genes. Lower: Distribution of normalized signal (RPKM) for ATAC-seq in wild type over protein-coding genes.

e) Snapshot representing normalized read counts (RPKM) for MBD9-3XFlag ChIP-seq, wild-type Flag ChIP-seq control, ATAC-seq, H2A.Z, and H3. Signal for ATAC-seq, H2A.Z, and H3 is a merge of two biological replicates.

f) Distribution of normalized H2A.Z ChIP-seq signal (RPKM) from merged replicates in wild type, mbd9-3, arp6-1, and arp6-1 mbd9-3 over MBD9-occupied genes.

g) Heatmap ordered based on k-means clustering of H2A.Z ChIP-seq signal in wild type (cluster n = 5) at MBD9-occupied genes. Shown are corresponding H2A.Z ratio plots for wild type over mbd9-3, arp6-1, and arp6-1 mbd9-3 mutants and MBD9-3X Flag ChIP-seq over wild-type flag control in log2 RPKM, ATAC-seq in wild type in RPKM, and H3K27me3 levels in wild type in RPKM. Source data are provided as Source Data file.
preferentially localized to the open chromatin regions of promoters and the transcription termination sites of active genes that have high levels of activating histone modifications (H3K4me3 and histone acetylation) and did not localize to genes repressed by the Polycomb Group Complex. These data suggest that the MBD9-containing SWR1 complex promotes H2A.Z deposition at the 5′ ends of highly expressed genes.

Regarding the interaction of MBD9 with the SWR1 complex, three possibilities exist based on our genetic and genomic observations: (1) MBD9 is always a member of the SWR1 complex but not always required, (2) MBD9 is part of a subset of all SWR1 complexes in Arabidopsis, and (3) MBD9 only transiently interacts with the SWR1 complex. Based on size exclusion chromatography of ARP6-containing SWR1 complex in the mbd9 mutant, Sijacic et al. concluded that MBD9 is not a core subunit of the Arabidopsis SWR1 complex. However, as noted by the authors, this does not exclude the possibility that MBD9 is part of a small subset of all SWR1 complexes in Arabidopsis. Given that we detected most components of the SWR1 complex in our MBD9 IP–mass spec, our genetic and genomic data best fit the model that MBD9 is part of a subset of the SWR1-containing complexes.

Surprisingly, although we detected many genes that lost H2A.Z in the mbd9-3 mutant, this loss did not result in major transcriptional changes. This may be because the loss of H2A.Z in the

Fig. 5 Chromatin features at MBD9-occupied genes. a Boxplots of average expression levels log2 (FPKM + 1) from RNA-seq in wild type for MBD9-occupied genes, MBD9 control genes, and protein-coding genes. Center line indicates the median, upper and lower bounds represent the 75th and the 25th percentile, respectively, whiskers indicate the minimum and the maximum, and outliers are represented as circles. b Distribution of normalized ATAC-seq levels (RPKM) over MBD9-occupied genes, MBD9 control genes, and protein-coding genes. c Distribution of normalized levels (RPKM) for H3K4me3, H3Ac, H3K27me3, and H3 ChIP-seq in MBD9-occupied genes, MBD9 control genes, and protein-coding genes.
short-day flowering time experiment, plants were grown at 22 °C (16 h dark, 8 h light). The following Arabidopsis mutant lines were used: mbd9-3 (SALK_039302), arpe-1 (SAIL_599_G03), and pic1-2 (SALK_003766C).

Quantitative real time PCR. Total RNA was prepared from whole seedlings (14 DAG) using the RNA Prep Pure Plant Kit (Tiangen Biotech, Beijing, China). Two micrograms of RNA was used for the preparation of cDNA using PrimeScript Reagent Kit with gDNA eraser (Takara, Japan). Signal detection, quantification, and normalization were done using Quant Studio 6 proprietary Software (Life Technologies, USA).

Co-immunoprecipitation. An anti-MBD9 polyclonal antibody was raised in rabbits using the 1941–2176 aa MBD9 peptide as an antigen. The antibody was coupled to magnetic beads using the Dynabeads antibody coupling kit (Invitrogen, USA) following the manufacturer’s recommendations; 7 µL of 3.12 mg mL⁻¹ were used per sample. Approximately 2 g of floral tissue was ground in liquid nitrogen, 10 ml IP buffer (50 mM Tris pH 7.6, 150 mM NaCl, 10 mM MgCl₂, 1% glycerol, 0.1% NP-40, 0.5 mM DTT, 1 µg mL⁻¹ pepstatin, 1 mM PMSF, 1 protease inhibitor cocktail (Roche, 14696200)) was added, the mix was incubated on ice for 20 min, and spun at 4000g for 10 min at 4 °C. The supernatant was filtered through a double layer of Miracloth. Anti-MBD9 antibody coupled to magnetic beads was added and incubated for 2 h at 4 °C with gentle rotation. Beads were washed six times with IP buffer, 5 min each wash at 4 °C with rotation, and then boiled. Western blotting and detection were performed with anti-Myc 9E10 mouse monoclonal antibody (Thermo Fisher Scientific, MA1-980) with 1:1000 dilution.

RNA-seq and analysis. Total RNA was extracted from 13 DAG shoots grown on 1% MS supplemented with 1% sucrose under long-day conditions. Four replicates, grown on separate plates, were collected for each genotype. RNA was extracted using the Direct-zol RNA Miniprep kit (Zymo). For RNA-seq, 1 µg of total RNA was used to prepare libraries using the TruSeq Stranded mRNA-Seq kit (Illumina). Reads were aligned to TAIR10 using TopHat by allowing up to two mismatches and mapping only to one location. FPKM values and differential gene expression were analyzed using cufflinks with default settings. GO term enrichment was determined using AgriGO.

Epitope-tagged transgenic ARP6 and MBDB9 lines. Full-length genomic DNA fragments containing ~1 kb (MBD9) or ~1.5 kb (ARP6) of promoter sequence, together with genomic gene sequences up to the annotated/major stop codon of MBDB9 and ARP6 were amplified using PCR (primers are listed in Supplementary Table 1). The PCR product was cloned into the pENTR/D vector (Invitrogen) and delivered into a modified pEG containing 3X FLAG and 9X MYC tags using the LR reaction (Invitrogen). The pEG destination vectors were transformed into Agrobacterium strain AGL01, followed by transformation using the floral dip method.

Affinity purification and mass spectrometry. Approximately 10 g of flowers from transgenic lines expressing T3 ARP6-3xFLAG or T4 MBD9-3xFLAG and 10 g of Col-0 plants as negative controls were ground on a fine powder using a RETCH homogenizer (3 min 30 Hz min⁻¹) and suspended in 30 mL of IP buffer. Tissue was further dounce homogenized until lump-free, then centrifuged for 20 min at 4000g and 4 °C. The lysate was filtered through two layers of Miracloth. Supernatant was incubated with 200 µL anti-FLAG M2 magnetic beads (MB823, Sigma), at 4 °C for 2 h. The bead-bound complex was washed once with 10 mL IP buffer, then four times (5 min rotating at 4 °C with 1.5 mL IP buffer), followed by a final wash with IP buffer without NP-40. The FLAG-IP was eluted twice with 300 µL 250 µg mL⁻¹ 3X FLAG peptide (Sigma, F4799) in TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl), mixing for 15 min at 4 °C. The eluted protein complexes were precipitated with trichloroacetic acid and subjected to mass spectrometric analyses.

The in case of ARP6-9xMyc and MBD9-9xMyc, monoclonal 9E10 coupled to magnetic beads was used (88842, Pierce). The bead-bound complexes were washed six times with 1 mL of IP buffer. For each wash, the beads were rotated at 4 °C for 5 min. For ARP6-9xMyc, proteins were released from the Streptavidin beads by 3C cleavage overnight at 5 °C. 3C-GST was removed by incubation with GST couple magnetic beads (Pierce, USA). For MBD9-9xMyc, proteins were eluted twice with 100 µL 8 M urea in 50 mM Tris pH 8.5, mixing for 15 min at 37 °C. The supernatant was TCA precipitated.

Genome-wide ChiP sequencing and library generation. For histone modification ChiPs, about 0.5 g of shoots were collected from 13 DAG seedlings grown on 1% MS plates supplemented with 1% sucrose under long-day conditions. Samples were crosslinked in vitro, sheared using Bioruptor Plus (Diagenode). Libraries were generated with NuGEN Ovation Ultra Low System V2 kit, according to the manufacturer’s instructions, and were sequenced on an Illumina HiSeq 4000 instrument. H2A.Z ChiP assays were performed using polyclonal antibodies specific to Arabidopsis H2A.Z HTA11 and HTA9. The antibodies were incubated in rabbits and further purified using the peptides described in Deal et al. For each ChiP we used 4 µL of 3.34 mg mL⁻¹ Abcam1791 (Abcam) antibody was used for
H3 ChiP assays (10 µl of 0.5 mg mL⁻¹) and active motif 39140 (Active Motif) was used for pan-acetyl H3 ChiP assays (5 µl of 1 mg mL⁻¹). For ChIP-seq, 13 DAG T4 MBdX FLAG and Col-0 seedlings grown on 1% MS were fixed in vivo using vacuum in 1% formaldehyde in 1× PBS for 20 min. Crosslinking was stopped with 0.1 M glycine in 1× PBS and the seedlings were kept on ice for 5 min. Tissue was patted dry and frozen at −80 °C. Tissue was homogenized to a fine powder using a RETCH homogenizer (2 min @ 30 Hz min⁻¹) and resuspended with Nuclear Isolation Buffer (50 mM Hepes, 1 M Sucrose, 5 mM KCl, 5 mM MgCl₂, 0.6% Triton X-100, 0.4 mM PMsF, 5 mM Benzanide, 1× protease inhibitor cocktail tablet (Roche, 14692600), lysate was filtered through one layer of Miracloth and centrifuged for 20 min at 2880 g and 4 °C. The pellet was resuspended with 1 mL Extraction buffer 2 (0.25 M sucrose, 10 mM Tris-HCl pH 8, 10 mM MgCl₂, 1% Triton X-100, 5 mM BME, 0.1 mM PMsF, 5 mM Benzanide, 1× protease inhibitor cocktail tablet (Roche, 14692600)), followed by centrifugation for 10 min at 12,000g and 4 °C. The pellet was then resuspended in 500 µL Extraction buffer 3 (1.7 M sucrose, 10 mM Tris-HCl pH 8, 2 mM MgCl₂, 0.15% Triton X-100, 5 mM BME, 0.1 mM PMsF, 5 mM Benzanide, 1× protease inhibitor cocktail tablet (Roche, 14692600)). To lysed nuclei, 1.7 mL of ChiP Dilution Buffer (1.1% Triton X-100, 1.2 mM EDTA) was added (1× Sigma-Aldrich) and incubation overnight with rotation at 4 °C. 50 µL Dynabeads (equal mix of Protein A and G; Invitrogen 1004D/10020D) was added to chromatin and incubated for an additional 2 h. The magnetic beads were washed with 1 mL of the following buffers for 5 min rotating at 4 °C: 2× with low salt buffer (150 mM NaCl, 0.2% SDS, 0.5% EDTA), 1× high salt buffer (500 mM NaCl, 0.5% Triton X-100, 2 mM EDTA), 2× with TE buffer (10 mM Tris pH 8, 1× LiCl Wash Buffer (250 mM LiCl, 1% Igepal, 1% sodium deoxycholate, 1× EDTA, 1× TE buffer), and 1× with TE buffer (10 mM Tris pH 8, 1× EDTA). The immunocomplex was eluted from the beads twice with 250 µL elution buffer (1% SDS, 10 mM EDTA, 0.1 M NaHCO₃), incubating for 20 min with shaking at 65 °C. Four hundred microliters of elution buffer was added to the input samples. A total of 20 µL 5 M NaCl was added to each tube; the crosslink was reversed by incubation at 65 °C overnight. Residual protein was degraded with 20 µg Prot K in 10 mM EDTA and 40 mM Tris pH 8 at 45 °C for 1 h followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The pellet was washed with 70% ETOH and resuspended in 50 µL molecular grade water. Libraries were prepared using the NextGen Ovation Ultra Low System V2 kit, according to the manufacturer’s instructions, and were sequenced on an Illumina HiSeq 4000 instrument.

**ChiP-seq analysis.** ChiP-seq fastq reads were aligned to the TAIR10 reference genome with Bowtie using default settings and allowing only uniquely mapping reads. Duplicated reads were removed using SAMtools. IGB genome browser was used to visualize the data and to generate snapshots. Normalized read coverage and DNA methylation are mutually antagonistic chromatin marks. *Nature* 453, 358–362 (2008).

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Author contributions
M.E.P. performed RNA-seq and ChIP-seq and generated sequencing libraries. Y.W. and L.X. developed ARP6-tagged lines and performed IP, co-IP, western blots, RT-PCRs, and genetic analyses. M.E.P. and B.N. developed MBD9-tagged lines. M.E.P., Z.Z. and W.L. performed data bioinformatics analysis. Z.Z. and W.L. performed ATAC-seq. S.F. performed high-throughput sequencing. S.R. and J.A.W. performed mass spectrometry. M.E.P., I.A., and S.E.J. wrote the manuscript. M.E.P., Z.W., I.A. and S.E.J. coordinated the research.

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