A Companion Cell–Dominant and Developmentally Regulated H3K4 Demethylase Controls Flowering Time in Arabidopsis via the Repression of FLC Expression

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Abstract

Flowering time relies on the integration of intrinsic developmental cues and environmental signals. FLC and its downstream target FT are key players in the floral transition in Arabidopsis. Here, we characterized the expression pattern and function of JMJ18, a novel JmJC domain-containing histone H3K4 demethylase gene in Arabidopsis. JMJ18 was dominantly expressed in companion cells; its temporal expression pattern was negatively and positively correlated with that of FLC and FT, respectively, during vegetative development. Mutations in JMJ18 resulted in a weak late-flowering phenotype, while JMJ18 overexpressors exhibited an obvious early-flowering phenotype. JMJ18 displayed demethylase activity toward H3K4me3 and H3K4me2, and bound FLC chromatin directly. The levels of H3K4me3 and H3K4me2 in chromatin of FLC clade genes and the expression of FLC clade genes were reduced, whereas FT expression was induced and the protein expression of FT increased in JMJ18 overexpressor lines. The early-flowering phenotype caused by the overexpression of JMJ18 was mainly dependent on the functional FT. Our findings suggest that the companion cell–dominant and developmentally regulated JMJ18 binds directly to the FLC locus, reducing the level of H3K4 methylation in FLC chromatin and repressing the expression of FLC, thereby promoting the expression of FT in companion cells to stimulate flowering.

Introduction

DNA is packaged as chromatin in eukaryotic cells. Nucleosomes, which consist of an octamer of four histones wrapped around 146 base pairs of DNA, are the fundamental unit of chromatin [1]. The flexible N-terminal tails of histones, which protrude from the nucleosome core particle, are subject to several types of covalent modification, including acetylation, methylation, phosphorylation, and ubiquitylation [2]. Each of these modifications is reversible and is required for the dynamic regulation of gene expression [3].

In vivo, the lysine residues in histones display three distinct methylation states: monomethylated (me1), dimethylated (me2), and tri-methylated (me3). These differences in methylation are important for the recognition of chromatin by chromatin modulators and for the recruitment of other modulators and regulators [4,5,6,7,8]. In addition, lysine methylation at different sites within the histone protein plays distinct roles in gene activation and repression [2,9]. For example, the methylation of H3K4 and H3K36 is correlated with gene activation, while transcriptional repression has been demonstrated in genomic regions with increased levels of H3K9 and H3K27 methylation [3,10,11,12]. Various developmental processes are regulated by histone methylation in animals and plants. For example, H3K27 methylation mediated by the PRC2 complex is required for embryonic development and stem cell identity in mammals and controls most steps in the development of Arabidopsis [9,13,14,15].

The methylation state of histone proteins is determined by the balance between methylation and demethylation, which is mediated by histone methyltransferases and demethylases, respectively [16,17,18]. However, the reversibility of histone methylation in vivo is the latest to be discovered compared to other covalent forms of histone modification. The amine oxidase LSD1 was the first histone demethylase found to demethylate H3K4me2 and H3K4me1 through an FAD-dependent oxidation reaction [18]. LSD1 demethylase family proteins are unable to remove methyl groups from tri-methylated lysines, suggesting the presence of other histone demethylases in eukaryotic cells [18]. More recently, a family of JmJC domain-containing proteins was characterized as histone demethylases which were able to reduce any one of the three histone lysine methylation states at several specific sites in yeast and animals [17,19,20,21]. These histone demethylases are involved in many biological processes in animals, including spermatogenesis, HOX gene regulation, and germ cell development [19,22,23].
Author Summary

Flowering is an important developmental transition during plant life cycle and the key process for production of the next generation. Flowering time is controlled by both intrinsic developmental and environmental signals. FLC and its target FT work as repressor and activator, respectively, to regulate flowering time in Arabidopsis; thus the regulation of FLC and FT expression is the key for the control of floral transition. Epigenetic modifications are critical for transcription regulation. Here, we show that a novel JmJC domain-containing histone H3K4 demethylase, JMJ18, is a key regulator for the expression of FLC and FT in companion cells and flowering time. JMJ18 is dominantly expressed in vascular tissue; its temporal expression pattern was developmentally regulated, and negatively and positively correlated with FLC and FT, respectively. JMJ18 mutation leads to weak late-flowering, while JMJ18 overexpressor exhibited an obvious early-flowering phenotype. JMJ18 binds to chromatin of FLC, represses its expression, and releases expression of FT in companion cells. Our results suggest that JMJ18 is a developmentally regulated companion cell–dominantly expressed signal to control flowering time by binding to FLC—reducing level of H3K4 methylation in FLC and repressing expression of FLC thereby promoting expression of FT in companion cells during vegetative development in Arabidopsis.

FLD and LDLs are the homologs of human LSD1 in Arabidopsis. They promote the floral transition by constitutively inhibiting the expression of FLC [24,25,26]. The Arabidopsis genome contains 21 JmJC family proteins [27]; however, only five of them have been characterized, and they have been found to be involved in RNA silencing, DNA methylation, flowering time control, circadian clock regulation, BR signaling and shoot regeneration in vitro [28,29,30,31,32,33,34,35,36].

Flowering at the appropriate time is the most important factor in achieving reproductive success. To produce the next generation, plants rely on intricate signaling pathways involving a variety of intrinsic factors, including developmental stage and age, and environmental cues such as photoperiod, temperature, and light quantity [37,38,39]. Thus, distinct environmental conditions modulate endogenous gene expression to ensure flowering at the correct time [12,40].

FLOWERING LOCUS C (FLC) encodes a MADS-box transcription factor and floral repressor that regulates flowering time in a dosage-dependent manner by integrating the vernalization, autonomous, PAF1 complex, and H2B ubiquitination pathways [11,41,42,43,44,45]. FLC and the functional locus FRIGIDA (FRI) act together to produce winter-annual Arabidopsis accessions, which must be exposed to cold temperature for several weeks to repress FLC expression and promote flowering in the following spring [46,47]. As endogenous factors, autonomous pathway genes consecutively repress FLC expression [48,49]. In addition, the FLC antisense transcript affects the expression of sense transcript, thereby influencing flowering time in Arabidopsis [30,51,52].

FLC expression is predominant in the shoot apical meristem (SAM); however, it is also expressed in vascular tissue in young leaves and the root tip [44,53,54]. FLC expressed in the SAM and leaf vascular tissue contributes to the control of flowering time in Arabidopsis [55]. During vernalization, VIN3 binds FLC chromatin, thereby repressing its expression in the SAM [53]. The components of the autonomous pathway repress, while those of the PAF1 complex activate, FLC expression in the SAM [11,37,43]. Thus, although the regulation of FLC expression in the SAM has been extensively studied, little is known about how FLC expression is regulated in leaf vascular tissue.

Histone modification plays crucial roles in the regulation of FLC expression in Arabidopsis. In FLC chromatin, H3K4 hyper-trimethylation and acetylation are associated with the activation of gene expression [43,56,57,59]. The methylation of H3K27 and H3K9 in FLC chromatin leads to the repression of FLC expression and is required for maintenance of the repression of FLC expression in plants growing in the following spring after vernalization [12,53,59]. In addition, H2B monoubiquitination decreases the level of H3K4me3 and H3K36me2, leading to the repression of FLC transcription and early flowering [11,45].

FLOWERING LOCUS T (FT), which is a component of the photoperiod pathway, coordinates signals from the vernalization, autonomous, PAF1 complex, and photoperiod pathways to promote flowering in response to increase in day length [60,61,62,63]. FT expression is restricted to leaf phloem companion cells [62]. FT travels from leaves to the SAM, where it interacts with the bZIP transcription factor FD to stimulate floral meristem initiation [64,65,66,67,68]. As a mobile and systemic signal, FT integrates photoperiod- and FLC-dependent pathways to control flowering time by regulating the expression of floral identity genes. It was shown previously that the companion cells specifically expressed FLC represses FT expression in leaf companion cells and delays the expression of its cofactor FD in the SAM [55]. Thus, FLC systemically blocks the function of FT to repress flowering. However, the regulation of FLC expression in companion cells for the control of floral development has not been characterized.

As mentioned above, several JmJC domain-containing proteins have been reported to be involved in flowering time control [28,29,33,69]. Among them, JMJ14 is a member of JARID family with H3K4 demethylase activity, and is involved in flowering time control through the repression of floral integrators [28,29,33,69]. It is not yet clear whether those floral integrator genes are the direct targets of JMJ14 [28,29,69]. ELF6 acts as a repressor in photoperiod pathway [31], and its closest homolog REF6 acts as a FLC repressor in flowering time regulation [31]. Recent results suggest that REF6 is a H3K27 demethylase; thus, FLC is not likely to be the direct target of REF6 [70]. No JmJC domain-containing histone demethylases that target FLC locus have yet been found.

In this paper, we describe the expression pattern and function of JMJ18, a novel JmJC domain-containing histone H3K4 demethylase in Arabidopsis. JMJ18 was predominantly expressed in phloem companion cells, and its level of expression increased during vegetative development. JMJ18 was found to promote the floral transition in Arabidopsis by binding FLC chromatin and demethylating H3K4 methylation, leading to the repression of FLC and enhanced expression of the downstream flowering activator FT in companion cells.

Results

JMJ18 demethylates histone H3K4me3 and H3K4me2 in vitro

JMJ18 (At1g30810) belongs to the evolutionarily conserved JARID1 family. Previous studies have shown that JARID1 family proteins demethylate H3K4 methylations in yeast, animal and Arabidopsis [28,29,33,69,71,72,73].

To determine whether JMJ18 exhibits histone demethylase activity, we expressed recombinant His-tagged JMJ18 and purified
the recombinant protein from insect cells (Figure S1A and S1B). A MALDI-TOF mass spectrometric analysis-based demethylation assay was used to detect the histone demethylation activity of JMJ18. Purified His-JMJ18 was incubated with a variety of histone peptides representing the tri- and di-methylated states of the four lysine residues in histone H3. As shown in Figure 1A, JMJ18 converted H3K4me3 to H3K4me2, but did not alter the H3K4me2 methylation state or the methylation status of any other lysine residue in the peptides (summarized in Figure S1C).

To investigate the demethylase activity of JMJ18 further, we purified two truncated versions of JMJ18, JMJ18_D141–144 and JMJ18_D141–158, in which the partial linker sequence between JmjN and JmjC was deleted (Figure 1B), and examined the histone demethylase activity of each using the assay described above. Similar to full-length JMJ18, the truncated proteins were H3K4-specific demethylases (Figure 1B and Figure S1C). Interestingly, both truncated forms of JMJ18 exhibited H3K4me3 and H3K4me2 demethylase activity, and they were able to convert H3K4me3 to H3K4me2 and H3K4me1 in the presence of H3K4me3 peptide (Figure 1B and Figure S1C). This suggests that the linker between JmjN and JmjC blocks the enzyme’s demethylase activity toward H3K4me2 in vitro. However, they were unable to demethylate H3K4me2 to H3K4me1 if H3K4me2 peptide was used as the substrate (Figure 1B and Figure S1C).

We further observed that the absence of α-ketoglutarate (α-KG) or presence of EDTA completely abolished the enzyme’s demethylase activity (Figure 1C), while a lack of Fe(II) strongly inhibited the enzyme’s activity (Figure 1C). Taken together, our results suggest that JMJ18 functions as an H3K4-specific demethylase in vitro, and that its activity is dependent on α-KG and Fe(II).

Characterization of jmj18 mutants

To assess the biological function of JMJ18, we obtained three T-DNA insertion lines from the ABRC [74]: jmj18-1 (SALK_073442), jmj18-2 (GABI_649D05), and jmj18-3 (SAIC_861_F02) (Figure 2A). Reverse transcription (RT)-PCR analysis revealed a lack of full-length JMJ18 mRNA in jmj18-1 and jmj18-2; however, a partial transcript was detected in both lines (Figure 2B). In the third line, the T-DNA was inserted into exon 4 (Figure 2A), and full-length mRNA was detected at a reduced level compared to that in wild-type plants, and the transcript was confirmed by sequencing, suggesting the existence of a knock-down allele (Figure 2B).

All three alleles exhibited a weak but reproducible late-flowering phenotype under long- (LD) and short-day (SD) conditions (Figure 2C, 2D and Table 1). This late flowering phenotype in jmj18-1 was complemented by JMJ18:JMJ18-GFP transformation (Figure S2). Knock-down lines for JMJ18 expression by using both double strand RNA interference (RNAi) and artificial micro-RNA (amiR) were generated, and these lines also exhibit weak late-flowering phenotype under LD conditions (Figure S3 and Table S1). To address the molecular mechanism underlying this phenotype, we examined the expression of three important floral regulators, FLC, CONSTANS (CO), and FT, by quantitative real-time PCR (qRT-PCR). No obvious change in expression compared to wild-type was detected at various vegetative developmental stages, but statistical significant for the expression of FLC and FT between wild-type and jmj18 mutants if any at later vegetative developmental stages (Figure 2E–2G and Figure S4). This suggests that the changes in gene expression are mild, which is consistent with the mild late-flowering phenotype in the jmj18 mutants and knock-down lines.
Figure 2. Characterization of three jmj18 mutants. (A) Structures of JMJ18 and its gene product. Arrows indicate the T-DNA insertion sites in the mutants. Introns are represented as lines, exons as filled black boxes, and untranslated regions as filled gray boxes. The primers used for transcript detection (P1–P3) are indicated under the schematic gene structure. (B) Transcript analysis of the jmj18 mutants by RT-PCR. Pairs of P1/P3 and P1/P2 were used for full-length and partial transcripts detection, respectively. The 11-day-old plants of each genotype were collected at dusk for RNA isolation. The jmj18 mutants exhibited a weak late-flowering phenotype under both LD (C) and SD (D) conditions. Bar = 2 cm. Relative expression levels of FLC (E), CO (F), and FT (G) in the jmj18 mutants. The expression level was normalized to that of ACTIN. RNA was isolated from eleven-day-old seedlings. The values are the mean and standard deviation from three independent experiments. There was no significant difference between wild-type and jmj18 mutants for the expression levels of FLC (E), CO (F) or FT (G) tested by Student’s t test (P<0.05).

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Table 1. jmj18 mutants exhibit late-flowering phenotype.

| Genotype | Days to visible buds | Days to first flower open | Rosette leaf no. | Caulin leaf no. | n  |
|----------|----------------------|--------------------------|------------------|----------------|----|
| LD       |                      |                          |                  |                |    |
| WT       | 23.1±2.3             | 31.8±1.3                 | 11.7±0.9         | 3.0±0.4        | 45 |
| jmj18-1  | 25.9±1.7*            | 33.5±1.4*                | 13.6±1.1*        | 3.1±0.6        | 45 |
| jmj18-2  | 26.6±1.5*            | 33.8±1.2*                | 13.5±0.8*        | 2.8±0.6        | 45 |
| jmj18-3  | 26.3±2.2*            | 33.8±1.5*                | 13.3±1.1*        | 2.9±0.6        | 45 |
| SD       |                      |                          |                  |                |    |
| WT       | 70.0±2.6             | 79.5±2.8                 | 60.7±2.6         | 8.6±0.5        | 12 |
| jmj18-1  | 74.3±2.5*            | 83.5±2.4*                | 65.3±2.4*        | 9.0±0.8        | 12 |
| jmj18-2  | 73.4±2.1*            | 82.6±1.5*                | 65.8±2.1*        | 9.6±0.9        | 12 |
| jmj18-3  | 73.8±2.3*            | 82.8±3.0*                | 66.2±2.4*        | 9.8±1.1        | 12 |

LD, long-day condition (16 h light, 22°C/8 h dark, 18°C, cycle). SD, short-day condition (8 h light, 22°C/16 h dark, 18°C, cycle). The values are the mean ± standard deviation. n indicates the plants number scored for phenotype analysis. Asterisks indicate the significant differences in the statistic analysis between WT and mutants using Student’s t test (P<0.05).

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Spatial expression pattern of \textit{JMJ18}

To gain a deeper understanding of the function of \textit{JMJ18} in \textit{Arabidopsis}, we examined its expression pattern using wild-type \textit{Arabidopsis} plants carrying a \textit{JMJ18} promoter-fused GUS reporter construct. The whole integenic region between \textit{JMJ18} and its upstream gene was first selected to be the promoter of \textit{JMJ18} (Figure S5A). GUS expression was detected in vascular tissue collected from the cotyledons, young leaves, and roots of seedlings (Figure 3A and 3B). Similarly, GUS was detected in the vascular tissue of adult leaves and flowers (Figure 3C and 3D). Similar expression pattern was obtained if the length of promoter was increased from 1.4 to 1.7 kb (Figure S5A–S5D).

Next, \textit{JMJ18} expression was examined in detail by microscopy. Longitudinal section analysis showed that \textit{JMJ18} expression was expressed in both the shoot and root phloem, as well as in protophloem, but not in the SAM in seedlings (Figure 3E and 3F). Further, \textit{JMJ18} expression was restricted to the cell files of protophloem in the root tip (Figure 3H and Figure S5E–S5J) and phloem companion cells in mature roots (Figure 3F and 3G). These results suggest that \textit{JMJ18} is predominantly expressed in companion cells.

The phloem cell-dominant expression of \textit{JMJ18} was further confirmed in transgenic plants expressing GFP-tagged \textit{JMJ18} driven by the control of the \textit{JMJ18} promoter (Figure 3I–3K). \textit{JMJ18-GFP} was expressed in the protophloem in root tip and phloem in other tissues (Figure 3I–3K and Figure S6A–S6C). We also found that \textit{JMJ18-GFP} was localized to the nucleus (Figure 3I and Figure S6D), which is consistent with its function as a histone demethylase.

To further verify the expression pattern of \textit{JMJ18}, the companion cell-specific \textit{SUC2} promoter [75,76] was used to drive \textit{JMJ18-GFP} expression in wild-type plants. The expression pattern of \textit{SUC2::JMJ18-GFP} was similar to that of \textit{JMJ18} under the control of the \textit{JMJ18} promoter (Figure 3K–3L). In addition, we generated \textit{JMJ18::JMJ18-RFP} and \textit{SUC2::JMJ18-GFP} double-transgenic plants and found that \textit{JMJ18-RFP} and \textit{JMJ18-GFP} were colocalized in the nuclei of companion cells (Figure 3M–3P). It was also observed that \textit{JM18-GUS} was also detected in pollen if the anther was stained longer time (Figure S5K–S5N). These results demonstrate that \textit{JM18} is expressed predominantly in phloem companion cells, similar to the floral integrator \textit{FT} in vegetative developmental stage [62].

The temporal expression pattern of \textit{JM18} is positively correlated with that of \textit{FT}

To determine the developmental expression profiles of \textit{JM18}, wild-type seedlings grown under LD conditions were harvested at dusk on days 6, 9, 12, and 15 for RNA isolation. The plants did not form floral meristems; day 15 was the last day before floral meristem formation based on our microscopic observations. \textit{JM18} was expressed at a low level in young seedlings; however, its expression progressively increased during vegetative development from day 6 to day 15 (Figure 4A). Thus, the expression of \textit{JM18} is developmentally regulated.

\textit{FT} expression was also examined using the same batch of materials. Consistent with previous data [60,61], \textit{FT} expression was weak in young seedlings; however, it increased steadily as the plants matured (Figure 4A). There was a significant positive correlation between the expression patterns of \textit{JM18} and \textit{FT} during vegetative development, with a correlation coefficient of 0.906 (Figure 4A).

\textit{JM18} overexpression promotes flowering through \textit{FT} expression

To further investigate the function of \textit{JM18}, we overexpressed \textit{JM18} using its endogenous promoter (\textit{JM18} promoter), the companion cell-specific \textit{SUC2} promoter, and the constitutively expressed \textit{CaMYC33} promoter of which is expressed in and outside of the companion cells in wild-type plants. All three types of overexpression lines exhibited earlier flowering compared to wild-type under inductive LD and non-inductive SD conditions (Figure 4B, 4C and Table 2). Overall, the \textit{SUC2::JM18-GFP} transgenic lines flowered earlier than the \textit{JM18::JM18-GFP} transgenic plants; however, the \textit{35S::JM18-GFP} transgenic lines flowered before either of the other two (Figure 4B, 4C and Table 2). This suggests that the overexpression phenotype was \textit{JM18} dose-dependent.

To verify this prediction, total protein was extracted from seven-day-old transgenic plants harvested at dusk, and the abundance of \textit{JM18} was assessed by Western blotting using anti-\textit{JM18} antibodies generated against recombinant \textit{JM18} in rabbits. Consistent with the early-flowering phenotype described above, the expression of \textit{JM18} in the transgenic plants was as follows (in order from lowest to highest): \textit{JM18::JM18-GFP}, \textit{SUC2::JM18-GFP}, and \textit{35S::JM18-GFP} (Figure 4E and Figure S7). Taking our flowering time and \textit{JM18} expression data together, the overexpression of \textit{JM18} promotes flowering in a \textit{JM18} dose-dependent manner.

The strong positive correlation between the \textit{JM18} and \textit{FT} spatio-temporal expression patterns (Figure 3, Figure 4A, and Figure S5) suggests that \textit{JM18} regulates \textit{FT} expression in companion cells to control floral development. To investigate this possibility, total RNA was isolated from the same batch of materials used to examine \textit{JM18} expression. \textit{FT} expression was increased to varying degrees in all three types of overexpression lines, and the degree of increase in each line was correlated with the abundance of \textit{JM18} and flowering time (Figure 4D, 4E and Table 2). Similar results were obtained for the expression of \textit{TFL1}, a homolog of \textit{FT}, in \textit{JM18} overexpression lines (Figure S8).

It has been shown that \textit{FT} is one of the most important components of \textit{florigen}, and its abundance determines flowering behavior [66,67,68]. Thus, we next examined the abundance of \textit{FT} in \textit{JM18} overexpression lines by Western blotting using anti-\textit{FT} antibodies generated against recombinant \textit{FT} in rabbits. The \textit{FT} level detected in each line was consistent with the mRNA level and abundance of \textit{JM18} (Figure 4D and 4E). These results indicate that \textit{JM18} induces \textit{FT} and its homologs such as \textit{TFL1} transcription and enhances their accumulation to accelerate the vegetative-to-reproductive transition.

The promotion of flowering by \textit{JM18} mainly depends on \textit{FT} function

To test whether \textit{FT} function is genetically necessary for facilitation of the vegetative-to-reproductive transition by \textit{JM18}, \textit{ft-11} was crossed with the \textit{SUC2::JM18-GFP} line. The mutation in \textit{FT} dramatically suppressed the early-flowering phenotype of the \textit{SUC2::JM18-GFP} plants (Figure 5A–5C), suggesting that \textit{FT} is required for the function of \textit{JM18} in flowering control.

In the photoperiod pathway, \textit{CO} is responsible for \textit{FT} induction, and \textit{FT} expression is obviously delayed in a \textit{CO} mutant background even under inductive LD conditions [60,61]. \textit{FD} is an \textit{SAM}-specific transcription factor that interacts with \textit{FT} in the \text{SAM} [64]. Both \textit{FT} and \textit{FD} are required for floral meristem formation [64,65]. The mutation of \textit{CO} or \textit{FD} also abolished the \textit{SUC2::JM18-GFP} early-flowering phenotype, such as...
**Figure 3. JMJ18s expressed predominantly in companion cells.** (A–H) Histochemical analysis of JMJ18, JMJ18-GFP transgenic plants. Ninety-one percent (22 out of 24 independent lines) of the plants exhibited a similar expression pattern. (A) Nine-day-old seedling. (B) Seven-day-old root. (C) Rosette leaves from a 24-day-old plant. (D) Flower. Bar = 1 mm in (A), (C), and (D), and 0.1 mm in (B). Longitudinal sections of the shoot apex (E) and root (F) from twelve-day-old plants. Bar = 50 μm. GUS staining of mature roots (G) and root tips (H) from seven-day-old seedlings. Bar = 50 μm. (I–K) GFP fluorescence in JMJ18, JMJ18-GFP transgenic plants. Eighty-five percent (17 out of 20 independent lines) of the JMJ18/JMJ18-GFP plants displayed a similar expression pattern. Seven-day-old seedlings, mature roots (I) and (K), and root tips (J). Bar = 50 μm. (L) GFP fluorescence in seven-day-old roots of SUC2:JMJ18-GFP transgenic plants. Bar = 50 μm. (M) to (P) JMJ18, JMJ18-RFP and SUC2:JMJ18-GFP double-transgenic plants exhibited JMJ18 expression in companion cells. All of the transgenic lines (13 independent lines) observed for the colocalization of JMJ18-GFP and -RFP displayed a similar colocalization pattern. GFP fluorescence (M), RFP fluorescence (N), bright-field image (O), and merged image (P). Bar = 25 μm.

**SUC2:JMJ18-GFP co-11 or SUC2:JMJ18-GFP fl-5, flowered at a similar time to co-11 or fl-5 (Figure 5A–5C).** In addition, we found that overexpression of JMJ18 in mutant background, such as SUC2:JMJ18-GFP fl-11, SUC2:JMJ18-GFP co-11 and SUC2:JMJ18-GFP fl-5, flowered slightly earlier than the single mutants fl-11, co-11, and fl-5, in terms of both flowering time and rosette leaf number (Figure 3A 5C). These genetic data demonstrate that promotion of the floral transition by JMJ18 mainly depends on functional FT.

The JmjN, JmjC, and zinc-finger domains are required for JMJ18 function

JMJ18 has four conserved domains: the JmjN, JmjC, zinc-finger, and FY-rich domains (Figure 2A). Due to the obvious early-flowering phenotype of JMJ18 overexpressors, we overexpressed truncated JMJ18 (Figure 6A and Figure S9A) in wild-type plants to determine the domain(s) in JMJ18 necessary for its function in planta. The early-flowering phenotype of 35S:JMJ18 (containing the entire JMJ18 CDS) was echoed by that of 35S:JMJ18-GFP (Figure 6B and 6C), suggesting that blocking of the free C-terminus of JMJ18 by GFP does not affect its function. 35S:NC2 (containing the JmjN, JmjC, and zinc-finger domains but lacking the FY-rich domain) transgenic plants exhibited a shorter life cycle than wild-type plants; however, the effect was weaker than that observed in 35S:JMJ18 plants (Figure 6B and 6C). Regardless, no early-flowering phenotype was observed in those plants overexpressing truncated JMJ18 with a deletion in both the zinc-finger and FY-rich domains (35S:NC) (Figure 6B and 6C). Northern blot analysis was used to determine whether these truncated transcripts were expressed in the transgenic plants. As shown in Figure 6D, all of the truncated transcripts were expressed. The transgenic plants overexpressing truncated JMJ18 with a deletion in both the JmjN and JmjC domains displayed similar flowering time compared to wild-type plants (Figure S9). Thus, these data suggest that the JmjN, JmjC, and zinc-finger domains are required for the function of JMJ18, while the FY-rich domain is not fully necessary for its function but affects its activity.

The 35S:T (encoding a truncated protein identical to that in jmj18-1 and containing the JmjN, JmjC, and zinc-finger domains) transgenic plants also displayed an obviously earlier floral transition compared to wild-type plants (Figure S9A). Figure S9B, which indicates that jmj18-1 and jmj18-2 are weak alleles. And the result is consistent with their weak early-flowering phenotype (Figure 2C, 2D and Table 1).

**JMJ18 represses the expression of FLC and MAFs**

H3K4 methylation is associated with gene activation; thus, H3K4 demethylases should work as gene repressors, rather than as activators. Therefore, FT is not likely to be the direct target of JMJ18. The target of JMJ18 could be an upstream repressor of FT. If this is the case, the floral repressor FLC is a good candidate. To examine this possibility, the effect of JMJ18 overexpression on FLC expression was examined in JMJ18 overexpressor plants. FLC expression was significantly repressed in all JMJ18 overexpressor lines examined, and the degree of repression was positively correlated with the abundance of JMJ18, but negatively with the level of FT (Figure 4E and Figure 7A). The other members of FLC clade genes (MAF1 to MAF5) were also repressed to different extent in JMJ18 overexpressors (Figure 7C); in comparison, there was no obvious change in the expression of CO, an upstream activator of FT (Figure 7B).

To analyze the temporal expression pattern of FLC and its relationship to that of JMJ18 during vegetative development, we measured FLC expression in plants grown under LD conditions on days 6, 9, 12, and 15 as described above. FLC expression was strong at the seedling stage, then decreased during vegetative development, reaching its lowest level before floral meristem formation on day 15 (Figure 7D). There was a strong negative correlation between the developmental expression patterns of JMJ18 and FLC during vegetative development (r = -0.950). Thus, we propose that JMJ18 is a repressor of FLC.

We generated jmj18-1 flc-3 to test whether JMJ18 interacts genetically with FLC. The FLC mutation mainly blocks the late-flowering phenotype of jmj18-1 (Figure 7E, 7G and Table S2). Thus, the mutation in FLC did not enhance the early-flowering phenotype of the JMJ18 overexpressors. In addition, overexpression of JMJ18 in flc, such as SUC2:JMJ18-GFP flc-3, flowered earlier than flc-3 and at almost the same time as SUC2:JMJ18-GFP (Figure 7F, 7G and Table S2), indicating that endogenous JMJ18 enhances flowering mainly by repressing FLC in wild-type plants, while overexpressing JMJ18 recognized FLC and MAFs as targets. Taken together, those results indicate that JMJ18 and FLC belong to the same genetic pathway in flowering time control, and JMJ18 functions in upstream of FLC.

**JMJ18 demethylates the chromatin of FLC and MAFs**

Our previous result suggested that JMJ18 functions as an H3K4-specific demethylase in vivo (Figure 1 and Figure S1). To verify the histone demethylase activity of JMJ18 in vivo, nucleoproteins were extracted from jmj18, our JMJ18 overexpressor lines, and wild-type plants. There was no obvious change in the levels of H3K4me3, H3K4me2, and H3K4me1 in wild-type, jmj18, and JMJ18 overexpressor plants on a global scale (Figure 8A and 8B). A slight decrease in H3K4me3, H3K4me2, and H3K4me1 was observed in JM18 overexpressors (Figure 8B), but no obvious difference was detected between wild-type and the other JMJ18 overexpressors (Figure 8B). Thus, endogenous JMJ18 may function as a gene-specific H3K4 demethylase in vivo, while overexpressed JMJ18 can target many other loci to demethylate H3K4me3 once it is expressed in many cell types.

To verify whether JMJ18 demethylates FLC chromatin, chromatin immunoprecipitation (ChIP) was done to detect the level of H3K4 methylation across the entire FLC chromatin region. In wild-type plants, the regions around the transcription start site (regions B4, B5, and B6) had higher levels of H3K4me3 than the other regions (Figure 9A and 9B), which is consistent with previous data [11,43]. However, the H3K4me3 level was obviously decreased in SUC2:JMJ18-GFP plants across the entire
generation for JMJ18:JMJ18-GFP and SUC2:JMJ18-GFP, and T3 generation for 35S:JMJ18-GFP. Two independent lines per transformation were shown. (C) JMJ18 overexpression promotes the floral transition under SD conditions. Bar = 2 cm. Fifty-eight-day-old plants were analyzed. (D) qRT-PCR analysis of FT mRNA expression in the transgenic plants. The expression level was normalized to that of ACTIN. Error bars indicate the standard deviation of three independent biological replicates. The 11-day-old plants were collected at dusk for the analysis. Asterisks indicate the significant difference between wild-type and overexpression lines using Student’s t test (P < 0.05). (E) Western blot analysis of JMJ18 and FT expression in the transgenic plants. Tubulin was used as a loading control. The 11-day-old plants were harvested at dusk for the analysis.

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Figure 4. Temporal expression pattern of JMJ18, JMJ18-induced FT expression, and the floral transition. (A) qRT-PCR analysis of JMJ18 and FT expression in plants grown under LD conditions. The expression level was normalized to that of ACTIN. Error bars indicate the standard deviation of three independent biological replicates. (B) JMJ18 overexpression promotes the floral transition under LD conditions. Bar = 2 cm. Twenty-five-day-old plants were analyzed. Twenty-one (23 out of 108), 33 (36 out of 108), and 19 (25 out of 108) percent of the independent lines for JMJ18:JMJ18-GFP, SUC2:JMJ18-GFP, and 35S:JMJ18-GFP, respectively, exhibited earlier flowering compared to wild-type plants. The plants selected for phenotypic analysis were homozygous with one insertion at T4

Histone Demethylase Controls Flowering

JMJ18 directly associates with FLC chromatin

JMJ18 controls FLC expression by modulating the H3K4 methylation level at FLC chromatin, suggesting that JMJ18 associates with the FLC locus and mediates the methylation of FLC chromatin directly. To investigate this possibility, ChIP was used to detect the binding of JMJ18 to the FLC locus. Due to the increased level of nonspecific binding of GFP to Arabidopsis chromatin under our experimental conditions, we used SUC2:JMJ18-myc transgenic plants instead of SUC2:JMJ18-GFP plants in our assay. The SUC2:JMJ18-myc transgenic plants exhibited similar phenotypes to the SUC2:JMJ18-GFP plants, including an early-flowering phenotype, altered FLC and FT expression, and reduced H3K4me3 and H3K4me2 levels across FLC chromatin (Figure S12). A myc-specific antibody was used to precipitate chromatin from wild-type and SUC2:JMJ18-myc plants. Compared to the wild-type plants, the FLC chromatin of the transgenic plants was significantly enriched with JMJ18 protein, with varying levels of occupancy across the entire FLC chromatin region, while, there was no obvious binding of JMJ18 to the chromatin of ACTIN, which was a negative control, compared with its binding to FLC chromatin (Figure 9F). Regions B3 and B6 exhibited the highest binding ability (Figure 9F), whereas the H3K4 methylation level was higher than in the other regions (Figure 9B and 9C), demonstrating that JMJ18 associates with FLC chromatin and that the binding of JMJ18 to FLC is necessary for the dynamic of H3K4 methylation in FLC chromatin.

JMJ18 overexpression suppresses the FRI late-flowering phenotype

FRI is the major determinant of ecotype differences in flowering time in Arabidopsis [46,47]. To examine the influence of JMJ18 on flowering time promotion, JMJ18 was overexpressed in FRI plants

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by the introduction of FRI into JMJ18::JMJ18-GFP, SUC2::JMJ18-GFP, and 35S::JMJ18-GFP transgenic lines via crossing, respectively. The overexpression of JMJ18 significantly suppressed the late-flowering phenotype of FRI in a JMJ18 dose-dependent manner (Figure 10A and Table 3).

Table 2. Over-expression of JMJ18 induces floral transition under long-day and short-day conditions.

| Genotype       | Days to visible buds | Days to first flower open | Rosette leaf no. | Cauline leaf no. | n  |
|----------------|----------------------|----------------------------|------------------|------------------|----|
| LD WT          | 23.9 ± 1.6           | 31.5 ± 2.0                 | 11.7 ± 1.4       | 2.2 ± 0.7        | 30 |
| JMJ18::JMJ18-GFP #18 | 20.1 ± 0.8                  | 28.0 ± 1.4                 | 9.5 ± 0.7        | 2.6 ± 0.5        | 28 |
| JMJ18::JMJ18-GFP #32 | 18.0 ± 0.8                  | 26.4 ± 0.9                 | 8.9 ± 0.6        | 2.8 ± 0.4        | 35 |
| SUC2::JMJ18-GFP #73 | 18.0 ± 0.5                   | 25.0 ± 0.7                 | 7.0 ± 0.8        | 3.1 ± 0.4        | 36 |
| SUC::JMJ18-GFP #93 | 17.8 ± 0.7                   | 24.8 ± 0.9                 | 6.7 ± 0.7        | 3.1 ± 0.5        | 30 |
| 35S::JMJ18-GFP #1 | 18.3 ± 0.8                   | 26.5 ± 0.8                 | 6.0 ± 0.7        | 2.3 ± 0.5        | 24 |
| 35S::JMJ18-GFP #2 | 18.3 ± 1.1                   | 25.9 ± 1.4                 | 5.6 ± 0.9        | 2.2 ± 0.7        | 26 |
| SD WT          | 65.4 ± 2.6           | 74.6 ± 2.4                 | 57.5 ± 3.3       | 8.3 ± 0.9        | 12 |
| JMJ18::JMJ18-GFP #18 | 59.7 ± 1.8                   | 68.7 ± 1.8                 | 46.6 ± 2.2       | 7.2 ± 0.7        | 12 |
| JMJ18::JMJ18-GFP #32 | 58.3 ± 1.7                   | 67.2 ± 1.5                 | 37.6 ± 2.4       | 6.8 ± 0.8        | 12 |
| SUC2::JMJ18-GFP #73 | 42.9 ± 2.8                   | 51.2 ± 3.4                 | 23.6 ± 2.2       | 5.6 ± 0.8        | 12 |
| JMJ18::JMJ18-GFP #93 | 45.2 ± 2.2                   | 50.3 ± 2.1                 | 19.8 ± 2.0       | 4.3 ± 0.8        | 12 |
| 35S::JMJ18-GFP #1 | 32.3 ± 3.0                   | 42.5 ± 1.8                 | 9.8 ± 1.6        | 2.3 ± 0.7        | 12 |
| 35S::JMJ18-GFP #2 | 33.3 ± 4.3                   | 43.0 ± 3.4                 | 9.5 ± 2.0        | 2.3 ± 0.5        | 12 |

LD, long-day condition (16 h light, 22 °C/8 h dark, 18 °C, cycle). SD, short-day condition (8 h light, 22 °C/16 h dark, 18 °C, cycle). The length of JMJ18 promoter is 1.4 kb. Values are the mean number ± standard deviation. n indicates the number of plant scored for each line.

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Figure 5. Functional FT is necessary for promotion of the floral transition by JMJ18. Promotion of the floral transition by JMJ18 is mainly dependent on FT, CO, or FD. (A) WT and SUC2::JMJ18-GFP are 24-day-old plants; ft-11, SUC2::JMJ18-GFP ft-11, co-11, and SUC2::JMJ18-GFP co-11 are 43-day-old plants. fd-5 and SUC2::JMJ18-GFP fd-5 are 35-day-old plants. SUC2::JMJ18-GFP line #73 was used in the experiment. Bar = 2 cm. The values in (B) and (C) are the mean ± standard deviation from at least 20 plants per genotype. Asterisks in (B) and (C) indicate the significant difference between marked plants from the two genotypes by Student’s t test (P<0.05). doi:10.1371/journal.pgen.1002664.g005

Figure 6. The JmjN, JmjC, and zinc-finger domains are necessary for the promotion of flowering by JMJ18. (A) Schemes display the structures of full-length JMJ18 protein as well as truncated versions used for overexpression test. (B) and (C) Flowering time phenotype for the JMJ18 overexpression lines. All of the plants in (B) were grown for 24 days under LD conditions. All of the 108 independent 35S::NC lines examined flowered at a similar time compared to wild type. Fifteen out of 108 35S::NCZ lines, 20 out of 108 35S::T lines, and 27 out of 108 35S::T lines displayed an early-flowering phenotype for the 35S::T lines and 35S::T lines, respectively. The overexpression of JMJ18 significantly suppressed the late-flowering phenotype of FRI in a JMJ18 dose-dependent manner (Figure 10A and Table 3). (D) Northern blot analysis of the expression of truncated JMJ18 in the transgenic lines. doi:10.1371/journal.pgen.1002664.g006
**Figure 7. JMJ18 acts as a repressor of FLC and MAFs.** (A) FLC expression was repressed in the JMJ18 overexpressor lines. Asterisks indicate the significant difference between wild-type and transgenic plants analyzed by Student’s t test (P < 0.05). (B) CO expression was not obviously changed in the JMJ18 overexpressor lines. There was no significant difference between wild-type and transgenic plants analyzed by Student’s t test (P > 0.05). The expression level was normalized to that of ACTIN in (A) to (C). (D) Temporal expression patterns of FLC and JMJ18 during vegetative development. The mRNA levels were normalized to that of ACTIN. The error bars indicate the standard deviation from three independent biological replicates. (E) to (G) Genetic analysis of JMJ18 and FLC in flowering time control. The SUCC2/JMJ18-GFP #73 was crossed to fcl-3. Twenty-eight- (E) and 24-day-old (F) plants were photographed. Bar = 2 cm. Asterisks indicate the significant difference analyzed by Student’s t test (P < 0.05).

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**Figure 8. Global H3K4 methylation levels in wild-type, jmj18, and JMJ18 overexpressor lines.** (A) Detection of the global H3K4me3, H3K4me2, and H3K4me1 levels in the wild-type and transgenic plants analyzed by Student’s t test (P < 0.05). The expression level was normalized to that of ACTIN in (A) to (C). (B) Detection of the global H3K4me3, H3K4me2, and H3K4me1 levels in the JMJ18 overexpressor lines.

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**Discussion**

JMJ18 is a histone H3K4 demethylase in Arabidopsis

Several JARID1 family proteins have been characterized as H3K4 demethylases in various organisms [9,28,29,33,69,71,72,73]. There are 6 members of JARID1 proteins in Arabidopsis, JMJ14 (at1g20400), JMJ15 (at2g34880), JMJ16 (at1g08620), JMJ17 (at1g63490), JMJ18 (at1g30810), and JMJ19 (at2g38950). To examine whether Arabidopsis JMJ18 is a real histone demethylase, we characterized its histone demethylase activity in vivo and in vitro. We examined the histone demethylase activity of JMJ18 in vitro by purifying recombinant JMJ18 from insect cells and analyzing it by MALDI-TOF mass spectrometry. In that analysis, JMJ18 exhibited histone H3K4-specific demethylase activity (Figure 1A, 1B, and Figure S1), which was dependent on Fe(II) and α-KG (Figure 1C). Additionally, JMJ18 was able to demethylate histone H3K4me3 to H3K4me2 and H3K4me1 (Figure 1A, 1B, and Figures S1C). This observation was confirmed in vivo using FLC and MAFs chromatin (Figure 9B–9E, Figures S11A–11B and S12E–S12F), and full-length JMJ18 lacks demethylase activity towards H3K4me2 to H3K4me1 (Figure 1A and Figure S1C), predominant expression in the SAM, while both the JMJ18 and SUCC2 promoters were specifically expressed in companion cells. Thus, this result supports the notions that JMJ18 promotes flowering in FRI by repressing FLC expression, and that FLC expressed in both the SAM and companion cells contributes to flowering time control. Consistent with our previous results (Figure 4D and Figure S12D) and early-flowering phenotype (Figure 10A and Table 3), the expression of FT in these transgenic lines was obviously activated (Figure 10D), indicating that JMJ18 represses FLC expression and induces FT expression in an FRI background as well.
indicating that JMJ18 favors H3K4me3 to H3K4me2 demethylation. Our results also indicate that JARID1 family proteins are conserved histone H3K4 demethylases from human to plants.

Interestingly, full-length JMJ18 only demethylated H3K4me3 to H3K4me2 (Figure 1A). However, truncated JMJ18 lacking the partial linker between the JmjN and JmjC domains exhibited demethylase activity toward both H3K4me3 and H3K4me2 (Figure 1B and Figure S1C). Taken together (Figure 9B–9E and Figure S12E–S12F), our results suggest that the linker between the JmjN and JmjC domains of JMJ18 blocks its H3K4me2 demethylase activity in vitro. This linker could not block the H3K4me2 demethylase activity of JMJ18 in vivo by functioning as a protein-interaction domain; however, this requires further study. At any rate, this observation raises an interesting question as to the structural function of the linker sequence in JMJ18 itself and within the JMJ18 complex.

**JMJ18 is a companion cell–predominant, developmentally regulated gene**

Cell- or tissue-specific expression is a significant part of enabling a gene to achieve its function in plant cell fate determination and development [64,65,78]. Since the transgenic plants carrying JMJ18:JMJ18-GFP transformation could complement the late-flowering phenotype of jmj18-1 (Figure S2), it was possible to characterize the expression pattern of JMJ18 in *Arabidopsis* based on JMJ18 promoter-reporter expression (Figure 3, Figures S5 and S6). We found that JMJ18:GUS and JMJ18:GFP were expressed in the vascular tissue of cotyledons, young leaves, adult leaves, hypocotyl and flowers (Figure 3A, 3C, 3D; Figures S5 and S6), not expressed in the SAM where FLC was expressed dominantly (Figure 3E). In the roots of the transgenic plants, signals for GUS and GFP were predominantly observed in protophloem at the root tip, and in phloem in other regions of the root (Figure 3B, 3F–3K; Figures S5 and S6D). In addition, different length of promoter-driven reporter exhibits similar expression pattern (Figure 3, Figures S5 and S6), indicating that JMJ18 is predominantly expressed in the phloem of vascular tissue during vegetative development. Recently, Hong et al. (2009) reported that the expression of JMJ18 (*Atjmj18*) in their study is expressed broader in root and leaf [79]. We constructed a same version of JMJ18

Figure 9. JMJ18 binds FLC chromatin and mediates the level of H3K4 methylation in FLC chromatin. (A) Schematic genomic structure of the FLC locus, and the probes used to measure the histone methylation level. Open boxes represent introns, while filled boxes represent exons. B1–B14 indicate the FLC regions recognized by the probes during the analysis of the H3K4 methylation level by ChIP. (B) ChIP analysis of the H3K4me3 level across FLC in wild-type and JMJ18 overexpressor plants. (C) ChIP analysis of the H3K4me2 level across FLC in wild-type and JMJ18 overexpressor plants. (D) and (E) ChIP analysis of the binding of JMJ18 to FLC chromatin in eleven-day-old plants. The enrichments were normalized to the input. ACTIN was used as a negative control. The values are the mean ± standard deviation from three biological replicates.

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Figure 10. JMJ18 overexpression suppresses the FRI late-flowering phenotype. (A) Flowering time in FRI wild-type and F1 plants from a cross between FRI wild-type and JMJ18 overexpressor plants. All of the plants were 48 days old at analysis except for 35S:JMJ18-GFP #1 × FRI F1, which was 38 days old. All plants were grown under LD conditions. Bar = 2 cm. Expression levels of FLC in eleven-day-old whole seedlings (B) and 26-day-old rosette leaves (C). (D) FT expression in eleven-day-old seedlings. The expression level was normalized to that of ACTIN. The values are the mean and standard deviation from three independent biological replicates. The lines crossed with FRI were: JMJ18:JMJ18-GFP #34, SUC2:JMJ18-GFP #73, and 35S:JMJ18-GFP #1. Asterisks in (B), (C) and (D) indicate the significant difference for the expression levels of FLC and FT between FRI and other genotypes analyzed by Student’s t test (P < 0.05).

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promoter-GUS fusion as Hong et al. (2009) and transformed it into the wild-type plants. We observed that all 21 independent lines transformed with 1.7 kb length promoter-GUS we examined exhibited similar expression pattern, and the two versions of promoter (1.4 and 1.7 kb in length) exhibit very similar expression patterns (Figure 3, Figures S5 and S6). One possibility for the discrepancy between the two studies is due to the over-staining of GUS of tissues in study of Hong et al. (2009).[79]

Phloem is composed of sieve elements and companion cells. An analysis of the cell-specific expression pattern of JMJ18:GFP in phloem indicated that JMJ18 is predominantly expressed in companion cells (Figure 3I, 3K, and Figure S5). This result was confirmed by examining the cellular localization of tagged JMJ18 in the phloem using Arabidopsis coexpressing JMJ18:JMJ18-RFP and SUC2:JMJ18-GFP. SUC2 is a companion cell-specific gene in Arabidopsis [76,80]. JMJ18-RFP and -GFP were colocalized in the same cell in the roots of the transgenic plants (Figure 3M–3P), indicating that JMJ18 is predominantly expressed in companion cells. Thus, our results reveal that JMJ18 is a companion cell-dominant histone H3K4 demethylase in Arabidopsis.

In addition, the expression of JMJ18 increased during vegetative development, reaching its high level before the formation of the floral meristem (Figure 4A). This suggests that JMJ18 encodes a companion cell-dominant, developmentally-regulated histone demethylase in Arabidopsis.

### JMJ18 regulates flowering time

To determine the role of JMJ18 in Arabidopsis development, we characterized three jmj18 T-DNA insertion mutants. All three mutants displayed a late-flowering phenotype under LD and SD conditions (Figure 2C, 2D and Table 1). The knock-down lines for JMJ18 using both RNAi and amiR also exhibit delayed flowering (Figure S3 and Table S1). The late-flowering phenotype in jmj18 is complemented by transforming JMJ18:JMJ18-GFP to jmj18 (Figure S2). However, the flowering phenotype was weak, and the changes in the expression of flowering marker genes in jmj18 were very mild (Figure 2E–2G, Figures S3 and S4).

To determine the reason for the weak phenotype of jmj18, we further characterized the three jmj18 alleles. We found that the T-DNA was inserted at the 3′-end of JMJ18, behind the JmjN, JmjC, and zinc-finger domains, in both jmj18-1 and jmj18-2 (Figure 2A). Partial transcripts of JMJ18 were detected in jmj18-1 and jmj18-2 (Figure 2B). In addition, the truncated JMJ18 in jmj18-1 and jmj18-2 was functional in planta (Figure 6B and 6C). For the third allele, although the T-DNA was inserted in the fourth exon of JMJ18, full-length JMJ18 mRNA was detected at reduced levels in jmj18-3 (Figure 2B). These results indicate that the three jmj18 alleles were weak mutants. Unfortunately, no other allele for jmj18 is currently available.

Table 3. JMJ18 overexpression suppresses the FRI late-flowering phenotype.

| Genotype               | Days to visible buds | Days to first flower open | Rosette leaf no. | Cauline leaf no. | n  |
|------------------------|----------------------|---------------------------|-----------------|-----------------|----|
| WT (Col-0)             | 222 ± 1.1            | 29.5 ± 1.4                | 11.7 ± 1.1      | 2.8 ± 0.7       | 29 |
| FRI                    | > 90                 |                           |                 |                 |    |
| JMJ18:JMJ18-GFP #32 × FRI F1 | 48.1 ± 2.9          | 58.5 ± 3.4                | 46.7 ± 2.1      | 7.2 ± 1.0       | 19 |
| SUC2:JMJ18-GFP #73 × FRI F1 | 37.4 ± 2.0          | 45.7 ± 2.0                | 38.1 ± 2.0      | 5.3 ± 1.0       | 21 |
| JMJ18:JMJ18-GFP #1 × FRI F1 | 33.1 ± 4.6           | 41.4 ± 4.4                | 15.4 ± 2.8      | 3.8 ± 0.8       | 14 |

The plants were grown under long-day condition. The values are the mean ± standard deviation. n indicates the plant number scored for phenotype analysis. The length of JMJ18 promoter is 1.4 kb.

However, the increase in JMJ18 activity caused by the overexpression of JMJ18 significantly enhanced flowering in Arabidopsis (Figure 4B, 4C; Figure 6B, 6C; Figure 10A; Table 2 and Table 3; and Figure S12A, S12B). This observation was true for all JMJ18 overexpressors driven by the constitutive CaMV35S promoter, companion cell-specific SUC2 promoter, and endogenous JMJ18 promoter (Figure 4B, 4C; Figure 6B, 6C; Figure 10A; Table 2 and Table 3; and Figure S12A, S12B). The above data suggest that JMJ18 is a flowering time regulator in Arabidopsis.

JMJ18 directly represses FLC expression and indirectly induces FT expression in companion cells to control flowering time in Arabidopsis

FLC is a central repressor of flowering in Arabidopsis that works in part through the repression of a companion cell-specific flowering activator, FT [55]. FLC is expressed in both the SAM and companion cells, and the expressed FLC in both tissues contributes to flowering time control in Arabidopsis [55]. The regulation of FLC expression in the SAM has been extensively studied [11,24,43,53]; however, little is known about the regulation of FLC expression in companion cells.

In the present work, we found that JMJ18 was predominantly expressed in companion cells in vegetative tissue (Figure 3, Figures S5 and S6). In addition, we confirmed that the expression pattern of JMJ18 was strongly negatively correlated with that of FLC, but strongly positively correlated with that of FT, during vegetative development in Arabidopsis (Figure 4A and Figure 7D). These results suggest that JMJ18 works as an endogenous developmental signal to regulate the expression of FLC and FT in the control of flowering time.

We further found that JMJ18 binds FLC chromatin (Figure 9F), and decreases the levels of H3K4me3 and H3K4me2 in FLC chromatin (Figure 9B, 9C; Figures S11A and S12F). In addition, an increase in JMJ18 abundance obviously decreased the mRNA levels of FLC and MAFs (Figure 4E; Figure 7A, 7C, 7D; Figure 10B, 10C; and Figure S12C), and obviously increased the expression of FT at both the mRNA and protein levels (Figure 4D, 4E; Figure 10D; Figure S12D). Furthermore, the promotion of flowering time by JMJ18 was mainly dependent on functional FT (Figure 5A–5C), but was not enhanced by mutation in FLC (Figure 7F, 7G, and Table 2). These results indicate that JMJ18 works as an endogenous, companion cell-dominant developmental signal that directly represses FLC expression and indirectly induces FT expression in companion cells to control flowering time during vegetative development in Arabidopsis. It was noted that overexpressed JMJ18-GFP in companion cells reduced both levels of H3K4me3 and H3K4me2 on FLC, but only H3K4me3 on MAFs (Figure 9B–9E and Figure S12E–S12F). The difference of the decrease in the levels of H3K4 methylation between FLC and
MAFs in JMJ18 overexpression line may suggest that JMJ18 is more important for the regulation of FLC than MAFs in Arabidopsis.

In Arabidopsis, the flowering transition is a complicated process. It needs to integrate the environmental cues and internal signals; in addition, these signals will be sensed and regulated in different tissues or organs in the plant [81,82]. There are two main tissues involved in the flowering control: the vascular tissue in the leaf and the SAM [37,83]. The character of the apical meristem is determined not only by the processes occurring in apical meristem, but also by the signals transmitted from vascular tissue. Thus, different flowering regulators should be functional and regulated in distinct tissues or steps, integrated in the SAM for the precise control of flowering time. As flowering regulators, the JmJC domain-containing demethylases are functional in both vascular tissue and SAM, and target different genes. Since REF6 is characterized as a H3K27 demethylase and represses FLC in both SAM and leaves, FLC can not be the direct target of REF6 [31,70]. However, its homolog ELF6, an H3K4 demethylase, is expressed in leaves and targets FT to repress flowering [28]. Another H3K4 demethylase, JMJ14, is restricted in leaves to repress flowering by decreasing the expression of the floral integrators including FT, SOC1, LFY and API [28,29,69]. In this report, we demonstrated that JMJ18 is a H3K4 demethylase and specially expressed in the vascular tissue (Figure 1, Figure 3, Figure 9; Figures S5, S6, S11, and S12E-S12F). The expression of JMJ18 is developmentally-regulated (Figure 4A). JMJ18 directly represses the expression of FLC and releases the expression of FT in vascular tissue (Figure 4, Figure 5, Figure 7, Figure 9, Figure 10, and Figure S12), then the released FT is transmitted from the vascular tissue to the SAM to stimulating flowering. These results suggest that plants may have evolved to the point where they use a family of proteins with different expression patterns which target various genes to integrate environmental cues and internal signals to precisely control flowering time. Thus, this study provides novel insight into the regulation of FLC expression in companion cells and the epigenetic control of the floral transition in Arabidopsis.

Methods

Plant materials and growth conditions

The Arabidopsis plants used in our experiments were of the Columbia-0 ecotype except for FRI. The seeds were first sterilized with 2.25% bleach, then washed three times with water, stratified for three days at 4°C, and then put on Murashige and Skoog (MS) medium (Sigma-Aldrich) containing 1% sucrose and 0.3% phytagel (Sigma-Aldrich). Following ten days of growth under LD (16 h of light, 22°C) or SD (8 h of light, 22°C) conditions at 50% relative humidity. For the observation of GFP fluorescence, roots were cut from seedlings grown on MS medium under LD conditions for seven days. For the observation of GFP fluorescence, roots were cut from seedlings grown on MS medium under LD conditions for seven days.

Histological and cytological analyses of JMJ18 expression

GUS staining was performed as described previously [86,87]. Twenty-four independent JMJ18:GUS T2 lines were used for histochemical analysis, with at least ten individual plants observed for each line. Images were taken using a stereomicroscope (Leica MZ16) or light microscope (Zeiss Imager M1).

For the observation of GFP fluorescence, roots were cut from seedlings grown on MS medium under LD conditions for seven days. For JMJ18:JMJ18-GFP, 20 independent lines were observed, with at least ten individual plants analyzed per line. Images
were collected using a Zeiss LSM 510 Meta confocal laser scanning microscope as described previously [90].

**Observation of the colocalization of JMJ18-GFP and -RFP**

JMJ18:JMJ18-RFP was constructed to pCAMBIA2300 and introduced into SUC2:JMJ18-GFP (in pCAMBIA1300) transgenic plants. The double transformants were selected on MS medium containing kanamycin and hygromycin. Transgenic lines expressing JMJ18-GFP and -RFP were selected and photographed using a Zeiss LSM 510 Meta confocal laser scanning microscope.

**Gene expression analysis**

Total RNA was isolated from the plant materials indicated in the text using RNAiso plus (Takara) and treated with RNA-free DNase (Promega) to remove all remaining DNA. Three micrograms of total RNA were used to synthesize first-strand cDNA with a reverse transcription kit (Fermentas). The cDNAs were diluted to 60 μl with sterilized water. One microliter of diluted cDNA was used for real-time PCR amplification with SYBR Premix Ex Taq (Takara). Three biological replicates were performed to calculate the mRNA abundance. Standard deviations were calculated from the replicates. The primers used were listed in Table S3.

**ChIP assay**

Plant tissue (1.5 g) was collected after growth under LD conditions for eleven days. ChIP was performed as described previously with three biological replicates [89,99]. The results are shown as absolute enrichment compared to the input or to total H3. The antibodies used were: anti-H3K4me3 (Upstate 07-030), -H3K4me2 (Upstate 07-030), and -c-myc (Sigma-Aldrich M4439). The primers used to measure the amount of DNA from the ChIP products were listed in Table S4.

**Preparation of anti-JMJ18 and -FT antibodies**

Purified recombinant JMJ18 from insect cells and FT from E. coli were used as antigens to immunize rabbits. Each rabbit was immunized once every fourteen days with 1 mg of protein. After four successive immunizations, the anti-serum was examined by ELISA and Western blotting using total protein from the mutant and overexpressor lines.

**Western blot analysis**

For immunoblotting, Arabidopsis seedlings were ground to a powder in liquid nitrogen then homogenized in extraction buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM MgCl2, 1% Nonident P-40, complete protease inhibitor [Roche], and 1 mM phenylmethylsulfonyl fluoride). The extracts were then centrifuged, the pellet removed, and the supernatant boiled in 6× SDS sample buffer. The proteins in the samples were separated by 0% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and detected using different antibodies. The antibody used for Tubulin detection was anti-β-Tubulin (Sigma-Aldrich T5168).

**Double-strand RNA and amiRNA interferences**

To knock-down the expression of JMJ18, a double strand RNA and artificial microRNA interference approaches were used. For a double strand RNA amiRNA, a unique 616 bp JMJ18 sequence was amplified by PCR. The gene-specific primers were: forward 5’-GGGTTACCTGCTAGAGGTCGTTCCTTTGGAACCTC-3’, reverse 5’-GGGGATCCGAGCTCCTGCACCTGACCCAATATAGCAAGGTTC-3’. The self-complementary hairpin RNA was constructed to pTCK303 as described by Wesley et al. (2001) [90].

Three primer pairs were designed through the web (http://wmi2.weigelworld.org/cgi-bin/mirnatools.pl?page = 1) for artificial microRNA interference assay. The primers are: amiR-a, 5’-GATGAGTCTTTAAATGCAGGAGCTCCTGCCTGTATTTTTCA-3’, amiR-b, 5’-GAGCCCTCTATTATTAAAGACGCTATTCCAAAAAGAAGTAGATTAGA-3’, amiR’s, 5’-GACGCCTCGATTTATAGACCTTCTACATATAAATGACTGGTATATG-3’, amiR*a, 5’-GAGAGGTCTTAAATGACGAGCTGACCCATCTACATATAATTA-3’. The constructs for amiRNA were established by following the protocols from Schwab et al. (2006).

The resulting three constructs were delivered to wild-type through agrobacterium–mediated transformation to generate JMJ18 amiRNA interference lines.

**Accession numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: ACTIN (At5g09810), CO (At5g15840), FLC (At5g10140), FT (At1g5480), FRI (At4g06500), JMJ18 (At1g30810), MAF1 (At1g77080), MAF2 (At5g65050), MAF3 (At5g65060), MAF4 (At5g65070), MAF5 (At5g65080), SUC2 (At1g22710) and TSF (At4g20370).

**Supporting Information**

**Figure S1** His-JMJ18 purification and characterization of its histone demethylase activity. (A) SDS-PAGE of purified recombinant His-JMJ18 from High5 insect cells. M, molecular standard; Total, total lysate; Input, cleared input. F1–F5, the fractions eluted from the nickel-affinity column. The numbers represent the molecular weights. (B) Purification of His-JMJ18 by size-exclusion chromatography. Top panel, UV absorbance; Bottom panel, results of the SDS-PAGE analysis of the purified and eluted recombinant His-JMJ18 fractions produced by size-exclusion chromatography. (C) Summary of the histone demethylase activity of His-JMJ18 in vivo.

**Figure S2** Complementation assay of jmj18-1 by JMJ18:JMJ18-GFP transformation. (A) The late-flowering phenotype of jmj18-1 was complemented by JMJ18:JMJ18-GFP transformation. Twenty-eight-day-old plants were photographed. Bar = 2 cm. (B) and (C) Statistical analysis the phenotype of complementary plants by days to first flower open (B) and rosette leaf number (C). At least 16 plants of each genotype were used for analysis. Asterisks indicate the significant differences analyzed by Student’s t test (P<0.05).

**Figure S3** Characterization of JMJ18 RNAi and amiR lines. The gene expression levels of JMJ18, FLC and FT in JMJ18 knock-down transgenic lines. RNAi: knock-down the expression of JMJ18 by double strands of RNA. amiR: knock-down the expression of JMJ18 by artificial microRNA. The numbers in parentheses indicate the amplification cycles.

**Figure S4** The expression levels of FLC and FT in jmj18 mutants at differential vegetative developmental stages. The expression levels of FLC at day 6 (A), 9 (C) and 15 (E), and FT at day 6 (B), 9 (D) and 15 (F) grown under long-day condition were measured. The eleven-day-old seedlings were collected at dusk at the day indicated. The expression level was normalized to that of ACTIN. Error bars indicate the standard deviation of three replicates. Asterisks indicate the significant difference between wild-type and jmj18 mutants analyzed by Student’s t test (P<0.05).
Figure S5  The expression pattern of JMJ18. (A) Two promoter regions selected for GUS constructs. The black filled boxes are shown exons, black lines for introns, and gray lines for intergenic sequence. (B) 7-day-old cotyledon. Bar = 1 mm. (C) 20-day-old rosette leaf. Bar = 1 mm. (D) 10-day-old root. Bar = 0.5 cm. (E–J) The marked regions of the root in (D). Bar = 0.1 mm. GUS staining in anther (K, M) and (L, N). 21 independent T2 lines were used for GUS staining. JMJ18/GUS transgenic plants were used in (A)–(L). All 21 independent lines we examined display the similar expression pattern for JMJ18. JMJ18/GUS transgenic plants were used in (M) and (N).

Figure S6  The expression pattern of JMJ18 in stem, hypocotyl and petal by using JMJ18-GFP and nuclear localization of JMJ18. JMJ18 was expressed in companion cells in different tissues. GFP fluorescence was detected in (A) the junction region between cotyledon and hypocotyl from 7-day-old seedling, where the vascular tissue branched, bar = 100 μm; (B) 7-day-old hypocotyl, bar = 50 μm and (C) petal, bar = 20 μm, respectively. (D) Nuclear localization of JMJ18. Bar = 20 μm.

Figure S7  JMJ18 in [M]J18 overexpression and wild-type plants. The 35S:JMJ18 transgenic plant was used to mark [M]J18 band, about 20% amount of the other samples was loaded for the sample from 35S:JMJ18 transgenic plant. The arrows indicated the JMJ18-GFP or JMJ18 bands. The upper panel: the gel was exposure for 1 min; the lower panel: the gel was exposure for 20 min. Tubulin was used as a loading control.

Figure S8  JMJ18 induced TSF expression. qRT-PCR analysis of TSF mRNA expression in the transgenic plants. The expression level was normalized to that of ACTIN. Error bars indicate the standard deviation of three independent biological replicates. Asterisks indicate the significant difference between wild-type and transgenic plants analyzed by Student’s t test (P<0.05).

Figure S9  Characterizing 35S::JMJ18 transgenic plants. (A) Schematic structures of full-length [M]J18 protein as well as [M]J18 2Y. (B) Flowering time distributions of wild-type and 35S::JMJ18 at T1 generation measured by rosette leaf number. Thirty-two wild-type and 108 independent transgenic plants were analyzed. (C) Flowering time of wild-type and 35S::JMJ18 transgenic plants counted by rosette leaf number. (D) JMJ18 2Y transcription levels in independent 35S::JMJ18 transgenic line, and the rosette leaf number for each plant was indicated.

Figure S10  JMJ18 do not affect the H3K4me3 modification levels at ACTIN, AtMu1 and AtSN1 loci. The H3K4me3 modification levels were detected in wild-type and SUC2::JMJ18-GFP plants. The results were normalized to the input. The values are the mean ± standard deviation from three biological replicates. Asterisk indicates the significant difference between wild-type and transgenic plants analyzed by Student’s t test (P<0.05).

Figure S11  JMJ18 decreases FLC/MAF: H3K4me3 levels. (A) Overexpression JMJ18 reduced H3K4me3 level at FLC locus in JMJ18 overexpression plant compared to wild-type plant. (B) ChIP analysis of H3K4me3 levels for other members of FLC clade genes. (C) The H3K4me3 levels were not obviously changed at ACTIN, AtMu1 or AtSN1 chromatin between wild-type and JMJ18 overexpression plants. The enrichments were normalized to total H3. The values are the mean ± standard deviation from three biological replicates.

Figure S12  Early-flowering phenotype of the SUC2::JMJ18-myc line. (A) Twenty-four-day-old SUC2::JMJ18-myc plants grown under LD conditions were shown. Bar = 2 cm. Thirty-three out of 108 independent SUC2::JMJ18-myc lines displayed the early-flowering phenotype. The independent line shown was used to analyze FLC and FT expression and for ChIP. (B) Flowering time in wild-type plants and the SUC2::JMJ18-myc line based on the number of rosette leaves at flowering. The values are the mean ± standard deviation from 20 plants. qRT-PCR analysis of FLC (C) and FT (D) expression. The expression level was normalized to that of ACTIN. The levels of H3K4me3 (E) and H3K4me2 (F) across the FLC genomic region in wild-type and SUC2::JMJ18-myc plants were determined. The values in (C) to (F) are the mean and standard deviation from three technical replicates.

Table S1  Knock-down JMJ18 expression leads to late-flowering phenotype. All the plants were grown in LD condition. The values are the mean ± standard deviation. n indicates the plants number scored for the analysis. Asterisks indicate the significant differences in the statistic analysis between wide type and mutants using Student’s t test (P<0.05).

Table S2  Genetic interaction between JMJ18 and FLC in flowering time control. All the plants were grown under long-day condition. The values are the mean ± standard deviation. n indicates the plant number scored for phenotype analysis.

Table S3  The primers used for detecting gene expression level.

Table S4  The primers used in ChIP assay.

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Author Contributions

Conceived and designed the experiments: L Ma, J Chai, S Cui, S Chen, H Yang. Performed the experiments: H Yang, Z Han, Y Cao, D Fan, H Li, H Mo, Y Feng, Z Wang, L Liu, Y Yue. Analyzed the data: H Yang, Z Han, L Ma. Contributed reagents/materials/analysis tools: L Ma, J Chai, S Cui. Wrote the paper: L Ma, H Yang.

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