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The 3′ processing of antisense RNAs physically links to chromatin-based transcriptional control

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Noncoding RNA plays essential roles in transcriptional control and chromatin silencing. At Arabidopsis thaliana FLC, antisense transcript quantitatively influences transcriptional output, but the mechanism by which this occurs is still unclear. Proximal polyadenylation of the antisense transcripts by FCA, an RNA-binding protein that physically interacts with RNA 3′ processing factors, reduces FLC transcription. This process genetically requires FLD, a homolog of the H3K4 demethylase LSD1. However, the mechanism linking RNA processing to FLD function had not been established. Here, we show that FLD tightly associates with LUMINIDEPENDENS (LD) and SET DOMAIN GROUP 26 (SDG26) in vivo, and, together, they prevent accumulation of monomethylated H3K4 (H3K4me1) over the FLC gene body. SDG26 interacts with the RNA 3′ processing factor FY (WDR33), thus linking activities for proximal polyadenylation of the antisense transcripts to FLD/SDG26-associated H3K4 demethylation. We propose this demethylation antagonizes an active transcription module, thus reducing H3K36me3 accumulation and increasing H3K27me3. Consistent with this view, we show that Polycomb Repressive Complex 2 (PRC2) silencing is genetically required by FCA to repress FLC. Overall, our work provides insights into RNA-mediated chromatin silencing.

Significance

RNA-mediated chromatin regulation is central to gene expression in many organisms. However, the mechanisms by which RNA influences the local chromatin environment are still poorly understood. Here, we show how RNA 3′ processing factors, which promote proximal polyadenylation of an Arabidopsis antisense transcript, physically associate with the chromatin modifiers FLD/SDG26. The chromatin modifiers exist in a protein complex that inhibits H3K4me1 and H3K36me3 accumulation. By antagonizing transcription, the FLD/SDG26-containing complex promotes H3K27me3 accumulation, reducing transcriptional initiation and elongation rates. This cotranscriptionally mediated chromatin silencing mechanism may be widely relevant for gene regulation in many organisms.

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processing of the COOLAIR transcripts with a chromatin modification complex that influences H3K4me1-H3K36me3 and transcriptional activity at the locus. By antagonizing transcription, FLD/LD/SDG26-containing complex promotes H3K27me3 accumulation, consistent with a requirement for Polycomb Repressive Complex 2 in the FCA-mediated repression of FLC. We propose that FLD/LD/SDG26 influences an active transcription module that antagonizes PRC2 function.

Results

FLD Associates with LD and SDG26. We previously performed a suppressor mutagenesis screen and identified FLD as one of the components required for FCA-mediated FLC regulation (11). To gain insights into how FLD represses FLC transcription, we used a proteomic approach to search for FLD interactors. We immunopurified FLD from a transgenic line expressing FLD tagged at the carboxyl terminus with FLAG-TAP epitopes (FLD-FLAG-TAP) (10). Mass spectrometric analyses of the FLD immunoprecipitation revealed that FLD tightly associates with LUMINIDEPENDENS (LD) and a SET domain protein, SDG26, in vivo (Fig. L4 and Dataset S1). Purifications from transgenic plants expressing GFP-tagged versions of each protein but not GFP only or Col-0 enriched the other two proteins of the complex (Fig. L4 and Datasets S2 and S3). The interaction between FLD and SDG26 was confirmed by communoprecipitation (co-IP) in stable transgenic lines (Fig. LB). Loss of LD or SDG26 caused a reduction in FLD protein levels (Fig. 1C and SI Appendix, Fig. S1). One possible explanation for this is that the interaction between FLD and LD/SDG26 may be required for FLD stability.

LD was one of the first flowering regulators to be cloned based on a late-flowering phenotype of a T-DNA insertion (12), but how its function connected to other autonomous pathway components was unclear. LD encodes a protein carrying an N-terminal homeodomain (SI Appendix, Fig. S24) and has been reported to bind DNA without sequence specificity (13). SDG26 is a close homolog of SDG8 (SI Appendix, Fig. S24), the major histone H3K36 methyltransferase in the Arabidopsis genome; however, in vitro and in vivo analysis so far has provided no evidence that SDG26 is an H3K36 methyltransferase. In fact, sdg26 mutants show an opposite (late-flowering) phenotype compared to sdg8 (early flowering) through opposite effects on FLC expression, suggesting different functions or indirect effects (14, 15). We tested the subcellular localization of FLD, LD, and SDG26 in stable transgenic lines and found that they are all nuclear-localized (SI Appendix, Fig. S2B).

LD and SDG26 Function Genetically in the Same Pathway as FLD and FCA. Similar to fld mutant, loss-of-function mutations of LD and SDG26 showed a late-flowering phenotype and increased FLC expression (Fig. 2 A–C). In order to dissect the genetic relationships between FLD, LD, and SDG26, we combined the mutations to create double mutants. The results showed that fld ld, fld sdg26, and ld sdg26 did not give any additional lateness (Fig. 2A) or increase in spliced FLC RNA levels (Fig. 2B), but did lead to higher unspliced FLC RNA levels (Fig. 2C), compared to the single mutants. The inconsistency between spliced and unspliced FLC suggests that, similar to Puf1C (16), FLD, LD, and SDG26 might have a concerted role in regulating the release of nascent FLC transcripts.

FLD has been shown to function in the same genetic pathway and downstream of FCA in that fld is not additive with fca with respect to flowering time, and fld suppressed the ability of FCA to down-regulate FLC (11). To test whether LD and SDG26 behave in the same way as FLD, we first combined ld and sdg26 with fca and found no additivity compared to fca with respect to flowering time (Fig. 2D) or FLC expression (Fig. 2 E and F). Combination of a 35S-FCA transgene, with and without the FLC activator FRIGIDA, with ld and sdg26 mutations then showed that both mutations compromised the effect of overexpressed FCA on FLC (Fig. 2G). Taken together, these data support that FLD, LD, and SDG26 exist in a complex that functions downstream of FCA to repress FLC expression.

SDG26 Transiently Interacts with the 3′ Processing Factor FY (WDR33). The strong genetic interactions between FLD/LD/SDG26 and FCA raised the question of how FCA function is molecularly linked to FLD. No in vivo physical interactions of FCA with 3′ processing factors or chromatin regulators had been found until our recent analysis using a technique termed cross-linked nuclear immunoprecipitation and mass spectrometry (CLNIP–MS) (9). We found FCA interacted with both RNA and a range of proteins and, in vivo, localizes to nuclear condensates that are highly dynamic (9). Those condensates are likely to concentrate 3′ processing factors and contribute to 3′-end processing of RNAs including COOLAIR (9). We reasoned that the interaction between the FLD/LD/SDG26-containing complex and FCA, if any, would also be transient and dynamic. To this end, we applied CLNIP–MS to SDG26. Surprisingly, we found that, in addition to finding FLD and LD with high peptide counts, some 3′ RNA processing factors were also detected (Fig. 3A and Dataset S4) in the SDG26 immunoprecipitation after cross-linking. These include FCA, as well as the RRM-containing protein FPA (8, 17), FY (18, 19), and Cleavage/Polyadenylation Specificity Factor 160 (CPSF160), all of which have been shown to associate with FCA and colocalize with FCA in the nuclear condensates (9).

![Figure 1](https://example.com/figure1.png)

Fig. 1. FLD forms a complex with LD and SDG26. (A) Table listing the number of unique peptides identified for FLD, LD, and SDG26 in FLAG-FLD, GFP-LD, and SDG26-GFP affinity purifications. Nontransgenic Col-0 was included in all purifications, and the transgenic line expressing GFP alone was included in GFP purifications as a negative control. The read counts from the negative controls were all zero for the listed proteins. (B) Co-IP in stable transgenic plants to detect the association of SDG26-GFP with FLD-FLAG-TAP. The FLD-FLAG-TAP transgenic line was crossed either with sdg26 mutant or SDG26-GFP transgenic line. F1-generation plants were used for co-IP. (C) The protein level of FLD-FLAG-TAP in the indicated genetic backgrounds as determined by Western blot. The numbers indicate three biological replicates. Ponceau staining served as a loading control.
Purifications from Col-0 or a transgenic plant expressing a 35S-GFP fusion did not retrieve any of those proteins (Dataset S4). We then set out to confirm the interaction between SDG26 and FY, using an FY antibody raised in rabbits against the native recombinant protein (20). Using an SDG26-FLAG-TAP transgenic line, we performed cross-linked nuclear immunoprecipitation of SDG26 and probed against FY. The result showed that FY was readily detected (Fig. 3B). Without cross-linking, neither FY nor any of the 3′ processing factors were found in the SDG26 immunoprecipitation (Dataset S3). CLNIP-MS of LD also identified FY and FPA (Fig. 3A and Dataset S5). These data suggest that the interactions between the FLD/LD/SDG26-containing complex and 3′ processing factors provide a physical link, so that, when 3′ RNA processing of proximal COOLAIR occurs, the FLD/LD/SDG26-containing complex is brought in to repress FLC transcription.

Loss of FLD/LD/SDG26 Results in Overaccumulation of H3K4me1 at FLC. Our mathematical modeling and experimental evidence have shown that FLD-mediated repression of FLC is achieved in a manner consistent with a coordinated reduction of transcription initiation and Pol II elongation rates (10). Whether and how this is connected to histone modifications is not fully understood. Arabidopsis has four homologs of human LSD1, including FLD, LDL1, LDL2, and LDL3 (21). The fld mutation led to a limited 1.5- to 2-fold increase of H3K4me2 on FLC (10, 11). More recently, the ldld mutation was shown to increase gene body H3K4me1, which correlated positively with gene expression (22). We therefore decided to analyze the effect of FLD, LD, and SDG26 mutations on H3K4me1 and H3K4me2 levels at FLC. Chromatin immunoprecipitation coupled with quantitative PCR (ChiP-qPCR) showed a small increase of H3K4me2 at 1 to 4 kb beyond the TSS of FLC in fld (Fig. 4A and C), consistent with previous reports (10, 11). Surprisingly, we observed a much more dramatic increase of H3K4me1 over the FLC gene body in fld (Fig. 4A and B). ld and sdg26 also significantly overaccumulated H3K4me1 (Fig. 4B), indicating a major role of the FLD/LD/SDG26-containing complex in inhibiting H3K4me1 accumulation through the demethylase activity of FLD. It is also noteworthy that sdg26 accumulated more H3K4me2 than fld (Fig. 4C), suggesting a role for the FLD/LD/SDG26-containing complex in a stepwise removal of H3K4me2 and H3K4me1, with each component contributing differently to this activity. fca-9 showed a large increase in H3K4me1 and a similar increase in H3K4me2 as sdg26, in agreement with FLD/LD/SDG26 functioning genetically downstream of FCA (SI Appendix, Fig. S3A–C). Given that SDG26 features a SET domain, a hallmark of histone methyltransferases, we sought to determine whether the FLD/LD/SDG26-containing complex, in addition to FLD-mediated demethylation, could also directly alter chromatin states through SDG26-mediated histone methylation. However, we failed to detect activity of SDG26 toward recombinant Arabidopsis nucleosomes in vitro for both heterologously expressed SDG26 or FLD/LD/SDG26 complex purified from Sf9 cells, nor for the endogenous FLD/LD/SDG26-containing complex purified via FLD-FLAG-TAP purification (SI Appendix, Fig. S4). Overall, these findings suggest demethylation of H3K4 is a major activity of the complex.

SDG8 Is Epistatic to FLD/LD/SDG26 to Activate FLC. H3K4me1 is enriched at enhancers as well as gene bodies in mammalian cells (23). Recent studies suggested that H3K4me1 might fine-tune, rather than tightly control, enhancer activity and function (24–26). In plants, H3K4me1 is mainly found in gene bodies, removal of which mediates transcriptional silencing (22). Interestingly, the CW domain of Arabidopsis SDG8, an H3K36me3 methyltransferase, preferentially binds H3K4me1 (27, 28), providing a mechanism to link H3K4me1 to delivery of the active histone modification H3K36me3. Consistent with this, we found loss of the FLD/LD/SDG26-containing complex, as well as FCA,
led to a large overaccumulation of H3K36me3 in the FLC gene body (Fig. 4D and SI Appendix, Fig. S3D), which mirrored the change of H3K4me1 (Fig. 4B and SI Appendix, Fig. S3B). In addition, H3K27me3, the mutually exclusive histone modification of H3K36me3, was greatly reduced in the fld-4, id, and fca mutants (Fig. 4E and SI Appendix, Fig. S3E). Consistent with this, SDG8 ChIP did not show signal on FLC in the Col-0 background (29), where H3K4me1 was kept at a very low level (Fig. 4B). The connection between H3K4me1 and H3K36me3 raised the possibility that FLD/LD/SDG26 repressed FLC via removal of H3K4 methylation, thereby inhibiting SDG8-mediated H3K36me3 and indirectly promoting the accumulation of H3K27me3. To test this possibility, we generated the fld sdg8 double mutant and found that the sdg8 mutation completely suppressed both the fld-induced higher expression of FLC (Fig. 5A and B) and the resulting delayed flowering time (Fig. 5C). This would suggest that the FLD/LD/SDG26 repression of FLC transcription involves inhibition of SDG8 function. In comparison, the sdg8 mutation largely, but not completely, reversed the expression of FLC (Fig. 5A and B) and flowering time (Fig. 5C) caused by fca-9, suggesting that FCA can, to a limited extent, also repress FLC via a pathway that is independent of FLD and SDG8.

**FCA Requires PRC2 to Silence FLC.** The above data support a model where the alternative 3’ processing of COOLAIR by FCA mediates the silencing of FLC by Polycomb Repressive Complex 2 (PRC2) via inhibiting an active transcription module consisting of H3K4me1, H3K36me3, and transcription, which antagonizes the deposition of H3K27me3 (30). We tested this model by asking whether PRC2 is required by FCA to silence FLC. We took advantage of an Arabidopsis progenitor line carrying a single insertion of a 35S::FCAγ transgene in combination with an active FRIGIDA allele, in an otherwise wild-type background, which we had used to identify mutations suppressing the ability of FCA to down-regulate FLC (11). This sensitized background enhances FLC derepression and so is an efficient way to screen for factors required for FCA function. A weak allele of clf, reduced in PRC2 H3K27me3 methyltransferase activity (31), was introduced into this 35S::FCAγ genotype. clf-81 strongly released FLC expression, much more than in the Col background (Fig. 5D and E), supporting that FCA requires PRC2 to silence FLC. In line with our findings, Tian et al. showed that CLF enrichment at the FLC locus requires FCA function (32).

**Discussion**

Studying the quantitative transcriptional regulation of the A. thaliana floral repressor FLC has led us into dissection of how alternative processing of antisense transcripts regulates local chromatin environment and thus transcriptional output (7). We find that dynamic interactions between RNA-binding proteins, 3’ processing factors, and the chromatin modifiers FLD/LD/SDG26 result in a chromatin state associated with low transcriptional initiation and slow elongation, marked by low H3K4me1, low H3K36me3, and high H3K27me3. Loss of any of the factors switches the locus to the opposing high transcriptional state, overaccumulation of H3K4me1 and H3K36me3 and reduction of H3K27me3. We propose that the FLD/LD/SDG26 exist in a complex that inhibits an active transcription module, so promoting the deposition of H3K27me3 (SI Appendix, Fig. S5). This process parallels with the cleavage and polyadenylation factor (CPF)-mediated facultative heterochromatin assembly in yeast (33), the exact mechanism of which is still unknown.

FCA associates dynamically with 3’ processing factors in FCA nuclear bodies (9). The fact that the interactions between SDG26 and 3’ processing factors were only detected after cross-linking suggested that the interactions are also dynamic, and raised the possibility that FLD/LD/SDG26 might colocalize in FCA nuclear bodies. LD, like FCA and FY, has been found to contain a prion-like domain (34) (SI Appendix, Fig. S2A), which was identified as a driver for ribonucleoprotein granule assembly.

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**A**

| Protein | AGI code | Mol. Mass | No. of unique peptides |
|---------|----------|-----------|------------------------|
| SDG26   | AT1G7610 | 55 kD     | 108 115 36 21          |
| FLD     | AT3G10390| 96 kD     | 68 79 61 35            |
| LD      | AT4G02560| 105 kD    | 80 91 134 83           |
| FY      | AT5G13480| 72 kD     | 1 3 1 0               |
| FCA     | AT4G16280| 92 kD     | 1 3 0 0               |
| FPA     | AT2G43410| 100 kD    | 23 22 5 2             |
| CPSF160 | AT5G51660| 158 kD    | 1 4 0 0               |

**B**

![Figure 3](image-url)  
**Fig. 3.** SDG26 transiently associates with 3’ processing factors. (A) A partial list of proteins identified by SDG26 and LD affinity purifications after cross-linking. Nontransgenic Col-0 and transgenic line expressing GFP alone were included as negative controls in both purifications, and the read counts were all zero for the listed proteins. (B) Co-IP in stable transgenic plants after cross-linking to detect the association of SDG26 with FY.

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**C**

![Figure 4](image-url)  
**Fig. 4.** Measurements of histone modification levels upon the loss of the FLD/SDG26 complex. (A) Schematic diagram showing FLC gene structure. Gray boxes represent untranslated regions, and black boxes represent exons. The other regions are represented by the black line. The arrow indicates the transcription start site (TSS). Short black lines indicate positions of primers used for qPCR amplification. (B–E) ChIP analysis of H3K4me1 (B), H3K4me2 (C), H3K36me3 (D), and H3K27me3 (E) levels at FLC in various genetic backgrounds. The letters on the x axis correspond to the positions indicated in A. Data are shown as relative to H3. Values are means ± SEM from three independent biological replicates.
An important question raised by this work is what is the active transcription module that FLD/LD/SDG26-containing complex inhibits. We were unable to find any histone methyltransferase activity in vitro for the FLD/LD/SDG26 complex (SI Appendix, Fig. S4), suggesting that additional components are required for the complex to exert its function. One tantalizing hypothesis is that the histone-modifying activity is tightly linked to the RNA polymerase II (Pol II) complex during transcription. Indeed, we detected Pol II subunits (e.g., NRPB1, NRPB2, and NRPB3) and factors involved in the regulation of transcription initiation and elongation (e.g., SPT5, SPT6, and SPT16) in the SDG26 CLNIP-MS list (Dataset S4). In addition, LD contains a PP1-AP–like domain shared with the transcription elongation factor TFIIS, suggesting a role for LD in transcriptional elongation (38). Further analysis of these possibilities will expand our understanding of how the RNA-binding protein FCA connects COOLAIR to antagonizing an active transcription module, thereby eventually leading to Polycomb silencing. Full dissection of this mechanism will reveal any further parallels between COOLAIR and Xist function, thus elaborating our evolutionary understanding of RNA-mediated chromatin silencing.

Materials and Methods

More detailed descriptions of the materials and methods used in this study are provided in the SI Appendix. A brief summary is provided here.

Plant Materials. The progenitor lines C2 and 35S::FCACol (11) and the mutants fdl-4 and fca-9 (11), sdg8 (39), and clf-81 (40) were described previously. The transfer-DNA (T-DNA) insertion line id-1 (CS876430) and sdg26-3 (GK-087812) were obtained from the Nottingham Arabidopsis Stock Centre.

Flowering Time Analysis. The flowering time was determined essentially as described (9). Briefly, plants were grown in long-day conditions, and the total leaf number (TLN) produced before the initiation of flowering was counted to measure variation in flowering time.

RNA Analysis. RNA analysis was performed as described previously (9). Briefly, total RNA was extracted, treated with DNase, and reverse-transcribed by SuperScript IV Reverse Transcriptase (Invitrogen) using gene-specific reverse primers. Quantitative reverse transcription and PCR (qPCR) analysis was performed on a LightCycler480 II (Roche), and qPCR data were normalized to UBC. Primer pairs for amplifying unspliced FLC, spliced FLC, and UBC are listed in SI Appendix, Table S1.

Immunoprecipitation and Immunoblot. Extracts were prepared and immunoprecipitated with either anti-FLAG M2 Magnetic Beads (Sigma-Aldrich; M8823) or GFP-Trap Magnetic Agarose (ChromoTek; gtma-10).

For immunoblot analysis, protein extracts or immunoprecipitates were separated by SDS/PAGE, transferred to PVDF membranes, and detected by GFP (Roche; no. 11814460001), FLAG (Sigma-Aldrich; F3165), or FY (20) antibodies.

Materials and Data Availability. Full lists of mass spectrometry are provided as Datasets S1–S5. All of the other raw data and materials that support the findings of this study are available from the corresponding authors upon reasonable request.

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Fig. 5. The genetic relationships of FCA and FLD with SDG8 and PRCL2. (A and B) Expression of spliced FLC (A) and unspliced FLC (B) relative to UBC in the indicated genotypes. Data are presented as the mean ± SD (n = 3). Asterisks indicate significant differences between the indicated plants (*P < 0.017, **P = 0.0043, ****P = 6.27105E-05, two-tailed t test). Each dot represents one biological replicate. (C) Flowering time of indicated plants (assayed as total leaf number, produced by the apical meristem before it switched to producing flowers) grown in a long-day photoperiod. Data are presented as the mean ± SD (n ≥ 10). Asterisks indicate significant differences between the indicated plants (****P ≤ 2.26769E-09, two-tailed t test). (D and E) Expression of spliced FLC (D) and unspliced FLC (E) relative to UBC in the indicated genotypes. Note that expression level in the mutant background was separately normalized to its corresponding wild-type background. Data are presented as the mean ± SD (n = 3). Asterisks indicate significant differences between the indicated plants (*P ≤ 0.0458, two-tailed t test). Each dot represents one biological replicate.
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