The arabidopsis epigenetic regulator ICU11 as an accessory protein of polycomb repressive complex 2

Citation for published version:
Bloomer, RH, Hutchison, CE, Bäurle, I, Walker, J, Fang, X, Perera, P, Velanis, C, Gümüs, S, Spanos, C, Rapp, M, Feng, X, Goodrich, J & Dean, C 2020, 'The arabidopsis epigenetic regulator ICU11 as an accessory protein of polycomb repressive complex 2', Proceedings of the National Academy of Sciences (PNAS). https://doi.org/10.1073/pnas.1920621117

Digital Object Identifier (DOI):
10.1073/pnas.1920621117

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Proceedings of the National Academy of Sciences (PNAS)

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
The Arabidopsis epigenetic regulator ICU11 as an accessory protein of Polycomb Repressive Complex 2

Rebecca H. Bloomer1,a, Claire E. Hutchison1,a, Isabel Bäurle1,b, James Walker1,a, Xiaofeng Fang1,a, Pumi Perera1,a, Christos N. Velanis1,b, Serin Gümüş1,a, Christos Spanos1,a, Juri Rappsilber1,e, Xiaoqi Feng1,a, Justin Goodrich2,b, and Caroline Dean2,c,d

1Department of Cell and Developmental Biology, John Innes Centre, Colney Lane, NR47UH Norwich, United Kingdom; 2Institute of Molecular Plant Sciences, University of Edinburgh, Max Born Crescent, EH9 3BF Edinburgh, United Kingdom; 3Faculty of Biotechnology, Hochschule Mannheim, 68163 Mannheim, Germany; 4Wellcome Centre for Cell Biology, University of Edinburgh, Max Born Crescent, EH9 3BF Edinburgh, United Kingdom; and 5Institute of Biotechnology, Technische Universität Berlin, 13355 Berlin, Germany

Contributed by Caroline Dean, May 22, 2020 (sent for review December 2, 2019; reviewed by Claudia Köhler and Jose Luis Micol)

Molecular mechanisms enabling the switching and maintenance of epigenetic states are not fully understood. Distinct histone modifications are often associated with ON/OFF epigenetic states, but how these states are stably maintained through DNA replication, yet in certain situations switch from one to another remains unclear. Here, we address this problem through identification of Arabidopsis INCURVATA11 (ICU11) as a Polycomb Repressive Complex 2 accessory protein. ICU11 robustly immunoprecipitated in vivo with PRC2 core components and the accessory proteins, EMBRYONIC FLOWER 1 (EMF1), LIKE HETEROCHROMATIN PROTEIN1 (LHP1), and TELOMERE_REPEAT BINDING FACTORS (TRBs). ICU11 encodes a 2-oxoglutarate-dependent dioxygenase, an activity associated with histone demethylation in other organisms, and mutant plants show defects in multiple aspects of the Arabidopsis epigenome. To investigate its primary molecular function we identified the Arabidopsis FLOWERING LOCUS C (FLC) as a direct target and found icu11 disrupted the cold-induced, Polycomb-mediated silencing underlying vernalization. icu11 prevented reduction in H3K36me3 levels normally seen during the early cold phase, supporting a role for ICU11 in H3K36me3 demethylation. This was coincident with an attenuation of H3K27me3 at the internal nucleation site in FLC, and reduction in H3K27me3 levels across the body of the gene after plants were returned to the warm. Thus, ICU11 is required for the cold-induced epigenetic switching between the mutually exclusive chromatin states at FLC, from the active H3K36me3 state to the silenced H3K27me3 state. These data support the importance of physical coupling of histone modification activities to promote epigenetic switching between opposing chromatin states.

epigenetic | ICU11 | Polycomb | chromatin

Epigenetic silencing is mediated by conserved histone-based mechanisms in many organisms. Polycomb Repressive Complex 2 (PRC2) delivers a H3K27me3-based silencing that plays major roles in developmental and environmental epigenetic regulation (1–3). The PRC2 core components are conserved between organisms and associate with more diverse PRC2 accessory proteins that recognize sequence-specific or chromatin features such as CpG islands (4–13). A well-studied PRC2 target in Arabidopsis is the floral repressor locus, FLOWERING LOCUS C (FLC) (14). The prolonged cold of winter epigenetically silences the locus enabling expression in the spring of the genes required for the meristem to adopt a floral fate (15–18). FLC silencing occurs in a two-step cis-mediated PRC2 switching mechanism mediated by distinct complexes. During the cold, a PRC2 containing SWINGER (SWN) and associated with the accessory proteins VERNALIZATION5 (VRN5) and VERNALIZATION INSENSITIVE3 (VIN3) nucleates H3K27me3 silencing at a small intragenic site and this confers a metastable silencing (19). After return to warm, a PRC2 containing CURLY LEAF (CLF) and LHP1 mediate spreading of H3K27me3 silencing across the gene body, and this confers long-term stable silencing (19). Resetting FLC expression then occurs as the embryos develop to ensure vernalization is required each generation, a process requiring the H3K27me3 demethylase, EARLY FLOWERING 6 (ELF6) (20).

Modeling of the mechanism at FLC has provided a generic view of how opposing chromatin states, marked by a transcriptionally active H3K36me3 state and a silenced H3K27me3 state, can provide epigenetic stability, yet switch from one to another (1, 15, 21). This mechanism involves positive feedbacks to maintain each state and nonlinearity in the interaction mechanisms (22). Physical coupling of methylation and demethylation activities has been proposed to contribute to cooperativity in the system, a prediction partly validated for the Arabidopsis system.

Significance

Epigenetic regulation of gene expression is associated with switching between chromatin states characterized by distinct histone modifications. Polycomb/Trithorax regulation involves the mutually exclusive H3K27me3/H3K36me3 modifications, but how these states are faithfully inherited through DNA replication, yet can switch from one to another, is still poorly understood. One mechanism that would aid switching is the association of histone methyltransferases with factors demethylating the opposing histone modification. Here, we show that an Arabidopsis 2-oxoglutarate-dependent dioxygenase, an activity associated with histone demethylation in other organisms, physically associates with the Polycomb Repressive Complex 2. We propose that physical association of histone methylation/demethylation activities will be generally important to coordinate switching between chromatin states.

Author contributions: R.H.B., C.E.H., and C.D. designed research; R.H.B., C.E.H., I.B., J.W., X. Fang, P.P., C.N.V., S.G., C.S., J.R., X. Feng, and J.G. performed research; R.H.B. analyzed data; and R.H.B. and C.D. wrote the paper.

Reviewers: C.K., Swedish University of Agricultural Sciences; and J.L.M., Universidad Miguel Hernández.

The authors declare no competing interest.

This open access article is distributed under Creative Commons Attribution License 4.0 (CC BY).

Data deposition: DNA methylation analysis data have been deposited in the Gene Expression Omnibus (GEO) repository (accession no. GSE151449).

3Present address: The New Zealand Institute for Plant and Food Research, 7608 Lincoln, New Zealand.

4Present address: William Harvey Research Institute, Charterhouse Square Barts and the London School of Medicine and Dentistry, Queen Mary University of London, EC1M 6BQ London, United Kingdom.

5Present address: Institute for Biochemistry and Biology, University of Potsdam, 14469 Potsdam, Germany.

*To whom correspondence may be addressed. Email: caroline.dean@jic.ac.uk.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1920621117/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1920621117

PNAS Latest Articles | 1 of 7
by the finding that the H3K27me3 demethylase ELF6 physically interacted in vivo with the H3K36me3 methyltransferase SET DOMAIN GROUP 8 (SDG8) (23). Whether there was a similar physical interaction linking H3K36me3 demethylation with the Polycomb methyltransferases was not known.

Here, through analysis of a Ds transposon-tagged Arabidopsis mutation showing pleiotropic developmental phenotypes we identify an activity associated with H3K36me3 demethylation. The mutation was initially called *wavy leaves and cotyledons furled back* (wlc-1) and was identified as an early flowering, deformed leaf mutant in the Landsberg erecta genotype (24). Cloning revealed the gene (At1g22950) corresponds to the recently described *ICU11* locus (25), so the mutant is hereafter referred to as icu11-3. *ICU11* is part of a small family of genes in the *Arabidopsis* genome with partially redundant functions. *icu11* mutants misexpress many developmental regulators, share many phenotypes with double mutants, and genetic analysis linked ICU11 activity with developmental regulators, share many phenotypes with *icu11* mutants, and genetic analysis linked ICU11 activity with PRC2 function (25). Here, we extend this understanding and show that ICU11 robustly associates with PRC2 components in plants, and when defective, PRC2-mediated repression is compromised. Through analysis of a direct target, the floral repressor locus FLC, we find that ICU11 facilitates H3K36me3 demethylation and promotes the switch to the Polycomb H3K27me3 silenced state. *icu11* also shows other subtle epigenetic changes, so we propose that perturbed histone demethylation causes increased transcriptional activity of ICU11 targets with direct effects on PRC2 silencing and indirect effects on the wider *Arabidopsis* epigenome.

**Results**

**Ds insertion into At1g22950 Results in Weak PcG Mutant Phenotypes.** The wlc-1 mutant, hereafter *icu11*-3, was identified in an *At* Ds transposon-tagging mutagenesis screen (24) through its phenotypes, which include small size, pronounced cotyledon and leaf curling and early flowering in short days (Fig. 1A and B). Subsequent cloning revealed it to be *ICU11*, a 397-amino acid protein of unknown function (At1G22950, TAIR10), with the InterPro EMBL-EBI protein domain database predicting an Fe²⁺/2-oxoglutarate-dependent dioxygenase (2OG) domain with homology to *Arabidopsis* prolyl-4-hydroxylases (P4Hs) and alpha ketoglutarate-dependent dioxygenases B (AlkB) (26). In *icu11*-3, the insertion of the transposed Ds into the fifth exon introduces a premature stop codon, truncating ICU11 before the predicted enzymatic domain (SI Appendix, Fig. S1A). In addition, the *icu11*-3 mutant expresses approximately fourfold less *ICU11* at the mRNA level (SI Appendix, Fig. S1B). Transformation of the *icu11*-3 mutant with genomic *ICU11* alone, or genomic *ICU11* with C-terminal enhanced green fluorescent protein (eGFP) or 3xHA fusion constructs complemented *icu11*-3, rescuing the observed morphological
Table 1. ICU11 associates with the PRC2

| Protein            | 35S::GFP     | icu11-3      | icu11-3      | 35S::GFP     | icu11-3      | Col-0  | 35S::GFP     | clf-50  | gSWN-GFP     |
|--------------------|--------------|--------------|--------------|--------------|--------------|--------|--------------|---------|--------------|
| Core PRC2          |              |              |              |              |              |        |              |         |              |
| ICU11              | 0-0          | 13-19        | 0-0          | 12-11        | 0-0          | 11-20  | 0-0          | 13-2    | 5-5          |
| EMF2               | 0-0          | 11-4         | 0-0          | 29-0         | 0-0          | 2-22   | 0-0          | 33-18   | 4-36         |
| MS1                | 0-0          | 12-6         | 0-0          | 25-2         | 0-0          | 3-2    | 6-18         | 2-0     | 22-18        |
| FIE                | 0-0          | 10-1         | 0-0          | 18-0         | 0-0          | 5-17   | 2-0          | 23-22   | 6-25         |
| SWN                | 0-0          | 10-0         | 0-0          | 27-0         | 0-0          | 0-26   | 0-0          | 11-0    | 21-68        |
| CLF                | 0-0          | 7-0          | 0-0          | 26-0         | 0-0          | 3-17   | 9-0          | 75-46   | 8-4          |
| Accessory          |              |              |              |              |              |        |              |         |              |
| EMF1               | 0-0          | 5-3          | 0-0          | 26-2         | 0-0          | 6-40   | 0-0          | 46-17   | 2-37         |
| LHP1               | 0-0          | 2-1          | 0-0          | 11-0         | 0-0          | 2-14   | 0-0          | 22-8    | 3-18         |
| TRB1               | 0-0          | 5-9          | 0-0          | 12-2         | 0-0          | 0-1    | 0-0          | 0-0     | 0-0          |
| TRB2               | 0-0          | 9-5          | 0-0          | 15-1         | 0-0          | 1-11   | 0-0          | 11-3    | 2-10         |
| TRB3               | 0-0          | 9-6          | 0-0          | 15-3         | 0-0          | 3-12   | 0-0          | 9-4     | 2-7          |
| VRN5               | 0-0          | 0-0          | 0-0          | 0-0          | 0-0          | 0-0    | 0-0          | 24-13   | 0-24         |
| VEL1               | 0-0          | 0-0          | 0-0          | 0-0          | 0-0          | 0-0    | 0-0          | 41-28   | 0-46         |

The numbers indicate uniquely identified peptides from each protein found by mass spectrometry in two independent experiments. The total number of peptides identified in each experiment is also indicated at the bottom of the columns. PRC2 core components are shown at the top, with accessory components below. Columns are paired with control on left and immunoprecipitated to the right. For example, 35S::GFP samples in column 1 were the control for the ICU11-GFP samples in column 2 in the ICU11-GFP analysis. 35S::GFP-CLF clf-50 and gSWN-GFP share the 35S::GFP control. The full list of proteins identified is presented as an excel sheet in Dataset S1.

defects (SI Appendix, Figs. S1C and S2 A and B) and the misexpression of target genes in young seedlings (SI Appendix, Figs. S1D and S2 C and D). In addition, revertants were generated through Ac-induced remobilization of Ds to generate wild-type (WT) plants and fully stable mutant plants; in the latter the excision had led to a frame shift in the coding sequence. ICU11 is widely expressed, appearing elevated during later plant development (SI Appendix, Fig. S1E). While pICU11::ICU11-eGFP constructs showed broad expression of the protein in roots, nuclear localization at the subcellular level and association with metaphase chromosomes (SI Appendix, Fig. S1F).

To investigate the early flowering of icu11-3 in short days, we compared the expression of the floral activator FT, a known PRC2 target, during early seedling development under short-day growth conditions. FT was up-regulated in icu11-3 compared with the progenitor line (C12b), with the overexpression phenotype becoming more pronounced with seedling age (Fig. 1C). To investigate the potential misregulation of PRC2 targets more generally, we performed quantitative RT-PCR (qRT-PCR) on RNA extracted from 14-d-old seedlings of both icu11-3 and the progenitor line (Fig. 1D) and found the icu11-3 mutant overexpresses a range of PRC2 target genes compared with WT, including the reproductive transition regulators FT and FLC, floral genes AG, AP1, and AP3, and the shoot meristem maintenance gene STM. We crossed icu11-3 with clf-2 and compared morphological and gene expression phenotypes in the single and double mutants. As found previously, double mutants exhibit severe morphological defects (Fig. I A) and higher up-regulation of the flowering targets FLC, FT, and AG compared with either single mutant alone (Fig. 1E), similar to combination of clf with mutations in other PRC2 accessory proteins such as lhp1 (27).

ICU11 Is a PRC2 Accessory Protein. To investigate ICU11 function further, we performed coimmunoprecipitation/mass spectrometry (coIP-MS) experiments using 3xHA- and GFP-tagged ICU11 proteins as bait. Our IP-MS revealed core PRC2 complex components CLF, SWN, FERTILIZATION INDEPENDENT ENDOSPERM (FIE), MULTICOPY SUPPRESSOR OF IRA1 (MSI), and EMBRYONIC FLOWER (EMF)2 and accessory proteins EMF1, LHP1, and TELOMERE-REPEAT-BINDING (TRB) 1–3, as ICU11 interactors. EMF1 and LHP1 have been found in several studies as direct interactors of PRC2 (28, 29), and TRBs have been found associated with CLF and SWN (11). JM14, a H3K4me3 demethylase (30), was also detected in the ICU11-enriched peptides. Reciprocal coIP-MS with CLF-GFP, SWN-GFP, and EMF1-FLAG confirmed the interaction between ICU11 and PRC2 (Table 1 and Dataset S1). Interestingly, we did not find ICU11 interacting with VIN3 or its homologs VRN5 and VEL1, reinforcing the view of distinct Polycomb complexes operating over different spatial and temporal timescales.

ICU11 as a Putative Histone Demethylase. To further investigate the role of ICU11 in epigenetic regulation we used Western blots to determine levels of histone modifications associated with active chromatin (H3K4me1, H3K4me2, H3K4me3; H3K36me3) and those associated with silenced chromatin (H3K27me3) in WT and icu11-3 14-d-old seedlings. We observed a small increase in H3K4me2/me3, and H3K36me3 in icu11-3 relative to WT (Fig. 2A and SI Appendix, Figs. S3 and S4). These data, together with the analysis of FLC derepression in the icu11-3 mutant, raised the possibility that a primary activity of ICU11 was demethylating of histone modifications associated with active chromatin states, for example, H3K4me2/3 and H3K36me3. Loss of one protein may destabilize the whole-protein complex that contains putative demethylases specific for different histone modifications. A combination of activities, e.g., ICU11 and JM14 in the whole complex would then link demethylation of active histone modifications at multiple sites on the histone tail. This linking of activities in vivo means in vitro analyses are the best way to define the specific activity of different demethylases. The 2OGD domain of ICU11 falls within the same enzymatic superfamily as Jumonji C-domain histone demethylases. Despite considerable effort using in vitro assays using recombinant protein and commercial hisetones, or by colocalization assays of demethylation and expressed ICU11 protein in Nicotiana benthamiana transient assays, we could not generate reproducible data to clearly support a direct H3K4 or K36me3 demethylase role for ICU11. This is not uncommon for this family of proteins and may indicate that ICU11 requires accessory proteins or specific post-translational modification for histone demethylase activity. It is also possible that ICU11 modifies another component of the complex, which then allosterically influences histone demethylation.

DNA Methylation Is Subtly Changed in icu11-3. The wide range of phenotypes in icu11 mutants prompted us to look for changes in other epigenetic marks. In plants, DNA methylation occurs in
CG, CHG, and CHH contexts (H = A, T, or C). CG methylation is broadly distributed, present in both gene bodies and repeat-rich regions; while CHG and CHH methylation are primarily found in transposable elements (31, 32). Southern blot analysis of genomic DNA digested with the differentially methylated-sensitive enzymes MspI (control) and methylation-sensitive enzyme HpaII indicates slightly reduced DNA methylation over centromeric repeats in icu11-3.

CG methylation over transposon sequences were slightly increased in the icu11-3 mutant (SI Appendix, Fig. S6B), but these slight changes in the non-CG contexts are consistent with the variations seen among plant tissues and plants grown in different environmental conditions due to the dynamics of non-CG methylation (33, 34). Overall, therefore these data agree with previous observations that ICU11 function does not have a large impact on DNA methylation (25). The observed effects may be due to indirect effects of increased transcription at ICU11 targets on the wider Arabidopsis epigenome.

**icu11 Perturbs the Vernalization Response in Arabidopsis.** To clarify the functional implications of the physical and genetic interaction of ICU11 with PRC2, we asked how the icu11 mutation affected vernalization, a well understood PRC2-regulated developmental transition. To set a high FLC expression state and therefore a vernalization requirement, we crossed icu11-3 to fca-1. icu11-3 fca-1 plants showed an increased variability in flowering compared to fca-1 (Fig. 3 A and B and SI Appendix, Table S4). Cold treatment of different durations revealed a clear vernalization defect in icu11-3 fca-1 plants; flowering was significantly delayed after 2 wk cold treatment (SI Appendix, Table S4) and was only marginally accelerated with longer cold. No difference in flowering was observed in the icu11-1 single mutant following vernalization (SI Appendix, Fig. S7).

We collected tissue before vernalization (NV), on the final cold day after 2, 4, or 6 wk cold (T0), and at 10-d intervals (T10, 20, 30) upon return of plants to warm, long-day conditions, allowing us to examine the expression of key regulatory genes in the vernalization-induced flowering pathway. Expression of FT in fca-1 and icu11-3 fca-1 mirrored the differences observed in flowering time. Without cold, FT was expressed at ∼10-fold higher levels in icu11 fca-1, even though expression of FLC (its repressor) was also high. This presumably reflects misregulation of all Polycomb target genes in an icu11 background (Fig. 3C). Following increasing periods of cold exposure, FT expression levels increased in WT plants, but less so in icu11 fca-1. In fca-1, FLC expression decreased quantitatively by prolonged cold and was maintained in the transcriptionally silent state after return to warm conditions. In contrast, in icu11-3 fca-1, FLC expression was approximately twofold higher prior to vernalization (Fig. 3D), and although expression was reduced by cold, this was not maintained following vernalization. We interpret this as reflecting a reduced ability for the locus to digitally switch to an epigenetically silenced state. Consistent with this, the icu11-3 mutant showed higher expression of the FLC antisense transcript COOLAIR during the cold (Fig. 3E), but expression of the cold-induced FLC repressor VIN3, another PRC2 target, was unaffected (Fig. 3F).

**ICU11 Functions Directly at FLC to Affect the Balance of Histone Modifications.** Vernalization-induced silencing of FLC is dependent on PRC2-mediated switching between bistable opposing epigenetic states: an active state, marked by high levels of H3K36me3 at the nucleation region of FLC; and a silent state, marked by a peak of H3K27me3 in the nucleation region that accumulates during cold exposure and spreads to cover the entire FLC locus upon return to the warm. We had previously shown that H3K4me3 at FLC follows transcription rather than functioning as the opposing chromatin state to H3K27me3 (1). Comparison of icu11-3 fca-1 compared to fca-1 over the vernalization time series was used to define the role of ICU11 in FLC epigenetic switching. As expected, H3K36me3 was lost from the nucleation region in fca-1 after 4 wk of cold exposure and replaced by a peak of H3K27me3 that spread to cover FLC after 10 d subsequent growth in warm long-day conditions (Fig. 4). In contrast, in icu11-3 fca-1 the H3K36me3 peak was higher

*Fig. 2. icu11-3 affects global histone methylation and DNA methylation at the pericentromeric cen180 repeats. (A) The icu11-3 mutant shows elevation of H3K4me3 and H3K4 methylation, but no change in H3K27me3 compared with WT C12b. Histones were prepared from a nuclear extract from 14-d-old seedlings and serially diluted; Western blots are shown with total histone H3 from the same extraction as loading controls. (B) Southern blot analysis of methylation at the pericentromeric cen180 repeat region. Genomic DNA digested with the methylation-insensitive restriction enzyme MspI (control) and methylation-sensitive enzyme HpaII indicates slightly reduced DNA methylation over centromeric repeats in icu11-3.*
before vernalization and was not lost from the nucleation region following cold exposure or subsequent warm growth conditions. This was coincident with an attenuated accumulation of H3K27me3 at the locus following cold (Fig. 4). Comparison of H3K27me3 and H3K36me3 levels at ACT7 and STM indicated that histone methylation was relatively stable across time points and between genotypes at control genes (SI Appendix, Fig. S8). Thus, ICU11 is required for the cold-induced epigenetic switch between the active H3K36me3 state to the silenced H3K27me3 state at FLC.

These analyses had been undertaken using icu11-3, a mutation in the Landsberg erecta (Ler) background. In Ler, a Mutator-like transposable element (MULE) in the first intron of FLC leads to low expression before vernalization compared with other alleles. We wanted to confirm the effect of ICU11 at FLC was on Polycomb switching rather than an influence FLC through an effect of the MULE. In the first intron of FLC, using a GFP-tagged ICU11 construct transformed into the icu11-3 fca-1 background. ICU11 was localized at the FLC 5′ end coincident with the H3K36me3 enrichment, both before vernalization and at the end of a 4-wk cold treatment (Fig. 4E). We also observed a small increase in ICU11 occupancy over the gene body following cold exposure or subsequent warm growth conditions.

To confirm our hypothesis that ICU11 does not influence FLC via an effect of the MULE, we utilized a pFLC::FLC-GUS reporter construct generated using the Col-0 FLC allele (pFLC::FLC<sub>Col-0</sub>-GUS), which lacks the MULE. This construct was crossed into the icu11-3 (Ler) background, and WT and icu11-3 siblings from an F2 population examined in nonvernalizing conditions. In icu11-3, FLC expression was strongly up-regulated before cold even in the absence of the MULE (Fig. 4E). We used a second Col-0 allele construct, pFLC::FLC<sub>Col-0</sub>-LUCIFERASE, to examine whether the FLC reactivation seen following vernalization was also independent of transposon derepression in icu11-3. The pFLC::FLC<sub>Col-0</sub>-LUCIFERASE construct was backcrossed into the icu11-3 fca-1 background. Paralleling our observation of the native FLC<sup>Ler</sup> allele in icu11-3 fca-1 (Fig. 3D), we found that FLC-LUC expression was higher in the icu11-3 background than in WT prior to

![Image](https://example.com/image.png)

**Fig. 3.** icu11-3 is defective in vernalization. (A and B) Flowering is significantly more asynchronous at all time points and significantly delayed in the icu11-3 fca-1 genotype after 2 wk vernalization (SI Appendix, Table S4). Days to flowering counted from the end of a pregrowth period of 14 d in warm conditions (NV), or from the end of pregrowth followed by a 2-, 4-, or 6-wk vernalization treatment. Bars represent mean and SEM (n = 20). (B) Flowering plants imaged at 25 d postvernalization. (C–F) Gene expression in the vernalization-requiring fca1 and icu11-3 fca-1 backgrounds. Gene expression is normalized to PP2A; error bars represent SEM for three biological replicates. (C) FT expression at 30 d posttreatment is higher in fca1 than icu11-3 fca-1. (D) Expression of the flowering repressor FLC is higher in icu11-3 fca1 than fca-1 before (NV), during (T0), and 10, 20, or 30 d after vernalization (T10–30), reactivating strongly in icu11-3 fca1 compared with the stable postvernalization repression observed in fca-1. Increasing vernalization time (2, 4, and 6 wk) leads to lower and more stably silenced FLC expression. (E) The antisense RNA COOLAIR is expressed at higher levels in icu11-3 fca1 than fca-1. (F) Expression of VIN3, a cold-induced PRC2 target, is not affected by the icu11-3 mutation.
vernalization, was reduced by cold, but that transcription reactivated on return to the warm (Fig. 4G). Thus, defective silencing of FLC in icu11-3 is PRC2-dependent and independent of the MULE insertion in FLCler.

Discussion
Here, through analysis of a mutant displaying a range of PcG-impaired phenotypes we identify the ICU11 Fe2+/2 oxoglutarate-dependent oxygenase activity that influences Arabidopsis PRC2 regulation. The large 2OGD superfamily in Arabidopsis, of which ICU11 is a member, contains prolyl-4-hydroxylases, AlkB DNA repair enzymes and enzymes integral to an array of biosynthetic processes (26). However, ICU11 and its most closely related sequences form a separate clade within the Arabidopsis genome (25), suggesting distinct functionality. Our analysis of global changes and specific defects in the vernalization silencing process suggests that impairment of ICU11 primarily influences active histone marks—especially H3K36me3. A 2-OGD is the catalytically active demethylation domain of Jumonji histone demethylases; however, we could not reproducibly show using in vitro assays that ICU11 has this biochemical activity. Notably, previous studies have similarly struggled to demonstrate recombinant activity in vitro despite clear functional evidence for their function in vivo (35, 36).

The subtle alterations in DNA methylation in the icu11-3 mutant were unexpected for a mutation predominantly associated with Polycomb silencing. Such pleiotropic changes have not previously been reported for PRC2 mutants in Arabidopsis; indeed, a comparable Southern blot analysis indicates that even
the severe PR2C elf swm double mutant maintains effective cen180 repeat methylation (37). Thus, we speculate that any DNA methylation icu11 phenotypes are linked to altered transcriptional activity. ICU11 may work specifically as an H3K36me3 demethylase, influencing directly PRC2 targets and then indirectly a larger set of targets. Alternatively, ICU11 may act as a promiscuous histone demethylase targeting distinct methylation types according to the specificity of its binding partners. However, the robust immune precipitation with PRC2 components, but not other chromatin regulators, argues against this. ICU11 may also influence methylation levels of trans factors. For example, the histone demethylase regulators, argues against this. ICU11 may also influence methyl-demethylase targeting distinct methylation types according to the targets. Alternatively, ICU11 may act as a promiscuous histone demethylase. ICU11 may work specifically as an H3K36me3 demethylase, repeat methylation (37). Thus, we speculate that any DNA methylation antagonizes PRC2-mediated H3K27 methylation. J. Biol. Chem. 286, 7983–7989 (2011).

3. L. J. Gaydos, A. Rechtsteiner, T. A. Egelhofer, C. R. Carroll, S. Strome, Antagonism of a polycomb target gene instructs its own epigenetic inheritance. Proc. Natl. Acad. Sci. U.S.A. 105, 644–649 (2008).

4. M. Calonje, R. Sanchez, L. Chen, Z. R. Sung, MEDEA, is required for epigenetic silencing of Arabidopsis thaliana. Plant Cell 20, 277–291 (2008).

5. Y. Zhou, B. Hartwig, G. V. James, K. Schneeberger, F. Turck, Complementary activities between MES-4 and Polycomb repressive complex 2 promote appropriate gene expression in the Arabidopsis meristem. EMBO J. 18, 485–488 (2016).

6. F. De Lucia, P. Crevillen, A. M. E. Jones, T. Greb, C. Dean, A PHD-polycomb repressive factor protein VIN3. Nature 427, 159–164 (2004).