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The Evening Complex and the Chromatin-Remodeling Factor PICKLE Coordinately Control Seed Dormancy by Directly Repressing *DOG1* in *Arabidopsis*

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ABSTRACT

Primary seed dormancy is acquired during seed development and maturation, which is important for plant fitness and survival. DELAY OF GERMINATION1 (DOG1) plays a critical role in inducing seed dormancy. *DOG1* expression increases rapidly during seed development, but the precise mechanism underlying this process remains elusive. In this study, we showed that mutants with a loss or reduced function of the chromatin-remodeling factor PICKLE (PKL) exhibit increased seed dormancy. PKL associates with *DOG1* chromatin and inhibits its transcription. We found that PKL physically interacts with LUX ARRHYTHMO (LUX), a member of the evening complex (EC) of the circadian clock. Furthermore, LUX directly binds to a specific coding sequence of *DOG1*, and DOG1 acts genetically downstream of PKL and LUX. Mutations in either *LUX* or *EARLY FLOWERING3* (*ELF3*) encoding another member of the EC led to increased *DOG1* expression and enhanced seed dormancy. Surprisingly, these phenotypes were abolished when the parent plants were grown under continuous light. In addition, we observed that loss of function of either *PKL* or *LUX* decreased H3K27me3 levels at the *DOG1* locus. Taken together, our study reveals a regulatory mechanism in which EC proteins coordinate with PKL to transmit circadian signals for directly regulating *DOG1* expression and seed dormancy during seed development.

Key words: seed dormancy, chromatin remodeling, clock, DOG1

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INTRODUCTION

Seed dormancy is an important agricultural trait that helps plants survive under unfavorable conditions and prevents preharvest seed sprouting. Primary seed dormancy is induced during maturation and is maintained for a certain period of time in mature seeds. After-ripening or environmental cues trigger the release of dormancy, which leads to germination and the beginning of a new plant life cycle (Bentsink and Koornneef, 2008; Finkelstein et al., 2008; Graeber et al., 2012; Nee et al., 2017; Honogaki, 2019). Seed dormancy is controlled by both endogenous, such as phytohormone signalings, and exogenous factors, such as light (Jiang et al., 2016; Shu et al., 2016; Ravindran and Kumar, 2019).

Early studies have identified many quantitative trait loci (QTL) that contribute to dormancy in *Arabidopsis thaliana*, rice (*Oryza sativa*), and wheat (*Triticum aestivum*) (Alonso-Blanco et al., 2003; Osa et al., 2003; Gu et al., 2006; Bentsink et al., 2010). Further genetic and molecular studies have uncovered many genes involved in regulating the induction and release of seed dormancy and germination (Graeber et al., 2012; Nonogaki, 2014; Shu et al., 2016). *DELAY OF GERMINATION1* (*DOG1*) is a

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major QTL that was discovered in a recombinant inbred *Arabidopsis* population, and variation in *DOG1* transcript levels between accessions contributes to natural variation for seed dormancy (Alonso-Blanco et al., 2003; Bentsink et al., 2006). *DOG1* encodes a protein with unknown function. It forms a homodimer and the protein level is important for its role in seed dormancy (Nakabayashi et al., 2012, 2015). DOG1 regulates seed germination in part through influencing miR156 and miR172 levels in lettuce (*Lactuca sativa*) (Huo et al., 2016). DOG1 binds to heme and interacts with the type 2C protein phosphatase ABA HYPERSENSITIVE GERMINATION1, inhibiting its regulation of seed dormancy (Nishimura et al., 2018). *DOG1* is subject to alternative polyadenylation, which leads to the production of multiple transcript variants encoding three protein isoforms (Nakabayashi et al., 2015; Cyrek et al., 2016). AtNTR1, a component of the spliceosome, is required for the splicing and expression of *DOG1* (Dolata et al., 2015). The noncoding antisense transcript *asDOG1* suppresses the expression of the *DOG1* sense transcript in *cis* during seed maturation (Fedak et al., 2016). In turn, *asDOG1* transcript levels are controlled by C-TERMINAL PHOSPHATASE-LIKE1-mediated alternative polyadenylation of the sense transcript (Kowalczyk et al., 2017). Two recent studies showed that ETHYLENE RESPONSE FACTOR12 and basic LEUCINE ZIPPER TRANSCRIPTION FACTOR67 either negatively or positively regulate *DOG1* expression during seed maturation (Bryant et al., 2019; Li et al., 2019).

The circadian clock plays key roles in integrating multiple environmental signals (such as light and temperature) into endogenous transcriptional reprogramming, which enables plants to anticipate changes and to gate their responses according to the time of day. In *Arabidopsis*, the circadian oscillator is composed of multiple interlocking loops that function in transcriptional and post-translational regulation (Harmer, 2009; Pruneda-Paz and Kay, 2010; Greenham and McClung, 2015). Three clock proteins, LUX ARRHYTHMO (LUX), EARLY FLOWERING3 (ELF3), and ELF4, comprise the evening complex (EC), a critical component in the regulation of circadian outputs (Nusinow et al., 2011; Mizuno et al., 2014). LUX is an MYB-domain-containing transcription factor that directly binds to DNA through the cognate LUX binding site ([LBS]; GATT/ACG) in its target genes. ELF3 acts as an adaptor linking ELF4 and LUX, which form a ternary transcriptional repression complex (Helfer et al., 2011; Nusinow et al., 2011). However, the questions whether and how the circadian clock directly regulates *DOG1* expression and seed dormancy remain unknown.

PICKLE (PKL) is an ATP-dependent chromatin-remodeling factor that affects the levels of trimethylation of histone H3 Lys 27 (H3K27me3) at loci involved in cell elongation (Jing et al., 2013; Zhang et al., 2014). PKL plays essential roles in regulating various developmental processes and environmental responses, including embryonic development, root meristem activity, photomorphogenesis, and thermomorphogenesis (Ogas et al., 1999; Fukaki et al., 2006; Perruc et al., 2007; Aichinger et al., 2011; Jing et al., 2013; Zha et al., 2017). Here, we show that PKL and the EC negatively regulate seed dormancy. Mutations in *PKL*, *LUX*, or *ELF3* lead to reduced germination of freshly harvested seeds. PKL physically interacts with LUX, which directly binds to the regulatory regions and mediates the circadian regulation of *DOG1*expression. Moreover, PKL is recruited to *DOG1* chromatin and affects H3K27me3 levels to inhibit its expression. Therefore, EC proteins act together with the chromatin-remodeling factor PKL to prevent seeds from becoming overly dormant by directly controlling *DOG1* transcription during seed development.

RESULTS

PKL Inhibits Primary Seed Dormancy

PKL is involved in regulating seed germination (Perruc et al., 2007). The *pkl-1* seeds in mature siliques showed a lower level of germination than the wild-type (Columbia-0 [Col-0]) control (Figure 1A), suggesting that PKL likely also plays a role in seed dormancy. To confirm this notion, we grew Col-0 and *pkl* plants under identical growth conditions and collected seeds at the same developmental stage. Freshly harvested seeds of various *pkl* mutant alleles, including *pkl-1*, *pkl-10*, *pkl-11*, and *pkl-12* (Jing et al., 2013, 2019), exhibited lower germination rates than Col-0 seeds (Figure 1B and 1C). The reduced germination of *pkl* seeds was not due to developmental defects, as they all fully germinated after cold stratification (Figure 1D). The germination rates of *pkl* seeds gradually increased with increasing storage period (Figure 1E). The expression of the *PKLp:GUS* (*β-GLUCURONIDASE*) reporter gene in transgenic *Arabidopsis* seeds was clearly observed in embryos and endosperm after 12 h of imbibition (Figure 1F). The phytochrome B (phyB) photoreceptor mediates red/far-red reversible seed germination (Jiang et al., 2016). However, mutations in *PKL* did not affect light-induced regulation of seed germination (Supplemental Figure 1). These observations suggest that PKL regulates primary seed dormancy.

PKL Associates with the *DOG1* Locus and Represses Its Transcription

DOG1, REVEILLE1 (RVE1), and RVE2 are essential for controlling seed dormancy (Jiang et al., 2016). To explore how PKL regulates seed dormancy, we examined *DOG1*, *RVE1*, and *RVE2* transcript levels in freshly harvested seeds after imbibition. *DOG1* transcript levels were approximately 13- and 6-fold higher after 12 and 24 h of imbibition, respectively, in *pkl-1* versus Col-0 (Figure 2A), suggesting that downregulation of *DOG1* during imbibition is less inhibited in *pkl* and that PKL inhibits *DOG1* expression. However, *RVE1* and *RVE2* transcript levels did not drastically differ between the *pkl* mutant and the wild type (Supplemental Figure 2A).

The status of seed dormancy is mainly balanced by the antagonistic effects of two phytohormones: gibberellin (GA) and abscisic acid (ABA) (Finkelstein et al., 2008). Indeed, the expression levels of several genes encoding catalytic enzymes of the GA and ABA biosynthesis pathways were altered in *pkl-1* compared with Col-0 (Supplemental Figure 3A and S3B). In addition, the transcript levels of several germination-related genes, including *LATE EMBRYOGENESIS ABUNDANT1* (), *DORMANCY-ASSOCIATED PROTEIN1* (), *ABA HYPERSENSITIVE GERMINATION1* (), and *1-CYSTEINE PEROXIREDOXIN1* () (Finkelstein et al., 2008; Rae et al., 2013), were higher in *pkl-1* than in Col-0 (Supplemental Figure 3C), which is consistent with the reduced germination rate of *pkl*.

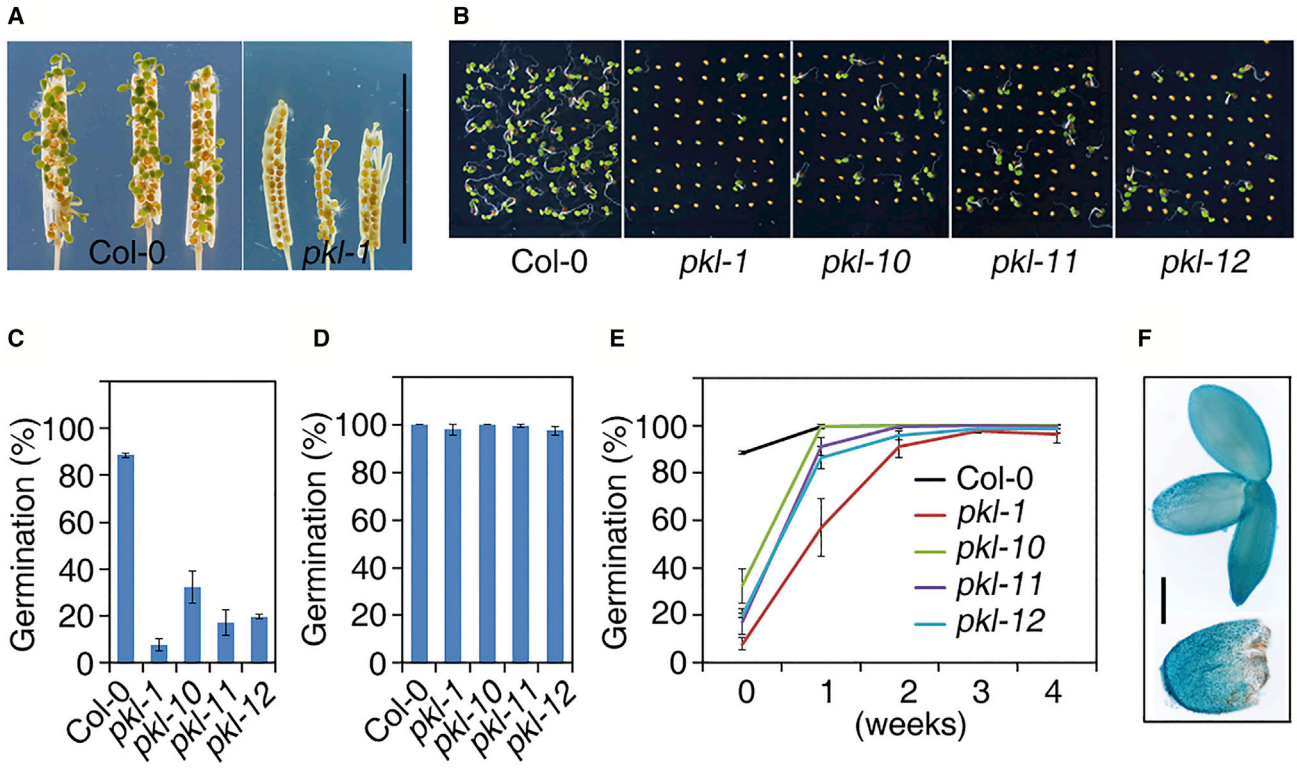

Figure 1. PKL Inhibits Seed Dormancy.

(A) Representative images of seed germination in mature siliques. Siliques at the same developmental stage were incubated on agar plates for 4 d. Scale bar, 1 cm.

(B) Germination of freshly harvested seeds on agar plates incubated under white light for 4 d.

(C) Germination frequency of the seeds shown in **(B)**.

(D) Germination frequency of seeds after 4°C stratification treatment for 3 d.

(E) Germination frequency of seeds stored at room temperature for up to 4 weeks.

In **(C)** and **(D)**, values denote the average ± SD of three replicates.

(F) *PKLp:GUS* expression in embryos and endosperm of freshly harvested seeds after 12 h of imbibition. Scale bar, 200 μm.

To explore the genetic relationship between PKL and DOG1, we generated a *pkl dog1* double mutant by crossing *pkl-1* with *dog1-2*. The reduced germination rate of *pkl-1* was largely suppressed in the *pkl dog1* double mutant (Figure 2B), suggesting that *DOG1* acts downstream of *PKL*. However, the germination rates of *pkl rve1* and *pkl rve2* were similar to those of the *pkl-1* single mutant (Supplemental Figure 2B), indicating that RVE1 and RVE2 do not genetically interact with PKL. Mutations in *phyB* also lead to increased seed dormancy (Jiang et al., 2016). Seeds of the *pkl phyB* double mutant had a much lower germination rate than those of the parental single mutant lines (Supplemental Figure 2C), suggesting that PKL and phyB function in parallel to repress seed dormancy.

PKL associates with the chromatin of its target loci (Jing et al., 2013; Zhang et al., 2014). We therefore performed a chromatin immunoprecipitation (ChIP) assay to investigate whether PKL binds to different chromatin regions of *DOG1* (Figure 2C). As shown in Figure 2D, the anti-PKL antibody pulled down significantly more chromatin from regions 3, 4, 5, and 6 of *DOG1* (spanning the exons and introns of this gene), but not its promoter regions (1 and 2), in Col-0 plants versus the *pkl* mutant, indicating that PKL associates with the gene body of *DOG1*.

PKL Physically Interacts with LUX

The chromatin-remodeling factor PKL interacts with multiple transcription factors via its central Helicase/ATPase domain (Jing et al., 2013; Zhang et al., 2014). We thus used this ATPase domain as the bait to screen for interacting factors in a yeast two-hybrid assay. The clock component LUX was identified in this screen and subjected to further investigation. To confirm the PKL-LUX interaction, we fused LUX with the B42 activation domain (AD) and full-length PKL or various PKL fragments with the LexA DNA-binding domain (BD) (Figure 3A). As shown in Figure 3B, BD-D6 containing the Helicase/ATPase domain of PKL and BD-D5 containing additional chromo-domains interacted with AD-LUX, as revealed by the presence of blue yeast colonies. BD-D3 and BD-D4 containing the PHD finger and/or chromo-domains did not interact with AD-LUX. Surprisingly, full-length PKL and PKL fragments D1, D8, and D9 failed to interact with AD-LUX. These results suggest that PKL interacts with LUX via its Helicase/ATPase domain and that the N-terminal PHD finger and the C-terminal portion of PKL likely prevent its contact with LUX in yeast cells. However, PKL and its fragments failed to interact with the two remaining EC components, ELF3 and ELF4 (Figure 3B). PKL also weakly interacted with CCA1, but not with LHY or TIMING OF CAB EXPRESSION1 (TOC1), in yeast cells (Supplemental Figure 4).

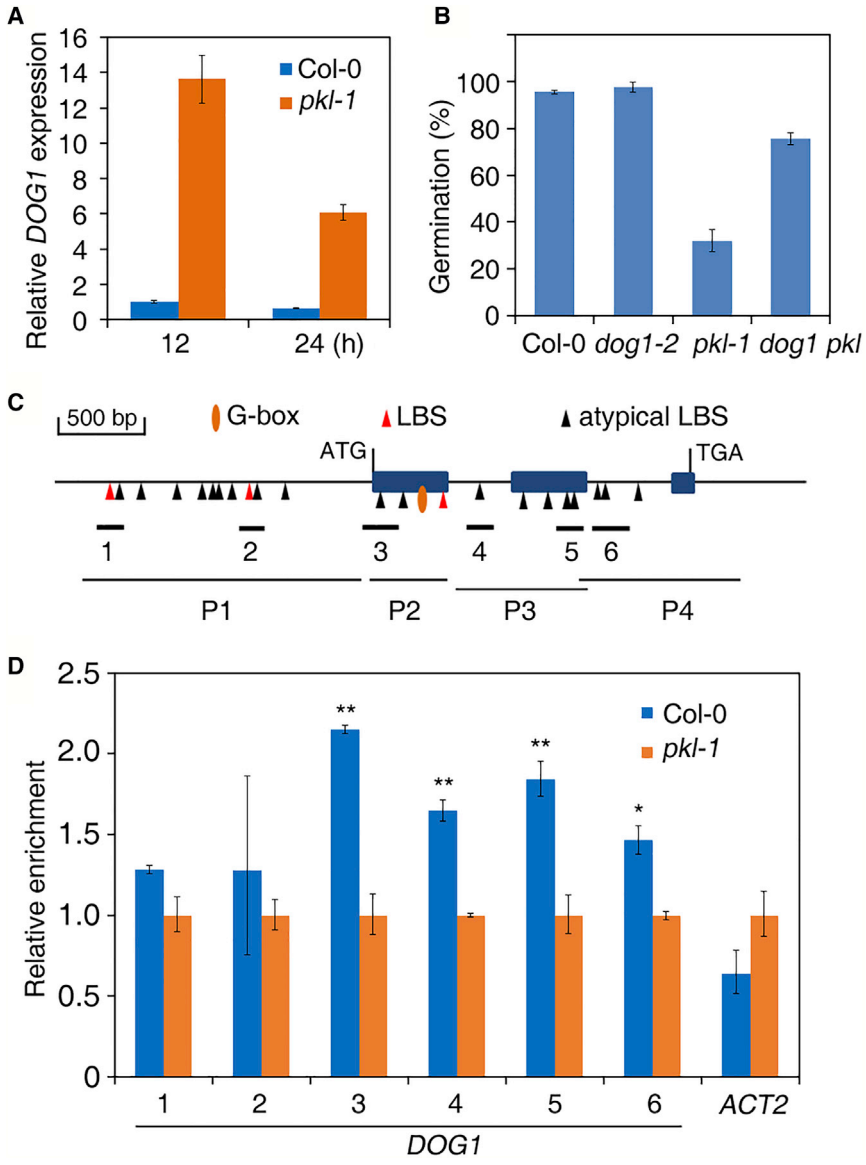

Figure 2. PKL Associates with *DOG1* Chromatin and Represses Its Expression.

(A) *DOG1* expression in freshly harvested seeds after 12 and 24 h of imbibition.

(B) Germination frequency of freshly harvested seeds incubated under white light for 4 d.

(C) Diagram of the *DOG1* locus. Black bars indicate exons. G-box, CACGTG; LBS, GATT/ACG. Numbers 1 to 6 indicate fragments subjected to amplification in the ChIP assays, and P1 to P4 denote regions used for the yeast one-hybrid assay.

(D) ChIP assay. PKL antibody was used to pull down different fragments of *DOG1*, shown in **(C)** and the *ACT2* control from Col-0 and *pkl-1* samples. Seedlings were grown under LD conditions for 5 d and samples were harvested at ZT4. Relative enrichment per antibody was normalized to the input value.

For **(A)**, **(B)**, and **(D)**, values denote the average ± SD of three biological replicates. Asterisks indicate significant differences from Col-0 using Student's *t*-test ($P < 0.01$).

Next, we performed an *in vitro* pull-down assay using purified His-D6 (containing the Helicase/ATPase domain of PKL) and GST-LUX or MBP-LUX recombinant proteins. The anti-GST antibody pulled down His-D6 when incubated with GST-LUX, but not with GST alone (Figure 3C). Similarly, the anti-MBP antibody pulled down His-D6 when incubated with MBP-LUX, but not with MBP alone (Figure 3D). We also generated constructs in which PKL was fused with the N terminus of luciferase (nLUC) and LUX was fused with the C terminus of luciferase (cLUC) and carried out a luciferase complementation imaging (LCI) assay in *Nicotiana benthamiana* leaves. Cotransformation of PKL-nLUC with LUX-cLUC led to strong LUC expression compared with the controls (Figure 3E). Taken together, these results confirm that PKL physically interacts with the EC by directly binding to LUX.

LUX Directly Binds the *DOG1* Chromatin

Multiple putative LBS *cis*-elements were present in the genomic region of *DOG1* (Figure 2C). We reasoned that the LUX transcription factor might directly bind to *DOG1*. To investigate this possibility, we performed a ChIP assay using anti-GFP antibody to probe *35S:GFP-LUX* transgenic lines versus the Col-0 control. Regions 4 and 5 of *DOG1* and two positive controls, *PSEUDO-RESPONSE REGULATOR7* (*PRR7*) and *PRR9*, but not the other regions of *DOG1* or the negative control *ACT2*, were significantly enriched with the anti-GFP antibody in *35S:GFP-LUX* plants compared with the wild type (Figure 4A and Supplemental Figure 5). We performed a yeast one-hybrid assay using various *DOG1* fragments (P1–P4, Figure 2C) linked to the *LacZ* reporter gene. The expression of *P2:LacZ* (position 1–432 bp) and *P3:LacZ* (459–1182 bp), but not *P1:LacZ* (−1584 to −15 bp) and *P4:LacZ* (1148–2234 bp), was activated by AD-LUX (Figure 4B). We then synthesized oligonucleotides containing the putative G-box or LBS motifs within regions P2 (P2-1 to P2-4) and P3 (P3-1 to P3-4) and constructed *LacZ* reporter vectors. As shown in Figure 4B and Supplemental Figure 6, AD-LUX specifically bound to P3-4 (containing GATACT and GATTCT) and activated *LacZ* expression. Finally, we purified MBP-LUX recombinant fusion proteins from *E. coli* and performed an electrophoretic mobility shift assay (EMSA). MBP-LUX fusion protein, but not MBP alone, bound to P3-4 oligonucleotides labeled with biotin and caused a mobility shift. The addition of unlabeled nucleotides drastically reduced the mobility shift signal (Figure 4C). These results demonstrate that LUX directly binds to *DOG1* at specific sequences.

LUX and ELF3 Inhibit *DOG1* Expression and Seed Dormancy

Next, we investigated DOG1 expression and seed dormancy in mutant alleles of *lux*, *elf3*, and *elf4*. *DOG1* transcript levels were higher in *lux-6* and *elf3-1*, but not *elf4-101*, compared with

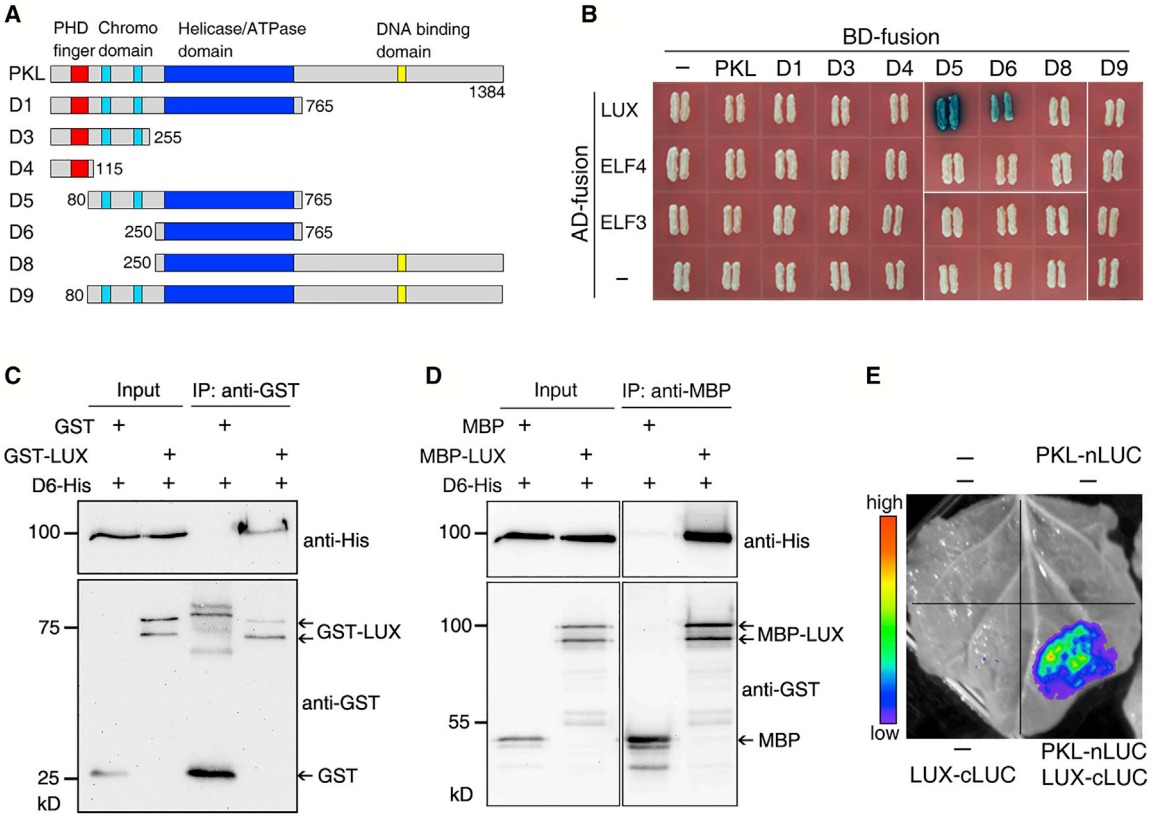

Figure 3. PKL Interacts with LUX.

(A) Diagram of the *PKL* domains and various deletions. Numbers indicate amino acid positions.

(B) Yeast two-hybrid assay. Full-length PKL and its deletion variants were fused with the LexA DNA-binding domain (BD-fusion), and LUX, ELF3, and ELF4 were tagged with the B42 activation domain (AD-fusion). Blue colonies denote protein–protein interactions.

(C and D) Pull-down assay. D6-His recombinant protein was incubated with GST-LUX **(C)** or MBP-LUX **(D)** and immunoprecipitated by anti-GST or anti-MBP antibodies, respectively.

(E) LCI assay. Full-length PKL was fused in-frame with the N terminus of LUC and LUX, ELF3, and ELF4 were fused in-frame the C terminus of LUC. Different plasmid compositions were cotransformed into *N. benthamiana* leaves.

Col-0 (Figure 4D). Strikingly, transient overexpression of *LUX* (*35S:LUX*) inhibited the expression of the *DOG1p:LUC* reporter gene (driven by the *DOG1* promoter and coding sequences) in *Arabidopsis* protoplasts (Figure 4E). The germination rates of newly harvested *lux-6*, *elf3-1*, and *elf3-7* seeds were lower than those of the wild type in both light and darkness (Figure 4F and 4G), suggesting that ELF3 and LUX are negative regulators of seed dormancy. However, *elf4-101* did not exhibit a distinct seed dormancy phenotype (Figure 4H). After cold stratification, the *lux*, *elf3*, and *elf4* seeds fully germinated, as did wild-type seeds (Figure 4I). Together, these results suggest that LUX and ELF3 repress *DOG1* expression and seed dormancy.

We also generated *elf3 dog1* and *lux dog1* double mutants and tested their genetic interactions. As shown in Figure 4J and 4K, the germination frequencies of *elf3 dog1* and *lux dog1* were similar to those of the *dog1* single mutant, indicating that DOG1 is epistatic to ELF3 and LUX.

LUX and PKL Regulate H3K27me3 Levels at the *DOG1* Locus

To investigate the molecular relevance of the PKL-LUX interaction, we performed a ChIP assay in the *pkl-1* and *lux-6* mutant backgrounds. The enrichment of PKL at genomic regions 4 and 5 of *DOG1* was significantly reduced in *lux-6*, as well as *pkl-1* (Figure 5A), indicating that the association of PKL with *DOG1* chromatin depends on LUX. The chromatin-remodeling factor PKL affects H3K27me3 levels of its target genes (Jing et al., 2013; Zhang et al., 2012, 2014). We therefore performed ChIP assays using an anti-H3K27me3 antibody to examine the enrichment of the H3K27me3 histone marker at the *DOG1* locus. In the wild type, H3K27me3 was relatively enriched in regions 1, 3, 4, and 5 of *DOG1*, whereas this enrichment was greatly reduced in *pkl* and *lux* (Figure 5B). The germination rates of *pkl lux* were lower than those of the single mutants (Supplemental Figure 7). These results suggest that PKL and LUX promote the association of H3K27me3 with *DOG1* chromatin, which is in agreement with the roles of these proteins in transcriptionally repressing *DOG1*.

LUX and ELF3 Affect the Circadian Output to Seeds

According to the *Arabidopsis* eFP browser database, *LUX*, *ELF3*, and *ELF4* transcript levels gradually increase during seed development and are sharply elevated in dry seeds (Winter et al., 2007). However, *PKL* expression was maintained at a stable level during seed development (Supplemental Figure 8). Intriguingly, *DOG1*

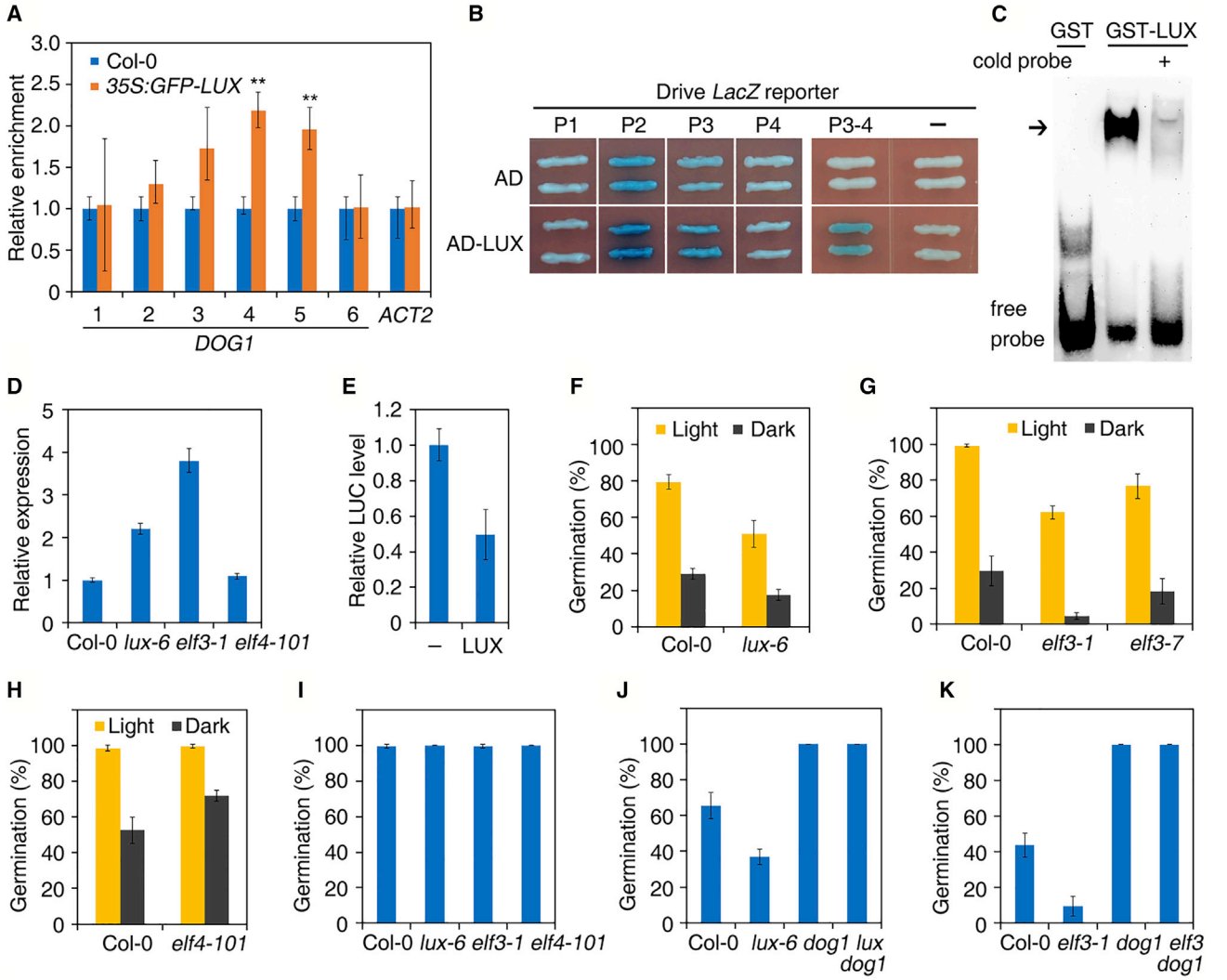

Figure 4. LUX Directly Binds the *DOG1* Chromatin, and LUX and ELF3 Inhibit Seed Dormancy.

(A) ChIP assay. GFP antibody was used to pull down different fragments of *DOG1* (shown in Figure 2C) and the *ACT2* control from Col-0 and *35S:GFP-LUX*. Seedlings were grown under LD conditions for 5 d, and samples were harvested at ZT3. Relative enrichment by GFP antibody was normalized to the input control. Asterisks indicate significant differences from Col-0 using Student's *t*-test ($P < 0.01$).

(B) Yeast one-hybrid assay. The *LacZ* reporter gene was driven by various *DOG1* fragments (shown in Figure 2C), and LUX protein was fused with the B42 activation domain.

(C) EMSA. MBP-LUX or MBP recombinant proteins were incubated with biotin-labeled P3-4 oligos of *DOG1* in the absence or presence of cold DNA oligo probes. Arrow indicates shifted protein–DNA band.

(D) *DOG1* expression in wild-type Col-0 and the *lux*, *elf3*, and *elf4* mutants. Seeds were imbibed for 12 h.

(E) Relative LUC activity of *DOG1p:LUC* in *Arabidopsis* protoplasts in the absence or presence of LUX.

(F–H) Seed germination rates of Col-0 and *lux* **(E)**, *elf3* **(F)**, and *elf4* **(G)** after 4 d of incubation in the light or dark.

(I) Seed germination rates of Col-0 and various mutants after 3 d of cold treatment.

(J and K) Germination rates of *lux dog1* **(J)** and *elf3 dog1* **(K)** double mutants and single mutants in the light.

For **(D)** and **(F)–(K)**, freshly harvested seeds were used. For **(A)** and **(D)–(K)**, values denote the average ± SD of three biological replicates.

transcript levels strongly increased during seed development and peaked at the curled cotyledon stage but decreased thereafter and remained low in dry seeds (Supplemental Figure 8). These data suggest that transcription of EC components and *DOG1* is temporally controlled during seed development and maturation.

Because the EC transmits circadian signals to regulate plant growth and responses (Nusinow et al., 2011; Hsu and Harmer, 2014), we grew seedlings under 12 h light/12 h dark cycles for 6 d and then transferred to continuous light (CL). *DOG1* expression displayed a circadian pattern and peaked at zeitgeber time 28 (ZT28) and then dropped afterward in Col-0 wild type; however, this expression pattern was disrupted in *lux* and *lux pkl* mutants (Figure 6A). Next, we examined the expression pattern of *DOG1* in the siliques of Col-0 and *lux elf3* grown under long-day (LD) conditions (16 h light/8 h dark). The developing siliques (5 d after pollination) were harvested every 4 h starting from the onset of light (ZT0). We found that the *DOG1* expression levels had peaks at ZT8 and ZT20 in Col-0 wild-type siliques, and were drastically increased in *lux elf3* and peaked at ZT4 and ZT16 (end of the day) (Figure 6B),

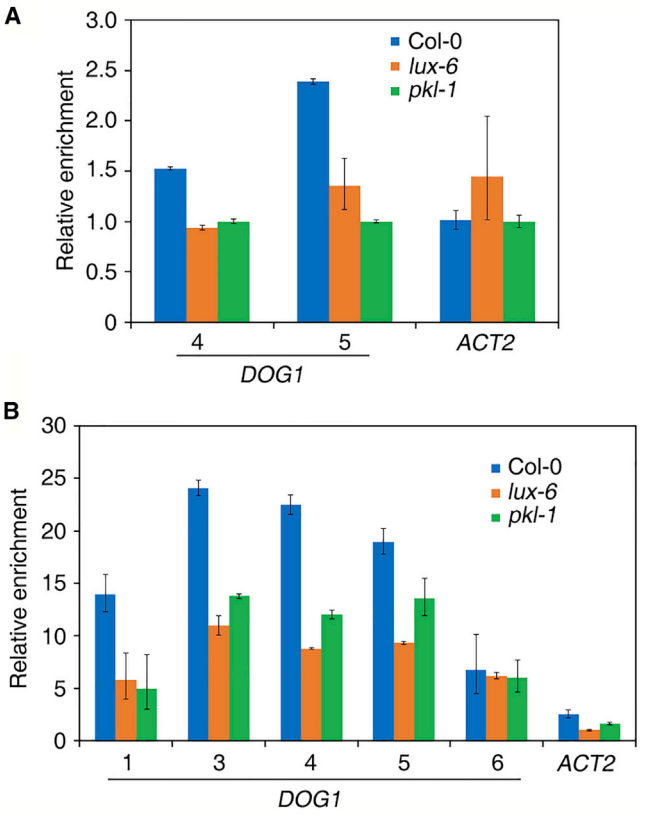

Figure 5. LUX and PKL Regulate H3K27me3 Levels at the *DOG1* Locus.

(A) ChIP assay. PKL antibody was used to pull down different fragments of *DOG1* (shown in Figure 2C) and the *ACT2* control from Col-0, *lux-6*, and *pkl-1* plants.

(B) ChIP assay. H3K27me3 antibody was used to pull down different fragments of *DOG1* and the *ACT2* control from Col-0, *lux-6*, and *pkl-1* plants. Seedlings were grown under LD conditions for 5 d and samples were harvested at ZT4. Relative enrichment using the H3K27me3 antibody was normalized to that using the H3 antibody. In all experiments, values denote average ± SD of three biological replicates.

suggesting that *DOG1* transcription exhibits photoperiodic regulation. Moreover, we investigated the effects of LUX and ELF3 on seed dormancy in response to circadian changes. We grew plants under LD conditions until flowering and maintained them under LD or transferred them to CL, and analyzed the mature dry seeds. The germination rates were much lower for *lux-6* and *elf3-7* seeds than for Col-0 seeds under LD conditions, whereas they were similar under CL conditions (Figure 6C). Finally, we isolated mRNA from the developing siliques of plants grown under both conditions. As expected, *DOG1* transcript levels were much higher in *lux-6* and *elf3-7* compared with Col-0 under LD, but only slightly higher in the mutants under CL (Figure 6D). These results suggest that LUX and ELF3 play important roles in gating circadian signals into seeds during development.

DISCUSSION

Many components that regulate the establishment of primary seed dormancy have been identified (Nonogaki, 2014; Shu et al., 2016). DOG1 is a pivotal regulator of the dormancy state of seeds, and DOG1 activity is negatively correlated with the seed germination rate (Nee et al., 2017). Therefore, *DOG1* expression must be precisely controlled during seed development and maturation. Here, by performing ChIP analysis, yeast one-hybrid assays, and EMSA, we demonstrated that the LUX transcription factor, a component of the EC, physically binds to a specific region of *DOG1* (Figure 4A–4C). Surprisingly, the core binding site of LUX is two atypical LBS motifs located in the second exon, but not in the promoter region, of *DOG1*. Consistently, the *DOG1* locus shows sequence variation in coding region among accessions (Bentsink et al., 2006). This binding site is close to the transcriptional start site of the noncoding antisense *asDOG1* sequence, suggesting that it is likely that LUX also directly regulates the expression of *asDOG1* (Fedak et al., 2016). In agreement with this notion, *asDOG1* transcription was downregulated in the *pkl-1* mutant (Supplemental Figure 9). A genome-wide binding study using ChIP coupled to sequencing also showed that *DOG1* is a target of EC components (Ezer et al., 2017). Hence, LUX and the EC are the direct upstream regulators of *DOG1*, which controls seed dormancy.

Several clock proteins, including CIRCADIAN CLOCK ASSOCIATED1, GIGANTEA, and TOC1, help integrate environmental signaling to mediate dormancy release, likely via the indirect regulation of ABA and GA biosynthesis (Penfield and Hall, 2009). Two recent studies showed that functional alleles of PSEUDO-RESPONSE REGULATOR7 and TOC1 are required for the induction of seed germination in response to daily temperature cycles (Arana et al., 2017). Furthermore, temperature-induced dormancy occurred more rapidly when the morning loop was compromised and delayed versus when the evening loop was compromised (Footitt et al., 2017). However, the precise role of the circadian clock in regulating seed dormancy has been unknown.

Here, we showed that LUX and ELF3 repress *DOG1* expression and seed dormancy and that DOG1 acts downstream of LUX and ELF3 (Figure 4), suggesting that the EC controls seed dormancy mainly through directly regulating *DOG1* expression. ELF4 itself might play a minor role in this process. Consistently, LUX acts as a transcriptional repressor to inhibit the expression of downstream genes (Helfer et al., 2011). However, *lux-2* and *lux-5* did not exhibit altered seed germination in a previous study (Penfield and Hall, 2009). This discrepancy might be due to the different mutant alleles and/or growth environments used in the two studies. Indeed, mutation of any member of the EC leads to arrhythmic cycles in plants (Helfer et al., 2011; Nagel and Kay, 2012).

Strikingly, we found that *lux* and *elf3* mutant seeds displayed high levels of *DOG1* expression and strong dormancy when the parent plants were grown under LD conditions, whereas this phenotype was largely diminished when the plants were grown under CL (Figure 6B and 6C), indicating that the exogenous photoperiod and/or endogenous circadian rhythms affect the establishment of primary seed dormancy and that this process is dependent (at least in part) on LUX and ELF3. Consistent with this notion, *LUX*, *ELF3*, and *ELF4* transcript levels were negatively correlated with that of *DOG1* during seed development (Supplemental Figure 8). We previously demonstrated that the

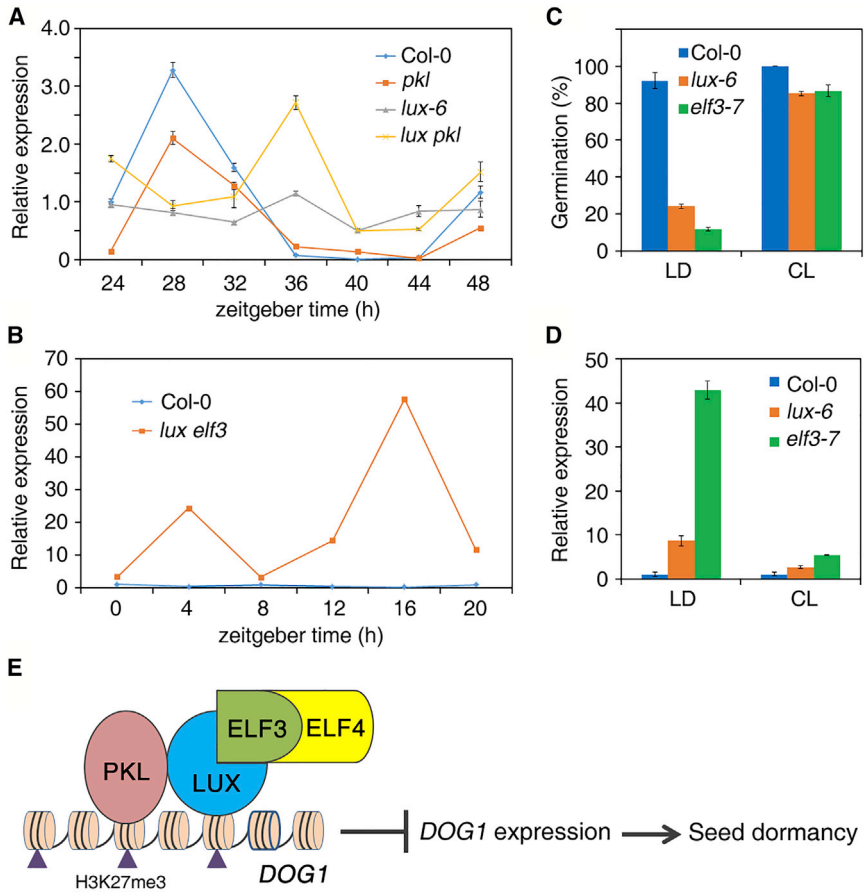

Figure 6. LUX and ELF3 Affect Circadian Output to Seeds.

(A) Relative *DOG1* expression in seedlings under free-running conditions. Seedlings were grown under 12 h light/12 h dark for 6 d followed by CL illumination for 24 h. Samples were harvested every 4 h from ZT24.

(B) Relative *DOG1* expression in developing siliques. Plants were grown under LD conditions, and siliques (6 d after pollination) were harvested every 4 h started from ZT0.

(C) Seed germination rate. Col-0, *lux*, and *elf3* plants were grown under LD conditions for 3 weeks and transferred to CL or kept at LD until seed maturation. Germination of freshly harvested seeds in the light was analyzed.

(D) Relative *DOG1* expression in developing siliques. Plants were grown under LD conditions, and siliques (8 d after pollination) were harvested at ZT8. For **(A)**, **(B)**, and **(D)**, data are the average ± SD of three biological replicates.

(E) A working model illustrating the roles of PKL and EC in controlling seed dormancy. LUX binds directly to a specific DNA sequence of *DOG1* and recruits PKL to the *DOG1* locus through their physical interaction. This interaction increases H3K27me3 levels on *DOG1* chromatin, thereby repressing its transcription and leading to reduced seed dormancy. Arrow indicates positive regulation and bar denotes negative regulation.

photoreceptor phytochrome B (phyB) plays a key role in regulating *DOG1* expression and seed dormancy (Jiang et al., 2016). A genome-wide ChIP sequencing study suggested that the EC plays a central role in coordinating endogenous and environmental signals (Ezer et al., 2017). It is likely that, at least, phyB and the clock oscillator integrate both environmental (light and temperature) and circadian signaling to seeds during the growth of the parent plant and seed development. Therefore, EC components play essential roles in transmitting the seasonal and photoperiodic signals that suppress seed dormancy through regulating *DOG1* transcriptional activity. We do not exclude the possibility that other clock component(s) might also contribute the circadian gating of seed dormancy. This mechanism might help plants maintain a relatively low seed dormancy state that confers proper germination for the next generation.

Transcriptional regulation of seed dormancy is also associated with chromatin restructuring (Nee et al., 2017). Chromatin-remodeling factors alter DNA histone contacts and the accessibility of genomic regions to the transcriptional machinery or transcription factors, thus playing crucial roles in regulating gene expression (Ho and Crabtree, 2010; Ho et al., 2013; Han et al., 2015). Our study demonstrated that LUX physically interacts with the chromatin-remodeling factor, PKL, and that recruitment of PKL to the chromatin region of *DOG1* depends on LUX (Figure 5). H3K27me3 levels at specific *DOG1* chromatin regions were greatly reduced in the *lux* and *pkl* mutants compared with the wild type (Figure 5C). Other enzymes involved in histone methylation, demethylation, or deacetylation also affect seed dormancy. For instance, mutants of the H3K4 histone methyltransferase ARABIDOPSIS TRITHORAXRELATED7 exhibit reduced seed dormancy, whereas mutants of the H3K9 histone methyltransferase SUVH4 show upregulation of *DOG1* and increased seed dormancy (Liu et al., 2007; Zheng et al., 2012). Consistently, double mutations in the H3K4 histone demethylases LYSINE SPECIFIC DEMETHYLASE-LIKE1 and 2 led to elevated *DOG1* expression and increased dormancy (Zhao et al., 2015). The levels of H3K27me3 and H3K4me3 at *DOG1* locus correlate with *DOG1* expression (Muller et al., 2012). Taken together, we propose that the EC uses LUX to bind to a specific *DOG1* genomic region and recruits PKL through a direct interaction, thereby increasing the level of the repressive marker H3K27me3 at the *DOG1* locus to inhibit its transcription (Figure 6D). It would be interesting to investigate whether the methyltransferase H3K27 and polycomb repressive complexes are involved in regulating *DOG1* expression and/or seed dormancy with PKL and the EC. However, in contrast to the current model, PKL interacts with the transcription factor ELONGATED HYPOCOTYL5 and inhibits the accumulation of H3K27me3 on the chromatin of cell-elongation-related genes, thereby promoting their expression during seedling deetiolation (Jing et al., 2013). Thus, the remodeler PKL might differentially affect H3K27me3 accumulation and modulate target gene expression by interacting with different transcription factors or regulators during diverse plant growth and developmental processes.

METHODS

Plant Materials and Growth Conditions

The *pkl-1*, *pkl-10*, *pkl-11*, *pkl-12*, *dog1-2*, *elf3-1*, *elf3-7*, *lux-6*, *elf4-101*, *rve1-2*, *rve2-1*, and *phyB-9* mutants, as well as the *PKLp:GUS* and *35S:GFP-LUX* transgenic lines, were described previously (Hicks et al., 2001; Khanna et al., 2003; Jing et al., 2013; Jiang et al., 2016; Zha et al., 2017; Zhang et al., 2018). All mutants and transgenic lines are of the Col-0 ecotype. Double mutant and transgenic plants were generated by genetic crossing, and homozygous lines were verified by PCR genotyping, antibiotic selection, and/or sequencing. Adult plants were grown side-by-side in soil in a growth chamber with regular irrigation at 22°C ± 2°C and 60%–70% humidity, under LD (16 h light/8 h dark, 100 μmol m^{-2} s^{-1}) conditions. Far-red, red, and white light was supplied by light-emitting diodes.

Seed Dormancy Test

To investigate seed dormancy, mature seeds at the same developmental stage were harvested, surface sterilized, plated on 0.6% agar (pH 5.7) under light, and then transferred to darkness or white light (80 μmol m^{-2} s^{-1}) for 4 d. Seeds with protruded radicals were considered to be germinated, and the germination frequency was used to determine the degree of seed dormancy. The viability of seeds in each batch was tested after cold stratification at 4°C for 3 d.

For the phyB-dependent germination assay, seeds were harvested and stored dry at room temperature for up to 5 months. After sterilization and plating (within 1 h), the seeds were irradiated with far-red light (3.5 μmol m^{-2} s^{-1}) for 5 min to inactivate phyB (phyB-off), followed by 5 min of red light (10 μmol m^{-2} s^{-1}) to activate phyB (phyB-on) (Jiang et al., 2016). All seeds were then incubated in the dark for 4 d and the germination frequency was determined. At least 100 seeds were used for each genotype per experiment, and three replicates were performed for statistical analysis.

GUS Staining

Seeds from *PKLp:GUS* (Jing et al., 2013) transgenic plants were imbibed for 12 h and incubated in the solution provided with the GUS Histochemical Kit (Real-Times) at 37°C for 6 h following the manufacturer's instructions. The embryos and endosperm were dissected and photographed under a dissecting microscope (Olympus).

Plasmid Construction

The regulatory or coding sequences or fragments of *PKL*, *ELF3*, *ELF4*, and *LUX* were amplified from Col-0 genomic DNA or cDNA using High-fidelity Pfu DNA Polymerase (Invitrogen). All primers used for cloning with the appropriate restriction sites are listed in Supplemental Table 1. The genes were cloned into the pEASY-Blunt vector and verified by sequencing. Various plasmids and vectors were digested with the corresponding restriction enzymes, followed by ligation and transformation into *E. coli* strain Trans-T1. The *PKL*, D8, and D9 fragments were inserted into pLexA (Clontech) to generate LexA-D8/D9. Full-length *ELF3*, *ELF4*, and *LUX* were cloned into yeast vectors pB42AD and pGBKT7 (Clontech), generating AD-ELF3/ELF4/LUX and GBD-ELF3/ELF4/LUX, respectively. The coding sequences of *ELF3*, *ELF4*, and *LUX* were also cloned into pUC19-cLUC (Chen et al., 2008), resulting in ELF3/ELF4/LUX-cLUC. LUX was cloned into pMAL-c5X-1 to produce MBP-LUX. The full-length *PKL* sequence was inserted into pUC19-nLUC (Chen et al., 2008) to generate PKL-nLUC. Various *DOG1* fragments, including P1 to P4, P2-1 to P2-4, and P3-1 to P3-4 were cloned into pLacZ-2μ (Lin et al., 2007), generating the corresponding *LacZ* reporter constructs. The promoter and coding sequences of *DOG1* were ligated into pCAMBIA1302-LUC, resulting in *DOG1p:LUC*.

The binary construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation and transformed into wild-type *Arabidopsis* via the floral dip method. Transgenic plants were selected on MS plates in the presence of 50 mg/l kanamycin or hygromycin. Homozygous lines were used in all experiments.

Transcriptional Analysis

Freshly harvested seeds were imbibed for 12 or 24 h as described in the figure legends. Total RNA was isolated from plants using a Universal Plant Total RNA Extraction Kit (BioTeke), and first-strand cDNA was synthesized from the RNA using reverse transcriptase (Invitrogen). The cDNA templates were amplified using an SYBR Premix ExTaq Kit (Takara) in a LightCycler 480 (Roche) following the manufacturer's instructions with primers listed in Supplemental Table 1. Three technical replicates were performed per sample, and relative expression levels were normalized to *IPP2*. Each experiment was performed at least three times with similar results, and representative data from a single experiment are shown.

ChIP Assay

For the ChIP reactions, procedures for crosslinking, chromatin isolation, sonication, and immunoprecipitation were performed as described (Bowler et al., 2004). In brief, 1.5 g of tissue from 5-d-old seedlings was harvested and fixed for 15 min in 1% formaldehyde under a vacuum. Chromatin was isolated and sonicated to produce ~500-bp DNA fragments. The chromatin samples were immunoprecipitated with anti-PKL (Jing et al., 2013), anti-GFP (Abcam, ab1218), or anti-H3K27me3 (Millipore, 07-449) antibodies. The precipitated DNA was subjected to phenol/chloroform extraction, precipitated in ethanol, and dissolved in water. The relative enrichment of each fragment was determined using the precipitated DNA samples by qPCR using SYBR Green PCR Master Mix. The ChIP assays were performed with three biological replicates. The primer pairs used for the ChIP assays are listed in Supplemental Table 1.

Yeast Two-Hybrid and One-Hybrid Assays

For the yeast two-hybrid assay, individual BD-fusion constructs were co-transformed with *LexAop:LacZ* (Clontech) reporter plasmids into yeast strain EGY48, and the AD-fusion constructs were transformed into yeast strain Ym4271. After mating, the transformants were grown on SD/-Trp-Ura-His dropout plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for color development. For the yeast one-hybrid assay, AD-LUX or AD control plasmids were cotransformed with various LacZ reporter constructs into yeast strain EGY48. The transformants were grown on SD/-Trp-Ura dropout plates with X-gal for color development. Blue yeast colonies indicated protein–protein or protein–DNA interactions.

Pull-Down Assay

The GST-LUX, MBP-LUX, and D6-His recombinant fusion proteins or GST and MBP controls were expressed in *E. coli* strain BL21 (DE3) and purified using Glutathione Sepharose 4B beads (for GST fusion, GE Healthcare), Dextrin Sepharose (for MBP fusion; GE Healthcare), or Ni-NTA Agarose (for His fusion; QIAGEN), respectively. Approximately 2 μg of purified bait proteins (GST-LUX or GST, MBP-LUX or MBP) and 2 μg of D6-His prey proteins were incubated in binding buffer (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, and 0.6% Triton X-100) for 2 h at 4°C. Following the addition of Glutathione Sepharose 4B or Dextrin Sepharose beads, the samples were incubated for 1 h. After washing with binding buffer, the precipitated proteins were eluted by heating the beads at 70°C for 5 min in 10 μl 10× SDS–PAGE loading buffer. The proteins were size-fractioned on a 10% gel and analyzed by immunoblotting with anti-GST (Abcam, ab19256), anti-MBP (Abcam, ab9084), or anti-His (TransGen, HT501) antibodies.

LCI Assay

The LCI experiments were carried out as described previously (Chen et al., 2008). The nLUC/cLUC fusion plasmids and conjugative P19 plasmid were introduced into *Agrobacterium* strains GV3101 and EHA105,

respectively. A single colony was transferred to Luria-Bertani medium and cultured overnight to OD_{600} = 0.6–0.8. The culture was pelleted, washed twice with transformation buffer (10 mM MES [pH 5.6], and 10 mM $MgCl_2$), and resuspended to a final OD_{600} of 1.5. Various nLUC/cLUC fusion constructs were mixed with an equal volume of P19. The bacteria were supplemented with 200 mM acetosyringone and incubated at 28°C for 3–5 h without shaking. The bacterial suspensions were infiltrated into fully expanded young *N. benthamiana* leaves with a needleless syringe. The plants were grown for 2 d under LD conditions. The infiltrated leaves were sprayed with 2 μM luciferase (dissolved in 0.02% Triton X-100) and incubated in the dark for 10 min before imaging. Luminescence was captured using a NightSHADE LB985 plant imaging system equipped with a CCD camera (Berthold Technologies). The experiments were repeated at least three times.

EMSA

MBP-LUX or MBP recombinant proteins were expressed in *E. coli* BL21 (DE3) cells and purified using Dextrin Sepharose. EMSA was performed using a LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions. The two complementary oligonucleotides were annealed, labeled with biotin, and incubated with recombinant proteins in the absence or presence of excess amounts of unlabeled wild-type oligonucleotides. The protein–DNA samples were separated on 5% polyacrylamide gels and the signals captured with a Chemiluminescence Imaging System (biostep).

Luciferase Transient Expression Assay

The LUX effector, *DOG1p:LUC* reporter, and *35S:GUS* control plasmids were cotransformed into *Arabidopsis* protoplasts, and LUC and GUS activity assays were performed as described previously (Tang et al., 2012). The relative reporter expression level was calculated as the LUC/GUS ratio.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

P.Z. and R.L. designed the research. P.Z., S.L., Y.L., L.Y., Y.J., and T.M. performed the research and analyzed the data. R.L. wrote the paper.

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REFERENCES

Aichinger, E., Villar, C.B.R., Di Mambro, R., Sabatini, S., and Köhler, C. (2011). The CHD3 chromatin remodeler PICKLE and polycomb group proteins antagonistically regulate meristem activity in the *Arabidopsis* root. Plant Cell **23**:1047–1060.

Alonso-Blanco, C., Bentsink, L., Hanhart, C.J., Blankestijn-de Vries, H., and Koornneef, M. (2003). Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. Genetics **164**:711–729.

Arana, M.V., Tognacca, R.S., Estravis-Barcalá, M., Sánchez, R.A., and Botto, J.F. (2017). Physiological and molecular mechanisms underlying the integration of light and temperature cues in *Arabidopsis thaliana* seeds. Plant Cell Environ. **40**:3113–3121.

Bentsink, L., and Koornneef, M. (2008). Seed dormancy and germination. Arabidopsis Book **6**:e0119.

Bentsink, L., Hanson, J., Hanhart, C.J., Blankestijn-de Vries, H., Coltrane, C., Keizer, P., El-Lithy, M., Alonso-Blanco, C., de Andrés, M.T., Reymond, M., et al. (2010). Natural variation for seed dormancy in *Arabidopsis* is regulated by additive genetic and molecular pathways. Proc. Natl. Acad. Sci. U S A **107**:4264–4269.

Bentsink, L., Jowett, J., Hanhart, C.J., and Koornneef, M. (2006). Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in *Arabidopsis*. Proc. Natl. Acad. Sci. U S A **103**:17042–17047.

Bowler, C., Benvenuto, G., Laflamme, P., Molino, D., Probst, A.V., Tariq, M., and Paszkowski, J. (2004). Chromatin techniques for plant cells. Plant J. **39**:776–789.

Bryant, F.M., Hugues, D., Hassani-Pak, K., and Eastmond, P.J. (2019). Basic LEUCINE ZIPPER TRANSCRIPTION FACTOR67 transactivates *DELAY OF GERMINATION1* to establish primary seed dormancy in *Arabidopsis*. Plant Cell **31**:1276–1288.

Chen, H.M., Zou, Y., Shang, Y.L., Lin, H.Q., Wang, Y.J., Cai, R., Tang, X.Y., and Zhou, J.M. (2008). Firefly luciferase complementation imaging assay for protein-protein interactions in plants. Plant Physiol. **146**:368–376.

Cyrek, M., Fedak, H., Ciesielski, A., Guo, Y.W., Sliwa, A., Brzezniak, L., Krzyczmonik, K., Pietras, Z., Kaczanowski, S., Liu, F.Q., et al. (2016). Seed dormancy in *Arabidopsis* is controlled by alternative polyadenylation of *DOG1*. Plant Physiol. **170**:947–955.

Dolata, J., Guo, Y.W., Kołowerzo, A., Smoliński, D., Brzyżek, G., Jarmołowski, A., and Świeżewski, S. (2015). NTR1 is required for transcription elongation checkpoints at alternative exons in *Arabidopsis*. EMBO J. **34**:544–558.

Ezer, D., Jung, J.H., Lan, H., Biswas, S., Gregoire, L., Box, M.S., Charoensawan, V., Cortijo, S., Lai, X.L., Stöckle, D., et al. (2017). The evening complex coordinates environmental and endogenous signals in *Arabidopsis*. Nat. Plants **3**:17087.

Fedak, H., Palusinska, M., Krzyczmonik, K., Brzezniak, L., Yatusevich, R., Pietras, Z., Kaczanowski, S., and Swiezewski, S. (2016). Control of seed dormancy in *Arabidopsis* by a cis-acting noncoding antisense transcript. Proc. Natl. Acad. Sci. U S A **113**:E7846–E7855.

Finkelstein, R., Reeves, W., Ariizumi, T., and Steber, C. (2008). Molecular aspects of seed dormancy. Annu. Rev. Plant Biol. **59**:387–415.

Footitt, S., Ölçer-Footitt, H., Hambidge, A.J., and Finch-Savage, W.E. (2017). A laboratory simulation of *Arabidopsis* seed dormancy cycling provides new insight into its regulation by clock genes and the dormancy-related genes *DOG1*, *MFT*, *CIPK23* and *PHYA*. Plant Cell Environ. **40**:1474–1486.

Fukaki, H., Taniguchi, N., and Tasaka, M. (2006). PICKLE is required for SOLITARY-ROOT/IAA14-mediated repression of ARF7 and ARF19 activity during *Arabidopsis* lateral root initiation. Plant J. **48**:380–389.

Graeber, K., Nakabayashi, K., Miatton, E., Leubner-Metzger, G., and Soppe, W.J.J. (2012). Molecular mechanisms of seed dormancy. Plant Cell Environ. **35**:1769–1786.

Greenham, K., and McClung, C.R. (2015). Integrating circadian dynamics with physiological processes in plants. Nat. Rev. Genet. **16**:598–610.

Gu, X.Y., Kianian, S.F., and Foley, M.E. (2006). Dormancy genes from weedy rice respond divergently to seed development environments. Genetics **172**:1199–1211.

Han, S.K., Wu, M.F., Cui, S.J., and Wagner, D. (2015). Roles and activities of chromatin remodeling ATPases in plants. Plant J. **83**:62–77.

Harmer, S.L. (2009). The circadian system in higher plants. Annu. Rev. Plant Biol. **60**:357–377.

Helfer, A., Nusinow, D.A., Chow, B.Y., Gehrke, A.R., Bulyk, M.L., and Kay, S.A. (2011). LUX ARRHYTHMO encodes a nighttime repressor of circadian gene expression in the *Arabidopsis* core clock. Curr. Biol. **21**:126–133.

Hicks, K.A., Albertson, T.M., and Wagner, D.R. (2001). EARLY FLOWERING3 encodes a novel protein that regulates circadian clock function and flowering in *Arabidopsis*. Plant Cell **13**:1281–1292.

Ho, K.K., Zhang, H., Golden, B.L., and Ogas, J. (2013). PICKLE is a CHD subfamily II ATP-dependent chromatin remodeling factor. Biochim. Biophys. Acta **1829**:199–210.

Ho, L., and Crabtree, G.R. (2010). Chromatin remodelling during development. Nature **463**:474–484.

Honogaki, H. (2019). Seed germination and dormancy: the classic story, new puzzles, and evolution. J. Integr. Plant Biol. **61**:541–563.

Hsu, P.Y., and Harmer, S.L. (2014). Wheels within wheels: the plant circadian system. Trends Plant Sci. **19**:240–249.

Huo, H.Q., Wei, S.H., and Bradford, K.J. (2016). DELAY OF GERMINATION1 (DOG1) regulates both seed dormancy and flowering time through microRNA pathways. Proc. Natl. Acad. Sci. U S A **113**:E2199–E2206.

Jiang, Z.M., Xu, G., Jing, Y.J., Tang, W.J., and Lin, R.C. (2016). Phytochrome B and REVEILLE1/2-mediated signalling controls seed dormancy and germination in *Arabidopsis*. Nat. Commun. **7**:12377.

Jing, Y.J., Zhang, D., Wang, X., Tang, W.J., Wang, W.Q., Huai, J.L., Xu, G., Chen, D.Q., Li, Y.L., and Lin, R.C. (2013). *Arabidopsis* chromatin remodeling factor PICKLE interacts with transcription factor HY5 to regulate hypocotyl cell elongation. Plant Cell **25**:242–256.

Jing, Y., Guo, Q., Zha, P., and Lin, R. (2019). The chromatin-remodeling factor PICKLE interacts with CONSTANS to promote flowering in *Arabidopsis*. Plant Cell Environ. **42**:2495–2507.

Khanna, R., Kikis, E.A., and Quail, P.H. (2003). EARLY FLOWERING 4 functions in phytochrome B-regulated seedling de-etiolation. Plant Physiol. **133**:1530–1538.

Kowalczyk, J., Palusinska, M., Wroblewska-Swiniarska, A., Pietras, Z., Szewc, L., Dolata, J., Jarmolowski, A., and Swiezewski, S. (2017). Alternative polyadenylation of the sense transcript controls antisense transcription of *DELAY OF GERMINATION 1* in *Arabidopsis*. Mol. Plant **10**:1349–1352.

Li, X., Chen, T., Li, Y., Wang, Z., Cao, H., Chen, F., Li, Y., Soppe, W.J.J., Li, W., and Liu, Y. (2019). ETR1/RDO3 regulates seed dormancy by relieving the inhibitory effect of the ERF12-TPL complex on *DELAY OF GERMINATION1* expression. Plant Cell **31**:832–847.

Lin, R.C., Ding, L., Casola, C., Ripoll, D.R., Feschotte, C., and Wang, H.Y. (2007). Transposase-derived transcription factors regulate light signaling in *Arabidopsis*. Science **318**:1302–1305.

Liu, Y.X., Koornneef, M., and Soppe, W.J.J. (2007). The absence of histone H2B monoubiquitination in the *Arabidopsis hub1 (rdo4)* mutant reveals a role for chromatin remodeling in seed dormancy. Plant Cell **19**:433–444.

Mizuno, T., Nomoto, Y., Oka, H., Kitayama, M., Takeuchi, A., Tsubouchi, M., and Yamashino, T. (2014). Ambient temperature signal feeds into the circadian clock transcriptional circuitry through the EC night-time repressor in *Arabidopsis thaliana*. Plant Cell Physiol. **55**:958–976.

Muller, K., Bouyer, D., Schnittger, A., and Kermode, A.R. (2012). Evolutionarily conserved histone methylation dynamics during seed life-cycle transitions. PLoS One **7**:e51532.

Nagel, D.H., and Kay, S.A. (2012). Complexity in the wiring and regulation of plant circadian networks. Curr. Biol. **22**:R648–R657.

Nakabayashi, K., Bartsch, M., Ding, J., and Soppe, W.J.J. (2015). Seed dormancy in *Arabidopsis* requires self-binding ability of DOG1 protein and the presence of multiple isoforms generated by alternative splicing. PLoS Genet. **11**:e1005737.

Nakabayashi, K., Bartsch, M., Xiang, Y., Miatton, E., Pellengahr, S., Yano, R., Seo, M., and Soppe, W.J.J. (2012). The time required for dormancy release in *Arabidopsis* is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds. Plant Cell **24**:2826–2838.

Nee, G., Xiang, Y., and Soppe, W.J.J. (2017). The release of dormancy, a wake-up call for seeds to germinate. Curr. Opin. Plant Biol. **35**:8–14.

Nishimura, N., Tsuchiya, W., Moresco, J.J., Hayashi, Y., Satoh, K., Kaiwa, N., Irisa, T., Kinoshita, T., Schroeder, J.I., Yates, J.R., et al. (2018). Control of seed dormancy and germination by DOG1-AHG1 PP2C phosphatase complex via binding to heme. Nat. Commun. **9**:2132.

Nonogaki, H. (2014). Seed dormancy and germination-emerging mechanisms and new hypotheses. Front. Plant Sci. **5**:233.

Nusinow, D.A., Helfer, A., Hamilton, E.E., King, J.J., Imaizumi, T., Schultz, T.F., Farré, E.M., and Kay, S.A. (2011). The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. Nature **475**:398–402.

Ogas, J., Kaufmann, S., Henderson, J., and Somerville, C. (1999). PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in *Arabidopsis*. Proc. Natl. Acad. Sci. U S A **96**:13839–13844.

Osa, M., Kato, K., Mori, M., Shindo, C., Torada, A., and Miura, H. (2003). Mapping QTLs for seed dormancy and the Vp1 homologue on chromosome 3A in wheat. Theor. Appl. Genet. **106**:1491–1496.

Penfield, S., and Hall, A. (2009). A role for multiple circadian clock genes in the response to signals that break seed dormancy in *Arabidopsis*. Plant Cell **21**:1722–1732.

Perruc, E., Kinoshita, N., and Lopez-Molina, L. (2007). The role of chromatin-remodeling factor PKL in balancing osmotic stress responses during *Arabidopsis* seed germination. Plant J. **52**:927–936.

Pruneda-Paz, J.L., and Kay, S.A. (2010). An expanding universe of circadian networks in higher plants. Trends Plant Sci. **15**:259–265.

Rae, G.M., David, K., and Wood, M. (2013). The dormancy marker *DRM1/ARP* associated with dormancy but a broader role in planta. Dev. Biol. J. **2013**, 632524.

Ravindran, P., and Kumar, P.P. (2019). Regulation of seed germination: the involvement of multiple forces exerted via gibberellic acid signaling. Mol. Plant **12**:24–26.

Shu, K., Liu, X.D., Xie, Q., and He, Z.H. (2016). Two faces of one seed: hormonal regulation of dormancy and germination. Mol. Plant **9**:34–45.

Tang, W.J., Wang, W.Q., Chen, D.Q., Ji, Q., Jing, Y.J., Wang, H.Y., and Lin, R.C. (2012). Transposase-derived proteins FHY3/FAR1 interact with PHYTOCHROME-INTERACTING FACTOR1 to regulate chlorophyll biosynthesis by modulating *HEMB1* during deetiolation in *Arabidopsis*. Plant Cell **24**:1984–2000.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., and Provart, N.J. (2007). An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One **2**:e718.

Zha, P., Jing, Y.J., Xu, G., and Lin, R.C. (2017). PICKLE chromatin-remodeling factor controls thermosensory hypocotyl growth of *Arabidopsis*. Plant Cell Environ. **40**:2426–2436.

Zhang, H., Bishop, B., Ringenberg, W., Muir, W.M., and Ogas, J. (2012). The CHD3 remodeler PICKLE associates with genes enriched for trimethylation of histone H3 lysine 27. Plant Physiol. **159**:418–432.

Zhang, D., Jing, Y.J., Jiang, Z.M., and Lin, R.C. (2014). The chromatin-remodeling factor PICKLE integrates brassinosteroid and gibberellin signaling during skotomorphogenic growth in *Arabidopsis*. Plant Cell **26**:2472–2485.

Zhang, Y.Y., Wang, Y., Wei, H., Li, N., Tian, W.W., Chong, K., and Wang, L. (2018). Circadian evening complex represses jasmonate-induced leaf senescence in *Arabidopsis*. Mol. Plant **11**:326–337.

Zhao, M.L., Yang, S.G., Liu, X.C., and Wu, K.Q. (2015). *Arabidopsis* histone demethylases LDL1 and LDL2 control primary seed dormancy by regulating DELAY OF GERMINATION 1 and ABA signaling-related genes. Front. Plant Sci. **6**:159.

Zheng, J., Chen, F.Y., Wang, Z., Cao, H., Li, X.Y., Deng, X., Soppe, W.J.J., Li, Y., and Liu, Y.X. (2012). A novel role for histone methyltransferase KYP/SUVH4 in the control of *Arabidopsis* primary seed dormancy. New Phytol. **193**:605–616.

Supplemental Data

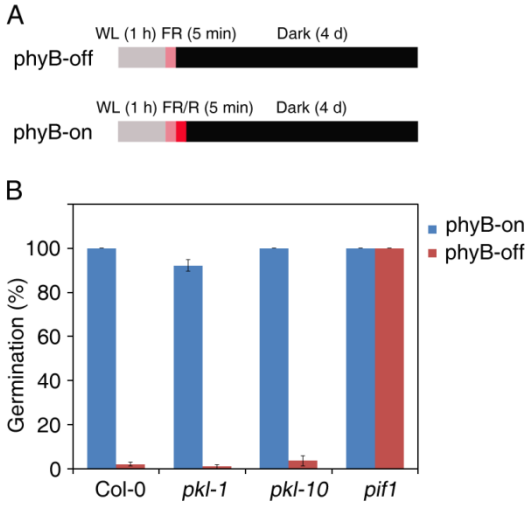

Figure S1. PKL is not involved in phyB-mediated seed germination.

(A) Light treatment. Post-harvest seeds were irradiated with white light (WL) for 1 hr (starting from seed sterilization) and were then exposed to far-red (FR) light for 5 min (phyB-off) or exposed to FR for 5 min followed by 5 min of red (R) light (phyB-on). Seeds were then incubated in darkness for 4 d and germination frequencies were recorded. **(B)** Germination rate of seeds under phyB-off and phyB-on conditions. Data are mean ± s.d. of three biological replicates.

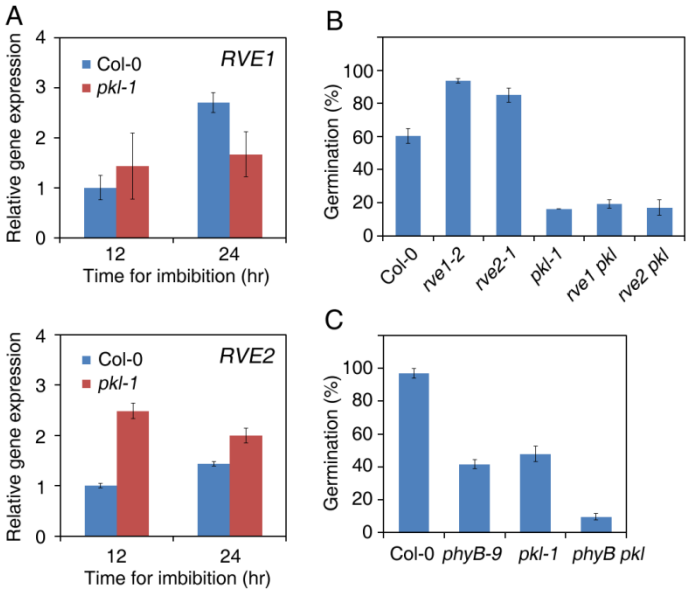

Figure S2. Genetic relationship between PKL and RVE1/RVE2 and phyB.

(A) *RVE1* and *RVE2* expression in Col-0 and *pkl-1* mutants after imbibition. **(B)** Germination frequencies of *rve1 pkl* and *rve2 pkl* with their single mutants and Col wild type under light. **(C)** Germination rate of Col-0, *phyB*, *pkl*, and *phyB pkl* under light. Freshly harvested seeds were used in these experiments. Data are mean ± s.d. of three biological replicates.

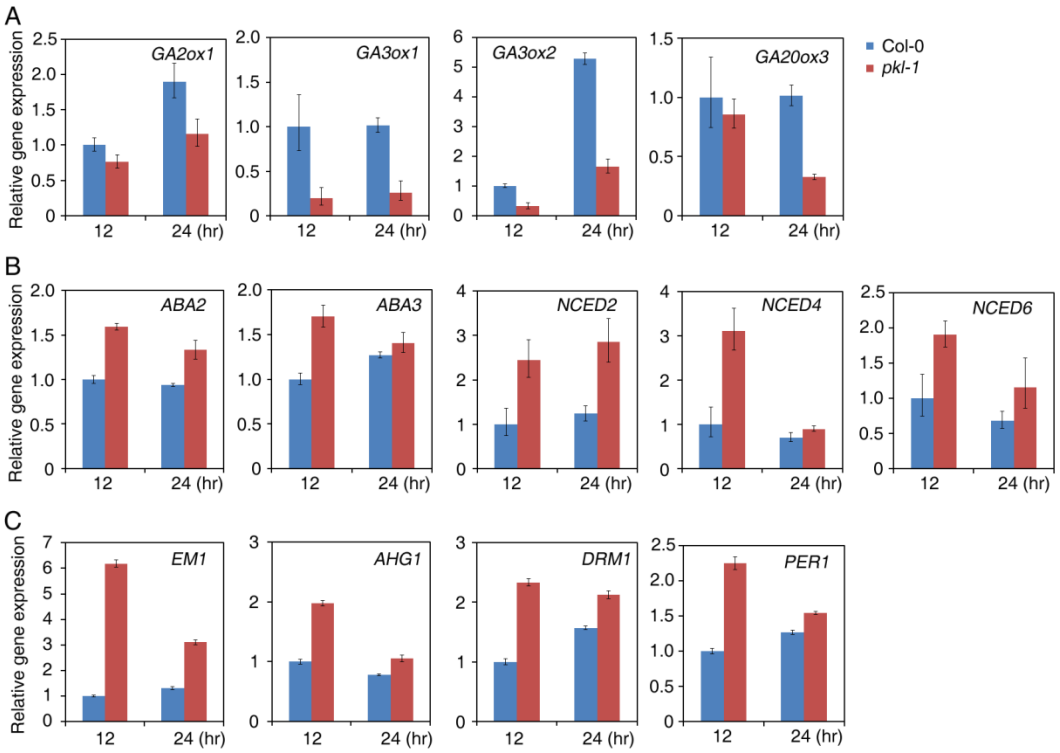

Figure S3. Expression of GA and ABA biosynthetic genes and germination-related genes.

The transcription levels of GA (A) and ABA (B) biosynthetic genes and dormancy-regulated genes (C) were examined in Col and *pkl-1* seeds after imbibition for 12 or 24 hr. Data are mean ± s.d. of three biological replicates.

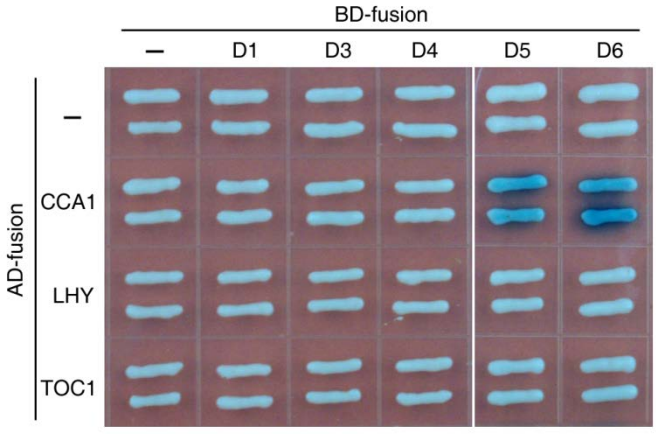

Figure S4. Yeast two-hybrid assay.

PKL fragments were fused with the LexA DNA binding domain (BD-fusion), and CCA1, LHY, and TOC1 were tagged with the B42 activation domain (AD-fusion). Blue colonies denote protein-protein interactions.

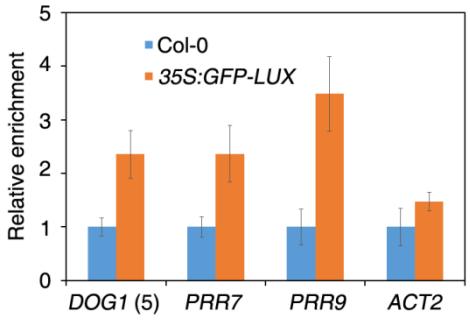

Figure S5. ChIP assay.

The experiment was performed similar as Figure 4A.

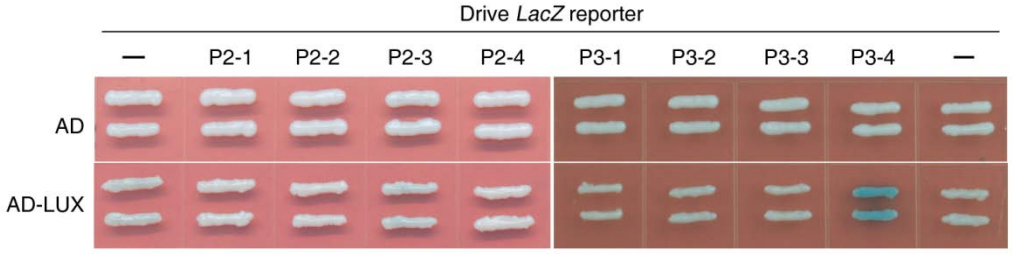

Figure S6. Yeast one-hybrid assay.

The data of *P3-4:LacZ* and the control are same as shown in Figure 4B.

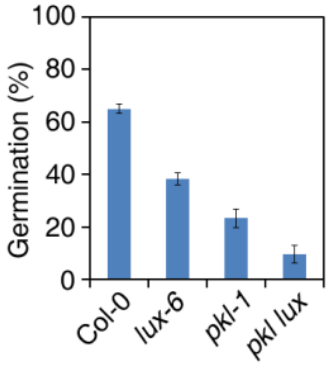

Figure S7. Seed dormancy phenotype of *pkl lux* double mutant.

Freshly harvested seeds were used in this experiment. Data are mean ± s.d. of three biological replicates.

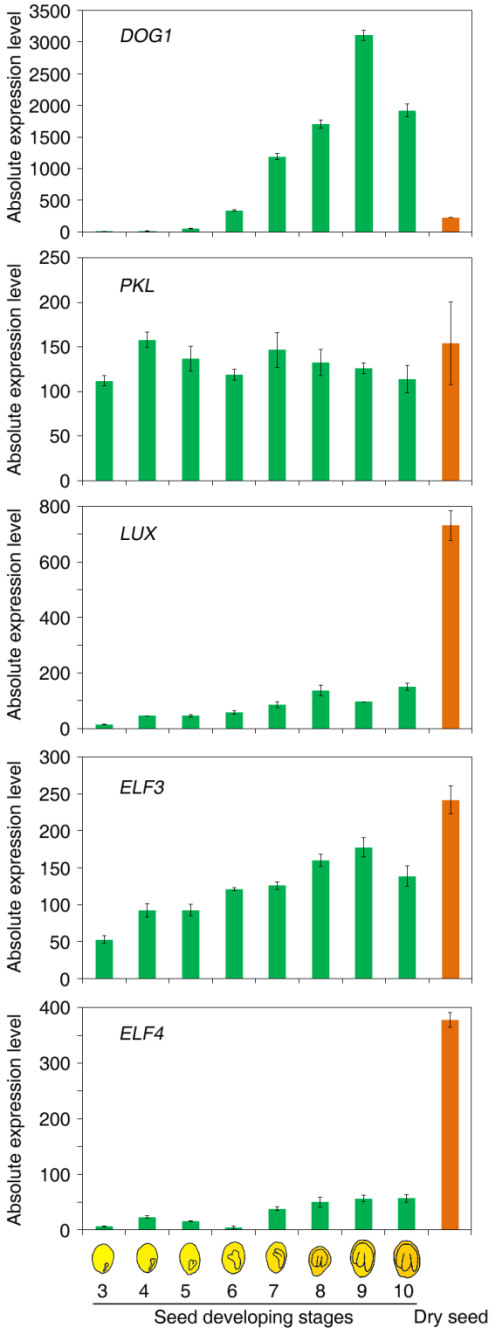

Figure S8. Gene expression pattern during seed development.

Data were collected from http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Winter et al., 2007). Absolute expression levels of *DOG1*, *PKL*, *LUX*, *ELF3*, and *ELF4* are shown.

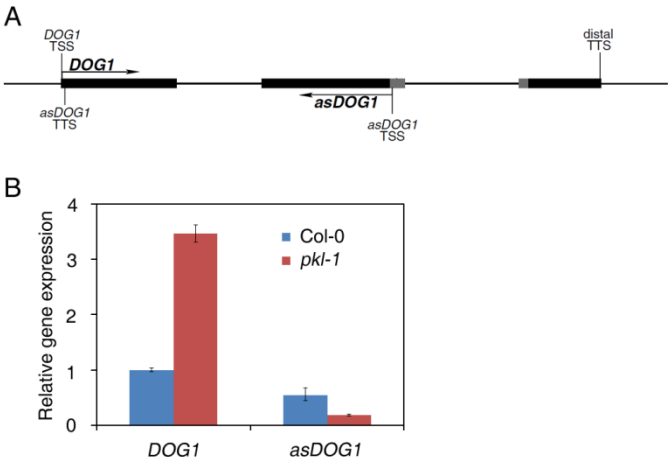

Figure S9. Expression of *DOG1* variants.

(A) Schematic diagram of *DOG1* organization (modified from Fedak et al., 2016). Black boxes, exon sequences; gray boxes, alternative exonic regions; arrows, sense and antisense transcripts. **(B)** Expression levels of *DOG1* and *asDOG1* in freshly harvested seeds after imbibition for 12 hr. Data are mean ± s.d. of three biological replicates.

Table S1. Primers used in this study.

Gene	Sequence (5'-3')	Notes
PCR genotyping for verifying mutants		
PKL	CTGTACTGCGTATATACGAG	*pkl-1*
	ATTACTGTATCAGCCGTTGC	
DOG1	ATGGGATCTTCATCAAAGAACATCGAAC	*dog1-2*
	GAATTTTGGTATAGATCTATGGTTCGGAATC	
LUX	GTGGAAGCGCAAATGAGAATTAAAA	*lux-6*
	ATGGCTTCTTCTGAAACATTTGATT	
ELF3	GTGAGTGATGAAGAGAGGGAA	*elf3-7*
	TTCTTTCCACACCACAAGG	
ELF4	ACCCCAATAGAGATGGGTTTG	*elf4-101*
	GGTAGTTAGTGCCCAGGTTCC	
RVE1	AACCAGTGTTTGATCCAGTCG	*rve1-2*
	CAAAGACCGCAGTTCAGATTC	
RVE2	CAAGGATCTCAATTTCTAACTGG	*rve2-1*
	TGACTTTTGTTGGTTCTTCTATGG	
Plasmid construction		
ELF3	GGATCCAATGAAGAGAGGGAAAGATGA	ELF3-cLuc
	GTCGACTTAAGGCTTAGAGGAGTCATAGC	
	CAATTGATGAAGAGAGGGAAAGATGA	AD-ELF3,

	GTCGACAGGCTTAGAGGAGTCATAGC	GBD-ELF3
ELF4	GGTACCATGAAGAGGAACGGCGAGAC GTCGACTTAAGCTCTAGTTCCGGCAGCAC	ELF4-cLuc
	GAATTCATGAAGAGGAACGGCGAGAC CTCGAGAGCTCTAGTTCCGGCAGCAC	AD-ELF4, GBD-ELF4
LUX	GGTACCATGGGAGAGGAAGTACAAATGAGC GTCGACATTCTCATTTGCGCTTCCACC	LUX-cLuc
	GAATTCATGGGAGAGGAAGTACAAATGAGC CTCGAGATTCTCATTTGCGCTTCCACC	AD-LUX, MBP-LUX, GBD-LUX
PKL	GGTACCATGAGTAGTTTGGTGGAGAGGC CTCGAGTCAATCAACGACCATGTTCTTTG	PKL-nLUC
	CAATTGATGGTTGACCATAAAAGAAATCCCA GGTACCTCGAGGCTAGCTCAATCAACGACCATGTT CTTTG	LexA-D8
	CAATTGATGCTTAAAGATGCTTCCGTGGAAA GGTACCTCGAGGCTAGCTCAATCAACGACCATGTT CTTTG	LexA-D9
DOG1	GAATTCGGTACCTCTAGACAAGATATTGTGGACCG CTTGT CCCGGGCTGATATAATGAGGAGTATTTTGAT	P1:LacZ
	GAATTCGGTACCTCTAGAATGGGATCTTCATCAAAG AACATCG CCCGGGTTCCTCAAAACCGAAATAACCGAAAAC	P2:LacZ
	GAATTCGGTACCTCTAGATTGGTTCGGTAGTCAGTA CGG CCCGGGCTTTCCTTCCTCTCCTCCGG	P3:LacZ
	GAATTCGGTACCTCTAGAGTAATGCCGGAGGAGAG GAAG CCCGGGCAAATCGCATTGAAGAGACTCAAATC	P4:LacZ
	AATTCCGCAACATCGACGGCTACGAATCTTCAGGT AAGGGTTTGGAC TCGAGTCCAAACCCTTACCTGAAGATTCGTAGCCG TCGATGTTGCGG	P2-1:LacZ
	AATTCATCGAACAAGCTCAAGATTCTTATCTCGAG TGGATGAGT TCGAACTCATCCACTCGAGATAAGAATCTTGAGCT TGTTCGATG	P2-2:LacZ
	AATTTCGTAAGTTAACGGGAAAAATCATCGGTGAT TTCAAAAAT TCGAATTTTTGAAATCACCGATGATTTTTCCCGTTA ACTTACGA	P2-3:LacZ
	AATTCGAACTATTATGCACCCACGTGGAACAGTCC TTTAGAGAAC TCGAGTTCTCTAAAGGACTGTTCCACGTGGGTGCA TAATAGTTCG	P2-4:LacZ

	AATTCTTATGACAAAAATAATAGATTCTTAGGTTT TATATTAAGTT TCGAAACTTAATATAAAACCTAAGAATCTATTATT TTTGTCATAAG	P3-1:LacZ
	AATTTGCGGAGCAGCTAGCTAAAATCAATGTGTTG CATGTAAAAAT TCGAATTTTTACATGCAACACATTGATTTTAGCTA GCTGCTCCGCA	P3-2:LacZ
	AATTCAAGAAGACGCAGCGGATATTCCCATCGCC ACTGTGGCTTAC TCGAGTAAGCCACAGTGGCGATGGGAATATCCGC TGCGTCTTCTTG	P3-3:LacZ
	AATTAAGGGTTGATACTTTAGCGAAGATCCTCGGG ATTCTATCTCC TCGAGGAGATAGAATCCCGAGGATCTTCGCTAAA GTATCAACCCTT	P3-4:LacZ
	GGTACCCAAGATATTGTGGACCGCTTGT GTCGACTTCCTCTCCTCCGGCATTAC	DOG1p:LUC
ChIP		
ACT2	GCCATCCAAGCTGTTCTCTC GCTCGTAGTCAACAGCAACAA	
DOG1	TGTGGACCGCTTGTCCATAAT AGGAAACCTTTGAGAGCCGT	1
	ATTTCTTTCCTCTTTAAAGAGG CGATCTAAGACTTACACGAG	2
	ACACAAACACGCAAACCAAAA TTGTTTGAGCTCAGGGATGC	3
	CGGTAGTCAGTACGGTGCG ACCGAACCCAACTTAATATAAAACC	4
	GTCGAGGCCGATAATCTAAG AGTTCCCCACTCATGCATCG	5
	GGAGAGGAAGGAAAGTAGTC CGCATTGCACCTTGCTACCA	6
PRR7	ACCCACCATTACACGTGTCAGT ACGTTCGAGAAGTTCCACGTCA	
PRR9	CCTGCGAAGCAGAGGACCACC AGCGGGCCTTCACTGAGCTGA	
RT-qPCR		
IPP2	GTGCTTTCTCTGGTACCGCT TACTTGCTGTCATGCCCCAC	
PKL	GCTCTTGGAAAAAGGAAGAGAAG CGCGACCCTTTCTTCTGTAC	
DOG1	AAGAAGACGCAGCGGATATT TTGTCGAGAGCTTGATCCAC	sense

	GACTGGAGCACGAGGACACT	antisense
	ACGTTAGGCTCTCCGACATT	
LUX	CGGTAATGTTGGAGTGCCGA	
	TGACTCAAACGCACCATTTCC	
ELF3	AGTTTCTCGTCGGGCTTTCA	
	TAAGCTCTAGTTCCGGCAGC	
ELF4	GCCATTGCCAATCAACAAAGAG	
	AGAGATCCGGTGATGCAGC	
RVE1	CTCCTCGTCCCAAGAGAAAG	
	GTGGACAACACAGAGGTTGG	
RVE2	CTAACCGGATCCAAGCTGAT	
	GAACCTAATCCATCTGAGCCA	
GA2ox1	CGGGTCCACTATTTCCAAGT	
	GTTCCTCGGTTTGATCCCTA	
GA3ox1	AAATGTGGTCCGAAGGTTTC	
	CATCAATTTCGATGCCAACT	
GA3ox2	AAGGTTTCACCGTTATTGGC	
	ACCTAATGCGAACCACATCA	
GA20ox3	GTGGTGAACATAGGCGACAC	
	CCCTTTCGGACATAGGAAGA	
ABA2	TCCAAGCATGCTGTTCTAGG	
	AAATGAGCCAAAGCGAGTTT	
ABA3	GTGATACGTTGGCCACTTTG	
	GACCCTGAACCATCCATTCT	
NCED2	AGCATAATCCTCTCCGGCTA	
	GGAGAATCTTGCTCGTGTGA	
NCED4	CATGTTGGATTTGGTTCTCG	
	CCAGGAACTTCGAACCATTT	
NCED6	ACCGGATTGTTTCTGTTTCC	
	ACGACGATAACTGGGTCTCC	
EM1	TCAAATGGTATGCGGTTATG	
	TATCACAAGTAAGACACGAAG	
AHG1	ACGACAATGACGGAGAACAA	
	TGAGATCAATGACGACCACA	
DYL1	CGGCTCCAACATCTTTGATA	
	CTCCTTGAGTCACCGCTGTA	
PER1	ACGGTGCCGAACCTAGAAGTG	
	GTATTTGGCCATCGCACCAAG	
LEC1	TCAAGATGAATCCAGTGTTG	
	CATCTTCACTTATACTGACC	
EMSA		
DOG1	AATTAAGGGTTGATACTTTAGCGAAGATCCTCGGG	
	ATTCTATCTCC	
	TCGAGGAGATAGAATCCCGAGGATCTTCGCTAAA	

GTATCAACCCTT