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EIN2-directed histone acetylation requires EIN3-mediated positive feedback regulation in response to ethylene

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Abstract

Ethylene is an important phytohormone with pleotropic roles in plant growth, development, and stress responses. ETHYLENE INSENSITIVE2 (EIN2) mediates the transduction of the ethylene signal from the endoplasmic reticulum membrane to the nucleus, where its C-terminus (EIN2-C) regulates histone acetylation to mediate transcriptional regulation by EIN3. However, no direct interaction between EIN2-C and EIN3 has been detected. To determine how EIN2-C and EIN3 act together, we followed a synthetic approach and engineered a chimeric EIN2-C with EIN3 DNA-binding activity but lacking its transactivation activity (EIN2C–EIN3DB). The overexpression of *EIN2C–EIN3DB* in either wild-type or in the ethylene-insensitive mutant *ein3-1 eil1-1* led to a partial constitutive ethylene response. Chromatin immunoprecipitation sequencing showed that EIN2C–EIN3DB has DNA-binding activity, indicating that EIN3DB is functional in EIN2C–EIN3DB. Furthermore, native EIN3 protein levels determine EIN2C–EIN3DB binding activity and binding targets in a positive feedback loop by interacting with EIN2C–EIN3DB to form a heterodimer. Additionally, although EIN3 does not direct affect histone acetylation levels in the absence of EIN2, it is required for the ethylene-induced elevation of H3K14Ac and H3K23Ac in the presence of EIN2. Together, we reveal efficient and specific DNA-binding by dimerized EIN3 in the presence of ethylene to mediate positive feedback regulation, which is required for EIN2-directed elevation of histone acetylation to integrate into an EIN3-dependent transcriptional activation.

Introduction

Ethylene, a gaseous plant hormone, is important for a myriad of physiological and developmental processes including seed germination, plant growth, fruit ripening, organ abscission, and senescence. It is also involved in responses to stresses such as drought, cold, flooding, and infections (Alonso et al., 1999; Pierik et al., 2006; Ju and Chang, 2015). The common aquatic ancestor of plants, which existed about 450 million years ago, possessed an ethylene signaling pathway that was similar to that of modern Arabidopsis (*Arabidopsis thaliana*; Ju et al., 2015). The receptors for ethylene are located at the endoplasmic reticulum (ER)

membrane (Chen et al., 2002). The factors involved in transmission of the signal from the ER membrane to the nucleus have been previously identified based on decades of genetic and molecular biology research (Chang et al., 1993; Kieber et al., 1993; Chao et al., 1997; Hua and Meyerowitz, 1998; Hua et al., 1998; Alonso et al., 1999; Chen et al., 2002; Huang et al., 2003; Grefen et al., 2008; Ju et al., 2012; Qiao et al., 2012; Wang and Qiao, 2019). ETHYLENE INSENSITIVE2 (EIN2) has been shown to be the key mediator in this process (Ju et al., 2012; Qiao et al., 2012). The Arabidopsis *EIN2* gene encodes a 1,294 amino acid protein with a predicted 12-fold hydrophobic transmembrane domain at its N terminus that shows a high sequence similarity to the conserved natural resistance-associated macrophage protein family of metal ion transporters (Alonso et al., 1999), although no metal transport activity has been shown for EIN2 to date (Alonso et al., 1999). In the presence of ethylene, the hydrophilic C terminus of EIN2 (EIN2-C) is dephosphorylated and cleaved from the rest of the protein, and cleaved EIN2-C translocates to the P body to suppress the translation of the two F-box proteins EIN3-BINDING F BOX PROTEIN1 (EBF1) and EBF2 (Li et al., 2015; Merchante et al., 2015), and to the nucleus to activate key transcription factors EIN3/EIN3-LIKE1 (EIL1)-dependent transcription (Qiao et al., 2012).

Recent studies revealed that the histone acetylation marks H3K14Ac and H3K23Ac are positively associated with gene expression in the response to ethylene, and that the ethylene-induced change in histone acetylation depends on EIN2 levels (Zhang et al., 2016b; Wang and Qiao, 2019; Wang et al., 2019). By ectopically expressing *EIN2-C* in an *ein2-5* mutant using clustered regularly interspaced short palindromic repeats (CRISPRs) combined with a dead version of the CRISPR-associated nuclease (dCas9), we demonstrated that EIN2-C directly regulates histone acetylation (Zhang et al., 2017). Detailed mechanistic studies showed that in the presence of ethylene, nucleus-localized EIN2-C interacts with EIN2 NUCLEAR-ASSOCIATED PROTEIN1 (ENAP1), a histone-binding protein, to associate with the chromatin of ethylene-responsive genes to regulate acetylation at H3K14 and H3K23, leading to EIN3-dependent transcriptional regulation (Zhang et al., 2016b, 2017).

It is now well established that many of the effects exerted by transcription factors in eukaryotes are through their interaction with co-regulators that modify the chromatin state, which results in a more open (in the case of activation) or a more closed (in the case of repression) conformation. In ethylene signaling, EIN3 and EIL1 are the key transcription factors that are necessary and sufficient for the ethylene response (Chao et al., 1997; Guo and Ecker, 2003; Chang et al., 2013). EIN3 has been shown to form a homodimer in the absence of DNA in vitro, and the EIN3 binding element was identified after analysis of genes such as *ETHYLENE RESPONSE FACTOR1* (*ERF1*) and *ETHYLENE RESPONSE DNA-BINDING FACTOR2* (*EDF2*), whose expression is highly upregulated by ethylene (Ohme-Takagi and Shinshi, 1990; Eyal et al., 1993; Meller et al., 1993; Sessa et al., 1995; Shinshi et al., 1995; Sato et al., 1996; Solano et al.,

1998). Our previous study demonstrated that EIN3 is partially required for the EIN2-directed elevation of histone acetylation in response to ethylene. Yet, how EIN3 is involved in EIN2-C-mediated histone acetylation, and how the regulation of histone acetylation integrates into EIN3-dependent transcriptional regulation in response to ethylene is still largely unknown. We have found that mutations in EIN3 lead to a decrease in both histone acetylation levels and the transcriptional activities (Zhang et al., 2016a). Both EIN2-C and EIN3 interact with ENAP1 (Zhang et al., 2016a); however, no direct interaction between EIN2-C and EIN3 has been detected by multiple approaches. Given that both transcriptional regulation and histone acetylation are impaired simultaneously in *ein3* mutants, it is not possible to uncouple the function of EIN3 in the regulation of histone acetylation and transcriptional regulation by using *ein3* mutants. In this study, to determine how EIN3 functions in EIN2-directed histone acetylation and transcriptional regulation in response to ethylene, we engineered a chimeric protein that fuses EIN2-C to the EIN3 DNA-binding domain (EIN2C–EIN3DB). The overexpression of *EIN2C–EIN3DB* in wild-type Arabidopsis (Col-0 accession) and *ein3-1 eil1-1* seedlings leads to a partial constitutive ethylene response. Chromatin immunoprecipitation sequencing (ChIP-seq) showed that EIN2C–EIN3DB binds DNA in vivo and that native EIN3 protein levels are crucial for EIN2C–EIN3DB binding activity and specificity. Pull-down and co-immunoprecipitation assays also demonstrated that EIN2C–EIN3DB interacts with native EIN3 both in vitro and in vivo, and that the DNA-binding activity of EIN2C–EIN3DB depends on the presence of native EIN3. By examining the levels of H3K14Ac and H3K23Ac in *ein3-1 eil1-1 EIN2*^{S645A} and *ein2-5 EIN3ox* seedlings, we found that EIN3 does not directly affect the levels of H3K14Ac and H3K23Ac in the absence of EIN2. However, EIN3 is required for the ethylene-induced elevation of H3K14Ac and H3K23Ac mediated by EIN2-C. In summary, in the presence of ethylene, efficient and specific DNA binding by a potentially dimerized EIN3 mediates a positive feedback regulation, which is required for EIN2-directed elevation of histone acetylation at H3K14Ac and H3K23Ac to integrate into an EIN3-dependent transcriptional regulation.

Results

A fusion of EIN2-C and the EIN3 DNA-binding domain can regulate the ethylene response

It has been proposed that transcription factors guide chromatin remodeling complexes to certain regions of the genome through their sequence-specific DNA-binding domains. However, how exactly EIN3 functions in the EIN2-dependent regulation of histone acetylation in response to ethylene is unknown. Our previous study has shown that both EIN2-C and EIN3 interact with ENAP1 (Zhang et al., 2016a), yet no direct interaction between EIN2-C and EIN3 has been detected by different approaches. To investigate whether and how the EIN3 DNA-binding domain plays such

a role, we fused the sequence encoding the EIN3 DNA-binding domain (*EIN3DB*) with the sequence encoding EIN2-C in both orientations (*EIN2C–EIN3DB* and *EIN3DB–EIN2C*; Figure 1A; Supplemental Figure 1A). Additionally, EIN2-C was fused with a EIN3DB point mutant carrying a K to N mutation at amino acid 245 (K245N) that completely abolishes EIN3 DNA-binding activity (Chao et al., 1997; Solano et al., 1998) in both orientations (*EIN2C–EIN3DB^{K245N}* and *EIN3DB^{K245N}–EIN2C*; Figure 1A; Supplemental Figure 1A). We then introduced all constructs into Col-0 and into the ethylene insensitive mutant *ein3-1 eil1-1*, which lacks both EIN3 and EIL1 (Alonso et al., 2003). About 20% of T$_1$ plants that carried wild-type *EIN3DB* (*EIN2C–EIN3DB* or *EIN3DB–EIN2C*) in both backgrounds showed a constitutive ethylene-responsive adult plant phenotype, as did about 20% of T$_1$ plants that carried *EIN3DB^{K245N}* (*EIN2C–EIN3DB^{K245N}* or *EIN3DB^{K245N}–EIN2C*) in Col-0 (Figure 1B; Supplemental Figure 1B). However, only 10% of T$_1$ plants with the *EIN3DB^{K245N}* transgenes (*EIN2C–EIN3DB^{K245N}* and *EIN3DB^{K245N}–EIN2C*) showed a constitutive ethylene-responsive phenotype in the *ein3-1 eil1-1* background (Figure 1B; Supplemental Figure 1B). Since *EIN3DB–EIN2C* and *EIN2C–EIN3DB* resulted in similar phenotypes with similar percentages of dwarf T$_1$ plants (Figure 1B; Supplemental Figure 1B), we performed subsequent analyzes using *EIN2C–EIN3DB* and *EIN2C–EIN3DB^{K245N}*.

We then obtained homozygous transgenic lines and examined the phenotypes of 3-day-old etiolated seedlings. Consistent with our observations in T$_1$ transgenic plants, overexpression of *EIN2C–EIN3DB* or *EIN2C–EIN3DB^{K245N}* led to a partial ethylene-responsive phenotype in Col-0 and *ein3-1 eil1-1* seedlings (Figure 1, C–E; Supplemental Figure S1, C–E). Interestingly, although EIN2C–EIN3DB and EIN2C–EIN3DB^{K245N} protein levels were comparable in both Col-0 and *ein3-1 eil1-1* (Supplemental Figure 1F), the partial constitutive ethylene-responsive phenotype observed in *EIN2C–EIN3DB^{K245N}* overexpression lines was weaker than that resulting from the overexpression of *EIN2C–EIN3DB* in both Col-0 and *ein3-1 eil1-1* (Figure 1, C–E; Supplemental Figure 1, C–E). Furthermore, the phenotype caused by the overexpression of *EIN2C–EIN3DB* or *EIN2C–EIN3DB^{K245N}* in *ein3-1 eil1-1* was weaker than in Col-0 (Figure 1, C–E; Supplemental Figure 1, C–E). To further confirm if the phenotype of *ein3-1 eil1-1 EIN2C–EIN3DB^{K245N}* and *ein3-1 eil1-1 EIN2C–EIN3DB* is ethylene related, we conducted a transcriptome deep sequencing analysis. Compared to the levels in Col-0 seedlings, more genes were differentially expressed in *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings (5,686 genes) than in *ein3-1 eil1-1 EIN2C–EIN3DB^{K245N}* seedlings (3,721 genes). About 40% of all ethylene-regulated genes evaluated had an altered expression level in *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings (Figure 1F), as compared to 33% in *ein3-1 eil1-1 EIN2C–EIN3DB^{K245N}* seedlings (Figure 1F). When further compared to *ein3-1 eil1-1*, we found 22% of ethylene-regulated genes with an altered expression in *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings (Supplemental Figure 1, G–J), against only 12% in

ein3-1 eil1-1 EIN2C–EIN3DB^{K245N} seedlings (Supplemental Figure 1, G–J). Most of the shared genes in *ein3-1 eil1-1 EIN2C–EIN3DB* vs. *ein3-1 eil1-1* were regulated in the same direction as those responding to ethylene in Col-0 (Supplemental Figure 1, H–J). These analyses confirmed the ethylene-responsive phenotype of *ein3-1 eil1-1 EIN2C–EIN3DB* and *ein3-1 eil1-1 EIN2C–EIN3DB^{K245N}* seedlings at a molecular level. The fact that fewer genes were differentially expressed upon expression of *EIN2C–EIN3DB^{K245N}* than upon expression of *EIN2C–EIN3DB* may possibly be due to the interruption of EIN3 DNA-binding activity, which could potentially lead to a weaker ethylene-responsive phenotype associated with the overexpression of *EIN2C-EIN3DB^{K245N}* (Figure 1F; Supplemental Figure 1, G–J).

EIN2C–EIN3DB retains DNA-binding activity and is enhanced by native EIN3

To evaluate EIN3 DNA-binding ability in the context of the engineered EIN2C–EIN3DB protein, we conducted chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) using an anti-EIN2-C antibody in *EIN2C–EIN3DB* (in Col-0), *ein3-1 eil1-1 EIN2C–EIN3DB*, *EIN2C–EIN3DB^{K245N}* (in Col-0), and *ein3-1 eil1-1 EIN2C–EIN3DB^{K245N}* seedlings treated with air (Figure 2A). We detected more than 1,000 genes whose promoters were bound by EIN2C–EIN3DB in *EIN2C–EIN3DB* seedlings (Figure 2A). In contrast, only about 100 genes had their promoters bound by EIN2C–EIN3DB in *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings (Figure 2A). The EIN2C–EIN3DB ChIP-seq signals from *EIN2C–EIN3DB* seedlings were significantly higher than those from *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings (Figure 2B). We obtained a similar result when comparing the ChIP-seq signals in shared peaks between the two genotypes (Figure 2C). No ChIP signals were detected in *EIN2C–EIN3DB^{K245N}* or *ein3-1 eil1-1 EIN2C–EIN3DB^{K245N}* seedlings. These results suggest that the DNA-binding domain of EIN2C–EIN3DB has DNA-binding activity and that K245 is necessary for this activity, which is consistent with previous studies (Chao et al., 1997; Solano et al., 1998). Our data also suggest that endogenous EIN3 potentially regulates EIN2C–EIN3DB binding activity.

These results, however, also raised a question: how can overexpression of *EIN2C–EIN3DB^{K245N}*, which has no DNA-binding activity, cause a weak ethylene responsive phenotype even in the absence of EIN3 and EIL1? We speculated that both EIN2C–EIN3DB and EIN2C–EIN3DB^{K245N} associate with ENAP1, allowing EIN2-C to regulate histone acetylation above a basal level, therefore leading to an alteration in the expression of a subset of ethylene-responsive genes. To test this hypothesis, we examined the interaction between ENAP1 with EIN2C–EIN3DB and ENAP1 with EIN2C–EIN3DB^{K245N}. As expected, we detected an interaction between ENAP1 and EIN2C–EIN3DB and with EIN2C–EIN3DB^{K245N} by yeast two hybrid, with the interaction between EIN2-C and ENAP1 serving as a positive control (Supplemental Figure 2A). We also compared the binding targets of EIN2C–EIN3DB and ENAP1: about 41% of EIN2C–
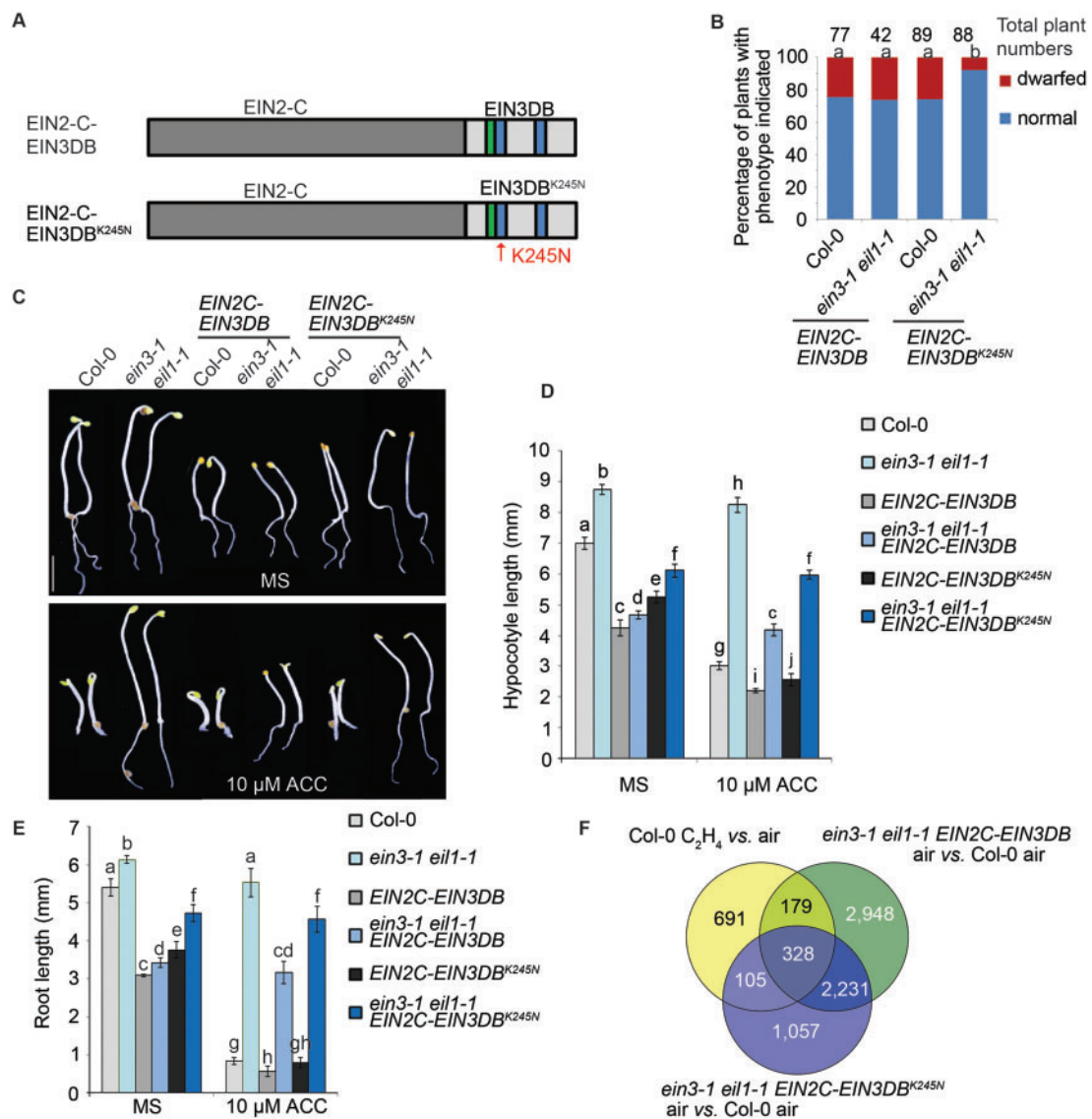
Figure 1 Overexpression of engineered EIN2–EIN3DB leads to a partial constitutive ethylene-responsive phenotype. (A) Diagrams showing the fusion of EIN2C with EIN3DB. Green indicates the proline-rich region; blue indicates the second and third basic motifs in EIN3DB. (B) The ratio of adult *EIN2C–EIN3DB* and *ein3-1 eil1-1 EIN2C–EIN3DB* plants that displayed a dwarf phenotype. Eight-week-old plants were grown under 16 h light and 8 h dark before investigation. Different letters indicate significant differences, determined by hypergeometric probability ($P < 0.05$). (C) The phenotypes of 3-day-old Col-0 and *ein3-1 eil1-1* seedlings that express *EIN2C–EIN3DB* or *EIN2C–EIN3DB^{K245N}*. Seedlings were grown on MS medium with or without 10-μM ACC. Bar indicates 2.5 mm. (D and E) Lengths of (D) hypocotyls and (E) roots of 3-day-old etiolated seedlings of the indicated phenotypes. Data are means ± SD of at least 30 seedlings. Different letters indicate significant differences, determined using one-way ANOVA (Analysis of Variation) Scheffe method ($P < 0.05$). (F) Venn diagrams showing ethylene-regulated genes that are differentially expressed in *ein3-1 eil1-1 EIN2C–EIN3DB* or *ein3-1 eil1-1 EIN2C–EIN3DB^{K245N}* seedlings compared to Col-0. Total RNA was prepared from 3-day-old etiolated seedlings treated with air. Differentially expressed genes were identified by reads per kilobase per million reads (RPKM) $\geqslant 1$ with twofold change and $q \leqslant 0.05$.

EIN3DB binding targets overlapped with those of ENAP1 (Supplemental Figure 2B), which further validated our hypothesis.

As more genes were bound by EIN2C–EIN3DB in Col-0 than in *ein3-1 eil1-1* seedlings, we speculated that the native EIN3 protein enhances the binding activity of EIN2C–EIN3DB. To test this idea, we compared EIN2C–EIN3DB ChIP-seq signals in Col-0 with those of *ein3-1 eil1-1* seedlings. There were significantly more binding targets identified in Col-0 seedlings (1,223) than in *ein3-1 eil1-1* seedlings (122;

Figure 2A). A chromatin immunoprecipitation quantitative polymerase chain reaction (ChIP-qPCR) assay further confirmed that there was increased enrichment of EIN2C–EIN3DB binding detected in the Col-0 background relative to *ein3-1 eil1-1* (Figure 2D). This observation strongly indicates that native EIN3 protein enhances EIN2C–EIN3DB binding.

Given the fact that EIN2-C is the key factor that regulates histone acetylation during the ethylene response (Zhang et al., 2017) and that EIN2C–EIN3DB confers an ethylene-responsive phenotype, we then conducted ChIP-qPCR of
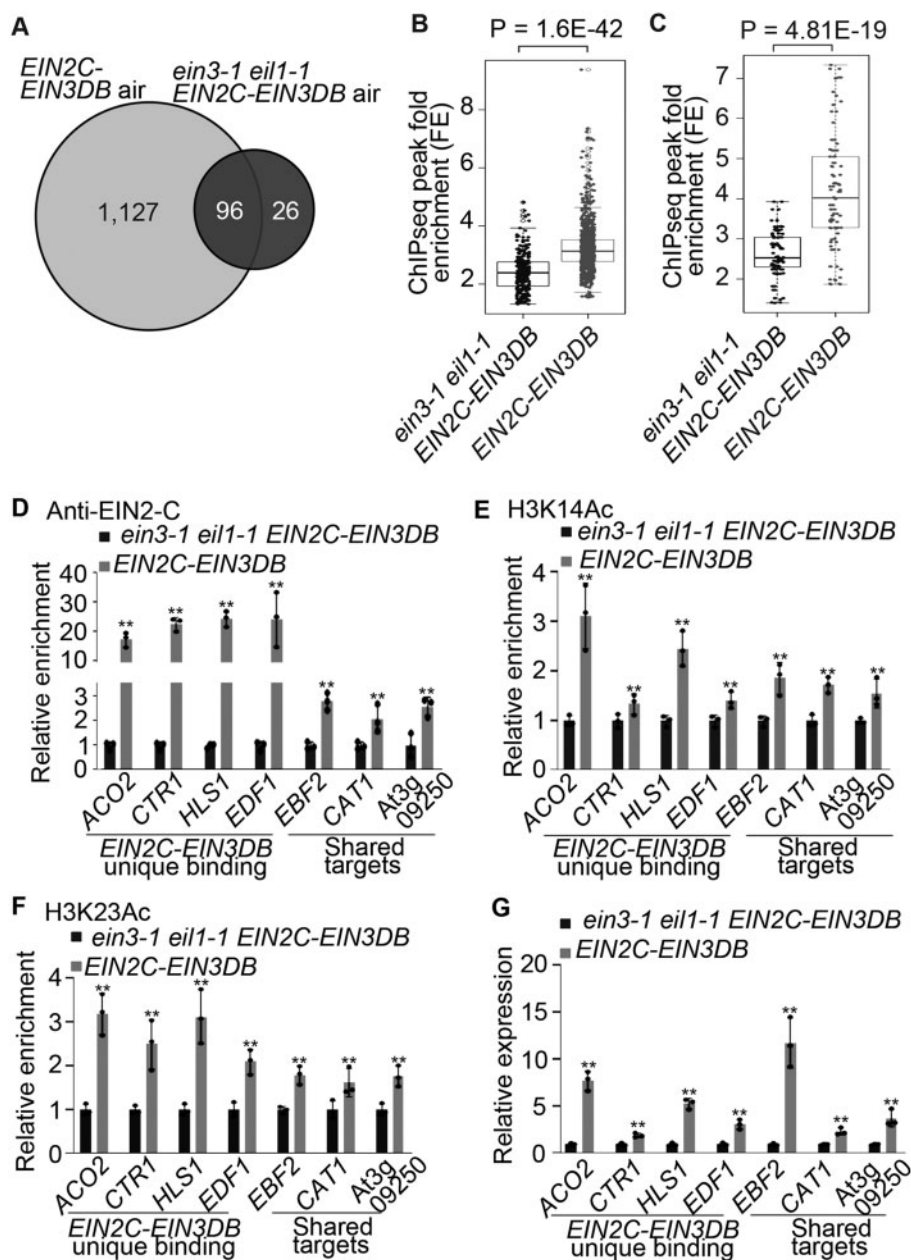
Figure 2 Endogenous EIN3 protein regulates EIN2C–EIN3DB binding activity, histone acetylation, and gene expression. (A) Venn diagram showing EIN2C–EIN3DB-bound targets in the Col-0 and *ein3-1 eil1-1* backgrounds. ChIP-seq was conducted on 3-day-old etiolated *EIN2C–EIN3DB* and *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings treated with air. (B and C) Boxplots and dotplots of peak signals in *EIN2C–EIN3DB* and *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings determined by analyses of (B) signals from the peaks identified in *EIN2C–EIN3DB* and *ein3-1 eil1-1 EIN2C–EIN3DB* or (C) signals from shared peaks. *P*-value was calculated by one-way ANOVA and indicated in the figure. (D–F) ChIP-qPCR assays of (D) the binding of EIN2C–EIN3DB, (E) H3K14Ac levels, and (F) H3K23Ac in selected loci from 3-day-old etiolated seedlings of the indicated backgrounds. Data represent relative fold changes. G, RT-qPCR quantification of gene expression in 3-day-old etiolated seedlings. Shared targets indicate binding targets that are shared between *ein3-1 eil1-1 EIN2C–EIN3DB* and *EIN2C–EIN3DB*. Each ChIP-qPCR or RT-qPCR was repeated at least three times. Values are means ± SD of three biological replicates. **P < 0.05 when compare to the control in Col-0 background by one-tailed unpaired *t* test.

H3K14Ac and H3K23Ac to examine the association between EIN2C–EIN3DB binding and histone acetylation levels. The levels of H3K14Ac and H3K23Ac were higher in *EIN2C–EIN3DB* seedlings than in *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings (Figure 2, E and F). Furthermore, reverse transcription (RT-qPCR) showed that gene expression is positively correlated with the levels of H3K14Ac and H3K23Ac (Figure 2G).

These data suggest that native EIN3 protein regulates EIN2C–EIN3DB binding and that the levels of H3K14Ac and H3K23Ac are positively associated with EIN2C–EIN3DB binding activity.

To further confirm the roles of native EIN3 protein in the regulation of EIN2C–EIN3DB binding activity, we compared the binding of EIN2C–EIN3DB under three conditions: in

ein3-1 eil1-1 EIN2C–EIN3DB seedlings without native EIN3 protein; in *EIN2C–EIN3DB* seedlings without ethylene treatment, corresponding to EIN3 basal levels; in *EIN2C–EIN3DB* after 4 h of ethylene treatment, when EIN3 has accumulated (Guo and Ecker, 2003; Potushak et al., 2003). We detected about 1,000 peaks in *EIN2C–EIN3DB* seedlings without ethylene treatment and about 1,500 binding peaks after ethylene treatment (Figure 3, A and B). We obtained a similar number of binding peaks (~130) in *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings with or without ethylene treatment, and differences in peak numbers or in ChIP-seq signals were not significant (Figure 3, A and B). The peak numbers and ChIP-seq signals were thus significantly higher in *EIN2C–EIN3DB* seedlings than in *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings (Figure 3, A and B). We then compared the EIN2C–EIN3DB binding targets in Col-0 seedlings with and without ethylene treatment (Figure 3C). Almost 50% of genes bound by

EIN2C–EIN3DB without ethylene treatment were also bound by EIN2C–EIN3DB with ethylene treatment (Figure 3C), and the enrichment of EIN2C–EIN3DB ChIP-seq signals were significantly higher with ethylene treatment than in the absence of ethylene treatment (Figure 3D), which indicates that EIN2C–EIN3DB binding strength is positively correlated with native EIN3 protein levels.

We then performed several motif searches: using the peak sequences bound by EIN2C–EIN3DB in Col-0 seedlings without ethylene treatment; with the peak sequences from the shared targets with ethylene treatment; with the peak sequences from the shared targets without ethylene treatment; and with the peak sequences specifically after ethylene treatment. No classical EIN3 binding element was identified from the peaks without ethylene treatment or from the peaks shared between with and without ethylene treatments (Supplemental Figure 3, A–C). However, we
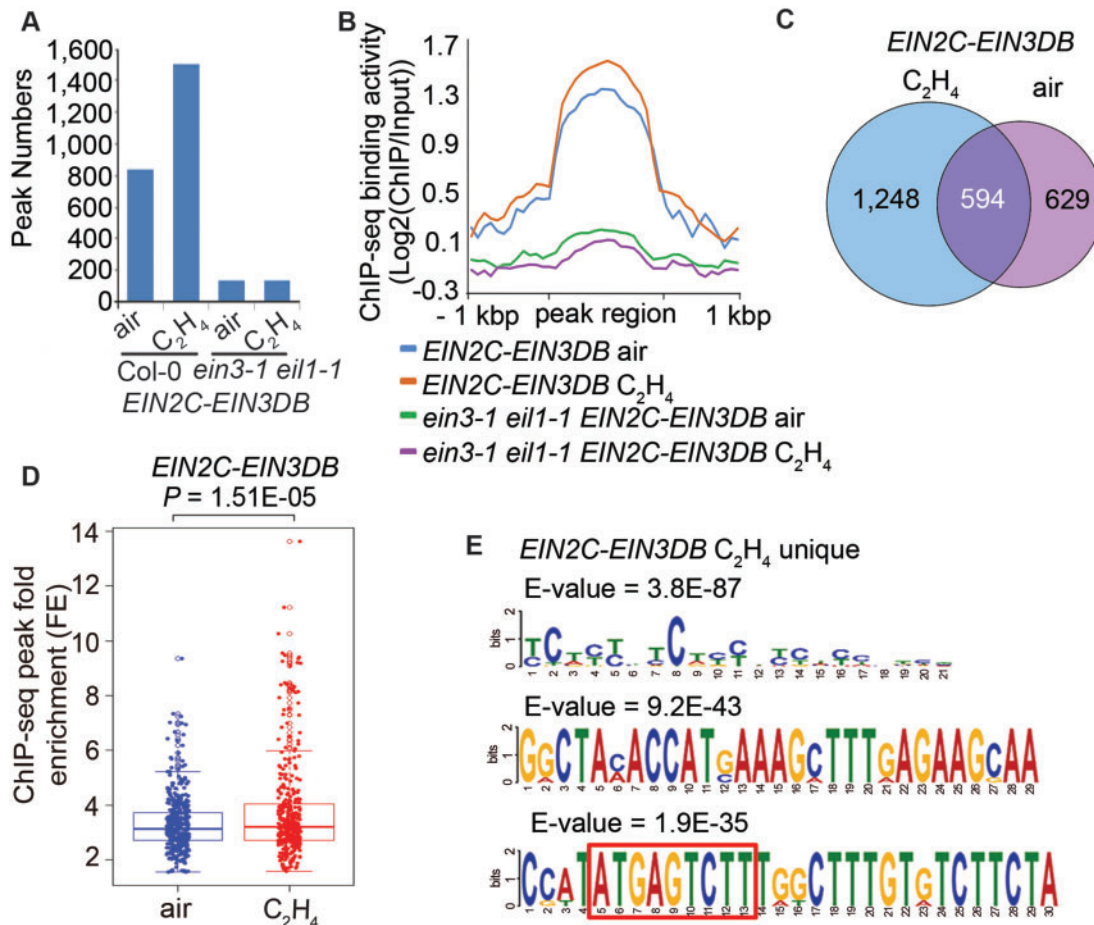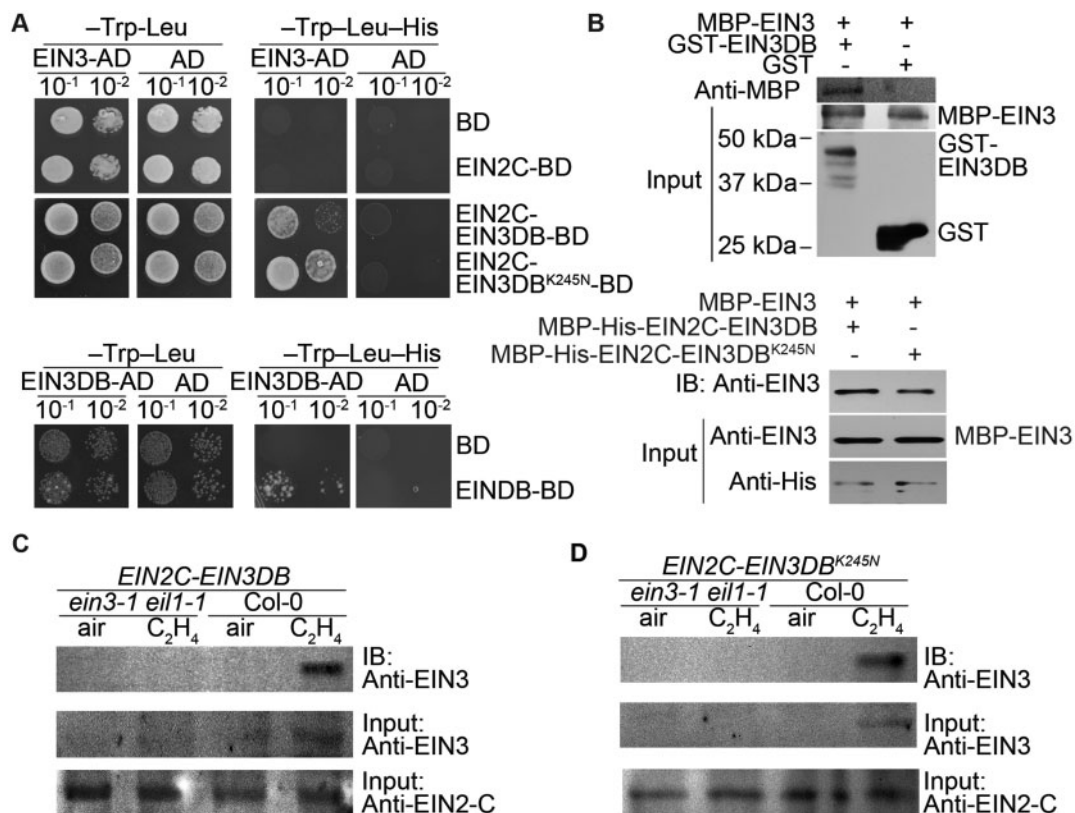

Figure 3 EIN2C–EIN3DB binding activity is positively associated with endogenous EIN3 protein levels. (A) Number of EIN2C–EIN3DB ChIP peaks identified from 3-day-old *EIN2C–EIN3DB* and *ein3-1 eil1-1 EIN2C–EIN3DB* etiolated seedlings treated with or without ethylene. (B) ChIP-seq signals of 1-kbp upstream and downstream of peak regions from *EIN2C–EIN3DB* and *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings treated with or without ethylene. (C) Venn diagrams showing the number of EIN2C–EIN3DB-bound genes in *EIN2C–EIN3DB* seedlings with and without ethylene treatment. (D) Boxplots and dotplots of ChIP-seq signal in *EIN2C–EIN3DB* seedlings with and without ethylene treatment. *P*-value was calculated by one-way ANOVA and indicated in the figure. (E) DNA-binding motifs identified from EIN2C–EIN3DB-bound regions uniquely targeted under ethylene treatment. E-values are indicated. Motifs were identified using MEME-ChIP (http://meme-suite.org/tools/meme-chip) by the analysis of 300-bp centered on each EIN2C–EIN3DB binding peak. Red box highlights the motif with similarity to the known EIN3 binding motif. The canonical EIN3 binding motif sequence is: (A/T)(T/C)G(A/C/T)A(T/C/G)(C/G)T(T/G).

did identify the previously established EIN3 binding element from the peaks sequences that specifically appeared upon ethylene treatment (Figure 3E; Chang et al., 2013). Taken together, these data strongly suggest that native EIN3 protein levels regulate EIN2C–EIN3DB binding specificity, and that the positive correlation between EIN3 protein levels and EIN2C–EIN3DB binding activity indicates the existence of a positive feedback regulation in response to ethylene.

EIN2C–EIN3DB interacts with EIN3 both in vivo and in vitro to form dimers

The positive correlation between EIN2C–EIN3DB binding and native EIN3 protein levels led us to speculate that native EIN3 interacts with EIN2C–EIN3DB. To test this hypothesis, we conducted yeast two-hybrid assays and in vitro pull-down assays. Both EIN2C–EIN3DB and EIN2C–EIN3DB^{K245N} interacted with EIN3 (Figure 4A, upper panel), demonstrating that the point mutation in the EIN3 DNA-binding domain has no effect on the interaction between EIN2C–EIN3DB and EIN3. Interestingly, we found that EIN3DB can form a homodimer (Figure 4A, lower panel), which indicates

that the EIN3DB moiety may potentially mediate the interaction between EIN3 and EIN3DB, although no such interaction was detected by yeast two-hybrid assay (Supplemental Figure 4A). To further test the interaction between EIN3 and EIN3DB, we conducted a pull-down assay using recombinant maltose-binding protein (MBP)-EIN3 and glutathione S-transferase (GST)-EIN3DB. The result showed that recombinant EIN3 interacts with EIN3DB (Figure 4B, upper panel), suggesting that EIN3DB is sufficient to mediate the interaction between EIN3 and EIN3DB. A previous study has shown that EIN3 can form dimers in yeast two-hybrid assays (Solano et al., 1998). However, it is normally impossible to dissect the contribution of EIN3 dimerization with EIN3 function in vivo because the EIN3 DNA-binding domain overlaps with the dimerization domain. Indeed, when dimerization is abolished, the DNA-binding activity of EIN3 will be largely reduced. However, EIN2C–EIN3DB provides a perfect tool to explore the role of EIN3 dimerization in vivo. We first examined the interaction between EIN3 and EIN2C–EIN3DB or EIN2C–EIN3DB^{K245N} by pull-down assay. Consistent with the yeast two-hybrid result, EIN3 interacted with both EIN2C–EIN3DB and

Figure 4 EIN3 interacts with EIN2C–EIN3DB to regulate its binding activity. (A) Yeast two-hybrid assays to examine the interaction between EIN3 and EIN2C–EIN3DB (upper panel), and the dimerization of EIN3DB (lower panel). Yeast cells co-expressing the indicated combinations of constructs were grown on nonselective (left) or selective (right) medium. Different dilutions of cultures indicated in the figure were inoculated on each spot. (B) Pull-down assays to examine the interaction between EIN3 and EIN3DB, EIN2C–EIN3DB, or EIN2C–EIN3DB^{K245N}. Reactions were performed using recombinant MBP-EIN3, GST-EIN3DB, and MBP-His fusion proteins (MBP-His-EIN2C-EIN3DB and MBP-His-EIN2C-EIN3DB^{K245N}). Proteins were separated by SDS–PAGE and detected with anti-EIN3 or anti-MBP antibody. (C and D) In vivo immunoprecipitation assay to examine the interaction between EIN3 and (C) EIN2C–EIN3DB or (D) EIN2C–EIN3DB^{K245N} in total protein extracts from 3-day-old etiolated seedlings of the indicated genotypes treated with or without ethylene.

EIN2C–EIN3DB^{K245N} (Figure 4B, a lower panel). We next evaluated these interactions by in vivo immunoprecipitation with an anti-EIN2 antibody in *EIN2C–EIN3DB*, *ein3-1 eil1-1 EIN2C–EIN3DB*, *EIN2C–EIN3DB^{K245N}*, and *ein3-1 eil1-1 EIN2C–EIN3DB^{K245N}* seedlings both with and without ethylene treatment. We detected an interaction between EIN3 and EIN2C–EIN3DB in *EIN2C–EIN3DB* seedlings as well as between EIN3 and EIN2C–EIN3DB^{K245N} in *EIN2C–EIN3DB^{K245N}* seedlings with the presence of ethylene (Figure 4, C and D). These interactions were confirmed by in vivo immunoprecipitation assays in Arabidopsis protoplasts derived from *ein2-5 EIN3ox* and *ein2-5* etiolated seedlings and transfected with *35Spro:EIN2C–EIN3DB* or *35Spro:EIN2C–EIN3DB^{K245N}* (Supplemental Figure 4, B and C).

EIN2 regulates histone acetylation in the absence of EIN3 and EIL1

To further investigate the function of EIN3 in EIN2-C-mediated regulation of histone acetylation during ethylene response, we introduced the previously generated *EIN2^{S645A}ox* transgene, in which the C terminus of EIN2 is constitutively localized to the nucleus and causes a constitutive ethylene-response phenotype (Qiao et al., 2012), into *ein3-1 eil1-1* mutants by crossing to generate *ein3-1 eil1-1 EIN2^{S645A}*. Etiolated seedlings for *ein3-1 eil1-1 EIN2^{S645A}* displayed a partial ethylene-responsive phenotype in shoots and roots when compared to that of *ein3-1 eil1-1* and Col-0 in the absence of ethylene treatment, and the phenotype of *ein3-1 eil1-1 EIN2^{S645A}* was not altered by ethylene exposure (Figure 5, A–C). Further testing showed that EIN2^{S645A} protein levels were similar in *EIN2^{S645A}* and in *ein3-1 eil1-1 EIN2^{S645A}* (Figure 5D). To study the association between the *ein3-1 eil1-1 EIN2^{S645A}* phenotype and ethylene responsiveness at a molecular level, we analyzed gene expression levels in *ein3-1 eil1-1 EIN2^{S645A}* and Col-0 seedlings by RNA-seq. About 7,000 genes were differentially expressed in *ein3-1 eil1-1 EIN2^{S645A}* seedlings when compared to Col-0 (Figure 5E), and about 38% (491/1,303) of ethylene-regulated genes were altered in *ein3-1 eil1-1 EIN2^{S645A}* seedlings compared to Col-0 seedlings (Figure 5E). When compared to *ein3-1 eil1-1*, we found that about 5,000 genes were differentially expressed in *ein3-1 eil1-1 EIN2^{S645A}* seedlings and that about 29% of ethylene-regulated genes were altered in *ein3-1 eil1-1 EIN2^{S645A}* seedlings (Supplemental Figure 5, A and B). We validated these results by RT-qPCR confirmed for selected genes (Supplemental Figure 5C), showing that sufficient levels of EIN2-C can alter the expression of a subset of ethylene-responsive genes in the absence of EIN3 and EIL1.

We next evaluated H3K14Ac and H3K23Ac levels for the subset of genes that was elevated in *ein3-1 eil1-1 EIN2^{S645A}* seedlings relative to *ein3-1 eil1-1* seedlings. H3K23Ac and H3K14Ac levels were significantly higher in *ein3-1 eil1-1 EIN2^{S645A}* seedlings than in *ein3-1 eil1-1*, but lower than in *EIN2^{S645A}* seedlings (Figure 5, F and G). This result suggests that in the absence of EIN3, EIN2-C acts to elevate H3K14Ac

and H3K23Ac levels, while EIN3 is important for ethylene-induced elevation in H3K14Ac and H3K23Ac.

We previously demonstrated that EIN2-C directly regulates histone acetylation by using the *gEBF2-dCAS9-EIN2C* transgene, in which EIN2-C is targeted to the promotor region of *EBF2*, a typical ethylene elevated gene, by the gRNA/dCAS9 system (Zhang et al., 2017). To further investigate the roles of EIN3 in the regulation of histone acetylation in the promoter regions of its target genes, we introduced *gEBF2-dCAS9-EIN2C* and *gETR2-dCAS9-EIN2C*, derived from another well-known EIN3 target (Chang et al., 2013), into *ein3-1 eil1-1* mutants by crossing. We found that in the absence of EIN3 and EIL1, seedlings displayed partial ethylene-responsive phenotypes (Figure 6, A–C; Supplemental Figure 6A), similar to those of *gEBF2-dCAS9-EIN2C* and *gETR2-dCAS9-EIN2C* in Col-0 or *ein2-5* mutant backgrounds (Zhang et al., 2017). H3K14Ac and H3K23Ac levels were elevated in both *ein3-1 eil1-1 gEBF2-dCAS9-EIN2C* and *ein3-1 eil1-1 gETR2-dCAS9-EIN2C* when compared to *ein3-1 eil1-1* seedlings (Figure 6, D and E); the expression of genes targeted by the guide RNA was also elevated (Supplemental Figure 6B). There was no significant ethylene-induced elevation in either H3K14Ac or H3K23Ac levels in *ein3-1 eil1-1 gEBF2-dCAS9-EIN2C* or *ein3-1 eil1-1 gETR2-dCAS9-EIN2C* seedlings (Figure 6, D and E). This result further confirmed that EIN2-C regulates histone acetylation in the absence of EIN3, while EIN3 is required for EIN2C-mediated elevation of H3K14Ac and H3K23Ac in response to ethylene.

EIN3 does not directly regulate histone acetylation in the absence of EIN2

To further study EIN3-regulated histone acetylation in the absence of EIN2, we introduced the EIN3 overexpression transgene in the *ein2-5* mutant to generate *ein2-5 EIN3ox* by crossing. The constitutive ethylene-responsive phenotype of *EIN3ox* seedlings was partially suppressed by *ein2-5*. However, these seedlings still displayed a partial ethylene-responsive phenotype when compared to the *ein2-5* mutant, whether with or in the absence of ethylene treatment (Figure 7, A–C; Supplemental Figure 7A). The expression of ethylene-responsive genes was significantly reduced in *ein2-5 EIN3ox* seedlings compared to the levels in *EIN3ox* seedlings, again with and without ethylene treatment (Figure 7D). In contrast, EIN3 protein levels were comparable in *EIN3ox* and in *ein2-5 EIN3ox* seedlings (Figure 7E). These results suggest that EIN2 plays a key role in EIN3-dependent transcriptional regulation.

We then examined H3K14Ac and H3K23Ac levels in the promoters of ethylene-responsive genes. The H3K14Ac and H3K23Ac levels in *ein2-5 EIN3ox* seedlings were reduced when compared to those in *EIN3ox* seedlings, while the levels were comparable to that in *ein2-5* (Figure 7, F and G), demonstrating that histone acetylation levels are not directly correlated with EIN3 protein levels in the absence of EIN2. Taken together, these results indicate that EIN3 is not sufficient to regulate histone acetylation in the absence of EIN2.
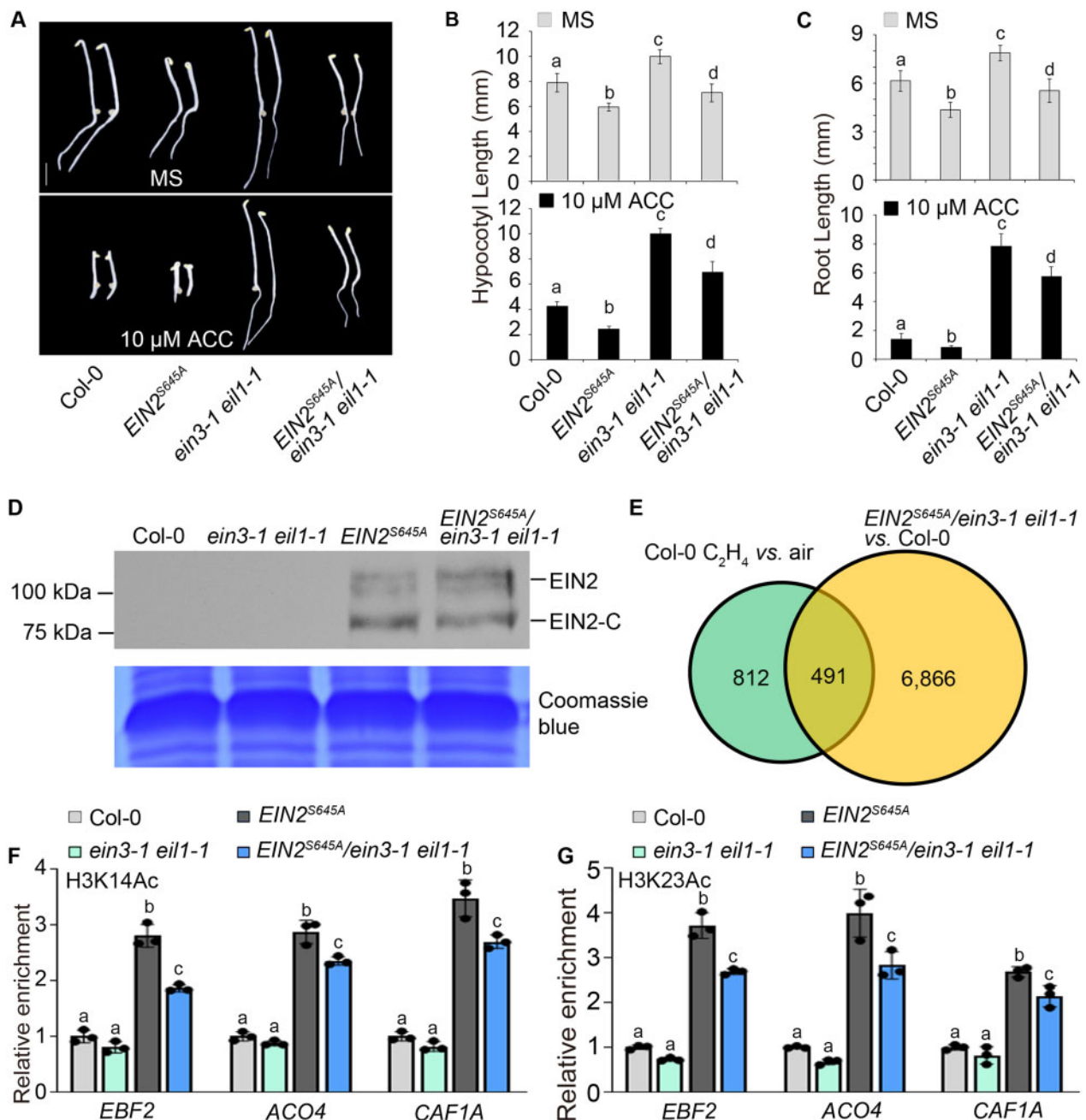
Figure 5 EIN2-mediated elevation of histone modifications is independent of EIN3. (A–C) The seedling phenotypes of Col-0, *ein3-1 eil1-1*, *EIN2^{S645A}*, and *ein3-1 eil1-1 EIN2^{S645A}* etiolated seedlings treated with or without 10-μM ACC. Different letters indicate significant differences determined using one-way ANOVA Scheffe method ($P < 0.05$). Bar indicates 2.5 mm.

However, EIN3 is required for the EIN2-mediated elevation in histone acetylation in response to ethylene.

Discussion

We determined that EIN2 interacts with the histone-binding protein ENAP1 (Zhang et al., 2016a) to regulate histone acetylation, and that EIN3 is potentially involved in the regulation of histone acetylation through its interaction with ENAP1. However, the role of EIN3 in histone modification is still largely unknown. We speculated that the DNA-binding

activity of EIN3 is important for EIN2-directed histone acetylation. Although both EIN2-C and EIN3 interact with ENAP1, no interaction between EIN2-C and EIN3 has been detected, thus it is almost impossible to directly study how EIN3 regulates EIN2-C-mediated histone acetylation in response to ethylene. In this research, we propose a new strategy to study how EIN2-C and EIN3 function together by fusing EIN2-C with the DNA-binding domain from EIN3 to create an EIN2-C variant with EIN3 DNA-binding activity. The synthetic protein EIN2C–EIN3DB provides a perfect system to achieve our goal because the protein preserves both
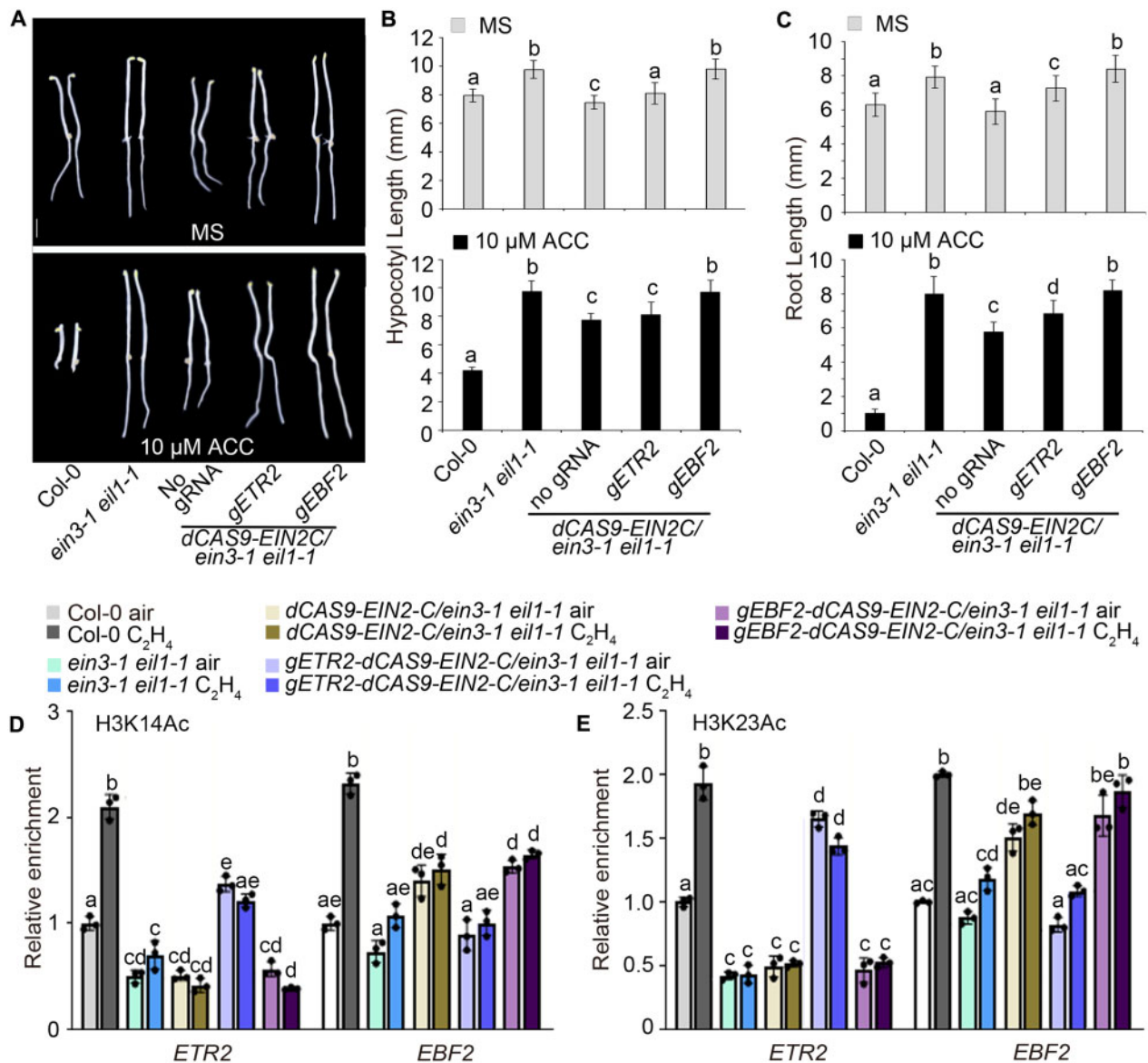
Figure 6 Histone acetylation induced by EIN2 does not depend on EIN3. (A) The phenotypes of 3-day-old *dCas9-EIN2-C/ein3-1 eil1-1*, *gETR2-dCas9-EIN2-C ein3-1 eil1-1*, and *gEBF2-dCas9-EIN2-C ein3-1 eil1-1* transgenic etiolated seedlings grown on medium with or without 10-μM ACC. Bar indicates 2.5 mm. (B and C) Lengths of hypocotyls (B) and roots (C) of 3-day-old etiolated seedlings grown with or without 10-μM ACC. Values are means ± SD of at least 30 seedlings. Different letters indicate significant differences determined using one-way ANOVA Scheffe method ($P <$ 0.05). (D–E) Levels of (D) H3K14Ac and (E) H3K23Ac in the guide RNA target loci in 3-day-old etiolated seedlings. Data are fold-changes relative to levels in Col-0 etiolated seedlings grown in air. Each ChIP-qPCR or RT-qPCR was repeated at least three times. Values are means ± SD of three biological replicates. Different letters indicate significant differences determined using one-way ANOVA Scheffe method ($P < 0.05$).

EIN3 DNA-binding activity and EIN2-C function. In addition, EIN2C–EIN3DB can interact with EIN3 through the EIN3 DNA-binding domain and largely fulfilled their typical functions. Thus, this approach provides a great opportunity to dissect the function of EIN3 in EIN2-mediated histone acetylation and gene expression in vivo. We believe this approach can also be applied more broadly to other proteins.

Here, we provide multiple lines of evidence showing that native EIN3 is important to determine the target for histone modification, and that the positive feedback loop mediated by dimerized EIN3 is required for the EIN2-mediated elevation of histone acetylation in response to ethylene. First, by

fusing EIN2-C to EIN3DB, we found that a mutation in EIN3DB that disrupts DNA binding (EIN3DB[K245N]) also leads to a weaker ethylene-responsive phenotype than with wild-type EIN3DB. Second, we found that EIN2C–EIN3DB DNA-binding activity is positively correlated with EIN3 protein levels and that the EIN2C–EIN3DB binding signal is correlated with H3K14Ac and H3K23Ac levels and with expression of ethylene-responsive genes. Third, we found that EIN3 and EIN2C–EIN3DB can interact and that the binding of EIN2C–EIN3DB to target genes is enhanced by native EIN3 and that the binding specificity also increased by native EIN3 in vivo. Fourth, in the absence of EIN3, overexpression
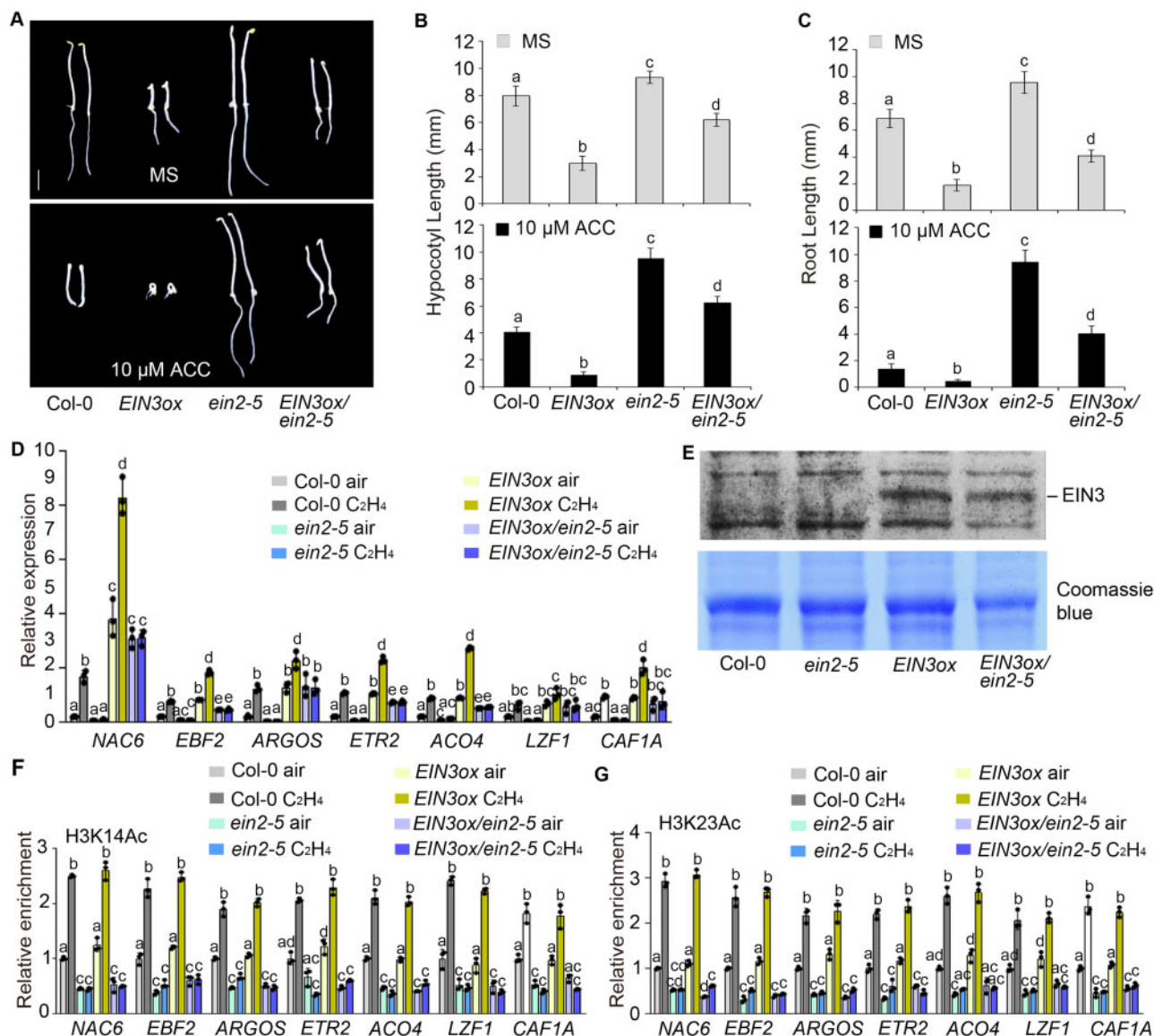
Figure 7 EIN3 does not directly regulate histone acetylation in the absence of EIN2. (A) The phenotypes of Col-0, *ein2-5*, *EIN3ox*, and *ein2-5 EIN3ox* etiolated seedlings treated with or without 10-μM ACC. Bar indicates 2.5 mm. (B and C) The lengths of (B) hypocotyls and (C) roots in 3-day-old etiolated seedlings for Col-0, *ein2-5*, *EIN3ox*, and *ein2-5 EIN3ox* grown with or without 10-μM ACC. Values are means ± SD of at least 30 seedlings. Different letters indicate significant differences determined using one-way ANOVA Scheffe method ($P < 0.05$). (D) RT-qPCR analysis of the indicated genes. Total RNAs were extracted from Col-0, *ein2-5*, *EIN3ox*, and *ein2-5 EIN3ox* 3-day-old etiolated seedlings treated with or without ethylene. Different letters indicate significant differences determined using one-way ANOVA Scheffe method ($P < 0.05$). (E) Immunoblot assay for EIN3 protein levels in Col-0, *ein2-5*, *EIN3ox*, and *ein2-5 EIN3ox* seedlings using anti-EIN3 antibody. Total proteins were extracted from 3-day-old etiolated seedlings. (F and G) ChIP-qPCR assays to examine (F) H3K14Ac and (G) H3K23Ac levels at the indicated loci in 3-day-old etiolated seedlings of Col-0, *ein2-5*, *EIN3ox*, and *ein2-5 EIN3ox* treated with or without ethylene. Data are fold-changes relative to Col-0 grown in air. Values are means ± SD of three biological replicates. Each ChIP-qPCR or RT-qPCR was repeated at least three times. Different letters indicate significant differences determined using one-way ANOVA Scheffe method ($P < 0.05$).

of EIN2-C can elevate histone acetylation compared to without EIN2-C; however, EIN3 is required for the ethylene-induced elevation of H3K14Ac and H3K23Ac levels. Finally, we provide evidence that EIN3 alone is not sufficient to regulate H3K14Ac or H3K23Ac levels in response to ethylene. Based on these results, we propose that in the presence of ethylene, the EIN2-C-induced elevation of H3K14Ac and H3K23Ac levels requires a positive feedback regulation

mediated by EIN3, potentially through its dimerization (Supplemental Figure 7B). This positive feedback regulation integrates EIN2-C-mediated histone acetylation regulation into EIN3-dependent transcriptional regulation in response to ethylene.

In the absence of EIN3, overexpression of *EIN2C–EIN3DB* resulted in a partial constitutive ethylene-responsive phenotype. More importantly, expression of approximately 30% of

ethylene-regulated genes was altered in *ein3-1 eil1-1 EIN2C–EIN3DB* seedlings. Meanwhile, transcriptional activation is a stepwise process that requires the creation and maintenance of an open chromatin structure, assembly of the preinitiation complex, and finally the transition to productive elongation. Most likely, in the absence of EIN3, EIN2-C interacts with ENAP1, which shares target genes with EIN3, to regulate histone acetylation, by creating an open chromatin structure that leads to an elevation above basal levels for a subset of genes. EIN3 potentially determines where the preinitiation complex forms for transcriptional regulation; therefore, in the absence of EIN3, EIN2C–EIN3DB binding efficiency and specificity are poor, and ethylene-induced transcription regulation is impaired.

Dimerization of transcription factors enhances DNA binding and increases binding specificity. For example, most auxin-responsive factors consist of an N-terminal B3-type DNA-binding domain, a variable middle region that can function as either an activation or repressor domain, and a C-terminal dimerization domain that is involved in protein-protein interaction. Dimerization mediated by the C-terminal domain determines binding site selection (Kim et al., 1997; Guilfoyle and Hagen, 2007, 2012; Boer et al., 2014; Hewezi et al., 2014). Consistent with this model, abscisic acid-responsive element binding factors determine abscisic acid-mediated transcriptional regulation of stress response genes, and the specificity of their transcriptional regulation is determined by dimerization among these factors (Choi et al., 2000; Uno et al., 2000; Jakoby et al., 2002). Interaction between EIN3 and other transcription factors such as JASMONATE ZIM DOMAIN, MYC2, OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59, and PHYTOCHROME INTERACTING FACTOR 3 participates in crosstalk with jasmonate and light signaling pathways (Zhu et al., 2011; He et al., 2017; Liu et al., 2017). EIN3 homodimerization was previously demonstrated in vitro, but it appears that binding DNA is not necessary for this dimerization (Solano et al., 1998). It should be noted that EIN3 dimerization has yet to be replicated in vivo. Given that the DNA-binding domain and the dimerization domain of EIN3 overlap, it is normally impossible to uncouple the dimerization and DNA-binding activity of EIN3 by mutating EIN3 to prevent its dimerization (Solano et al., 1998). The EIN2C–EIN3DB fusion protein provides here a unique opportunity to address the question of in vivo relevance of this dimerization, because the protein preserves both EIN3 DNA-binding activity and EIN2-C function. The EIN2C–EIN3DB fusion protein can also interact with EIN3 through the EIN3 DNA-binding domain. Although we observed no DNA binding for EIN2-C using an anti-EIN2-C antibody under a standard ChIP-seq method, we succeeded in detecting DNA binding by EIN2C–EIN3DB using an anti-EIN2-C antibody. Therefore, we identified EIN2C–EIN3DB binding targets without interference from native EIN2 and EIN3 proteins by performing our analyses in the *ein3-1 eil1-1* mutant background. Importantly, we observed an interaction between EIN2C–EIN3DB and EIN3 in vivo and examined how native EIN3 protein works to regulate EIN2C–EIN3DB binding in various backgrounds. Our results clearly demonstrated that the binding specificity of EIN2C–EIN3DB is enhanced by EIN3, and that the interaction of EIN2C–EIN3DB with EIN3 plays an important role in histone acetylation at H3K14Ac and H3K23Ac of target genes.

Due to the tight link between histone modification and transcriptional regulation, it is a great challenge to dissect how transcription factors and their transcriptional activity effect histone modifications in vivo. The synthetic approach of using chimeric proteins to reconstruct a signaling pathway provides a powerful tool for functional dissection and has been widely applied to studies in cell signaling transduction, plant growth and development. For example, in order to test whether the recruitment of the chromatin-remodeling complex BRAHMA (BRM)/SPLAYED (SYD) leads to the induction of MONOPTEROS (MP, also called AUXIN RESPONSE FACTOR 5) target genes, the authors tethered the chromatin remodeling complex to MP target genes by fusing the DNA-binding domain to BUSHY, a shared component of the BRM and the SYD chromatin remodeling complex. The chimeric protein can rescue the flower primordium initiation phenotype in the hypomorph *mp-S319* mutant and thus carries out the function(s) of its wild-type counterpart (Wu et al., 2015). LONG HYPOCOTYL 5 (HY5) is an important transcription factor that plays pleotropic roles in light signaling. To determine its function, Burko et al. applied the synthetic approach to analyze chimeric proteins between HY5 and either a constitutive transcriptional activator (VP16) or repressor (SRDX) in Arabidopsis and tomato (*Solanum lycopersicum*; Burko et al., 2020). They demonstrated that the primary activity of HY5 is to promote transcription (Burko et al., 2020). The CRISPR-dCas9 system has been used as a synthetic transcriptional regulator to direct the expression of a target gene or multiple genes by fusing dCas9 with a transcription repression domain such as the KRAB domain, or via fusion with transcription activation domains such as VP16 (Konermann et al., 2015; Park et al., 2017; Parsi et al., 2017; Moradpour and Abdulah, 2020). Yet, the chimeric proteins generated by this synthetic approach potentially will have some problems: the 3D protein structure of the chimera may potentially be altered relative to the native protein and modify its function. Therefore, the evaluation on the proteins by a genetic approach is also necessary and critical to its success.

Materials and methods

Plant growth conditions

Arabidopsis thaliana seeds were surface sterilized in 50% bleach with 0.01% Triton X-100 for 15 min and washed five times with sterile, double-distilled H_2O before plating on Murashige and Skoog (MS) medium (4.3-g MS salts, 10-g sucrose, pH 5.7, 8 g phyto agar per liter) with or without the

addition of 10-μM 1-aminocyclopropane-1-carboxylic acid (Sigma), the biosynthetic precursor for ethylene. After 3–4 days of stratification at 4°C in the dark, the plates were wrapped in foil and kept at 24°C in an incubator before the seedling phenotypes were analyzed. For propagation, seedlings were transferred from plates to soil (Pro-mix-HP) and grown to maturity at 22°C under 16 h light (F32T8/SPP50/ECO 5000K, SKU: GE-66350, General Electric)/8-h dark cycles. For all gene expression assays, protein level assays, RNA-seq, and ChIP-seq assays, ethylene treatments of Arabidopsis seedlings were performed by growing seedlings on MS plates in air-tight containers in the dark for 4 h, supplied with either a flow of hydrocarbon-free air (Zero grade air, AirGas) or hydrocarbon-free air with 10 p.p.m. ethylene, as previously described (Kieber et al., 1993; Zhang et al., 2018). For hypocotyl and root length measurements, 3-day-old seedlings were scanned using an Epson Perfection V700 Photo scanner, and hypocotyl and root lengths were measured using Image J (https://imagej.nih.gov/ij/).

Cloning of *EIN2C–EIN3DB, EIN3DB–EIN2C, EIN2C–EIN3DB^{K245N}, and EIN3DB^{K245N}–EIN2C*

All primers are listed in Supplemental Table 1.

For *EIN2C–EIN3DB*, the *EIN2-C* sequence was PCR-amplified by Phusion High-Fidelity DNA Polymerase (M0530S, NEB) using the primers EIN2-kpn1 and EIN2CEIN3DB-R, the EIN3DB sequence was amplified using the primers EIN2CEIN3DB-F and EIN3DB-sal1, using the first strand Arabidopsis cDNA from Col-0 as template. The PCR products were further purified, mixed together and used as template for PCR using the primers EIN2-kpn1 and EIN3DB-sal1 to generate *EIN2C–EIN3DB*. For *EIN3DB–EIN2C*, the *EIN3DB* sequence was amplified using the primers EIN3DB-kpn1 and EIN3DB–EIN2C-R, the *EIN2-C* sequence was amplified using the primers EIN3DBEIN2C-F and EIN2C-sal1, using the first strand Arabidopsis cDNA from Col-0 as template. The PCR products were further purified, mixed together, and used as template for PCR using the primers EIN3DB-kpn1 and EIN2C-sal1 to generate *EIN3DB–EIN2C* sequence. For *EIN2C–EIN3DB^{K245N}*, the DNA fragments were amplified using the primers EIN2-kpn1 and EIN3K245N-R, EIN3K245N-F, and EIN3DB-sal1, using the *EIN2C–EIN3DB* fragment as template, and the PCR products were further purified, mixed together and used as template for PCR using EIN2-kpn1 and EIN3DB-sal1 to generate *EIN2C–EIN3DB^{K245N}*. For *EIN3DB^{K245N}–EIN2C*, the DNA fragments were amplified using the primers EIN3DB-kpn1 and EIN3K245N-R, EIN3K245N-F, and EIN2C-sal1, using the *EIN3DB–EIN2C* fragments as template, and the PCR products were further purified, mixed together and served as template for PCR using EIN3DB-kpn1 and EIN2C-sal1 to generate *EIN3DB^{K245N}–EIN2C*. The *EIN2C–EIN3DB*, *EIN3DB–EIN2C*, *EIN2C–EIN3DB^{K245N}*, *EIN3DB^{K245N}–EIN2C* PCR fragments were further digested by Kpn1 and Sal1 and cloned into the pCHF3 vector.

RNA extraction and real-time PCR

Total RNA was extracted using the RNeasy Plant Kit (Qiagen) from 3-day-old etiolated seedlings treated for 4 h with air or ethylene gas, as described above. First strand cDNA was synthesized using Superscript III First-Strand cDNA Synthesis Kit (Invitrogen). Real-time quantitative PCR was performed with the LightCycler 480 SYBR Green I Master (Roche) following the manufacturer's instructions. PCR reactions were performed in triplicate on a Roche 96 LightCycler. Expression levels were normalized to *ACT2*. Primers used for qPCR are listed in Supplemental Table 1.

RNA-seq processing and analysis

Three-day-old etiolated seedlings treated with air or ethylene gas for 4 h were harvested, and total RNA was extracted using Plant RNA Purification Reagent (Invitrogen) as described previously (Qiao et al., 2012). Total RNA (4 μg) was used to prepare RNA-seq libraries using TruSeq RNA Library Prep Kit (Illumina; Zhang et al., 2016a, 2016b; Wang et al., 2017). Multiplexed libraries were sequenced on an Illumina NextSeq. Raw RNA-seq reads were aligned to the TAIR10 genome release using TopHat version 2.0.9 (Kim et al., 2013; Wang et al., 2018) with default parameters. Differentially expressed genes were identified using Cufflinks version 2.1.1 following the workflow with default parameters described previously (Trapnell et al., 2012). The genes showing $|\log_2(\text{fold-change})| > 1$, reads per kilobase per million reads values > 1 and $q < 0.05$ were considered to be differentially expressed.

Yeast two-hybrid assays

Yeast two-hybrid assays were performed using the ProQuest^{TM} Two-Hybrid System (Invitrogen) following a previously published method (Zhang et al., 2016b). Briefly, the coding sequences of *EIN2-C, EIN3DB, EIN2C–EIN3DB,* and *EIN2C–EIN3DB^{K245N}* were introduced into the pDBLeu vector, and the coding sequences of *EIN3* and *ENAP1* were introduced into pEXP-AD502. The resulting pDBLeu and pEXP-AD502 constructs were co-transformed into yeast (*Saccharomyces cerevisiae*) strain AH109. The transformants were grown on SD–Trp–Leu medium or SD–Trp–Leu –His medium. Growth on SD–Trp–Leu–His medium indicates interaction between the corresponding proteins.

Immunoblots

Total proteins were extracted from 3-day-old etiolated seedlings with extraction buffer (50-mM Tris–HCl, pH 8.0, 150-mM NaCl, 1-mM EDTA, 0.1% Triton X-100, and protease inhibitor cocktail). After incubation on ice for 30 min, plant extracts were centrifuged at 15,000g for 15 min at 4°C. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto a nitrocellulose membrane. The membrane was blocked using 1 × TBST (20-mM Tris, 150-mM NaCl, 0.1% Tween-20) with 5% w/v dry milk and was probed with the indicated primary antibodies (diluted in 1 × TBST with 5% w/v dry milk). After washing three times with 1 × TBST

buffer (15 min each), the membrane was then probed with secondary goat anti-rabbit (Bio-Rad 170-6515) or goat anti-mouse (Bio-Rad 170-6516) antibodies conjugated with horseradish peroxidase. The signals were detected by a chemiluminescence reaction using the SuperSignal kit (Pierce). Polyclonal anti-EIN2-C (Qiao et al., 2009, 2012) and anti-EIN3 antibodies developed in house according to Guo and Ecker, 2003 were used at dilutions of 1:2,000.

Pull-down assays

The coding sequences of *EIN2C–EIN3DB* and *EIN2C–EIN3DB^{K245N}* were introduced into the pVP13 vector (to generate MBP-His-EIN2C-EIN3DB and MBP-His-EIN2C-EIN3DB^{K245N}). These MBP-His fusion proteins were purified using amylose resin (New England Biolabs) and re-purified using Ni-NTA Agarose (Qiagen) before being washed using pull-down buffer (50-mM Tris–HCl, pH 8.0, 150-mM NaCl, 0.5-mM EDTA, 0.5% Tween-20, and protease inhibitor cocktail). GST-EIN3DB was purified using Glutathione Sepharose (GE Healthcare). MBP-tagged EIN3 (MBP-EIN3, purified using amylose resin) was added and incubated for 1 h at 4°C. After washing five times with pull-down buffer, Sepharose beads were collected by brief centrifugation (2,000 g, 2 min) and then resuspended in protein extraction buffer (50-mM Tris–HCl, pH 8.0, 150-mM NaCl, 1-mM EDTA, 0.1% Triton X-100, and protease inhibitor cocktail). Proteins were separated by SDS–PAGE and detected with the anti-EIN3 at a 1:2,000 dilution (Guo and Ecker, 2003) or anti-MBP (NEB; catalog number E8032S, lot number 0101501) antibody at a 1:25,000 dilution.

Immunoprecipitation assays

Total proteins extracted from *EIN2C–EIN3DB*, *ein3-1 eil1-1 EIN2C–EIN3DB*, *EIN2C–EIN3DB^{K245N}*, and *ein3-1 eil1-1 EIN2C–EIN3DB^{K245N}* plants were immunoprecipitated using anti-EIN2-C antibody (Qiao et al., 2012). Samples were incubated overnight at 4°C and washed three times before analysis by immunoblot as above.

ChIP-seq and ChIP-qPCR

Briefly, 3-day-old etiolated seedlings treated with air or ethylene for *EIN2C–EIN3DB* (one replicate representing the mixed tissues from two independent collections of pooled seedlings) and *ein3-1 eil1-1 EIN2C–EIN3DB* (two biological replicates representing two independent collections from pooled seedlings) were harvested and cross-linked in 1% formaldehyde, and the chromatin was isolated and sonicated using Bioruptor (setting H, 0.5 min on and 2 min off, 25 cycles at 4°C; put tubes on ice for 1 min every five cycles). The indicated antibodies were added to the sonicated chromatin, followed by incubation overnight to precipitate bound DNA fragments (Zhang et al., 2016b). Two-microgram antibodies were used for each sample, including anti-EIN2-C antibody (Qiao et al., 2012), anti-H3K14Ac (Millipore; catalog number 07-353; lot number 3259085), and anti-H3K23Ac (Millipore; catalog number 07-355; lot number 3249938). DNA was eluted and amplified by PCR with primers corresponding to

genes of interest. Primers used for ChIP-qPCR are listed in Supplemental Table 1.

Chromatin-immunoprecipitated DNA was sequenced using an Illumina NextSeq platform according to standard operating procedures. Initial quality-control analysis was performed using FastQC (Andrews, 2013). The genome-wide average depth of coverage obtained for each library is provided in Supplemental Table 2. Single-end reads were first mapped to the Arabidopsis genome (TAIR10; Lamesch et al., 2012) using bowtie software (version 0.7.1; Langmead et al., 2009) with default parameters. Peaks significantly enriched in ChIP-seq tags were identified using MACS2 (version 2.1.0.20150603; Zhang et al., 2008). Peaks within 2 kbp of a gene body were assigned to the gene. Genes present in both biological replicates were retained as binding targets. Peaks were merged when the maximum gap between two peaks was < 200 bp, and shared peaks were used for subsequent analysis. R (version 3.2.2) scripts were used to generate of Venn diagrams.

Accession numbers

The Arabidopsis Genome Initiative numbers for the genes mentioned in this article are as follows: *EIN2* (At5g03280); *ENAP1* (At3g11100); *EIN3* (At3g20770); *EIL1* (At2g27050); *ERF1*, (At3g23240); *EDF2* (At1g68840); *ACT2* (At3g18780); *ETR2*, (At3g23150); *EBF2* (At5g25350); *NAC6* (At5g39610); *ARGOS* (At3g59900); *ACO4* (At1g05010); *LZF1* (At1g78600); *CAF1A* (At3g44260); *ACO2* (At1g62380); *CTR1* (At5g03730); *HLS1* (At4g37580); *EDF1* (At1g25560); *CAT1* (At1g20630); NTF2 family member (At3g09250). RNA-seq and ChIP-seq data reported in this study have been deposited in the Gene Expression Omnibus database under Accession Number GSE138154.

Supplemental data

Supplemental Figure 1. Engineered EIN3DB–EIN2C leads to partial ethylene-responsive phenotype.

Supplemental Figure 2. Interaction test between ENAP1 and EIN2C–EIN3DB.

Supplemental Figure 3. Motifs identified from EIN2C–EIN3DB binding targets in different conditions.

Supplemental Figure 4. Yeast two hybrid assay to examine interaction between EIN3 and EIN3DB.

Supplemental Figure 5. The roles of EIN2^{S645A} in the absence of EIN3 and EIL1.

Supplemental Figure 6. Immunoblots to detect the expression of dCas9-EIN2-C protein levels.

Supplemental Figure 7. Model of EIN3 mediated positive feedback regulation in EIN2-directed histone acetylation and transcriptional regulation

Supplemental Table 1. Primers used in this paper.

Supplemental Table 2. Aligned reads from chromatin immunoprecipitation sequencing libraries.

Supplemental Data Set 1. ANOVA tables.

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