

1 **Maize EHD1 is Required for Kernel Development and Vegetative Growth**  
2 **through Regulating Auxin Homeostasis**

3 Yafei Wang<sup>1,2</sup>, Wenwen Liu<sup>2</sup>, Hongqiu Wang<sup>1,2</sup>, Qingguo Du<sup>2</sup>, Zhiyuan Fu<sup>1</sup>, Wen-Xue  
4 Li<sup>2,\*</sup> and Jihua Tang<sup>1,\*</sup>

5 <sup>1</sup>National Key Laboratory of Wheat and Maize Crop Science, Collaborative  
6 Innovation Center of Henan Grain Crops, College of Agronomy, Henan Agricultural  
7 University, Zhengzhou, 450002, China;

8 <sup>2</sup>National Engineering Laboratory for Crop Molecular Breeding, Institute of Crop  
9 Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China

10

11 \*To whom correspondence should be addressed:

12 Jihua Tang

13 Tel: +86-371-63558377

14 E-mail: [tangjihua1@163.com](mailto:tangjihua1@163.com)

15

16 Wen-Xue Li

17 Tel: +86-10-82105799

18 E-mail: [liwenxue@caas.cn](mailto:liwenxue@caas.cn)

19

20 **Running title:** ZmEHD1-mediated CME regulates maize auxin homeostasis

21

22

23

24

25

26

27

28

29

30

31 **Abstract**

32 The roles of EHDs in clathrin-mediated endocytosis (CME) in plants are poorly  
33 understood. Here, we isolated a maize mutant, designated as *ehd1*, which showed  
34 defects in kernel development and vegetative growth. Positional cloning and  
35 transgenic analysis revealed that *ehd1* encodes an EHD protein. Internalization of the  
36 endocytic tracer FM4-64 was significantly reduced in *ehd1* mutant and *ZmEHD1*  
37 knock-out mutants. We further demonstrated that *ZmEHD1* and *ZmAP2*  $\sigma$  subunit  
38 physically interact in the plasma membranes. Cellular IAA levels were significantly  
39 lower in *ehd1* mutant than in wild-type maize. Auxin distribution and *ZmPIN1a*-YFP  
40 localization were altered in *ehd1* mutant. Exogenous application of 1-NAA but not  
41 GA3 rescued the seed germination and seedling emergency phenotypic defects of  
42 *ehd1* mutants. Taken together, these results indicate that *ZmEHD1* regulates auxin  
43 homeostasis by mediating CME through its interaction with the *ZmAP2*  $\sigma$  subunit,  
44 which is crucial for kernel development and vegetative growth of maize.

45

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

## 61 **Introduction**

62 Endocytosis, which can be clathrin-dependent or clathrin-independent, is the  
63 internalization of plasma membrane (PM) proteins or uptake of extracellular materials  
64 for transport to the endosome (Murphy et al., 2005). Clathrin-mediated endocytosis  
65 (CME) is the major gateway into plant cells (Fan et al., 2015). Although detailed  
66 information is available on the roles of endocytosis in animals, researchers have only  
67 recently documented that CME is critical for various developmental processes in  
68 plants, including cell polarity determination (Van et al., 2011; Wang et al., 2013), male  
69 reproductive organ development (Blackbourn and Jackson, 1996; Kim et al., 2013),  
70 gametophyte development (Backues et al., 2010), and embryogenesis (Fan et al., 2013;  
71 Kitakura et al., 2011). CME is also important in plant responses to abiotic and biotic  
72 stresses by internalizing of PM-resident transporters or receptors, such as the boron  
73 transporter (BOR1) (Takano et al., 2010), the iron-regulated transporter (IRT1)  
74 (Barberon et al., 2011), the auxin efflux transporter PIN-FORMED (PINs)  
75 (Dhonukshe et al., 2007), the brassinosteroid receptor BRASSINOSTEROID  
76 INSENSITIVE 1 (BRI1) (Di et al., 2013), the bacterial flagellin receptor FLAGELIN  
77 SENSING 2 (FLS2) (Robatzek et al., 2006), and the ethylene-inducing xylanase  
78 receptor (LeEIX2) (Bar and Avni, 2009).

79 CME is a complex process that can be divided into at least four steps: the  
80 budding of a vesicle, the packaging of cargo into the vesicle, the release of the mature  
81 vesicle from the PM, and the fusing of the vesicle with the endosome (Sigismund et  
82 al., 2012). Because clathrin cannot directly bind to the PM or to cargoes, the  
83 formation of clathrin-coated endocytic vesicles initiates the association of adaptor  
84 protein complex 2 (AP2) with the PM, and thus AP2 plays a central role in the  
85 initiation of CME. The AP2 complex forms a heterotetrameric complex containing  
86 two large subunits ( $\beta$ 1-5 and one each of  $\gamma/\alpha/\delta/\epsilon/\zeta$ ), a medium subunit ( $\mu$ 1-5), and a  
87 small subunit ( $\sigma$ 1-5) (McMahon and Boucrot, 2011). Our understanding of AP2 in  
88 plants has been enhanced by the following recent findings: knockdown of the two  
89 *Arabidopsis* *AP2A* genes or overexpression of a dominant-negative version of the  
90 medium AP2 subunit, *AP2M*, was shown to significantly impair BRI1 endocytosis

91 and to enhance brassinosteroid signaling (Di et al., 2013);  $\alpha$  and  $\mu$  adaptins were  
92 demonstrated to be crucial for pollen production and viability, as well as elongation of  
93 staminal filaments and pollen tubes by modulating the amount and polarity of PINs  
94 (Kim et al., 2013);  $\sigma$  adaptin was found to play important roles in the assembly of a  
95 functional AP2 complex, and *ap2*  $\sigma$  loss-of-function *Arabidopsis* exhibited defects in  
96 multiple aspects of plant growth and development (Fan et al., 2013).

97 In addition to the classical AP2 complex, other accessory proteins, including the  
98 C-terminal Eps15 homology domain (EHD) proteins, also connect the cargo and  
99 membrane lipid to form the CME vesicle. The EHD family contains one member in  
100 *Drosophila* and *C. elegans* and four orthologs in mouse and human. All members  
101 contain an EHD with two calcium-binding helix-loop-helix motifs (EF-hands), a  
102 P-loop with a predicted ATP/GTP binding site, a dynamin-N motif with a  
103 nucleotide-binding domain, and a coil-coil region (Bar et al., 2008). The involvement  
104 of EHD-containing proteins in CME was described in detail in mammalian cells, in  
105 which RME1 was demonstrated to be a key player controlling the recycling of  
106 internalized receptors from the endocytic recycling compartment to the PM (Lin et al.,  
107 2001). By homologous cloning, two *EHD* genes were isolated from *Arabidopsis* (Bar  
108 et al., 2008). The two *AtEHD* proteins regulated endocytosis in distinct patterns.  
109 Over-expression of *AtEHD2* had an inhibitory effect on endocytosis, while  
110 down-regulation of *AtEHD1* caused retardation of entry of endocytosed material into  
111 plant cells (Bar et al., 2008). However, the mechanisms by which EHD family  
112 members linked to CME in plants have not been characterized.

113 Maize ranks first in total production among major staple cereals and is also an  
114 important raw material for biofuel and many other industrial products (McLaren, 2005).  
115 As an important model system for basic biological research, maize has contributed  
116 significantly to our knowledge of plant development and evolution, and this  
117 understanding has been used to elucidate the developmental mechanisms of maize  
118 kernel morphogenesis. The earliest genetic studies performed in maize included  
119 analyses of defective kernel (*dek*) mutants. Although several hundred *dek* mutants  
120 have been isolated, relatively few corresponding genes have been molecularly cloned

121 because embryo-lethal alleles are difficult to study (Scanlon and Takacs, 2009).

122 To better understand maize endosperm filling and maturation, we characterized a  
123 maize mutant *ehd1* that is impaired in kernel development and vegetative growth.  
124 Positional cloning of *ehd1* and transgenic analysis identified the causative locus as a  
125 gene that encodes an EHD-containing protein and that is herein designated as  
126 *ZmEHD1*. We show that *ZmEHD1* is involved in CME through interaction with the  
127 *ZmAP2*  $\sigma$  subunit. We further demonstrate that *ZmEHD1* affects the gene expression  
128 involved in auxin-related processes and that exogenous 1-NAA application rescues  
129 the fertility of the *ehd1* mutant. Our results indicate that, by regulating auxin  
130 homeostasis, *ZmEHD1*-mediated CME is crucial for kernel development and  
131 vegetative growth of maize.

132

## 133 **Results**

### 134 **The *ehd1* mutant is impaired in kernel development and vegetative growth**

135 The *ehd1* mutant was originally isolated as a shrunken kernel mutant in the screening  
136 of natural mutagenesis defective in the filling of maize grains. At 14 days after  
137 pollination (DAP), the kernels of *ehd1* homozygous mutant (*ehd1/ehd1*) and the  
138 wild-type (WT) (*EHD1/EHD1* and *EHD1/ehd1*) resembled each other (Figure 1A). At  
139 16 DAP, the WT kernels were canary-yellow while the *ehd1* mutants were ivory-white  
140 (Figure 1A). At maturity, both the endosperm and embryo of *ehd1* mutant were  
141 shrunken (Figure 1B), which led to a significant reduction in the 100-kernel weight  
142 (Figure 1C). The 100-kernel weight of the WT (26.9 g) was 3-times greater than that  
143 of the *ehd1* mutant (Figure 1C).

144 To analyze the kernel development of the *ehd1* mutant and the WT, we examined  
145 immature embryos at 20 DAP by light microscopy. Longitudinal sections indicated  
146 that embryo development was slower in the *ehd1* mutant than in the WT (Figure 1D).  
147 Endosperm development and texture were also observed by light microscopy.  
148 Compared to WT endosperm cells, those of the *ehd1* mutant had less dense cytoplasm  
149 and fewer starch granules (Figure 1E).

150 A paper-culture system was used to measure the germination rates of the WT and

151 the *ehd1* mutant. The germination rate of the *ehd1* mutant was only about 3%, which  
152 was far lower than that of the WT (Figure 2A). Relative to the WT seedlings, the  
153 germinated *ehd1* seedlings had shorter and fewer primary roots (Figure 2B). Most of  
154 the *ehd1* seedlings died before the first two leaves had completely expanded (Figure  
155 2C). These results indicated that *ZmEHD1* is essential for maize kernel development  
156 and vegetative growth.

157 The mutant was crossed to an inbred line Xun9058. The hybrid F<sub>1</sub> displayed  
158 normal kernel as Xun9058. Due to the low germination rate of the *ehd1* mutant, the  
159 normal kernels from F<sub>2</sub> ears were planted. Among the 244 F<sub>2</sub> individuals, 79 had the  
160 normal kernel phenotype (*EHD1/EHD1*), and 165 had the segregation phenotype  
161 (*EHD1/ehd1*). The segregation ratio followed 1:2 theoretical ratio predicted by a  
162 Chi-square test at a 0.05 level (Supplemental Table 1). For each F<sub>2</sub> individual with  
163 kernel phenotype segregation, the pattern of shrunken kernel phenotype to normal  
164 kernel phenotype fit a 1:3 segregation ratio (Supplemental Table 2). A F<sub>3</sub> population  
165 with 376 individuals derived from normal kernels of F<sub>2</sub> selfed ears was also planted to  
166 validate the kernel phenotype segregation. Among them, 130 individuals had kernel  
167 phenotype (*EHD1/EHD1*), and 246 had the segregation phenotype (*EHD1/ehd1*)  
168 (Supplemental Table 1). These results indicate that this shrunken kernel mutant is  
169 controlled by a single recessive gene.

170

### 171 **Positional cloning of *ZmEHD1***

172 Preliminary genetic mapping of *ZmEHD1* was carried out by BSA using the F<sub>2</sub>  
173 population, which contained 165 individuals that differed in seed size in the ears. The  
174 *ZmEHD1* gene was first mapped to a 2,339-kb region between the simple sequence  
175 repeat (SSR) marker umc1650 (17 recombinants) and umc1716 (27 recombinants) on  
176 the long arm of chromosome 4 (Figure 3A). Because the germination rate of the *ehd1*  
177 mutant was rather low, expanded F<sub>3</sub> mapping population containing ~53,000 normal  
178 kernels (*EHD1/EHD1* and *EHD1/ehd1*) was obtained to increase mapping resolution  
179 as described by Zuo et al. (2015). Thirteen new polymorphic SSR markers were  
180 developed (Supplemental Table 3), and *ZmEHD1* was eventually mapped to a ~6-kb

181 region between the SSR markers RM7 (56 recombinants) and RM13 (5 recombinants)  
182 (Figure 3A). This region contains two predicted open reading frames (ORFs) (Figure  
183 3A). Sequence analysis revealed three deletions in the 5' UTR and eight  
184 single-nucleotide polymorphisms (SNPs) in the ORF of GRMZM2G052740 (Figure  
185 3B). Among the eight SNPs in the ORF of GRMZM2G052740, three led to amino  
186 acid replacement between the *ehd1* mutant and the WT (Figure 3B). In addition to  
187 GRMZM2G052740, GRMZM2G052720 was also located in this region. Its  
188 candidacy as *ZmEHD1* gene was excluded because there was no sequence difference  
189 between the WT and the *ehd1* mutant. Thus, we inferred that GRMZM2G052740 was  
190 the candidate gene for the *ZmEHD1* locus.

191 The genomic DNA sequence of GRMZM2G052740 spanned ~4.4-kb and  
192 generated a transcript that included 16 exons (Figure 3B). The corresponding  
193 2,209-bp cDNA sequence encoded a polypeptide of 547 amino acids with a molecular  
194 mass of ~61 kD (Supplemental Figure 1). BLASTP analysis in GenBank indicated  
195 that GRMZM2G052740 is closely related to the *Arabidopsis* EHD-containing proteins,  
196 AtEHD1 and AtEHD2 (Supplemental Figure 1). The ZmEHD1 protein shares the  
197 typical structure of the EHD family; it has an EH domain with two calcium-binding  
198 EF-hands (15-39 aa and 49-84 aa), a P-loop (GQYSTGKT), a dynamin-type guanine  
199 nucleotide-binding domain, and a coil-coil domain (Figure 3C).

200 According to the publicly available maize microarray database ([www.qteller.com](http://www.qteller.com),  
201 Waters et al., 2011), *ZmEHD1* is expressed in various tissues and its mRNA  
202 abundance is high in immature cobs (V18), embryos, and endosperms at 12 and 14  
203 DAP. To verify the microarray data, we performed quantitative real-time RT-PCR  
204 using RNA isolated from various maize tissues, including roots, leaves, stems,  
205 endosperms, and embryos, and the results confirmed the public microarray data  
206 (Supplemental Figure 2). We also isolated RNA from the endosperms of the *ehd1*  
207 mutant and the WT at 15, 20, 25, and 35 DAP to assess *ZmEHD1* expression levels.  
208 The transcripts of *ZmEHD1* were down-regulated during endosperm development in  
209 both the *ehd1* mutant and the WT (Figure 3D). Unexpectedly, *ZmEHD1* mRNA  
210 abundance at all sampling times in the *ehd1* mutant was higher than in the WT (Figure

211 3D), indicating that the mutated *ZmEHD1* might be only partly functional.

212

213 **Validating that *ZmEHD1* is the causative locus by *ZmEHD1* loss-of-function**  
214 **mutants**

215 To determine whether *ZmEHD1* is responsible for the EHD1 locus, we used the  
216 CRISPR/Cas9 system to generate *ZmEHD1* loss-of-function mutant (KO). The given  
217 gene sequencing in *ZmEHD1* loss-of-function mutants revealed a deletion of 775-bp  
218 or 785-bp fragment at the coding sequence, which resulted in a frameshift  
219 (Supplemental Figure 3). *ZmEHD1* loss-of-function also led to decreased germination  
220 rate as well as retarded vegetative development (Figure 4A and 4B).

221 An allelism test was performed by crossing KO-1 F<sub>1</sub> (*KO*<sup>+/-</sup>) and *ehd1*  
222 heterozygotes (*ehd1*<sup>+/-</sup>). The shrunken kernel phenotype (*KO/ehd1*) and the WT  
223 phenotype (*EHD1/EHD1*; *EHD1/KO*; *EHD1/ehd1*) in the hybrid F<sub>1</sub> ears displayed a  
224 1:3 segregation ratio (Figure 4C, 4D and Supplemental Table 4), indicating that *KO*  
225 cannot complement *ehd1*. Meanwhile, the germination rate of the *KO/ehd1* mutant  
226 was much lower than that of the WT (Figure 4E). These results indicate that *ZmEHD1*  
227 is the locus affected by *ehd1*.

228

229 **The *ehd1* and KO mutant are defective in endocytosis**

230 EHD has usually been identified among proteins involved in endocytosis, vesicle  
231 transport, and signal transduction (Lin et al., 2001). To test whether the *ZmEHD1*  
232 protein is involved in CME in maize, we examined the uptake of  
233 N-(3-triethylammonium-propyl)-4-(4-diethylaminophenyl)hexatrienyl pyridinium  
234 dibromide (FM4-64), a commonly used endocytosis tracer, in the *ehd1* mutant and the  
235 WT. Because maize seedlings are relatively large, we could not place the whole plant  
236 on the microscope platform as was previously done with 3-day-old *Arabidopsis* plants  
237 (Fan et al., 2013). Instead, we detached the similar parts of roots from the *ehd1*  
238 mutant and the WT seedlings that had been treated with 5 μM FM4-64 for 10 min to  
239 monitor the FM4-64 endocytosis. At 30 minutes after labeling, FM4-64-labelled  
240 fluorescent puncta were detected in 28% of the WT root cells (21 of 75 cells), while



241 no FM4-64-labelled fluorescent puncta were detected in *ehd1* root cells (0 of 90 cells)  
242 (Figure 5A). Although FM4-64-labelled fluorescent puncta could be detected in root  
243 cells of both the WT and the *ehd1* mutant at 60 minutes after labeling,  
244 FM4-64-labelled fluorescent puncta were detected in only 19% of the *ehd1* root cells  
245 (22 of 117 cells) but in 45% of the WT root cells (45 of 101 cells) (Figure 5A). The  
246 effects of ZmEHD1 on endocytosis were further confirmed by the delayed  
247 internalization of FM4-64 in *ZmEHD1* knock-out mutants (Figure 5B).

248 To further investigate whether ZmEHD1 was involved in the vesicle traffic  
249 pathway, we inhibited endocytic recycling of FM-64 using the fungal toxin brefeldin  
250 A (BFA). The accumulation of FM-64 in BFA bodies was clearly observed in the WT  
251 when treated with BFA (Figure 5C and 5D). In contrast, the aggregation of FM-64 in  
252 BFA bodies remarkably decreased in the *ehd1* mutant and *ZmEHD1* knock-out  
253 mutants (Figure 5C and 5D). Compared to the WT, the sizes of FM-64 labeled BFA  
254 bodies in the *ehd1* mutant and *ZmEHD1* knock-out mutants decreased by ~67% and  
255 ~76%, respectively (Fig. 5C and 5D). These results suggested that ZmEHD1 is  
256 important in endocytic transport.

257

### 258 **Subcellular localization of ZmEHD1 and ZmEHD1<sub>mut</sub>**

259 To determine the subcellular localization of ZmEHD1 protein, yellow fluorescent  
260 protein (YFP) fused N-terminally to ZmEHD1 was transiently expressed in tobacco  
261 (*N. benthamiana*) leaves under the control of the *CaMV 35S* promoter. In tobacco  
262 epidermal cells expressing the YFP-ZmEHD1 fusion protein, the YFP signal was  
263 mainly detected in the PM (Supplemental Figure 4A). The localization was confirmed  
264 by staining lipid membranes with the red fluorescent probe, FM4-64 (Supplemental  
265 Figure 4A). We also cloned the *ZmEHD1* CDS from the *ehd1* mutant and examined  
266 the subcellular localization of ZmEHD1<sub>mut</sub> protein. YFP-ZmEHD1<sub>mut</sub> signals were  
267 aggregated and colocalized with FM4-64 signals in tobacco epidermal cells  
268 (Supplemental Figure 4B). This phenomenon was consistently observed in different  
269 biological replicates. Thus, we deduced that the mutation in ZmEHD1 affected the  
270 subcellular localization of ZmEHD1.

271 **ZmEHD1 interacts with the ZmAP2  $\sigma$  subunit in yeast and plant**

272 In its drastically reduced fertility and endocytosis, the *ehd1* mutant resembles  
273 *Arabidopsis ap2*  $\sigma$  mutants, in which CME and multiple stages of plant development  
274 are impaired (Fan et al., 2013). We hypothesized that ZmEHD1 is involved in CME  
275 by virtue of its interaction with the ZmAP2  $\sigma$  subunit. To test this hypothesis, we  
276 cloned the cDNA sequence of the maize AP2  $\sigma$  subunit (GRMZM2G052713) and  
277 introduced it into *Arabidopsis ap2*  $\sigma$  loss-of-function mutant (Fan et al., 2013).  
278 *Arabidopsis ap2*  $\sigma$  homozygous for the T-DNA insertion was identified by PCR  
279 (Supplemental Figure 5A). RT-PCR showed that *ZmAP2*  $\sigma$  subunit was expressed in  
280 the *Arabidopsis ap2*  $\sigma$  mutant (Supplemental Figure 5B). The transformation of the  
281 *Arabidopsis ap2*  $\sigma$  mutant with *ZmAP2*  $\sigma$  subunit rescued the developmental defects,  
282 such as reduced leaf size and fertility (Supplemental Figure 5C). These results  
283 suggested that *ZmAP2*  $\sigma$  subunit has similar functions as *AtAP2*  $\sigma$  subunit in CME.

284 The potential interaction between ZmEHD1 and the ZmAP2  $\sigma$  subunit was first  
285 evaluated by a biomolecular fluorescence complementation (BiFC) assay in tobacco  
286 leaves and maize mesophyll protoplasts. The ZmEHD1, ZmEHD1<sub>mut</sub>, and ZmAP2  $\sigma$   
287 subunit constructs were introduced into *N. benthamiana* mesophyll cells or maize  
288 mesophyll protoplasts by *A. tumefaciens*-mediated infiltration. The vitality of the *N.*  
289 *benthamiana* mesophyll cells was determined by propidium iodide. Reconstituted  
290 YFP signals were observed in the PM when both nYFP-ZmAP2  $\sigma$  subunit and  
291 cYFP-ZmEHD1 constructs were co-expressed (Figure 6A and Supplemental Figure 6).  
292 Reconstituted YFP signals were also observed when the nYFP-ZmAP2  $\sigma$  subunit and  
293 cYFP-ZmEHD1<sub>mut</sub> constructs were co-expressed (Figure 6A and Supplemental Figure  
294 6). Consistent with the subcellular localization of ZmEHD1<sub>mut</sub>, the reconstituted YFP  
295 signals were diffused throughout the cells when the nYFP-ZmAP2  $\sigma$  subunit and  
296 cYFP-ZmEHD1<sub>mut</sub> constructs were co-expressed (Figure 6A and Supplemental Figure  
297 6). This was further verified by the localization of ZmAP2 $\sigma$  subunit in WT maize and  
298 *ehd1* mutant (Supplemental Figure 7).

299 We also used a split-ubiquitin membrane-based yeast two-hybrid system to  
300 verify the interaction between the ZmAP2  $\sigma$  subunit and ZmEHD1 as well as

301 *ZmEHD1<sub>mut</sub>*. Both *ZmEDH1* and *ZmEHD1<sub>mut</sub>* interacted directly with *ZmAP2*  $\sigma$   
302 subunit in yeast. The interaction resulted in survival in a medium lacking His and  
303  $\beta$ -galactosidase activity (Figure 6B).

304 Pull-down experiments using purified His-*ZmEHD1*/His-*ZmEHD1<sub>mut</sub>* to isolate  
305 GST-*ZmAP2*  $\sigma$  subunit also confirmed the physical interaction between  
306 *ZmEHD1*/*ZmEHD1<sub>mut</sub>* and *ZmAP2*  $\sigma$  subunit. His-*ZmAP2*  $\sigma$  subunit was clearly  
307 detected in the pull-down fraction (Figure 6C). These results strongly suggested that  
308 *ZmEHD1* directly interacts with *ZmAP2*  $\sigma$  subunit at the plasma membranes in  
309 maize.

310

### 311 **Transcriptome profiling of the *ehd1* mutant**

312 To gain insight into the molecular events involved in the *ZmEHD1*-mediated  
313 signaling pathway, we compared the whole-transcriptome profiles of endosperms of  
314 the *ehd1* mutant and the WT at 15 DAP using RNA-seq. Each sample was represented  
315 by two biological replicates, and the libraries were sequenced by Illumina  
316 high-throughput sequencing technology. These four RNA libraries yielded more than  
317 0.5 billion raw reads, and approximately 97% of the raw reads remained after adaptor  
318 polluted reads, Ns reads, and low quantity reads were trimmed. Of the remaining  
319 reads, more than 0.12 billion could be perfectly mapped to maize B73 RefGen\_V3.27  
320 ([ftp://ftp.ensemblgenomes.org/pub/plants/release-27/fasta/zea\\_mays](ftp://ftp.ensemblgenomes.org/pub/plants/release-27/fasta/zea_mays)). Sequences that  
321 could not be mapped to the maize genome were discarded, and only those perfectly  
322 mapped were analyzed further. The abundance of each gene was expressed by reads  
323 per kilo base million mapped reads (RPKM) (Wagner et al., 2012). We calculated the  
324 Pearson's correlation coefficients (*R* value) of the two biological replicates for each  
325 treatment to investigate the variability between the replicates. The *R* value of both  
326 comparisons exceeded 0.99 (Supplemental Figure 8), indicating a high correlation  
327 between biological replicates.

328 Based on the criteria that the Log<sub>2</sub> fold-change ratio was  $\geq 1$  and that the  
329 adjusted P value was  $\leq 0.05$ , 4,760 genes, including *ZmEHD1*, were identified as  
330 differentially expressed genes (DEGs) by DEGseq software (Wang et al., 2010).

331 Among the DEGs, 2,208 genes were up-regulated and 2,552 genes were  
332 down-regulated in the *ehd1* mutant relative to the WT (Supplemental Table 5). The  
333 results of RNA-seq were confirmed by quantitative real-time RT-PCR. In agreement  
334 with our RNA-seq data, the expression levels of randomly selected  
335 GRMZM2G088273, GRMZM2G346897, GRMZM2G067929, and  
336 GRMZM2G418119 were lower in the *ehd1* mutant than in the WT (Supplemental  
337 Figure 9A). As expected, GRMZM2G156877 and GRMZM2G420988 were  
338 expressed at higher levels in the *ehd1* mutant than in WT (Supplemental Figure 9A),  
339 demonstrating the reliability of our RNA-seq data. We then performed Gene Ontology  
340 (GO; <http://bioinfo.cau.edu.cn/agriGO/>) analysis to determine the molecular events  
341 related to the DEGs. GO analysis indicated that the 4,760 DEGs were highly enriched  
342 for biological processes involved in response to stress (GO:0006950,  $P = 1.2e^{-12}$ ),  
343 integral to membrane (GO:0016021,  $P = 1e^{-5}$ ), intracellular membrane-bounded  
344 organelle (GO:0043231,  $P = 0.0002$ ), vacuole (GO:0005773,  $P = 0.0007$ ), starch  
345 metabolic processes (GO:0005982,  $P = 1.6e^{-5}$ ), and carbohydrate metabolic processes  
346 (GO:0005975,  $P = 4.16e^{-5}$ ).

347 Interestingly, some of the DEGs have known or presumed functions associated  
348 with auxin-mediated signaling pathway (GO:0009734,  $P = 1.2e^{-25}$ ) and response to  
349 auxin stimulus (GO:0009733,  $P = 2.1e^{-25}$ ), including AUXIN RESPONSE FACTOR  
350 (ARF), AUX/IAA transcription factor, small auxin up-regulated RNA (SAUR),  
351 indole-3-acetaldehyde oxidase, auxin transporters, and efflux carrier (Table 1). We  
352 randomly chose four of the DEGs listed in Table 1 (GRMZM2G082943,  
353 GRMZM2G365188, GRMZM5G809195, and GRMZM2G019799) and validated  
354 their difference in expression levels in the *ehd1* mutant vs. the WT by quantitative  
355 real-time RT-PCR (Supplemental Figure 9B). These results indicated that *ZmEHD1*  
356 might affect auxin homeostasis in maize.

357

### 358 **Auxin distribution and ZmPIN1a-YFP localization in the *ehd1* mutant**

359 To test the hypothesis that *ZmEHD1* might affect auxin homeostasis in maize, we first  
360 explored the gravitropic response by examining mesocotyl-coleoptiles. We placed the

361 upright mesocotyl-coleoptiles in the horizontal direction. In contrast to less than 4 h  
362 for the recovered vertical growth of WT, it took about 5 h for the *ehd1* mutant and  
363 *ZmEHD1* knock-out mutants to recover vertical growth under the same condition  
364 (Figure 7A). The free IAA content in kernels of the *ehd1* mutant and the WT at 15  
365 DAP were determined. The free IAA content in the kernels of the *ehd1* mutant was  
366 2,558 pg mg<sup>-1</sup> FW, which was ~30% lower than that in the WT (Figure 7B).

367 To further demonstrate that *ZmEHD1* regulates auxin homeostasis in maize, we  
368 first crossed the auxin-responsive *ZmDR5::RFP* reporter maize with *ehd1* mutant to  
369 examine the auxin distribution in the root tips of *ehd1* mutant. The RFP signals were  
370 significantly reduced in the root caps in *ehd1* mutant (Figure 7C). We also crossed the  
371 *ZmPIN1a::YFP* line with *ehd1* mutant. The YFP signals in the roots of *ehd1* mutant  
372 showed more diffuse localization than that in *PIN1a::YFP EHD1* line, and could be  
373 detected in root cortex (Figure 7D). These results suggested that auxin homeostasis  
374 was altered in *ehd1* mutant.

375

### 376 **1-NAA application rescues the fertility of the *ehd1* mutant**

377 To further verify that the rather low fertility of the *ehd1* mutant and *ZmEHD1*  
378 knock-out mutant was caused by auxin homeostasis, we attempted to rescue the  
379 phenotypic defects of *ehd1* mutant and *ZmEHD1* knock-out mutants by exogenous  
380 application of the active auxin compound, 1-naphthaleneacetic acid (1-NAA). The  
381 seeds of the *ehd1* mutant and *ZmEHD1* knock-out mutants were rinsed in water or in  
382 four increasing concentrations of 1-NAA for 12 h, and then germinated in the  
383 paper-culture system. Little or no phenotypic rescue of the *ehd1* mutant was observed  
384 when 30 mg L<sup>-1</sup> 1-NAA or water alone was applied (Figure 8A). However, the  
385 germination rate of *ehd1* mutant and *ZmEHD1* knock-out mutant was significantly  
386 increased when treated with low concentrations of 1-NAA (Figure 8A). Also, when  
387 treated with low concentrations of 1-NAA, the *ehd1* mutant and *ZmEHD1* knock-out  
388 mutant survived after its first two leaves had completely expanded (Figure 8B). In  
389 contrast to IAA, exogenous GA3 had no effect on the germination of the *ehd1* mutant  
390 and *ZmEHD1* knock-out mutant (Supplemental Figure 10). Overall, these results

391 demonstrated that the phenotypic defects of the *ehd1* mutant could be rescued by  
392 exogenous application of 1-NAA.

393

### 394 **Discussion**

395 In the present research, we characterized the *ehd1* mutant, which is impaired in kernel  
396 development and vegetative growth, and used positional cloning and transgenic  
397 validation to verify that the *ehd1* gene encodes an EHD protein. The results of an  
398 FM4-64 uptake experiment, split-ubiquitin membrane-based yeast two-hybrid system,  
399 BiFC and pull-down assay indicated that ZmEHD1 is involved in CME through its  
400 interaction with the ZmAP2  $\sigma$  subunit. Additionally, the transcriptome profiling of the  
401 *ehd1* mutant, the auxin distribution and ZmPIN1a-YFP localization in the *ehd1*  
402 mutant, and the rescue of the mutant phenotypes following the exogenous application  
403 of 1-NAA revealed that the ZmEHD1-mediated endocytosis mainly affects auxin  
404 homeostasis in maize.

405 In *Arabidopsis*, AP2  $\sigma$  subunit is primarily recruited from the cytoplasm to the  
406 PM to initiate clathrin-coated endocytic vesicle formation (Fan et al., 2013; Fan et al.,  
407 2015). The developmental defects of *ap2*  $\sigma$  loss-of-function *Arabidopsis*, such as  
408 reduced leaf size and fertility, could be rescued by *ZmAP2*  $\sigma$  cDNA, suggesting that  
409 *ZmAP2*  $\sigma$  subunit has similar functions as *AtAP2*  $\sigma$  subunit in CME. Microarray data  
410 revealed that the AP2  $\sigma$  subunit is ubiquitously expressed in various tissues  
411 throughout maize development (Sekhon et al., 2011), which is consistent with the  
412 expression pattern of *ZmEHD1*. Based on the direct interaction between the ZmAP2 $\sigma$   
413 subunit and ZmEHD1 at the PM as demonstrated by BiFC, pull-down assay and  
414 split-ubiquitin membrane-based yeast two-hybrid system in the current study, we  
415 suspected that ZmEHD1 contributes to CME by interacting with the ZmAP2  $\sigma$   
416 subunit.

417 The naturally occurring *ehd1* mutant had amino acid substitutions in three  
418 positions. Of these, the V/A substitution was in a linker region. The other two  
419 mutations were in the coil-coil domain of ZmEHD1. The coil-coil domain was  
420 responsible for protein-protein interactions (Bar et al., 2009; Sharma et al., 2008).

421 Here, we showed that the subcellular location of ZmEHD1<sub>mut</sub> was obviously different  
422 from that of ZmEHD1. Though ZmEHD1<sub>mut</sub> could directly interact with ZmAP2 $\sigma$   
423 subunit, the PM was not the main location for interaction between ZmEHD1<sub>mut</sub> and  
424 the ZmAP2 $\sigma$  subunit. The impaired clathrin-coated endocytic vesicle formation  
425 and/or the recycling of the ZmAP2  $\sigma$  subunit from the endosome to the PM should be  
426 the major contributor to phenotypes observed in the *ehd1* mutant.

427 *Arabidopsis ARF2* controls seed size by repressing cell proliferation (Schruff et  
428 al., 2006); in rice, activation of *BIG GRAINI* (BG1) significantly improves grain size  
429 by regulating auxin transport (Liu et al., 2015); *TGW6*, an IAA-glucose hydrolase,  
430 negatively regulates endosperm development of rice (Ishimaru et al., 2013). Analyses  
431 of a seed-specific viable maize mutant, defective endosperm 18 (*de18*), demonstrated  
432 that the *ZmYUC1* gene (which is critical for IAA biosynthesis) is essential for normal  
433 endosperm development in maize (Bernardi et al., 2012). Thus, auxin biosynthesis,  
434 transport and signaling might coordinate to regulate vegetative and reproductive  
435 development in maize (Gallavotti et al., 2008; Li and Li, 2016). The current results  
436 with maize show that *ARFs*, indole-3-acetaldehyde oxidase, auxin transporters, and  
437 efflux carrier are differentially expressed in the *ehd1* mutant vs. the WT; that the  
438 concentration of free IAA is lower in the *ehd1* mutant than in the WT; that the auxin  
439 distribution and ZmPIN1a-YFP localization were altered in the *ehd1* mutant; and that  
440 exogenous application of 1-NAA rescues the phenotypes of the *ehd1* mutant. These  
441 results suggest that the growth defects of the *ehd1* mutant result from the loss of auxin  
442 homeostasis.

443

## 444 **Materials and methods**

### 445 **Plant materials**

446 The ZmPIN1a::YFP and ZmDR5::mRFP lines were kindly provided by Lixing Yuan  
447 (China Agricultural University). The maize mutant *ehd1* was isolated by screening for  
448 natural mutants defective in grain filling. To construct mapping population, the mutant  
449 was crossed with inbred line Xun9058 in the winter of 2011 in Sanya, Hainan  
450 Province. Xun9058 is the male parent of the elite hybrid Xundan20 and has been



451 widely used in the breeding of hybrid maize in China. F<sub>2</sub> individuals were obtained by  
452 selfing the F<sub>1</sub> plants in the summer of 2012 in Zhengzhou, Henan Province. The site  
453 (113°42'E, 34°48'N) is located in central China and has an average annual  
454 temperature of 14.3 °C and an average annual rainfall of 640.9 mm. A F<sub>2</sub> population  
455 with 165 individuals was used to map the preliminary location of *ZmEHD1* gene.  
456 Normal kernels from F<sub>3</sub> segregation ears were used for fine mapping of the candidate  
457 gene. The F<sub>3</sub> population contains ~53,000 individuals. A small piece of each F<sub>3</sub> kernel  
458 was chipped and genotyped before planting. At harvest stage, the ear phenotype was  
459 investigated to verify the kernel segregation of each individual. Two markers, bnlg589  
460 and RM1, were closely linked to the locus to determine recombined individuals.

461 From F<sub>2</sub> generation, the normal kernels from segregation ears were analyzed by  
462 closely linked marker with *ZmEHD1* gene and the recombined individuals were selfed.  
463 This process was continued for five generations to construct nearly isogenic lines  
464 (NILs). The NILs were used as WT.

465

#### 466 **Molecular markers**

467 Bulked segregant analysis (BSA) was used to detect the genetic linkage of the  
468 *ZmEHD1* gene (Michelmore et al., 1991) and 1082 SSR markers from the maize  
469 genome database ([www.maizegdb.org](http://www.maizegdb.org)) were tested for polymorphism in the two  
470 parents and the two bulks. To develop new markers for fine mapping, the sequence of  
471 the B73 reference genome between markers bnlg589 and umc2289 on chromosome 4  
472 was downloaded from MaizeGDB (<http://www.maizegdb.org/>). SSR-Hunter software  
473 was used find simple sequence repeats (SSRs). After BLAST was performed  
474 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), SSRs with single copy were developed to  
475 new markers with PRIMER 5.0. The newly designed SSRs were tested by  
476 polyacrylamide gel electrophoresis (PAGE) for polymorphism in the *ehd1* mutant,  
477 Xun9058, pooled samples of normal kernels (*EHD1/EHD1* and *EHD1/ehd1*) or  
478 shrunken kernels (*ehd1/ehd1*). SSRs with polymorphism were used for subsequent  
479 fine mapping. The sequences of the primers used for mapping are listed in  
480 Supplemental Table 3.



## 481 **Transgenic functional validation**

482 The CRISPR/Cas9-mediated *ZmEHD1* editing was performed as described by Xing et  
483 al. (2014) with some modifications. In brief, two gRNAs that direct target sequences  
484 located at nucleotides 16 and 791 of *ZmEHD1* were produced using primers listed in  
485 Supplemental Table 3. Thereafter, the fragments were cloned into the pBUE411  
486 vector using the *BsaI* restriction site by T4 Ligase reaction. The plasmid contained the  
487 *Streptomyces hygroscopicus* phosphinothricin acetyltransferase gene (*bar*) under the  
488 control of a *CaMV 35S* promoter and was electroporated into *A. tumefaciens* EHA105.  
489 Immature embryos of maize inbred line ZCC01 were transformed by co-cultivation  
490 with EHA105 at Life Science and Technology Center of China National Seed Group  
491 Co., LTD. Transformants were selected with gradually increasing concentrations of  
492 Bialaphos. T<sub>2</sub> homozygous lines were sequenced to ensure that *ZmEHD1* was  
493 knocked out.

494 For *Arabidopsis* transformation, the cDNAs of *ZmAP2*  $\sigma$  subunit without 3' UTR  
495 were amplified by PCR. The corresponding products were introduced into the  
496 pENTR<sup>TM</sup>/D-TOPO vector (Invitrogen) and cloned into pMDC32 by LR reactions  
497 (Invitrogen). The plasmid was electroporated into *A. tumefaciens* GV3101 and was  
498 transformed into *Arabidopsis* by the floral dip method (Clough and Bent, 1998).  
499 Transgenic plants were selected with the use of 35  $\mu\text{g ml}^{-1}$  hygromycin. The  
500 sequences of the primer pairs used in the experiments are listed in Supplemental  
501 Table 3.

502

## 503 **Expression analysis of *ZmEHD1***

504 Total RNA was extracted from the WT and the *ehd1* mutant with the RNeasy plant  
505 mini kit. The RNA was used for the synthesis of first-strand cDNA with SuperScript  
506 III first-strand synthesis supermix (Invitrogen). Quantitative real-time RT-PCR was  
507 carried out in an ABI 7500 system using the SYBR Premix Ex Taq<sup>TM</sup> (perfect real  
508 time) kit (TaKaRaBiomedicals), with 18S rRNA and *Actin* as controls. Primers  
509 designed to detect the transcription level of *ZmEHD1* were listed in Supplemental  
510 Table 3. The relative expression level was calculated using the comparative Ct

511 method. Each experiment was replicated at least three times.

512

### 513 **Subcellular localization of ZmEHD1**

514 The full-length *ZmEHD1* and *ZmEHD1<sub>mut</sub>* coding region was amplified from the WT  
515 and the *ehd1* mutant. To generate the *ZmEHD1* expression plasmids, the 1644-bp  
516 fragments were cloned into the pCPB vector, which was fused to an YFP protein in  
517 the N terminus using the *Bam*HI restriction site by In-Fusion reaction. The plasmid  
518 was electroporated into *A. tumefaciens* GV3101 and was transiently expressed in  
519 tobacco epidermal cells as described previously (Li et al., 2008). To stain the PM of  
520 epidermal cells, 5  $\mu$ M FM4-64 was infiltrated into tobacco leaves for 3 h before  
521 observation. YFP/FM-64 images were collected with a Zeiss LSM700 confocal  
522 microscope.

523

### 524 **Split-ubiquitin yeast two-hybrid assay**

525 The split-ubiquitin two-hybrid system was used to detect the interactions between  
526 membrane proteins. The assay was carried out according to the instructions provided  
527 with the DUALmembrane Kit (Dualsystems Biotech). The full-length coding regions  
528 of the *ZmAP2*  $\sigma$  subunit, *ZmEHD1* and *ZmEHD1<sub>mut</sub>* were amplified and cloned into  
529 the vectors pST3-STE and pPR3-N NubG using the *Sfi*I restriction site by In-Fusion  
530 reaction. Vectors were co-transformed into yeast strain NMY51. The interactions were  
531 assessed by the growth of yeast colonies on synthetic minimal medium containing 7.5  
532 mM 3-aminotriazole without Leu, Trp, His and Ade and also by chloroform overlay  
533  $\beta$ -galactosidase plate assay (Duttweiler, 1996).

534

### 535 **Bimolecular fluorescence complementation (BiFC) assays**

536 The coding sequences of the *ZmAP2*  $\sigma$  subunit, *ZmEHD1* and *ZmEHD1<sub>mut</sub>* were  
537 amplified using specific primers listed in Table S1, and were cloned into the  
538 pSPYNE-35S or pSPYCE-35S binary vectors using the *Bam*HI restriction site. The  
539 various combinations of *ZmAP2*  $\sigma$  subunit, *ZmEHD1*, and *ZmEHD1<sub>mut</sub>* expression  
540 vectors were transiently expressed in 3-week-old *N. benthamiana* leaves by

541 *Agrobacterium*-mediated infiltration (strain EHA105). YFP images were obtained 3  
542 days after infiltration with a Zeiss LSM700 confocal microscope.

543

#### 544 **Pull-down analysis**

545 The full-length coding sequences of *ZmEHD1*, *ZmEHD1<sub>mut</sub>* and *ZmAP2*  $\sigma$  subunit  
546 were individually subcloned into the pET-28a(+) and pGEX-4T-1 vectors using the  
547 *Bam*HI restriction site by In-Fusion reaction. The resulting constructs were verified by  
548 sequencing. His-ZmEHD1, His-ZmEHD1<sub>mut</sub>, GST and GST-ZmAP2  $\sigma$  subunit were  
549 expressed in *Escherichia coli* BL21.

550 For the *in vivo* pull-down analysis, 10  $\mu$ g of purified His-ZmEHD1,  
551 His-ZmEHD1<sub>mut</sub> were incubated with GST-ZmAP2  $\sigma$  subunit or GST bound to  
552 glutathione-Sepharose<sup>TM</sup> 4B (GE Healthcare) for 4 h at 4 °C on a rotary shaker.  
553 Precipitated beads were washed six times with washing buffer. Washed beads were  
554 boiled with 50  $\mu$ L of 1  $\times$  SDS sample buffer for 5 min and subjected to SDS-PAGE  
555 and immunoblot analysis.

556

#### 557 **FM4-64 internalization assay**

558 FM4-64 internalization assays were carried out to evaluate the rate of endocytosis as  
559 described by Fan et al. (2013) with some modifications. WT and *ehd1* seedlings were  
560 incubated in half-strength Hoagland's nutrient solution containing 5  $\mu$ M FM4-64 for  
561 10 min at room temperature. The roots were then cut and transferred to glass slides.  
562 The FM4-64 internalization was monitored at indicated durations at room temperature  
563 with a Zeiss LSM700 confocal microscope.

564

#### 565 **RNA-seq analysis**

566 At 15 DAP, total RNA was extracted from the endosperms of the *ehd1* mutant and the  
567 WT with TRIZOL reagent (Invitrogen), and 3  $\mu$ g of total RNA was used as input  
568 material for construction of the RNA libraries. The RNA-sequencing libraries were  
569 constructed with NEBNext® Ultra<sup>TM</sup> RNA Library Prep Kit for Illumina® (NEB,  
570 USA). In brief, mRNA was purified from total RNA using poly-T oligo-attached

571 magnetic beads. The enriched mRNA was then fragmented. The random hexamers  
572 were used for first-strand cDNA synthesis. After second-strand cDNA synthesis,  
573 terminal repair, and poly(A) tail and sequencing oligonucleotide adaptors ligation, the  
574 fragments were purified and subsequently amplified by PCR. The insert size was  
575 assessed with the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA).  
576 Finally, the libraries of inserting cDNAs with 200-bp in size were generated and  
577 sequenced with the IlluminaHiSeq 2500 platform (ANROAD, Beijing, China).

578 The raw reads were produced after exclusion of low quality reads and 5' and 3'  
579 adaptor contaminants. The unique RNAs were aligned to the maize RefGen\_V3.27  
580 ([ftp://ftp.ensemblgenomes.org/pub/plants/release-27/fasta/zea\\_mays](ftp://ftp.ensemblgenomes.org/pub/plants/release-27/fasta/zea_mays)). Only perfectly  
581 matching sequences were considered for further analysis. The count information was  
582 used to determine normalized gene expression levels as RPKM (Wagner et al., 2012).  
583 Multiple testing with the Benjamini-Hochberg procedure for false discovery rate  
584 (FDR) was taken into account by using an adjusted P-value. Genes were considered to  
585 be differentially expressed if the Log<sub>2</sub> fold-change ratio was  $\geq 1$  and if the adjusted P  
586 value was  $< 0.05$  according to the DEGseq method (Wang et al., 2010).

587

### 588 **Free IAA analysis**

589 At 15 DAP, kernels of the WT and the *ehd1* mutant were collected and frozen in  
590 liquid nitrogen. A 200-mg (fresh weight) sample of WT and *ehd1* kernels was finely  
591 ground in liquid nitrogen and then extracted with 1 ml of methanol containing  
592 antioxidant and <sup>2</sup>H<sub>2</sub>-IAA (internal standard, CDN Isotopes) at 4°C for 24 h. After  
593 centrifugation, the extract was purified with an Oasis Max solid phase extract  
594 cartridge (150 mg/6 cc; Waters). IAA was quantified using UPLC-MS/MS consisting  
595 of a UPLC system (ACQUITY UPLC, Waters) and a triple quadruple tandem mass  
596 spectrometer (Quattro Premier XE, Waters) as described by Wang et al. (Wang et al.,  
597 2015). Four independent biological replicates and two technical repeats were  
598 performed for the WT and the *ehd1* mutant.

599

### 600 **Phytohormone treatments**

601 For phytohormone treatments, WT and *ehd1* mutant seeds were immersed in water  
602 and the indicated concentrations of 1-NAA for 12 h or GA3 for 24 h. The seeds were  
603 then kept at 22 °C in the dark for 120 h for germination. A seed was scored as  
604 germinated if its radicle protruded through the seed coat.

605

#### 606 **Additional information**

#### 607 **Funding**

<b>Funder</b>	<b>Grant reference number</b>	<b>Author</b>
National Key Research and Development Program of China	2016YFD0101002	Wen-Xue Li
National Basic Research Program of China Grant	31370303	Jihua Tang

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

608

#### 609 **Author contributions**

610 J.T. and W.X.L. designed the research; Y.W., W.L., H.W., Q.D. and Z.F. performed the  
611 research; W.X.L. and J.T. analyzed the data and wrote the article.

612

#### 613 **References**

614 Backues SK, Korasick DA, Heese A, Bednarek SY. 2010. The *Arabidopsis*  
615 dynamin-related protein2 family is essential for gametophyte development. *Plant*  
616 *Cell* **22**:3218-3231. DOI: <https://doi.org/10.1105/tpc.110.077727>, PMID:  
617 20959563

618 Bar M, Aharon M, Benjamin S, Rotblat B, Horowitz M, Avni A. 2008. AtEHDs,  
619 novel *Arabidopsis* EH-domain-containing proteins involved in endocytosis.  
620 *Plant J.* **55**:1025-1038. DOI: <https://doi.org/10.1111/j.1365-313X.2008.03571.x>,  
621 PMID: 18547399

622 Bar M, Avni A. 2009. EHD2 inhibits ligand-induced endocytosis and signaling of the

- 623 leucine-rich repeat receptor-like protein LeEix2. *Plant J.* **59**:600-611. DOI :  
624 <https://doi.org/10.1111/j.1365-313X.2009.03897.x>, PMID: 19392695
- 625 Bar M, Sharfman M, Schuster S, Avni A. 2009. The coiled-coil domain of EHD2  
626 mediates inhibition of LeEix2 endocytosis and signaling. *PLoS ONE* **4**:e7973.  
627 DOI: <https://doi.org/10.1371/journal.pone.0007973>, PMID:19936242
- 628 Barberon M, Zelazny E, Robert S, Conéjéro G, Curie C, Friml J, Vert G. 2011.  
629 Monoubiquitin-dependent endocytosis of the iron-regulated transporter 1 (IRT1)  
630 transporter controls iron uptake in plants. *PNAS* **108**:E450-E458. DOI:  
631 <https://doi.org/10.1073/pnas.1100659108>, PMID: 21628566
- 632 Bernardi J, Lanubile A, Li QB, Kumar D, Kladnik A, Cook SD, Ross JJ, Marocco A,  
633 Chourey PS. 2012. Impaired auxin biosynthesis in the defective endosperm18  
634 mutant is due to mutational loss of expression in the *ZmYuc1* gene encoding  
635 endosperm-specific YUCCA1 protein in maize. *Plant Physiol.* **160**:1318-1328.  
636 DOI: <https://doi.org/10.1104/pp.112.204743>, PMID: 22961134
- 637 Blackbourn HD, Jackson AP. 1996. Plant clathrin heavy chain: sequence analysis and  
638 restricted localization in growing pollen tubes. *Journal of Cell Science*  
639 **109**:777-786. PMID: 8718669
- 640 Clough SJ, Bent AF. 1998. Floral dip: a simplified method for  
641 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.*  
642 **16**:735-743. DOI: <https://doi.org/10.1046/j.1365-313x.1998.00343.x>, PMID:  
643 10069079
- 644 Dhonukshe P, Aniento F, Hwang I, Robinson DG, Mravec J, Stierhof YD, Friml J.  
645 2007. Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in  
646 *Arabidopsis*. *Current Biology* **17**:520-527. DOI:  
647 <https://doi.org/10.1016/j.cub.2007.01.052>, PMID: 17306539
- 648 Di Rubbo S, Irani NG, Kim SY, Xu ZY, Gadeyne A, Dejonghe W, Vanhoutte I,  
649 Persiau G, Eeckhout D, Simon S, Song K, Kleine-Vehn J, Friml J, De Jaeger G,  
650 Van Damme D, Hwang I, Russinova E. 2013. The clathrin adaptor complex AP-2  
651 mediates endocytosis of BRASSINOSTEROID INSENSITIVE1 in *Arabidopsis*.  
652 *Plant Cell* **25**:2986-2997. DOI: <https://doi.org/10.1105/tpc.113.114058>, PMID:

- 653 23975899
- 654 Duttweiler HM. 1996. A highly sensitive and non-lethal beta-galactosidaseplate assay  
655 for yeast. *Trends in Genetics* **12**:340-341. DOI:  
656 [https://doi.org/10.1016/S0168-9525\(96\)80008-4](https://doi.org/10.1016/S0168-9525(96)80008-4), PMID: 8855661
- 657 Fan L, Hao H, Xue Y, Zhang L, Song K, Ding Z, Botella MA, Wang H, Lin J. 2013.  
658 Dynamic analysis of *Arabidopsis* AP2  $\sigma$  subunit reveals a key role in  
659 clathrin-mediated endocytosis and plant development. *Development*  
660 **140**:3826-3837. DOI: <https://doi.org/10.1242/dev.095711>, PMID: 23924631
- 661 Fan L, Li R, Pan J, Ding Z, Lin J. 2015. Endocytosis and its regulation in plants.  
662 *Trends in Plant Science* **20**:388-397. DOI:  
663 <https://doi.org/10.1016/j.tplants.2015.03.014>, PMID: 25914086
- 664 Gallavotti A, Barazesh S, Malcomber S, Hall D, Jackson D, Schmidt RJ, McSteen P.  
665 2008. Sparse inflorescence1 encodes a monocot-specific YUCCA-like gene  
666 required for vegetative and reproductive development in maize. *PNAS*  
667 **105**:15196-15201. DOI: <https://doi.org/10.1073/pnas.0805596105>, PMID:  
668 18799737
- 669 Ishimaru K, Hirotsu N, Madoka Y, Murakami N, Hara N, Onodera H, Kashiwagi T,  
670 Ujiie K, Shimizu B, Onishi A, Miyagawa H, Katoh E. 2013. Loss of function of  
671 the IAA-glucose hydrolase gene TGW6 enhances rice grain weight and increases  
672 yield. *Nature Genetics* **45**:707-711. DOI: <https://doi.org/10.1038/ng.2612>, PMID:  
673 23583977
- 674 Kim SY, Xu ZY, Song K, Kim DH, Kang H, Reichardt I, Sohn EJ, Friml J, Juergens G,  
675 Hwang I. 2013. Adaptor protein complex 2-mediated endocytosis is crucial for  
676 male reproductive organ development in *Arabidopsis*. *Plant Cell* **25**:2970-2985.  
677 DOI: <https://doi.org/10.1105/tpc.113.114264>, PMID: 23975898
- 678 Kitakura S, Vanneste S, Robert S, Löffke C, Teichmann T, Tanaka H, Frimla J. 2011.  
679 Clathrin mediates endocytosis and polar distribution of PIN auxin transporters in  
680 *Arabidopsis*. *Plant Cell* **23**: 1920-1931. DOI:  
681 <https://doi.org/10.1105/tpc.111.083030>, PMID: 21551390
- 682 Li N, Li Y. 2016. Signaling pathways of seed size control in plants. *Current Opinion*



- 683        *in Plant Biology* **33**:23-32. DOI: <https://doi.org/10.1016/j.pbi.2016.05.008>,  
684        PMID: 27294659
- 685    Li WX, Oono Y, Zhu J, He XJ, Wu JM, Iida K, Lu XY, Cui X, Jin H, Zhu JK. 2008.  
686        The *Arabidopsis NFYA5* transcription factor is regulated transcriptionally and  
687        posttranscriptionally to promote drought resistance. *Plant Cell* **20**:2238-2251.  
688        DOI: <https://doi.org/10.1105/tpc.108.059444>, PMID: 18682547
- 689    Lin SX, Grant B, Hirsh D, Maxfield FR. 2001. Rme-1 regulates the distribution and  
690        function of the endocytic recycling compartment in mammalian cells. *Nature*  
691        *Cell Biology* **3**: 567-572. DOI: <https://doi.org/10.1038/35078543>, PMID:  
692        11389441
- 693    Liu L, Tong H, Xiao Y, Che R, Xu F, Hu B, Liang C, Chu J, Li J, Chu C. 2015.  
694        Activation of Big Grain1 significantly improves grain size by regulating auxin  
695        transport in rice. *PNAS* **112**: 11102-11107. DOI:  
696        <https://doi.org/10.1073/pnas.1512748112>, PMID: 26283354
- 697    McLaren JS. 2005. Crop biotechnology provides an opportunity to develop a  
698        sustainable future. *Trends in Biotechnology* **23**: 339-342. DOI:  
699        <https://doi.org/10.1016/j.tibtech.2005.04.004>, PMID: 15978317
- 700    McMahon HT, Boucrot E. 2011. Molecular mechanism and physiological functions of  
701        clathrin-mediated endocytosis. *Nature Reviews Molecular Cell Biology* **12**:  
702        517-533. DOI: <https://doi.org/10.1038/nrm3151>, PMID: 21779028
- 703    Michelmore RW, Paran I, Kesseli V. 1991. Identification of markers linked to disease  
704        resistance genes by bulked segregant analysis: a rapid method to detect markers  
705        in specific genomic region is by using segregating population. *PNAS* **88**:  
706        9828-9832. DOI: <https://doi.org/10.1073/pnas.88.21.9828>, PMID: 1682921
- 707    Murphy AS, Bandyopadhyay A, Holstein SE, Peer WA. 2005. Endocytic cycling of  
708        PM proteins. *Annual Review of Plant Biology* **56**: 221-251. DOI:  
709        <https://doi.org/10.1146/annurev.arplant.56.032604.144150>, PMID: 15862095
- 710    Robatzke S, Chinchilla D, Boller T. 2006. Ligand-induced endocytosis of the pattern  
711        recognition receptor FLS2 in *Arabidopsis*. *Genes & Development* **20**: 537-542.  
712        DOI: <https://doi.org/10.1101/gad.366506>, PMID: 16510871



- 713 Scanlon MJ, Takacs EM. 2009. Kernel biology. In J Bennetzen, S Hake, eds,  
714 Handbook of Maize, Its Biology. Springer, New York, pp 121-129.
- 715 Schruff MC, Spielman M, Tiwari S, Adams S, Fenby N, Scott RJ. 2006. The AUXIN  
716 RESPONSE FACTOR 2 gene of Arabidopsis links auxin signalling, cell division,  
717 and the size of seeds and other organs. *Development* **133**: 251-261. DOI:  
718 <https://doi.org/10.1242/dev.02194>, PMID: 16339187
- 719 Sekhon RS, Lin H, Childs KL, Hansey CN, Buell CR, de Leon N, Kaeppler SM. 2011.  
720 Genome-wide atlas of transcription during maize development. *Plant J* **66**:  
721 553-563. DOI: <https://doi.org/10.1111/j.1365-313X.2011.04527.x>, PMID:  
722 21299659
- 723 Sharma M, Naslavsky N, Caplan S. 2008. A role for EHD4 in the regulation of early  
724 endosomal transport. *Traffic* **9**: 995-1018. DOI:  
725 <https://doi.org/10.1111/j.1600-0854.2008.00732.x>, PMID: 18331452
- 726 Sigismund S, Confalonieri S, Ciliberto A, Polo S, Scita G, Di Fiore PP. 2012.  
727 Endocytosis and signaling: cell logistics shape the eukaryotic cell plan.  
728 *Physiological Reviews* **92**: 273-366. DOI:  
729 <https://doi.org/10.1152/physrev.00005.2011>, PMID: 22298658
- 730 Takano J, Tanaka M, Toyoda A, Miwa K, Kasai K, Fuji K, Onouchi H, Naito S,  
731 Fujiwarab T. 2010. Polar localization and degradation of *Arabidopsis* boron  
732 transporters through distinct trafficking pathways. *PNAS* **107**: 5220-5225. DOI:  
733 <https://doi.org/10.1073/pnas.0910744107>, PMID: 20194745
- 734 Van Damme D, Gadeyne A, Vanstraelen M, Inze D, VanMontagu MC, De Jaeger G,  
735 Russinova E, Geelen D. 2011. Adaptin-like protein TPLATE and clathrin  
736 recruitment during plant somatic cytokinesis occurs via two distinct pathways.  
737 *PNAS* **108**: 615-620. DOI: <https://doi.org/10.1073/pnas.1017890108>, PMID:  
738 21187379
- 739 Wagner GP, Kin K, Lynch VJ. 2012. Measurement of mRNA abundance using  
740 RNA-Seq data: RPKM measure is inconsistent among samples. *Theory Biosci*  
741 **131**: 281-285. DOI: <https://doi.org/10.1007/s12064-012-0162-3> PMID:  
742 22872506

- 743 Wang B, Chu J, Yu T, Xu Q, Sun X, Yuan J, Xiong G, Wang G, Wang Y, Li J. 2015.  
744 Tryptophan-independent auxin biosynthesis contributes to early embryogenesis  
745 in *Arabidopsis*. *PNAS* **112**: 4821-4826. DOI:  
746 <https://doi.org/10.1073/pnas.1503998112>, PMID: 25831515
- 747 Wang C, Yan X, Chen Q, Jiang N, Fu W, Ma B, Liu J, Li C, Bednarek SY, Pan J. 2013.  
748 Clathrin light chains regulate clathrin-mediated trafficking, auxin signaling, and  
749 development in *Arabidopsis*. *Plant Cell* **25**: 499-516. DOI:  
750 <https://doi.org/10.1105/tpc.112.108373>, PMID: 23424247
- 751 Wang L, Feng Z, Wang X, Wang X, Zhang X. 2010. DEGseq: an R package for  
752 identifying differentially expressed genes from RNA-seq data. *Bioinformatics* **26**:  
753 136-138. DOI: <https://doi.org/10.1093/bioinformatics/btp612>, PMID:19855105
- 754 Waters AJ, Makarevitch I, Eichten SR, Swanson-Wagner RA, Yeh CT, Xu W,  
755 Schnable PS, Vaughn MW, Gehring M, Springer NM. 2011. Parent-of-origin  
756 effects on gene expression and DNA methylation in the maize endosperm. *Plant*  
757 *Cell* **23**: 4221-4233. DOI: <https://doi.org/10.1105/tpc.111.092668>, PMID:  
758 22198147
- 759 Xing HL, Dong L, Wang ZP, Zhang HY, Han CY, Liu B, Wang XC, Chen QJ. 2014. A  
760 CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology*  
761 **14**: 327. DOI: <https://doi.org/10.1186/s12870-014-0327-y>, PMID: 25432517
- 762 Zuo W, Chao Q, Zhang N, Ye J, Tan G, Li B, Xing Y, Zhang B, Liu H, Fengler KA,  
763 Zhao J, Zhao X, Chen Y, Lai J, Yan J, Xu M. 2015. A maize wall-associated  
764 kinase confers quantitative resistance to head smut. *Nature Genetics* **47**: 151-157.  
765 DOI: <https://doi.org/10.1038/ng.3170>, PMID: 25531751

766  
767  
768  
769  
770  
771  
772

773 **Table 1** Differentially expressed genes involved in auxin-related processes in the *ehd1*  
774 mutant.

Gene ID	Annotation <sup>1</sup>	Log <sub>2</sub> Fold-change ( <i>ehd1</i> vs. WT)	Adjusted P value
GRMZM2G126260	PIN10a	-5.53	0.0007
GRMZM2G149481	Auxin transporter-like protein 3	3.40	0.0023
GRMZM2G127949	Auxin transporter-like protein 1	1.94	2.10e <sup>-16</sup>
GRMZM2G019799	Indole-3-acetaldehyde oxidase-like	1.39	0.0006
GRMZM2G082943	ZAR9	-2.46	2.04e <sup>-12</sup>
GRMZM2G065244	Auxin-induced in root cultures protein 12-like	1.01	0.0158
GRMZM2G079957	AUX17	-1.17	0.0061
GRMZM2G130953	AUX22	1.80	0.0004
GRMZM2G081158	Auxin response factor 25-like	1.36	2.61e <sup>-6</sup>
GRMZM2G475263	Auxin response factor 1	1.15	7.27e <sup>-11</sup>
GRMZM2G028980	Auxin response factor 6 isoform X1	1.53	1.62e <sup>-12</sup>
GRMZM5G808366	Auxin response factor 5	1.99	0.0038
GRMZM2G137413	Auxin response factor 14	2.06	5.04e <sup>-41</sup>
GRMZM2G064371	Auxin binding protein-like protein	1.38	0.0001
GRMZM2G365188	SAUR23	-3.37	0.0004
GRMZM2G414727	SAUR56	-2.95	1.12e <sup>-26</sup>
GRMZM2G059138	SAUR33	-2.01	7.63e <sup>-8</sup>
GRMZM2G121309	IAA7	1.16	5.26e <sup>-8</sup>
GRMZM2G030465	IAA9	3.19	0.0146
GRMZM2G077356	IAA13	2.90	9.83e <sup>-18</sup>
GRMZM5G809195	IAA14	-2.12	0.0046
GRMZM2G115357	IAA24	-1.25	8.09e <sup>-7</sup>
GRMZM2G048131	IAA26	inf	0.0002

775 <sup>1</sup>Annotation is based on maize IDs

776 **Figure Legends**

777 **Figure 1. Phenotypes of the maize *ehd1* mutant kernels.** (A) F<sub>2</sub> ears of *ehd1* ×  
778 Xun9058 at 14 or 16 days after pollination (DAP). The red arrows indicated the *ehd1*  
779 kernels. Scale bars = 1 cm. (B) The mature kernels of wild type (WT) and *ehd1*  
780 mutant randomly selected from F<sub>2</sub> ears of *ehd1* × Xun9058. Scale bars = 0.5 cm. (C)  
781 100-grain weight of the WT and the *ehd1* mutant. Values are means and standard  
782 errors (n=4). \*\* indicates a significant difference (P < 0.01) between the WT and the  
783 *ehd1* mutant. (D) Comparison of WT and *ehd1* embryos at 20 DAP. The sections  
784 stained with Safranin and Fast Green. Scale bars = 5 mm. (E) Microstructure of WT  
785 and *ehd1* endosperms at 15 DAP. The sections stained with fuchsin. Scale bars = 5  
786 mm.

787

788 **Figure 2. *ZmEHD1* is required for normal growth and development of maize.** (A)  
789 Germination of the WT and the *ehd1* mutant. Values are means and standard errors of  
790 approximately 100 seeds from three independent experiments. (B) Root number and  
791 root elongation of the WT and the *ehd1* mutant. Values are means and standard errors  
792 (n=5). \*\* indicates a significant difference (P < 0.01) between the WT and the *ehd1*  
793 mutant. (C) Phenotypes of WT and *ehd1* seedlings (n=30). Representative photograph  
794 is shown.

795

796 **Figure 3. Map-based cloning of *ZmEHD1*.** (A) Schematic representation of the  
797 positional cloning of *ZmEHD1* gene on chromosome 4. The SSR markers, umc1716  
798 and umc1650, were used for rough mapping. Recombinants are indicated in  
799 parentheses below each SSR marker. (B) Gene structure of *ZmEHD1*. Black boxes  
800 indicate exons, and lines between black boxes represent introns. The positions of  
801 mutations were marked. The mutations that lead to the amino acid change between the  
802 *ehd1* mutant and the WT are indicated with red letters. (C) Schematic representation  
803 of the predicted structure of *ZmEHD1*. The regions encoding the potential protein  
804 domains are shown. The positions of mutations on coil-coil domain were marked by  
805 asterisks. (D) Real-time RT-PCR detection of *ZmEHD1* gene transcripts in

806 endosperms of the WT maize and the *ehd1* mutant at 15, 20, 25, and 30 DAP.  
807 Quantifications were normalized to the expression of 18S rRNA. Values are means  
808 and standard errors (n=3). \*\* indicates a significant difference ( $P < 0.01$ ) between the  
809 WT and the *ehd1* mutant.

810

811 **Figure 4. Transgenic validation of *ZmEHD1*.** (A) Germination of inbred line  
812 ZZC01 (WT) and *ZmEHD1* knock-out mutants (KO). Values are means and standard  
813 errors of approximately 100 seeds from three independent experiments. An LSD test  
814 was used to assess differences between WT and *ZmEHD1* knock-out mutant. \*\*,  $P <$   
815 0.01 (t-test), significant difference between WT and KO mutants. (B) Phenotypes of  
816 WT and KO mutants (n=20). Representative photograph is shown. (C) Heterozygous  
817 *KO(+/-) × ehd1(+/-)* were used in an allelism test. The red arrows indicated the *ehd1*  
818 kernels. (D) The mature kernels of WT and *KO/ehd1* mutant randomly selected from  
819 ears of *KO(+/-) × ehd1(+/-)*. Scale bars = 0.5 cm. (E) Germination of WT and  
820 *KO/ehd1* mutant. Values are means and standard errors of approximately 140 seeds  
821 from three independent experiments. An LSD test was used to assess differences  
822 between WT and *KO/ehd1* mutant. \*\*,  $P < 0.01$  (t-test), significant difference between  
823 WT and *KO/ehd1* mutant.

824

825 **Figure 5. Endocytosis of FM4-64 is reduced in the *ehd1* and in *ZmEHD1***  
826 **knock-out mutants.** Three dimensional reconstructions of z-stacks (60  $\mu$ M with  
827 2.4- $\mu$ M steps) were obtained in the *ehd1* (A) and in *ZmEHD1* knock-out mutant (B).  
828 FM-64 labeled BFA bodies in the *ehd1* mutant (C) and in *ZmEHD1* knock-out  
829 mutants (D). Representative photographs at indicated durations are shown. Scale bars  
830 = 10  $\mu$ m. Numbers below the photographs indicate the rate of FM4-64-labelled  
831 fluorescent puncta (% and number of FM4-64-labelled fluorescent puncta/total cell  
832 number). \*\* indicates a significant difference from the WT at  $P < 0.01$  according to a  
833 *t*-test.

834

835 **Figure 6. *ZmEHD1* directly interacts with the *ZmAP2*  $\sigma$  subunit in plants and**

836 **yeast.** (A) Interaction between ZmEHD1 and the ZmAP2  $\sigma$  subunit in the leaves of *N.*  
837 *benthamiana* as observed by BiFC. The photographs were taken in the dark field for  
838 yellow fluorescence. Propidium iodide (PI) was used to determine the vitality of the  
839 cells. Representative photographs are shown. Scale bars = 10  $\mu$ m. (B) Interaction  
840 between ZmEHD1 and the ZmAP2  $\sigma$  subunit as indicated by split-ubiquitin yeast  
841 two-hybrid assays. The ZmAP2  $\sigma$  subunit was used as the fused bait protein (AP2  
842  $\sigma$ -Cub), and ZmEHD1 was used as the fused prey protein (NubG-ZmEHD1). The  
843 presence or absence of His or X-Gal is indicated. (C) Pull-down assays for the  
844 interaction between ZmEHD1 and the ZmAP2  $\sigma$  subunit. The ZmEHD1/ ZmEHD1<sub>mut</sub>  
845 in the pull-downed fraction was detected by immunoblot using anti-His antibody.

846

847 **Figure 7. Auxin distribution and ZmPIN1a-YFP localization were altered in the**  
848 ***ehd1* mutant.** (A) The response of horizontally placed mesocotyl-coleoptiles to  
849 gravity is delayed in *ehd1* mutant (n=15). (B) Free IAA contents in WT maize and  
850 *ehd1* mutant kernels at 15 DAP. Error bars represent standard errors (n=4). \*\* indicates  
851 a significant difference from the WT at P < 0.01 according to a *t*-test. (C) The  
852 fluorescence of ZmDR5::mRFP in WT and *ehd1* mutant. Scale bars = 50  $\mu$ m. (D) The  
853 fluorescence of ZmPIN1a::YFP in WT and *ehd1* mutant. Scale bars = 50  $\mu$ m. cc,  
854 central cylinder; me, root meristem; rc, root cap; co, cortex.

855

856 **Figure 8. Rescue of the *ehd1* phenotype by exogenous 1-NAA.** (A) Germination of  
857 the *ehd1* mutant ZmEHD1 knock-out mutants (KO) after treatment with water alone  
858 or with different concentrations of 1-NAA. Values are means and standard errors of  
859 approximately 100 seeds from three independent experiments. An LSD test was used  
860 to assess differences between treatments. Means with the same letter are not  
861 significantly different at P < 0.01. (B) Phenotypes of *ehd1* and KO seedlings treated  
862 with different concentrations of 1-NAA.

863

864 **Supplemental Table 1. The ear performance of F<sub>2</sub> and F<sub>3</sub> individuals evaluated in**  
865 **the fields.**

866 **Supplemental Table 2. The kernel performance of F<sub>2</sub> population evaluated in the**  
867 **fields.**

868

869 **Supplemental Table 3. Oligos used as primers in the experiment.**

870

871 **Supplemental Table 4. Allelism test between *KO*<sup>+/-</sup> and *ehd1*<sup>+/-</sup>**

872

873 **Supplemental Table 5. List of genes with significant expression changes.**

874

875 **Supplemental Figure 1. The alignment with AtEHD1, AtEHD2, ZmEHD1 and**  
876 **ZmEHD<sub>mut</sub>. The motifs are underlined.**

877

878 **Supplemental Figure 2. Tissue pattern of *ZmEHD1* transcript accumulation.**

879

880 **Supplemental Figure 3. The observed target deletion of *ZmEHD1* in *ZmEHD1***  
881 **loss-of-function mutants.**

882

883 **Supplemental Figure 4. The different subcellular localization of ZmEHD1**  
884 **protein (A) and ZmEHD1<sub>mut</sub> protein (B). Representative photographs are shown.**

885

886 **Supplemental Figure 5. Rescue of *Arabidopsis ap2*  $\sigma$  mutant by the *ZmAP2*  $\sigma$**   
887 **cDNA. (A) Identification of *ap2*  $\sigma$  T-DNA insertion mutant. (B) Semi-quantitative**  
888 **RT-PCR analysis of *ZmAP2*  $\sigma$  transcript levels in the Col, *ap2*  $\sigma$  mutant and**  
889 ***35S::ZmAP2*  $\sigma$  transgenic plants. (C) Three week-old and five week-old of WT (left),**  
890 ***ap2*  $\sigma$  mutant (middle) and *35S::ZmAP2*  $\sigma$  transgenic plants.**

891

892 **Supplemental Figure 6. Interaction between ZmEHD1 and the ZmAP2  $\sigma$  subunit**  
893 **in maize mesophyll protoplasts observed by BiFC. The photographs were taken in**  
894 **the dark field for yellow fluorescence. Representative photographs are shown. Scale**  
895 **bars = 10  $\mu$ m.**

896

897 **Supplemental Figure 7. The different subcellular localization of ZmAP2  $\sigma$**   
898 **subunit in WT maize and *ehd1* mutant.** Representative photographs are shown.  
899 Scale bars = 10  $\mu$ m.

900

901 **Supplemental Figure 8. Pearson's correlation coefficients (*R* value) of biological**  
902 **replicates in the WT maize and the *ehd1* mutant.**

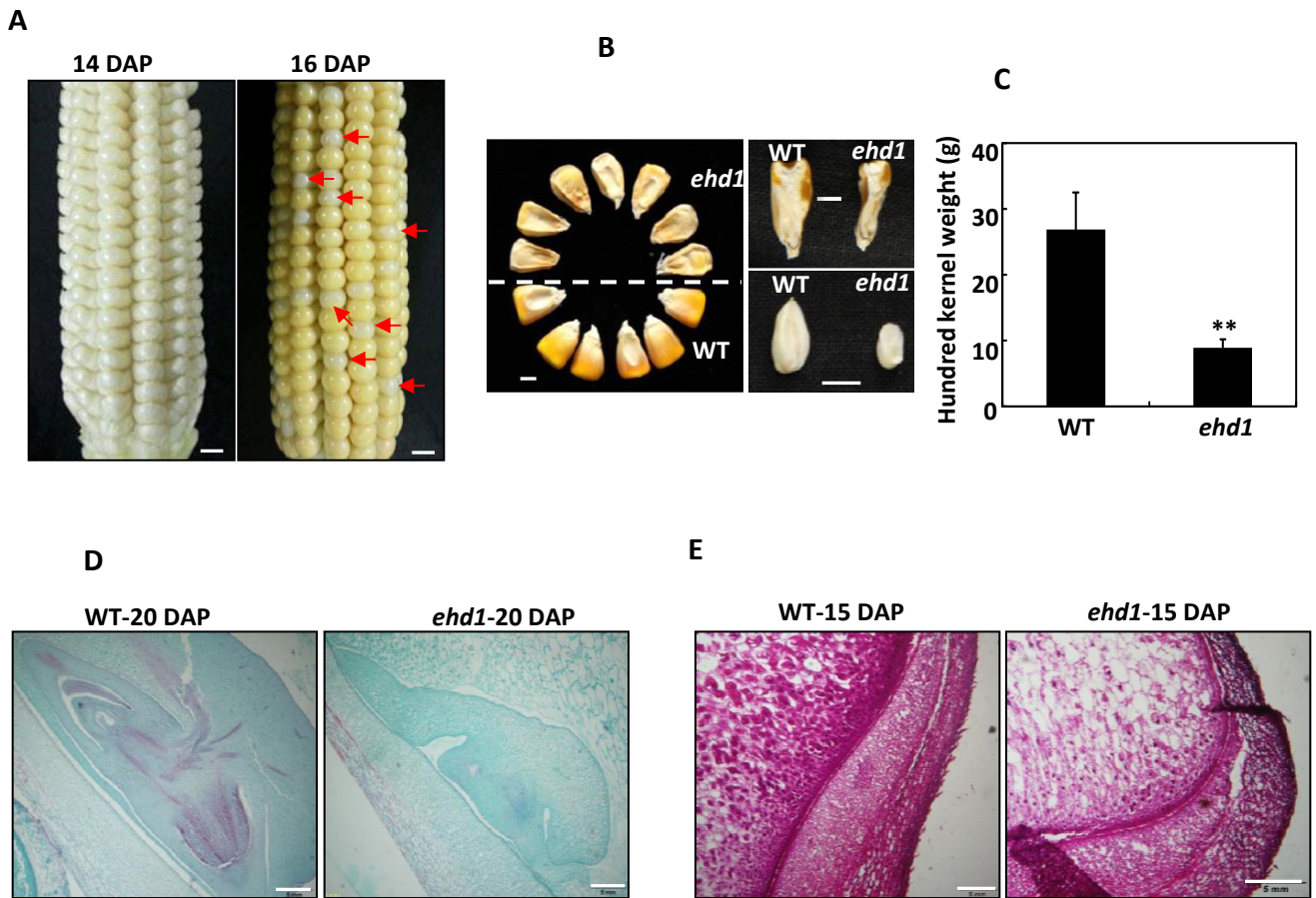
903

904 **Supplemental Figure 9. Validation of RNA-Seq by real-time RT-PCR.** (A) Genes  
905 listed in Supplemental Table 5. (B) Genes listed in Table 1.

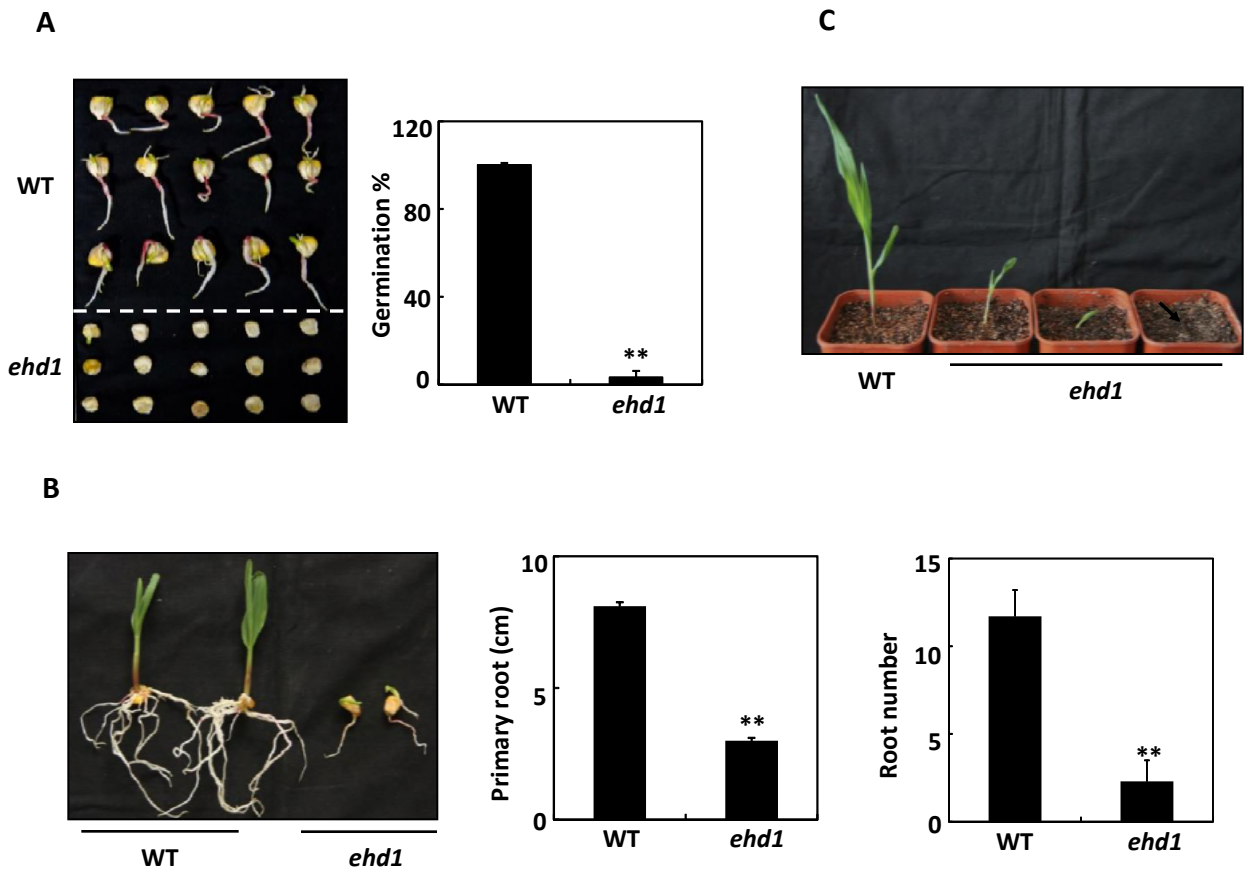
906

907 **Supplemental Figure 10. Germination of the *ehd1* mutant (A) and *ZmEHD1***  
908 **knock-out mutant (B) treated with water alone or with different concentrations**  
909 **of GA3.** Values are means and standard errors of approximately 150 seeds from three  
910 independent experiments.

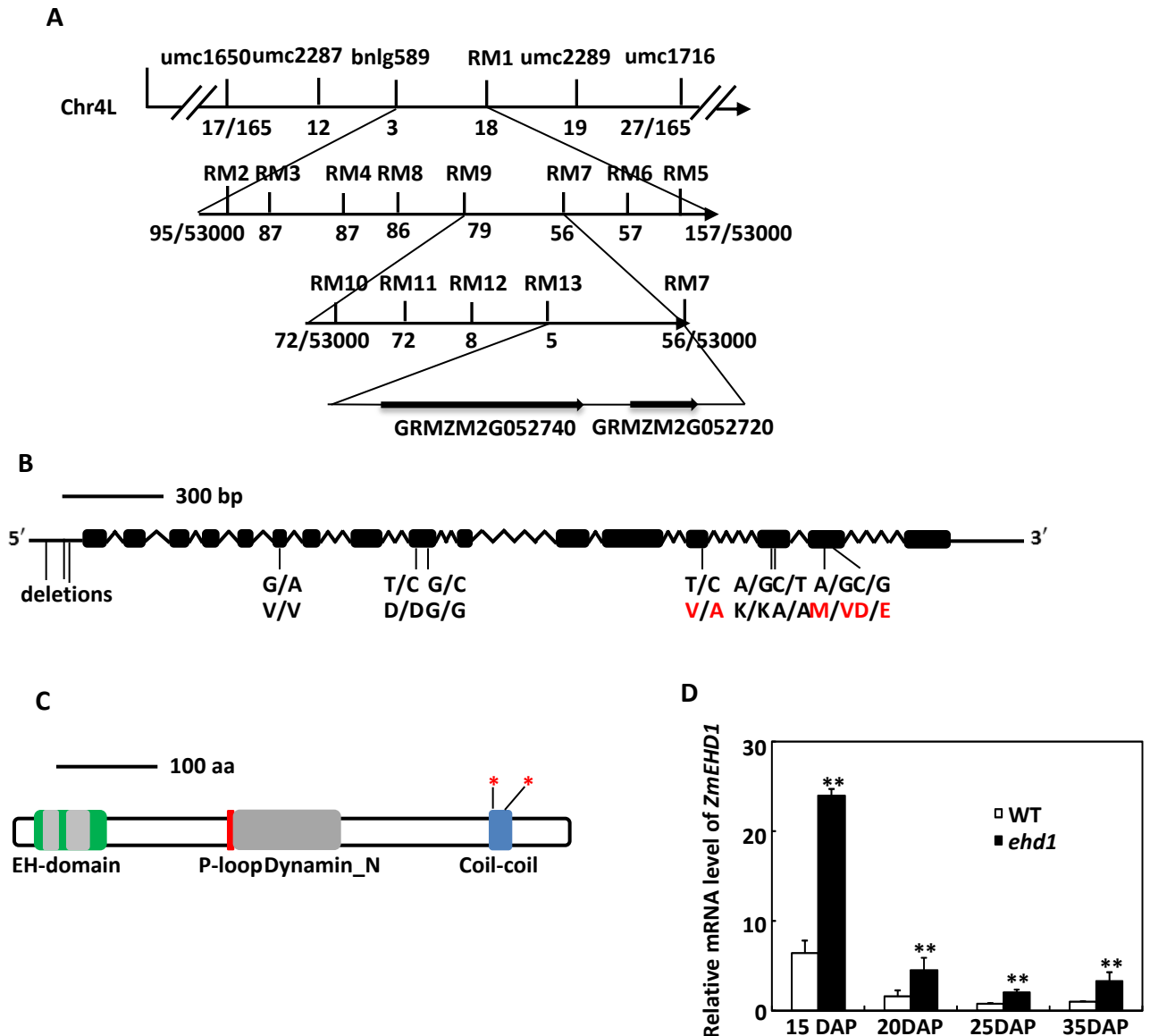




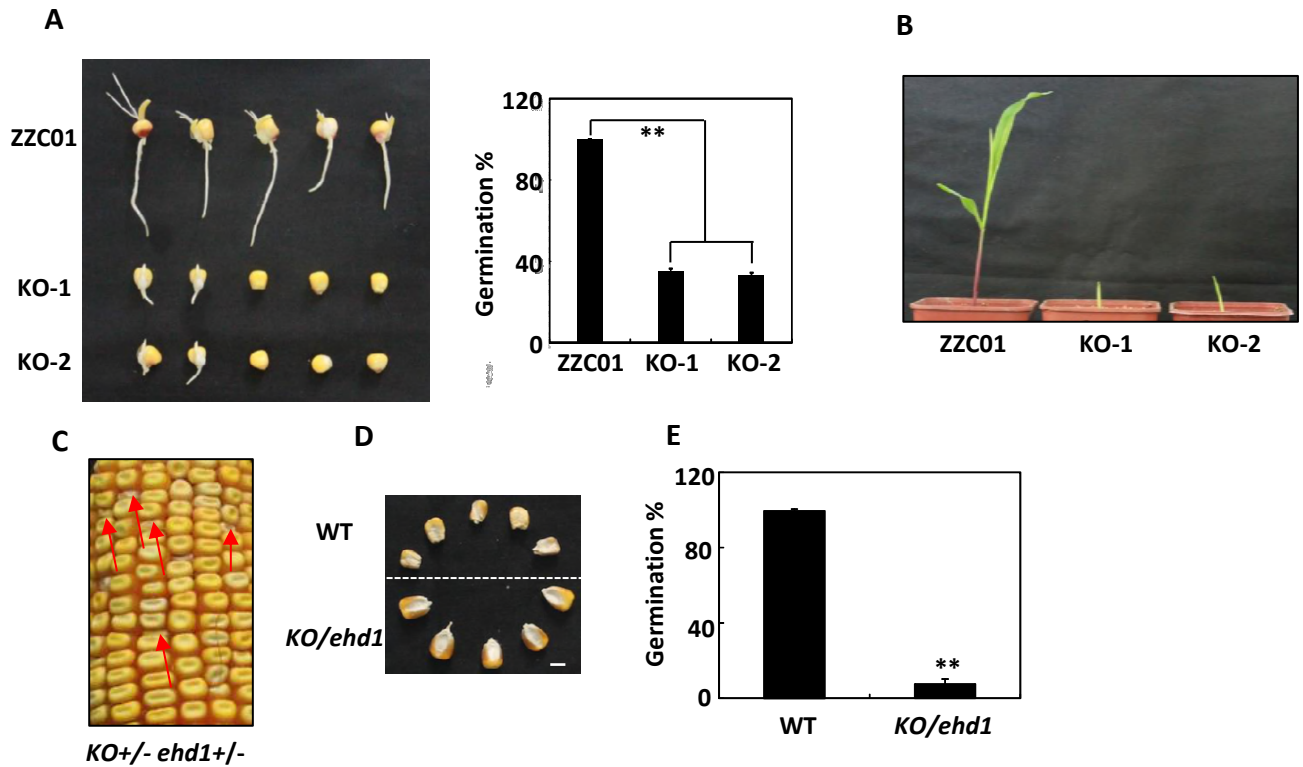
**Figure 1. Phenotypes of the maize *ehd1* mutant kernels.** (A) F<sub>2</sub> ears of *ehd1* × Xun9058 at 14 or 16 days after pollination (DAP). The red arrows indicated the *ehd1* kernels. Scale bars = 1 cm. (B) The mature kernels of wild type (WT) and *ehd1* mutant randomly selected from F<sub>2</sub> ears of *ehd1* × Xun9058. Scale bars = 0.5 cm. (C) 100-grain weight of the WT and the *ehd1* mutant. Values are means and standard errors (n=4). \*\* indicates a significant difference (P < 0.01) between the WT and the *ehd1* mutant. (D) Comparison of WT and *ehd1* embryos at 20 DAP. The sections stained with Safranin and Fast Green. Scale bars = 5 mm. (E) Microstructure of WT and *ehd1* endosperms at 15 DAP. The sections stained with fuchsin. Scale bars = 5 mm.



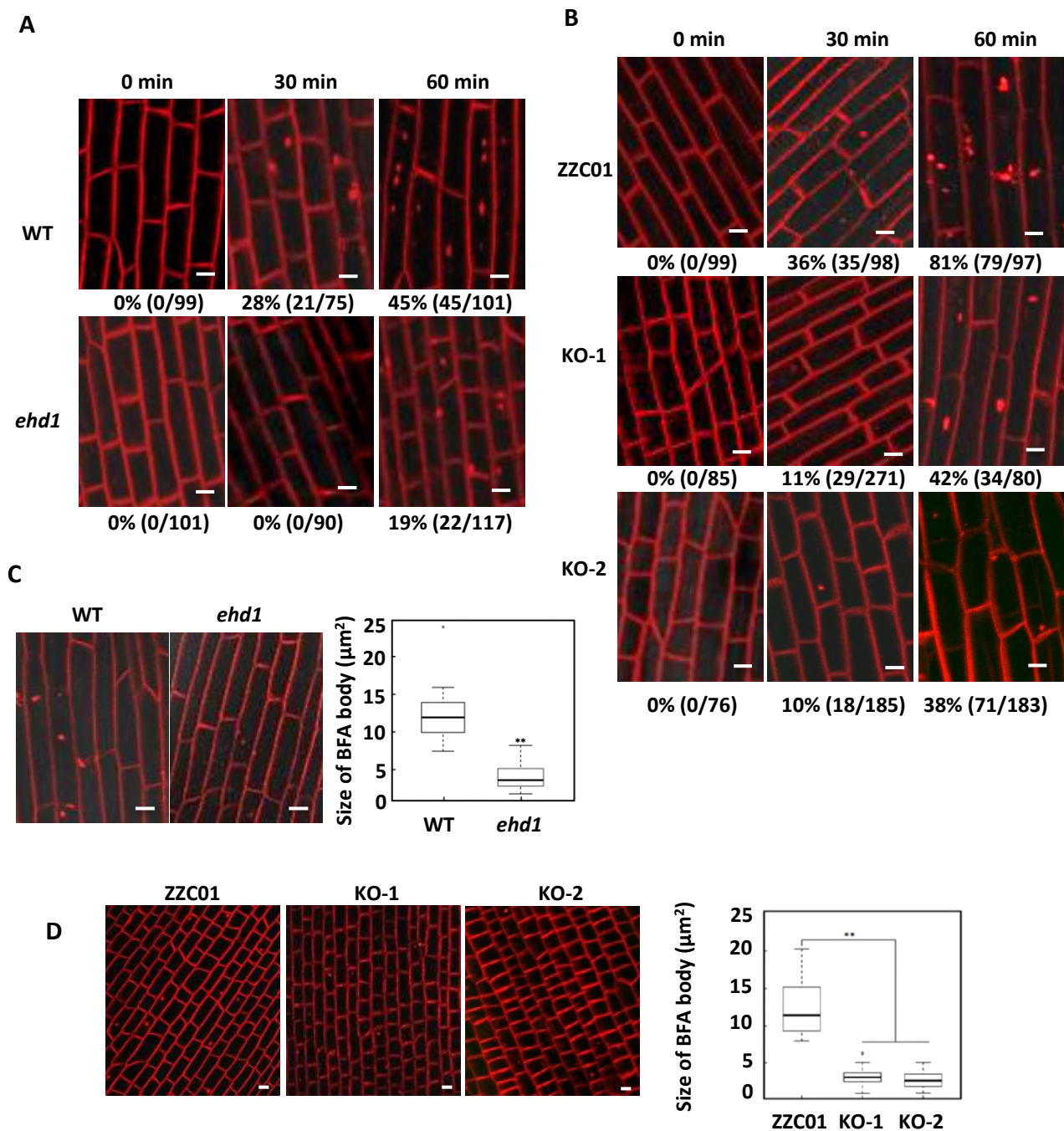
**Fig. 2 *ZmEHD1* is required for normal growth and development of maize.** (A) Germination of the WT and the *ehd1* mutant. Values are means and standard errors of approximately 100 seeds from three independent experiments. (B) Root number and root elongation of the WT and the *ehd1* mutant. Values are means and standard errors ( $n=5$ ). \*\* indicates a significant difference ( $P < 0.01$ ) between the WT and the *ehd1* mutant. (C) Phenotypes of WT and *ehd1* seedlings ( $n=30$ ). Representative photograph is shown.



**Fig. 3 Map-based cloning of *ZmEHD1*.** (A) Schematic representation of the positional cloning of *ZmEHD1* gene on chromosome 4. The SSR markers, umc1716 and umc1650, were used for rough mapping. Recombinants are indicated in parentheses below each SSR marker. (B) Gene structure of *ZmEHD1*. Black boxes indicate exons, and lines between black boxes represent introns. The positions of mutations were marked. The mutations that lead to the amino acid change between the *ehd1* mutant and the WT are indicated with red letters. (C) Schematic representation of the predicted structure of *ZmEHD1*. The regions encoding the potential protein domains are shown. The positions of mutations on coil-coil domain were marked by asterisks. (D) Real-time RT-PCR detection of *ZmEHD1* gene transcripts in endosperms of the WT maize and the *ehd1* mutant at 15, 20, 25, and 30 DAP. Quantifications were normalized to the expression of 18S rRNA. Values are means and standard errors (n=3). \*\* indicates a significant difference (P < 0.01) between the WT and the *ehd1* mutant.



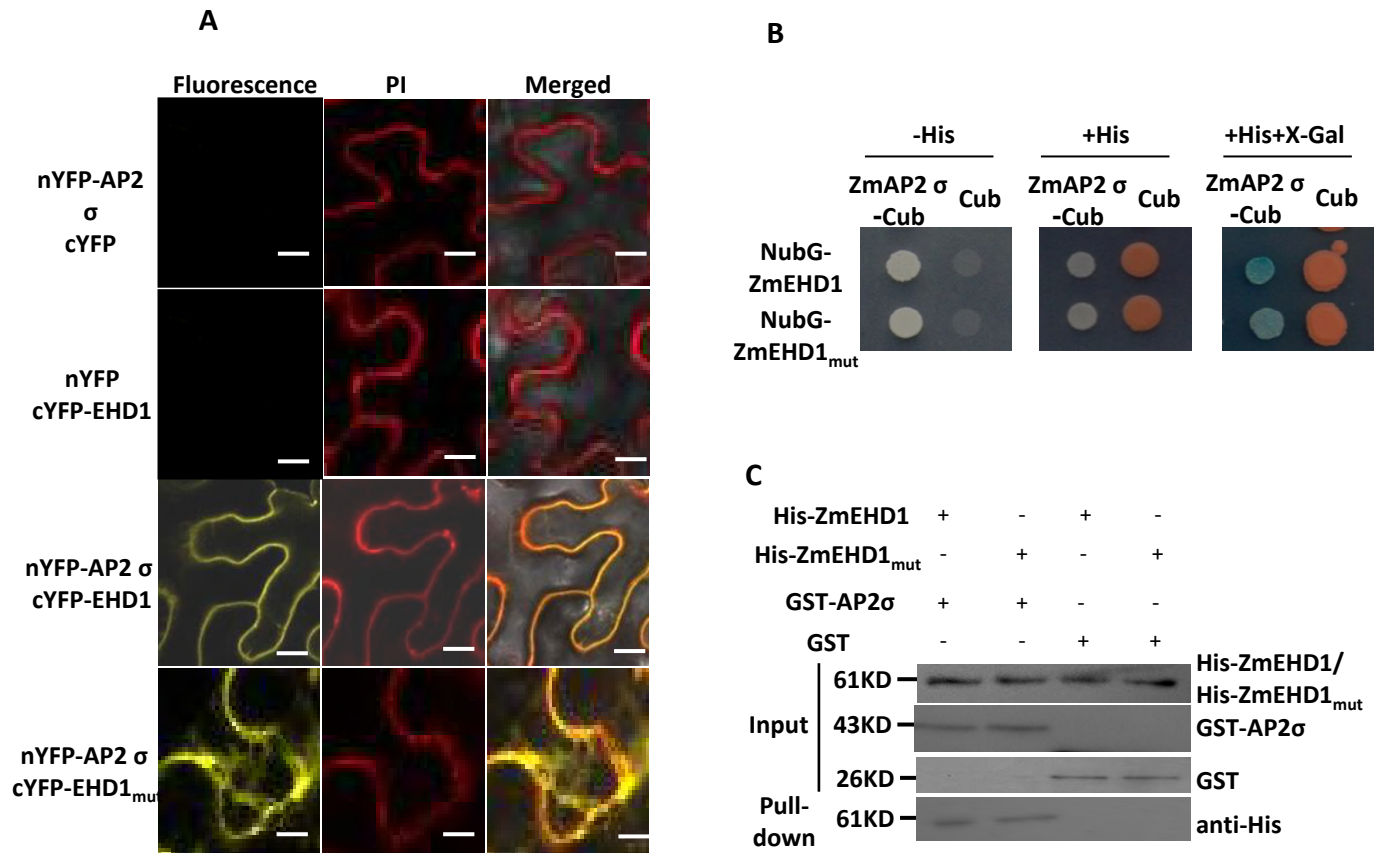
**Fig. 4 Transgenic validation of *ZmEHD1*.** (A) Germination of inbred line ZCC01 (WT) and *ZmEHD1* knock-out mutants (KO). Values are means and standard errors of approximately 100 seeds from three independent experiments. An LSD test was used to assess differences between WT and *ZmEHD1* knock-out mutant. \*\*,  $P < 0.01$  (t-test), significant difference between WT and KO mutants. (B) Phenotypes of WT and KO mutants ( $n=20$ ). Representative photograph is shown. (C) Heterozygous *KO(+/-) × ehd1(+/-)* were used in an allelism test. The red arrows indicated the *ehd1* kernels. (D) The mature kernels of WT and *KO/ehd1* mutant randomly selected from ears of *KO(+/-) × ehd1(+/-)*. Scale bars = 0.5 cm. (E) Germination of WT and *KO/ehd1* mutant. Values are means and standard errors of approximately 140 seeds from three independent experiments. An LSD test was used to assess differences between WT and *KO/ehd1* mutant. \*\*,  $P < 0.01$  (t-test), significant difference between WT and *KO/ehd1* mutant.



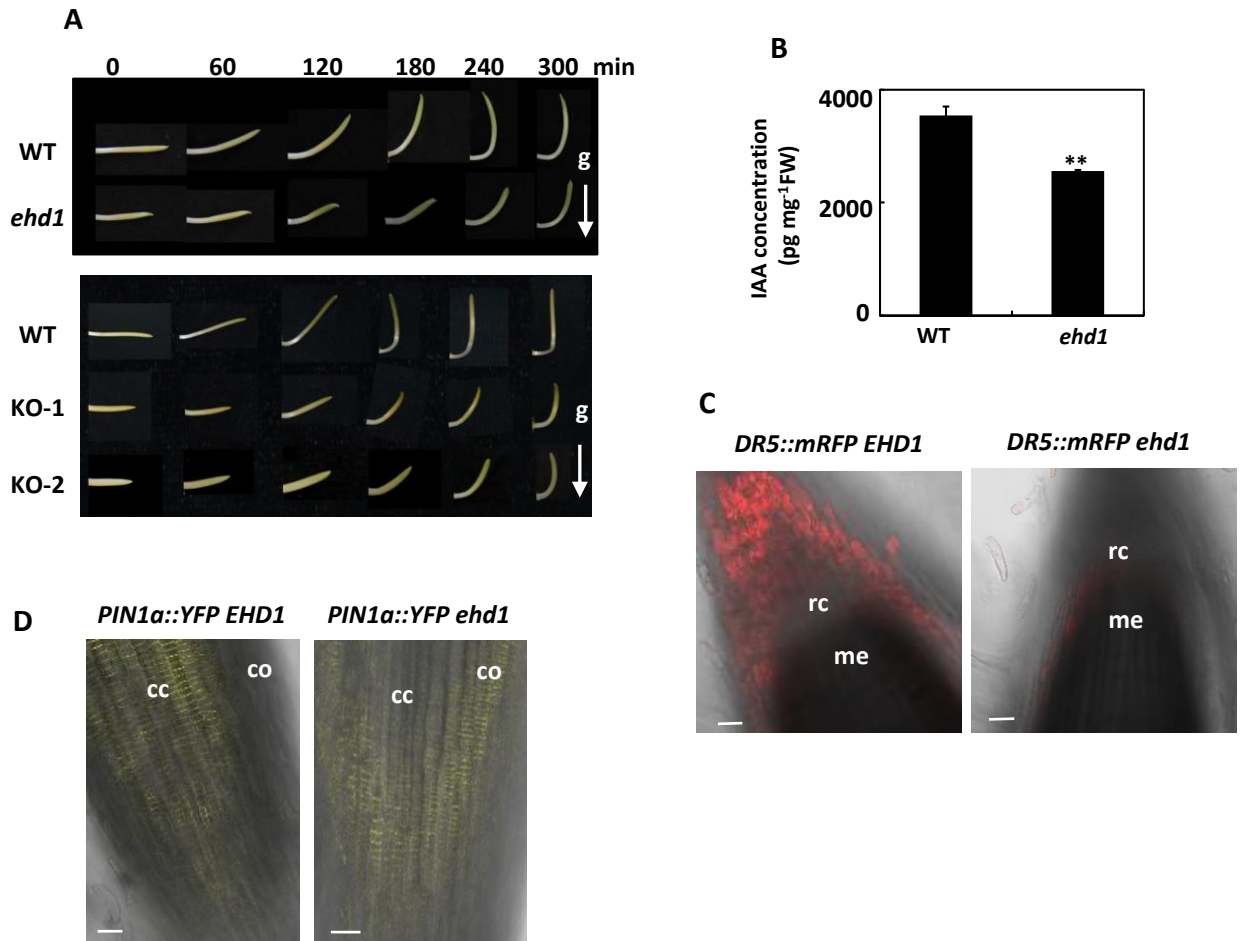
**Fig. 5 Endocytosis of FM4-64 is reduced in the *ehd1* and in *ZmEHD1* knock-out mutants.**

Three dimensional reconstructions of z-stacks (60  $\mu\text{M}$  with 2.4- $\mu\text{M}$  steps) were obtained in the *ehd1* (A) and in *ZmEHD1* knock-out mutant (B). FM-64 labeled BFA bodies in the *ehd1* mutant (C) and in *ZmEHD1* knock-out mutants (D). Representative photographs at indicated durations are shown. Scale bars = 10  $\mu\text{m}$ . Numbers below the photographs indicate the rate of FM4-64-labelled fluorescent puncta (% and number of FM4-64-labelled fluorescent puncta/total cell number). \*\* indicates a significant difference from the WT at  $P < 0.01$  according to a *t*-test.

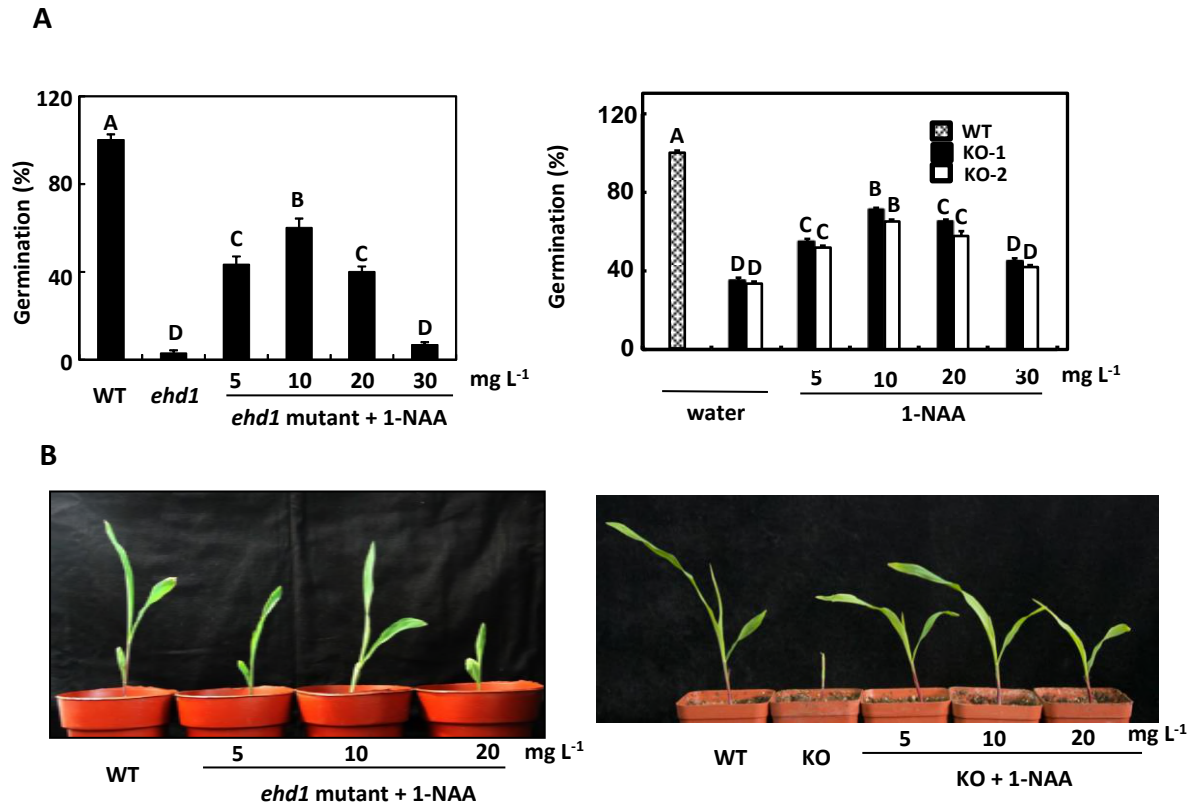




**Figure 6. ZmEHD1 directly interacts with the ZmAP2  $\sigma$  subunit in plants and yeast. (A)** Interaction between ZmEHD1 and the ZmAP2  $\sigma$  subunit in the leaves of *N. benthamiana* observed by BiFC. The photographs were taken in the dark field for yellow fluorescence. Propidium iodide (PI) was used to determine the vitality of the cells. Representative photographs are shown. Scale bars = 10  $\mu$ m. **(B)** Interaction between ZmEHD1 and the ZmAP2  $\sigma$  subunit as indicated by split-ubiquitin yeast two-hybrid assays. The ZmAP2  $\sigma$  subunit was used as the fused bait protein (AP2  $\sigma$ -Cub), and ZmEHD1 was used as the fused prey protein (NubG-ZmEHD1). The presence or absence of His or X-Gal is indicated. **(C)** Pull-down assays for the interaction between ZmEHD1 and the ZmAP2  $\sigma$  subunit. The ZmEHD1/ ZmEHD1<sub>mut</sub> in the pull-downed fraction was detected by immunoblot using anti-His antibody.



**Figure 7. Auxin distribution and ZmPIN1a-YFP localization were altered in the *ehd1* mutant.** (A) The response of horizontally placed mesocotyl-coleoptiles to gravity is delayed in *ehd1* mutant (n=15). (B) Free IAA contents in WT maize and *ehd1* mutant kernels at 15 DAP. Error bars represent standard errors (n=4). \*\* indicates a significant difference from the WT at  $P < 0.01$  according to a *t*-test. (C) The fluorescence of ZmDR5::mRFP in WT and *ehd1* mutant. Scale bars = 50  $\mu$ m. (D) The fluorescence of ZmPIN1a::YFP in WT and *ehd1* mutant. Scale bars = 50  $\mu$ m. cc, central cylinder; me, root meristem; rc, root cap; co, cortex.



**Figure 8. Rescue of the *ehd1* phenotype by exogenous 1-NAA.** (A) Germination of the *ehd1* mutant *ZmEHD1* knock-out mutants (KO) after treatment with water alone or with different concentrations of 1-NAA. Values are means and standard errors of approximately 100 seeds from three independent experiments. An LSD test was used to assess differences between treatments. Means with the same letter are not significantly different at  $P < 0.01$ . (B) Phenotypes of *ehd1* and KO seedlings treated with different concentrations of 1-NAA.