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

Yafei Wang^{1,2}, Wenwen Liu², Hongqiu Wang^{1,2}, Qingguo Du², Zhiyuan Fu¹, Wen-Xue Li^{2,*} and Jihua Tang^{1,*}

¹National Key Laboratory of Wheat and Maize Crop Science, Collaborative Innovation Center of Henan Grain Crops, College of Agronomy, Henan Agricultural University, Zhengzhou, 450002, China;

²National Engineering Laboratory for Crop Molecular Breeding, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China

*To whom correspondence should be addressed:

Jihua Tang

Tel: +86-371-63558377

E-mail: tangjihua1@163.com

Wen-Xue Li

Tel: +86-10-82105799

E-mail: liwenxue@caas.cn

Running title: ZmEHD1-mediated CME regulates maize auxin homeostasis

Abstract

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

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 (β 1-5 and one each of $\gamma/\alpha/\delta/\epsilon/\zeta$), a medium subunit (μ 1-5), and a
 87 small subunit (σ 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); α and μ 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); σ adaptin was found to play important roles in the assembly of a
95 functional AP2 complex, and *ap2* σ 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* σ 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

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

The mutant was crossed to an inbred line Xun9058. The hybrid F₁ displayed normal kernel as Xun9058. Due to the low germination rate of the *ehd1* mutant, the normal kernels from F₂ ears were planted. Among the 244 F₂ individuals, 79 had the normal kernel phenotype (*EHD1/EHD1*), and 165 had the segregation phenotype (*EHD1/ehd1*). The segregation ratio followed 1:2 theoretical ratio predicted by a Chi-square test at a 0.05 level (Supplemental Table 1). For each F₂ individual with kernel phenotype segregation, the pattern of shrunken kernel phenotype to normal kernel phenotype fit a 1:3 segregation ratio (Supplemental Table 2). A F₃ population with 376 individuals derived from normal kernels of F₂ selfed ears was also planted to validate the kernel phenotype segregation. Among them, 130 individuals had kernel phenotype (*EHD1/EHD1*), and 246 had the segregation phenotype (*EHD1/ehd1*) (Supplemental Table 1). These results indicate that this shrunken kernel mutant is controlled by a single recessive gene.

170

171 **Positional cloning of *ZmEHD1***

Preliminary genetic mapping of *ZmEHD1* was carried out by BSA using the F₂ population, which contained 165 individuals that differed in seed size in the ears. The *ZmEHD1* gene was first mapped to a 2,339-kb region between the simple sequence repeat (SSR) marker umc1650 (17 recombinants) and umc1716 (27 recombinants) on the long arm of chromosome 4 (Figure 3A). Because the germination rate of the *ehd1* mutant was rather low, expanded F₃ mapping population containing ~53,000 normal kernels (*EHD1/EHD1* and *EHD1/ehd1*) was obtained to increase mapping resolution as described by Zuo et al. (2015). Thirteen new polymorphic SSR markers were 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 closed 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,
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 F₁ (*KO*+/-) and *ehd1*
222 heterozygotes (*ehd1*+/-). The shrunken kernel phenotype (*KO/ehd1*) and the WT
223 phenotype (*EHD1/EHD1*; *EHD1/KO*; *EHD1/ehd1*) in the hybrid F₁ 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 μ M 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

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

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

257

258 **Subcellular localization of ZmEHD1 and ZmEHD1_{mut}**

To determine the subcellular localization of ZmEHD1 protein, yellow fluorescent protein (YFP) fused N-terminally to ZmEHD1 was transiently expressed in tobacco (*N. benthamiana*) leaves under the control of the *CaMV 35S* promoter. In tobacco epidermal cells expressing the YFP-ZmEHD1 fusion protein, the YFP signal was mainly detected in the PM (Supplemental Figure 4A). The localization was confirmed by staining lipid membranes with the red fluorescent probe, FM4-64 (Supplemental Figure 4A). We also cloned the *ZmEHD1* CDS from the *ehd1* mutant and examined the subcellular localization of ZmEHD1_{mut} protein. YFP-ZmEHD1_{mut} signals were aggregated and colocalized with FM4-64 signals in tobacco epidermal cells (Supplemental Figure 4B). This phenomenon was consistently observed in different biological replicates. Thus, we deduced that the mutation in ZmEHD1 affected the subcellular localization of ZmEHD1.

271 **ZmEHD1 interacts with the ZmAP2 σ subunit in yeast and plant**

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

284 The potential interaction between ZmEHD1 and the ZmAP2 σ subunit was first
285 evaluated by a biomolecular fluorescence complementation (BiFC) assay in tobacco
286 leaves and maize mesophyll protoplasts. The ZmEHD1, ZmEHD1_{mut}, and ZmAP2 σ
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 σ 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 σ subunit and
293 cYFP-ZmEHD1_{mut} constructs were co-expressed (Figure 6A and Supplemental Figure
294 6). Consistent with the subcellular localization of ZmEHD1_{mut}, the reconstituted YFP
295 signals were diffused throughout the cells when the nYFP-ZmAP2 σ subunit and
296 cYFP-ZmEHD1_{mut} constructs were co-expressed (Figure 6A and Supplemental Figure
297 6). This was further verified by the localization of ZmAP2 σ 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 σ subunit and ZmEHD1 as well as

301 ZmEHD1_{mut}. Both ZmEDH1 and ZmEHD1_{mut} interacted directly with ZmAP2 σ
302 subunit in yeast. The interaction resulted in survival in a medium lacking His and
303 β -galactosidase activity (Figure 6B).

304 Pull-down experiments using purified His-ZmEHD1/His-ZmEHD1_{mut} to isolate
305 GST-ZmAP2 σ subunit also confirmed the physical interaction between
306 ZmEHD1/ZmEHD1_{mut} and ZmAP2 σ subunit. His-ZmAP2 σ subunit was clearly
307 detected in the pull-down fraction (Figure 6C). These results strongly suggested that
308 ZmEHD1 directly interacts with ZmAP2 σ 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). 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 Log₂ fold-change ratio was ≥ 1 and that the
329 adjusted P value was ≤ 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

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

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

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

To further verify that the rather low fertility of the *ehd1* mutant and *ZmEHD1* knock-out mutant was caused by auxin homeostasis, we attempted to rescue the phenotypic defects of *ehd1* mutant and *ZmEHD1* knock-out mutants by exogenous application of the active auxin compound, 1-naphthaleneacetic acid (1-NAA). The seeds of the *ehd1* mutant and *ZmEHD1* knock-out mutants were rinsed in water or in four increasing concentrations of 1-NAA for 12 h, and then germinated in the paper-culture system. Little or no phenotypic rescue of the *ehd1* mutant was observed when 30 mg L⁻¹ 1-NAA or water alone was applied (Figure 8A). However, the germination rate of *ehd1* mutant and *ZmEHD1* knock-out mutant was significantly increased when treated with low concentrations of 1-NAA (Figure 8A). Also, when treated with low concentrations of 1-NAA, the *ehd1* mutant and *ZmEHD1* knock-out mutant survived after its first two leaves had completely expanded (Figure 8B). In contrast to IAA, exogenous GA3 had no effect on the germination of the *ehd1* mutant 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 σ 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 σ 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* σ loss-of-function *Arabidopsis*, such as
408 reduced leaf size and fertility, could be rescued by *ZmAP2* σ cDNA, suggesting that
409 *ZmAP2* σ subunit has similar functions as *AtAP2* σ subunit in CME. Microarray data
410 revealed that the AP2 σ 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 σ
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 σ
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).

Here, we showed that the subcellular location of ZmEHD1_{mut} was obviously different from that of ZmEHD1. Though ZmEHD1_{mut} could directly interact with ZmAP2 σ subunit, the PM was not the main location for interaction between ZmEHD1_{mut} and the ZmAP2 σ subunit. The impaired clathrin-coated endocytic vesicle formation and/or the recycling of the ZmAP2 σ subunit from the endosome to the PM should be the major contributor to phenotypes observed in the *ehd1* mutant.

Arabidopsis ARF2 controls seed size by repressing cell proliferation (Schruff et al., 2006); in rice, activation of *BIG GRAIN1* (BG1) significantly improves grain size by regulating auxin transport (Liu et al., 2015); *TGW6*, an IAA-glucose hydrolase, negatively regulates endosperm development of rice (Ishimaru et al., 2013). Analyses of a seed-specific viable maize mutant, defective endosperm 18 (*de18*), demonstrated that the *ZmYUC1* gene (which is critical for IAA biosynthesis) is essential for normal endosperm development in maize (Bernardi et al., 2012). Thus, auxin biosynthesis, transport and signaling might coordinate to regulate vegetative and reproductive development in maize (Gallavotti et al., 2008; Li and Li, 2016). The current results with maize show that *ARFs*, indole-3-acetaldehyde oxidase, auxin transporters, and efflux carrier are differentially expressed in the *ehd1* mutant vs. the WT; that the concentration of free IAA is lower in the *ehd1* mutant than in the WT; that the auxin distribution and ZmPIN1a-YFP localization were altered in the *ehd1* mutant; and that exogenous application of 1-NAA rescues the phenotypes of the *ehd1* mutant. These results suggest that the growth defects of the *ehd1* mutant result from the loss of auxin homeostasis.

Materials and methods

Plant materials

The ZmPIN1a::YFP and ZmDR5::mRFP lines were kindly provided by Lixing Yuan (China Agricultural University). The maize mutant *ehd1* was isolated by screening for natural mutants defective in grain filling. To construct mapping population, the mutant was crossed with inbred line Xun9058 in the winter of 2011 in Sanya, Hainan 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. F₂ individuals were obtained by
 452 selfing the F₁ 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 F₂ population
 455 with 165 individuals was used to map the preliminary location of *ZmEHD1* gene.
 456 Normal kernels from F₃ segregation ears were used for fine mapping of the candidate
 457 gene. The F₃ population contains ~53,000 individuals. A small piece of each F₃ 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 F₂ 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 Bulk 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) 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 ZZC01 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. T₂ homozygous lines were sequenced to ensure that *ZmEHD1* was
 493 knocked out.

494 For *Arabidopsis* transformation, the cDNAs of *ZmAP2* σ subunit without 3' UTR
 495 were amplified by PCR. The corresponding products were introduced into the
 496 pENTRTM/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 TaqTM (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

method. Each experiment was replicated at least three times.

Subcellular localization of ZmEHD1

The full-length *ZmEHD1* and *ZmEHD1_{mut}* coding region was amplified from the WT and the *ehd1* mutant. To generate the *ZmEHD1* expression plasmids, the 1644-bp fragments were cloned into the pCPB vector, which was fused to an YFP protein in the N terminus using the *Bam*HI restriction site by In-Fusion reaction. The plasmid was electroporated into *A. tumefaciens* GV3101 and was transiently expressed in tobacco epidermal cells as described previously (Li et al., 2008). To stain the PM of epidermal cells, 5 μ M FM4-64 was infiltrated into tobacco leaves for 3 h before observation. YFP/FM-64 images were collected with a Zeiss LSM700 confocal microscope.

Split-ubiquitin yeast two-hybrid assay

The split-ubiquitin two-hybrid system was used to detect the interactions between membrane proteins. The assay was carried out according to the instructions provided with the DUALmembrane Kit (Dualsystems Biotech). The full-length coding regions of the *ZmAP2* σ subunit, *ZmEHD1* and *ZmEHD1_{mut}* were amplified and cloned into the vectors pST3-STE and pPR3-N NubG using the *Sfi*I restriction site by In-Fusion reaction. Vectors were co-transformed into yeast strain NMY51. The interactions were assessed by the growth of yeast colonies on synthetic minimal medium containing 7.5 mM 3-aminotriazole without Leu, Trp, His and Ade and also by chloroform overlay β -galactosidase plate assay (Duttweiler, 1996).

Bimolecular fluorescence complementation (BiFC) assays

The coding sequences of the *ZmAP2* σ subunit, *ZmEHD1* and *ZmEHD1_{mut}* were amplified using specific primers listed in Table S1, and were cloned into the pSPYNE-35S or pSPYCE-35S binary vectors using the *Bam*HI restriction site. The various combinations of *ZmAP2* σ subunit, *ZmEHD1*, and *ZmEHD1_{mut}* expression 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_{mut}* and *ZmAP2* σ 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_{mut}, GST and GST-ZmAP2 σ subunit were
549 expressed in *Escherichia coli* BL21.

550 For the *in vivo* pull-down analysis, 10 μ g of purified His-ZmEHD1,
551 His-ZmEHD1_{mut} were incubated with GST-ZmAP2 σ subunit or GST bound to
552 glutathione-SepharoseTM 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 μ 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 μ 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 μ 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® UltraTM 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). 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 Log₂ fold-change ratio was ≥ 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 ²H₂-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

Funder	Grant reference number	Author
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

leucine-rich repeat receptor-like protein LeEix2. *Plant J.* **59**:600-611. DOI :
<https://doi.org/10.1111/j.1365-313X.2009.03897.x>, PMID: 19392695

Bar M, Sharfman M, Schuster S, Avni A. 2009. The coiled-coil domain of EHD2
mediates inhibition of LeEix2 endocytosis and signaling. *PLoS ONE* **4**:e7973.
DOI: <https://doi.org/10.1371/journal.pone.0007973>, PMID:19936242

Barberon M, Zelazny E, Robert S, Con G, Curie C, Friml J, Vert G. 2011.
Monoubiquitin-dependent endocytosis of the iron-regulated transporter 1 (IRT1)
transporter controls iron uptake in plants. *PNAS* **108**:E450-E458. DOI:
<https://doi.org/10.1073/pnas.1100659108>, PMID: 21628566

Bernardi J, Lanubile A, Li QB, Kumar D, Kladnik A, Cook SD, Ross JJ, Marocco A,
Chourey PS. 2012. Impaired auxin biosynthesis in the defective endosperm18
mutant is due to mutational loss of expression in the *ZmYuc1* gene encoding
endosperm-specific YUCCA1 protein in maize. *Plant Physiol.* **160**:1318-1328.
DOI: <https://doi.org/10.1104/pp.112.204743>, PMID: 22961134

Blackbourn HD, Jackson AP. 1996. Plant clathrin heavy chain: sequence analysis and
restricted localization in growing pollen tubes. *Journal of Cell Science*
109:777-786. PMID: 8718669

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for
Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.*
16:735-743. DOI: <https://doi.org/10.1046/j.1365-313x.1998.00343.x>, PMID:
10069079

Dhonukshe P, Aniento F, Hwang I, Robinson DG, Mravec J, Stierhof YD, Friml J.
2007. Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in
Arabidopsis. *Current Biology* **17**:520-527. DOI:
<https://doi.org/10.1016/j.cub.2007.01.052>, PMID: 17306539

Di Rubbo S, Irani NG, Kim SY, Xu ZY, Gadeyne A, Dejonghe W, Vanhoutte I,
Persiau G, Eeckhout D, Simon S, Song K, Kleine-Vehn J, Friml J, De Jaeger G,
Van Damme D, Hwang I, Russinova E. 2013. The clathrin adaptor complex AP-2
mediates endocytosis of BRASSINOSTEROID INSENSITIVE1 in *Arabidopsis*.
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 σ 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

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

Gene ID	Annotation ¹	Log ₂ 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 ⁻¹⁶
GRMZM2G019799	Indole-3-acetaldehyde oxidase-like	1.39	0.0006
GRMZM2G082943	ZAR9	-2.46	2.04e ⁻¹²
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 ⁻⁶
GRMZM2G475263	Auxin response factor 1	1.15	7.27e ⁻¹¹
GRMZM2G028980	Auxin response factor 6 isoform X1	1.53	1.62e ⁻¹²
GRMZM5G808366	Auxin response factor 5	1.99	0.0038
GRMZM2G137413	Auxin response factor 14	2.06	5.04e ⁻⁴¹
GRMZM2G064371	Auxin binding protein-like protein	1.38	0.0001
GRMZM2G365188	SAUR23	-3.37	0.0004
GRMZM2G414727	SAUR56	-2.95	1.12e ⁻²⁶
GRMZM2G059138	SAUR33	-2.01	7.63e ⁻⁸
GRMZM2G121309	IAA7	1.16	5.26e ⁻⁸
GRMZM2G030465	IAA9	3.19	0.0146
GRMZM2G077356	IAA13	2.90	9.83e ⁻¹⁸
GRMZM5G809195	IAA14	-2.12	0.0046
GRMZM2G115357	IAA24	-1.25	8.09e ⁻⁷
GRMZM2G048131	IAA26	inf	0.0002

775 ¹Annotation is based on maize IDs

776 Figure Legends

777 **Figure 1. Phenotypes of the maize *ehd1* mutant kernels.** (A) F₂ 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 F₂ 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 μ M with
827 2.4- μ 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 μ 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* σ subunit in plants and**

836 **yeast.** (A) Interaction between ZmEHD1 and the ZmAP2 σ 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 μ m. (B) Interaction
840 between ZmEHD1 and the ZmAP2 σ subunit as indicated by split-ubiquitin yeast
841 two-hybrid assays. The ZmAP2 σ subunit was used as the fused bait protein (AP2
842 σ -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 σ subunit. The ZmEHD1/ ZmEHD1_{mut}
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 μ m. (D) The
853 fluorescence of ZmPIN1a::YFP in WT and *ehd1* mutant. Scale bars = 50 μ 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 F₂ and F₃ individuals evaluated in**
865 **the fields.**

866 **Supplemental Table 2. The kernel performance of F₂ 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*+/- and *ehd1*+/-**

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_{mut}. 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_{mut} protein (B). Representative photographs are shown.**

885

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

891

892 **Supplemental Figure 6. Interaction between ZmEHD1 and the ZmAP2 σ 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 μ m.**

896

897 **Supplemental Figure 7. The different subcellular localization of ZmAP2 σ**
 898 **subunit in WT maize and *ehd1* mutant.** Representative photographs are shown.
 899 Scale bars = 10 μ 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) F₂ 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 F₂ 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 μM with 2.4- μM 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 μm . 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 σ subunit in plants and yeast. (A) Interaction between ZmEHD1 and the ZmAP2 σ subunit in the leaves of *N. benthamiana* as 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 μ m. (B) Interaction between ZmEHD1 and the ZmAP2 σ subunit as indicated by split-ubiquitin yeast two-hybrid assays. The ZmAP2 σ subunit was used as the fused bait protein (AP2 σ -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 σ subunit. The ZmEHD1/ ZmEHD1_{mut} 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 μ m. (D) The fluorescence of ZmPIN1a::YFP in WT and *ehd1* mutant. Scale bars = 50 μ 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.