RESEARCH ARTICLE

The transcription factors ZmNAC128 and ZmNAC130 coordinate with Opaque2 to promote endosperm filling in maize

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Short Title: Three core TFs control endosperm filling

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Abstract

Endosperm filling in maize (*Zea mays*), which involves nutrient uptake and biosynthesis of storage reserves, largely determines grain yield and quality. However, much remains unclear about the synchronization of these processes. Here, we comprehensively investigated the functions of duplicate NAC-type transcription factors, namely ZmNAC128 and ZmNAC130, in endosperm filling. The gene-edited double mutant *zmnac128 zmnac130* exhibits a poorly filled kernel phenotype such that the kernels have an inner cavity. RNA sequencing and protein abundance analysis revealed that the expression of many genes involved in the biosynthesis of zein and starch is reduced in the filling endosperm of *zmnac128 zmnac130*. Further DNA affinity purification and sequencing combined with chromatin-immunoprecipitation quantitative PCR and promoter transactivation assays demonstrated that ZmNAC128 and ZmNAC130 are direct regulators of three (*16*-, *27*-, and *50-kD*) *γ-zein* genes and six important starch metabolism genes (*Brittle2* [*Bt2*], *pullulanase-type starch debranching enzyme* [*Zpu1*], *granule-bound starch synthase 1* [*GBSS1*], *starch synthase 1* [*SS1*], *starch synthase IIa* [*SSIIa*], and *sucrose synthase 1* [*Sus1*]). ZmNAC128 and ZmNAC130 recognize an additional cis-element in the *Opaque2* (*O2*) promoter to regulate its expression. The triple mutant *zmnac128 zmnac130 o2* exhibits extremely poor endosperm filling, which results in more than 70% of kernel weight loss. ZmNAC128 and ZmNAC130 regulate the expression of the transporter genes *sugars will eventually be exported transporter 4c* (*ZmSWEET4C*), *sucrose and glucose carrier 1* (*ZmSUGCAR1*), and *yellow stripe-like2* (*ZmYSL2*) and in turn facilitate nutrient uptake, while O2 plays a supporting role. In conclusion, ZmNAC128 and ZmNAC130 cooperate with O2 to facilitate endosperm filling, which involves nutrient uptake in the basal endosperm transfer layer (BETL) and the synthesis of zeins and starch in starchy endosperm (SE).

IN A NUTSHELL

Background: Cereal endosperms store starch and proteins in grains and play a critical role in determining grain yield and quality. Maize (*Zea mays*), with its sizable grain, is an excellent model for studying cereal endosperm development. Endosperm filling involves two synchronized processes: maternal-to-endosperm nutrient transfer and storage reserve biosynthesis. Several important nutrient transfer-related genes have been identified, and the regulation of storage reserve biosynthesis has been broadly studied in maize. Notably, the transcription factor-encoding genes *Opaque2* (*O2*), *ZmNAC128*, and *ZmNAC130* exhibit strong expression in the filling endosperm. However, how these three transcription factors co-regulate the synchronization of these endosperm-filling processes remains elusive.

Question: What are the roles of ZmNAC128 and ZmNAC130 in endosperm filling? How do they function together with O2 in this process?

Findings: ZmNAC128 and ZmNAC130 directly regulate the expression of all *γ-zein* genes (encoding storage proteins) and multiple important starch metabolism genes, making them pivotal coordinators of grain quality and yield. Furthermore, ZmNAC128 and ZmNAC130 directly regulate the expression of *O2*, and together these three transcription factors synergistically activate their own expression through autoregulation and physical interactions. ZmNAC128 and ZmNAC130 also regulate the expression of vital transporter genes responsible for facilitating nutrient transfer from the mother plant to the endosperm. This regulatory mechanism enhances nutrient uptake, with O2 playing a supportive role.

Next steps: We plan to identify additional co-regulatory factors involved in endosperm filling to gain a comprehensive understanding of endosperm filling, from nutrient uptake to the biosynthesis of storage reserves.

Introduction

Cereal grain endosperms, which serve as the main storage site of starch and proteins in kernels, account for approximately 90% of kernel weight (Flint-Garcia et al., 2009). The endosperm supports embryogenesis and seed germination during early seedling development, while the endosperm is also a main source of food, feed, and

industrial raw materials worldwide (Flint-Garcia, 2017; Hannah and Boehlein, 2017; Huang et al., 2021). Among cereals, maize (*Zea mays*) has the largest caryopsis, making it an excellent model species for investigating endosperm development (Sabelli and Larkins, 2009). Like that of other cereal grains, maize endosperms start to develop after double fertilization, wherein one sperm fertilizes the diploid central cell to initiate the formation of the triploid endosperm (Sabelli and Larkins, 2009).

Endosperm development involves three typical physiological stages: early, middle, and mature stages. During the early stage, the fertilized endosperm undergoes coenocyte formation, cellularization, and differentiation (Olsen, 2001). Seven distinguishable compartments are formed by approximately 8 d after pollination (DAP): starchy endosperm (SE), aleurone (AL), embryo surrounding region (ESR), basal endosperm transfer layer (BETL), basal-intermediate zone (BIZ), conducting zone (CZ), and sub-aleurone (SA) (Olsen, 2001; Becraft and Gutierrez-Marcos, 2012; Leroux et al., 2014; Zhan et al., 2017; Dai et al., 2021). Endosperm cell fate is largely determined during this stage. Starting at approximately 8-10 DAP, endosperm development is accompanied by the coordination of maternal-to-filial nutrient transfer in the BETL and the synthesis of storage reserves in the SE, which ultimately determine the yield and quality of the kernel. Subsequently, kernels gradually dehydrate and mature.

In recent years, spatiotemporal high-resolution transcriptome analyses and advanced functional genomic studies have greatly advanced our understanding of endosperm development in maize (Xin et al., 2013; Chen et al., 2014; Zhan et al., 2015; Yi et al., 2019; Li and Wu, 2020). For instance, the BETL at the base of the endosperm is the gateway through which nutrients are transported to the central SE, a process that is facilitated by the BIZ and CZ compartments (Chourey and Hueros, 2017). When mutated, BETL-specific sugar transfer-related genes including *Miniature1* (*ZmMN1*, encoding CELL WALL INVERTASE2) (Cheng et al., 1996), *Sugars will eventually be exported transporter 4c* (*ZmSWEET4c*) (Sosso et al., 2015), and *Sucrose and glucose carrier 1* (*ZmSUGCAR1*, also referred to as *ZmNPF7.9*

126 [*Nrt1/Ptr family 7.9*] and *ZmMN2*) largely block mother-to-endosperm sugar transfer
127 and the synthesis of storage reserves (Guan et al., 2020; Wei et al., 2021; Zhou et al.,
128 2021; Yang et al., 2022a). Recently, the *shrunken4* (*sh4*) mutant was shown to harbor
129 a mutation in the oligopeptide metal transporter YELLOW STRIPE-LIKE2
130 (ZmYSL2), which predominantly exists in the AL and BETL compartments (He et al.,
131 2021; Chao et al., 2022). Like mutants with defects in sugar transporter genes
132 expressed in the BETL, the *sh4* mutant also exhibits a shrunken-kernel phenotype,
133 suggesting that ZmYSL2 is critical for endosperm filling. MYB-RELATED
134 PROTEIN1 (MRP1) is an important transcription factor (TF) for BETL development
135 and function (Gomez et al., 2002; Gomez et al., 2009); loss-of-function mutants are
136 needed to determine its role during grain filling.

137 The SE compartment occupies the largest part of the endosperm and is the site of
138 the biosynthesis and deposition of storage reserves during the filling stage.
139 Ultramicroscopy observations of filling-stage and mature endosperms revealed that
140 the SE is filled with two types of storage bodies: starch granules (SGs) and protein
141 bodies (PBs) (Sabelli and Larkins, 2009). SGs are formed by the orderly assembly of
142 starch in amyloplasts (Hannah and Boehlein, 2017). Starch accounts for
143 approximately 70% of dry kernel weight, so its content is closely related to yield. PBs,
144 which are insoluble and spherical, are formed by the synthesis and deposition of zeins
145 (the most abundant seed storage proteins in maize) within the lumen of the rough
146 endoplasmic reticulum (Lending and Larkins, 1989; Larkins et al., 2017). Zeins lack
147 several essential amino acids (lysine, tryptophan, and methionine), which greatly
148 affects seed protein quality (Larkins et al., 2017). Temporal high-resolution
149 transcriptome analyses of endosperm development have indicated that genes encoding
150 zeins and enzymes participating in starch biosynthesis are specifically and strongly
151 expressed during the filling stage (Chen et al., 2014). This suggested that common
152 TFs or regulators might contribute to their synchronized biosynthesis.

153 The first TF identified related to zein biosynthesis was Opaque2 (O2), which
154 directly regulates the expression of *22-kD α-zein* through the recognition of the

O2-box cis-element in its promoter (Schmidt et al., 1987; Schmidt et al., 1990). Analysis of differentially expressed genes (DEGs) in the *o2* mutant, combined with genome-wide chromatin immunoprecipitation followed by deep sequencing (ChIP-seq), revealed that O2 regulates the expression of most zein genes, with the exception of the *16-kD γ-zein* gene (Li et al., 2015; Zhan et al., 2018). ZmNAC128 and ZmNAC130 were recently shown to regulate the expression of *16-kD γ-zein* via their ability to bind to a specific cis-element (ACGCAA) in its promoter (Zhang et al., 2019), although it remains unknown whether or how these two NACs regulate the expression of other zein genes. The 27-kD γ-zein is involved in the initiation of PB formation (Wu et al., 2010), and its expression is regulated by a complex series of TFs, including BASIC LEUCINE ZIPPER22 (ZmbZIP22) (Li et al., 2018), PROLAMIN-BOX BINDING FACTOR1 (PBF1) (VicenteCarbajosa et al., 1997; Wu and Messing, 2012), O2 HETERODIMERIZING PROTEINS (OHP1 and OHP2) (Pysh et al., 1993; Zhang et al., 2015), and O2 (Li et al., 2015). Upon the interaction with O2, ZmMADS47, a MADS box-containing TF, binds to the CATGT motif in the promoters of *α-zein* and *50-kD γ-zein* for their transactivation (Qiao et al., 2016). In general, O2 appears to be a core TF that regulates the expression of zein genes through additive and synergistic interactions with multiple TFs, including ZmbZIP22, PBF1, OHP1, OPH2, and ZmMADS47.

Among the characterized TFs regulating the expression of zein genes, some also directly regulate the expression of genes encoding enzymes in the starch biosynthesis pathway. Our previous studies demonstrated that O2 coordinates the expression of important starch metabolism genes, including *starch synthase III* (*SSIII*), the three major sucrose synthases (*sucrose synthase Sh1* [*Shrunken1*], *sucrose synthase 1* [*Sus1*], and *Sus2*), and *pyruvate orthophosphate dikinase* (*PPDK*) (Zhang et al., 2016; Deng et al., 2020). Knockdown of *ZmNAC128* and *ZmNAC130* via RNA interference (RNAi) significantly downregulated the expression of multiple starch metabolism genes. Among these genes, *Brittle2* (*Bt2*), which encodes the adenosine diphosphate glucose pyrophosphorylase (AGPase) small subunit, is a direct downstream target of

ZmNAC128 and ZmNAC130 (Zhang et al., 2019).

Although ZmNAC128 and ZmNAC130 are also important coordinators of the synchronized synthesis of zeins and starch, their gene regulatory networks (GRN) remain largely unknown. Additionally, Opaque11 (O11), a hub TF for endosperm development, also directly regulates the expression of carbohydrate-related genes (*starch synthase V* [*SSV*] and *pyruvate kinase*) (Feng et al., 2018). Moreover, NAKED ENDOSPERM1 (NKD1) and NKD2 directly or indirectly regulate the expression of zein genes, and are also predicted to act as transcriptional activators of *starch synthase 1* (*SS1*), *sugary1* (*Su1*), and *waxy* (*Wx*) (Gontarek et al., 2016). We do not yet clearly understand the transcriptional regulation of starch metabolism genes in the endosperm. One way to fill this gap is to identify additional TFs that regulate starch metabolism via forward or reverse genetics; another complementary approach is to determine the GRN of the identified TFs, such as ZmNAC128 and ZmNAC130 or NKD1 and NKD2, by DEG analysis in their respective mutants combined with global cis-element characterization. Such an approach successfully revealed the GRN of O2 and PBF1 through the combined analysis of DEGs and genome-wide ChIP-seq by different groups (Li et al., 2015; Zhan et al., 2018; Ning et al., 2022).

In general, endosperm filling has been intensively investigated over the past few decades, and progress has been made in understanding the regulation of storage proteins and starch, nutrient transfer, and initiation of filling. However, the synchronization of these events during grain filling remains poorly understood. *O2*, *ZmNAC128*, and *ZmNAC130* are the most highly expressed TF-encoding genes in the filling-stage endosperm (Chen et al., 2014). O2 has been comprehensively investigated in recent decades, but ZmNAC128 and ZmNAC130 remain poorly understood. This study aims to shed light on the roles of ZmNAC128, ZmNAC130, and O2 in regulating endosperm filling. These roles encompass the coordination of zeins and starch synthesis, maintaining their own high expression levels, and regulating the expression of critical transporter genes in the BETL

Results

ZmNAC128 and ZmNAC130 are essential for endosperm filling

Our previous study found that knockdown of expression of *ZmNAC128* and *ZmNAC130* via RNA interference (RNAi) caused a shrunken kernel phenotype with 30% of kernel weight loss, while a *zmnac130* mutant of a five-amino acids deletion within the conserved NAC domain of its encoding protein did not lead to any apparent kernel phenotype. Meanwhile, ZmNAC128 and ZmNAC130 could recognize the same cis-element ACGCAA to co-regulate the expression of *16-kD γ-zein* and *Bt2*, which suggests their functional redundancy (Zhang et al., 2019). However, the contribution by ZmNAC128 and ZmNAC130 to endosperm filling remains largely unknown.

Therefore, knockout mutants of *ZmNAC128* and *ZmNAC130* were generated by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9)-mediated gene editing in this study. We obtained three independent knockout mutants, each of which had a different frameshift mutation: *zmnac128-1*, *zmnac128-2*, *zmnac128-3*, *zmnac130-1*, *zmnac130-2*, and *zmnac130-3* (Supplemental Figure S1). The six single mutants exhibited normal cob and kernel phenotypes similar to the wild type (WT) KN5585. However, all double mutants of different allele combinations of *zmnac128* and *zmnac130* exhibited the same poorly filled-kernel phenotype (Supplemental Figure S1). Among these knockout mutants, *zmnac128-1*, *zmnac130-1*, and *zmnac128-1 zmnac130-1*, which were abbreviated as *zmnac128*, *zmnac130*, and *zmnac128 zmnac130* hereafter, were used for subsequent studies.

Observations of kernel longitudinal sections revealed a cavity within the endosperm of *zmnac128 zmnac130* and its peripheral endosperm became floury, while kernel phenotypes of the single mutants *zmnac128* and *zmnac130* were the same as that of the WT (Figure 1A). Scanning electron microscopy (SEM) observations indicated that the SGs are smaller in the endosperm of *zmnac128 zmnac130* than in that of the WT (Figure 1A). The hundred-kernel weight (HKW) of *zmnac128*

zmnac130 was 56.8% lower than that of the WT, while the HKW of *zmnac128* and *zmnac130* was not significantly reduced (Figure 1B). Although their RNAi mutants (*nacRNAi*) also caused 30% of kernel weight loss compared to the corresponding nontransgenic (NT) kernels (Zhang et al., 2019), it is obvious that knockout of *ZmNAC128* and *ZmNAC130* lead to even lower kernel weight and more severe kernel phenotypes than that of *nacRNAi*.

Furthermore, we analyzed the protein and starch contents in mature kernels via near-infrared (NIR) spectroscopy analysis (Figure 1C). The starch contents in the kernels of the two single mutants were slightly but significantly reduced, while the protein content in the kernels of *zmnac128* was also slightly reduced with a significant difference. However, the contents of protein and starch in the kernels of *zmnac128 zmnac130* decreased by more than 50% compared to those of the WT and were also lower than those of *nacRNAi* (Zhang et al., 2019). Therefore, these knockout mutants generated in this study are a prerequisite for comprehensively investigating the function of ZmNAC128 and ZmNAC130 in the endosperm filling. These results clearly pointed out that ZmNAC128 and ZmNAC130 redundantly promote endosperm filling, as their simultaneous loss of function severely impaired the accumulation of storage reserves.

RNA-seq combined with DAP-seq facilitates the functional dissection of ZmNAC128 and ZmNAC130

DEG analysis of *zmnac128 zmnac130* indicates the enrichment of zein and starch metabolism

To study the GRN of ZmNAC128 and ZmNAC130, we conducted RNA sequencing (RNA-seq) analysis using total RNA extracted from 16-DAP endosperms of WT, *zmnac128*, *zmnac130*, and *zmnac128 zmnac130* in the KN5585 background. We identified DEGs in the three mutants relative to the WT based on the selection criteria of a fold-change (FC) ≥ 2 and a *p*-value ≤ 0.05. Only 80 and 2 DEGs were detected in *zmnac128* and *zmnac130*, respectively. However, this number rose to 2,686, with 1,529 upregulated and 1,157 downregulated genes in *zmnac128 zmnac130*

(Figure 2A and Supplemental Dataset S1). This result further supported the functional redundancy of the two NACs.

We performed an enrichment analysis of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways based on the DEGs in *zmnac128 zmnac130*. We observed two significantly enriched GO terms for molecular function, one of which was "nutrient reservoir activity" (GO:0045735, *p*-value = 2.17E–06), which mainly included zein family genes (Figure 2B). Indeed, the expression of all zein family genes was significantly downregulated to varying extents in *zmnac128 zmnac130* (Supplemental Table S1). Moreover, multiple carbohydrate metabolism-related pathways were enriched (Figure 2B). These results suggested that ZmNAC128 and ZmNAC130 play crucial roles in the biosynthesis of zeins and starch.

DAP-seq reveals genome-wide binding features of ZmNAC128 and ZmNAC130

DNA affinity purification and sequencing (DAP-seq) is a recently developed method of genome-wide TF-binding site identification, which is fast, inexpensive, and more easily scaled than ChIP-seq and bypasses the difference of sampling stage (Bartlett et al., 2017 and Galli et al., 2018). In this study, we conducted the DAP-seq assay by incubation of recombinant purified ZmNAC128 and ZmNAC130 proteins and the use of a DNA library prepared from genomic DNA extracted from 16-DAP endosperms of the reference inbred line B73. The Model-based Analysis of ChIP-Seq (MACS) (Zhang et al., 2008) under the threshold of q-value ≤ 0.05 revealed 14,211 peaks for ZmNAC128 and 9,261 peaks for ZmNAC130 (Supplemental Dataset S2), of which 7,082 peaks were common between the two NACs. We determined that approximately 60% of all the peaks bound by the two NACs were in the genic region approximately 3 kb from the transcription start site (TSS), of which more than 40% (4,811 genes) were within 1 kb of the TSS (Figure 2C). Further analysis showed that the peaks were strongly concentrated immediately upstream of the TSS (Figure 2D), consistent with the function of ZmNAC128 and ZmNAC130 as TFs.

To identify putative target genes of ZmNAC128 and ZmNAC130, these 4,811 genes with NAC-binding peaks around 1 kb of TSS were overlapped with DEGs of *zmnac128 zmnac130* versus the WT in the KN5585 genetic background. Venn diagram results showed that 248 upregulated and 250 downregulated genes overlapped as putative direct targets (Supplemental Figure S2 and Supplemental Dataset S3). These identified genes were further compared with DEGs of *zmnac128 zmnac130* in the KN5585 × B73 background of this study, of which 138 downregulated and 64 upregulated genes were common to both genetic backgrounds (Supplemental Dataset S4). They included multiple plant hormone-related genes, TFs, putative transporters, sugar metabolism genes, and so on. Among them, the two amino acid metabolism genes, *proline oxidase* (Zm00001eb022980) and *lysine-ketoglutarate reductase/saccharopine dehydrogenase 1* (*LKR/SDH1*, Zm00001eb192910), are not only the potential targets of ZmNAC128 and ZmNAC130 but also the direct targets of O2 (Li et al., 2015 and Zhan et al., 2018).

ZmNAC128 and ZmNAC130 bind to the promoters of *16-kD γ-zein* and *Bt2*

16-kD γ-zein and *Bt2* were previously identified as direct target genes of the two NACs (Zhang et al., 2019). Visualization of the DAP-seq data by the Integrated Genomics Viewer (IGV) showed that the identified cis-element ACGCAA was surrounded by binding peaks for ZmNAC128 and ZmNAC130 in the promoters of *16-kD γ-zein* and *Bt2* (Figure 2E). This suggested that our DAP-seq data are robust for the detection of potential target genes. We thus explored the genome-wide cis-elements bound by the two NACs via the motif discovery tool MEME-ChIP. The most significant cis-element bound by ZmNAC128 and ZmNAC130 was ACGCAA (E-value < 5.7E–149) (Figure 2F), which was identical to the previously identified cis-element in the promoters of *16-kD γ-zein* and *Bt2* (Zhang et al., 2019). In addition to this motif, multiple candidate binding motifs were detected by DAP-seq (Supplemental Figure S3). These candidates were highly identical either in the two independent replicates or between ZmNAC128 and ZmNAC130, suggesting that the two NACs also recognize other cis-elements in addition to the ACGCAA motif.

Although multiple enriched elements are commonly detected in the DAP-seq analysis of ZmNAC128 and ZmNAC130, some of them could be binding motifs for their cofactors. For instance, the core motif 'ACGT' recognized by bZIP-type TFs (such as O2) is also enriched in the DAP-seq of ZmNAC128 and ZmNAC130, but it requires additional investigation.

27-kD and *50-kD γ-zein* are direct targets of ZmNAC128 and ZmNAC130

Consistent with the downregulated expression of all zein family genes in *zmnac128 zmnac130* (Supplemental Table S1), overall zein protein abundance was also lower in mature kernels of *zmnac128 zmnac130*, with the 16-kD γ-zein protein being nearly undetectable (Supplemental Figure S4). These results thus supported the notion that, in addition to O2, ZmNAC128 and ZmNAC130 are also core TFs involved in zein gene expression. To investigate the regulation of ZmNAC128 and ZmNAC130 on the expression of other zein genes, we examined the DAP-seq data in detail. We observed peaks corresponding to the binding of ZmNAC128 and ZmNAC130 in the promoters of the other two γ-zein genes, *27-kD* and *50-kD γ-zein* (Figure 3A). We detected the conserved motif ACGCAA around the binding peak regions in the promoters at –1,421/–1,464 and –461 bp upstream from the start codon of *27-* and *50-kD γ-zein* genes, respectively (Figure 3A).

To test the binding of ZmNAC128 and ZmNAC130 to the fragments containing ACGCAA in the two promoters, we performed the ChIP-qPCR assay. For this assay, we used an antibody against the Flag tag and the 16-DAP endosperms of the transgenic lines of 3×Flag-tagged ZmNAC128 and ZmNAC130 that are driven by the *27-kD γ-zein* promoter (Supplemental Figure S5). The ChIP-qPCR results verified the binding relationship of ZmNAC128 and ZmNAC130 in the two γ-zein gene promoters revealed by our DAP-seq analysis (Figure 3B). A dual-luciferase reporter (DLR) assay was thus performed to assess the transactivation activities of ZmNAC128 and ZmNAC130 for the expression of firefly luciferase (LUC)-encoding gene driven by the two promoters in maize leaf protoplast cells. Compared to the empty vector control, LUC activities driven by the *27-kD γ-zein* promoter could be

significantly increased by the co-transformation of 35S promoter-driven *ZmNAC128* or *ZmNAC130*. Similarly, LUC activities driven by the *50-kD γ-zein* promoter were also significantly increased by ZmNAC128 or ZmNAC130 (Figure 3C). Similar to the *16-kDa γ-zein* promoter (Zhang et al., 2019), LUC activities driven by the two promoters were not stronger under the co-expression of both ZmNAC128 and ZmNAC130 (Figure 3C).

Combining these results with our previous findings (Zhang et al., 2019), we concluded that ZmNAC128 and ZmNAC130 are direct transcriptional regulators of all three (*50-*, *27-*, and *16-kD*) *γ-zein* genes. We previously found that the PB number is significantly reduced in the filling endosperm of *nacRNAi* lines (Zhang et al., 2019). As γ-zein proteins are essential for PB formation, ZmNAC128 and ZmNAC130 are likely to play important roles in PB formation. There were no apparent DAP-seq peaks in other zein gene promoters, so the expression of other zein genes is possibly regulated by their co-factors.

ZmNAC128 and ZmNAC130 directly regulate the expression of multiple important starch metabolism genes

Reduced starch content and enrichment of the starch and sucrose metabolism KEGG pathways among the observed DEGs of *zmnac128 zmnac130* suggest that the two NACs may directly regulate expression of genes involved in starch metabolism. We previously found that the expression of multiple important starch metabolism genes was downregulated in the *nacRNAi*, but only *Bt2* was characterized as the direct target gene of ZmNAC128 and ZmNAC130 (Zhang et al., 2019).

To study which starch metabolism genes are the direct targets of ZmNAC128 and ZmNAC130, we emphasized the commonly downregulated genes in the 16-DAP endosperms of both *zmnac128 zmnac130* and *nacRNAi* (Table 1). In all seven downregulated genes in the *nacRNAi* (Zhang et al., 2019), six genes were also detected to be significantly downregulated in *zmnac128 zmnac130*, including *Bt2* (Zm00001eb176800), *pullulanase-type starch debranching enzyme* (*Zpu1*, Zm00001eb088740), *SS1* (Zm00001eb376100), *Sus1* (Zm00001eb392880),

granule-bound starch synthase 1 (*GBSS1*, also named *waxy* [*Wx*], Zm00001eb378140), *starch branching enzyme IIb* (*SBEIIb*, also named *amylose extender1* [*Ae1*], Zm00001eb242610) (Supplemental Dataset S5). *Starch branching enzyme 1* (*SBE1*, Zm00001eb228530) was detected to be transcriptionally downregulated only in the *nacRNAi* (Zhang et al., 2019), but its encoded protein was not reduced in *zmnac128 zmnac130* compared to the WT (Figure 4A).

Meanwhile, transcriptome analysis of *zmnac128 zmnac130* found that another two important starch metabolism genes also significantly downregulated, including *isoamylase-type starch-debranching enzyme1* (*ISA1*, also named *sugary1* [*Su1*], Zm00001eb242610) and *starch synthase IIa* (*SSIIa*, also named *sugary2* [*Su2*], Zm00001eb279740) (Supplemental Dataset S5). Therefore, the complete elimination of *ZmNAC128* and *ZmNAC130* through CRISPR/Cas9-mediated knockout caused the downregulated expression of more important starch metabolism genes than their RNAi-mediated knockdown.

To detect the effects of ZmNAC128 and ZmNAC130 on starch metabolism, we performed a suite of immunoblotting assays with antibodies against the major starch metabolism enzyme proteins in 20-DAP endosperms of *zmnac128 zmnac130* and the WT (Figure 4A). Consistent with the pronounced reduction in *Zpu1* and *Bt2* transcripts, their corresponding protein accumulation also decreased by nearly 80% in *zmnac128 zmnac130* compared to the WT. Moreover, the accumulation of four starch biosynthesis enzymes, including GBSS1, SS1, SSIIa, and ISA1 decreased by more than 50% in *zmnac128 zmnac130* compared to the WT. The accumulation of multiple other enzymes (AGPase large subunit shrunken2 [Sh2], Sh1, SBEIIb, SSIII, SSV, and ADP-glucose transporter brittle endosperm 1 [Bt1]) decreased by 20%–50% in *zmnac128 zmnac130* compared to the WT. Taken together, these results indicated that almost all the detected proteins accumulated to lower levels in *zmnac128 zmnac130* than in the WT. This could be one main cause for the dramatic reduction in starch content and kernel weight of *zmnac128 zmnac130*.

To investigate which genes were directly regulated by ZmNAC128 and

ZmNAC130, we did the DAP-seq and promoter analysis. In these eight downregulated starch metabolism genes in *zmnac128 zmnac130*, seven genes (*Bt2*, *Zpu1*, *GBSS1*, *SS1*, *SSIIa*, *Sus1*, and *SBEIIb*) were found to have binding peaks of ZmNAC128 and ZmNAC130 in their promoters (Table 1). Besides *Bt2* (Figure 2E), the promoters of another five genes (*Zpu1*, *GBSS1*, *SS1*, *SSIIa*, and *Sus1*) also contained the conserved cis-element ACGCAA at positions –1, –394/–692, –2,664, –875/–970, and –1,613/–1,627 upstream from their corresponding TSS, respectively (Figure 4B). Sh1, Sh2, SSIII, SSV, and Bt1 exhibited reduced protein accumulation without a corresponding decrease in transcript level. Notably, although the promoters of four of these genes (*Sh1*, *Sh2*, *SSIII*, and *SSV*) contain the element ACGCAA, no binding peaks were detected in any of the four promoters (Table 1 and Supplemental Figure S6).

In contrast, despite the absence of the conserved cis-element ACGCAA in the promoters of *Bt1* and *SBEIIb*, binding peaks were still detected in both promoters (Table 1), indicating that NACs can bind to these promoters even in the absence of their canonical target sequence. ChIP-qPCR further verified the binding relationships of ZmNAC128 and ZmNAC130 for these five promoters of *Zpu1*, *GBSS1*, *SS1*, *SSIIa*, and *Sus1* (Figure 4C). Finally, DLR assays were performed to detect the transactivation activities of ZmNAC128 and ZmNAC130 with respect to the five promoters. LUC activities driven by the four promoters of *Zpu1*, *GBSS1*, *SS1*, and *SSIIa* were significantly increased by the co-transformation of 35S promoter-driven ZmNAC128 and ZmNAC130 (Figure 4D). The *Sus1* promoter was slightly transactivated only by ZmNAC130 but not ZmNAC128. This suggested that although ZmNAC128 and ZmNAC130 can bind to this promoter, its transactivation potentially requires the involvement of other TFs. Similarly, the LUC activities driven by these five promoters were not stronger upon co-expression of ZmNAC128 and ZmNAC130. Because both the transcript levels and protein accumulation of *Bt2*, *Zpu1*, *GBSS1*, *SS1*, and *SSIIa* were significantly reduced in *zmnac128 zmnac130*, ZmNAC128 and ZmNAC130 function as core TFs for starch metabolism through the direct regulation

of these starch metabolism genes, i.e., *Bt2*, *Zpu1*, *GBSS1*, *SS1*, *SSIIa*, and *Sus1*.

Because carbohydrate-related pathways were generally enriched in DEGs in *zmnac128 zmnac130*, we also analyzed the expression of genes involved in the three primary carbohydrate metabolism pathways: the pentose phosphate pathway, glycolysis, and the tricarboxylic acid cycle. In contrast to the overall downregulated expression of starch metabolism genes, many genes in these three pathways were upregulated in *zmnac128 zmnac130* (Supplemental Dataset S5). The upregulated expression of many genes involved in these three primary carbohydrate metabolism pathways seems to indicate negative feedback due to a sharp decrease in starch metabolism in the endosperm of *zmnac128 zmnac130*, but this hypothesis needs to be investigated.

Regulatory and physical interactions of ZmNAC128 and ZmNAC130 with O2

Among known endosperm filling-related TF genes, *O2*, *ZmNAC128*, and *ZmNAC130* are the most highly expressed in the filling-stage endosperm (Chen et al., 2014). Based on the three RNA-seq datasets from the *ZmNAC128* and *ZmNAC130* knockdown lines and knockout mutants in this study and our previous publication (Zhang et al., 2019), we observed that the expression of the *O2* gene is consistently downregulated in *nacRNAi* and *zmnac128 zmnac130* compared to the corresponding NT and WT, respectively (Supplemental Figure S7). Importantly, only a few DEGs were shared across studies, one of which was *O2*, making this TF a candidate target gene of ZmNAC128 and ZmNAC130.

ZmNAC128 and ZmNAC130 directly regulate the expression of *O2*

To test whether the two NACs regulate the expression of *O2*, we further measured the transcript level and protein accumulation of *O2* in the endosperms of *zmnac128 zmnac130* across the entire filling stages from 12- to 28-DAP. RT-qPCR analysis showed that the transcript levels of *O2* decreased by nearly 50% on average in the endosperm of *zmnac128 zmnac130* throughout the whole filling stage (Figure 5A). Likewise, the protein accumulation of O2 significantly decreased by 60%–80% in endosperms of *zmnac128 zmnac130* throughout the whole filling stage (Figure 5B

474 and Supplemental Figure S8). These findings support a role for ZmNAC128 and
475 ZmNAC130 in maintaining the high expression of *O2* in the endosperm across the
476 whole filling stage.

477 To know whether ZmNAC128 and ZmNAC130 directly regulate the expression
478 of *O2*, we first examined the DAP-seq data. There were apparent peaks corresponding
479 to regions bound by ZmNAC128 and ZmNAC130 in the *O2* promoter (Figure 5C).
480 The peak encompassing binding region contains the maize ABSCISIC ACID
481 INSENSITIVE3 transcription factor 19 (ZmABI19)-binding RY motif (CATGCATG),
482 the O2/O11-binding core motif (ACGT), and three candidate
483 ZmNAC128/130-binding motifs (GTACGT, CTAGCTA, and TTGCTT) (Figure 5C).
484 To ascertain which candidate motif in the *O2* promoter can be recognized by
485 ZmNAC128 and ZmNAC130, we conducted an electrophoretic mobility shift assay
486 (EMSA) involving recombinant His-ZmNAC128 and His-ZmNAC130 and five
487 biotin-labeled probes covering a 151-bp region from –510 to –360 bp upstream from
488 the start codon of *O2* gene (Figure 5D). Only the P3 fragment was bound by
489 ZmNAC128 and ZmNAC130, to form retarded bands in the gel, and ChIP-qPCR also
490 confirmed this binding relationship (Figure 5E). The binding specificity of
491 ZmNAC128 and ZmNAC130 was furthermore verified through the addition of 50×
492 and 200× unlabeled P3 probes in the reactions, which caused a progressive loss of
493 retarded bands (Supplemental Figure S9).

494 Subsequently, GTACGT in the P3 was subjected to point mutation analysis. Any
495 point mutation could abolish the binding interaction of ZmNAC128 and ZmNAC130,
496 confirming GTACGT as an additional cis-element (Figure 5D). Finally, a DLR assay
497 was conducted to determine the transactivation ability of ZmNAC128 and
498 ZmNAC130 with respect to the *O2* promoter. Compared to the empty vector control,
499 LUC activities driven by the *O2* promoter were significantly increased by
500 ZmNAC128 or ZmNAC130, and the co-expression of the two NACs resulted in a
501 slightly but significantly stronger transactivation compared to each individual NAC
502 alone (Figure 5F). These results indicated that these two NACs play crucial roles in

503 directly activating the high expression of *O2*.

504 **Physical interactions of these three TFs**

505 To test the physical interaction between the three TFs, we performed a series of
506 *in vitro* and *in vivo* protein-protein interaction assays: pull-down, bimolecular
507 fluorescence complementation (BiFC), luciferase complementation imaging (LCI),
508 and co-immunoprecipitation (Co-IP) assays. The pull-down assay results showed that
509 glutathione S-transferase (GST)-tagged O2 interacts with His-tagged ZmNAC128 but
510 not ZmNAC130, while GST-tagged ZmNAC130 interacts with His-tagged
511 ZmNAC128 (Figure 6A). For BiFC and LCI assays, we cloned the coding sequences
512 of each TF-encoding gene in-frame with the sequence encoding the N-terminal or
513 C-terminal half of yellow fluorescent protein (YFP) or LUC, after which we
514 transformed the resulting constructs into Agrobacterium (*Agrobacterium tumefaciens*)
515 strain GV3101.

516 Unlike for the negative control with no visible signals, we detected YFP signals
517 and LUC activity in the *Nicotiana benthamiana* leaves co-infiltrated with *ZmNAC128*
518 and *O2* constructs as well as in those co-infiltrated with the *ZmNAC128* and
519 *ZmNAC130* constructs (Figure 6B, Supplemental Figure S10 and S11). We also
520 conducted a Co-IP assay in which we cloned *ZmNAC128* and *ZmNAC130* in-frame
521 with a sequence encoding 6×Myc, while *O2* and *ZmNAC130* were cloned in-frame
522 with a 3×Flag sequence, after which each construct was transiently co-infiltrated in *N.*
523 *benthamiana* leaves. We detected a band of the expected molecular weight for
524 Myc-ZmNAC128 in the protein extracts immunoprecipitated with anti-Flag
525 antibodies from leaves co-expressing *Flag-O2* (or *Flag-ZmNAC130*) and
526 *Myc-ZmNAC128* (Figure 6C). These results demonstrated that ZmNAC128 interacts
527 with O2, ZmNAC130, and itself, while ZmNAC130 interacts with ZmNAC128 and
528 itself.

529 **Combined effects of ZmNAC128, ZmNAC130, and O2 on the regulation of**
530 **endosperm filling**

531 To investigate the combined effects of the three TFs on the endosperm filling, we

generated a triple mutant *zmnac128 zmnac130 o2*. The two loss-of-function mutants of *O2* were identified by screening an ethyl methanesulfonate (EMS)-mutagenized B73 library and were backcrossed to B73 for several generations. We named the two mutant alleles *o2-1*, which has a premature stop codon in the first exon, and *o2-2*, which has an acceptor splice site mutation between the first intron and the second exon (Supplemental Figure S12). In this study, *o2-1* was used for crossing with *zmnac128 zmnac130*.

We isolated homozygous mutants for *zmnac128 zmnac130*, *o2*, and *zmnac128 zmnac130 o2* from the offspring of the same F_2 cob resulting from the *zmnac128 zmnac130* × *o2* cross. The triple mutant *zmnac128 zmnac130 o2* exhibited a more poorly filled kernel phenotype than did *zmnac128 zmnac130* (Figure 7A). Compared to that of the NT segregated from *zmnac128 zmnac130* × *o2* cross , the HKWs of *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2* decreased by 26%, 55%, and 73%, respectively (Figure 7B). Compared to the NT, the mature kernels of *zmnac128 zmnac130 o2* exhibited an 80% decrease in starch content and a 50% decrease in protein content (Figure 7C).

We further performed RNA-seq analysis of 16-DAP endosperms from *o2*, *zmnac128 zmnac130*, *zmnac128 zmnac130 o2*, and the NT. Principal component analysis showed that the transcriptome of *zmnac128 zmnac130 o2* is more similar to that of *zmnac128 zmnac130* than *o2* (Figure 8A). The number of DEGs was slightly higher in *zmnac128 zmnac130 o2* than in *zmnac128 zmnac130* but was twofold higher than in *o2* (Figure 8B). A Venn diagram of the DEGs also illustrated that more than 60% of all the DEGs were shared between *zmnac128 zmnac130 o2* and *zmnac128 zmnac130*, while less than 30% of DEGs were shared between *zmnac128 zmnac130 o2* and *o2* (Figure 8C). This suggested that the two NACs have stronger effects on endosperm filling than O2.

As these three TFs significantly impacted the transcriptome of filling-stage endosperms, it is likely that their shared downstream TFs play crucial roles in their GRN. We first detected their co-transactivation in their own promoters by a group of

DLR assays. O2 was previously reported to exhibit auto-transactivation (Lohmer et al., 1991 and Yang et al., 2022). Our results demonstrated that the *O2* promoter exhibited a stronger transactivation effect in the presence of all three TFs than when they acted individually, indicating a synergistic effect of their co-expression (Supplemental Figure S13). Transcriptome analysis indicated that *ZmNAC130* but not *ZmNAC128* is significantly downregulated in the *o2* mutant (Supplemental Figure S14A). DLR results revealed that O2 can transactivate the promoter of *ZmNAC130*, but not that of *ZmNAC128*, which is consistent with their respective expression patterns in *o2* (Supplemental Figure S14B).

Similar to the *O2* promoter, the promoters of *ZmNAC130* and *ZmNAC128* were found to be strongly activated by the co-expression of all three TFs (Supplemental Figure S14B). In addition, several TFs have been reported as the direct downstream genes of O2, including *bZIP G-box binding factor 1* (*GBP1*), *MYBR13*, *bZIP17*, and *NKD2* (Li et al., 2015 and Zhan et al., 2018). To detect which ones can also be the target genes of ZmNAC130 and ZmNAC128, we analyzed binding peaks of ZmNAC128 and ZmNAC130 in these four gene promoters. Only the *MYBR13* promoter was apparently bound by both ZmNAC128 and ZmNAC130 (Supplemental Figure S15A). DLR results indicated that all three TFs were capable of individually transactivating the *MYBR13* promoter, with the highest LUC activity observed in the co-expression of the three TFs (Supplemental Figure S15B). Consistent with the promoter transactivation, the expression of *MYBR13* was the lowest in *zmnac128 zmnac130 o2* (Supplemental Figure S15C), hinting at their synergistic regulation.

Combined effects of the three TFs on the regulation of zein synthesis

GO term enrichment analysis of the 581 DEGs common among the three mutants revealed that “nutrient reservoir activity” (GO: 0045735, *p*-value = 1.8E–18) was the most significantly enriched GO term (Supplemental Figure S16). This is consistent with the strong influence of O2, ZmNAC128, and ZmNAC130 on the expression of zein family genes. The expression of all zein genes was lower in *zmnac128 zmnac130 o2* than in *zmnac128 zmnac130* or *o2* (Supplemental Dataset S6). Except for 27-kD

γ-zein, most zein proteins were barely visible, as shown by Coomassie Brilliant Blue staining (Figure 8D). In view of the transactivation of O2 on the promoters of *27-kD γ-zein* and *50-kD γ-zein*, our further detection indicated that ZmNAC128 and ZmNAC130 can coordinate with O2 to enhance the transactivation of the promoter of *50-kD γ-zein* but not that of *27-kD γ-zein* (Supplemental Figure S17). Therefore, the synergistic effect of these three TFs on the expression of most zein genes seems similar to that of O2, PBF1, and OHPs, which do not involve direct transcriptional regulation (Zhang et al., 2015).

Combined effects of the three TFs on the regulation of starch synthesis

Consistent with the dramatic reduction in starch contents in mature *zmnac128 zmnac130 o2* kernels, starch accumulation decreased in the filling-stage endosperms of *zmnac128 zmnac130 o2* compared to *zmnac128 zmnac130* and *o2*, as evidenced by potassium iodide staining (Supplemental Figure S18). However, the expression of most starch metabolism genes and other carbohydrate metabolism genes was downregulated to a similar extent in *zmnac128 zmnac130 o2*, *zmnac128 zmnac130*, and *o2* (Supplemental Dataset S7). We further performed the immunoblotting assay to detect the protein accumulation of starch metabolism enzymes in 20-DAP endosperms from NT, *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2*. Consistent to the previous findings that O2 directly regulates the expression of *SSIII* and *PPDK* (Zhang et al., 2016), these two proteins and SSV were reduced in both *o2* and *zmnac128 zmnac130 o2* (Figure 8E). Zpu1, GBSS1, SS1, Bt2, and SSIIa were reduced in both *zmnac128 zmnac130* and *zmnac128 zmnac130 o2* (Figure 8E). The protein accumulation of more starch metabolism enzymes was lower in *zmnac128 zmnac130 o2* than in *zmnac128 zmnac130* or *o2*. Therefore, the two NACs and O2 separately regulated the expression of different starch metabolism genes. This could be one major cause for the relatively low amount of starch metabolism in *zmnac128 zmnac130 o2*.

Regulation of sugar and nutrient transport from maternal to filial tissue: ZmNAC128, ZmNAC130, and O2 regulate the expression of key transporter

genes in the BETL

Our RNA *in situ* hybridization results showed that *ZmNAC128* and *ZmNAC130* are expressed not only in the SE but also in the BETL (Figure 9A). This suggested that the two NACs possibly regulate the expression of nutrient transfer-related genes in the BETL. To date, two sugar transporters (ZmSWEET4c and ZmSUGCAR1, which are also responsible for K^+ uptake), one cell wall invertase (ZmMN1), and one zinc transporter (ZmYSL2) have been characterized as playing crucial roles in mother-to-endosperm transport in the BETL (Chao et al., 2022; Cheng et al., 1996; Guan et al., 2020; He et al., 2021; Sosso et al., 2015; Wei et al., 2021; Yang et al., 2022a; Zhou et al., 2021).

Transcriptome analysis of the whole 16-DAP endosperms, including the BETL, revealed that the expression of *ZmSWEET4c*, *ZmSUGCAR1*, *ZmYSL2*, and *ZmMN1* was significantly reduced by 8.7-, 7.2-, 6.2-, and 4.8-fold in *zmnac128 zmnac130* compared to the WT, respectively (Figure 9B). DAP-seq analysis indicated that there are peaks corresponding to regions bound by ZmNAC128 and ZmNAC130 in the promoters of *ZmSWEET4c* and *ZmSUGCAR*, but not in the promoters of *ZmMN1* and *ZmYSL2* (Figure 9C). We further found the cis-element GTACGT consistently around the peak binding regions in the *ZmSWEET4c* and *ZmSUGCAR1* promoters (Figure 9C). The ChIP-qPCR results confirmed the binding of ZmNAC128 and ZmNAC130 to the two promoters (Figure 9D). We found three GTACGT motifs in the *ZmSUGCAR* promoter, whereas ZmNAC128 and ZmNAC130 recognized only the second motif-containing region (Figure 9D).

Typically, the protein-DNA interactions could be disrupted by the flanking DNA sequence around the binding motif. For instance, there are two ACGCAA elements within a 1,000 bp region of the *Bt2* promoter upstream from the start codon, but ZmNAC128 and ZmNAC130 recognize only the one near the start codon (Zhang et al., 2019). Although our DAP-seq analysis did not find the peaks bound by ZmNAC128 and ZmNAC130 in the *ZmYSL2* promoter, ChIP-qPCR showed the interaction of ZmNAC128 and ZmNAC130 with the GTACGT-centered fragment in

the *ZmYSL2* promoter (Figure 9D). This result suggested that our DAP-seq might have missed some targets. We finally performed DLR assays to detect the transactivation of ZmNAC128 and ZmNAC130 on these four promoters. Indeed, ZmNAC128 or ZmNAC130 significantly enhanced the LUC activities driven by the promoters of *ZmSWEET4c*, *ZmSUGCAR1*, and *ZmYSL2* but not by the *ZmMN1* promoter, while their LUC activities were not higher upon co-expression of ZmNAC128 and ZmNAC130 (Figure 9E).

In view of the more severe endosperm-filling defect of *zmnac128 zmnac130 o2*, we also investigated whether O2 is involved in the regulation of the four genes (*ZmSWEET4c*, *ZmSUGCAR1*, *ZmYSL2*, and *ZmMN1*). Transcriptome analysis showed that the expression of only *ZmYSL2* was significantly downregulated by twofold in *o2*, although the expression of all four genes was downregulated in *zmnac128 zmnac130 o2* (Supplemental Figure S19A). DLR results indicated that O2 could enhance LUC activities driven by the promoters of *ZmSWEET4c* and *ZmSUGCAR1*. Meanwhile, ZmNAC128 and ZmNAC130 together with O2 could further enhance the transactivation activities driven by the promoters of *ZmSWEET4c*, *ZmSUGCAR1*, and *ZmYSL2* (Supplemental Figure S19B). Taken together, these results suggested that O2 and the two NACs synergistically co-activate these three transporter-encoding genes.

Combined effects of these three TFs on sugar and nutrient uptake

To detect whether the mutations of the three core TFs affect kernel nutrient uptake, we measured the contents of soluble sugars and elements transported by ZmSWEET4c, ZmSUGCAR1, and ZmYSL2 in the mature dry kernels of the NT, *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2*. The levels of soluble sugars (glucose, fructose, and sucrose) in the kernels could be affected by multiple factors, including BETL-related uptake ability and carbohydrate metabolism and accumulation. The levels of glucose, fructose, and sucrose were significantly increased in the kernels of *o2* compared to the NT (Figure 10A). These results are consistent with those corresponding to the previous determination of soluble sugars in *o2* kernels (Zhang et al., 2016), which is related to decreased starch biosynthesis.

However, although starch accumulation is lower in *zmnac128 zmnac130* than *o2*, the levels of soluble sugars are also lower in *zmnac128 zmnac130*.

In particular, the sucrose level is significantly decreased in *zmnac128 zmnac130* compared to the NT, which is similar to the effects that occur when *ZmSUGCAR1* is mutated (Yang et al., 2022). Conversely, the levels of these three soluble sugars are much higher in *zmnac128 zmnac130 o2* than *zmnac128 zmnac130* and *o2*, which is similar to what occurs in mutants due to the blockage of starch biosynthesis. This reflects the importance of the three core filling TFs in starch synthesis. Although the evaluation of sugar uptake is relatively complex, the loss-of-function mutations of *ZmNAC128* and *ZmNAC130* indeed affect kernel sugar uptake, especially for sucrose.

ZmSUGCAR1 and ZmYSL2 can transport K and Zn from maternal to endosperm respectively, so element analysis was also performed on the mutant kernels. Although there was no statistically significant change in the K level between *zmnac128 zmnac130* and the NT, a significant reduction in the K level was observed in *zmnac128 zmnac130* compared to *o2*.(Figure 10A). The mutation of *ZmNAC128* and *ZmNAC130* affects the expression of *ZmSUGCAR1* and in turn its transport ability for K and sucrose. The level of Zn, but not Fe, is significantly decreased in *zmnac128 zmnac130* compared to the NT and *o2*. This is consistent with the fact that ZmYSL2 is responsible for the uptake of Zn rather than Fe (Chao et al., 2022), while the Zn level was observed to be even lower in *zmnac128 zmnac130 o2* than *zmnac128 zmnac130* (Figure 10A).

Overall, O2, ZmNAC128, and ZmNAC130 affect the uptake of soluble sugars, Zn, and K potentially partly through the regulation of the expression of *ZmSWEET4c*, *ZmSUGCAR1*, and *ZmYSL2* (Figure 10B). By itself, O2 seems to have a very weak effect on the uptake of these nutrients, but it can enhance the function together with ZmNAC128 and ZmNAC130 to affect the transport abilities of ZmSWEET4c, ZmSUGCAR1, and ZmYSL2.

Discussion

The molecular basis of the genetic redundancy between *ZmNAC128* and *ZmNAC130*

ZmNAC128 and *ZmNAC130* are a pair of duplicated genes that arose from the allotetraploidization of maize (Zhang et al., 2019). The previous study suggested the functional redundancy of ZmNAC128 and ZmNAC130 because they regulate the expression of the same downstream genes (*16-kD γ-zein* and *Bt2*) by recognizing a common binding motif ACGCAA (Zhang et al., 2019). This study demonstrated the genetic redundancy of ZmNAC128 and ZmNAC130 by generating their knockout mutations. It is generally accepted that the two copies of duplicated genes can be retained by functional divergence or stronger functions (such as robustness) in the evolution (Huang et al., 2023). Undoubtedly, ZmNAC128 and ZmNAC130 commonly exert stronger functions in endosperm filling.

ZmNAC128 and ZmNAC130 shared high sequence similarities in both NAC and transactivation domains (Zhang et al., 2019), hinting at their similarities in structure and function. Indeed, the single mutations of *ZmNAC128* and *ZmNAC130* have little effect on the transcriptome (such as few DEGs) of filling endosperm, but their double mutant leads to thousands of DEGs. Protein-protein interaction experiments also indicated that these two NACs can form homo- or hetero-dimer. So when one NAC is absent, the other NAC can form a homodimer to function in filling endosperms. DLR assays were used to assess which kind of interaction exerts stronger functions, either homo- or hetero-dimerization. With the addition of *16-kD γ-zein* and *Bt2* (Zhang et al., 2019), the total fourteen direct downstream genes of ZmNAC128 and ZmNAC130 have been verified by their promoter transactivation. In general, the co-expression of ZmNAC128 and ZmNAC130 does not have stronger transactivation in many of these promoters than either of them alone, indicating that the two kinds of dimers have roughly equivalent abilities to activate the expression of their target genes.

On the other hand, although the single mutants *zmnac128* and *zmnac130* have no visible kernel phenotypes, their kernel starch contents are slightly reduced compared to the WT. It suggests that the absence of one NAC seems to have a weak effect on

their dimerization or polymerization in endosperm cells. Indeed, the co-expression of O2, ZmNAC128, and ZmNAC130 exhibited the highest transactivation activities on the promoters of many of their common target genes in all combinations. And according to the interaction of O2, ZmNAC128, and ZmNAC130, it is supported that ZmNAC128 and ZmNAC130 should form a heterodimer and interact with other co-factors (such as O2) to more effectively regulate complex endosperm filling (Figure 10B).

O2, ZmNAC128, and ZmNAC130 coordinate the synchronized biosynthesis of zeins and starch

The regulation of zein gene expression during endosperm filling has been comprehensively investigated (Dai et al., 2021; Yang et al., 2023). This study further broadens the understanding of the transcriptional regulation of zein gene expression. Unlike other TFs regulating the expression of zein genes, ZmNAC128 and ZmNAC130 specifically regulate the expression of all three (*16-*, *27-*, and *50-kD*) *γ-zein* genes and mediate the expression of other zein genes. However, with the exception of that of 16-kD γ-zein, the accumulation of other zein proteins is not apparently reduced in *zmnac128 zmnac130*. This is very similar to the regulatory ability of O2 on the expression of zein family genes. Although O2 regulates the expression of most zein genes (excluding *16-kD γ-zein*) (Li et al., 2015; Zhan et al., 2018), the accumulation of only α-zeins is apparently reduced in *o2*. The two NACs and O2 are the major regulators of the expression of *16-kD γ-zein* and *α-zein*, respectively, while they have minor effects on the regulation of other zein gene expression.

Although the protein accumulation of O2 is largely reduced in *zmnac128 zmnac130*, α-zein accumulation is not apparently reduced in this mutant. This suggested that partial expression of *O2* is sufficient to activate the expression of *α-zein* genes, while other TFs also possibly compensate for the reduction of O2 in *zmnac128 zmnac130*. In addition, all three TFs can transactivate the expression of the *27-kD γ-zein* gene, although the accumulation of the 27-kD γ-zein protein is not

apparently reduced in the triple mutant. Indeed, multiple TFs have been characterized to regulate the expression of *27-kD γ-zein* (Li et al., 2015; Li et al., 2018; Pysh et al., 1993; VicenteCarbajosa et al., 1997; Wu and Messing, 2012; Zhang et al., 2015), supporting the idea that 27-kD γ-zein is essential for PB formation and endosperm filling. The other TFs thus could compensate for the absence of O2, ZmNAC128, and ZmNAC130. Nevertheless, most zein proteins are almost undetectable in *zmnac128 zmnac130 o2*, demonstrating the core function of these three TFs in the regulation of zein synthesis.

Moreover, ZmNAC128 and ZmNAC130 directly regulated the expression of at least six important starch metabolism genes. The transcript and protein levels of other major starch metabolism genes were also reduced to varying degrees in *zmnac128 zmnac130*, indicating that ZmNAC128 and ZmNAC130 are critical TFs for starch metabolism. ZmNAC128 and ZmNAC130 play even more important roles than O2 in the synchronized biosynthesis of zein and starch. In agreement with this notion, compared with *zmnac128 zmnac130*, *zmnac128 zmnac130 o2* showed less accumulation of storage reserves and more poorly filled kernels. Moreover, these three TFs have no universally synergistic effect on the expression of their common target genes for the biosynthesis of starch and zeins. Perhaps the accumulation of the two major storage components in the endosperm is synergistically regulated through a more complex mechanism by the three core TFs, as they modulate the expression of hundreds or thousands of genes, thus imposing hierarchical regulation on other aspects of development.

Regulatory mechanism underlying the high expression of *O2*, *ZmNAC128*, and *ZmNAC130* during the endosperm-filling stage

Although O2 is a well-known core TF in filling endosperm, the mechanism of regulating *O2* expression is rather unclear. Two recent studies demonstrated that *ZmABI19* and *ZmbZIP29* are highly expressed in the early stage of kernel development and play initial roles in grain filling (Yang et al., 2021; Yang et al., 2022b). ZmABI19 initiates the expression of *O2* and *ZmNAC130*, while ZmbZIP29

and ZmABI19 synergistically transactivate the expression of *O2* upon abscisic acid treatment. Because *ZmABI19* and *ZmbZIP29* are expressed during the early stage, they function mainly as initial regulators of *O2* expression. Auto-activation of *O2* may play a role in activating its expression during the filling stage (Lohmer et al., 1991; Yang et al., 2021), but it is difficult to quantify this contribution. This investigation illustrated how ZmNAC128 and ZmNAC130 act as major regulators to control the expression of more than 50% of transcripts and proteins of *O2* across the entire filling stage via direct regulation. Moreover, the transactivation activity of O2 can be further enhanced in the presence of both NACs and O2. Therefore, these three TFs clearly play a decisive role in regulating the high expression of *O2* in the endosperm throughout the whole filling stage.

According to this investigation, although O2 also regulates the expression of *ZmNAC130*, it does not appear to be the core regulator for the expression of *ZmNAC128* and *ZmNAC130*. Because the contribution of auto-activation is hard to quantify with respect to the expression of *ZmNAC128* and *ZmNAC130*, their high expression in filling endosperm needs to be further investigated. Nevertheless, a hierarchical regulatory circuit among these three core TFs is a prerequisite for activating their high expression and endosperm filling.

The function of putative orthologs of ZmNAC128 and ZmNAC130 in the grains of other species

Grain filling involves two synchronized processes, i.e., maternal-to-filial nutrient transport and storage component synthesis. However, it was previously unknown whether the expression of the genes involved in these two distinct processes is coordinated by common TFs. Recent advances in the functional genomics of maize kernels prompted us to investigate the link between nutrient uptake and storage reserve synthesis. For instance, the maize nutrient uptake-related genes *ZmSWEET4c*, *ZmSUGCAR1*, and *ZmYSL2* are partly or specifically expressed in the filling endosperm, which is highly consistent with the expression pattern of genes responsible for the synthesis of starch and zeins. Here, we found that ZmNAC128 and

ZmNAC130 regulate the expression of important filling-related genes in both the BETL and the SE, which in turn facilitate nutrient uptake and storage reserve synthesis in the endosperm (Figure 10B).

There are no reports on the mechanism underlying the coordination of mother-to-endosperm nutrient transfer and storage reserve biosynthesis during grain filling in other cereals, such as rice (*Oryza sativa*) and wheat (*Triticum aestivum*). OsNAC20, OsNAC26, and TaNAC019, which are homologous to ZmNAC128 and ZmNAC130, were recently shown to coordinate the biosynthesis of starch and storage proteins in the endosperm (Liu et al., 2020; Wang et al., 2020; Gao et al., 2021), although it has yet to be tested whether they are involved in nutrient uptake. Based on DEG analysis of *OsNAC20*, *OsNAC26*, and *TaNAC019* mutants (Supplemental Figure S20), we preliminarily found that the expression of TraesCS6D02G012100 (a homolog of *ZmSWEET4c*) (Sosso et al., 2015) and TraesCSU02G13020 (a homolog of *ZmSUGCAR1*) (Yang et al., 2022a) is significantly downregulated in the *TaNAC019* mutant.

Like maize, wheat also has a typical transfer cell layer that mediates nutrient transfer from the mother plant to the grain. We hypothesize that TaNAC019 might also be involved in the regulation of endosperm nutrient uptake, although this hypothesis needs to be investigated. However, *OsSWEET4c*, *OsNPF7.9*, and *GRAIN INCOMPLETE FILLING1* (*GIF1*) (Wang et al., 2008) were not among the DEGs identified in the double mutant for *OsNAC20* and *OsNAC26*, indicating that their expression is not significantly altered in the mutant. This suggested that OsNAC20 and OsNAC26 are unlikely to be involved in the regulation of nutrient uptake, but this hypothesis also needs to be investigated.

In summary, ZmNAC128 and ZmNAC130 coordinate with O2 to promote endosperm filling, from nutrient uptake in the BETL to the synthesis of starch and zeins in the SE, while regulate their own expression (Figure 10B). Therefore, our findings greatly broaden our understanding of grain filling and have potential importance for improving grain-filling efficiency in breeding.

Materials and methods

Plant materials and growth conditions

The CRISPR/Cas9-mediated knockout lines of *ZmNAC128* and *ZmNAC130* were generated in the maize (*Zea mays*) KN5585 background by Agrobacterium (*Agrobacterium tumefaciens*)-mediated transformation as previously described (Frame et al., 2011). The T_1 plants were backcrossed to KN5585 and then self-pollinated to produce homozygous knockout lines without the CRISPR/Cas9 cassette, as determined by PCR identification. The two loss-of-function mutant lines for *O2* were identified from an EMS-mutagenized maize inbred B73 population. *o2-1* harbors a premature termination codon at the +417 position downstream from the first codon ATG. *o2-2* is an acceptor splice site mutation at the boundary between the first intron and the second exon. The *o2-1* mutant used in this study was backcrossed twice to the WT B73 and self-pollinated several times. The triple mutant *zmnac128 zmnac130 o2* was generated from an F_2 ear of *zmnac128 zmnac130* ×*o2-1*. The other three genotypes, NT, *o2*, and *zmnac128 zmnac130*, were also identified from the same F_2 ear. For the transgenic materials of the *27-kD γ-zein* promoter-driving *ZmNAC128* or *ZmNAC130* expression, the 3′ terminus for each gene was linked to a short nucleotide sequence encoding the 3×FLAG tag. The expression cassettes were transformed into the modified binary vector pTF102 with the GFP driven by the *10-kD δ-zein* promoter as the visible selection marker (Wu and Messing, 2012). The transgenic lines were also generated in the KN5585 background through Agrobacterium-mediated transformation. All maize materials were planted in the field of Hefei (Anhui Province, China) and Sanya (Hainan Province, China). *Nicotiana benthamiana* plants were cultivated in a chamber maintained at a constant temperature of 21°C. The humidity level inside the chamber was maintained at 50%-60%, and the plants were exposed to a 16-hour light cycle each day. The primers for construction and genotyping are listed in Supplemental Dataset S8.

Prediction of starch and protein content in the mature kernels

Mature dry kernels were milled into flour and then filtered with a 50-mesh stainless steel screen. The starch and protein content of the milled flour was predicted using a pre-constructed calibration model based on MPA-type Fourier transform near-infrared spectrophotometer (Bruker, Germany). The model calibration was based on 30 different varieties of maize flour samples, of which starch and protein content were determined by the Amyloglucosidase-α-Amylase Method (AOAC 996.11) and the Kjeldahl method (Lynch and Barbano, 1999), respectively. These sample spectra were collected on the MPA spectrophotometer and the modeling algorithm was partial least squares regression. The coefficient of determination (R^2) of starch and protein models were 0.826 and 0.984, respectively, and root-mean-square error of cross-validation (RMSECV) values were 0.533 and 0.123, respectively. The mature kernel flour of each material was divided into four portions for measurement. To calculate the percentage of starch and protein in a single kernel per genotype, the values were further calculated based on the kernel weight.

Determination of soluble sugars in the mature kernels

The determinations were performed according to the methods as previously reported (Deng et al., 2020). 50 mg of different fresh or matured maize endosperm powder were extracted with 2 mL of deionized water. After incubating the tubes for 30 min on ice, the supernatants were moved to a new 2 mL centrifuge tube after centrifugation at 13, 000 g for 15 min at 4 °C and then filtered with the 0.22 μm filter. The resulting filtered liquid was diluted tenfold with deionized water for measurement. The determinations were performed on the ion chromatography (ICS5000, Thermo Fisher Scientific) using a CarboPac PA-20 column (Nagamine and Komae, 1996). The mobile phase consisted of solvent A (deionized water) and solvent B (200 mM NaOH).

Iodine staining starch in the developing endosperm

The 16- and 20-DAP fresh kernels were longitudinally cut in half with a single-edge blade and the cut kernels were placed in the iodine solution (0.3 g of I_2 and 2 g of KI in 100 ml of deionized water in an amber glass bottle to protect from

light at room temperature) for 3 min. Immediately, the staining kernels were rinsed with deionized twice, and then observed and photographed under a stereomicroscope (LEICA S9i).

RNA *in situ* hybridization

The 12-DAP B73 kernels were used for RNA *in situ* hybridization. cDNA fragments of *ZmNAC128* and *ZmNAC130* were amplified, and their antisense and sense RNA probes were synthesized by *in vitro* transcription using T7 RNA polymerase with DIG RNA Labeling Mixture (Roche). Tissue processing and *in situ* hybridization experiments using 10-mm sections were performed according to the methods described previously (Zhang et al., 2015). A light microscope (Leica DM500) was used to observe paraffin-embedded sections of filling-stage kernels.

Element profiling

The elemental analysis was conducted by inductively coupled plasma mass spectrometry (ICP-MS) as previously described (Chao et al, 2022). This analysis was conducted in the mature kernels of *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2* as well as the corresponding wild type. Six dry mature kernels of each genotype were ground into powder by a tomized grinder with a grinding tank (material: polytetrafluoroethylene) and beads (material: Jargonia) manufactured by Jingxin Industry Co., Ltd., Shanghai. 2-5 mg of each sample was used for ICP-MS analysis. The elemental content by ICP-MS was measured following the method as previously described (Chao et al, 2022) and all the solid samples were normalized with a heuristic algorithm using the best-measured elements as previously described (Lahner et al, 2003).

Electron- and light-microscope observation

To observe endosperm texture and starch granules of the wild type and mutants, mature kernels were placed in a drying oven at 45℃ for at least 24 h and then cut along the longitudinal axis of the kernel. Subsequently, the endosperm texture and starch granules in the longitudinal section were observed under a scanning electron microscope (ZEISS Gemini SEM 500), following the instructions outlined on the

ZEISS Microscopy website (https://www.zeiss.com/microscopy/en/home.html). For kernel phenotype observations, the longitudinal sections of kernels were observed under a stereomicroscope (LEICA S9i).

Protein extraction, polyclonal antibody generation, and immunoblotting assay

Zein and non-zein proteins from endosperm and kernels were extracted as described previously (Zhang et al., 2015). The protein concentration was determined with a Compat-Able Protein Assay Preparation Reagent Kit and a BCA Protein Assay Kit (Pierce) according to the standard procedures. 10 μg of non-zein proteins in each sample was loaded for immunoblotting assays with the corresponding antibodies as described previously (Zhang et al., 2015). The antibody against O2 protein was previously generated (Yang et al., 2021). For the production of antibodies against starch metabolism enzymes in this study, their partial cDNAs (encoding 60-200 amino acids) were amplified by PCR and cloned into the pET51b expression vector. The recombinant plasmids were transformed into *Escherichia coli* BL21 Rosetta 2 (DE3) competent cells. Bacteria were grown at 37 °C until the absorbance at 600 nm (OD600) reached 0.6. Protein production was induced with 0.1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG). Bacteria were cultured at 37 °C for an additional 5 h before pelleting by centrifugation at 4,000 g. The fusion protein was purified on a Ni-NTA His Bind Resin (Novagen). The eluted proteins were dialyzed in PBS five times, and about 6 mg was sent to Shanghai Orizymes Biotech Company to produce the antibodies. Detailed information on antigens and their efficacy and specificity are in Supplemental Figure S21 and Supplemental Table S2.

Protein purification, pull-down, and Co-IP assay

For the pull-down assay, O2 and ZmNAC130 were fused to a GST tag, and ZmNAC128 and ZmNAC130 were fused to a His-TF tag. The encoding plasmids were transformed into *E. coli* Rosetta, and recombinant proteins were extracted and purified. GST, GST-ZmNAC128, and GST-ZmNAC130 were incubated with glutathione beads (GE Healthcare) at 4°C for 2–3 h; the beads were incubated with His-ZmNAC128 and His-ZmNAC130 at 4°C for 2 h. After washing and elution, the

proteins were separated by SDS-PAGE and subjected to immunoblot analysis with anti-GST (ProteinTech, No. 10000-0-AP, 1:5,000), anti-His (TransGen Biotech, HT501, 1:5,000), and anti-NAC (Shanghai Orizymes Biotech Company, PAB191213, 1:5,000).

For the Co-IP assay, O2 and ZmNAC130 were fused to 3xFlag, and ZmNAC128 and ZmNAC130 were fused to a 6xMyc tag. The encoding plasmids were transformed into Agrobacterium strain GV3101 and co-expressed in *Nicotiana benthamiana* leaves. Total proteins were extracted in IP buffer (150 mM NaCl, 12.7 mM KCl, 25 mM Na_2HPO_4, .5 mM KH_2PO_4 1, 10% [v/v] glycerol, 0.01 mM EDTA, 0.05% [v/v] NP-40, 1 mM PMSF, 5 mM DTT, and 1× protease inhibitor cocktail) at 4°C for 1 h and then incubated with Flag beads (Sigma–Aldrich, M8823-1 mL) at 4°C for 2–3 h. The beads were washed three to four times with washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% [v/v] NP-40). After washing, the eluted proteins were separated by SDS-PAGE and subjected to immunoblot analysis with anti-Myc (Cell Signaling Technology, 2278S, 1:2,000) and anti-Flag (Sigma–Aldrich, F1804, 1:2,000) antibodies. The above protein bands were visualized using a Tanon-5200M imaging system. The primers are listed in Supplemental Dataset S8.

BiFC and LCI assays

O2, ZmNAC128, and ZmNAC130 were fused with the N-terminal or C-terminal half of YFP or luciferase (LUC). The encoding plasmids were transformed into Agrobacterium strain GV3101 and then co-expressed in *N. benthamiana* leaves. YFP fluorescence was visualized using confocal microscopy (ZEISS980; Carl Zeiss). YFP signals were excited at 514 nm, and emission was detected in the range of 520-545 nm with an intensity value of approximately 0.5% and a gain value of around 600. For LCI assays, leaf samples were infiltrated with 1× luciferin, and luciferase signals were detected using a Tanon-5200M imaging system. The primers are listed in Supplemental Dataset S8.

RNA extraction and reverse transcription quantitative PCR

Total RNA was extracted from 12-, 16-, 20-, 24-, and 28-DAP endosperm using

TRIzol reagent (Invitrogen) and purified with an RNeasy Mini Kit after DNase I digestion (Qiagen). First-strand cDNA was then generated with a SuperScript III First Strand Kit (Invitrogen). RT-qPCR was performed with SYBR Green (Takara) on a CFX Connect Real-Time PCR system (Bio-Rad) according to the standard operating manual. The comparative CT (ΔΔCT) method was employed for the relative quantification of gene expression (Livak and Schmittgen, 2001), with *ACTIN* serving as the reference. The primers are listed in Supplemental Dataset S8.

RNA-seq

The details for RNA-seq were as described previously. Total RNA was extracted from 16-DAP maize kernel endosperm using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and quality of total RNA (from two or three independent groups of endosperms for each genotype) were determined on a NanoDrop™ 2000/2000c Spectrophotometer. Sequencing libraries were generated by Novogene Co., Ltd. using a NEBNext®Ultra™ RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations; index codes were added to attribute sequences to each sample. The index-coded samples were clustered using a HiSeq 4000 PE Cluster Kit (Illumina) according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on an Illumina HiSeq 4000 platform as 150-bp paired-end reads. The clean reads were aligned to the B73 reference genome (RefGen_v5) through the HISAT2 program (Kim et al., 2015). Differentially expressed genes (DEGs) were identified as meeting the criteria of an absolute value of $\log_2$(fold-change) > 1 and adjusted *p*-value < 0.05 by the R package DESeq (Love et al., 2014).

DAP-seq

The *in vitro* DAP-seq was performed as described previously, with some minor modifications (Allen et al., 2006; O'Malley et al., 2016; Bartlett et al., 2017). Halo-tagged transcription factors (O2, ZmNAC128, and ZmNAC130) and recombinant proteins were produced in a TNT Coupled Wheat Germ Extract System (Promega). Genomic DNA was extracted from 8-, 12-, and 16-DAP maize kernels

using a modified cetyltrimethylammonium bromide (CTAB) extraction method, purified with a FastPure Gel DNA Extraction Mini Kit (Vazyme), and sonicated to ~200-bp fragments. After end-repair and adenylation, the fragmented gDNA was then purified using AMPure® XP Beads (Beckman Coulter). The Halo-tagged-TFs and Halo proteins were bound to Magne HaloTag Beads (Promega) and subsequently incubated with the fragmented gDNA library for 1 h on a rotator at room temperature. The beads were then washed three times, and the eluted DNA was ligated to an indexed adaptor for sequencing analysis. For peak analysis, the mapped reads and peak files were examined using Integrative Genomics Viewer. Enriched motifs were identified by MEME motif discovery software (http://meme-suite.org/). The primers are listed in Supplemental Dataset S8.

ChIP-qPCR

The 16-DAP developing endosperms of *ZmNAC128-3×Flag* and *ZmNAC130-3×Flag* transgenic lines were fixed with 1% (v/v) formaldehyde (Sigma) in phosphate buffer as described previously (Yang et al., 2021). Antibodies against 3×Flag peptide (F1804, Sigma) and Rabbit IgG control (Sigma, 12-370) were used for immunoprecipitation. The experimental procedure is the same as described previously (Yang et al., 2021; Li et al., 2015). The immunoprecipitated DNA was used for qPCR experiments of target DNA fragments which were performed with SYBR Green (Takara) on a CFX Connect Real-Time PCR system (Bio-Rad) according to the standard operating manual. Relative enrichment of target DNA fragments was calculated by analyzing the immunoprecipitated samples of anti-Flag compared to that of anti-IgG. *ACTIN* is the internal reference gene in the detected samples with three biological replicates. The primers are listed in Supplemental Dataset S8.

EMSA

Oligonucleotide probes of the *O2* promoter were synthesized and labeled with biotin at the 3′ end with a Biotin 3′ End DNA Labeling Kit (Thermo) according to the standard procedures. Each probe was mixed with purified recombinant protein at 25°C for 20 min in reaction buffer (20 μL) containing 10× binding buffer, 50% (v/v)

glycerol, 100 mM $MgCl_2$, 1 μg/μL poly(dI-dC), 50 mM KCl, and 1% (v/v) NP-40. Biotin-labeled DNA was detected according to the instructions of the LightShift Chemiluminescent EMSA Kit (Thermo). The luminescence was visualized on a Tanon-5200M imaging system.

DLR assay

Maize protoplasts were prepared from the leaves of two-weeks seedlings of the inbred B73. The protoplast isolation, polyethylene glycol (PEG)-calcium transfection of plasmid DNA, and protoplast culture were described previously (Zhang et al., 2015). The vector pRI101 (Clontech) was used for the expression of *O2*, *ZmNAC128*, and *ZmNAC130* under the control of the 35S promoter. The transient expression vector pGreenII 0800-LUC was used to generate the reporter constructs by cloning the promoters of different target genes upstream of LUC. The LUC/REN activity ratio was measured using a Dual-luciferase Reporter Assay System (Promega). The primers are listed in Supplemental Dataset S8.

Statistical analysis

Data processing of means, standard deviations, and *p*-values was performed with Microsoft Excel (2016): AVERAGE, STDEV.S, and Student's *t*-test, respectively. The one-way ANOVA test in the SPSS software was employed to determine whether there are significant differences among three or more groups. Immunoblotting signals in each band were quantified by ImageJ (https://imagej.nih.gov/ij/). The *p*-value and false discovery rate (FDR) of differentially expressed genes in the RNA-seq data were calculated by the DESeq2R package (v 1.28.1 with default arguments). The raw data and detailed statistical analysis are in Supplemental Dataset S9.

Accession numbers

ZmNAC128: Zm00001eb126890; *ZmNAC130*: Zm00001eb334180; *O2*: Zm00001eb301570; *16-kD γ-zein*: Zm00001eb099950; *27-kD γ-zein*: Zm00001eb313800; *50-kD γ-zein*: Zm00001eb313790; *Sus1*: Zm00001eb392880; *SSV*: Zm00001eb191890, *Bt2*: Zm00001eb176800; *SSIIa*: Zm00001eb279740; *GBSSI*: Zm00001eb378140; *SS1*: Zm00001eb376100; *Zpu1*: Zm00001eb088740; *SSIII*:

Zm00001eb413290; *Sh2*: Zm00001eb159060; *SBEIIb*: Zm00001eb242610; *PPDK1*: Zm00001eb287770; *PPDK2*: Zm00001eb349810; *Sh1*: Zm00001eb374090; *ISA1*: Zm00001eb174590; *Bt1*: Zm00001eb235570; *ZmSWEET4c*: Zm00001eb236810; *ZmSUGCAR1*: Zm00001eb304390; *ZmYSL2*: Zm00001eb248990; *ZmMN1*: Zm00001eb083790; *ACTIN*: Zm00001eb348450. The RNA-seq and DAP-seq data in this article are available from the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the series entries PRJNA913969 for RNA-seq data and PRJNA914049 for DAP-seq data.

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Author contributions

Z.Z. supervised the project. Z.Z., E.C., J.H., and D.P. designed the experiments and wrote the manuscript. E.C., H.Y., J.H., and D.P. performed the experiments. P.Z., S.P., X.W., J.W., and C.J. assisted with the field work, material sampling, and identification. Z.C. and D.C. measured and analyzed the element levels in the kernels. Z.X. and Y.W. analyzed seed composition. Y.W. helped conduct the project.

Tables

Table 1. Summary of promoter analysis of starch metabolism genes and their expression in *nacRNAi* and *zmnac128 zmnac130*.

Gene name	Binding peaks	Cis-element ACGCAA	*nacRNAi*		*zmnac128 zmnac130*	
			mRNA	Protein	mRNA	Protein
Bt2	Y	Y	down.	reduced	down.	reduced
Zpu1	Y	Y	down.	n.d.	down.	reduced
GBSS1	Y	Y	down.	n.d.	down.	reduced
SS1	Y	Y	down.	n.d.	down.	reduced
Sus1	Y	Y	down.	n.d.	down.	n.d.
SBEIIb	Y	N	down.	n.d.	down.	reduced
SSIIa	Y	Y	n.s.	n.d.	down.	reduced
ISA1	N	N	n.s.	n.d.	down.	reduced
Sh2	N	Y	n.s.	n.d.	n.s.	reduced
Sh1	N	Y	n.s.	n.d.	n.s.	reduced
SSIII	N	Y	n.s.	n.d.	n.s.	reduced
SSV	N	Y	n.s.	n.d.	n.s.	reduced
Bt1	Y	N	n.d.	n.d.	n.s.	reduced
SBE1	N	N	down.	n.d.	n.s.	n.s.

Note: The data of nacRNAi from Zhang et al., 2019. The data of *zmnac128 zmnac130* from this study. Y, Yes (binding peaks detected in the promoter); N, No (ACGCAA

element absent in the promoter); down., downregulated; n.d., no detection; n.s., not significantly downregulated or reduced.

Figure legends

Figure 1. Knockout mutation of *ZmNAC128* and *ZmNAC130* causes poorly filled kernel phenotypes.

(A) Kernel phenotype of KN5585 (wild type, WT), *zmnac128*, *zmnac130*, and *zmnac128 zmnac130*. Top, mature kernel longitudinal sections; middle and bottom, scanning electron microscope of kernel longitudinal sections shown above. Middle, kernel peripheral region; bottom, kernel inner region.

(B) The hundred kernel weight (HKW) of mature kernels of the three mutants compared to the WT. g, gram.

(C) NIR analysis conducted to determine the percentage of protein and starch accounting for kernel weight. The calibration of the NIR model utilized mature kernel flours from various maize varieties, as explained in detail in the 'Materials and Methods' section. The protein or starch content in each kernel is expressed as a percentage (%) of kernel weight, calculated by comparing NIR-derived value to kernel weight. Data represent means ± standard deviation (SD) of at least three independent samples for each genotype (**B** and **C**). Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

Figure 2. RNA-seq combined with DAP-seq investigating the direct targets of ZmNAC128 and ZmNAC130.

(A) Number of upregulated and downregulated expressed genes in *zmnac128*, *zmnac130*, and *zmnac128 zmnac130*, compared to the WT. DEGs were identified based on fold-change ≥ 2 and p-value ≤ 0.05.

(B) Significantly enriched GO terms and KEGG pathways based on DEGs of *zmnac128 zmnac130*. GO:0046982 (protein heterodimerization activity); GO:0045735 (nutrient reservoir activity); ko00010 (glycolysis/gluconeogenesis); ko00500 (starch

and sucrose metabolism); ko01200 (carbon metabolism); ko00620 (pyruvate metabolism); ko00710 (carbon fixation in photosynthetic organisms); ko00520 (amino sugar and nucleotide sugar metabolism); ko00630 (glyoxylate and dicarboxylate metabolism); ko00030 (pentose phosphate pathway); ko00052 (galactose metabolism); ko01230 (biosynthesis of amino acids); ko00260 (glycine, serine and threonine metabolism); ko01212 (fatty acid metabolism).

(C) Distribution of ZmNAC128 and ZmNAC130 binding regions in the maize genome. **(D)** Metaplots showing the distribution of ZmNAC128 and ZmNAC130 binding peaks per 100-bp bin corresponding to the –1,000- to +2,000-bp region flanking the transcription start site (TSS).

(E) IGV shows the distribution of ZmNAC128- and ZmNAC130-binding peaks in the promoters of *16-kD γ-zein* and *Bt2*. For ZmNAC128, ZmNAC130, and IgG (control), two independent experiments were performed. Aligned reads are indicated in red (ZmNAC128), orange (ZmNAC130), or gray (IgG). The y-axis (or peaks) represents the number (or accumulation) of the aligned reads. The relative positions of the element ACGCAA are marked by arrowheads.

(F) The most significant elements of ZmNAC128 and ZmNAC130 according to MEME-ChIP analysis.

Figure 3. Verification of *27-kD γ-zein* and *50-kD γ-zein* as the direct targets of ZmNAC128 and ZmNAC130.

(A) IGV shows the peaks bound by ZmNAC128 and ZmNAC130 in these two promoters. The relative positions of the element ACGCAA are marked by arrowheads.

(B) ChIP-qPCR detects the *in vivo* binding activities of ZmNAC128 and ZmNAC130 to these two promoters. The red lines under the arrowheads in (**A**) indicate the region detected by ChIP-qPCR. ChIP products of IgG were used as a negative control. *ACTIN* was used as an internal control.

(C) DLR detects the transactivation activities of ZmNAC128 and ZmNAC130 on the two promoters. The results were also shown in **Supplemental Figure S17**. Data represent means ± SD of three independent samples for each test (**B** and **C**).

Significance differences (***$p < 0.001$) were determined by a Student's *t*-test (**B**). Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$) (**C**).

Figure 4. Verification of starch metabolism genes as direct targets of ZmNAC128 and ZmNAC130.

(A) Immunoblotting of protein accumulation for the major starch metabolism enzymes in 20-DAP endosperms of *zmnac128 zmnac130* and WT. The 20 ng non-zein proteins per lane were loaded, while ACTIN served as the loading control. The original and intact images were shown in **Supplemental Figure S21**. Three independent samples for each material were performed and quantified by the ImageJ software. For each starch metabolism enzyme, protein abundance in *zmnac128 zmnac130* was normalized to the corresponding WT, which was set to 1.

(B) IGV shows binding peaks in the promoters of genes transcriptionally downregulated in *zmnac128 zmnac130*. The relative positions of the cis-element ACGCAA are marked by arrowheads.

(C) ChIP-qPCR detects the *in vivo* binding activities of ZmNAC128 and ZmNAC130 to the five promoters. The red lines under the arrowheads in (**B**) indicate the region detected by ChIP-qPCR.

(D) DLR detects the transactivation activities of ZmNAC128 and ZmNAC130 on these five promoters. Data represent means ± SD of three independent samples for each test (**A**, **C,** and **D**). Significance differences (**$p < 0.01$ and ***$p < 0.001$) were determined by a Student's *t*-test (**A** and **C**). Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$) (**D**).**Figure 5. ZmNAC128 and ZmNAC130 regulate the expression of *O2* in the filling endosperm.**

(A) RT-qPCR of *O2* expression in the filling endosperms of *zmnac128 zmnac130* and WT. Relative expression was normalized to *ACTIN*. Data represent means ± SD of three independent samples for each time point except for two samples at 28 DAP. Statistical significance was determined by a Student's *t*-test. *$p < 0.05$, **$p < 0.01$,

1274 ***$p < 0.001$.

1275 **(B)** Immunoblotting of O2 protein in the filling endosperms of *zmnac128 zmnac130*
1276 and WT. The 20 ng non-zein proteins per lane were loaded. The quantification was
1277 shown in **Supplemental Figure S8**.

1278 **(C)** IGV shows the peaks bound by ZmNAC128 and ZmNAC130 in the *O2* promoter.
1279 The relative region covering four candidate elements, CATGCATG, GTACGT,
1280 CTAGCTA, and TTGCTT, are marked by a red line in the promoter.

1281 **(D)** EMSA detects an additional cis-element of ZmNAC128 and ZmNAC130 in the
1282 *O2* promoter. Upside: five biotin-labeled probes in the region of -510 to -360
1283 upstream from the start codon are listed, and candidate cis-elements in the probe
1284 sequences are highlighted in red font. Underside: the 6-bp GTACGT box in the P3
1285 probe (WT) and three mutant probes were produced by 2-bp mutations in the 6-bp
1286 box.

1287 **(E)** ChIP-qPCR detects the *in vivo* binding activity of ZmNAC128 and ZmNAC130
1288 to the *O2* promoter. A red line in the *O2* promoter in (**C**) indicates the region detected
1289 by ChIP-qPCR.

1290 **(F)** DLR detects the transactivation activities of ZmNAC128 and ZmNAC130 on the
1291 *O2* promoter. Data represent means ± SD of three independent samples for each test
1292 (**E** and **F**). Significance differences (**$p < 0.01$ and ***$p < 0.001$) were determined
1293 by a Student's *t*-test (**E**). Different lowercase letters indicate significant differences
1294 according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$)
1295 (**F**).

1296 **Figure 6. *In vitro* and *in vivo* protein interaction assays show the physical**
1297 **interaction of O2, ZmNAC128, and ZmNAC130.**

1298 **(A)** GST pull-down detects the interactions between recombinant purified proteins of
1299 O2, ZmNAC128, and ZmNAC130.

1300 **(B)** BiFC detects the interactions between O2, ZmNAC128, and ZmNAC130.
1301 Negative controls in **Supplemental Figure S11**. BF, bright field. Scale bars, 50 μm.

1302 **(C)** Co-IP detects the interactions between O2, ZmNAC128, and ZmNAC130. The

indicated plasmids (harboring Flag or Myc tags) were transformed and subsequently co-expressed in *N. benthamiana* leaves. The Flag-fused proteins were immunoprecipitated, and the precipitates were probed with anti-Flag or anti-Myc antibodies.

Figure 7. The triple mutation of *O2*, *ZmNAC128*, and *ZmNAC130* causes a more poorly filled kernel phenotype.

(A) Mature cobs and kernel longitudinal sections of *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2* compared to the NT in the KN5585 × B73 background.

(B) The hundred-kernel weight (HKW) analysis of the four genetic materials.

(C) Determination of protein and starch in the mature kernels from the four genetic materials. Data represent means ± SD of at least three independent samples for each genotype (**B** and **C**). Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$) (**B** and **C**).

Figure 8. O2, ZmNAC128, and ZmNAC130 synergistically affect the expression of zeins and starch metabolism enzymes.

(A) Principal component analysis based on transcriptome data of the four genetic materials. There were three independent samples for each genotype.

(B) Number of DEGs in *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2* compared to the NT. DEGs were identified based on fold-change ≥ 2 and *p*-value ≤ 0.05.

(C) Venn diagram shows the overlap between these DEGs.

(D) Coomassie Brilliant Blue staining SDS-PAGE of zein proteins in mature kernels. The total zein proteins loaded in each lane are equal to 200 μg of mature kernel flour. Each band was indicated by the corresponding type of zein. 50γ, 50-kD γ-zein; 27γ, 27-kD γ-zein; 22α, 22-kD α-zein; 19α, 19-kD α-zein; 16γ, 16- kD γ-zein; 15β, 15-kD β-zein; 10δ, 10-kD δ-zein.

(E) Immunoblotting of protein accumulation of starch metabolism enzymes in the 20-DAP endosperm. The 20 ng non-zein proteins for each lane were loaded, while

ACTIN was the loading control.

Figure 9. ZmNAC128 and ZmNAC130 regulate the expression of *ZmSWEET4c*, *ZmSUGCAR1*, and *ZmYSL2*.

(A) RNA *in situ* hybridization of *ZmNAC128* and *ZmNAC130* in 12-DAP B73 kernels. Antisense probes were used to detect the spatial expression of *ZmNAC128* and *ZmNAC130* transcripts. Sense probes were the negative controls. SE, starchy endosperm; AL, aleurone; BETL, basal endosperm transfer layer. Scale bars, 100 μm.

(B) Expression levels of *ZmSWEET4c*, *ZmSUGCAR1*, *ZmYSL2*, and *ZmMN1* in the 16-DAP endosperms of *zmnac128 zmnac130* versus WT in the KN5585 background. Expression levels are shown as FPKM (fragments per kilobase of exon per million mapped reads). Statistical significance was determined by DESeq2R. $^{**}p < 0.01$; $^{***}p < 0.001$.

(C) IGV shows the peaks bound by ZmNAC128 and ZmNAC130 in the four promoters. The relative positions of the element GTACGT are marked by arrowheads.

(D) ChIP-qPCR detects the in vivo binding activities of ZmNAC128 and ZmNAC130 to the promoters of *ZmSWEET4c*, *ZmSUGCAR1*, and *ZmYSL2*. The red lines under the arrowheads in (**C**) indicate the region detected by ChIP-qPCR. Data represent means ± SD of three independent samples for each test. Statistical significance was determined by a Student's *t*-test. $^{**}p < 0.01$; ns, not significant.

(E) DLR detects the transactivation activities of ZmNAC128, ZmNAC130, and O2 on the four promoters. The data was also shown in **Supplemental Figure S19B** combined with the transactivation of O2, ZmNAC128, and ZmNAC130 on the four promoters. Data represent means ± SD of three independent samples for each test. Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

Figure 10. Mutations in *O2*, *ZmNAC128*, and *ZmNAC130* influence soluble sugar and element levels in kernels, and a working model of these three TFs facilitates endosperm filling.

(A) Determination of soluble sugars (glucose, fructose, and sucrose) and elements (Zn,

Fe, and K) in the mature kernels of NT, *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2* in the KN5585 × B73 background. Data represent means ± SD of three independent samples in sugar measurement and six independent samples in element measurement for each genotype. Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

(B) A working model of O2, ZmNAC128, and ZmNAC130 facilitates endosperm filling. Left panel, a cartoon illustration depicts the cooperation of the three TFs to promote endosperm filling. Maternal nutrients are transported via the BETL into the starchy endosperm for synthesis and deposition of storage reserves (starch and zeins). Right panel, the major targets of ZmNAC128 and ZmNAC128 have been characterized in this study except for *16-kD γ-zein* and *Bt2* which have been previously investigated (Zhang et al., 2019). Arrows indicate direct transcriptional regulation.

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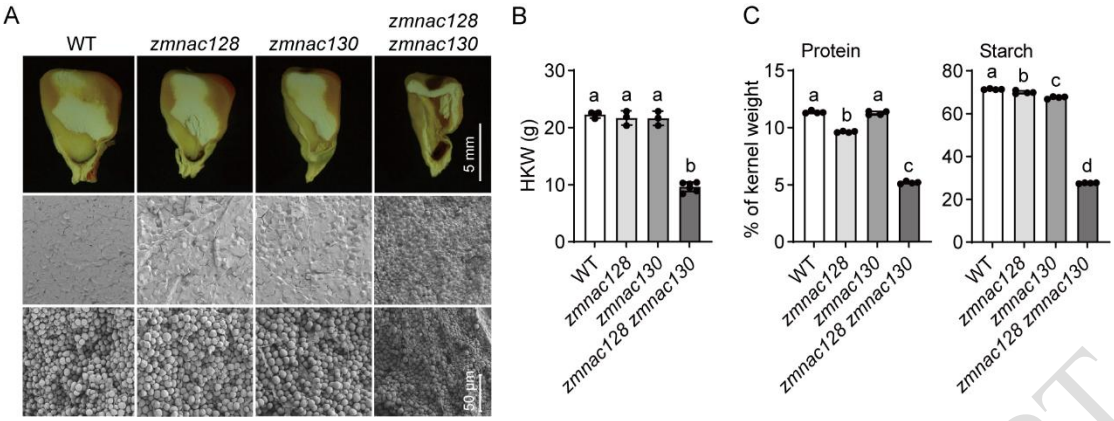

Figure 1. Knockout mutation of *ZmNAC128* and *ZmNAC130* causes poorly filled kernel phenotypes.

(A) Kernel phenotype of KN5585 (wild type, WT), *zmnac128*, *zmnac130*, and *zmnac128 zmnac130*. Top, mature kernel longitudinal sections; middle and bottom, scanning electron microscope of kernel longitudinal sections shown above. Middle, kernel peripheral region; bottom, kernel inner region.

(B) The hundred kernel weight (HKW) of mature kernels of the three mutants compared to the WT. g, gram.

(C) NIR analysis conducted to determine the percentage of protein and starch accounting for kernel weight. The calibration of the NIR model utilized mature kernel flours from various maize varieties, as explained in detail in the 'Materials and Methods' section. The protein or starch content in each kernel is expressed as a percentage (%) of kernel weight, calculated by comparing NIR-derived value to kernel weight. Data represent means ± standard deviation (SD) of at least three independent samples for each genotype (**B** and **C**). Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

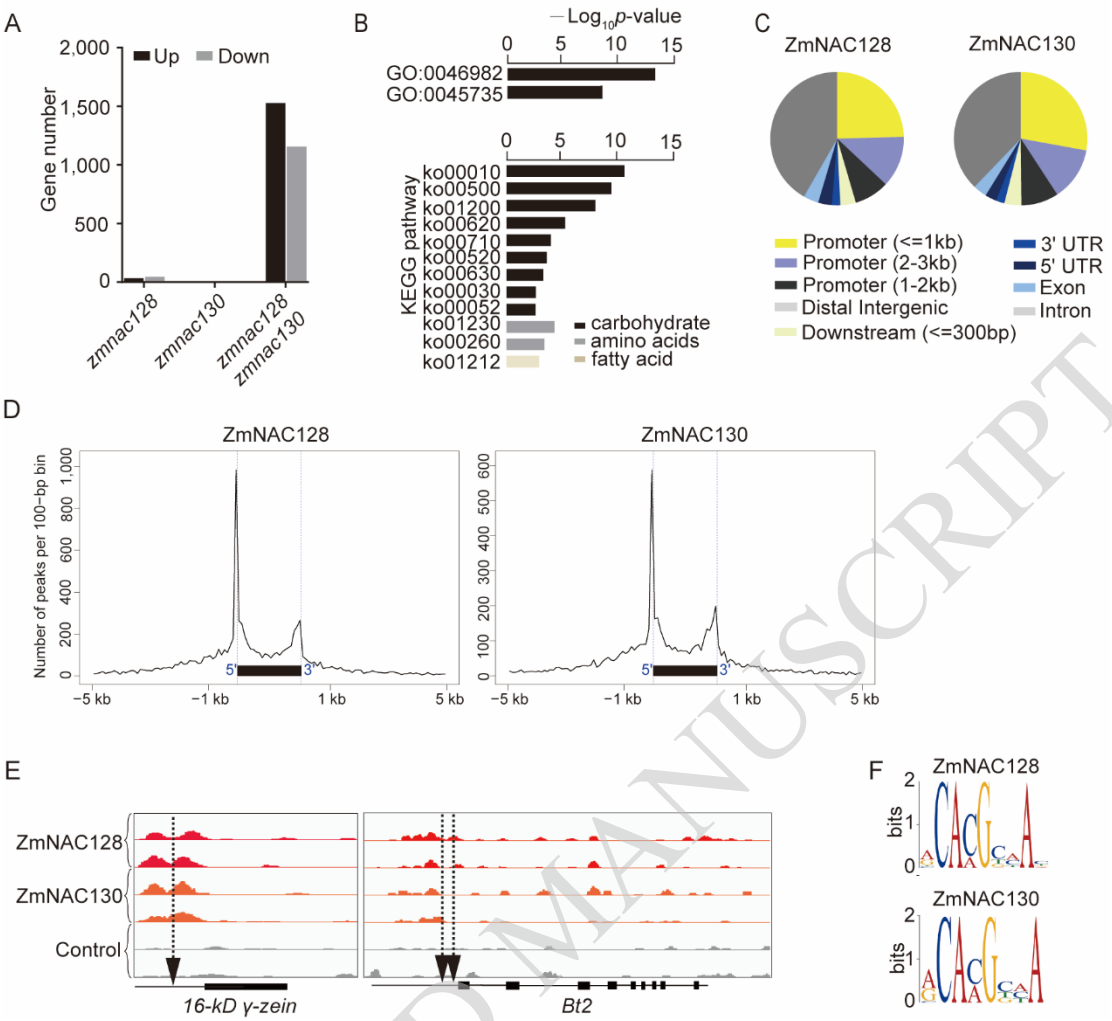

Figure 2. RNA-seq combined with DAP-seq investigating the direct targets of ZmNAC128 and ZmNAC130.

(A) Number of upregulated and downregulated expressed genes in *zmnac128*, *zmnac130*, and *zmnac128 zmnac130*, compared to the WT. DEGs were identified based on fold-change ≥ 2 and *p*-value ≤ 0.05.

(B) Significantly enriched GO terms and KEGG pathways based on DEGs of *zmnac128 zmnac130*. GO:0046982 (protein heterodimerization activity); GO:0045735 (nutrient reservoir activity); ko00010 (glycolysis/gluconeogenesis); ko00500 (starch and sucrose metabolism); ko01200 (carbon metabolism); ko00620 (pyruvate metabolism); ko00710 (carbon fixation in photosynthetic organisms); ko00520 (amino sugar and nucleotide sugar metabolism); ko00630 (glyoxylate and dicarboxylate metabolism); ko00030 (pentose phosphate pathway); ko00052 (galactose metabolism); ko01230 (biosynthesis of amino acids); ko00260 (glycine, serine and threonine metabolism); ko01212 (fatty acid metabolism).

(C) Distribution of ZmNAC128 and ZmNAC130 binding regions in the maize genome. **(D)** Metaplots showing the distribution of ZmNAC128 and ZmNAC130 binding peaks per 100-bp bin corresponding to the –1,000- to +2,000-bp region flanking the transcription start site (TSS).

(E) IGV shows the distribution of ZmNAC128- and ZmNAC130-binding peaks in the promoters of *16-kD γ-zein* and *Bt2*. For ZmNAC128, ZmNAC130, and IgG (control), two independent

experiments were performed. Aligned reads are indicated in red (ZmNAC128), orange (ZmNAC130), or gray (IgG). The y-axis (or peaks) represents the number (or accumulation) of the aligned reads. The relative positions of the element ACGCAA are marked by arrowheads. **(F)** The most significant elements of ZmNAC128 and ZmNAC130 according to MEME-ChIP analysis.

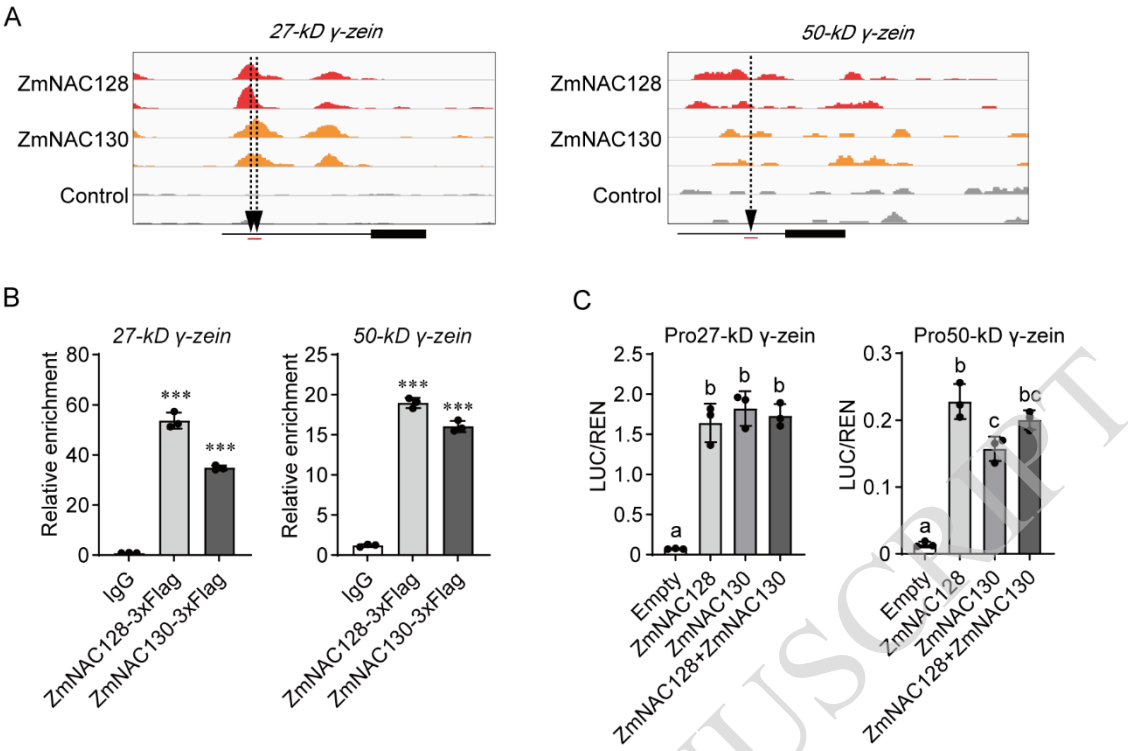

Figure 3. Verification of *27-kD γ-zein* and *50-kD γ-zein* as the direct targets of ZmNAC128 and ZmNAC130.

(A) IGV shows the peaks bound by ZmNAC128 and ZmNAC130 in these two promoters. The relative positions of the element ACGCAA are marked by arrowheads.

(B) ChIP-qPCR detects the *in vivo* binding activities of ZmNAC128 and ZmNAC130 to these two promoters. The red lines under the arrowheads in (**A**) indicate the region detected by ChIP-qPCR. ChIP products of IgG were used as a negative control. *ACTIN* was used as an internal control.

(C) DLR detects the transactivation activities of ZmNAC128 and ZmNAC130 on the two promoters. The results were also shown in **Supplemental Figure S17**. Data represent means ± SD of three independent samples for each test (**B** and **C**). Significance differences (***p < 0.001) were determined by a Student's *t*-test (**B**). Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test (p < 0.05) (**C**).

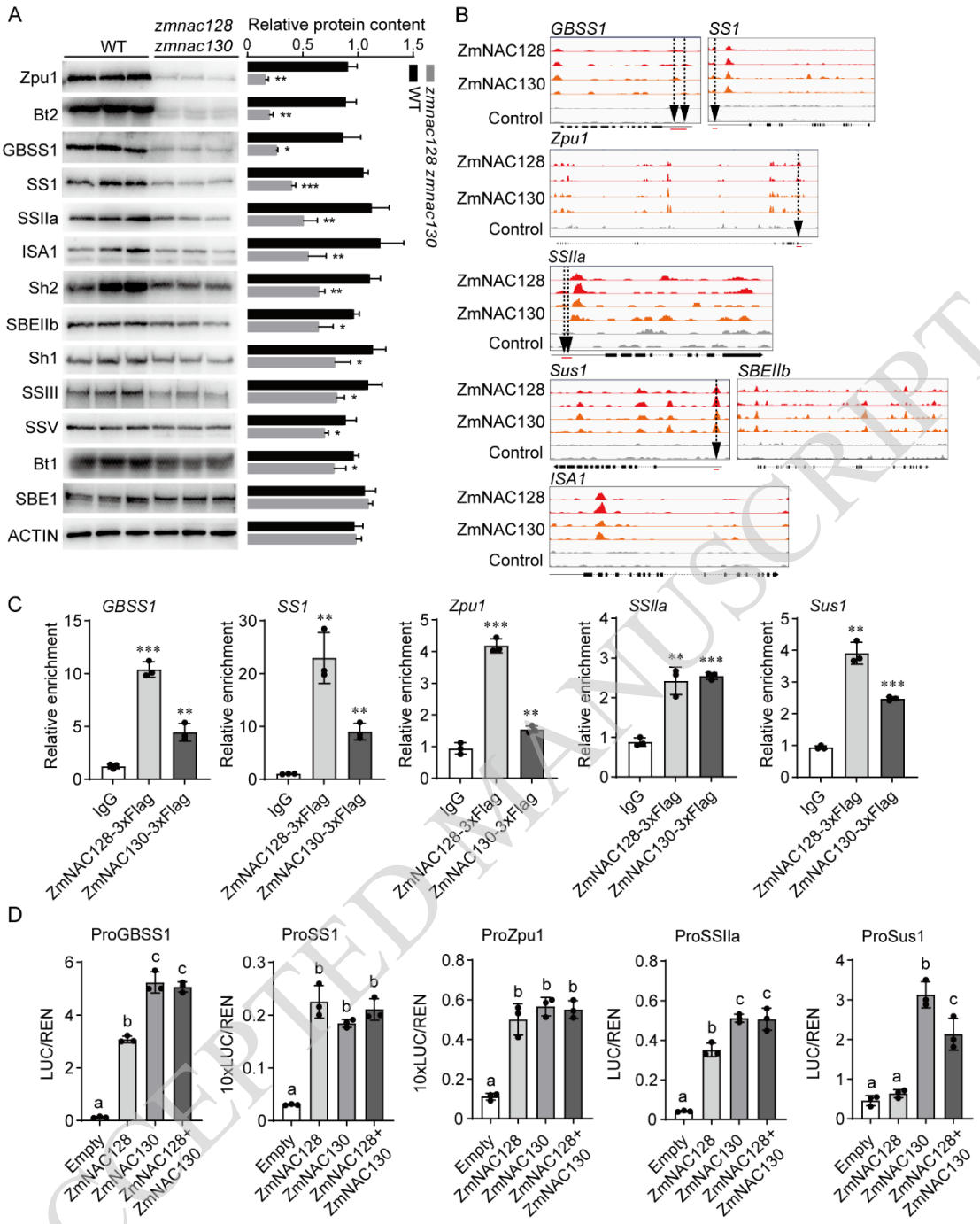

Figure 4. Verification of starch metabolism genes as direct targets of ZmNAC128 and ZmNAC130.

(A) Immunoblotting of protein accumulation for the major starch metabolism enzymes in 20-DAP endosperms of *zmnac128 zmnac130* and WT. The 20 ng non-zein proteins per lane were loaded, while ACTIN served as the loading control. The original and intact images were shown in **Supplemental Figure S21**. Three independent samples for each material were performed and quantified by the ImageJ software. For each starch metabolism enzyme, protein abundance in *zmnac128 zmnac130* was normalized to the corresponding WT, which was set to 1.

(B) IGV shows binding peaks in the promoters of genes transcriptionally downregulated in *zmnac128 zmnac130*. The relative positions of the cis-element ACGCAA are marked by arrowheads.

(C) ChIP-qPCR detects the *in vivo* binding activities of ZmNAC128 and ZmNAC130 to the five promoters. The red lines under the arrowheads in (**B**) indicate the region detected by ChIP-qPCR.

(D) DLR detects the transactivation activities of ZmNAC128 and ZmNAC130 on these five promoters. Data represent means ± SD of three independent samples for each test (**A, C,** and **D**). Significance differences (**$p < 0.01$ and ***$p < 0.001$) were determined by a Student's *t*-test (**A** and **C**). Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$) (**D**).

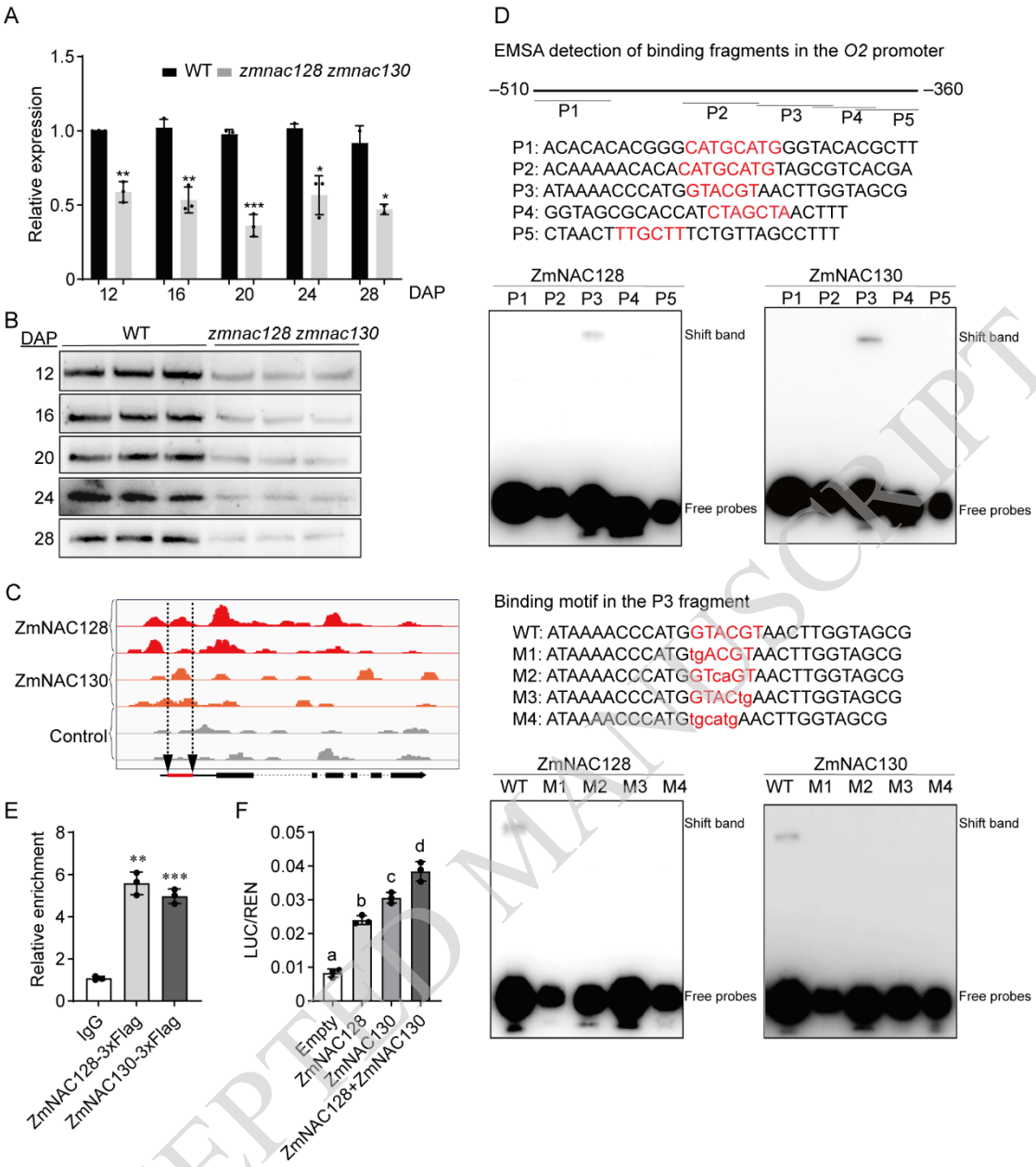

Figure 5. ZmNAC128 and ZmNAC130 regulate the expression of *O2* in the filling endosperm.

(A) RT-qPCR of *O2* expression in the filling endosperms of *zmnac128 zmnac130* and WT. Relative expression was normalized to *ACTIN*. Data represent means ± SD of three independent samples for each time point except for two samples at 28 DAP. Statistical significance was determined by a Student's *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

(B) Immunoblotting of O2 protein in the filling endosperms of *zmnac128 zmnac130* and WT. The 20 ng non-zein proteins per lane were loaded. The quantification was shown in **Supplemental Figure S8**.

(C) IGV shows the peaks bound by ZmNAC128 and ZmNAC130 in the *O2* promoter. The relative region covering four candidate elements, CATGCATG, GTACGT, CTAGCTA, and TTGCTT, are marked by a red line in the promoter.

(D) EMSA detects an additional cis-element of ZmNAC128 and ZmNAC130 in the *O2* promoter. Upside: five biotin-labeled probes in the region of -510 to -360 upstream from the start codon are listed, and candidate cis-elements in the probe sequences are highlighted in red font. Underside: the 6-bp GTACGT box in the P3 probe (WT) and three mutant probes were produced by 2-bp mutations in the 6-bp box.

(E) ChIP-qPCR detects the *in vivo* binding activity of ZmNAC128 and ZmNAC130 to the *O2* promoter. A red line in the *O2* promoter in (**C**) indicates the region detected by ChIP-qPCR.

(F) DLR detects the transactivation activities of ZmNAC128 and ZmNAC130 on the *O2* promoter. Data represent means ± SD of three independent samples for each test (**E** and **F**). Significance differences (**$p < 0.01$ and ***$p < 0.001$) were determined by a Student's *t*-test (**E**). Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$) (**F**).

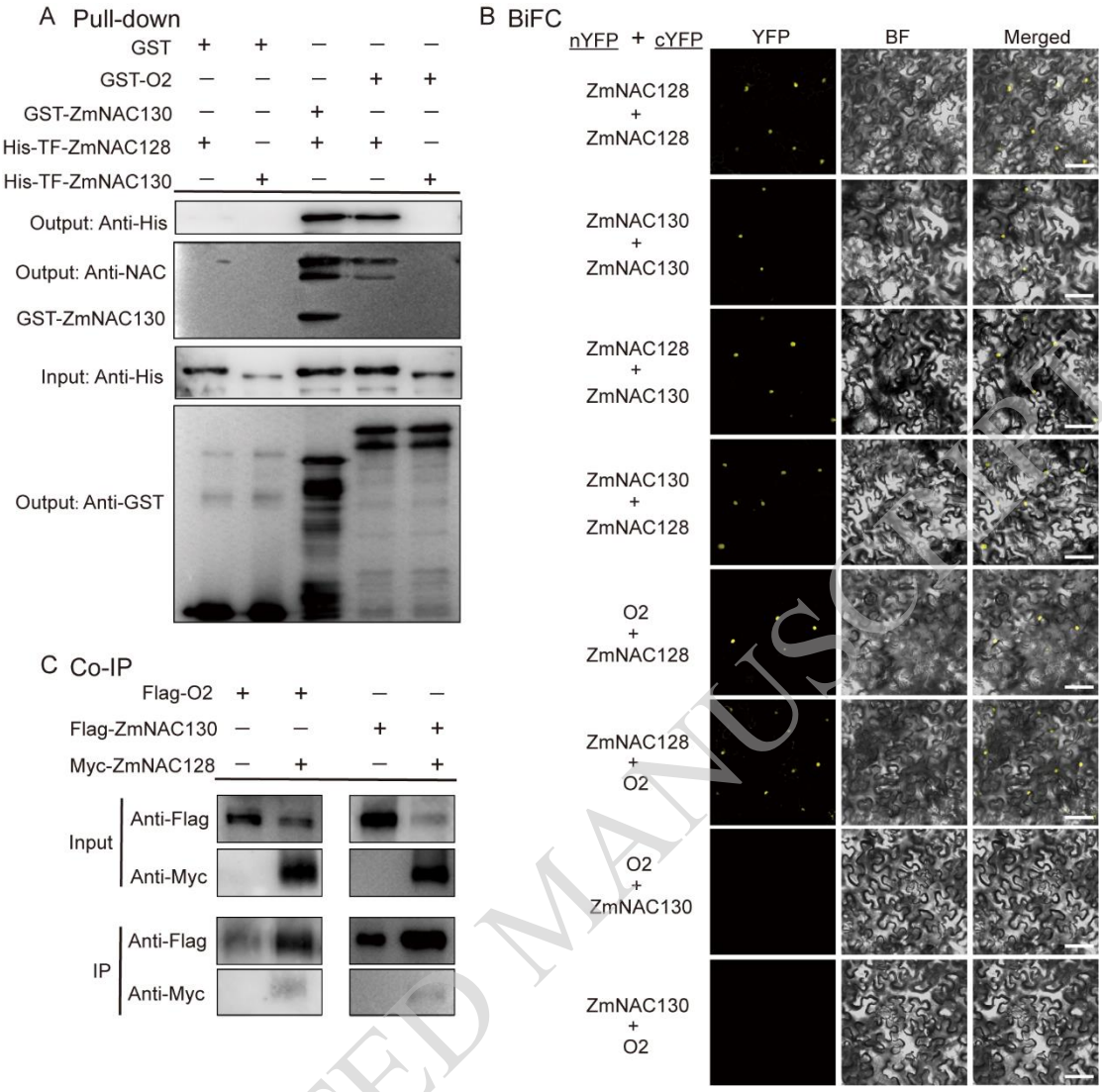

Figure 6. *In vitro* and *in vivo* protein interaction assays show the physical interaction of O2, ZmNAC128, and ZmNAC130.

(A) GST pull-down detects the interactions between recombinant purified proteins of O2, ZmNAC128, and ZmNAC130.

(B) BiFC detects the interactions between O2, ZmNAC128, and ZmNAC130. Negative controls in **Supplemental Figure S11**. BF, bright field. Scale bars, 50 μm.

(C) Co-IP detects the interactions between O2, ZmNAC128, and ZmNAC130. The indicated plasmids (harboring Flag or Myc tags) were transformed and subsequently co-expressed in *N. benthamiana* leaves. The Flag-fused proteins were immunoprecipitated, and the precipitates were probed with anti-Flag or anti-Myc antibodies.

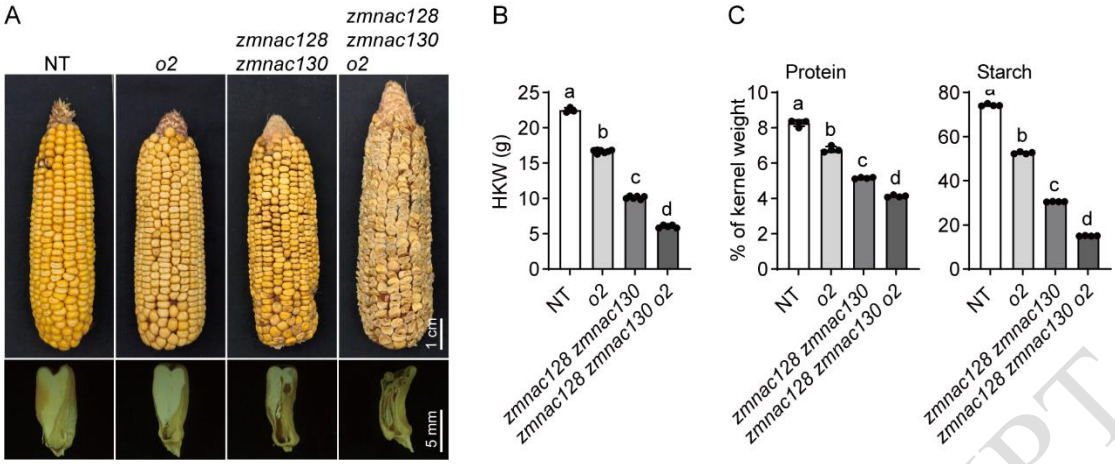

Figure 7. The triple mutation of *O2*, *ZmNAC128*, and *ZmNAC130* causes a more poorly filled kernel phenotype.

(A) Mature cobs and kernel longitudinal sections of *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2* compared to the NT in the KN5585 × B73 background.

(B) The hundred-kernel weight (HKW) analysis of the four genetic materials.

(C) Determination of protein and starch in the mature kernels from the four genetic materials. Data represent means ± SD of at least three independent samples for each genotype (**B** and **C**). Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$) (**B** and **C**).

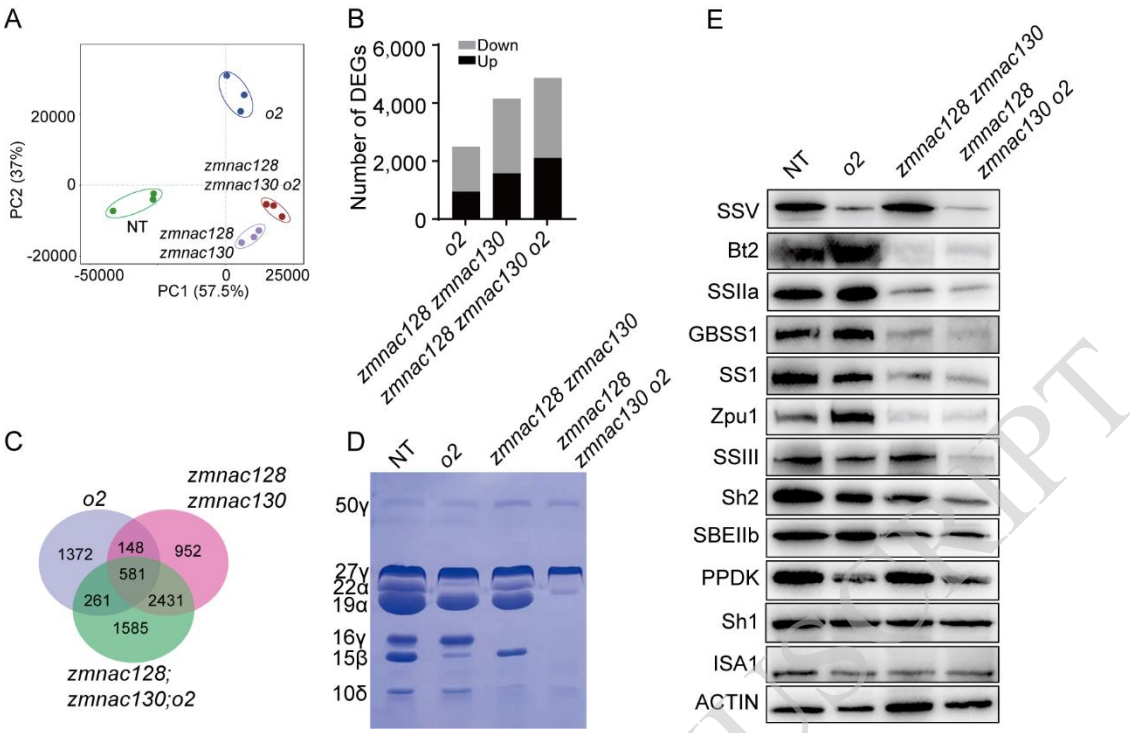

Figure 8. O2, ZmNAC128, and ZmNAC130 synergistically affect the expression of zeins and starch metabolism enzymes.

(A) Principal component analysis based on transcriptome data of the four genetic materials. There were three independent samples for each genotype.

(B) Number of DEGs in *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2* compared to the NT. DEGs were identified based on fold-change ≥ 2 and *p*-value ≤ 0.05.

(C) Venn diagram shows the overlap between these DEGs.

(D) Coomassie Brilliant Blue staining SDS-PAGE of zein proteins in mature kernels. The total zein proteins loaded in each lane are equal to 200 μg of mature kernel flour. Each band was indicated by the corresponding type of zein. 50γ, 50-kD γ-zein; 27γ, 27-kD γ-zein; 22α, 22-kD α-zein; 19α, 19-kD α-zein; 16γ, 16- kD γ-zein; 15β, 15-kD β-zein; 10δ, 10-kD δ-zein.

(E) Immunoblotting of protein accumulation of starch metabolism enzymes in the 20-DAP endosperm. The 20 ng non-zein proteins for each lane were loaded, while ACTIN was the loading control.

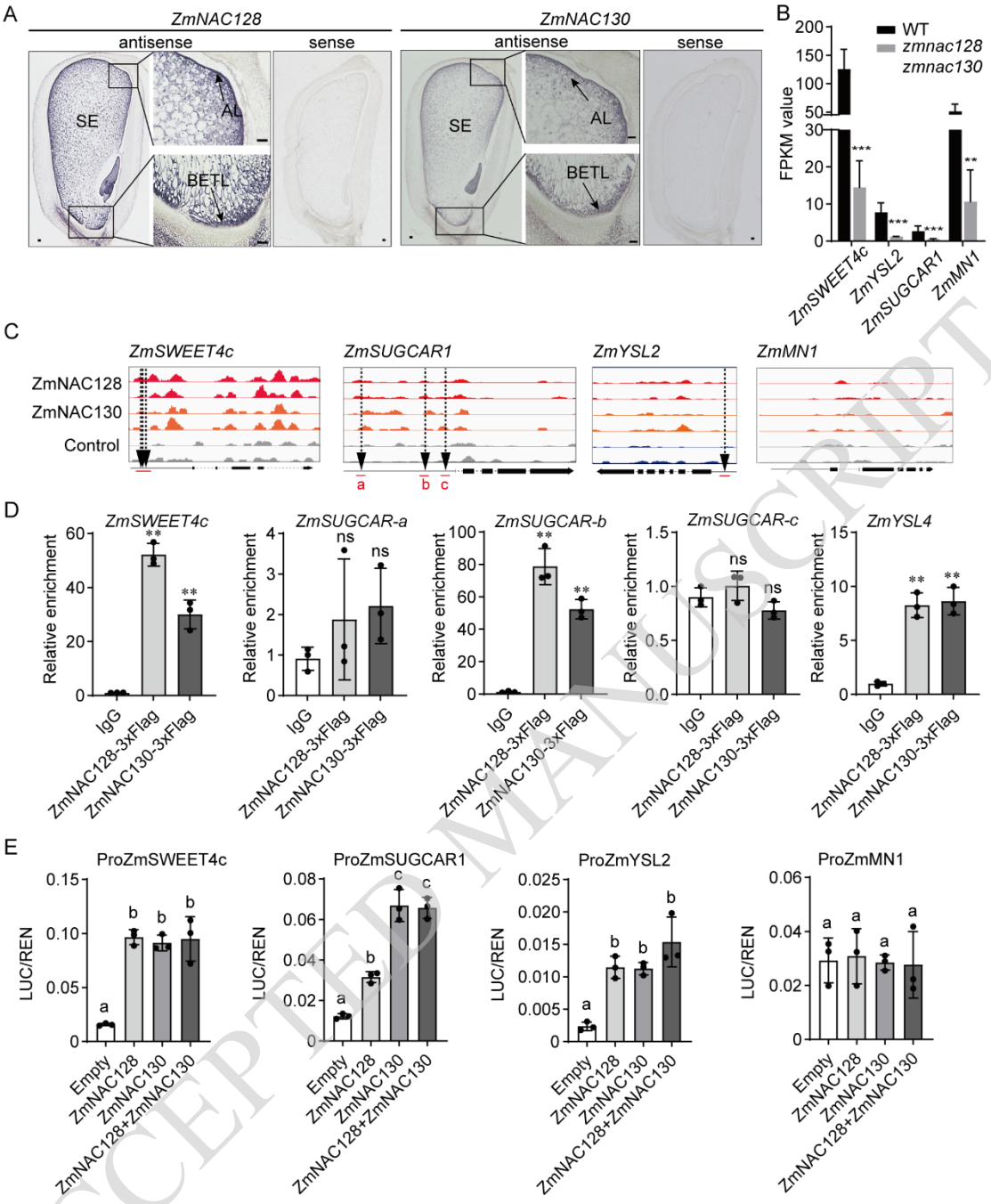

Figure 9. ZmNAC128 and ZmNAC130 regulate the expression of *ZmSWEET4c*, *ZmSUGCAR1*, and *ZmYSL2*.

(A) RNA *in situ* hybridization of *ZmNAC128* and *ZmNAC130* in 12-DAP B73 kernels. Antisense probes were used to detect the spatial expression of *ZmNAC128* and *ZmNAC130* transcripts. Sense probes were the negative controls. SE, starchy endosperm; AL, aleurone; BETL, basal endosperm transfer layer. Scale bars, 100 μm.

(B) Expression levels of *ZmSWEET4c*, *ZmSUGCAR1*, *ZmYSL2*, and *ZmMN1* in the 16-DAP endosperms of *zmnac128 zmnac130* versus WT in the KN5585 background. Expression levels are shown as FPKM (fragments per kilobase of exon per million mapped reads). Statistical significance was determined by DESeq2R. $^{**}p < 0.01$; $^{***}p < 0.001$.

(C) IGV shows the peaks bound by ZmNAC128 and ZmNAC130 in the four promoters. The relative positions of the element GTACGT are marked by arrowheads.

(D) ChIP-qPCR detects the *in vivo* binding activities of ZmNAC128 and ZmNAC130 to the promoters of *ZmSWEET4c*, *ZmSUGCAR1*, and *ZmYSL2*. The red lines under the arrowheads in (**C**) indicate the region detected by ChIP-qPCR. Data represent means ± SD of three independent samples for each test. Statistical significance was determined by a Student's *t*-test. $^{**}p < 0.01$; ns, not significant.

(E) DLR detects the transactivation activities of ZmNAC128, ZmNAC130, and O2 on the four promoters. The data was also shown in **Supplemental Figure S19B** combined with the transactivation of O2, ZmNAC128, and ZmNAC130 on the four promoters. Data represent means ± SD of three independent samples for each test. Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

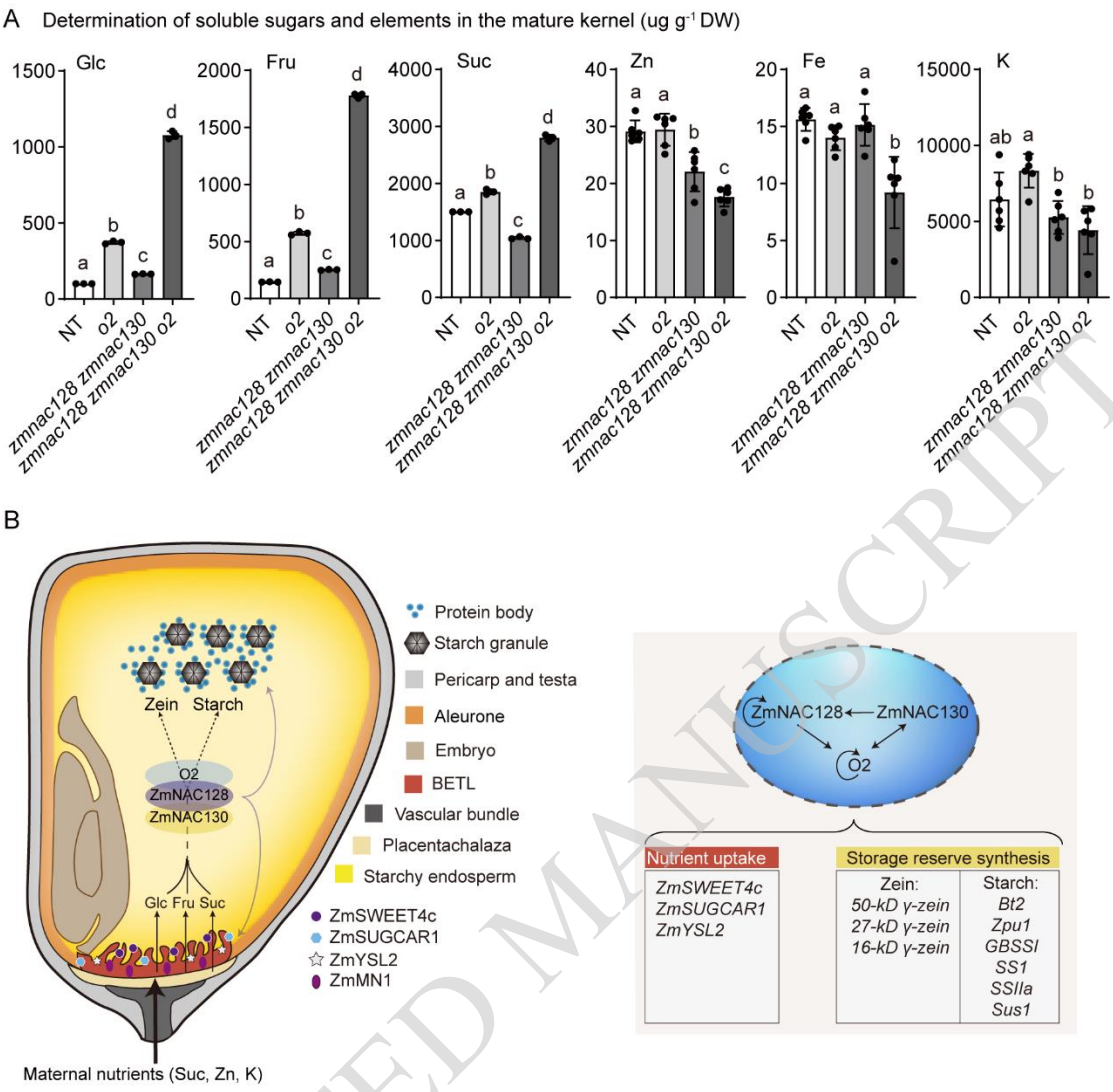

Figure 10. Mutations in *O2*, *ZmNAC128*, and *ZmNAC130* influence soluble sugar and element levels in kernels, and a working model of these three TFs facilitates endosperm filling.

(A) Determination of soluble sugars (glucose, fructose, and sucrose) and elements (Zn, Fe, and K) in the mature kernels of NT, *o2*, *zmnac128 zmnac130*, and *zmnac128 zmnac130 o2* in the KN5585 × B73 background. Data represent means ± SD of three independent samples in sugar measurement and six independent samples in element measurement for each genotype. Different lowercase letters indicate significant differences according to a one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

(B) A working model of O2, ZmNAC128, and ZmNAC130 facilitates endosperm filling. Left panel, a cartoon illustration depicts the cooperation of the three TFs to promote endosperm filling. Maternal nutrients are transported via the BETL into the starchy endosperm for synthesis and deposition of storage reserves (starch and zeins). Right panel, the major targets of ZmNAC128 and ZmNAC128 have been characterized in this study except for *16-kD γ-zein* and *Bt2* which have been previously investigated (Zhang et al., 2019). Arrows indicate direct transcriptional regulation.

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