RESEARCH ARTICLE

NKD Transcription Factors are Central Regulators of Maize Endosperm Development

Bryan C. Gontarek\textsuperscript{a,b}, Anjanasree K. Neelakandan\textsuperscript{b}, Hao Wu\textsuperscript{b} and Philip W. Becraft\textsuperscript{a,b,c,1}

\textsuperscript{a}Plant Biology Program, Iowa State University, Ames, Iowa 50011
\textsuperscript{b}Genetics, Development & Cell Biology Department, Iowa State University, Ames, Iowa 50011
\textsuperscript{c}Agronomy Department, Iowa State University, Ames, Iowa 50011
\textsuperscript{1}Corresponding Author: becraft@iastate.edu.

Short title: NKD Control of Endosperm Development

One sentence summary: The NKD transcription factors regulate diverse and key pathways in developing maize endosperm, including cell fate, storage compound accumulation and seed maturation.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Philip W. Becraft (becraft@iastate.edu).

ABSTRACT

NAKED ENDOSPERM1 (NKD1) and NKD2 are duplicate INDETERMINATE DOMAIN (IDD) transcription factors important for maize (Zea mays) endosperm development. RNAseq analysis of the \textit{nkd1 nkd2} mutant endosperm revealed that NKD1 and NKD2 influence 6.4\% of the transcriptome in developing aleurone and 6.7\% in starchy endosperm. Processes regulated by NKD1 and NKD2 include gene expression, epigenetic functions, cell growth and division, hormone pathways and resource reserve deposition. The NKD1 and NKD2 proteins bind a consensus DNA sequence of TTGTCGT with slightly different properties. This motif was enriched in the promoters of gene transcripts differentially expressed (DE) in mutant endosperm. DE genes with a NKD binding motif in the 5′ promoter region were considered as likely direct targets of NKD1 and NKD2 regulation and these putative direct target genes were notably enriched for storage proteins. Transcription assays demonstrate that NKD1 and NKD2 can directly regulate gene transcription, including activation of \textit{opaque2} and \textit{viviparous1} promoters. NKD2 functions as a negative regulator of \textit{nkd1} transcription, consistent with previously reported feedback regulation. NKD1 and NKD2 can homo- and heterodimerize through their ID domains. These analyses implicate NKD1 and NKD2 as central regulators of gene expression in developing maize endosperm.
INTRODUCTION

Cereal endosperm nourishes the developing embryo and germinating seedling, composes a major portion of human and livestock diets, and has important industrial applications. Maize endosperm consists of seven cell types as defined by histology and gene expression patterns (Leroux et al., 2014; Zhan et al., 2015). In mature kernels, the major cell types include starchy endosperm (SE), basal endosperm transfer (BET) cells and aleurone (AL) (Becraft and Gutierrez-Marcos, 2012). SE cells constitute the majority of the endosperm mass and function in nutrient reserve storage, primarily starch and protein. BET cells transport nutrients from maternal tissue into the developing endosperm. AL is important for digestion and remobilization of stored reserves during germination as well as mineral storage and pathogen defense (Stewart et al., 1988; Fath et al., 2000; Jerkovic et al., 2010).

Endosperm development begins with the formation of the coenocyte followed by cellularization. Subsequent cell differentiation involves the perception and response to positional cues that specify the different cell fates (Becraft and Asuncion-Crabb, 2000; Geisler-Lee and Gallie, 2005; Gruis et al., 2006). Maturation entails genomic endoreduplication and the accumulation of resource reserves in SE, acquisition of desiccation tolerance in AL, culminating in programmed cell death of the SE and BET cells, metabolic quiescence of the AL, and desiccation of the grain as a whole (Sabelli and Larkins, 2009; Becraft and Gutierrez-Marcos, 2012).

The duplicate genes naked endosperm1 (nkd1) and nkd2 encode INDETERMINATE DOMAIN (IDD) proteins (Yi et al., 2015), and the nkd1 nkd2 double mutant shows pleiotropic effects, including multiple layers of peripheral endosperm cells with compromised cell identity, decreased anthocyanin accumulation, opaque and floury endosperm texture, decreased carotenoid accumulation, decreased kernel dry weight, and occasional vivipary (Becraft and Asuncion-Crabb, 2000; Yi et al., 2015). These phenotypes indicate that nkd1 and nkd2 functions are required for cell patterning and differentiation, resource reserve deposition and seed maturation. In wild type (WT),
nkd1 and nkd2 transcripts accumulate in both AL and SE, consistent with the pleiotropic phenotype (Yi et al., 2015).

IDD proteins are a plant-specific family of transcription factors (TFs) whose members function in a broad range of developmental and signaling processes (Morita et al., 2006; Welch et al., 2007; Tanimoto et al., 2008; Hassan et al., 2010; Feurtado et al., 2011; Ogasawara et al., 2011; Seo et al., 2011b; Cui et al., 2013; Wu et al., 2013; Yoshida et al., 2014; Yoshida and Ueguchi-Tanaka, 2014; Jost et al., 2016). The IDD is composed of a nuclear localization signal and four highly conserved tandem zinc fingers: one standard C2H2 zinc finger, one irregular C2H2 zinc finger, and two irregular CCHC zinc fingers. There are 17 IDD family members in maize, some with tissue-specific expression differences (Colasanti et al., 2006; Sekhon et al., 2013; Yi et al., 2015). The founding member of the IDD family, INDETERMINATE1 (ID1), controls flowering time in maize (Colasanti et al., 1998). ID1 localizes to the nucleus and binds an 11 base pair DNA consensus sequence of TTTGTCGTTTT which NKD1 (IDDveg9) can also bind, although with different specificity (Kozaki et al., 2004; Wong and Colasanti, 2007; Yi et al., 2015).

In this study, we analyze NKD1 and NKD2 function in developing maize endosperm. We identify the DNA binding specificities of NKD1 and NKD2, discern that NKD1 and NKD2 proteins can homo- and heterodimerize through their IDDs, and demonstrate they regulate transcription by binding DNA. RNA sequencing (RNAseq) analysis of nkd1 nkd2 versus WT endosperm revealed that NKD1 and NKD2 regulate widespread processes, including nutrient reserve deposition as well as cell growth and proliferation. Analyses of transcriptomic data lead to functional predictions that were validated by phenotypic analyses of nkd1 nkd2 mutants.

RESULTS

Identification of Endosperm Gene Transcripts Regulated by NKD1 and NKD2
To identify genes and biological processes directly or indirectly regulated by NKD1 and NKD2 (NKD1/2) in developing endosperm, a transcriptomic analysis was undertaken to identify gene transcripts differentially expressed (DE) between WT and \textit{nkd1 nkd2} mutant. Laser capture microdissection (LCM) coupled with RNAseq was previously performed on AL and SE cells from WT (B73 inbred) versus \textit{nkd1 nkd2} mutant endosperms at 15 days after pollination (DAP) (Yi et al., 2015). These 50-base paired-end reads were re-aligned and mapped to the maize reference genome (B73 RefGen-V3, GSE61057). A total of $1.14 \times 10^9$ raw reads were generated, with a minimum of $256 \times 10^8$ per tissue, summed over 3 biological replicates (Supplemental Table 1). Reads that uniquely mapped to the genome and were properly paired were selected for further analysis, which ranged from 40.7% to 72.6% of the raw reads per tissue. Expression of 34,014 genes was detected in AL cells whereas 32,629 genes were expressed in SE. Among these, 31,792 genes were expressed in all endosperm tissues sampled (WT and \textit{nkd1 nkd2} mutant; AL and SE).

Differential expression (DE) analysis comparing \textit{nkd1 nkd2} mutant to WT identified 2,188 DE genes in AL and 2,193 in SE (adjusted p-value $\leq 0.01$) (Figure 1; Supplemental Table 2; Supplemental Datasets 1 and 2). An MA plot of gene expression changes did not reveal any systemic bias in the data (Supplemental Figure 1A). Multidimensional scaling (MDS) analysis of gene expression variance among samples revealed that AL exhibited lesser variance than SE (Supplemental Figure 1B). Furthermore \textit{nkd1 nkd2} genotype had a greater overall impact on gene expression than did tissue type. Transcript abundance among the DE genes ranged from highs with mean normalized read counts up to $8 \times 10^6$ to lows of less than 10. Among DE genes, 63.48% had decreased transcript abundance in mutant AL and 58.28% had decreased transcript abundance in mutant SE. The AL and SE DE datasets showed statistically significant overlap and similar transcript fold change trends (P<0.001; Z-score of 80.8).

A total of 935 genes were DE between WT and mutant in both tissues (Figure 1A). To validate the RNAseq data, 15 DE genes were tested by qRT-PCR on independent, unamplified RNA from 15 DAP WT and \textit{nkd1 nkd2} AL. The qRT-PCR expression values
showed a positive correlation ($R^2=0.7036$) with the RNA sequencing log$_2$ fold changes, indicating minimal bias in LCM-RNA sequencing results (Figure 1C). Eight of these transcripts exhibited statistically significant DE as expected based on the RNAseq analysis (Supplemental Figure 1C). Verified transcripts showing increased expression in \textit{nkd1 nkd2 AL} included cell cycle related genes (\textit{tubulin1, cell division cycle2-like, actin-1, proliferating cell nuclear antigen2}) and genes annotated as involved in epigenetic regulation (e.g., one encoding FASCIATA1-LIKE). Transcripts showing decreased mutant expression included the AL cell identity marker \textit{phospholipid transfer protein homolog1} \cite{Gruis2006}, and genes related to nutrient reserve accumulation (\textit{granule bound starch synthase, prolamin-box binding factor1}).

\textit{nkd1 and nkd2 Regulate Genes Involved in Diverse Processes}

To determine biological processes regulated by \textit{nkd1} and \textit{nkd2}, pathway analyses were performed on DE genes using Gene-Ontology (GO), MaizeCyc, and CornCyc \cite{Monaco2013}. Complete lists of significantly enriched GO terms are shown in Tables 1 and 2 and individual genes within each pathway are reported in Supplemental Datasets 1 and 2. There were notable effects on genes functioning in the regulation of gene expression, including 80 TFs with altered expression in mutant AL and 86 in SE (Figure 2). Epigenetic related factors were also affected, showing a general trend of increased expression in both mutant AL and SE. Thirty-seven epigenetic related factors were DE in AL and 23 in SE. Cell division and cell growth pathways trended to increased expression in the mutant AL. Thirty-three cell division-related factors were DE in AL and 18 in SE, while 17 cell-growth factors were DE in AL and 17 in SE. The AL and SE each also showed wide ranging effects on hormone-related pathways including biosynthesis, signaling and response in systems including auxin, abscisic acid (ABA), ethylene, jasmonate, brassinosteroids, cytokinins and gibberellins (Figure 2; Supplemental Figures 2 and 3).

Pathways involved in the metabolism or accumulation of storage compounds generally showed decreased transcript levels in \textit{nkd1 nkd2 endosperm} (Figure 2; Supplemental
Figures 2 and 3). Notably, many pathways involved in carbohydrate metabolism were affected. The mutant SE and AL both exhibited decreased expression of genes functioning in starch/glycogen biosynthesis, sucrose biosynthesis, and sucrose, galactose, and glycerol degradation, while mutant AL also showed decreased expression of gluconeogenesis genes. Protein metabolism was also affected, with mutant SE and AL both showing decreased expression of genes for storage protein accumulation (nutrient reservoir activity) and amino acid biosynthesis, while mutant SE also showed decreased expression of chorismate biosynthesis genes. Mutant AL also displayed decreased expression of lipid metabolism pathways, including linoleate biosynthesis and triacylglycerol degradation (Supplemental Figure 2A). SE showed decreased expression of genes involved in nutrient transport pathways (Figure 2). Of final note, genes involved in defense response were altered in both AL and SE, with an overall decrease in defense response-related gene expression in the mutant (Supplemental Figures 2C and 3C). Functional characterization performed by evaluating GO overrepresentation produced results consistent with pathway analyses, with AL DE genes enriched for GO-terms related to storage proteins (nutrient reservoir activity), sugar metabolism, epigenetics, microtubule related processes and cell division (Table 1). SE GO-terms were enriched for DNA replication, nucleolus, plastid and cell wall (Table 2).

Pathway analysis suggested that nkd1 and nkd2 may regulate cellular transport activities. BET cells are sites of intensive nutrient transport from the maternal plant tissue into the developing endosperm (Thompson et al., 2001) and decreased BET cell transport activity would be consistent with the decreased kernel weight and storage reserve accumulation in the mutant (Table 3). Because BET tissues were not included in the transcriptomic analysis, potential NKD1 NKD2 regulation of BET gene expression was tested using qRT-PCR on BET cell marker genes. RNA was isolated from the lower quarter of 12 DAP WT and nkd1 nkd2 endosperms. The expression of maternally expressed gene1 (meg1, p=0.027), and basal endosperm transfer layer2/bap2 (betl2, p=0.0195) were significantly increased in nkd1 nkd2 mutant, while basal endosperm
transfer layer1 (betl, p=0.482) and myb related protein1 (mrp1, p=0.1445) were not significantly different (Supplemental Figure 4). These results suggest that nkd1 and nkd2 likely regulate transcription in BET cells but are not required for BET cell differentiation.

Effects of the nkd1 nkd2 Mutant on Endosperm Composition

The nkd1 nkd2 mutant was previously reported to cause decreased total kernel weight and opaque endosperm texture (Yi et al., 2015), and transcriptomic analysis suggested alterations in starch and storage protein pathways, prompting examination of seed composition. WT and nkd1-Ds nkd2-Ds0297 mutant kernels from the same F2 segregating ears were compared. Mutants contained less vitreous endosperm than WT (Figure 3A) and showed a 41.8% decrease of total kernel dry weight with a 50.8% reduction in endosperm (Table 3). The nkd1-Ds nkd2-Ds0297 mutant kernels showed a 32.5% reduction in total starch abundance as determined by amylglucosidase digestion quantified by colorimetric glucose oxidase-peroxidase (GOPOD) assays (Table 3). Some of the DE gene transcripts were implicated in determining starch structure (Figure 2), of which glucan branch chain length distribution is a major component. Glucan branch chain length abundance was measured by fluorophore-assisted carbohydrate electrophoresis of de-branched endosperm starch (O’Shea and Morell, 1996). Difference plots of nkd1 nkd2 mutant minus WT starch chain length abundance show the mutant starch had a decrease in the frequency of branch chains of 6 and 27-28 glucose units, while chains 13-18 glucose units in length were significantly increased in abundance (Figure 3B).

Starch grain morphology was examined with scanning electron microscopy (SEM). Whereas the WT starch granules had smooth surfaces and were nearly spherical in shape, mutant granules had distinctly pitted surfaces and were more irregularly shaped, sometimes appearing faceted (Figure 3C and 3D; Supplemental Figure 5). Further, bisected mutant starch grains often showed irregular hollow cores with internal pitting, while WT grains were nearly solid with a small hollow center (Figure 3E and 3F).
Protein content in WT versus $nkd1$-$Ds$; $nkd2$-$Ds0297$ mutant endosperm was estimated from total nitrogen content measured using Dumas N combustion (Schindler and Knighton, 1999). There was a 52.11% reduction in total protein content in endosperm of mutant segregants ($p=0.0054$, Table 3).

Fluorescently tagged transgenes were available for several genes DE in $nkd1$ $nkd2$ mutant endosperm, including 2 storage protein genes, $floury2$ ($fl2$) (Coleman et al., 1997), $globulin3$ ($glb3$) (Woo et al., 2001) and a putative ABA response gene responsive to $aba17$ ($rab17$) (Kizis and Pages, 2002). The $nkd1$-$R$ $nkd2$-$R$ mutant alleles were introduced into FL2-RFP, GLB3-RFP and RAB17-YFP transgenic lines, which consist of fluorescent proteins translationally fused to the maize gene coding sequences controlled by their native regulatory elements (Mohanty et al., 2009). Mature kernels were sampled from segregating F2 ears and epifluorescence imaging demonstrated that each reporter showed decreased fluorescence intensity in mutant compared to WT (Figure 4). Thus, decreased expression levels of these transgenic reporter proteins were consistent with the RNAseq analysis of native gene transcripts.

Many variables can influence grain yield and composition but principal component analysis (PCA) clearly identified genotype as the primary contributor to the observed variance in these traits. Genotype explained 99.9% of the variance for endosperm weight and total protein, as well as for starch content and branch length, while the remaining 0.1% of variance was explained by independent cob, or independent kernel for protein and starch, respectively (Supplemental Figure 6).

**Identification of NKD1 and NKD2 Target DNA Binding Sequences**

To identify DNA binding sequences for NKD1 and NKD2 proteins, selection and amplification binding (SAAB) was undertaken. Columns were generated with the conserved DNA-binding ID domains of NKD1 (NKD1ID) and NKD2 (NKD2ID). Each protein was bacterially expressed as a GST fusion and purified proteins were covalently bound to ester agarose columns. The identity of purified recombinant protein was confirmed by SDS PAGE and MS/MS (Supplemental Figure 7). A column of purified
GST from the empty vector served as a negative control for nonspecific binding. Libraries enriched for DNA-sequences that interact with the NKD1ID proteins were generated by passing random double-stranded oligonucleotides over each protein column for 6 cycles of selection and amplification. After cloning and sequencing selected oligos, a total of 77 unique sequences were recovered for NKD1 and 54 for NKD2. Motif analysis of the recovered sequences identified an 8-bp binding consensus sequence (BCS) of \([TA]-T-[TCG]-G-T-[CGA]-G-T\) for NKD1 produced from 27 of the recovered sequences (Figure 5A; Supplemental Table 3). For NKD2, a 6bp BCS of \(T-G-T-[CT]-G-[TG]\) was identified from 19 recovered sequences, which was similar to the NKD1 BCS (Figure 5B; Supplemental Table 4). No sequences were recovered from the GST negative controls.

Electrophoretic mobility shift assays (EMSAs) were performed to further examine the DNA binding properties of the NKD1 and NKD2 ID domains. Oligonucleotide probes were designed based on frequency of base occupancy in the SAAB selected BCSs (Supplemental Figure 8A and B). To test specific DNA base requirements, each base was substituted individually and assayed with both the NKD1ID-GST and NKD2ID-GST proteins. The GST protein tag purified from the empty vector showed no interaction with any of the oligonucleotide probes (Supplemental Figure 8C and E). Relative binding affinity was determined for each mutant by comparing the amount of shifted probe relative to the non-mutant BCS probe. For NKD1ID, binding was abolished by a substitution of cytosine in position 8, and strongly decreased by a substitutions of thymine at position 4 or adenine at position 5 (Figure 5C and 5D; Supplemental Figures 8E and 9). Base substitutions at positions 2 and 7 produced moderate decreases in binding and substitutions at positions 3 and 6 produced slight decreases in binding affinity. Although position 1 appeared selected by SAAB, a cytosine substitution did not decrease binding affinity. Taken together, NKD1ID recognizes a 7-bp BCS of \(T-T-G-T-C-G-T\) (NKDcore). NKD2ID recognizes the same 7bp NKDcore BCS but with different binding tolerances for off consensus base substitutions in positions 2, 3, 4, 6 and 8. (Figure 5C and 5F; Supplemental Figure 8E). Although position 2 was not selected by
NKD2ID SAAB, binding was abolished in EMSA by a cytosine substitution. Binding was also abolished by a substitution in position 4, and strongly decreased by a substitution in position 5. Moderate decreases in DNA binding were observed for substitutions in positions 7 and 8. Competition EMSA with mutant probes showed binding to the BCS was specific and confirmed that NKD1ID and NKD2ID have different tolerances for sequence variants at positions 2 and 8 (Figure 5E and 5G).

NKD1 and NKD2 Direct Target Gene Prediction and Motif Enrichment

To determine likely direct targets of NKD1 and NKD2, proximal promoter regions (PPRs) of all genes with transcripts detected in SE and AL were searched for the presence of NKD1, NKD2, and NKDcore BCSs using the MEME suite motif analysis tool Find Individual Motif Occurrences (FIMO) (Bailey et al., 2015). Sequence elements shown by EMSA not to bind NKD1 or NKD2 were removed from the analysis. NKDID binding to six of the variant BCSs identified by FIMO were confirmed by EMSA, verifying the efficacy of the FIMO analysis for predicting in vitro binding ability (Figure 5C, 5D and 5F; Supplemental Figure 8E). A total of 32,621 PPRs were identified from the 15 DAP AL transcriptome, including 2,135 DE genes and 31,389 PPRs were identified from the SE transcriptome, including 2,192 DE genes (Supplemental Dataset 3). The occurrences of NKD BCSs are summarized in Table 4, Supplemental Figure 10, and Supplemental Dataset 3. Due to the relatedness among the identified BCSs, there was considerable overlap among the FIMO-identified promoter elements (Supplemental Figure 10). Furthermore, because only 6 bp were selected for NKD2 while 8 bp were selected for NKD1, there are considerably more potential NKD2 sites throughout the genome. Nearly half the predicted NKD1 and NKDcore sites are included among the NKD2 predicted sites and all the predicted NKD1 sites are included among the NKDcore sites. To test whether NKD BCSs were enriched in the promoters of genes DE in the nkd1 nkd2 mutant endosperm, the proportion of PPRs with one or more BCS among DE
genes was compared to that of the 15 DAP transcriptome. In AL, NKD2 and NKDcore motifs were significantly enriched in DE genes, while the NKD1 motif was not (Table 4; Supplemental Dataset 3). Conversely, in SE, the NKD1 motif was enriched in DE genes, while NKD2 and NKDcore motifs were not significant (Table 4; Supplemental Dataset 3). BCS enrichment controls consisted of three 'shuffle motifs' each for NKD1 and NKD2 generated by randomly rearranging bases in the BCSs. Enrichment was not detected for any of the 'shuffle motifs', indicating that the enrichment of the NKD motifs was specific (Supplemental Figure 11).

Predicted Direct Target Genes are Enriched for Storage Proteins

To determine pathways directly transcriptionally regulated by NKD1 and NKD2, a GO enrichment analysis was performed by comparing occurrences of GO terms among predicted direct target genes to the endosperm-expressed genes. For NKD2 AL, GO enrichment was observed for nutrient reservoir activity (storage proteins), starch biosynthesis, nucleosome and chromatin regulation, and processes related to plastids, vacuoles, and microtubules (Table 5; Supplemental Dataset 4). For NKDcore AL, GO enrichment was detected for nutrient reservoir activity (Table 5; Supplemental Dataset 4). GO enrichment was not detected for NKD1 AL target genes. In the SE, GO enrichment was detected for NKD1 target genes functioning in external encapsulating structure, cell wall and apoplast (Table 6; Supplemental Dataset 4). For NKD2 SE, GO enrichment was detected for genes in nutrient reservoir activity, cell wall, and plastid related ontologies (Table 6; Supplemental Dataset 4). GO enrichment was detected for NKDcore SE genes functioning in cell wall and external encapsulating structure (Table 6; Supplemental Dataset 4).

NKD1 and NKD2 Proteins Dimerize via the ID Domain

To determine if NKD1 and NKD2 can dimerize as shown for other IDD protein family members (Seo et al., 2011b; Yoshida et al., 2014), bimolecular florescence complementation (BiFC) and pull-down assays were performed. For BiFC, the N- or C-terminal portion of the YFP protein was fused to the C-termini of the NKD1 and NKD2
proteins. Both full-length NKD1 and NKD2 proteins as well as truncated proteins containing only the ID domains were tested. Constructs were biolistically introduced into onion epidermal cells with a 35S:mCherry construct co-bombarded to identify transiently transformed cells. For the full-length constructs, reconstituted YFP fluorescence was detected, indicating homodimerization of NKD1 and NKD2, as well as heterodimerization between NKD1 and NKD2 (Figure 6A). Likewise for the ID domain truncations, NKD1ID and NKD2ID could homo- and heterodimerize (Supplemental Figure 12). Control bombardments consisting of combinations of NKD full length-nYFP+ empty vector cYFP or NKD full length-cYFP+ empty vector nYFP did not produce YFP fluorescence indicating that YFP reconstitution was dependent on interactions among the NKD1 and NKD2 proteins (Figure 6 and Supplemental Figure 12).

Pull-down assays were performed on NKD1 and NKD2 full-length and ID domain proteins expressed in E. coli cells with a C-terminal 6x His-tag or a N-terminal GST-tag. Co-pull downs were performed by mixing lysates of NKD-6x His and GST-NKD proteins, precipitating protein complexes using Ni resin for 6x His and immunoblotting with GST antibodies, or precipitating with Glutathione Sepharose for GST tags and immunoblotting with 6x His antibodies. Co-precipitation was observed in all combinations of NKD1 and NKD2 full-length proteins, but not with the empty vector controls (Figure 6B; Supplemental Figure 13). Likewise, for the ID domain truncated protein constructs, co-precipitation was observed for all NKD1ID and NKD2ID combinations (Figure 6C; Supplemental Figure 13). Thus, NKD1 and NKD2 homo- and heterodimerize, and these interactions are mediated by residues in the ID domain.

**NKD1 and NKD2 Function as Transcription Factors**

To test the transcriptional regulatory activity of NKD1 and NKD2, transcription assays were designed using the promoter regions of select predicted direct targets of NKD1 and NKD2. Reporter constructs contained the promoters of interest cloned upstream of the firefly luciferase (LUC) coding sequence. Promoter regions tested included *xylanase inhibitor protein 1 (zmX1P-1_pro:LUC)*, *opaque2 (o2_pro:LUC)*, *22KD zein protein 22.1*.
(zp22.1<sub>pro</sub>:LUC), nkd1 (<i>nkd1</i><sub>pro</sub>:LUC), viviparous1 (<i>vp1</i><sub>pro</sub>:LUC), jasmonate induced protein (<i>JIP</i><sub>pro</sub>:LUC), mother of FT-like (<i>MTF</i><sub>pro</sub>:LUC) and WRKY transcription factor 29 (wrky29<sub>pro</sub>:LUC). Effector constructs contained the NKD1 or NKD2 cDNAs under the regulation of the cauliflower mosaic virus 35S promoter (35S<sub>pro</sub>:NKD1, 35S<sub>pro</sub>:NKD2). The empty effector vector (35S<sub>pro</sub>:null) was used as a control. A 35S promoter:Renilla luciferase construct (35S<sub>pro</sub>:RLUC) served as the internal normalization standard. The reporter, effector, and normalization constructs were co-introduced into protoplasts of <i>nkd1-Ds; nkd2-Ds</i>0297 mutant endosperms using PEG-calcium transfection. Transcriptional activity for each effector and test promoter construct pair was determined by comparing the firefly/renilla luminescence ratio to the 35S<sub>pro</sub>:null effector. Transcriptional activation was detected for the NKD1 effector from the zmX1P-1<sub>pro</sub> (p<0.0001), o2<sub>pro</sub> (p=0.0409) and <i>vp1</i><sub>pro</sub> reporter constructs (p= 0.038; Figure 7B). No significant transcriptional regulation was observed for NKD1 effector from the zp22.1<sub>pro</sub> or <i>nkd1</i><sub>pro</sub> reporters (p=0.3368, p=0.1008, respectively; Figure 7B). Transcriptional activation was detected for the NKD2 effector from the zmX1P-1<sub>pro</sub> (p=0.0009), o2<sub>pro</sub> (p=0.0025), zp22.1<sub>pro</sub> (p=0.0014), and the <i>vp1</i><sub>pro</sub> reporters (p=0.0317, Figure 7B). Transcriptional repression was detected for NKD2 effector from the <i>nkd1</i><sub>pro</sub> reporter (p=0.0140, Figure 7B). No significant transcriptional activation or repression was detected for NKD1 or NKD2 from the JIP<sub>pro</sub>, MTF<sub>pro</sub> or wrky29<sub>pro</sub> reporters (p>0.05).

The relative luciferase ratios for each reporter were compared between NKD1 and NKD2 effectors. NKD1 showed significantly higher activity from the zmX1P-1<sub>pro</sub> (p< 0.001) and o2<sub>pro</sub> (p=0.048) reporters, while NKD2 transcriptional activities were higher for zp22.1<sub>pro</sub> (p< 0.001) and <i>vp1</i><sub>pro</sub> (p= 0.020) reporters. For the <i>nkd1</i><sub>pro</sub> (p=0.025) reporter, NKD2 effector repressed activity while activation by NKD1 was not statistically significant (Figure 7B). These results suggest possible functional differences between NKD1 and NKD2 transcriptional activities, consistent with observed differences in DNA binding specificities (Figure 5; Supplemental Figure 8). Overall, these results were consistent with direct target gene predictions and confirmed NKD1 and NKD2 function to regulate gene transcription.
To test whether NKD1 and NKD2 require their BCS to regulate transcription, a mutant vp1 promoter construct (vp1-mutpro:LUC), was cloned with the thymine in the 7th position of the second NKDcore BCS substituted with a cytosine (TTGTCG to TTGTCGC). This substitution abrogated binding in EMSAs (Figure 5D and 5F). Transcriptional activation of the vp1-mutpro:LUC reporter construct was not detected for NKD1 effector (p=0.3120; Figure 8B), while NKD2 was still able to activate transcription (p=0.0377). The level of vp1-mutpro reporter activity was decreased compared to WT vp1pro for both NKD1 and NKD2 effectors (p=0.0139, p=0.0437, respectively). Also, transcriptional activity from the vp1-mutpro reporter was significantly different between NKD1 and NKD2 effectors (p=0.007). These results demonstrate that NKD1 and NKD2 transcriptional activity is mediated by binding to their BCS in the promoter regions of target genes, validates the bioinformatic predictions of NKD direct target genes, and further indicate NKD1 and NKD2 have differences in transcriptional activity.

DISCUSSION

Functions of NKD1 and NKD2 in developing endosperm

In this study, we revealed the functions of the NKD1 and NKD2 transcription factors in maize endosperm development by analyzing the AL and SE transcriptomes of WT compared to nkd1 nkd2 double mutant. Previous genetic analysis indicated that nkd1 and nkd2 are largely redundant with nkd1 single mutants, producing a mild opaque phenotype and no discernable nkd2 mutant phenotype (Yi et al., 2015). Therefore, we chose to focus on the double mutant for this study. Pathway analyses showed NKD1 and NKD2 are regulators of diverse biological processes including cell growth, division and differentiation, hormone metabolism and signaling, resource reserve deposition, and seed maturation. These results were largely consistent with the nkd1 nkd2 mutant’s pleiotropic endosperm phenotype (Becraft and Asuncion-Crabb, 2000; Yi et al., 2015) which provides biological validity to the transcriptomic and bioinformatic analyses.
NKD1 and NKD2 are required to restrict AL to a single cell layer and to promote AL cell identity (Becraft and Asuncion-Crabb, 2000; Yi et al., 2015). Consistent with these functions, NKD1 and NKD2 were found to regulate genes associated with cell cycle, cell growth and division. NKD1 and NKD2 are predicted to be direct transcriptional repressors of retinoblastoma-related1 and mitotic cyclin 3B-like (Figure 2; Supplemental Datasets 1, 2 and 3). The defective kernel1 (dek1) gene is required for aleurone differentiation (Becraft et al., 2002; Lid et al., 2002) and regulates cell wall orientation and cell division plane via the regulation of cell cycle (Liang et al., 2015). These results suggest the regulation of cell division is important for aleurone development and that the multiple peripheral cell layers in the nkd1 nkd2 mutant may result in part from the misregulation of cell proliferation genes.

Grain weight of nkd1 nkd2 mutants was decreased compared to WT, which is likely related to NKD1 and NKD2 functions in multiple aspects of resource reserve deposition including starch and storage protein accumulation. Starch is the major product for grain yield, and starch structure can greatly affect physical properties that influence end use. The nkd1 nkd2 mutant endosperm displayed a reduction in total starch abundance as well as altered glucan chain length distribution. Decreased starch accumulation is consistent with decreased expression of starch biosynthetic genes (Table 3; Figure 2). Notably, transcript levels of shrunken-2 (sh2) and brittle-2 (bt2) were both decreased (Figure 2); these genes encode subunits of ADP-glucose pyrophosphorylase, which catalyzes a rate-limiting step in starch biosynthesis (Tuncel and Okita, 2013). Other key starch biosynthetic genes showing decreased transcript abundance included sucrose synthase2, waxy1, starch synthase1 (ss1) and sugary2 (su2) (Figure 2). Likewise, altered glucan chain length distribution (Figure 3B) could result from decreased expression of amylose-extender1 (ae1), which encodes starch branching enzyme IIb (BEIIb) (Nishi et al., 2001), and ss1 and su2 genes, which catalyze glucan chain elongation. Direct target analysis predicted that NKD1 and NDK2 are direct transcriptional activators of ss1, su1 and wx1 (Supplemental Dataset 3).
The nkd1 nkd2 mutant starch granules showed irregular morphology that could not be
obviously attributed to specific genes (Figures 2, 3C and 3D). While WT starch granules
are nearly spherical, mutants showed a mild faceting somewhat reminiscent of other
starch mutants like opaque5 or various starch branching mutants (Myers et al., 2011;
Zhao et al., 2015). The pitted surface of nkd1 nkd2 mutant starch granules resembled
starch grains subjected to amylase digestion (Dhital et al., 2014). Given the propensity
for vivipary in nkd1 nkd2 mutant kernels and decreased ABA signaling, it is possible that
amyrase expression is prematurely activated (Yi et al., 2015). Also, the irregular hollow
core of nkd1 nkd2 mutant starch granules is fascinating, and it is unclear whether this
might be caused by internal amylase digestion or whether this reflects aberrant starch
grain initiation.

Pathway analyses indicated that NKD1 and NKD2 promote storage protein
accumulation, both directly and indirectly, consistent with decreased storage protein
content in the nkd1 nkd2 mutant. NKD2 activated transcription from the 22KD zein
protein 22.1 promoter (Figure 7B), while NKD1 and NKD2 both activated transcription of
the o2 promoter. Furthermore, prolamin binding factor1 (pbf1) is a predicted direct
target of NKD1 and NKD2. O2 and PBF1 are TFs both well known to promote
expression of zein storage protein genes (Zhang et al., 2015; Zhang et al., 2016). The
FL2-RFP and GLB3-RFP transgenes showed decreased expression in nkd1 nkd2
mutant endosperm corroborating the positive regulation of storage protein gene
expression by NKD1 and NKD2 (Table 3, 5 and 6; Figure 4A and 4B).

NKD1 and NKD2 promote carotenoid accumulation because mutant endosperm is pale
yellow, sometimes almost white. While carotenoid biosynthesis did not meet the
pathway criterion of having at least 3 DE genes, 2 key genes showed decreased
expression in the mutant; yellow endosperm1 (y1) encodes phytoene synthase and
viviparous5 (vp5) encodes phytoene desaturase (Buckner et al., 1996; Hable et al.,
1998). Both represent rate-limiting steps in carotenoid biosynthesis and mutations in
either gene cause carotenoid deficiency. Carotenoids are precursors for ABA
biosynthesis, so this is also likely to contribute to the vivipary phenotype. Additionally,
the *nkd1 nkd2* mutant showed decreased expression of *aldehyde oxidase1* and 2 genes encoding enzymes that catalyze the final step in ABA synthesis (Seo et al., 2000), which is also consistent with vivipary and the decreased expression of ABA response pathways (Figure 2; Supplemental Datasets 1 and 2).

The *nkd1 nkd2* mutant was originally identified based on its anthocyanin-deficient phenotype (Becraft and Asuncion-Crabb, 2000), and our analysis suggests the regulation of anthocyanin biosynthesis is indirect. The *colored1 (r1)* and *colored aleurone1 (c1)* genes encode transcription factors that together activate expression of anthocyanin biosynthetic genes, and VP1 is a transcriptional activator of *c1* (Cone, 2007). NKD1 and NKD2 directly activate *vp1* transcription and are predicted to directly activate *r1* (Figure 7B; Supplemental Datasets 1, 2 and 3).

A phenotype we have not systematically evaluated is the proclivity of *nkd1 nkd2* mutant kernels to develop fungal infections. It is unclear whether this is a direct mutant defect or an indirect effect caused by soft texture or impaired seed maturation. However, the defense response pathway showed decreased activity in the *nkd1 nkd2* mutant endosperm (Supplemental Figures 2 and 3) and genome-wide promoter scan analysis suggests NKD1 and NKD2 are direct positive regulators of defense responses (Supplemental Datasets 1, 2 and 3). The aleurone is the outermost cell layer of the endosperm and the only cell type alive at maturity; thus, it is fundamentally important for defense to pathogens (Jerkovic et al., 2010). Among predicted direct targets was *xylanase inhibitor protein-1* (XIP-1), which is involved in fungal defense (Moscetti et al., 2013) and whose promoter showed transcriptional activation by NKD1 and NKD2 (Figure 7B). These results implicate NKD1 and NKD2 as potentially having a direct regulatory role in mediating defense.

**NKD1 and NKD2 are key regulators of endosperm gene expression**

NKD1 and NKD2 regulate widespread gene expression in developing endosperm and direct target analysis revealed approximately 6% of maize gene promoters contain a NKDcore BCS. 80 DE genes in AL and 86 in SE were TF-related, with 36 and 33,
respectively, predicted as direct NKD targets (Figure 2; Supplemental Datasets 1 to 4). Additionally, epigenetic regulation was enriched in GO terms of DE genes. Taken together, NKD1 and NKD2 appear to function as central regulators in the gene networks governing multiple aspects of maize endosperm development.

Several key TFs were shown or predicted to be direct targets of NKD regulation, including O2, PBF1, VP1 and R1. O2 is an important promoter of zein storage protein gene expression, significant for its importance in quality protein maize with enhanced lysine content (Gibbon and Larkins, 2005). PBF1 is a Dof TF that also promotes expression of storage protein genes (Marzábal et al., 2008). O2 binds a DNA motif known as GCN4 while PBF1 binds the prolamin box. These motifs are frequently combined into a bifactorial ‘endosperm box’ conserved among storage protein genes in all cereals (Marzábal et al., 1998). Recent analyses showed O2 and PBF1 also function broadly, controlling multiple classes of zeins, as well genes involved in carbon and nitrogen metabolism, pathogen and stress responses, as well as other TFs (Li et al., 2015; Zhang et al., 2016). Comparison of the direct target genes of O2 with NKD1 and NKD2 revealed 23 genes related to resource reserve deposition in common (Supplemental Tables 5 and 6). Furthermore, GO terms associated with protein synthesis and storage were overrepresented in both NKD and O2 regulated genes (Supplemental Tables 7 and 8), suggesting O2, NKD1 and NKD2 may function together to regulate storage protein accumulation. VP1 (ABI3 ortholog) is a B3 TF required for ABA responses to promote seed maturation and inhibit germination (Suzuki et al., 2003). As mentioned, VP1 is also required for anthocyanin accumulation in the aleurone via its transcriptional activation of the c1 gene, which encodes a myb TF (Cone, 2007). C1 heterodimerizes with R1, a bHLH TF, to activate expression of structural genes in anthocyanin biosynthesis (Cone, 2007). The r1 gene is also predicted to be directly regulated by NKD1 and NKD2. The c1 and r1 genes are historically important in early genetic studies by McClintock and others.

It was previously reported that the nkd genes were subject to feedback regulation because nkd1 transcript levels increased in a nkd2 single mutant and vice versa (Yi et
Here we showed that NKD2 repressed expression of the \textit{nkd1} gene promoter; thus, the observed feedback appears to be due to direct transregulation between the \textit{nkd1} and \textit{nkd2} duplicate factors. The \textit{nkd1} and \textit{nkd2} genes were recently shown to be direct targets of transcriptional activation by DOF3 (Qi et al., 2016), hereafter referred to as DOF36 (GRMZM2G137502) in accordance with NCBI and MaizeGDB annotations. RNAi knockdown of \textit{dof36} generated similar effects as the \textit{nkd1 nkd2} double mutant including abnormal starch deposition and multiple layers of compromised aleurone cells. Some of the same genes involved in sugar and starch metabolism were identified as direct (and indirect) targets for DOF36 as for NKD1 and NKD2. Interestingly, \textit{dof36} transcript shows decreased expression in \textit{nkd1 nkd2} mutant (Supplemental Dataset 1), although it was not identified as a direct target. This indicates \textit{dof36} belongs to the same GRN as \textit{nkd1} and \textit{nkd2}, and that \textit{dof36} expression is reinforced by feedback, albeit indirect, from \textit{nkd} genes.

\textbf{Functions of NKD1, NKD2 and other IDD family members}

NKD1 and NKD2 bind DNA and regulate transcription via a consensus motif (TTGTCGT) similar to the ID1 binding site (TTTGTCGTTTT) although shorter (Kozaki et al., 2004). Despite 97\% amino acid identity (118 of 122 residues) between their ID domains, NKD1 and NKD2 showed differences in their DNA-binding and transcriptional regulation activities. Mutation of position 1 in the NKDcore motif to C decreased binding by NKD1 but abolished NKD2 binding, whereas mutation of position 7 to C abolished NKD1 binding but NKD2 could still bind (Figure 5). In transcription assays, NKD1 showed higher activity than NKD2 on the XIP-1 and \textit{o2} promoters, whereas NKD2 showed greater activation of \textit{vp1} and \textit{zp22.1} promoters (Figure 7). On the \textit{nkd1} promoter, NKD2 repressed transcription, whereas NKD1 activated, though not statistically significant. Whether these functional differences relate to differences in DNA binding activity or interactions with unknown cofactors remains to be explored, as do the functional differences between NKD1 and NKD2 on a genome-wide scale. These
differences indicate that NKD1 and NKD2 are not completely redundant at the molecular level and that they have undergone subfunctionalization since the most recent maize genome duplication event that generated these loci.

Different IDD protein family members function directly as DNA-binding transcriptional regulators, indirectly as co-factors for other transcription factors, or both. DELLA proteins, such as Arabidopsis RGA1, are GRAS family TFs that regulate gene expression in response to GA signalling, among other things. RGA1 activates transcription of SCARECROW-LIKE3 (SCL3) via interaction with any of 5 IDD proteins, AtIDD3, -4, -5, -9, and -10. The IDD proteins bind DNA and function as scaffolds for the DELLA proteins, which lack intrinsic DNA binding activity (Yoshida et al., 2014; Yoshida and Ueguchi-Tanaka, 2014). SCL3 is another GRAS protein that interacts with the IDD proteins to competitively inhibit the binding and action of RGA1. This provides feedback to modulate gene expression responses to GA signalling. Similarly, the IDD protein JACKDAW (JKD) regulates transcription of SCARECROW (SCR) in Arabidopsis root development. JKD activity is enhanced by the GRAS protein SHORTROOT (SHR), as well as by the target gene product, SCR, another GRAS protein (Ogasawara et al., 2011). Further, the activity of JKD is also modulated by interactions with other IDD proteins, MAGPIE and BALDIBIS (Long et al., 2015; Ogasawara et al., 2011). In addition to modulating transcriptional activity of this complex, these interactions prevented intercellular trafficking of the mobile protein SHR.

We found NKD1 and NKD2 could each homodimerize as well as heterodimerize with one another. The functional significance of this is unknown and we do not yet know whether they can dimerize with other IDD family members. Arabidopsis IDD14 also homo- and heterodimerizes, and this dimerization modulates function (Seo et al., 2011b). Under standard laboratory temperatures, IDD14α binds DNA and regulates transcription of target genes, but under cold temperatures the IDD14 gene produces an alternatively spliced transcript that encodes a product, IDD14β, that lacks the DNA-binding domain. IDD14β can heterodimerize with IDD14α and decreases DNA binding activity.
IDD gene family members are implicated in many biological functions including the regulation of carbohydrate metabolism, gravitropism, seed germination, lateral organ morphogenesis, cellular patterning, flowering time and hormone signaling (Morita et al., 2006; Welch et al., 2007; Tanimoto et al., 2008; Hassan et al., 2010; Feurtado et al., 2011; Ogasawara et al., 2011; Seo et al., 2011a; Cui et al., 2013; Wu et al., 2013; Yoshida et al., 2014; Yoshida and Ueguchi-Tanaka, 2014; Jost et al., 2016). It is striking how many similar processes are regulated by the nkd genes during seed development. For example, Arabidopsis IDD8 and IDD14 transcriptionally regulate carbohydrate metabolism and starch accumulation by modulating expression of some of the same target genes as NKD1 and NKD2, such as ss1 (Seo et al., 2011b; Seo et al., 2011a) (Figures 2, 3 and 4A).

An intriguing functional analogy is with IDD proteins (a.k.a. BIRD proteins) in Arabidopsis root development. Four IDD proteins, JKD, BALDIBIS (BIB), NUTCRACKER (NUC) and MAGPIE, have overlapping functions in the specification of the cortical cell layer, and jkd bib double mutants contained extra layers of cells with indistinct identity (Ogasawara et al., 2011; Long et al., 2015). The normal pattern of cell division is mediated in part through the transcriptional repression of the CYCLIND6 gene (Sozzani et al., 2010; Long et al., 2015). Similarly, in nkd1 nkd2 mutants of maize, the normally single layer of aleurone cells is replaced by multiple layers of cells with compromised identity. Further, scarecrow-like1 (scl1) is predicted to be directly activated by NKD1 and NKD2, while the cell cycle-related genes retinoblastoma-related1 and mitotic cyclin 3B-like are predicted to be negatively regulated by NKD1 and NKD2 (Figure 2; Supplemental Dataset 3). Future work will seek to resolve potential mechanistic conservation among these processes.

METHODS

Plant Materials
Plants were grown in the field at Iowa State University experimental farms near Ames, Iowa for RNA isolation and quantitative RT-PCR and for analysis of kernel composition, starch structure and morphology and expression of transgenic fluorescent markers (FL2-RFP, GLB3-RFP, RAB17-YFP). Protoplast isolation was performed on developing endosperm from plants grown in the greenhouse at 28°C under natural lighting, supplemented with high-intensity sodium halide lamps for 16 hours.

The nkd1-R and nkd2-R alleles and all the fluorescent marker transgenes were backcrossed into the B73 inbred background at least 4 generations and B73 served as the WT control. The nkd1-Ds and nkd2-Ds0297 alleles arose and were maintained in a W22 background and W22 was used for the WT control.

**LCM-RNAseq maize v3 Transcriptome Assembly and DE Analyses**

The LCM-RNAseq was previously reported and involved isolation of RNA from 3 biological replicates of AL and SE cells from B73 WT and nkd1 nkd2 mutant kernels (Yi et al., 2015). The RNAseq reads were aligned to maize reference genome B73 V3 assembly (AGPv3) using Tophat 2 (version 2.1.0; http://ccb.jhu.edu/software/tophat/index.shtml) with default settings. The sequence aligned files (SAM/BAM format) were analyzed using coverageBed in the BEDTools package; (Quinlan and Hall, 2010) for coverage of transcript models reported in the filtered gene set (Zea_mays.AGPv3.28.gff3.gz) to generate an integer count of transcript data for each sample. Differentially expressed genes (DEGs) were identified using DESeq2 (Love et al., 2014) at an adjusted P value cut off of 0.01. MA plots were generated using the M (log intensity ratios or fold change) values against the A (mean average or mean normalized read count between the two samples). Multidimensional scaling (MDS) analysis was performed to assess the relative similarity in the sample data, wherein the information contained in the DE datasets was transformed into distance matrices in two dimensions. The number of genes expressed in any endosperm cell type was computed based on the presence of a detected read in any
one of the replicates. The ‘Genesect’ web analysis tool (Katari et al., 2010) was used for testing the statistical significance of overlap between two DE data sets.

**RNA isolation and Quantitative RT-PCR**

Aleurone was hand dissected from 15 DAP endosperms and tissues collected from 10 kernels off the same cob were pooled to constitute one biological replicate. Three replicates were sampled from independent cobs for each genotype, WT and homozygous *nkd1-R nkd2-R* mutant. Total RNA was extracted as described (Wang et al., 2012). Samples of 12 DAP pooled BETL enriched endosperm was also collected from three independent cobs and RNAs extracted from 5 pooled kernels from the same cob constituted a single biological replicate. For BETL qRT-PCR, samples were enriched for BETL by endosperm dissecting the approximately 1/3 basal end of each kernel. The RNA samples were subjected to DNAase treatment using RQ1 RNase-free DNase (Promega) and 2µg of RNA was reverse transcribed to cDNA with SuperScript™ III Reverse Transcriptase (Invitrogen) using the manufacturer’s protocol. The ‘PrimerQuest Tool’® ([http://www.idtdna.com/Primerquest/Home/Index](http://www.idtdna.com/Primerquest/Home/Index)) was used to design gene specific RT-PCR primers corresponding to coding region that spans an intron, overlaps an intron-exon junction, or in some instances including 3’ untranslated regions. Supplemental Table 9 shows the gene-specific primer sequences and the respective amplicon sizes.

Quantitative RT-PCR was performed using iQ SYBR Green Master Mix (BioRad) on an Applied Biosystems StepOnePlus™ Real-Time PCR System with 250nM of gene-specific primers and cDNA template. The thermal cycle applied was 95°C for 10min, 40 cycles of 95°C for 30s, 58°C for 30s, 72°C for 30s, followed by dissociation curve analysis. Melting curves of samples were examined for the absence of multiple peaks/non-specific amplification and those of non-template control samples were checked for possible primer dimers. Threshold cycle (CT) was automatically calculated for each reaction using the StepOnePlus qRT-PCR machine default parameters. Data were normalized to the expression level of transcript for UBIQUITIN CONJUGATING...
ENZYME (GRMZM2G132759 for AL qRT and GRMZM2G027378 for BETL qRT), and fold changes in nkd1 nkd2 mutant were computed relative to the WT control using the comparative threshold cycle (2^ΔCT) method (Livak and Schmittgen, 2001).

Construction of GST-tag and 6x His expression vectors

The glutathione S-transferase (GST)-Tag, and 6x histidine (His) NKD1 and NKD2 fusion proteins were constructed by cloning the full-length coding sequences (CDS) or ID domains into pGEX-4T (GE Healthcare Life Sciences) and pET-34b (Novagen) respectively using primers listed in Supplemental Table 10. Protein expression constructs were transformed into ORIGAMI (NOVAGEN) or BL21 (Promega) chemically competent cells. Protein expression was induced with IPTG and NKD-GST or NKD-6xHis were purified using Glutathione Sepharose 4B (GE Healthcare) or HIS-Select HF Nickel Affinity Gel resins (Sigma P6611), respectively, according to the manufacturer’s instructions. Fusion protein identity was confirmed by immunoblotting with GST antibody (Thermofisher MA4-004-HRP) and His antibody (Thermofisher MA1-21315-HRP) and by QSTAR MS/MS (Supplemental Figure 7).

Selection and amplification binding (SAAB)

SAAB was performed using purified NKD1ID-GST and NKD2ID-GST fusion proteins as described (Kozaki et al., 2004) with the following alterations: the library of randomly synthesized dsDNA was generated via Klenow fill-in reaction of a single stranded oligo library. Oligos had 5’ flanking sequence cagggtcgctggtacgaa and 3’ flanking sequence cgtaccagcgacctg with 20 random bases in between (cagggtcgctggtacgaa[N20]ttcgtaccagcgacctg). Forward (cagggtcgctggtacgaa) and reverse (cagggtcgctggtacgaa) primers were used to PCR amplify selectant oligos after each round of selection. NKD1ID and NKD2ID GST proteins were purified as described above and were covalently cross-linked to active ester agarose Affi-Gel 10 (BIO-RAD 1536099) following manufacturer’s instructions and loaded into columns (BioRad). The library of random oligos in binding buffer (described below) was passed over NKDID columns, washed, eluted and PCR amplified as previously described (Kozaki et al.,
After six cycles of selection and amplification, selectant oligo libraries were blunt end cloned into pGEM-T Easy (Promega) and sequenced. Primer sequences were trimmed and SAAB selected sequences were then analyzed for a recurring pattern (motif) with MEME-suit tool Multiple Em for Motif Elicitation (Bailey et al., 2015) using parameters previously described (Kozaki et al., 2004).

**Electrophoretic mobility shift assays (EMSA)**

NKD1ID-GST, NKD2ID-GST and empty vector GST proteins were purified as described above. 5’ biotinylated oligos (Integrated DNA Technologies) and were made double stranded by annealing with reverse complement oligos listed in Supplemental Table 11. EMSA was performed as previously described (Kozaki et al., 2004) with the following alterations: 100 ng of purified NKD1-GST, NKD2-GST, or empty vector GST fusion proteins were incubated in binding buffer (10 mMTris–HCl (pH 7.5), 75 mMNaCl, 1 mM DTT, 6% glycerol, 1% BSA, 1% Nonidet P - 40, poly[d(I,C)] and 10 µM ZnCl) with 50 fmol of 5’ biotin-labeled binding consensus sequence (BCS) or point mutated BCS variant oligos. Purified GST from induction of the empty vector served as a negative control for nonspecific interactions. After incubation on ice for 30 minutes, loading dye (without EDTA) was added and samples were loaded onto a 10% native polyacrylamide gel and run in Tris-borate buffer (without EDTA) on ice. DNA and DNA-protein complexes were transferred onto an ImmobilonNy+ membrane and UV cross linked. Biotin labeled oligos were detected with a LightShift™ Chemiluminescent EMSA Kit (Thermo Scientific 20148). Relative binding affinity for each oligo was determined by the intensity of the shifted oligo relative to unmutated BCS quantified with ImageJ.

Competition EMSA was performed using the biotinylated WT BCS oligo with the addition of 50, 100 and 500 fold excess (relative to labeled) non-biotinylated (unlabeled) WT or point mutant oligoes. Sequences are listed in Supplemental Table 11. Oligonucleotide probes were added to the binding reaction before the addition of NKD1ID-GST, NKD2ID-GST, or GST proteins.

**NKD Motif Enrichment Analysis**
The DEG proximal promoter regions (PPRs) were searched for the presence of NKD1, NKD2, and NKDcore BCSs via the Meme Suite tool Find Individual Motif Occurrences (FIMO; http://meme-suite.org/tools/fimo) using NKD1, NKD2 SAAB-MEME derived BCS probability matrices and the NKDcore motif. PPR was defined as -600 bp upstream of the transcriptional start site (TSS) extending to the TSS, and PPR sequences were downloaded from GRAMENE BIOMART (http://ensembl.gramene.org/biomart/martview/, last assessed 1/3/2016). Motif enrichment was determined by comparing the number of genes in the AL or SE transcriptome with one or more motif in its PPR to the number of DE genes in nkd1 nkd2 mutant with one or more motif in its PPR by use of Fisher’s Exact Test. The AL and SE transcriptomes were defined as all genes detected in either B73 or nkd1 nkd2 mutant LCM RNAseq datasets with 1 or more read counts. To control for the specificity of NKD BCS enrichment, three shuffle control SAAB-MEME BCS were generated for NKD1 and NKD2 from SAAB-MEME selected sequences using the MEME Suit shuffle sequence option (http://meme-suite.org/tools/meme). Enrichment was determined as described above. Direct target GO enrichment was determined by comparing the number of GO terms in DE AL or SE genes with one or more motif in PPR to the number of GO terms in the in the AL or SE transcriptome via agriGO Singular Enrichment tool (http://bioinfo.cau.edu.cn/agriGO/, last assessed 6/14/16).

Gene Ontology (GO) Enrichment and RNAseq Pathway Analysis

GO term enrichment was determined by comparing the number of GO terms in DEGs to the number of GO terms in the endosperm transcriptomes via agriGO Singular Enrichment Analysis (SEA) tool with default parameters and a critical cutoff value of FDR $\leq 0.2$ (Genome version Zea mays AGPv3.30, http://bioinfo.cau.edu.cn/agriGO/, last access on 6/14/2016). The AL and SE transcriptomes were defined as all genes detected with 1 or more read counts in RNAseq. The AL transcriptome was compared to the AL DEGs and the SE transcriptome to the SE DEGs. Fisher exact test p-values were calculated by agriGO using default parameters. Pathway analysis was performed on DEGs using MaizeCyc, CornCyc, and gene ontology (GO) tools and databases.
Pathways with a significant cutoff value of 3 nodes were analyzed for up or down regulation by overlaying differentially expressed gene log2 fold change expression onto each pathway. For non-metabolic pathways, gene ontology was used with the maize sequence v3 ontologies (agriGO v3.3 http://bioinfo.cau.edu.cn/agriGO/, maizeGDB, http://www.maizegdb.org/). Pathways were visualized using heatmaps generated by the R pheatmap program.

**Epifluorescence Microscopy**

The *nkd-R* alleles were crossed into GL3B-RFP, FL2-RFP, and RAB17-YFP transgenic lines (MAIZE CELLGENOMICS DATABASE, http://maize.jcvi.org). Transgenic individuals were selected by LIBERTY herbicide and self-pollinated to produce *nkd-R* segregating cobs. Mature kernels were harvested and sectioned using a Leica Vibratome or by hand. Three kernels each, from the same segregating cob, of WT and *nkd-R* within each transgenic line were viewed using an Olympus BX-60 microscope under brightfield or epifluorescence. Tissues were visualized by autofluorescence using a Chroma™ narrow violet (NV) filter (excitation 400–410 nm, dichroic mirror and barrier filter, 455 nm). YFP was observed with a Chroma™ EYFP filter set (excitation 495 nm, dichroic barrier filter 515 nm, emission 540 nm) and mCherry was observed using a Chroma mCherry filter set (excitation 560 nm, dichroic barrier filter 600 nm, emission 635 nm). Micrography was performed with a Jenoptik C-5 camera and constant gain and exposure time settings were used for each filter set to compare expression of each respective transgene reporter protein in WT versus *nkd1* *nkd2* mutant kernels. Standard PCR genotyping of transgene and *nkd1-R* and *nkd2-R* alleles was performed using primers described (Yi et al., 2015) to confirm kernel genotypes.

**Endosperm Starch Extraction, Quantification and Chain Length Distribution Assay**

WT (W22) and *nkd1-Ds; nkd2-Ds0297* kernels were collected from the same segregating ear, with six individual kernels of each genotype serving as biological replicates. Mature kernels were soaked in 0.45% (w/v) sodium metabisulfite at 50°C
overnight. Pericarp and embryo were removed and the total endosperm starch was isolated. The extraction procedure (Dinges et al., 2001) was modified as follows: the endosperm starch was washed with chilled deionized water twice and with chilled 80% ethanol once, and centrifuged 3,000Xg at 4°C for 10 min after each liquid addition. The final pellet was dissolved in 100% dimethyl sulfoxide (DMSO) and boiled in water bath for 1 hour.

To measure endosperm starch content, DMSO-dissolved starch was diluted 10-fold with amyloglucosidase buffer (0.1 m sodium acetate, pH 5, and 5 mm calcium chloride), and digested by amyloglucosidase (60 units/reaction, Megazyme E-AMGDF100), followed by incubation at 50°C for 100 min, producing glucose. Glucose was measured by a GOPOD Assay Kit (Megazyme K-GLUC) following the manufacturer’s instructions to determine the weight of starch.

For glucan chain length distribution, less than 1 mg of starch was precipitated in 5 volumes of 100% ethanol at 4°C overnight, followed by centrifugation at 13,000Xg for 10 min. The pellet was re-solubilized in deionized water and pH was adjusted to 4.5 with 0.5 M sodium acetate. Starch was de-branched with 4 units of isoamylase (Megazyme E-ISAMY) at 42°C overnight. The chain length distribution of each sample was analyzed via Dionex HPAEC-PAD system (Thermo Fisher Scientific, Sunnyvale, CA) as described (Dinges et al., 2001).

**Scanning Electron Microscopy**

For scanning electron microscopy (SEM), 6 WT and 6 nkd1 nkd2 mutant mature kernels from F2 nkd1-Ds; nkd2-Ds0297 segregating ears were used. Kernel genotypes were verified by standard PCR genotyping using primers described in Yi et al., 2015. Mature kernels were cracked and freshly planed with a razor blade, cleaned with ethanol, and placed on a specimen stub with a carbon coated adhesive. Specimen stubs were then painted with silver paint and air dried for 10 minutes at room temperature. Kernels were sputter coated with gold and images with a digital JEOL 5800LV scanning electron microscope.
Total Pericarp, Endosperm, Embryo, and Seed Dry Weight Analysis

For pericarp, endosperm, embryo and total seed weight analysis, 31 WT and 31 nkd1
nkd2 mutant mature kernels from the same F2 segregating nkd1-Ds; nkd2-Ds0297
segregating ear were randomly selected. This was done on three independent ears for
a total of 93 WT kernels and 93 nkd1 nkd2 mutant kernels. Mature kernels were
imbibed in double distilled water for 12 hours and were frozen solid at 4°C and thawed
to allow for efficient dissection. Pericarp, endosperm, and embryos were then dissected
into pools of tissue from 31 kernels and placed in a 55°C oven for 36 hours to re-
desiccate the materials. Samples were then weighed to obtain pooled kernel pericarp,
endosperm, and embryo dry weights. Total seed weights were determined by adding
the pericarp, endosperm and embryo dry weights together for each independent ear.
Average pericarp, endosperm, embryo and total seed weights per kernel for WT and
nkd1 nkd2 mutant were determined and statistical analysis was performed using a
Student's t-test.

Total Nitrogen and Protein Analysis

For total nitrogen analysis, 10 WT and 10 nkd1-Ds; nkd2-Ds0297 kernels were
randomly selected from an F2 segregating nkd1-Ds; nkd2-Ds0297 ear. This process
was repeated twice for a total of 3 randomly selected 10 WT and 10 nkd1-Ds; nkd2-
Ds0297 kernel pools from the same ear. This process was performed on a total of three
independent ears representing 90 WT and 90 nkd1-Ds; nkd2-Ds0297 kernels for a total
of 9 WT and 9 nkd1 nkd2 mutant kernel 10 kernel pools. Each of the 10 WT and 10
nkd1 nkd2 mutant kernel pools were imbibed in double dilute water for 12 hours, frozen
at -20°C and thawed to allow efficient dissection. Endosperm dissections were then
performed and 10-kernel pools and were ground in a mortar and pestle with liquid
nitrogen to a particle size of less than 1 mm in diameter. Ground samples were placed
in a 55°C oven for 48 hours to re-desiccate the materials, then weighed. Total nitrogen
content was determined from 0.15 grams of each of the 9 WT and 9 nkd1 nkd2 mutant
pooled endosperm samples using the Dumas N combustion procedure with a Leco
Truspec CN analyzer and Elementar Variomax CNS analyzer. Protein content (%) per sample was determined by multiplying % nitrogen content by 6.25. Total grams protein per sample was determined by multiplying % total protein by dry weight. Sample means were compared using Student's t-test.

**Principle Component Analysis**

Principal component analysis (PCA) was performed using the MetaboAnalyst 3.0 web tool (Xia et al., 2015). Due to experimental design differences, PCA was performed separately for endosperm weight combined with the total protein to account for the effects of independent ears (cob) and for starch branch chain length distribution combined with the total starch to account for the effect of independent kernels within the same segregating ear.

**Bimolecular Fluorescence Complementation Assay (BiFC)**

BiFC assays were performed as described (Citovsky et al., 2006). *nkd1* and *nkd2* ID domain and full-length CDS were cloned in-frame to the N-terminal half and the C-terminal half of the yellow florescent protein (YFP) in vectors pSAT1A-nEYFP-N1 and pSAT1A-cEYFP-N1, respectively, using primers listed in Supplemental Table 12. A 35S:mCherry construct was generated by cloning the mCherry CDS into a modified pSAT1A vector from which the EYFP fragment had been removed. The 35S:mCherry, nYFP-NKD and cYFP-NKD constructs were biolistically introduced into onion epidermal cells and incubated in the dark for 24-36 h. 35S:mCherry was co-bombarded with each experiment and used as an internal control to identify transiently transformed cells. Expression of the mCherry marker and reconstitution of YFP florescence was observed by epifluorescence microscopy as described above.

**Reciprocal Co-Pull Down**

Pull-down assays were performed using the NKD-GST and NKD-6x HIS fusion proteins. Fusion protein expression was induced by IPTG for two hours and total soluble protein extracts were collected as previously described (Kozaki et al., 2004).
NKD-GST and NKD-6x His tagged total soluble protein extracts were mixed and
incubated in PBS buffer with 0.15 mM PMSF for 1 h at room temperature with gentle
rotation. Samples were then passed though Glutathione Sepharose 4B (GE
Healthcare) or HIS-Select HF Nickel Affinity Gel resins (Sigma), then washed with GST
wash buffer (1x phosphate-buffered saline, pH 7.4) or 6x His wash buffer (50 mM
phosphate buffer pH 7.0, 300 mM NaCl, 1mM imidizole), respectively. Proteins were
eluted with GST elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) or
6x His elution buffer (50 mM phosphate buffer pH 7.0, 300 mM NaCl, 150 mM
imidizole) and detected by SDS-PAGE immunoblotting using GST antibody (product #
MA4-004-HRP) or His antibody (product # MA1-21315-HRP).

**Protoplast Isolation and Transformation**

To avoid confounding effects from endogenous NKD1 and NKD2 protein, 18-20 DAP
nkd1-Ds; nkd2-Ds0297 kernels were used for aleurone protoplast isolation as
described (Bethke and Jones, 2001). Aleurone peels from 30-40 kernels (~0.5g) of the
same ear were harvested and placed immediately in 15 ml of TVL solution (0.3 M
sorbitol; 50 mM CaCl$_2$). 20 ml of Enzyme solution (0.5 M sucrose, 10 mM MES-KOH
[pH 5.7], 20 mM CaCl$_2$, 40 mM KCl, 1% Cellulase Onozuka R-10, 1% Macerozyme
R10) were then added and the tissue shaken at 35 rpm at room temperature for 16-18
hours. Protoplasts were collected by passing through 10 micro meter nylon mesh
(spectrum labs) with W5 solution (154 mM NaCl, 125 mM CaCl$_2$, 5 mM KCl, 2 mM MES
[pH 5.7]). Protoplasts were recovered from the nylon mesh by rinsing with 15 ml of W5
solution followed by centrifugation for 5 minutes at 60 g. Protoplast pellets were gently
re-suspended in 15 ml of fresh W5 solution, centrifuged for 5 minutes at 60 g, then re-
suspended 1 ml MMg solution (0.4 M mannitol, 15 mM MgCl$_2$, 4 mM MES [pH 5.7]).
Protoplast integrity and quantification was determined by optical visualization on a light
microscope.

Protoplast transformation was performed following the protocol outlined by Bethke and
Jones (2001). For each transformation, 100 µl of fresh protoplasts (~2x10^4) were
added to a 2 ml microfuge tube. Effector, reporter and normalization plasmids in 10 μl total volume (10-20 μg DNA total) were added and 110 μl of PEG solution (40% weight/volume PEG 4000, 0.2 M mannitol, 100 mM CaCl₂) was added, gently mixed, and incubated at room temperature for 10 min without shaking. W5 solution (400 μl) was added to stop the transfection process, tubes were centrifuged at 100 g for 2 min at room temperature, and protoplast pellets gently re-suspended in 1 ml WI solution (0.5 M mannitol, 4 mM MES [pH 5.7], 20 mM KCl) and incubated at room temperature on a rotator for 14 h.

Transcriptional Activity Assays

To test the transcriptional activity of NKD1 and NKD2, a series of reporter, effector and normalization constructs (Figure 8A) were designed and cloned using primers listed in Supplemental Table 13. For reporter vectors, the promoter regions of select putative direct targets of NKD were cloned upstream of the firefly luciferase coding sequence in the pGL3 vector (Promega). The proximal promoter regions of xylanase inhibitor protein 1 (zmX1P-1, GRMZM2G328171), opaque2 (o2, GRMZM2G015534), zein protein 22.1 (zp22.1, GRMZM2G044625), nkd1 (GRMZM2G074773), viviparous1 (vp1, GRMZM2G133398), jasmonate induced protein (JIP, GRMZM2G112238), Mother of FT-like (MTF, GRMZM2G059358) and WRKY transcription factor 29 (wrky29, GRMZM2G040298) constituted the reporter plasmids (Supplemental Table 14). For the effector constructs, 35Spro:NKD1 and 35Spro:NKD2, nkd1 and nkd2 CDSs were cloned into a modified pSAT1A vector described above using primers listed in Supplemental Table 13. The empty vector was used as 35S:null control. The normalization construct was generated by cloning the Renilla luciferase CDS sequence into a modified pSAT1A vector using primers listed in Supplemental Table 13. The reporter, effector, and normalization constructs were co-transformed into nkd1-Ds/nkd1- Ds0297; nkd1-Ds; nkd2-Ds0297 AL protoplasts as described above. Sets of transactivation assays were performed on protoplasts from the same isolation and protoplasts from independent isolations constituted biological replicates. A control using the 35S-null construct as effector was included for each set of assays. A total of three biological replicates were
performed for each treatment. Firefly and Renilla luciferase activity assays were performed using a dual luciferase assay System kit following the manufacturer's recommended instructions (Promega E1910). Luminescence was measured using a microplate reader (BioTek) and three technical replicates were used for each assay.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: RNAseq reads were deposited in NCBI Gene Expression Omnibus accession number GSE61057. Gene model numbers are provided for differentially expressed genes in Supplemental Dataset 1 for the aleurone and Supplemental Dataset 2 for the starchy endosperm.

Supplemental Data

Supplemental Figure 1. Maize V3 LCM-RNAseq transcriptome assembly and DE gene confirmation.

Supplemental Figure 2. Disrupted pathways in nkd1 nkd2 mutant aleurone.

Supplemental Figure 3. Disrupted pathways in nkd1 nkd2 mutant starchy endosperm.

Supplemental Figure 4. BETL qRT-PCR.

Supplemental Figure 5. nkd1 nkd2 mutant starch granule SEM.

Supplemental Figure 6. Principal component analysis of nkd1 nkd2 mutant and wild-type resource reserve datasets.

Supplemental Figure 7. Verification of NKD-ID GST fusion proteins.

Supplemental Figure 8. EMSA controls and additional tested mutant probes.

Supplemental Figure 9. Scanning mutagenesis EMSA overexposure.
Supplemental Figure 10. Overlap in NKD motifs and predicted direct target genes.

Supplemental Figure 11. NKD BCS enrichment shuffled controls.

Supplemental Figure 12. BiFC controls.

Supplemental Figure 13. Reciprocal tag co-pull downs.

Supplemental Table 1. Summary of RNA sequencing reads.

Supplemental Table 2. Summary of gene expression analysis in endosperm cell types.

Supplemental Table 3. NKD1 SAAB selected sequences.

Supplemental Table 4. NKD2 SAAB selected sequences.

Supplemental Table 5. NKD1, NKD2 and O2 aleurone co-regulated genes.

Supplemental Table 6. NKD1, NKD2 and O2 starchy endosperm co-regulated genes.

Supplemental Table 7. Enriched gene ontologies in NKD1, NKD2 and O2 aleurone co-regulated genes.

Supplemental Table 8. Enriched gene ontologies in NKD1, NKD2 and O2 starchy endosperm co-regulated genes.

Supplemental Table 9. Primer sequences used for qRT-PCR.

Supplemental Table 10. Primers used to generate NKD GST and 6x His tag expression constructs.

Supplemental Table 11. Oligonucleotides used in electrophoretic mobility shift assays (EMSA).

Supplemental Table 12. Primers used for cloning of constructs used in BiFC.

Supplemental Table 13. Primers used for cloning of transcription assay constructs.
Supplemental Table 14. Constructs cloned in this study.

Supplemental Dataset 1. nkd1 nkd2 mutant aleurone differentially expressed genes and pathway analyses.

Supplemental Dataset 2. nkd1 nkd2 mutant starchy endosperm differentially expressed genes and pathway analyses.

Supplemental Dataset 3. NKD direct target gene analyses.

Supplemental Dataset 4. NKD direct target gene ontologies and enrichment.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

P.W.B., B.C.G., A.K.N., and H.W. designed experiments. A.K.N. performed LCM (Yi et al., 2015), RNAseq transcriptome assembly and differential expression analyses. A.K.N. and B.C.G. performed qRT-PCR and transgene fluorescent microscopy experiments. B.C.G. performed RNAseq DE gene enrichment and pathway analyses, seed composition analyses, principle component analyses (PCA), SAAB and MEME analyses, EMSAs, NKD direct target and enrichment analyses, BiFC and Co-IP assays, transcription assays and cloned all constructs used in the manuscript. H.W. and B.C.G. performed starch branch chain length distribution and kernel starch content
experiments. B.C.G was the main contributor to the writing of the manuscript and
A.K.N., H.W. and P.W.B. also contributed to writing.

REFERENCES


the transition to germination by regulating light and hormonal signaling during seed maturation.


jointly stabilize tissue boundaries by confining the cell fate regulator SHORT-ROOT and
contribute to fate specification. Plant Cell 27, 1185-1199.


FIGURE LEGENDS

Figure 1. Summary of Transcriptomic Analysis

(A) Shared and unique differentially expressed (DE) genes in nkd1-R nkd2-R mutant aleurone (AL) and starchy endosperm (SE).

(B) Proportion of genes with ≥1 read counts detected in RNAseq (expressed) to number of DE genes in AL and SE.

(C) Scatter plot of RNAseq transcript abundance fold change values in nkd1-R nkd2-R mutant AL relative to WT AL (on the X-axis) against RT-PCR expression values (on Y axis) for the 15 RNAseq DE genes in nkd1 nkd2 mutant AL tested by qRT-PCR.

Figure 2. Pathway Analysis of Differentially Expressed Gene Transcripts

(A) and (B) Log2 fold change heat maps of differentially-expressed genes functioning in selected disrupted pathways in nkd1 nkd2 mutants. (A) aleurone and (B) starchy endosperm.
Figure 3. Disrupted Nutrient Reserve Deposition in nkd1 nkd2 Mutant Endosperm

(A) Opaque endosperm phenotype in WT and nkd1-Ds; nkd2-Ds0297 mutant kernels from an F2 segregating ear

(B) Difference plots of nkd1-Ds; nkd2-Ds0297 mutant minus WT mean starch branch chain length abundance. "**" denotes statistically significant differences (p<0.05) determined by Student’s t-test.

(C-F) Scanning electron microscopy (SEM) of starch grains of mature segregating nkd1-Ds nkd2-Ds0297 mutant (C,E) and WT (D,F) kernels. Arrows indicate hollow core in bisected starch granules.

Figure 4. Marker Transgene Expression in WT and nkd1 nkd2 mutant endosperm.

(A) to (C) Florescence microscopy using narrow violet-broad range (NV) filter to visualize autofluorescence and an mCherry filter to visualize (A) FL2-RFP and (B) GL3B-RFP or a YFP florescence filter for (C) RAB17-YFP. AL indicates the aleurone layer and SE indicates the starchy endosperm. Size bars = 100 μm

Figure 5. NKD1 and NKD2 DNA Binding

(A) and (B) SAAB-MEME derived binding consensus sequences (BCS) for NKD1ID (A) and NKD2ID (B).

(C) Oligonucleotide sequences used as probes for electrophoretic mobility shift assays (EMSAs). WT contains the consensus binding sequence, while M1 to M8 contain base substitutions indicated by magenta lettering. Binding, relative to WT for a given protein is indicated by +++ (>66%) ++ (33 to 66%) + (<33%) and – (no detected shift).
to (G) EMSAs. (D) and (F) show EMSA of consensus (WT) and mutant probes for
NKD1-ID (D) and NKD2-ID (F) proteins. (E) and (G) Competition assays using labeled
WT probe, and 50, 100 or 500-fold excess of unlabeled WT, M2 or M8 oligonucleotide,
incubated with NKD1ID (E) or NKD2ID (G) protein. + indicates reaction with no
competitor, – indicates negative control with GST protein instead of NKD. Overexposed
images, additional EMSAs and negative controls using purified GST are shown in
Supplemental Figures 8 and 9.

Figure 6. NKD1 and NKD2 Protein BiFC and Co-Pull Down

(A) Transient bimolecular fluorescence complementation (BiFC) assays in onion
ejdermal cells for full-length NKD1 and NKD2 proteins. Vectors used for each BiFC
assay are listed to the left of each row. Arrows designate nuclei viewed under
differential interference contrast (DIC). mCherry fluorescence marks transient cells
transformation and YFP fluorescence indicates a positive protein-protein interaction.
Control bombardments containing NYFP + NKD1-CYFP or NKD2-NYFP + CYFP did not
produce YFP fluorescence. Size bars = 100μm. Additional controls are shown in
Supplemental Figure 12.

(B) and (C) Co-pulldown immunoblots for full-length NKD1 and NKD2 proteins (B) and
NKD1-ID and NKD2-ID proteins (C). Affinity column and antibody (Ab) used for each
assay are indicated at the top of each panel. Total soluble bacterial lysates (inp) were
immunoblotted as a positive control for protein expression. The first set of control lanes
show no cross reaction between NKD-GST protein with anti-6xHis antibody or NKD-
6xHis protein with anti-GST antibody.

Figure 7. NKD1 and NKD2 Transcription Assays

(A) Schematic of constructs transfected into aleurone protoplasts for transient reporter
assays of NKD1 and NKD2 transregulatory activity on selected direct target promoters.
Effector, reporter and normalization constructs were cotransfected. The Null construct was substituted for the effector construct as a negative control. See Supplemental Table 14 for details on promoters used for each reporter construct.

**Figure 8. A NKD Binding Motif is Required for Transcriptional Activation**

**(A)** Reporter constructs used to test the requirement of a NKD binding site for transcriptional activation of the *vp1* promoter by NKD1 and NKD2. The mutant *vp1* promoter construct (*vp1*-mut*pro*:LUC) was cloned with the thymine in the 7th position of the second NKDcore BCS in the *vp1*:*LUC construct substituted with a cytosine (TTGTCG*T* to TTGTCG*C*).

**(B)** Activities of NKD1 and NKD2 on expression of WT and mutant *vp1* promoter constructs. Reporter and effector constructs are listed. Error bars represent standard errors of the means. * designates statistically significant (p=0.05, Student’s t-test) difference relative to control (35S*pro*:null) or between NKD1 (35S*pro*:NKD1) and NKD2 (35S*pro*:NKD2) effectors.
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<td>GO:0004553</td>
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<td>hydrolase activity, hydrolyzing O-glycosyl compounds</td>
<td>42</td>
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<tr>
<td>GO:0005875</td>
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<td>15</td>
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<td>GO:0046983</td>
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<td>58</td>
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<td>GO:0016769</td>
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<tr>
<td>GO:0031226</td>
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<tr>
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<tr>
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<td>GO term</td>
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<td>Description</td>
<td>DE</td>
<td>SE</td>
<td>SE Tran.</td>
<td>p-value</td>
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<td>56</td>
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<tr>
<td>GO:0005730</td>
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<td>nucleolus</td>
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<td>membrane-enclosed lumen</td>
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<td>755</td>
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<td>GO:0070013</td>
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<td>747</td>
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<td>324</td>
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<td>747</td>
<td>0.00075</td>
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<td>GO:0009507</td>
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<td>579</td>
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<td>0.18</td>
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<td>GO:0031981</td>
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<td>613</td>
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<td>3160</td>
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<tr>
<td>GO:0030312</td>
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<td>external encapsulating structure</td>
<td>60</td>
<td>596</td>
<td>0.0042</td>
<td>0.2</td>
</tr>
<tr>
<td>GO:0044435</td>
<td>C</td>
<td>plastid part</td>
<td>105</td>
<td>1128</td>
<td>0.0031</td>
<td>0.2</td>
</tr>
<tr>
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<td>C</td>
<td>intracellular organelle part</td>
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<tr>
<td>GO:0005576</td>
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<td>extracellular region</td>
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<td>467</td>
<td>0.0041</td>
<td>0.2</td>
</tr>
<tr>
<td>GO:0032993</td>
<td>C</td>
<td>protein-DNA complex</td>
<td>16</td>
<td>105</td>
<td>0.0028</td>
<td>0.2</td>
</tr>
</tbody>
</table>

1. Number of genes associated with each GO term that are differentially expressed between WT and nkd1 nkd2 starchy endosperm.
2. Total number of genes in each GO category expressed in the starchy endosperm transcriptome.
3. Fisher’s exact test for GO term enrichment.
4. False discovery rate.
<table>
<thead>
<tr>
<th>Seed Component</th>
<th>WT (+/- se)</th>
<th>nkd1 nkd2 (+/- se)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total seed weight (mg)</td>
<td>220.16 (1.49)</td>
<td>128.12 (4.52)</td>
<td>1.01E-03</td>
</tr>
<tr>
<td>Pericarp (mg)</td>
<td>11.34 (0.7)</td>
<td>9.61 (0.41)</td>
<td>0.12</td>
</tr>
<tr>
<td>Endosperm (mg)</td>
<td>186.45 (1.39)</td>
<td>91.67 (3.74)</td>
<td>4.72E-04</td>
</tr>
<tr>
<td>Embryo (mg)</td>
<td>22.38 (0.52)</td>
<td>26.84 (0.86)</td>
<td>0.02</td>
</tr>
<tr>
<td>Endosperm protein (mg)</td>
<td>16.63 (1.05)</td>
<td>7.96 (0.53)</td>
<td>5.39E-03</td>
</tr>
<tr>
<td>Endosperm nitrogen (%)</td>
<td>1.58 (0.05)</td>
<td>1.72 (0.12)</td>
<td>0.34</td>
</tr>
<tr>
<td>Endosperm starch (mg)</td>
<td>108.19 (5.75)</td>
<td>73.02 (2.98)</td>
<td>2.97E-05</td>
</tr>
</tbody>
</table>

*p Student's t-test
### Table 4. NKD BCS Enrichment in Differentially Expressed Gene Promoters

<table>
<thead>
<tr>
<th>Motif</th>
<th>DE Genes</th>
<th>Transcriptome</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKD1</td>
<td>194</td>
<td>3084</td>
<td>p=0.70</td>
</tr>
<tr>
<td>NKD2</td>
<td>1050</td>
<td>11200</td>
<td>p ≤ 0.01</td>
</tr>
<tr>
<td>NKDcore</td>
<td>816</td>
<td>10684</td>
<td>p ≤ 0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Motif</th>
<th>DE Genes</th>
<th>Transcriptome</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKD1</td>
<td>217</td>
<td>2930</td>
<td>p ≤ 0.01</td>
</tr>
<tr>
<td>NKD2</td>
<td>1059</td>
<td>15222</td>
<td>p=0.95</td>
</tr>
<tr>
<td>NKDcore</td>
<td>696</td>
<td>9970</td>
<td>p=0.982</td>
</tr>
</tbody>
</table>

*A Fisher’s exact test

The number of each motif’s occurrence in aleurone and starchy endosperm differentially expressed (DE) and RNaseq detected (Transcriptome) proximal promoter regions was determined. Motif enrichment in DE genes was assessed relative to the transcriptome.
Table 5. Enriched GO Terms in Aleurone Direct Target Genes

<table>
<thead>
<tr>
<th>GO term</th>
<th>Ontology</th>
<th>Description</th>
<th>DE AL Genes$^1$</th>
<th>AL Transcriptome$^2$</th>
<th>p-value$^1$</th>
<th>FDR$^1$</th>
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</thead>
<tbody>
<tr>
<td>GO:0045735</td>
<td>F</td>
<td>nutrient reservoir activity</td>
<td>22</td>
<td>56</td>
<td>2.80E-18</td>
<td>1.70E-15</td>
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<tr>
<td>GO:0032993</td>
<td>C</td>
<td>protein-DNA complex</td>
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<td>GO:0000786</td>
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<td>nucleosome</td>
<td>13</td>
<td>101</td>
<td>3.40E-05</td>
<td>0.0077</td>
</tr>
<tr>
<td>GO:0034728</td>
<td>P</td>
<td>nucleosome organization</td>
<td>14</td>
<td>107</td>
<td>1.40E-05</td>
<td>0.0085</td>
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<tr>
<td>GO:0006334</td>
<td>P</td>
<td>nucleosome assembly</td>
<td>14</td>
<td>107</td>
<td>1.40E-05</td>
<td>0.0085</td>
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<tr>
<td>GO:0031497</td>
<td>P</td>
<td>chromatin assembly</td>
<td>14</td>
<td>109</td>
<td>1.80E-05</td>
<td>0.0085</td>
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<tr>
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<td>P</td>
<td>protein-DNA complex assembly</td>
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<td>110</td>
<td>2.00E-05</td>
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<tr>
<td>GO:0006333</td>
<td>P</td>
<td>chromatin assembly or disassembly</td>
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<td>111</td>
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<td>GO:0006323</td>
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<td>DNA packaging</td>
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<td>135</td>
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<tr>
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</table>
GO:0005875  C microtubule associated complex  9  92  0.0037  0.17
GO:0009579  C thylakoid  29  513  0.0049  0.17
GO:0044434  C chloroplast part  55  1125  0.0044  0.17
GO:0015630  C microtubule cytoskeleton  14  189  0.0048  0.17
GO:0044422  C organelle part  135  3190  0.0058  0.18
GO:0044446  C intracellular organelle part  135  3187  0.0056  0.18
GO:0004553  F hydrolase activity, hydrolyzing O-glycosyl compounds  24  371  0.0019  0.19

GO:0045735  F nutrient reservoir activity  21  56  6.50E-19  3.50E-16

The number of each GO term occurrence in differentially expressed (DE) aleurone (AL) genes with a motif in proximal promoter region and in RNAseq AL detected (Transcriptome) genes was determined. GO enrichment in DE genes with a motif was assessed relative to the transcriptome. GO enrichment was not detected for NKD1 AL direct target genes. See Supplemental Datasets 3 and 4 for further details.

1 Number of putative direct target genes expressed in the aleurone associated with each GO term.
2 Total number of genes in each GO category expressed in the aleurone transcriptome.
3 Fisher's exact test for GO term enrichment.
4 False discovery rate.
Table 6. Enriched GO Terms in Starchy Endosperm Direct Target Genes

<table>
<thead>
<tr>
<th>GO term</th>
<th>Ontology</th>
<th>Description</th>
<th>DE Genes</th>
<th>SE Transcriptome</th>
<th>p-value $^3$</th>
<th>FDR $^4$</th>
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<td>GO:0030312</td>
<td>C</td>
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<td>12</td>
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<td>0.0011</td>
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<td>GO:0005618</td>
<td>C</td>
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<td>12</td>
<td>579</td>
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NKD2

<table>
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<th>Description</th>
<th>DE Genes</th>
<th>SE Transcriptome</th>
<th>p-value $^3$</th>
<th>FDR $^4$</th>
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<tr>
<td>GO:0009536</td>
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<td>plastid</td>
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<td>2023</td>
<td>3.80E-05</td>
<td>0.0097</td>
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<td>1111</td>
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</tr>
<tr>
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<td>external encapsulating structure</td>
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<td>596</td>
<td>0.0012</td>
<td>0.1</td>
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NKD

<table>
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<th>GO term</th>
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<th>Description</th>
<th>DE Genes</th>
<th>SE Transcriptome</th>
<th>p-value $^3$</th>
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</tr>
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<td>27</td>
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</tbody>
</table>

The number of each GO term occurrence in differentially expressed (DE) starchy endosperm (SE) genes with a motif in proximal promoter region and in RNAseq SE detected (Transcriptome) genes was determined. GO enrichment in DE genes with a motif was assessed relative to the transcriptome. See Supplemental Datasets 3 and 4 for further details.

1 Number of putative direct target genes expressed in the starchy endosperm associated with each GO term.
2 Total number of genes in each GO category expressed in the starchy endosperm transcriptome.
3 Fisher’s exact test for GO term enrichment.
4 False discovery rate.
Figure 1. Summary of Transcriptomic Analysis

(A) Shared and unique differentially expressed (DE) genes in nkd1-R nkd2-R mutant aleurone (AL) and starchy endosperm (SE).

(B) Proportion of genes with ≥1 read counts detected in RNAseq (expressed) to number of DE genes in AL and SE.

(C) Scatter plot of RNAseq transcript abundance fold change values in nkd1-R nkd2-R mutant AL relative to WT AL (on the X-axis) against RT-PCR expression values (on Y axis) for the 15 RNAseq DE genes in nkd1 nkd2 mutant AL tested by qRT-PCR.
Figure 2. Pathway Analysis of Differentially Expressed Gene Transcripts (A) and (B) Log₂ fold change heat maps of differentially-expressed genes functioning in selected 
disrupted pathways in nkd1 nkd2 mutants. (A) aleurone and (B) starchy endosperm.
Figure 3. Disrupted Nutrient Reserve Deposition in nkd1 nkd2 Mutant Endosperm

(A) Opaque endosperm phenotype in WT and nkd1-Ds; nkd2-Ds0297 mutant kernels from an F2 segregating ear.

(B) Difference plots of nkd1-Ds; nkd2-Ds0297 mutant minus WT mean starch branch chain length abundance. "**" denotes statistically significant differences (p<0.05) determined by Student's t-test.

(C-F) Scanning electron microscopy (SEM) of starch grains of mature segregating nkd1-Ds nkd2-Ds0297 mutant (C,E) and WT (D,F) kernels. Arrows indicate hollow core in bisected starch granules.
Figure 4. Marker Transgene Expression in WT and nkd1 nkd2 mutant endosperm.

(A) to (C) Fluorescence microscopy using narrow violet-broad range (NV) filter to visualize autofluorescence and an mCherry filter to visualize (A) FL2-RFP and (B) GL3B-RFP or a YFP fluorescence filter for (C) RAB17-YFP. AL indicates the aleurone layer and SE indicates the starchy endosperm. Size bars = 100 µm.
Figure 5. NKD1 and NKD2 DNA Binding

(A) and (B) SAAB-MEME derived binding consensus sequences (BCS) for NKD1ID (A) and NKD2ID (B).

(C) Oligonucleotide sequences used as probes for electrophoretic mobility shift assays (EMSAs). WT contains the consensus binding sequence, while M1 to M8 contain base substitutions indicated by magenta lettering. Binding, relative to WT for a given protein is indicated by +++ (>66%) ++ (33 to 66%) + (<33%) and – (no detected shift).

(D) to (G) EMSAs. (D) and (F) show EMSA of consensus (WT) and mutant probes for NKD1-ID (D) and NKD2-ID (F) proteins. (E) and (G) Competition assays using labeled WT probe, and 50, 100 or 500-fold excess of unlabeled WT, M2 or M8 oligonucleotide, incubated with NKD1ID (E) or NKD2ID (G) protein. + indicates reaction with no competitor, – indicates negative control with GST protein instead of NKD. Overexposed images, additional EMSAs and negative controls using purified GST are shown in Supplemental Figures 8 and 9.
Figure 6. NKD1 and NKD2 Protein BiFC and Co-Pull Down

(A) Transient bimolecular fluorescence complementation (BiFC) assays in onion epidermal cells for full-length NKD1 and NKD2 proteins. Vectors used for each BiFC assay are listed to the left of each row. Arrows designate nuclei viewed under differential interference contrast (DIC). mCherry fluorescence marks transient cells transformation and YFP fluorescence indicates a positive protein-protein interaction. Control bombardments containing NYFP + NKD1-CYFP or NKD2-NYFP + CYFP did not produce YFP fluorescence. Size bars = 100 μm. Additional controls are shown in Supplemental Figure 12.

(B) and (C) Co-pulldown immunoblots for full-length NKD1 and NKD2 proteins (B) and NKD1-ID and NKD2-ID proteins (C). Affinity column and antibody (Ab) used for each assay are indicated at the top of each panel. Total soluble bacterial lysates (inp) were immunoblotted as a positive control for protein expression. The first set of control lanes show no cross reaction between NKD-GST protein with anti-6xHis antibody or NKD-6xHis protein with anti-GST antibody.
Figure 7. NKD1 and NKD2 Transcription Assays

(A) Schematic of constructs transfected into aleurone protoplasts for transient reporter assays of NKD1 and NKD2 transregulatory activity on selected direct target promoters. Effector, reporter and normalization constructs were cotransfected. The Null construct was substituted for the effector construct as a negative control. See Supplemental Table 14 for details on promoters used for each reporter construct.

(B) Relative luciferase activities. Reporter activity (Firefly LUC) is shown in proportion to the normalization standard (Renilla LUC). Error bars represent standard errors of the means for three biological replicates. Reporter and effector constructs used for each assay are listed. * designates statistically significant difference (p=0.05, Student’s t-test) relative to control (35S pro:NULL) or between NKD1 (35S pro:NKD1) and NKD2 (35S pro:NKD2) effectors.
Figure 8. A NKD Binding Motif is Required for Transcriptional Activation

(A) Reporter constructs used to test the requirement of a NKD binding site for transcriptional activation of the vp1 promoter by NKD1 and NKD2. The mutant \( vp1 \) promoter construct (\( vp1\text{-mut}_\text{pro}:\text{LUC} \)) was cloned with the thymine in the 7th position of the second NKDcore BCS in the \( vp1\text{pro}:\text{LUC} \) construct substituted with a cytosine (TTGTCGT to TTGTCGC).

(B) Activities of NKD1 and NKD2 on expression of WT and mutant vp1 promoter constructs. Reporter and effector constructs are listed. Error bars represent standard errors of the means. * designates statistically significant (p=0.05, Student’s t-test) difference relative to control (35S\( \text{pro} :\text{null} \)) or between NKD1 and NKD2 effectors.


Sozzani, R., Cui, H., Moreno-Risueno, M.A., Busch, W., Van Norman, J.M., Vernoux, T., Brady, S.M., Detwile, W., Murray, J.A.H., and


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