Kzp Controls Canonical wnt8 Signaling to Modulate Dorsoventral Patterning during Zebrafish Gastrulation*

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During vertebrate embryonic development, the body axis formation requires the action of Wnt signals and their antagonists. Zygotic canonical wnt8 expression appears exclusively at the ventrolateral margin and mediates Wnt/β-catenin activities to promote posterior and ventral cell fate. However, the mechanisms involved in the initiation of zygotic wnt8 signals are poorly understood. Here, we identify a novel, maternally derived transcription factor, Kzp (Kaiso zinc finger-containing protein), as an important determinant for the initiation of zygotic Wnt signals in zebrafish. Kzp is a DNA-binding transcription factor that recognizes specific consensus DNA sequences, 5′-(t/a/g)(t/a/ctg)ctgcc-3′, through zinc fingers and controls the initiation of zygotic wnt8 expression by directly binding to the wnt8 promoter during zebrafish embryonic development. Depletion of Kzp strongly dorsalized embryos, which was characterized by the expansion of dorsal gene expression. Overexpression of Kzp caused posteriorization.

These phenotypes were highly similar to ones induced by wnt8 depletion or overexpression and were rescued by alteration of wnt8 activity. Thus, our results provide the first insight into the mechanism involved in the initiation of zygotic canonical Wnt signals by a maternally derived transcription factor.

Gastrulation, including the fate specification and coordinated movements of three germ layers, leads to the formation of the primary body plan during early vertebrate embryonic genesis (1, 2). Structures of the posterior vertebrate body are derived from the ventral lateral tissue, an undifferentiated group of cells producing signals, predominantly bone morphogenetic proteins (BMPs) and Wnt proteins, which maintain ventral cell fate, whereas the structures of the anterior body are derived from dorsal tissue, which produces antagonistic signals (3–5). A large body of evidence shows that the dorsal organizer is generated by maternal β-catenin proteins translocating specifically to the nuclei of the dorsal margin, which activates the zygotic expression of dorsal-specific genes, including boz, chordin, and dickkopf1 (1). As for the generation of the ventral organizer, there is evidence from studies on smad5 mutants that zygotic Bmp genes are activated by maternal BMP signals (6). However, there is relatively little known about the mechanisms that initiate the canonical Wnt proteins, which are another ventral organizer signaling protein family.

Canonical Wnt ligands belong to a large family of secreted, cysteine-rich glycoproteins that play crucial roles in many aspects of embryonic development and human diseases (7, 8). In vertebrates, maternal Wnt/β-catenin signaling promotes dorsal axis formation during the blastula stage (9), whereas zygotic Wnt/β-catenin signaling is required for promoting ventroposterior development and restricting dorsal mesodermal development (10, 11). Among the zygotic canonical wnt genes, the wnt8 ligand is expressed exclusively at the ventrolateral margin (12, 13) and is the strongest signal for the induction and maintenance of ventral lateral cell fate. Embryos that have a homozygous wnt8 mutant show severe phenotypes that are characterized by an expansion of the dorsal organizer and a significant reduction of the ventral posterior structure (14). Numerous studies have examined the transcription mechanisms of Wnt signaling cascades. Although the downstream transduction molecules of the Wnt signaling pathway have been studied extensively (7, 8), the control of zygotic wnt expression is poorly understood, particularly the mechanisms involved in the initiation and direct regulation of wnt ligand transcription during embryonic development. For instance, inhibition of the mesoderm inducing signal nodal activity leads to a reduction in wnt8 expression (13, 15). Moreover, the overexpression of fgf8a (fibroblast growth factor 8a) mRNA in Xenopus results in ectopic wnt8 expression (16). These phenotypes may be derived from the mesoderm induction effects of nodal or FGF signal activities. BMP signaling has also been shown to positively regulate wnt8 expression in Xenopus (17). The T-box transcription factor ntl regulates wnt8 expression during the late stage (18, 19). goosecoid can bind to the wnt8 promoter through consensus binding sites and repress its expression in the dorsal organizer (20). Published data show that the regulation activities of wnt8 expression occur during the late stage of the zygotic wnt8 expression program. However, there is still no evidence that an endogenous transcription factor can directly bind to the wnt8 promoter and initiate zygotic wnt8 expression.

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3 The abbreviations used are: BMP, bone morphogenetic protein; CE, convergent extension; MO, morpholino oligonucleotide; CAST, cyclic amplification of select target; Kzp-BS, Kzp-binding site.
In this study, we show that a novel transcription factor, Kzp, directly binds to the wnt8 promoter and controls the initiation and maintenance of wnt8 transcription as a mechanism for regulating the primary body axis formation. Depletion of the Kzp protein leads to gastrulation defects, including convergent extension (CE) movement and dorsoventral patterning abnormalities, whereas overexpression inhibits eye formation. These are similar to the phenotypes caused by Wnt8 depletion or overexpression, respectively. Mechanistically, we demonstrate that Kzp is a DNA-binding protein that specifically interacts with the wnt8 promoter, which contains multiple consensus Kzp-binding sites, and controls the initiation and maintenance of zygotic wnt8 expression. A reduction in Kzp activity dramatically inhibits wnt8 transcription and blocks wnt8 expression induced by FGF signaling. Our results suggest that the regulation of wnt8 expression by Kzp is critical for embryonic patterning.

**EXPERIMENTAL PROCEDURES**

**Constructs and Primers**—kzp cDNA was amplified with primers and subcloned into phBlueScript SK to make antisense and sense probes for *in situ* hybridization. The coding region of *kzp* was cloned into pcDNA-FLAG for mRNA synthesis and mammalian cell expression. The upstream region of *wnt8* was cloned into pBluescript SK to make antisense morpholino oligonucleotides (MOs). Zebrafish *wnt8.2* cDNAs in pcDNA3 for testing the effectiveness of wnt8 MOs targeting zkpa mRNA. Standard control MO was used as an injection control. The sequences and doses of wnt8 MOs targeting zebrasia wnt8 ORF1 and ORF2 were used as described (14).

**Cell Transplantation**—For cell migration experiments, cells from donor embryos injected with control MO and FITC-dextran (*M* 10,000, Invitrogen) or with *kzp* MO and rhodamine-dextran (*M* 10,000, Invitrogen) were transplanted into wild-type host embryos as described (22).

**Cell Tracking**—For tracing lateral mesodermal cells, embryos at the one-cell stage were injected with a mixture of MO and *kaede* mRNA (50 pg). Then, at the shield stage (~6–6.5 h post-fertilization), lateral mesodermal cells were labeled by converting the fluorescent spectrum of the *Kaede* protein from green to red fluorescence by exposure to 440 mm red light for ~1 min (23). Photoconversion was done at ×200 on a Zeiss Axioskop with a specially machined field diaphragm with a pinhole aperture of ~50 μm. Migration of these red cells continued to be monitored to the one-somite stage.

**Identification of Kzp-binding DNA Sequences**—The cyclic amplification of select target (CAST) assay was performed with bacterially expressed GST-Kzp zinc fingers as described (24). Briefly, an 59-mer DNA was synthesized beginning with 5’-TGTGGCCCTTGGACACTGACGAGGGAG-3’; followed by a stretch of 15 totally random bases and ending with the sequence 5’-TCGTCACCGAATGTTTCCAGTT-3’. The oligonucleotides were double-stranded by annealing with a reverse primer and extension for 30 min at 72 °C with *Taq* DNA polymerase. 50 ng of double-stranded DNA was incubated with 0.1 μg of purified GST-Kzp zinc finger fusion protein. The binding sequences were pulled down with 10 μl of glutathione-agarose beads (GE Healthcare). The selected double-stranded DNA was amplified by PCR. The products were used for the second CAST cycle. Eight sequential rounds of CAST were performed. The final PCR products were cloned into the pGEM-T Easy vector for sequencing. 43 clones from Kzp-ZFN and 12 clones from Kzp-ZFC were sequenced, and consensus DNA-binding sequences were visually examined.

**Whole-mount in Situ Hybridization**—*In situ* hybridization was performed as described previously (25). gsc, ntl, and wnt8 cDNAs were a kind gift from Dr. Bo Xiao (Sichuan University, Chengdu, China); hgg, dlk3, chordin, and eve1 were from Dr. Anming Meng (Tsinghua University, Beijing, China); and flh was from Dr. Lingfei Luo (Southwest University, Chongqing, China).

**Cell Culture and Transfection**—HEK293T cells were grown in DMEM plus 10% FBS. DNA transfection into HEK293T cells were performed using Lipofectamine (Invitrogen). The antibody against the FLAG tag was purchased from Abcam. Nonradioactive EMSA—GST-Kzp-ZFN and GST-Kzp-ZFC proteins were bacterially expressed and purified following the manufacturer’s protocol (GE Healthcare). EMSA was performed as described by Aimee Kenoyer (Protocol Online). The 5’-biotin-labeled oligonucleotides were synthesized by Invitrogen. The sequences of the oligonucleotides used were as follows: Kzp-BS, 5’-CTCGAC-
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AACCTTGTGCAACCGCATTTGTTCC-3'; K mut1, 5'-CTCGACAGGGCCTGCAACCGCATTTGTTCC-3'; K mut2, 5'-CTCGACACGGGTAGTGCAACCGCATTTGTTCC-3'; K mut3, 5'-CTCGACACGGGTAGTGCAACCGCATTTGTTCC-3'; and wnt8 promoter Kzp-binding element, 5'-AATTGTGATTAGTGCACATTTGTTGG-3'.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed as described (26). About 200 embryos were injected with Kzp-FLAG plasmid at the one-cell stage and harvested at the shield stage. The harvested embryos were cross-linked with 1% formaldehyde, homogenized with a 1-ml Dounce tissue grinder, and then sonicated in 500 μl of lysis buffer (150 mM NaCl, 25 mM Tris (pH 7.5), 5 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate) to generate 300–500-bp fragments. Fragmented chromatin was immunoprecipitated with 2 μg of each antibody. Precipitated DNA was purified and used for PCR. The primer pair used for the amplification of zebrafish wnt8 intron 1 was 5'-ACCTATGTTCAGAAAAACCAACTAA-3' and 5'-TAGCCGTGTAGGTGTAGGTGA-3'; the primers flanking the binding region of the wnt8 promoter were 5'-AAACATGTCGCTTACATG-3' and 5'-AAACATGTCGCTTACATG-3'.

RESULTS

Kzp Is Maternally Expressed in Zebrafish Embryos—We cloned the kzp gene from a zebrafish expression library by antibody screening and found that the zebrafish Kzp protein contains eight Krüppel-like C2H2-type zinc fingers, three of which are highly homologous to DNA-binding zinc fingers found in the Kaiso and BTB/POZ zinc finger proteins (supplemental Fig. S1) (27). Kzp also lacks the known functional domains of zinc finger proteins, including the BTB domain, which is found in the Kaiso and BTB/POZ zinc finger proteins (28). These findings suggest that Kzp does not belong to any of the known protein families and is a novel DNA-binding protein. Consistent with this hypothesis, the Kzp protein was found to localize to nuclei when transfected into HEK293T cells (Fig. 1A).

Whole-mount in situ hybridization revealed dynamic expression of kzp during zebrafish embryonic development (Fig. 1B). Kzp was maternally loaded, and the zygotic transcripts were distributed uniformly throughout the gastrula cells up to 24 h post-fertilization, at which point kzp mRNA was expressed in the anterior neural tissue at high levels. Subsequently, kzp mRNA was detected in the mid-hind brain boundary, forebrain, and eyes. RT-PCR analysis confirmed a similar temporal expression pattern (Fig. 1C).

Kzp Modulates Ventral Posterior Patterning—The expression pattern of Kzp suggested that the protein may function in the early embryonic development of zebrafish. Therefore, we wished to determine Kzp function by knocking down endogenous expression of Kzp with two non-overlapping, translation-blocking antisense MOs in zebrafish embryos. Both MOs dramatically inhibited the protein production of mRNA transcribed from a microinjected DNA construct containing the kzp gene fused to GFP (supplemental Fig. S2) and produced similar phenotypes in zebrafish embryos (Fig. 2, B–G).

In addition, the phenotypes induced by the 5'-UTR MO were effectively rescued by the co-injection of synthetic FLAG-tagged kzp mRNA lacking the morpholino target (Fig. 2J). These data demonstrated that the two MOs efficiently and specifically blocked kzp mRNA translation.

At 24 h post-fertilization, embryos that received kzp MO exhibited a reduction or loss of tail structure (Fig. 2, B–G, and supplemental Fig. S3). Almost all of the reduced tails in kzp MO-injected embryos curved dorsally, and the weakly reduced tails exhibited a loss of the ventral fin. These phenotypes are characteristics of embryonic dorsalization and resemble the phenotypes seen in mutant embryos that lack BMP or canonical Wnt signaling. To explore these phenotypes in more detail, we analyzed the expression of a set of dorsal- or ventral-specific genes. As shown in Fig. 2, the ex-
Expression domains of the dorsal-specific genes *chordin* (72.7%, \( n = 33 \)), *floating head* (74.2%, \( n = 31 \)), and *goosecoid* (62.5%, \( n = 32 \)) were expanded mediolaterally at the shield stage, whereas the domains of the ventral-specific genes *eve1* (70.8%, \( n = 48 \)) and *tbx6* (78.5%, \( n = 41 \)) were decreased in *kzp* MO-injected embryos (Fig. 2, J–U; data not shown). In contrast to the *kzp* MO phenotypes, the overexpression of *kzp* posteriorized the zebrafish embryos. Injection of *kzp* mRNA induced eyeless phenotypes, which are the features of posteriorized embryos. In embryos that received 200 pg of *kzp* mRNA at the one-cell stage, 24% of the embryos lost one eye, and 11% lost both eyes (\( n = 81 \)). Moreover, 31 and 14% of the embryos that received 300 pg of *kzp* mRNA lost one eye or both eyes, respectively (\( n = 42 \)). However, only 12% of the embryos injected with 300 pg of GFP mRNA lost one eye (\( n = 88 \)) (supplemental Fig. S4). Taken together, these results suggest that *kzp* modulates the normal dorsoventral and anteroposterior patterning of zebrafish embryos.

### Kzp Controls CE Movements

In addition to dorsalization, we also noted that *kzp* MO-injected embryos had a shorter and wider anteroposterior axis than control MO-injected embryos at the tail bud stage. The morphological abnormalities were highlighted by the altered expression patterns of several marker genes as a result of the loss of *kzp* activity. The *ntl* expression domain in the posterior axial mesoderm was shorter but wider in *kzp* MO-injected embryos (76%, \( n = 25 \)) (Fig. 3A, panels A7–A9). It was also apparent that injection of *kzp* MO led to less convergence and extension of the paraxial and lateral mesoderm, as shown by the alteration of the expression patterns of *myoD* (60.8%, \( n = 23 \)) (Fig. 3A, panel A12) and *papc* (79.4%, \( n = 34 \)) (panels A10 and A11). The *dlx3* expression pattern indicated that the neural plate was much broader in *kzp* MO-injected embryos (76%, \( n = 25 \)) (Fig. 3A, panels A7–A9).

These phenotypes are frequently seen in embryos that have cell movement defects (29, 30). To address whether cell
movement, the CE movement, was affected in \textit{kzp}-depleted embryos, we performed cell tracing studies of the migration of lateral mesodermal cells that were labeled at the shield stage with the green-to-red photoconverting protein Kaede (Fig. 3B). The labeled cells in control MO-injected embryos clearly converged toward the dorsal midline. In contrast, the labeled cells in \textit{kzp} morphants showed less convergence (76.5%, \(n = 17\)) (Fig. 3C). Similar phenotypes were also observed by time-lapse analysis of cell movement (data not shown). To further characterize how \textit{kzp} affects mesodermal cell movement, we performed cell transplantation experiments. Lateral mesodermal cells from the control MO-injected embryos and \textit{kzp} MO-injected embryos were labeled with green and red fluorescence, respectively, and cotransplanted into the lateral mesoderm of a wild-type host embryo. As shown in Fig. 3E, at the end of gastrulation, all of the donor lateral mesodermal cells began to migrate toward the dorsal side and formed two overlapped strips along the antero-posterior axis near the midline, suggesting that the \textit{kzp}-depleted lateral mesodermal cells recovered their dorsal convergence ability when transplanted into wild-type embryos. However, the strip of \textit{kzp} MO-injected cells was much wider and shorter than that of the control cells (87.5%, \(n = 8\)), which was consistent with the altered expression pattern of the paraxial mesoderm marker \textit{myoD} in \textit{kzp} morphant embryos (Fig. 3A, panel A12). Together, these analyses revealed a critical role of \textit{Kzp} in gastrulation movements. Specifically, \textit{Kzp} acts cell-non-autonomously during the dorsal migration and cell-autonomously during the cell intercalation of the lateral mesoderm (Fig. 3F).

\textit{Kzp} Binds to Specific DNA Sequences—The phenotypes caused by the depletion of \textit{Kzp} indicate that the \textit{Kzp} protein
has a critical function during zebrafish embryonic gastrulation. The fact that three zinc fingers at the N terminus of the Kzp protein (Kzp-ZFN) are homologous to DNA-binding domains within BTB/POZ proteins and Kaiso (supplemental Fig. S1) suggests that Kzp is a DNA-binding protein. To test this hypothesis, we performed a CAST assay (24) using the bacterially expressed GST-Kzp-ZFN or GST-Kzp-ZFC fusion protein (Fig. 4A). The results showed that the Kzp zinc finger domains at the N terminus (Kzp-ZFN) of the protein bound to the 10-bp DNA fragment 5′- (t/a/g)t(a/t/g)nctgcca-3′ (referred to as the Kzp-binding site (Kzp-BS)) (Fig. 4B). Kzp-ZFC did not specifically bind to DNA (data not shown). Notably, the 5′-ctgcca-3′ sequence of the binding fragment was consistent in 41 sequenced clones that were derived from the Kzp-ZFN-based assays. In addition, we found that the 5′-tta-3′ sequence could be substituted with other nucleotides to some extent (Fig. 4B).

To further characterize the DNA-binding specificity of Kzp, we performed EMSA competition experiments. As shown in Fig. 4C, shifted bands were observed when Kzp-BS and GST-Kzp-ZFN were incubated together but not when incubated with GST. Moreover, these complexes were diminished with the addition of excess unlabeled wild-type probe but not with a probe lacking the Kzp-BS. The binding specificity was also confirmed using EDTA as a zinc-chelating agent to abrogate the formation of the complex (Fig. 4C). In addition, to determine the nucleotide sequence that was sufficient for Kzp binding, a series of oligonucleotides with base substitutions within the Kzp-BS were synthesized and used in EMSA as competitors. The mut2 and mut3 oligonucleotides,
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A table is shown with columns for gene, cDNA microarray (Ctrl vs. kzp MO log ratio), and RT-PCR (Ctrl MO, kzp MO).

**FIGURE 5.** Kzp controls the initiation and maintenance of wnt8 expression. A, microarray data and RT-PCR analysis of selected kzp-responsive genes. B, expression of wnt8.2 in control (Ctrl) MO- or kzp MO-injected embryos at various stages. C, expression of wnt8.2 in wild-type or kzp overexpressing embryos at the shield stage. Embryos in B and C are lateral views with the anterior to the top. D, expression of wnt8.1 in wild-type, kzp MO-, or kzp DNA-injected embryos at the shield stage. Upper panels are lateral views with the anterior to the top, lower panels are dorsal views with the anterior to the top. E, expression of wnt8.2 in shield stage embryos that received MO or/and mRNA as indicated. Upper panels are lateral views with the anterior to the top, and lower panels are dorsal views with the anterior to the top.

which contained three base substitutions in the middle and 3′-regions of the Kzp-BS, respectively, did not compete for binding, and excess amounts of mut1, which contained three base substitutions in the 5′-region, retained competition ability (Fig. 4D), indicating that the 5′-ctgcca-3′ sequence of the Kzp-BS has a critical role in Kzp binding. Together, these results suggest that Kzp is a specific DNA-binding factor that may regulate gene expression in vivo.

Zygotic wnt8 Is a Direct Target of Kzp—To assess the gene expression regulated by Kzp in zebrafish embryos, we performed a two-color cDNA microarray hybridization assay to compare the difference in expression of 15,000 DNA tags between embryos carrying either control MO or kzp MO at the shield stage (data not shown). The results showed that kzp MO caused a decrease in the expression of 1562 tags and an increase in the expression of 2371 tags. To verify the initial screening data, we performed RT-PCR and quantified the mRNA levels of 10 genes that were increased or decreased in the embryos in which Kzp levels were reduced. The mRNA levels detected by RT-PCR were consistent with the results of the cDNA profiles (Fig. 5A) except for wnt5 expression, suggesting that Kzp is able to control gene expression in zebrafish embryos.

In the microarray data, we noted that wnt8 and its downstream target genes, including sp5l, cdx4, and tbx6, were dramatically down-regulated, which was consistent with the in situ hybridization results and was further confirmed by RT-PCR analysis (Fig. 5A). In zebrafish, wnt8 signaling plays a key role in ventroposterior cell fate induction and tail outgrowth promotion (11). Loss of function of the wnt8 locus by either mutation or MO injection results in a dorsalized phenotype that is characterized by an enlarged gastrula organizer and reduced tail structure (14). Overexpression of wnt8 causes eyeless or headless phenotypes. The similarities in the phenotypic changes between the loss of Wnt8 and Kzp (supplemental Fig. S3) (14), together with the coherence of the down-regulated expression of wnt8 and its targets in Kzp-disrupted embryos, raised the possibility that wnt8 may be a Kzp target gene. To examine this hypothesis, we performed a time-lapse analysis of wnt8 expression in embryos injected with kzp MO by in situ hybridization. The wnt8 locus in zebrafish contains two transcripts, wnt8.1 and wnt8.2, and the wnt8.2 expression pattern overlaps with wnt8.1. Therefore, we first analyzed the expression pattern of the wnt8.2 transcript. In wild-type embryos, zygotic wnt8.2 expression started at the sphere stage (~4 h post-fertilization) when the mRNA was located in the ventral portion of the blastula and yolk syncytial layer (Fig. 5B). However, the expression of wnt8.2 mRNA was dramatically reduced or even lost in kzp MO-injected embryos (73.3%, n = 45). We also observed a reduced expression of wnt8.2 mRNA in kzp morphants at the 30% epiboly, shield, and 80–90% epiboly stages (Fig. 5B). Similarly, kzp had the same effect on the transcription of wnt8.1 mRNA (Fig. 5D). In agreement with these data, the overexpression of kzp, by in-
jection of either mRNA or DNA vectors, induced the ectopic expression of both \textit{wnt8} transcripts (Fig. 5, C and D). Therefore, these results suggest that Kzp is critical for the zygotic expression of both \textit{wnt8} transcripts.

Previous studies have shown that FGF signals can induce the expression of mesodermal marker genes, including \textit{wnt8} and its downstream target genes (16). To gain further insight into the mechanism by which Kzp regulates \textit{wnt8} expression, we studied the function of Kzp in FGF-mediated \textit{wnt8} transcriptional activation. Treatment of embryos with an excess amount of \textit{fgf8a} mRNA induced ectopic \textit{wnt8} expression (65.9%, \( n = 41 \)) (Fig. 5E). In contrast, the same dose of \textit{fgf8a} mRNA did not induce \textit{wnt8} expression in \textit{kzp} MO-injected embryos (71.6%, \( n = 61 \)) (Fig. 5E), suggesting that Kzp is required for the activation of \textit{wnt8} expression induced by other factors.

Sequence analysis of the \textit{wnt8} promoter in zebrafish revealed three Kzp-BS-like sequences, one of which was identical to the Kzp-BS that was derived from the CAST experiments (Fig. 6A). To test whether Kzp can bind the \textit{wnt8} promoter through these conserved sequences \textit{in vivo}, we performed ChIP analysis of the \textit{wnt8} promoter in embryos at the shield stage. Ectopic expression of FLAG-tagged Kzp efficiently immunoprecipitated the \textit{wnt8} proximal promoter region that contained one Kzp-BS in zebrafish embryos (Fig. 6A). In contrast, Kzp did not show any specific affinity for the first intron of \textit{wnt8}, a region that does not contain Kzp-binding sequences. Furthermore, EMSA experiments confirmed that Kzp readily associated with the sequence-specific Kzp oligonucleotides derived from the \textit{wnt8} promoter (Fig. 6B). Therefore, these analyses suggest that Kzp can specifically interact with the \textit{wnt8} promoter.

\textbf{Wnt8 Functions downstream of Kzp to Maintain Ventrolateral Cell Fate}—Because the expression of \textit{wnt8} is directly regulated by Kzp and because the phenotypes of embryos in which \textit{wnt8} is manipulated are similar to those with altered \textit{kzp} expression, we asked whether \textit{wnt8} is required for Kzp functions. We overexpressed \textit{kzp} and simultaneously knocked down \textit{wnt8} expression with a pair of MOs (\textit{wnt8} MOs) that have been shown to be able to block translation of \textit{wnt8} mRNA (14). As shown in Fig. 7A, 12.6% of the embryos injected with \textit{kzp} mRNA lacked both eyes, and 27.6% of the embryos lacked one eye or exhibited a cyclopic phenotype. Interestingly, the co-injection of \textit{kzp} mRNA with \textit{wnt8} MOs rescued the eyeless phenotypes. The percentage of co-injected embryos that lacked one or both eyes decreased to 12.6 and 1.1%, respectively, suggesting that the inhibition of \textit{wnt8} activity dramatically suppressed the \textit{kzp}-induced eyeless phenotypes. In zebrafish, overexpression of \textit{wnt8.1} mRNA at the one-cell stage induces both the ventral-specific gene \textit{eve1} and

\begin{figure}[h]
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\caption{\textbf{Kzp directly binds to the \textit{wnt8} promoter and activates its transcription.} A, upper panel, schematic representation of the \textit{wnt8} promoter. Two pairs of PCR primers were designed to flank the most proximal Kzp-BS within the \textit{wnt8} promoter and fragment in intron 1, respectively. Lower panel, ChIP analysis of \textit{kzp} in zebrafish embryos. \textit{IP}, immunoprecipitation. B, gel shift analysis of the binding between \textit{kzp} and the \textit{kzp}-BS within the \textit{wnt8} promoter. C, model showing that \textit{kzp} binds to the \textit{wnt8} promoter and regulates transcription of both ORFs.}
\end{figure}
dorsal-specific gene *chordin* and causes hyperdorsalization phenotypes. Whereas, the overexpression of *wnt8.2* induces only *eve1* and does not induce *chordin* and therefore lacks dorsalization effects (31). Therefore, we chose *wnt8.2* for further rescue experiments. As expected, the co-injection of *wnt8.2* mRNA with *kzp* MO efficiently restored the expression of the ventral-specific genes *eve1* (64.1%, n = 64) and *tbx6* (64.5%, n = 48) and partially rescued the posterior defects (Fig. 7B) in the *kzp* MO-injected embryos. Together, these genetic interaction data indicate that *wnt8* is a major effector in *Kzp*-mediated phenotypes during zebrafish gastrulation.

**DISCUSSION**

In this study, we have demonstrated that *Kzp* has an essential role in the promotion of ventroposterior patterning in zebrafish embryos. The depletion of *Kzp* leads to gastrulation defects, including CE movement and dorsoventral patterning defects, whereas the overexpression of *Kzp* inhibits eye formation. Mechanistically, we have shown that *Kzp* is a DNA-binding protein that specifically interacts with DNA fragments containing the sequence 5'-((t/a)g)(t/a)gctgcca-3'. In addition, we have shown that *Kzp* activates zygotic *wnt8* expression by directly binding to the *wnt8* promoter and modulates ventroposterior cell fate.

*Kzp-Wnt8 Maintains Ventrolateral Cell Fate*—In our studies, we found that *Kzp* contributes to the modulation of dorsoventral cell fate predominantly though the regulation of *wnt8* expression. Interference with *Kzp* function using MO injection dramatically reduced the expression levels of the ventral mesoderm marker *tbx6*. In addition, the introduction of *wnt8* mRNA was able to rescue the *tbx6* expression in *kzp* MO-injected embryos, suggesting that Wnt8 acts downstream of *Kzp* in the regulation of *tbx6* expression. Previous studies have shown that the formation of the ventrolateral mesoderm also requires the T-box transcription factors (e.g., the products of the *ntl* and *spt* genes) (32, 33). In *ntl/spt* double mutants, the *tbx6* expression is almost completely absent (34). Injection of *kzp* MOs did not significantly affect the expression of *ntl* and *spt* during the gastrulation stages (data not shown), suggesting that *Kzp*-Wnt8 regulates dorsoventral patterning through a parallel mechanism with that of *ntl/spt*.

The *Kzp* protein also modulates CE movement. Previous studies have shown that overexpression of *wnt8.2* results in a cyclopic eye, which is a distinct phenotype induced by the activation of the β-catenin pathway (14) and suggests that *wnt8.2* has a role in the regulation of CE movement. Therefore, *wnt8* may also transduce the signaling of *Kzp*-mediated convergence movement. However, the inhibition of both *wnt8* genes by co-injection of *wnt8* MOs did not lead to a significant disruption of convergence movement as evaluated by the cell tracing assay (supplemental Fig. S5), indicating that Wnt signaling does not act downstream of *Kzp* for CE movement.
Therefore, a detailed analysis will be required to reveal the mechanism by which Kzp controls gastrulation movements. Kzp Controls the Expression of Two wnt8 Genes—The wnt8 locus in zebrafish contains two open reading frames, wnt8.1 and wnt8.2. Zygotic expression of both wnt8 genes is initiated at the sphere stage and occurs on the ventral side of the yolk syncytial layer and blastula margin (Fig. 5B; data not shown). Subsequent to this stage of development, the two wnt8 genes are expressed in different manners. Although they are generally both coexpressed in the non-axial marginal zone, wnt8.2 is also expressed in the marginal axial mesoderm and in anterior epiblasts. Because a functional promoter has been identified between the wnt8.1 and wnt8.2 coding regions, the expression of wnt8.2, at least in its own specific domain, may be driven by this unique promoter element (31). Therefore, the divergence in the wnt8 gene expression pattern may be a reflection of the utilization of different regulation mechanisms. For example, ntl is needed for the maintenance of wnt8 expression from the mid-gastrulation to the somite stage. However, the ntl expression pattern overlaps with wnt8.1 but not wnt8.2. Therefore, ntl lacks the spatial capacity to regulate the expression of wnt8.2 in the anterior epiblast. We have shown that Kzp binds to the wnt8 promoter and regulates expression of both ORFs (Fig. 6C). Moreover, loss of Kzp activity causes not only the reduced expression of both wnt8.1 and wnt8.2 in their shared domains but also a reduction of wnt8.2 expression within its specific domain, suggesting that a common mechanism regulates the expression of both wnt8 ORFs. In addition, it is also reasonable to hypothesize that the loss of endogenous wnt8.1 expression in the domain unique to Kzp is due to either a transcription suppression mechanism specific to wnt8.1 transcription or an activation mechanism specific to wnt8.2, or both.

Kzp Is an Initiating Regulator of wnt8—A few factors have been shown to activate the expression of canonical wnt genes. However, the studies to date have addressed the mechanisms that regulate wnt8 expression during only the late stage but not the initiation stage of development in zebrafish. Zygotic expression of both wnt8 genes is initiated at the sphere stage and occurs on the ventral side of the yolk syncytial layer and blastula margin. Previous studies have shown that FGF signals can induce wnt8 expression. At the sphere stage, fgf genes are expressed in the dorsal margin. In agreement with these data, FGF target genes or a responsive reporter was first expressed in the dorsal margin at the sphere stage and then began to express throughout the margin from the 30% epiboly stage onward. Therefore, these data suggest that FGF signaling is not linked to the initiation of wnt8 transcription. T-box family members have been reported to be involved in the regulation of wnt8 expression. In ntl mutant or ntl MO-injected embryos, wnt8 expression is reduced at the mid-gastrulation stage, but not before this time point, suggesting that ntl is not a determination factor for the initiation of wnt8 expression. Another T-box gene, tbx6, was reported to be able to activate wnt8 expression in Xenopus. However, these functional analyses used a constitutively active (Tbx6VP16) and a dominant-negative (Tbx6EnR) mutant, which may not reflect endogenous tbx6 function (18). In fact, wild-type tbx6 RNA can act as a competitive inhibitor of ntl-mediated transcriptional activation. Moreover, the injection of tbx6 antisense MOs in zebrafish causes only mild axial defects and does not decrease wnt8 expression (35) *data not shown). These data indicate that tbx6 is not necessary for the regulation of wnt8 expression. In addition, these regulation factors are also controlled by zygotic Wnt signaling.

Kzp regulates zygotic wnt8 expression at the sphere stage, which is the point of development when zygotic wnt8 expression first appears, suggesting that Kzp contributes to the initiation of wnt8 signaling. Kzp is a maternally derived and widely expressed transcription factor that is capable of inducing ectopic wnt8 expression, whereas endogenous wnt8 expression is restricted to a specific domain. The inconsistency between kzp and wnt8 expression patterns indicates that kzp requires additional spatial-specific factors that either express or activate within the defined wnt8 expression domain for the activation of wnt8 expression. Another possible explanation is that the specific expression pattern is established largely by suppressive mechanisms, as mentioned in a previous study (30).

Kzp Belongs to a Novel Krüppel Zinc Finger Protein Family—The BTB/POZ domain in the Kaiso family of proteins tends to localize the protein to the heterochromatin, whereas Kzp does not localize to these regions. In addition, Kaiso functions as a transcription repressor and is involved in the control of the non-canonical Wnt pathway to regulate Xenopus gastrulation movements (36). In contrast, Kzp acts as a transcription activator, and the overexpression or depletion of Kzp does not alter the mRNA level of wnt11. Because the Kzp-binding sites within the wnt8 promoter are similar to the core Kaiso DNA-binding sequence, CTGCA (37), we further tested whether Kaiso can recognize these sites and therefore regulate the activity of the wnt8 promoter. However, neither the overexpression nor reduction of Kaiso activity had an effect on the expression of wnt8 mRNA (data not shown), suggesting that Kaiso does not contribute to the regulation of wnt8 expression. In agreement with these observations, the modulation of Kaiso activity in kzp morphant embryos did not rescue the phenotypes (data not shown). These findings indicate that the gene regulatory functions of Kzp are different from those of Kaiso during embryonic gastrulation.

Kzp contains a DNA-binding domain that is homologous to Kaiso, and the Kzp binding sequences match five of the eight core Kaiso DNA-binding sequences, suggesting that the Kaiso activity in zygotic gastrulation. Moreover, the injection of tbx6 antisense MOs in zebrafish causes only mild axial defects and does not decrease wnt8 expression (35) *data not shown). These data indicate that tbx6 is not necessary for the regulation of wnt8 expression. In addition, these regulation factors are also controlled by zygotic Wnt signaling.
body was able to recognize a specific protein in HeLa cells (data not shown). The subsequent analysis of the identified protein is currently ongoing. Therefore, to date, we cannot conclude whether the zebrafish kzp gene is the ortholog of mammalian zBTB4.

In summary, our study has shown that Kzp has an essential role in the promotion of ventroposterior fate and in the limitation of the lateral extent of the gastrula organizer. Depletion of Kzp leads to dorsalized phenotypes, whereas overexpression inhibits eye formation, which is quite similar to the phenotypes induced by wnt8 signaling perturbations (14). We have also provided several lines of evidence that demonstrate that Kzp directly binds to the wnt8 promoter through specific binding elements and controls wnt8 expression during gastrulation. We conclude that the kzp gene acts upstream of the canonical wnt8 signaling pathway and that the Kzp-Wnt8 axis plays a critical role in dorsoventral patterning during zebrafish gastrulation.

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