The BMP ligand Pinhead together with Admp supports the robustness of embryonic patterning

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Vertebrate embryonic dorsoventral axis is robustly stable in the face of variations in bone morphogenetic protein (BMP) signaling. However, the molecular mechanism behind this robustness remains uncharacterized. In this study, we show that zebrafish Pinhead, together with Admp, plays an important compensatory role in ensuring the robustness of axial patterning through fine-tuning of BMP signaling. pinhead encodes a BMP-like ligand expressed in the ventrolateral margin of the early gastrula. Transcription of pinhead and admp is under opposing regulation, where pinhead depletion results in a compensatory increase in admp transcription and vice versa, leading to normal axis formation in pinhead or admp mutants. Expression of pinhead and admp is directly repressed by the BMP/Smad pathway. When BMP signals were inhibited or excessively activated, pinhead/admp expression changed accordingly, allowing for self-regulation. Thus, this study reveals a negative feedback loop between BMP signaling and pinhead/admp that effectively stabilizes embryonic patterning by buffering against fluctuations in BMP signaling.

INTRODUCTION

Bone morphogenetic proteins (BMPs), originally identified by their ability to induce ectopic bone formation, are multifunctional extracellular polypeptides that belong to the transforming growth factor–β (TGF–β) superfamily (1). Secreted BMP ligands bind as dimers to type I and type II receptors on the cell surface. The type II receptors become phosphorylated and then activate the type I receptors, which in turn phosphorylate the regulatory Smads (Smad1/5/8) (2). These phosphorylated Smads form complexes with Smad4, which then translocate into the nucleus to regulate the expression of BMP target genes (2). In zebrafish,bmp2b andbmp7a, which function as BMP heterodimers that activate Smad1/5, are initially expressed throughout the blastoderm shortly after the midblastula transition (3). BMP signaling in dorsal regions is subsequently attenuated by the BMP antagonist Chordin (Chd), and then a BMP signaling gradient forms along the dorsoventral (DV) axis and patterns tissues with high levels ventrally and low levels dorsally during late blastula stages and before the onset of gastrulation (3, 4). Although many positive and negative regulators of BMP signaling have been identified during early embryonic development (4), the molecular network that generates and maintains the BMP gradient is still not well characterized.

The formation of a morphogen gradient is a dynamic process and is influenced by the kinetics of morphogen production, diffusion, and degradation. During embryonic development, the formation of a morphogen gradient is often challenged by signaling component-level fluctuations, temperature differences, size variations, and/or unequal distributions of components between daughter cells (5). Therefore, morphogen gradients should be reproducibly formed with robust stability from one embryo to the next (5). Specifically, a robust resistance of DV axis formation to perturbations has been observed in various vertebrate embryos during classic grafting and ablation experiments. When grafted to the ventral-most part of a host embryo, where the BMP signal is maximally activated, the Spemann-Mangold organizer of Xenopus and the embryonic shield of zebrafish retain their ability to induce a secondary body axis at the site of the graft (6, 7). Furthermore, even when an amphibian blastula is bisected into dorsal and ventral halves, the dorsal half can give rise to a well-proportioned half-sized embryo (8). In addition, avian embryos can compensate for the removal of the organizer (Hensen’s node) during the primitive streak stage as evidenced by the reappearance of organizer markers (9). These observations support the idea that self-regulation occurs on the dorsal side of vertebrate embryos. On the other hand, transplantation of zebrafish ventral margin cells into animal poles induces the formation of secondary tails, indicating the existence of a tail organizer (10). Therefore, both the ventral and dorsal sides of vertebrate embryos are involved in self-regulation of DV patterning, but the underlying mechanism ensuring the robust BMP activity gradient remains one of the great unsolved mysteries in developmental biology.

In zebrafish embryos, ventral BMP signaling maintains expression of the vox/vent/ved transcriptional repressors, which restrict the expression of dorsal-promoting genes, including chd (11). In Xenopus, the expression of chd is negatively regulated by BMP4 (8). Therefore, the BMP signal gradient controlled by ventral BMP ligands and their dorsally secreted antagonist Chd is theoretically unstable, where a small change in the BMP signals or Chd expression would cause severe defects in the DV body plan (12). A BMP-like protein, anti-dorsalizing morphogenetic protein (Admp), is uniquely expressed in and secreted by the dorsal organizer (13, 14). Admp associates with Chd and facilitates Chd degradation (15). The expression of admp is repressed by BMP signals, and a depletion of the ventral BMP signals will increase admp expression, thus allowing the regeneration of a new BMP signal gradient. Therefore, Admp is an appealing candidate for ensuring the stabilization of DV patterning (8). Unexpectedly, knockdown of admp in Xenopus or zebrafish using morpholinos (MOs) only causes mild dorsalization (8, 12, 16, 17), and the distribution of the Chd protein remains largely unchanged (17), suggesting that there are other BMP-like members that compensate for the loss of Admp function. In addition, the dorsal halves of split Xenopus embryos still retain substantial DV polarity when

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BMP4 and BMP7 are depleted (8), indicating that unidentified BMP-like members may function in the newly induced ventral side and are transcriptionally up-regulated to compensate for the loss of BMP ligands.

The precursor proteins of BMP family members consist of three parts: an N-terminal signal peptide that targets the protein to the secretory pathway, a prodomain that mediates proper folding, and a C-terminal mature peptide containing seven highly conserved cysteines, i.e., cysteine knots, that form intramolecular disulfide bonds (18). In addition, an Arg-X-X-Arg sequence motif in the prodomain of the precursor proteins is hydrolyzed by serine proteinases to form mature C-terminal proteins that are subsequently secreted (18). There are at least 20 structurally and functionally related BMPs, including Decapentaplegic, Screw, and Glassbottom-boat in Drosophila and BMP2/4, BMP5/6/7/8, and BMP9/10 in vertebrates. Most of these BMPs play critical roles in embryogenesis and organ morphogenesis (19–21). The characterization of previously unknown BMP members involved in embryonic development will be interesting and provide key insights into this developmental pathway.

The novel gene pinhead was originally isolated from a functional knockdown screen searching for genes involved in nervous system development and is expressed in the anterior neural plate of Xenopus neurula as a key regulator of head development (22). pinhead is located immediately upstream of admp in the genomes of various animals, ranging from arthropods to vertebrates (22, 23). This genomic configuration of pinhead and admp is important for mutually exclusive expression of these genes in Ciona embryos, which lack a structure homologous to the vertebrate organizer (23). In gastrulating Xenopus embryos, pinhead is expressed in an arc around the blastopore with a distinct gap corresponding to the dorsal mesoderm, which implies a possible role in the embryonic body plan (22).

In this study, we demonstrated that Pinhead is a secreted BMP-like ligand expressed in the ventrolateral margin and has ventralizing functions in the zebrafish embryonic body plan. Similar to Admp, Pinhead was also found to promote metalloproteinase-mediated Chd degradation. Expression of pinhead was notably increased in response to the inhibition or depletion of admp and vice versa. This “seesaw”-like expression of pinhead and admp establishes a well-orchestrated alternative mechanism for the robust generation of the DV axis. This is evidenced by the normal DV polarity exhibited by pinhead or admp mutants alongside the marked dorsalization displayed when both of these genes are absent. Last, the expression of pinhead and admp is negatively regulated by BMP signaling, where this negative feedback loop between BMP signaling and pinhead/admp is important for buffering against fluctuations in dynamic BMP signaling during DV axis formation. Therefore, we propose an alternative mechanism to ensure stable axis formation that couples pinhead and admp with system control based on opposing regulation of BMP signaling and pinhead/admp expression. This work will provide important insights into the mechanisms of robustness in organisms by the self-regulating BMP activity gradient.

RESULTS

Zebrafish pinhead is a ventralizing gene expressed in the ventrolateral margin

Expression of pinhead and admp occurs in a mutually exclusive manner during Ciona and Xenopus embryonic development (22, 23). Admp is a BMP-like protein with important functions in the embryonic body plan (13, 14, 16). However, the developmental role of pinhead in DV patterning remains unknown. To gain insight into the functions of zebrafish pinhead [NM_205587.1, National Center for Biotechnology Information (NCBI)] during embryogenesis, we firstly characterized its expression during early embryonic development using whole-mount in situ hybridization (WISH) with an antisense probe. As shown in Fig. 1A, pinhead transcripts were undetectable before and during the sphere stage. Soon afterward, pinhead was expressed around the marginal zone but not the dorsal mesoderm, as indicated by co-staining with goosecoid (gsc) (Fig. 1, A and B). During the midgastrulation stages, the expression of pinhead transcripts appeared in a DV gradient in the blastoderm margin (Fig. 1A). Abundant pinhead transcripts were consistently observed in the presomitic mesoderm, but not the axial mesoderm, at the bud stage and during somitogenesis (Fig. 1A and fig. S1A). pinhead transcripts were no longer detectable after the segmentation stages (fig. S1B). These data indicate that zebrafish pinhead may play a role in establishing ventral cell fates during early embryonic development.

Next, the effects of pinhead overexpression on embryogenesis were assessed by injecting mRNA synthesized in vitro into one-cell stage embryos. Embryos injected with different amounts of pinhead mRNA exhibited dose-dependent ventralized phenotypes, characterized by the loss of dorsoanterior structures and the expanded ventral tissues at 24 hours post-fertilization (hpf) (Fig. 1, C and D), suggesting that Pinhead protein has ventralizing activity. In contrast to the impaired function of BMP2b by the addition of six amino acids at its C terminus in swirl mutants (24), the ventralizing activity of Pinhead was not obviously affected when a hemagglutinin (HA) epitope tag was fused to the carboxy end (Fig. 1D). Moreover, ectopic expression of Xenopus pinhead (NM_203534.1, NCBI) in zebrafish embryos generated similar ventralized phenotypes but did not result in macrocephaly (fig. S1, C and D), which had been observed in pinhead-overexpressed Xenopus embryos, suggesting an additional function of pinhead in the development of Xenopus nervous system (22).

In addition to the morphological changes, we also assessed the expression of several dorsal and ventral markers in embryos injected with 100 pg of pinhead mRNA. During the gastrula stages, injection of pinhead mRNA greatly reduced the expression of dorsal markers, including chd and gsc (Fig. 1, E and G). By contrast, the expression of the ventral markers eve1 and vent was notably expanded in response to injection of pinhead mRNA (Fig. 1, F and G). In addition, embryos injected with pinhead mRNA had a much smaller dorsal neuroectoderm (as indicated by sox3 expression), as well as an expanded ventral nonneural ectoderm (as indicated by gata2 expression) at 75% epiboly stage (Fig. 1H). At later stages, overexpression of pinhead in zebrafish embryos resulted in a slight expansion of the blood cell population within the intermediate cell mass, which is derived from the ventral mesoderm (Fig. 1I). On the basis of these observations, we conclude that Pinhead has ventralizing functions in the zebrafish embryonic body plan.

The pinhead gene encodes a functional BMP ligand

Zebrafish pinhead encodes a 316-amino acid protein with a predicted hydrophobic N-terminal signal sequence (fig. S1E). Because Pinhead is a ventralizing factor and predicted to be secreted, we speculated that it is a BMP-like ligand. To address this hypothesis, we compared the sequences of Pinhead and several zebrafish BMP members, including BMP2b, BMP4, BMP7a, and Admp. Although the sequence
Fig. 1. Overexpression of pinhead ventralizes zebrafish embryos. (A) Expression of pinhead in wild-type zebrafish embryos was analyzed by whole-mount in situ hybridization. 128-Cell and sphere stages, lateral views; 30% epiboly (ep) and shield stages, animal pole views with dorsal to the right, and dorsal views with animal pole at the top; and 75% epiboly and bud stages, lateral views with dorsal to the right, and dorsal views with animal pole at the top. In the last panel, the embryo is slightly tilted upward to expose the tail bud. (B) Double in situ hybridization of pinhead (dark blue) and gsc (red) expression at the shield stage. In the upper panel, the animal pole view shows the relative positions of the pinhead and gsc domains. In the lower panel, the dorsal view displays pinhead and gsc transcripts in embryos at exclusive domains. (C and D) Embryos were injected with different doses of gfp or pinhead mRNAs. Representative pictures of different classes and statistical data are shown in (C) (lateral views with anterior to the left) and (D). Scale bar, 100 μm. (E and F) The expression of dorsal (E, chd and gsc) and ventral (F, eve1 and vent) marker genes in gfp and pinhead mRNA–injected embryos at the indicated stages. (E) Animal views with dorsal side to the right in upper panels, and dorsal views with animal pole at the top in lower panels. (F) Animal views with dorsal to the right. (G) Expression levels of several dorsal-ventral genes were analyzed at the shield stage by real-time quantitative (qPCR). The expression levels of β-actin were used as a reference to normalize the amount of mRNAs in each sample. Error bars indicated SD. Asterisks indicated statistical significance of difference, *P < 0.05; **P < 0.01, Student’s t test. (H) Analysis of the expression patterns of the dorsal neuroectoderm marker sox3 and ventral nonneural ectoderm marker gata2 at the 75% epiboly stage. Lateral views with the dorsal side pointing to the right. (I) The expression pattern of gata1 in gfp and pinhead mRNA-injected embryos. Lateral views with anterior to the left.
of Pinhead displays little similarity to the other BMP ligands, it does contain a number of features characteristic of BMP proteins, including a consensus Arg-X-X-Arg proteolytic processing site and six characteristic cysteine residues conservatively located in the mature carboxyl terminal domain (fig. S1F).

To examine the biochemical properties of Pinhead, we expressed Pinhead-HA protein in human embryonic kidney (HEK) 293T cells and studied the conditioned medium produced by the transfected cells (Pinhead CM). Immunoprecipitation of Pinhead CM revealed that the Pinhead protein was present in the medium (Fig. 2A). To analyze the secretion rate of Pinhead, we treated HEK293T cells expressing Pinhead-HA with the protein synthesis inhibitor, cycloheximide (CHX), and then immunoprecipitated Pinhead proteins in conditioned medium and cell lysates at different time points, respectively. We found that about 30% of Pinhead proteins were secreted within 4 hours, and more than 90% of Pinhead proteins were present in the medium after 12 hours of CHX treatment (Fig. 2, B and C). In addition, after 8 hours of CHX treatment, we detected more than 90% of Pinhead proteins in the CM produced by the suspended cells dissociated from the gastrula embryos injected with pinhead-HA mRNA (fig. S1, G and H), suggesting that Pinhead proteins can be more effectively processed in and secreted from zebrafish embryonic cells.

To further demonstrate that Pinhead is a secreted protein in vivo, we examined whether Pinhead is secreted in zebrafish embryos by injecting mRNA encoding plasma membrane–localized mCherry-CAXX protein and Pinhead-GFP protein, in which green fluorescent protein (GFP) was fused to the C-terminal end of Pinhead, into one-cell stage embryos. The Pinhead-GFP fusion protein has a ventralizing activity similar to untagged Pinhead, as injection of equimolar amounts of pinhead-gfp and pinhead mRNAs resulted in similar percentages of ventralized embryos at 24 hpf (fig. S1, I and J). As expected, Pinhead-GFP protein was primarily intercellular at the shield stage (Fig. 2D). We also injected pinhead-gfp mRNA together with rhodamine-dextran into one marginal blastomere at the 16- to 32-cell stages. At later stages, the descendant cells could be indicated by rhodamine fluorescence. At the shield stage, we observed obvious GFP fluorescence at the periphery of the rhodamine-positive and rhodamine-negative cells and even the cells far away from the progeny of the injected blastomere (Fig. 2E). Immunoprecipitation of CM produced by the suspended cells from embryos injected with gfp or pinhead-gfp mRNAs showed that it was not GFP protein but Pinhead-GFP that could be detected in the medium (fig. S1K), ruling out the possibility that the high mobility of the protein observed in zebrafish embryos is due to a substantial amount of free GFP. Thus, these results suggest an efficient secretion and a long-range diffusion of Pinhead proteins in the developing embryos.

The mature form of Pinhead is one conserved cysteine residue less than other BMP ligands (fig. S1F). Therefore, we next examined whether secreted Pinhead protein could form covalent dimers that had been proved to be essential for downstream signaling events (3). Pinhead-HA proteins were enriched from the CM by immunoprecipitations and then subsequently separated on reducing and nonreducing SDS–polyacrylamide gel electrophoresis (PAGE), respectively. Immunoblotting analysis showed a single SDS-resistant band with an apparent molecular weight of about 70 kDa under nonreducing conditions, which migrated much more quickly under reducing conditions (Fig. 2F), implicating that most of the mature Pinhead proteins exist as disulfide-linked dimers in vivo. To determine whether secreted Pinhead activates an intracellular signaling cascade, we measured the phosphorylation levels of Smad1/5/8 in Hep3B cells in the presence and absence of Pinhead CM. We found that stimulation with recombinant BMP4 or Pinhead CM substantially enhanced Smad1/5/8 phosphorylation, and combining BMP4 and Pinhead CM further promoted this phosphorylation (Fig. 2G). By contrast, Smad2 phosphorylation (p-Smad2) in Hep3B cells was induced by incubations with TGF-β but not Pinhead CM (Fig. 2H).

In addition, overexpression of pinhead in embryos had no effects on p-Smad2 expression (Fig. 2H). These data not only demonstrate that Pinhead specifically triggers the BMP pathway but also rule out the possibility that Pinhead ventralizes embryos by inhibiting Nodal signaling, which is required for the formation of the organizer and the dorsal axial structures. Moreover, Pinhead binds to and signals through BMP receptors, as Pinhead CM–induced Smad1/5/8 phosphorylation was totally abolished in the presence of the selective BMP type I receptor inhibitor dorsomorphin or DMH1, and overexpressed Pinhead was coimmunoprecipitated with BMP type I receptors ALK2, ALK3, ALK6, and ALK8 (Fig. 2, I and J). We were not expecting to find an association between Pinhead and TGF-β type I receptor ALK5 (Fig. 2I). However, this Pinhead-ALK5 association may not have biological significance, as Pinhead proteins did not induce phosphorylation of Smad2 in Hep3B cells and zebrafish embryos (Fig. 2H).

In zebrafish embryos, injection of 100 pg of pinhead-HA mRNA promoted phosphorylation of Smad1/5/8 during gastrulation (Fig. 2K). Pinhead protein efficiently coimmunoprecipitated with the BMP antagonist Chd and Noggin1 (Fig. 2, L and M). The pinhead overexpression–induced DV defects in the shield-stage embryos, such as the reduction in gsc expression and the expansion of eve1 expression, were eliminated by coinjecting 10 pg of chd mRNA (Fig. 2, N and O). Consistent with these observations, at 24 hpf, injection of chd mRNA well rescued the ventralized morphology in Pinhead-overexpressing embryos (fig. S1L). Together, these findings indicate that Pinhead is a functional BMP ligand during zebrafish embryo development.

The pinhead and admp genes repress one another and compensatorily function in DV patterning

To examine the in vivo functions of pinhead, we generated a pinhead mutant by targeting exon 1 with the CRISPR-Cas9 system. The mutant was named phΔ49, as there was a 49–base pair (bp) deletion that led to the loss of the translational start site (fig. S2A). We further generated the maternal–zygotic mutant by incrossing homozygous pinhead mutants. In situ hybridization experiments revealed an obvious decrease in pinhead transcripts in the phΔ49 mutants, providing further evidence that this mutant is a null allele of the pinhead gene (fig. S2B). Unexpectedly, ph49A9 embryos had normal morphologies at the end of gastrulation and at 24 hpf (fig. S2, C and D). In addition, we found no DV pattern defects in ph49A9 mutants for typically expressed dorsal and ventral genes (fig. S2, E and F). Previous studies indicate that a compensatory network may be activated to buffer against deleterious mutations, which was not observed after translational or transcriptional knockdown (25). Therefore, knockdown experiments were performed using an antisense MO (ph MO) that interfered with translation by targeting the pinhead sequence and efficiently blocking the production of the Pinhead-GFP fusion protein in embryos (fig. S2G). However, injection of 5 ng of ph MO into wild-type embryos did not result in any obvious DV defects (fig. S2, H and I). Therefore, the loss of pinhead does
Fig. 2. Pinhead is a secreted functional BMP ligand. (A) The CM from HEK293T cells transfected with Pinhead-HA was examined using an immunoprecipitation assay. Pinhead levels in cell lysate were examined by Western blot as a positive control. Underlying data can be found in data file S1. (B and C) HEK293T cells were transfected with Pinhead-HA plasmids. Twenty-four hours later, cells were treated with CHX (20 μg/ml) for the indicated times. Then, the CM and CHX-treated cells were harvested for immunoblotting (B). Pinhead-HA protein levels were quantified and normalized to tubulin (mean ± SD, three independent biological repeats; C). Underlying data can be found in data file S1. (D and E) Pinhead-GFP fusion proteins were efficiently secreted from zebrafish embryonic cells. In (D), 50 pg of pinhead-GFP mRNA and 50 pg of mCherry-CAAX mRNA were coinjected into embryos at the one-cell stage. In (E), 10 pg of pinhead-GFP mRNA together with rhodamine-dextran was injected into one marginal blastomere at the 16- or 32-cell stage. All embryos were imaged using a Nikon A1R+ confocal microscope at the shield stage. Scale bar, 10 μm. (F) Pinhead-HA proteins were enriched from the CM by immunoprecipitation and then subsequently separated on reducing and nonreducing SDS-PAGE. Underlying data can be found in data file S1. (G and H) Hep3B cells were treated with Pinhead CM alone or together with BMP4 (G) or TGF-β1 (H) for 1 hour and then harvested for Western blots with the indicated antibodies. The expression of β-actin was analyzed as a loading control. In (H), wild-type embryos treated with 25 μM SB431542 (SB) from the 16-cell stage and embryos injected with 100 pg of gfp or pinhead mRNA at the one-cell stage were also harvested at the shield stage and subjected to immunoblotting. Underlying data can be found in data file S1. (I) Hep3B cells were treated with Pinhead CM alone or together with the indicated BMP type I receptor inhibitors for 4 hours and then harvested for Western blot with the indicated antibodies. Note that Pinhead CM-induced Smad1/5/8 phosphorylation notably decreased in the presence of BMP type I receptor inhibitors. Underlying data can be found in data file S1. (J) Pinhead binds to BMP type I receptors. HEK293T cells were transfected as indicated with expression plasmids encoding Flag-tagged Pinhead and HA-tagged BMP type I receptors and harvested for immunoprecipitation with an anti-HA antibody. Underlying data can be found in data file S1. (K) Western blots of total lysates from embryos injected with 100 pg of pinhead-HA mRNA. Underlying data can be found in data file S1. (L) Extracellular Pinhead interacts with Chd (L) and Noggin (M). CM were prepared from HEK293T cells transfected with indicated plasmids. Immunoprecipitation assays were performed using an anti-Flag antibody. Underlying data can be found in data file S1. (N) Overexpression of chd rescues Pinhead-induced DV defects. Embryos were injected with 100 pg of pinhead mRNA alone or together with 10 pg of chd mRNA at the one-cell stage and collected at the shield stage for in situ hybridization. (O) Expression levels of gsc and eve1 were analyzed at the shield stage by real-time qPCR. Error bars indicated SD. *P < 0.05; **P < 0.01, Student’s t test. NS, not significant.
not disturb the formation of the DV axis, and an additional signal may be present in the embryo to compensate for the lack of pinhead.

The genes pinhead and the BMP ligand-encoding admp exist in tandem in the genomes of various animals, including zebrafish. These two genes have diametrically opposed expression patterns in the trunk epidermis in gastrulating Ciona embryos (23). We speculate that admp is an ideal candidate for buffering the loss of pinhead. In support of this hypothesis, contrary to the narrowed expression of admp in pinhead-deficient Ciona embryos (23), we found admp expression to be up-regulated in zebrafish ph49 mutants at the 30% epiboly and shield stages (Fig. 3A). To exclude the possibility that the increase in admp expression was merely an adaptation for gene loss, we injected 5 ng of ph MO into wild-type embryos. We found that admp expression also greatly increased in the pinhead morphants (Fig. 3B), suggesting that admp expression is repressed by pinhead. We also generated a null allele of admp with an 11-bp deletion in exon 1 (adΔ11) (fig. S3, A and B). Mild dorsalization phenotypes were observed in knockdown experiments with admp MO in Xenopus and zebrafish (8, 12, 16, 17). By contrast, the morphol and DV polarity were not affected in adΔ11 maternal-zygotic mutants compared to the wild-type control (fig. S3, C to F). It had been reported that admp morphants exhibited a notable enlargement of gsc expression domain and an evident diminution of eve1 expression (14). Unexpectedly, we did not observe any marked changes in the expression of dorsal-ventral markers in embryos injected with 3 ng of admp MO (fig. S3, G and H), which had previously been used (14). This inconsistency may be due to the different experimental conditions between the studies. pinhead expression was evidently expanded in adΔ11 mutants and admp morphants (Fig. 3, C and D). These results reveal that pinhead and admp are expressed in a “seesaw”-like fashion through opposing transcriptional regulation in the embryonic body plan.

As shown in Fig. 3E, coimmunoprecipitation experiments revealed a steady binding of secreted Pinhead and Bamp1a, a Xolloid-related metalloproteinase that plays a pivotal role in proteolytic cleavage of Chd in zebrafish (26). The association of Pinhead with Bamp1a led us to examine whether Pinhead regulates BMP1a-mediated Chd degradation. Compared with the effects of the corresponding untagged proteins, overexpression of Admp-HA or Chd-Flag in wild-type embryos caused similar or slightly alleviated DV polarity defects at 24 hpf (fig. S4, A to D), suggesting that the addition of C-terminal epitopes has no obvious impact on their activities. Then, BMP1a, Chd, Admp, and Pinhead proteins were prepared by collecting the corresponding CM produced by transfected HEK293T cells. When BMP1a was coinubcated with Chd, we detected a decrease in Chd protein, where adding Admp further facilitated this cleavage (Fig. 3F), suggesting that the secreted BMP1a functions well in our biochemical system. Pinhead was then coinubcated with BMP1a and Chd in vitro, which promoted a reduction in Chd levels (Fig. 3G). These observations demonstrate that, similar to Admp, Pinhead promotes metalloproteinase-mediated Chd degradation.

To further confirm the roles of pinhead and admp in the formation of DV polarity in zebrafish embryos, we deleted the pinhead gene in the adΔ11 mutants using the CRISPR-Cas9 system. One mutant was obtained with an identical 49-bp deletion in the pinhead gene in the adΔ11 background. The phA49−/−;adΔ11−/− embryos develop normally and are viable and fertile, but phA49−/−;adΔ11−/− embryos began to die 36 hpf, with a few surviving up to adulthood. Homozygous pinhead and admp double-mutant embryos were generated by crossing the surviving adults. Most of the phA49;adΔ11 double mutants had an ovoid shape at the bud stage and a clearly shortened posterior trunk and reduced yolk extension at 24 hpf, all of which are characteristic of dorsalization (Fig. 3, H and I). Furthermore, although injection of pinhead MO or admp MO into wild-type embryos did not lead to observable DV polarity defects at 24 hpf, coinjection of these MOs generated a dorsalized phenotype very similar to that of phA49;adΔ11 double mutants (fig. S5A), excluding the potential CRISPR-Cas9 off-target effects.

The dorsalization phenotypes in phA49;adΔ11 double mutants were further confirmed by the expression of several dorsal and ventral markers. As shown in Fig. 3I, we observed a marked expansion in the dorsal markers chd and gsc in phA49;adΔ11 embryos, while the expression of these markers remained unchanged in the phA49 and adΔ11 single mutants compared to the wild-type embryos. Meanwhile, the expression of the ventral marker eve1 was nearly abolished in phA49;adΔ11 embryos (Fig. 3K). Genetic deletion of these two genes consistently caused enlargement of dorsal-related tissues, including the prechordal plate (indicated by gsc) and notochord (indicated by ntl) (fig. S5B). There was also a large decrease in the blood cells located in the intermediate cell mass in phA49;adΔ11 mutants (fig. S5C). It has been reported that dorsalized embryos exhibit a slightly widened adaxial domain and expanded somite due to loss of swirl/bmp2b (11, 24). However, phA49;adΔ11 mutants showed reduced presomitic mesoderm (indicated by papc) at the bud stage (fig. S5D), which might reflect a role of pinhead and admp in somitogenesis, as pinhead transcripts were highly enriched in the presomitic mesoderm at the end of gastrulation (Fig. 1A). In addition, at the shield stage, phosphorylation of Smad proteins and the BMP gradient decreased in the phA49;adΔ11 embryos (Fig. 3, L and M). Consistent with previous reports that bmp expression is maintained through autoregulatory feedback loops (24, 27), we observed that, compared to the wild-type embryos at the shield stage, phA49;adΔ11 double mutants displayed reduced expression of bmp2b, bmp4, and bmp7a, which were not obviously changed in phA49 and adΔ11 single mutants (fig. S5E). In addition, by knockdown of admp in phA49 mutants or injection of pinhead MO into adΔ11 embryos, we observed similar dorsalization phenotypes, including the changes in the expression of dorsal and ventral markers (fig. S6, A to D), the repression of Smad1/5/8 phosphorylation (fig. S6, E and F), and the destruction of the BMP activity gradient (fig. S6, G and H). These results further confirm that pinhead and admp function together to regulate the formation of the BMP activity gradient and DV patterning in early zebrafish embryos.

It is well established that Admp functions in DV axis formation by enhancing Chd degradation (12, 15). Moreover, Admp overexpression could enhance the ventralized phenotype of dik homozygous mutants, a mutant allele of chd (28), suggesting a role for Admp in BMP signal activation through a Chd-independent manner. It has been proved that Admp has BMP-like activity and signals via the ALK2 receptor (8). Given that Pinhead has similar functional properties to Admp, we asked whether Pinhead functions as a secreted scaffold aiding in Chd degradation and a ligand involved in activating BMP signaling during zebrafish DV patterning. We first examined the expression levels of endogenous Chd in shield-stage phA49 and adΔ11 mutants by Western blot analysis using a previously validated antibody (17). We found that the endogenous Chd protein heavily accumulated in phA49;adΔ11 double mutants compared to wild-type or single-mutant embryos (fig. S7A). In contrast, the expression of Chd...
Fig. 3. Pinhead and Admp repress one another and compensatorily function in DV patterning. (A and B) The expression pattern of admp in wild-type, pinheadΔ49 homozygous mutant, and 5-ng pinhead MO (ph MO)–injected embryos at the indicated stages. (C and D) The expression pattern of pinhead in wild-type, admpΔ11 homozygous mutant, and 3-ng admp MO (ad MO)–injected embryos at the indicated stages. (E) Pinhead binds to BMP1a. HEK293T cell–produced Pinhead-HA was coincubated with or without BMP1a-Myc–containing medium at 4°C for 12 hours. The interactions between Pinhead and BMP1a were analyzed with an anti-HA antibody. Underlying data can be found in data file S1. (F and G) Admp or Pinhead promotes BMP1a-mediated Chd degradation. HEK293T cell–produced Chd-Flag protein was incubated with the indicated CM at 4°C for 12 hours, and then analyzed by Western blot with an anti-Flag antibody. Underlying data can be found in data file S1. (H and I) Morphological defects in phΔ49;adΔ11 double-mutant embryos. Note the ovoid shape at the bud stage (H) and the shortened posterior trunk at 24 hpf (I) in phΔ49;adΔ11 double mutants. Scale bar, 100 μm. (J and K) The expression patterns of dorsal and ventral markers in wild-type and indicated mutant embryos. Note that the double depletion of pinhead and admp results in evidently increased expression of dorsal markers chd and gsc (J) and reduced expression of ventral marker eve1 (K) at the shield stage. (L and M) Wild-type and indicated mutant embryos were harvested at the shield stage for Western blots (L) and immunofluorescence assays (M) with the indicated antibodies. Note the distinct decrease in Smad1/5/8 phosphorylation in the phΔ49/adΔ11 double mutants. Scale bar, 50 μm. Underlying data can be found in data file S1.
evidently decreased upon injection of pinhead or admp mRNA (fig. S7B). To avoid the influence of changes in endogenous Chd expression induced by DV defects, we further examined the expression of exogenous Chd-HA protein in phΔ49:adΔ11 double mutants and wild-type embryos. All the embryos were injected with the same amount of chd-HA mRNA (50 pg) at the one-cell stage. Western blot results showed that the expression level of Chd-HA was up- or down-regulated upon depletion or overexpression of pinhead/admp (fig. S7, C and D). These results indicate that Pinhead can facilitate Chd degradation in vivo.

We next tested whether Pinhead also has a role in DV patterning when chd and bmp2b are depleted. As previously reported, injection of bmp2b MO into wild-type embryos caused a severe dorsIALIZED morphology at 24 hpf, while the interference with chd function by MO generated a clear ventralized phenotype (fig. S7E) (29, 30). As expected, double depletion of chd and bmp2b gave a nearly normal morphology (fig. S7E). Injection of 100 pg of pinhead mRNA into chd/bmp2b-depleted embryos led to a ventralized phenotype, which was slightly serious in embryos injected with the same amount of admp mRNA (fig. S7E). Thus, similar to Admp, Pinhead might also regulate the establishment of DV regionalization via its BMP-like activity.

On the basis of these results, overexpression of either pinhead or admp would be expected to compensate for the loss of these two genes. Injection of 5 pg of bmp2b mRNA into the phΔ49:adΔ11 mutant embryos efficiently reversed the dorsIALIZED morphologies (fig. S7F). Injection of either 100 pg of pinhead mRNA or 50 pg of admp mRNA also considerably alleviated the DV defects in the phΔ49:adΔ11 mutants (fig. S7F). Thus, the consistency in the molecular nature and the opposite transcriptional regulation of Pinhead and Admp provides an important compensatory mechanism by which to maintain stability of axial patterning when one of these genes is disrupted.

### BMP signaling negatively regulates pinhead and admp expression

In Ciona gastrulas, pinhead is expressed in the posterior ventral epidermal cells, while admp is expressed in the dorsal epidermis. Their mutually exclusive expression is regulated at the chromatin level by a cis-acting mechanism that is widely conserved between animals (23). When the cis-acting repression is relieved, ectopic admp expression is observed in the ventral region (23). Expression of admp greatly increased in the phΔ49 mutant but did not spread into the ventral lateral regions, suggesting that the microdeletion in the phΔ49 mutant did not alter the chromosomal conformation around the gene locus (Fig. 3, A and B). Repression of admp by BMP signaling and the BMP-like activity of Pinhead prompted us to examine whether the up-regulation in admp expression in the phΔ49 mutants was due to a transient decline in BMP signaling induced by Pinhead deficiency.

As shown in Fig. 4 (A and B), wild-type embryos injected with bmp2b MO or treated with the BMP inhibitor dorsomorphin or DMH1 had remarkably increased expression of admp. Conversely, injection of bmp2b mRNA led to a reduction in admp expression (Fig. 4, C and E). In addition, the expression of pinhead was negatively regulated by BMP signaling, as knockdown of bmp2b induced and high levels of bmp2b inhibited its transcription (Fig. 4, A, B, D, and E). The increased expression of admp and pinhead in the corresponding mutants was reduced to a lower level than that in wild-type embryos by injection of 10 pg of bmp2b mRNA (Fig. 4, F to H), indicating a complete compression of the compensatory expression of pinhead or admp upon BMP2b overexpression. Therefore, we conclude that BMP signals negatively regulate pinhead and admp transcription. In addition, wild-type embryos injected with 10 pg of bmp2b mRNA displayed various ventralized phenotypes ranging from mild to severe at 24 hpf (fig. S8, A and B). Upon bmp2b mRNA injection, the phΔ49 and adΔ11 single mutants exhibited a similar ventralized morphology (fig. S8, A and B), further suggesting that the compensatory expression of pinhead or admp can make up for the gene loss in the corresponding mutants.

On the basis of the “seesaw”-like expression patterns of pinhead and admp, we speculate that, when the expression of one is disturbed in embryos, BMP signaling will temporarily be lower and expression of the other gene will be subsequently promoted to support the self-regulation of the BMP signaling levels for the embryonic body plan. To address this issue, MOs targeting pinhead or admp were injected into Tg(BRE:EGFP) embryos, in which a GFP reporter can reveal the dynamic changes in BMP activity during embryonic development (31). In support of our hypothesis, the results of real-time quantitative polymerase chain reaction (PCR) analysis revealed an early partial loss of BMP activity in the morphants, which was dynamically compensated before the shield stage (Fig. 4I). The expression of admp in phΔ49 mutants or pinhead in adΔ11 mutants was gradually elevated compared to that in wild-type embryos (Fig. 4, J and K). The promotion of admp and pinhead expression may well be due to the temporary reduction in BMP activity in the single-mutant embryos, as coinjection of 10 pg of bmp2b mRNA notably suppressed the elevation of their expression (Fig. 4, J and K).

**Smad proteins directly bind to pinhead and admp enhancers**

To further investigate whether BMP/Smad signaling directly represses pinhead and admp transcription, we amplified 1336 bp of the admp and 1516 bp of the pinhead promoter regions upstream of the translation start site of each gene and fused them to GFP cDNA to create reporter constructs (named −1336-ad-P-GFP and −1516-ph-P-GFP, respectively). The upstream sequence of admp drove GFP expression on the dorsal side of shield stage embryos, recapitulating endogenous expression of admp (Fig. 5A). Next, we generated serial truncations of the admp promoter and injected them into embryos. We found that the truncated promoter containing the −633-bp upstream sequence (−633-ad-P-GFP) exhibited transcriptional activity in the dorsal region similar to the full-length promoter, while the −210-ad-P-GFP construct lost the ability to express GFP (Fig. 5A), suggesting that the region between −633 and −210 bp is an enhancer essential for admp expression. To identify potential BMP/Smad-responsive elements in this enhancer, we injected the −633-ad-P-GFP construct (100 pg) into wild-type and phΔ49 mutant embryos. The expression of −633-ad-P-GFP was augmented in DMH1-treated wild-type and phΔ49 mutant embryos (Fig. 5B), suggesting that the enhancer responds well to BMP signals. To quantitatively analyze the transcriptional regulation of admp by BMP signal, we generated a luciferase reporter plasmid (−633-ad-P-Luc) by subcloning the −633-bp upstream sequence of admp into pGL3-Basic vector. As expected, 3- or 4.5-fold enhanced transcriptional activity of −633-ad-P-Luc was observed in pinhead defective or DMH1-treated embryos (Fig. 5C). Similarly, a BMP signal-responsive enhancer that specifically drove reporter gene expression in the ventral and lateral margin of the gastrulas was identified between −431 and −225 bp in the pinhead promoter (Fig. 5, D to F).
Smad proteins physically interact with the promoters of their target genes to regulate gene expression. Specifically, Smad1/5 proteins, the intracellular downstream mediators of BMP signaling, directly bind to the GC-rich elements in BMP/Smad target promoters. There are two and four potential Smad1/5-bound GC-rich elements in the *admp* and *pinhead* enhancers, respectively. The expression of *pinhead* and *admp* is repressed by BMP signaling. Embryos injected with 10 pg of *bmp2b* mRNA showed a decrease in *admp* and *pinhead* expression at the shield stage. Overexpression of *bmp2b* in mutants showed a significant inhibition of *admp* and *pinhead* expression.
Fig. 5. Smad1 binds to and suppresses the enhancers of pinhead and admp. (A) The −633- to −210-bp region of the admp promoter was an important enhancer driving GFP expression in the dorsal side. Wild-type embryos were injected with various constructs as indicated at the one-cell stage and photographed at the shield stage. Animal pole views with dorsal to the right. Scale bar, 100 μm. (B and C) Responses of the admp enhancer to BMP signal inhibition. Wild-type and phΔ49 mutant embryos were injected with 100 pg of the −633-ad-P-GFP (B) or −633-ad-P-luc (C) reporter construct at the one-cell stage and treated with or without 5 μM DMH1 from the 1K cell stage to the shield stage. Scale bar, 100 μm. ***P < 0.001, Student's t test. (D and E) Ventral and lateral margin expression of the pinhead promoter was determined by an enhancer located between −431- and −225-bp upstream of the transcription start site (D), which was heightened at the shield stage in adΔ11 mutant and wild-type embryos treated with DMH1 (E). Animal pole views with dorsal to the right. Scale bar, 100 μm. (F) The expression of −431-ph-P-luc in indicated embryos was examined by luciferase measurement. The relative luciferase activity in each sample was the mean with SD from three independent experiments. ***P < 0.001, Student's t test. (G and H) Purified Smad1 bound specifically to admp probe B (ad Probe B) (G) and mutations in probe B abolished Smad binding (H). DNA-protein complexes were analyzed with a polyacrylamide gel and visualized by Typhoon FLA9500 Scanner. (I and J) Purified Smad1 directly bound to the wild-type pinhead probe B (ph Probe B) (G) but not the mutated probe (H). Electrophoretic mobility shift assays were performed similarly as in (G) and (H). (K to N) The mutated promoter reporters (−633-ad-MTB-GFP, −633-ad-MTB-luc, −431-ph-MTB-GFP, and −431-ph-MTB-luc) showed a higher transcriptional activity than the corresponding wild-type reporter. Wild-type embryos were injected with 100 pg of indicated reporter constructs at the one-cell stage. At the shield stage, these embryos were photographed (K and M) or subjected to luciferase assays (L and N). Scale bar, 100 μm. ***P < 0.001, Student's t test. (O and P) Smad binding was essential for BMP signal–mediated suppression of admp (O) and pinhead (P) transcription. Mutated reporter constructs (50 pg) were injected into wild-type and indicated mutant embryos at the one-cell stage, respectively. Wild-type embryos injected with −633-ad-MTB-GFP or −431-ph-MTB-GFP reporter were treated with or without DMH1 from the 1K cell stage. Note that these mutated reporters lost their ability to respond to BMP inhibition. Scale bar, 100 μm.
A and B). To explore the ability of Smad proteins to bind to these sites, we performed electrophoretic mobility shift assays using purified Smad1 proteins and synthesized probes containing presumptive Smad-binding sites. Smad1 specifically bound to admp probe B (ad Probe B) but not to the mutated probe with the “GGCGCC” to “AAAAAA” substitutions within the putative Smad1/5-binding site (Fig. 5, G and H, and fig. S9A). Meanwhile, Smad1 also bound to pinhead probe B (ph Probe B) but not its mutant (Fig. 5, I and J, and fig. S9B). When the proper mutagenesis were introduced into each enhancer, the mutated promoters (100 pg of each) exhibited a remarkable increase of their transcriptional activities in wild-type embryos (Fig. 5, O and P). Together, these results demonstrate that Smad1/5 binds to the enhancers 11 or admp mutants (Fig. 5, O and P).

Together, these results demonstrate that Smad1/5 binds to the pinhead enhancers and admp enhancers to repress their transcription in response to BMP signaling.

The pinhead and admp genes provide a dual protection system for the robustness of embryonic patterning

Embryonic DV patterning displays substantial resistance to experimental perturbations and Admp has been proposed to aid in the self-regulation of the BMP signaling gradient and the regeneration of normal DV structures (8, 12). Because pinhead and admp are expressed under feedback regulations in a “seesaw”-like fashion and have compensatory functions in DV patterning, we presumed that both of these genes might be involved in the robust stability of axial patterning through fine-tuning of BMP signaling. To investigate whether pinhead and admp contribute to the buffering of BMP activity profiles against variations in gene dosage, we introduced bmp2b MO into phΔ49 or adΔ11 mutants and phΔ49adΔ11 embryos injected with a rescuing amount of pinhead mRNA (improved phΔ49adΔ11 mutants). As shown in Fig. 6A, injection of 1 ng of bmp2b MO induced a mild increase of chd expression in most wild-type, phΔ49, and adΔ11 embryos and caused a much stronger expansion of this dorsal marker gene in nearly 70% of the improved phΔ49adΔ11 mutants, where the DV defects were significantly rescued by pinhead overexpression (Fig. 6, A and B). In response to bmp2b MO injection, the expression domain of chd even extended to the ventral regions in about 20% improved phΔ49adΔ11 mutants, suggesting a much more severe dorsalized phenotype (Fig. 6, A and B). Consistent with this, injection of 1 ng of bmp2b MO caused a more severe decrease in expression of the ventral marker eve1 in improved phΔ49adΔ11 mutants compared to the control embryos (Fig. 6, A and C). These bmp2b morphants displayed different dorsalized morphologies (C1 to C5) at 24 hpf (Fig. 6, D and E). About 80% of the improved phΔ49adΔ11 embryos showed a normal DV morphology (Fig. 6E). Upon injection of 1 ng of bmp2b MO, above 20% of wild-type embryos and pinhead or admp single mutants showed some mild cases of dorsalization defects (C1 and C2), whereas all of the improved phΔ49adΔ11 embryos exhibited more severely dorsalized phenotypes (C3 to C5; Fig. 6E). Furthermore, a reduced amount of injected bmp2b MO (0.3 or 0.1 ng) led to marginal dorsalized phenotypes in wild-type and single-mutant embryos, while these subdose injections resulted in hyperdorsalization in improved phΔ49adΔ11 mutants (Fig. 6, F and G). These findings indicate that pinhead and admp cooperatively confer robust resistance to the decrease of BMP signaling during DV patterning.

Because the expression of pinhead and admp notably decreased in bmp2b overexpression embryos (Fig. 4, C to E), these two genes might also play important roles when embryos are challenged with excessive BMP activity. If this deduction is correct, reduced expression of pinhead and admp should dampen the elevated BMP activity to some extent due to impeded Chd degradation and depletion of these two genes should further stabilize BMP activity profiles during DV patterning. Consistent with these predictions, injection of 10 pg of bmp2b mRNA led to severe ventralized phenotypes in above 90% wild-type, phΔ49, and adΔ11 embryos, which were obviously alleviated in about 40% improved phΔ49adΔ11 mutants (Fig. 6, H to J). Moreover, our study proved that, similar to Admp, Pinhead also functions in DV patterning via its BMP-like activity (fig. S7E). To explore whether the BMP-like activity of Pinhead and Admp is involved in the robustness of embryonic patterning, we examined the expression patterns of gsc and eve1 in chd-depleted embryos at the shield stage. We observed that, upon chd depletion, the improved phΔ49adΔ11 mutants showed a lower rate of severe ventralized phenotype than wild-type, phΔ49, and adΔ11 embryos (fig. S10, A to C), indicating that the over-activation of BMP signaling induced by chd deletion can be appeased through genetic inactivation of both pinhead and admp. These results also imply that Pinhead and Admp could act as BMP ligands to stabilize embryonic DV patterning. Collectively, these data demonstrate that pinhead and admp serve as a dual protection system for the robustness of embryonic patterning by buffering against disturbances in the dynamic BMP signaling (Fig. 6K).

DISCUSSION

Robustness is a ubiquitous property in organisms that allows a system to maintain its functions despite external and internal perturbations. These robust biological traits are often selected through evolution and facilitate evolvability (33, 34). One of the best-studied models of robustness in embryonic development is the normal production of gastrula DV patterns after experimental perturbations (5–10). However, the molecular nature of this self-regulating pattern remains one of the most challenging areas that remain to be delineated in developmental biology. It is well known that a BMP signaling gradient is established through ventral BMP signals and their dorsally expressed antagonists. Chd, which forms along the DV axis to pattern tissues (4). Admp, a BMP-like protein expressed as part of the feedback regulation of the Chd/BMP system on the dorsal side of gastrulating embryos, is an appealing candidate in ensuring embryonic self-regulation (8, 12). Robustness can be enhanced by an “alternative” or “fail-safe” mechanism, where multiple means achieve a specific function, and the failure of one of them can be overcome by the others (33). However, whether there is an alternative mechanism to support the substantial DV polarity remains to be determined.

A previous study revealed that zebrafish admp morphants have an almost normal distribution of Chd protein (17). Consistent with this observation, the morphology and DV polarity are not affected in the adΔ11 mutants generated in this present study, suggesting that the loss of Admp function may be quickly compensated for by other BMP-like members. Xenopus ONT1, an olfactomedin-class secreted protein, was demonstrated to contribute to the robust stability of axial patterning with Admp in a synergistic manner (12).
Fig. 6. The *pinhead* and *admp* genes function together to buffer against fluctuations in BMP activity. (A to C) The expression patterns of dorsal and ventral markers in the indicated embryos injected with *bmp2b* MO. The expression patterns of *chd* and *eve1* were categorized as shown in (A), and the ratios were presented in (B) and (C). Note that injection of 1 ng of *bmp2b* MO into improved *pha49;ad411* mutant embryos (injected with 100 pg of *pinhead* mRNA) led to much more severe dorsalized phenotypes at the shield stage than wild-type control and single-mutant embryos. (D to G) Double depletion of *pinhead* and *admp* strongly enhanced *bmp2b* MO-induced dorsalization. Indicated embryos were injected with different doses of *bmp2b* MO (1, 0.3, or 0.1 ng) at the one-cell stage and imaged at 24 hpf. Representative dorsalized morphologies (C1 to C5) are shown in (D), and their ratios are shown in (E) to (G). Scale bar, 100 μm. (H to J) Double depletion of *pinhead* and *admp* stabilized DV patterns when BMP activity was elevated. Indicated embryos were injected with 10 pg of *bmp2b* mRNA at the one-cell stage and harvested at the shield stage for in situ hybridization with *chd* and *eve1* probes (H). Their ratios of the categorized expression patterns of *chd* and *eve1* are shown in (I) and (J). (K) Proposed model of the alternative mechanism for the robustness of embryonic patterning that couples *pinhead* and *admp* with system control based on opposing regulation of BMP signaling and *pinhead/admp* expression. In wild-type embryos, the expression of *pinhead* and *admp* is repressed by each other and by BMP/Smad pathways (the left panel). The negative feedback loop between *pinhead/admp* and BMP signals plays an important role in buffering against fluctuations in dynamic BMP signaling during DV axis formation (left panel). Meanwhile, when the function of *pinhead* or *admp* decreased or failed, the “seesaw”-like expression of these two genes will provide a well-orchestrated alternative mechanism for embryonic self-regulation (the middle and right panels). The arrows with dashed outlines in the middle and right panels indicate the quick up-regulation of *pinhead* or *admp* expression to compensate for the genetic loss of the other gene.
However, unlike Admp protein, ONT1 has no BMP ligand activity and acts as a secreted scaffold that enhances Chd degradation by facilitating enzyme-substrate association. The attenuation of ONT1 causes an increase in Admp expression but still leads to dorsalization phenotypes in Xenopus embryos (12), suggesting that ONT1 and Admp have non-overlapping functions during DV axis formation. In this study, our data indicate that zebrafish pinhead encodes a secreted BMP ligand with ventralizing functions during zebrafish embryo development. pinhead mutants had no DV pattern defects because the enhanced expression of admp fully compensated for the gene loss. Conversely, pinhead also responds to the decrease or depletion of admp to stabilize axial formation. Therefore, the “seesaw”-like expression of pinhead and admp establishes a well-coordinated alternative mechanism for the robust generation of the DV axis. In addition, similar to Admp, Pinhead acts as a scaffold that promotes metalloproteinase-mediated Chd degradation. Thus, this alternative mechanism is mediated by the remarkable molecular similarities between Pinhead and Admp.

In Ciona embryos, pinhead is expressed in the posterior ventral epidermal cells, and MO-mediated gene knockdown experiments revealed that pinhead functions in the DV axis formation of the trunk epidermis (23). In Xenopus embryos, pinhead is expressed in the anterior neural plate of the neurula and genetic manipulation by MO injection showed that pinhead is a key regulator of head development (22). Because of a lack of expression of pinhead in the epidermal cells and the neural plate during zebrafish embryo development, it is reasonable that we found no defects in the formation of epidermal and neural tissues in our pinhead mutants. An important unanswered question in developmental biology is how a self-differentiating morphogenetic field is established in the developing embryos. Classic embryological studies have demonstrated that admp expression is repressed by BMP signaling, and transcriptional up-regulation of admp plays a key role in compensating for the depletion of ventrally expressed BMPs (8,28). Likewise, pinhead expression is remarkably increased in embryos injected with bmp2b MO or treated with BMP inhibitors, while it notably decreased in embryos overexpressing bmp2b. Although a rescuing amount of pinhead mRNA had been introduced into pinhead and admp double mutants, these embryos were more fragile to disturbances in the dynamic BMP signaling gradient, indicating that the opposing transcriptional regulation between pinhead/admp and ventral BMP signals serves as a negative feedback mechanism and is responsible for the robust pattern formation. On the basis of these observations, we hypothesize a new framework, where the alternative mechanism is coupled with system feedback controls to ensure embryonic self-regulation. This working hypothesis is further reinforced by the identification of functional Smad1/5 binding elements in pinhead and admp enhancers. However, it has been reported that admp expression is decreased in pinhead morphants, and pinhead expression is suppressed in admp-depleted Ciona embryos during DV axis formation of the trunk epidermis (23). Furthermore, BMP signaling has no effect on admp expression but is required for pinhead expression in Ciona embryos (23). Therefore, it is possible that the effects of BMP signaling on pinhead and admp expression may be context dependent.

Expression of admp has been observed in the dorsal organizer (13, 14). ONT1, which has been proposed to stabilize axial formation by restricting Chd activity, is also expressed on the dorsal side (12). In addition to the ventral and lateral region, bmp2b is also expressed in the dorsal organizer, where organizer-derived BMP2b represses chd transcription and helps control the Chd gradient during gastrulation of zebrafish embryos (17). It will be interesting to investigate whether organizer-specific bmp2b functions in the robust stability of DV patterning. However, our study showed that the expression of bmp2b in both the ventrolateral region and the dorsal organizer was not affected by single depletion of pinhead or admp, suggesting that bmp2b may not be involved in the compensatory mechanism for the robustness of embryonic patterning.

Our study revealed that pinhead is expressed in the ventrolateral margin of zebrafish gastrulas. The mutually exclusive expression and shared functions of pinhead and admp suggest that Pinhead can diffuse to the dorsal side as an extracellular signaling molecule, which is indirectly confirmed by the compensatory effects of pinhead up-regulation in admp mutants. We observed an efficient secretion and a long-range diffusion of Pinhead-GFP fusion proteins in the developing embryos. In embryos, a subset of extracellular secreted factors, such as xolloid-related, twisted gastrulation, and crossveinless 2, function ventrally to promote BMP signaling through a variety of ways. The expression of these genes is positively regulated by BMPs (4, 11), ruling out any potential compensatory roles they may have buffering morphogen profiles against variations. Meanwhile, BAMBI and Sizzled, secreted feedback BMP antagonists, are expressed on the ventral side as part of the BMP synexpression group and shape the BMP signaling gradient (35–38). These BMP inhibitors may play additional roles ensuring reproducible DV patterns in the face of natural fluctuations (4). However, considering normal epidermal DV patterning occurs in BMP4/7-depleted dorsal halves of split Xenopus embryos and the formation of extra tails when cells from the ventral margin are transplanted into the animal pole of host zebrafish embryos (8, 10), an additional BMP-like member is likely up-regulated in the ventral region to compensate for deficiencies in BMP activity. Whether expression of pinhead is induced in the bisected embryos or the grafts to preserve the ventral identity remains to be determined.

In summary, this present study suggests that pinhead and admp serve as an alternative mechanism of embryonic self-regulation, where the functions of these two genes can be restored by modular feedbacks when one component fails. It is important to note that this alternative mechanism is coupled with system control on the basis of the opposing regulation of BMP signaling and pinhead/admp expression for coping with environmental perturbations. Pinhead is the only known BMP member expressed in the ventrolateral region that is suppressed by BMP signaling. However, no expressed sequence homologous to pinhead has been found in birds and mammals (22, 23). The identification of other BMP members with functions overlapping with Admp in animals that have lost pinhead gene over the course of evolution will be important for increasing our understanding of the molecular mechanism underlying self-regulative DV patterning.

MATERIALS AND METHODS

Zebrafish strains

Embryos and adult fish were raised and maintained under standard laboratory conditions. Wild-type embryos were obtained from natural matings of Tubingen zebrafish. Studies in this manuscript involving zebrafish embryo collection and analyses were in full compliance with the Institutional Animal Care and Use Committee at the Institute of Zoology, Chinese Academy of Sciences (permission number IOZ-13048).
Generation of zebrafish mutant lines using Cas9/gRNA system

Mutant pinhead and admph lines were generated using the CRISPR-Cas9 system as previously described (39). Two guide RNAs (gRNAs) were designed to target the sequences 5′-GGAGTTGTTGGCTC-GTG-3′ and 5′-GGTGGAGGCGAGTCAGG-3′ within the first exon of the pinhead and admph loci, respectively. The Cas9 mRNA and gRNA were synthesized in vitro and co-injected into one-cell stage wild-type embryos. For screening of the mutant alleles, the genomic regions surrounding gRNA-targeted sequences were amplified by PCR with the following primers: for pinhead mutants, 5′-CATGGTTGAT AAACAAAGGCC-3′ (forward) and 5′-GAA-ATACGTGAAATGCTGAACGT-3′ (reverse); for admph mutants, 5′-TCAGATCATCTCCCGAAGACCTCC-3′ (forward) and 5′-TTAT-CTTACATTGCTGGAAGAG-3′ (reverse). The amplified DNA fragment was purified for enzymatic digestion with T7 endonuclease I (M0302, New England BioLabs) or subjected to Sanger sequencing.

Confirmed founders were crossed to wild-type animals to raise F1 carriers for each mutant. Homozygous mutants were obtained by incrossing F1 fish carrying mutated genomic DNA. adph homozygous mutant embryos were injected with Cas9 mRNA and pinhead gRNA to generate germline mutants for pinhead in an adph mutant background. F1 animals were obtained by crossing the founders and adph homozygous mutants. Homozygous phadph/adph double mutants were obtained from the offspring of the phadph/adph 1/2 adults.

Constructs

Zebrafish pinhead was cloned into pCS2(+) vectors containing a C-terminal HA or FLAG tag for eukaryotic expression. For Pinhead-GFP, the sequence encoding GFP protein was inserted downstream of the pinhead coding sequence with an “EFLQDIIDGPSGLE” linker separating the fluorescing protein and the fluorescing peptide. C-terminal epitope-tagged Admp, Chd, Noggin1, and Bmp1a were cloned in pcS2-Flag, pcS2-HA, or pcS2-Myc vectors. All the resulting constructs were confirmed by sequence analysis.

Cell lines and transfections

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum in a 37°C humidified incubator with 5% CO₂. Transfection was performed using LipoFectamine 2000 (11668019, Invitrogen) according to the manufacturer’s instructions. Once the HEK293T cells transfected with constructs expressing secreted proteins, such as Pinhead, Chd, and BMP1a, reached 80% confluency, they were washed three times with phosphate-buffered saline (PBS) and cultured in serum-free DMEM for 24 hours. CM was then collected from each sample and centrifuged at 3000g for 5 min, filtered through a 0.22-μm filter, and concentrated to 10% of the original volume using Centriplus concentrators (Amicon).

RNA and MO microinjection

Capped mRNAs were synthesized in vitro for pinhead, pinhead-HA, pinhead-GFP, mCherry-CAAX, admph, bmp2b, and gfp from the corresponding linearized plasmids using the mMessage mMachine kit (Ambion). The following MOs were synthesized by Gene Tools and resuspended in nuclease-free water: ph MO (5′-ACTGACAC-CAGTAAATCCATAGCC-3′), admph MO (ad MO; 5′-TGGACAAACATTGAAGAACATGTC-3′) (14), bmp2b MO (5′-CGC-GGCCAGGACCAGCATGATC-3′) (30), and chd MO (5′-ATCACAGCAGCCCCCTCCATCCATC-3′) (29). The mRNA and MOs were injected into the yolk, while plasmid DNA was injected into the cytoplasm of one-cell stage embryos.

RNA probe synthesis and WISH

Digoxigenin–uridine triphosphate–labeled and fluorescein-labeled antisense RNA probes were transcribed using the MEGAScript Kit (Ambion) according to the manufacturer’s instructions. WISH was performed according to previously published methods (39). For double in situ hybridization, anti–digoxigenin-peroxidase (POD) (11633716001, Roche) and anti–fluorescein-POD (11426346910, Roche) were used as primary antibodies to detect digoxigenin-labeled pinhead probe and fluorescein-labeled gsc probe, respectively.

Embryonic treatment

To block BMP signaling, embryos were treated with 10 μM dorsomorphin (P5499, Sigma) or 5 μM DHM1 (D8946, Sigma) at the 1K-cell stage in the dark, and then were collected at the shield stage for WISH.

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed to check mRNA expression levels of the tested genes. Total RNA was extracted with Trizol (Invitrogen) and complementary DNAs were synthesized with high-efficiency reverse transcriptase ReverTra Ace (Toyobo). A Biorad CFX96 PCR system was employed to perform real-time PCR using SYBR Premix Ex Taq dye (Takara). The primer sequences were as follows: for β-actin, 5′-ATGGATGATGAAATTGCGG-3′ (forward) and 5′-ACCATCAGCAGTCCATACG-3′ (reverse); for gsc, 5′-GACGACAACCAGAACCATT-3′ (forward) and 5′-TCTTGAAGCATCGACCTTTTTC-3′ (reverse); for chd, 5′-TAGACTGTGTTAAGGATGTCC-3′ (forward) and 5′-CCATGAAGTCTCTATGACATC-3′ (reverse); for eveI, 5′-GGCAAGTGGCAGCCGACCCCTTACT-3′ (forward) and 5′-GTAGTGTAGGGAGCAGGG-3′ (reverse); for vent, 5′-GAATTTCCTAGTGAGAGGTCT-3′ (forward) and 5′-TCTTACTCGCAGGTTTGG-3′ (reverse); for pinhead, 5′-GATTCAGTGAATGATGATG-3′ (forward) and 5′-TCTTACGCACTACATC-3′ (reverse); for admph, 5′-TCAGTGTGATGATGATG-3′ (forward) and 5′-GTACTGCGACATGCAG-3′ (reverse); for gfp, 5′-TGAATTCATGCACCCCCGCAA-3′ (forward) and 5′-GGAGATGTTTGCGGTCCTTGGAA-3′ (reverse).

Immunoprecipitation and Western blot analysis

For immunoprecipitation assays, HEK293T cells were transfected with the indicated plasmids. Cells were harvested 48 hours after transfection and lysed with TNE lysis buffer [10 mM tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, and 0.5% Nonidet P-40] containing a protease inhibitor cocktail. Immunoprecipitation assays were performed on lysates and collected CM as previously described (39).

For immunoblotting, affinity-purified anti-Flag (F2555, Sigma), anti-HA (CW0092A, CW), anti-Myc (M047-3, MBL), anti–phospho-Smad1/5/8 (9511, Cell Signaling Technology), anti-Smad1/5/8 (ab72504, abcam), anti–phospho-Smad2/3 (3101, Cell Signaling Technology), anti-Smad2/3 (3102, Cell Signaling Technology), anti-Chd (GTX128209, GeneTex), and anti–β-actin (SC1615, Santa Cruz Biotechnology) antibodies were used.
Immunofluorescence analysis
Embryos were collected at the shield stage, fixed in 4% paraformaldehyde overnight, washed with PBS containing 0.1% Tween 20 for 30 min, blocked with 1% bovine serum albumin for 1 hour at room temperature, and then incubated with anti–phospho-Smad1/5/8 (1:200; 9511, Cell Signaling Technology) for 24 hours at 4°C. After washing with PBS three times for 5 min each, the samples were incubated with a donkey anti-rabbit secondary antibody conjugated to DyLight 594 (1:500; 711-585-152, Jackson ImmunoResearch) overnight at 4°C. All immunofluorescence images were captured using a Nikon A1R+ confocal microscope with the same settings for all samples within each experiment.

Prokaryotic expression and protein purification
An in-frame insertion of the cDNA of the full human Smad1 gene with a glutathione S-transferase (GST) tag into the plasmid pGEX-4 T-1 was performed, and the resulting recombinant plasmid was transformed into Escherichia coli strain BL21. GST-tagged Smad1 protein expression was induced using 1 mM isopropyl-β-D-thiogalactopyranoside, and the resulting protein was purified with glutathione Sepharose 4B beads (GE Healthcare) according to the manufacturer’s instructions. Protein was digested by thrombin and dialyzed against buffer I [10 mM tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), and 0.2 M NaCl] at 4°C overnight. Proteins were brought to a final concentration of 200 µg/ml.

Electrophoretic mobility shift assays
Electrophoretic mobility shift assays were performed as follows. Mixtures of purified Smad1 (10 ng) protein and 0.5 ng of carboxyfluorescein (FAM)–labeled probes were incubated at room temperature for 30 min in a 10-µl reaction volume. The reaction buffer contained 25% glycerol, 50 mM KCl, 0.5 mM EDTA, 10 mM DTT, and 5 mM tris-HCl and had a pH of 8.0. The mixtures were resolved on a 6% nondenaturing polyacrylamide gel (60:1 acrylamide-to-bisacrylamide ratio) containing 5% glycerol in 0.5× tris-borate EDTA buffer. The gels were visualized using a Typhoon FLA9500 Scanner.

Dual luciferase reporter assays
For detection and quantification of pinhead or admp promoter activity in zebrafish embryos, the luciferase reporter construct DNA was mixed with Renilla luciferase reporter DNA in a ratio of 10:1. Wild-type and mutant embryos were injected with 100 pg of the DNA mixture at the one-cell stage. To inhibit BMP signal transduction, embryos were treated with or without 5 µM DMH1 at the 1K-cell stage and then lysed with passive lysis buffer at the shield stage for detecting luciferase activities. Each luciferase reporter assay was performed in triplicate, and the data represent the mean ± SD of three independent biological repeats after normalization to Renilla activity.

Statistical analysis
Student’s t tests (two-tailed, unequal variance) were performed to analyze all datasets (Microsoft Excel software). At a minimum, experiments were performed in triplicate. All the group values are expressed as the mean ± SD. Results were considered statistically significant at P < 0.05.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/12/eaau6455/DC1
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