RESEARCH ARTICLE SUMMARY

DEVELOPMENTAL BIOLOGY

Maternal Huluwa dictates the embryonic body axis through β-catenin in vertebrates

Lu Yan*, Jing Chen*, Xuechen Zhu*, Jiawei Sun, Xiaotong Wu, Weimin Shen, Weiying Zhang, Qinghua Tao†, Anming Meng†

INTRODUCTION: The formation of the body axis in vertebrate embryos relies on the formation of the dorsal organizer at the onset of gastrulation, which is called the Spemann-Mangold organizer in frogs and the embryonic shield in zebrafish. Previous studies have indicated that nuclear β-catenin signaling is essential for induction of the dorsal organizer.

RATIONALE: Maternal Wnt ligand/receptor signaling has been proposed for stabilization and nuclear transportation of cytoplasmic β-catenin during early blastulation for the dorsal organizer formation. It is unknown whether β-catenin signaling is activated for organizer formation by Wnt-independent mechanisms.

RESULTS: In a spontaneous maternal-effect mutant line of zebrafish, none of the maternal mutant embryos formed the dorsal organizer at the shield stage, nor did they have a head or other dorsoanterior tissues at later stages of development. Through positional cloning and candidate gene testing, we found the mutant gene to be a previously uncharacterized locus that we designate *huluwa* (*hwa*). The defects of M*hwa* mutants could be fully rescued by overexpression of wild-type *hwa* mRNA. Using antisense oligodeoxynucleotides or morpholino, we found that maternal depletion of *Xenopus hwa* transcript in oocytes also causes loss of the body axis and dorsal tissues in the derived embryos. Zebrafish Hwa protein consists of 294 amino acids comprising a 23–amino acid extracellular domain, a 23–amino acid transmembrane domain, and a 248–amino acid intracellular domain. Immunostaining revealed that Hwa protein is located on the plasma membrane of blastomeres only in a region during zebrafish early blastulation in which β-catenin is translocated into nuclei. By performing rescue experiments in *hwa* mutant embryos using different *hwa* mutant mRNAs, we found that the transmembrane domain and the intracellular ^{164}VPPNSP^{169} and ^{184}SLRRSST^{190} motifs are essential for Hwa activity. Overexpression of β-*catenin* efficiently rescued the defects in zebrafish M*hwa* mutants. Ectopic expression of *hwa* mRNA efficiently induced a secondary axis in zebrafish and *Xenopus* embryos, which absolutely required β-catenin. Although mammalian homologs of zebrafish Hwa have not yet been identified, we found that transfection of zebrafish Hwa in human HEK293T cells enhances β-catenin signaling. Zebrafish Hwa could directly bind to human Axin protein, and transfection of zebrafish *hwa* into mammalian cells promoted Axin degradation with participation of tankyrases. Consistent with in vitro data, levels of Axin1 and Axin2 proteins in M*hwa* mutant embryos were up-regulated, and overexpression of the dominant negative form *Axin1ΔRGS* or *Axin1ΔDIX* of Axin1 in M*hwa* mutant embryos could rescue the dorsal organizer and the body axis. Therefore, we conclude that maternal Hwa is absolutely required for the formation of the dorsal organizer and the body axis by protecting β-catenin from Axin-mediated degradation in vertebrate embryos.

Overexpression of the Wnt antagonist gene *DKK1*, the dominant negative Wnt8a form *dnwnt8*, or the dominant negative LRP5 form *LRP5ΔC* in zebrafish embryos neither disrupted the dorsal organizer nor blocked the organizer- and body axis–inducing activity of ectopic *hwa*; treatment with the Wnt inhibitor Wnt-C59 also had no such effects. *Xenopus* embryos derived from oocytes depleted of *lrp6* or *wnt11b* transcript could form the full body axis after being injected with *hwa* mRNA at the four-cell stage.

CONCLUSION: Maternal Hwa protein in vertebrate embryos is essential for the dorsal organizer and body axis formation, which activates β-catenin signaling during early blastulation in a Wnt ligand/receptor-independent fashion. ■

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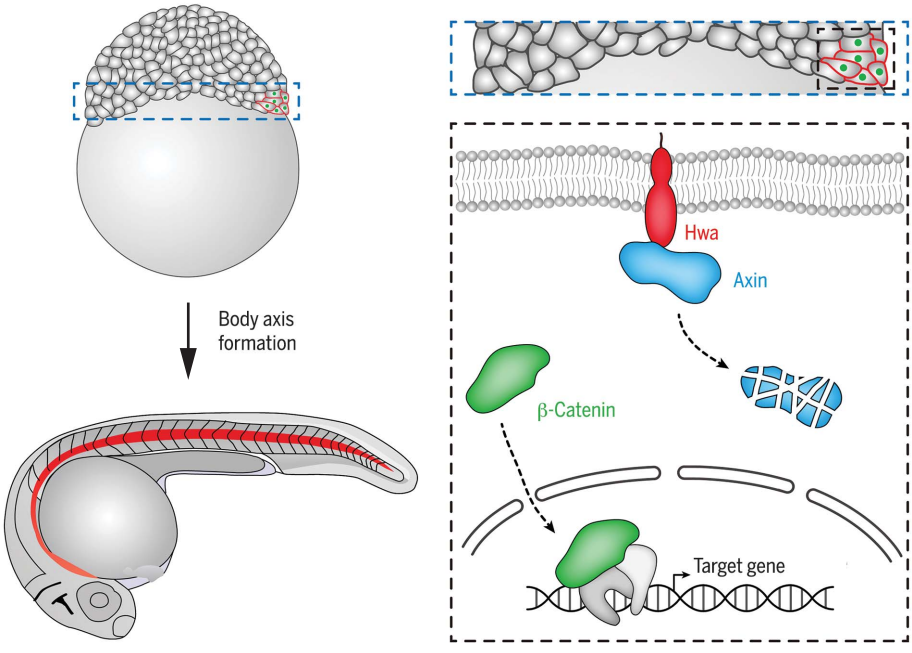

Huluwa (Hwa) is essential for the organizer and body axis formation. Hwa protein is located on the plasma membrane of the prospective dorsal blastomeres in the zebrafish blastula. Hwa binds to and promotes the degradation of Axin in a way independent of Wnt ligand/receptor signaling, resulting in stabilization and nuclear translocation of β-catenin for activating organizer-specific target gene expression. The notochord (red) is an organizer-derived tissue.

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Cite this article as L. Yan *et al.*, *Science* **362**, eaat1045 (2018). DOI: 10.1126/science.aat1045

RESEARCH ARTICLE

DEVELOPMENTAL BIOLOGY

Maternal Huluwa dictates the embryonic body axis through β-catenin in vertebrates

Lu Yan[1]*, Jing Chen[1]*, Xuechen Zhu[2]*, Jiawei Sun[1], Xiaotong Wu[1], Weimin Shen[1], Weiying Zhang[1], Qinghua Tao[2]†, Anming Meng[1]†

The vertebrate body is formed by cell movements and shape change during embryogenesis. It remains undetermined which maternal signals govern the formation of the dorsal organizer and the body axis. We found that maternal depletion of *huluwa*, a previously unnamed gene, causes loss of the dorsal organizer, the head, and the body axis in zebrafish and *Xenopus* embryos. Huluwa protein is found on the plasma membrane of blastomeres in the future dorsal region in early zebrafish blastulas. Huluwa has strong dorsalizing and secondary axis–inducing activities, which require β-catenin but can function independent of Wnt ligand/receptor signaling. Mechanistically, Huluwa binds to and promotes the tankyrase-mediated degradation of Axin. Therefore, maternal Huluwa is an essential determinant of the dorsal organizer and body axis in vertebrate embryos.

The form of the vertebrate embryo changes markedly through extensive morphogenetic movements during development. Before fertilization, an animal-vegetal axis forms within the oocyte, with the maternal nucleus closer to the animal end and (in some species) most of the yolk in the vegetal hemisphere. After fertilization in *Xenopus* and zebrafish, maternal dorsal determinants in the vegetal pole are transported to the future dorsal side through microtubule bundles, to subsequently stabilize β-catenin and allow its nuclear translocation. β-Catenin is the key effector of canonical Wnt signals in the future dorsal blastomeres (*1*) and it induces the formation of the organizer, which acts as a signaling center for correct embryonic patterning and gastrulation cell movements (*2*–*7*). The dorsal organizer is fully formed at the onset of gastrulation, which is called the Spemann-Mangold organizer in frogs and the embryonic shield in zebrafish. Subsequently, two major body axes form: the rostral-caudal (or anteroposterior) axis and the dorsoventral axis.

Loss-of-function studies in frogs, fish, and mice demonstrate that β-catenin plays an essential role in the formation of the dorsal organizer and the anteroposterior axis (*8*–*13*). In the absence of Wnt signaling, cytoplasmic β-catenin forms a complex with the β-catenin destruction complex components Axin, Gsk3β, APC, and CK1 and is subjected to degradation (*14*). Upon binding of Wnt ligand to the Wnt receptors Frizzled and Lrp5/6, Dishevelled proteins participate in a signal transduction pathway to release cytoplasmic β-catenin, allowing its nuclear translocation and activation of target gene transcription. However, accumulating evidence suggests that β-catenin can also be released in a Wnt ligand/receptor-independent fashion. For instance, epidermal growth factor, hepatocyte growth factor, insulin-like growth factor, and platelet-derived growth factor have been found to activate β-catenin via the kinases Akt, Erk, or C-Abl, resulting in dissociation of β-catenin from the E-cadherin adherens complex, inactivation of Gsk3β, or displacement of Axin and Gsk3β (*15*–*20*).

It is widely believed that the role of β-catenin during embryonic development is dependent on Wnt ligand/receptor signaling (*15*–*20*). One supporting observation is that a maternal Dishevelled protein appears to be actively transported to the future dorsal side in early *Xenopus* embryos (*21*); however, Dishevelled proteins can have other functions in addition to their implication in Wnt/β-catenin signaling (*22*). In the zebrafish *wnt8* zygotic mutant *Df(LG14)wnt8*^{w8}, the embryonic shield is expanded at the shield stage and

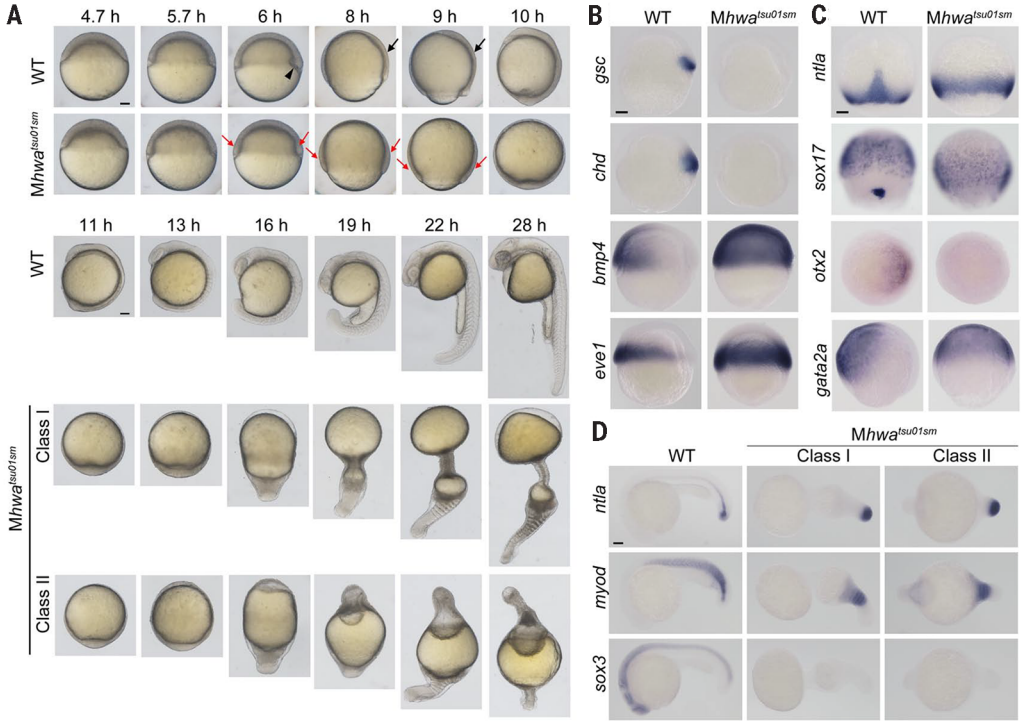

Fig. 1. Mhwa^{tsu01sm} mutant phenotype. (**A**) Morphogenesis of live wild-type (WT) and mutant embryos. The black arrowhead and arrows indicate the dorsal organizer and the thickening body axis, respectively; the red arrows in mutants indicate the thicker blastodermal marginal zone. See also movie S1. (**B** and **C**) Germ layer marker expression detected by WISH during gastrulation. Orientation: *ntla* and *sox17*, dorsal views with animal pole to the top at 75% epiboly stage; others, lateral views (*gsc*, *chd*, *bmp4*, *eve1*, and *gata2a*) or animal-pole view (*otx2*) with dorsal to the right. (**D**) Marker expression at 24 hpf. Lateral views with anterior to the left. Scale bars, 100 μm.

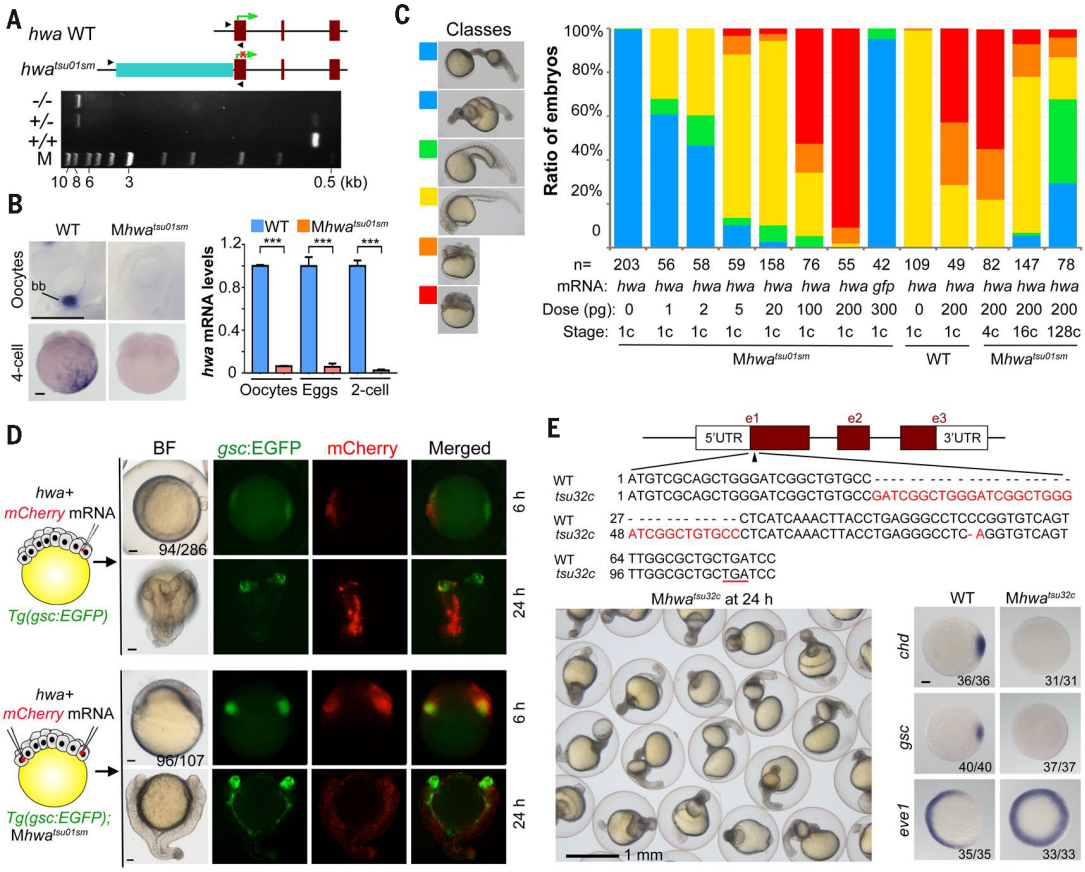

Fig. 2. Identification and verification of *tsu01sm* mutant gene. (**A**) Top: Illustration of the zebrafish *hwa* wild-type and *tsu01sm* mutant alleles. Arrowheads indicate the positions of PCR primers. The green arrow indicates the translation start site. Bottom: Electrophoretic result of PCR products in wild-type, heterozygous, and homozygous mutant embryos. M, molecular weight markers. (**B**) *hwa* mRNA detection by WISH (left) and by qRT-PCR (right). bb, Balbiani body in stage I oocyte. For qRT-PCR, oocytes are a pool of stage I–III oocytes and eggs are squeezed from females. The relative *hwa* mRNA levels are averages (±SEM) from three independent experiments, normalized to *eif4g2a* levels. ***$P < 0.001$. Scale bars, 100 μm. (**C**) M*hwa*^{tsu01sm} mutant embryos form the body axis or are dorsalized by *hwa* overexpression. Left: Morphology of classified embryos at 24 hpf. Right: Ratios of embryos in different classes. (**D**) Induction of secondary body axis by ectopic *hwa* mRNA overexpression. One blastomere of 32-cell stage *Tg(gsc:EGFP)* transgenic embryos or two opposite blastomeres of 32-cell stage *Tg(gsc:EGFP);*M*hwa*^{tsu01sm} mutant embryos were injected with 50 pg of *hwa* and 50 pg of *mCherry* mRNAs, as illustrated at left. Embryos were observed dorsally (top) or laterally (bottom) at 6 hpf under a fluorescence dissection microscope, and those with two dorsal organizers (EGFP-positive) were observed again at 24 hpf. The ratio of embryos with double organizers is indicated. Scale bars, 100 μm. (**E**) Generation of a *hwa* mutant allele by Cas9 knockout. Top: Illustration of the mutant allele with an underlined premature stop codon. Bottom left: A group of M*hwa*^{tsu32c} mutant embryos at 24 hpf. Bottom right: Alteration of the dorsal markers *gsc* and *chd* and the ventral marker *eve1* at shield stage; scale bar, 100 μm.

posterior/ventral tissues are lost at later stages, suggesting a role for zygotic Wnt/β-catenin signaling in restricting the organizer size and dorsal development (*23*, *24*). The role of *Xwnt8* in constraining dorsal cell fate during late blastulation has also been reported in *Xenopus* (*25*). Depletion of zygotic *Wnt3A* or *Lrp5* in mouse embryos results in truncation of the trunk, supporting an important role of Wnt signaling in the development of posterior tissues (*26*–*28*). In *Xenopus*, the use of antisense oligodeoxynucleotides to deplete *wnt11b* or *lrp6* mRNA in oocytes leads to delayed gastrulation and the loss of axial structures (*29*, *30*), which could be ascribed to decreased cytoplasmic β-catenin levels during oocyte maturation as opposed to after fertilization. It is unclear whether β-catenin is stabilized for the organizer and body axis formation by maternal Wnt ligand/receptor signaling from fertilization to early blastulation stages.

Here, we identified a maternal-effect mutant line, *tsu01sm*, that we have named *huluwa* (*hwa*). Unlike previously known β-catenin–related, maternal-effect mutants showing variable degrees of ventralization in zebrafish (*10*, *31*–*37*), all *huluwa* maternal mutant embryos lack the dorsal organizer and fail to form the head and other dorsoanterior tissues. We demonstrate that *huluwa* encodes a transmembrane protein and acts as an essential determinant of the vertebrate body axis formation by activating β-catenin in a Wnt ligand/receptor-independent fashion.

M*hwa* mutant embryos lack a body axis

The maternal-effect *tsu01sm* mutants were found during normal breeding of Tübingen fish and were then used to establish mapping families and preservation families. As described below, some mutant embryos look like calabashes (bottle gourds). We then named the mutant line *huluwa*, the Chinese equivalent of calabash boy. (In the Chinese animation TV series *Calabash Brothers*, seven calabash brothers each have a special power to defeat monsters.) *hwa*^{tsu01sm} zygotic mutants (*Zhwa*$^{-/-}$) from *hwa*^{tsu01sm} heterozygous fish crosses undergo normal development and grow to adulthood. When *Zhwa* mutant adult females mate with wild-type or heterozygous male fish, all resultant embryos (M*hwa* or M*Zhwa*) develop normally during cleavage and blastula stages but lack the embryonic shield at the shield stage (Fig. 1A). The mutant phenotype is stable among batches and generations. Through morphological assessment at 24 hours post-fertilization (hpf), these embryos can be categorized into two classes: class I, calabash-like shape with the yolk splitting into two parts and a tail at the vegetal pole; class II, onion-like shape with one yolk protrusion at the original animal pole and a tail-like protrusion at the vegetal pole (Fig. 1A and movie S1). Both classes of embryos lack a thick body axis on the yolk ball.

As revealed by whole-mount in situ hybridization (WISH), the dorsal organizer markers *gsc*

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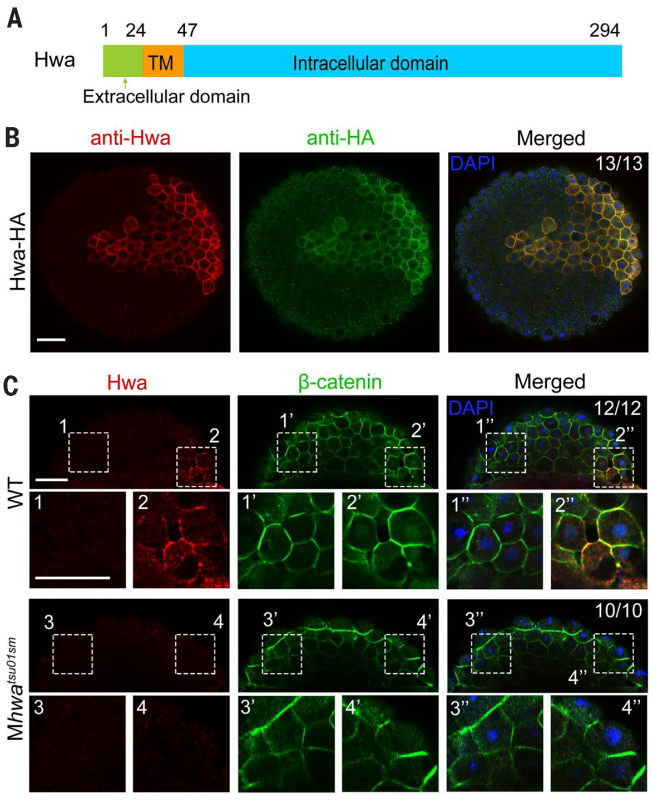

Fig. 3. Hwa is localized on the plasma membrane of dorsal blastomeres. (**A**) Illustration of zebrafish Hwa protein structure. Positions of residues at the start of the domain are indicated except the last residue position. (**B**) Detection of exogenous Hwa-HA by immunostaining using anti-Hwa antibody: 50 pg of *hwa-HA* mRNA was injected into one blastomere of eight-cell stage M*hwa*^{tsu01sm} mutant embryos, and the embryos were collected at 4 hpf for immunostaining with anti-HA and anti-Hwa antibodies. Nuclei were also stained with 4′,6-diamidino-2-phenylindole (DAPI). Embryos were imaged by confocal microscopy in animal pole view. Note that exogenous Hwa-HA is enriched on the plasma membrane. Scale bar, 50 μm. (**C**) Location of endogenous Hwa protein. Zebrafish wild-type and M*hwa*^{tsu01sm} mutant embryos were collected at the 512-cell stage for coimmunostaining of endogenous Hwa and β-catenin. Embryos were imaged by confocal microscopy and are shown in lateral view with the animal pole to the top. In all 12 wild-type embryos with β-catenin in nuclei of the dorsal blastomeres, endogenous Hwa protein is enriched on the plasma membrane in the same region (top panel). In M*hwa* mutants (bottom panel), Hwa signal is absent on the plasma membrane and no blastomeres have nuclear β-catenin. For each embryo, two numbered areas are enlarged for better view. The ratio of embryos with the representative pattern is indicated. Scale bars, 50 μm.

and *chd* are eliminated in M*hwa*^{tsu01sm} mutants at the shield stage, whereas the ventral markers *bmp4* and *eve1* are expanded (Fig. 1B), indicative of embryonic ventralization. The mesodermal marker *ntla* and the endodermal marker *sox17* are expressed in mutant embryos at the shield stage, but expression of *ntla* and *myod* (a myogenic marker) is detected only in the tail-like region at 24 hpf (Fig. 1, C and D). Mutant embryos do not express the anterior neuroectoderm marker *otx2* at the shield stage, nor do they express the central nervous system marker *sox3* at 24 hpf (Fig. 1, C and D). Instead, the epidermis marker *gata2a* is expanded throughout the ectoderm in mutants at the shield stage. These data suggest that in the absence of maternal *hwa*, the mesendodermal fate is specified and the ectoderm completely transforms into the epidermis fate.

During gastrulation in wild-type embryos, marginal cells committed to the mesendodermal fate internalize, and the internalized cells in the lateral and ventral margin migrate dorsoanteriorly to form a thick body axis along the dorsal midline. A blastodermal margin zone close to the margin in M*hwa* mutants (Fig. 1A, red arrows) is thicker than the other blastodermal region during gastrulation, which suggests that blastodermal marginal cells have internalized. As shown in movie S1, blastodermal cells in M*hwa* mutants internalize and migrate vegetally without dorsally converging during gastrulation, and some internalized cells move toward the animal pole until halting and aggregating at variable positions on the yolk to form a furrow during the segmentation period. If the furrow is formed at an equatorial position, the calabash shape (class I) is created; if the furrow is closer to the animal pole, the onion shape (class II) appears. By observing dynamic migration of enhanced green fluorescent protein (EGFP)–labeled mesodermal cells in *Tg(efnb2b:EGFP)*;M*hwa*$^{-/-}$ embryos and endodermal cells in *Tg(sox17:EGFP)*:M*hwa*$^{-/-}$ embryos (figs. S1 and S2 and movies S2 and S3), we confirmed that mesendodermal cells in mutants undergo internalization but fail to converge dorsally. Furthermore, transplantation experiments revealed that transplanted M*hwa* mutant cells and wild-type cells converge normally in wild-type host embryos during gastrulation but are unable to converge dorsally in M*hwa* mutant embryos (fig. S3 and movie S4). Taken together, these data suggest that the failure of gastrulation cell convergence in M*hwa* mutants accounts for the failure to form the dorsal organizer, head, and trunk. It has been proposed that during zebrafish gastrulation, high Bmp levels inhibit the convergence and extension of internalized mesendodermal precursors, but their vegetal migration is promoted (*38*). In M*hwa* mutants, up-regulated expression of *bmp4* across the dorsoventral axis with loss of the gradient (Fig. 1B) may account for the failure of cell convergence.

The *hwa* locus is responsible for the mutant phenotype

By combining positional and candidate cloning, we found that in M*hwa*^{tsu01sm}, the uncharacterized locus *si:dkey-121h17.7* on chromosome 21 carries a 7313–base pair (bp) insertion element at position +118 upstream of the first exon (Fig. 2A). *hwa* transcripts were enriched in the Balbiani body in wild-type stage I oocytes and in the vegetal pole region upon fertilization (Fig. 2B and fig. S4A). During the early cleavage period, *hwa* transcripts in the vegetal pole region were transported toward the animal pole from one side of the yolk (indicated by arrows in fig. S4A), but transcripts appeared to be distributed evenly in blastomeres. *hwa* mRNA levels decreased at 2 hpf (fig. S4, A and B), indicating maternal expression. However, *hwa* transcripts were undetected in M*hwa* mutant oocytes or early mutant embryos by WISH, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays revealed low levels of *hwa* mRNA in mutants, which suggests that *hwa*^{tsu01sm} is a null allele.

Next, we performed rescue experiments. Injection of 5 to 20 pg of *hwa* mRNA into M*hwa* mutant embryos at the one-cell stage efficiently restored the body axis at 24 hpf, and higher doses led to embryonic dorsalization of both mutant and wild-type embryos (Fig. 2C). In addition, *hwa* overexpression also rescued the expression of the dorsal marker *gsc* and the ventral marker *eve1* (fig. S5). Some of the rescued mutant embryos could survive to adulthood. Interestingly, injection of *hwa* mRNA into a single blastomere of mutant embryos at four-cell, 16-cell, or 128-cell stages also produced a rescuing effect (Fig. 2C and fig. S5B). Injection of *hwa* mRNA into one blastomere of the 16- to 32-cell stage wild-type embryos induced a secondary organizer/body axis, and injection of *hwa* mRNA into two distant blastomeres of M*hwa*^{tsu01sm} mutant embryos at 16- to 32-cell stages efficiently induced two organizers/body axes (Fig. 2D). These data indicate that Hwa has potent organizer/body axis–inducing activity and that the dorsalizing activity of maternal *hwa* may not depend on asymmetrical transportation of dorsal determinants after fertilization.

To further confirm the function of *hwa*, we used Cas9 technology to generate the *hwa* mutant line *hwa*^{tsu32c}, in which exon 1 of the *hwa* locus harbors a 33-bp insertion, a 1-bp deletion, and a 1-bp substitution allowing a premature stop codon to appear at the 36th codon. M*hwa*^{tsu32c} mutant embryos at 24 hpf exhibited a calabash- or onion-like shape without the major body axis in addition

to alterations of the dorsal and ventral marker expression pattern (Fig. 2E). The phenotype of M*hwa*^{tsu32c} is identical to that of M*hwa*^{tsu01sm} mutants.

The predicted Hwa protein contains a 23–amino acid extracellular domain, a 23–amino acid transmembrane domain, and a 248–amino acid intracellular domain (Fig. 3A). However, no regions of Hwa protein share high homology with any known protein motifs in the prediction databases. An antibody recognizing an extracellular region of zebrafish Hwa could detect overexpressed hemagglutinin (HA)–tagged Hwa by immunofluorescence in zebrafish embryos (Fig. 3B), showing an enrichment on the plasma membrane. Endogenous Hwa was detected around the 512-cell stage using this antibody. We found that endogenous Hwa is enriched on the plasma membrane of blastomeres only in a small region in which β-catenin is translocated into nuclei and in which the dorsal organizer will form (Fig. 3C). In contrast, no M*hwa*^{tsu01sm} embryos (n = 10) displayed Hwa protein on the plasma membrane in any region (Fig. 3C). These results suggest that, like nuclear β-catenin, Hwa protein is present in future dorsal blastomeres.

By performing rescue experiments in *hwa* mutant embryos using different *hwa* mutant mRNAs, we found that the transmembrane domain and an 84–amino acid intracellular domain (positions 137 to 220) of H*wa* protein are essential for function (fig. S6, A and B). Fine mapping identified two essential motifs, ^{164}VPPNSP^{169} (Val-Pro-Pro-Asn-Ser-Pro) and ^{184}SLRRSST^{190} (Ser-Leu-Arg-Arg-Ser-Ser-Thr) (fig. S6C).

Potential homologs of zebrafish Hwa protein have been identified in sea cucumber (urochordate), *Xenopus laevis* (amphibian), and lizard (reptile), which share a pairwise identity of 17.3 to 34% in amino acid sequence (fig. S7). The VPPNSP motif of zebrafish Hwa is conserved in frog and lizard Hwa, whereas its SLRRSST motif is only conserved in bony fish Hwa (fig. S6C). We have not found Hwa homologs in mammals, most likely because of the extremely low homology with Hwa proteins in the lower organisms.

Next, we examined the developmental role of the *Xenopus hwa* gene. We found that *Xenopus hwa* transcripts are present in granules at the vegetal cortex of oocytes and move dorsally along with cortical rotation after fertilization (Fig. 4A) so that its transcripts are highly enriched in two dorsal blastomeres at the four-cell stage (Fig. 4B). The *hwa* mRNA level in one-cell stage embryos (stage 1) was much lower than that of oocytes, suggesting a quick turnover after fertilization (Fig. 4C). Injection of *hwa* mRNA into one ventral blastomere of the four-cell stage *Xenopus* embryos induced a secondary axis (Fig. 4D) and caused an extra expression domain of the organizer markers *nodal3.1*, *chordin*, and *goosecoid* with inhibition of the ventral markers *wnt8a* and *ventx1.2* (Fig. 4E). Use of antisense oligodeoxynucleotides or morpholino to deplete *hwa* in *Xenopus* oocytes resulted in embryos lacking the body axis and dorsal tissues with reduced dorsal marker expression but enhanced ventral marker expression (Fig. 4F and figs. S8 and S9). These defects could be efficiently rescued by co-injection with a modified *hwa* mRNA that may not be targeted by either antisense oligodeoxynucleotides or morpholino (figs. S8 and S9). These results indicate an evolutionarily conserved function of maternal Hwa. We note that injection of *hwa* morpholino into fertilized eggs had no effect on embryonic development, implying that maternal Hwa protein may play a key role.

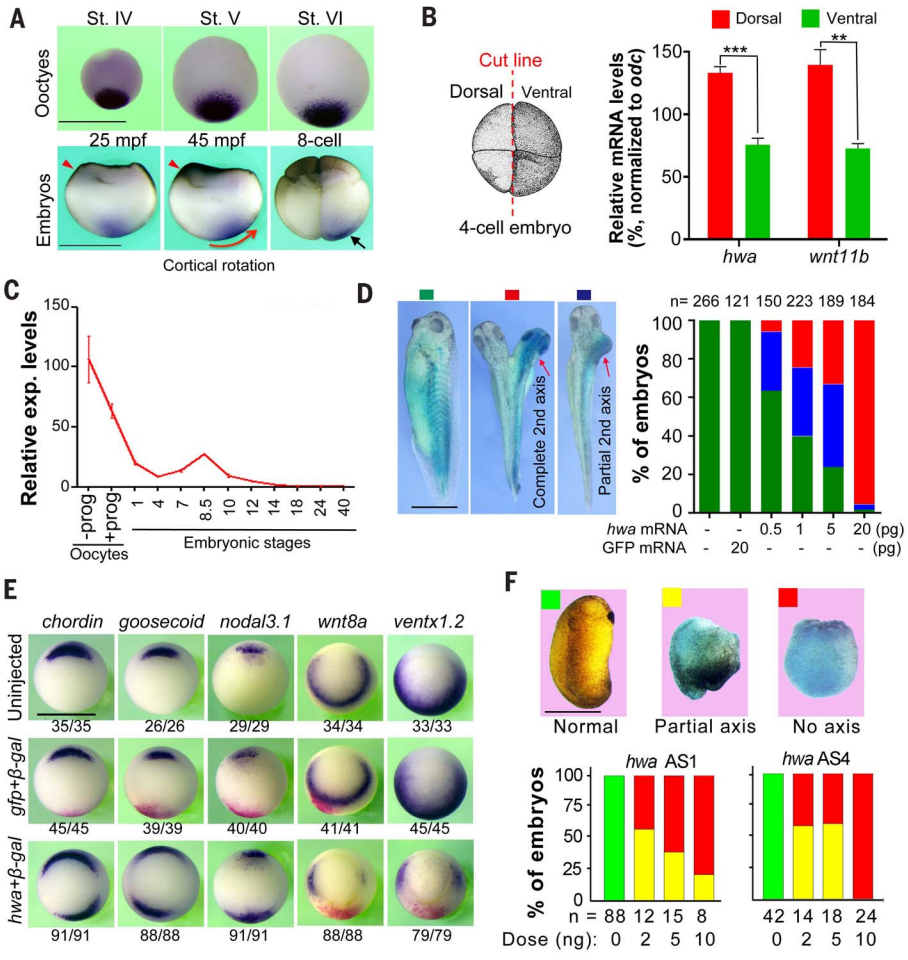

Fig. 4. Roles of *Xenopus hwa* during embryonic body axis formation. (**A**) Expression of *hwa* in oocytes and embryos by WISH detection. Red arrowheads indicate the sperm entry site. St, stage; mpf, minutes post-fertilization. The red arrow shows the cortical rotation direction; the black arrow indicates asymmetrically located *hwa* transcripts. (**B**) Quantification of *hwa* transcripts in two dorsal and two ventral blastomeres at four-cell stage by qRT-PCR. Left: Illustration of cutting of embryos. Right: qRT-PCR result showing average levels from three technical repeats (±SEM) normalized to *odc* level. The dorsal marker *wnt11b* serves as an indicator. $^{**}P < 0.01$, $^{***}P < 0.001$. (**C**) Quantification of *hwa* mRNA levels by qRT-PCR at the indicated stages; prog, progesterone. *hwa* expression level is averaged from technical repeats (±SEM) and normalized to *odc* level at the indicated stages. (**D**) Induction of secondary axis by *hwa* in *Xenopus* embryos. One ventral blastomere of four-cell stage embryos was injected with different doses of *hwa-myc* or 20 pg of *GFP* mRNA plus 200 pg of β-*gal* mRNA and observed at stage 32. Left: Classes of embryos. Right: Ratios of classified embryos. (**E**) Organizer and ventral marker expression patterns detected by WISH at stage 10 (vegetal view). One ventral blastomere of four-cell stage embryos was injected with 20 pg of *hwa* or 100 pg of *GFP* mRNA plus 200 pg of β-*gal* mRNA. The ratio of embryos with the representative pattern is indicated. (**F**) Maternal knockdown effect of *hwa*. Oocytes were injected with different doses of antisense oligodeoxynucleotides (AS1 or AS4) and the resulting embryos were observed and categorized at stage 24. All scale bars, 1 mm.

Hwa function is dependent on β-catenin but independent of Wnt ligand/receptor signaling

Given that β-catenin is essential for body axis formation and its coexistence with Hwa in the future dorsal blastomeres in zebrafish early blastulas (Fig. 3), we investigated the functional relationship between Hwa and β-catenin. Immunofluorescence results showed that β-catenin is present on the plasma membrane but not in nuclei of any blastomeres in M*hwa*^{tsu01sm} mutants at

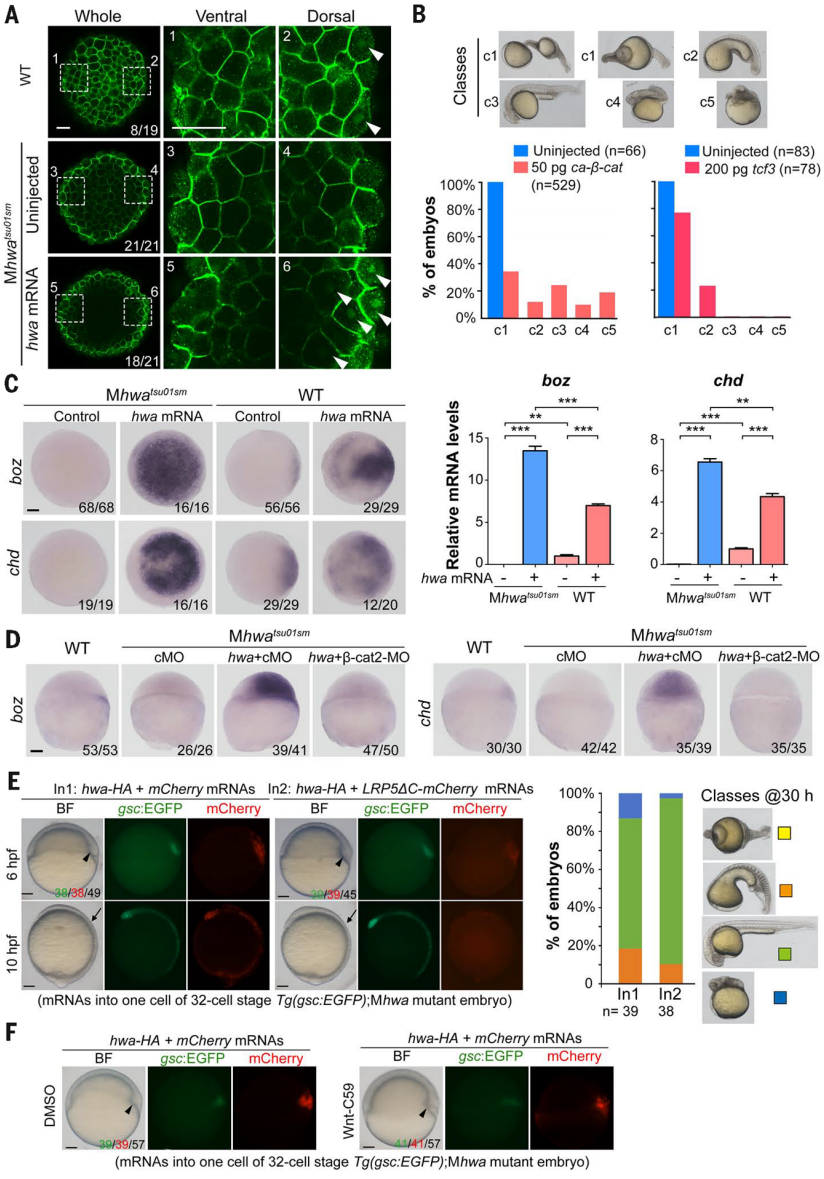

Fig. 5. *hwa* can function through β-catenin in a Wnt ligand/receptor-independent manner in zebrafish embryos. (**A**) Immunostaining of β-catenin. After injection of 50 pg of *hwa* mRNA at one-cell stage, embryos were immunostained for β-catenin at 512-cell stage and observed by confocal microscopy. Whole embryos are viewed from the animal pole; two opposite marginal areas are viewed at a higher magnification. The ratio of embryos with the representative pattern is indicated. (**B**) Recovery of the body axis in M*hwa*^{tsu01sm} embryos by *ca-β-catenin* or *tcf3* overexpression. Embryos were injected at one-cell stage and observed at 24 hpf. The embryos are categorized into five classes (top panel); ratios are shown below. (**C**) *hwa* overexpression induces the expression of the β-catenin target genes *boz* and *chd*. Wild-type or M*hwa*^{tsu01sm} embryos were injected with 50 pg of *hwa*-HA mRNA at the one-cell stage and collected at 4 hpf for WISH or qRT-PCR analysis. Left: WISH results (animal-pole views). Right: qRT-PCR results showing averages from three independent experiments (±SEM). $^{**}P < 0.01$, $^{***}P < 0.001$. (**D**) *hwa* overexpression fails to induce *boz* and *chd* in β-*cat2* morphants. Wild-type or M*hwa*^{tsu01sm} embryos were injected with 50 pg of *hwa-HA* mRNA together with 20 ng of control MO (cMO) or β-cat2-MO at the one-cell stage and subjected to WISH at 4 hpf. Embryos are laterally viewed. (**E** and **F**) Inhibition of Wnt ligand/receptor signaling has no effect on *hwa*'s organizer/axis-inducing activity. (E) One blastomere of *Tg(gsc:EGFP)*;M*hwa*^{tsu01sm} embryos at about 32-cell stage was injected with 50 pg of *hwa* plus 200 pg of *mCherry* (In1) or *LRP5ΔC-mCherry* mRNA (In2). Left: Embryos are shown at 6 and 10 hpf. Embryo ratios are expressed as GFP$^+$/mCherry$^+$/total. Right: Bar graph showing ratios of classes of 30-hpf embryos growing from GFP$^+$ 6-hpf embryos. Note that the class I mutant phenotype did not show in any injected embryos and served only for comparison. (F) *Tg(gsc:EGFP)*;M*hwa*^{tsu01sm} embryos injected with *hwa* and *mCherry* mRNAs as in (E) were incubated in DMSO or 20 μM Wnt-C59. The organizer and body axis are indicated by arrowheads and arrows, respectively. Scale bars, 10 μm (A), 100 μm (all others).

the 512-cell stage, and that nuclear β-catenin is recovered by injection of *hwa* mRNA (Fig. 5A). Overexpression of mRNA encoding the constitutively active (ca) β-catenin in M*hwa*^{tsu01sm} mutants could restore the embryonic body axis and even dorsalize the embryos, whereas *tcf3* overexpression had little rescue effect (Fig. 5B). The expression of the β-catenin target genes *boz* and *chd* was eliminated in M*hwa*^{tsu01sm} mutants, which could be recovered by injection of *hwa* mRNA (Fig. 5C). Note that *hwa* overexpression in the mutants induced higher levels of *boz* and *chd* than in wild-type embryos, which suggests that M*hwa* mutants have a larger number of cells that are responsive to activity of ectopic Hwa relative to wild-type embryos. When β-*catenin2* was silenced, however, the expression of *boz* and *chd* could not be induced by *hwa* mRNA overexpression in M*hwa*^{tsu01sm} mutants (Fig. 5D). These data suggest that Hwa functions through β-catenin.

It has been proposed that during early vertebrate embryogenesis, β-catenin stabilization in dorsal blastomeres depends on Wnt ligand/receptor-mediated signaling (*39*–*41*), but compelling genetic evidence is lacking. In zebrafish, *wnt8a* is believed to be an essential maternal Wnt ligand for the formation of the organizer (*42*). However, a recent study showed that M*wnt8a* mutants have no defects in axis formation and that MZ*wnt8a* mutants display an anterodorsalized phenotype similar to zygotic *wnt8a* mutants (*43*). Given the existence of many maternally expressed Wnt ligands including *wnt8a* and *wnt6a* (*42*, *43*), it is possible that several Wnt ligands work cooperatively to activate β-catenin in early embryos. Nonetheless, we decided to investigate the requirement of maternal Wnt ligands/receptors for dorsal organizer and body axis formation as well as *hwa* function in these aspects. We found that in M*hwa*^{tsu01sm} mutant embryos at the one-cell stage and 4 hpf, the mRNA levels of *wnt8a*, β-*catenin2*, *axin1*, *axin2*, *dvl2*, *lrp5*, *lrp6*, *fzd3b*, *fzd5*, *fzd7b*, and *fzd8b*, which are highly expressed in oocytes (*44*), are comparable to those of wild-type embryos (fig. S10); this suggests that Wnt ligand/receptor signaling, even if activated, is not sufficient for organizer and body axis formation in the absence of Hwa. Furthermore, zebrafish embryos injected at the one-cell stage with *dnwnt8a-mCherry* (encoding mCherry fused to a dominant negative form of Wnt8a), *DKK1-EGFP* (encoding human DKK1-EGFP fusion protein), or *LRP5ΔC-mCherry* mRNA (encoding mCherry fused to human LRP5 with a truncation of the intracellular domain) or treated with the Wnt inhibitor Wnt-C59 all exhibit a typical dorsalized phenotype instead of a ventralized phenotype at 24 hpf and show no obvious reduction of the dorsal marker genes *boz* and *chd* (figs. S11 to S14). The *dnwnt8a*-induced dorsalized phenotype was also reported by Lu *et al.* (*42*), but a complete loss of *chd* expression in a *dnwnt8a*-overexpressing embryo was shown at the sphere stage (4 hpf) [figure 2C of (*42*)], which we did not observe in our assays (fig. S11C).

This inconsistency may be due to the different experimental conditions between the studies. In our study, DKK1-EGFP and DnWnt8a-mCherry proteins in the injected embryos become fluorescently visible as early as the two- to eight-cell stages (figs. S11A and S12A) and thus should be capable of antagonizing existing maternally derived Wnt ligands/receptors. Thus, maternal Wnt/receptor signaling may not be required for organizer formation from the period of fertilization to the onset of gastrulation during zebrafish embryogenesis. Conversely, the dorsalizing and axis-inducing activity of ectopic *hwa* mRNA in zebrafish embryos is not prevented by the coexpression of *dnwnt8a-mCherry*, *DKK1-EGFP*, or *LRP5ΔC-mCherry* or by Wnt-C59 treatment (Fig. 5, E and F, and figs. S11 to S14). Therefore, Hwa can function in a Wnt ligand/receptor-independent fashion.

We next assessed the overexpression of *hwa* or *wnt8* during *Xenopus* oocyte maturation. We found that overexpression slows down the post-fertilization degradation of cytosolic β-catenin accumulated in oocytes (Fig. 6A), which in turn suggests that Hwa helps to stabilize cytosolic β-catenin. When β-catenin MO was injected into *Xenopus* one-cell stage embryos followed by *hwa* mRNA injection into one blastomere at the four-cell stage, no body axis formed (Fig. 6B), confirming that β-catenin is required for Hwa function. Ectopic *hwa* or *wnt8* in *Xenopus* embryos efficiently induced a secondary axis, and the induction of *wnt8* but not *hwa* activity was inhibited by coexpression of *dkk1* (Fig. 6C). As demonstrated earlier (*29*, *30*), depletion of *lrp6* or *wnt11b* mRNA in *Xenopus* oocytes by antisense oligodeoxynucleotides leads to a loss of the body axis and reduction of dorsal organizer marker expression with increased ventral marker expression in the resulting embryos (fig. S15). However, when one blastomere at the four-cell stage was injected with *hwa* mRNA, these embryos derived from oocytes depleted of *lrp6* or *wnt11b* mRNA could form the full body axis and restore dorsal organizer gene expression (Fig. 6, D and E, and fig. S15). These data suggest that *Xenopus* Hwa can also function in a Wnt ligand/receptor-independent fashion.

Hwa promotes Axin degradation

Given that Axin is the rate-limiting component of the β-catenin destruction complex because of its low concentration (*45*, *46*), we wanted to know whether Hwa acts on Axin. Transfection of zebrafish *hwa* into human embryonic kidney (HEK) 293T cells stimulated the expression of the Wnt/β-catenin responsive Topflash reporter and increased the amount of β-catenin in the cytosol (fig. S16). Next, we tested the potential interaction between zebrafish Hwa and mouse Axin in HEK293T cells and detected Myc-Axin2 in the Hwa-Flag immunoprecipitate (Fig. 7A), whereas Hwa and Axin1 did not coimmunoprecipitate. However, an association of Hwa-Flag and Axin1-HA was detected in the presence of XAV939 (Fig. 7B), an inhibitor of tankyrases (Tnks) that mediate poly-ADP ribosylation (PARsylation) and subsequent degradation of Axin (*47*). Western blotting results of whole-cell lysates showed a reduction of Axin1-HA and Myc-Axin2 levels in HEK293T cells when zebrafish Hwa was coexpressed (Fig. 7, A and B). To assess whether Hwa directly binds to Axin, we used the His-AXIN1 (N351) protein, which contains the N-terminal 351 amino acids of human AXIN1, and purified the intracellular domain [GST-Hwa(ΔN46)] of zebrafish Hwa after *Escherichia coli* expression. We found that GST-Hwa(ΔN46) can be pulled down by His-AXIN1 (N351) (Fig. 7C),

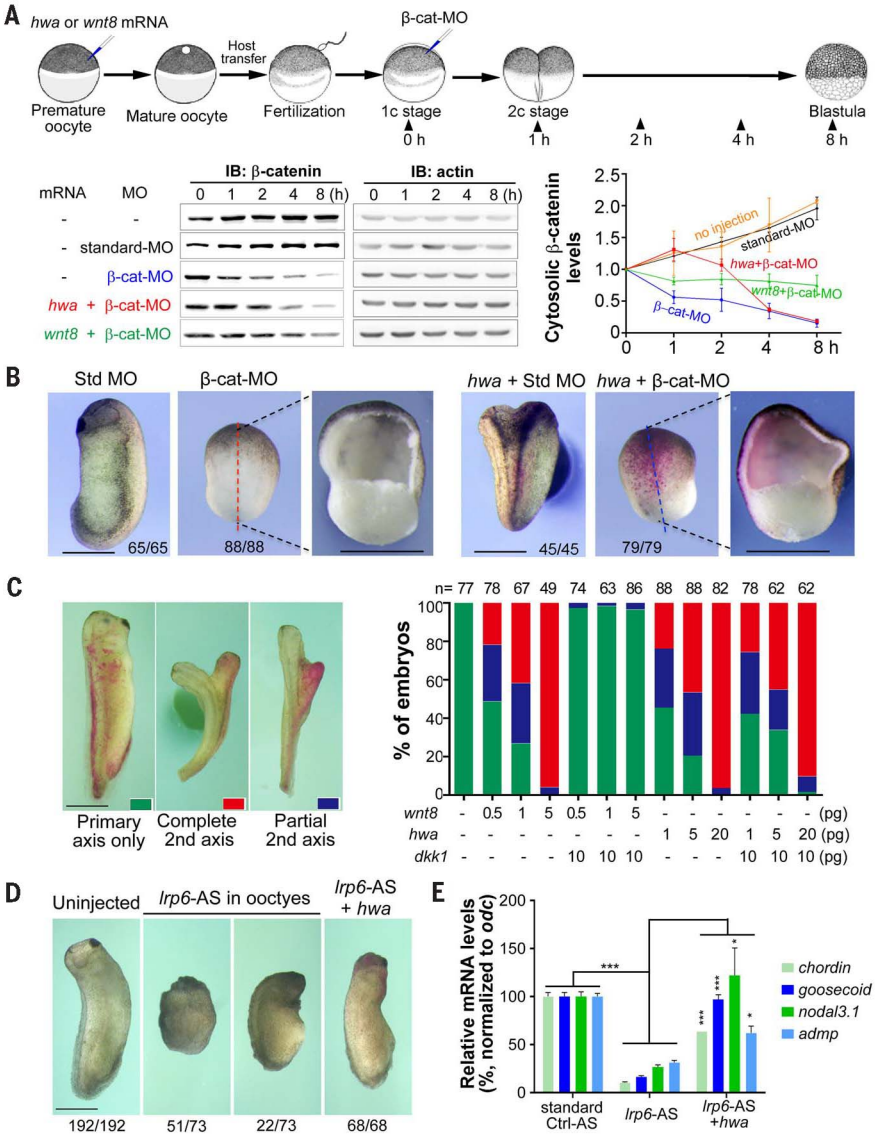

Fig. 6. *hwa* can function through β-catenin in a Wnt ligand/receptor-independent manner in *Xenopus* embryos. (**A**) *hwa* or *wnt8* overexpression delays cytosolic β-catenin degradation. Top: Illustration of experiment design (dosage: *hwa* or *wnt8* mRNA, 200 pg; β-catenin MO or standard MO, 20 ng). Bottom: Immunoblotting results and quantification of cytosolic β-catenin levels. For immunoblotting, membrane fraction in embryo lysate was removed using digitonin buffer. Quantitative data are derived from three experiments. (**B**) *hwa* fails to induce the body axis in embryos depleted of β-catenin. Embryos were first injected with 20 ng of β-cat-MO or standard MO at one-cell stage and then injected with 200 pg of *hwa* and 200 pg of β-*gal* mRNAs into one ventral blastomere at four-cell stage, and were observed at stage 26. (**C**) *dkk1* overexpression inhibits secondary axis induction of *wnt8* but not *hwa*. mRNAs were injected into one ventral blastomere of four-cell stage embryos. Left: Categories of embryos at stage 32. Right: Ratios of embryos in each category. (**D**) *hwa* overexpression induces the body axis of embryos derived from oocytes depleted of *lrp6*. Oocytes were injected with 6 ng of *lrp6* antisense oligodeoxynucleotide (*lrp6*-AS) and one blastomere of the resulting embryos at two-cell stage was injected with 20 pg of *hwa* mRNA. Embryos were observed at stage 28 (left panel). (**E**) Embryos from the experiment shown in (D) were also collected at stage 10 for RT-PCR analysis of dorsal markers. qRT-PCR results are based on four technical repeats. **P* < 0.05, ****P* < 0.001. Scale bars, 1 mm.

(N351) protein, which contains the N-terminal 351 amino acids of human AXIN1, and purified the intracellular domain [GST-Hwa(ΔN46)] of zebrafish Hwa after *Escherichia coli* expression. We found that GST-Hwa(ΔN46) can be pulled down by His-AXIN1 (N351) (Fig. 7C),

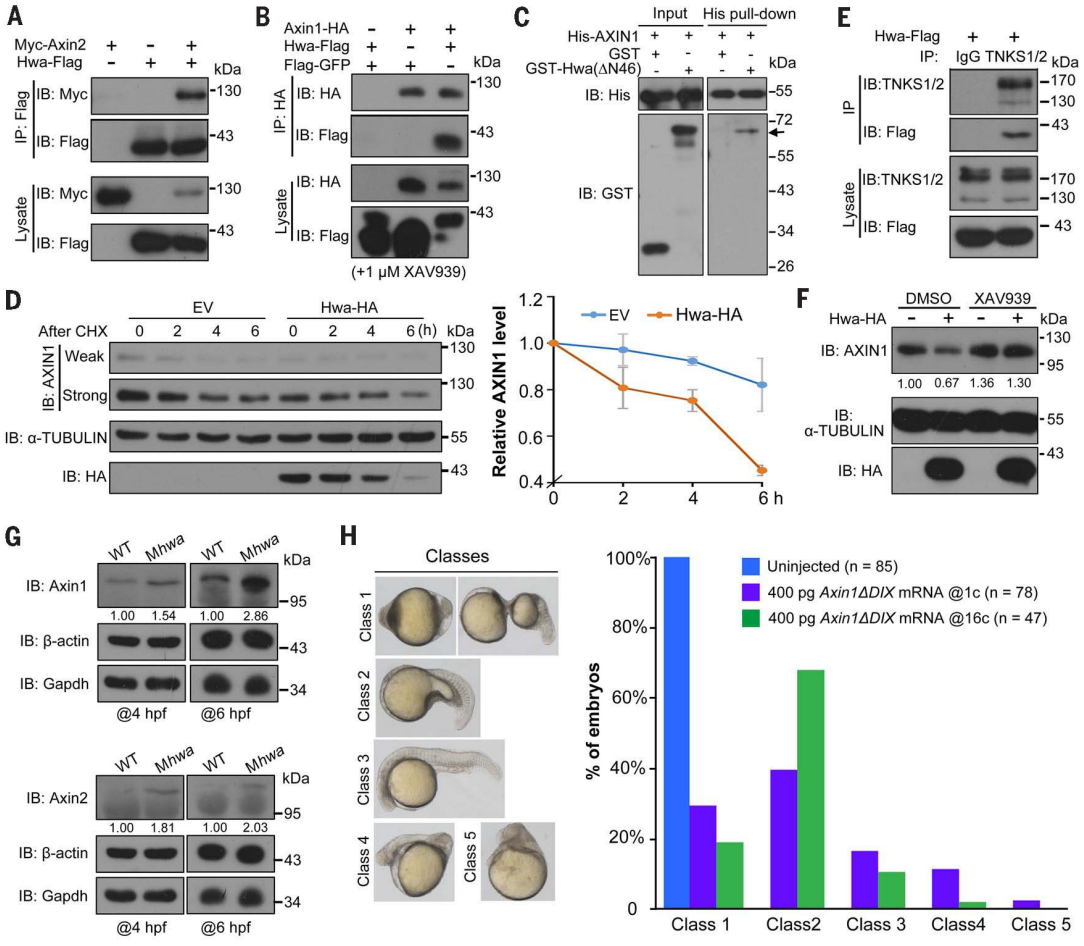

Fig. 7. Hwa binds to and promotes the degradation of Axin. (**A** and **B**) Hwa-Flag physically interacts with Myc-Axin2 (A) and Axin1-HA (B) in HEK293T cells. Zebrafish Hwa and mouse Axin1 and Axin2 were used. XAV939 is a tankyrase inhibitor. Note that Axin1 and Axin2 levels are reduced when zebrafish Hwa is expressed. (**C**) Direct binding of Hwa with AXIN1. His-AXIN1 (N351) protein of human AXIN1 origin was incubated with GST-Hwa(ΔN46) protein of zebrafish origin expressed in *E. coli*. GST-Hwa (indicated by an arrow) is detected by Western blotting in the His-AXIN1 precipitates. (**D**) Hwa-HA promotes degradation of endogenous AXIN1 in HEK293T cells. Left: Cells transfected with empty vector (EV) or Hwa-HA were treated with cycloheximide (CHX; 100 μg/ml) and harvested for immunoblotting at indicated time points. Right: Dynamic change of AXIN1 protein level (normalized to α-TUBULIN), shown as mean ± SD. (**E**) Hwa-Flag binds to endogenous TNKS1/2 in HEK293T cells. (**F**) Hwa-promoted degradation of endogenous AXIN1 in HEK293T cells is prevented by inhibition of tankyrases. Six hours after transfection, cells were treated with DMSO or 1 μM XAV939 for 18 hours. Relative AXIN1 levels (normalized to α-TUBULIN) are indicated. (**G**) Immunoblotting reveals an increase of endogenous Axin1 and Axin2 proteins in M*hwa*^{tsu01sm} mutants at 4 and 6 hpf. Relative levels are indicated. (**H**) *Axin1ΔDIX* overexpression rescues the body axes of M*hwa*^{tsu01sm} mutants. *Axin1ΔDIX* mRNA was injected into one-cell stage mutants or into one blastomere of 16-cell stage mutants. Embryos at 24 hpf were categorized into five classes (left) and the ratio of embryos in each class is shown (right).

which indicates a direct physical interaction of Hwa with Axin.

To confirm the effect of Hwa on Axin protein stability, we examined the degradation of existing AXIN1 protein in HEK293T cells treated with cycloheximide (CHX), a translation inhibitor. Degradation of endogenous AXIN1 protein was accelerated by Hwa-HA expression (Fig. 7D). Hwa-HA in HEK29T cells associated with endogenous TNKS1/2 (Fig. 7E), and XAV939 treatment efficiently blocked Hwa-induced degradation of endogenous AXIN1 (Fig. 7F). These data suggest that Hwa recruits Axin and Tnks to facilitate Tnks-mediated Axin PARsylation and degradation, thereby preventing the formation of the β-catenin destruction complex.

As expected, immunoblotting results showed an increase of endogenous Axin1 and Axin2 levels in zebrafish M*hwa*^{tsu01sm} mutant embryos at 4 and 6 hpf (Fig. 7G). By contrast, overexpression of *hwa* in zebrafish M*hwa*^{tsu01sm} embryos or in wild-type *Xenopus* embryos led to a marked reduction of Axin protein levels (fig. S17). It has been reported that the DIX domain of the Axin protein is required for its activity when forming the β-catenin destruction complex (*48*) and that overexpression of *axinΔDIX* lacking the DIX domain in the zebrafish dorsal axis–defective mutant *ichabod* can recover the dorsal tissues (*49*). Injection of *Axin1ΔDIX* mRNA into one-cell stage M*hwa*^{tsu01sm} mutants or into one blastomere of 16-cell stage M*hwa*^{tsu01sm} mutants rescued or dorsalized the embryonic body in more than 70% of the mutants at 24 hpf (Fig. 7H). Furthermore, overexpression of *Axin1ΔRGS*, which lacks the RGS domain and can act as a dominant negative form (*50*), rescued the embryonic body in approximately 10% of M*hwa*^{tsu01sm} mutants (fig. S18), showing a less efficient rescue effect relative to *Axin1ΔDIX* overexpression. These in vivo data support the notion that Hwa exerts the body axis–inducing effect by promoting Axin degradation.

Discussion

We have identified the transmembrane protein Hwa as a maternal determinant of the dorsal organizer and body axis formation in vertebrates (Fig. 8). Hwa protein is located on the plasma membrane of the future dorsal blastomeres in zebrafish early blastulas (Fig. 3C), in which β-catenin is translocated into the nucleus. Hwa appears to promote Axin degradation and stabilize cytosolic β-catenin during early embryonic development in a Wnt ligand/receptor-independent fashion.

Previous studies in *Xenopus* embryos, which resulted from oocytes depleted of *wnt11b* or *lrp6* mRNA using antisense oligodeoxynucleotides, suggested that Wnt ligand/receptor-mediated signaling is essential for β-catenin activation and dorsal axis formation during early embryogenesis (*29*, *30*). Our results show that the inhibition of Wnt ligand/receptor signaling during early embryogenesis does not impair the organizer and body axis formation (Fig. 5, E and F, and figs. S11 to S14), and instead results in a dorsalized phenotype that is typically ascribed to loss of zygotic Wnt signaling. Our observations are consistent with the finding that the overexpression of dominant

negative *lrp6* in *Xenopus* embryos does not hinder axis formation (*51*).

In zebrafish and *Xenopus* embryos, nuclear β-catenin is not detected until early blastula stages (*5*). We found that injection of *hwa* mRNA into one blastomere of 128-cell stage M*hwa* mutant embryos is able to rescue the body axis, although the rescue efficiency is lower than for earlier injections (Fig. 2C). In mice, although β-catenin signaling is activated through oocyte maturation and embryonic development, inhibition or overactivation of β-catenin does not influence embryonic development prior to implantation (*52*, *53*). These data raise the possibility that β-catenin signaling prior to early blastulation may not be essential for the formation of the dorsal organizer and body axis.

Hwa is a transmembrane protein, and zebrafish Hwa contains an extracellular domain of only 23 residues. Overexpression of a *hwa* mutant construct lacking the extracellular domain rescued M$hwa^{tsu01sm}$ mutants (fig. S6, A and B); therefore, Hwa is unlikely to be a ligand-binding receptor. We have also shown that Hwa may not functionally depend on Wnt receptors during early embryonic development. However, we cannot exclude the possibility that Hwa exerts its dorsalizing activity in concert with other signaling pathways. The mechanisms underlying Hwa function need to be explored further.

Materials and methods

Zebrafish

Tübingen (TU) and India strains were used. Embryos were raised at 28.5°C and staged as described (*54*). Fish maintenance followed the institutional animal care and use committee (IACUC) protocol, with the approval by the Tsinghua University Animal Care and Use Committee. Zygotic homozygous ($Zhwa^{tsu01sm/tsu01sm}$) mutant offspring derived from crosses between heterozygous fish develop and grow normally. M$hwa^{tsu01sm}$ mutant embryos derived from crosses between $Zhwa^{tsu01sm/tsu01sm}$ females and wild-type males are phenotypically identical to MZ$hwa^{tsu01sm}$ mutants generated by $Zhwa^{tsu01sm/tsu01sm}$ female/male crosses. Using a mapping strategy described by Zhou *et al.* (*55*), the mutant gene was initially mapped in a F_2 population established by crossing TU mutation carriers with India fish to the 1.2-Mb region between the markers CH73-29C22 and DKEY-93M18 on chromosome 21. Examination of the expression of 35 candidate genes residing in that region identified the uncharacterized gene *si:dkey-121h17.7* as the mutant gene, which was further confirmed by mRNA rescue experiments. To genotype $hwa^{tsu01sm}$ mutants by PCR, the upper primer (5′-AAAGTGGCTTCTGGCGACAT-3′) and the lower primer (5′-AAGTACTGGTGAGTTCGGCG-3′) were used. hwa^{tsu32c} mutants were generated by CRISPR/Cas9 approach. The guide RNA was designed to target the first exon of *si:dkey-121h17.7* (Ensembl Gene ID: ENSDARG00000094542). The target sequence including the PAM (underlined) is GCCCTCATCAAACTTACCTGAGG. Primers used for genotyping hwa^{tsu32c} were 5′-CACACCGAAGACACCCTGAT-3′ (upper primer) and 5′-CCTCTGGCGGGTGGAATATG-3′ (lower primer). The transgenic lines *Tg(efnb2b:EGFP)*^{tsu2014} (*56*, *57*), *Tg(sox17:EGFP)*^{s870} (*58*), and *Tg(gsc:EGFP)* (*59*) were described previously. For inhibition of Wnt signaling, zebrafish embryos were treated with Wnt-C59 (10 or 20 μM) from the one-cell stage to the desired stage.

The *Xenopus laevis* animals (J strain) were obtained from Nasco Inc. (Wisconsin, USA) and maintained according to the IACUC protocol. Approval was provided by the Tsinghua University Animal Care and Use Committee.

Whole-mount in situ hybridization (WISH) and immunofluorescence

WISH for zebrafish embryos was performed following the standard protocol. WISH for *Xenopus* embryos followed the previous procedures (*60*). The linearized plasmid was used as the template for in vitro synthesizing digoxigenin-UTP–labeled antisense RNA probe. Embryos after WISH were immersed in glycerol and photographed with a Nikon SMZ1500 stereomicroscope.

Whole-mount immunofluorescence was carried out based on the standard protocol using the following primary antibodies: rabbit anti–β-catenin antibody (C2206, Sigma, 1:1000 dilution) or mouse anti–β-catenin antibody (2677S, CST, 1:200 dilution), anti-HA antibody (sc-7392, Santa Cruz, 1:200 dilution). The rabbit anti-Hwa antibody was customized by Fantibody using a synthesized peptide (MSQLGSAVPSSNLPE) derived from the extracellular domain of zebrafish Hwa protein. For nuclear β-catenin staining, embryos were treated with 2 M hydrochloric acid for 30 min after rehydration and equilibrated with 100 mM Tris-HCl (pH 8.5) for 15 min. For Hwa immunostaining, the tyramide signal amplification (TSA)–based immunodetection protocol was used (*61*). After rehydration, embryos were treated with 0.3% H_2O_2 for 30 min. An HRP-conjugated goat anti-rabbit antibody (D110058, BBI, 1:200) was used as the secondary antibody. After six times of washing, using PBST with 0.1% Triton X-100, embryos were incubated in TSA reagent (TSA-Cy5, PerkinElmer, 1:50) for 1 hour at room temperature. Then, embryos were washed and mounted for observation. Immunostained embryos were photographed using a Zeiss 710 META confocal laser scanning microscope.

Morpholino, antisense oligodeoxynucleotides, and mRNA injection

Morpholino methods to knock down zebrafish β-*catenin2* and mismatched control morpholino were as described (*62*). Sequences of morpholinos and oligodeoxynucleotides for *Xenopus* genes are listed in table S1. Antisense morpholino for knocking down *Xenopus* β-*catenin* and oligodeoxynucleotides for *Xenopus wnt11b* and *lrp6* genes were as described (*9*, *29*, *30*). All morpholinos were purchased from Gene Tools LLC. Capped mRNAs were synthesized with the mMessage mMachine kit (Ambion), purified with RNeasy

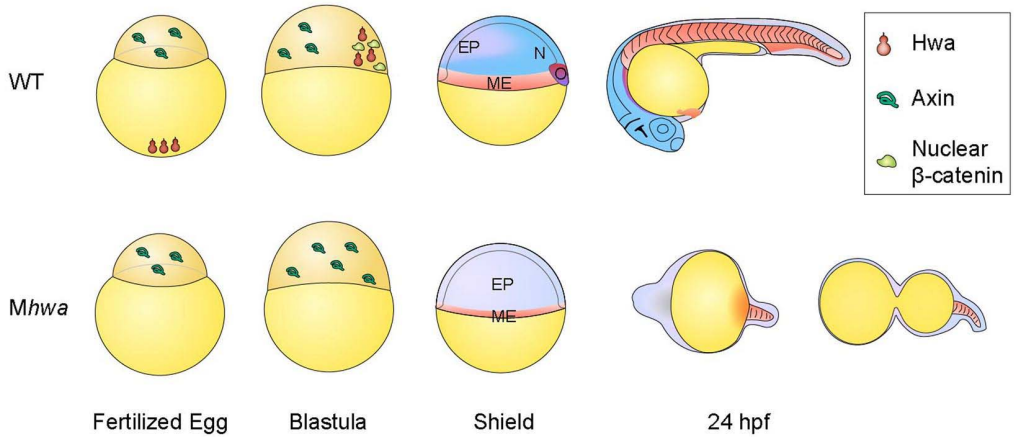

Fig. 8. Working model of Hwa in the zebrafish embryo. In the wild-type fertilized egg, *hwa* mRNA is localized in the vegetal pole region, whereas Axin and β-catenin proteins are located in the cytoplasm. During early blastulation, Hwa protein is located in the dorsal blastomeres, where it locally promotes Axin degradation and results in the stabilization and nuclear transportation of β-catenin. At the onset of gastrulation, the dorsal organizer, also known as the embryonic shield, is formed by the action of β-catenin. The organizer regulates the cell fates along the dorsal-ventral and anterior-posterior axes as well as cell movements during gastrulation, which leads to the formation of the main body. In the absence of *hwa* (as in M*hwa* mutants), cytosolic β-catenin is destroyed by the Axin-containing machinery. As a result, the organizer is not formed and the head, neural tissues, and body axis do not develop, but there are residual mesendodermal tissues in the tail-like region. O, organizer; N, neuroectoderm; EP, epidermis; ME, mesendoderm.

mini kit (Qiagen) according to the manufacturers' instructions, and injected into the yolk or the cytoplasm at the desired stage as elucidated in the main text.

Knockdown of Xenopus maternal genes through host transfer technique

Xenopus embryos depleted of maternal *hwa*, *wnt11b*, or *lrp6* were obtained through host transfer technique as described (*63*, *64*). Briefly, meiosis I (G_2/M)–arrested oocytes were isolated from small lobes of ovary through manual defolliculation using watchmaker's forceps and cultured in oocyte culture medium (OCM, glutamine plus L15 supplemented with 4% BSA and antibiotics). Isolated oocytes injected with or without antisense oligos or mRNA were cultured for 48 hours and then matured with 2 μM progesterone for 12 hours at 18°C. Mature oocytes were labeled with vital dyes (neutral red, Bismarck brown, Nile blue) and then transferred into the body cavity of an egg-laying female anesthetized with tricaine methanesulfonate (MS222, A5040, Sigma). The female recovered from the surgery was reared in 1.2× MMR (Marc's Modified Ringer) solution for 4 to 6 hours for egg collection. Eggs were then fertilized with sperm suspension. Fertilized eggs were dejellied using 2% cysteine in 0.1× MMR (pH 8.0) followed by thorough wash with 0.3× MMR. For microinjection after fertilization, eggs were cultured in 0.3× MMR + 4% Ficoll400. Microinjected embryos were transferred into fresh 0.3× MMR at stage 8 and cultured to the desired stages.

Embryonic cell transplantation

To investigate the cell migration behavior of M*hwa*^{tsu01sm}, lateral margin cells from wild-type or M*hwa*^{tsu01sm} embryos at 6 hpf were transplanted to the corresponding position in the host embryos. Donor cells were labeled by EGFP via injection of 100 pg *EGFP* mRNA at the one-cell stage. Embryos were embedded in 0.5% low–melting point agarose; time-lapse imaging started at about 8 hpf.

Live imaging

Zebrafish embryos were placed in a temperature-controlled sample holder and live-imaged under a Zeiss 710 META or Olympus FV1200 confocal microscope. GFP channel images are 3D views of z-stacks. For axis induction visualization, embryos were mounted in 5% methyl cellulose (Sigma) and photographed under an Olympus MVX10 fluorescence macro zoom microscope at indicated stages. For time-lapse movies showing the early development, embryos were photographed using a Nikon Eclipse Ti microscope and a Motic SMZ-161 stereoscope. Movies were processed with Imaris software 7.2.3 (Bitplane) and Adobe Photoshop CC.

Immunoblotting and immunoprecipitation

HEK293T cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin in a 37°C humidified incubator with 5% CO_2. Plasmids were transfected with polyethylenimine (23966, Polysciences) or VigoFect (T001, Vigorous) according to the manufacturers' instructions. For Topflash Luciferase reporter assay, the internal control pRL-TK plasmid was co-transfected. Coimmunoprecipitation and immunoblotting were performed essentially as described (*56*, *65*). Treatment of cultured cells with the tankyrase inhibitor XAV939 was performed as described (*47*). Briefly, cells were cultured in DMEM containing 1 μM XAV939 from 6 hours after transfection and harvested at 24 hours after transfection. To investigate the degradation of endogenous Axin1, CHX (100 μg/ml) was added to the culture medium at 30 hours after transfection and cells were then harvested for immunoblotting at desired time points. The band intensity was assessed using ImageJ software.

Immunoblotting using *Xenopus* embryos was performed as described (*66*). Briefly, embryos at desired stages were lysed with Digitonin buffer (0.15 mg/ml Digitonin in PBS pH7.6) or RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Protein amount equivalent to one embryo for each sample was loaded onto PAGE/SDS gel. β-Actin was detected as the loading control.

In vitro pull-down assay

GST and GST-Hwa(ΔN46) protein were produced in *E. coli* BL21 (DE3). Briefly, when OD_{600} reached 0.8 to 1.0, 1 mM IPTG was added to induce protein synthesis. After 3 hours of induction at 37°C, cells were harvested and sonicated. The cell lysates were centrifuged at 12,000*g* at 4°C for 40 min. GST protein present in the supernatant was purified using glutathione Sepharose 4B resin (GE Healthcare). To purify GST-Hwa(ΔN46) present in the pellet (in the form of inclusion bodies), the pellet was suspended and washed sequentially with wash buffer I [50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 2 M urea], wash buffer II [50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 3% Triton X-100], and wash buffer III [50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 2 M guanidine hydrochloride], each followed by centrifugation at 1500*g* at 4°C for 30 min. After final wash, the precipitate was dissolved in lysis buffer [50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 2 mM DTT, 8 M urea] at room temperature for 30 min and dialyzed at 4°C in dialysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 5% glycerol, 1% Triton X-100]. The purified protein had a purity of >90% as examined by Coomassie Brilliant Blue G-250 staining. *E. coli*–derived N-terminal 6× His-tagged human AXIN1 (amino acids 1 to 351) was purchased from Proteintech (Ag10079).

For detection of Hwa/AXIN1 interaction, the His-AXIN1 protein was incubated with GST or GST-Hwa(ΔN46) in 1 ml of dialysis buffer for 2 hours at 4°C. Then, ~10 μl of Ni-NTA-Agarose resin (30310, Qiagen) was added to the mixture and incubated at 4°C overnight. Finally, the resin was washed four times, 5 to 10 min each, dissolved in 2× SDS loading buffer, and examined by immunoblotting with anti-GST and anti-His antibodies.

Quantitative RT-PCR

Zebrafish embryos, oocytes, or eggs were collected at desired stages. Total RNA isolation, cDNA synthesis, and qRT-PCR were performed as described (*67*). Expression levels were normalized to the reference gene *eif4g2a* for zebrafish gene expression assays. The sequences of the qRT-PCR primers for zebrafish genes are listed in table S2.

For qRT-PCR analysis of *Xenopus* genes, oocytes or embryos at desired stages were lysed with extraction buffer [50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.5% SDS, and Proteinase K (0.25 mg/ml)], extracted with acidic phenol/chloroform, and precipitated with 70% isopropanol. RNA pellets were then washed with 70% alcohol and air-dried. Purified RNA was resuspended in pure water; 2 μg of total RNA for each sample was used to synthesize cDNA. All quantitative PCR data were acquired using the BIOER qPCR machine (Hangzhou, China) following the manufacturer's instruction. The expression level of *odc* was used as the control reference for all samples. The sequences of qRT-PCR primers for *Xenopus* genes are listed in table S2.

Plasmid constructs

Full-length coding sequence and 3′UTR of *hwa* (XM_698288.8) was amplified and cloned into the EZ-T vector (GenStar) for making antisense RNA probe. The ORF of *Xenopus hwa* with an HA-tag at its 3′ end was cloned into pCS107 vector using restriction enzymes *EcoR*I/*Xho*I. The expression constructs Myc-tagged Axin2 (mouse) and β-catenin (mouse), HA-tagged Axin1 (mouse), and LRP5ΔN (human) were all based on pCS2(+) vector and were obtained from W. Wu, Tsinghua University. For zebrafish *hwa* expression constructs, full-length or partial *hwa* coding sequence was amplified and cloned into pCS2(+) vector, with addition of HA, Flag, or Myc tag in frame at the C terminus. Hwa tagged with HA or Flag was functional, as indicated by an efficient rescue effect on *hwa* mutant embryos. The construct pCS2(+)-LRP5ΔC-mCherry was made by fusing human LRP5 lacking the intracellular domain to mCherry, which was used as a dominant negative form of LRP5; pCS2(+)-Axin1ΔDIX and pCS2(+)-Axin1ΔRGS were constructed by deleting the DIX domain (amino acids 750 to 832) and the RGS domain (amino acids 88 to 211) of the mouse Axin1 from HA-tagged Axin1, respectively. For the production of zebrafish Hwa(ΔN46) protein, the coding sequence encoding amino acids 47 to 294 plus the His tag was cloned into the pGEX-6p-1 expression vector with GST fusion at the N terminus.

Antibodies and drugs

The following antibodies and reagents were used: anti-Flag (F1804, Sigma; M185-3L, MBL), anti–c-Myc (sc-40, Santa Cruz), anti-HA antibody (sc-7392, Santa Cruz), anti-GFP (sc-9996, Santa Cruz), anti-Axin1 (2087S, CST), anti–β-catenin (8480S, CST), anti–non-p-β-catenin (8814S, CST), anti-Axin2 (LS-C345085, LifeSpan BioSciences), anti-Tankyrase1/2 (sc-365897, Santa Cruz), anti-His

(AE003, Abclonal), anti-GST (AF0174, Beyotime), anti–α-tubulin (T6199, Sigma), anti–β-actin (AC026, Abclonal), anti-GAPDH (TA-08, ZSGB-BIO), XAV939 (13596, Cayman), CHX (C8030, Solarbio), and Wnt-C59 (S7037, Selleck). Antibodies used for immunoblotting of *Xenopus* embryo lysates included anti–β-catenin (2206, Sigma), anti-HA (11867423001, Roche), and anti–β-actin (BE0021, Easy Bio).

Statistical analysis

Significance of differences between means was analyzed using Student's *t* test. Data for statistical analysis are expressed as mean ± SEM unless otherwise stated.

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ACKNOWLEDGMENTS

We thank S. Su and W. Wu (Tsinghua University), Y.-G. Chen (Tsinghua University), and J. Yuan (Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences) for providing experimental materials and methods. We are also grateful to other members of the Meng laboratory for help and discussion and the staff at the Cell Facility in Tsinghua Center of Biomedical Analysis for technical assistance. **Funding:** National Natural Science Foundation (31330052, 31590832, and 31571531), the Tsinghua University Initiative Scientific Research Program (20141081299), and China Postdoctoral Science Foundation (2017M620748). **Author contributions:** L.Y. found and characterized *huluwa*^{tsu01sm} mutants, generated *huluwa*^{tsu32c} mutants, performed embryonic analyses, analyzed the data, and contributed to manuscript preparation; J.C. mapped the *hwa* gene, performed embryonic and biochemical analyses, analyzed the data, and contributed to manuscript preparation; X.Z. performed all *Xenopus*-related experiments with Q.H.T.'s supervision; Q.H.T. analyzed the *Xenopus*-related data; J.S., X.W., W.S., and W.Z. helped to carry out experiments; and A.M. conceived the project, analyzed the data, and wrote the manuscript. **Competing interests:** None. **Data and materials availability:** All data are available in the main text or the supplementary materials.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/362/6417/eaat1045/suppl/DC1
Figs. S1 to S18
Tables S1 and S2
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24 January 2018; accepted 27 September 2018
10.1126/science.aat1045

Science

Maternal Huluwa dictates the embryonic body axis through β-catenin in vertebrates

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Science **362** (6417), eaat1045.
DOI: 10.1126/science.aat1045

Maternal factor sets axis

The vertebrate body form changes from the round shape of the fertilized egg to a cylindrical shape when the body plan is established. However, it is unknown whether a maternal factor controls this body axis formation. Yan *et al.* identified such a maternal factor and named it Huluwa. Loss of maternal Huluwa, a transmembrane protein, in zebrafish or frog eggs resulted in embryos that lacked the body axis and were missing the head and dorsoanterior tissues. Huluwa promoted Axin degradation, likely independent of Wnt ligand –receptor signaling, to protect β-catenin from degradation and induce body axis development during embryogenesis.

Science, this issue p. eaat1045