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# Maternal control of embryonic dorsal organizer in vertebrates☆

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ABSTRACT

The establishment of the body axis and developmental blueprint in embryos has remained to be a central question in developmental biology, captivating scientists for centuries. A milestone in this field was achieved in 1924 when Hans Spemann and Hilde Mangold discovered the dorsal organizer for embryonic body axis formation in amphibians. Since then, extensive studies have demonstrated that the dorsal organizer is evolutionarily conserved in vertebrates. This organizer functions as a signaling center, directing adjacent cells toward specific fates and orchestrating pattern formation to establish the embryonic axis. After 70 years since the discovery of the organizer, studies in different model animal species had revealed that locally activated β-catenin signaling during blastulation plays an indispensable role in organizer induction. Then, efforts have been made to identify initiators of β-catenin activation in blastulas. Now, it appears that maternal Huluwa, a transmembrane protein, is a bona fide organizer inducer at least in teleost fish and frog, which can activate downstream signaling pathways, including but probably not limited to β-catenin pathway. More studies are needed to decode the complete molecular network controlling organizer induction.

## 1. Introduction

Vertebrate development begins with a single fertilized egg that undergoes extensive morphogenetic and molecular transformations to give rise to a fully developed organism. The most critical period for cell fate specification and body patterning occurs from the middle blastula to gastrulation stages. During this time, coordinated signals and extensive cell movements establish the foundational body plan and fate map. Among these processes, the dorsal organizer plays a pivotal role in guiding the formation of the embryonic body axis (Bouwmeester, 2001; Cousin, 2019).

In the 1920s, Hans Spemann (winner of the Nobel Prize for Physiology or Medicine in 1935) and Hilde Mangold first identified and named the embryonic organizer in amphibian embryos (Spemann and Mangold, 1924). Through transplantation experiments in salamander and *Xenopus* embryos, they demonstrated that tissue from the dorsal lip of the blastopore could induce a secondary body axis when grafted onto another embryo, coining the term "organizer", now known as the Spemann–Mangold organizer. Subsequent research identified equivalents of the Spemann–Mangold organizer in various vertebrates, including the Hensen's node in avians, the embryonic shield in fish, and the node in mammals (Fig. 1)(Boettger et al., 2001; Saude et al., 2000; Shih and Fraser, 1996; Waddington, 1933; Zhou et al., 1993).

The dorsal organizer, a localized group of cells in early gastrula embryos, serves as a crucial signaling center directing dorsal fate specification and primary body axis formation. Through secreted proteins and intracellular transcription factors, it orchestrates developmental cues necessary for body plan establishment (Anderson and Stern, 2016). In the absence of the organizer, embryos fail to develop a body axis, exhibiting ventralized phenotypes devoid of dorsal and anterior structures.

Maternal and zygotic signaling pathways, including Wnt/β-catenin, BMP and Nodal, tightly regulate dorsal organizer formation. According to the "default model" (Hemmati-Brivanlou and Melton, 1997), several maternal factors primarily induce ventralizing signals during blastulation. Rather than actively inducing dorsal fates, dorsalizing factors from the organizer acts by antagonizing ventralizing signals (Baker et al., 2010; Leung et al., 2003). While the genetic control of axis formation has been extensively reviewed (Hikasa and Sokol, 2013; Jones and Mullins, 2022; Langdon and Mullins, 2011; Schier and Talbot, 2005), this review

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revisits maternal control of the dorsal organizer, especially recent discoveries in activation of maternal β-catenin signaling on the future dorsal side.

## 2. Maternally derived dorsal determinants

In addition to zygotic genome products, maternal factors are crucial for organizer induction in fish and frog. Among these, the most extensively studied and essential maternal signal is β-catenin signaling (Carron and Shi, 2016; Guger and Gumbiner, 1995; Kelly et al., 2000; Wylie et al., 1996). Compelling evidence demonstrates that maternal β-catenin accumulates specifically in the nuclei of dorsal blastomeres, where it activates organizer-specific genes, such as *bozozok*, *squint* and *chordin* in zebrafish, as well as *siamois* and *nodal-related 3* in *Xenopus* (Schneider et al., 1996). Loss-of-function studies in zebrafish, frogs, and mice further confirm the indispensable role of β-catenin signaling in dorsal organizer formation (Haegel et al., 1995; Heasman et al., 1994; Heasman et al., 2000; Huelsken et al., 2000; Kelly et al., 2000). These findings consolidate the concept that the organizer formation is induced by maternally derived critical factors, commonly referred to as maternal dorsal determinants (DDs) in zebrafish and *Xenopus*.

### 2.1. Dorsal determinants and asymmetric translocation

Mature *Xenopus* and zebrafish eggs are radially symmetrical along the animal-vegetal (AV) axis, with dorsal determinants initially localized at the vegetal pole. Removal of the vegetal pole prior to the first cleavage results in ventralized embryos, supporting the vegetal pole localization of dorsal determinants (Mizuno et al., 1999; Ober and Schulte-Merker, 1999; Shao et al., 2017).

The asymmetrical transport of dorsal determinants following fertilization is essential for organizer formation and dorsal fate specification (Fig. 2). This concept is strongly supported by both biochemical and genetic evidence. Treatments such as UV irradiation, low temperatures, or treatment with nocodazole (a compound that suppresses the assembly of microtubule bundles) disrupt this asymmetrical transport process, resulting in dorsal-deficient (or called ventralized) phenotypes in both zebrafish and *Xenopus* (Elinson and Rowning, 1988; Jesuthasan and Stahle, 1997). The importance of asymmetrical transport of dorsal determinants is further underscored by several maternal-effect zebrafish mutants, such as *brom bones* (*hnrnp1*), *tokkaebi* (*syntabulin*), *hecate* (*grip2a*) and *kif5ba* (Campbell et al., 2015; Ge et al., 2014; Mei et al., 2009; Nojima et al., 2010; Oh and Houston, 2017).

### 2.2. Dorsal determinants and β-catenin signaling

A key outcome of the directional transport of dorsal determinants is the activation of β-catenin signaling in dorsal marginal blastomeres (Kelly et al., 2000; Liao et al., 2006; Schneider et al., 1996). Endogenous β-catenin protein was found to be enriched dorsally at the two-cell stage in *Xenopus* embryos, coinciding with the establishment of the dorsal-ventral axis (Larabell et al., 1997). In the zebrafish, two maternal β-catenin genes, *ctnnb1* and *ctnnb2*, are expressed. In *ichabod* mutants, the absence of maternal *ctnnb2* transcripts results in loss of the embryonic shield and body axis. Overexpression of *ctnnb2* can rescue the *ichabod* mutant phenotype, whereas manipulation of upstream components of the canonical Wnt/β-catenin signaling pathway fails to phenocopy this rescue effect (Kelly et al., 2000). Similarly, in mice, embryos lacking β-catenin exhibit abnormal visceral endoderm patterning prior to gastrulation and fail to form the primitive streak (Haegel et al., 1995; Huelsken et al., 2000). These findings indicate an essential role of β-catenin signaling in dorsal organizer formation.

What, then, is the relationship between dorsal determinants and β-catenin activation? A straightforward hypothesis posits that β-catenin itself functions as the dorsal determinant, accumulating dorsally via asymmetrical transport. However, experimental evidence challenged this view. The subcortical vegetal cytoplasm from β-catenin-depleted *Xenopus* embryos retains the ability to induce a secondary axis

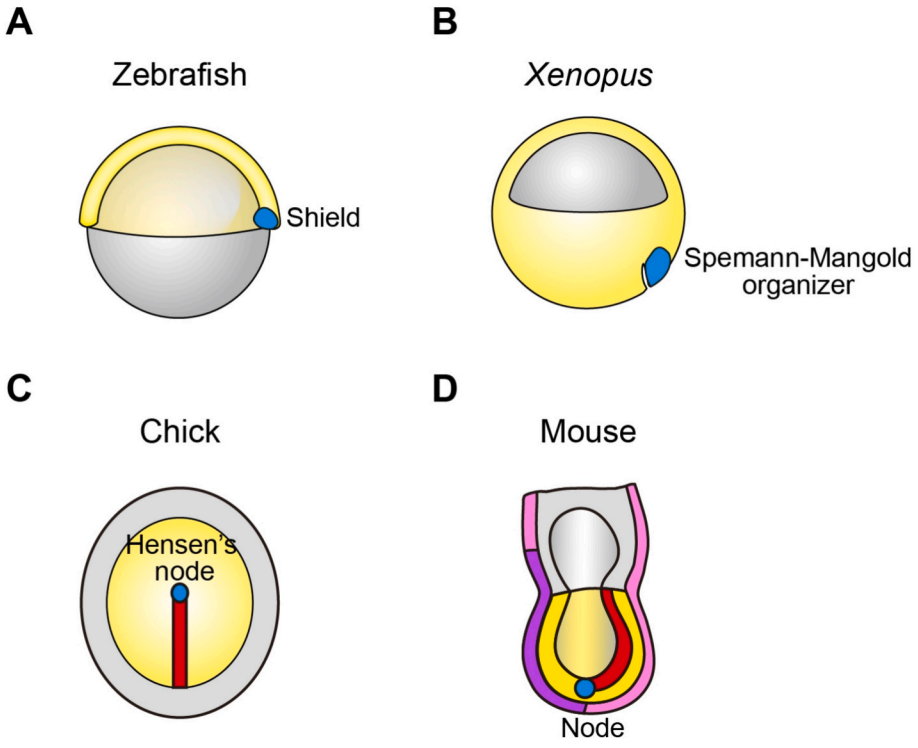


**Fig. 1.** Dorsal organizers in vertebrates. Embryonic shield in zebrafish (A), Spemann-Mangold organizer in *Xenopus* (B), Hensen's node in chick (C), and Node in mouse embryo (D) are marked with blue. Primitive streak in chick(C) and mouse (D) embryo are labeled in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

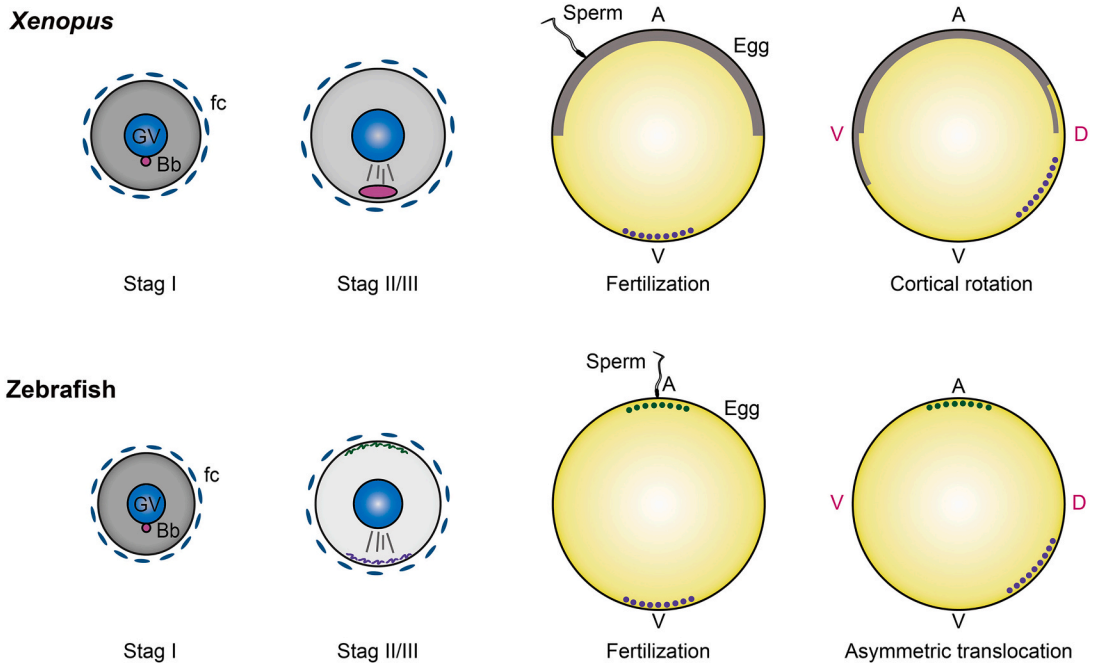


**Fig. 2.** Deposition and asymmetrical transport of dorsal determinant(s) in zebrafish and *Xenopus* embryos. Different stages of oocytes or eggs are shown in each panel. Purple indicates Bb (Stage I) or the vegetal pole localized dorsal determinant(s) (Stage II/III). Green and brown dots indicate maternal factors deposited at the animal and vegetal region, respectively. GV, Germ vesicle; Bb, Balbiani body; fc, follicle cell; A, animal pole; V, vegetal pole; V (red), ventral side; D (red), dorsal side. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Marikawa and Elinson, 1999). This observation implies that there may exist other dorsal determinants except β-catenin in the subcortical vegetal cytoplasm.

## 3. Discoveries of bona fide dorsal determinants

For decades, researchers have dedicated to understand the identity and nature of these dorsal determinants that trigger localized activation of maternal β-catenin signaling in the future dorsal region. While several potential activators/determinants have been proposed, these findings have remained controversial until the discovery of Huluwa.

### 3.1. Extracellular Wnt ligands

As critical stimulators of the canonical Wnt/β-catenin signaling pathway, Wnt ligands have been hypothesized to trigger maternal β-catenin signaling during organizer formation (Hikasa and Sokol, 2013; Langdon and Mullins, 2011; Schier and Talbot, 2005). Early evidence supporting the role of Wnt/β-catenin signaling in body axis determination emerged from gain-of-function experiments, where overexpression of Wnt mRNA led to the formation of an ectopic organizer (McMahon and Moon, 1989; Smith and Harland, 1991). Similarly, overexpression of *lrp6* alone or in combination with *wnt5a* induces axis duplication in *Xenopus* (Tamai et al., 2000). In agreement, antisense oligodeoxynucleotide-mediated depletion of *wnt11b* or *lrp6* mRNA in *Xenopus* oocytes results in loss of the dorsal organizer and axial structures (Kofron et al., 2007; Tao et al., 2005). Further studies indicate that Wnt11b and Wnt5 can form heterodimer to activate β-catenin signaling (Cha et al., 2008). However, overexpression of dominant-negative Wnt11 (DnWnt11) in *Xenopus* embryos or depletion of maternal and zygotic *wnt11* (MZ*wnt11*) in zebrafish embryos does not impact organizer induction, but disrupts convergent extension movements during gastrulation (Heisenberg et al., 2000). Moreover, a recent study demonstrates that maternal mutation of *wnt11b* results in reduced cortical rotation in *Xenopus* embryos, suggesting that Wnt11b may affect the distribution of dorsal determinants, rather than constituting the dorsal determinant itself (Houston et al., 2022). Furthermore, overexpressing *wnt11* alone is insufficient to induce ectopic body axis formation in zebrafish and *Xenopus*, suggesting that *wnt11* is unlikely to be the dorsal determinant for β-catenin activation during organizer formation.

In the zebrafish, maternal *wnt8a*, localized at the vegetal pole in eggs and asymmetrically translocated after fertilization, has been supposed to activate β-catenin signaling (Lu et al., 2011). Overexpression of *wnt8* leads to nuclear accumulation of β-catenin and ectopic expression of organizer-specific genes in both *Xenopus* and zebrafish, indicating that *wnt8a* has the potential to activate β-catenin signaling. However, zebrafish zygotic *wnt8a* mutants (Z*wnt8*$^{w8/w8}$) display an expanded organizer at the shield stage and a truncated trunk at 24 h post fertilization, suggesting that zygotic Wnt/β-catenin signaling restricts organizer size and regulates non-axial mesoderm patterning (Ramel et al., 2005; Ramel and Lekven, 2004). Additionally, a study by Masahiko Hibi' group found that maternal *wnt8a* mutants (M*wnt8a*$^{-/-}$) show no defects in dorsal-axis formation, and depletion of maternal *wnt8a* enhances the truncation phenotype in zygotic *wnt8a* mutant (Z*wnt8a*$^{-/-}$), indicating that maternal *wnt8a* supports zygotic *wnt8a* function but is dispensable for dorsal organizer induction (Hino et al., 2018). Consistently, Xwnt8 in *Xenopus* has also been reported to restrict the dorsal fate during late blastulation (Christian and Moon, 1993).

Besides, *wnt6a* has been suggested as another potential dorsal determinant in the zebrafish responsible for dorsal organizer induction (Hino et al., 2018). However, this finding is primarily supported by overexpression experiments, and additional genetic knockout or knockdown evidence is required to validate its role as a dorsal determinant. Additionally, overexpression of Wnt antagonists, such as Dkk1 and Frzb, which block the function of endogenous zygotic Wnt/β-catenin signaling and ectopic Wnt ligands, has minimal impact on body axis induction (Glinka et al., 1998; Wang et al., 1997). Therefore, whether Wnt ligands themselves directly contribute to dorsal organizer and body axis induction remains largely questionable. One possibility is that the canonical Wnt signaling is active in regulating the transcription of maternal dorsal determinants or in asymmetrical distribution of dorsal determinants during oocyte maturation.

### 3.2. Intracellular dorsalizing factors

Beyond Wnt ligands, an intriguing alternative hypothesis proposes that dorsal organizer induction may involve a Wnt ligand/receptor-independent mechanism, directly activating intracellular components of the Wnt/β-catenin pathway. Supporting this hypothesis, intracellular kinases such as Akt, Erk, and c-Abl can facilitate β-catenin signal activation by promoting the release of β-catenin from the E-cadherin complex, inhibiting GSK3β, or displacing Axin (Cross et al., 1995; Desbois-Mouthon et al., 2001; Fernandez et al., 2014; Jeong et al., 2018; Ji et al., 2009; Yang et al., 2006). Besides, two intracellular components of the canonical Wnt/β-catenin pathway, Gsk3-binding protein (GBP/Frat1) and Dishevelled (Dsh/Dvl), are found to transport to the prospective dorsal side during cortical rotation, thereby stabilizing b-catenin in future dorsal blastomeres (Dominguez and Green, 2000; Miller et al., 1999; Salic et al., 2000; Weaver et al., 2003; Yost et al., 1998).

Both GBP and Dsh can induce complete ectopic dorsal axes when overexpressed in *Xenopus* embryos (Sokol et al., 1995; Yost et al., 1998). GBP has been reported to be required for axis formation in *Xenopus* embryos as its antisense knockdown prior to fertilization in oocytes results in embryonic ventralization (Yost et al., 1998). However, this could be ascribed to its role in modulating Gsk3β activity during the early stages of dorsal determinant transport, shortly after fertilization (Shao et al., 2012). However, overexpression of dominant-negative Dsh, which strongly inhibits induction of secondary body axis by wild-type *Xdsh* mRNAs, has no effect on primary axis formation (Sokol, 1996). In the zebrafish, simultaneous mutations of maternal and zygotic *dvl2* and *dvl3a*, two most abundantly expressed maternal *dvl* genes, did not cause defective dorsal fate specification or loss of the head and anterior tissues, implying that *dvl* genes might be dispensable for ß-catenin activation during early development (Xing et al., 2018). Further studies suggest that neither Dsh nor GBP alone acts as the primary β-catenin stabilizing factor (Marikawa and Elinson, 1999). It seems more likely that GBP, Dsh, or both are not genuine dorsal determinants, but instead regulators of dorsal determinants movements (Weaver and Kimelman, 2004).

### 3.3. Discovery of Huluwa

The precise nature of the endogenous dorsalizing activity responsible for dorsal organizer formation in vertebrates remains unclear for a long time, particularly it is unsure if this activity originates from Wnt ligands or intracellular components. Several years ago, our group found the zebrafish maternal mutant, M*hwa*$^{tsu01sm}$, in which the transcription of the *huluwa* (*hwa*) gene is completely shut down due to the insertion of a 7.3-kb DNA element (likely a retrotransposon sequence) into the *huluwa* promoter (Yan et al., 2018). Maternal *huluwa* transcripts at the vegetal pole are transported to the future dorsal side upon fertilization, underscoring its moving character of dorsal determinant. The Huluwa protein has an N-terminal 23-residue extracellular domain, a 21-residue single transmembrane domain and a 250-residue intracellular domain, being located on the plasma membrane of the presumptive dorsal blastomeres with nuclear β-catenin in early blastulas. All of M*hwa* mutant embryos show the absence of the embryonic shield at the onset of gastrulation and a complete loss of the body axis at 24 h postfertilization (hpf), the most severely ventralized phenotypes. Microinjection of *huluwa* mRNA into two opposite blastomeres of 16/32-cell stage M*hwa* mutants efficiently induces two complete body axes in over 90 % of injected embryos at 24 hpf, indicating an extremely potent organizer-inducing capacity. These observations demonstrate that maternal Huluwa is both necessary and sufficient for dorsal organizer induction and body axis formation. Thus, maternal Huluwa is a dorsal organizer inducer.

## 4. Huluwa signaling

As a newly identified novel protein, Huluwa's function in determining dorsal organizer formation in vertebrates is assured, but its signaling pathway has not be fully explored.

### 4.1. Evo-devo insights into Huluwa function

From an evolutionary perspective, Huluwa homologs are broadly present in chordates, including amphioxus (Cephalochordata), sea squirt (Urochorda), lamprey (Cyclostomata), shark (Chondrichthyes), frog (Amphibia), and lizard (Reptilia). The essential role of *huluwa* in dorsal organizer and body axis formation in *Xenopus laevis* is supported by the findings that depletion of *huluwa* transcripts by antisense oligos in oocytes leads to loss of the organizer and body axis, and that microinjection of *huluwa* mRNA in two ventral blastomeres of 4-cell stage embryos efficiently induces secondary body axis (Yan et al., 2018). This pivotal role of *huluwa* in organizer formation has since been independently validated in *Xenopus* (Azbazdar and De Robertis, 2024; Tejeda-Munoz and De Robertis, 2022; Zhu et al., 2021). Remarkably, overexpression of *huluwa* mRNA from lower organisms such as sea squirt and amphioxus can partially rescue the body axis in zebrafish M*hwa*$^{tsu01sm}$ mutant embryos (Li et al., 2024). These observations suggest a functional conservation of the Huluwa protein across subphyla of the phylum Chordata. However, we must be aware that the Huluwa mechanism may be only one of ancient mechanisms for body axis induction in bilateria.

The formation of the embryonic body axis in annual killifish was reported to occur without Huluwa-mediated prepatterning. Instead, Nodal and β-catenin pathways are repurposed to coordinate cellular aggregation and axis formation (Abitua et al., 2024). It remains elusive how Nodal and β-catenin pathways are activated in annual killifish. Interestingly, the *huluwa* gene in annual killifish (GRZ strain) appears to be a pseudogene, encoding a truncated, non-functional protein, whereas non-annual killifish (MZM strain) possesses a functional *huluwa* gene. It is unclear whether the abandonment of Huluwa function in the GRZ strain represents an adaptation to its short lifespan and high-stress environment or reflects an alternative compensatory mechanism for body axis formation.

It seems that the *Huluwa* gene in aves or mammalia has been lost during evolution. One possibility is that dysfunction of this gene is fatal for reproduction so that species with poorer fecundity such as aves and mammals had to discard it during evolution. However, it is likely that Huluwa function has been replaced by mild factors with similar function in birds and mammals.

### 4.2. Downstream effectors of Huluwa signaling

In both zebrafish and frog, the axis-inducing activity of ectopic *huluwa* is largely inhibited by knockdown of *ctnnb2* (Yan et al., 2018). On the other hand, overexpression of *huluwa* results in accumulation of nuclear β-catenin. The dorsalizing and axis-inducing activity of ectopic *huluwa* is unaffected by the co-expression of *dnwnt8a-mCherry*, *DKK1-EGFP,* or *LRP5ΔC-mCherry,* nor by Wnt-C59 treatment in zebrafish. These observations suggest that β-catenin signaling is activated during dorsal organizer formation by Huluwa rather than by Wnt ligand/receptor signaling. Mechanistically, Huluwa protein directly binds to and promotes the degradation of Axin through TNKS1/2-mediated PARsylation (poly-ADP-ribosylation), thereby stabilizing cytosolic β-catenin (Fig. 3).

At least two pieces of evidence raise the possibility that β-catenin is not a sole intracellular effector of Huluwa signaling during dorsal organizer formation. First, unlike M*hwa*$^{tsu01sm}$ mutant embryos, all of which lack the body axis completely, only 34 % of zebrafish *ichabod* mutants lacking nuclear β-catenin have no body axis at all, while the remaining do have variable degrees of head and trunk tissues (Bellipanni et al., 2006; Kelly et al., 2000). Second, overexpression of ectopic constitutively active β-catenin in M*hwa*$^{tsu01sm}$ mutants restores a full body axis only in about one quarter of microinjected mutants, which is in sharp contrast to a rescue efficiency of over 90 % with injection of

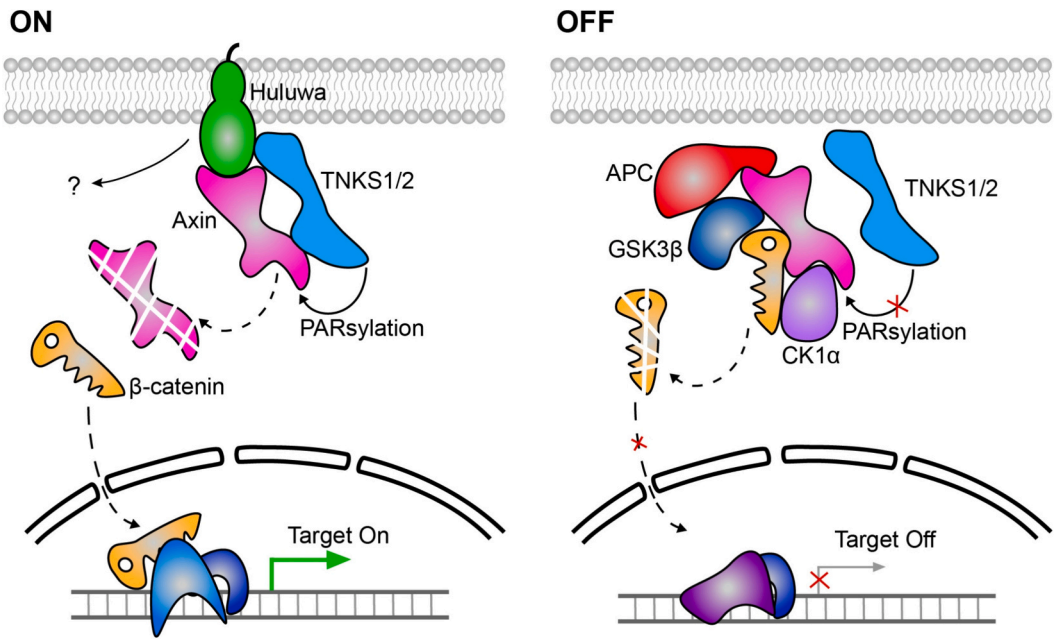


**Fig. 3.** Huluwa/β-catenin signaling pathway. The transmembrane protein Huluwa binds to and promotes the degradation of Axin, which is mediated by TNKS1/2 through PARsylation. Subsequently, stabilized cytosolic β-catenin translocate into nucleus to activate downstream target genes by binding to Tcf/Lef transcription activators. Huluwa may also transduce the signal to other unknown downstream effectors. In M*hwa* mutants, β-catenin in the cytoplasm is degraded by the APC-Axin-GSK3β-CK1α destruction complex, and downstream target genes could not be activated.

wildtype *huluwa* mRNA (Yan et al., 2018). Therefore, we hypothesize that there may exist other effectors or pathways downstream of Huluwa signaling.

### 4.3. Regulators of Huluwa signaling

Our recent study has identified the Ser168 residue within a conserved PPNSP motif as an important phosphorylation switch for Huluwa function (Li et al., 2024). The zebrafish Huluwa with alanine substitution of Ser168 loses the organizer-inducing and β-catenin stabilizing activities. Ser168 of Huluwa is likely subjected to phosphorylation by multiple cell cycle-related kinases, including Cdk2, Cdk16 and GSK3β. This finding raises several interesting questions: how multiple kinases are coordinated to regulate Huluwa activation; how phosphorylated Huluwa is dephosphorylated; whether its phosphorylation and dephosphorylation are regulated spatially and temporally.

Our previous work revealed a specific location of Huluwa protein in presumptive dorsal blastomeres in zebrafish early blastulas (Yan et al., 2018). An interesting question is how this asymmetrical distribution is achieved. Zhu et al. (2021) identified maternal E3 ubiquitin ligase ZNRF3 as a key negative regulator of Huluwa in *Xenopus* embryos (Zhu et al., 2021). Overexpression of a dominant negative form of Znrf3 ($Znrf3^{\Delta RING}$) in one ventral blastomere of four-cell stage embryos induces a secondary body axis. ZNRF3 mediates Huluwa ubiquitination and subsequent lysosomal degradation presumably in embryonic regions outside the Spemann-Mangold organizer, preventing the expansion of the organizer. On the other hand, De Robertis' group highlights the role of endolysosomal trafficking and lysosome function in enhancing Huluwa/β-catenin signaling in the dorsal region of *Xenopus* blastulas for the organizer formation (Azbazdar and De Robertis, 2024; Tejeda-Munoz and De Robertis, 2022). As a central player for the dorsal organizer formation, Huluwa's expression and activity should be precisely regulated at multiple levels. We are still at the beginning stage of understanding regulation of Huluwa signaling.

## 5. Concluding remarks

The dorsal organizer was first discovered a century ago, but the molecular mechanisms underlying its induction are not fully understood yet. In lower vertebrates such as fish and frog, it is clear that the dorsal organizer is controlled by maternal factors, among which Huluwa may be a master. In another word, information or programs for dorsal organizer induction are deposited in oocytes/eggs in lower vertebrates, which may be called prepatterning mode. The avian Henson's node and mammalian node are considered as the equivalents of the Spemann-Mangold organizer, which are located at the tip of the primitive streak (Stern, 2024). In these species, the formation of the node involves coordinated Wnt, Activin/Nodal and BMP gradients (Morgani and Hadjantonakis, 2020; Robb and Tam, 2004). It remains unknown whether any maternal factors are required for primitive streak/node formation in avian or mammalian species. It is possible that the primitive streak/node is induced wholly by zygotic signals, which may be called zygotic patterning mode. It will be interesting to know how the dorsal organizer formation switches from the preset model to the induction model during evolution.

## CRediT authorship contribution statement

**Jing Chen:** Writing – original draft, Visualization, Funding acquisition, Conceptualization. **Anming Meng:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization.

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