

Maternal Huluwa regulates postfertilization microtubule array organization for asymmetrical transport of dorsal determinants in zebrafish

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Abstract

The dorsal organizer, essential for vertebrate embryonic axis formation, is induced by microtubule-mediated transport of maternal determinants. Maternal Huluwa (Hwa) has been identified as an essential organizer inducer in zebrafish and frogs, functioning at midblastula stages to activate β -catenin signaling in the preorganizer. It remains unknown if maternal Hwa functions at or before fertilization. Here, we report that maternal Hwa protein is critical for organizing the vegetal parallel microtubule array immediately after fertilization in zebrafish. Hwa protein and mRNA are enriched at the vegetal pole and facilitate microtubule network formation, enabling asymmetrical transport of dorsal determinants. Loss of maternal Hwa disrupts this microtubule architecture and abrogates mRNA transport, revealing a self-reinforcing mechanism where Hwa regulates its own asymmetrical distribution. Our findings establish a dual-phase model of dorsal specification: Hwa initially governs symmetry breaking through postfertilization microtubule organization and later on activates β -catenin signaling at blastula stages. This work provides fundamental insights into how the key maternal factor regulates the organizer and body axis formation at different developmental stages.

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 dorsal organizer, known as the Spemann and Mangold organizer in *Xenopus* and as the shield in zebrafish, plays a pivotal role in instructing the formation of the embryonic body axis ^{1,2}. In the absence of the organizer, embryos fail to develop a body axis, exhibiting ventralized phenotypes devoid of dorsal and anterior structures.

The formation of the dorsal organizer is critically dependent on the microtubule-mediated asymmetrical transport of maternal dorsal determinants ^{1,2}. In zebrafish and *Xenopus*, these determinants, initially localized at the vegetal pole, are directionally translocated to the future dorsal side along a transient vegetal parallel microtubule (VPM) array that forms between ~15 and 30 minutes postfertilization (mpf) ^{3,4,5,6}. This transport is essential for breaking embryonic radial symmetry and establishing the dorsal-ventral axis ⁷. The integrity of this microtubule network is paramount, as its disruption through chemical agents (e.g., nocodazole) or mutations in maternal-effect genes prevents dorsal determinant delivery, resulting in ventralized embryos without the head and other anterior structures ^{8,9}. Specifically, the cargo linker protein Syntabulin is essential for this transport, as evidenced by the ventralized phenotype of the *tokkaebi* (*syntabulin*) mutant ¹⁰. Importantly, *syntabulin* mutants maintain a normal VPM network but fail to transport dorsal determinants, highlighting its specific role as a cargo adapter rather than a microtubule organizer. Furthermore, the motor protein Kif5ba, the microtubule-associated protein Grip2a, and the midbody component Prc1l are required for organizing and bundling VPM, and their maternal mutations lead to body axis defects ^{11,12,13}. The formation of the VPM bundle network in zebrafish is known to depend on Ca^{2+} signaling during egg activation, rather than sperm entry or the fertilization process itself ^{9,14}. Studies have identified the RNA-binding protein hnRNPI as another crucial regulator

since its deficiency impairs calcium signaling during egg activation and disrupts VPM formation. It is proposed that abnormal calcium signaling affects cortical granule release, leading to their accumulation in the cortex and subsequent compromise of microtubule network assembly¹⁵. The importance of microtubule regulation is further underscored by the fact that GSK-3 promotes the correct orientation of vegetal MTs early in development¹⁶, and defects in the Nanog-cyp11a1-P5 axis also disrupt microtubule assembly¹⁷. Thus, a functional microtubule network is not merely a passive transport system but a fundamental, actively assembled structure that is indispensable for the initiation of organizer formation and subsequent embryonic patterning.

While numerous maternal mutants exhibiting dorsoventral patterning defects have been identified, they often display heterogeneous and inconsistent ventralized phenotypes, implying potential genetic redundancy or residual inductive signals. A significant advance was made with the characterization of the maternal-effect mutant *tsu01sm* in our laboratory, which bears a mutation in the *huluwa* (*hwa*) gene¹⁸. This mutant exhibits a completely penetrant and severely ventralized phenotype, attributed to the absence of maternal *hwa* mRNA. *hwa* mRNA is vegetally localized in the oocyte and fertilized egg, is asymmetrically transported upon fertilization. Combined with the result that overexpression on two sides of the embryo at the 32-cell stage can induce the formation of two individual body axes, *hwa* has been proposed as one of the dorsal determinants. In 512-cell stage embryos, endogenous Hwa proteins are mainly asymmetrically distributed on the cell membrane of cells in the future dorsal side of the embryo. Hwa protein is phosphorylated at Ser168 by multiple kinases and activated, enhancing Tnks1/2-mediated degradation of the Axin protein, thus stabilizing β -catenin in the cytosol for entering the nucleus to activate downstream organizer genes^{18, 19, 20}. Apart from the induction of dorsal organizer formation by Hwa/ β -catenin signaling at blastula stages, it remains unclear whether Hwa has other biological functions at earlier developmental stages.

In this study, we demonstrate that maternal Hwa protein and mRNAs stored in eggs are critical for the organization of the VPM array before the first cell cleavage completion after fertilization. The absence of maternal Hwa disrupts this microtubule architecture and abrogates the asymmetrical transport of vegetal mRNAs. We define a previously unrecognized mechanism through which dorsal determinant governs the symmetry-breaking events through regulating its own asymmetrical transport by regulating the postfertilization microtubule network, which later on induces the dorsal organizer by Hwa/ β -catenin signaling at the blastula stage.

Results

Vegetal microtubule organization is impaired in *Mhwa*^{*tsu01sm*} mutants

As disruption of the VPM network gives rise to ventralized phenotypes^{9, 11, 12, 13, 15, 16} mimicking *Mhwa* mutants, we examined the microtubule network in 20-mpf fertilized eggs via immunofluorescence. In wild type (WT) embryos, a robust VPM array was evident. Strikingly, this array failed to form in all

Mhwa^{*tsu01sm*} mutant embryos examined (Fig. 1a). Microtubules in mutants appeared disorganized, with impaired bundling and alignment. In contrast, the lateral cortical microtubule networks were comparable between WT and mutant embryos (Fig. 1b). We also analyzed the furrow microtubule array (FMA) during the late stage of the first cleavage, a non-centrosomal structure involved in cytokinesis, and found no discernible differences between WT and *Mhwa*^{*tsu01sm*} mutants (Fig. 1c), which conforms to normal cleavage and epibolic processes in *Mhwa* mutants¹⁸. This observation indicates that Hwa specifically governs vegetal parallel microtubule organization. Furthermore, while *Mgrip2a* and *Mkif5ba* mutants exhibit variable microtubule phenotypes^{11, 12}, the VPM disorganization in *Mhwa*^{*tsu01sm*} was fully penetrant, usually observed in every embryo. Collectively, these results demonstrate a requirement of maternal Hwa for forming the VPM array.

In some zebrafish maternal-effect mutants, persistent cortical granules (CGs) are thought to physically impede microtubule array formation^{11, 15}. To test whether this mechanism explains the *Mhwa*^{*tsu01sm*} phenotype, we labeled CGs with Wheat Germ Agglutinin (WGA). Results showed that CGs were rapidly and completely exocytosed upon egg activation in *Mhwa*^{*tsu01sm*} mutants, indistinguishable from WT (Fig. 1d, e). Quantification revealed no significant delay in CG release. Thus, the microtubule defect in *Mhwa*^{*tsu01sm*} mutants is not secondary to impaired CG exocytosis.

The absence of *hwa* mRNA in *Mhwa*^{*tsu01sm*} mutants makes it impossible to observe the transport of endogenous *hwa* mRNAs in *Mhwa* mutants. We then examined the localization of *wnt8a*, *grip2a*, and *sybu* in early embryos by *in situ* hybridization (Fig. 1f-h). In WT, all three transcripts were vegetally localized at the 1-cell stage. By the 2-cell stage, transcripts of *wnt8a* and *grip2a*, but not *sybu*, underwent asymmetrical translocation. In *Mhwa*^{*tsu01sm*} mutants, *sybu* RNA was initially localized but appeared more dispersed (Fig. 1h). While *grip2a* translocation occurred normally, the asymmetrical transport of *wnt8a* was abolished (Fig. 1f-i). Real time quantitative PCR (RT-qPCR) confirmed that the overall mRNA levels of these genes were unchanged in *Mhwa*^{*tsu01sm*} mutant embryos at 1-cell stage (Fig. 1j). Examination of additional vegetally-localized mRNAs revealed further transport defects, including loss of asymmetry or weakened vegetal localization (Supplementary Fig. 1). Taking these observations together, we propose that maternal Hwa facilitates the establishment of embryonic polarity by orchestrating the vegetal microtubule network, which in turn enables the proper asymmetrical transport of key maternal factors.

Maternal Hwa protein is present in eggs

To understand how *hwa* regulates the microtubule network, we set out to determine whether the Hwa protein is present at or before the 1-cell stage. We hypothesized that, if only *hwa* mRNA but no Hwa protein exist in eggs, blocking *hwa* mRNA translation after fertilization should cause ventralized phenotypes resembling *Mhwa* mutants. To test this idea, we designed a morpholino (MO) to block *hwa* mRNA translation (Fig. 2a). Co-injection of *hwa*-GFP mRNA with the *hwa*-MO into 1-cell WT embryos resulted in a marked reduction of GFP fluorescence (Fig. 2b) and *hwa*-MO effectively suppressed the body axis rescue effect of exogenous *hwa* mRNA in *Mhwa* mutants (Fig. 2c), which confirm the *hwa*-

MO's efficacy in inhibiting *hwa* mRNA translation. We then injected *hwa*-MO into 1-cell stage WT embryos, and found unaltered expression of the dorsal organizer marker *gsc* at 6 hours postfertilization (hpf) and normal embryonic morphology at 24 hpf (Fig. 2d). This result raised a possibility that a functional pool of maternal Hwa protein is already present at the 1-cell stage. To test this possibility, we performed the Oocyte Microinjection *in situ* (OMIS)²¹ and injected *hwa*-MO into stage III oocytes of WT females to block *hwa* mRNA translation during oocyte maturation, followed by analyzing the resulted embryos after natural spawning (Fig. 2e). In this scenario, we observed marked reduction of *gsc* expression at 6 hpf and ventralized phenotypes at 24 hpf in a proportion of embryos (Fig. 2f), supporting the idea that Hwa protein is synthesized in oocytes and required for organizer induction after fertilization.

We performed immunofluorescence many times to detect endogenous Hwa protein in oocytes and embryos during cleavage period but failed. It is most likely that its level is too low to be detected. Then, we performed mass spectrometry of fertilized egg lysate after vitellogenin clearance and antibody enrichment (Fig. 2g, top). We successfully identified specific Hwa peptides (Fig. 2g, bottom). Thus, both maternal *hwa* transcripts and protein are present in eggs.

Maternal Hwa protein is required for VPM formation

The fact that *hwa* is not transcribed in *hwa*^{*tsu01sm*} mutant oocytes¹⁸ makes it difficult to assess the contribution of maternal *hwa* mRNA or protein to the postfertilization VPM network formation. Then, we tried to create new *hwa* mutant lines with different mutational features. Using CRISPR/Cas9 technology, we generated two new mutant alleles, i.e., *hwa*^{*tsu+1*}, which encodes a truncated peptide of only 80 amino acids (AA) due to a premature stop codon, and *hwa*^{*tsu-30*}, which would produce a protein lacking 10 amino acids within the transmembrane domain (Fig. 3a, and Supplementary Fig. 2a). We confirmed that overexpressed Hwa^{*tsu-30*}-GFP in HeLa cells was not enriched on the plasma membrane (Supplementary Fig. 2b) and its overexpression in *Mhwa*^{*tsu01sm*} mutant embryos failed to rescue the body axis (Supplementary Fig. 2c). Besides, a previously reported *hwa*^{*S168A*} mutant allele, which leads to a serine-to-alanine substitution at residue 168 (S168A) and abrogates phosphorylation at this site²⁰, was also used for comparison. Like *Mhwa*^{*tsu01sm*} mutants, *Mhwa*^{*tsu+1*}, *Mhwa*^{*tsu-30*}, or *Mhwa*^{*S168A*} mutants exhibited a fully penetrant ventralized phenotype at 24 hpf, characterized by radial symmetry and a lack of body axis (Fig. 3b). RT-qPCR and *in situ* hybridization confirmed that *hwa* mRNA in all three mutants was retained at levels comparable to that in WT (Fig. 3c) and correctly localized to the vegetal cortex at the 1-cell stage (Fig. 3d). We then examined the vegetal microtubule network at 20 mpf (Fig. 3e). Strikingly, both *Mhwa*^{*tsu+1*} and *Mhwa*^{*tsu-30*} mutants displayed completely disorganized VPM, phenocopying *Mhwa*^{*tsu01sm*} mutants. In contrast, approximately half of the *Mhwa*^{*S168A*} mutants showed disorganization of VPM. These data together suggest that Hwa protein stored in egg is necessary for postfertilization VPM formation.

Our previous study suggests that Hwa is capable of inducing organizer and body axis during early blastulation by activating β -catenin¹⁸. We generated the *ctnnb2*^{tsu17-4} mutant line using CRISPR/Cas9 technology, which carried a 17-bp insertion and a 4-bp deletion in the third exon of the *ctnnb2* locus and thus gave rise to a premature stop codon (Supplementary Fig. 3a). Like *ichabod* mutants lacking *ctnnb2* transcripts^{22, 23}, all of *Mctnnb2*^{tsu17-4} mutants exhibited variable degrees of ventralized phenotypes at 24 hpf (Supplementary Fig. 3b). However, most of *Mctnnb2*^{tsu17-4} mutant fertilized eggs had normal VPM network (Fig. 3e). This result suggests that maternal Hwa protein regulates the VPM formation most likely independently of β -catenin. WT embryos transiently treated with nocodazole (NOC) at the one-cell (1c) stage showed disruption of the VPM arrays with failed asymmetrical transport of maternal determinants such as *wnt8a* and *hwa* mRNAs (Fig. 3f, g) and consequently failed to form the body axis at 24 hpf (Fig. 3g). Injection of *hwa* mRNA into 8-cell stage NOC-pretreated embryos had a rescue effect on the body axis (Fig. 3h). This result validates that Hwa can function as a body axis inducer at later stages. Collectively, our findings demonstrate that Hwa contributes to dorsal organizer formation in two temporally and mechanistically distinct phases. First, maternal Hwa protein orchestrates the establishment of the VPM network at the 1-cell stage independently of β -catenin, which is a prerequisite for the asymmetrical transport of dorsal determinants. Subsequently, the transported Hwa facilitates the nuclear translocation of β -catenin at the blastula stage to initiate dorsal-specific gene expression.

Maternal Hwa localizes to the vegetal pole to regulate the VPM network

To visualize location of Hwa protein in fertilized eggs, we made transgenic lines that expressed GFP or Hwa-GFP fusion proteins, in which the coding region of the transgene was inserted between a 1.4 kb *hwa* promoter region plus 5'UTR and *hwa*-derived 3'UTR (h3'U) (Fig. 4a). Results showed that at the 1c stage, GFP protein in *Tg(hwa:GFP)* (i.e., *Tg1*) or *Tg(hwa:GFP-h3'U)* (i.e., *Tg4*) embryos was mainly accumulated in the animal-pole cytoplasm with some retained in the yolk, and Hwa-GFP fusion protein in *Tg(hwa:hwa-GFP)* (i.e., *Tg2*) embryos appeared accumulated in the yolk (Fig. 4b). In contrast, Hwa-GFP fusion protein in *Tg(hwa:hwa-GFP-h3'U)* (i.e., *Tg3*) embryos was apparently enriched at the 1c stage in the vegetal-pole region with a GFP-fluorescent vertical stream towards the animal pole and the vegetal Hwa-GFP shifted to one-side as observed at 2-cell and 256-cell stages (Fig. 4c), which may simulate behavior of endogenous maternal Hwa. It appears that correct localization of Hwa protein at the vegetal pole upon fertilization at least partially depends on the existence of the *hwa*'s 3'UTR.

Next, we asked how the transgene mRNA is distributed in the above transgenic embryos. *In situ* hybridization results revealed that *GFP* mRNA in *Tg1* embryos and *hwa-GFP* mRNA in *Tg2* embryos were localized in the cytoplasm but undetectable at the vegetal pole at the 1c stage whereas *hwa-GFP* mRNA in *Tg3* embryos and *GFP* mRNA in *Tg4* embryos were obviously localized in the vegetal-pole region (Fig. 4d), which suggest an essential role of *hwa*'s 3'UTR in vegetal-pole localization. Examination of *hwa-GFP* transcripts in *Tg3* embryos from 2-cell to 256-cell stages revealed its asymmetrical transportation, which mimicked endogenous *hwa* mRNA (Fig. 4e). Thus, 3'UTR of *hwa* mRNA is necessary for its correct location and postfertilization movement.

To test functional activity of transgenic Hwa-GFP protein, we introduced the transgene *hwa:hwa-GFP* (*Tg2*) or *hwa:hwa-GFP-h3'U* (*Tg3*) into *Mhwa*^{*tsu01sm*} mutants. These transgenes were expressed at comparable levels at the 1c stage (Fig. 4f) and capable of partially rescuing the dorsal axis deficiency at 24 hpf with better effect from *hwa:hwa-GFP-h3'U* (Fig. 4g). Interestingly, *hwa:hwa-GFP-h3'U* was able to restore the vegetal microtubule network in some (4/18) of *Mhwa* mutants while the other one had no rescue effect at all (Fig. 4h). These data together suggest that the vegetal pole enrichment of maternal Hwa protein upon fertilization at the 1-cell stage, directed by its mRNA, is a prerequisite for its function in organizing the postfertilization VPM network and later forming the dorsal-ventral axis.

Hwa protein-mRNA complexes undergo phase transition

Confocal microscopic examination of Hwa-GFP protein in the vegetal-pole cortex of *Tg(hwa:hwa-GFP-h3'U)* embryos at the 1c stage found its existence as puncta (Fig. 5a), suggesting the formation of Hwa-GFP condensates. Bioinformatic analysis revealed that Hwa contains a large intrinsically disordered region from 60th to 237th residues (Fig. 5b), a feature mediating multivalent interactions during phase transition. To test the phase separation potential of Hwa, His-GFP-Hwa fusion proteins with different mutated Hwa forms (Fig. 5c) were expressed in *E. coli* and purified, followed by in vitro phase separation assay in the presence of the crowding agent PEG8000^{24, 25}. We found that His-GFP-Hwa(ICD), which contained the Hwa's intracellular domain including the IDR, could form numerous microscale puncta depending on PEG8000 concentrations, whereas His-GFP protein remained diffuse under identical conditions (Fig. 5d, e). The puncta formation of His-GFP-Hwa(47-294) was concentration-dependent and influenced by ionic strength (Supplementary Fig. 4a). His-GFP-Hwa protein, which contained the Hwa's IDR only, formed many more and larger puncta than His-GFP-Hwa(ICD) under the same conditions (Fig. 5f, g). In contrast, His-GFP-Hwa(ICDDIDR), which contain the ICD deleted of the IDR, could hardly form condensate droplets (Fig. 5f, g). When transiently overexpressed in HeLa cells, Hwa(ICD)-GFP was present in nuclei as many distinct puncta while Hwa(ICDDIDR)-GFP formed few puncta (Supplementary Fig. 4b). Fluorescence recovery after photobleaching (FRAP) experiments demonstrated that Hwa(ICD)-GFP puncta were mobile and exhibited liquid-like properties (Supplementary Fig. 4c, d), and time-lapse imaging captured fusion of smaller puncta into larger ones (Supplementary Fig. 4e) (Supplementary Video 1 and 2). These observations collectively indicate that the intrinsic multivalent interaction property of Hwa protein in liquid is driven primarily by its IDR.

Given that both Hwa protein and its mRNA localize at the vegetal pole of the fertilized egg, we investigated if they can interact for phase transition. First, combined fluorescence *in situ* hybridization (FISH) and immunofluorescence confirmed that in 1c stage *Tg(hwa:hwa-GFP-h3'U)* embryos, Hwa-GFP protein was indeed co-localized with *hwa* mRNA in the vegetal-pole region (Fig. 5h). Second, in vitro RNA immunoprecipitation (RIP) revealed physical association of His-GFP-Hwa(ICD) protein with synthetic full-length *hwa* mRNA (Supplementary Fig. 4f). Third, in vitro phase separation assay indicated that the addition of Cy3-labeled full-length *hwa* mRNA, but not *GFP* mRNA, significantly promoted the formation of Hwa condensates (Fig. 5i, j). Finally, injection of RNase A into stage V *Tg(hwa:hwa-GFP-h3'U)* oocytes markedly reduced the number and size of Hwa-GFP condensates at the vegetal pole following

fertilization (Fig. 5k, l). These data suggest that Hwa protein and *hwa* mRNA together form protein-RNA condensates. The formation of such condensates at the vegetal pole of the fertilized egg may ensure a high local concentration of Hwa for efficiently nucleating the parallel microtubule arrays that establish embryonic asymmetry.

Correct *hwa* mRNA localization in oocytes and fertilized eggs is guided by Igf2bp3

Transcripts of *hwa* are localized in the Balbiani body (Bb) of stage I oocytes and maintain an asymmetrical distribution pattern during oocyte maturation¹⁸. To further determine the importance of *hwa* mRNA correct location in organizing the postfertilization VPM network, we set out to identify proteins associating with *hwa* transcripts in oocytes. To this end, we performed an RNA pull-down assay using the *hwa* 3'UTR as the bait and stage I/II oocyte lysate as prey pool, and identified 467 prey proteins. Cross-referencing these prey proteins with a published proteome of zebrafish Bb-enriched proteins²⁶ revealed an overlap of 85 candidates. Among these, 16 were known RNA-binding proteins, with Igf2bp3 being the most abundant (Fig. 6a). RNA pull-down assays using lysates from HEK293T cells overexpressing zebrafish Igf2bp3 confirmed its robust interaction with the *hwa* 3'UTR (Fig. 6b). Deletion mapping indicated that the first 240 nucleotides of the 3'UTR are critical for this interaction. Reciprocally, RIP assays demonstrated a specific binding of Igf2bp3 protein to the *hwa* 3'UTR *in vivo* (Fig. 6c).

To test the functional significance of this interaction, we generated two *igf2bp3* mutant lines, i.e., *igf2bp3*^{tsu+1} and *igf2bp3*^{tsu-17}, both of which introduced a premature stop codon (Supplementary Fig. 5a). Zygotic *igf2bp3*^{tsu+1} and *igf2bp3*^{tsu-17} mutants could survive to adulthood, allowing production of their maternal mutants. *Migf2bp3*^{tsu+1} mutants at the 1c stage had an *igf2bp3* mRNA level comparable to that in WT embryos while *igf2bp3* mRNA level in *Migf2bp3*^{tsu-17} mutants was reduced by 32.77% (Fig. 6d). We noted that some of *Migf2bp3*^{tsu-17} and *Migf2bp3*^{tsu+1} embryos experienced abnormal cleavage during cleavage period (Supplementary Fig. 5b), which were also seen in other maternal *igf2bp3* mutant lines^{27, 28}. At 24 hpf, about 30-60% of mutant embryos died and a small fraction of mutants displayed severely ventralized phenotypes, which varied among individual mutant females (Fig. 6e, and Supplementary Fig. 5c). These observations suggest that maternal *igf2bp3* has pleiotropic effects on early embryonic development. Examination of *hwa* mRNA distribution by WISH revealed that, in contrast to enrichment in Bb of WT stage I oocytes, *hwa* mRNA in stage I *igf2bp3*^{tsu-17} mutant oocytes was diffusive in the cytoplasm (Fig. 6f). At the 1c stage, *hwa* mRNA was more restrictive in WT embryos but was generally more diffusive in all kinds of *Migf2bp3* mutants (Fig. 6g). As examined in *Migf2bp3*^{tsu-17/tsu-17} mutants at the 2c stage, *hwa* mRNA distribution were not visibly shifted towards one side (Fig. 6g). In line with abnormal distribution of *hwa* mRNA in maternal *igf2bp3* mutants, immunofluorescence analysis revealed disorganized VPM arrays in a significant fraction of *Migf2bp3* mutants (Fig. 6h). These observations imply that the asymmetrical localization of *hwa* mRNA during oogenesis is mediated by Igf2bp3, providing a critical prerequisite for organizing the postfertilization VPM network.

We hypothesized that interruption of the postfertilization VPM network in *Migf2bp3* mutants would cause inefficient transport of the dorsal determinants including Hwa from the vegetal pole yolk to the animal-pole blastomeres after fertilization, ultimately resulting in abnormal development such as defective body axis patterning at later stages. To test this idea, we directly injected *hwa* mRNA (50 pg per embryo) into the cytoplasm of 1c stage *Migf2bp3*^{tsu+1/tsu-17} mutants and observed dorsalized phenotypes in the majority of injected embryos and a few embryos with normal body axis at 24 hpf (Fig. 6i). However, injection of *igfbp2* mRNA (200 pg per embryos) into the cytoplasm of 1c stage *Mhwa*^{tsu01} mutants was unable to induce the body axis or dorsalized phenotypes at 24 hpf (Fig. 6i). Thus, abnormal development of *Migf2bp3* mutant embryos may be partially ascribed to improper location and transport of maternal *hwa* mRNA and protein.

Hwa orchestrates vegetal microtubule network through multiple mechanisms

To elucidate how Hwa regulates the VPM network, we performed a detailed super-resolution microscopic analysis of this structure in WT and *Mhwa*^{tsu01sm} mutant embryos at 20 mpf (Fig. 7a). Quantitative analysis of microtubule orientation revealed severe disruption of the VPM organization in mutants (Fig. 7b-d). Whereas WT embryos exhibited a coherent microtubule array with low angular dispersion (15.42° on average), the mutant array was profoundly disorganized, displaying near-random orientation (48.46° dispersion on average) (Fig. 7d). Examination of microtubule length disclosed longer continuous microtubule segments of the arrays in WT than in *Mhwa*^{tsu01sm} embryos (Fig. 7e). Analysis of microtubule bundling showed that WT embryos contained thicker bundles composed of more aligned individual microtubules (43.98% bundles with more than one microtubule). In contrast, *Mhwa*^{tsu01s} mutants exhibited reduced bundling capacity, with a very low proportion (11.03%) of bundles containing more than one microtubule (Fig. 7f). Spatial distribution analysis further demonstrated that in WT embryos, microtubules were tightly associated with the cortex, concentrated within 3 µm of the cell surface. Conversely, *Mhwa*^{tsu01s} mutants showed dispersed microtubule networks extending up to 6 µm from the cortex, resulting in closer proximity to yolk granules (Fig. 7g). These observations demonstrate that Hwa is essential for governing multiple aspects of microtubule organization, including their alignment, stability, and bundling, suggesting multifaceted regulatory mechanisms.

To gain molecular insights into the early function of Hwa, we performed RNA sequencing of 1c stage *Mhwa*^{tsu01s} mutant embryos. Analysis of differentially expressed genes revealed significant alterations in maternal mRNA populations. Gene Ontology cellular component (GO-CC) analysis showed marked downregulation of cytoskeleton and microtubule-associated genes in *Mhwa*^{tsu01sm} mutants (Fig. 7h). Concurrently, KEGG pathway analysis identified significant suppression of MAPK signaling pathway components (Fig. 7i), the role of which in microtubule regulation has been previously reported^{14, 29}. Supporting this finding, pharmacological inhibition of MAPK signaling in WT fertilized eggs phenocopied the disrupted VPM organization (Fig. 7j). These results position MAPK signaling as a key downstream effector of Hwa-mediated postfertilization microtubule organization. We next asked whether Hwa might also exert direct control over the microtubule cytoskeleton by interacting with core microtubule-

associated proteins. We focused on proteins known to be involved in VPM formation, such as Kif5ba, Grip2a, Prc1l and GSK3 in zebrafish^{11, 12, 13, 16}, and Trim36 in *Xenopus*^{30, 31}, in addition to other microtubule regulators (Kif23, Apc, Mapre1b and Stmn1a)^{32, 33, 34, 35, 36}. Co-immunoprecipitation assays demonstrated that Hwa interacted with Kif5ba, Grip2a, Gsk3, Trim36, Kif23 and Apc when these were transfected into HEK293T cells (Fig. 7k, l). Since the roles of Kif5ba and Grip2a in zebrafish VPM organization are well established, we functionally tested the novel candidates Trim36, Kif23, and Apc by injecting mRNAs encoding their dominant-negative mutants into zebrafish oocytes to disrupt their activity. For Trim36, we introduced point mutations (C217A/H220A) in the conserved B-box 2 domain, which are known to disrupt its function in *Xenopus*³⁰. For Apc, we used a C-terminal truncated construct (amino acids 1–1000) lacking the microtubule-binding domain³⁴. For Kif23, we employed a deletion mutant (amino acids Δ 24-434) lacking the N-terminal kinesin motor domain³³. Results showed that overexpression of Trim36 and Apc dominant-negative variant disrupted the formation of the VPM network (Fig. 7m) but Kif23(Δ 24-434) overexpression had no effect. These observations imply that maternal Hwa signaling may regulate multiple microtubule regulators to organize the postfertilization VPM network in zebrafish.

Discussion

In this study, we demonstrate that *hwa* mRNA and protein stored in mature oocytes play a previously unrecognized role in organizing the highly ordered VPM arrays upon fertilization and this role is also necessary for embryonic axis specification at later developmental stages. In WT oocytes, maternal *hwa* mRNA is anchored to the vegetal cortex via its 3' UTR. Locally translated Hwa protein forms condensates with its mRNA, reinforcing its vegetal pole enrichment. At this site, Hwa may directly interact with microtubule-associated proteins and modulates signaling pathways such as MAPK, thereby ensuring the formation of parallel, bundled microtubule arrays upon fertilization. Consequently, maternal determinants including Hwa itself are asymmetrically transported along these microtubules, leading to preferential Hwa mRNA/protein accumulation in blastomeres on one side of the embryo. By contrast, *hwa*^{tsu01sm} mutant oocytes lack both *hwa* mRNA and protein and subsequent fertilized egg fail to form properly aligned, bundled microtubules at the vegetal pole. This defect disrupts the directional transport of maternal dorsal determinants and ultimately impairs embryonic polarity establishment and body axis formation (Supplementary Fig. 6). Thus, our findings redefine the functional timeline of Hwa: first in microtubule-mediated transport of maternal factors upon fertilization and then in activating organizer-specific β -catenin signaling during blastula stages.

A notable phenotypic distinction emerges when comparing maternal mutants that affect the vegetal microtubule array. Mutations in *grip2a* or *kif5ba* disrupt postfertilization parallel microtubule bundle formation and cause embryonic ventralization at later stages, yet these phenotypes exhibit variable expressivity, with embryos showing differing degrees of microtubule disorganization and ventralization^{11, 12}. In stark contrast, *Mhwa*^{tsu01sm} mutants display a highly uniform, fully penetrant phenotype: all mutant embryos exhibit complete disorganization of the vegetal microtubule network and consistently

develop the most severely ventralized morphology by 24 hpf. Moreover, whereas microtubule defects in *nanog* mutants can be rescued by pregnenolone (P5) supplementation¹⁷, restoring the microtubule network in *Mhwa* mutants requires local reintroduction of Hwa itself. Importantly, although overexpression of other microtubule-associated genes such as *kif5ba*, *grip2a*, and *prc1l* fails to induce dorsal organizer formation, maternal Hwa uniquely integrates the capacity to organize the VPM network with the ability to initiate dorsal axis specification.

RNA sequencing of *Mhwa*^{*tsu01sm*} mutant embryos at the 1-cell stage revealed transcriptomal alterations with downregulation of genes related to cytoskeleton, metabolism and some signaling pathways (Fig. 7h, i). This observation suggests that Hwa protein is functional during oocyte maturation. Given that the postfertilization VPM organization in *Mctnnb2* mutants appears unaffected, Hwa protein may not regulate transcription during oogenesis by activating β -catenin signaling. It will be interesting to identify Hwa's downstream signaling pathways in oocytes in the future. We showed that Hwa protein has the capacity to physically associate with some microtubule regulators (Fig. 7l). It remains unknown if Hwa regulates posttranslational modifications or activity of these microtubule regulators.

Based on our findings that Igf2bp3 regulates the vegetal localization of *hwa* mRNA, together with existing literature, we propose that Igf2bp3 plays a fundamental role in establishing mRNA polarity during oogenesis. In zebrafish maternal *igf2bp3* mutants, while the overall levels of maternal mRNAs remain largely unaffected, the specific vegetal localization of several key transcripts, including *dazl*, *wnt8a*, and as we now demonstrate *hwa*, is disrupted in 1-cell embryos²⁷. This fact supports a model in which Igf2bp3 is specifically required for the recruitment of a subset of maternal mRNAs into the Bb during early oogenesis, thereby ensuring their subsequent anchoring at the vegetal cortex. Both the initial recruitment into the Bb and the subsequent maintenance of vegetal localization during oocyte maturation appear to be critical for the precise cortical anchoring of *hwa* mRNA. The conservation of this mechanism is highlighted by studies in *Xenopus*, where Igf2bp3 has been shown to mediate the vegetal localization of *Vg1* mRNA in 1-cell embryos, further supporting its evolutionarily conserved role in mRNA spatial organization³⁷.

Methods

Zebrafish culture Zebrafish Tübingen line was maintained in the Meng Lab. The laboratory animal facility was accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International), and the IACUC (Institutional Animal Care and Use Committee) of Tsinghua University approved all animal protocols used in this study. Fish were maintained at 28.5 °C. One female and one male fish were separated in a mating tank at night and allowed their mating by removing the separator next morning at a desired time, followed by collecting and incubate fertilized eggs to desired stages. Stage V oocytes were squeezed from female and maintained in oocyte culture medium (90% Leibovitz's L-15 medium (Gibco), 0.5 mg/ml BSA (Amresco)) until activation with Holtfreter's solution at the desired time.

Capped mRNAs were synthesized with mMESSAGE mMACHINE™ SP6 (AM1340, Thermo Fisher), purified with the RNeasy Mini kit (74104, Qiagen) according to the manufacturers' instructions, and injected into the yolk at 1-cell stage. For microtubule disruption, embryos at 5 mpf were treated with 250 nM nocodazole for 10 min. To inhibit MAPK signaling, stage V oocytes were incubated with 10 μ M PD0325901 for 1.5 hours prior to activation. Tol2-mediated transgenesis was conducted utilizing the previously established system³⁸. A 1479 bp promoter sequence upstream of the zebrafish *hwa* transcription start site was amplified to drive targets genes maternal expression. Zebrafish knock out mutants was generated by the CRISPR/Cas9 system as described before¹⁸. The primers for mutant identification were listed at Supplementary table 1.

Plasmid constructs HA tagged wild-type Hwa expression plasmids was previously reported¹⁸. Zebrafish *igf2bp3*, *grip2a*, *prc1l*, *kif5b*, *gsk3b*, *trim36*, *apc (1-1000)*, *mapre1b*, *stmn1a* and *kif23* were cloned and constructed into pCS2 backbone with a Flag tag to the N-terminal or C-terminal of the coding sequence, respectively. Hwa-GFP was constructed into pCS2 backbone with GFP tag to the C-terminal. Deletion mutations were introduced into the expression plasmids via a PCR-based point mutation strategy. For bacterial expression and protein purification, Hwa was subcloned into the pRSF-1b vector with an N-terminal His-GFP tag.

Cell culture, immunoblotting and coimmunoprecipitation HeLa and HEK293T cells were cultured at 37 °C with 5% CO₂ in DMEM supplemented with 10% FBS (HyClone), 100 U/ml penicillin and 100 mg/ml streptomycin. Transfections were performed with VigoFect (T001, Vigorous) according to the manufacturer's instructions. Western blots and coimmunoprecipitation were performed as previously described¹⁸. The following commercial antibodies were used in this study: anti-Flag (M185-3L, MBL; AE092, Abclonal), anti-HA (sc-7392, Santa Cruz).

RNA extraction and gene expression analysis Total RNA was isolated from embryos at specified developmental stages using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized from 2 μ g of total RNA using the SuperScript VILO Master Mix (Life Technologies). Real time quantitative PCR (RT-qPCR) was performed in triplicate with SYBR Green PCR Master Mix (Applied Biosystems) following the manufacturer's protocol. Transcript levels were normalized to β -actin unless otherwise noted. Primer sequences used for RT-qPCR are listed in Supplementary Table 1.

Oocyte microinjection *in situ* (OMIS) The OMIS procedure was adapted from established protocols to enable the injection of stage III oocytes within adult female zebrafish²¹. Briefly, 5-12 months old females, pre-screened for high fecundity, were anesthetized with 550 μ g/ml tricaine (Sigma, A5040). A small incision (~4-6 mm) was made on the abdominal side to expose the ovary. Approximately 0.2 nL of microinjection solution, containing MO or mRNA, with rhodamine B (Sigma, R8881) as a tracer in a physiological salt buffer, was pressure-injected into individual stage III oocytes. The abdominal wound was subsequently sutured, and the female was revived in antibiotic-supplemented fish water. To obtain embryos derived from the injected oocytes, the female was paired with a male on the evening of the

second day. Spawning was induced the following morning, and successfully manipulated embryos were identified and selected based on rhodamine B fluorescence for subsequent analysis.

Immunostaining For cell line Immunostaining, cells were fixed in 4% PFA for 30 min. After extensive washing with PBS, cells were incubated in blocking buffer (3% BSA, 0.1% TritonX-100 in PBS) for 1 h at RT and then incubated with primary antibodies in blocking buffer at 4°C overnight. Cells were washed three times for 15 mins each, and incubated with DAPI and secondary antibodies in in blocking buffer for 1 h at RT. Cells were washed three times for 15 mins each. For whole-mount IF of microtubules, eggs and embryos were fixed with microtubule staining buffer (80 mM K-PIPES pH 6.8, 5 mM EGTA, 1 mM MgCl₂, 3.7% formaldehyde, 0.25% glutaraldehyde, 0.2% Triton X-100) for 4-5 h at RT. Staining was performed immediately. Anti- β -tubulin (MAB3408, Millipore) was diluted at 1:500, Alexa Fluor 488 and Alexa Fluor 568 (Molecular Probes) secondary antibodies were diluted at 1:400. Before imaging, embryos were oriented in glass-bottom dishes using 1.5% low-melting-point agarose for optimal positioning. Fluorescent images were acquired using Olympus FV3000 lasers scanning confocal microscope or Multimodality Structured Illumination Microscopy (Multi-SIM). Three-dimensional reconstruction of microtubules and measurement of their length were performed using Imaris software (version 10.1). To quantify microtubule alignment and angular dispersion, image data were analyzed with the Directionality plugin in ImageJ. For the cortical granules (CGs) exocytosis test, over 30 ovulated eggs at 1 mpa (minutes post activation), 5 mpa, and 10 mpa in Holtfreter's solution were collected and fixed with 4% paraformaldehyde overnight before further steps. CGs were visualized by staining embryos with 50 μ g/ml IFluor 488-Wheat Germ Agglutinin (WGA) (I3300, Solarbio) as previously described^{39, 40}. TRITC-Phalloidin (40734ES75, YEASEN) for F-actin staining. Fluorescent images were acquired using Olympus FV3000 lasers scanning confocal microscope and analyzed with the ImageJ.

Whole mount *in situ* hybridization (WISH) and fluorescence *in situ* hybridization (FISH) Zebrafish embryos that reached the desired stages were fixed in 4% paraformaldehyde. The linearized plasmids or PCR-amplified DNA fragments were used as templates for in vitro synthesis of Digoxigenin or Fluorescein -UTP labeled antisense RNA probes. The primers for antisense probe templates were listed at Supplementary table 2. WISH was performed essentially as before¹⁸. FISH combined with antibody staining was performed as reported⁴¹. WISH images were acquired using Nikon stereomicroscope (SMZ1500), while FISH images were acquired using Olympus FV3000 lasers scanning confocal microscope.

Identification of endogenous Hwa protein Approximately 5,000 dechorionated embryos at the one-cell stage were thoroughly lysed in cell lysis buffer supplemented with protease inhibitors. The lysate was first processed using a 50-kDa molecular weight cut-off (MWCO) ultrafiltration device to remove proteins larger than 50 kDa. The resulting flow-through was subsequently concentrated using a 10-kDa MWCO device, yielding a fraction hypothesized to contain the Hwa protein. To enhance detection sensitivity for the potentially low-abundance Hwa, the concentrated fraction was subjected to immunoprecipitation using a commercially purified anti-Hwa antibody as reported before¹⁸. The resulting immunoprecipitate was then prepared for LC-MS/MS analysis.

His-GFP-Hwa protein purification The His-GFP-tagged Hwa protein was expressed in *Escherichia coli* strain BL21(DE3). Protein expression was induced with 1 mM IPTG at an OD600 of 0.6-0.8, followed by incubation at 37°C for 4 h. As the protein was primarily localized in inclusion bodies, the cell pellet was resuspended in TBS supplemented with 1 mM PMSF and lysed by sonication on ice. The insoluble fraction was collected by centrifugation at 12,000 ×g for 30 min at 4°C. The resulting pellet was subsequently washed with TBS containing 2 M urea and then solubilized in TBS with 6 M urea for 30 min at room temperature. The filtrate was incubated with Ni-NTA resin that had been pre-equilibrated with TBS containing 6 M urea for 1 h at 4°C. The resin was sequentially washed with TBS containing 6 M urea and 1 M NaCl, followed by TBS with 6 M urea and 20 mM imidazole. The target protein was eluted twice using TBS with 6 M urea and 250 mM imidazole. The eluate was dialyzed overnight at 4°C against phase separation buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) with three buffer changes. Precipitates formed during dialysis were removed by centrifugation. The supernatant was analyzed or stored at –80°C for subsequent experiments.

In vitro phase separation assay Proteins dissolved in a buffer containing 20 mM HEPES, pH 7.4, 500 mM NaCl were mixed, and the concentration of NaCl was adjusted to 150 mM with a buffer containing 20 mM HEPES, pH 7.4. The mixture was treated immediately with PEG8000, and the concentration of NaCl was further adjusted to 150 mM NaCl and then puncta formation was examined. For imaging, puncta were observed either on a glass slide or in a glass-bottom cell culture dish for fluorescence imaging.

Fluorescence recovery after photobleaching (FRAP) analysis FRAP experiments were performed on a Nikon A1 HD25 confocal microscope equipped with a 100×/1.45 NA oil-immersion objective at room temperature. Cells were transfected 24 hours prior to imaging. Defined regions of interest (ROIs) were photobleached, and fluorescence recovery was monitored by collecting images every 7 seconds. Fluorescence intensity within the bleached ROI was measured, normalized to the pre-bleach intensity, and analyzed using GraphPad Prism software.

RNA pull-down assay RNA pull-down assays were performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (20163, Thermo Fisher Scientific) according to the manufacturer's instructions with minor modifications. Briefly, biotin-labeled RNA transcripts (target and antisense control) were synthesized in vitro. For each reaction, 1-2 µg of labeled RNA was diluted in RNA Structure Buffer, heated to 95°C for 2 minutes, and then incubated on ice for 3 minutes to facilitate proper secondary structure formation. The folded RNAs were then mixed with pre-washed Streptavidin Magnetic Beads and incubated at room temperature for 30 minutes with gentle rotation to allow immobilization. Subsequently, the RNA-bound beads were incubated with 500 µg of pre-cleared zebrafish stage I/II oocyte lysate, prepared in IP Lysis Buffer supplemented with RNase inhibitor and protease inhibitor cocktail, for 60 minutes at 4°C with constant mixing. After extensive washing with Wash Buffer, the proteins specifically bound to the bait RNA were eluted with Elution Buffer. The eluates were then analyzed by mass spectrometry for unbiased identification. For experiments examining Igf2bp3, HEK293T cells were transfected with the relevant plasmid 36 hours before lysis. Cell lysates were then subjected to the RNA pull-down procedure described above, and bound proteins were detected by western blotting.

RNA Immunoprecipitation (RIP) Assay RNA immunoprecipitation was performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (17-700, Millipore) following the manufacturer's protocol. HEK293T cells were transfected 36 hours before. Then cell lysed using the supplied RIP Lysis Buffer containing protease and RNase inhibitors. The cell lysate was incubated with magnetic beads pre-conjugated with 5 µg of specific antibody against the target protein or normal IgG as a negative control for 2 hours at 4°C with gentle rotation. After extensive washing with RIP Wash Buffer, the immunoprecipitated complexes were treated with Proteinase K to digest proteins. RNA was then isolated from the complexes by phenol-chloroform extraction and ethanol precipitation. The purified RNA was subsequently reverse-transcribed into cDNA, and the enrichment of specific target RNAs was analyzed by quantitative PCR (qPCR). Input samples, representing 10% of the total lysate used for each immunoprecipitation reaction, were processed in parallel for normalization.

RNA sequencing and data analysis Total RNA was isolated from *Mhwa*^{tsu01sm} mutant and wild-type embryos at 10 mpf (n=50 embryos per genotype; three biological replicates each) using TRIzol reagent (Invitrogen). cDNA was amplified from 1 µg of total RNA using the Single Cell Full-Length mRNA-Amplification Kit (N712, Vazyme). Sequencing libraries were then constructed with the TruePrep DNA Library Prep Kit V2 for Illumina (TD503, Vazyme). Paired-end 150-bp sequencing (PE150) was performed on an Illumina HiSeq platform by Novogene. Clean reads were aligned to the zebrafish reference genome (GRCz11) using STAR (v2.7.8a). The gene expression level was calculated by feature Counts v2.0.3. P value ≤ 0.05 and |log₂FC| > 2 were considered significant. Gene Ontology (GO) and KEGG pathway enrichment analyses were performed on significantly downregulated genes using the DAVID web tool. The raw RNA-seq data generated in this study have been deposited in the CNCB Genome Sequence Archive (GSA) under accession number CRA036720.

Statistical analysis Statistical analyses were performed using Prism (GraphPad Software) to generate curves or bar graphs. An average from multiple samples was expressed as mean ± SD (standard deviation). Two-tailed unpaired t test was used for statistical analysis of two groups of samples. *Chi-square* was used to evaluate the statistical significance of mRNA shifted. *P< 0.05, **P< 0.01, ***P< 0.001 and ****P< 0.0001. P> 0.05 was considered not significant (ns).

Declarations

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Author Contributions

X.L. and A.M. designed the study and wrote the manuscript. X.L. performed the majority of the experiments and data analysis. F.C. generated the *ctnnb2* knock-out zebrafish line. Y.L. helped to generate the transgenic zebrafish lines. A.T. assisted in data analysis. T.L. provided key reagents. J. C. contributed to the experimental design and provided knock-in zebrafish lines. A.M. conceived and supervised the project. X.W. supervised the project and helped analyze the data.

Competing Interests

The authors declare no potential conflicts of interest.

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Figures

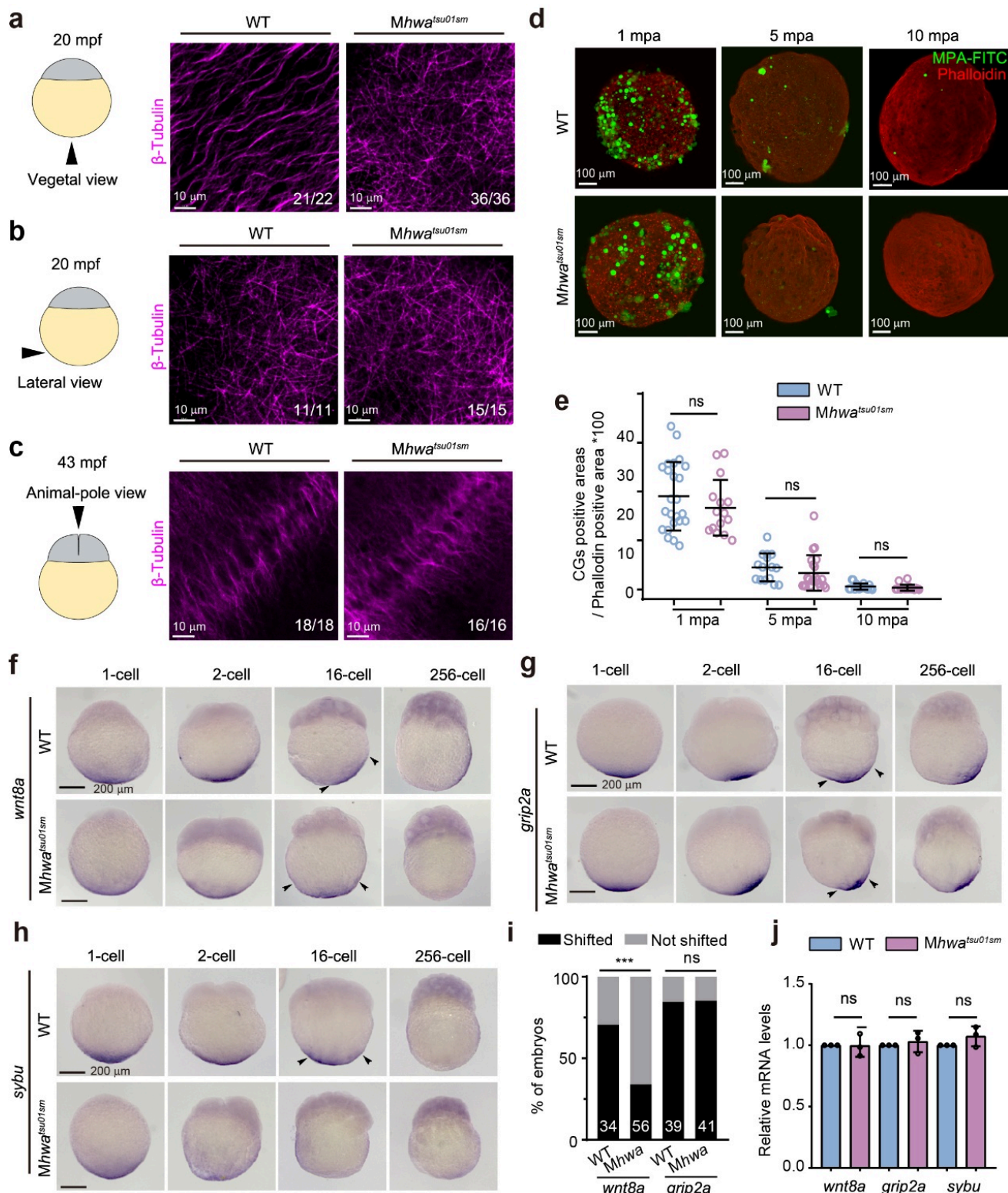


Figure 1

Abnormal vegetal parallel microtubule arrays in *Mhwa^{tsu01sm}* mutants.

a-c, Representative confocal immunofluorescent images of β -Tubulin showing microtubule organization in wild-type (WT) and *Mhwa^{tsu01sm}* mutant embryos at 20 mpf. Imaging direction was indicated on the left and the ratio of embryos with the representative pattern. **d** and **e**, Representative confocal images (**d**)

and number (**e**) of cortical granules labeled with Wheat Germ Agglutinin (WGA, green) and actin (phalloidin, red) in WT and *Mhwa^{tsu01sm}* mutant activated eggs. mpa, minutes post-activation. For quantification, GC number was counted from 24, 15, 15, 22, 17, 17 activated eggs in each group and data were presented as mean $\pm$ SD, two-tailed unpaired Student's t-test; ns, not significant. **f-h**, Dynamics of indicated mRNA location shift (examined by WISH) during early development. Arrowheads denoted RNA localization domains at the 16-cell stage. **i**, Proportion of embryos with dorsal translocation of indicated RNA at 45 mpf. Sample sizes were indicated; Chi-square test was performed; ***P= 0.0006; ns, not significant. **j**, Relative mRNA levels of *wn t8a*, *grip2a*, and *sybu* in indicated embryos at 1-cell stage. Data were mean $\pm$ SD.; two-tailed unpaired Student's t-test; ns, not significant.

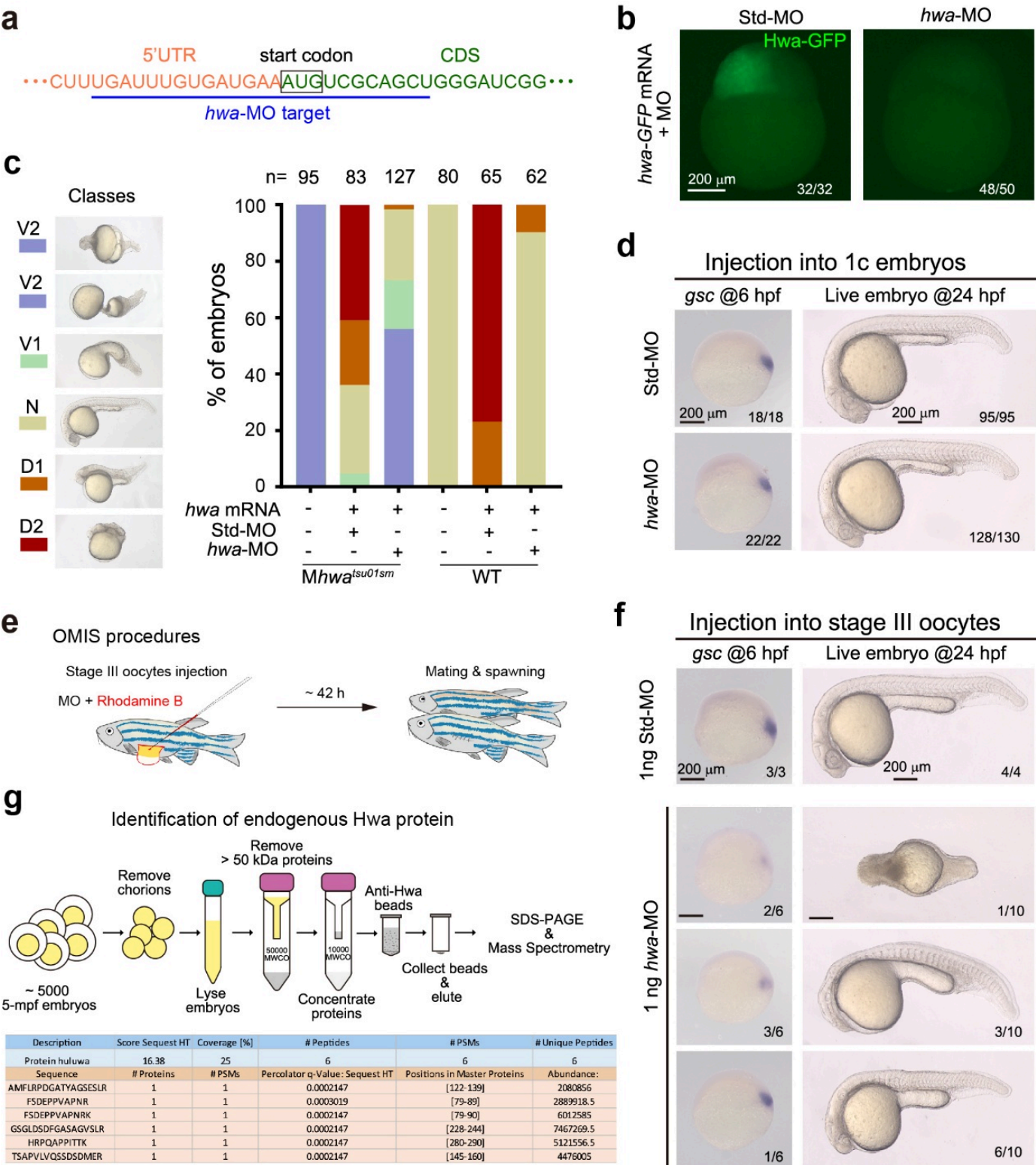


Figure 2

Maternal Hwa protein is present at the 1-cell stage.

a, Schematic of the *hwa* morpholino (MO) targeting sequence. The 5' untranslated region and (5'UTR) and coding sequence (CDS) of *hwa* mRNA were indicated by different colors. **b**, Demonstration of *hwa*-MO effectiveness. One-cell stage WT embryos were co-injected with *hwa*-GFP mRNA and MO, followed

by fluorescence observation at 3 hpf. The proportion of embryos with the representative pattern were indicated. **c**, Phenotypic changes of 24-hpf stage WT or *Mhwa*^{*tsu01sm*} embryos injected with *hwa* mRNA or/and *hwa*-MO at 1c stage. Left, phenotype classes: N, normal; V1, moderately ventralized; V2, most severely ventralized; D1, mildly dorsalized; D2, severely dorsalized. Right, ratio of embryos in different classes. Injection dose per embryo: 50 pg *hwa-Flag* mRNA, or/and 10 ng MO; n, number of observed embryos. **d**, Postfertilization *hwa* knockdown unaltered dorsoventral patterning in WT embryos. Embryos were injected at the dose of 15 ng and examined for *gsc* expression by WISH and for morphological changes at 24 hpf. The ratio of embryos with the representative pattern was indicated. **e**, Schematic overview of the oocyte microinjection *in situ* (OMIS) procedure. **f**, Effect of *hwa* knockdown in WT stage III oocytes. Oocytes were injected with 1 ng MO via OMIS and resulted embryos were examined later for *gsc* expression and morphological changes. The number of embryos with the representative patterns were indicated. **g**, Identification of Hwa protein in WT fertilized eggs by mass spectrum. Top, illustration of strategy; bottom, identified Hwa-derived peptides.

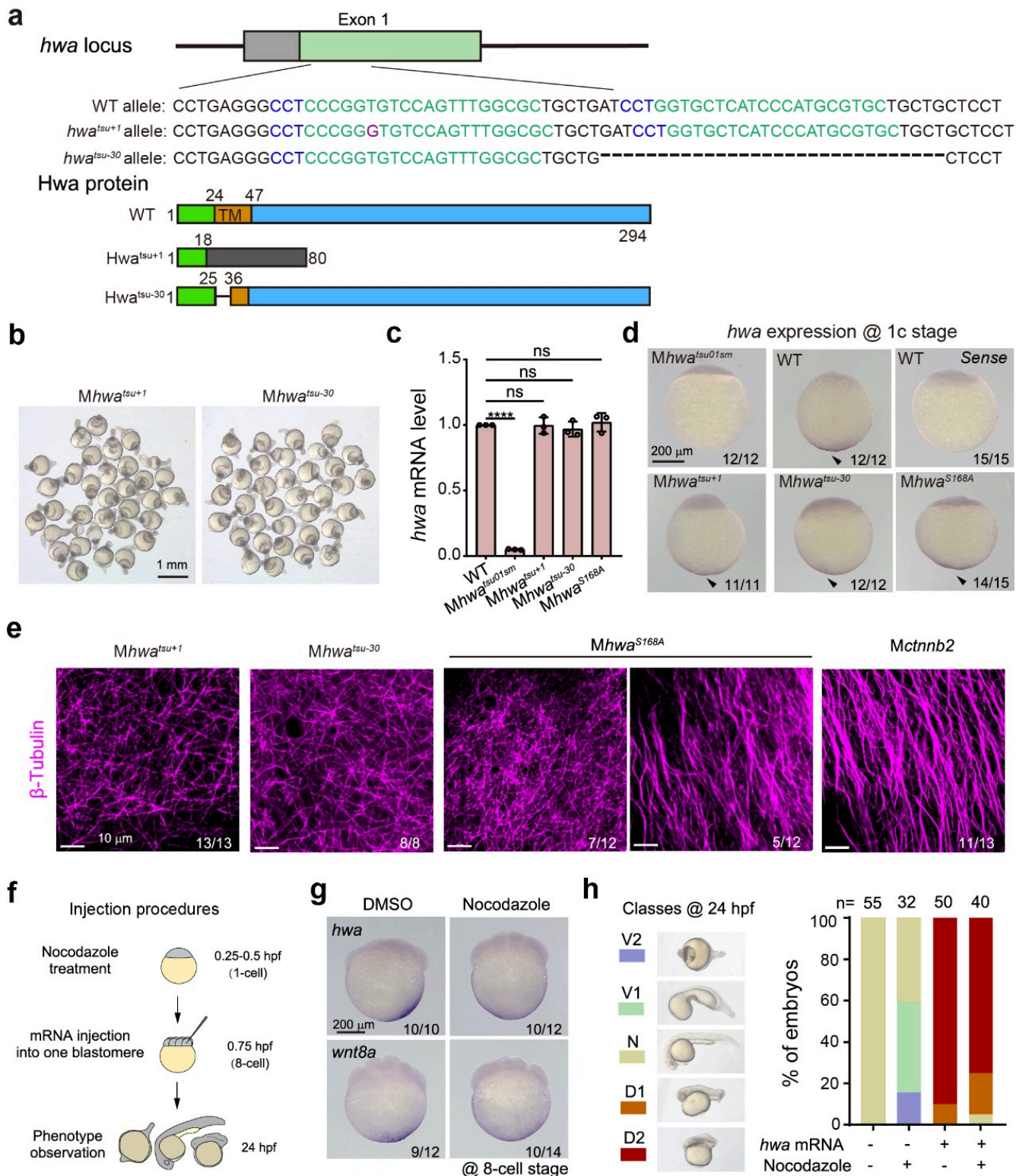


Figure 3

Maternal Hwa protein functions for VPM network formation.

a, Generation of new *hwa* mutant alleles by CRISPR/Cas9 technology. Mutated DNA and putative protein sequences of two new mutant alleles were shown. TM, the transmembrane domain. **b**, Group images of Mhwa^{tsu+1} and Mhwa^{tsu-30} mutant embryos at 24 hpf. All mutants showed completely absence of the dorsal axis. **c**, Relative *hwa* mRNA levels examined by RT-qPCR analysis in indicated embryos at the 1c

stage. Data were mean $\pm$ SD; two-tailed unpaired t-test; ns, not significant; ****P < 0.0001. **d**, Localization of *hwa* mRNA in indicated embryos at the 1c stage. The ratio of embryos with representative pattern was shown with arrowheads pointing to the vegetal pole area. **e**, Organization of vegetal cortical microtubules detected by immunofluorescence of β -Tubulin in indicated maternal mutants at 20 mpf. Embryos were imaged from the vegetal pole by confocal microscopy. The ratio of embryos with the representative pattern was shown. **f**, Experimental scheme of nocodazole treatment combined with mRNA injection. **g**, Examination of *hwa* and *wnt8a* transcript location by WISH in nocodazole-treated embryos at the 8-cell stage. The ratio of embryos with the representative pattern was indicated. **h**, Rescue effect of *hwa*-Flag mRNA overexpression (20 mg per embryo in nocodazole-treated embryos. Left, phenotypic classification with N for normal, V for ventralized and D for dorsalized. Right, the ratio of embryo classes. n, total number of observed embryos.

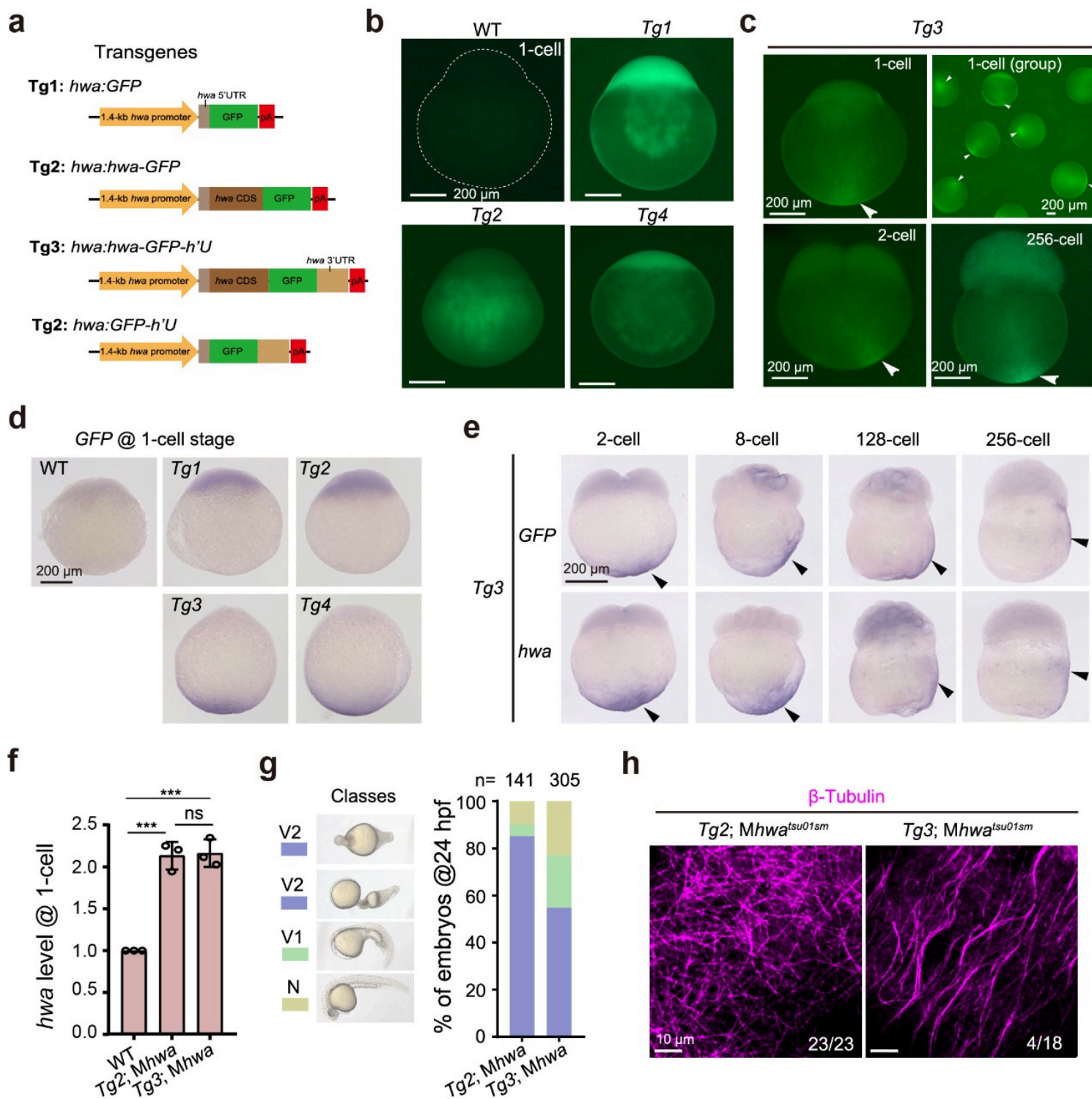


Figure 4

Detection of maternally expressed transgenic Hwa-GFP protein and mRNA.

a, Diagrams of transgenes. pA, polyadenylation signal. **b**, **c**, Representative fluorescent images of 1-cell stage transgenic embryos. Arrowheads indicated vegetally enriched Hwa-GFP in *Tg3* embryos (**c**). **d**, Transgene mRNA distribution in indicated transgenic embryos, detected by WISH using a *GFP* antisense probe. **e**, Distribution of transgenic *GFP* and *hwa* mRNAs in *Tg3* transgenic embryos at indicated stages. Arrowheads indicated asymmetrical shift of the transcripts. **f**, qRT-PCR detection of *hwa* mRNA levels in

WT embryos and *Tg2;Mhwa*^{*tsu01sm*} and *Tg3;Mhwa*^{*tsu01sm*} transgenic mutant embryos at the 1c stage. Data were mean ± SD; two-tailed unpaired *t*-test; ns, not significant; *****P* < 0.0001. Fluorescence imaging of *Tg3* embryos at indicated stages. Scale bar, 200 μm. **g**, Ratio of embryos with a representative phenotype (left panel) in *Tg2;Mhwa*^{*tsu01sm*} and *Tg3;Mhwa*^{*tsu01sm*} transgenic mutant embryos at 24 hpf. *n*, the number of observed embryos. **h**, Organization of vegetal cortical microtubules detected by immunofluorescence of β-Tubulin in *Tg2;Mhwa*^{*tsu01sm*} and *Tg3;Mhwa*^{*tsu01sm*} transgenic mutant embryos at 20 mpf. The ratio of embryos with the representative pattern was indicated.

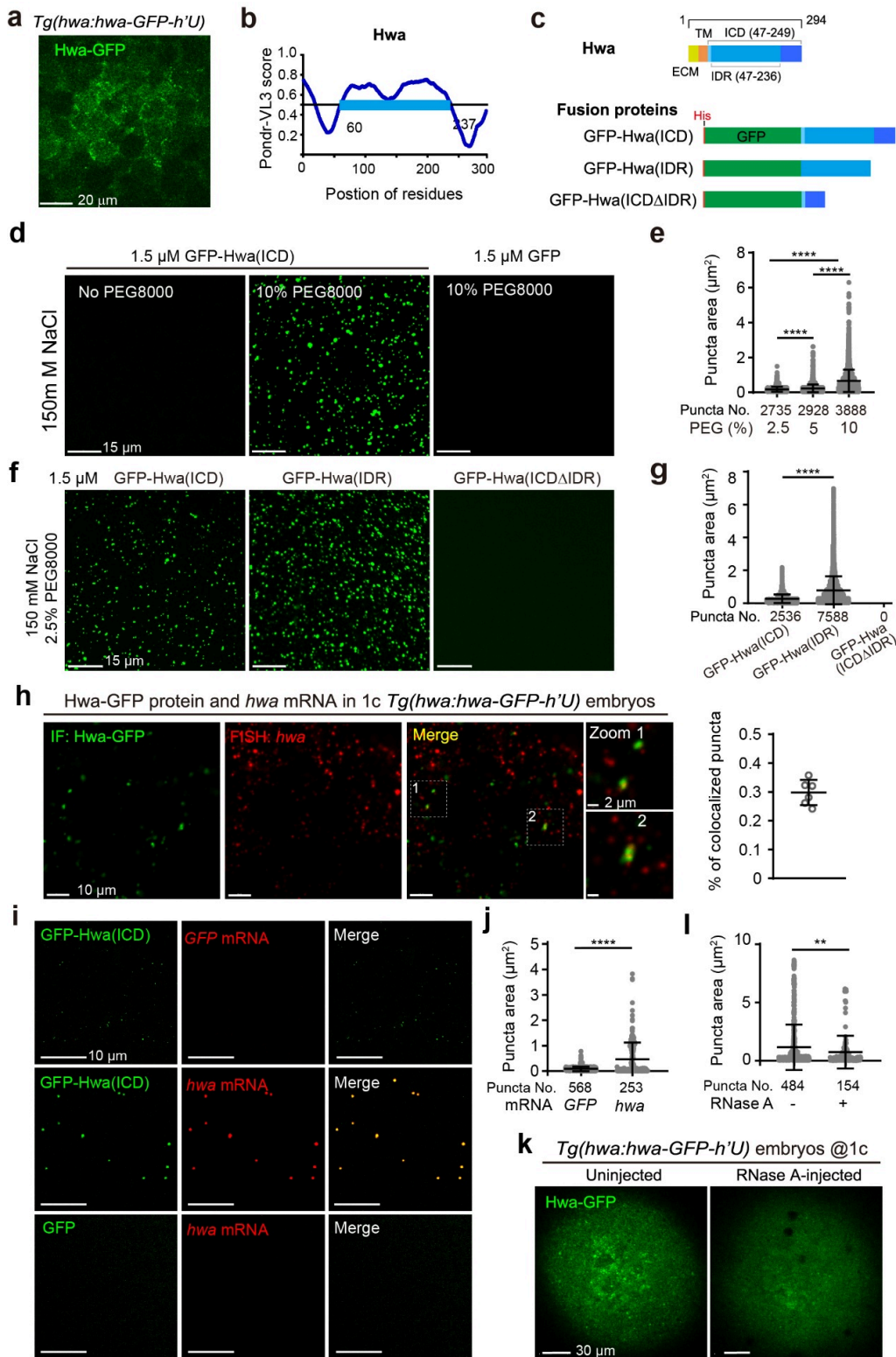


Figure 5

Hwa protein interacts with its mRNA to form condensates.

a, Condensates of Hwa-GFP protein in *Tg(hwa:hwa-GFP-h'U)* fertilized eggs. The fertilized egg was viewed from the vegetal pole. **b**, Prediction of the disordered regions in Hwa protein (POND-R). The region spanning 60th to 237th residues showed high disorder propensity. **c**, Composition of Hwa protein and its mutant variants. ECM, extracellular domain; TM, transmembrane domain; ICD, intracellular domain; IDR,

intracellular disordered region. **d** and **e**, Representative images showing formed droplet formation (**d**) of His-GFP-Hwa(ICD) and statistical data (**e**). Purified proteins were used. **f** and **g**, Droplet formation of Hwa variant proteins (**f**) and quantification (**g**). **h**, Colocalized Hwa-GFP protein and *hwa* mRNA in 1-cell stage *Tg(hwa:hwa-GFP-h'U)*transgenic embryos (vegetal view). Hwa-GFP was detected by immunofluorescence with GFP antibody and *hwa* (and *hwa-GFP*) mRNA was detected by fluorescent *in situ* hybridization (FISH) using *hwa* antisense probes. Left panel, representative images. Zooms 1 and 2 showed enlarged areas. Right, percentage of colocalized puncta in 6 embryos. **i** and **j**, Effect of interaction between GFP or GFP-Hwa(ICD) proteins and *GFP* or *hwam*RNA on in vitro droplet formation. Purified protein and in vitro synthesized mRNA were used. Left, representative confocal images; right, statistical results. For statistical analyses in (**e**, **g**, and **i**), two-tailed unpaired *t*-test was performed with **** $P < 0.0001$. **k** and **l**, Effect of RNA treatment on Hwa-GFP puncta in *Tg(hwa:hwa-GFP-h'U)*transgenic embryos at the 1c stage. Representative images were viewed from the vegetal pole (**k**) and statistical data from 3 embryos were shown (**l**). RNase A was injected into stage V oocytes and then in vitro fertilization was done. Two-tailed unpaired *t*-test was performed with ** $P = 0.0043$.

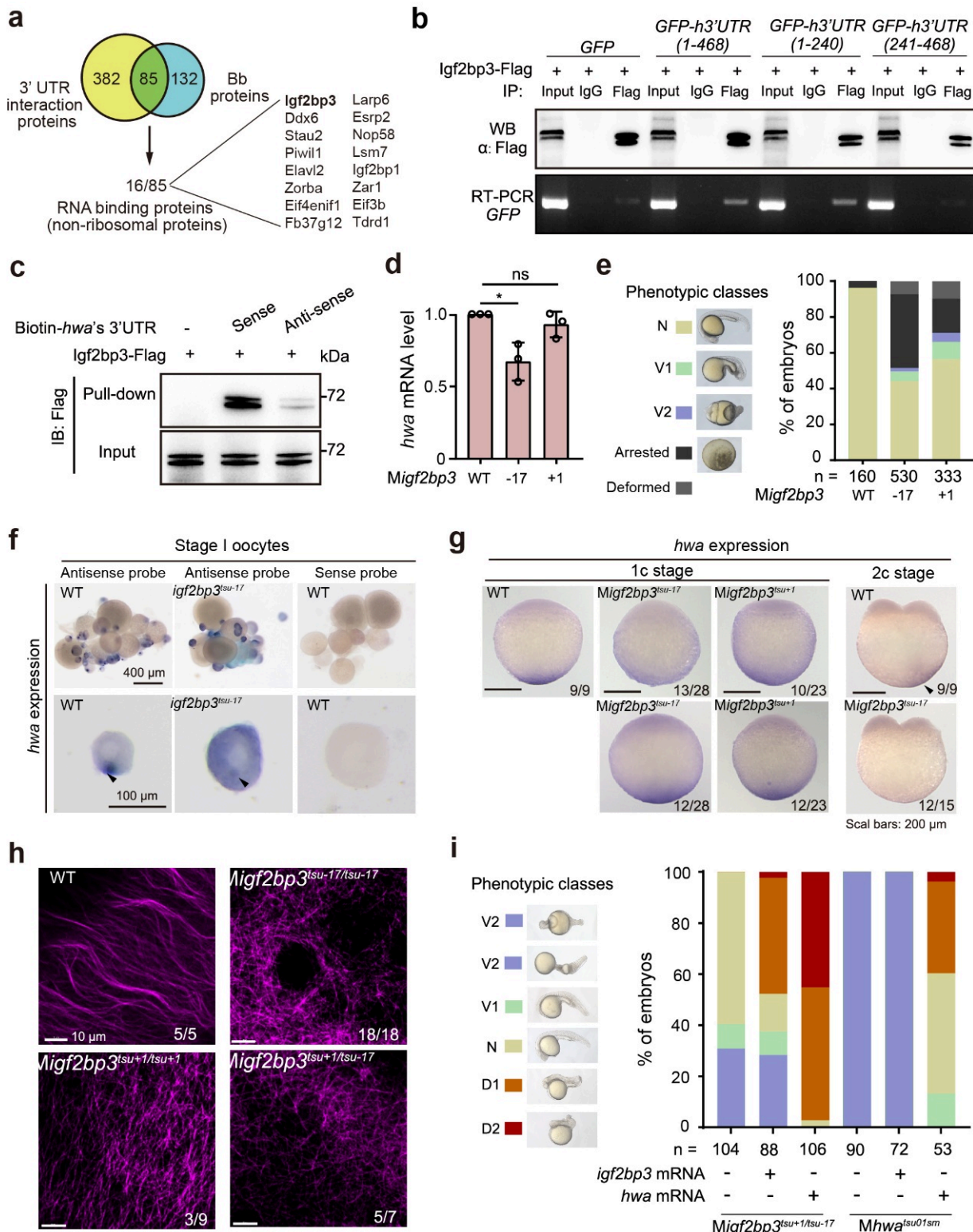


Figure 6

Proper distribution of *hwa* mRNA in oocytes and fertilized eggs depends on Igf2bp3.

a, Proteins pulled-down using *hwa* 3'UTR from stage I/II oocytes. 16 RNA-binding proteins among *hwa* RNA-bound Bb proteins were highlighted on the right according to their abundance. **b** and **c**, Interaction between Igf2bp3 and *hwa* 3' UTR validated by RNA immunoprecipitation (RIP) (**b**) and RNA pull-down (**c**).

For RIP, zebrafish *Igf2bp3* tagged with Flag as well as *GFP* or *GFP-hwa* 3'UTR variants were transfected into HEK293T cells, followed by immunoprecipitation of Igf2bp3-Flag and RT-PCR detection of *GFP* mRNA; For RNA pull-down, biotin-labeled *hwa* 3'UTR was used to pull down Igf2bp3 overexpressed in zebrafish embryos. **d**, Relative *hwa* mRNA level in *Migf2bp3* mutants of different lines at the 1c stage, detected by RT-qPCR. Two-tailed unpaired *t*-test was performed with * for $P = 0.0499$ and ns for nonsignificant. **e**, Ratios of embryos with different phenotypes in different maternal *igf2bp3* mutant lines at 24 hpf. Left, classification of mutants; right, ratio. n, number of observed embryos. Data were pooled from offsprings of several mutant females. **f**, *hwa* mRNA location in WT or *igf2bp3*^{tsu-17/tsu-17} mutant oocytes, examined by WISH. Note diffusive distribution of *hwa* mRNA in mutant oocytes. **g**, *hwa* mRNA location in 1c and 2c stage embryos. Note that *hwa* transcripts were more diffusive in mutants than in WT embryos at the 1c stage and were not shifted in mutants at the 2c stage. **h**, Organization of the VPM in *Migf2bp3* fertilized eggs at 20 mpf, indicated by immunofluorescent β -Tubulin. **i**, Rescue effect of *hwa* or *igf2bp3* overexpression on ventralized phenotypes of *Migf2bp3*^{tsu+1/-17} or *Mhwa*^{tsu01sm} mutants. Mutant embryos at the 1c stage were injected with *hwa* mRNA (50 pg/embryo) or *igf2bp3* mRNA (200 pg/embryo) and observed for morphology at 24 hpf. Left, phenotype classification; right, ratio of embryos. n, number of observed embryos.

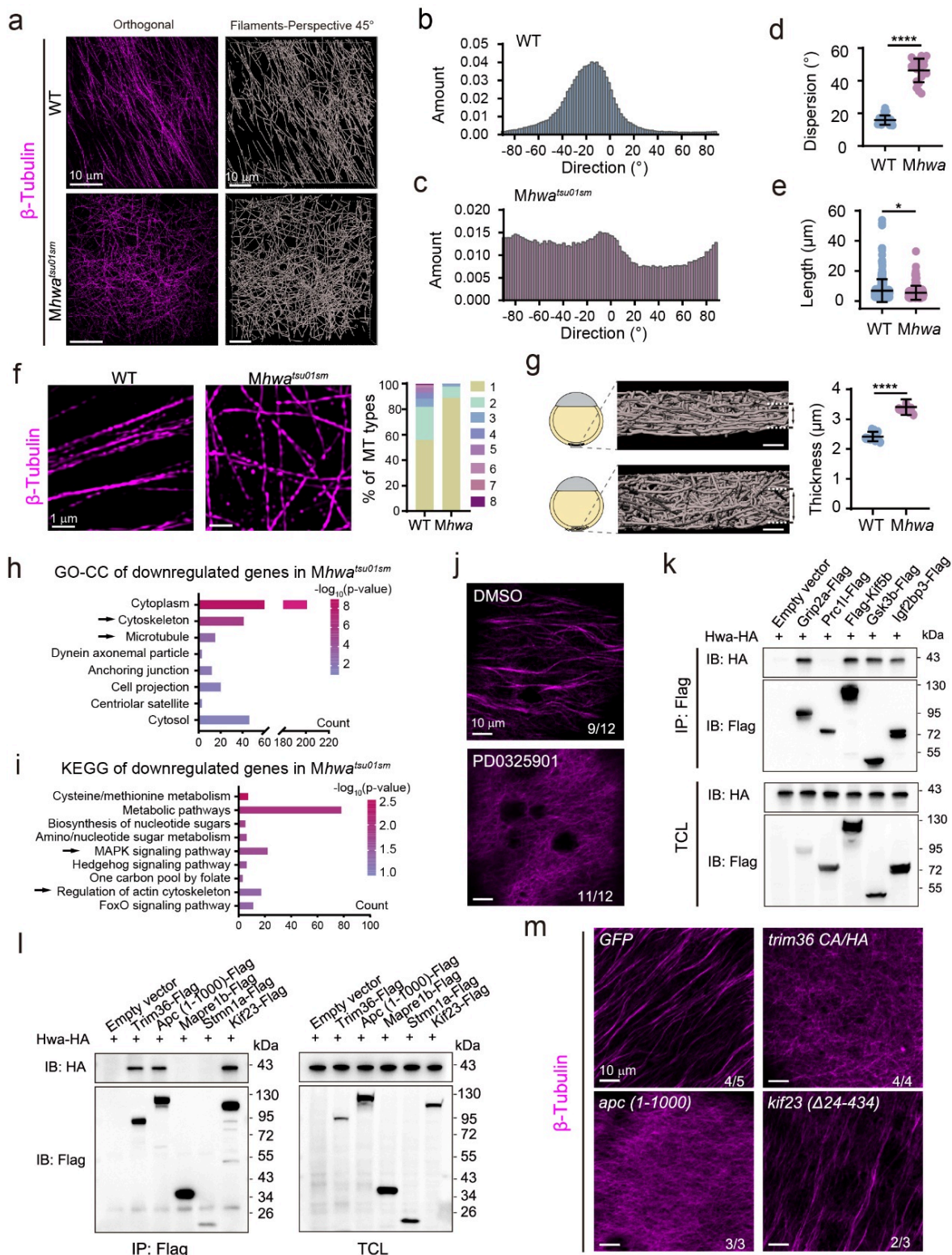


Figure 7

Hwa orchestrates the VPM network likely through multiple mechanisms.

a, Super-resolution microscopy images (left) and Filaments analysis with 45° perspective (right) of the VPM network in WT and *Mhwa^{tsu01sm}* embryos at 10 mpf. **b–c**, Representative orientation of microtubules (one embryo for each group) in WT (**b**) and *Mhwa^{tsu01sm}* (**c**) embryos. **d**, Angular dispersion

measurements showing severely disorganized microtubules in mutants compared to the coherent WT array (n = 20 embryos). **e**, Microtubule length in WT and *Mhwa*^{tsu01sm} embryos. 298 and 153 microtubules were measured in 4 embryos for each group. **f**, Proportions of microtubule bundles with different number (1-8) of microtubules. Left, representative images; right, ratios (n = 6 embryos). **g**, Spatial distribution of cortical microtubules. Left, illustration of observed cortical area; middle, representative side view of 3D-reconstructed microtubules; right, thickness of cortex-associated microtubules (n = 10 embryos). **h** and **i**, Gene Ontology cellular component (GO-CC) (**h**) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (**i**) analyses of downregulated genes in 1c stage *Mhwa*^{tsu01sm} mutants based on RNA-seq data. **i**, reveals significant suppression of MAPK signaling components in *Mhwa*^{tsu01sm} mutants. **j**, Inhibition of MAPK signaling in oocytes disrupts microtubule organization following egg activation. Stage V oocytes were treated with 5 µM PD0325901 for 1 h prior to egg activation. **k** and **l**, Co-immunoprecipitation assays demonstrate physical interaction between Hwa and indicated microtubule-associated proteins expressed in HEK293T cells. **m**, Effects of by overexpression dominant-negative variants of indicated Hwa-interacting proteins on the VPM organization. mRNA (150 pg per oocyte) encoding an indicated protein was injected into stage III oocytes by OMIS and α -tubulin was immunostained after fertilization. Representative images were shown with proportion indicated.

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