Vegetally localized *Xenopus trim36* regulates cortical rotation and dorsal axis formation

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Specification of the dorsoventral axis in *Xenopus* depends on rearrangements of the egg vegetal cortex following fertilization, concomitant with activation of Wnt/β-catenin signaling. How these processes are tied together is not clear, but RNAs localized to the vegetal cortex during oogenesis are known to be essential. Despite their importance, few vegetally localized RNAs have been examined in detail. In this study, we describe the identification of a novel localized mRNA, *trim36*, and characterize its function through maternal loss-of-function experiments. We find that *trim36* is expressed in the germ plasm and encodes a ubiquitin ligase of the Tripartite motif-containing (Trim) family. Depletion of maternal *trim36* using antisense oligonucleotides results in ventralized embryos and reduced organizer gene expression. We show that injection of *wnt11* mRNA rescues this effect, suggesting that Trim36 functions upstream of Wnt/β-catenin activation. We further find that vegetal microtubule polymerization and cortical rotation are disrupted in *trim36*-depleted embryos, in a manner dependent on Trim36 ubiquitin ligase activity. Additionally, these embryos can be rescued by tipping the eggs 90° relative to the animal-vegetal axis. Taken together, our results suggest a role for Trim36 in controlling the stability of proteins regulating microtubule polymerization during cortical rotation, and subsequently axis formation.

**KEY WORDS:** *Xenopus*, Dorsal axis, Wnt, Microtubules, Cortical rotation, Trim, Ubiquitylation

**INTRODUCTION**

Understanding the establishment of the embryonic body axis from a symmetrical egg has been a long-standing problem in developmental biology. In amphibian eggs, axial determination requires a dorsally directed rotational movement of the egg cortex (cortical rotation) during the first cell cycle (Elinson and Rowning, 1988; Vincent et al., 1986). Cortical rotation is driven by a parallel array of microtubules beneath the cortex (Elinson and Rowning, 1988), and the degree of rotation is correlated with the size and extent of Spemann organizer formation, the main determinant of dorsal fate in the embryo (Gerhart et al., 1989). Cortical rotation is thought to transport axial determinants to the dorsal side (Kageura, 1997; Marikawa et al., 1997) along the microtubule array, resulting in activation of the Wnt/β-catenin signaling pathway and in the dorsal stabilization of β-catenin (reviewed by Weaver and Kimelman, 2004).

Wnt/β-catenin signaling is clearly sufficient for axis formation (McMahon and Moon, 1989; Sokol et al., 1995; Yost et al., 1996). The requirement for Wnt activity in vertebrates was first demonstrated through the depletion of maternal β-catenin mRNA using antisense oligonucleotides (Heasman et al., 1994). β-catenin-depleted embryos are ventralized, lacking dorsal tissues of all three germ layers, including the neural tube, notochord and somites. Additional maternal depletion studies showed that other Wnt/β-catenin components are also required for proper axis formation (Belenkaya et al., 2002; Houston et al., 2002; Kofron et al., 2001; Sumanas et al., 2000; Yost et al., 1998). Despite the importance of both cortical rotation and Wnt/β-catenin signaling in axis determination, how these two processes are initiated and their interrelationships are not clearly understood.

Recent studies have shown that the Wnt ligand Wnt11 plays a crucial role in axis formation and maternal Wnt/β-catenin signaling (Tao et al., 2005). *wnt11* mRNA is vegetally localized during oogenesis to the region of the germ plasm (Kloc and Etkin, 1995; Ku and Melton, 1993), which is a subcellular aggregation of mitochondria, membranous organelles and ribonuceloproteins that is required for establishing the germline (reviewed by Houston and King, 2000b). Germ plasm is anchored in the cortex and is displaced dorsally by cortical rotation, thus distributing *wnt11* mRNA to the dorsal side (Tao et al., 2005). Depletion of maternal *wnt11* mRNA using antisense oligonucleotides results in ventralized embryos, and molecular epistasis experiments demonstrate that Wnt11 lies upstream of β-catenin (Tao et al., 2005).

An alternative model suggests that Wnt/β-catenin signaling is achieved by intracellular transport of β-catenin stabilizing agents dorsally along vegetal microtubules (Weaver and Kimelman, 2004). Evidence supporting this model comes from cytoplasmic transplantation experiments (Kageura, 1997), the visualization of Dishevelled (Miller et al., 1999) and Gbp (also known as Frat1) (Weaver et al., 2003) movement during cortical rotation and the ventralization phenotype observed upon depletion of maternal gbp (Yost et al., 1998). However, these models are not mutually exclusive, as the enrichment of Wnt activators on the dorsal side might sensitize these cells to ongoing Wnt signaling. Either model would require the vegetal microtubule array; however, the mechanisms that form this array and the extent of its involvement in Wnt signaling are not well understood.

The formation of vegetal microtubule arrays during the first cell cycle is conserved in many organisms (Eyal-Giladi, 1997), whereas cortical rotation per se and *wnt11* localization and function in axis formation might be features specific to basal fish and amphibians. Recent studies have shown that the germ-plasm-localized mRNA *fatvg* (*adipophilin*), which encodes a lipid-vesicle-associated protein, is required for axis formation by regulating the movement of vesicles during cortical rotation (Chan et al., 2007). These data, together with the role of localized *wnt11*, suggest that germ-plasm-localized molecules might play
significant roles in axis formation as well as in germ-cell formation. To better understand the functions of localized mRNAs, we undertook the identification and functional analysis of novel vegetally localized mRNAs in *Xenopus*. In this work, we describe one such novel localized mRNA, the *Xenopus* homolog of *tripartite motif-containing 36* (trim36).

Trim36 is a member of the large Tripartite motif-containing protein family [known as the Trim or RBCC family (reviewed by Meroni and Diez-Roux, 2005)]. Trim proteins are defined by a conserved domain architecture, consisting of an N-terminal RING finger domain, in combination with adjacent B-box-type zinc fingers and a coiled-coil motif. Mammalian Trim36 localizes to microtubules in cultured cells and is thought to have a role in acrosome exocytosis (Kitamura et al., 2003; Kitamura et al., 2005). Here we show that, in *Xenopus*, *trim36* mRNA is localized vegetally in the oocyte and is enriched in the germ plasm and adult germ cells. Interestingly, we find that depletion of maternal *trim36* results in ventrIALIZED embryos. We show that Trim36 does not regulate Wnt signal transduction directly, that Trim36 is required for polymerization of cortical microtubules during cortical rotation and that ubiquitin ligase activity is likely to be required for this function.

These data provide evidence that Trim36 is essential for positioning the dorsalizing Wnt signal in early development and further suggest that proteins encoded by vegetally localized mRNAs are important in the control of microtubule polymerization or stabilization in the vegetal cortex.

**MATERIALS AND METHODS**

**Oocytes and embryos**

Oocytes were manually defolliculated and cultured in a modified oocyte culture medium [OCM; 70% L-15 media, 0.04% BSA, 1 mM GlutaMAX media, 1.0 μg/ml gentamicin, pH 7.6-7.8 (Heasman et al., 1991)] at 18°C. Embryos were obtained as described (Sive et al., 2000; Kerr et al., 2008). For tipping experiments, eggs were de-jellied and sorted within 20 minutes of fertilization; the colored eggs were then transferred to a Nitex mesh dish containing 5% Ficoll in 0.3 M sucrose/NaCl/5 mM MgCl2containing 5% Ficoll in 0.3× MMR. Individual eggs were oriented with the sperm entry point uppermost and were left in place until the first cleavage. The buffer was gradually changed to 0× MMR and embryos were cultured to the tailbud stage.

**Plasmids**

A full-length cDNA for *trim36* in the vector pCMV-Sport6 was obtained commercially (Open Biosystems) and the insert size was confirmed by restriction enzyme digestion. The coding region (CDS) for *trim36* was amplified by PCR and cloned into pCR8/GW/TOPO (Invitrogen). Clones were verified by sequencing and selected clones in the 5′-L1 to L2-3′ orientation were inserted via recombinase into a pCS2+ Gateway-converted vector (custom vector conversion kit; Invitrogen). Details of the Gateway plasmid are available upon request. Template DNA for sense transcription from *trim36* in pCS2+ was prepared by NoI digestion and was used for proteinase K digestion. Capped messenger RNA was synthesized using SP6 mRNAMESSAGE mMACHINE Kits (Ambion).

**Antisense oligos and host transfer**

Antisense oligodeoxynucleotides (oligos) used were HPLC-purified phosphorothioate-phosphodiester chimeric oligos (Integrated DNA Technologies) with the following sequences (* indicates phosphorothioate linkages): *trim36-3, 5′-TCTCCAGGGTCATGCTGAC*GC*+T-F (nt 408-426); *trim36-4, 5′-G*GC*CTATACCTTCTCGC*GC*+C-3′ (nt 506-523); *trim36-6, 5′-CCGCTCTTCTCCATCGCGCTTGT-G (nt 198-222); β-catenin 303, 5′-T*GC*CTTCCGCTGGTGC*GC*+C-3′ (Heasman et al., 1994).

Oocytes were injected in the vegetal pole (doses of oligos are indicated in the text) and cultured for 24 hours at 18°C before being matured by treatment with 2.0 μM progesterone. For rescue experiments, mRNAs were injected immediately prior to progesterone treatment. Matured oocytes were colored with vital dyes, transferred to egg-laying host females, recovered and fertilized essentially as described (Heasman et al., 1991).

**Analysis of gene expression using real-time RT-PCR**

Total RNA was prepared from oocytes and embryos using proteinase K and then treated with RNase-free DNase as described (Houston and Wylie, 2005). Real-time RT-PCR was carried out using the LightCycler 480 System (Roche Applied Science). Samples were normalized to *ornithine decarboxylase (ode)* levels and relative expression values were calculated against a standard curve of control cDNA. Samples lacking reverse transcriptase in the cDNA synthesis reaction failed to give specific products. The data shown are representative individual experiments using unreplicated samples; experiments were repeated at least twice with similar results. Primer sequences and detailed protocols are available upon request.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was performed essentially as described (Sive et al., 2000; Kerr et al., 2008). Template DNAs for in vitro transcription were prepared by digestion, followed by transcription, with appropriate restriction enzymes and polymerases: *trim36-pcmsgsport6 (Salt/T7), nr3* (a gift from R. Harland, University of California-Berkeley, Berkeley, CA, USA; EcoRI/T7), *eomes and myod* (gifts from J. Gurdon, The Gurdon Institute, Cambridge, UK; EcoRI/T3 and BamHI/SP6, respectively), and *sizzled* (from M. Kirschner, Harvard Medical School, Boston, MA, USA).sense plasmid 16688, BamHI/T3). Antisense RNA probes labeled with digoxigenin-11-UTP (Roche) were synthesized using polymerases and reaction buffers from Promega.

**Immunostaining**

Whole-mount immunostaining for microtubules and cytokeratins was performed using previously described methods (Elinson and Rowning, 1988). Fluorescence was visualized on a Leica DMI3000B inverted microscope using a 63× dry objective (Leica Microsystems). Antibodies were α-tubulin mouse antibody, clone DM1A (ascites 1:200, Sigma) and cytokeratin mouse antibody, clone clone 1h5 (supernatant 1:5, DSHB). Secondary antibodies were goat anti-mouse conjugated with Alexa-488 or -564 (Invitrogen/Molecular Probes) diluted in PGT (1×PBS, 1% goat serum, 0.5% Triton X-100; 1:500). Whole-mount staining for β-catenin was performed essentially as described (Schneider et al., 1996), using a rabbit anti-β-catenin antibody (C2206, Sigma, 1:200).

For immunostaining of sections, samples were fixed and embedded in polyethylene glycol 400 diesterate (PEDS wax, Polysciences) with 10% cetyl alcohol (l-hexadecanol), modified from Godsave et al. (Godsave et al., 1984). Sections were immunostained as described (Houston and Wylie, 2003). Tor70 (a gift from Richard Harland) was used at a 1:6 dilution; 12/101 (DSHB) was used at a 1:6 dilution. Detection was performed as described above. For histology, sections were stained with Hematoxylin and Eosin (H&E).

Guinea pig Trim36 antibodies were raised against purified *Xenopus* Trim36 protein (Cocalico Biological). The *trim36* CDS was cloned into the pcDEST17 vector (Invitrogen) by Gateway recombination to generate a fusion protein N-terminally tagged with six histidines (6-His). Recombinant protein was produced in bacteria following the manufacturer’s instructions, and refolded from inclusion bodies as described (Houston and King, 2000a). The 6-His-tagged Trim36 protein was then purified over a nickel column (His GraviTrap, GE Healthcare), eluted with 300 mM imidazole and dialyzed against PBS.

**Ubiquitylation assays**

Embryos were injected at the 2-cell stage with full-length or mutant *trim36* mRNAs, HA-ubiquitin (a gift from S. Piccolo, University of Padua, Italy), or both (1 ng of each), and cultured to stages 9-10. Samples were lysed in cell lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA (pH 8.0), 1% Triton X-100, with protease and/or phosphatase inhibitors) and immunoprecipitated using Trim36 antisera. The resulting complexes were electrophoresed and immunoblotted as described (Yokota et al., 2003). Samples were blotted against HA-ubiquitin (mouse anti-HA clone 3F10, 1:500, Roche Applied Science) and against Trim36 (1:2000).
RESULTS
Trim36 is encoded by a vegetally localized mRNA in Xenopus

We set out to identify novel mRNAs localized to the vegetal cortex of *Xenopus* oocytes. To accomplish this, we isolated total mRNA from hand-dissected vegetal cortex pieces (Elinson et al., 1993) and intact whole oocytes, generated labeled cDNA and probed Affymetrix GeneChip arrays in duplicate. These data were analyzed for RNAs enriched in the cortex relative to the whole oocyte. The details and full results of this screen will be described elsewhere. We selected one of the most highly enriched genes from this screen for further functional analysis, *tripartite motif-containing 36* (*trim36*; UniGene ID Xl.6926), which is enriched by ~25-fold in the cortex compared with the whole oocyte (data not shown).

We verified the enrichment of *trim36* in the cortex by RT-PCR (data not shown) and obtained a full-length EST through a commercial source. This full-length *trim36* EST (from *Xenopus laevis*) encodes a cDNA of ~4000 nucleotides (nt), with a 2200 nt coding region (CDS) preceded by several in-frame stop codons (see Fig. S1 in the supplementary material). Conceptual translation of the cDNA identified a methionine residue in a favorable initiation context and yielded a putative protein of 733 amino acids. Amino acid sequence analysis revealed characteristic domains classifying this protein as a member of the large tripartite motif-containing family (Trim), namely an N-terminal RING-finger domain followed by two B-box zinc-finger domains and a coiled-coil domain (reviewed by Meroni and Diez-Roux, 2005) (see Fig. S1 in the supplementary material). The C-termini of Trim proteins can include a variety of different functional domains, such as bromo- or NHL-domains, or in the case of Trim36, a B30.2/SPRY domain of uncertain function. Trim36 also contains a COS motif, which groups this protein within a subclass of Trim proteins that localize to microtubules (Short and Cox, 2006). Amino acid sequence alignments using the conceptual Trim36 protein sequence indicated strong similarity to human and mouse Trim36 proteins (also known as mouse Harpin, and human RBC728 or RNF98; ~75% identity) (see Fig. S1 in the supplementary material) and revealed that it is 95% identical to the *Xenopus tropicalis* Trim36 protein. Comparisons across species using the Metazome database (http://www.metazome.com/) found that the *Xenopus tropicalis trim36* gene occupies a conserved position with respect to surrounding genes compared to other tetrapods, suggesting orthology (data not shown).

Analysis of *trim36* mRNA expression during early development further confirmed its vegetal cortex localization and also showed that *trim36* is enriched in the germ plasm of oocytes and early embryos. Temporally, *trim36* is abundant maternally, but decreases throughout development, as assessed by RT-PCR (Fig. 1A). Spatially, *trim36* mRNA is enriched in the mitochondrial cloud/Balbiani body of stage I oocytes (Fig. 1B), the origin of germ-plasm material (Heasman et al., 1984). In fully grown stage VI oocytes, *trim36* is localized in a compact expression domain in the vegetal cortex (Fig. 1C). *trim36* RNA was further detected in the germ plasm of fertilized eggs and 4- to 8-cell-stage embryos, clustered in yolk-free islands along vegetal cleavage furrows (Fig. 1D). During neurula stages, *trim36* RNA is expressed outside the germ plasm in the developing neural tube and is enriched at the midbrain-hindbrain boundary (Fig. 1E).

In situ hybridization on sectioned blastulae and gastrulae revealed *trim36* transcripts associated with the germ plasm during gastrulation, including perinuclear localization in stage 11 embryos (Fig. 1F,G), a hallmark of the germ plasm. *trim36* RNA was undetectable in the primordial germ cells (PGCs) of post-gastrulation embryos, including migrating PGCs at the tailbud stage (data not shown). We also examined the testis, as mammalian *trim36* homologs are implicated in acrosome function, and found robust *trim36* staining in spermatogenic cells of the adult testis (Fig. 1H). Overall, our expression data show that *trim36* is localized to the germ plasm of oocytes and early embryos, and is expressed in the developing nervous system and in adult germ cells.

Depletion of maternal *trim36* causes reduced dorsal axis structures

Although the enrichment of *trim36* in the germ plasm was suggestive of a role in PGC formation, other germ-plasm RNAs, such as *wnt11* (Tao et al., 2005) and *fatvg* (Chan et al., 2007), have been implicated in dorsoventral axis formation. To examine the role of *trim36* in early development, we injected antisense *trim36* mRNAs into one-cell-stage embryos, including migrating PGCs at the tailbud stage (data not shown). We also examined the testis, as mammalian *trim36* homologs are implicated in acrosome function, and found robust *trim36* staining in spermatogenic cells of the adult testis (Fig. 1H). Overall, our expression data show that *trim36* is localized to the germ plasm of oocytes and early embryos, and is expressed in the developing nervous system and in adult germ cells.

Fig. 1. Expression of *trim36* in Xenopus. (A) RT-PCR for *trim36* at different Nieuwkoop and Faber (NF) stages; ‘–RT’ was processed in the absence of reverse transcriptase. ornithine decarboxylase (odc) was included as a loading control. (B-E) Whole-mount in situ using antisense *trim36* probe. (B) Stage I oocytes, (C) stage VI (left) and stage IV (right) oocytes, (D) 4-cell embryos (vegetal view) and (E) neurula embryos (dorsal/anterior view). (F-H) In situ for *trim36* on sections; insets in F and G are low-power views, inset in H is *trim36* sense probe. (F) Stage 7 sagittal section, (G) stage 11 sagittal section and (H) adult testis.
embryos derived from uninjected sibling oocytes. These trim36-depleted embryos eventually completed gastrulation, but in the majority of cases failed to form neural plates and formed large cysts beneath the animal pole. By the late neurula and early tailbud stages, trim36-depleted embryos were radially ventralized in the extreme cases, or formed partial axes lacking anterior structures in the less severe cases (Fig. 2B,C). The effects of trim36 oligo injection were highly consistent over many experiments, resulting in ~82% of injected embryos with ventralized phenotypes (80/98; 81.6%), compared with a very low incidence of ventralization in uninjected embryos (1/117; 0.9%). We counted the distribution of phenotypes (see key) in trim36-depleted embryos from two experiments.

Since differentiated dorsal structures require signals from the organizer, we examined the extent of organizer specification in gastrula-stage trim36-depleted embryos using in situ hybridization and RT-PCR (Fig. 3). In trim36-depleted embryos, the notochord expression domain of eomesodermin (eomes) was lost (Fig. 3A,B), and the expression of myod, an early molecular marker for somites, was reduced (Fig. 3C,D). Organizer markers nodal-related 3 (nr3) (Fig. 3E,F) and noggin (not shown) were severely reduced in early gastrula-stage embryos. Since nr3 is a direct target of Wnt signaling in the early embryo (Smith et al., 1995), we also examined the dorsal stabilization of β-catenin directly by immunostaining. β-catenin was enriched in the dorsal nuclei of control embryos (Fig. 3G; see Fig. S2A,B in the supplementary material), whereas this staining was reduced or absent in trim36-depleted embryos (Fig. 3H; see Fig. S2C,D in the supplementary material). Consistent with these results, real-time RT-PCR analysis of gene expression in trim36-depleted embryos showed much reduced levels of direct Wnt target genes siamois and nr3, and a concomitant increase in szl expression, which is a ventral marginal zone marker at this stage (Fig. 3I). Furthermore, activity of the Wnt/β-catenin reporter, TOPflash, was reduced by ~50% in trim36-depleted gastrulae, further supporting the deficiency in Wnt/β-catenin signaling at this stage (see Fig. S2E in the supplementary material).

To confirm the specificity of these phenotypes, we injected a subset of trim36-depleted oocytes with trim36 mRNA prior to their maturation and fertilization. Injection of trim36 mRNA reduced the proportion of ventralized embryos to ~35% (15/43; 34.9%; Fig. 4A-C). These rescued embryos also showed partial restoration of wild-type levels of organizer markers sia and nr3 at the gastrula stage.
Trim36 in axis formation

**Fig. 4. Specificity of trim36 depletion.** (A-C) Phenotypes of representative control uninjected (Un, A), trim36-depleted (trim36–, B) and rescued (trim36– + trim36 RNA, C) embryos. (D) Quantitative real-time PCR of dorsal (sia and nr3) and ventral (szl) markers in rescue experiments (green bars). N.D., not determined.

**Fig. 5. trim36-depleted embryos are rescued by wnt11 mRNA.** trim36 was depleted by the injection of antisense oligos into oocytes and 50 pg wnt11 mRNA was injected prior to fertilization by the host-transfer method. gsc (A,C,E) and nr3 (B,D,F) expression in stage 10.5 embryos; (A,B) uninjected; (C,D) trim36-depleted (trim36–); and (E,F) trim36-depleted + 50 pg wnt11 mRNA (trim36– + wnt11). (G) Histogram showing the distribution of phenotypes (see key) in uninjected embryos, trim36-depleted embryos and trim36-depleted embryos rescued by wnt11 injection (from two experiments). (H,I) Quantitative real-time PCR analysis of nr3 expression in control, depleted and rescued embryos injected with 50 pg wnt11 (H) or 1.0 ng dngsk3b (I) (green bars).

**Wnt/β-catenin signal transduction is not dependent on Trim36**

We next tested the hypothesis that Trim36 might be required within the Wnt signal transduction cascade. Preliminary experiments showed that ubiquitous expression of high doses of trim36 (>300 pg) caused defects in embryo integrity, leading to disaggregation of the embryos during gastrulation and to epidermal lesions (see Fig. S4A,B in the supplementary material). Lower doses had no visible effect on development, and we failed to induce secondary axis formation by injecting trim36 into ventral vegetal blastomeres, a well-characterized effect of Wnt ligands and signaling molecules (data not shown). Trim36 was not sufficient to activate Wnt signaling in animal cap assays (see Fig. S4C in the supplementary material) and also failed to rescue β-catenin-depleted embryos (see Fig. S4D in the supplementary material).

We next stimulated Wnt/β-catenin signaling in trim36-depleted embryos to determine the activity of Wnt ligands and effectors in the absence of trim36. Oocytes were injected with the trim36-3 oligo and were subsequently injected with wnt11 mRNA prior to host transfer and fertilization. The resulting embryos were analyzed at the gastrula stage for Wnt target gene expression by RT-PCR and in situ hybridization, and at the tailbud stage by phenotype comparisons. In at least three separate experiments, injection of wnt11 restored the expression of dorsal markers (gsc and nr3; Fig. 5A-F,H), as well as reducing the incidence of axis deficiency (Fig. 5G). Some embryos were mildly dorsalized at a moderate wnt11 dose, whereas higher doses caused severe dorsalization (see Fig. S5 in the supplementary material). wnt11 also rescued notochord formation in two out of three embryos examined by histology (see Fig. S5 in the supplementary material). Similar results were seen using wnt8 (data not shown) and dominant-negative gsk3β (dngsk3β, Fig. S1), further supporting the idea that Trim36 acts upstream of Wnt pathway activation. The expression of dorsal markers could also be rescued by injection of the BMP antagonist noggin (see Fig. S6 in the supplementary material), which functions downstream of Wnt/β-catenin signaling, suggesting that other pathways controlling dorsoventral patterning are not affected by trim36 depletion. Overall, these data suggest that Trim36 is not required for Wnt/β-catenin signal transduction per se, but that it might lie upstream of or parallel to Wnt/β-catenin signaling.

**Trim36 is required for vegetal microtubule array formation and cortical rotation**

Since Trim36 and other Trim proteins with a COS motif associate with microtubules (Short and Cox, 2006), we investigated a possible role for Trim36 in microtubule function during cortical rotation. We generated trim36-depleted eggs through the host-transfer procedure and immunostained against α-tubulin at ~80 minutes post...
fertilization, a time when robust microtubule arrays are present and cortical rotation is occurring (Elison and Rowning, 1988). As controls, we included uninjected sibling oocytes, as well as β-catenin-depleted oocytes, which are also ventralized (Heasman et al., 1994) but should have normal cortical rotation. These controls showed robust, normal organization of microtubules arrays (Fig. 6A,C; ~90% normal; n=30 for each). By contrast, trim36-depleted eggs either lacked microtubule organization completely or exhibited fragmented microtubules in a loose organization (Fig. 6B; 77% affected; n=22). As an additional control to ensure that trim36-depleted eggs were indeed activated and were otherwise developing normally, we stained for cytokeratin in the vegetal cortex. Cytokeratins form parallel arrays similar to microtubules upon activation (Klymkowsky et al., 1987) and, importantly, these arrays appear independently of microtubules (Houliston and Elison, 1991). Consistent with this idea, we found that uninjected, β-catenin- and trim36-depleted eggs all exhibited normal cytokeratin arrays (Fig. 6D-F; 100% normal; n=16 for each). In a separate series of experiments, we tested the specificity of this microtubule effect by examining trim36-depleted eggs that were rescued by injection of trim36 mRNA. In these experiments, the incidence of aberrant or absent microtubules was reduced in rescued eggs (46% affected; n=15; Fig. 6I) compared with trim36-depleted siblings from the same experiment (74% affected, n=15; Fig. 6H), indicating that the disruption of microtubules is indeed specific to trim36 depletion.

We next examined the extent of cortical rotation more directly using time-lapse imaging. For simplicity, we made short time-lapse movies of prick-activated oocytes stained with DiOC6(3), which labels the mitochondria associated with the germ plasm and can be used to follow cortical rotation movements in living eggs (Quaas and Wylie, 2002; Savage and Danilchik, 1993). We treated uninjected and trim36-depleted oocytes with progesterone to induce maturation and 20 hours later we stained them with DiOC6(3) and prick-activated them. At 50 minutes after pricking, we collected images over 10 minutes from different eggs and assembled these into movies. In movies of uninjected eggs, the germ-plasm islands were seen moving across the oocyte (see Movie 1 in the supplementary material; Fig. 6P,Q shows individual frames), whereas in time-lapse videos of trim36-depleted eggs, germ-plasm islands remained stationary or moved slowly (see Movie 2 in the supplementary material; Fig. 6R,S shows individual frames). These results were seen in all of the videos of individual trim36-depleted eggs (n=4), from two separate experiments, demonstrating that normal cortical rotation does not occur in the absence of trim36.

Trim36 depletion is rescued by tipping/gravity

Inclining, or tipping, eggs by 90° can rescue the deficiencies in dorsal axis formation resulting from microtubule disruption during the period of cortical rotation (Scharf and Gerhart, 1980; Scharf and Gerhart, 1983). In order to determine whether the effect of trim36 depletion on microtubule array formation is sufficient to explain the loss of the dorsal axis, we tested whether orienting trim36-depleted eggs by 90° could rescue normal dorsal development. As controls, we included uninjected eggs and β-catenin-depleted eggs within the same host-transfer experiment. β-catenin depletion does not affect microtubule formation and is required for the ultimate outcome of cortical rotation; we thus expected that these eggs would remain ventralized following inclination. We further expected that if Trim36 were primarily required for microtubule polymerization, then tilting would be able to rescue dorsal development.

We obtained uninjected, trim36- and β-catenin-depleted eggs by host transfer and quickly sorted them into dishes containing Ficoll buffer. Eggs were then manually tipped 90° relative to the animal-vegetal axis and left in this position until the first cleavage. A subset of each experimental group was placed in the same Ficoll buffer without tipping. These untipped eggs developed as expected; resulting in normal uninjected embryos and ventralized trim36- and β-catenin-depleted embryos (10/12 and 8/8 ventralized, respectively; Fig. 6J,L,N). From two separate tipping experiments, we recovered a lower proportion of affected trim36-depleted...
Trim36 ubiquitin ligase activity is required for microtubule polymerization

Many Trim proteins have ubiquitin ligase activity (Meroni and Diez-Roux, 2005), and this has recently been shown for human TRIM36 (Miyajima et al., 2009). To gain insight into Trim36 function, we asked whether Xenopus Trim36 has ubiquitin ligase activity and whether this activity is required for microtubule regulation. Since the putative substrates for Trim36 are unknown, we first tested the ability of Trim36 to catalyze its own ubiquitylation, a property of many ubiquitin ligases. Trim36 mRNA was overexpressed in embryos in the presence or absence of an mRNA encoding hemagglutinin (HA)-tagged ubiquitin. Trim36 protein was immunoprecipitated and the resulting complexes were immunoblotted with HA antibodies. We detected numerous high molecular weight bands in immunoprecipitates from co-injected embryos probed against HA-ubiquitin (Fig. 7A), indicating that ubiquitin conjugation had occurred. Blotting of the immune complexes with Trim36 antisera confirmed that equivalent amounts of Trim36 protein were recovered in each case (Fig. 7A).

To determine the role of Trim36-dependent ubiquitin ligase activity during cortical rotation, we compared the ability of wild-type and ubiquitylation-deficient Trim36 forms to rescue the depletion of maternal trim36. The RING finger and B-box 2 domains have been implicated in mediating the substrate recognition and ubiquitin ligase activity of Trim proteins (Meroni and Diez-Roux, 2005). We made point mutations in conserved cysteine and histidine residues within the B-box 2 domain of Trim36 (C217A/H220A) that were implicated in the activity of a related Trim protein, Trim5α (Javanbakht et al., 2005). This mutant was inactive in gain-of-function assays. We confirmed that the C217A/H220A mutant was deficient in auto-ubiquitylation activity, as shown by a reduced amount of ubiquitin immunoprecipitated along with mutant Trim36 (Fig. 7B) compared with wild-type Trim36.

We next depleted oocytes of trim36 by antisense oligo injection, followed by rescue with either trim36 or trim36-C217A/H220A mRNAs. In two separate experiments, fertilized eggs rescued with wild-type trim36 showed normal microtubule array formation in at least half the cases, and had normal nr3 levels. By contrast, depleted embryos injected with trim36-C217A/H220A did not show either normal microtubule polymerization (Fig. 7C-F) or nr3 levels (Fig. 7G). Overall, these data suggest that the ability of Trim36 to ubiquitylate target proteins is necessary for the normal polymerization and alignment of microtubules during cortical rotation.

DISCUSSION

trim36 is localized to the germ plasm and encodes a ubiquitin ligase

We identified trim36 in a microarray screen for RNAs enriched in the vegetal cortex of Xenopus oocytes. The RNA for trim36 localizes to the mitochondrial cloud of stage I oocytes and is found in a pattern identical to that of germ plasm up to stage 11 of embryogenesis. Although we did not specifically address germ-cell formation in this study, it is possible that Trim36 also functions in specifying primordial germ cells. Targeted interference with Trim36 function in germ-plasm-containing cells could address these questions, although morpholino inhibition of zygotic Trim36 function disrupts morphogenesis and somite arrangement (Yoshigai et al., 2009), making the study of primordial germ cells problematic.

RING finger domain-dependent E3-ubiquitin ligase function is characteristic of Trim proteins (reviewed by Meroni and Diez-Roux, 2005). Our results, and those of a recent study on human TRIM36 (Miyajima et al., 2009), show that Trim36 has ubiquitin ligase activity both in vivo and in vitro, although the targets of Trim36 ubiquitylation have not yet been identified. Interestingly, a mutant Trim36 with substitutions in the B-box domain, which mediates substrate recognition, was unable to rescue dorsal axis formation, suggesting that Trim36 could not be recruited to the relevant protein complexes.
A subset of Trim proteins (including Trim36 and Trim18) contain a COS motif, which confers localization to microtubules (Short and Cox, 2006). Interestingly, TRIM18 mutations in Opitz syndrome patients result in the accumulation of PP2A in the microtubule fraction, leading to the dysregulation of microtubule dynamics (Trockenbacher et al., 2001). By analogy, Trim36 could regulate the microtubule-associated fraction of a protein involved in microtubule stability or organization, and potentially Wnt signaling.

**Trim36 is required for cortical rotation and axis determination**

Trim36-depleted embryos do not form a normal vegetal microtubule array, and do not undergo cortical rotation. These effects of Trim36 depletion can be rescued by re-orienting the eggs prior to the first cleavage by 90°, a treatment that overcomes the effects of UV irradiation. Our results thus suggest that Trim36 is not required for the synthesis of a component needed for dorsal axis formation. Furthermore, Wnt11 rescues trim36 depletion, ruling out a role for Trim36 in the regulation of the core Wnt/β-catenin signaling components.

It is intriguing that injection of Wnt11 into trim36-depleted oocytes, which would be expected to generate spatially unrestricted expression, results in the rescue of regional organizer gene expression and a single axis (Fig. 5). Wnt signaling induces a number of autoregulatory molecules, so it is possible that small differences in the abundance of injected Wnt could be regulated to result in a single active domain. Alternatively, Brown et al. (Brown et al., 1994) have shown that mature eggs have cryptic asymmetry, which could bias where the axis forms in the presence of a uniform signal. Taken together, our data suggest a model in which Trim36 is required to stabilize or align microtubules in the vegetal cortex region, resulting in the proper distribution of wnt11 mRNA and/or β-catenin stabilizing agents in the embryo.

Our results are reminiscent of data obtained by depletion of another germ-plasm-localized mRNA, fatvg (Chan et al., 2007), but with several important differences. fatvg-depleted embryos fail to undergo cortical rotation (microtubules were not examined) and exhibit stabilized β-catenin at the vegetal pole, which is essentially a phenocopy of UV irradiation (Darras et al., 1997; Medina et al., 1997). Our results differ, as we see an overall loss of β-catenin and Wnt target gene expression, in addition to a loss of cortical rotation. It is widely assumed that microtubules have a role solely in transporting dorsalizing molecules; however, our results suggest that the transport and activation of dorsalizing molecules might be closely coupled. A more thorough and consistent comparison of the phenotypic effects of disrupting microtubules and/or directional transport is needed to better understand how these processes are linked.

Is the role of Trim36 in axis formation conserved? The polymerization of microtubules at the vegetal pole of fertilized eggs has been observed in many chordate species (Eyal-Giladi, 1997). The Trim36 protein sequence is well conserved among vertebrates; however, our work is the first to describe its function in early development. It will be of interest to discover whether Trim36 has a wider role in early embryonic patterning, or whether it is required only in animals with cortical rotation. In either case, comparison of Trim36 function will help to better understand the complex problem of symmetry breaking in eggs.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/18/3057/DC1

**References**


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