Syntabulin, a motor protein linker, controls dorsal determination

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SUMMARY
In amphibian and teleost embryos, the dorsal determinants (DDs) are believed to be initially localized to the vegetal pole and then transported to the prospective dorsal side of the embryo along a microtubule array. The DDs are known to activate the canonical Wnt pathway and thereby promote the expression of genes that induce the dorsal organizer. Here, by identifying the locus of the maternal-effect ventralized mutant tokkaebi, we show that Syntabulin, a linker of the kinesin I motor protein, is essential for dorsal determination in zebrafish. We found that syntabulin mRNA is transported to the vegetal pole during oogenesis through the Bucky ball (Buc)-mediated Balbiani body-dependent pathway, which is necessary for establishment of animal-vegetal (AV) oocyte polarity. We demonstrate that Syntabulin is translocated from the vegetal pole in a microtubule-dependent manner. Our findings suggest that Syntabulin regulates the microtubule-dependent transport of the DDs, and provide evidence for the link between AV and dorsoventral axis formation.

KEY WORDS: Dorsal determination, Syntabulin, Kinesin, Microtubules, Balbiani body, Bucky ball, Zebrafish

INTRODUCTION
During early vertebrate embryogenesis, the dorsal organizer plays an important role in the formation of the dorsoventral (DV) and anteroposterior (AP) body axes. The molecular mechanisms that control the zygotic gene cascades that initiate the organizer have been elucidated (De Robertis, 2006; De Robertis et al., 2000; Gerhart et al., 1989; Hibi et al., 2002; Schier and Talbot, 2005). However, the mechanisms that initially specify the dorsal axis and induce zygotic genes that are required for dorsal organizer formation are not fully understood. In amphibian and teleost embryos, specification of the dorsal axis begins soon after fertilization. In Xenopus, the egg cortex rotates relative to the sperm entry point during the first cell cycle (cortical rotation). During this process, small particles and organelles are transported along a microtubule array that is oriented with the plus ends towards the prospective dorsal side (Elinson and Rowning, 1988; Houliston and Elinson, 1991). In zebrafish, the sperm enters at the animal pole. A parallel microtubule array forms at the vegetal pole of the yolk ~20 minutes post-fertilization (mpf) (Jesuthasan and Stahle, 1997). Disruption of the microtubule array by treatment with nocodazol, cold or UV irradiation prior to the 32-cell stage leads to loss of the dorsal organizer and to the ventralization of embryos (Jesuthasan and Stahle, 1997). Removal of the vegetal yolk mass at the early 1-cell stage also results in severely ventralized embryos (Mizuno et al., 1999; Ober and Schulte-Merker, 1999). These studies established the hypothesis that the dorsal determinants (DDs) are initially localized to the vegetal pole and then transported along microtubules to the prospective dorsal side, where they are incorporated by dorsal blastomeres and promote dorsal organizer formation. Activation of the canonical Wnt pathway in Xenopus and zebrafish embryos results in ectopic formation or expansion of the dorsal organizer, whereas its inhibition impairs dorsal axis formation and reduces dorsal-specific gene expression (reviewed by Hibi et al., 2002). These data place canonical Wnt pathway function downstream of the DDs. In zebrafish, the β-catenin accumulates in the nuclei of dorsal blastomeres by the 128-cell stage and in the dorsal blastoderm and dorsal yolk syncytial layer of mid-blastula stage embryos (Dougan et al., 2003; Schneider et al., 1996). Thus, the DDs activate canonical Wnt signaling in the dorsal blastomeres by the 128-cell stage. A β-catenin and Tcf/Lef complex is thought to regulate zygotic expression of dorsal-specific genes, including the homeobox gene dharna (dha, also known as bozozok) and the nodal-related gene ndr1 (also known as squint) (Dougan et al., 2003; Leung et al., 2003; Ryu et al., 2001; Shimizu et al., 2000a), which function in dorsal organizer formation (Shimizu et al., 2000a; Sirotkin et al., 2000). Thus, the DDs induce the expression of these dorsal-specific genes through activation of the canonical Wnt pathway and thereby promote dorsal organizer formation.

Genetic studies in zebrafish have revealed maternal factors that are involved in dorsal determination. The recessive maternal-effect mutant ichabod shows impaired maternal expression of β-catenin 2 (ctnb2; one of two β-catenin genes in zebrafish), defective dorsal organizer formation and severe ventralization (Bellipanni et al., 2006; Kelly et al., 2000). The maternal-effect mutant brom bones, which has a nonsense mutation disrupting hnRNP I (ptbp1a –
Zebrafish Information Network), shows egg activation defects, disorganized vegetal microtubule array formation, and subsequently displays ventralization (Mei et al., 2009). Furthermore, a ubiquitin ligase, trim36, is localized to the vegetal cortex of Xenopus oocytes, and when depleted causes defective vegetal microtubule array formation and ventralization (Cuykendall and Houston, 2009). Cumulatively, these data support the hypothesis that microtubule-dependent transport of DDs plays an essential role in canonical Wnt pathway activation and dorsal axis determination in teleost and amphibian embryos.

The DDs are present at the vegetal pole of eggs prior to fertilization and therefore achieve their vegetal pole localization during oogenesis. In Xenopus and zebrafish oocytes, mRNAs localize to the oocyte vegetal pole via overlapping transport pathways. The early pathway involves recruitment of mRNAs, including germine-specific transcripts to the Balbiani body (Kloc et al., 2001; Kloc and Etkin, 1995; Kloc et al., 1996; Wilk et al., 2005), an evolutionarily conserved aggregate of organelles and molecules that is present in early oocytes (Guraya, 1979; Kloc et al., 2004). Balbiani body formation is controlled by Bucky ball (Buc). Oocytes lacking Buc function fail to localize mRNAs to the vegetal pole in early and late stage oocytes, and show defective animal-vegetal (AV) polarity (Bontems et al., 2009; Marlow and Mullins, 2008). However, the relationship between the Balbiani body-dependent RNA transport system and vegetal pole localization of the DDs has not been fully elucidated.

The zebrafish maternal-effect recessive mutation tokkaebi (tkk) affects the formation of dorsal structures (Nojima et al., 2004). Embryos obtained from tkk homozygous females (tkk embryos) show severely ventralized phenotypes. Accumulation of B-catenin in the dorsal nuclei at mid-blastula stages is impaired in tkk embryos, indicating that tkk functions upstream of activation or stabilization of B-catenin in the maternal Wnt signaling pathway. In this report, we show that the tkk locus encodes a zebrafish ortholog of Syntabulin, a linker molecule that attaches cargo to the motor protein kinesin I in neuronal axons (Cai et al., 2005; Cai et al., 2007; Su et al., 2004). We demonstrate that syntabulin RNA is transported to the vegetal pole during oogenesis through the Buc-mediated Balbiani body-dependent pathway. We further show that Syntabulin protein translocates from the vegetal pole in a microtubule-dependent manner during early embryogenesis. Our findings suggest that Syntabulin is involved in the vegetal pole localization and microtubule-dependent transport of the DDs, and that Syntabulin links oocyte AV polarity and embryonic DV polarity.

**MATERIALS AND METHODS**

**Positional cloning**

tkk heterozygous fish were crossed with wild-type India fish (Knapik et al., 1996) to generate F1 families. Homozygous tkk female fish were raised from the F1 cross and identified based on the ventralized phenotypes of their offspring. Since embryos from tkk female fish with India genetic background show relatively mild phenotypes and low penetrance (Nojima et al., 2004), phenotypic analysis was performed at least twice. Genomic DNA from mutant females was used to carry out segregation analyses with SSLP markers. z1543 on chromosome 16 was found to be close to the tkk locus (one recombination event out of 280 meiosis). Using the PCR primers for z1543 as a probe, BAC DKEY-97P23 was obtained. Through BAC end sequencing and rescreening of BAC clones, seven BAC clones (DKEY-22J10, 42F24, 9G17, 96G15, 71M14, 106H17, 97P23) were found to cover the tkk locus (see Fig. 2A). Further linkage analyses revealed that four BAC clones (DKEY-42F24, 9G17, 96G15, 71M14) contained the tkk locus. The inserts in these BAC clones were sequenced by a shotgun sequencing procedure and 12 genes were found in this region. The sequence and gene information in the region were also confirmed by the current zebrafish genome data (http://www.sanger.ac.uk/Projects/D_rerio/).

Expression of the 12 candidate genes in ovary or in 1-cell stage embryos was analyzed by RT-PCR (see Table S1 in the supplementary material). The cDNAs of syntabulin and nine other maternally expressed candidates were isolated from wild-type and tkk 1-cell stage embryos by RT-PCR and cloned into pBluescript II SK+ (Stratagene). A 3′ terminal fragment of the insertion in the syntabulin promoter was isolated by inverse PCR with primers 5'-AGATTTTAATCTGGAACAAGACAAA-3' and 5'-GTGTTTATGTAATGCAATTTTTAC-3', as described (Kawakami et al., 2004). The insertion was detected by PCR with primer1, 5'-GATCCATTTCCCAACTTTTCTTAGC-3' and primer2, 5'-AGATTATTATCTGGAACAAAAAGACAAA-3' (see Fig. 2G). The cDNA, genomic and insert sequences of zebrafish syntabulin were deposited in the DDBJ databank under the accession numbers AB517946, AB517947 and AB517948, respectively. For phenotypic analysis, tkk embryos were obtained from crosses of young (3 month old) homozygous male and female fish in the TL genetic background (Nojima et al., 2004). For time course analysis, in vitro fertilization was performed (Westferfield, 1995). For studies of bucky ball mutants, buc+/buc (Dosh et al., 2004; Marlow and Mullins, 2008) and buc+/buc (Bontems et al., 2009) alleles were used.

**RT-PCR**

Quantitative (q) RT-PCR analysis was conducted to examine the expression of syntabulin, the other candidate genes and Wnt genes in wild-type and tkk embryos, using an ABI Prism 7000 (Applied Biosystems) or LightCycler 480 (Roche) with Power SYBR Green PCR Master Mix (Applied Biosystems) or SYBR Green I Master (Roche), respectively. Primers used were: syntabulin, 5'-AGTAGGTGGCGCAAAATG-3' and 5'-TTCTCGCCCTGGAATACCTTCTT-3'; zdhhc3, 5'-AGTACGGTCCAGGACACGTG-3' and 5'-GAAGCGAGAACCAGGTTGATGATC-3'; e(3), 5'-GGTTCGGTGTAATCCTCCAAAAGG-3' and 5'-GGCAAGAGGCTCTTTATCTCTG-3'; aggA, 5'-GGGCTGAGCGTCTGGTTTG-3' and 5'-TCGAGCGTCTGGTTTG-3'; and 5'-TGCAGCCTTGACGACTTTCTT-3'; for primer information for the other candidate genes, see Table S2 in the supplementary material. All PCR assays were performed in triplicate. The relative mRNA level of the averaged CT (cycle threshold, ABI Prism 7000) or CP (crossing point, LightCycler 480) was quantitated and normalized to gapdh. For detection of syntabulin in wild-type and bucky ball mutant oocytes (see Fig. 6), oocytes were dissected from AB, buc+/buc and buc+/buc female fish. Fifty stage I/II or III oocytes were manually sorted for RNA isolation and cDNA preparation, qRT-PCR was carried out using the Realplex2 System (Eppendorf) with Power SYBR Green PCR Master Mix. Primers used were: syntabulin, 5'-CACACTGTGACGACCTGGAAAC-3' and 5'-TCCCTCGGATTGGAATAGGAGAAG-3'; ef1α, 5'-GGCTGAGCGTCTGGTTTG-3' and 5'-ACGCGTCTGGTTTG-3'; for primer information for the other candidate genes, see Table S2 in the supplementary material. All PCR assays were performed in triplicate. The relative mRNA level of the averaged CT was quantitated and normalized to ef1α.

In situ hybridization

Whole-mount in situ hybridization was performed as described previously (Thisse and Thisse, 1998) except that hybridization was at 65°C. BM purple AP substrate (Roche) was used as the substrate for alkaline phosphatase. dharna (boozok) (Yamana et al., 1998) and dusp6 (mkp3) detection was described (Tsang et al., 2004). For the syntabulin probe, the 3′UTR of syntabulin was amplified by PCR using the primers 5'-GCTCTAGATAGGGCGGCTACCTACACCCTAC-3' and 5'-ACGGCTGCATTTTCTGTTTATGATCC-3' and 5'-CCAAGTTGTCTTCTTCTCCTCG-3'. The relative mRNA level of the averaged CT was quantitated and normalized to ef1α.
For β-catenin and Syntabulin staining, embryos were fixed with 4% PFA in PBS for 24 hours at 4°C. For microtubule staining, embryos were fixed with a microtubule-stabilizing buffer: 80 mM potassium Pipes pH 6.8, 5 mM EGTA, 1 mM MgCl2, 3.7% formaldehyde, 0.25% glutaraldehyde, 0.2% Triton X-100 (Schoeder and Gard, 1992). The dechorionated embryos were washed with PBS containing 0.1% Triton X-100 (PBS-T) and blocked with 1% bovine serum albumin (BSA) in PBS-T for 1 hour. Detection of β-catenin was as described previously (Nofima et al., 2004), and the immune complexes were visualized with diaminobenzidine (DAB, Sigma). For detection of Syntabulin, the samples were incubated with anti-Syntabulin antibody solution (anti-Syn1, 1/50 dilution of hybridoma supernatant; anti-Syn2, 1/500 of hybridoma supernatant; anti-SynP1, 1/500 of affinity-purified antibody) at 4°C overnight. For detection of microtubules, the samples were incubated with mouse monoclonal anti-β-tubulin antibody solution (1/1000, Chemicon KMX-1). After four washes with PBS-T, the samples were incubated with secondary antibodies [1/500, Alexa Fluor 555 goat anti-mouse or goat anti-rabbit IgG (H+L), Molecular Probes]. The DAB signals (β-catenin) and fluorescent images (Syntabulin) were obtained by AxioPlan2 imaging. Microtubule images were taken with an LSM5 Pascal laser-scanning inverted microscope (Carl Zeiss) and constructed from z-stack sections by a 3D projection program associated with the microscope.

**Plasmid construction and transgenesis**

The Tol2 rescue plasmids were constructed using Gateway technology (Invitrogen) in pT2KDest-Raf6, which is derived from pT2KXIG (Kawakami et al., 2004). Further information about the plasmids is available on request. The promoter of zona pellucida protein C (zpc), which is derived from zoella, was described previously (Onichtchouk et al., 2003). The promoter of zona pellucida protein C (zpc) was described previously (Onichtchouk et al., 2003). The promoter of zona pellucida protein C (zpc) was derived from zp0.5GFP, was described previously (Onichtchouk et al., 2003). The promoter of zona pellucida protein C (zpc) was derived from zp0.5GFP, was described previously (Onichtchouk et al., 2003).

**Antibodies**

To raise monoclonal and polyclonal antibodies against Syntabulin, glutathione S-transferase (GST) fusion proteins containing amino acids 14-98 (Syn1 antigen) or amino acids 102-271 (Syn2 antigen) of Syntabulin, or a His-tagged protein containing amino acids 1-120 of Syntabulin were generated in E. coli. The GST fusion and His-tagged proteins were purified and used for immunization. For generating monoclonal antibodies, BALB/c rabbits were immunized with the His-tagged protein six times. The antibody was purified using an affinity column that was covalently conjugated to the antigen.

**Transfection, immunoprecipitation and immunoblotting**

HEK293T cells in a 6-cm dish were transfected using HilyMax (DOJINDO Laboratories). The cells were lysed at 24 hours post-transfection in 1 ml of lysis buffer: 10 mM HEPES-HCl pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA, protease inhibitor cocktail (Nacalai). The lysates were precipitated with anti-HA antibody (3F10, Roche), resolved by 5-20% SDS-PAGE (SuperSep, Wako, Japan), and blotted with either anti-HA (3F10, Roche) or anti-Myc (9E10, SantaCruz) antibodies. The proteins were detected with an HRP-conjugated rabbit anti-rat IgG (H+L) (Zymed) or HRP-conjugated anti-mouse IgG (Mouse TrueBlot, eBioscience) antibody, using a chemiluminescence system (Western Lightning, Perkin Elmer Life Science). For detection of Syntabulin in embryos, 200 embryos were lysed in 700 µl of lysis buffer by grinding on ice. The lysates were cleared by centrifugation and immunoprecipitated with anti-Syntabulin antibodies (1 µl of ascites of monoclonal antibodies).

**Treatment with microtubule inhibitors**

Stock solutions of nocodazole (Sigma; 2 mg/ml in DMSO) and colchicine (Sigma; 100 mg/ml in ethanol) were diluted to 1 µg/ml or 0.4 mg/ml, respectively, in embryo medium (Westerfield, 1995). Embryos were placed in inhibitor solutions with their chorion intact, just after in vitro fertilization, for the times indicated.

**Electron microscopy**

Electron microscopy (see Fig. S2 in the supplementary material) was carried out essentially as described previously (Shimizu et al., 2005; Yonemura et al., 2002).

**Statistics**

Statistical analyses were performed by two-tailed Student’s t-test using GraphPad Prism 5. P<0.01 was considered significant.

**RESULTS**

**Positional cloning of the tokkaberi gene reveals a novel role for Syntabulin in dorsal axis formation**

_tkkaberi (tkk)_ is a maternal–effect recessive mutation that disrupts a gene required for dorsal axis formation in zebrafish. The progeny of _tkk_ homozygous females (_tkk_ embryos) display severely ventralized phenotypes, which are more severe in the TL genetic background (Nofima et al., 2004). We reassessed the expression of the earliest known zygotic dorsal-specific genes and nuclear accumulation of β-catenin in _tkk_ embryos obtained after backcrossing with TL fish. Approximately 80% of _tkk_ embryos from young females showed complete ventralization at the pharyngula stage (24 hours post- fertilization (hpf); class C1, Fig. 1A). In _tkk_ embryos, expression of the earliest known dorsal-specific genes _dharma_ (dha) and _dual-specificity phosphatase 6_ (dusp6), also known as _mitogen-activated protein kinase phosphatase 3_ (mkp3), two direct targets of the canonical Wnt pathway (Ryu et al., 2001; Tsaung et al., 2004; Yamanaka et al., 1998), was absent or strongly reduced at mid-to-late blastula stage (4 hpf, Fig. 1H-K). Dorsal accumulation of β-catenin at the 256-cell stage was observed in wild-type, but not in _tkk_, embryos (Fig. 1F,G). A parallel microtubule array that is thought to play an important role in dorsal determination (Jesuthasan and Stahlhe, 1997) was present at the vegetal pole at 20 mpf in wild-type (Fig. 1L) and in _tkk_ (Fig. 1M) embryos. Electron microscopy revealed a similar composition of organelles, including ribosomes at the vegetal pole, of wild-type and _tkk_ embryos at 20 mpf (see Fig. S2 in the supplementary material), indicating that the vegetal yolk is not significantly affected in _tkk_ embryos. Together, these data suggest that the _tkk_ gene product is dispensable for vegetal pole microtubule formation, but functions in early embryogenesis or oogenesis to promote nuclear accumulation of β-catenin and expression of zygotic genes required for dorsal organizer formation, including _dha._

To reveal how the _tkk_ gene product contributes to dorsal determination, we carried out positional cloning. We mapped the _tkk_ locus to a 600 kb region of chromosome 16 that contains 12 potential _dha_.
analysis, we found that the maternal expression of one candidate gene was strongly reduced in ttk embryos (Fig. 2B and see Table S1 in the supplementary material). The gene encodes the zebrafish ortholog of Syntabulin (Su et al., 2004), based on strong sequence similarity to human syntabulin (see Fig. S3 in the supplementary material) and on synteny between the ttk chromosomal region in zebrafish and the human syntabulin chromosomal region (chromosome 8, data not shown).

Expression of zebrafish syntabulin remained strongly reduced at 24 hpf in ttk as compared with wild-type embryos (see Fig. S4 in the supplementary material). Whole-mount in situ hybridization revealed vegetal pole localization of syntabulin mRNA in wild-type eggs and embryos through the 16-cell stage (Fig. 2C-E; see Fig. S4 in the supplementary material), and its absence in ttk eggs and embryos (Fig. 2F). By genomic PCR and Southern blot analyses, we found an insertion in the syntabulin promoter region (232 bp upstream of the transcription initiation site) in the ttk mutant genome (Fig. 2G and see Fig. S5 in the supplementary material). The 3′ sequence of the insertion shows similarities to various genome regions and to the pol-like protein of snail and Drosophila retrotransposons (accession numbers ABN58714, AAA70222) at the amino acid level, implying that the insertion is a transposable element. Our expression data suggest that this insertion in the promoter region strongly suppresses transcription of the syntabulin gene.

**Maternal Syntabulin is sufficient to rescue ventralization of ttk mutant progeny**

To address whether the strong reduction of syntabulin expression causes the ventralization of ttk embryos, we attempted to rescue the mutant phenotype by injecting syntabulin mRNA into 1-cell stage embryos. Supplying syntabulin after egg activation was not sufficient to rescue ventralization in ttk mutant progeny (data not shown). The failure of these rescue attempts, and the localization of syntabulin transcripts at the vegetal pole of early embryos (Fig. 2C-E), suggest that Syntabulin functions in the zygote during early cleavage stages or earlier during oogenesis. With this in mind, we generated transgenic fish that express syntabulin in ttk oocytes (Fig. 3). We constructed the rescue plasmid in the Tol2 vector (Kawakami et al., 2004) to contain the syntabulin cDNA, the first intron and 3′ UTR, the zona pellucida protein C (zpc; zpcx) promoter, which drives maternal expression (Onichtchouk et al., 2003) (Fig. 3A). The rescue plasmid and transposase RNA were injected into embryos obtained from crosses of relatively old ttk homozygotes, the offspring of which show only mild, or no, ventralized phenotypes (Nojima et al., 2004). These fish were raised to adulthood (see Fig. S6 in the supplementary material). Maternal expression of the transgene was observed in a subset of progeny from these ttk mutant females based on GFP visualization at the 1-cell stage, prior to activation of zygotic gene expression. In total, from three crosses 80.7% of GFP-negative (non-transgenic) ttk embryos were completely ventralized, and only 9.3% showed normal DV axis defects, and provide evidence that reduced syntabulin expression in oocytes causes ventralization of ttk progeny.

**Exon-intron structure of syntabulin is required for vegetal pole localization and proper dorsal determination**

The 3′ UTR of germline-specific mRNAs, such as vasa, nanos and dazl, is necessary and sufficient for their vegetal pole localization (Kosaka et al., 2007). In ttk embryos rescued by maternal expression of syntabulin mRNA containing the first intron and 3′ UTR, the mRNA was not vegetally restricted, but was instead distributed throughout the embryos (Fig. 3E,F). Furthermore, EGFP-3′ UTR RNA was not localized to the vegetal pole in transgenic wild-type or ttk zygote-stage embryos (Fig. 4A-E). These data suggest that the 3′ UTR of syntabulin is not sufficient for its vegetal pole localization. In Drosophila oocytes, oskar mRNA localization at the posterior pole, which is required for oogenesis, is controlled in part by splicing (Hachet and Ephrussi, 2004). Therefore, we examined whether the exon-intron structure of the syntabulin gene is required for the vegetal localization of...
syntabulin mRNA in zebrafish. The syntabulin gene is composed of five exons and four introns (Fig. 4F). We constructed a rescue plasmid that contains all the exons and introns, and inserted the 2A peptide sequence of porcine teschovirus-1 (PTV1) and Venus (a YFP variant) cDNA between the coding region and the 3′/H11032 UTR (Fig. 4G). Transgenic embryos were generated by co-injection with transposase RNA (see Fig. S6 in the supplementary material). Of Venus-negative (non-transgenic) tkk embryos, 78.3% showed complete ventralization and 13% showed normal DV patterning, whereas only 30.1% of Venus-positive (transgenic) tkk embryos displayed complete ventralization and 59.4% displayed normal DV patterning (Fig. 4H). This result indicates that this exon/intron-containing plasmid rescued the tkk phenotypes more efficiently than the plasmid containing the cDNA, first intron and 3′/H11032 UTR (data not shown).

Microtubule-dependent transport of Syntabulin

Syntabulin is a linker protein that attaches cargo to kinesin I, and in neurons Syntabulin functions in cargo transport to the synaptic terminal along microtubules (Cai et al., 2005; Cai et al., 2007; Su et al., 2004) (see Fig. S3 in the supplementary material). We found that kif5b, which encodes the heavy chain of kinesin I, is expressed in zebrafish oocytes (see Fig. S7 in the supplementary material). When HA-tagged Kif5b and Myc-tagged Syntabulin were co-expressed in human embryonic kidney (HEK) 293T cells, Syntabulin co-immunoprecipitated with Kif5b (Fig. 5A). These data indicate that zebrafish Syntabulin can interact with the motor protein kinesin I that functions to transport cargo along microtubules.

We next examined the localization of Syntabulin protein. We raised one polyclonal and two monoclonal antibodies against Syntabulin (see Fig. S8 in the supplementary material). All the antibodies detected Syntabulin at the vegetal pole in the wild type but not in tkk embryos from just after fertilization through 20 mpf (Fig. 5B-G and see Fig. S8C,D in the supplementary material). After 20 mpf, when microtubule array formation occurs at the vegetal pole (Jesuthasan and Stahle, 1997), Syntabulin redistributed to one side of the vegetal pole (Fig. 5H-J). This biased localization of Syntabulin was detected through 50 mpf (the 2-cell stage, Fig. 5J). Furthermore, Syntabulin remained at the vegetal pole in embryos.
treated with nocodazole or colchicine, which are inhibitors of microtubule formation (Fig. 5M-O), indicating that syntabulin translocation is microtubule dependent. After 60 mpf, Syntabulin was not detected by whole-mount immunohistochemistry (Fig. 5K), nor in whole embryonic lysates by immunoprecipitation and immunoblotting (Fig. 5L), although syntabulin mRNA persisted at the vegetal pole through the 16-cell stage (90 mpf, Fig. 2E and see Fig. S4A in the supplementary material). These data indicate that the inability to detect Syntabulin after 60 mpf was most likely due to its degradation, rather than to protein diffusion or a decline in syntabulin mRNA abundance. Together, our findings reveal that the localization and stability of Syntabulin are tightly controlled after fertilization until the protein is no longer detected around the 2-cell stage.

**syntabulin RNA is transported to the vegetal pole through the Buc-mediated Balbiani body-dependent pathway**

Some vegetal RNAs, including germline-specific gene products, are transported to the vegetal pole through the Balbiani body (Kloc et al., 2001; Kloc and Etkin, 1995; Kloc et al., 1996; Wilk et al., 2005), which requires maternal Buc function for its formation (Bontems et al., 2009; Marlow and Mullins, 2008). As syntabulin mRNA localizes to the vegetal pole of eggs and early embryos, we investigated when syntabulin achieves its vegetal pole localization and whether this involves the Buc-mediated Balbiani body-dependent pathway in oocytes. During oogenesis, syntabulin mRNA was detected in the Balbiani body of wild-type stage Ib oocytes (compare Fig. 6A with 6B,E), but was not localized in buc mutant oocytes (Fig. 6C,D,F). This was not due to reduced expression or degradation of syntabulin mRNA, as similar levels were detected by qRT-PCR in wild-type and buc mutant stage I-III oocytes (Fig. 6G).

In unfertilized buc mutant eggs, syntabulin mRNA showed circumferential localization, indicating that the later localization of syntabulin to the vegetal pole depends on its proper localization in the Balbiani body of early oocytes (Fig. 6H-J). These data suggest that vegetal pole localization of syntabulin mRNA is mediated through the Buc-mediated Balbiani body-dependent pathway, which is essential for AV polarity in oocytes.

**DISCUSSION**

**Buc-mediated Balbiani body-dependent transport of syntabulin RNA**

Syntabulin mRNA localization to the vegetal pole of unfertilized eggs and early stage embryos is established during oogenesis (Fig. 2C-E and Fig. 6). In early stage wild-type oocytes, syntabulin transcripts localize to the Balbiani body (Fig. 6B,E). Balbiani body localization of syntabulin mRNA was disrupted in buc mutant oocytes (Fig. 6L), which lack the Balbiani body and show aberrant localization of vegetal RNAs during establishment of oocyte AV polarity (Bontems et al., 2009; Dosch et al., 2004; Marlow and Mullins, 2008). These data indicate that, after syntabulin RNA is transcribed, it is transported to the vegetal pole through the Balbiani...
body pathway during oogenesis (Fig. 7). The Balbiani body-dependent RNA transport system is shared by germline-specific RNAs (Kloc et al., 2001; Kloc and Etkin, 1995; Kloc et al., 1996; Kosaka et al., 2007; Wilk et al., 2005). Despite their common localization in early oocytes, important differences exist between syntabulin and germline-specific mRNA localization in late stage oocytes and in embryos. First, after the initial localization of germline-specific mRNAs to the Balbiani body during oogenesis, some of these transcripts, such as dazl (Maegawa et al., 1999; Kosaka et al., 2007), remain at the vegetal pole, whereas others, such as vasa (Knaut et al., 2000), redistribute around the circumference of the oocyte cortex or, such as nanos (Draper et al., 2007), are found throughout late stage oocytes. Despite their distinct positions in late stage oocytes, the germline-specific mRNAs are transported to the vegetal pole until the 16-cell stage, and was not transported to the cleavage furrows or to the primordial germ cells (Fig. 2E and see Fig. S4 in the supplementary material). Second, whereas the 3'UTR of syntabulin RNA in wild-type and buc activated eggs. In activated eggs from wild type, syntabulin shows polarized localization (H; 100% m; 37 from two females). In activated eggs from buc106 (I; 100%, n=44 from three females) and buc43 (J; 100%, n=8 from one female) mutants, the transcripts are present in early and late stage oocytes. (H-J) Localization of syntabulin RNA in wild-type and buc activated eggs. In activated eggs from wild type, syntabulin shows polarized localization (H; 100% m; 37 from two females). In activated eggs from buc106 (I; 100%, n=44 from three females) and buc43 (J; 100%, n=8 from one female) mutants, syntabulin is detected around the circumference. Arrows indicate localization of syntabulin at the vegetal pole in wild type and its circumferential expansion in buc eggs. nuc, nucleus; Bb, Balbiani body. Scale bars: 50 μm.

as vasa (Knaut et al., 2000), redistribute around the circumference of the oocyte cortex or, such as nanos (Draper et al., 2007), are found throughout late stage oocytes. Despite their distinct positions in late stage oocytes, the germline-specific mRNAs are transported to the animal pole during egg activation, and subsequently localize to the distal ends of the blastomere cleavage furrows, where germ plasm is thought to be present during early cleavage stages (Hashimoto et al., 2004; Kosaka et al., 2007; Maegawa et al., 1999; Suzuki et al., 2000). By contrast, syntabulin mRNA remained at the vegetal pole until the 16-cell stage, and was not transported to the cleavage furrows or to the primordial germ cells (Fig. 2E and see Fig. S4 in the supplementary material). Second, whereas the 3' UTR of syntabulin RNA in wild-type and buc activated eggs. In activated eggs from wild type, syntabulin shows polarized localization (H; 100% m; 37 from two females). In activated eggs from buc106 (I; 100%, n=44 from three females) and buc43 (J; 100%, n=8 from one female) mutants, syntabulin is detected around the circumference. Arrows indicate localization of syntabulin at the vegetal pole in wild type and its circumferential expansion in buc eggs. nuc, nucleus; Bb, Balbiani body. Scale bars: 50 μm.

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When ttk embryos were rescued with the plasmid containing the cDNA, first intron and 3' UTR of syntabulin, the transgene-derived RNA was not enriched at the vegetal pole (Fig. 3E), implying that vegetal pole localization of syntabulin RNA is not strictly required for dorsal determination. Alternatively, because syntabulin RNA was present throughout the embryo, it is possible that the amount of syntabulin RNA at the vegetal pole was sufficient for dorsal determination. Moreover, the rescue data suggest that additional factors contribute to localizing the DDs to the vegetal pole. Importantly, RNA from the plasmid containing the syntabulin exon-intron structure was enriched at the vegetal pole and rescued the ttk phenotypes more efficiently (Fig. 4K). Therefore, vegetal localization of syntabulin RNA might ensure that Syntabulin protein is positioned to localize the DDs at the vegetal pole to facilitate their robust transport to the prospective dorsal side.

Recently, a Balbiani body-localized ubiquitin ligase, Trim36, was identified that regulates microtubule polymerization or stability in the vegetal cortex of *Xenopus* (Cuykendall and Houston, 2009). Depletion of Trim36 leads to ventralization in *Xenopus* embryos (Cuykendall and Houston, 2009). Notably, like other Balbiani-localized transcripts, trim36 associates with the germ plasm in *Xenopus* embryos (Cuykendall and Houston, 2009). Our data identify syntabulin as a novel Balbiani body-localized mRNA. We find that unlike other Balbiani body-localized mRNAs, syntabulin is not a component of germ plasm in embryos, but instead mediates localization and transport of the DDs.

**Role of Syntabulin in dorsal determination**

Removal of the vegetal yolk or disruption of microtubule array formation during zygote or early cleavage stages perturbs the development of dorsal structures in zebrafish (Jesuthasan and Stahle, 1997; Mizuno et al., 1999; Ober and Schulte-Merker, 1999). These studies established the hypothesis that the DDs, initially localized to the vegetal pole, are subsequently transported to the prospective dorsal side along microtubules, where they are incorporated by dorsal blastomeres to activate zygotic gene expression cascades required for the formation of the dorsal organizer.

**The link between AV oocyte polarity and DV axis formation**

The DDs initially localize to the vegetal pole of embryos. It is anticipated that AV oocyte polarity is linked to dorsal determination, although this possibility has not been formally tested. We show that syntabulin gene products, which are necessary for dorsal determination, localize to the vegetal pole in a Buc-mediated Balbiani body-dependent manner. Our findings provide evidence that links early AV polarity in oocytes to DV embryonic axis formation. Since Syntabulin functions as a cargo linker (Cai et al., 2005; Cai et al., 2007; Su et al., 2004), we hypothesize that Syntabulin localizes the DDs to the vegetal pole during oogenesis and/or in early stage zygotes (Fig. 7).
How are the DDs transported to the dorsal blastomeres after their release from Syntabulin protein? Inhibition of microtubule formation at 8- or 16-cell stages leads to defective organizer formation (Jesuthasan and Stahlhe, 1997). Vegetal microtubules are only transiently detected in zygotes before the first cleavage, but are not detected at the 8- or 16-cell stage (Jesuthasan and Stahlhe, 1997). At these later stages, long microtubule arrays emanate from the marginal blastomeres (Jesuthasan and Stahlhe, 1997; Solnica-Krezel and Driever, 1994). Whether these microtubule arrays contribute to DD transport to the dorsal blastomeres requires further study. Our findings reveal two steps in DD transport from the vegetal pole to the dorsal blastomeres. Initially, the DDs are localized within the vegetal yolk. The first phase involves asymmetric translocation of the DDs via the vegetal microtubules, followed by their release from the vegetal portion of the yolk. Second, the DDs are transported to, and incorporated by, the prospective dorsal blastomeres. Syntabulin is essential for the first phase of DD transport (Fig. 7).

Dorsal determinants

In zebrafish, transcripts of the nodal-related gene ndr1 localize asymmetrically in a microtubule-dependent manner, which predicts the dorsal side of the embryos, and antisense morpholino-mediated knockdown of ndr1 affects the formation of dorsal structures (Gore et al., 2005). In contrast to syntabulin transcripts, which are localized to the vegetal pole of early oocytes and embryos, ndr1 mRNA is uniformly distributed throughout oocytes and localizes to the animal pole during egg activation before the first cleavage (Gore and Sampa, 2002). During this time in development, syntabulin mRNA and protein remain in the vegetal yolk (Fig. 2C,E and Fig. 6). Later, during early cleavage stages, ndr1 mRNA localizes asymmetrically within the blastomeres of the 2- to 4-cell stage embryo (Gore et al., 2005). At this time, maternal Syntabulin protein is no longer detected in the embryo (Fig. 5I,K). Moreover, asymmetric localization of ndr1 is β-catenin independent (Gore et al., 2005), and Nodal signaling is not required before the mid-blastula transition (Hagos and Dougan, 2007; Hagos et al., 2007), after β-catenin is detected in the nuclei of dorsal blastomeres (Fig. 1) (Dougan et al., 2003). Although it is possible that Syntabulin is involved in ndr1 mRNA transport, the reports discussed above and our spatial-temporal analysis of Syntabulin localization and function suggest that the mechanisms that mediate the translocation of ndr1 and the Wnt pathway-activating DDs are distinct. The notion that distinct mechanisms contribute to translocation of the DDs and ndr1 is compatible to a model whereby Syntabulin transports the DDs to activate β-catenin, which then induces essential zygotic regulators of axis formation, including ndr1 (Bennett et al., 2007; Dougan et al., 2003; Shimizu et al., 2000b).

In Xenopus, maternal Wnt11 and Wnt5a function in dorsal determination (Cha et al., 2008; Tao et al., 2005). Wnt11 mRNA localizes to the Balbiani body (Kloc and Etkin, 1995) and to the vegetal pole (Ku and Melton, 1993) of Xenopus oocytes. Wnt11 protein is enriched dorsally in cleavage-stage Xenopus embryos (Schoeder et al., 1999). Depletion of maternal wnt11 from late stage Xenopus oocytes leads to dorsal axis defects (Tao et al., 2005). In zebrafish, expression of wnt11/wnt11r and wnt5a/b genes is weak or below detection in 1-cell stage embryos, as compared with gastrula and/or pharyngula stages (see Fig. S9 in the supplementary material) when these Wnts function in cell migration (Heisenberg et al., 2000; Matsui et al., 2005; Rauch et al., 1997). Maternal zygotic wnt11 (silverblick) mutant embryos do not exhibit disrupted DV axis formation (Heisenberg et al., 2000). Injection of wnt5a/b and/or wnt11/wnt11r RNA just after fertilization impairs gastrulation movements (data not shown), but neither affected dha expression in wild-type embryos nor rescued dha expression in ttk embryos (see Fig. S10 in the supplementary material), suggesting negligible contributions of these Wnts to Syntabulin-mediated dorsal determination. Furthermore, overexpression of the Wnt signaling components Dishevelled 3 (Dv3), Glycogen synthase kinase (Gsk) binding protein (Gbp), dominant-negative Axin1 or dominant-negative Gsk3β rescued and/or induced hyper-dorsalization in ttk embryos; by contrast, Wnt8, Wnt3a or Frizzled 8b did not efficiently dorsalize ttk embryos (Nojima et al., 2004). These data suggest that Syntabulin functions downstream of Wnt receptors and upstream of β-catenin, in agreement with the hypothesis that Syntabulin does not transport Wnt RNAs or proteins. In Xenopus, Dishevelled (Dv1) and Gbp undergo microtubule- and/or motor protein-dependent transport and contribute to dorsal axis determination (Miller et al., 1999; Weaver et al., 2003; Yost et al., 1998). In early zebrafish embryos, biased localization of Dv12, Dv13 and Gbp was not detected (data not shown). Therefore, zebrafish and Xenopus may utilize different DDs, or there are DDs that are shared among vertebrate species that remain to be discovered. In either case, the answer to this question requires the isolation and identification of the DDs. As a linker protein that attaches cargo, presumably including the DDs, to kinesin I, Syntabulin might facilitate identification of the elusive DDs.

In summary, the regulation of dorsal determination via microtubule dynamics and association with motor and adaptor proteins is shared among zebrafish and amphibians. Our findings provide a missing component of the microtubule-dependent DD transport machinery. Moreover, our finding that syntabulin, an essential regulator of dorsal axis formation, relies on early AV oocyte polarity for its localization provides new insight into the link between the first embryonic axis and dorsal determination in zebrafish.

Acknowledgements

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Table S1. Expression of candidate genes near the zebrafish tkk locus

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name/symbol</th>
<th>Expression at 1 cell</th>
<th>Expression in ovary</th>
<th>Expression level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>glycerol-3-phosphate dehydrogenase 1-like</td>
<td>Yes</td>
<td>Yes</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>hypothetical protein (NM_212698)</td>
<td>Yes</td>
<td>Yes</td>
<td>78</td>
</tr>
<tr>
<td>3</td>
<td>zinc finger, DHHC-type containing 3, zdhhc3 (BC076087)</td>
<td>Yes</td>
<td>Yes</td>
<td>88</td>
</tr>
<tr>
<td>4</td>
<td>estrogen receptor-binding fragment-associated gene 9</td>
<td>Yes</td>
<td>Yes</td>
<td>62</td>
</tr>
<tr>
<td>5</td>
<td>novel serine/threonine protein kinase</td>
<td>No</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>syntabulin</td>
<td>Yes</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>gdf6a</td>
<td>Yes</td>
<td>Yes</td>
<td>80</td>
</tr>
<tr>
<td>8</td>
<td>enhancer of yellow gene, e(y)2 (NM_001002427)</td>
<td>Yes</td>
<td>Yes</td>
<td>105</td>
</tr>
<tr>
<td>9</td>
<td>hypothetical protein (BC150421)</td>
<td>Yes</td>
<td>Yes</td>
<td>150</td>
</tr>
<tr>
<td>10</td>
<td>thyrotropin-releasing hormone receptor</td>
<td>No</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>similar to transmembrane protein 74 (XM_001921419)</td>
<td>Yes</td>
<td>Yes</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>tetratricopeptide repeat domain 35</td>
<td>Yes</td>
<td>Yes</td>
<td>78</td>
</tr>
</tbody>
</table>

Expression of genes near the tkk locus in 1-cell stage tkk embryos and in tkk mutant ovary as assessed by RT-PCR. Among the 12 candidate genes, ten were expressed maternally. Their expression was further examined by qRT-PCR. The expression level of each gene in tkk embryos, compared with the level in wild-type embryos, is indicated. ND, not determined.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GACTGAAGCTCATCTCGGACATCCCCATAGGACACTGATC</td>
</tr>
<tr>
<td>2</td>
<td>AGCGGAACCATCTTCTTCTCTGAAATCATCAACTGTGGCATCTGATCCTGATCCGGCTCAGCTTAA</td>
</tr>
<tr>
<td>4</td>
<td>GCACCTGCTTTGGGATCTTATTCATCTGATCCGGCTCAGCTTAA</td>
</tr>
<tr>
<td>5</td>
<td>GACTCAGTTCTAAATCCGGAGACCACTGATCCTATTGCAAGACGTAATGCCCCTGTTT</td>
</tr>
<tr>
<td>7</td>
<td>AGGCATGGCAAAAAAGCTCAAATCCAGTGTCCAATCCAGTTGATCCGGCTCAGCTTAA</td>
</tr>
<tr>
<td>8</td>
<td>TGCGCCCTTGATCTGAGATGAGCTTTGCTCCATCCAGCTATTGATCCGGCTCAGCTTAA</td>
</tr>
<tr>
<td>9</td>
<td>ATGGAGAACGGCACTTTCAGCAACGGTCATAAAGGCGACCTTTCAGCAACGGAAGATGAGCTTTGCTCCATCCAGCTATTGATCCGGCTCAGCTTAA</td>
</tr>
<tr>
<td>10</td>
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</tr>
<tr>
<td>11</td>
<td>TGAGAAAAGTGACGTGGGCTTACTCCAGCTATTGATCCGGCTCAGCTTAA</td>
</tr>
<tr>
<td>12</td>
<td>GAGGGGTGCTCTGAGATGAGCTTTGCTCCATCCAGCTATTGATCCGGCTCAGCTTAA</td>
</tr>
</tbody>
</table>

The primers for gene 3 (zdhhc3), gene 6 (syntabulin) and gene 8 (ely2) are described in Materials and methods.