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Maternal *syntabulin* is required for dorsal axis formation and is a germ plasm component in *Xenopus*

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ABSTRACT

In amphibians and teleosts, early embryonic axial development is driven by maternally deposited mRNAs and proteins, called dorsal determinants, which migrate to the presumptive dorsal side of the embryo in a microtubule-dependent manner after fertilization. Syntabulin is an adapter protein that binds to kinesin KIF5B and to the transmembrane protein Syntaxin1. In zebrafish, a mutation in Syntabulin causes complete embryo ventralization. It is unknown whether Syntabulin plays an analogous role during early development of other species, a question addressed here in *Xenopus laevis*. *in situ* hybridization of *syntabulin* mRNA was carried out at different stages of *Xenopus* development. In oocytes, *syntabulin* transcripts were localized to the vegetal cortex of large oocytes and the mitochondrial cloud of very young oocytes. We extended the zebrafish data by finding that during cleavage *Xenopus syntabulin* mRNA localized to the germ plasm and was later expressed in primordial germ cells (PGCs). This new finding suggested a role for Syntabulin during germ cell differentiation. The functional role of maternal *syntabulin* mRNA was investigated by knock-down with phosphorothioate DNA antisense oligos followed by oocyte transfer. The results showed that *syntabulin* mRNA depletion caused the complete loss of dorso-anterior axis formation in frog embryos. Consistent with the ventralized phenotype, *syntabulin*-depleted embryos displayed severe reduction of dorsal markers and ubiquitous transcription of the ventral marker *sizzled*. Syntabulin was required for the maternal Wnt/β-Catenin signal, since ventralization could be completely rescued by injection of *β-catenin* (or *syntabulin*) mRNA. The data suggest an evolutionarily conserved role for Syntabulin, a protein that bridges microtubule motors and membrane vesicles, during dorso-ventral axis formation in the vertebrates.

1. Introduction

A fundamental question in developmental biology is how embryonic body axes are established from an initially symmetrical egg. This developmental event has been extensively studied in Amphibians, particularly in the frog *Xenopus laevis*, in which the dorso-ventral (D-V) polarity is established soon after fertilization. Sperm entry triggers a series of cytoplasmic rearrangements, known as cortical rotation, which occur before the end of the first cleavage (reviewed in Gerhart et al., 1989; Houston, 2012; Weaver and Kimelman, 2004). During cortical rotation, the outer or cortical vegetal cytoplasm rotates about 30° relative to the inner core cytoplasm in the opposite direction to the sperm entry point, towards the presumptive dorsal side. Externally, this can be followed by the formation of a less pigmented dorsal crescent. Cortical rotation requires the polymerization of microtubules associated to the cortex of the egg, forming parallel bundles with their plus ends oriented away from the sperm entry point (Gerhart et al., 1989; Houliston and Elinson, 1991). Experimental inhibition of cortical microtubule polymerization by nocodazole or ultraviolet (UV) irradiation prevents cortical rotation and ventralizes the frog embryo, causing reduced or missing dorsoanterior structures and expansion of ventral tissues (Scharf and Gerhart, 1983; Elinson and Rowning, 1988).

Cortical rotation coincides with the translocation of vegetally deposited mRNAs, proteins and organelles called maternal determinants, which are transported by kinesin motor proteins towards the plus ends of the cortical microtubules, causing their relocation to the prospective embryonic dorsal side (Gerhart et al., 1989; De Robertis et al., 2000; Weaver and Kimelman, 2004; Houston, 2012). Dorsal determinants are endowed with dorsalizing activity, as shown by their ability to rescue the primary dorsal axis in UV-ventralized embryos and to induce a secondary axis when transplanted into ventral blastomeres of wild type embryos (Holowacz and Elinson, 1993; Kageura, 1997; Marikawa et al., 1997). Thus, the translocation of dorsalizing factors from the vegetal pole to the dorsal side establishes the site where the Spemann Organizer will form at later stages of development (De Robertis et al., 2000; Houston, 2012).

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The molecular nature of dorsal determinants remains one of the big mysteries in developmental biology. However, it is clear that the effects of dorsal determinants is to activate the canonical Wnt pathway on the dorsal side of the early embryo, leading to stabilization of the transcriptional co-activator β-Catenin which translocates into the nuclei of dorsal cells (Schneider et al., 1996; Larabell et al., 1997). Several components of the Wnt/β-Catenin signaling pathway have been found to play essential roles in the establishment of *Xenopus* D-V axis. These include the Wnt ligands Wnt11 and Wnt5A (Tao et al., 2005; Cha et al., 2008), the receptors Frizzled7 (Sumanas et al., 2000) and Lrp6 (Kofron et al., 2007), and cytoplasmic proteins such as Disheveled (Dvl) (Sokol et al., 1995; Miller et al., 1999), Glycogen Synthase Kinase 3 (GSK3)-Binding Protein (GBP) (Yost et al., 1998; Dominguez and Green, 2000) and β-Catenin (Heasman et al., 1994, 2000). Interestingly, Green Fluorescent Protein (GFP)-tagged versions of both Dvl and GBP proteins were observed to translocate to the prospective dorsal side during cortical rotation, in association to vesicle-like organelles (Rowning et al., 1997; Miller et al., 1999; Weaver et al., 2003).

Despite the realization of the pivotal role of early Wnt signaling in establishing the initial D-V axis in the frog embryo, a mechanistic link connecting dorsal determinant translocation to Wnt signaling activation is still missing. It has recently been found that Wnt signaling requires the endosomal membrane trafficking machinery in cultured cells and *Xenopus* animal caps (Taelman et al., 2010). When Wnt ligand binds to its receptors, the complex is internalized carrying with it Glycogen Synthase Kinase 3 (GSK3), which becomes sequestered from the cytosol into multivesicular bodies, resulting in the stabilization of proteins such as β-Catenin (Taelman et al., 2010; Dobrowolski and De Robertis, 2011). In addition to GSK3, other components of the activated LRP6 receptor complex such as Dvl-2, Axin and phospho-β-Catenin are also sequestered inside MVBs (Vinyoles et al., 2014). Given that Dvl-containing organelles are transported dorsally (Miller et al., 1999) and the connection between Wnt signaling and endosomal membrane trafficking, proteins that may link membrane vesicles to the microtubule machinery are of great interest.

A new player in the formation of the dorsal axis has been identified by the Hibi laboratory while studying a zebrafish maternal-effect mutation called *tokkaebi* (*tkk*) (Nojima et al., 2004). *Tkk* mutant embryos exhibit a severely ventralized phenotype, lacking all dorso-anterior tissues, and accumulated less β-Catenin in the nuclei of dorsal cells. Importantly, the ventralized phenotype was rescued by overexpression of components of the Wnt pathway such as β-Catenin and Dvl3 (Nojima et al., 2004). The *tkk* mutation was mapped to the gene encoding for Syntabulin, a Kinesin I linker protein (Nojima et al., 2010). Kinesins are microtubule motors that transport cargo, including membrane vesicles, to the plus-end of microtubules. Interestingly, Syntabulin has also been identified as a protein that binds to the transmembrane protein Syntaxin1 (Su et al., 2004). In neurons, Syntabulin binds to the Kinesin family member 5B (KIF-5B), connecting it to the microtubules, and to Syntaxin1-containing vesicles (Cai et al., 2005, 2007). In neurons, depletion of Syntabulin blocks transport of presynaptic precursor vesicles and mitochondria along the axon to the synaptic terminal, revealing its requirement for the formation of functional synapses (Bury and Sabo, 2011; Cai et al., 2005, 2007; Ma et al., 2009).

Although the precise molecular mechanism of how zebrafish Syntabulin regulates dorsal determination is still unknown, its mRNA has been found to be localized to the vegetal pole of the egg. After fertilization, a Syntabulin antibody showed that this protein translocated from the vegetal pole to the prospective dorsal side of zebrafish zygotes in a microtubule-dependent manner (Nojima et al., 2010). These observations suggested that Syntabulin might be a protein essential for the transport of putative determinants that activate the dorsalizing Wnt signal.

In the present study, we investigated the expression and biological function of the *Xenopus syntabulin* homolog. *Syntabulin* transcripts were found to be associated with the mitochondrial cloud of early stage I oocytes and then localized to the vegetal cortex of fully grown oocytes, as is the case in zebrafish. In addition, we found that *syntabulin* mRNA was associated with germ plasm islands during cleavage, and was expressed in primordial germ cells (PGCs) at later stages. The association between *syntabulin* and germ plasm/PGCs had not been reported previously. Antisense DNA oligo-mediated knockdown of maternal *syntabulin* caused radially symmetrical ventralization of frog embryos, with complete loss of dorso-anterior structures, showing a striking similarity to *tkk* mutant embryos. Notably, *syntabulin*-depleted embryos could be rescued not only by *syntabulin* mRNA but also by injection of *β-catenin* mRNA, reinforcing the idea that maternally stored transcripts of *syntabulin* are required for the early Wnt signal which triggers dorsal axis formation.

2. Material and methods

2.1. Xenopus syntabulin cloning and antisense oligos

A commercially available full-length *Xenopus syntabulin* clone (clone ID BC079762, Source BioScience) was identified. For maternal rescue experiments this clone, which contains the 5′ and 3′ untranslated (UTR) sequences, was subcloned into pCS2+ between the *Cla*I and *Stu*I sites (pCS2-syntabulin-UTR^{+}), and mRNA synthesized with SP6 polymerase after linearization with *Not*I. For probe preparation the coding sequence was amplified by PCR and subcloned into pCS2+, between the *Cla*I and *Xho*I restriction sites. Primer information and cloning details are available upon request. For antisense probe synthesis, pCS2-*syntabulin*-UTR^{-} plasmid was linearized with *Cla*I and transcribed with T7 polymerase. For *syntabulin* maternal knockdown, we used the following phosphorothioate-modified DNA antisense oligos (Integrated DNA Technologies, Inc.):

AS6 : G∗C∗T∗ACTGGCAGATGA∗A∗A∗C

AS10 : T∗T∗C∗TGCTTCCCTGTC∗T∗T∗C

where asterisks mark phosphorothioate modifications. Oligos were resuspended in sterile, filtered water at a concentration of 1 μg/μl.

2.2. Oocyte injection and host transfer method

Fully grown (stage VI) oocytes were manually defolliculated and cultured in Oocyte Culture Medium (OCM, 70% Leibovitz L-15, 0.004% BSA, 1X Pen-Strep, pH adjusted to 7.6–7.8 with NaOH) as described elsewhere (Mir and Heasman, 2008; Olson et al., 2012). 4 to 5 ng of *syntabulin*-specific phosphorothioate antisense oligos were injected aiming for the center of the oocyte vegetal hemisphere, and cultured for 48–72 h at 18 °C before transplantation into the peritoneum of host females as follows. Oocytes were matured overnight (10–12 h) with 2 μM of Progesterone at 18 °C, stained with vital dyes and finally introduced into hCG-stimulated host females using the technique of host transfer (Mir and Heasman, 2008; Olson et al., 2012). We used a modification of this protocol, by employing albino females as hosts. This allowed easy identification of transferred eggs while not affecting the egg fertilization rate, which ranged from 30% to 80% depending on the quality of donor oocytes. Collected laid eggs were fertilized with 200–300 μl of a sperm suspension in 1 × Marc's Modified Ringers (MMR, 0.1 M NaCl, 2.0 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.4). To improve fertilization rate, transferred eggs were covered with a few drops of 0.3 × MMR right after sperm addition and flooded with 0.1 × MMR only after animal pigment contraction was detected. Embryos were maintained in

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Table 1
Primers used in this study.

Gene	Forward	Reverse	Ta (°C)	Use
Syntabulin	GGGCCAACAACATTAATAGACTTGAC	CAACAGCCACGCATCATGGC	53	sq RT-PCR
H4	CGGGATAACATTCAGGGTATCACT	ATCCATGGCGGTAACTGTCTTCCT	53	sq RT-PCR
ODC	CAGCTAGCTGTGGTGTGG	CAACATGGAAACTCACACC	55	qRT-PCR
Siamois	AAGATAACTGGCATTCCTGAGC	GGTAGGGCTGTGTATTTGAAGG	55	qRT-PCR
Xnr3	CGAGTGCAAGAAGGTGGACA	ATCTTCATGGGGACACAGGA	55	qRT-PCR
Chordin	GTTGTACATTTGGTGGGAA	ACTCAGATAAGAGCGATCA	55	qRT-PCR
Sizzled	GTCTTCCTGCTCCTCTGC	AACAGGGAGCACAGGAAG	55	qRT-PCR
Syntabulin	GAGAAGCCAAATTCCAACAGG	GATCTGCCTTCATTATTTGCTTTG	55	qRT-PCR

Sq RT-PCR=semiquantitative RT-PCR; qRT-PCR=quantitative RT-PCR; Ta=annealing temperature.

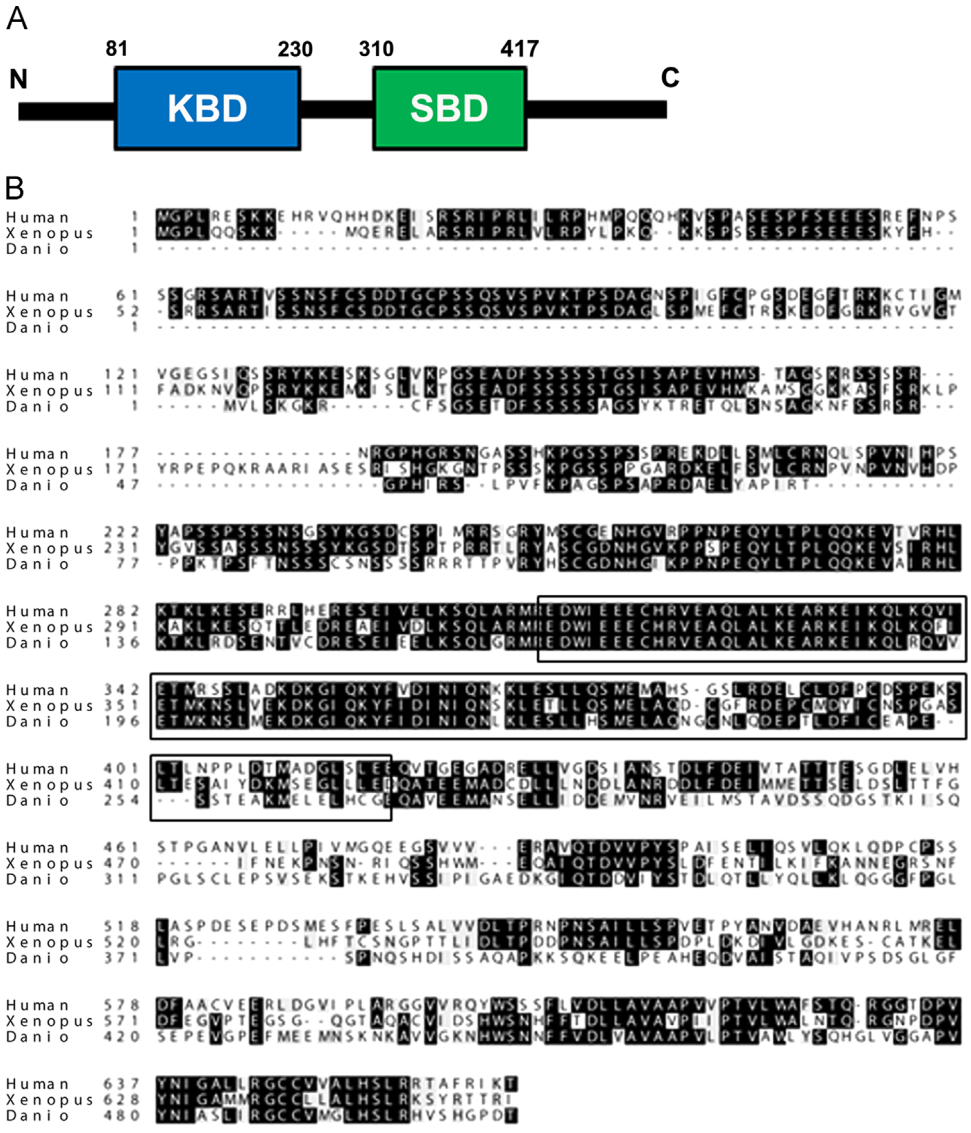

Fig. 1. *Xenopus* Syntabulin: Protein structure and sequence comparison among vertebrate orthologs. (A) Schematic diagram of Syntabulin showing the kinesin binding domain (KBD) and the syntaxin binding domain (SBD). Numbers refer to the amino acids comprising the respective domains in the human sequence. (B) Amino acid sequence comparison among three different vertebrate Syntabulin orthologs (from human, frog and zebrafish) reveals a high degree of homology, especially in the region that binds to the SNARE protein Syntaxin1 (boxed). The multiple sequence alignment of amino acid sequences was generated using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). The black and gray shaded boxes indicate identical and similar amino acid residues, respectively.

0.1 × MMR and sorted by color, each dye corresponding to different experimental conditions. The vital dyes used were Neutral Red, Bismark Brown and Nile Blue (Sigma-Aldrich) as described by Mir and Heasman (2008). Developmental stages were determined according to Nieuwkoop and Faber (1994). Unfertilized eggs or abnormally cleaving embryos were removed from all batches.

2.3. mRNA injections and in situ hybridization

For synthetic mRNA synthesis, pCS2- β-*catenin-myc* (Yost et al., 1996) or pCS2-*syntabulin*-UTR^{+} were linearized with *Not*I and transcribed with the mMessage mMachine SP6 kit (Ambion). For *syntabulin* rescue experiments, oocytes were first injected

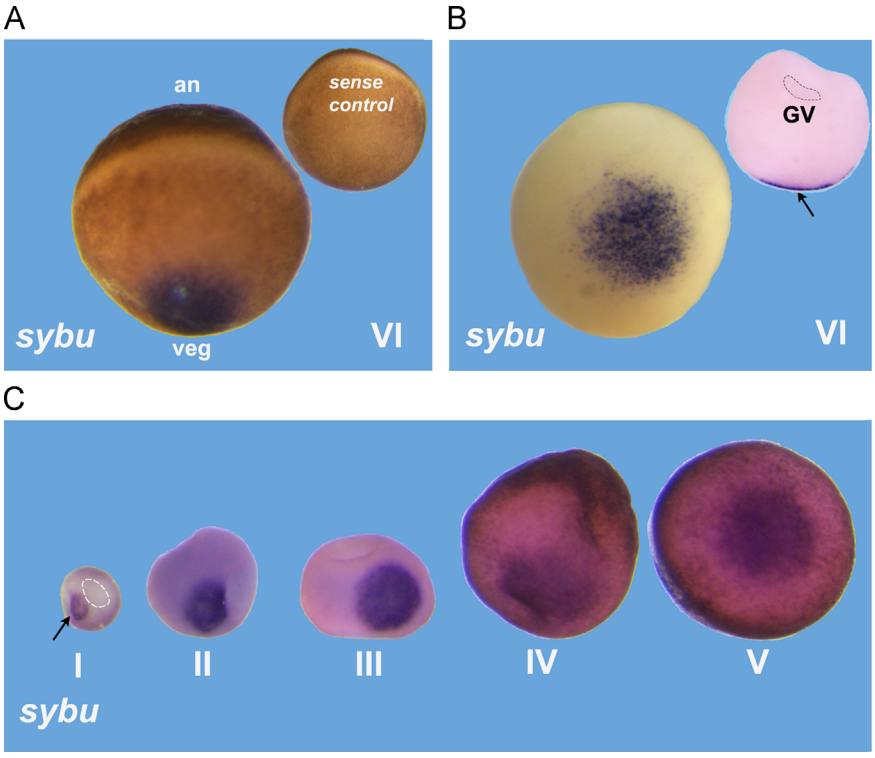

Fig. 2. *Xenopus syntabulin* is a vegetally localized maternal mRNA. (A) Whole-mount *in situ* hybridization on stage VI oocytes using an antisense probe showing that maternal *syntabulin* (*sybu*) transcripts are localized at the center of the vegetal pole. The inset shows the sense probe negative control. (B) *In situ* hybridization on albino stage VI oocytes showing that *syntabulin* transcripts form a granular pattern in the vegetal cortex; note arrow in the hemisection shown in the inset. The germinal vesicle is indicated by a dotted line in inset. (C) *In situ* hybridization of *sybu* at different stages of oogenesis (I–V) showing that maternal transcripts localize early (stage I, arrow) in the mitochondrial cloud, and in the cortex of the vegetal pole at later stages. Dotted line in stage I oocyte marks the nucleus. An = animal pole; Veg = vegetal pole; GV = germinal vesicle.

vegetally with 4 ng of *syntabulin* phosphorothioate antisense oligo. After 24 h of incubation, when the antisense oligo is known to be mostly degraded (Hulstrand et al., 2010), 250 pg of *syntabulin*-UTR^{+} mRNA were injected vegetally. For *β-catenin* rescue experiments, *syntabulin*-depleted embryos were obtained using the host transfer method and injected with 80 pg/blastomere of *β-catenin-myc* mRNA into the two dorsal blastomeres at 4-cell stage. For whole-mount *in situ* hybridization, embryos were dechorionated and fixed at the appropriate stage in MEMFA (0.1 M MOPS, pH 7.4, 1 mM EGTA, 2 mM $MgSO_4$ and 3.7% formaldehyde, overnight at 4 °C) and dehydrated in ethanol 100% A detailed protocol for *in situ* hybridization is available at http://www.hhmi.ucla.edu/derobertis/index.html. Stained embryos were finally bleached overnight in methanol:H_2O_2 (2:1) to enhance contrast.

2.4. Liberase treatment

For some applications, such as oocyte *in situ* hybridization, small clumps of oocytes were chemically defolliculated with the Liberase TM Research Grade enzyme (Roche 05401127001) as described in Halley-Stott et al. (2010). After dissociation, oocytes at different stages of oogenesis (I–VI) were washed several times in 1 × Modified Barth's Saline (MBS, 88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM HEPES, pH 7.8, 2.5 mM $NaHCO_3$), fixed overnight in MEMFA, and processed for *in situ* hybridization. Note that oocytes for host transfer were manually defolliculated.

2.5. Total RNA extraction and RT-PCR

Total RNA was extracted from groups of 5 oocytes or 3 embryos with the RNeasy Mini Kit (QIAGEN), following manufacturer's instructions. 2 μg of total RNA was used for cDNA synthesis with random hexamer primers, using the AffinityScript Multi-Temp Reverse Transcriptase (Agilent). cDNA was then used for semi-quantitative or quantitative RT-PCR. The latter was performed on a Stratagene Mx 3000 P thermal cycler, using the 2X Brilliant II Sybr Green QPCR Master Mix (Agilent). Expression levels were normalized to those of *ornithine decarboxylase* (*ODC*) mRNA. Primer sequences and thermal conditions are listed in Table 1.

3. Results

3.1. Xenopus syntabulin is a conserved maternal gene localized to the vegetal cortex

The motor linker protein Syntabulin is characterized by an amino-terminal kinesin binding domain (KBD) and a central syntaxin binding domain (SBD) (Cai et al., 2005, 2007; Su et al., 2004) (Fig. 1A), which enables it to connect syntaxin1-containing vesicle cargoes to kinesin motor proteins for microtubule-dependent transport. *Xenopus laevis* Syntabulin protein shares an overall high degree of sequence homology with other vertebrate orthologues, such as human and zebrafish (Fig. 1B). The sequence similarity is especially high in the SBD (boxed sequence in Fig. 1B), where it reaches 70% identity with both human and zebrafish proteins, indicating a possible involvement in similar biological processes.

Since zebrafish *syntabulin* mRNA is maternally inherited and deposited in the vegetal pole of fish egg (Nojima et al., 2010), we investigated whether *Xenopus syntabulin* shared a similar maternal localization. *Syntabulin* mRNA expression pattern was determined by whole mount *in situ* hybridization of *Xenopus* oocytes, and revealed maternal accumulation of *syntabulin* transcripts at the center of the

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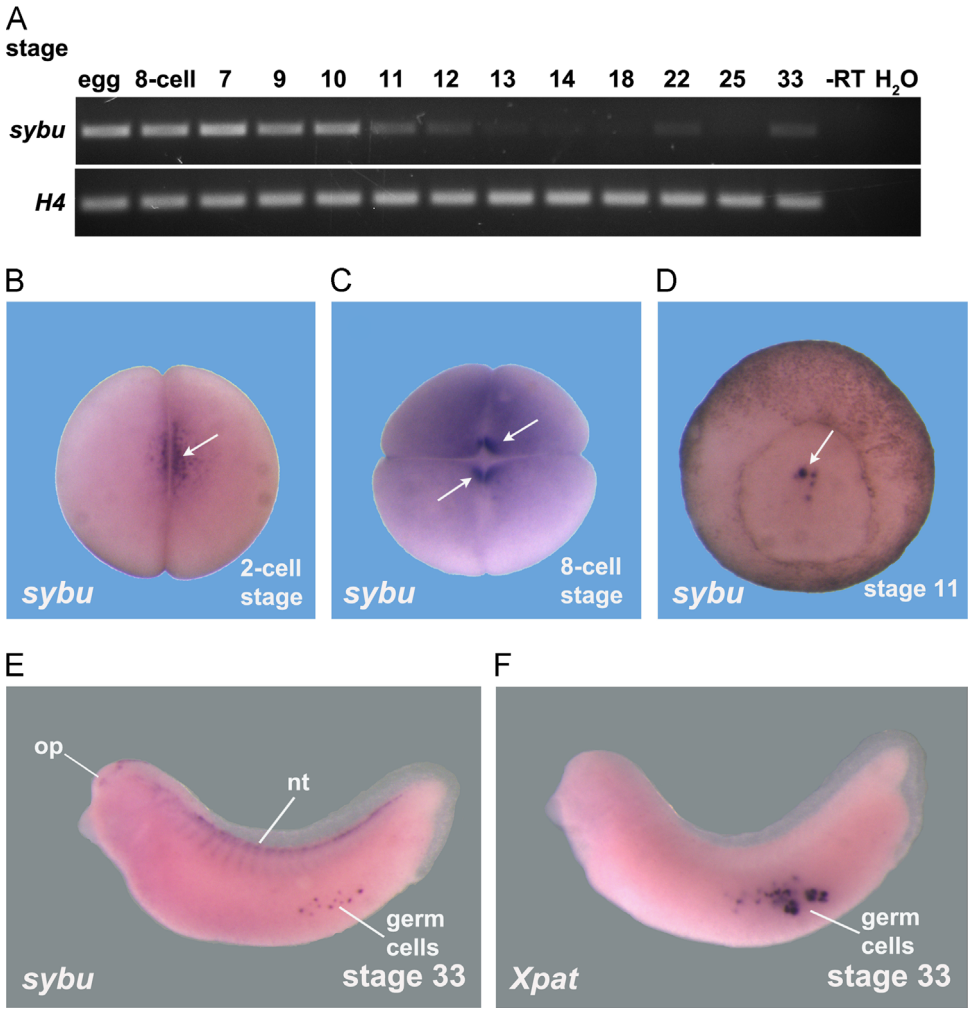

Fig. 3. Maternal *syntabulin* mRNA is present during early stages of development, and associates with germ plasm and germ cells. (A) Semiquantitative RT-PCR showing *syntabulin* (*sybu*) mRNA levels at different developmental stages. *Histone 4* (*H4*) mRNA was used as loading control. (B, C) In the cleaving embryo, *syntabulin* mRNA associates to germ plasm islands, that occupy a characteristic position close to the cleavage furrow (arrows). (D) During gastrulation, *syntabulin* transcripts are detected in a small subset of endodermal cells (arrow), corresponding to the known location of germ cell precursors. (E) At stage 33, *syntabulin* is detected in different tissues such as the olfactory placode (op), the neural tube (nt) and the germ cells. (F) Expression pattern of an established germ cell marker, *Xpat*, at stage 33. Note the similar localization between *syntabulin* and *Xpat* in the germ cell territory.

vegetal pole (Fig. 2A). Interestingly, *syntabulin* staining appeared as granules exclusively associated to the vegetal cortex (Fig. 2B), which is characteristic of germ plasm components (Houston, 2013). In stage I oocytes, *syntabulin* mRNA localized around the mitochondrial cloud (also known as Balbiani body), which represents the source of the germinal granules (Heasman et al., 1984), and remained associated to the vegetal cortex for the rest of oogenesis (Fig. 2C). This suggests that *Xenopus syntabulin* localizes vegetally using the Messenger Transport Organizer (METRO) pathway (Kloc and Etkin, 1995). Zebrafish *syntabulin* is also associated with the Balbiani body in early oocytes (Nojima et al., 2010).

3.2. *Xenopus syntabulin is associated with germ plasm and with germ cells*

To better understand the *syntabulin* expression pattern, we performed RT-PCR analyses on frog embryos at different developmental stages. As shown in Fig. 3A, *syntabulin* mRNA levels were detected until gastrulation, at which point it began to decrease to undetectable levels at neurula stages (stages 13–18), but expression reappeared at low levels at tailbud stage. In cleaving embryos, *syntabulin* mRNA coalesced forming clusters near the cleavage furrow on the vegetal hemisphere (Fig. 3B, C, see arrows). This same localization pattern is followed by the germ plasm required for germ cell differentiation and migration (Houston, 2013; Savage and Danilchik, 1993). We did not notice D-V displacement of these transcripts during early cleavage. During mid-gastrulation, *syntabulin* expression was found in scattered cells at the apex of the yolk plug (Fig. 3D), similarly to the pattern of the germ cell determinants *Dead End* (Horvay et al., 2006), *Dazl* (Houston and King, 2000) and *Grip2* (Tarbashevich et al., 2007). To determine that *syntabulin* is expressed in PGCs, we compared its expression at stage 33 with that of the well-known germ cell marker, *Xpat* (Hudson and Woodland, 1998). In addition to being expressed in the neural tube and olfactory placodes at tailbud stage, *syntabulin* was also expressed in scattered cells localized along the posterior endoderm (Fig. 3E). This closely resembled the expression pattern of *Xpat*, which is found at the same location (compare Fig. 3E to F). Altogether, these data indicate that *syntabulin* is a germ plasm component and is later expressed in PGCs.

3.3. *Syntabulin is required for establishing the dorsal axis and functions upstream of Wnt/β-Catenin signaling*

To address whether *Xenopus syntabulin* is involved in dorsal axis development, we conducted maternal loss of function experiments

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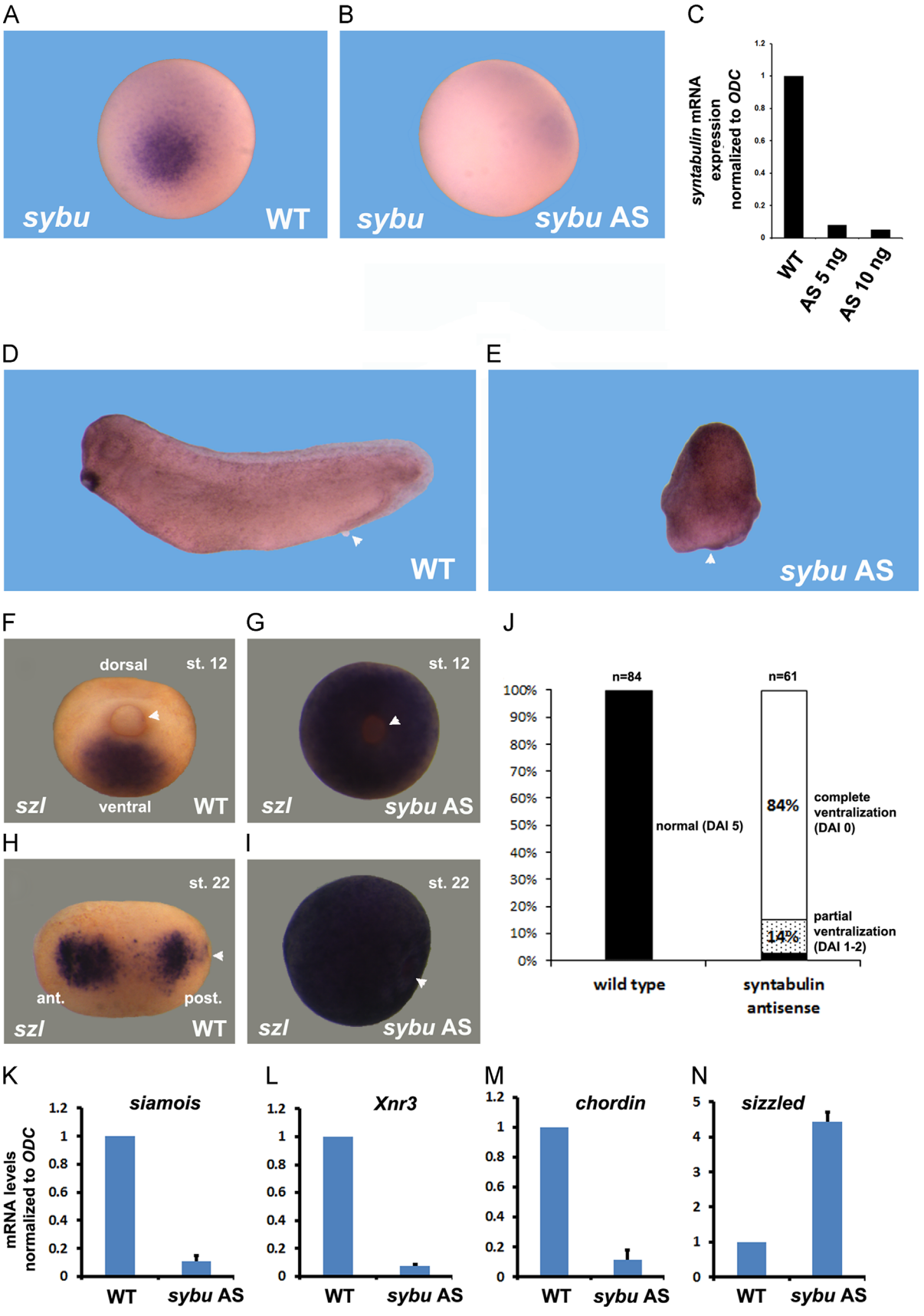
A
sybu
WT
B
sybu
sybu AS
C
syntabulin mRNA expression normalized to ODC
1.2
1
0.8
0.6
0.4
0.2
0
WT
AS 5 ng
AS 10 ng
D
WT
E
sybu AS
F
dorsal
st. 12
szl
ventral
WT
G
st. 12
szl
sybu AS
H
st. 22
ant.
post.
szl
WT
I
st. 22
szl
sybu AS
J
n=84
n=61
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
normal (DAI 5)
84%
complete ventralization (DAI 0)
14%
partial ventralization (DAI 1-2)
wild type
syntabulin antisense
K
siamois
mRNA levels normalized to ODC
1.2
1
0.8
0.6
0.4
0.2
0
WT
sybu AS
L
Xnr3
1.2
1
0.8
0.6
0.4
0.2
0
WT
sybu AS
M
chordin
1.2
1
0.8
0.6
0.4
0.2
0
WT
sybu AS
N
sizzled
5
4
3
2
1
0
WT
sybu AS

using phosphorothioate antisense DNA oligos to knockdown maternal *syntabulin* mRNA (Heasman et al., 1991). *Xenopus* oocytes are particularly amenable to this system, because of their high content in maternal RNase H which allows for rapid degradation of selected mRNAs (Olson et al., 2012). Stage VI oocytes were isolated and injected with phosphorothioate-modified DNA antisense oligos. In this study we identified two antisense oligos targeting *syntabulin* mRNA, designed as AS6 and AS10, that were effective in reducing *syntabulin* mRNA levels. Both antisense oligos caused similar phenotypes, but AS6 gave the most consistent results and was used in the experiments that follow. To assess the effectiveness of knockdown, oocytes were collected 48 h after antisense oligo injection and processed for *in situ* hybridization and qRT-PCR. *Syntabulin* mRNA staining disappeared from the vegetal pole of the oocyte (Fig. 4A, B), and qRT-PCR analyses confirmed that *syntabulin* mRNA depletion was efficient (Fig. 4C).

To assess whether *syntabulin* knock-down caused axis defects, injected oocytes were transferred into egg-laying frogs. Antisense-injected embryos displayed a radially ventralized phenotype, lacking all dorso-anterior structures, when compared to wild type uninjected controls (Fig. 4D, E). Furthermore, *syntabulin*-depleted embryos showed a radial expansion of the ventral marker *sizzled* (Fig. 4F–I) (Lee et al., 2006) confirming the expansion of ventral tissues. The penetrance of the radially ventralized phenotype was consistently high, reaching 84% of total embryos ($n=61$) (Fig. 4J). Ventralization was confirmed by qRT-PCR, showing a strong decrease of dorsal markers (*siamois*, *Xnr3* and *chordin*) and a 4-fold increase of *sizzled* (Fig. 4K–N).

In zebrafish, *syntabulin* mutants can be rescued by activating the canonical Wnt pathway, suggesting that *syntabulin* operates upstream or in parallel of Wnt/β-Catenin (Nojima et al., 2004). To test whether this was the case in *Xenopus*, *syntabulin*-depleted embryos were injected with *β-catenin* mRNA in the two dorsal blastomeres at 4-cell stage. *Syntabulin* knockdown abolished the expression of the dorsal neural marker *Sox2*, except for a faint expression domain around the blastopore (Fig. 5A, B), and injection of *β-catenin* mRNA rescued *Sox2* expression to levels comparable to those of wild type controls (Fig. 5C). Dorso-anterior structures were rescued by vegetal injection of 250 pg of *Xenopus syntabulin* mRNA into oocytes (24 h after antisense oligo injection to allow for its degradation, Hulstrand et al., 2010), showing that the phenotype was specific for Syntabulin, as well as being required for early β-Catenin signaling (Fig. 5D–G). *Syntabulin* or *β-catenin* mRNA also caused the rescue of *chordin* expression (Fig. 6A–D) and the repression of ectopic *sizzled* transcripts (Fig. 6E–H) at gastrula stages. These data show that *Xenopus syntabulin* is required for dorsal axis specification and that it acts through the early canonical Wnt signaling pathway.

4. Discussion

Two main findings are reported here. First, *Xenopus syntabulin* mRNA was not only localized to the vegetal pole of the oocyte but also to the germ plasm of early embryos. Second, maternal depletion of *syntabulin* mRNA at the very end of oogenesis was sufficient to eliminate dorsal axis formation by the early Wnt/β-Catenin signal. The role of Syntabulin in the establishment of D-V axis was discovered through zebrafish genetics (Nojima et al., 2004). Analysis of the *tkk* recessive mutant revealed an insertion of a transposable element in the *syntabulin* promoter region, strongly reducing its expression (Nojima et al., 2010). Mutant fish embryos displayed a range of ventralized phenotypes, characterized by reduced expression of dorsal markers and decreased nuclear β-Catenin accumulation in dorsal cells (Nojima et al., 2010). Importantly, components of Wnt/β-Catenin signaling could rescue the phenotype (Nojima et al., 2004), suggesting that Syntabulin may be involved in the regulation of early maternal Wnt signaling, perhaps by participating in the transport of some still unknown dorsal determinant that activates canonical Wnt signaling dorsally. We have extended these results to *Xenopus laevis* using maternal depletion of *syntabulin* mRNA with antisense phosphorothioate DNA oligos (Mir and Heasman, 2008; Olson et al., 2012). Degradation of *syntabulin* mRNA resulted in embryos completely lacking dorsal structures and a corresponding radial expansion of ventral *sizzled*-positive tissues. Importantly, dorsal injection of *β-catenin* was sufficient to restore normal D-V patterning, suggesting that Syntabulin functions in a molecular pathway of dorsal axis formation shared by both fish and frogs.

In zebrafish maternal mutants, which lack *syntabulin* mRNA throughout oogenesis, ventralization might result from a requirement of Syntabulin for the transport of other molecules to the oocyte vegetal cortex. However, the *Xenopus* knockdown experiments make this possibility unlikely. Oogenesis in *Xenopus* takes 6–8 months, culminating in a full grown oocyte (stage VI) in which animal-vegetal asymmetries are fixed (Houston, 2013). Our DNA antisense oligo experiments were performed on stage VI oocytes, and an incubation of only 48 h after microinjection was sufficient to generate ventralized embryos with high penetrance. It seems likely that *syntabulin* mRNA translation might be triggered by progesterone-induced egg maturation or fertilization, so that the newly synthesized protein is specifically required for the transport of dorsal determinants during cortical rotation. Syntabulin may be required at high concentrations at the time and place in which it is utilized. It will be interesting to determine whether other components of the microtubule/kinesin machinery are similarly localized.

The *Xenopus* oocyte contains many vegetally localized mRNAs (Weeks and Melton 1987; King et al., 2005), several of which encode proteins involved in mRNA translational regulation. One of these mRNAs, *dead end*, is required for D-V patterning (Mei et al., 2013). Dead end is thought to function by activating the translation of Trim36, a vegetally localized RING finger ubiquitin ligase that controls microtubule polymerization and, like *dead end*, is required for dorsal axis formation (Cuykendall and Houston, 2009). In future, it would be interesting to test whether *syntabulin* mRNA translation is also regulated by Dead end.

In *Xenopus* oocytes, vegetal mRNAs rely on two alternative mechanisms to achieve their localization: the early Messenger Transport Organizer (METRO) pathway and the late pathway (reviewed in Houston, 2013; King et al., 2005; Kloc and Etkin, 2005;

Fig. 4. Depletion of maternal *syntabulin* causes embryo ventralization and decreases the expression of dorsal genes. (A) Wild type oocyte showing normal *syntabulin* expression. (B) 91% ($n=12$) of oocytes injected with 10 ng of phosphorothioate-modified DNA antisense oligo targeting *syntabulin* (*sybu* AS) showed no expression of the maternal mRNA 48 h after microinjection. Hybrids between mRNA and DNA are efficiently degraded by RNase H. (C) Depletion of *syntabulin* in injected oocytes assessed by quantitative RT-PCR (qPCR). Groups of five oocytes were used. Injection of different amount of *sybu* AS (5 or 10 ng) caused a strong decrease of mRNA levels in a dose-dependent fashion. WT indicates uninjected oocytes. (D, E) Representative wild type and *sybu* AS-injected embryos obtained from host transfer experiments. Note the complete absence of axial structures in *sybu*-depleted embryos. (F) In wild type embryos, the ventral marker *sizzled* (*szl*) is expressed in a ventral domain at stage 12. (G) Age-matched *sybu*-depleted embryos (*sybu*-AS) display radial ectopic *szl* expression, indicating strong expansion of ventral tissues. (H) In wild type embryos at stage 22, *szl* has an anterior and a posterior domain of expression. (I) *sybu*-depleted embryos have a radially expanded *szl* ventro-posterior expression domain. Strong expansion of ventral tissue was observed in 70% of the embryos ($n=36$). Arrowheads point to the blastopore. (J) Quantification of the ventralization phenotype after *syntabulin* depletion. Note the high penetrance of complete ventralization (dorso-anterior index 0) in antisense injected embryos (84%); data from 4 independent host transfer experiments ($n=61$). (K–N) qRT-PCR analyses showing significant decreases in the dorsal markers *siamois*, *Xnr3* and *chordin*, and a strong increase of the ventral marker *sizzled* after maternal knockdown of *syntabulin*. Error bars indicate the standard error from three independent host transfer experiments.

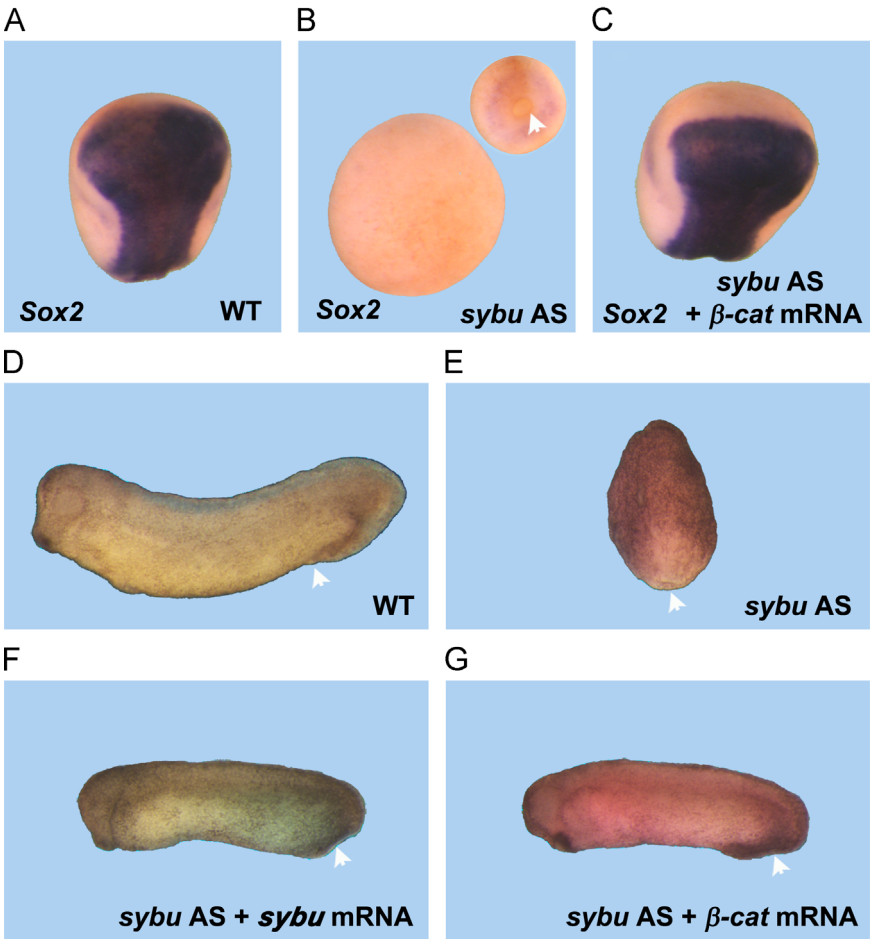

Fig. 5. The Syntabulin maternal depletion phenotype is rescued by *sybu* mRNA or by *β-catenin* mRNA. (A) Expression of the neural plate marker *Sox2* in wild type early neurula embryos, $n=16/16$. (B) *Syntabulin* maternal depletion eliminated almost all *Sox2* expression, with the exception of a weak residual ring of expression around the blastopore (inset, arrowhead), $n=15/15$. (C) Injection of *β-catenin* mRNA at 4-cell stage efficiently rescued expression of *Sox2* in *sybu* depleted host transfer embyos, $n=12/12$. (D–G) The sybu AS ventralized phenotype was rescued both by *syntabulin*-UTR^{+} mRNA ($n=7$) and by *β-catenin* mRNA ($n=12$) at the tailbud stage.

Mowry and Cote, 1999). In the METRO pathway, mRNAs accumulate within a region of the Balbiani body, also called the mitochondrial cloud, in stage I oocytes (Chang et al., 2004), and then translocate to the vegetal cortex through a directional expansion of the mitochondrial cloud itself (Wilk et al., 2004). Interestingly, many mRNAs localized through the METRO pathway are also components of the germ plasm and involved in germ cell differentiation (Kloc et al., 1998).

As in *Xenopus*, in zebrafish the Balbiani body serves as an organizing center for vegetal mRNA localization, and this system requires the product of the *bucky ball* gene (reviewed in Langdon and Mullins, 2011). Zebrafish *syntabulin* mRNA localizes to the Balbiani body in early oocytes, but in *bucky ball* mutants it shows diffuse unlocalized staining instead (Nojima et al., 2010). In stage I *Xenopus* oocytes, *syntabulin* mRNA localized to the periphery of the mitochondrial cloud from where they translocated to the vegetal pole, indicating the METRO pathway is used.

A novel finding in our study is that *syntabulin* maternal mRNA becomes associated with the germ plasm at 2–4 cell stage and with germ cells at gastrula and tailbud stages. Increasing lines of evidence show that some germ plasm-associated mRNAs, such as *Fatvg* (a lipid droplet-associated protein likely involved in intracellular vesicle trafficking) and *dead end* have dual activities, being required for both germ cell differentiation and embryonic D-V axial development (Chan et al., 2001, 2007; Horvay et al., 2006; Mei et al., 2013). Although not investigated in this study, our expression data suggest that Syntabulin may also have a role in germ cell specification, an intriguing possibility that should be tested in future. The fact that Fatvg, Dead end and perhaps Syntabulin, function in D-V pattern as well as in germ cell specification raises the possibility of an ancient signaling system. In *Drosophila*, the translational repressor *nanos* is essential for both antero-posterior abdominal patterning and germ cell determination (Gavis and Lehmann, 1992; Forbes and Lehmann, 1998). In *Xenopus*, *nanos1* (previously called *Xcat2*) mRNA localizes to the vegetal cytoplasm of the oocyte. Nanos1 is essential for germ cell differentiation, but a requirement for embryonic patterning has not been reported (Mosquera et al., 1993; Lai et al., 2011, 2012). The relationship between vegetal cytoplasm, germ cell determination and body patterning represents an interesting developmental problem for future studies.

Which are the factors that might interact with Syntabulin in the oocyte? A well-known binding partner in nerve cells is Syntaxin1 (Su et al., 2004), belonging to the family of the Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor (SNARE) transmembrane proteins that mediate synaptic vesicle membrane fusion and neurotransmitter release (for a review, see Rizo and Südhof, 2012). Interestingly, western blot analysis using a polyclonal anti-syntaxin1 antibody has revealed the presence of a still unidentified syntaxin protein in the *Xenopus* oocyte (Fili et al., 2001). However, to date it has not been determined which isoform is expressed and whether it has a role in early development. In *Drosophila*, Syntaxin1A was shown to be involved in the release of exosomes containing Wingless (Wg) and Wntless/Evenness Interrupted (Wls/Evi), which are required for neuromuscular junction synapses

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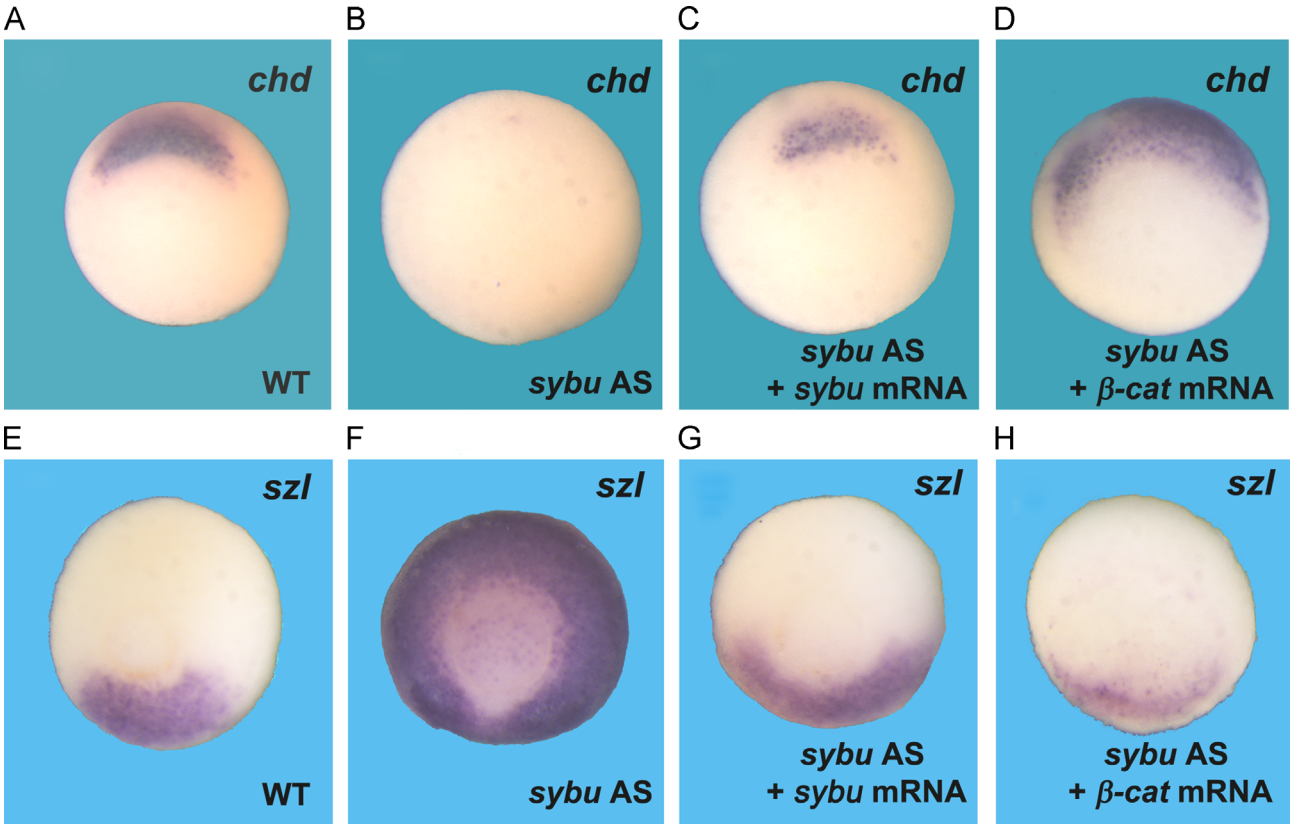

Fig. 6. Rescue of D-V patterning genes in Syntabulin-depleted embryos by *syntabulin* or *β-catenin* mRNA injection. (A) Expression of the Spemann organizer marker *chordin* ($n=13/13$) in wild type embryos at stage 10. (B) *Chordin* is eliminated by maternal depletion of *syntabulin* ($n=9/9$). (C) Injection of *sybu* mRNA partially rescues its own depletion ($n=6/10$), demonstrating specificity of the knock-down. (D) *chordin* expression is rescued, and somewhat expanded with respect to wild type, by *β-catenin* mRNA ($n=14/14$). (E–H) Maternal depletion of *syntabulin* increases expression of the ventral marker *sizzled* and this is counteracted by the injection of *syntabulin*-UTR^{+} or *β-catenin* mRNAs.

(Packard et al., 2002; Koles and Budnik, 2012; Koles et al., 2012). Syntabulin may provide a link between microtubules and membrane trafficking during the generation of the Wnt/β-Catenin signal (Taelman et al., 2010; Dobrowolski and De Robertis, 2011; Vinyoles et al., 2014). Further experiments will be required to understand the relationship between Syntabulin, a protein bound to microtubule motors, and the elusive dorsal determinants of the *Xenopus* egg.

Author disclosure statement

The authors declare that there are no conflicting financial interests.

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