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ORIGINAL ARTICLE

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Xenopus Tetraspanin-1 regulates gastrulation movements and neural differentiation in the early *Xenopus* embryo

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Abstract The tetraspanin family of four-pass transmembrane proteins has been implicated in fundamental biological processes, including cell adhesion, migration, and proliferation. Tetraspanins interact with various transmembrane proteins, establishing a network of large multimolecular complexes that allows specific lateral secondary interactions. Here we report the identification and functional characterization of *Xenopus* Tetraspanin-1 (xTspan-1). At gastrula and neurula, xTspan-1 is expressed in the dorsal ectoderm and neural plate, respectively, and in the hatching gland, cement gland, and posterior neural tube at tailbud stages. The expression of xTspan-1 in the early embryo is negatively regulated by bone morphogenetic protein (BMP) and stimulated by Notch signals. Microinjection of *xTspan-1* mRNA interfered with gastrulation movements and reduced ectodermal cell adhesion in a cadherin-dependent manner. Morpholino knock-down of endogenous xTspan-1 protein revealed a requirement of xTspan-1 for gastrulation movements and primary neurogenesis. Our data suggest that xTspan-1 could act as a molecular link between BMP signalling and the regulation of cellular interactions that are required for gastrulation movements and neural differentiation in the early *Xenopus* embryo.

Key words tetraspanin · BMP · Notch · gastrulation movements · *Xenopus* · cadherin · adhesion

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Introduction

The basic body plan of vertebrate embryos is established in the course of gastrulation, when mesendodermal cells involute into the blastocoel. Subsequently, temporal and spatial coordinated cell movements bring the cells of all three germ layers into their proper positions from which they form complex tissues and organs. In amphibians, the first sign of this highly dynamic process is the formation of the dorsal blastopore lip, better known as the Spemann organizer that has been shown to express several growth factor antagonists (De Robertis and Bouwmeester, 2001; Niehrs, 2004). In particular, determination of the dorsoventral, or back to belly, embryonic body axis is dependent by the generation of a functional bone morphogenetic protein (BMP) activity gradient that is established by the secretion of BMP antagonist from the Spemann organizer, including chordin, noggin, and follistatin (De Robertis and Kuroda, 2004; Stern, 2005).

Various genetic studies in vertebrate and invertebrate species have revealed a central role of the non-canonical Wnt signalling pathway in the regulation of directed cell migration (Kühl et al., 2001; Keller, 2002). In particular, convergent extension movements, leading to a mediolateral narrowing and anteroposterior lengthening of the *Xenopus* and zebrafish embryo, are controlled by components of the planar cell polarity (PCP) pathway (Goto and Keller, 2002; Wallingford et al., 2002; Wedlich, 2002; Formstone and Mason, 2005). Interestingly, genetic studies in zebrafish demonstrated an important function of the BMP activity gradient in the determination of embryonic domains that undergo distinct convergent extension movements. Although the molecular mechanisms remain unknown, it has been suggested that BMP might regulate convergent extension movements independently of its essential function in cell fate determination (Myers et al., 2002).

Tetraspanins have been implicated in the regulation of cell migration, adhesion, and signalling in normal and pathological conditions, like tumor progression and metastasis (Boucheix and Rubinstein, 2001; Hemler, 2003). The tetraspanin family is characterized by four transmembrane domains. The second extracellular loop contains characteristic amino acid motifs that distinguish the tetraspanin family from other four-pass transmembrane proteins (Hemler, 2001; Kitadokoro et al., 2001; Stipp et al., 2003). Tetraspanins interact with various membrane proteins, including growth factor receptors, leukocyte receptors, and integrins, the main extracellular matrix receptors and have been proposed to form multimolecular complexes or microdomains in the cell membrane (Berditchevski, 2001; Hemler, 2003; Stipp et al., 2003; Tarrant et al., 2003). A central function of tetraspanin proteins might be the establishment of a network of tetraspanin microdomains to organize additional proteins into signalling complexes (Stipp et al., 2003; Yunta and Lazo, 2003).

Several of the over 30 vertebrate tetraspanins are expressed in the nervous system and have been implicated in synapse formation, neurite outgrowth, and glial cell proliferation (Hemler, 2003). Mutations of TM4SF2 were identified in 10% of X-linked mental retardation patients (Zemni et al., 2000) and CD81 mutant mice have increased numbers of astrocytes and microglia (Geisert et al., 2002). Thus, tetraspanin deficient mice display phenotypes that reveal specific, non-redundant functions (Hemler, 2003; Tarrant et al., 2003; Wright et al., 2004).

We have isolated a *Xenopus laevis* homolog of Tspan-1/Net-1 (Todd et al., 1998; Serru et al., 2000). *Xenopus* Tetraspanin-1 (xTspan-1) is expressed dorsally at gastrula and neurula stages. The transcription of *xTspan-1* is repressed by BMP signals and stimulated by activated Notch signalling. Microinjection of *xTspan-1* mRNA interfered with gastrulation movements and ectodermal cell adhesion that was rescued by co-injection of *C*- and *E-cadherin* mRNA. Loss-of-function experiments, using *xTspan-1*-specific morpholino oligomers, revealed a requirement of xTspan-1 for gastrulation movements in the whole embryo, as well as Activin-treated ectodermal explants. In addition, the depletion of endogenous xTspan-1 protein interfered with primary neurogenesis. These studies demonstrate an essential function of a *Xenopus* homolog of Tspan-1 in the regulation of morphogenesis and neurogenesis in the early *Xenopus* embryo.

Materials and methods

Embryo manipulations

Xenopus embryos obtained by *in vitro* fertilization were maintained in 0.1 modified Barth medium (Sive et al., 2000) and staged according to Nieuwkoop and Faber (1994). Ectodermal explants were excised at stage 9 and cultured in 0.5 MMR saline until sibling embryos reached the indicated stage. The treatment of ectodermal explants with recombinant Activin protein (R&D) and LiCl-treatment of whole embryos at the 32-cell stage was performed as previously described (Sive et al., 2000; Oelgeschläger et al., 2003). To facilitate the separation of the ectodermal and mesodermal cell layers from neurula stage embryos, the dorsal embryonic fragments were incubated in CMFM. For dissociation and reaggregation assays, animal cap explants were excised at stage 9 and dissociated in CMFM (Sive et al., 2000) for 10 min. The outer layer was removed and the equivalent of five uninjected control caps and two microinjected caps mixed in CMFM. Calcium and magnesium was added to final concentrations of 1 and 2 mM, respectively, and the cells allowed to reaggregate for 3 hr. The aggregates were fixed for 1 hr in MEMFA (Sive et al., 2000), incubated in Tissue-Tek (Sakura, Torrance, CA) at 4°C overnight, slowly cooled down to −80°C as described by Shariatmadari et al. (2001) and frozen sections analyzed by fluorescence microscopy.

DNA constructs and mRNA synthesis

A full-length cDNA coding for xTspan-1 was obtained from RZPD (IMAGE Consortium cDNA clone # 6639112; Lennon et al., 1996) and the ORF sequence verified by comparison with the full-length cDNA sequence of LOC496318 (BC088907). The ORF was amplified by PCR and cloned into the *Eco*RI site of pCS2+. For the xTspan–GFP (green fluorescent protein) construct, a fragment encoding the xTspan-1 ORF fused to a C-terminal FLAG-Tag epitope was PCR amplified and cloned in frame with EGFP of pEGFP-N1 (Clontech, Mountain View, CA) using *Eco*RI and *Bam*HI and subsequently cloned into the *Eco*RI–*Xba* sites of pCS2+. For the xTspan–GFP construct containing the 5′-untranslated region (5′-UTR), a *Kpn*I–*Not*I fragment of xTspan–GFP was cloned into the xTspan-1 EST cDNA. The expression vectors for E-cadherin and the Notch ICD were kindly provided by Dr. D. Wedlich and Dr. R. Kopan. For mRNA synthesis pCS2-xTspan-1, pCMV-xTspan–GFP (+UTR), pCS2-E-Cadherin, pCS2-Notch-ICD, pCMV-Sport6-C-Cad (Accession # CA788543) were linearized with *Not*I, pCS2–xTspan–GFP with *Apa*I and mRNA synthesis performed using the mMessage mMachine Sp6 kit (Ambion, Austin, TX).

Morpholino oligomers

Morpholino oligomeres were obtained from Gene Tools (Philomath, OR), resuspended at 1 mM in water and 2 nl of a 250 μM solution were microinjected radially at the two- to four-cell stage or into single dorsal-animal blastomeres at eight- to 16-cell stage. The sequences for the morpholinos were: standard control morpholino: 5′-CCTCTTACCTCAGTTACAATTTATA-3′TMO1 5′-ATTGTTCTGGCTGAAAGTTCCCTCA-3′, TMO2 5′-TGATAAACGAGAAACACCCCATTGT-3. Both *xTspan-1*-specific morpholinos generated similar phenotypes and in all the experiments shown, TMO1 was used. Inhibition of xTspan-1 translation was performed with 100 ng expression vector using the Promega (Madison, WI) Rabbit Reticulocyte System in the absence or presence of 2.5 μM morpholino oligomer. To test for inhibition of xTspan-1 translation *in vivo*, 50 pg of the xTspan–GFP or Tspan–GFP (+5′-UTR) mRNA was injected animally at the eight-cell stage alone or together with 2 nl morpholino oligomer and the fluorescence analyzed at stage 11.

Reverse transcriptase polymerase chain reaction and whole mount *in situ* hybridization

RT-PCR was performed as described (Oelgeschläger et al., 2003). The primers were for *xTspan-1* 5′-TCCACCTACTACAGCAACTACC-3′ (forward) and 5′-CTGCCGAATGAGGTAGAGGAGC-3′ (reverse) and for *E-cadherin*, 5′-GATGAAGAA-