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Gtpbp2 is required for BMP signaling and mesoderm patterning in *Xenopus* embryos

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ABSTRACT

Smad proteins convey canonical intracellular signals for activated receptors in the TGFβ superfamily, but the activity of Smads and their impact on target genes are further regulated by a wide variety of cofactors and partner proteins. We have identified a new Smad1 partner, a GTPase named Gtpbp2 that is a distant relative of the translation factor eEf1a. Gtpbp2 affects canonical signaling in the BMP branch of the TGFβ superfamily, as morpholino knockdown of Gtpbp2 decreases, and overexpression of Gtpbp2 enhances, animal cap responses to BMP4. During *Xenopus* development, *gtpbp2* transcripts are maternally expressed and localized to the egg animal pole, and partitioned into the nascent ectodermal and mesodermal cells during cleavage and early gastrulation stages. Subsequently, *gtpbp2* is expressed in the neural folds, and in early tadpoles undergoing organogenesis *gtpbp2* is expressed prominently in the brain, eyes, somites, ventral blood island and branchial arches. Consistent with its expression, morpholino knockdown of Gtpbp2 causes defects in ventral–posterior germ layer patterning, gastrulation and tadpole morphology. Overexpressed Gtpbp2 can induce ventral–posterior marker genes and localize to cell nuclei in *Xenopus* animal caps, highlighting its role in regulating BMP signaling in the early embryo. Here, we introduce this large GTPase as a novel factor in BMP signaling and ventral–posterior patterning.

Introduction

The Transforming Growth Factor β (TGFβ) superfamily regulates a diverse set of biological processes, such as cell proliferation, adhesion, migration, apoptosis, differentiation embryonic pattern formation and organogenesis (Ten Dijke et al., 2002; Shi and Massagué, 2003; Feng and Derynck, 2005; Schier and Talbot, 2005). Ligands in the TGFβ superfamily bind to particular combinations of serine/threonine kinase receptors that signal through Smad and non-Smad-dependent pathways (Moustakas and Heldin, 2005, 2012). In canonical mode, ligand-bound receptors activate R-Smads to signal downstream to target genes. R-Smad signaling is grouped into two distinct branches, with Smads1/5/8 conveying BMP/GDF signals and Smads2/3 operating under Activin/Nodal/TGFβ.

Genes encoding TGFβ signaling components, and many of their functions, are well conserved throughout the metazoa, regulating embryonic development in animals as diverse as ancient diploblast lineages (cnidaria and ctenophora) through complex triploblasts (chordata). In vertebrate embryos in particular, Nodal/Vg1 and BMP-related pathways provide essential signals that induce and pattern the primary germ layers, regulate tissue morphogenesis and left–right asymmetry, and affect cellular pluripotency, differentiation, growth and death. TGFβ signals often act in concert with FGF and Wnt signaling in these developmental processes. In *Xenopus* embryos in particular, mesoderm and endoderm are induced by Nodals, Vg1 and Derriere ligands, acting together with FGFs, and early tissue patterning is achieved by BMPs alongside Wnt and FGF signals (Heasman, 2006; Kimelman, 2006; Itasaki and Hoppler, 2010). In the ectoderm, different levels BMP signaling triggers differentiation of the epidermis, neural crest, sensory placodes and neural tissues (DeRobertis and Kuroda, 2004; Vonica and Brivanlou, 2006; Rogers et al., 2009).

Because of their importance in embryonic development and tissue homeostasis, a variety of mechanisms have evolved to regulate the activity of TGFβ pathways at all levels, from ligand production and extracellular regulation, through receptors, signal transducers and transcriptional cofactors (Itoh and ten Dijke, 2007). To explore regulation at the level of signal transduction, we sought to identify new partners of BMP/Smad signaling by performing yeast two-hybrid screens with Smad1 (Zhu et al., 1999), which retrieved several TGFβ signaling regulators, including

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Smurf1 (Zhu et al., 1999; Thomsen, 2013), Eps15r (Callery et al., 2012), and XMan (Osada et al., 2003; our unpublished results). Another factor we retrieved is Gtpbp2, a large GTPase distantly related to the translational regulators eEf1a1, Gspt1 (eRF3) and Hbs1-like (Kudo et al., 2000). No function has been ascribed to Gtpbp2, although it has shown to be expressed in developing mouse embryos (Watanabe et al., 2000; Kudo et al., 2000). Gtpbp2 has a conserved yet distinct paralog, Gtpbp1, that regulates mRNA 3′ end-processing, but Gtpbp2 appears to lack that function (Woo et al., 2011). Here we show that Gtpbp2 interacts directly with Smad1, can potentiate BMP signaling and activate BMP target genes, is required for embryonic responses to BMP signaling, and is essential for normal ventral–posterior mesodermal patterning.

Materials and methods

cDNA isolation and constructs

A partial clone corresponding to the C terminus of Gtpbp2 was retrieved from a yeast two-hybrid screen done with a *Xenopus* oocyte cDNA library (Clontech) using Smad1 as bait (Zhu et al., 1999). Full length EST for gtpbp2 (DT061674) was obtained from Resgen Inc. For mRNA synthesis and expression in cultured cells, Gtpbp2 isoforms including one with mismatches at morpholino binding sites were amplified by PCR and subcloned into pCS2-HA or pCS2. The HindIII-XbaI fragment of pCS2-HA for each construct was then subcloned into pCDNA3.1NotI. Final constructs were linearized with NotI, and mRNAs were made using an mMESSAGE mMACHINE T7 Kit (Ambion). Deletion constructs for Gtpbp2ΔN and Gtpbp2ΔC were made by PCR from parent vector; 3xHA-Gtpbp2-pcDNA3.1NotI. N′ Cherry tagged versions of Gtpbp2 isoforms were made by cloning Cherry sequence into XhoI sites of 3xHA-Gtpbp2 parent vectors, thereby replacing the 3xHA tag. C′-flag-*Xenopus* Smad1 was cloned by PCR addition of a C terminal flag tag into pCS2. Flag–MH1 and Flag–MH2 were derived from this parental construct by PCR deletion of excluded sequences. All PCRs were performed using Platinum Pfx polymerase (Invitrogen) with low cycle number (< 18 cycles). Flag-xsmad1, and flag-xsmad4 were previously described. (Thomsen, 1996; Zhu et al., 1999).

Morpholino design and injection

Xenopus embryos were collected and microinjected as described previously (Alexandrova and Thomsen, 2006). Morpholino oligonucleotides (MOs) were supplied by GeneTools Inc., as follows, M2: TCCCCCTGACTGGCACGGAATGCCC, M1: CGCGGCTCCATCCCACCGGCCCTG, 5mis-to-M1: CcCGGgTCCATgCCACCcGCCgTG. *Xenopus* is an allotetroploid organism in which most of the genes, if not all, are coded from duplicated copies (Uno et al., 2013). Morpholinos were designed to target both copies of Gtpbp2.

Immunoprecipitation and western blots

Full-length and deletion constructs of HA tagged Gtpbp2 isoform b were co-transfected with full length Flag-tagged Smad1, or deletion constructs of Flag–Smad1, to Hek293T cells using transfection reagent Fugene6 (Roche). Cells were lysed 24 h after transfection with PBS containing 1% Triton X-100, 2 mM EDTA, 1 mM Na_3VO_4 and complete protease inhibitors (Roche). Immunocomplexes were precipitated and washed according to the Flag-M2 Beads protocol (Sigma). Anti-HA-HRP (Roche) (1:500) and anti-Flag M2 (Sigma) (1:2000) followed by anti-mouse-HRP antibody (Sigma) (1:5000) were used to detect HA–Gtpbp2 forms and Flag–Smad1 constructs, respectively. A Flag-GFP construct was used as a balancer in DNA transfections into cultured cells.

In situ hybridization and developmental RT-PCR

A fragment corresponding to positions 609–1510 of *Xenopus gtpbp2* reference sequence NM_001099909 was PCR amplified, and cloned into pGEMT-Easy (Promega). The gtpbp2 RNA in situ probe was made from the pGEMT-Easy construct linearized with ApaI, transcribed with SP6 RNA polymerase, and labeled with digoxygenin-UTP (Roche). Whole mount in situ RNA hybridization was performed as described (Harland, 1991). in situ hybridization on sections was performed as described (Ciau-Uitz et al., 2000). Real-time quantitative PCR was performed with a LightCycler 480 System (Roche) to determine the developmental expression of gtpbp2, using primer pair: GTACGCTCTGGAGCCTGATG and TGTCTGCACCGACCTTCTCT. Digoxygenin (dig) labeled in situ probes for analysis of morphant embryos were made as described previously (Alexandrova and Thomsen, 2006).

Xenopus animal cap assays and quantitative RT-PCR

Synthetic mRNAs or MOs were injected into the animal pole of 2-cell stage embryos at doses indicated in the figures and text. The total amount of synthetic mRNA injected was held constant by balancing with GFP mRNA. Animal caps were excised at blastula stage 8, cultured in 0.5 × MMR, and harvested at Nieuwkoop and Faber stage 11 (mid-gastrula) or 18 (late neurula). Ten animal caps per each treatment were pooled and total RNA was extracted as described (Alexandrova and Thomsen, 2006), followed by cDNA synthesis with Superscript II Reverse Transcriptase (Invitrogen) using oligo-d(T)16–20 primers (Invitrogen). Real-time quantitative PCR (qPCR) was performed with the LightCycler 480 (Roche), using primer sequences and conditions as described (Xanthos et al., 2002; ⟨http://www.hhmi.ucla.edu/derobertis/⟩). Marker gene expression levels in cultured animal cap explants were normalized to an endogenous control gene (*ornithine decarboxylase*, ODC), and then plotted as a percentage of the level of endogenous gene expression in one embryo (set as 100%).

Luciferase reporter gene assay

Reporter assays were done by injecting 100 ng Vent2-Luc reporter (Hata et al., 1998) together with 50 ng TK-RL reporter as internal control to normalize the reporter activity, and 20–40 ng of M1 or 40 ng 5-base mismatched (5mis), morpholinos designed against Gtpbp2. Animal caps were explanted at stage 8 and harvested at stage 11. Ten animal caps or embryos per each treatment were pooled and extracts were prepared and analyzed using a Dual-Glo Luciferase Assay System (Promega).

Immunoflourescence and imaging:

mCherry-tagged *gtpbp2a* (2 ng) and *membrane localized GFP* (5 pg) synthetic mRNA were co-injected into the animal pole of 2-cell embryos, and then gently fixed at gastrula stage 11 in 1% paraformaldehyde in PBS for 15 min, washed 3 times in PBS, and then costained with 2 mg/ml 4,6-diamino-2-phenylindole (DAPI). Embryos were then imaged using a 10 × objective on a Zeiss fluorescence microscope (Motorized Axio Imager Z1) with Apo-Tome attachment.

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