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Smurf1 regulates neural patterning and folding in *Xenopus* embryos by antagonizing the BMP/Smad1 pathway

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Abstract

The ubiquitin ligase Smurf1 can target a handful of signaling proteins for ubiquitin-mediated proteasomal destruction or functional modification, including TGF-β receptors, Smads, transcription factors, RhoA and MEKK2. Smurf1 was initially implicated in BMP pathway regulation in embryonic development, but its potential role in vertebrate embryogenesis has yet to be clarified. Here we demonstrate that inhibition of Smurf1 in *Xenopus laevis* embryos with an antisense morpholino oligonucleotide or a dominant-negative protein disrupts early development, with the nervous system being the principal target. Smurf1 is enriched on the dorsal side of gastrula stage embryos, and blocking Smurf1 disturbs neural folding and neural, but not mesoderm differentiation, enhances BMP/Smad1 signaling, and elevates phospho-Smad1 levels in the dorsal ectoderm. We conclude that in *Xenopus* embryos, the BMP pathway is a major physiological target of Smurf1, and we propose that in normal development Smurf1 cooperates with secreted BMP antagonists to limit BMP signaling in dorsal ectoderm. Our data also reveal a novel role for Smurf1 and Smad1 in neural plate morphogenesis.

Keywords: Smurf1; Smad1; BMP; Ubiquitin ligase; Neural tube; Neural folding; Neural patterning; Signal transduction; Embryo; *Xenopus laevis*

Introduction

Smurf1 is a member of the HECT class of E3 ubiquitin ligases, and it is evolutionarily conserved from *Drosophila* through man (Zhu et al., 1999; Podos et al., 2001; Ebisawa et al., 2001). Smurf1 and the related Smurf2 are characterized by an N-terminal phospholipid binding or C2 domain, two or three WW domains that bind PPXY consensus motifs in partner proteins and substrates, and a C-terminal catalytic HECT domain (Zhu et al., 1999; Pickart, 2001a). Ubiquitin ligases catalyze transfer of ubiquitin from an E2, ubiquitin-conjugating enzyme, onto target proteins that results in their proteasomal or lysosomal degradation, or regulates their subcellular localization, trafficking or protein–protein interactions (Pickart, 2001a, b). We originally isolated Smurf1 as a Smad1-interacting factor by a yeast two-hybrid screen (Zhu et al., 1999).

Smad1 is a signal transducer in the canonical bone morphogenetic protein (BMP) signal transduction pathway that plays an important role in several events during vertebrate embryonic development: (1) the patterning of the ventro-lateral mesoderm; (2) the decision between epidermal and neural cell fate, in which high activity of Smad1/5 specifies epidermis, intermediate activity specifies the "neural border" fates (e.g. neural crest and cement gland), and in the absence of BMP/Smad1 signaling, neural induction takes place; (3) dorsoventral patterning of the neural tube, wherein BMPs are responsible for differentiation of dorsal neuronal subtypes (Dale and Wardle, 1999; Harland, 2000; Hill, 2001; De Robertis and Kuroda, 2004; Chizhikov and Millen, 2005; Wilson and Maden, 2005).

BMP signaling commences when homo- or heterodimers bind a complex of type I and type II Ser/Thr kinase receptors, Smads 1, 5 or 8 (Smad1/5/8) get phosphorylated and activated, bind to the co-partner Smad4 and translocate

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as a complex to the nucleus where they regulate target gene transcription (Lutz and Knaus, 2002). The BMP/Smad1 pathway can be negatively regulated at several levels: by extracellular BMP antagonists such as Noggin and Chordin, pseudoreceptors (e.g. BAMBI), inhibitory Smads, MAP kinases and Smad ubiquitylation regulatory factors or Smurfs (reviewed by von Bubnoff and Cho, 2001; Lutz and Knaus, 2002; De Robertis and Kuroda, 2004).

We have shown that Smurf1 can ubiquitylate and down-regulate Smad1/5 (Zhu et al., 1999; see below), but it also has a number of other potential targets that depend on the cell. For example, in C2C12 and 2T3 cells, Smurf1 can suppress BMP/Smad5 signaling and osteoblast differentiation by ubiquitylating Smad5 (Ying et al., 2003) or the osteoblast-specific transcription factor Cbfα1/Runx2 (Zhao et al., 2003, 2004; Kaneki et al., 2006). In overexpression assays, Smurf1 can target the TGF-β type I receptor (TBRI), BMP type I receptor (ALK6), Smad4 and inhibitory Smad7 for proteasomal degradation (Moren et al., 2005; Ebisawa et al., 2001; Suzuki et al., 2002; Murakami et al., 2003; Zhu et al., 1999 supplementary data). Furthermore, endogenous Smurf1-dependent ubiquitylation can trigger degradation of the small GTPase RhoA to affect cell protrusive activity and polarity (Wang et al., 2003), neurite outgrowth (Bryan et al., 2005) or epithelial cell tight junction dissolution in TGF-β-induced epithelial–mesenchymal transition (Ozdamar et al., 2005).

By misexpressing Smurf1 in *Xenopus* embryos, we previously found that Smurf1 can cause incomplete secondary axis formation by dorsalizing ventral marginal zone tissue, and Smurf1 can neuralize embryonic ectodermal explants (Zhu et al., 1999). However, a loss-of-function analysis of Smurf1 in *Xenopus* embryos is needed to reveal which, if any, of these phenomena are relevant *in vivo*. Smurf1 loss-of-function studies have been accomplished in *Drosophila* and mouse, with somewhat different results. *Drosophila* maternal-zygotic *dSmurf* mutants display enhanced and prolonged DPP/BMP signaling (Podos et al., 2001) as a consequence of stabilized phospho-MAD, the activated *Drosophila* homolog of vertebrate Smad1/5 (Liang et al., 2003). In contrast, Smurf1 knockout (KO) mice do not have developmental defects, but are characterized by an age-dependent increase in bone mass through enhanced osteoblast activity (Yamashita et al., 2005). Although osteoblasts from these mice are sensitized to BMP signaling, Smurf1 does not directly affect the levels of Smad1 or BMP receptors. Instead, MEKK2 is stabilized and activates JNK. The mouse results in particular raise the question of whether or not Smurf1 targets the BMP/Smad1 pathway under physiological situations in developing vertebrate embryos.

Here we report that blocking endogenous Smurf1 in *Xenopus* embryos with an antisense morpholino oligonucleotide (MO), or a dominant-negative mutant protein, disrupts neural folding and patterning, greatly affecting head development. We show that up-regulation of the BMP/Smad1 signaling pathway is the underlying cause of the knockdown phenotypes.

Materials and methods

Embryo manipulations, in situ hybridization, morpholino oligos and synthetic mRNAs

Xenopus laevis embryos were obtained by standard *in vitro* fertilization, de-jellied in 2% cysteine pH8.0, microinjected and incubated for several hours in 3% ficoll+0.5×MMR+10 μg/ml gentamycin and grown in 0.1×MMR+10 μg/ml gentamycin thereafter. 30–40 ng Smurf1 MO or 0.10–0.20 ng *Smurf1CA* mRNA was injected into the dorsal-animal region of the 4–8 cell stage embryos, unless indicated otherwise. All stages are according to Nieuwkoop and Faber (1967). Animal caps were excised at stage 8; prospective neural ectoderm (NE) and ventral ectoderm (VE) were excised at stage 10.25 as 60°-wide sectors on either the dorsal or the ventral side, from the animal pole to the bottom of the pigmented zone. The explants were cut and cultured in 0.5×MMR+10 μg/ml gentamycin until the sibling embryos reached the appropriate stage. *In situ* hybridization was as previously described (Harland, 1991) using BM purple as a chromogenic substrate (Roche). Template plasmids for making probes were pCS2-Smurf1, pGEM-Pax6, pBS-SK-Otx2, pBS-KS+En2, pGEM-Krox20, pBS-NCAM, pCS2-reverse-Msx1, pBS-SK-XNkx2.2, pBS-Sox2 and pBS-Xep. Morpholino oligo sequences are: standard control MO-5′cctcttacctcagttacaatttata3′, Smurf1 MO-5′attcgacatccctccaaacgccg3′ (Gene Tools, LLC, Philomath, OR). Full-length *X. laevis* Smad6 and zebrafish *Danio rerio* zSmurf1 were obtained as EST clones (I.M.A.G.E. consortium, clone ID numbers 6317366 and 5915182, respectively) and verified by sequencing. zSmurf1 was subcloned into pCS2 using *Eco*RI and *Xba*I sites. Capped synthetic mRNAs were *in vitro* transcribed using mMESSAGE mMACHINE kits (Ambion) from the following linearized plasmids: pCS2-Smurf1, pCS2-Smurf1CA, pCS2-zSmurf1, pCS2-Smad1, pSPYS-Chordin, pCS2-BMP4, pCMV-SPORT6-Smad6.

In vitro translation, Western blot analysis, antibodies and phalloidin staining

Smurf1 protein was *in vitro* translated using TnT T7/SP6 Coupled Reticulocyte Lysate system (Promega) in the presence of control or Smurf1 MO and [^{35}S] methionine, according to the manufacturer's protocol. Half of each reaction was resolved by 8% SDS-PAGE, visualized by autoradiography and quantified using NIH Image software. For Western blot analysis, three total embryos (Fig. 2C) or 15 explants (Fig. 11) were lysed and the proteins were resolved by 8% SDS-PAGE. A Smurf1 monoclonal antibody (Wang et al., 2003) was used at 1:4 dilution; β-tubulin antibody was used at 1:20,000 dilution (Accurate Chemical and Scientific Corporation); P-Smad1/5 antibody was used at 1:200 dilution (Cell Signaling Technology, Inc.); AF680 goat anti-rabbit and AF800 goat anti-mouse secondary antibodies were used at 1:2000 dilution (Molecular Probes). Resolved proteins were visualized and quantified using the Odyssey Infrared Imager (LI-COR, Inc.). F-actin staining was done using 1:100 AF488-phalloidin (Molecular Probes) on stage 18 embryos fixed 30 min in MEMPFA.

Quantitative RT-PCR

Total RNA was extracted from 10–15 animal caps or one wt embryo in the presence of 0.25 μg/ml proteinase K, phenol/chloroform extracted and ethanol precipitated, treated with DNaseI and phenol/chloroform extracted/ethanol precipitated again. 1 μg of total RNA was used to synthesize cDNA and 1/30 of the resulting cDNA was used in each RT-PCR reaction. The primers to *Xagr2* (Novoselov et al., 2003), 5′gaaccagctgatattgatcatttg3′ (upstream) and 5′ aatggtctccttcatacaccac3′ (downstream), were used at the following conditions: 95°C/10 s, 55°C/5 s, 72°C/12 s, acquisition temperature 79°C. The primers to *XAG1*, 5′ctgactgtccgatcagac3′ (upstream) and 5′gagttgcttctctggcat3′ (downstream), were used at the following conditions: 95°C/10 s, 55°C/6 s, 72°C/12 s, acquisition temperature 85°C. Primers and cycling conditions for *ODC*, *N-CAM*, *Wnt8*, *Vent1*, *GATA6* and α*T4-globin* were previously described (Kofron et al., 1999; Xanthos et al., 2001; Tao et al., 2005). Quantitative RT-PCR using LightCycler System (Roche Applied Science) was previously described (Kofron et al., 1999). We used 1:1, 1:10, 1:100 and 1:1000 dilutions of the cDNA from a wt embryo to generate standard curves. Expression levels of all genes were