

# *Tsukushi* controls ectodermal patterning and neural crest specification in *Xenopus* by direct regulation of BMP4 and X-delta-1 activity

Sei Kuriyama<sup>1,2</sup>, Giuseppe Lupo<sup>3</sup>, Kunimasa Ohta<sup>1,4,\*</sup>, Shin-ichi Ohnuma<sup>5</sup>, William A. Harris<sup>3</sup> and Hideaki Tanaka<sup>1,2</sup>

In *Xenopus*, ectodermal patterning depends on a mediolateral gradient of BMP signaling, higher in the epidermis and lower in the neuroectoderm. Neural crest cells are specified at the border between the neural plate and the epidermis, at intermediate levels of BMP signaling. We recently described a novel secreted protein, *Tsukushi* (*TSK*), which works as a BMP antagonist during chick gastrulation. Here, we report on the *Xenopus TSK* gene (*X-TSK*), and show that it is involved in neural crest specification. *X-TSK* expression accumulates after gastrulation at the anterior-lateral edges of the neural plate, including the presumptive neural crest region. In gain-of-function experiments, *X-TSK* can strongly enhance neural crest specification by the dorsolateral mesoderm or *X-Wnt8* in ectodermal explants, while the electroporation of *X-TSK* mRNA in the lateral ectoderm of embryos after gastrulation can induce the expression of neural crest markers in vivo. By contrast, depletion of *X-TSK* in explants or embryos impairs neural crest specification. Similarly to its chick homolog, *X-TSK* works as a BMP antagonist by direct binding to BMP4. However, *X-TSK* can also indirectly regulate *BMP4* mRNA expression at the neural plate border via modulation of the Delta-Notch signaling pathway. We show that *X-TSK* directly binds to the extracellular region of X-delta-1, and modulates Delta-dependent Notch activity. We propose that *X-TSK* plays a key role in neural crest formation by directly regulating BMP and Delta activities at the boundary between the neural and the non-neural ectoderm.

**KEY WORDS:** Ectoderm, Neural crest, Neural plate, Epidermis, X-TSK, BMP, Notch, *Xenopus*

## INTRODUCTION

Vertebrate neural crest cells originate at the border between the neural plate and the epidermis in the embryonic ectoderm. After their specification, neural crest cells migrate extensively along defined pathways, ultimately giving rise to diverse cell types that include the peripheral nervous system, melanocytes and the craniofacial skeleton (Hall, 1999; Le Douarin and Kalcheim, 1999). Several factors secreted from the neural plate, non-neural ectoderm or mesoderm show neural crest inducing activity in ectodermal explants, when applied alone or in combination. Yet, how the neural crest is specified at the junction between the neural plate and the epidermis remains unclear.

It has been proposed that bone morphogenetic protein (BMP) signaling plays an important role in specification of cell fates at the neural plate/ectodermal border in mouse, chick and frog embryos (Kanzler et al., 2000; Liem et al., 1995; Marchant et al., 1998). During neural development in *Xenopus*, BMPs are first expressed in the whole ectoderm, but they are then gradually downregulated in the presumptive neural plate, while BMP antagonists such as *Noggin*, *Chordin* and *Follistatin* are secreted from the dorsal axial mesendoderm (reviewed by Sasai and De Robertis, 1997). Several in vitro experiments suggest that a gradient of BMP activity in the

ectoderm is established by interactions between BMPs expressed in the ectoderm and BMP inhibitors secreted from the axial mesendoderm, resulting in the specification of the neural crest at a specific threshold of BMP signaling levels (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Nguyen et al., 1998). However, neural crest formation is specifically restricted to the posterior border of the neural plate, excluding the anterior neural plate (Hopwood et al., 1989; Mayor et al., 1995). This cannot be explained by the BMP gradient alone, and posteriorizing factors such as Wnts, fibroblast growth factors (FGF) and retinoic acid (RA), are required for this anteroposterior localization (Villanueva et al., 2002). Activation of Wnt signaling in the *Xenopus* embryo or in neuralized animal cap induces neural crest specification, while Wnt inhibition by either *Wnt8* knockdown or overexpression of a dominant-negative *Tcf3* abrogates neural crest specification in zebrafish (LaBonne and Bronner-Fraser, 1998; Lewis et al., 2004; Mayor et al., 1995; Tan et al., 2001). These results led to the two-signal model, suggesting that the neural crest is specified in the posterior neural plate border by the intersection of canonical Wnt signaling with moderate levels of BMP signaling (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Villanueva et al., 2002). Recently, the Notch signaling pathway has also been shown to play an important role in neural crest specification, by regulating the expression of *BMP4* and the BMP target gene *Msx1* at the lateral edge of the neural plate (Glavic et al., 2004).

Here, we show that the *Xenopus Tsukushi* gene (*X-TSK*) is strongly expressed at the border of the neural plate at the time of neural crest specification, and that its expression in the ectoderm is regulated by BMP signaling. By biochemical analysis and overexpression assays in *Xenopus*, we show that: (1) *X-TSK* works as a BMP antagonist by direct binding to BMPs; (2) *X-TSK* modulates activation of Notch signaling by directly binding to X-delta-1 extracellular region. Furthermore, *X-TSK* can regulate

<sup>1</sup>Division of Developmental Neurobiology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan. <sup>2</sup>The 21st Century COE program "Cell Fate Regulation Research and Education Unit", Kumamoto University, Kumamoto 860-0811, Japan. <sup>3</sup>Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK. <sup>4</sup>PRESTO, JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan. <sup>5</sup>Department of Oncology, The Hutchison/MRC Research Centre, University of Cambridge, Hills Road, Cambridge CB2 2XZ, UK.

\* Author for correspondence (e-mail: ohta9203@gpo.kumamoto-u.ac.jp)

BMP4 transcription indirectly via modulation of the Notch signaling pathway. Both gain- and loss-of-function assays demonstrate that X-TSK plays a crucial role in patterning of the ectoderm and especially in neural crest specification. We argue that X-TSK functions in the crosstalk of BMP and Notch signaling pathways at the boundary between the neural and non-neural ectoderm to determine the correct specification of the neural crest progenitors.

## MATERIALS AND METHODS

### Screening and RT-PCR

A *Xenopus* cDNA library at stage 24 (high density cDNA filter 725, RZPD) was screened using *C-TSK* cDNA as a probe to isolate *X-TSK* cDNA (GenBank Accession Number, AB176536).

For RT-PCR, total RNA was isolated by RNeasy Kit (QIAGEN) and TRIzol purification system (Invitrogen). The obtained total RNA was treated with DNase (Invitrogen) for removing genome DNA contamination, and first-strand cDNA was synthesized using oligo d(T)<sub>12-18</sub> primer and Superscript III reverse transcriptase (Invitrogen). PCR was performed with the Expand Long template PCR system 3 (Roche), with the following primers: *Xslug*-U 5'-CAATGCAAGAACTGTTCC-3'; *Xslug*-D 5'-TC-TAGGCAAGAATTGCTC-3'; *XAG-1* (Sive and Bradley, 1996); *XBF-1U*, 5'-TCAACAGCCTAATGCCTGAAGC-3'; *XBF-1D*, 5'-GCCGTCCAC-TTTCTTATCGTCG-3'; *Xotx2* (Blitz and Cho, 1995); *Sox2* (De Robertis et al., 1997); *Msx1* (Tribulo et al., 2003); *BMP4* (Dale et al., 1992); *XK81* (LaBonne and Bronner-Fraser, 1998); *ODC* (Agius et al., 2000); *Cardiac actin* (Stutz et al., 1986); *Sox9* and *Zic5* (Monsoro-Burq et al., 2003); *XESR-1* (Wittenberger et al., 1999); *X-notch-1U*, 5'-TCCTGATTATATTG-CTTATCCGAGT-3'; and *X-notch-1D*, 5'-TTACAGAAGTGTTAACA-GCAACAACA-3'.

### Embryological methods, in situ hybridization and immunostaining

*Xenopus* embryos were obtained as previously described (Newport and Kirschner, 1982) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For animal cap assays, mRNA was injected into the animal pole of two- or four-cell stage embryos. Animal caps (ACs) were dissected from stage 8-9 embryos in 1× MBS and cultured in 0.5× MBS until early neurula (stage 14), mid neurula (stage 17) or early tailbud (stage 21/22) stages, depending on the experiment. For dissections of ventral marginal zone (VMZ) or dorsal marginal zone (DMZ) explants, embryos were marginally injected into ventral or dorsal blastomeres at the four-cell stage with *X-TSK*, *BMP4* or *BMP4* + *X-TSK* mRNAs. After culturing to the early gastrula stage (stage 10-10\*), VMZ or DMZ explants (comprising about 60° of the VMZ or DMZ) were dissected in 1× MBS and cultured in 0.5× MBS until stage 24-26. Dorsal lateral marginal zone (DLMZ)-AC conjugates were prepared as described by Bonstein et al. (Bonstein et al., 1998).

Whole-mount in situ hybridization was performed as previously described (Harland, 1991; Shain and Zuber, 1996). Pigmented embryos and explants were bleached after the color reaction (Mayor et al., 1995). The following probes were used: *Xdlx3* (Woda et al., 2003); *ADAM13* (Alfandari et al., 1997); *XAG-1* (Bradley et al., 1996); *XK81* (Jonas et al., 1985); *Sox2* (Mizuseki et al., 1998); *Xrx1* (Casarosa et al., 1997); *N-tubulin* (Richter et al., 1988); cardiac actin (Mohun et al., 1984); *Xotx2* (Pannese et al., 1995); *Xotx5b* (Vignali et al., 2000); *X-ESR-1* (Wettstein et al., 1997). *X-delta-1* was cloned independently (Kiyota et al., 2001). *Xbmp4* cDNA was subcloned into pBSSK (-) from a *BMP4* expression vector. *Xslug*, *Sox9*, *Zic5*, *Msx-1*, *Hairy2A* and *Trp-2* cDNAs were isolated by RT-PCR.

We raised polyclonal antibody against X-TSK in rabbits (QIAGEN). The specificity of this antibody was checked by SDS-PAGE and western blot. Myc-His tagged X-TSK and X-TSK protein in the lysate of embryo can be detected in blot (data not shown). For double or triple staining, the hybridized embryo was sectioned by cryostat, and detected by anti-X-TSK antibody with Cy3-conjugated secondary antibody (Jackson Immuno Research).

### Microinjection of mRNA, short inhibitory double strand RNA (siRNA) or morpholino oligonucleotides

Capped mRNAs were synthesized from linearized plasmid templates with the mMessage Machine kit (Ambion). Embryos were injected with 500-4000 pg mRNA/embryo at the two- or four-cell stage in 0.1× MMR with

4% Ficoll and kept in it for a few hours. They were then transferred into 0.1× MMR for subsequent culturing. The following RNAs were made as previously described (Kuriyama and Kinoshita, 2001): *X-TSK* (pCS2+*X-TSK*), truncated BMP receptor (tBR) (Graff et al., 1994), *Chordin* (Sasai et al., 1994), *BMP4* (Fainsod et al., 1994), *Xnr1* (Jones et al., 1995), *Notch* Intracellular domain, *X-delta-1* and dominant-negative form of *Delta* (Chitnis et al., 1995), *Suppressor of hairless* DNA binding mutant, and *Su(H)/Notch ankyrin repeats (Ank)* fusion construct (Wettstein et al., 1997).

We used the computer program provided on Dr Gregory Hannon's website (<http://www.cshl.edu/gradschool/hannon.html>) to predict the short-hairpin-siRNA sequence for gene silencing of *X-TSK*. Myc-His tagged X-TSK was transfected into COS-7 cells with either a control or X-TSK siRNA-expressing vector and downregulation of X-TSK protein synthesis was checked by western blotting. After selecting the effective sequences, we used a X-TSK siRNA designed for the following sequence: 5'-GATACCTCGATCTGGATCTC-3'. C-TSK siRNA, which degrades *C-TSK* mRNA effectively, was used as control siRNA (Ohta et al., 2004). C-TSK mRNA was used for a rescue experiment of X-TSK siRNA. The applicable region of the *C-TSK* sequence was 5'-GATACCTCCTACTTGGATTG-3'. In another loss-of-function experiment, we used a morpholino antisense oligo (MO) against *X-TSK*, designed to target the following sequence: 5'-TCTAACAATGGCTCTTCTCTCTTGG-3' (Gene Tools). To distinguish the effect of MO on lateral expression of X-TSK from MO injection to deplete the organizer expression (Ohta et al., 2004), we injected it into ventral border of animal dorsal blastomere at the eight-cell stage corresponding to future D121 and D122 in the cell fate map of 32-cell stage embryo (Moody, 2000).

### Western blotting and immunoprecipitation

Myc-His tagged X-TSK, Myc-His tagged C-TSK, a Flag tagged BMP4 plasmids or a Flag tagged X-delta-1 extracellular domain plasmids were transfected into COS-7 cells with LipofectAMINE 2000 (Invitrogen) and the supernatants were harvested after 96 hours culture in serum-free Opti-MEM (Invitrogen). The immunoprecipitation experiment was performed as previously described (Ohta et al., 2004). Myc-tagged and Flag-tagged proteins were detected after blotting using an anti-Myc antibody 9E10 or anti-Flag antibody M2 (Sigma), respectively.

### *Xenopus* mRNA electroporation

mRNA electroporation was performed as described by Sasagawa et al. (Sasagawa et al., 2002). mRNA (200 nl) of X-TSK (2 µg/ µl) and GFP (1 µg/ µl), or only GFP (3 µg/ µl) were injected into the space under the vitelline membrane, and electroporated at stage 11.5. The position of electroporation was at the border between the lightly pigmented dorsal ectoderm and darkly pigmented lateral ectoderm. Electroporated embryos were selected under the fluorescent microscope for GFP expression at stage 14, fixed at the stage 15/16, then analyzed by whole-mount in situ hybridization.

### Staining of the branchial arch cartilages

The same procedure was used as previously described (Berry et al., 1998). Samples were fixed overnight in 4% paraformaldehyde/PBS, dehydrated in 95% ethanol and stained with Alcian Blue solution [95% ethanol:acetic acid:0.3% Alcian Blue in 70% ethanol (8 ml:2 ml:1 ml)]. After the staining, samples were left in 95% ethanol for 2 days and then stained with Alizarin Red solution [0.1% Alizarin Red in 95% ethanol:1% potassium hydroxide (0.25 ml:10 ml)] for 2 days. After 6 hours incubation in 1% potassium hydroxide, samples were transferred into 100% glycerol solution for image acquisition.

## RESULTS

### Isolation and expression pattern of *Xenopus* TSK

Chick Tsukushi (*C-TSK*) consists of twelve leucine-rich repeats (LRRs) and two cysteine-rich clusters at both termini and belongs to the small leucine-rich proteoglycan (SLRP) family (Ohta et al., 2004). A *Xenopus* tailbud cDNA library was screened using *C-TSK* as a probe, obtaining a cDNA fragment encoding for a predicted protein of 338 amino acid residues, which corresponds to the



*Xenopus* TSK gene (*X-TSK*). We have previously reported about other Tsukushi homologues in zebrafish, mouse and human (Ohta et al., 2004), all of which have similar characteristics of structure. At the amino acid level, X-TSK shares 59% identity with C-TSK, and 48–49% identity with zebrafish, mouse and human TSK.

Maternal *X-TSK* expression is observed in the animal hemisphere from unfertilized eggs to early gastrula embryos (Ohta et al., 2004). However, at the early neurula stage (stage 13), *X-TSK* expression is hardly detectable in the presumptive neural plate region, and restricted to the non-neural ectoderm (Fig. 1A), where its levels increase by stage 14, especially in the presumptive anterior neural fold (Fig. 1C). At these stages, the spatiotemporal expression pattern of *X-TSK* closely resembles that of *Xbmp4* (Fig. 1D) (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995) and BMP target genes, such as *Xdlx3* (Fig. 1B) (Woda et al., 2003).

In addition, *X-TSK* is expressed in the prospective cranial neural crest, in a similar domain to that of *Xslug* (Fig. 1E,F) (Mayor et al., 1995). Sections of hybridized embryos at these stages revealed that *X-TSK* is expressed in a broad area overlapping with the neural fold, but it is hardly observed inside the neural plate or in the underlying mesoderm (Fig. 1I), while *Xslug* expression is restricted to the neural crest region (Fig. 1J). We immunostained *Xslug* hybridized embryos with an anti-*X-TSK* antibody on sections (Fig. 1K,L). *X-TSK* protein is expressed in the superficial layer of the epidermis and the neural crest region, and also in the proximal edge of the neural crest region in the same layer of the *Xslug* expression domain (Fig. 1L, arrowhead). At the early tailbud stage (stage 23), *X-TSK* expression is observed in cranial neural crest cells, the dorsal retina and the lens placode (Fig. 1G). Comparison with *Xbmp4* showed that *X-TSK* has a more distinct expression pattern at this stage (Fig. 1H). In the migrating cranial neural crest, *X-TSK* expression is observed strongly in the mandibular crest segment, weakly in the distal tip of the hyoid crest segment, and in the anterior and posterior branchial

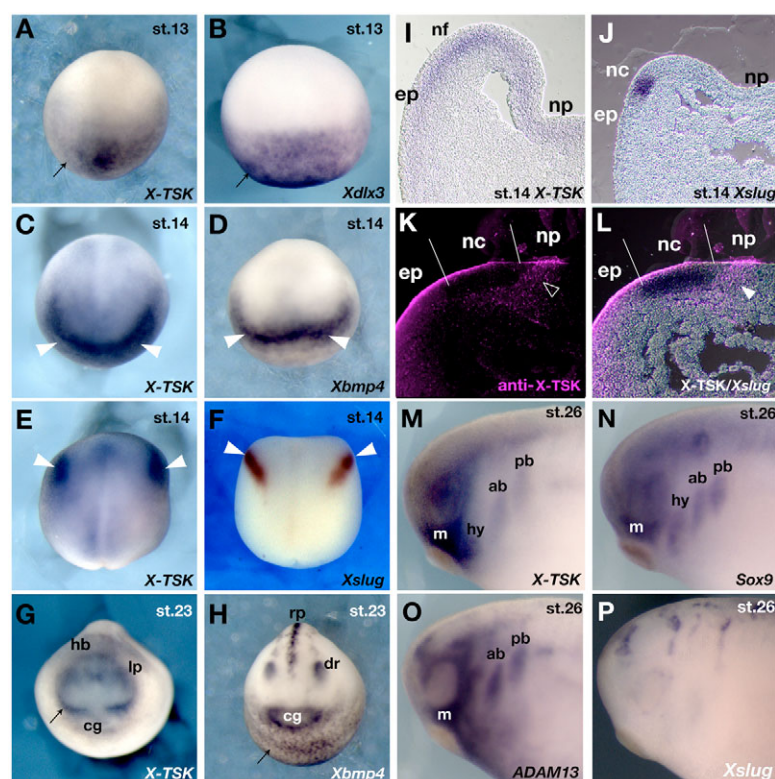
crest segments (Fig. 1M). This expression is similar to that of *Sox9* (Fig. 1N) (Spokony et al., 2002) and *ADAM13* (Fig. 1O) (Alfandari et al., 1997), while *Xslug* is downregulated in neural crest cells after they leave the neural tube (Fig. 1P).

### *X-TSK* expression is regulated by BMP4 signaling

As the early expression domains of *X-TSK* and *Xbmp4* in the ectoderm partially overlap, we speculated that *X-TSK* expression might be under the control of BMP signaling. To test this hypothesis, we analyzed *X-TSK* expression in stage 14 animal caps from embryos injected at the two-cell stage with either 0.5 ng of the BMP antagonist *Chordin* mRNA or 1 ng of truncated, dominant-negative, *BMP2/4* receptor (*tBR*) mRNA. In accordance with its expression in the non-neural ectoderm (Fig. 2I), *X-TSK* was clearly expressed in uninjected caps (Fig. 2D,G), which have epidermal character as shown by expression of the epidermal marker *XK81* (Fig. 2A). By contrast, inhibition of BMP signaling by injection of *Chordin* or *tBR* mRNAs downregulated *X-TSK* expression, similar to *XK81* (Fig. 2B,E,H). In caps co-injected with 0.5 ng *Chordin* and 1 ng of *BMP4* mRNAs, both *XK81* and *X-TSK* expression were restored (Fig. 2C,F). Similar results were obtained by semi-quantitative RT-PCR (Fig. 2J). In particular, *Chordin*-injected caps showed dose-dependent upregulation of *X-TSK* expression when increasing amounts of *BMP4* mRNA were co-injected with *Chordin* mRNA, at both stages 14 and 17. These results indicate that BMP activity positively regulates *X-TSK* expression in the ectoderm.

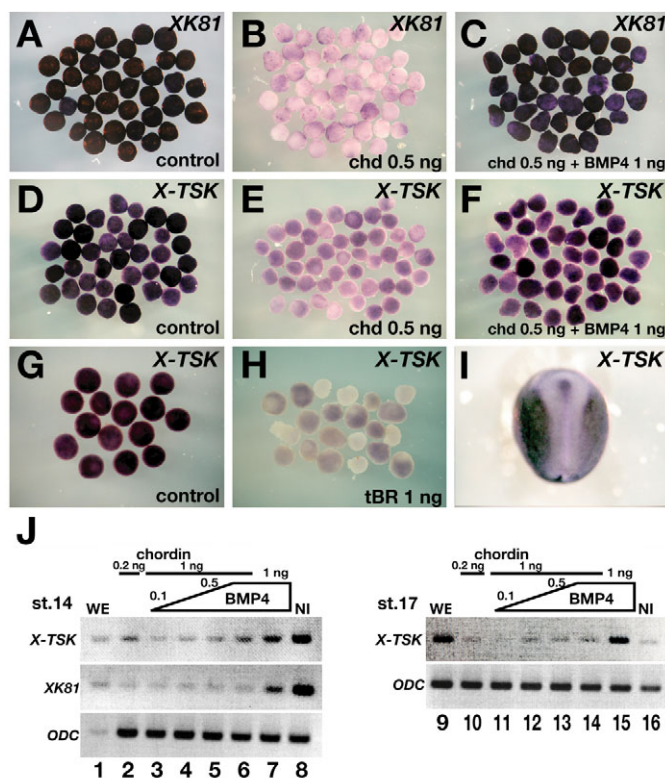
### *X-TSK* antagonizes BMP4 activity and directly binds to BMP proteins in vitro

In a previous study, we showed that *C-TSK* has dorsalizing activity when overexpressed in *Xenopus* embryos, which is due to its ability to bind to BMPs directly and antagonize BMP signaling in the extracellular space (Ohta et al., 2004). In addition, C-TSK expression partially overlaps with BMP expression only at the



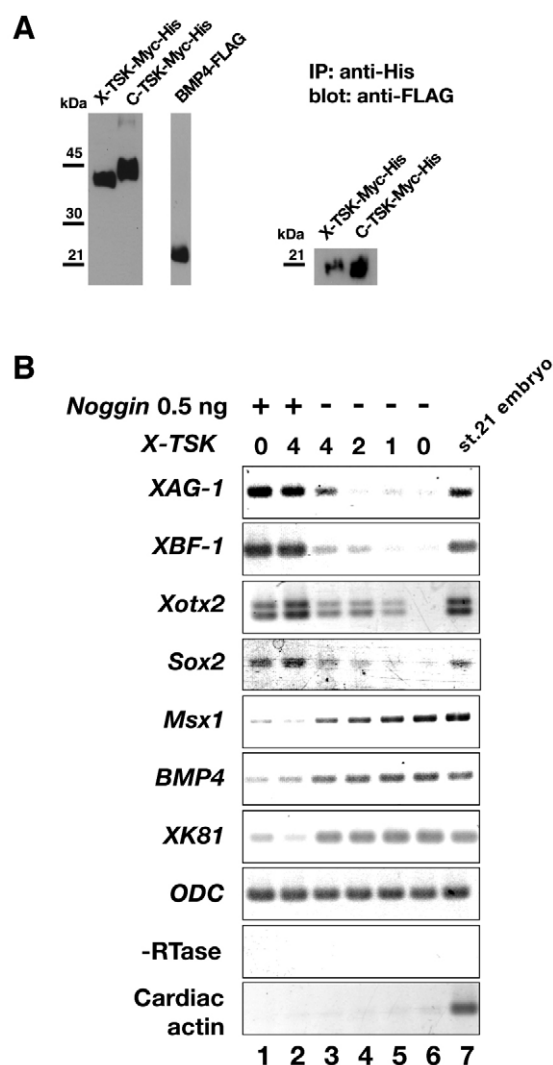
**Fig. 1. Expression patterns of *X-TSK* and marker genes during development.** Results of whole-mount in situ hybridization. (A,B) Stage 13, anterior views; dorsal is upwards. (A) *X-TSK* and (B) *Xdlx3*. Both genes are expressed in the presumptive epidermal region (arrows). (C,D) Stage 14, anterior views. (C) *X-TSK* and (D) *Xbmp4*. Both genes are expressed in the anterior neural plate fold (arrowheads).

(E,F) Stage 14, dorsal views; anterior is upwards. (E) Bilateral expression of *X-TSK* outside the neural plate (arrowheads). (F) *Xslug* expressed in premigratory neural crest cells (arrowheads). (G,H) Stage 23, anterior views. (G) Expression of *X-TSK* in the hindbrain (hb), lens placode (lp) and cranial neural crest derivative (arrow), but not in the epidermis or cement gland (cg). (H) Expression of *Xbmp4* in the roof plate (rp), dorsal retina (dr), cement gland and epidermis (arrow). (I,J) Sections of hybridized embryos. Expression of *X-TSK* (I) and *Xslug* (J) in the anterior neural plate region in stage 14 embryos. *X-TSK* is expressed in the anterior neural fold (nf) and epidermal region (ep) (I), while *Xslug* is expressed in a narrow area of the neural crest (nc; J). (K,L) Section at the cranial neural crest level at stage 16. Lines indicate the border of each area. (K) *X-TSK* protein localization. (L) The merged image of K and the image of *Xslug* hybridized embryo. *X-TSK* is localized on the surface ectoderm and in the proximal edge of neural crest cells (arrowheads). (M) *X-TSK* expression at stage 26 was found in mandibular neural crest (m), anterior branchial crest (ab) and posterior branchial crest (pb), and very weakly in hyoid neural crest segments (hy). (N) *Sox9*, (O) *ADAM 13* and (P) *Xslug* expression at stage 26.



**Fig. 2. X-TSK expression requires BMP signaling.** Results of the animal cap (AC) assays. (A-H) Animal caps were prepared from stage 8-9 embryos injected with 0.5 ng *Chordin* (B,E), 0.5 ng *Chordin* + 1 ng *BMP4* (C,F), 1 ng *truncated BMP-receptor* (*tBR*) (H), or none of the above (controls; A,D,G). ACs were harvested at the time equivalent to stage 14 and hybridized with *XK81* (A-C) or *X-TSK* (D-H) probes. *Chordin* downregulated *XK81* expression (B), which was rescued by *BMP4* (C). The expression of *X-TSK* was repressed by *Chordin* (E), but rescued by *BMP4* (F). (H) Truncated BMP receptors repressed the expression of *X-TSK*. (I) *X-TSK* expression in a normal embryo at stage 17. (J) RT-PCR analysis with ACs harvested at stages 14 (lanes 1-8) and 17 (lanes 9-16). *ODC* is used as an internal control. *X-TSK* expression in the ACs was recovered by increasing amounts of *BMP4*. *BMP4* overexpression induced *X-TSK* expression at both stages. WE, whole embryo; NI, non-injected ACs.

posterior marginal zone and the posterior primitive streak during chick gastrulation. However, the expression of *X-TSK* largely overlaps with *BMP4* in the ectoderm of *Xenopus* neurula embryos, and is controlled by *BMP4*. Thus, to confirm whether these dorsalizing and anti-BMP activities are conserved in *X-TSK*, we performed overexpression experiments of *X-TSK* mRNA in *Xenopus* embryos, and found that *X-TSK* can dorsalize ventral mesoderm both in ventral marginal zone explants and in animal caps injected with low doses of *Xnr1* mRNA, as detected by explant elongation and induction of cardiac actin expression (see Fig. S1 in the supplementary material). In addition, in co-injection experiments, *X-TSK* can antagonize the ventralizing effects of *BMP4* overexpression in dorsal marginal zone explants (see Fig. S1 in the supplementary material). To examine whether *X-TSK* can bind to



**Fig. 3. X-TSK functions as a BMP antagonist in *Xenopus*.**

(A) Western blot analysis of X-TSK-Myc-His, C-TSK-Myc-His and XBMP4-Flag protein. Each protein was detected by anti-tag antibody. Co-immunoprecipitation of X-TSK-Myc-His or C-TSK-Myc-His and XBMP4-Flag. After immunoprecipitation, bound BMP4 was detected by immunoblotting with anti-Flag antibody. IP, immunoprecipitation. (B) RT-PCR analysis of X-TSK, Noggin or a mixture of both. The numbers above the columns show the amount of X-TSK mRNA in each cap (ng). Plus or minus indicates co-injection or not of Noggin (0.5 ng).

*BMP4* directly, we carried out an immunoprecipitation assay between *BMP4* and *X-TSK* or *C-TSK* proteins (Fig. 3A). When Myc-tagged *X-TSK* or *C-TSK* was reacted with FLAG-tagged *BMP4*, immunoprecipitation of both proteins with nickel chelating resins pulled down *BMP4*. These data indicate the direct binding of *X-TSK* to *BMP4*. Thus, the basic molecular characteristics of *Tsukushi* are also conserved in the *Xenopus* homologue.

### **X-TSK overexpression induce the identities of peripheral area of anterior neural plate in animal caps**

*BMP* signaling has a crucial role in mediolateral patterning of the ectoderm (Knecht and Harland, 1997; Wilson et al., 1997). To further test the ability of *X-TSK* as a *BMP* antagonist, we



performed animal cap assays and analyzed the induction of the dorsoanterior ectodermal markers. Using whole-mount in situ hybridization, we could detect strong induction of cement gland markers, but weak induction of neural markers, in *X-TSK*-injected caps (see Fig. S2 in the supplementary material). We then analyzed *X-TSK*-injected caps by semi-quantitative RT-PCR, which provides more sensitivity than in situ hybridization (Fig. 3B). In these experiments, *X-TSK* induced the cement gland marker *XAG-1*, the telencephalic marker *XBF-1*, the forebrain and cement gland marker *Xotx2*, and the pan-neural marker *Sox2* in a dose-dependent manner (Fig. 3B, lanes 3-5). Interestingly, *X-TSK* overexpression had only a weak, if any, effect on the expressions of *BMP4* and its target gene *Msx1* (Fig. 3B). Comparison with the effects of another BMP antagonist, *Noggin*, showed that, even at high doses of injected mRNA (4 ng), the effects of *X-TSK* mRNA on the induction of cement gland/neural markers and on the repression of *BMP4* and *Msx1* were clearly weaker than those of *Noggin* mRNA. Co-overexpression of *X-TSK* and *Noggin* also slightly reinforced the effects of *Noggin* (Fig. 3B, lanes 1,2). These effects were direct and not due to the induction of dorsal mesoderm, as no activation of cardiac actin was detected in the injected caps. Altogether, these data indicate that, while BMP antagonists such as *Noggin* can strongly inhibit BMP activity and strongly neuralize ectodermal explants, *X-TSK* overexpression causes a moderate repression of BMP signaling. Thus, *X-TSK* may induce peripheral neural plate character in the ectoderm via direct induction of intermediate levels of BMP signaling.

### Loss of X-TSK function inhibits neural crest formation in vivo

Several studies in *Xenopus* have suggested that intermediate levels of BMP signaling are necessary for neural crest specification at the neural plate border (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998). Thus, it was interesting to see, as described above, that *X-TSK* is a BMP antagonist strongly expressed in the presumptive cranial neural crest, which can promote intermediate levels of BMP signaling in ectodermal explants. To determine the role of *X-TSK*, we performed loss-of-function experiments using siRNA (Zhou et al., 2002). The inhibitory effect of *X-TSK* siRNA (*X-TSK-si*) sequence was confirmed by the inhibition of *X-TSK*-Myc protein production after co-transfection in COS cells (data not shown). *X-TSK-si* was also effective in reducing the levels of endogenous *X-TSK* mRNA in *Xenopus* embryos, as shown by RT-PCR (Fig. 4C). Although the endogenous expression of *X-TSK* increased from stage 13 in uninjected embryos, this increase was completely prevented in embryos injected with *X-TSK-si*. Injection of *X-TSK-si* into one animal blastomere at the four-cell stage (0.3-0.5 pmol/cell) caused abnormal neural fold formation in the neural plate and increased pigmentation at the site where the neural fold is normally formed (Fig. 4A,B).

We then analyzed the effects on *X-TSK* depletion on ectodermal patterning using molecular markers after co-injection of *X-TSK-si* with  $\beta$ -gal mRNA (see Table 1). The expression of the neural crest markers *Sox9*, *Zic5* and *Xslug* was inhibited in the  $\beta$ -gal positive area (Fig. 4E,G,L). No effects were detected after injection of *C-TSK* siRNA (*C-TSK-si*) (Fig. 4D,F). In addition, while the expression of the neural plate markers *Sox2* and *Xrx1* were not affected by *X-TSK-si* injection (Fig. 4H,J), the expression of the epidermal marker *XK81* was enhanced (Fig. 4I) to the same extent of the increased pigmentation as shown in Fig. 4B. In these embryos, no changes were detectable in the expression of *Xbmp4* (Fig. 4K). These effects were rescued by co-injection of both 500

**Table 1. Effects of *X-TSK* depletion on the expression of various genes**

Sample/probe	Normal	Expanded or ectopic	Diminished
<i>C-TSK</i> siRNA ( <i>C-TSK-si</i> ) 0.5 pmol			
<i>Sox9</i>	26	0	0
<i>Xslug</i>	28	0	0
<i>Zic5</i>	25	0	0
<i>Hairy2A</i>	39	0	0
<i>Msx-1</i>	45	0	1
<i>XK81</i>	21	1	2
<i>Xrx1</i>	9	0	0
<i>X-TSK</i> siRNA ( <i>X-TSK-si</i> ) 0.5 pmol			
<i>Sox9</i>	15	0	51 (77.3%)
<i>Xslug</i>	17	0	39 (69.6%)
<i>Zic5</i>	3	0	17 (85.0%)
<i>Hairy2A</i>	8	0	28 (77.8%)
<i>Msx-1</i>	16	0	37 (69.8%)
<i>Sox2</i>	24	0	0
<i>XK81</i>	15	37 (71.2%)	0
<i>Xbmp4</i>	11	1	0
<i>Xrx1</i>	16	0	1
Control morpholino (CMO) 5 ng			
<i>Sox9</i>	14	0	0
<i>X-TSK</i> morpholino ( <i>X-TSK-MO</i> ) 5 ng			
<i>Sox9</i>	11	0	23 (67.6%)
<i>Xslug</i>	16	0	39 (70.9%)

*C-TSK* siRNA (*C-TSK-si*), *X-TSK* siRNA (*X-TSK-si*) was injected into unilateral blastomeres at the four-cell stage. Embryos showing identical effects on the injected side were counted at stage 15.

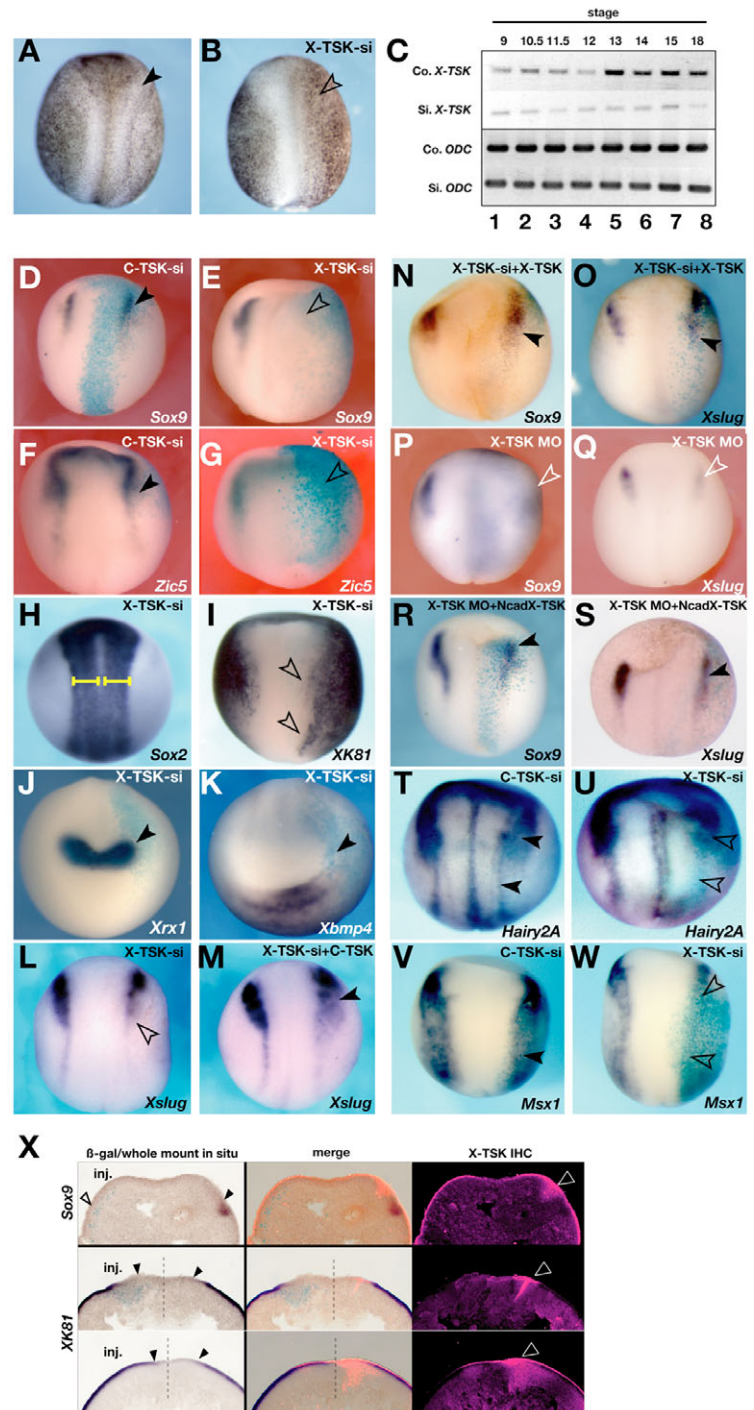
pg *C-TSK* mRNA (Fig. 4L,M) or 250 pg *X-TSK* mRNA (Fig. 4N,O), indicating that these phenotypes are specifically caused by the depletion of the TSK protein (Table 2). Previously, we performed morpholino oligonucleotide (MO) injections into the prospective dorsal midline of the embryo to determine the effects of *X-TSK* depletion on the Spemann organizer and neural induction. In those experiments, we showed that *X-TSK* MO impairs anterior neural plate specification, as shown by a reduction in the expression domain of *Sox2* and *Xrx1* (Ohta et al., 2004). To confirm siRNA effects and to avoid indirect effects due to abnormal organizer formation, we performed MO injections into the prospective lateral ectoderm, as described in the Materials and methods section. In these conditions, both *Sox9* and *Xslug* were diminished by *X-TSK* MO injection (Fig. 4P,Q). These effects were rescued by the injection of N-cad-*X-TSK* mRNA, where the N-terminal region of *X-TSK*, including the initiation codon and the signal peptides, were replaced by a myc-tagged N-cadherin signal peptide (Fig. 4R,S; see also Table 2). We also examined the effects of *X-TSK* depletion on the expression of *Hairy2A* and *Msx1*, which identify a pre-neural crest region before neural crest specification (Glavic et al., 2004; Tribulo et al., 2003). Both *Hairy2A* and *Msx1* were downregulated by *X-TSK-si* (Fig. 4U,W), but not *C-TSK-si* (Fig. 4T,V). To confirm the relationship between *X-TSK* protein reduction and the observed phenotypes, we sectioned the embryos hybridized with neural crest markers and immunostained them with an anti-TSK antibody (Fig. 4X). In the embryos showing downregulation of *Sox9* and upregulation of *XK81* after *X-TSK-si* injection, *X-TSK* protein expression in the neural crest was reduced on the injected side, while *X-TSK* endogenous expression was detectable in the proximal edge of the neural crest region on the control side (Fig. 4X, arrowheads). Morphological and molecular analysis at later stages of development confirmed that, in agreement

with the reduction of early neural crest markers at neurula stages, inhibition of X-TSK function strongly repressed formation of neural crest derivatives such as melanocytes and the branchial arch cartilages (see Fig. S3 in the supplementary material). Altogether, these results suggest that X-TSK is required at an early step of neural crest specification upstream of *Hairy2A* and *Msx1*, and that in the absence of TSK function in the ectoderm the presumptive neural crest region is at least partially specified as epidermis.

### X-TSK is required in the ectoderm for neural crest formation, and can induce neural crest specification in cooperation with the dorsolateral mesoderm or *XWnt-8*

As previously described, dorsolateral marginal zone (DLMZ) explants dissected from early gastrula embryos at stage 10.25–10.5 induced expression of the neural crest marker *Sox9* in conjugated animal caps grown to the late neurula stage (Fig. 5A; 78.9%,  $n=38$ )

**Fig. 4. Effects of X-TSK depletion using siRNA on ectodermal patterning.** (A) A normal *Xenopus* embryo at stage 17; the arrowhead shows the neural fold. (B) Stage 17; an embryo injected with X-TSK siRNA (X-TSK-si). The arrowhead shows a flattened neural fold and enhanced pigmentation. (C) RT-PCR analysis of embryos injected with siRNA radially into all blastomeres at the four-cell stage. The embryos were harvested at the appropriate stages. *ODC* is used as an internal control. Co, Control embryo; Si, X-TSK-si-injected embryo. (D–S) Whole-mount in situ hybridization of injected samples at stage 15. The injected samples are indicated in the upper right-hand corner, and the probes are in the lower right-hand corner. All pictures show the injected side on the right. The results are described in Table 1, except M–O, R, S. (D,F) An embryo injected with C-TSK siRNA (C-TSK-si). The expression of *Sox9* and *Zic5* was unchanged (arrowheads). (E,G) An embryo injected with X-TSK siRNA (X-TSK-si). The expression *Sox9* or *Zic5* was not observed (arrowheads). (H) *Sox2* expression was not disturbed in the X-TSK-si-injected embryo. The bars indicate the width of neural plates. (I) Epidermal keratin was activated in the X-TSK-si-injected side (arrowheads). (J) *Xrx1* expression was not disturbed in the X-TSK-si-injected side (arrowhead). (K) *Xbmp4* expression was not activated (arrowhead). (L,M) Results of the rescue experiment with co-injection of X-TSK-si and mRNAs. (L) X-TSK-si (0.5 pmol) injection diminished *Xslug* expression (arrowhead). (M) X-TSK-si (0.5 pmol) and C-TSK mRNA (0.5 ng) were injected into one blastomere of a stage 2 embryo. *Xslug* expression was weak, but regionally rescued (arrowhead). (N,O) X-TSK-si (0.5 pmol) and X-TSK mRNA (250 pg) were injected. (N) *Sox9* expression was slightly extended alongside  $\beta$ -gal staining (arrowhead). (O) *Xslug* expression was restored to the same level as control side (arrowhead). (P,Q) An embryo injected with a morpholino oligo of X-TSK (X-TSK MO) (5 ng). Neural crest marker expression, *Sox9* (P) and *Xslug* (Q) levels were decreased. (R,S) X-TSK MO phenotype is restored by X-TSK in which the signal peptide is replaced with N-cadherin signal peptide. The sequence around the initiation codon, which is a MO target, is swapped for the *N-cadherin* sequence. (R) *Sox9* expression was observed alongside  $\beta$ -gal-positive cells (arrowhead). (S) *Xslug* expression was restored (arrowhead). (T–W) The pre-neural crest genes in injected embryos. (T,V) C-TSK-si did not change the expression of *Hairy2A* or *Msx-1* (arrowheads). (U,W) X-TSK-si diminished *Hairy2A* and *Msx-1* expression in the cranial to trunk lateral neural plate (open arrowheads). (X) Triple staining sections. *Sox9* and *XK81* phenotypic embryos are sectioned, and stained using anti-X-TSK antibody. (Top) *Sox9* expression is missing in the  $\beta$ -gal-positive region (left, open arrowhead) and neural crest expression of X-TSK has also disappeared in the injected side (left). Normal expression of X-TSK protein was observed as red fluorescence (right, triangle). (Middle) The border of epidermis is indistinguishable in anterior neural plate area; X-TSK protein levels are diminished in  $\beta$ -gal-positive region. (Bottom) The trunk region of epidermis is clearly enhanced on the injected side (arrowheads). Arrowheads indicate the epidermal borders. X-TSK protein was seen only in the non-injected side (right, triangle).





(Bonstein et al., 1998). *Sox9* induction was much reduced in X-TSK-si-injected caps conjugates with DLMZ explants (Fig. 5B; 15.9%,  $n=44$ ), but not in uninjected caps conjugated with X-TSK-si-injected DLMZ (Fig. 5C; 75%,  $n=28$ ). Expressions of the neural crest marker, *Zic5* and *Xslug* were also decreased in a dose-dependent manner in X-TSK-si-injected caps after conjugation with DLMZ, as detected by RT-PCR analysis (Fig. 5G). By contrast, the expression of *Sox9* was strongly increased in conjugates of DLMZ with animal caps overexpressing X-TSK mRNA (Fig. 5A,D; 100%,  $n=15$ ), while it was decreased in uninjected caps conjugated with X-TSK-overexpressing DLMZ (Fig. 5A,E; 41.6%,  $n=24$ ). These results indicate that X-TSK expression in the ectoderm enhances the response to neural crest inducing signals derived from DLMZ, while X-TSK overexpression in the dorsolateral mesoderm has the opposite effect. As X-TSK overexpression in the mesoderm has a dorsalizing effect (see Fig. S1 in the supplementary material), this opposite effect may be explained by dorsalization of the DLMZ.

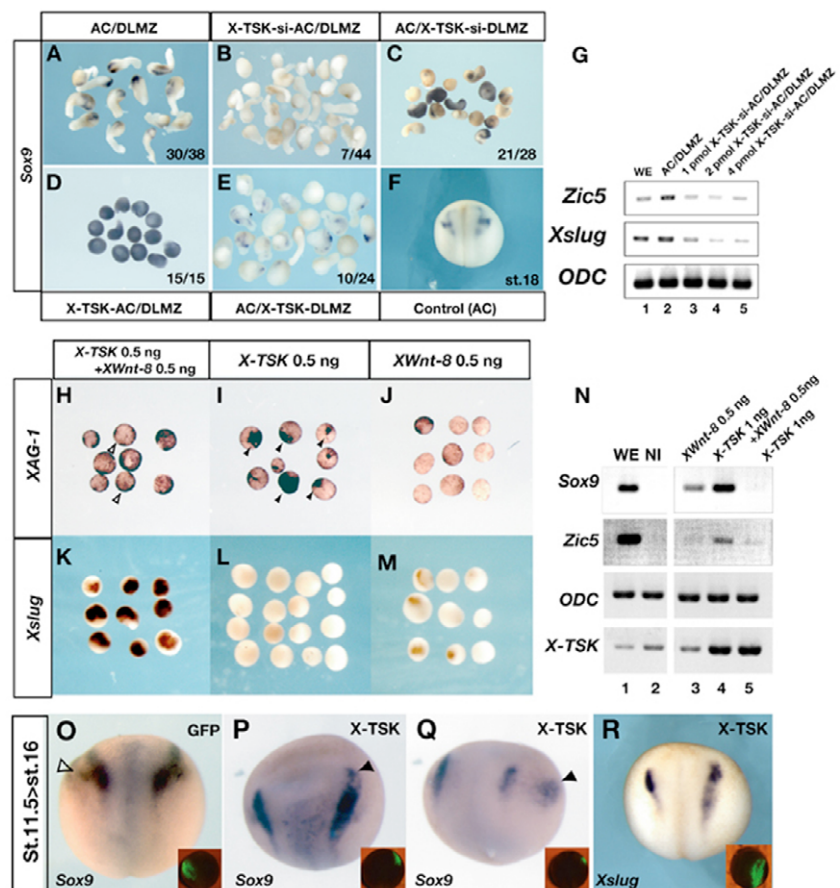
In *Xenopus* embryos, neural crest specification requires the action of posteriorizing signals such as Wnts, FGFs and retinoic acid acting on cells with intermediate levels of BMP signaling at the border of the neural plate (Villanueva et al., 2002). These signals may be at least partially produced by the DLMZ (Bang et al., 1999; Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005; Wu et al., 2003). *XWnt-8* overexpression induces ectopic neural crest markers in vivo

(LaBonne and Bronner-Fraser, 1998), and it can induce neural crest specification in animal caps in cooperation with BMP antagonists (*Chordin* or *Noggin*) (Christian et al., 1991; LaBonne and Bronner-Fraser, 1998). Therefore, we tested whether X-TSK could induce neural crest specification in cooperation with *XWnt-8*. X-TSK-injected caps induced the cement gland marker *XAG-1* (Fig. 3B, Fig. 5I), but did not show any significant expression of the neural crest marker *Xslug* (Fig. 5L), while *XWnt-8*-injected caps showed weak expression of both *XAG-1* and *Xslug* (Fig. 5J,M). By contrast, when X-TSK and *XWnt-8* were co-overexpressed, *Xslug* was strongly induced, while *XAG-1* expression was reduced compared with X-TSK-injected caps (Fig. 5H,K). RT-PCR analysis confirmed that co-injection of X-TSK and *XWnt-8* significantly enhanced expression of the neural crest markers *Sox9* and *Zic5*, compared with single X-TSK or *XWnt-8* injection (Fig. 5N). Therefore, the BMP antagonistic activity of X-TSK is sufficient for neural crest specification in cooperation with *XWnt-8*.

### The misexpression of X-TSK after gastrulation can induce neural crest markers in the epidermal area

In *Xenopus*, mRNA injections often show only earlier functions of the corresponding gene, preventing the analysis of later roles for the same gene. For example, while X-TSK or *Chordin* overexpression by mRNA injections into early embryos represses neural crest formation (see below), overexpression of a hormone-inducible

**Fig. 5. X-TSK induces the neural crest when in combination with Wnt.** (A-E) Conjugate assay of ACs (stage 8-9) and the dorsolateral marginal zone (DLMZ) (stage 10.5) analyzed by whole-mount in situ hybridization with a *Sox9* probe. The numbers of phenotypic explants are described in the lower right-hand corners. (A) *Sox9* expression in the conjugate of a non-injected animal cap and non-injected DLMZ. (B) The conjugate of X-TSK-si-injected AC and DLMZ; *Sox9* expression is decreased. (C) The conjugate of AC and X-TSK-si-injected DLMZ. (D) The conjugate of X-TSK overexpressing AC and DLMZ; *Sox9* is expressed in all explants. (E) The conjugate of AC and X-TSK expressing DLMZ. (F) The expression of *Sox9* at the same stage as ACs. (G) RT-PCR analysis of the AC/DLMZ conjugate assay. X-TSK-si (0-4 pmol) was injected into the animal blastomeres evenly. (H-M) Whole-mount in situ hybridization analysis of the animal cap assay. ACs were prepared from stage 8-9 embryos injected with 0.5 ng X-TSK + 0.5 ng *XWnt-8* (H,K), 0.5 ng X-TSK (I,L), or 0.5 ng *XWnt-8* (J,M). ACs were harvested at the time equivalent to stage 21/22 and hybridized with *XAG-1* (H-J) and *Xslug* (K-M) probes. (N) RT-PCR analysis of the animal cap assay. *ODC* was used as an internal marker. The amount of injected X-TSK mRNA was 1 ng/embryo, which is enough to induce anterior markers. *XWnt-8* mRNA (0.5 ng) was co-injected with X-TSK. X-TSK induced neural crest marker expression in combination with *XWnt-8*. (O-R) The embryos are transfected by the mRNA electroporation at stage 11.5 after gastrulation. The distribution of GFP fluorescence is indicated in the inset of each figure. (O) *Sox9* expression in GFP mRNA transfected embryo. (P) A variation of X-TSK transfected embryo. *Sox9* expression is extended to anterior side (arrowhead). (Q) A variation of X-TSK transfected embryo. *Sox9* expression is ectopically observed (arrowhead), but endogenous expression was interfered by ectopic expression of X-TSK. (R) *Xslug* expression is also affected.



**Table 2. Rescue experiments on the *X-TSK-si/X-TSK* MO phenotype**

Sample/probe	Normal	Expanded or ectopic	Half-rescued	Diminished
<i>X-TSK</i> siRNA ( <i>X-TSK-si</i> ) 0.5 pmol+C- <i>TSK</i> 500 pg				
<i>Xslug</i>	18	8	8	5 (12.8%)
<i>X-TSK</i> siRNA ( <i>X-TSK-si</i> ) 0.5 pmol+ <i>X-TSK</i> 250 pg				
<i>Sox9</i>	9	2	7	5 (21.7%)
<i>Xslug</i>	5	3	6	2 (12.5%)
<i>X-TSK</i> morpholino ( <i>X-TSK-MO</i> ) 5 ng+Ncad- <i>X-TSK</i> 500 pg				
<i>Sox9</i>	14	0	6	1 (4.8%)
<i>Xslug</i>	18	0	9	6 (18.2%)
<i>X-TSK</i> siRNA ( <i>X-TSK-si</i> ) 0.5 pmol+ <i>Chordin</i> 100 pg				
<i>Sox9</i>	3	0	19	0
<i>Xslug</i>	8	0	11	0

Compared with the phenotype of the *X-TSK-si*-injected embryo, embryos with restored expression by injection of a BMP antagonist were categorized as weakly rescued.

version of the BMP antagonist Smad6 causes ectopic neural crest induction when its activity is induced after stage 9 (Wawersik et al., 2005). Thus, BMP antagonistic activity is required for neural crest formation after gastrulation. To study *X-TSK* function in vivo at these later stages, we performed mRNA electroporation in stage 11.5 *Xenopus* embryos (Fig. 5O–R). In these conditions, overexpression of *X-TSK* in the anterior neural fold extended the expression domain of *Sox9* (Fig. 5P, arrowhead), and induced ectopic expression in the epidermal region (Fig. 5Q, arrowhead), while GFP mRNA electroporation had no effect (Fig. 5O, arrowhead). *Xslug* expression was also enhanced in the electroporated side (Fig. 5R). The electroporation of *X-TSK* mRNA after early neurula stage 13 caused a slight enhancement of the cranial-facial neural crest at stage 21 (data not shown). These data strongly suggest that *X-TSK* upregulation in the lateral ectoderm after gastrulation during normal development (Fig. 1C,M) is involved in cranial neural crest specification.

### Overexpression experiments suggest that *X-TSK* can modulate BMP4 transcription and regulate activation of Notch signaling

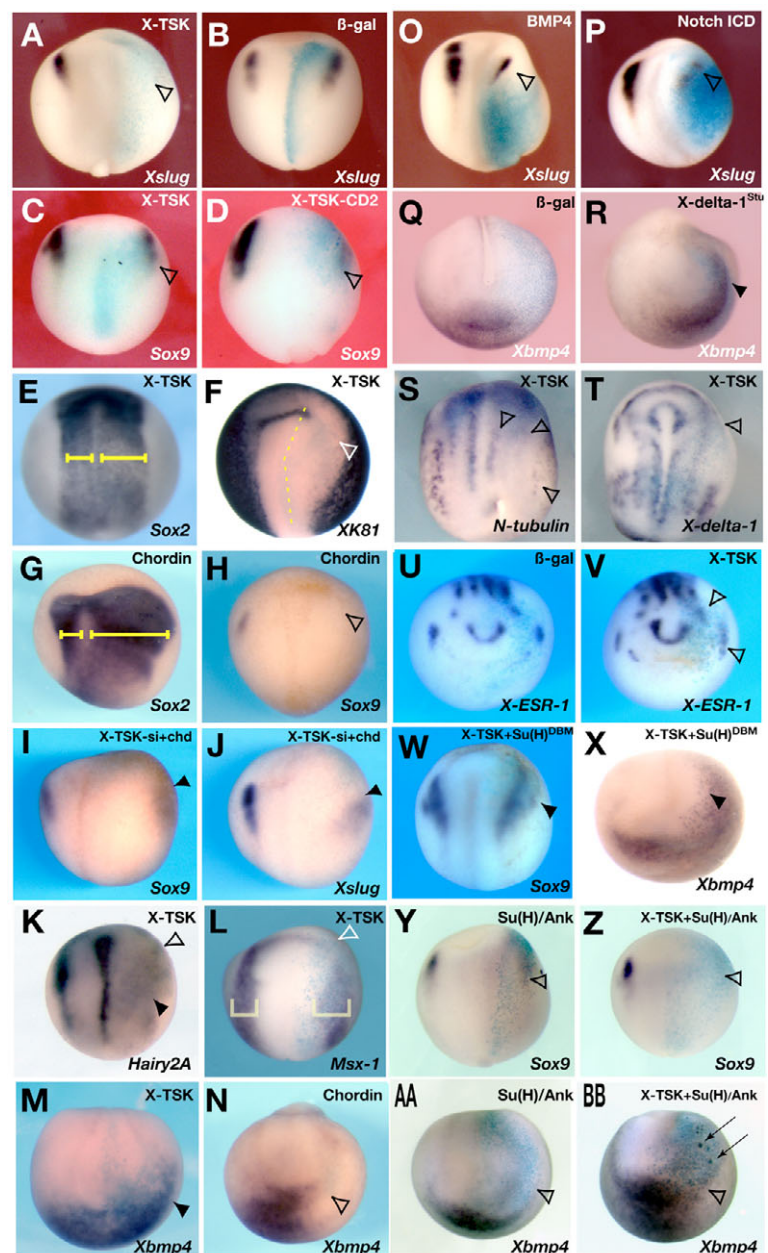
In this study, gain-of-function and loss-of-function analyses clearly suggest the importance of *X-TSK*-mediated BMP antagonism in ectodermal patterning and neural crest specification. To shed light on *X-TSK* activity during ectodermal development, we performed additional overexpression experiments in *Xenopus* embryos. When *X-TSK* mRNA was injected into one dorsoanimal blastomere of four-cell stage embryos (1 ng/cell; co-injected with 200 pg of  $\beta$ -gal mRNA as a lineage tracer), the expression of the neural crest marker *Xslug* was suppressed on the injected side, while  $\beta$ -gal alone did not cause any specific phenotype (Fig. 6A,B). *Sox9* expression was also suppressed by *X-TSK*, and even more strongly by a membrane-bound form of *X-TSK* (*X-TSK-CD2*), obtained after fusion with the CD2 transmembrane domain (Chang et al., 2001) (Fig. 6C,D). The fact that the effects of both the secreted and membrane-bound forms of *X-TSK* were very similar, and that the secreted form of *X-TSK* affected only the area immediately around the  $\beta$ -gal-stained region, suggest that *X-TSK* acts as a short-range factor and does not disperse far away. Unilateral injection of *X-TSK* also caused the expansion of the neural plate marker *Sox2* (Fig. 6E), the inhibition of the epidermal marker *XX81* (Fig. 6F), and a downregulation of *Hairy2A* and *Msx-1* in the presumptive

neural crest territory (Fig. 6K) (Table 3).  $\beta$ -Gal mRNA did not change the expression of *Hairy2A* and *Msx-1* (data not shown) (Table 3). These effects were dose dependent. Similar to *X-TSK* overexpression, injection of *Chordin* mRNA, another BMP antagonist, also caused an expansion of the neural plate (Fig. 6G), and the inhibition of neural crest formation (Fig. 6H). These data confirm that *X-TSK* acts as a BMP antagonist in vivo, although less efficiently than *Chordin*. However, *Chordin* was not effective in restoring neural crest gene expression in *X-TSK*-depleted embryos (Fig. 6I,J), suggesting that the function of *X-TSK* in neural crest formation may not be simply explained by its anti-BMP activity.

In support of the above interpretation, we found that *Chordin* inhibited *Xbmp4* expression in the lateral ectoderm (Fig. 6N), while *X-TSK* overexpression enhanced it (Fig. 6M), and that *BMP4* overexpression also caused a repression of neural crest formation (Fig. 6O). It has been shown that Notch signaling and its target gene *Hairy2A* are involved in neural crest specification in *Xenopus*, by regulating the expression of *BMP4* and *Msx-1* at the lateral border of the neural plate (Glavic et al., 2004). In fact, similar to the results described by Glavic et al. (Glavic et al., 2004), overexpression of *Notch-ICD* mRNA into early embryos repressed *Xslug* expression (Fig. 6P), while overexpression of *X-delta-1<sup>Suu</sup>*, a dominant-negative ligand of Notch, caused upregulation of *Xbmp4* expression (Fig. 6R). As these effects are similar to those of *X-TSK* overexpression, we then examined in more detail whether the effects of *TSK* overexpression are mediated by modulation of the Notch signaling pathway. We first checked the effects of *TSK* overexpression on primary neurogenesis, which is known to be controlled by Notch signaling (Chitnis et al., 1995). Analogous with what has previously been shown for Notch overexpression (Chitnis et al., 1995; Chitnis and Kintner, 1996), *TSK* overexpression repressed the expression of *N-tubulin*, a marker of differentiated neurons, and that of *X-delta-1* (Fig. 6S,T). Moreover, *X-TSK* overexpression upregulated the Notch-target gene, *X-ESR-1* (Fig. 6V). Finally, we found that the repression of neural crest specification by *TSK* overexpression could be overcome by co-injection with *Su(H)<sup>DBM</sup>* mRNA, which acts as an antagonist of Notch signaling (Wettstein et al., 1997) (Fig. 6W) (Table 3), while *Su(H)<sup>DBM</sup>* mRNA alone very slightly enhanced *Sox9* expression (data not shown) (Table 3). By contrast, co-overexpression of *X-TSK* with *Su(H)/Ank*, which works as an agonist of Notch signaling as described by Wettstein et al. (Wettstein et al., 1997), strongly inhibited *Xslug* and *Sox9* (Fig. 6Z, data not shown) (Table 3). *Su(H)/Ank* alone also inhibited the expression of neural crest markers, though more weakly than *Notch ICD* (Fig. 6Y) (Table 3). However, *Xbmp4* upregulation by *X-TSK* overexpression in the lateral ectoderm could not be rescued by co-injection of *Su(H)<sup>DBM</sup>* mRNA (Fig. 6X) (Table 3), consistent with the fact that the same effect is obtained after overexpression of *X-delta-1<sup>Suu</sup>* (Fig. 6R). By contrast, the upregulation of *Xbmp4* expression by *X-TSK* overexpression could be partially prevented by co-injection of *Su(H)/Ank* mRNA, though some patches of ectopic *Xbmp4* expression in the lateral area of  $\beta$ -gal-positive cells were still detectable (Fig. 6BB, arrows). *Su(H)/Ank* mRNA alone inhibited *Xbmp4* expression very weakly (Fig. 6AA, arrowhead). Altogether, these results suggest that *TSK* overexpression has complex, bimodal effects on the regulation of Notch signaling. Specifically, the repression of neural crest markers and primary neurogenesis appear to be consistent with enhanced Notch signaling, while the activation of *Xbmp4* transcription seems to be related to diminished Notch signaling.



**Fig. 6. X-TSK interacts with the Notch signaling pathway.** Whole-mount in situ hybridization analysis of mRNA-injected embryos. Injected samples are indicated in the upper right-hand corners; probes are indicated in the lower right-hand corner. (A,C) Neural crest markers *Xslug* (A) or *Sox9* (C) disappeared or diminished (arrowheads) with X-TSK (1 ng) overexpression. (B) The  $\beta$ -gal mRNA (0.5 ng) injection had no effect. (D) X-TSK-CD2 mRNA (1 ng) showed a similar effect to that in C. (E) *Sox2* expression expanded laterally (right bar) in the X-TSK (1 ng)-injected side. (F) Epidermal keratin levels diminished. The anterior border disappeared on the injected side (open arrowhead). (G) An embryo injected with *Chordin* (100 pg). The *Sox2*-expressing neural plate is expanded (right bar). (H) An embryo injected with *Chordin* (100 pg). Expression of the neural crest marker *Sox9* diminished (arrowhead). (I,J) X-TSK-si (0.5 pmol) and *Chordin* mRNA (100 pg). Faint expression of *Sox9* (I) or *Xslug* (J) was observed in the far lateral side (arrowheads). (K) *Hairy2A* expression is downregulated on X-TSK-injected side, though it appears to spread over a broader domain (arrowhead and open arrowhead). (L) X-TSK mRNA (1 ng) injection resulted in decreased expression of *Msx-1* (arrowhead) though the width of its expression area (right bracket) becomes wider than in the control side. (M) An embryo injected with X-TSK (1 ng). *Xbmp4* expression increased outside the injected side (arrowhead). (N) An embryo injected with *Chordin* (100 pg). *Xbmp4* expression disappeared around the  $\beta$ -gal-positive cells (arrowhead). (O) *BMP4* (0.5 ng) mRNA and (P) *Notch* ICD (0.5 ng) injections caused reduction of *Xslug* expression (arrowheads). (Q)  $\beta$ -Gal mRNA injection did not affect *Xbmp4* expression. (R) *X-delta-1<sup>Stu</sup>* injected areas are marked by  $\beta$ -gal staining (light blue), the arrowhead indicates ectopic *Xbmp4* expression (dark purple). (S,T) An embryo injected with X-TSK. (S) Two out of the three strips of *N-tubulin* expression disappeared (arrowheads). (T) *X-delta-1* expression disappeared on the outer border of the neural crest (arrowhead). (U)  $\beta$ -gal did not affect *X-ESR-1* expression. (V) X-TSK mRNA (1 ng) injection resulted in expanded peripheral expression of *X-ESR-1* on the injected side (arrowheads). (W,X) Co-injection of 1 ng X-TSK and 0.5 ng *Su(H)<sup>DBM</sup>* mRNAs. (W) Diffused signals of *Sox9* were observed around the arrowhead. (X) *Xbmp4* expression was extended towards the  $\beta$ -gal-positive side (arrowhead). (Y) *Sox9* expression was reduced in *Su(H)/Ank* 500 pg injected side (arrowhead). (Z) *Sox9* expression was completely missing in X-TSK 1 ng+ *Su(H)/Ank* 500 pg injected side (open arrowhead). (AA) *Xbmp4* expression was very weakly inhibited in *Su(H)/Ank* 500 pg injected side (open arrowhead). (BB) The patchy expression of *Xbmp4* was observed in X-TSK 1 ng+ *Su(H)/Ank* 500 pg injected side (arrows). The expansion of *Xbmp4*-expressing regions was not observed (open arrowhead).



### X-TSK modulates Notch signaling activation by direct binding to X-delta-1

Our results suggest that X-TSK overexpression can modulate Notch signaling in the neural crest region. In order to determine whether there is a direct interaction between X-TSK and the Notch signaling pathway, we stained Notch-transfected cells and Delta-transfected cells with TSK-myc tagged protein and anti-myc antibody. Binding of TSK protein to Delta-transfected cells, but not to Notch-transfected cells, was detectable (data not shown). To confirm this binding, we performed immunoprecipitation experiments, and found that the X-TSK protein precipitates the X-delta-1 extracellular domain (Fig. 7A). This result suggests that X-TSK modulates Notch signaling by binding to Delta in the extracellular space.

To clarify whether TSK works as an activator or an inhibitor of Notch signaling, we performed animal cap experiments by using the Notch-target gene *XESR-1* expression as a readout for the activation of the Notch signaling pathway (Fig. 7B). As the expression of *X-delta-1* in early gastrula stages is localized to the marginal zone, uninjected animal caps express Notch, but not *X-delta-1* (Wittenberger et al., 1999). Thus, Notch activation does not normally occur in isolated animal caps (Kiyota and Kinoshita, 2002). We then activated Notch signaling in animal caps by injection of *X-delta-1* mRNA, and assessed the effects of X-TSK gain- or loss-of-function on this activation. Synthetic mRNAs and/or X-TSK MO were injected into four-cell stage embryos, animal caps were excised from stage 8.5 embryos, and analyzed by RT-PCR at stage 9.5. As

expected, both *Notch ICD* mRNA and *X-delta-1* mRNA caused induction of *XESR-1* (Fig. 7B, lanes 3, 4). By contrast, co-injection of *X-TSK* with *X-delta-1* caused a much lower induction of *XESR-1* compared with *X-delta-1* alone, while *X-TSK* alone could not induce *X-ESR1* (Fig. 7B, lanes 4-6). Remarkably, co-injection of X-TSK-MO together with *X-delta-1* also strongly blocked X-ESR1

activation by *X-delta-1* (Fig. 7B, lanes 7, 8). Taken together, these data suggest that the endogenous levels of X-TSK present in uninjected animal caps (Fig. 2D,J) are required for activation of Notch signaling by *X-delta-1*. Conversely, higher levels of TSK, as those resulting from X-TSK overexpression, can cause inhibition of *X-delta-1* activity, suggesting that X-TSK may differentially modulate *X-delta-1* activity in a dose-dependent manner in vivo.

Table 3. Gene expression in X-TSK overexpressing embryos

Sample /probe	Normal	Expanded or ectopic	Diminished
X-TSK 250 pg			
Sox9	8	0	5
Sox2	3	10	1
XK81	5	0	6
X-TSK 500 pg			
Sox9	1	0	6
Sox2	4	1	0
Xslug	5	0	14 (73.7%)
Xbmp4	6	22 (78.6%)	0
Xrx1	9	17 (60.7%)	2
Xotx2	14	35 (71.4%)	0
Six3	3	22 (88.0%)	0
X-TSK 1 ng			
Sox9	11	0	41 (78.8%)
Xslug	3	0	22 (88.0%)
Sox2	11	44 (80.0%)	0
Xbmp4	16	22 (57.9%)	0
XK81	7	0	26 (78.8%)
N-tubulin	16	1	49 (74.2%)
X-ESR-1	10	22 (57.9%)	6
X-delta-1	18	3	60 (74.1%)
MyoD	26	5	9
Hairy2A	16	0	39 (70.9%)
Msx-1	12	0	40 (76.2%)
β-gal 500 pg			
Sox9	14	0	0
Xslug	16	1	1
Sox2	19	0	0
Xbmp4	62	2	4
XK81	15	0	2
Xrx1	37	0	3
Xotx2	14	0	0
Six3	37	1	2
N-tubulin	28	0	0
X-ESR-1	35	5	2
X-delta-1	44	0	1
Hairy2A	38	0	0
Msx-1	39	0	0
X-TSK 1 ng+Su(H) <sup>DBM</sup> 500 pg			
Sox9	0	16 (84.9%)	3
Xbmp4	7	16 (69.6%)	0
Su(H) <sup>DBM</sup> 500 pg			
		Weakly enhanced	
Sox9	15	12	0
Su(H)/Ank 500 pg			
		Weakly enhanced	Weakly diminished
Sox9	11	3	9
Xslug	10	4	12
Xbmp4	14	2	7
X-TSK 1 ng+Su(H)/Ank 500 pg			
Sox9	13	0	28 (68.3%)
Xslug	4	0	32 (88.9%)
Xbmp4	18	19 (51.3%)	0

X-TSK mRNA (250 pg-1 ng), β-gal mRNA (500 pg), other RNAs or their mixtures were injected into unilateral blastomeres at the four-cell stage. Embryos showing identical effects on the injected side were counted at stage 15.

DISCUSSION

In this paper, we describe the *Xenopus* homolog of the *Tsukushi* gene. The present results suggest that *X-TSK* is required for proper ectodermal patterning and neural crest specification, acting both as a BMP antagonist and as a modulator of *Notch* signaling.

Signaling activities of X-TSK

*X-TSK* encodes for a secreted protein belonging to the SLRP family. Although some members of this family, such as *decorin*, have previously been shown to bind TGF-β and modulate its activity

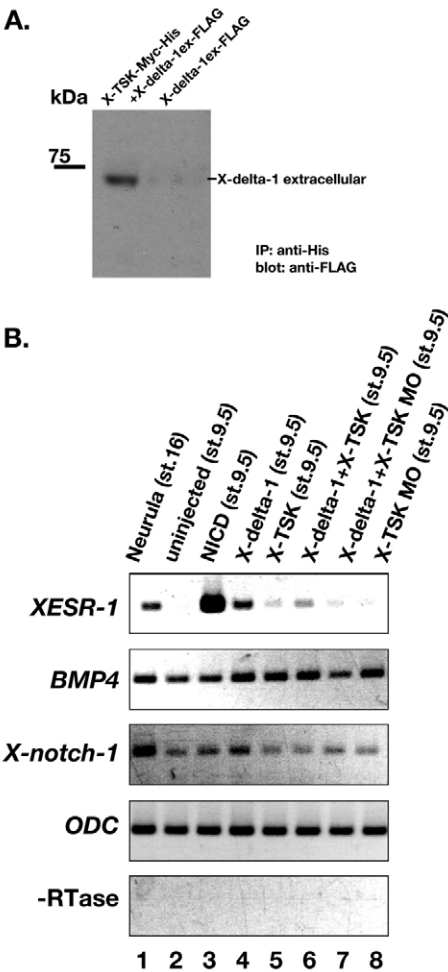


Fig. 7. A molecular interaction between X-TSK and X-delta-1. (A) Immunoprecipitation of X-delta-1 extracellular domain with X-TSK. (B) RT-PCR analysis of acute *XESR-1* activation by Notch canonical pathway with or without X-TSK. Animal caps are excised at stage 8.5 and analyzed at stage 9.5. As a positive control, the normal neurula was used (lane 1). Notch ICD (NICD, 1 ng), 1 ng X-delta-1 mRNA, 2 ng X-TSK mRNA, 10 ng X-TSK MO and mixtures thereof were injected into all blastomere at the four-cell stage.



(Yamaguchi et al., 1990), their role during embryonic development was not clear. We recently described the chick TSK homolog and showed that it works as a BMP antagonist during chick gastrulation (Ohta et al., 2004), while another SLRP member, Biglycan, has also been shown to modulate BMP activity during *Xenopus* early development (Moreno et al., 2005). Similar to its chick counterpart, X-TSK works as a BMP antagonist, as indicated by overexpression experiments in *Xenopus* embryos and in vitro assays. In fact, X-TSK overexpression can dorsalize ventral mesoderm (see Fig. S1 in the supplementary material), and it can induce cement gland and neural tissue, but not the dorsal mesodermal marker cardiac actin in animal caps (Fig. 3, see Fig. S2 in the supplementary material), thus mimicking the effects of other known BMP inhibitors, such as *Chordin*, *Noggin* or a dominant-negative BMP receptor (Sasai et al., 1995; Sive and Bradley, 1996). In whole embryos, X-TSK overexpression can enhance dorsoanterior fates and repress ventrolateral fates, and these effects are similar to those produced by *Chordin* overexpression (Fig. 6G). The opposite effect, namely a reduction of dorsal and an expansion of ventral structures, is observed after X-TSK depletion (Fig. 4) (Ohta et al., 2004). Finally, X-TSK can antagonize the ventralizing activity of *BMP4* in mesodermal explants, and it can bind BMP proteins in vitro. Altogether, these data indicate that X-TSK is endowed with a clear BMP antagonistic activity.

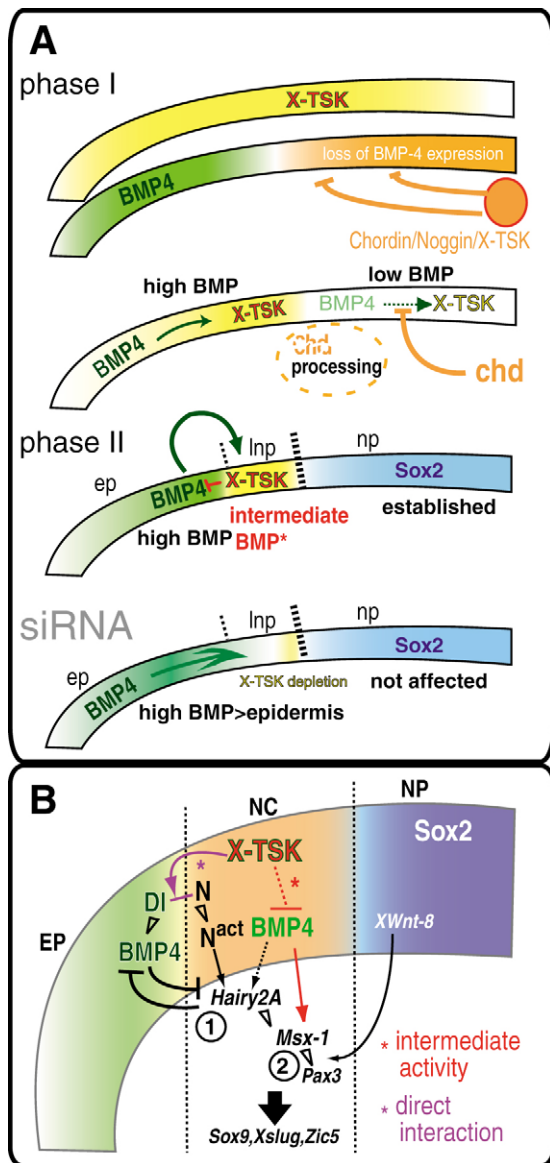
However, not all the effects of TSK gain or loss of function can be simply ascribed to its anti-BMP function. In particular, although chick TSK could efficiently rescue the reduction in neural crest formation caused by X-TSK depletion, *Chordin* could not (Fig. 4M-O,R,S, Fig. 6I,J). Moreover, although *Chordin* overexpression repressed *BMP4* expression in the ectoderm, X-TSK overexpression had the opposite effect (Fig. 3B, Fig. 6M,N). These data suggest that X-TSK has some signaling activities that are different from BMP antagonism. Thus, it is interesting to see (Figs 6 and 7) that modulation of the Notch signaling pathway is also involved in X-TSK function, as evidenced by the direct binding of X-TSK to X-delta-1 in vitro (Fig. 7A). Moreover, both *Notch ICD* and X-TSK had similar effects on neurogenesis (Chitinis et al., 1995), neural crest formation (Glavic et al., 2004) and the expression of the Notch-target gene *X-ESR-1* (Wettstein et al., 1997). Finally, suppression of neural crest formation by X-TSK overexpression was efficiently rescued by a dominant-negative *Su(H)*, which inhibits Notch signaling, but not by an active form of *Su(H)* (Fig. 6). Together, these data clearly suggest that X-TSK may also work, at least in part, via activation of Notch signaling. However, the effect of X-TSK on *BMP4* expression in the anterior peripheral region of the neural plate is similar to that of a dominant-negative Notch ligand, *X-delta-1<sup>Stu</sup>* (Glavic et al., 2004), and this X-TSK effect can be prevented, at least in part, by an active form of *Su(H)*, but not by a dominant-negative *Su(H)* (Fig. 6). This suggests that X-TSK overexpression may also cause inhibition of Notch signaling at the neural/epidermal border. To gain more insight into the interaction between X-TSK and X-delta-1, we performed gain- and loss-of-function experiments of X-TSK in isolated animal caps where Notch signaling was simultaneously activated by *X-delta-1* overexpression (Fig. 7B). Surprisingly, we found that while the endogenous levels of X-TSK in isolated animal caps are necessary for X-delta-1 activity, higher X-TSK levels resulting from X-TSK overexpression inhibits X-delta-1 function. Although further work will be needed to dissect the biochemical mechanism by which X-TSK exerts these dual effects on X-delta-1 activity, these results identify X-TSK as a new modulator of the Notch signaling pathway, which works by directly binding X-delta-1 in the extracellular space.

## X-TSK is required for ectodermal patterning and neural crest specification

The work described in this paper uncovers a novel function for TSK family members, i.e. the control of neural crest specification. During gastrulation and neurulation, X-TSK expression is downregulated in the middle of the neural plate, while it remains in the non-neural ectoderm and it accumulates to strong levels at the neural plate border (Fig. 1). This is the region where the neural crest is specified, and therefore it was reasonable to see that both X-TSK gain and loss of function affected neural crest formation. In particular, neural crest specification was inhibited in X-TSK-depleted embryos, which also showed an expansion of the epidermal ectoderm and, depending on the approach, a reduction of the neural plate (Fig. 4) (Ohta et al., 2004). In these experiments, the observation that the neural plate was reduced in embryos injected with a X-TSK-targeted morpholino (Ohta et al., 2004), but not siRNA, may be explained with the fact that siRNA did not apparently affect X-TSK levels during gastrulation, when X-TSK is expressed in the dorsal ectoderm and mesoderm, while the morpholino may be already effective at these stages (Ohta et al., 2004). By contrast, X-TSK overexpression after gastrulation caused an expansion of the neural crest (Fig. 6). In addition, though X-TSK on its own was not able to induce neural crest markers in animal caps, it could strongly cooperate in this process with the dorsolateral mesoderm or *XWnt-8*, both of which provide a posteriorizing signal required for neural crest specification (Fig. 5) (Villanueva et al., 2002). These experiments also showed that, consistent with its expression pattern at the neural plate border (Fig. 1), X-TSK function is required in the ectoderm for neural crest specification.

How does X-TSK control neural crest specification? Clearly, its BMP antagonistic activity is likely to play a role. In fact, other BMP inhibitors, such as *Chordin* or *Noggin*, can mimic X-TSK ability to induce neural crest markers in cooperation with *XWnt-8* (LaBonne and Bronner-Fraser, 1998; Mayor et al., 1995). In addition, both gain- and loss-of-function analysis suggest that X-TSK has a more general role in the mediolateral patterning of the ectoderm, which is best explained with its anti-BMP function. A specific gradient of BMP signaling is well-known to be required to specify the neural plate, neural crest and epidermal domains in the *Xenopus* ectoderm (Marchant et al., 1998). Therefore, one possibility is that the BMP-inhibitory activity of X-TSK, localized at the neural plate border and in the non-neural ectoderm, is essential for the proper shaping of the BMP gradient required for ectodermal patterning, and that the action of BMP antagonists secreted from the dorsal midline, such as *Chordin* and *Noggin*, is not sufficient in this respect. This would be in agreement with previous observations that removal of the dorsal marginal zone does not prevent neural crest specification, though it strongly affects neural plate induction (Marchant et al., 1998).

The fact that another BMP antagonist, namely *Chordin*, could not efficiently rescue the neural crest reduction in X-TSK-depleted embryos (Fig. 6I,J) suggested the interesting possibility that X-TSK may also control neural crest specification via alternative mechanisms distinct from direct BMP antagonism through protein-protein interaction. First, different from other BMP antagonists, X-TSK can upregulate *BMP4* expression in the non-neural ectoderm, while BMP signaling can positively regulate X-TSK expression. In addition, as described above, we found clear indications that X-TSK may also work in neural crest specification via modulation of the Notch signaling pathway. Remarkably, it has been already shown that Notch signaling plays a role in neural crest specification and that it can regulate expression of *BMP4* and genes associated with BMP signaling in the presumptive neural crest region (Glavic et al., 2004).



**Fig. 8. A model of consecutive steps of neural crest specification.** (A) A proposed model of sequential steps of neural crest specification. (Phase I) During gastrulation, midline BMP antagonists inhibit *BMP4* expression and specify the neural plate (orange). After this, *X-TSK* expression is repressed in the presumptive neural plate (yellow), while it is maintained in the lateral ectoderm by *BMP4* signaling. (Phase II) After stage 12.5, *X-TSK* expression is upregulated in the presumptive neural crest region, which has intermediate levels of *BMP* signaling. (siRNA) In *X-TSK* depleted embryo, *BMP* signaling levels (green) are increased in the *TSK* expressing-region. Under these conditions, epidermal fates are expanded up to the neural plate margin (blue), while the neural crest region (yellow) is repressed. (B) Molecular network of neural crest specification. *X-TSK* inhibits *BMP4* (red asterisk) and modulates Notch signaling via direct binding to *X-delta-1* (purple asterisk). Notch signaling can regulate the expression of *BMP4*, and *BMP* and Notch signaling interact to control the expression of *Hairy2A* and *Msx1*. Timing of Notch activation alters its effect on *BMP4* expression (see Glavic et al., 2004) (black asterisk). Finally, caudalizing signals such as *XWnt-8* converge on this network downstream of *Msx1* to control the activation of *Pax3* expression and neural crest cell specification within the *Msx1*-expressing region. The interactions indicated by '1' and '2' have been reported previously (Glavic et al., 2004; Monsoro-Burq et al., 2005).

Moreover, during *Xenopus* development, it has been described that Notch and its target gene *Hairy2A* are expressed in the neural crest territory, while the Notch ligands, Delta and Serrate, are expressed in the cells surrounding the prospective crest cells (Glavic et al., 2004). An attractive hypothesis is that, by directly interacting with both *BMP4* and *X-delta-1* in the extracellular space at the neural/epidermal border, and by indirectly regulating *BMP4* transcription in this region, *X-TSK* may work as a crucial molecular intersection between *BMP* and Notch signaling in the territory where the neural crest is specified.

Our results also suggest that *X-TSK* may differentially modulate *X-delta-1* activity in a dose-dependent manner, by activating it at lower *X-TSK* levels and repressing it at higher *X-TSK* levels. On this respect, it is interesting to note that the distribution of *TSK* expression levels shows some correlation with the localization of the neural crest. For example, the neural crest is specified at the lateral neural border, where *X-TSK* expression is lower, and excluded from the anterior neural border, where *X-TSK* expression is higher (Fig. 1A,C). Moreover, the proximal edge of the neural crest in the lateral neural border overlaps with a region of higher *X-TSK* expression, while *X-TSK* expression inside the neural crest domain is lower (Fig. 1K,L; Fig. 4X). As Notch is expressed in the whole neural plate, while *X-delta-1* is expressed both at the anterior neural boundary and at both the proximal and the distal edges of the neural crest in the lateral neural boundary (Glavic et al., 2004), the expression pattern of Notch and Delta can only partially account for the specific localization of the neural crest region. Furthermore, although caudalizing signals such as Wnt, FGF and RA are thought to be responsible for the localized induction of the neural crest at the lateral, but not the anterior, neural border, some FGFs and RA-producing enzymes, such as FGF8 and Raldh2, are expressed in the anterior neural border from early neurula stages (Lupo et al., 2005). Therefore, it is tempting to speculate that the presence of higher levels of *X-TSK* in the anterior neural border and in the proximal edge of the neural crest in the lateral neural border, compared with the neural crest itself, might be an additional mechanism to restrict the boundaries of the neural crest domain.

### A model of consecutive steps neural crest specification

Previously, two-signal models of neural crest specification were proposed (LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002), which suggested the requirement of intermediate levels of *BMP* signaling and posteriorization factors. Based on the results presented in this paper, we propose a model of consecutive steps in neural crest specification that contemplates the dynamic expression pattern of *X-TSK* and sequential molecular interaction during ectodermal development.

First, during gastrulation, *X-TSK* is expressed in the whole animal ectoderm and in the dorsal mesoderm (Ohta et al., 2004). Dorsal midline signals, such as *Chordin*, *Noggin* and *X-TSK*, bind to *BMPs* and directly inhibit their activity in the dorsal ectoderm (Sasai et al., 1994) (Fig. 8A, phase I), leading to the specification of the neural plate at these stages (reviewed by Sasai and De Robertis, 1997).

Subsequently, *X-TSK* expression is downregulated in the presumptive neural plate, while it is maintained and enhanced in the ectoderm flanking the neural plate (phase II). At these stages, in the presumptive neural crest region, *X-TSK* controls *BMP* activity through direct binding to *BMPs* and it also modulates *BMP* transcription, possibly via modulation of the Notch signaling



pathway (Fig. 8B). Acting via simultaneous modulation of BMP and Notch signaling, TSK regulates the establishment of a pre-neural crest region expressing *Hairy2A* and *Msx1*. Finally, posteriorizing signals such as *XWnt-8* converge on this molecular network downstream of *Msx1*, to control the activation of *Pax3* expression and the specification of the neural crest within the *Msx1*-positive region (Monsoro-Burq et al., 2005) (Fig. 8B).

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/175/DC1>

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