Development 128, 3381-3393 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV4529

Tumorhead, a *Xenopus* gene product that inhibits neural differentiation through regulation of proliferation

Chuan Fen Wu[1], Hisashi Nakamura[1], Agnes Pui-Yee Chan[1], Yi-Hong Zhou[1], Tongyu Cao[1,*], Jian Kuang[2], Siew-Ging Gong[3], Guangan He[2] and Laurence D. Etkin[1,‡]

[1]Department of Molecular Genetics, University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, USA
[2]Department of Oncology, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, USA
[3]Department of Orthodontics and Pediatric Dentistry, University of Michigan School of Dentistry, Ann Arbor, MI 48109, USA
*Present address: Department of Cell Biology, Baylor College of Medicine Houston, TX, USA
‡Author for correspondence (e-mail: lde@mdanderson.org)

Accepted 7 June 2001

SUMMARY

Tumorhead (TH) is a novel maternal gene product from *Xenopus laevis* containing several basic domains and a weak coiled-coil. Overexpression of wild-type TH resulted in increased proliferation of neural plate cells, causing expansion of the neural field followed by neural tube and craniofacial abnormalities. Overexpressed TH protein repressed neural differentiation and neural crest markers, but did not inhibit the neural inducers, pan-neural markers or mesodermal markers. Loss of function by injection of anti-TH antibody inhibited cell proliferation. Our data are consistent with a model in which tumorhead functions in regulating differentiation of the neural tissues but not neural induction or determination through its effect on cell proliferation.

Key words: Neural differentiation, *Xenopus laevis*, Maternal genes, Oocyte, Cell proliferation

INTRODUCTION

Embryonic development is a complex process involving the coordinated integration of spatial and temporal signals that result in the specification, patterning and differentiation of the embryonic germ layers. Recently, in *Xenopus laevis*, several maternal transcription factors have been discovered that directly effect this process. These include Tcf-3, which, in complex with β-catenin, patterns the mesoderm into dorsal and ventral regions (Molenaar et al., 1996), VegT, which is responsible for germ layer specification (Zhang et al., 1998), and Xnf7, which regulates dorsal and ventral patterning of the mesoderm (Reddy et al., 1991; El-Hodiri et al., 1997). Therefore, it is clear that many of the key components that regulate the initial steps of development often involve maternal factors.

The molecular basis of the specification of the ectoderm in vertebrates is still not fully understood. However, it has been well established that signaling through the bone morphogenetic protein (BMP) pathway plays an important role in ectoderm differentiation. Exposure of naïve ectoderm to BMP4 results in the formation of epidermis, while inhibition of BMP signaling by interaction with organizer-specific molecules such as chordin (Sasai et al., 1995; Piccolo et al., 1996), cerberus (Bouwmeester et al., 1996), noggin (Smith and Harland, 1992; Zimmerman et al., 1996), Xnr-3 (Smith et al., 1995), follistatin (Hemmatti-Brivanlou et al., 1994), and gremlin (Hsu et al., 1998) results in entry into the neural pathway (Sasai and De Robertis, 1997). Thus, two key steps in the specification and differentiation of the nervous system are induction through the influence of these organizer specific genes followed by differentiation that is initiated by a large number of early zygotically expressed genes such as Zic2 (Brewster et al., 1998), geminin (Kroll et al., 1998), Opal (Kuo et al., 1998) Zic-related-1, Xsox2 (Mizuseki et al., 1998) and Zic3 (Nakata et al., 1997). Expression of these genes is followed by proneural and neurogenic genes, including several basic HLH gene products that appear to regulate differentiation and patterning of the nervous system (Ferreiro et al., 1994; Chitnis and Kintner, 1995; Ma et al., 1996; Ma et al., 1997; Sommer et al., 1996; Lo et al., 1997; Chen et al., 1998; Lee et al., 1995; Chitnis and Kintner, 1996).

In our analysis of maternally expressed genes that regulate early development we have identified a novel cDNA, tumorhead (TH). Overexpression of TH results in expansion of the neural field by proliferation of cells that are already committed to the neural pathway. Cells in which TH was overexpressed, while activating the early neural markers such as Sox-2 (Mizuseki et al., 1998) and NCAM (Kintner and Melton, 1987), did not express markers typical of neural and neural crest differentiation. TH did not affect genes such as chordin or noggin, which are involved in neural induction. Loss of function by injection of anti-TH antibody inhibited cell proliferation. Our data are consistent with a model in which

TH functions in regulating differentiation of the neural tissues but not neural induction or determination through its effect on cell proliferation.

MATERIALS AND METHODS

Tumorhead cDNA isolation

A 1.2 kb fragment of tumorhead cDNA was first isolated from an oocyte library in a yeast two-hybrid screen with another maternal gene product Xnf7 (Reddy et al., 1991). The interaction between the two appears to be specific, based on several specificity tests. The functional significance of the interaction is not known. To isolate full-length of tumorhead cDNA, a λZAPII directional *Xenopus* oocyte cDNA library (provided by Dr Peter Klein, University of Pennsylvania) was further screened with random-primed ^{32}P-labeled probe generated from this 1.2 kb fragment.

Expression plasmids

To generate expression plasmids, all inserts were subcloned into pCS2+MT vector (provided by Dave Turner, University of Michigan) in the same frame as the Myc tag. pCS2+MT-TH contains the full-length TH cDNA; pCS2+MT-THΔ174 contains THΔ174 insert. SGP plasmid was used to generate green fluorescent protein (GFP) mRNA. To test if Myc tag affects TH function, the fragment encoding the Myc tag was removed from pCS2+MT-TH plasmid, generating the pCS2-TH plasmid. mRNA transcribed from this plasmid used the TH start codon for translation. Synthetic capped mRNAs were prepared according to El-Hodiri et al. (El-Hodiri et al., 1997).

Northern blot analysis

RNAs were extracted from various stage embryos using the guanidiumthiocyanate/phenol-chloroform method (Chomczynski and Sacchi, 1987). RNAs were separated on 4% formaldehyde/1.2% agarose gels, transferred to Hybond N+ nylon membrane (Amersham) in 10× SSC, fixed by 2 hours baking at 80°C. Blots were hybridized with random-primed ^{32}P-labeled TH cDNA *Eco*RI fragment (1.2 kb) as a probe. The filter was exposed to X-ray film for 2 days at −70°C.

Microinjection of embryos

Embryo handling and RNA injection were carried out according to El-Hodiri et al. (El-Hodiri et al., 1997). 0.1-2 ng of TH mRNA was injected into one or two blastomeres of *Xenopus* embryos at two-, four- or eight-cell stage, together with mRNA for green fluorescent protein (GFP) as a lineage tracer. As there are reports that the presence of a Myc epitope tag may interfere with the function of some proteins, we used constructs with and without the Myc tag. Both constructs gave identical results.

Whole-mount in situ hybridization and histology

Whole-mount in situ hybridization was carried out as previously described (Kloc and Etkin, 1999) and (Harland, 1991). Digoxigenin-labeled probes were generated from the following plasmids: pMX363 Xslug (Mayor et al., 1995); pBS-Xtwi18 (Hopwood et al., 1989); pBS(sk)-chordin (Sasai et al., 1994); pBluescript(ks)-N-CAM (a gift from Dr Paul A. Krieg); pBluescript(ks)-SOX2 subcloned from pCS2-SOX2 (Mizuseki et al., 1998); pBluescript(ks)-ZCR1, subcloned from pCS2-ZCR1 *Xenopus* Zic-related-1 (Mizuseki et al., 1998); *Xenopus* Zic3 X1 clone (Brett Casey, Baylor School of Medicine); pBluescript(ks)-neuroD, subcloned from *Xenopus* neuroD clone pCS2+MTx12A (a gift from Dr Jacqueline E. Lee); neural-specific type β-tubulin, p24-10 (Richter et al., 1988); pXnot10 (von Dassow et al., 1993). The histology was carried out as previously described (Kelly et al., 1991).

Animal cap RT-PCR assay

RNA (1-2 ng) was injected into the animal pole of embryos at the two-cell stage. The animal cap explants were removed at the late blastula stages and allowed to grow until the control embryos reached neurula stages. To induce mesoderm and neural gene expression, recombined human Activin A protein (provided by NIH) was added at a concentration of 5 ng/ml. Total RNA was then extracted and analyzed with RT-PCR. Primers were designed using information from the *Xenopus* Molecular Resources Web Page (Peter Vise, University of Texas). The RT-PCR assay was carried out according to Wilson and Hemmati-Brivanlou (Wilson and Hemmati-Brivanlou, 1995) except for plakoglobin (PG) whose primers were designed with reference to Kofron et al. (Kofron et al., 1999). Quantitative RT-PCR was carried out using a LightCycler™ System (Roche).

Hydroxyurea and aphidicolin treatment

Embryos were injected at eight-cell stage with TH mRNA. Hydroxyurea and aphidicolin (HUA) were added at stage 10.5, according to Harris and Hartenstein (Harris and Hartenstein, 1991).

Whole-mount immunocytochemistry

Xenopus embryos were injected with 2 ng of TH mRNA into dorsal or ventral animal blastomeres at eight-cell stage. At stage 14/15, the injected embryos were fixed with Dent's fixative overnight and bleached with 10% H_2O_2 in Dent's fixative. Anti phosphorylated histone H3 antibody (Upstate Biotechnology, Lake Placid, NY) and anti c-Myc antibody (Oncogene™ Research Product) were use at a concentration of 5 μg/ml; anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) was used at a dilution of 1:2000; anti-mouse IgG conjugated with FITC (Boehringer Mannheim) was used as a dilution of 1:200. Nuclear staining was performed with Hoechst 33258 at a concentration of 5 μg/ml in PBS. Whole-mount immunostaining was performed as previously described (Kloc and Etkin, 1998; Carl and Klymkowsky, 1999).

Injection of purified antibody and rescue experiments

Affinity purified anti-TH antibody (Ab GN114 or Ab GN9629) was injected into a single animal blastomere at the four to 16-cell stage. To trace the antibody distribution, rhodamine-conjugated dextran (M_r, 10,000, Molecular Probe, Eugene, OR) was injected with the antibody at 2-4 mg/ml. The embryos were photographed at gastrula stages. Rescue experiment were performed by injection of 1-3 ng of Myc-TH mRNA with 0.5 ng of Myc-GFP mRNA (a lineage tracer) into one of the blastomeres at the two-cell stage. At the 16-cell stage, embryos were injected with 1.5-3 ng of Ab114 antibody mixed with rhodamine-dextran. The embryos were analyzed for rescue at blastula and gastrula stages. To test for the antibody specificity we injected embryos with a mixture of Myc-tagged TH protein and anti-TH antibody. TH protein was produced by injection of TH mRNA into oocytes. The injected oocytes were cultured at 18°C for 1-2 days. The protein was extracted according to Kuang et al. (Kuang et al., 1989) and affinity purified with anti Myc-epitope tag antibody conjugated with agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), according to Harlow and Lane (Harlow and Lane, 1988). The purified protein was dialyzed against PBS and concentrated at 0.1 mg/ml. The protein was mixed with the antibody 1 hour before injection at 4°C. The protein-antibody mixture was injected into one of the blastomeres at animal hemisphere of *Xenopus* embryos at 8-cell stage and photographed at stage 8.

5′ RACE

5′ RACE was carried out using the Marathon cDNA Amplification Kit (CLONTECH, Palo Alto, CA). Total RNA was isolated from stage VI *Xenopus* oocytes. Poly(A)$^+$ RNA was isolated using Dynabeads Oligo (dT)25 (DYNAL, INC, Lake Success, NY). The TH-specific reverse primer was 310 basepairs downstream of the 5′ end sequence shown in Fig. 1A.

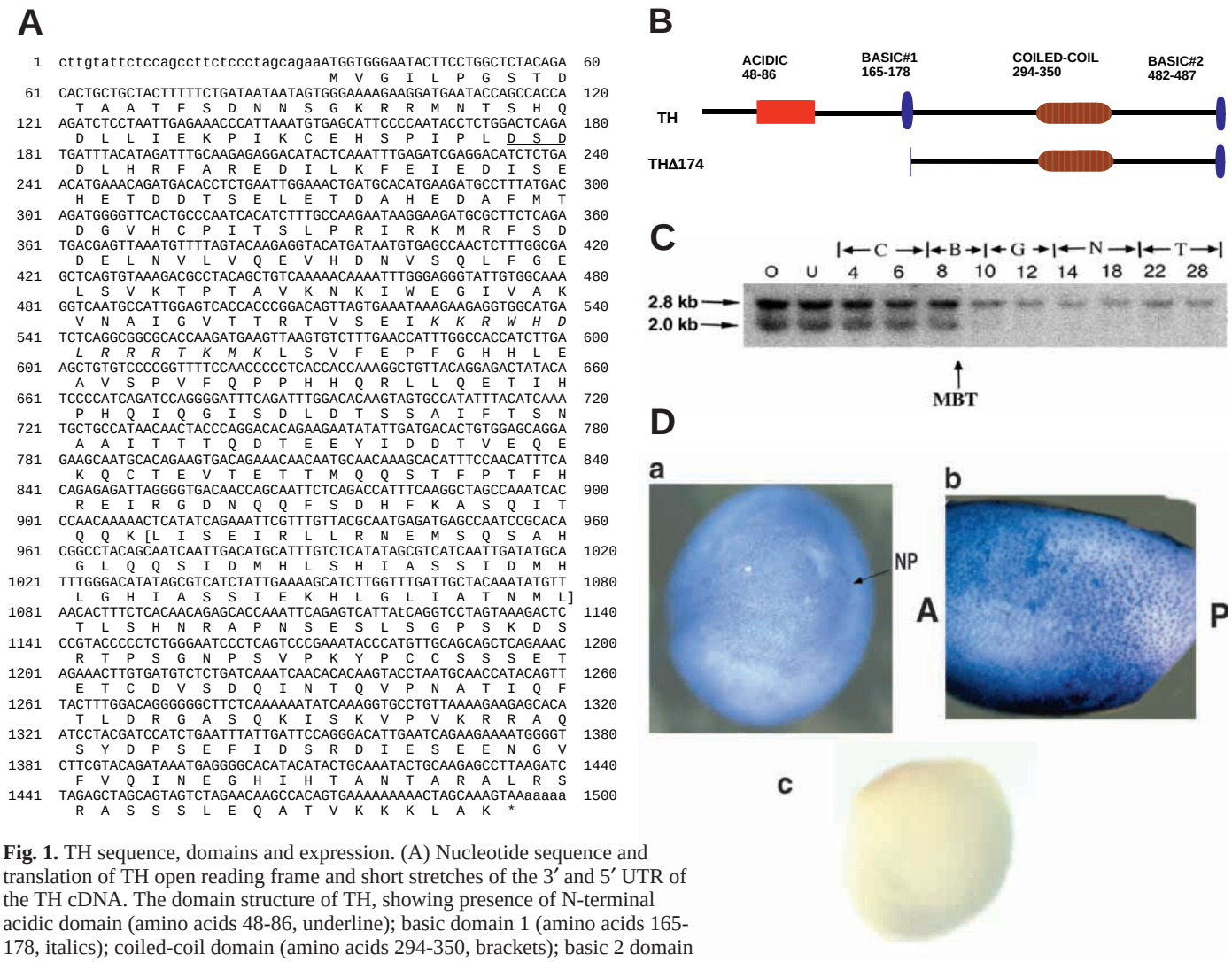

Fig. 1. TH sequence, domains and expression. (A) Nucleotide sequence and translation of TH open reading frame and short stretches of the 3′ and 5′ UTR of the TH cDNA. The domain structure of TH, showing presence of N-terminal acidic domain (amino acids 48-86, underline); basic domain 1 (amino acids 165-178, italics); coiled-coil domain (amino acids 294-350, brackets); basic 2 domain (amino acids 482-487). The asterisk indicates the stop codon. (B) Constructs used in the overexpression experiments. Construct THΔ174 consists of a deletion of the N-terminal 174 amino acids, removing the acidic domain and 10 amino acids of the basic domain. (C) Northern blot of TH mRNA from different developmental stages. Total mRNA (6 μg) was loaded into each lane and probed with TH. Dumont stages (Dumont, 1972) are shown at the top of each lane as well as stage designations. B, blastula; C, cleavage; G, gastrula; MBT, midblastula transition; N, neurula; T, tailbud. (D) Immunostaining of stage 17 (a) and stage 23 (b) embryos with anti-TH antibody. (c) A control embryo stained with the TH preimmune serum. A and P represent anterior and posterior; NP is the neural plate.

RESULTS

Cloning and domain structure of TH

A partial TH cDNA was cloned from an oocyte library in a screen for maternal proteins involved in embryonic patterning. Using one of the TH cDNAs as a probe, we rescreened an oocyte cDNA library and obtained the full-length TH cDNA. This cDNA is 2.8 kb in length and possesses an open reading frame of 1461 nucleotides (487 amino acids; Fig. 1A). There was a stop codon at the 12th amino acid upstream of the first ATG. Analysis of the open reading frame showed that TH possesses several putative domains, including a highly acidic domain (amino acids 48-86) that contains 40% acidic residues, a basic domain 1 (amino acids 165-178); a basic domain 2 (amino acids 482-487) and a coiled coil domain (amino acids 294-350) that is likely to be involved in protein-protein interaction (Fig. 1B).

Northern blot analysis of TH mRNA showed the presence of 2.8 kb and 2.0 kb transcripts (Fig. 1C). Both are maternally expressed, with the larger transcript persisting throughout embryogenesis (to stage 28) while the smaller one was detected until the mid-blastula transition (MBT), after which there was a precipitous decline. TH protein shows a complex pattern of behavior: shuttling between the nucleus and cytoplasm in a developmental stage specific manner. Fig. 1D shows an example of a neurula stage embryo with most of the TH protein in the cytoplasm, whereas stage 23 tailbud embryo has TH protein in the nucleus. The regulation of TH compartmentalization will require further analysis.

Overexpression of TH results in hyperplasia of ectodermal germ layer derivatives, giving rise to neural tube and craniofacial defects

A common way to test the function of an unknown gene in

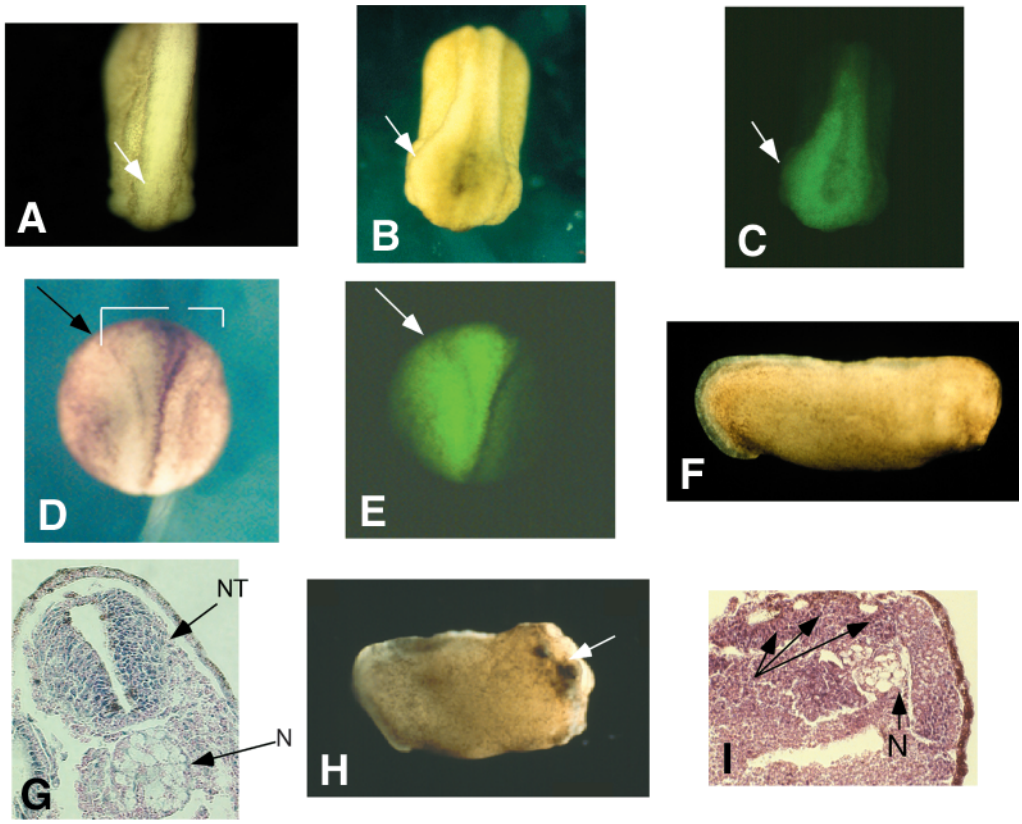

Fig. 2. Phenotype produced by overexpression of TH. (A-I) TH overexpression in two- to four-cell stage embryos. TH mRNA (0.1-2 ng) was injected into one or both blastomeres of embryos along with GFP mRNA as a lineage tracer. (A) Control stage 23 embryo injected with Δ174TH mRNA (see Fig. 1B for constructs), which does not produce an abnormal phenotype. (B,C) Stage 23 embryo injected with wild-type TH mRNA into both blastomeres at the two-cell stage, showing the distribution of the protein (GFP) in C and the abnormal neural tube phenotype in B (arrow). (D,E) Stage 16 embryo showing the expansion of the neural plate area in the region where TH was overexpressed (arrow and bracket). (D) Brightfield. (E) Distribution of co-injected GFP (arrow). (F,G) Whole-mount (F) and section (G) of a control stage 27 embryo showing the normal notochord (N) and neural tube structures (NT). (H,I) An embryo injected with TH at the two-cell stage. (H) Stage 25 embryo injected with wild-type TH at the two-cell stage. Notice the craniofacial abnormalities and the accumulation of pigment in H (arrow). (I) Histological section of the embryo in showing the abnormal neural tube structure (arrows) and the near normal notochord (N).

Xenopus is to overexpress the protein of interest by injection of its cognate mRNA. Therefore, we injected 100 pg-2 ng of the wild-type TH mRNA into one of two blastomeres at the two-cell stage or into specific blastomeres of four- and eight-cell embryos. As a control, we injected the same amounts of a deletion mutant (THΔ174) that lacks the N-terminal domain. In most experiments, we co-injected mRNA encoding the green fluorescent protein (GFP) as a lineage tracer.

Fig. 2A shows a stage 23 control embryo that was injected with THΔ174 at the two-cell stage. These appear normal as were 93% of THΔ174 injected embryos (Table 1). Fig. 2B,C shows an embryo that was injected with full-length TH mRNA in the dorsal blastomeres at the four-cell stage. Fig. 2B is a bright field image showing abnormalities in the dorsal anterior region of the neural folds, which did not close properly and were greatly enlarged. These types of embryos will develop into tadpoles with gross neural tube defects. Fig. 2C shows the location of the GFP protein whose mRNA was co-injected with the TH mRNA in the embryo in Fig. 2B. There was a close correspondence between the location of the GFP and the abnormalities in the embryo. The lower doses (100-500 pg) resulted in less dramatic phenotypes with mild neural tube defects; the higher does (500 pg-2 ng) resulted in the most severe phenotypes. Fig. 2D,E shows an embryo injected into one of the dorsal animal blastomeres at the four-cell stage in which there was an expansion of the neural plate area on the injected side. Injection of TH mRNA into the dorsal blastomeres at the two-cell stage resulted in severe abnormalities in the neural tube and the anterior region including lack of eyes, craniofacial abnormalities and accumulation of large aggregates of pigment cells (Fig. 2H) when compared with a control stage 27 embryo (Fig. 2F). In addition to the effects on the neural structures, we also saw thickenings of the epidermis (Fig. 2H).

Histological analysis (Fig. 2I) showed that there was a grossly abnormal neural tube-like structure characterized by

Table 1. Summary of TH overexpression phenotype

Substance injected*	Number of embryos			
	Total injected‡	Survived	TH phenotype (%)	Normal phenotypes (%)
TH RNA	647	574	540 (94)	32 (6)
Δ174-TH RNA	382	357	24 (7)	333 (93)
H_2O	98	92	4 (4)	88 (96)

*0.1-1 ng of RNA was injected, or 10 nl H_2O as a control.
‡The numbers include all injections into dorsal and ventral animal blastomeres.

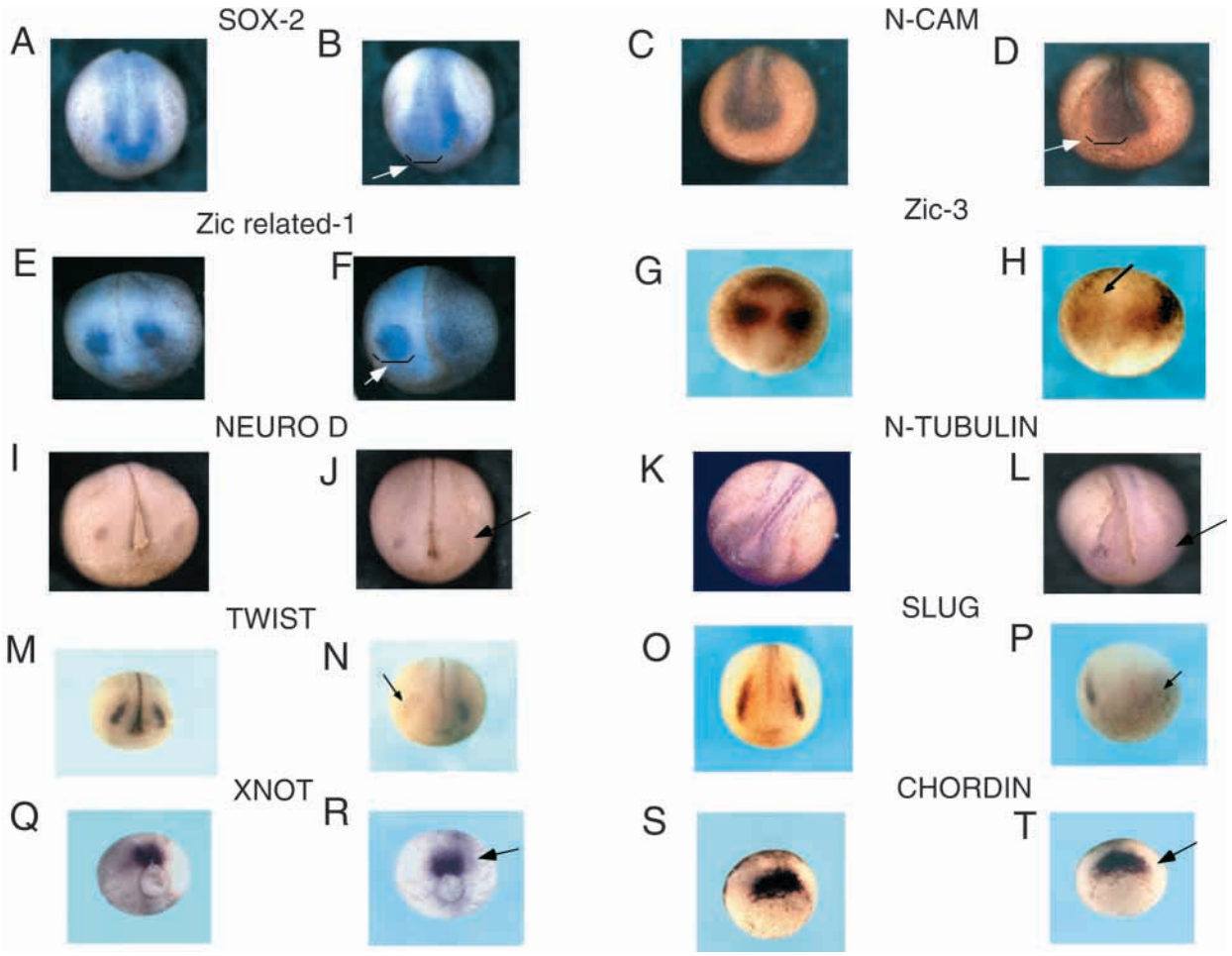

Fig. 3. Effect of TH overexpression on molecular markers. (A-H) Effect of TH on expression of early neural markers. (A) Control embryo at stage 15 showing normal in situ hybridization pattern of Xsox-2. (B) Xsox-2 expression is expanded on side of embryo that is overexpressing TH (arrow and brackets). (C) Control embryo at stage 16 showing normal in situ hybridization pattern of NCAM. (D) NCAM expression is expanded in TH overexpressing region (arrow and brackets). (E) Normal pattern of Zic-related-1 mRNA in stage 17 embryo. (F) Expanded region of Zic-related-1 expression in TH-injected embryo (arrow and brackets). (G) Normal pattern of Zic3 mRNA in stage 14 embryo. (H) Inhibition of Zic3 in region overexpressing TH (arrow). (I-L) Effect of TH overexpression of late neural markers: (I) control stage 18 embryo hybridized with NeuroD probe; (J) inhibition of NeuroD on side of embryo overexpressing TH (arrow); (K) control stage 18 embryo showing the pattern of expression of N-tubulin mRNA; (L) inhibition of N-tubulin on side of embryo overexpressing TH (arrow). (M-T) Effect of TH on neural crest and mesoderm markers: (M) control stage 18 embryo probed by in situ hybridization with the Xtwist probe showing normal pattern of twist mRNA; (N) inhibition of twist in region overexpressing TH (arrow); (O) control embryo showing the expression of Xslug mRNA; (P) inhibition of Xslug mRNA in region overexpressing TH (arrow); (Q) normal pattern of Xnot mRNA expression in stage12 embryo; (R) normal pattern of Xnot expression in embryo overexpressing TH (arrow); (S) normal pattern of chordin mRNA expression in stage 10.5 embryo; (T) normal pattern of chordin expression in area overexpressing TH (arrow).

disorganization and extreme hyperplasia of the cells comprising the neural tube, when compared with control embryos (Fig. 2G). In fact, in Fig. 2I, it appears that several small neural tube-like structures were attempting to organize. This may be due to the disorganization of the underlying notochord, which can be seen pushed to one side of the embryo by the expanded neural tube cells. Visual inspection of these animals suggests that there was not a major increase in cells contributing to notochord or somite tissues. In all of the overexpression experiments, we detected the typical TH phenotype in 94% of the embryos in which the GFP was expressed in the ectodermal derivatives (including the neural and epidermal cells; Table 1).

Overexpression of TH inhibits neural differentiation markers but not pan-neural markers such as NCAM and Sox-2

To further characterize the effect of TH on neural specification and differentiation, we analyzed a series of molecular markers normally expressed by the neural or neural crest cells. These included the pan-neural markers Xsox-2 (Mizuseki et al., 1998) and NCAM (Kintner and Melton, 1987), the early neural marker Zic-related-1 (Mizuseki et al., 1998), the neural differentiation markers Zic-3 (Nakata et al., 1997), NeuroD (Lee et al., 1995), N-tubulin (Richter et al., 1988), and the neural crest markers Xslug (Mayor et al., 1995) and Xtwist (Hopwood et al., 1989). In situ analysis in Fig. 3A-F shows

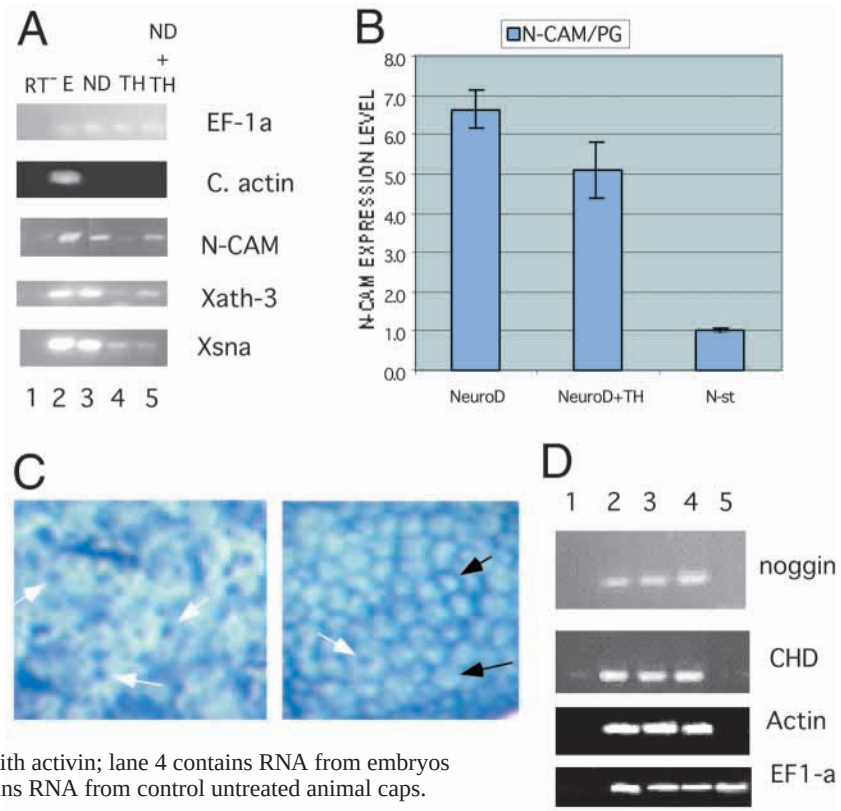

Fig. 4. Effect of TH on expression of neural markers in animal caps. (A) RT-PCR analysis of RNA isolated from animal caps overexpressing NeuroD (lane 3) showing induction of neural markers NCAM, Xath-3 and Xsnail (Xsna). TH alone did not induce any neural markers (lane 4); Co-injection of both NeuroD mRNA and TH mRNA resulted in an inhibition of the differentiated and neural crest markers, Xath-3 and Xsnail, respectively, but did not inhibit the pan-neural marker NCAM (lane 5). Lane 1 is an RT-negative control, while lane 2 is a control lane of embryo RNA. (B) Quantitative RT-PCR showing the levels of NCAM mRNA expression in NeuroD-, and NeuroD- and TH-injected animal caps. N-st are non-injected caps. (C) Animal caps that were immunostained with anti-TH antibody. The panel on the left shows control animal caps; the panel on the right shows animal caps from embryos injected with NeuroD mRNA at the four- to eight-cell stage. Black arrows indicate the cell periphery; white arrows, nuclei. (D) The effect of TH on animal caps induced with activin. Animal caps from embryos that were injected with TH mRNA into the animal blastomeres or uninjected embryos were dissected at stage 8 and treated with activin. Lane 1 is an RT-negative control; lane 2 contains RNA from embryos as a positive control; lane 3 contains RNA from animal caps treated with activin; lane 4 contains RNA from embryos overexpressing TH and treated with activin; lane 5 contains RNA from control untreated animal caps.

that the pan-neural markers Xsox-2 (Fig. 3A,B), N-CAM (Fig. 3C,D) and the early neural marker Zic-related-1 (Fig. 3E,F) were not inhibited by TH, but instead their expression was expanded along with the expansion of the neural field.

However, the early neural differentiation marker Zic3 (Fig. 3G,H) and the later neural differentiation markers NeuroD (Fig. 3I,J) and N-tubulin (Fig. 3K,L) were inhibited in regions overexpressing TH. Inhibition of these markers was not restricted to any specific region of the neural plate but occurred wherever TH was overexpressed. Inhibition was also observed for the neural crest markers Xtwist and Xslug (Fig. 3M-P). Overexpressed TH also failed to affect the mesodermal marker Xnot (Fig. 3Q,R) and the neural inducer chordin (Fig. 3S,T). In all experiments, lineage tracers were used and in all cases overlapped with the region where gene expression was either expanded or inhibited (data not shown).

Overexpression of TH in animal caps does not induce neural markers

Genes encoding proteins such as neurogenin (Ma et al., 1996), NeuroD, and Zic 3 have the ability to induce neural markers when overexpressed in naïve animal caps. Therefore, to determine if TH had the potential to induce neural markers, we injected 1-2 ng of TH mRNA into the animal hemisphere region of two or four-cell embryos, isolated the animal caps at stage 8, and cultured them until the equivalent of the late neurula-tailbud stage of embryogenesis. RNA was extracted and subjected to RT-PCR analysis for several different neural markers (e.g. NCAM, Xath-3 (Takebayashi et al., 1997), Xsnail (Essex et al., 1993) and the mesodermal marker actin). Fig. 4A (lane 4) shows that none of these markers was induced in TH-injected animal caps. This suggests that TH does not have the ability to direct animal cap cells into the neural or mesodermal pathways.

TH inhibits neural differentiation but not neural development in animal caps

Based on the knowledge that overexpressed TH did not induce neural markers in isolated animal caps and that it inhibited several neural and neural crest markers when overexpressed in embryos, we hypothesized that it may function to repress neural differentiation. To further support this hypothesis, we co-expressed NeuroD and TH together in animal caps and analyzed the ability of TH to inhibit induction of neural markers by NeuroD. Fig. 4A shows that, as expected, neither NeuroD nor TH had any effect on the induction of the mesodermal marker actin (Fig. 4A, lanes 3 and 4). However, NeuroD induced the pan-neural marker NCAM, the basic helix-loop-helix gene Xath-3 (which functions in neural differentiation) and the neural crest marker Xsnail (Fig. 4A, lane 3). When NeuroD and TH were co-expressed in animal caps, TH inhibited the induction of both Xath3 and Xsna, but did not inhibit the induction of NCAM (Fig. 4A, lane 5).

We have showed that overexpressed TH co-expressed in animal caps with NeuroD inhibited neural differentiation markers but not the pan-neural protein NCAM (Fig. 4A). We wanted to quantitate the expression of NCAM in this experiment with the idea that perhaps TH, as is the case in embryos, would expand the neural field as indicated by a corresponding increase in NCAM expression. RT-PCR of NCAM mRNA using the Light cycler revealed, instead, a small decrease in NCAM levels in NeuroD/TH-injected animal caps compared with those injected with only NeuroD (Fig. 4B). This result, while not supporting the idea that there is an increase in

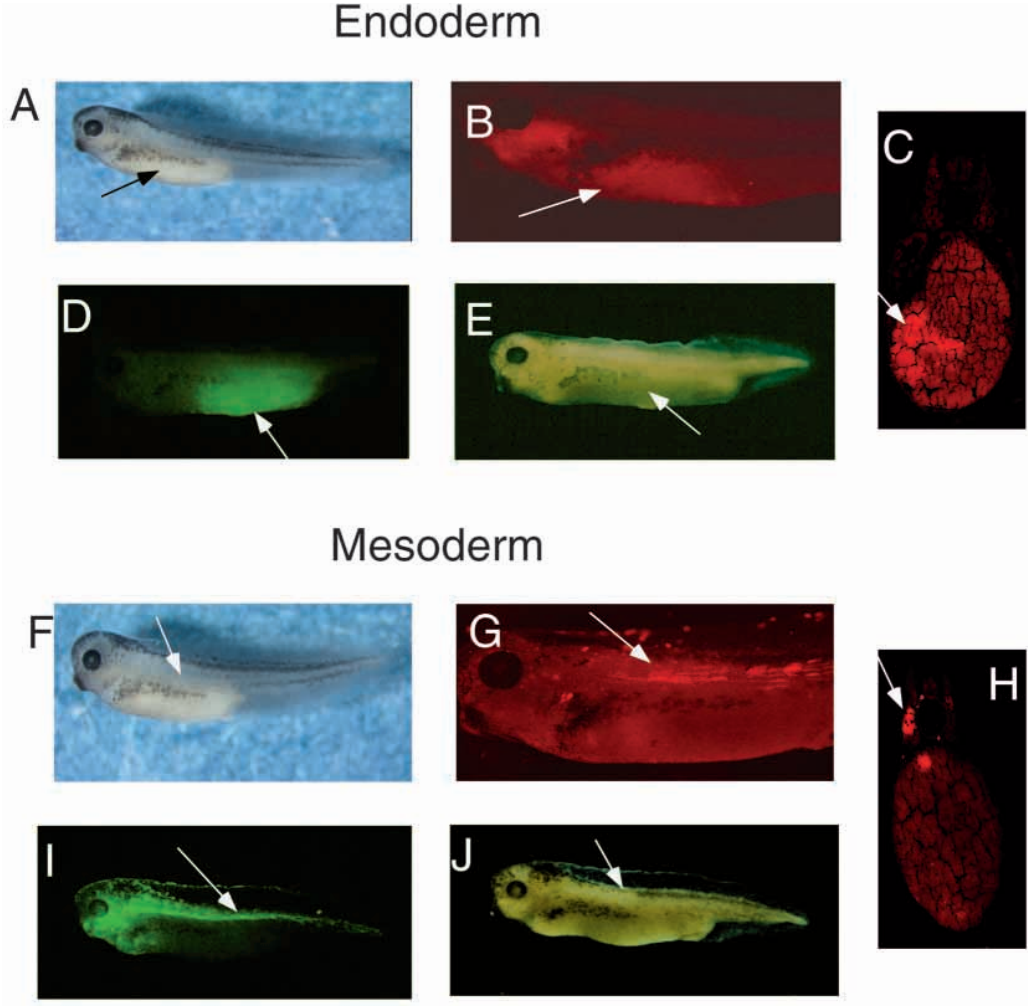

Fig. 5. Effect of TH overexpression on the endoderm and mesoderm. (A-E) Embryos that were injected at the four-cell stage with TH RNA into blastomeres giving rise to the endoderm. (A) Embryo in which TH was overexpressed in the endoderm in which we did not detect any abnormalities in endodermally derived structures. (B) The same embryo showing the rhodamine dextran lineage tracer co-injected with the TH mRNA. (C) Section of the embryo in A and B showing the rhodamine dextran and the normal nature of the endodermal cells (arrow) overexpressing TH. Arrows in A and B indicate the approximate position of the section in C. Arrow in C indicates to the endodermal cells. (D,E) Embryo injected with a mixture of TH and GFP mRNAs at the four-cell stage. (D) The distribution of the GFP. (E) A light microscopy image showing that the endodermal region of the tadpole appears normal. The arrows in D,E point to the region overexpressing the GFP and TH. (F-J) The effect of overexpressing TH in mesoderm. (F-H) The same embryo co-injected with TH mRNA and rhodamine dextran at the four-cell stage into a blastomere giving rise to the mesoderm. (F) Light microscopy image. (G) The distribution of the lineage tracer, rhodamine dextran in the axial muscle. (H) A section of the same embryo. The arrows indicate the same position in the tadpole in each panel. (I,J) A tadpole in which GFP and TH mRNAs were co-injected into the four-cell stage embryo. The TH is overexpressed in the notochord. In both instances there were no abnormalities detected within the mesodermally derived tissues, which were overexpressing TH.

cell numbers in the TH/NeuroD injected animal caps, agrees with our findings that TH does not effect neural development. This is consistent with a model placing TH function after the initial steps of ectodermal germ layer specification and neural induction but before neural differentiation.

Another interesting question is what is the effect of NeuroD-induced differentiation of animal caps on the TH gene product? Therefore, we analyzed the TH protein in control nontreated animal caps and in animal caps injected with NeuroD. Fig. 4C shows that while TH protein was present in both sets of animal caps, those overexpressing NeuroD showed TH localization predominantly at the cell periphery, while the controls showed a nuclear localization. This result suggests that the subcellular localization of TH protein is an important component of its functioning.

Overexpression of TH did not induce defects in the mesoderm or endoderm, and did not interfere with neural inducers chordin and noggin

We tested whether or not TH had any affect on mesoderm or endoderm by injecting TH mRNA into the blastomeres giving rise to these germ layers at the eight-cell stage. Fig. 5 shows TH overexpressed in the endoderm (Fig. 5A-E) or mesoderm (Fig. 5F-J). Fig. 5A is a light microscopy view of a tadpole injected with TH mRNA into a blastomere at the eight-cell stage, which gives rise to predominantly endodermal structures. Fig. 5B shows the distribution of the co-injected lineage tracer rhodamine dextran and Fig. 5C shows a section of this embryo. There were no detectable abnormalities in the endoderm. Fig. 5D,E shows another example in which TH was co-injected with GFP mRNA. Again, there were no abnormalities detected within the endoderm. Fig. 5F-H shows a tadpole injected at the eight-cell stage with TH mRNA and rhodamine dextran into a blastomere that gives rise to the somites. No abnormalities were detected. Fig. 5I,J shows another tadpole overexpressing TH in the notochord in which there were no abnormalities. These data suggest that overexpressed TH at these levels does not cause defects in derivatives of the endodermal or mesodermal germ layers.

Another way to induce mesoderm formation is to treat stage

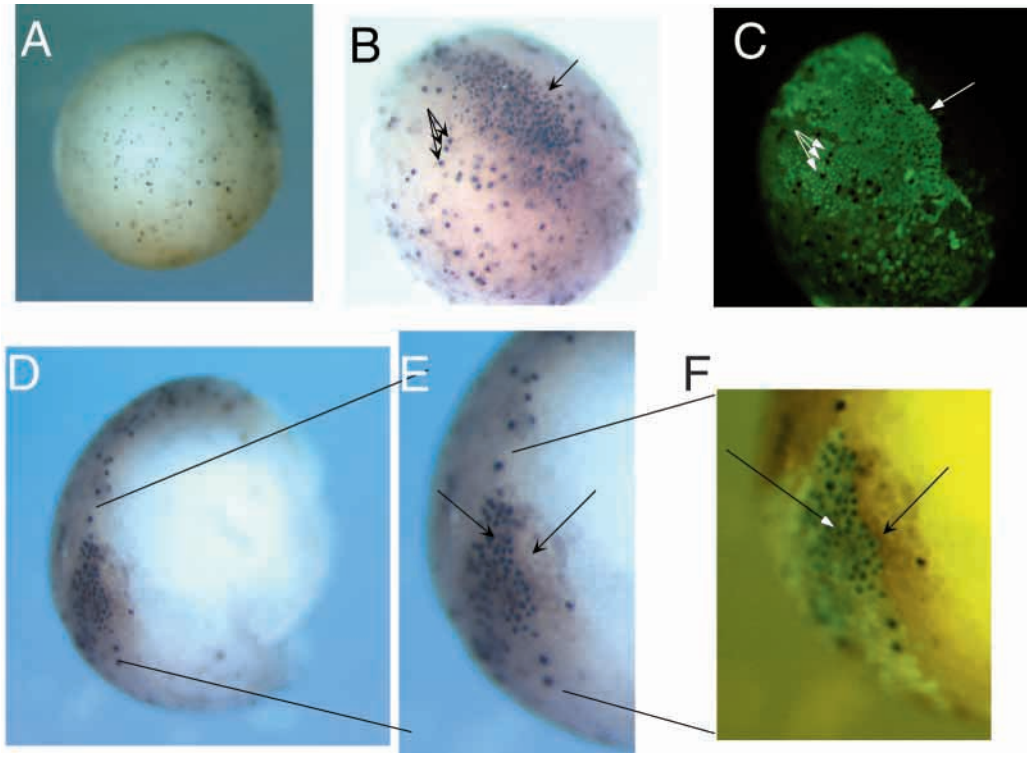

Fig. 6. Overexpression of TH increases cell proliferation. Embryos were injected with Myc-tagged TH mRNA into one blastomere of the four-cell stage embryo. Stage 14 embryos were immunostained with an antibody against phosphorylated histone H3 to detect mitotic cells. (A) Control embryo showing the normal distribution of mitotic cells. (B) Stage 14 treated embryo showing a distinct patch of mitotic cells in one region of the embryo. (C) The same embryo as in B immunostained with the Myc tag antibody to detect the distribution of exogenous TH protein. The arrows on the left point to H3 positive nuclei as landmarks. The arrow on the right points to the border of the TH-expressing cells, which coincides with the border of the mitotically active cells. (D-F) The same embryo as in B, dissected to show the coincident expression of TH with the mitotically active cells. (D) Light microscopy view of the dissected embryo. (E) A higher magnification view. (F) The embryo viewed under u.v. light to detect the fluorescein labeled Myc-tagged TH protein and partial light to visualize the distribution of the H3-positive nuclei. The white arrow points to the area showing of both the TH protein (green) and the H3-positive nuclei. The black arrow points to the region not expressing TH and not showing the H3-positive marker. These cells are all of the same germ layer.

8 animal caps with activin. RT-PCR analysis shows that over expressed TH did not effect cardiac actin, chordin or noggin expression in animal caps of embryos that were injected with TH and treated with activin (Fig. 4D). These results support the conclusion that overexpressed TH affects only the ectodermal derivatives and does not induce abnormalities in the mesodermal or endodermal germ layers. In addition, they also indicate that TH does not interfere with the initial steps of neural induction that are regulated through the organizer-specific genes chordin and noggin, which have important roles in neural induction.

The TH overexpression phenotype is due to induction of proliferation

There are several alternative mechanisms whereby TH could produce a phenotype that includes an enlarged neural plate and neural tube. These include TH affecting cell motility (resulting in aggregations of large numbers of cells whose fate is altered and now contribute to the neural plate region) or it could also affect cell proliferation. There are several ways to determine if cell proliferation is responsible for an increase in cell number. One approach is to immunostain embryos with the mitosis-specific antibody that recognizes phosphorylated histone H3. This is a reliable reagent for detecting cells in mitosis, so we used it to perform whole-mount immunostaining of embryos that were injected with TH mRNA in specific blastomeres at the four-cell stage. Fig. 6A shows the distribution of mitotic cells in a control stage 14 embryo immunostained with the anti-phospho H3 antibody. Fig. 6B shows a similar stage embryo that was injected with TH mRNA at the four-cell stage. It is clear that there is a large patch of cells that are in mitosis. Fig. 6C shows the same embryo as in Fig. 6B that was immunostained with an anti-Myc tag antibody that recognizes the exogenous TH protein. The overexpressed TH protein overlaps the region where there is an increase in mitotic cells. This embryo was analyzed further by dissecting it in half. Fig. 6D,E is a light microscopy view of the dissected embryo, showing that the mitotic cells are on the surface epithelium of the embryo. Viewing the same region under u.v. light to detect the exogenous TH shows that those cells undergoing mitosis contain the TH protein (Fig. 6F). This result clearly demonstrates a high correlation between the presence of the overexpressed TH protein and an increase in cell proliferation. In addition, Fig. 6C,F suggest that Myc-TH protein is in nuclei of non-dividing cells and in the cytoplasm of dividing cells.

The TH overexpression phenotype can be rescued by inhibiting proliferation with HUA

To further substantiate that TH induces proliferation we used the drugs hydroxyurea and aphidicholin (HUA), which inhibit cell proliferation to attempt to rescue embryos overexpressing TH. The rationale was that if cell aggregation or cell-fate conversion were responsible for increased cell number in TH injected embryos, then treatment with these agents should have no effect. However, if the increase in cell number was due to an increase in proliferation, then we would anticipate that TH-injected embryos would not exhibit an expanded neural plate region. Indeed, Fig. 7A,B shows that embryos overexpressing

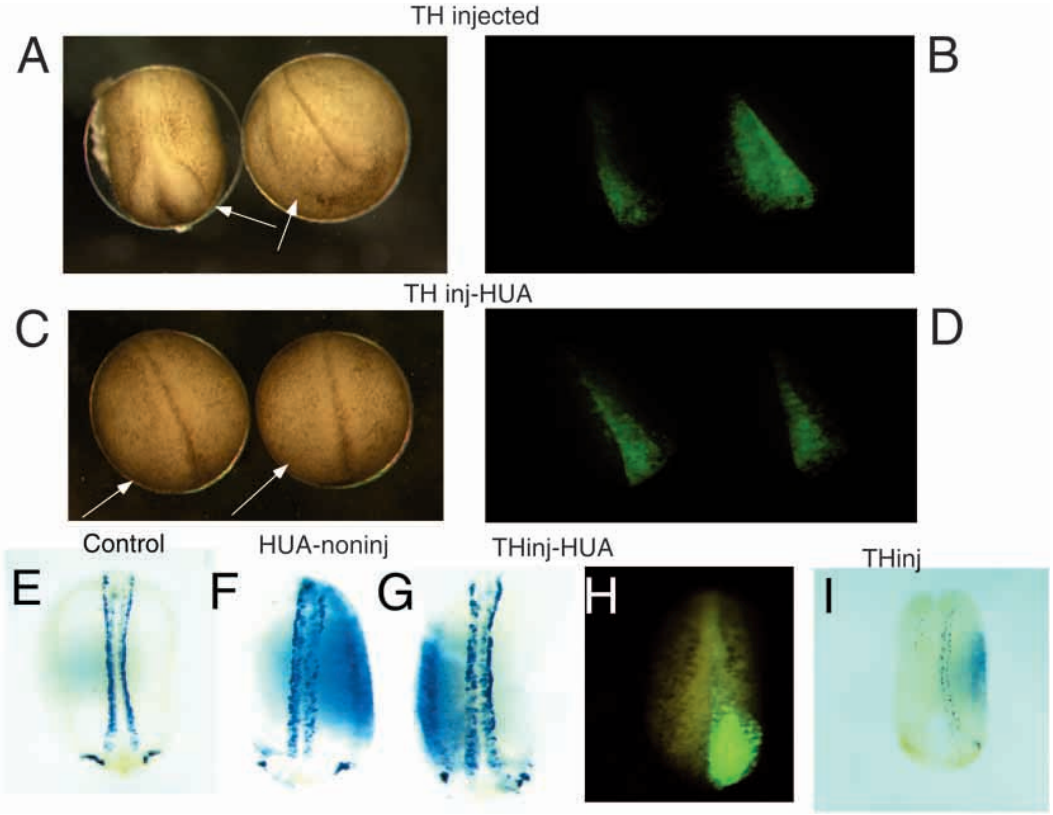

Fig. 7. The TH phenotype is due to proliferation of cells. Embryos injected with TH mRNA were raised until stage 10.5. At that time one group was treated with HUA to inhibit cell proliferation while the control group was not treated. (A,B) Control embryos showing the distribution of GFP (B) and the abnormally large neural folds (A, arrows). (C,D) Treated embryos showing that by inhibiting cell proliferation the enlarged neural folds were reduced to normal size (arrows). (E) Non-injected embryo analyzed by in situ hybridization for N-tubulin. (F) Non-injected embryo treated with HUA and analyzed by in situ hybridization for the expression of N-tubulin. (G) N-tubulin expression in an embryo injected with TH and treated with HUA showing rescue of molecular phenotype. (H) Analysis of GFP in embryo shown in G. (I) TH-injected embryo analyzed for N-tubulin expression showing inhibition without HUA treatment.

TH that were not treated showed the expected phenotype, while those embryos treated with a mixture of HUA from stage 10 onwards did not exhibit the expansion of the neural plate (Fig. 7C,D). This result supports the conclusion that the morphological aspects of the TH phenotype are the result of an increase in cell proliferation; however, it is also important to determine if the HUA treatment rescued the molecular phenotype.

To test this, we analyzed the expression of the neural differentiation marker N-tubulin in stage 23 embryos. Fig. 7F shows that the expression of N-tubulin was not interrupted in embryos treated with HUA but not injected with TH. Fig. 7G shows that N-tubulin expression was also rescued by HUA treatment in embryos where the morphology was also rescued. Fig. 7H shows the distribution of GFP in the embryo shown in 7F. Fig. 7I is a control embryo showing that TH overexpression results in a complete loss of N-tubulin expression in half of the embryo when injected into one blastomere at the two-cell stage. This result supports the hypothesis that increased cell proliferation was a major factor in keeping TH overexpressing cells from differentiating.

Injection of anti-TH antibodies inhibits cleavage in embryos

The overexpression phenotype showing an increase in cell number and the rescue of this phenotype with HUA suggested that TH functions through regulation of cell proliferation. To further test this we performed a loss-of-function experiment in which we injected affinity-purified anti-TH antibody into individual blastomeres of cleavage stage embryos. The rationale was that loss of function of TH may inhibit cleavage in embryos. Fig. 8 shows that injection of the antibody into individual blastomeres of a four-cell stage embryo inhibited cell division (Fig. 8A-C). Fig. 8B shows the effect early in a morula stage embryo, whereas Fig. 8C shows the effect when embryos reached the gastrula stage. There was no effect caused by the injected TH antibody after heat inactivation prior to injection (Fig. 8D; Table 2). The localization of the injected antibody was confirmed by using rhodamine-conjugated dextran as a lineage tracer. The results of the antibody injection using both the peptide antibody (GN114) and an antibody against the recombinant TH protein (ab9629) are summarized in Table 2. Both antibodies inhibited cleavage to the same extent.

Two important controls for this type of experiment consist of testing for the antibody specificity and also the specificity of the loss of TH function. For the former control we used TH protein that was affinity-purified from oocytes injected with

Table 2. Anti-TH antibody loss of function and rescue

	Number of embryos		
Substance injected*	Total	Normal phenotype	Cleavage inhibited phenotype
Rabbit IgG	81	81	0
GN114 (peptide Ab)	100	8	92
9629 (protein Ab)	73	0	73
Heat inactivated TH Ab (90°C, 5 minutes)	72	72	0
TH mRNA and GN114 Ab	18	10	8
myc-TH and GN114 Ab	18	18	0

*1.5 ng.

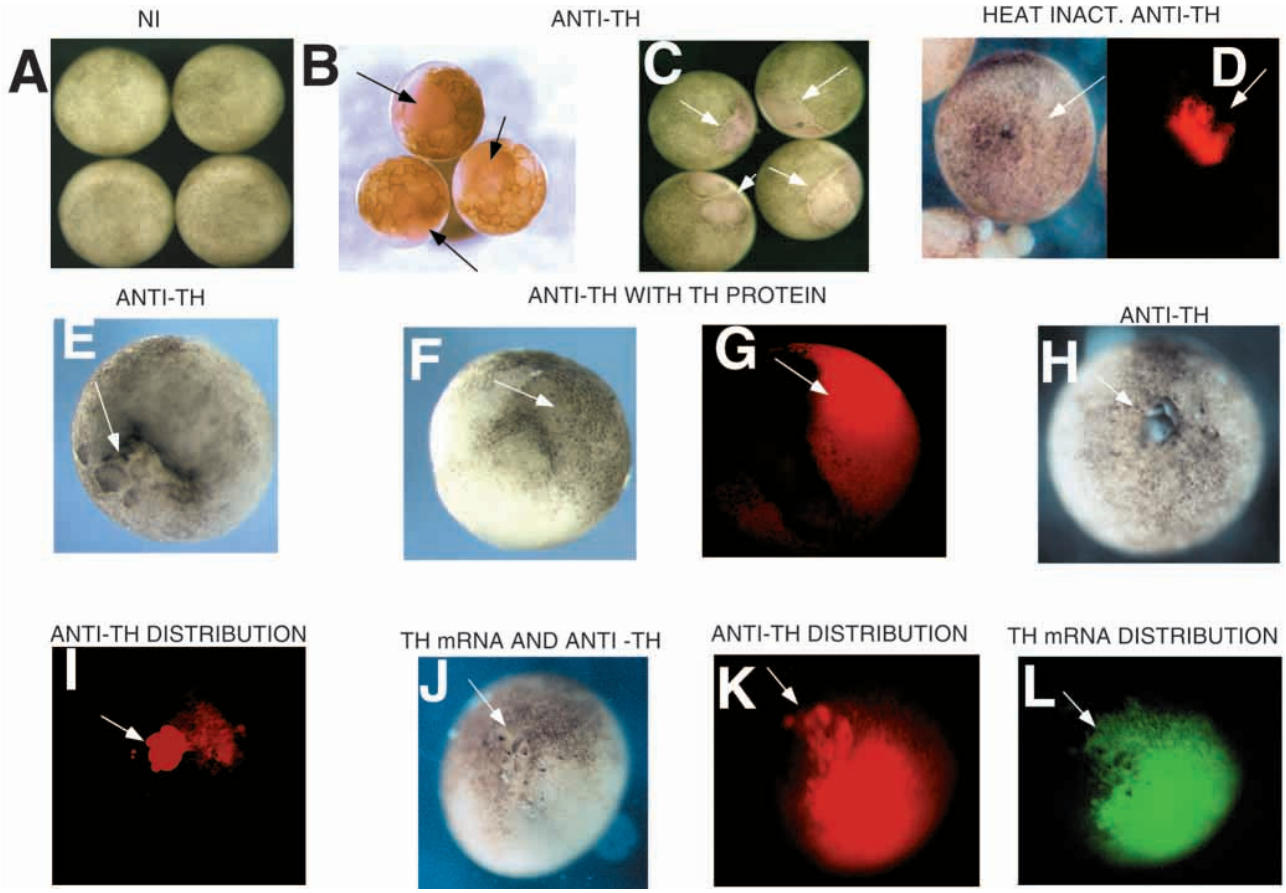

Fig. 8. Loss-of-function analysis of TH. (A) Non-injected stage 9 embryos. (B,C) Stage 7 and stage 9 embryos, respectively, injected with the anti-TH antibodies and rhodamine-derived dextran at the four-cell stage. This was photographed under both u.v. and incandescent light to show the presence of the rhodamine in injected blastomeres (arrows). (D) On the left is a stage 9 embryo injected with heat-inactivated anti-TH antibody. On the right co-injected rhodamine dextran (arrows) shows the location of the injected antibody. (E) Stage 8 embryo injected with anti-TH antibody at the 16 cell stage. The arrow indicates the enlarged blastomeres. (F) Stage 8 embryo injected with a mixture of the anti-TH antibody and purified TH protein. (G) u.v. microscopy image of the same embryo as in F, showing the distribution of the injected mixture (arrows). (H,I) Stage 8 embryo injected with anti-TH antibody at the 16-cell stage showing large blastomeres (arrow in H) and showing location of the antibody (arrow in I). (J,K,L) Stage 8 anti-TH antibody injected embryo rescued by injection of TH mRNA showing normal size blastomeres (arrow in J) in area where the TH mRNA was injected. (K) Distribution of the anti-TH antibody in the embryo, as indicated by rhodamine-conjugated dextran. (L) Distribution of TH mRNA, as indicated by presence of GFP in embryo. The cells in which there is less TH mRNA (arrow) appear larger than those receiving larger amounts of the mRNA.

TH mRNA to mix with the anti-TH antibody. This mixture was injected into blastomeres of embryos. Fig. 8 showed that the antibody alone induced inhibition of cleavage (Fig. 8E); however, the mixture of antibody and TH protein rescued the phenotype (Fig. 8F,G; Table 2).

The second control involved rescue of the phenotype by injection of TH mRNA. This involved pre-injection of TH mRNA at the two-cell stage to allow enough protein to be synthesized, followed by a second injection of the anti-TH antibody at the 16-cell stage. The mRNA was co-injected with GFP mRNA as a lineage tracer, while the antibody was co-injected with rhodamine-conjugated dextran. Fig. 8H shows the antibody alone injected embryos with the large blastomeres. Fig. 8I shows that the distribution of the antibody as determined by rhodamine dextran lineage tracing overlapped this area. Fig. 8J-L shows an mRNA rescued embryo (summarized in Table 2). Notice that in the areas in which the TH mRNA (Fig. 8L) and anti-TH antibody (Fig. 8K) overlap to the greatest extent the cells are normal size. Areas where there is less overlap show slightly larger cells. The results clearly show that, indeed, the phenotype was rescued by injection of TH mRNA. Taken together, these results demonstrate that TH functions by regulating cell proliferation.

DISCUSSION

TH is a novel gene

We have cloned a cDNA encoding a novel maternal gene product, TH, that functions in the differentiation of neural tissues. TH joins a growing list of maternal products that function in patterning, specification or differentiation of the embryonic germ layers. These include maternally expressed VegT, which functions in the establishment of several

embryonic germ layers (Zhang et al., 1998), and Tcf-3 (Molenaar et al., 1996) and Xnf7 (El-Hodiri et al., 1997; Dreyer and Hausen, 1983; Miller et al., 1991; Gong et al., 1995), which function in patterning of the mesoderm. All of these appear to be transcription factors and are likely to regulate a specific set of zygotically expressed genes at the MBT.

TH inhibits neural differentiation markers

A crucial event in neural induction involves the expression of noggin and chordin in Spemann's organizer (Smith and Harland, 1992; Sasai et al., 1994). Both of these interact and inhibit the function of BMP resulting in the schism of the ectoderm into regions in which BMP activity is inhibited, resulting in initiation of the neural pathway and regions in which BMPs are active, and in initiation of the epidermal pathway (Sasai and DeRobertis, 1997). Neural differentiation proceeds in an orderly fashion in which a series of early neural markers are expressed within the neural epithelium to promote neural determination or differentiation.

When overexpressed, TH has the effect of inhibiting a group of neural differentiation markers including the early marker Zic-3, the late markers NeuroD and N-tubulin, and the neural crest markers Xtwist and Xslug. This inhibition is accompanied by an increase in the size of the neural field. Interestingly, the genes for the pan-neural proteins NCAM and Xsox-2, and the early neural marker Zic related-1 are not inhibited but instead their expression boundary is expanded to overlap the region overexpressing TH.

It is also important to note that TH alone was unable to induce neural determination or differentiation in animal cap cells. However, it did inhibit the ability of neurogenic factors such as NeuroD to induce neural differentiation markers but not to promote neural determination. Overexpression of TH also did not effect the ability of activin to induce neural inducers chordin or noggin in animal caps. We interpret these results as indicating that overexpression of TH inhibits differentiation, but not determination or induction of cells within the neural pathway. We also found that overexpressed TH also affected the epidermis by producing abnormal growths (data not shown).

The effect of TH overexpression is strictly on cells derived from the ectodermal germ layer. TH had no effect on mesodermal or endodermal derivatives. Why TH has a germ layer-specific effect during early embryogenesis, even though it is ubiquitous, is not known. However, we speculate that co-factors may be necessary for its functioning that might be limited to this germ layer during specific stages of development.

TH may function in regulating cell proliferation

Our data suggest that overexpressed TH produces its phenotype by maintaining cells in a hyperproliferative state, which inhibits their differentiation. Several lines of evidence support this possibility. The first is that overexpression of TH did not change the fate of animal cap cells. The second line of evidence is that cell counting data showed that there was a two- to threefold increase in cell number in regions overexpressing TH (Wu and Etkin, unpublished observations). Third, regions overexpressing TH show an increase in staining with the anti-phosphorylated H3 antibody. This antibody is a marker for cell proliferation. Fourth, treatment of embryos overexpressing TH with HUA reduced the enlargement of the neural folds. However, if TH inhibited differentiation through maintaining cells in the cell cycle we would expect that treatment of TH overexpressing embryos to restore the activity of the neural markers such as N-tubulin. Indeed, this was the case. These results strongly support the function of TH in proliferation.

Further support of the involvement of TH with proliferation comes from the loss-of-function analysis. We found that injection of anti-TH antibodies inhibited cell proliferation in embryos. This effect could be rescued by co-injection of TH protein and also by injection of TH mRNA. Therefore, the anti-TH antibody inhibition appears to be a specific effect of inhibiting TH function. Our results suggest that overexpressed TH inhibits neural differentiation by maintaining cells in a proliferative state.

The observation that TH does effect cell proliferation is an important difference between TH and other gene products such as geminin (Kroll et al., 1998) and Zic-3 (Mariani and Harland, 1998; Bourguignon et al., 1998) that enlarge the neural plate but do so by changing cell fate and not proliferation. However, it is similar to that of XOptx2 which does increase eye size through proliferation, although there is evidence that it may also effect cell fate of midbrain cells to retina (Zuber et al., 1999; Bernier et al., 2000). As pointed out by Zuber et al. (Zuber et al., 1999) this is unusual for embryos that have holoblastic cleavage and may indicate that rapidly dividing cells such as those of the neural plate have greater access to nutrients, thus allowing them to maintain their normal cell size. In addition, a high dose of XBF-1 has recently been shown to promote the proliferation of neuroectodermal cells, while a low dose inhibits ectodermal proliferation (Hardcastle and Papalopulu, 2000). The effect of overexpressed TH on cell proliferation suggests the possibility that it may function in regulation of cell cycle events.

The dramatic shift in TH protein localization from the cell periphery to the nucleus in a defined temporal and spatial pattern suggest that it may modulate a signal from the cell surface to the nucleus. TH is cytoplasmic in cells that are rapidly dividing. This includes cells of the early embryo prior to the MBT, cells undergoing hyperplasia that contain exogenous TH and cells in various regions of the embryo likely to be dividing rapidly. It is nuclear in cells that may be dividing more slowly such as those after the MBT. However, as proliferation picks-up again after the gastrula stage (Hartenstein, 1989), TH protein is predominantly cytoplasmic at the cell periphery. These data support the possibility that TH is cytoplasmic in cells that are dividing while it is nuclear in cells that stop dividing to differentiate. Whether or not TH is the principle cause of the cell cycle slow down and the initiation of differentiation is not clear; however, based on the fact that the overexpressed TH protein remains cytoplasmic in the cells undergoing hyperplasia, it may be a key component in the pathway.

We thank members of the Etkin laboratory for their careful reading of the manuscript and Dr Malgosia Kloc for technical advice. We also thank Ms Junlin Qi for performing the 5′ RACE. We would like to thank C. Kintner, J. Lee, B. Casey, P. Klein, Y. Sasai, P. Krieg, Igor Dawid, Tom Sargent, D. Turner and D. Melton for gifts of reagents used in this study. This work was partially supported by a grant from the March of Dimes (LDE) and by NIH (LDE). We acknowledge

support from core grant (CA 16672) DNA sequencing facility and peptide synthesis facility.

REFERENCES

Bernier, G., Panitz, F., Zhou, X., Hollemann, T., Gruss, P. and Pieler, T. (2000). Expanded retina territory by midbrain transformation upon overexpression of Six6 (Optx2) in Xenopus embryos. *Mech. Dev.* **93**, 59-69.

Bourguignon, C., Li, J. and Papalopulu, N. (1998). XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in Xenopus competent ectoderm. *Development* **125**, 4889-4900.

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Brewster, R., Lee, J. and Ruiz i Altaba, A. (1998). Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. *Nature* **393**, 579-583.

Carl, T. and Klymkowsky, M. W. (1999). Visualizing endogenous and exogenous proteins in *Xenopus laevis*. In *A Comparative Methods Approach to the study of Oocytes and Embryos* (ed. J. D. Richter), pp. 291-315. New York: Oxford University Press.

Chen, Z. F., Paquette, A. J. and Anderson, D. J. (1998). NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. *Nat. Genet.* **20**, 136-142.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-766.

Chitnis, A. and Kintner, C. (1995). Neural induction and neurogenesis in amphibian embryos. *Perspect. Dev. Neurobiol.* **3**, 3-15.

Chitnis, A. and Kintner, C. (1996). Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in Xenopus embryos. *Development* **122**, 2295-2301.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Dreyer, C. and Hausen, P. (1983). Two-dimensional gel analysis of the fate of oocyte nuclear proteins in the development of Xenopus laevis. *Dev. Biol.* **100**, 412-425.

Dumont, J. (1972). Oogenesis in *Xenopus laevis* (Daudin). Stages of oocytes in laboratory maintained animals. *J. Morphol.* **136**, 153-180

El-Hodiri, H. M., Shou, W. and Etkin, L. D. (1997). xnf7 functions in dorsal-ventral patterning of the Xenopus embryo. *Dev. Biol.* **190**, 1-17.

Essex, L. J., Mayor, R. and Sargent, M. G. (1993). Expression of Xenopus snail in mesoderm and prospective neural fold ectoderm. *Dev. Dyn.* **198**, 108-122.

Ferreiro, B., Kintner, C., Zimmerman, K., Anderson, D. and Harris, W. A. (1994). XASH genes promote neurogenesis in Xenopus embryos. *Development* **120**, 3649-3655.

Gong, S. G., Reddy, B. A. and Etkin, L. D. (1995). Two forms of Xenopus nuclear factor 7 have overlapping spatial but different temporal patterns of expression during development. *Mech. Dev.* **52**, 305-318.

Hardcastle, Z. and Papalopulu, N. (2000). Distinct effects of XBF-1 in regulating the cell cycle inhibitor p27(XIC1) and imparting a neural fate. *Development* **127**, 1303-1314.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Harlow, E and Lane, D. (1988). *Antibodies, A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Harris, W. A. and Hartenstein, V. (1991). Neuronal determination without cell division in Xenopus embryos. *Neuron* **6**, 499-515.

Hartenstein, V. (1989). Early neurogenesis in Xenopus: the spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. *Neuron* **3**, 399-411.

Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell*. **77**, 283-295.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). A Xenopus mRNA related to Drosophila twist is expressed in response to induction in the mesoderm and the neural crest. *Cell* **59**, 893-903.

Hsu, D. R., Economides, A. N., Wang, X., Eimon, P. M. and Harland, R. M. (1998). The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Mol. Cell* **1**, 673-683.

Kelly, G. M., Eib, D. W. and Moon, R. T. (1991). Histological preparation of Xenopus laevis oocytes and embryos. *Methods Cell Biol.* **36**, 389-417.

Kintner, C. R. and Melton, D. A. (1987). Expression of Xenopus N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99**, 311-325.

Kloc, M. and Etkin, L. D. (1998). Apparent continuity between the messenger transport organizer and late RNA localization pathways during oogenesis in Xenopus. *Mech. Dev.* **73**, 95-106.

Kloc, M. and Etkin, L. D. (1999). Analysis of Localized RNAs in *Xenopus* oocytes. In *A Comparative Methods Approach to the Study of Oocytes and Embryos* (ed. J. D. Richter), pp. 256-278. New York: Oxford University Press.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osdada, S., Wright, C., Wylie, C. and Heasman, J. (1999). Mesoderm induction in *Xenopus* is a zygotic event regulated by maternal VegT via TGFβ growth factors. *Development* 126, 5759-5770.

Kroll, K. L., Salic, A. N., Evans, L. M. and Kirschner, M. W. (1998). Geminin, a neuralizing molecule that demarcates the future neural plate at the onset of gastrulation. *Development* **125**, 3247-3258.

Kuang, J., Zhao, J., Wright, D. A., Saunders, G. F. and Rao, P. N. (1989). Mitosis-specific monoclonal antibody MPM-2 inhibits Xenopus oocyte maturation and depletes maturation-promoting activity. *Proc. Natl. Acad. Sci. USA* **86**, 4982-4986.

Kuo, J. S., Patel, M., Gamse, J., Merzdorf, C., Liu, X., Apekin, V. and Sive, H. (1998). Opl: a zinc finger protein that regulates neural determination and patterning in Xenopus. *Development* **125**, 2867-2882.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N. and Weintraub, H. (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* **268**, 836-844.

Lo, L., Sommer, L. and Anderson, D. J. (1997). MASH1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells. *Curr. Biol.* **7**, 440-450.

Ma, Q., Kintner, C. and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87**, 43-52.

Ma, Q., Sommer, L., Cserjesi, P. and Anderson, D. J. (1997). Mash1 and neurogenin1 expression patterns define complementary domains of neuroepithelium in the developing CNS and are correlated with regions expressing notch ligands. *J. Neurosci.* **17**, 3644-3652.

Mariani, F. V. and Harland, R. M. (1998). XBF-2 is a transcriptional repressor that converts ectoderm into neural tissue. *Development* **125**, 5019-5031.

Mayor, R., Morgan, R. and Sargent, M. G. (1995). Induction of the prospective neural crest of Xenopus. *Development* **121**, 767-777.

Miller, M., Reddy, B. A., Kloc, M., Li, X. X., Dreyer, C. and Etkin, L.D. (1991). The nuclear-cytoplasmic distribution of the Xenopus nuclear factor, xnf7, coincides with its state of phosphorylation during early development. *Development* **113**, 569-575.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S. and Sasai, Y. (1998). Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* **125**, 579-587.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* **86**, 391-399.

Nakata, K., Nagai, T., Aruga, J. and Mikoshiba, K. (1997). Xenopus Zic3, a primary regulator both in neural and neural crest development. *Proc. Natl. Acad. Sci. USA* **94**, 11980-11985.

Piccolo, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-598.

Reddy, B. A., Kloc, M. and Etkin, L. (1991). The cloning and characterization of a maternally expressed novel zinc finger nuclear phosphoprotein (xnf7) in Xenopus laevis. *Dev. Biol.* **148**, 107-116.

Richter, K., Grunz, H. and Dawid, I. B. (1988). Gene expression in the embryonic nervous system of Xenopus laevis. *Proc. Natl. Acad. Sci. USA* **85**, 8086-8090.

Sasai, Y. and De Robertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. *Dev. Biol.* **182**, 5-20.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.

Sasai, Y., Lu, B., Steinbeisser, H. and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. *Nature* **377**, 757.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-840.

Smith, W. C., McKendry, R., Ribisi, S., Jr and Harland, R. M. (1995). A nodal-related gene defines a physical and functional domain within the Spemann organizer. *Cell* **82**, 37-46.

Sommer, L., Ma, Q. and Anderson, D. J. (1996). neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol. Cell. Neurosci.* **8**, 221-241.

Takebayashi, K., Takahashi, S., Yokota, C., Tsuda, H., Nakanishi, S., Asashima, M. and Kageyama, R. (1997). Conversion of ectoderm into a neural fate by ATH-3, a vertebrate basic helix-loop-helix gene homologous to Drosophila proneural gene atonal. *EMBO J.* **16**, 384-395.

von Dassow, G., Schmidt, J. E. and Kimelman, D. (1993). Induction of the Xenopus organizer: expression and regulation of Xnot, a novel FGF and activin-regulated homeo box gene. *Genes Dev.* **7**, 355-366.

Wilson, P. A. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376**, 331-333.

Zhang, J., Houston, D. W., King, M. L., Payne, C., Wylie, C. and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in Xenopus embryos. *Cell* **94**, 515-524.

Zimmerman, L. B., De Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.

Zuber, M. E., Perron, M., Philpott, A., Bang, A. and Harris, W. A. (1999). Giant eyes in Xenopus laevis by overexpression of XOptx2. *Cell* **98**, 341-352.