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Tumorhead distribution to cytoplasmic membrane of neural plate cells is positively regulated by *Xenopus* p21-activated kinase 1 (X-PAK1)

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+ : The authors wish to dedicate their work to the memory of Dr. Laurence D. Etkin.

Abstract:

Tumorhead (TH) regulates neural plate cell proliferation during Xenopus early development, and gain or loss of function prevents neural differentiation. TH shuttles between the nuclear and cytoplasmic/cortical cell compartments in embryonic cells. In this study, we show that subcellular distribution of TH is important for its functions. Targeting TH to the cell cortex/membrane potentiates a TH gain of function phenotype and results in neural plate expansion and inhibition of neuronal differentiation. We have found that TH subcellular localization is regulated, and that its shuttling between the nucleus and the cell cortex/cytoplasm is controlled by the catalytic activity of p21-activated kinase, X-PAK1. The phenotypes of embryos that lack, or have excess, X-PAK1 activity mimic the phenotypes induced by loss or gain of TH functions, respectively. We provide evidence that X-PAK1 is an upstream regulator of TH and discuss potential functions of TH at the cell cortex/cytoplasmic membrane and in the nucleus.

Keywords : Tumorhead, p21-activated kinase, neural plate, proliferation, differentiation, actin cytoskeleton.

1. Introduction

Development of the vertebrate nervous system involves morphogenetic cell movements, controlled cell proliferation, cell cycle withdrawal and neuronal differentiation. Neural tube formation is the result of the elongation of the neural plate along the body axis, followed by neural fold elevation and neural fold closure at the dorsal midline (Poznanski et al., 1997; Wallingford et al, 2002). Deregulation of these events can lead to neural tube defects with devastating effects on nervous system function (Smith and Schoenwolf, 1997; Davidson and Keller, 1999; Colas and Schoenwolf, 2001).

In Xenopus, the elongation of the neural plate requires convergent extension (CE) movements, that are driven by mediolateral intercalation of the midline mesoderm and neural plate cells. These movements are mainly under the control of non-canonical Wnt signaling pathways that resembles the planar cell polarity (PCP) pathway, first uncovered in Drosophila. The multifunctional protein Dsh is central to both canonical and non-canonical Wnt pathways (Mlodzik, 2002; Veeman et al, 2003; Wallingford and Habas, 2005) and its overexpression results in neural tube closure defects (Wallingford et al, 2002). In the same pathway, the Xenopus ortholog of Drosophila PCP gene strabismus (stbm) tightly regulates CE movements in posterior neural tissue (Darken et al, 2002; Goto and Keller, 2002).

While the pathways that regulate CE (convergent extension) movements have been extensively studied, the mechanisms that regulate neural fold elevation and neural tube closure are only now beginning to be unveiled. For example, the actin binding protein Shroom is essential for neural tube morphogenesis (Haigo et al, 2003) and has been proposed to regulate cellular and tissue morphology in embryos (Hildebrand, 2005). In Xenopus, Shroom induces the apical constriction of neural plate cells by controlling the cellular distribution of a contractile actomyosin network (Hildebrand, 2005) through the Rap1 GTPase (Haigo et al., 2003).

During neurulation, Xenopus neural ectoderm cells that are active in proliferation undergo a stepwise activation of different sets of genes that will eventually lead to exit of cell cycle and to neuronal differentiation. These steps are usually refered to as competence, specification, commitment and differentiation. Specification distinguishes the future neural plate cells from non neural ectoderm. The expression of the Sox 1-3 subfamily of HMG box transcription factors marks these neural progenitors and controls their proliferation (Bylund et al, 2003; Graham et al, 2003). During commitment, the proneural gene X-Ngnr-1 (neurogenin), a basic helix-loop-helix (bHLH) transcription factor, is expressed in a subset of cells distributing between the three stripes of primary neurons on each side of the posterior neural plate, and commits neuronal precursor cells (Ma et al., 1996). X-Ngnr-1 drives the expression of a number of genes, such as p27 Xic1 which promotes cell cycle exit (Vernon et al, 2003) and the bHLH transcription factor NeuroD, that initiates the differentiation program (Lee et al, 1995; Bertrand et al, 2002). X-Ngnr-1 also activates Notch signaling, mediating lateral inhibition to prevent neighbouring cells from developing into neurons (Chitnis et al, 1995; Bellefroid et al, 1996, Wettstein et al, 1997).

Recently, the understanding of the mechanisms that coordinate cell cycle withdrawal and the beginning of differentiation during neural development took a big step forward with the identification of Brg-1 as an interacting protein of Neurogenin and NeuroD. Brg-1 is the ATPase catalytic subunit of the SWI/SNF chromatin remodeling complex and is required for neuronal differentiation. Brg-1 inhibition results in an increase of the proliferating neural plate cells. Indeed, Brg-1 mediates the transcriptional activity of Neurogenin and NeuroD (Seo et al, 2005a). Moreover Geminin (Gem), another regulator of cell differentiation (Kroll et al, 1998; Luo et al, 2004) was shown to directly interact with Brg-1, preventing its interaction with BHLH transcription factors X-Ngnr-1 and NeuroD, impeding the transactivation of their target genes. It has been proposed that Gem, which is downregulated before neuronal differentiation, fine tunes the timing of cell cycle exit and differentiation (Seo et al, 2005b).

Neural tube morphogenesis in Xenopus embryos is also controlled by the maternal coiled-coil protein Tumorhead (TH). We previously reported that TH regulates proliferation of neural plate cell precursors and that impeding its functions by overexpression or loss of function prevents neuronal differentiation (Wu et al, 2001 ; 2003). We observed that the elongated shape of neural tube cells was lost in embryos lacking TH, indicating that TH might control cytoskeleton rearrangements. This hypothesis is strengthened by the observation that expression of TH in *S. pombe* cells induces severe morphological defects (Wu et al, 2004) that are characterized by ovoid, enlarged and sometimes mutiseptated cells. These cells appear to

have partially lost their ability to polarize cortical F-actin, and have disorganized microtubules. In this study, TH distribution to the fission yeast cell cortex was dependent on Shk1 kinase activity (Wu et al., 2004).

Shk1 is the yeast homolog of p21-activated kinase 1 (PAK1), a member of the group I PAK serine/threonine kinases that regulate a broad range of cell signaling networks (for review see, Hofman et al, 2004; Zhao and Manser, 2005; Kumar et al, 2006). PAKs are important regulators of the actin and microtubule cytoskeleton (Sells et al, 1997; Banerjee et al, 2002; Wittmann et al, 2004) and regulate cell motility (Sells et al, 1999). They are involved in cell cycle control, transcriptional regulation and cell proliferation. Recently, PAK functions in cell transformation and cancer cell invasiveness have also been found (For review, see Kumar et al, 2006). PAK1 and PAK3 are highly expressed in the brain and spinal cord (Burbelo et al, 1999). PAK1 regulates neurite outgrowth (Nikolic et al., 1998; Hayashi et al., 2002; Bryan et al., 2004) and netrin induced growth cone expansion in the CNS (Shekarabi et al, 2005). In Xenopus, we previously reported that X-PAK1 regulates the G2/M cell cyle transition and prevents oocytes from entering the apoptotic program (Faure et al., 1997; Faure et al., 1999). X-PAK1 is also involved in myosin II dependent cell fragmentation (Poitras et al., 2003; Bisson., 2003).

Since, our TH studies in *S. pombe* suggested a functional link between TH and PAK1, we decided to investigate whether TH and X-PAK1 pathways are functionally related during early neural development in Xenopus embryos. In the present work, we show that the subcellular distribution of TH, in neural plate cells, is dynamic and that targeting TH to the cell cortex enhanced its functions. We provide strong evidence that X-PAK1 regulates TH subcellular localization. Indeed, the expression of X-PAK1 mutants largely mimics TH-induced defects in neural development, and prevents neuronal differentiation of neural plate cells. Finally, we show that expression of a TH-CAAX mutant rescues the neural fold elevation inhibited by the loss of X-PAK1 activity. Altogether, these results lead us to propose that X-PAK1 and TH belong to the same functional pathway.

2. Materials and Methods

2.1. Expression plasmids

The full-length X-PAK1 cDNA (Faure et al, 1997) was subcloned in pEGFP *Eco*R1 site and pCS2 plasmids (D. Turner and R. Rupp, Fred Hutchinson Cancer Center, Seattle, WA) (Promega). X-PAK1/KR dead and X-PAK1/DE constitutive kinase mutants were constructed by site-directed mutagenesis (Faure et al., 1997). The following changes were made: K to R at residue 279 located in the putative ATP binding site for the kinase dead mutant and S to D and S to E at residues 402 and 403 for the DE constitutive mutant, respectively. All constructs were verified by sequencing. pEgfp-Cdc42V12, used for cell transfection, was previously described (Cau et al, 2001).

To generate pCS2-THGFP, an *Eco*R1 site was introduced into pCS2-TH plasmid (Wu et al., 2001) after the last codon of TH, to generate pCS2-TH-R1 plasmid by polymerase chain reaction (PCR). a *Hind*3-*Eco*R1 fragment of pCS2-TH-R1 was then inserted into pCS2*mt-SGP (provided by Dr. Micheal Klymkowsky, The University of Colorado at Boulder, Boulder, CO). pCS2-1MT-TH was generated from pCS2+MT-TH (Wu et al., 2001), and pCS2-1MT-TH-CAAX was generated from pCS2+MT-TH-CAAX by cutting off the fragment encoding five copies of the c-myc epitope tag using the *Cla*1 and *Nco*1 restriction enzymes; the blunted end of the *Cla*1 site was then religated with the blunted end of the *Nco*1 site. To create pCS2+MT-TH-CAAX, the TH open reading frame (ORF) followed by a sequence encoding a CAAX box was amplified using PCR with primers and linkers for ligation into pCS2+MT. The sense oligo, GTCA**GAATTC**AATGGTCGGTATTCTACCTG, encoded the first five codons of TH preceded by an *Eco*RI site, whereas the antisense oligo, AGTCA**CTCGAG**TTACATGATTACGCACTTTGCTAGTTTTTTTTTCAC, annealed to the 3' end of the TH ORF and inserted a sequence encoding the CVIM amino acid sequence (CAAX box), a stop codon, and an *Xho*I site after the last codon. The amplified PCR product was then ligated into the *Eco*RI/*Xho*I sites of pCS2+MT.

2.2. Antibodies, Western blots, and kinase activities

Monoclonal antibodies to Myc (9E10, OP10L, Calbiochem, EMD Biosciences), γ-tubulin (GTU 488) and vinculin (HVIN1) are from SIGMA, polyclonal antibodies to gfp are from Torrey Pines Biolabs Inc.

Antibodies against the N-terminal or the C-terminal amino acid domain of X-PAK1 were made and immunopurified in the lab (Faure et al., 1997). For Western blots, 100 μg of cell extracts was loaded on SDS-PAGE. For measuring kinase activity, endogenous and mutant X-PAK1 were immunoprecipitated from 5 x 10^6 cells using X-PAK1 antibodies and kinase activity measured as described in Cau et al. (2000) using MBP as a substrate.

2.3. In vitro transcription

All plasmids for mRNA injection were linearized using *Not*1 restriction enzyme for in vitro transcription templates. Capped synthetic mRNAs were generated using SP6 polymerase and the mMESSAGE mMACHINE kit (Cat.# 1340, Ambion, Austin, TX) according to the manufacturer's instructions.

2.4. Embryo manipulation and injection

To obtain *Xenopus* embryos, eggs were collected from human chorionic gonadotropin-induced female frogs and fertilized in vitro. Embryos were dejellied 30 min after fertilization in 2% cysteine and injected in modified Barth's solution. Embryos were staged as described by Nieukoop and Faber (1967). To target one side of the future neural field, mRNA was injected into one of the two dorsal/animal blastomeres at the 8-cell stage (Dale and Slack, 1987). The amount of mRNA injected were: THGFP, 100pg; X-PAK1/DE, 150pg; X-PAK1/KR, 1ng; 1MT-TH, 1ng; 1MT-TH-CAAX, 750pg. To compare the loss of TH with the loss of X-PAK1, 20ng of TH morpholino antisense oligonucleotide (TH-MO, Wu et al., 2003) was injected in some experiments. To identify the injected cells of the embryo, fluorescein-conjugated dextran (D-1820; Molecular Probes, Eugene, OR) was injected together with the RNA at 0.2 mg/ml.

2.5. Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as described previously (Kloc and Etkin, 1994; Harland, 1991). Digoxigenin-labeled Sox2, Xslug, and neural-specific type β-tubulin (N-tubulin) antisense RNA probes were generated from pBluescript (ks)-SOX2 (Wu et al., 2001), pMX363Xslug (Mayor et al., 1995) and p24-10 (Richter et al., 1988) plasmids.

2.6. Whole-mount immunocytochemistry

To detect the endogenous X-PAK1 protein distribution in the neural field at the neurula stages, albino *Xenopus* embryos at different stages were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at room temperature. The vitelline envelope was removed manually after fixation. Embryos were then treated with 0.5% Triton X-100/PBS for 5 min and washed three times with PBS. Next, embryos were incubated at 67^{o}C for 1 h to inactivate endogenous phosphatases and washed 5 times with PBS. Following the washing, embryos were blocked with 20% heat-inactivated goat-serum-3% bovine serum albumin-Tris buffered saline (TBS) for 2 h. Antibody incubations were carried out in blocking solution overnight at 4°C. Following each antibody incubation, embryos were washed for 2 h in 0.1% Tween 20-TBS with six changes. The anti-X-PAK1 primary antibody (Faure et al, 1997) was affinity-purified and used at a dilution of 1:200. Alkaline-phosphatase-conjugated goat anti-rabbit IgG Fc fragment (Cat.# 401371, Calbiochem, EMD Biosciences, La Jolla, CA) secondary antibody was used at 1:4000 dilution. Rabbit IgGs (1-5006; Sigma) were used as a control of primary antibody at 1 μg/ml.

2.7. Immunohistochemistry and immunofluorescence analyses

Embryos were fixed with 4% paraformaldehyde in PBS for 2 h at room temperature. After fixation, the neural plates were dissected from the embryos, treated with 0.5% Triton X-100/PBS for 5 min, and processed for immunohistochemistry. Anti-c-myc (Ab-1) mouse monoclonal antibody (9E10) (OP10L, Calbiochem, EMD Biosciences) was used at 1 μg/ml, anti phospho histone H3 (Upstate Biotechnology) was used at 3 μg/ml. Alexa Fluor 555-conjugated goat anti-mouse IgG (A-21422, Molecular probes) secondary antibody was used at 1:2000 dilution. To visualize the cell boundaries, F-actin was stained with

rhodamine-conjugated phalloidin. A Zeiss LSM510 confocal microscope was used to analyze the stained samples.

3. Results

3.1. Targeting TH to the cytoplasmic membrane in neural plate cells enhances its function.

We previously observed that TH subcellular localization may be temporally regulated in developing embryos between the cell periphery and the nucleus. Moreover, the distribution of TH at the cell periphery/cytoplasmic membrane may be correlated with cells which are proliferating (Wu et al, 2001 ; Wu et al, 2003 and our unpublished data).

These observations led us to study whether TH activity might be dependent upon its distribution. To investigate this question, we compared the phenotypes induced during neural development by a membrane-targeted TH versus wild type TH. A CAAX-tailed TH (TH-CAAX) mutant was engineered by fusing a membrane localization signal, Cys-Val-Ile-Met (CAAX) to the C-terminus of wild type TH (Fig. 1A). CAAX sequence has been extensively used in the past to study functions of membrane-anchored proteins through farnesylation of the cysteine in the CAAX box (for review, see Casey, 1992; Clarke, 1992). 8-cell stage embryos were microinjected either with N-terminal Myc tagged wild type TH (TH, 1ng) or N-terminal Myc tagged CAAX- TH (TH-CAAX, 750pg) mRNAs along with lineage tracer fluorescein-conjugated dextran into one side of the future neural field. Under these conditions, TH-CAAX was expressed at a lower level in embryos, as assessed by western blot analyses (Fig1.A). The induced phenotypes were scored at gastrula and neurula stages (Fig1. B and C) according to the following criteria: normal development, gastrulation defects, mild and severe neural phenotypes (as illustrated above the graph, Fig.1C).

Both TH-CAAX and TH expressing stage 18-19 embryos exhibit abnormal development of the nervous system compared to controls (Fig. 1B). However, TH-CAAX expressing embryos display a lower rate of normal development (14% vs. 30%), a higher rate of gastrulation defects (43% vs. 10%) and a higher rate of severe neural phenotypes including neural expansion, abnormal pigmentation, and failure of proper neural tube closure (62% vs. 28%) than TH expressing embryos (Fig.1C). Thus, although expressed at a lower level, TH-CAAX induced more severe neural development phenotypes than TH**.**

Using confocal microscope analyses of dissected neural plates, we next verified that the TH-CAAX induced phenotype correlates with a different distribution of the protein, in neural plate cells (Fig.1D). As we have previously shown (Wu et al, 2001), TH protein distributes between the cytoplasm and the cytoplasmic membrane and the nucleus of neural plate cells (Fig.1D and Fig.3). In contrast, the TH-CAAX mutant concentrates mostly at the cytoplasmic membrane, as almost no nuclear staining is observed (Fig. 1D). Thus, our data indicate that targeting TH to the cytoplasmic membrane and/or preventing its entry into the nucleus induces a more severe neural phenotype than expressing TH which can shuttle between cell compartments. This suggests that TH-CAAX acts as a constitutively active form of TH, and highlights the functional importance of the regulation of TH shuttling between the nuclear and cytoplasmic/membrane compartments.

3.2. Endogenous X-PAK1 is expressed in the neural field at neurula stages.

Regulation of TH targeting to specific subcellular compartment is required to control neural development of Xenopus embryos. Our previous studies demonstrated that expressed TH in *S. pombe* cells distributes to the cell cortex and septum and that its cortical localization is under the control of Shk1, the *S. pombe* p21 activated kinase homolog (PAK) (Wu et al, 2004). In order to study a potential relationship between TH and X-PAK1 signaling pathways during neurulation in Xenopus, we first wanted to ensure that the endogenous PAK1 kinase and TH are expressed in the same territories during neural development. X-PAK1 temporal expression during early development (Faure et al, 1997, Islam et al, 2000) and late spatial distribution in the ear and lateral line, were previously reported (Islam et al, 2000). However its spatial distribution during early development is still lacking. We examined, X-PAK1 distribution from gastrula to tailbud stages using whole-mount immunostaining with affinity purified X-PAK1 antibody (Faure et al., 1997). At gastrula stages, X-PAK1 is essentially ubiquitous (Fig. 2A, stage 11+ animal/vegetal). During

neurulation, X-PAK1 is expressed throughout the epidermal and neural ectoderm, with some enrichment in the neural field (Fig. 2A, b and c, stage 16). At tailbud stages, in agreement with a previous report (Islam et al, 2000), we observed a decrease in the expression levels of X-PAK1 in the epidermis of the body trunk (Fig. 2A, b and c, stage 24) and an increased expression in the eye vesicle (ev) and branchial arches (ba). Thus, expression of X-PAK1 kinase in the neural field is compatible with a potential regulatory function of endogenous TH in neural plate cells.

3.3. Catalytic activity of X-PAK1 regulates TH distribution in neural plate cells.

Next, we studied whether expression of X-PAK1 kinase mutants interferes with TH distribution in neural plate cells. Using sequence homology between Xenopus and human PAK1 kinases, we engineered X-PAK1/DE (Ser/Asp402-Thr/Glu403), a mutant on the autophosphorylation site, in the catalytic loop of the kinase and X-PAK1K/R (Lys/Arg279), a mutant in its ATP binding site. These mutations were previously shown to produce constitutively active and dominant negative hPAK1, respectively (Tang et al., 1998). We first tested the catalytic activity of the wild type (X-PAK1wt) and PAK1 mutant kinases, 24 hours after transfection into Xenopus XL-2 cells (Fig. 2B). Immunoblotting of the total cell extracts for GFP-X-PAK1 expression (upper panel) and for gamma-tubulin as a loading control (middle panel), shows that the X-PAK1/KR mutant was better expressed than X-PAK1/DE and X-PAK1wt. X-PAK1 mutant kinases were immunoprecipitated together with the endogenous X-PAK1 from cell lysates and were tested for kinase activity using MBP as a substrate. In the absence of any cell stimulation, only a basal kinase activity was detected in X-PAK1/KR and X-PAK1wt expressing cells. In contrast, although X-PAK1/DE was expressed at lower levels, it is a constitutively active kinase, as seen by at least a 20 fold increase of MBP phosphorylation by this mutant (Fig. 2B, bottom panel).

We then tested whether X-PAK1/KR acts as a dominant negative mutant, as was previously reported for its human counterpart (Tang et al., 1997). To do so, we measured the activation of endogenous X-PAK1 kinase following expression of an active form of Cdc42 GTPase (Cdc42V12 mutant), with or without simultaneous expression of the X-PAK1/KR mutant (Fig. 2C). Total cell lysates were tested for loading accuracy using vinculin antibodies (bottom panel) and for gfp-XPAK1/KR and gfp-Cdc42V12 expression using gfp antibodies (middle panel). X-PAK1 activity immunoprecipitated from total cell lysates was tested against MBP as a substrate (top panel). In the absence of cell stimulation, no endogenous X-PAK1 kinase activity is detected. Similarly, in cells expressing X-PAK1/KR mutant, as seen in panel B, no MBP kinase activity can be measured. In contrast, and as expected, expression of Cdc42V12 strongly activates endogenous X-PAK1 kinase activity. Coexpression of gfp-XPAK1/KR downregulates Cdc42V12 induced endogenous PAK1 activation. Thus, X-PAK1/KR mutant acts as a dominant negative mutant for endogenous X-PAK1 activation.

We next investigated whether gain or loss of X-PAK1 catalytic activity, using X-PAK1/DE and X-PAK1/KR mutants, could affect TH cellular distribution in neural plate cells. In this set of experiments, we used a GFP-tagged TH (THGFP) construct as a marker for microscopy. Very low doses of THGFP mRNA (100 pg), that do not induce a deleterious phenotype, (Wu et al., 2001), were either injected alone, or with X-PAK1/DE (150pg) or X-PAK1/KR (1ng) mRNAs in one of the two dorsal/animal blastomeres at the 8-cell stage to target one side of the future neural field (Dale and Slack, 1987). The distribution of THGFP in cells of the dissected neural plates was observed by confocal microscopy (Fig.3). Confocal images are shown as a reconstituted stack (A,A',A'') and at five positions (B-F,B'-F',B ''-F '') along the apical/basal axis of the neural plate epithelial cells. Along this axis, THGFP is associated to the apical cell membrane, to the cytoplasm and to some extent to the lateral membrane (Fig.3 C-D) and is concentrated in the nuclei located in the basal half of the cell (Fig.3 E-F).

In X-PAK1/KR expressing cells, THGFP could no longer be detected at the apical cell surface nor at the lateral cell membrane (Fig.3 B'-F'). Instead, all the TH protein accumulated in the cytoplasm and cell nuclei. In contrast, cytoplasmic TH, as well as cortical and lateral cell membrane bound TH, appeared reproducibly enriched, and extended further from the apical surface, in X-PAK1/DE expressing cells (see arrowheads in Fig.3 B''-D''). Western blot analyses revealed that the overall TH protein level was not affected by the expression of X-PAK1 mutants (data not shown).

In summary, our results indicate that expression of the catalytic activity of X-PAK1 enhances THGFP binding to the apical cortex and lateral cell membrane of neural plate epithelial cells, (or impedes nuclear transport/retention) mimicking the subcellular localization we observed for the TH-CAAX mutant (Fig.1D). In contrast, kinase-dead X-PAK1/KR mutant impedes the membrane association of THGFP (Compare

Fig.3 panel C' with C and C'' and panel D' with D and D'') and increases its nuclear localization. Taken together, our results first strengthen our previous observations that TH distribution between the cytoplasm, the cell cortex and the nucleus is regulated (Wu et al, 2001) and secondly identify X-PAK1 as a regulator of TH shuttling in the neural plate epithelial cells.

3.4. X-PAK1 and TH function in epithelial neural plate cells proliferation

Since TH induces neural defects characterized by an expansion of the neural plate and the inhibition of expression of neuronal differentiation markers (Wu et al, 2001), we wondered whether X-PAK1 might also regulate neural development. To address this question, we studied whether expression of X-PAK1 mutants in embryos interferes with neural plate evolution and the expression of neuronal markers.

8-cell stage embryos were microinjected with 150pg of X-PAK1/DE, 1ng of X-PAK1/KR, 700pg of TCAAX mRNAs as described above and the neural phenotypes were scored at neurula stages. As shown in Fig. 4A, the neural plate region is enlarged in the X-PAK1/DE expressing embryos, and this phenomenon is accompanied by an abnormal pigmentation of the neural field area compared to control embryos (compare control with X-PAK1/DE stage 15). The enlarged neural plate and the completely formed neural fold are best seen at stages 16-17 and 19-20 (broken lines help to visualize the neural fold). This phenotype is similar to that induced by TH overexpression (Wu et al., 2001) and TH-CAAX expression (Fig.1 and data not shown). In contrast, in kinase-dead X-PAK1/KR expressing embryos, the neural field on the injected side remained flat, indicating that the neural fold did not elevate under these conditions (Fig. 4A, compare control and X-PAK1/KR stage 16-17 and 19-20). This phenotype is similar to the phenotype observed following the loss of TH following injection of TH morpholino antisense oligonucleotides (TH-MO) (Wu et al., 2003).

To better visualize neural fold elevation, the neural plates of X-PAK1/DE, PAK1/KR , TH-CAAX and TH-MO injected embryos were dissected and examined by confocal microscopy (Fig. 4B). The cells that are packed in the contraction region of the neural plate are best seen using F-actin staining. In the control side of the neural plate, the contracting cells form an F-actin-enriched striped-structure corresponding to the cells packing in this region (Fig. 4B, all control panels are on the right side) . The same structure is seen on the X-PAK1/DE injected side (stage 16-17), confirming the morphological analyses of neurula embryos (Fig. 4A). However, several differences exist between X-PAK1/DE expressing cells and the control cells . First, on the X-PAK1/DE injected side, the contraction area is wider, indicating that contracting cells spread more. Second, in this area the F-actin staining appears less intense, indicating that fewer contracting cells are present. Indeed, observation of F-actin on transversal sections (ZX slice positions, on top of panels) shows that cell compaction/ contraction does not occur properly on the X-PAK1/DE injected side (compare the actin enrichment on the control side with the less intense and broader staining on the X-PAK1/DE side). This is confirmed by comparing ZY sections of the anterior posterior slices of control and X-PAK1/DE expressing cells.

The same phenotype, characterized by a somewhat disorganized actin rich striped-structure, is observed in the dissected neural plates of TH-CAAX expressing embryos (stage 16-17) (Fig.4B). In contrast, in X-PAK1/KR (stage 15/16) and TH-MO (stage 16-17) expressing neural plate cells, no cortical F-Actin enrichment is observed, as best seen in the transversal sections on top and right panels. These results confirm our observations that the cells do not pack nor fold and that the neural plate remains flat when either endogenous X-PAK1 activity or TH expression are inhibited. Thus, both X-PAK1 and TH expression regulate neural plate cell morphology changes during neural fold elevation.

We previously reported that overexpression of TH caused an expansion of Sox2 expression (a marker of proliferating neural progenitors, Bylund et al., 2003; Graham et al., 2003; and Seo et al., 2005a) that was correlated with the neural field expansion observed (Wu et al., 2001). Similarly, loss of TH, by TH-MO injection, caused a reduction of Sox2 expression territory (Wu and Etkin, unpublished data).

To further compare the effect of X-PAK1 and TH expression on neural plate cells proliferation, we analyzed Sox2 expression in neural plate cells expressing TH-CAAX or X-PAK/DE and TH-MO or X-PAK1/KR. The different mRNAs and TH-MO were injected into one side of the future neural field of the embryo at the 8-cell stage (Fig. 5A, arrows, left sides). Sox2 expression was analyzed by *in situ* hybridization at neurula stages 20-22. In control embryos, Sox2 expression and intensity was similar on either side of the neural field (Fig. 5A). In embryos expressing X-PAK1/KR and TH-MO, the intensity of Sox2 expression strongly decreased on the injected side, compared to the non injected side and, as seen before, the neural fold did not elevate in these embryos (Fig. 4A, 5A). In contrast, X-PAK1/DE and TH-

CAAX expression resulted in a phenotype characterized by an expanded neural plate on the injected that coincided with a broader Sox2 expression territory (Fig. 5A).
To study whether neural field expansion or flat neural plate phenotypes reflects a change in the number of cells, we counted cells in equivalent areas of injected (right side of the panels) and control (left side of the panels) dissected neural plate epithelia. X-PAK1/KR mRNAs and TH-MO were injected as described above. Neural plates were dissected between stage 15 and 16. Cell size and number were analyzed by staining the F-actin network with rhodamine-conjugated phalloidin, and the injected side of the embryo was visualized by a fluorescein-dextran tracer. In the representative experiment shown in Fig.5B, the average size of epithelial neural plate cells is bigger in the region injected with TH-MO compared to the corresponding area on the control side (TH-MO, Fig.5B). Consequently, cell counting of the same surface of control and injected cells gave a ratio of 1.5 (control side, 60 cells/TH-MO-injected side, 40 cells) further indicating that loss of TH decreases the ability of neural plate cells to proliferate. Interestingly, expression of X-PAK1/KR protein induced an effect similar to TH-MO. Indeed, neural plate cells expressing X-PAK1/KR appear larger than cells on the control side (X-PAK1/KR, Fig. 5B) and cell number counting gave a 1.41 ratio (control side, 83 cells/XPAK1/KR-injected side, 59 cells), indicating that X-PAK1/KR expression impedes neural plate cell proliferation. The ratios that we obtained in the different experiments (from 6 and 5 embryos respectively), ranged between 1. 3 and 1.5. Thus, loss of TH and expression X-PAK1/KR downregulate cell proliferation at a similar rate. In support of this conclusion, injection of lineage tracer alone neither affected cell size nor number since cell counting of the same area in control and dextran-injected regions gave a ratio of 1.03 (Fig. 5B, bottom panel).
To more directly analyze and compare the effects of X-PAK1 and TH on cell proliferation, dissected neural plates from X-PAK1/KR, X-PAK1/DE and TH-CAAX injected embryos were immunostained with the cell proliferation marker phospho histone H3 (PH3). A representative experiment is shown in figure 5C. Both in TH-CAAX and X-PAK1/DE injected side of the neural plate, PH3 positive cells were detected at a higher density relative to the control non injected side, visualized by the absence of fluorescent dextran tracer. In contrast, the X-PAK1/KR injected side of the neural plate was characterized by a lower density of PH3 positive cells relative to the control non injected side. The counting of PH3 positive cells is not an absolute marker of the proliferation state, since lengthening/shortening of mitosis, as well as delays in entry or exit of mitosis, will have an impact on positive PH3 cells. Currently, we do not know whether the number of PH3 positive cells may be biased by such mechanism. However, taken together with Sox2 in situ hybridization (Fig.5A) and ratios of number of cells per area (Fig. 5B), our PH3 staining results show that X-PAK1/KR and TH-MO interfere with neural fold elevation at least in part by downregulating proliferation of neural cell progenitors. In contrast, X-PAK1/DE and TH-CAAX expression, which allow abnormal neural fold elevation, result in upregulation of neural cell progenitors. This phenotype is characterized by an expansion of sox2 positive territory and an increased number of phosphohistone H3 positive cells.
Nevertheless, alternate mechanisms than cell proliferation could also be involved in the regulation by PAK and TH of neural plate cell morphology. PAKs were shown in many models to regulate, through numerous substrates, cytoskeleton rearrangements and cell morphology changes. Such rearrangements are mandatory for neural fold elevation. Moreover, morphological changes of neural plate cells in the fold region also involve modifications of their adhesion properties. We previously reported the regulation of adhesion during gastrulation convergent extension movements by subgroup 2 PAKs (Faure et al, 2005). In this report, we did not investigate further whether such regulation of these pathways may be relevant. This would indeed be very interesting to test. Thus, we cannot rule out, at this point, that PAK and/or TH might also regulate some aspects of the cytoskeleton rearrangements that occur during neural fold elevation.

3.5. X-PAK1 catalytic activity regulates neural differentiation

Both loss and overexpression of TH in Xenopus embryos prevent neuronal differentiation (Wu et al., 2001; Wu et al., 2003). To further compare X-PAK1 and TH pathways during neural development, we studied whether X-PAK1 mutants would interfere with the expression of the neural crest marker Xslug (Mayor, et al.,1995), and the differentiated neuron marker N-tubulin (Richter et al, 1988). We analyzed Xslug expression at stage 22 (Fig. 6A) and N-tubulin expression at stages 22-24 (Fig. 6B) by whole-mount *in situ* hybridization. In control embryos, Slug mRNAs, whose expression starts at stage 12/13, localizes laterally to the neural plate. In stage 22 control embryos, the neural crest cells start to migrate and X-Slug

expression correlates with the distribution in three broad streams of neural crest cells on each side of the midline (Fig. 6A). In contrast, in TH-MO and X-PAK1/KR and also in X-PAK1/DE and TH-CAAX injected sides (left sides, arrows), Slug expression is decreased, but not completely inhibited, compared to the non injected sides (right sides, arrowheads). Insets show magnification of Slug staining in TH-CAAX and X-PAK1/DE expressing embryos.

In control embryos, N-tubulin mRNA is distributed symmetrically on both sides of the dorsal midline, and as a patch at the posterior region of the eye vesicles (Fig. 6B). Neuronal tubulin expression is impeded by TH overexpression (Wu et al, 2001) and, as expected, we confirm here that TH-CAAX also prevents the transcription of the N-tubulin gene (Fig. 6B). Moreover, when X-PAK1/DE RNA is injected on the whole side of the neural field, N-tubulin mRNA expression is completely abolished (Fig. 6B). A similar inhibition of N-tubulin mRNA was induced by expressing the kinase dead X-PAK1/KR mutant and TH-MO (Fig. 6B). Therefore, both gain and loss of X-PAK1 kinase activity inhibit neuronal differentiation, as we had previously observed for gain and loss of TH protein (Wu et al., 2001; 2003).

In summary, we showed that expression of either X-PAK1/DE or TH-CAAX in early embryos upregulate the proliferation of neural plate cells and inhibit their differentiation. Loss of TH, or inhibition of endogenous X-PAK1 activity, downregulate neural plate cell proliferation and also inhibits their differentiation. We believe that the loss of differentiation under the gain and loss of TH and X-PAK1 might be the result of different processes. We hypothesize that when cells are prevented to fully withdraw from cell cycle (X-PAK1/DE and TH-CAAX), the differentiation program promoted by X-NeuroD is inhibited. In contrast, in X-PAK1/KR and TH-MO expressing embryos, since the right number of proliferative neural precursors can not be reached, the differentiation can not start.

These results, combined with our previous findings that X-PAK1 activity regulates TH cellular distribution, led us to study whether the X-PAK1 signaling pathway might be common to the TH signaling pathway.

3.6. TH-CAAX rescues X-PAK1/KR induced neural fold elevation defects.

We reasoned that TH is probably downstream of X-PAK1 in a signaling pathway, since its subcellular localization is regulated by X-PAK1 activity. To study this hypothesis, we tested whether TH or TH-CAAX expression rescues X-PAK1/KR induced inhibition of neural fold elevation and neural differentiation. Embryos were injected at 4 to 8 cell stages with X-PAK1/KR mRNAs (1ng) alone or together with increasing amounts of TH (from 500pg to 1.5ng) or TH-CAAX mRNA (from 100pg to 700pg). We carefully analyzed the morphology of the developing embryos. For batches of embryos coinjected with X-PAK1/KR mRNAs (1ng) and TH mRNAs (1ng), neural fold elevation was rescued in approximately 50% of the embryos (n=42). For embryos coinjected with X-PAK1/KRmRNAs (1ng) and TH-CAAX mRNAs (700pg), we observed a complete rescue of the neural fold elevation (100% of the embryos, n= 46) as summarized in Fig. 7A. These embryos batches were chosen to analyse Sox2 expression by in situ hybrization (Fig.7B), neural fold elevation by F-actin staining of dissected neural plates (Fig.7C), neural differentiation by expression of Slug and N-tubulin neuronal markers by in situ hybridization (Fig.7D). Results of representative experiments are presented in Fig.7B-C.

TH-CAAX expression rescued the X-PAK1/KR dependent inhibition of neural fold formation and Sox2 expression in embryos (Fig.7B-C). In these embryos, Sox2 area of expression could be more or less enlarged accompanied by some expansion of the neural plate. This indicates that a very tight balance of activity is required to obtain a completely wild-type phenotype (compare X-PAK1/KR to X-PAK1/KR+CAAX injected left sides (arrows), Fig.7B). F-actin staining of the dissected neural plates confirmed that TH-CAAX expression rescued cell contraction in the neural fold (Fig.7C). In some embryos (data not shown), the contraction area could be wider and less organized, as observed for TH-CAAX and X-PAK1/DE (see Fig. 4B), indicating that TH-CAAX activity was expressed above normal.

We next analyzed, by in situ hybridization using N-Tubulin and Slug markers, whether the neural differentiation program was also rescued in these embryos. Fig.7D shows that neither Xslug nor N-tubulin expression could be restored by expressing TH-CAAX together with X-PAK1/KR. We previously proposed that TH tight regulation of neural plate cell proliferation was a prerequisite for cell differentiation (Wu et al, 2001). In that previous work, neural plate expansion induced by TH was rescued by treating the embryos with a mixture of hydroxyurea and aphidicholin. Under these conditions, N-tubulin expression was also rescued. In the set of experiments described in this report, the failure of TH-CAAX to rescue differentiation in X-PAK1/KR expressing embryos may reflect our inability to fully restore a normal neural plate with the right number of neural plate cell progenitors. Alternatively, PAK are multifunctional kinases involved in the

regulation of many different pathways. It is therefore possible that inhibition of endogenous PAK, that prevents TH localization and function, might also prevent some other downstream target(s) from carrying out their function(s) in neuronal differentiation independently of the PAK/TH pathway.

4. Discussion

During neurulation, convergent extension movements couple narrowing (convergence) and elongation (extension) of the future neural plate. Neural plate cell morphology evolves by elongating along the dorso-ventral axis to become columnar. Apical constriction of the elongated neural plate cells allows neural plate bending and results in neural fold elevation and fusion (reviewed in Schoenwolf and Smith, 1990; van Straaten *et al.,* 1993). In parallel, expression of transcription factors regulate cell cyle withdrawal and initiation of differentiation. The coordination of these events is not fully understood.
In Xenopus embryos, Tumorhead (TH) regulates neural plate cells proliferation and also their morphology (Wu et al., 2001; Wu et al., 2003). In this study, we analyzed in depth how TH carries out its functions, and which signaling pathway it belongs to.

4.1. TH activity depends on its subcellular distribution and TH localization is controlled by X-PAK.

We previously observed that TH distributes between the nuclear and cytoplasmic/cortical compartments in embryonic cells and becomes enriched in the nuclei of cells withdrawing from the cell cycle (Wu et al., 2001; and our unpublished observations). Here, we analyzed the consequences of targeting TH to the cell cortex using TH-CAAX expression in early embryos. We report that TH and TH-CAAX expressing embryos induce similar neural phenotypes but the most severe abnormalities developed when TH is membrane-targeted. Thus, nuclear TH and cytoplasmic/cortical TH are likely to interact with different partners, and the understanding of the regulation of TH shuttling would help to identify the signaling cascade to which it belongs.
In fission yeast, TH binding to the cell cortex is dependent on the kinase activity of *S. pombe* p21-activated kinase, Shk1 (PAK1) (Wu et al., 2004). Mammalian PAKs also regulate the distribution of a number of target genes, allowing their localized activity. For instance, PAK6 regulates nuclear translocation of the stimulated androgen receptor (Schrantz et al, 2004). In *Xenopus*, X-PAK1 is expressed in the same regions as TH during neurogenesis, and we show in this report that expression of X-PAK1 mutants perturbed TH distribution in epithelial neural plate cells. An active PAK1 kinase enhanced TH cell membrane/cortex localization and binding to the apical edge of the neuroepithelial cells, while a dominant negative mutant induced its dramatic accumulation in the nuclear compartment.
In TH-CAAX expressing embryos, neural plate expansion correlates with an enlarged population of neural plate cells progenitors positive for sox-2, and the pigmentation in the outer layer of polarized epithelial cells is disrupted. Since pigment granules in these cells are apically localized (Fesenko et al, 2000; Merriam et al, 1983), the change of pigmentation may correlate with changes of cell polarity. This observation is consistent with the dramatic actin and microtubule cytoskeleton rearrangements and consequent cell shape changes and polarity abnormalities observed following TH expression in fission yeast (Wu et al, 2004). It could also explain the gastrulation defects, characterized by an open blastopore, observed in a number of TH-CAAX expressing embryos. Such defects, often associated with the impairment of convergent extension movements that require changes in embryonic cell shape and adhesive properties, were shown to be controlled during gastrulation by X-PAK5 (Faure et al, 2005). Neural plate cell shape appeared also to be modified by X-PAK1 mutants, since we constantly observed, in X-PAK1/KR expressing cells, a reduction in the distance between the apical surface of the cells and the largest section of the nuclei (compare Fig. 3 panel E':5μM from the apical surface to panel E: 7μM from the apical surface), indicating that apical/basal length may be altered.
The actin binding protein Shroom, by positioning apically an actomyosin network, plays a crucial function in the apico-basal heightening and apical constriction of cells required for neural fold elevation and neural tube fusion (Haigo et al, 2003, Hildebrand et al, 2005). hPAK1 regulates myosin light chain

phosphorylation (Wirth et al, 2003) and hPAK1 regulated translocation of myosin II-B to the cell cortex is required for its activity (Even-Faitelson et al, 2005). Potential cross talk between Shroom and X-PAK1 pathways could indeed exist., since our results show that both X-PAK1 kinase activity and TH overexpression regulate the cells behaviour in the contraction region of the neural plate. In that regard, it would be of interest to study, in more depth, the actomyosin network and contractile activity of neural plate cells expressing active PAK1.

4.2. X-PAK1 and TH induce similar neural phenotypes and function in the same pathway.

In the present study, we identified X-PAK1 as an upstream regulator of TH. Indeed, inhibition of endogenous X-PAK1 activity in embryos, through the expression of a dominant negative X-PAK1 mutant, prevents neural fold elevation and decreases the neural precursor population in the Sox-2 expression region. Moreover, the increased size of neural plate cells and a diminished staining of the mitotic marker phosphohistone H3 in the neural field of X-PAK1/KR expressing embryos strongly indicate that inhibition of X-PAK1 induces a decrease of the proliferation of neural precursor cells. In contrast, active X-PAK1 induces an expansion of the neural field, which correlates with an increase of Sox-2 positive neural progenitors. Modulating positively or negatively the kinase activity of X-PAK1 in neural plate cells, by expressing X-PAK1 mutants, also prevents expression of the neuronal differentiation marker N-tubulin. This indicates that X-PAK1 mutant expression faithfully mimics TH gain- and loss of function-induced phenotypes. Finally, we show that TH and X-PAK1 belong to the same functional pathway, and that TH functions downstream of X-PAK1, since the X-PAK1/KR neural fold elevation defect is rescued by expressing the TH-CAAX mutant. Although we cannot rule out, at this point that X-PAK1 may have other targets in neural plate cells, our results suggest that X-PAK1 controls neural plate cell proliferation/differentiation and morphology at least through the regulation of TH subcellular localization.
Such functions of PAKs in the regulation of cell proliferation and differentiation were previously reported in the mammary epithelium. Indeed, kinase-dead human PAK1 (hPAK1) expression during mammary gland development impaired functional differentiation of mammary epithelia (Wang et al, 2003), while kinase activation of hPAK1 is accompanied by widespread hyperplasia (Wang et al, 2002). Xenopus PAK3 is also involved in cell cycle withdrawal and differentiation during neurogenesis. Unlike X-PAK1, X-PAK3 appears to function as a proneural gene product under the control of X-Ngr-1 (Souopgui et al, 2002) and its expression pattern is restricted to the same regions as the neuronal differentiation marker N-tubulin (Souopgui et al, 2002). In contrast to X-PAK1, its activity results in cell cyle exit. It is thus likely that the two kinases control different signaling pathways.
How X-PAK1 and TH are co-regulated to affect neural development is indeed a matter of debate and we wish to discuss below different models that we are currently investigating in the lab.

4.3. A model of TH and X-PAK1 functions during neural development

X-PAK1 controlled shuttling of TH regulates neural fold elevation and cell proliferation. Does TH activity regulate various signaling cascades in the different cellular compartments? Or does its retention in one cellular compartment merely represent a way to sequester the protein and keep it away from its partners? Even though we can not discern between these possibilities at the moment, we can hypothesize, that TH or TH/PAK may carry out different functions at the cell cortex and in the nucleus. It is tempting to speculate that TH cortical localization is required for cell morphological changes, while its nuclear retention would be required for the inhibition of proliferative state and induction of differentiation.
In fission yeast, TH expression was shown to deregulate actin and microtubule cytoskeleton (Wu et al, 2004). Thus, active X-PAK1 controlled distribution of TH at the neuroepithelial cell cortex might regulate some aspects of the morphological changes required for these cells to become columnar and/or polarized. Indeed in the course of this study, we noticed that on the X-PAK1/DE-injected side of embryos, the contraction region of the neural fold is wider. Analysis of F-actin staining on Z sections demonstrates that contraction and compaction of X-PAK1/DE expressing cells in the neural plate is much reduced. PAKs are active at the cell cytoplasmic membrane and phosphorylate a number of their substrates to regulate actin (Vadlamundi et al, 2002) and microtubule (Wittmann et al, 2004) dynamics. In drosophila, inhibition of dPAK results in disruption of the leading edge cytoskeleton and interferes with dorsal closure (Conder et al, 2004). PAK substrates which explain their functions in the control of cell shape remodeling remain to

be identified. TH could be an X-PAK1 target in neuroepithelial cells, since we found that it is a good *in vitro* substrate for X-PAK1 (our unpublished observations). We are currently trying to identify X-PAK1 phosphorylation sites on TH, in order to study the requirement for TH phosphorylation for its shuttling in neural plate cells. Then, the functional importance of these phosphorylations for the cell morphological changes during neural tube development will be assessed.

How could TH function in the nuclei? TH is a SANT domain protein (AAs 104-174). The identification of SANT domains was based on their homology to the c-myb DNA binding domain. They are involved in chromatin remodeling and transcriptional control through their interactions with histone N-terminal tails (Boyer et al, 2002). Several SANT domain proteins belong to the SWI/SNF chromatin remodeling complexes. In yeast, Swi3P SANT domain is required for the SWI/SNF complex function. These complexes have an ATPase catalytic subunit which disrupts histone-DNA interaction and induces nucleosome displacement (Whitehouse et al, 1999). This chromatin remodeling allows regulation of transcriptional activities associated with development, cellular differentiation, and proliferation (Kassabov et al, 2003). In Xenopus the catalytic subunit of the Swi-SNF, Brg-1 regulates neuronal differentiation by mediating the transcriptional activity of Ngnr-1 and NeuroD (Seo et al, 2005a). Interestingly, loss of Brg1 induces an expansion of the population of sox2 positive neural progenitors and repressed the expression of the differentiation marker N-tubulin (Seo et al, 2005a). Thus loss of Brg-1 and by extension, loss of function of the SWI/SNF complex perfectly mimics TH-CAAX phenotype (prevention of nuclear translocation) during Xenopus neurogenesis. Moreover, a recent study reported that loss of Brg-1 also regulates cell shape by increasing cell volume and size (Hill et al, 2004). Finally, Brg-1 can regulate actin cytoskeleton dynamics by affecting the RhoA signalling pathway (Asp et al, 2002).

hPAK1 was recently shown to regulate nuclear transport of several targets. Indeed, hPAK1 mediated phosphorylation of the transcription factor snail allows its nuclear translocation and its repression of E-Cadherin transcription (Yang et al, 2005). hPAK1 mediated phosphorylation of estrogen receptor was proposed to regulate the expression of ER target genes (Wang et al, 2002). Moreover, it was recently shown that hPAK1 possesses nuclear localization signals (NLS) and can itself associate to chromatin and modulate transcription activity (Singh et al, 2005). Finally, it was found that *S. cerevisiae* Cla4p (a PAK member) interacts with the Rad54, *S. cerevisiae* SWI/SNF ATPase (Goehring et al, 2003) , in a genetic screen.

Taking these data together, we could envision that X-PAK1 mediated shuttling of the SANT domain containing protein TH might participate in some aspect of the chromatin remodeling and cell morphology regulation mediated by SWI/SNF complexes during neurogenesis. We are currently investigating whether the two proteins are translocated together in neural plate cell nuclei, and the putative interactions of X-PAK1 and TH with SWI/SNF proteins.

In summary, we show that TH regulation of neural differentiation in Xenopus embryos is regulated by its subcellular distribution and is under the control of X-PAK1. Although future work will enable a more complete understanding of how the two proteins function, we hypothesize that TH and X-PAK1 may have a cortical function in regulating cytoskeleton dynamics and chromatin remodeling functions required for expression of specific neural genes.

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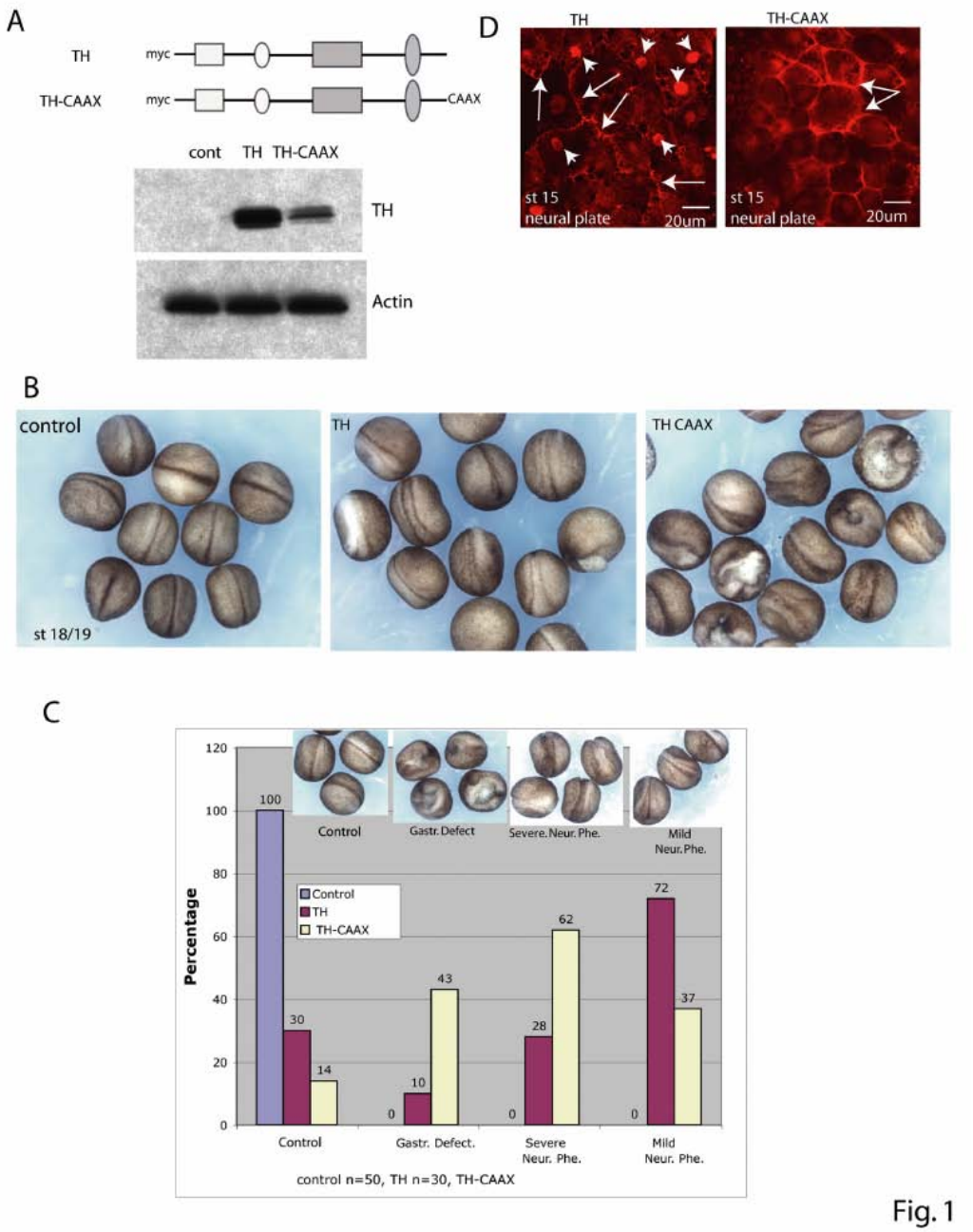

Fig. 1

Fig. 1. Targeting TH to cell membrane enhances TH function.
(A). Diagram of TH and TH-CAAX constructs. Western blot of stage 15 embryos extracts, non injected (con) or injected with Myc tagged TH: TH mRNA (1ng, TH), TH-CAAX mRNA (750 pg, TH-CAAX). Western blot were treated with myc monoclonal antibodies (Top panel). The same amount of embryo extracts were loaded again and the western blot probed with anti actin antibodies (bottom panel), to ensure equivalent amount of proteins were loaded. TH-CAAX is expressed at a lower level than TH.
(B). Photomicrographs showing the morphology of the control embryos and the phenotypes induced in embryos injected in one side of neural field with 1ng of TH mRNA or with 750pg of TH-CAAX mRNA.
(C). Graphs showing phenotypes quantification of TH-CAAX with TH. Inset photomicrographs illustrate phenotypes for each group of the graph. The gastrulation defective embryos are excluded from counting for the neural phenotype embryos. The experiment shown is representative of the results obtained in three distinct experiments.
(D). Cells in dissected neural plates were analyzed by indirect immunofluorescence using anti-myc monoclonal antibody. At the neural plate stage, while 1MT-TH is both at the cell cytoplasmic membrane (TH, arrows) and in the nucleus (TH, arrowheads), TH-CAAX is mainly accumulated at the cell membrane (TH-CAAX, arrows). Gastr Defect stands for gastrulation defects; Serve Neur Phe stands for severe neural phenotyes; Mild Neur Phe stands for mild neural phenotypes.

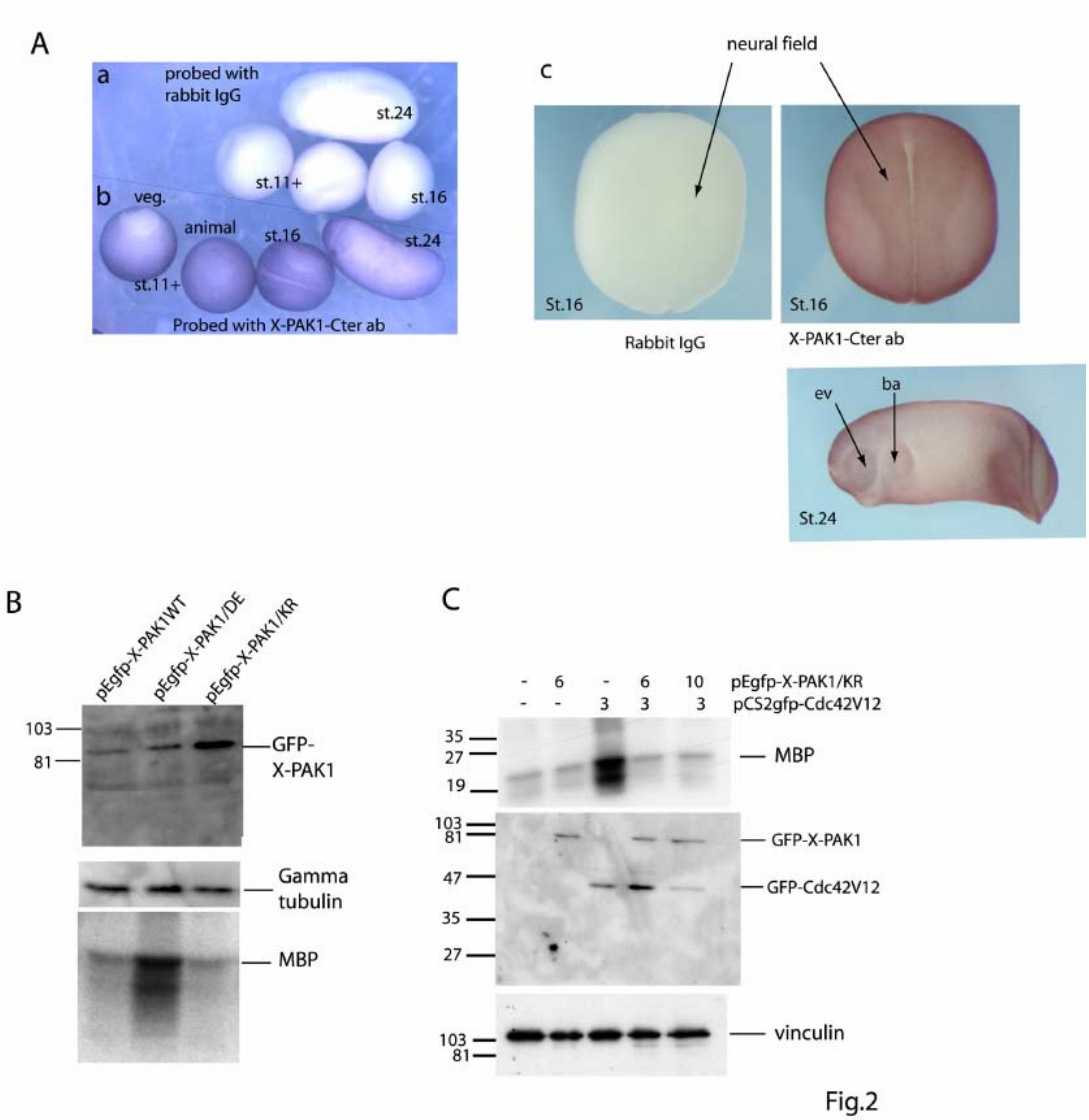

Fig.2

Fig. 2. X-PAK1 expression in embryos and characterization of X-PAK1 kinase activity.

(A). Endogenous X-PAK1 protein is expressed in the neural field during neurulation. (a-c). Photomicrographs of whole-mount immunostaining of different staged albino embryos. a. embryos immunostained with commercial rabbit IgG to ensure no /or low background from IgG *per se.* b. Embryos immunostained with anti-X-PAK1 antibody. At gastrula stages, X-PAK1 is essentially ubiquitous (b, st.11+, compare the vegetal view with the animal view). At neurula stages, X-PAK1 is ubiquitous with an enrichment in the neural field (b, stage 16 embryo and c, pointed out by the arrow). At tailbud stages, X-PAK1 level in the body epidermis of the embryo is decreased (b, st. 24 embryo). c. Enlarged photograph of stage 16 embryos treated with commercial rabbit IgG (left) or X-PAK1 antibodies (right), showing the staining of neural field (pointed out by the arrow). Enlarged photograph of stage 24 embryo stained with X-PAK1 antibodies and showing the enrichment of the protein in the eye vesicle and branchial arches. St. stands for stage; veg. stands for vegetal view; animal stands for animal view ; ev stands for eye vesicle ; ba. Stands for branchial arches.

(B). X-PAK1/DE acts as a constitutively active kinase. XL-2 cells were transfected for 24hrs with pEgfp-X-PAK1WT, pEgfp-X-PAK1/DE, or pEgfp-X-PAK1/KR respectively. Top panel : Western blot of transfected cells extract using gfp polyclonal antibodies, GFP-X-PAK1/KR is expressed at a higher level than GFP-X-PAK1WT and GFP-X-PAK1/DE. Middle panel : Same blot reprobed with ˜tubulin antibodies showing the protein loading. Bottom panel: Kinase activity of X-PAK1 immunoprecipitated from XL-2 cell extracts (using X-PAK1-Nter antibodies) is tested using MBP as a substrate. GFP-X-PAK1/DE kinase is constitutively active (middle lane of bottom panel).

(C). X-PAK1/KR acts as a dominant negative kinase and blocks endogenous X-PAK1 activation. XL-2 cells were transfected respectively with the amounts indicated of pCS2gfp-Cdc42V12, pEgfp-X-PAK1/KR, or mock control. Top panel: Kinase activity of X-PAK1 immunoprecipitated from XL-2 cells (using X-PAK1-Nter antibodies) is tested using MBP as a substrate. Expression of GFP-Cdc42V12 up-regulates the kinase activity of endogenous X-PAK1 (lane 3). Expression of GFP-X-PAK1/KR abolished this up-regulation (lane 4-5). Middle panel : Western blot of transfected cell extract using gfp polyclonal antibodies showing protein levels of GFP-Cdc42V12 and GFP-X-PAK1/KR. Bottom panel : Same blot reprobed with anti-vinculin antibodies to show protein loading.

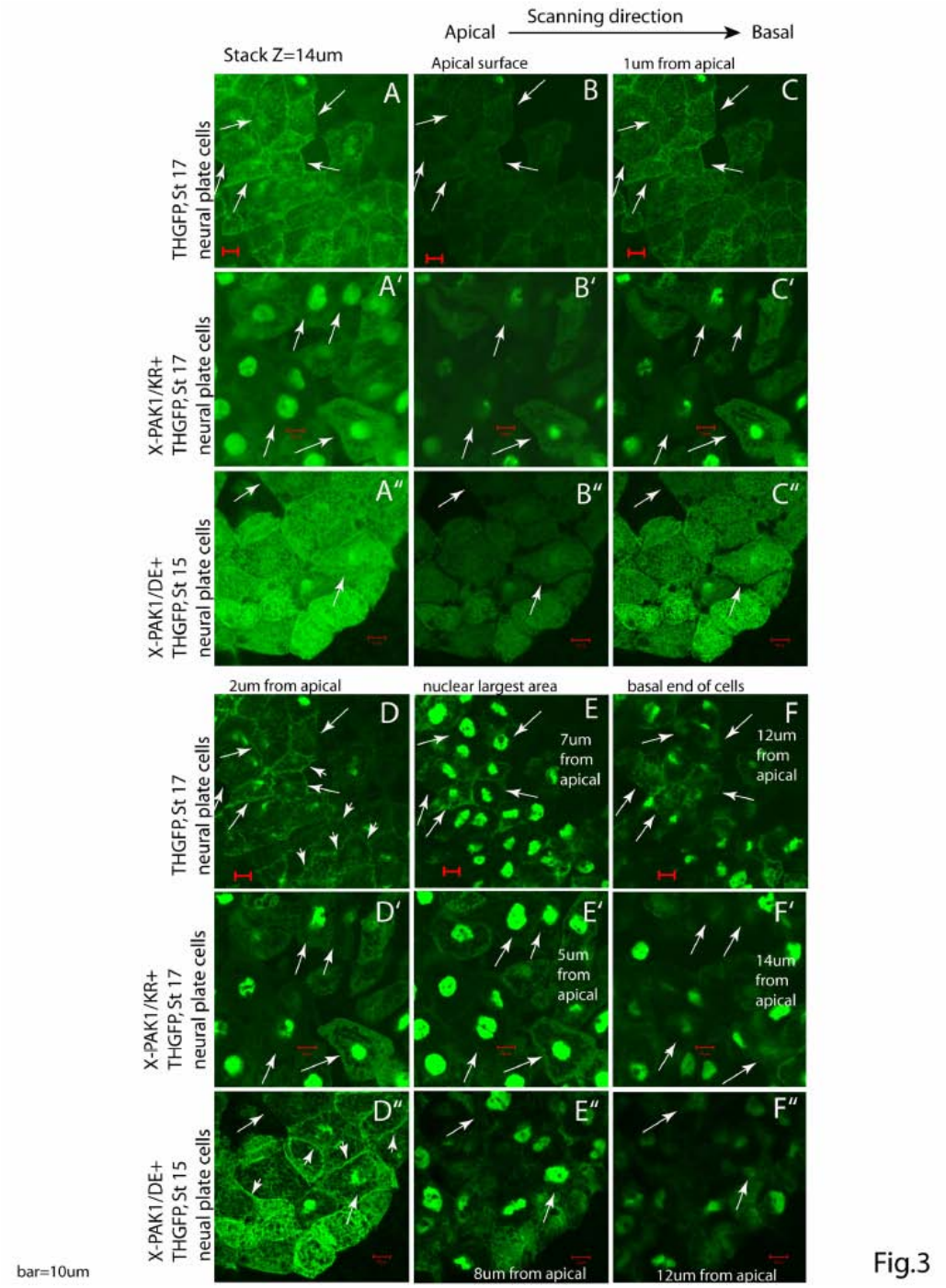

Fig.3

Fig. 3. X-PAK1 catalytic activity positively regulates TH distribution to cell cortex/membrane.
A-F" are confocal photomicrographs of cells in neural plates that were dissected from embryos respectively microinjected with THGFP, THGFP+X-PAK1/KR, or THGFP+X-PAK1/DE mRNAs into one side of the future neural field at the 8-cell stage as described in Material and Methods. THGFP protein localization is visualized in green. The neural plates are observed as reconstituted image from the stacks (A, A', and A"), at the apical surface (B, B' and B"), 1μm from the apical surface (C, C', and C"), 2μm from the apical surface (D, D', and D"), at the maximium area of nuclei (E, E', and E"), and at the basal end of the epithelial neural plate cells (F, F' and F", pointed by arrows). In cells only expressing THGFP (A-F), THGFP is at the apical cortex (A), the lateral cell membrane (C-D, pointed by arrowheads), and in the nucleus, which is at the basal half of the cell (E). In cells expressing both THGFP and X-PAK1/KR, THGFP is no longer at the apical cortex (A'), nor at lateral cell membrane (C'-D'), but accumulates in the nucleus (A'-F'). In cells expressing both THGFP and X-PAK1/DE, THGFP accumulates more to the apical cortex (A"-C") and the lateral cell membrane (D", pointed by arrows). Arrows indicate cells that can be traced from the apical surface to the basal end; arrowheads indicate THGFP at the cell cytoplamic membrane. Bar=10μm.

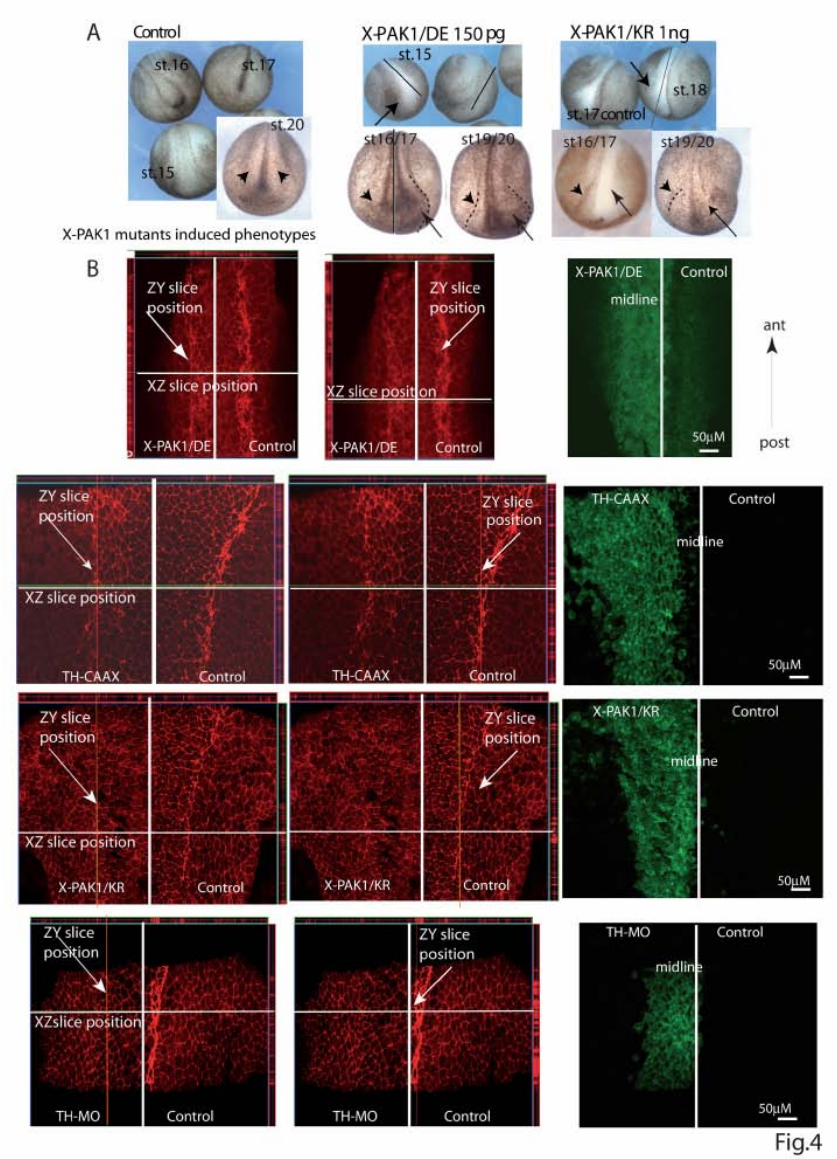

Fig.4

Fig. 4. Morphological phenotypes induced by X-PAK1 mutants. X-PAK1 and TH mutants regulate the contracting area of neural plate cells.

(A). X-PAK1 induces phenotypes in developing embryos similar to TH induced phenotypes. Broken lines allow a better visualization of the neural fold. Control : control embryos at stages 15-20. X-PAK1/DE : Embryos injected with X-PAK1/DE on one side of the future neural field (arrows), showing enlarged neural field and abnormal pigmentation. Enlarged embryos (st16-17 and st19-20) show the expansion of neural plate and neural fold elevation in the injected side (arrow) compared to the non- injected side (arrowhead), compare also with the enlarged control embryo. 28 from 30 injected embryos displayed this phenotype. X-PAK1/KR : X-PAK1/KR injected embryos, showing the inhibition of neural fold elevation and change in pigmentation in the injected side (arrows). Enlarged embryos show that neural fold did not elevate in the injected side (arrow), compared to control side (arrowhead). 33 of 41 injected embryos displayed this phenotype.

(B). Expression of active X-PAK1/DE and TH-CAAX reduced the packing of contracting cells in the neural plate epithelial cells. In all panels, left side is the injected side ; right side is control. X-PAK1/KR and TH-MO expression prevent neural fold elevation. Micrographs shown are representative of phenotypes observed in three separate experiments. Confocal photomicrographs of dissected mid-region neural plate from X-PAK1/DE, TH-CAAX, X-PAK1/KR and TH-MO injected embryos are shown. For each construct injected, there are three panels : F-actin is stained by rhodamine-conjugated phalloidin (left and middle panels); The green fluorescent tracer fluorescein-conjugated dextran indicates the injected side (right panels). The thick vertical white line, in every panel, segregates the injected from non-injected side. The thin red vertical lines indicate the ZY slice position, the horizontal white lanes indicate the XZ position. Z sections of XY slices are on the right end side of the panels. Z sections of YZ slices are on the top of the panels.

In the neural plate control sides, the contracting cells are packed along a stripe parallel to the dorsal midline. This is easily visualized by the intense F-actin staining. In X-PAK1/DE and TH-CAAX expressing embryos, the neural plate contraction area does form, however the F-actin staining is less intense (compare the Z section). On X-PAK1/KR and TH-MO expressing sides of the neural plate, cells remain well spread out and did not contract, as seen with F-actin staining.

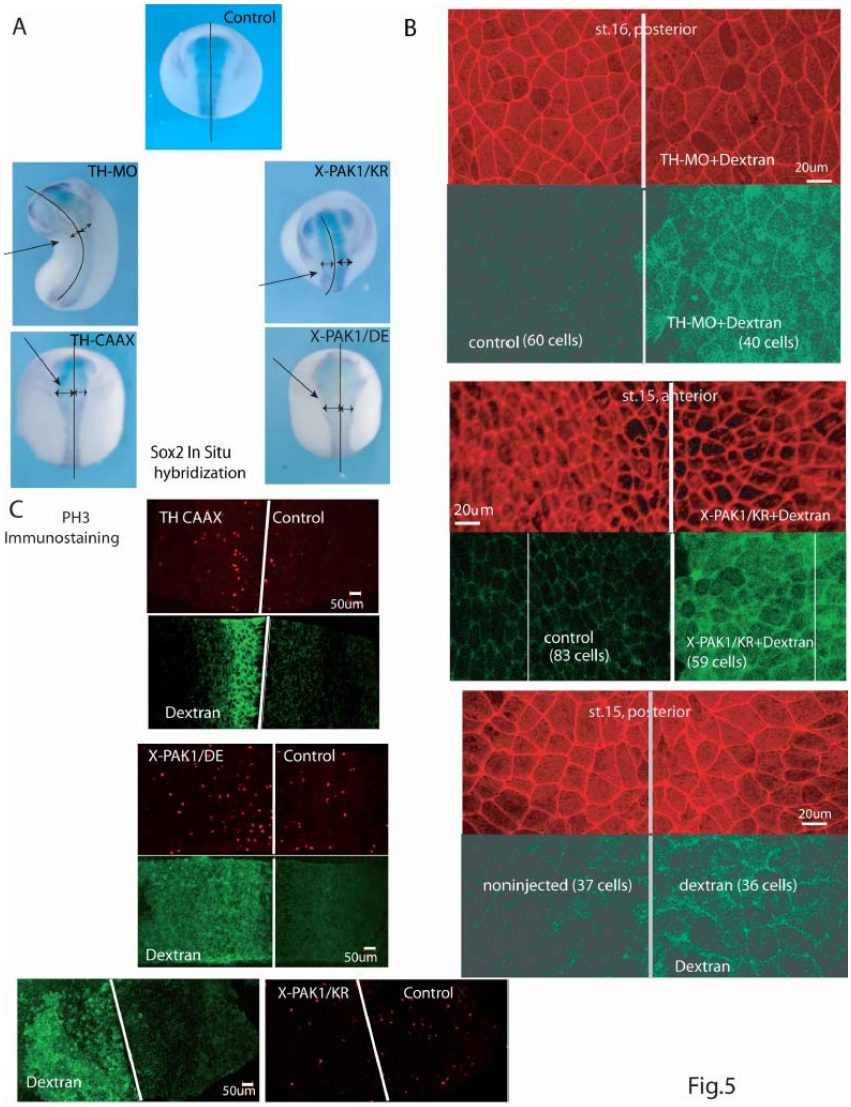

Fig. 5. X-PAK1 and TH have the same affect on neural plate cell proliferation.
Panel A. Photomicrographs of Sox2 *in situ* hybridization of embryos (representative from 3 different sets of injections). Injected sides are left (arrows). All embryos are viewed from the dorsal/anterior. Control embryos express sox2 in both sides of neural plate (control). In TH-MO and X-PAK1/KR expressing embryos, Sox2 is very poorly expressed and the neural field remains flat compared to control sides. In TH-CAAX and X-PAK1/DE, sox2 expression territory is broader and the neural fold formed in comparison to the non injected side. Compare the width of the sox2 positive territories with the doubleheads arrows.
Panel B. Representative confocal photomicrographs (of three separate experiments) of neural plate cells expressing control, TH-MO and X-PAK1/KR. Injected sides are on the right of the panels.
F-Actin staining of neural plate cells. F-actin is stained by rhodamine-conjugated phalloidin; the fluorescein-conjugated dextran marker is green. Top panels : neural plate dissected from TH-MO injected embryos. The average size of cells in the TH-MO-injected side is larger than cells in the control side. Cell counting of the total area reveals that there are 50% more cells in control compared to the TH-MO injected side. Middle panels : neural plate dissected from X-PAK1/KR injected embryos. Cell counting was performed in the defined area. The average cell size is larger in X-PAK1/KR injected side than in the control. In the experiment depicted, 43% more cells are counted on the control compared to X-PAK1/KR-injected side. Bottom panels : neural plate dissected from embryos injected with lineage tracer. The cell size and number in both injected and control sides are similar. Note that cells size should be compared between, the injected and the non injected side, since cells size is dependent of the region (anterior to posterior) of the neural plate that was dissected and analyzed.
Panel C : phosphohistone H3 (PH3) immunostaining of dissected neural plates. Injected sides are left in each panel. Embryos were injected with TH-CAAX, X-PAK1/DE and X-PAK1/KR. Neural plates were dissected and immunostained with PH3 antibodies. Green fluorescent dextran marker indicates the injected side. The ratio of PH3 positive cells on the X-PAK1/DE expressing side versus control side and TH-CAAX expressing side versus control side is clearly above 1. In contrast, in X-PAK1/KR expressing side versus control side the ratio of H3 positive cells is below 1. These micrographs shown are representative of the PH3 immunostaining observed in three separate experiments.

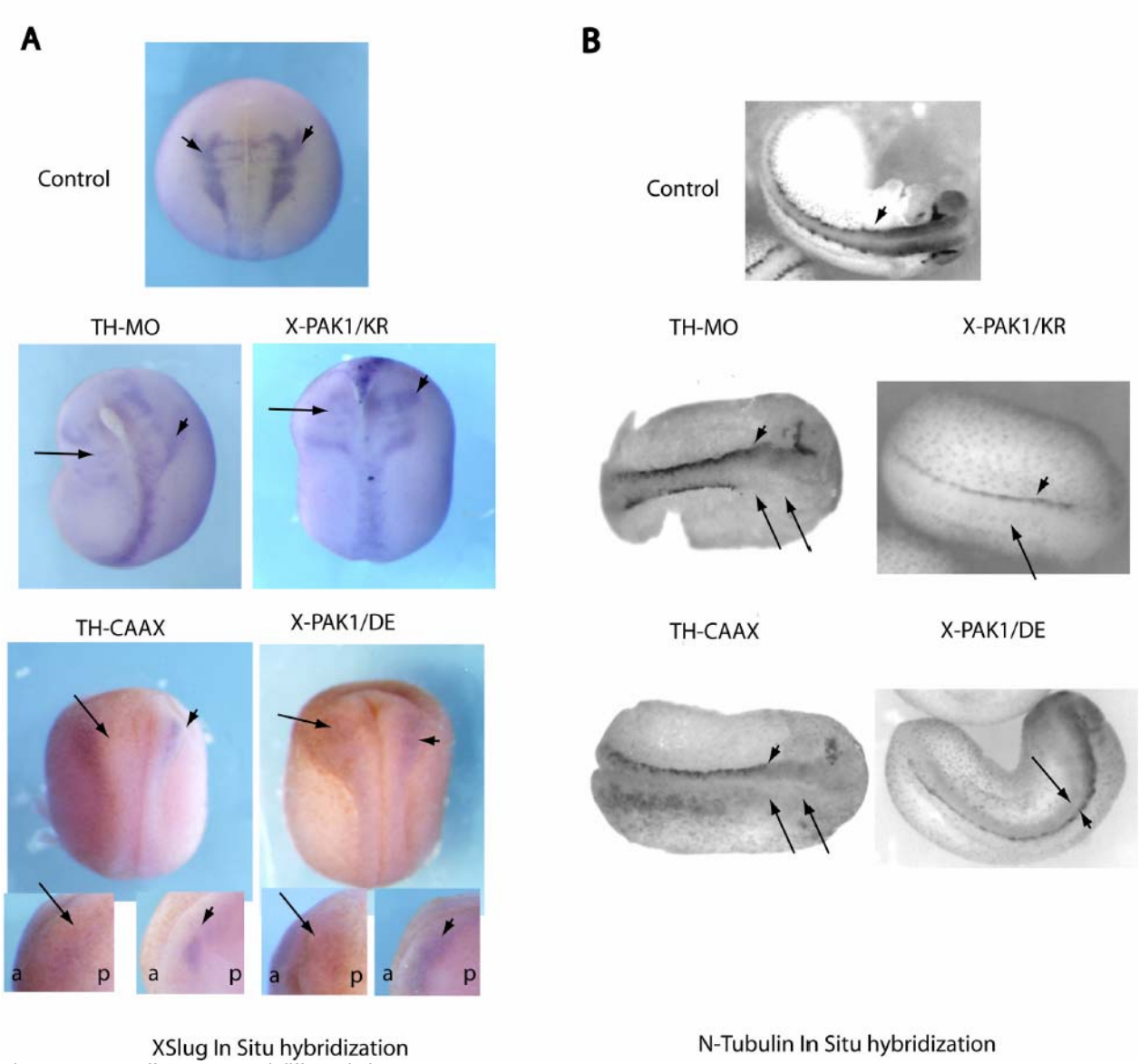

Fig. 6. X-PAK1 affects neuronal differentiation.

A: Photomicrographs of *in situ* hybridization of embryos (stage 22) with Xslug antisense RNA probe. XSlug is an early neural crest specific marker that is first expressed in two patches lateral to the neural plate. Dorsal views of embryos injected with TH-MO or X-PAK1/KR and with X-PAK1/DE or TH-CAAX. Arrows indicate the injected sides, arrowheads indicate the control side. In all injected sides XSlug expression is repressed. To better visualize the repression of Slug in TH-CAAX and X-PAK1/DE expressing embryos, higher magnification of lateral views (anterior (a) to posterior (p) of microphotograph taken on the injected and non injected sides are shown in the insets.

B : Photomicrographs of *in situ* hybridization of embryos with N-tubulin antisense RNA probe, which marks the differentiated neurons. Control embryos at stage 25 from dorsal views. The differentiated neurons are arranged as a stripe on each side of the dorsal midline (arrowhead); Dorsal views of stage 24/23 embryos injected with X-PAK1/DE or TH-CAAX. The neuron stripe is present in the control side (arrowhead) and absent in the injected side (arrows). Dorsal views of stage 22/23 embryos injected with X-PAK1/KR or TH-MO have a N-tubulin positive neuron stripes in the control side (arrowheads), this expression is absent in the injected side (arrows). Thus, expression of X-PAK1/DE and X-PAK1/KR, like expression of TH-CAAX and injection of TH-MO, inhibit neuron differentiation.

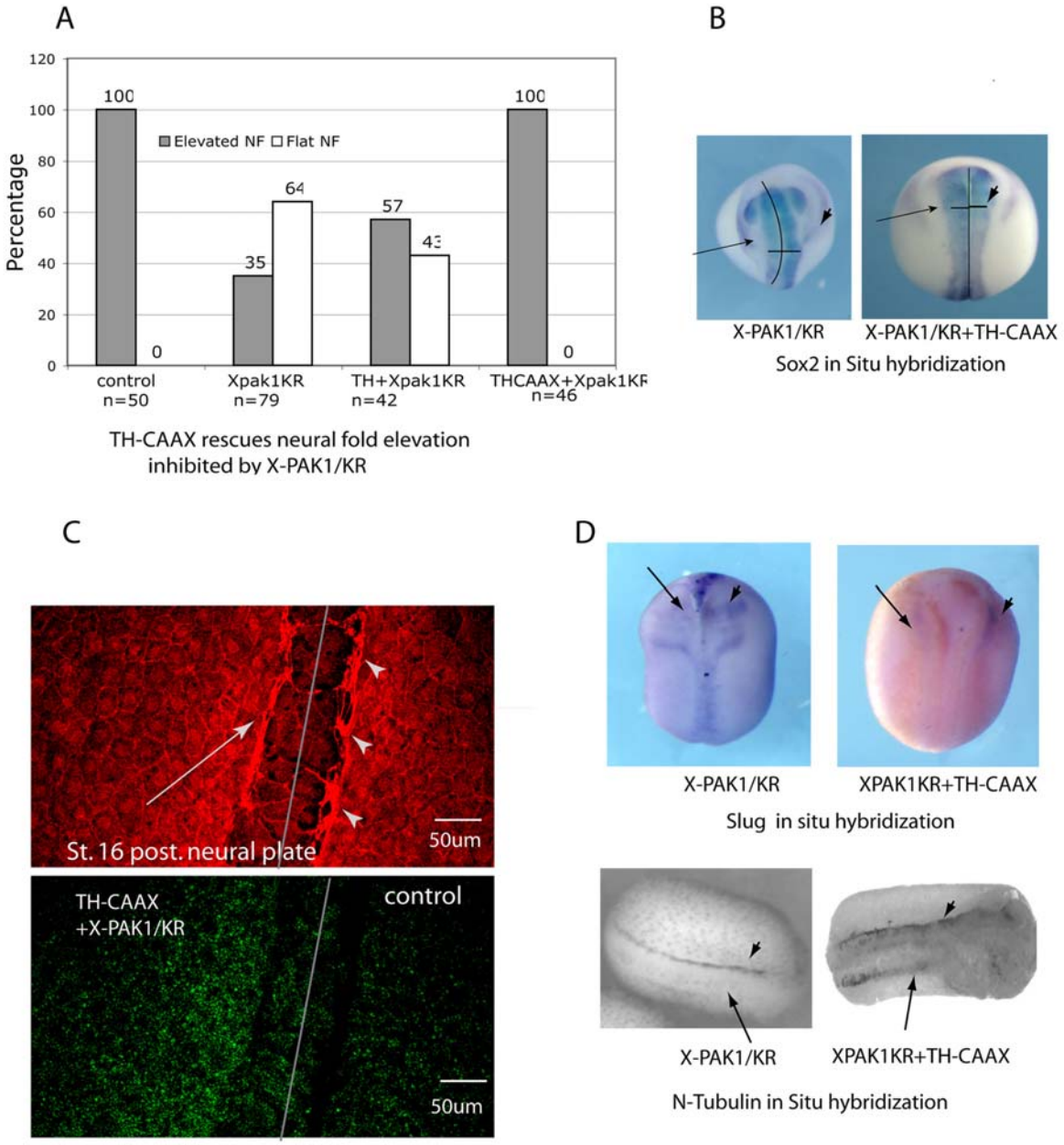

Fig. 7 :TH-CAAX rescues neural fold formation inhibited by X-PAK1/KR.
Panel A, Graph : quantification of neural fold rescue in the different coinjection experiments. The neural fold elevation of embryos was analyzed with a dissecting microscope.
Panel B : Photomicrographs of Sox2 *in situ* hybridization of embryos injected by X-PAK1/KR or a mixture of X-PAK1/KR+TH-CAAX mRNAs. Embryos are viewed from the dorsal/anterior. Sox2 is repressed in the X-PAK1/KR injected side compared to contro side (left embryo) . Sox2 is expressed in both control side (arrowhead) and X-PAK1/KR+TH-CAAX injected side (arrows) of the neural plate (right embryo). Sox2 expression territory is slightly broader and the neural fold formed in the injected side (arrows) by comparison to the non injected side (arrowheads).
Panel C : Representative confocal photomicrographs (of three separate experiments) of neural plate cells from an embryo injected with the same mixture of TH-CAAX and X-PAK1/KR mRNAs as in panel B. F-Actin staining of neural plate cells. F-actin is stained by rhodamine-conjugated phalloidin; the fluorescein-conjugated dextran marker is green. TH-CAAX rescues the contraction structure inhibited by X-PAK1/KR (injected side, pointed by the arrow).
Panel D : Photomicrographs of X-Slug and N-tubulin *in situ* hybridization of embryos injected with X-PAK1/KR (left embryos) or the same mixture of TH-CAAX+X-PAK1/KR mRNAs (right embryos) as in panel B. Arrows show that neither XSlug nor N-Tubulin expression are rescued when TH-CAAX is coepressed with X-PAK1/KR on the injected side compared to the the control side (arrowheads).