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mKlf7, a potential transcriptional regulator of TrkA nerve growth factor receptor expression in sensory and sympathetic neurons

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SUMMARY

Development of the nervous system relies on stringent regulation of genes that are crucial to this process. TrkA, the receptor for nerve growth factor (NGF), is tightly regulated during embryonic development and is essential for the survival and differentiation of neural crest-derived sensory and sympathetic neurons. We have previously identified a mouse *TrkA* enhancer and have characterized several *cis* regulatory elements that are important for appropriate TrkA expression in vivo. We now report the cloning of a novel gene encoding a Kruppel-like factor from a mouse dorsal root ganglion expression library. This Kruppel-like factor, named mKlf7, binds specifically to an Ikaros core binding element that is crucial for in vivo *TrkA* enhancer function. Using in situ hybridization, we demonstrate that *mKlf7* is coexpressed with *TrkA* in sensory and sympathetic neurons during embryogenesis and in adulthood. These data are consistent with the idea that mKlf7 may directly regulate *TrkA* gene expression in the peripheral nervous system.

Key words: Enhancer, Kruppel-like factor, Neurotrophin, Neural crest, TrkA, Gene regulation, Mouse

INTRODUCTION

Neural development is a complex process that involves the generation of diverse cell types assembled into functional circuits. Neuronal cell fate specification is determined by positional information, cell-cell interaction, extracellular and intracellular signaling events, transcription factor cascades, and ultimately, differential expression of neuronal genes. Different types of transcription factors such as basic helix-loop-helix (bHLH) proteins, homeodomain proteins and zinc-finger proteins have been identified to play important roles in neuronal determination and differentiation (Anderson, 1994; Anderson and Jan, 1997; Jan and Jan, 1990; Jan and Jan, 1993; Nolo et al., 2000; Pfaff and Kintner, 1998). Despite advances in understanding of transcriptional regulation in neural development, it is likely that additional, as yet unidentified transcription factors participate in this complex and highly regulated process. A challenge remains to ascribe physiologically relevant target genes for many of the identified regulatory transcription factors.

The nerve growth factor (NGF) family of neurotrophins (NTs) and their receptors play fundamental roles in the developing nervous system (Levi-Montalcini, 1987; Lewin and Barde, 1996; Snider, 1994). Besides NGF, this neurotrophin family also includes brain-derived neurotrophic factor (BDNF), NT3, and NT4/5. These NTs interact specifically with the Trk family of receptor tyrosine kinases (Bothwell, 1995; Parada et al., 1992). NGF binds TrkA, NT3 preferentially binds TrkC, while BDNF and NT4/5 bind TrkB. As target-derived trophic factors, neurotrophins are broadly expressed in both neural and non-neural innervation target tissues while Trk receptors are primarily expressed in neurons (Klein et al., 1990; Martin-Zanca et al., 1990; Tessarollo et al., 1993). *TrkA* (*Ntrk1* – Mouse Genome Informatics) is specifically expressed in neural crest-derived sensory and sympathetic neurons in the peripheral nervous system (PNS), including dorsal root ganglia (DRG), trigeminal ganglia (TG), superior and jugular ganglia, and sympathetic ganglia (Martin-Zanca et al., 1990; Schecterson and Bothwell, 1992). In the central nervous system (CNS), *TrkA* expression is limited to a few populations of cells including cholinergic neurons in the basal forebrain and striatum, and some interneurons in the spinal cord (Holtzman et al., 1992; Liebl et al., 2001; Sobreviela et al., 1994). Consistent with cell culture and pharmacological experiments, the generation of *TrkA* and *NGF* knockout mice directly demonstrates the requirement of NGF- and TrkA-mediated signaling in the development of peripheral sensory and sympathetic neurons (Snider, 1994). In either *NGF*- or *TrkA*-null mice, about 70-80% of dorsal root ganglion (DRG) neurons, 70% of trigeminal neurons and 99% of superior cervical ganglion (SCG) neurons are lost through apoptosis (Crowley et al., 1994; Smeyne et al., 1994). In the CNS, TrkA appears to be required for the normal maturation of basal forebrain and striatal cholinergic neurons although most of these neurons survive in *TrkA*-null mice for the lifespan of the mutant animals (Fagan et al., 1997).

Although *TrkA* is expressed in specific areas of the nervous system, little is known about the forces that control such stereotypic expression. To date, the only known gene implicated in regulating *TrkA* expression encodes the POU domain transcription factor Brn3a (Pou4f1 – Mouse Genome Informatics; Huang et al., 1999). In *Brn3a*-null mice, the onset of *TrkA* expression in trigeminal ganglia at embryonic day (E) 11.5 is normal, but no *TrkA* expression is detectable after E17.5. Therefore, although *Brn3a* may be important for the maintenance of *TrkA* expression, it apparently is not required for initiation of *TrkA* expression in sensory ganglia (Huang et al., 1999). It remains to be tested whether *Brn3a* regulates *TrkA* expression directly during late development.

To better understand *TrkA* expression and to identify transcription factors that are critical for sensory and sympathetic neuron specification, we isolated a mouse *TrkA* enhancer and functionally characterized the *cis* regulatory elements within this genomic fragment using transgenic mouse embryos (Ma et al., 2000). Functional analysis revealed that *TrkA* expression is coordinately controlled by multiple *cis* elements including consensus DNA-binding core sequences defined for the transcription factor families DELTAEF, HAND, MZF, Ikaros, E box-binding protein, ETS and AP1. Therefore, in vivo, multiple transcription factors binding to these *cis* elements cooperate to regulate *TrkA* expression (Ma et al., 2000).

In this study, we identify a novel gene encoding a Kruppel-like zinc-finger protein isolated through expression cloning from a mouse E13.5 DRG expression library. We have named this gene *mKlf7* (murine Kruppel-like factor 7) based on its significant sequence homology to human *UKLF* (ubiquitous Kruppel-like factor), also called *KLF7* by the Human Gene Nomenclature Committee (Matsumoto et al., 1998; Turner and Crossley, 1999). We provide evidence that mKlf7 is a transcription factor that may directly regulate *TrkA* expression in vivo. Because *mKlf7* gene expression precedes that of *TrkA* and continues into adulthood, it is further possible that *mKlf7* is required for both initiation and maintenance of *TrkA* gene expression.

MATERIALS AND METHODS

Expression cloning

A cDNA expression library was constructed using mRNA isolated from mouse E13.5 dorsal root ganglia and directionally cloned into the *Eco*RI/*Xho*I sites of the lambda ZAP Express vector (Stratagene) (J. Merenmies, K. Vogel and L. F. P., unpublished). This expression library was screened using oligonucleotide probes as described (Singh et al., 1988). The probes contained three copies of the wild-type Ikaros2, MZF2 or HAND sites from the mouse *TrkA* minimal enhancer. The sequences are Ikaros2(3×), 5′-*AATT* GAA AAA TAG T**GG GA**G AGA AGA GTC GAA AAA TAG TGG GAG AGA AGA GTC GAA AAA TAG TGG GAG AGA AGA GTC-3′; MZF2(3×), 5′-*AATT* CAG AAC CT**G GGG** AGA AAAA CAG AAC CTG GGG AGA AAA ACA GAA CCT GGG GAG AAA AA-3′; and HAND(3×), 5′-*AATT* AAC GCT CT**C CAG** ACC CTA GT AAC GCT CTC CAG ACC CTA GTA ACG CTC TCC AGA CCC TAGT-3′. One copy of each binding site is underlined and the core sequence for each binding site is highlighted in bold. The 5′ overhang of each oligonucleotide (italics) was added to facilitate labeling with radioactive nucleotides and cloning into the pHisi-1 vector (Clontech, see below), respectively. Each probe was labeled with [α-^{32}P]-dATP and [α-^{32}P]-dCTP using the Klenow enzyme in the presence of cold dGTP and dTTP. Probes were then purified using Sephadex G-25 spin column (Boehringer Mannheim). After three rounds of screening, positive lambda phages were purified and the cDNA inserts were excised in vivo using helper phages (Stratagene) and sequenced.

Cellular localization of mKlf7 proteins

The coding region of *mKlf7* was amplified by PCR using primers to generate an *Eco*RI site at the N terminus and an *Xho*I site at the C terminus. The PCR product was cloned into the pGemTeasy vector (Promega) and sequenced. The *mKlf7* fragment was then released by *Eco*RI/*Xho*I digestion and cloned into *Eco*RI/*Xho*I sites of the vector pEGFP-C2 (Clontech) to generate the construct pEGFP-Klf7 expressing GFP-Klf7 fusion proteins. Human embryonic kidney 293 (HEK293) cells and mouse neuroblastoma-rat glioma hybrid 108 (NG108) cells were plated on poly-D-lysine coated coverslips and subsequently transfected with 1 μg of pEGFP-Klf7 or pEGFP-C2 plasmid using Fugene 6 reagents (Boehringer Mannheim). 48 hours after transfection, cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at 4°C, washed with PBS twice and incubated with 1 μg/ml 4,6-diamidino-2 phenylinodole (DAPI) for 30 minutes at 4°C. Cells were then washed with PBS three times and coverslips were mounted onto glass slides. Cells were viewed using a fluorescence microscope.

Expression and purification of recombinant mKlf7 proteins

The cDNA fragment encoding the C-terminal DNA-binding domain (amino acid 213-301) of mKlf7 was amplified by PCR using primers to generate an *Eco*RI site at the N terminus and an *Xho*I site at the C terminus. The PCR product was cloned into pGemTeasy and sequenced. The mKlf7 fragment was released and subcloned into the *Eco*RI/*Xho*I sites of pGEX4T1 vector (Amersham) to generate the construct pGEX-Klf7C expressing GST-Klf7(213-301) fusion proteins. pGEX-Klf7C was transformed into *Escherichia coli* BL21 cells. Cells were grown at 37°C in LB/ampicilin until OD_{600} was about 0.6 and isopropylthio-β-galactoside (IPTG) was added at a final concentration of 1 mM. Cells were further incubated for 2 hours, harvested and GST-Klf7(213-301) proteins were purified using Glutathione-Sepharose 4B beads (Pharmacia). To use as controls, plasmids encoding GST, GST-p75 intracellular domain fusion protein (GST-p75ICD), or GST-Brn3a were used to transform BL21 cells; recombinant proteins were purified using Glutathione-Sepharose 4B beads. The GST-p75ICD plasmid and the GST-Brn3a plasmid were kindly provided by Drs Moses Chao and Mengqing Xiang, respectively. p75 is the low-affinity receptor for NGF.

Gel mobility shift assay (EMSA)

The EMSA protocol was as described (Lei et al., 1998). Each probe contains one copy of wild-type Ikaros2 (5′-GAA AAA TAG T**GG GA**G AGA AGA GTC-3′), MZF2 (5′-CAG AAC CT**G GGG** AGA AAA A-3′) or HAND (5′-AAC GCT CT**C CAG** ACC CTA GT-3′) site from the *TrkA* minimal enhancer. The core sequence of each binding site is highlighted in bold. The sequence of the Ikaros2 mutant oligonucleotide is 5′-GAA AAA TAG T**AA AG**G AGA AGA GTC-3′ in which the core sequence was changed to **AAAG**. Probes were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase and purified using Sephadex G25 spin columns. In a 20 μl reaction, GST-Klf7(213-301) or GST-p75ICD proteins were incubated with 6.7 μl Buffer S (75 mM Hepes, pH 7.9, 37.5 mM $MgCl_2$, 30 μM $ZnSO_4$, 60% glycerol and 0.3% Tween 20), 1 μg poly(dI-dC)-poly(dI-dC), 1 μg bovine serum albumin (BSA) and 20,000 cpm of an appropriate probe. The salt concentration was adjusted to 100 mM NaCl for each reaction. Samples were incubated at room temperature for 20 minutes and loaded onto a 4.5% non-denaturing polyacrylamide gel, separated in 0.5× TBE at 120 V for 3 hours. The gel was dried, exposed to Kodak X-OMAT AR films and analyzed by autoradiograph. For competition experiments, cold oligonucleotides were added 15 minutes prior to the addition of the labeled probe.

Yeast transformation and one-hybrid assays

Ikaros2(3×), MZF2(3×) and HAND(3×) oligonucleotides were cloned into the *Eco*RI/*Xba*I sites of the pHisi-1 vector, respectively. The pHisi-1 vector contains the *HIS3* reporter gene downstream of a *HIS3* minimal promoter (Clontech). Each construct was confirmed by DNA sequencing and integrated into the *HIS3* locus of yeast strain YM4271 (Clontech) to generate the reporter strains YM4271-IK, YM4271-MZF or YM4271-HAND. The coding region of *mKlf7* was cloned into the vector pGADT7 (Clontech) to generate the construct pGAD-Klf7 encoding the Gal4 activation domain-Klf7 fusion protein. pGADT7 and pGAD-Klf7 were transformed into YM4271, YM4271-IK, YM4271-MZF or YM4271-HAND and selected on SD-Leu plates. Single colonies from each transformed strain were streaked onto SD-His-Leu plates to test the expression of the *HIS3* reporter gene.

Northern blots and RT-PCR

Total RNA was isolated from adult mouse tissues using TRIZOL (Gibco BRL) following the manufacturer's instruction. An estimated 10 μg of denatured total RNA from each sample was electrophoresed on a 1.5% formaldehyde/agarose gel and transferred to a Hybond N+ nylon membrane (Amersham). Hybridization was performed with the NorthernMax prehybridization/hybridization buffer (Ambion). Probes were generated by random priming with [α-^{32}P]-dCTP (RediPrime kit, Amersham) using as templates cDNAs for *mKlf7* and *G3PDH* (*Gapd* – Mouse Genome Informatics) After the initial hybridization with the *mKlf7*-specific probe and autoradiograph, the blots were stripped of the remaining radioactivity by rinsing the membrane twice with a boiling 0.5% sodium dodecyl sulfate (v/w) solution and rehybridized with a *G3PDH*-specific probe to compare the quantity of RNA loaded.

For RT-PCR, total RNAs isolated from E8.5 embryos or E13.5 DRGs were used as templates to synthesize cDNAs using the Superscript II reverse transcriptase and oligo-dT primers (Gibco-BRL). The PCR program was as follows 1 minute at 94°C, 1 minute at 59°C, 2 minutes at 72°C; repeated for 25 cycles with a final extension of 5 minutes at 72°C. The gene-specific primers used in the PCR reactions were TrkA, sense 5′-TCA GCA CCG AGA GTG ATG TGT GGA GCTT-3′, antisense 5′-GGA TCC TAG CCC AGA ACG TCC AGG TAA CTC GGT-3′; mKlf7, sense 5′-TTT CCT GGC AGT CAT CTG CAC-3′, antisense 5′-GGG TCT GTT TGT TTG TCA GTC TGTC-3′; and G3PDH, sense 5′-ACC ACA GTC CAT GCC ATC AC-3′, antisense 5′-TCC ACC ACC CTG TTG CTG TA-3′. The PCR product of *mKlf7*, from −301 to −52 relative to the start codon, is highly divergent among Klf family members.

Single neuron cDNA library and PCR

E13.5 DRG neurons were dissected and plated as described (Vogel et al., 1995). Neurons were kept in culture for 2 days in media containing either NGF (10 ng/ml) or NT3 (10 pg/ml). The DRG neuronal plates were washed with PBS without Ca^{2+}/Mg^{2+} and single neurons were picked under a microscope and injected into 500 μl PCR tubes each containing 4 μl of ice-cold cDNA lysis buffer. Single-cell cDNA synthesis and amplification were performed and checked as described (Dulac and Axel, 1995). To determine the pattern of gene expression in NGF- or NT3-dependent single neurons, 1 μl of amplified cDNA from each single cell was used as the template for PCR with primers specific for the genes of interest. The PCR program was the same as used in the RT-PCR. PCR products were analyzed on a 1% agarose gel. Sequences for the gene-specific primers were as follows: TrkB, sense 5′-GGA TGG AGA TCA CAG AGG GT-3′, antisense 5′-AGA GGC AAA TGG GTG ACT TG-3′; TrkC, sense 5′-ATG GTG TGA GGT GGG AGG AC-3′, antisense 5′-TTG TAT GTG TAG CAG GCA CT-3′; CGRP (calcitonin gene related peptide), sense 5′-GCA GCC TCC AGG CAG-3′, antisense 5′-GAA GGT CCC TGC GGC G-3′; mKlf7, sense 5′-GCA CAG TGA CGT TGA AAC TGG TG-3′, antisense 5′-TGG TCA GAC CTG GAG AAA CAC CTG-3′; and NCAM (neural cell adhesion molecule), sense 5′-GAA GGA GGG ATG GAC TCC AC-3′, antisense 5′-TTG AAC ACA AGT ATT CTG AC-3′. The primers for TrkA and G3PDH were the same as described previously.

Tissue preparation

Timed pregnant ICR female mice were sacrificed at various stages to obtain embryos. Gestational age was calculated by taking the morning of the appearance of the mother's vaginal plug as E0.5. Embryos of E8.5 to E15.5 were dissected and fixed in 4% paraformaldehyde (PFA) overnight, dehydrated in 30% sucrose and 10 μm-thick sections were cut in a cryostat. For neonatal trunk sections, postpartum (P)0 pups were perfused with 4% PFA, fixed in 4% PFA overnight, dehydrated in 30% sucrose and 10 μm sections were collected. Adult brains were quickly dissected out after the animals were perfused with 4% PFA, fixed overnight with 4% PFA, dehydrated in 30% sucrose and 10 μm sections were collected. Adult trigeminal ganglia were dissected out, fixed overnight with 4% PFA, processed and embedded in paraffin, and 5 μm serial sections were collected using a microtome.

Mice with a targeted deletion in *TrkA* were maintained as described (Liebl et al., 1997). To obtain DRG sections, neonatal pups from the mating of *TrkA* heterozygous mice were perfused with 4% PFA, their spinal columns dissected out, fixed in Bouin's solution overnight, washed extensively with 70% ethanol, processed and embedded in paraffin. Sections (10 μm) were collected using a microtome. The tail DNA from each pup was used for genotyping by Southern blots (data not shown).

In situ hybridization

Cryostat or paraffin sections were processed for in situ hybridization as described (Martin-Zanca et al., 1990; Nef et al., 1996). [α-^{35}S]-CTP-labeled radioactive antisense cRNAs were produced by in vitro transcription with the T7 RNA polymerase using the Riboprobe Combination System (Promega). *mKlf7* antisense cRNA probe was synthesized using as a template the 1.3 kb fragment comprising the entire cDNA shown in Fig. 1A. *TrkA* antisense cRNA probe was synthesized using a 454 bp fragment encoding the mouse TrkA extracellular domain as the template. After in situ hybridization, slides were counterstained with Hematoxylin for bright field views. Both bright-field and dark-field micrographs were taken using a digital camera. In Fig. 7, the sections were digitally photographed using phase-contrast and dark-field microscopy sequentially, and the dark field images were processed in Adobe Photoshop to display the in situ signals as green particles. The dark field images were then superimposed onto the phase-contrast images to show the cellular morphology and the localization of the in situ signals.

RESULTS

Cloning of *mKlf7*

Several consensus *cis* regulatory elements within the mouse *TrkA* enhancer are crucial for its specific activity in sensory and sympathetic neurons during mouse embryogenesis (Ma et al., 2000). These *cis* elements include, among others, two Ikaros sites (Ikaros1 and Ikaros2), two MZF sites (MZF1 and MZF2) and a HAND site. To identify transcription factors present in relevant tissues that may directly bind these DNA elements and potentially regulate endogenous *TrkA* expression, we screened a mouse E13.5 DRG cDNA expression library using a mixture of three [^{32}P]-labeled oligonucleotide probes, each containing three copies of the Ikaros2, MZF2 or HAND consensus binding sites. After three rounds of hybridization, six positive lambda phage clones were identified. Sequence analysis of the cDNA inserts indicated two identical clones containing a 903

base pair open reading frame (ORF) preceded by termination codons. This ORF encodes a novel murine Kruppel-like zinc-finger transcription factor (Fig. 1A). The overall amino acid sequence identity between this protein and human UKLF, also known as KLF7, is over 97% (Matsumoto et al., 1998; Turner and Crossley, 1999). Therefore, we named this murine gene *mKlf7*, based on the significant sequence conservation.

mKlf7 protein has a calculated molecular weight of 33.3 kDa and an estimated isoelectic point (pI) of 8.82. Similar to UKLF, the primary structure of mKlf7 protein consists of three distinct domains (Fig. 1B). The N-terminal region (amino acid residues 1-76) is negatively charged and enriched in serines and glutamic acids. This region is identical to the N-terminal region of human UKLF, which was shown to be a transactivation domain (Matsumoto et al., 1998). The central region of mKlf7 (residues 77-211) is enriched in hydrophobic residues and serines, which may serve as a protein interaction domain. The amino acid identity between mKlf7 and UKLF in this region is 94.8%. A putative leucine-zipper motif (residues 98-119) is conserved in both UKLF and mKlf7. The C-terminal region of mKlf7 (residues 212-301) is a potential DNA-binding domain consisting of three zinc fingers of the Cys_2-His_2 type. This region is identical in both mKlf7 and UKLF. The nuclear localization signal KKRVHR identified in human UKLF is also conserved in mKlf7 (residues 214-219), just upstream of the first zinc finger (Matsumoto et al., 1998).

mKlf7 is a nuclear protein that binds the Ikaros2 site on the *TrkA* enhancer

To test whether the *mKlf7* gene encodes a nuclear protein, the full-length *mKlf7* cDNA was fused in frame with a DNA fragment encoding the green fluorescent protein (GFP). Cellular localization of the GFP-Klf7 fusion protein was monitored by GFP fluorescence and DAPI staining of transfected HEK293 and NG108 cells (Fig. 2). GFP-Klf7 proteins accumulated exclusively in the nuclei of both HEK293 and NG108 cells after transfection, while control GFP proteins were detected in the entire cell bodies of transfected cells. These data confirm that mKlf7 is a nuclear protein.

mKlf7 was initially cloned by screening an embryonic DRG expression library using a mixture of three probes including Ikaros2, MZF2 and HAND binding sites. To determine which *cis* element binds mKlf7, GST-Klf7C, which contains the C-terminal DNA-binding domain (amino acid residues 213-301) of mKlf7 fused in frame with GST (glutathione-S-transferase), was expressed and purified from *E. coli* (data not shown). Recombinant proteins GST-Klf7C or GST-p75ICD (GST-p75 intracellular domain) as a control, were incubated with Ikaros2, MZF2 or HAND probes in gel shift reactions (Fig. 3A). As shown, GST-KLF7C binds specifically to the Ikaros2 site but not to either the MZF2 site or the HAND site. Similar to GST-p75ICD, several additional proteins including GST and GST-Brn3a, fail to bind these probes (data not shown). Thus, mKlf7 exhibits specificity among the three functional sites in the *TrkA* enhancer, even though both Ikaros2 and MZF2 sites are conserved binding sites for transcription factors containing Kruppel-type zinc fingers in

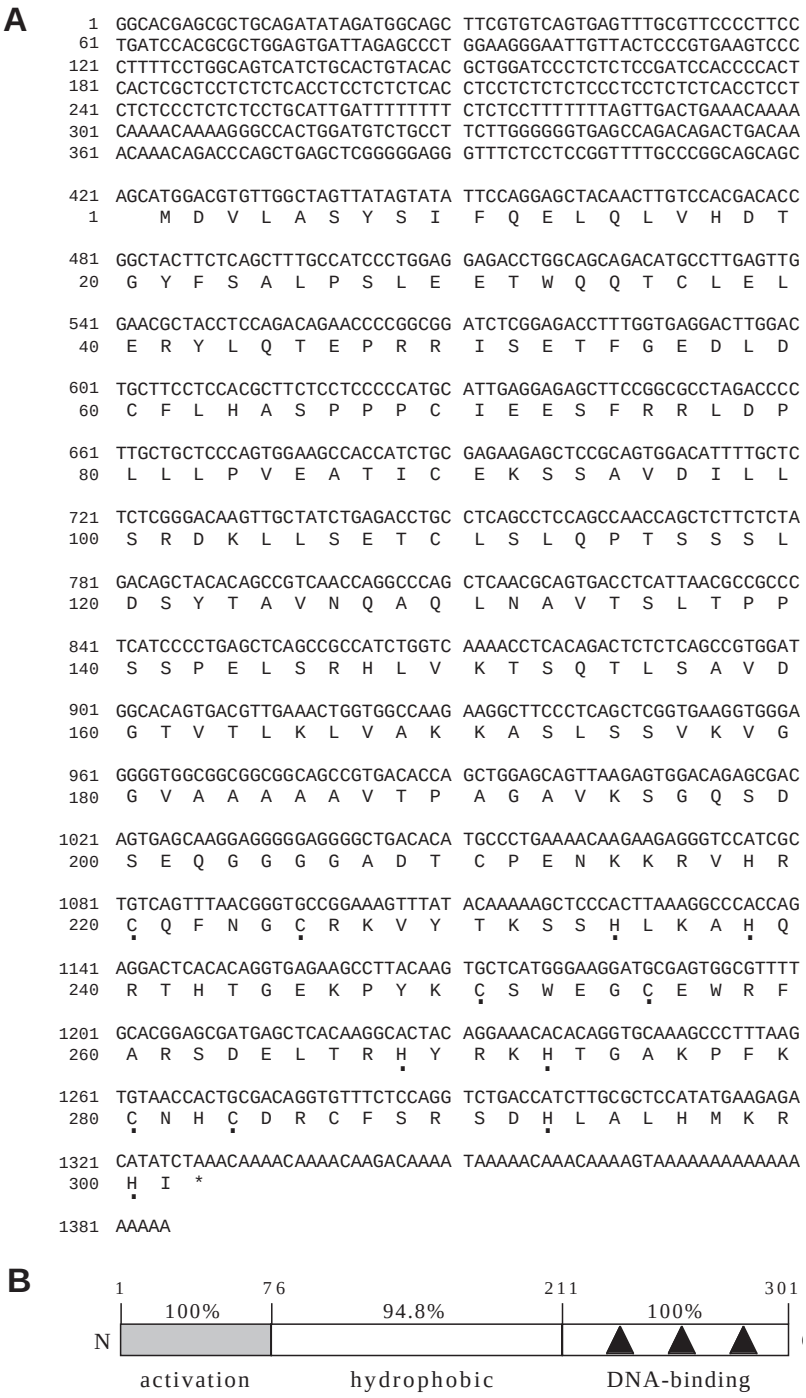

Fig. 1. Sequence analysis of *mKlf7* cDNA and protein. (A) Nucleotide and deduced amino acid sequences. Cysteines and histidines of the zinc fingers are highlighted by black circles underneath. (B) Domain structure of the mKlf7 protein. The triangles indicate the three zinc fingers of the C-terminal DNA-binding domain. The amino acid identity between mKlf7 and human UKLF for each region is also shown. The sequence of *mKlf7* gene has been deposited in the GenBank (Accession Number, AF338369).

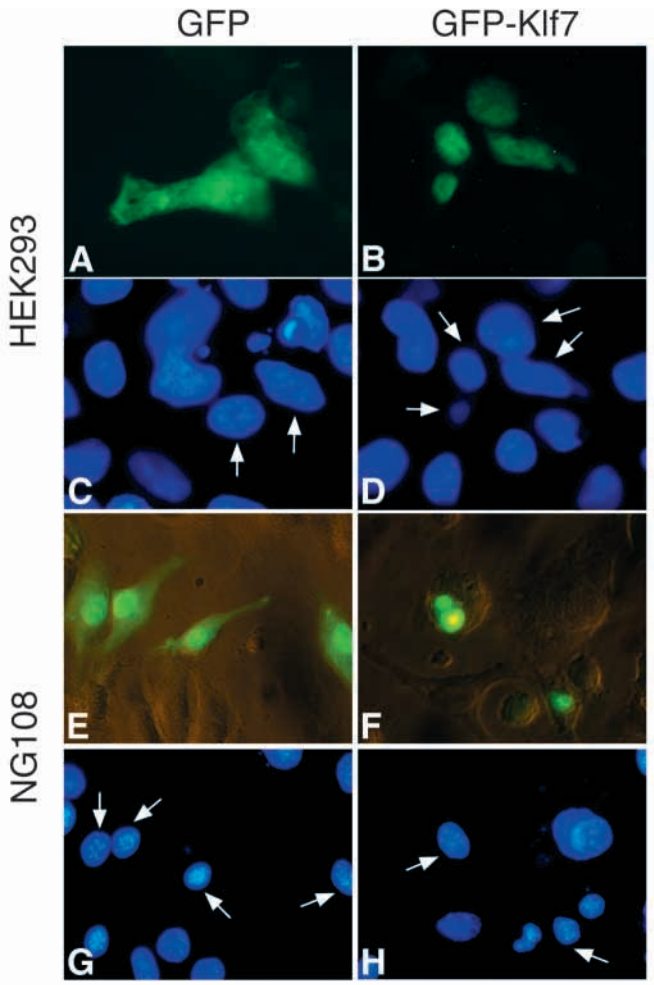

Fig. 2. Nuclear localization of mKlf7. (A,C,E,G) Cells transfected with pEGFP-C2. (B,D,F,H) Cells transfected with pEGFP-Klf7. (A,B) GFP autofluorescence, (C,D,G,H) DAPI staining of the nuclei, and (E,F) the combination of GFP autofluorescence and light microscopy are shown. The arrows indicate the nuclei of corresponding GFP-positive cells. Cell lines are indicated on the left-hand side of the figure.

their DNA-binding domains (Georgopoulos et al., 1992; Georgopoulos et al., 1997; Hromas et al., 1991). Furthermore, unlabeled oligonucleotides containing a wild-type Ikaros2 site, but not an MZF2 site, effectively competes with the Ikaros2 probe for binding GST-Klf7C (Fig. 3B). In contrast, Ikaros2 oligonucleotides harboring mutations in the Ikaros2 core sequence that has previously been shown to disrupt in vivo function of the *TrkA* enhancer (Ma et al., 2000), failed to compete for binding GST-Klf7C (Fig. 3B). These results indicate that the zinc-finger domain of mKlf7 is capable of binding the wild-type Ikaros2 site in vitro. Moreover, the decreased affinity between mKlf7 and the mutant Ikaros site probably accounts for the reduced activity observed for a *TrkA* enhancer bearing the same mutated Ikaros2 site in vivo.

To further establish the interaction of mKlf7 with the Ikaros2 consensus sequence, a yeast one-hybrid approach was used (Fig. 3C). Triple copies of either Ikaros2, MZF2 or HAND sequences were cloned upstream of a minimal promoter-driven *HIS3* reporter gene and the resulting reporter plasmids were integrated into the host strain YM4271. Plasmids encoding either the GAL4 activation domain (Gal4AD) alone or the Gal4AD-Klf7 fusion protein were transformed into these reporter strains and activation of the *HIS3* gene was analyzed by growth on SD-Leu-His plates. Gal4AD-Klf7 was able only to specifically activate expression of *HIS3* from the Ikaros2 site. Both the MZF2-driven reporter and the HAND-driven reporter exhibited 'leaky' expression in the presence of Gal4AD. However, no additional activation was detected in the presence of Gal4AD-Klf7. These results are consistent with the gel shift data and further support that mKlf7 binds the Ikaros2 site of the *TrkA* enhancer both in vitro and in vivo.

mKlf7 expression is neural specific and coincides with *TrkA*

TrkA is specifically expressed in neural crest-derived sensory and sympathetic ganglia during development (Martin-Zanca et al., 1990; Phillips and Armanini, 1996; Schecterson and Bothwell, 1992; Wright and Snider, 1995). Expression of *TrkA* in DRG begins at E9.5, peaks at E13.5, and remains constant during late development (Martin-Zanca et al., 1990). Transcription activators of the *TrkA* gene must be expressed in these tissues with appropriate timing. To examine whether mKlf7 could be a physiologically relevant transcription factor for regulating *TrkA* expression in vivo, expression of *mKlf7* was analyzed by in situ hybridization (Fig. 4). Similar to *TrkA* at E13.5, *mKlf7* was strongly and specifically expressed in dorsal root, trigeminal and superior XI/X complex ganglia (superior and jugular ganglia). Expression of *mKlf7* at this stage was also apparent in the CNS including developing brain and spinal cord.

One key feature of a putative *TrkA* regulatory gene would be that its expression precedes the onset of *TrkA* expression. We therefore compared the expression patterns of *mKlf7* and *TrkA* during early embryogenesis (Fig. 5). The earliest expression of *TrkA* has been reported at E9.5 (Martin-Zanca et al., 1990). We observed expression of *mKlf7* at E8.5, preceding that of *TrkA* by in situ hybridization (data not shown) and by RT-PCR (Fig. 5A). *mKlf7* transcripts were present at E8.5 when *TrkA* transcripts could not be detected. Fig. 5B-I shows oblique transverse sections of an E10.5 embryo and sagittal sections of an E11.5 embryo. At E10.5, *mKlf7* is expressed in trigeminal ganglion, the VII-VIII neural crest complex, and the subventricular neuroepithelium of both forebrain and hindbrain regions. *TrkA* expression is also apparent in trigeminal ganglion, the VII-VIII neural crest complex, and the subventricular neuroepithelium of the hindbrain. At E11.5, the expression of *TrkA* and *mKlf7* is stabilized in the neural crest derived sensory nervous system (compare Fig. 4 with Fig. 5). Thus *mKlf7* gene expression coincides with that of *TrkA* in neural crest-derived structures as well as in the neuroepithelium of the hindbrain, an area of *TrkA* expression that has not been previously described.

With progressing development, *TrkA* expression becomes restricted with no detectable expression in neuroepithelium after E10.5 and reduced expression in maturing cranial Ganglia VII and VIII (Fig. 6A,B). At E15.5, *mKlf7* exhibits continued coexpression with *TrkA* but also retains expression in additional neural regions including brain, spinal cord, retinal neuroepithelium and the inferior XI/X complex (nodose and petrosal ganglia) (Fig. 6C,D).

An additional functionally prominent area of *TrkA* expression is in sympathetic ganglia that acquire NGF/TrkA dependence for

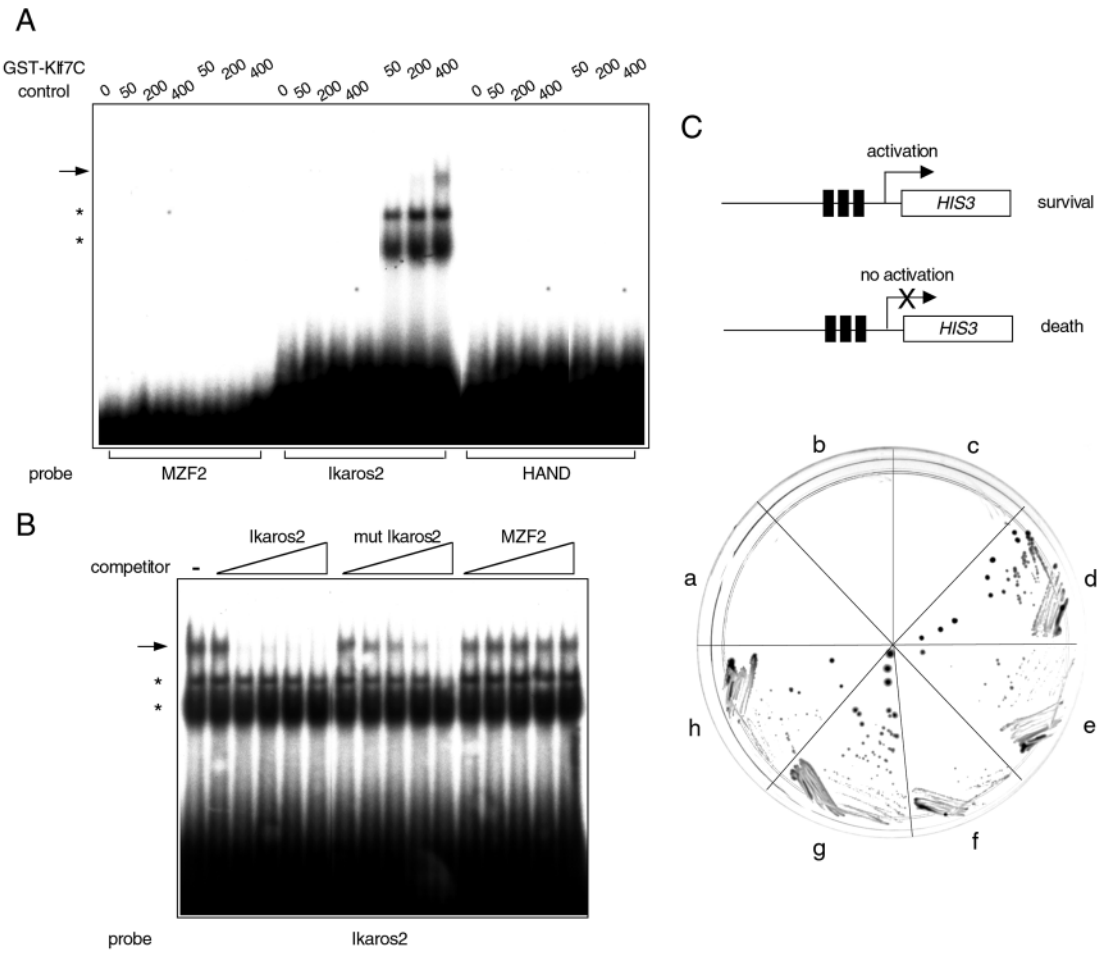

Fig. 3. mKlf7 binds to the Ikaros2 site of the *TrkA* minimal enhancer. (A) Increasing amounts (in ng) of purified GST-Klf7C or GST-p75ICD ('control') were incubated with the oligonucleotide probe containing either an Ikaros2 site, an MZF2 site, or a HAND site and analyzed by EMSA. (B) Increasing amounts (1×, 5×, 10×, 20× and 100×) of cold oligonucleotides containing either a wild-type Ikaros2 site, a mutant Ikaros2 site ('mut Ikaros'), or a wild-type MZF2 site were added to the gel shift reactions to compete with the wild-type Ikaros2 probe for binding GST-Klf7C. The arrows in A,B indicate the specific DNA-protein complex and the asterisks indicate nonspecific complexes. (C) Single colonies from various yeast reporter strains containing the plasmid pGADT7 or pGAD-Klf7 were streaked on SD-Leu-His plates to test expression of the *HIS3* reporter gene. (Part a) YM4271 transformed with pGADT7; (Part b) YM4271 with pGAD-Klf7; (part c) YM4271-IK with pGADT7; (part d) YM4271-IK with pGAD-Klf7; (part e) YM4271-MZF with pGADT7; (part f) YM4271-MZF with pGAD-Klf7; (part g) YM4271-HAND with pGADT7; and (part h) YM4271-HAND with pGAD-Klf7.

survival in late development (Crowley et al., 1994; Levi-Montalcini, 1987; Smeyne et al., 1994). We found that *mKlf7* was coexpressed with *TrkA* in sympathetic ganglia at P0 (Fig. 6E-H).

TrkA expression is maintained in adult neural crest-derived sensory and sympathetic ganglia (Kaplan et al., 1991; Phillips and Armanini, 1996). To examine whether *mKlf7* coexpression is also retained, serial sections from trigeminal ganglia of adult mice were hybridized with *TrkA* and *mKlf7* probes, respectively (Fig. 7A,B). The expression patterns suggest that *mKlf7* is coexpressed with *TrkA* in sensory nociceptive neurons. In addition, *mKlf7* is apparently expressed in all other neurons that do not express *TrkA*, but not in Schwann cells. Indeed, similar experiments using *TrkB*- (*Ntrk2* – Mouse Genome Informatics) and *TrkC*- (*Ntrk3* – Mouse Genome Informatics) specific probes demonstrate that *mKlf7* is also expressed in TrkB-positive neurons and TrkC-positive neurons (data not shown). An alternative way to examine the scope of *mKlf7* expression in sensory ganglia was to generate single cell cDNA libraries from DRG neurons cultured in either NGF or NT3. In culture, NGF supports the survival of TrkA-expressing nociceptive neurons, while NT3 supports only TrkC-expressing proprioceptive neurons (Liebl et al., 1997). As shown in Fig. 7C, *G3PDH* and a panneuronal marker *NCAM* (*Ncam* – Mouse Genome Informatics) are detected in all neuronal libraries but not in the control libraries, while *TrkA* and *TrkC* expression display appropriate NGF or NT3 dependence, further validating the reliability and specificity of this approach. CGRP is a marker for TrkA-positive neurons. *mKlf7* transcripts are present not only in NGF-dependent, TrkA- and CGRP-positive neurons, but also in NT3-dependent, TrkC-expressing neurons.

To gain clear confirmation about the scope of *mKlf7* expression in sensory neurons, compared with *TrkA*, we made use of *TrkA*-null mice that lose all TrkA-expressing, NGF-dependent sensory neurons (Smeyne et al., 1994). *TrkA* expression is completely abolished in *TrkA*-null DRG whereas *mKlf7* expression is

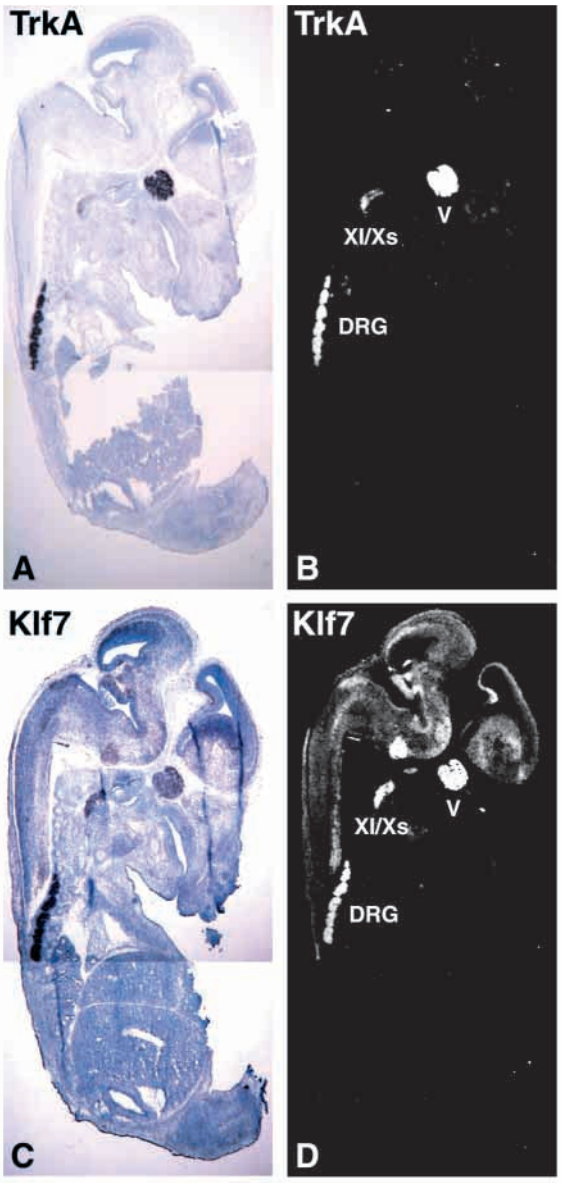

Fig. 4. Expression of *mKlf7* and *TrkA* genes at E13.5. Sagittal sections of an E13.5 embryo hybridized with the TrkA probe (A,B) or mKlf7 probe (C,D). Both bright field images (A,C) and dark field images (B,D) are shown. DRG, dorsal root ganglion; V, trigeminal ganglion (the fifth ganglion); XI/Xs, ninth/tenth superior complex.

retained at considerably reduced levels (Fig. 8). Consistent with Fig. 7, these data lead us to conclude that *mKlf7* is expressed in all DRG and trigeminal sensory neurons including TrkA-positive neurons.

mKlf7 is not coexpressed with *TrkA* in the adult CNS

We next examined *mKlf7* expression in adult tissues by northern blot analysis. *mKlf7* transcripts are detected in brain, heart, spleen and lung but not in liver, skeletal muscle, kidney or testis (Fig. 9A, and data not shown). The size of the *mKlf7* mRNA is approximately 8.5 kb, similar to that of human *UKLF* mRNA. Fig. 9B demonstrates that *mKlf7* transcripts are present in various regions of adult brain and spinal cord. To gain a better understanding of *mKlf7* expression in adult brain and to examine whether coexpression with *TrkA* is maintained in the CNS, we performed comparative in situ hybridization (Fig. 9C-F). As previously described (Holtzman et al., 1992; Sobreviela et al., 1994), cholinergic neurons of the basal forebrain express *TrkA* (Fig. 9C,E). The identity of these TrkA-expressing cells was confirmed by positive staining with anti-ChAT (choline acetyltransferase) antibodies (data not shown). However, *mKlf7* expression is not detected in this region (Fig. 9D,F). The lack of *mKlf7* expression in mature basal forebrain cholinergic neurons implies that mKlf7 may not be involved in the expression of *TrkA* in the CNS. However, it remains to be determined whether *mKlf7* is present in basal forebrain cholinergic neurons during the generation and early maturation of these cells.

Consistent with the northern blot analysis (Fig. 9B), *mKlf7* is detected in many areas of the CNS, including the cortex, hippocampus and cerebellum by in situ hybridization (Fig. 9G,H). As in the PNS, *mKlf7* is mostly, if not exclusively, expressed in neurons in the CNS. For example, in cortex (Fig. 9G), *mKlf7* is expressed strongly in neuronal layers II-VI but weakly in layer I, which contains mostly non-neuronal cells. In the cerebellum, the *mKlf7* transcript is abundant in granular neurons but absent in the white matter (Fig. 9H).

Taken together, the present results identify mKlf7 as a *TrkA* enhancer-binding protein that is confined to the nervous system during development. In both developing and mature nervous systems, *mKlf7* gene is expressed primarily in neurons, and in all *TrkA*-expressing neurons in the PNS.

DISCUSSION

mKlf7 is a *TrkA* enhancer-binding protein

Although the highly stereotypic expression of the TrkA-NGF receptor was reported more than a decade ago (Martin-Zanca et al., 1990), until recently, little was known about the molecular basis for regulation of its expression (Ma et al., 2000). Even less information has been available about the transcription factors that regulate such temporal- and spatial-specific expression. Brn3a is the only transcription factor previously identified to modulate *TrkA* expression (Huang et al., 1999). The available information implicates *Brn3a* in maintenance of *TrkA* gene expression in sensory neurons but not in initiation of expression, as *TrkA* expression initiates normally in *Brn3a* knockout mice (Huang et al., 1999). In addition, no consensus Brn3a-binding site is present in the *TrkA* minimal enhancer that is sufficient for driving specific expression of *TrkA* in sensory and sympathetic neurons (Ma et al., 2000).

Other candidate genes for regulating *TrkA* expression may include transcription factors that are important for the generation and differentiation of sensory and sympathetic neurons that eventually become NGF dependent for their survival. Several bHLH genes including *Mash1* (*Ascl1* – Mouse Genome Informatics) neurogenin 1 (*Ngn1*; *Neurod3* – Mouse Genome Informatics), neurogenin 2 (*Ngn2*; *Atoh4* – Mouse Genome Informatics), and the homeobox gene *Phox2b* (*Pmx2b* – Mouse Genome Informatics) are essential for the generation of sensory or sympathetic neurons (Fode et al., 1998; Guillemot et al., 1993; Ma et al., 1998; Ma et al., 1999; Pattyn et al., 1999). *Mash1* is expressed in sympathetic precursors and no sympathetic neurons are generated in

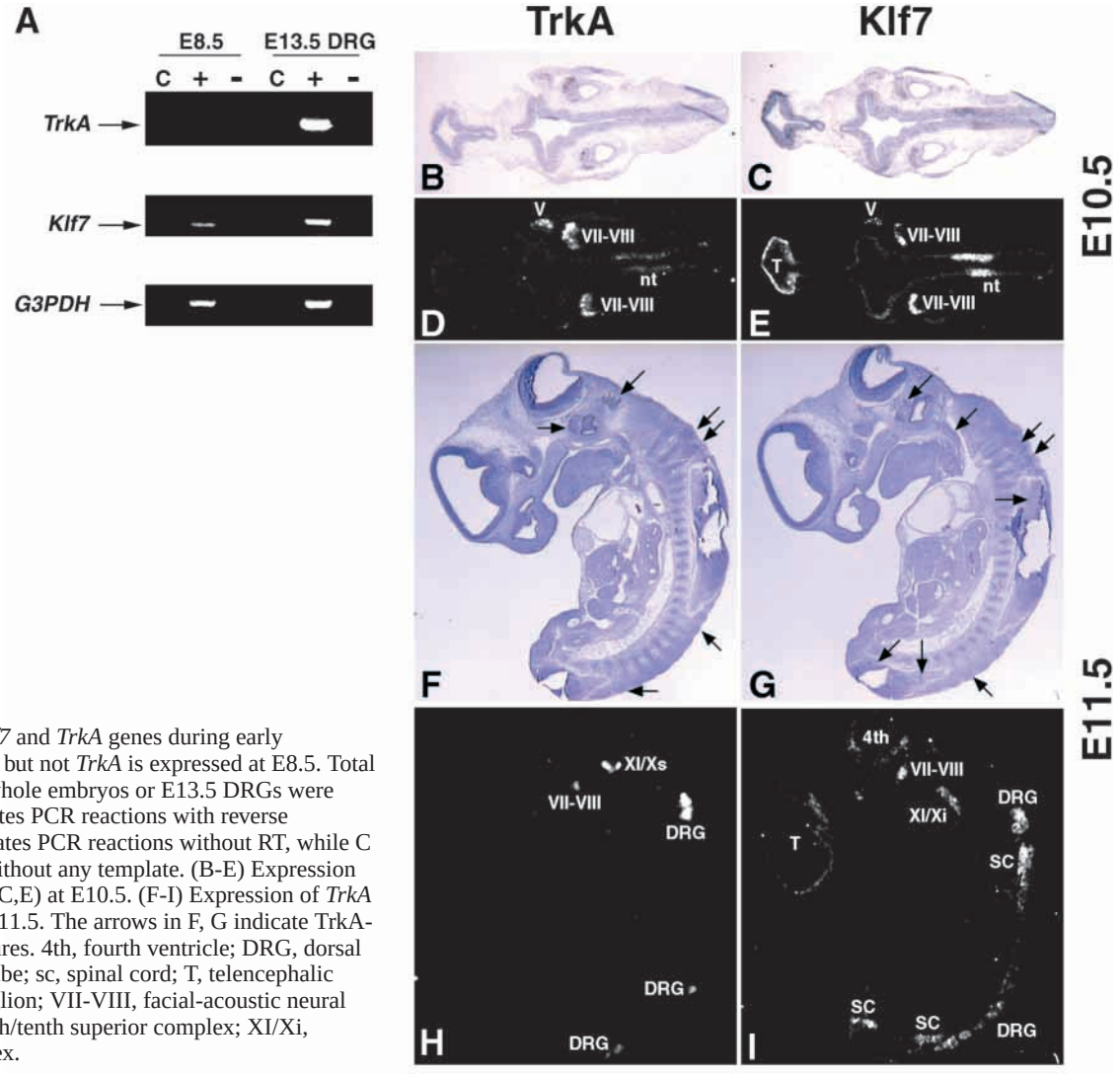

Fig. 5. Expression of *mKlf7* and *TrkA* genes during early embryogenesis. (A) *mKlf7* but not *TrkA* is expressed at E8.5. Total RNA isolated from E8.5 whole embryos or E13.5 DRGs were used for RT-PCR. + indicates PCR reactions with reverse transcription (RT), – indicates PCR reactions without RT, while C indicates PCR reactions without any template. (B-E) Expression of *TrkA* (B,D) and *mKlf7* (C,E) at E10.5. (F-I) Expression of *TrkA* (F,H) and *mKlf7* (G,I) at E11.5. The arrows in F, G indicate TrkA- and mKlf7-positive structures. 4th, fourth ventricle; DRG, dorsal root ganglion; nt, neural tube; sc, spinal cord; T, telencephalic vesicle; V, trigeminal ganglion; VII-VIII, facial-acoustic neural crest complex; XI/Xs, ninth/tenth superior complex; XI/Xi, ninth/tenth inferior complex.

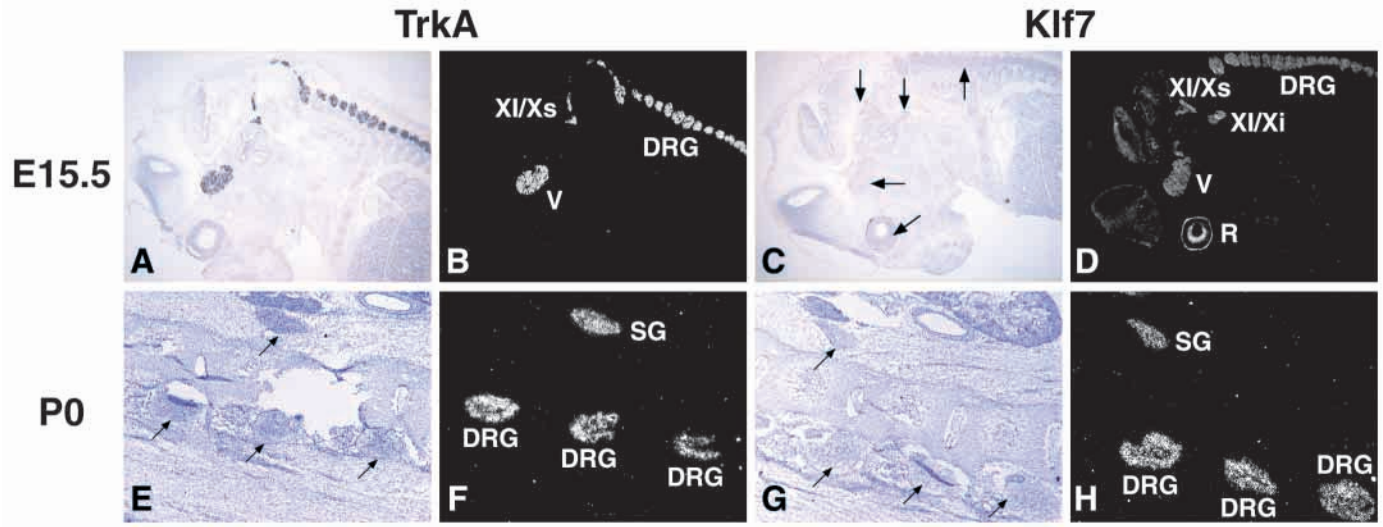

Fig. 6. Expression of *mKlf7* and *TrkA* genes during late embryogenesis. (A-D) Sagittal sections of an E15.5 embryo hybridized with the TrkA probe (A,B) or mKlf7 probe (C,D). Only the head region of each section was shown. (E-H) Sagittal sections of a neonatal pup hybridized with the TrkA probe (E,F) or mKlf7 probe (G,H). The trunk region of each section was shown. The arrows in C, E, G indicate TrkA- and mKlf7-positive ganglionic structures. DRG, dorsal root ganglion; R, retinal neuroepithelium; SG, sympathetic ganglion; V, trigeminal ganglion; XI/Xs, ninth/tenth superior complex; XI/Xi, ninth/tenth inferior complex.

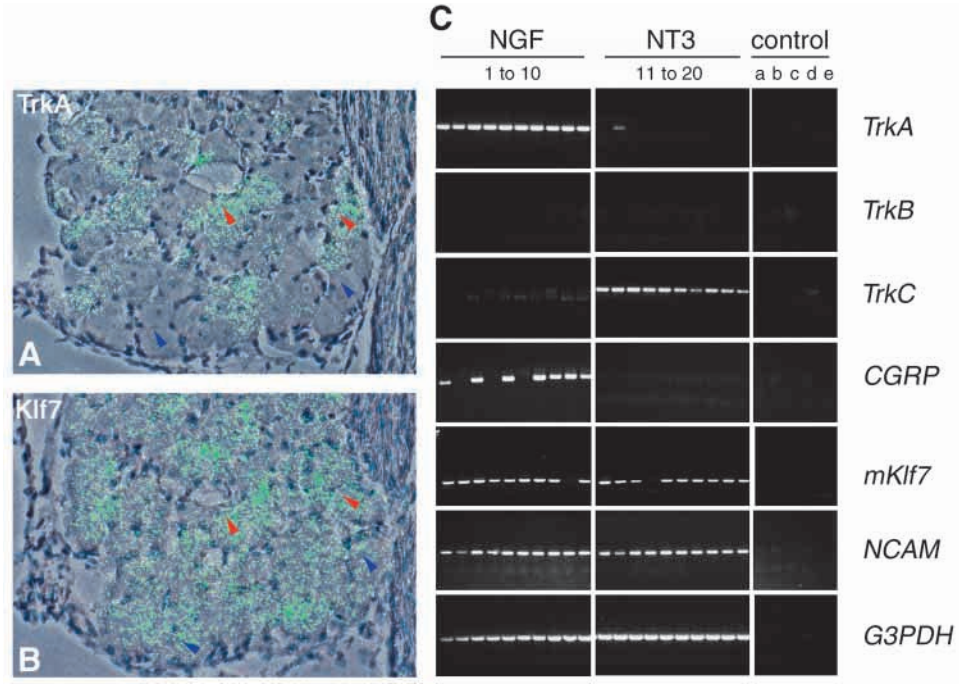

Fig. 7. Coexpression of *mKlf7* and *TrkA* in sensory neurons. (A,B) Adjacent sections from an adult trigeminal ganglion hybridized with TrkA (A) or mKlf7 (B) probes. The in situ signals were shown as green particles. The red arrowheads indicate two representative neurons expressing both *TrkA* and *mKlf7* while the blue arrowheads indicate two representative neurons expressing *mKlf7* but not *TrkA*. (C) *mKlf7* is expressed in both TrkA-positive neurons and TrkC-positive neurons. Single cell cDNA libraries were prepared from NGF- or NT3-dependent DRG neurons and expression of *mKlf7* and other marker genes in these single neurons was analyzed by PCR. PCR reactions with no template (e) or with libraries prepared in the absence of any cell as the templates (a-d) were used as controls.

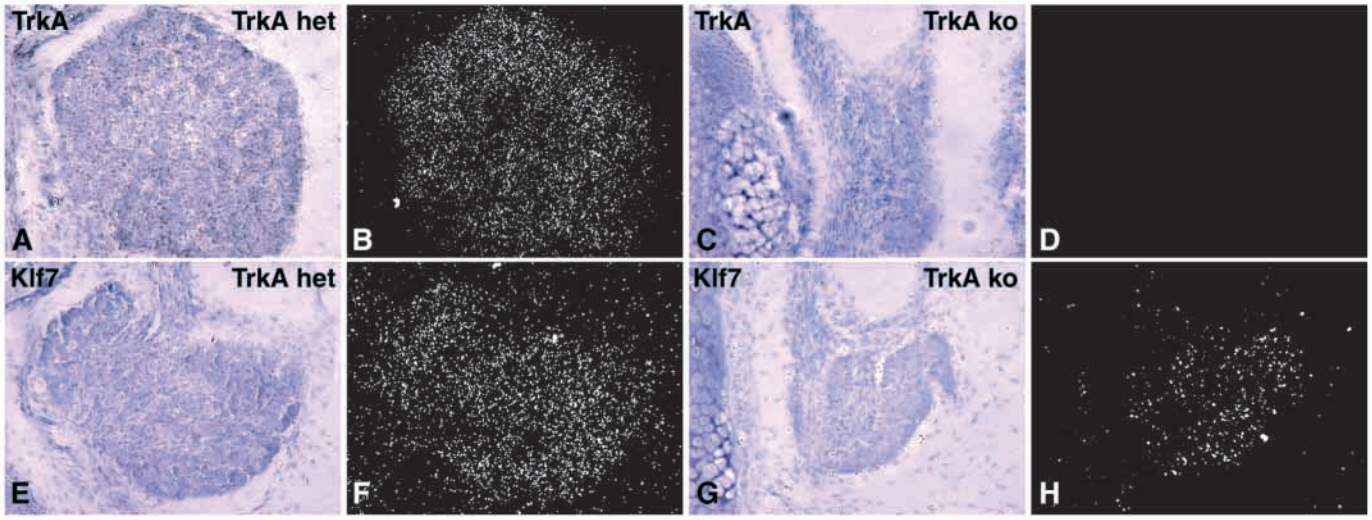

Fig. 8. *mKlf7* is expressed in TrkA-positive neurons in the DRG of P0 mice. Sections of spinal columns from *TrkA*-heterozygous (A,B,E,F) or *TrkA*-null (C,D,G,H) neonatal pups were hybridized with the TrkA probe (A-D) or mKlf7 probe (E-H). A single DRG is shown in each panel.

Mash1-null mice (Guillemot et al., 1993; Johnson et al., 1990; Lo et al., 1991). Sympathetic development in these mutant mice is arrested at embryonic day 10.5 (E10.5) (Guillemot et al., 1993). Similarly, *Phox2b* is expressed in sympathetic ganglia during early development and no sympathetic neurons are formed in *Phox2b* null mice at E13.5 (Pattyn et al., 1999). Because *TrkA* is not expressed in wild-type sympathetic neurons until E17, the failure of sympathetic neuron generation in *Mash1*- and *Phox2b*-null mice must occur in a *TrkA*-independent manner. Furthermore, neural crest-derived sensory neurons are normal in *Mash1*- and *Phox2b*-null mice (Guillemot et al., 1993; Pattyn et al., 1999). These results suggest that it is unlikely that either *Mash1* or *Phox2b* regulates *TrkA* expression. *Ngn1* and *Ngn2* are expressed in sensory ganglia during early gangliogenesis (Ma et al., 1999). In DRG, *Ngn2* is transiently expressed from E8.75 to E10.5, while *Ngn1* is expressed from E9 to E13, overlapping with the generation of TrkA-positive DRG neurons. *Ngn1* is required for generation of TrkA-positive neurons, whereas *Ngn2* is required for the generation of TrkB-positive and TrkC-positive neurons (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999). It is possible that the loss of TrkA-positive DRG neurons in *Ngn1*-null mice occurs in a TrkA-independent fashion and therefore the apparent lack of *TrkA* expression in these mutant mice may be a consequence of general neuronal death rather than transcriptional downregulation of *TrkA*. To date, no compelling data exist to determine whether *Ngn1* has a direct or indirect impact on TrkA expression.

The bHLH gene *NeuroD* (*Neurod1* – Mouse Genome Informatics) is also expressed in DRG and trigeminal ganglia

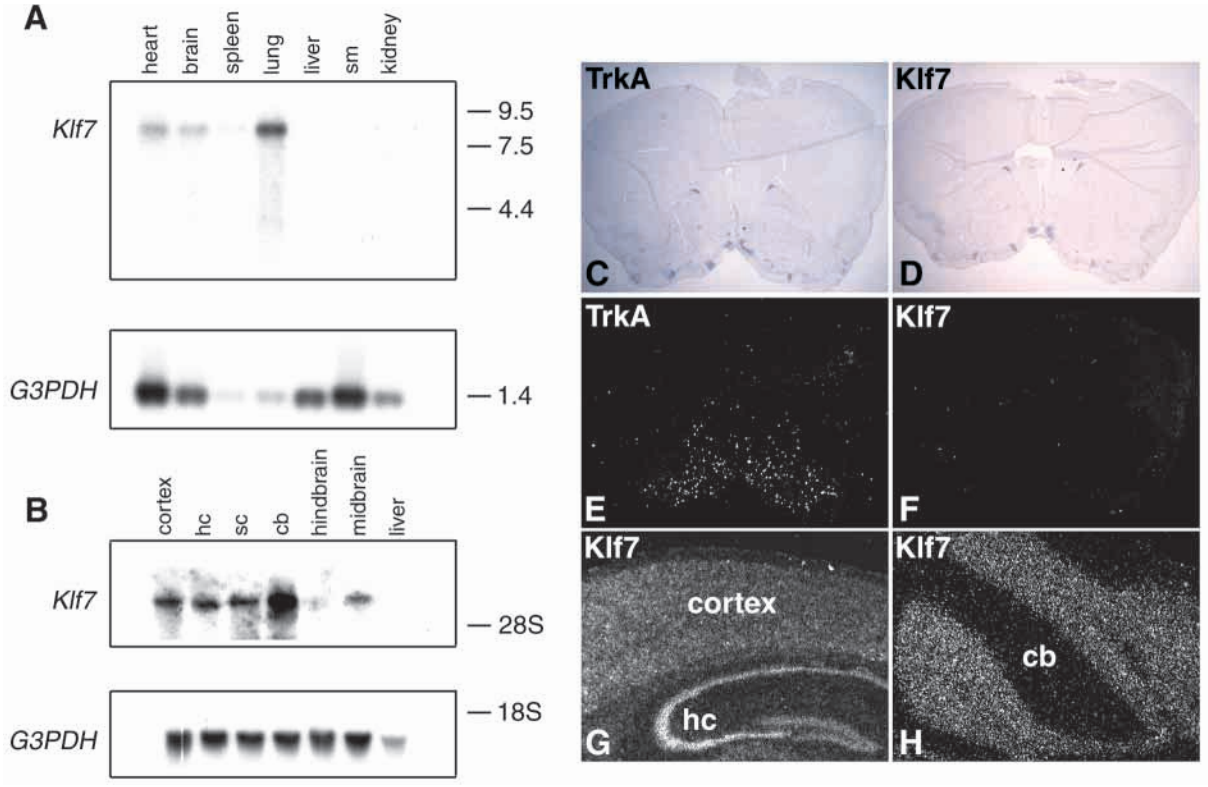

Fig. 9. Expression of *mKlf7* in adult tissues. (A) Northern blot analysis of adult mouse tissues. The molecular weight of *mKlf7* mRNA was estimated using an RNA size marker (indicated to the right). (B) Northern blot analysis of various regions of the adult CNS. The positions of 28S and 18S rRNA are indicated. (C-F) Adult brain coronal sections hybridized with the TrkA probe (C,E) or the mKlf7 probe (D,F). (G,H) Dark field pictures of adult brain coronal sections hybridized with the mKlf7 probe. cb, cerebellum; hc, hippocampus; sc, spinal cord; sm, skeletal muscle.

during early development (Lee et al., 1995). *NeuroD* is implicated in differentiation of neurons and pancreatic beta cells and *NeuroD*-null mice die shortly after birth, owing to severe neonatal diabetes (Lee et al., 1995; Naya et al., 1997). It is unknown whether sensory neurons depend on *NeuroD* for differentiation, although *NeuroD* is required for differentiation of cerebellar and hippocampal granular neurons (Miyata et al., 1999). Another bHLH gene *dHAND* (*Hand2* – Mouse Genome Informatics) is expressed in sympathetic neurons during early development (Srivastava et al., 1995). *dHAND* is sufficient to induce formation of sympathetic neurons when it is expressed ectopically in chick embryos using retroviruses (Howard et al., 2000). However, it is unknown whether *TrkA* is regulated by *dHAND*, although our previous functional analysis indicates a role for HAND-related transcriptional factors in *TrkA* regulation (Ma et al., 2000).

In this study, mKlf7 was identified as a *TrkA* enhancer-binding protein. The N-terminal domain of human UKLF, identical to the same region of mKlf7, is a potent transactivation domain (Matsumoto et al., 1998). Although both Ikaros2 and MZF2 sites share similar core sequences for transcription factors containing Kruppel-type zinc fingers (Georgopoulos et al., 1992; Georgopoulos et al., 1997; Hromas et al., 1991), mKlf7 does not interact with the MZF2 site. This is probably due to the diverging sequences flanking the core binding sites. This result demonstrates the specificity of the interaction between mKlf7 and the Ikaros2 *cis* element. Moreover, the decreased interaction between mKlf7 protein and the mutant Ikaros2 site is consistent with the severely decreased activity observed for the *TrkA* enhancer bearing the same mutation in vivo (Ma et al., 2000). Apparently, mutations introduced into the core sequence of the Ikaros2 *cis* element did not completely disrupt the interaction between mKlf7 and the mutated binding site (Fig. 3B). Therefore, it is possible that the *TrkA* enhancer bearing mutations that abolish the interaction between the Ikaros2 *cis* element and mKlf7 may be completely inactive in vivo.

mKlf7 is restricted in the developing nervous system

Kruppel-like factors constitute a family of transcription factors defined by their conserved C-terminal DNA-binding domains, each consisting of three Cys_2His_2 type of zinc fingers that are also present in the *Drosophila melanogaster* developmental regulator Kruppel (Schuh et al., 1986; Turner and Crossley, 1999). The GC-box-binding protein Sp1 was the first mammalian Kruppel-like factor identified (Kadonaga et al., 1987). So far, at least 18 different mammalian Kruppel-like factors have been reported (Turner and Crossley, 1999; Uchida et al., 2000). Related transcription factors have also been found in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Turner and Crossley, 1999). Many transcription factors containing Kruppel-type zinc fingers have also been reported but not included in the Kruppel-like factor gene family because of the different number and location of the zinc fingers in these proteins.

Although similar in their DNA-binding domains, Kruppel-like factors are divergent in other regions. Furthermore, they

differ in their expression patterns in vivo (Turner and Crossley, 1999). This family of transcription factors plays important roles in the control of tissue-specific genes as well as ubiquitously expressed 'housekeeping' genes. The physiological functions of several Kruppel-like factors have been directly demonstrated by gene knockout studies. *Sp1* is widely expressed and *Sp1*-null mice are embryonic lethal with reduced expression of target genes encoding thymidine kinase and methyl-CpG-binding protein MeCP2 (Kadonaga et al., 1987; Marin et al., 1997). *EKLF* (erythroid Kruppel-like factor; *Klf1* – Mouse Genome Informatics) is highly expressed in erythropoetic tissues and *EKLF*-null mice develop fatal anemia (Perkins et al., 1995). EKLF is an activator of the β-globin locus control region and essential for the erythropoietin-induced hemoglobin production in vivo (Gillemans et al., 1998; Spadaccini et al., 1998). *LKLF* (lung Kruppel-like factor; *Klf2* – Mouse Genome Informatics) is expressed at high levels in lung but also abundant in many other tissues. Besides being required for lung development, LKLF is also important for T-cell survival and blood vessel formation (Kuo et al., 1997a; Kuo et al., 1997b; Wani et al., 1999). *GKLF* (gut-enriched Kruppel-like factor; *Klf4* – Mouse Genome Informatics) is expressed in epithelial cells of the epidermis and essential for the barrier function of the skin (Segre et al., 1999; Shields et al., 1996). Therefore, the phenotypes of the Klf gene family knockout mice are consistent with the predominant expression sites of these genes in vivo.

Although *UKLF*, the human homolog of *mKlf7*, is widely expressed in all human tissues examined (Matsumoto et al., 1998), our study identifies *mKlf7* as the first mammalian Kruppel-like factor that is specifically expressed in the nervous system during development.

mKlf7 is coexpressed with *TrkA* in sensory and sympathetic neurons through embryogenesis and into adulthood

Our results indicate *mKlf7* expression precedes *TrkA* expression. From E10.5 to adulthood, *mKlf7* is coexpressed in all sensory and sympathetic ganglia with *TrkA*. Of particular note, two novel areas that transiently express *TrkA* are identified in this study: the VII-VIII neural crest complex at E10.5 and E11.5, and the spinal neuroepithelium at E10.5. Strikingly, *mKlf7* is coexpressed in these sites, again supporting the idea of a functional link between expression of this transcription factor and *TrkA*. The in situ data using serial sections of adult trigeminal ganglia and the single neuron expression study clearly demonstrate that *mKlf7* is coexpressed with *TrkA* in nociceptive sensory neurons. It is also apparent that *mKlf7* is expressed in other sensory neurons that do not express *TrkA*. This is supported by the reduced expression of *mKlf7* in the *TrkA*-null DRG.

In summary, the specific interaction between mKlf7 protein and the Ikaros2 regulatory element of the *TrkA* enhancer, and the striking coexpression patterns of *mKlf7* and *TrkA* in sensory and sympathetic neurons support the hypothesis that *mKlf7* may directly regulate *TrkA* expression in vivo. Because *mKlf7* is also expressed in areas that do not express *TrkA*, other potential target genes of *mKlf7* remain to be identified. In particular, the presence of *mKlf7* in *TrkB*- and *TrkC*-expressing sensory neurons may indicate a role for this transcription factor in the regulation of all Trk gene family members in sensory neurons. *In vivo* mutational, as well as molecular and genetic studies will determine the scope of the biological functions of *mKlf7* during neural development.

Note added in proof

Recent work by Laub et al. describes similar expression results to those found in this study (Laub et al., 2001).

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