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Research paper

Krüppel-like transcription factor 8 (*Klf8*) is expressed and active in the neurons of the mouse brain

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ABSTRACT

Krüppel-like transcription factor 8 (KLF8) is a transcription factor suggested to be involved in various cellular events, including malignant cell transformation, still its expression in the adult rodent brain remained unknown. To analyze *Klf8* in the mouse brain and to identify cell types expressing it, a specific transgenic $Klf8^{Gt1Gaj}$ mouse was used. The resulting *Klf8* gene-driven β-galactosidase activity was visualized by X-gal histochemical staining of the brain sections. The obtained results were complemented by in situ RNA hybridization and immunohistochemistry. *Klf8* was highly expressed throughout the adult mouse brain gray matter including the cerebral cortex, hippocampus, olfactory bulb, hypothalamus, pallidum, and striatum, but not in the cerebellum. Immunofluorescent double-labeling revealed that KLF8-immunoreactive cells were neurons, and the staining was located in their nucleus. This was the first study showing that *Klf8* was highly expressed in various regions of the mouse brain and in particular in the neurons, where it was localized in the cell nuclei.

1. Introduction

The mammalian Krüppel-like factor (KLF) family includes 17 family members, DNA binding transcriptional regulators involved in the control of different cell processes including cell proliferation, differentiation, apoptosis, angiogenesis, oncogenic transformation and development (Dynan and Tjian, 1983; Miller and Bieker, 1993; van Vliet et al., 2006; Kaczynski et al., 2003; Bieker, 2001). Their modular structure is based on three characteristic domains preserved even in evolutionary distant homologues (Oates et al., 2001; Huber et al., 2001; De Graeve et al., 2003). Post-translational modifications of the highly variable N-terminal activation domain are believed to underlie KLF ability to act as either an activator or repressor of transcription. The DNA binding domain is a C-terminal region with three highly conserved Cys_2/His_2 (C_2/H_2) zinc fingers (ZFs) and nuclear localization signal (NLS) is adjacent to this domain.

Krüppel-like factor 8 (KLF8, also known as ZNF741 or BKLF3) was initially isolated from K562 cells (human immortalized myelogenous leukemia cell line) and described as ubiquitously expressed transcription factor (van Vliet et al., 2006). KLF8 cellular function and expression can be positively regulated by other transcription factors like KLF1 (Eaton et al., 2008) or signaling molecules like Src and PI3K downstream of FAK (Zhao et al., 2003; Wang et al., 2008; Cox et al., 2006; Ding et al., 2005). Moreover it can be negatively regulated by KLF3 transcription factor (Eaton et al., 2008) and post-translational sumoylation (Wei et al., 2006). Molecular mechanisms underlying KLF8 physiological function as transcription regulator are currently uncertain; it may exert dual function on gene transcription dependent on the context of the target genes. KLF8 can recruit the C-terminal binding protein (CtBP) co-repressor to its N-terminal pro-val-asp-leu-ser (PVDLS) repression motif to repress different genes such as *KLF4* and *E-cadherin* (van Vliet et al., 2006; De Graeve et al., 2003; Wei et al., 2006; Wang et al., 2007; Hu et al., 2007; Zhang et al., 2005). It can also be a transcription activator by binding to CACCCGT-box promoter sequence through highly conserved C-terminal C_2H_2 zinc fingers (Zhao et al., 2003; Wang et al., 2008; Wei et al., 2006).

As a Focal Adhesion Kinase (FAK) signaling effector KLF8 can contribute to cell cycle progression by directly binding and activating cyclin D1 gene promoter (Zhao et al., 2003; Wang et al., 2008; Wei et al., 2006; Urvalek et al., 2010). Recent findings support the involvement of KLF8 in malignant cell transformation, epithelial-to-mesenchymal transition, migration, and tumor cell invasion (Wang et al., 2007, 2008; Wang and Zhao, 2007; Schnell et al., 2012). High expression of *Klf8*

Abbreviations: FAK, Focal Adhesion Kinase; KLF, Krüppel-like factor; NLS, nuclear localization signal; ZF, zinc finger.

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was shown in developing neural tube of mouse embryos (Ćurlin et al., 2002).

While an evidence of KLF8 importance in oncology is emerging in the last years, its function in both health and disease remains to be resolved. In an attempt to contribute to this issue, we analyzed *Klf8* expression in the central nervous system using a mouse model with gene trap modification of *Klf8* gene.

2. Materials and methods

2.1. Animals

Experiments were carried out on five wild type mice (C57Bl/6NCrl) and on the four mice of the gene trap line $Klf8^{Gt1Gaj}$. The age of the mice included in the experiments was 4–8 months. All animal procedures were approved by the University of Zagreb School of Medicine Ethics Committee and are in accordance with the Ethical Codex of Croatian Society for Laboratory Animal Science. All animals were allowed ad libitum access to food and water.

2.1.1. Genetically modified mice

The transgenic mouse line $Klf8^{Gt1Gaj}$ mouse was produced using a large-scale gene trap approach (Skibinski et al., 2005; Thomas et al., 2000; Gajovic et al., 1998). The gene trap procedure and the characterization of the mouse model was done as previously described (Ćurlin et al., 2002; Gajovic et al., 1998) and it will be shortly presented here. The gene trap vector used was *pKC199bgeo* containing the splice acceptor sequence from the mouse *Hoxc9* gene located upstream of the promoterless *βgeo* (fused *lacZ* and *neoR*), which generated both β-galactosidase reporter and neomycin resistance (Thomas et al., 2000). The vector was introduced into the mouse embryonic stem cells by electroporation. After selection for 10 days by 250 mg/ml G418 (GIBCO BRL, Gaithersburg, MD, USA), resistant clones were isolated and stained with X-gal for the presence of β-galactosidase. The positive clones were used for morula aggregation and after the germ line transmission the resulting mouse line was expanded by successive mating with C57Bl/6Crl mice. The particular gene trap event was selected according to the restricted expression pattern of *lacZ* reporter in the developing nervous system at E11.5. The expression was visualized by histochemical detection of β-galactosidase, due to the lacZ transcription driven by promoter of the endogenous gene in which the gene trap vector was inserted. The integration of gene trap vector into the mouse ES cell genome was random, therefore the endogenous gene affected by the gene trap mutation had to be identified. The known sequence of the inserted gene trap vector enabled the amplification and identification of the target gene by 5′ and 3′ RACE (rapid amplification of cDNA ends) method (Skibinski et al., 2005). BLAST search showed that the obtained cDNA sequence represented an already known gene, referred as *Klf8* (GenBank NM_173780; 21). The heterozygous mice were genotyped by PCR of tail genomic DNA.

2.2. Detection of β-galactosidase activity

Mice were anesthetized with intraperitoneal injections of 2.5% Avertin (Sigma Aldrich, St. Louis, MO, USA), transcardially perfused with phosphate buffered saline (PBS), followed by PBS buffered 2% formaldehyde (Sigma Aldrich, St. Louis, MO, USA) and 0.2% glutaraldehyde (Sigma Aldrich, St. Louis, MO, USA) (pH 7.4). Mouse brains were carefully dissected and postfixed with the same fixative mixture for 1 h on ice. After rinsing in PBS, mouse brains were sliced into 100 μm thick coronal and sagittal sections using a vibratome. Brain sections were incubated in the staining solution, containing 0.5 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate and 0.01% Igepal (Sigma Aldrich, St. Louis, MO, USA) in 0.01 M PBS (pH 7.4) under light protection at 37 °C overnight. The specimens were rinsed with PBS and cleared in ascending concentrations of glycerol in PBS at 4 °C, and visualized under the stereo microscope (Olympus SZH10, Olympus Optical Co. Ltd, Tokyo, Japan).

2.3. In situ RNA hybridization

The mice were sacrificed by cervical dislocation and the brains dissected in sterile conditions and instantly frozen in isopentane (2-methylbutane) for 60 s and stored at 80 °C till usage. The frozen brains were cut on a cryostat (Leica CM3000, Leica Instruments GmbH, Germany) into 20 μm thin sections and thaw-mounted on poly-L-lysine coated slides (Menzel-Glaser GmbH, Germany). After fixation by immersion in ice cold 2% PFA for 10 min, sections were rinsed twice at room temperature in 0.1 M PBS (pH 7.4) and acetylated with

Table 1
Regional distribution of Klf8 expression in the adult mouse brain assessed by X-gal staining (X-gal), in situ RNA hybridization (ISH) and immunohistochemistry (IHC).

Region	X-gal	ISH	IHC
Telencephalon			
Allocortex			
Piriform cortex	+++	+++	+++
Entorhinal cortex	−/+	−/+	−/+
Isocortex			
Temporal			
Lamine II, III, IV	+++	+++	+++
Lamine I, V, VI	−/+	+	+
Motor			
Lamine II, III, IV	+++	+++	+++
Lamine I, V, VI	−/+	+	+
Somatosensory			
Lamine II, III, IV	+++	+++	+++
Lamine I, V, VI	−/+	+	+
Parietal			
Lamine II, III, IV	+++	+++	+++
Lamine I, V, VI	−/+	+	+
Visual			
Lamine II, III, IV	+++	+++	+++
Lamine I, V, VI	−/+	+	+
Ectorhinal	−/+	+	+
Perirhinal	−/+	+	+
Hippocampal formation			
CA1–CA4	+++	++	+++
Dentate gyrus	−	++	+
Stiatum			
Dorsal region	++	++	++
Caudatoputamen	++	+	++
Ventral striatum	++	+	++
Nucleus accumbens	++	+	++
Pallidum			
Medial septal complex	++	++	++
Substantia inominata	+	++	++
Globus pallidus	+	++	++
Diencephalon			
Epithalamus			
Medial habenula	++	++	+++
Thalamus			
Paraventricular nucleus	++	++	+++
Intermediodorsal nucleus	++	++	+++
Centrolateral nucleus	++	++	+++
Mediodorsal nucleus	++	++	+++
Laterodorsal nucleus	++	+	+++
Lateroposterior nucleus	++	+	++
Hypothalamus	+		
Dorsomedial nucleus	++	++	+++
Arcuate nucleus	++	+++	+++
Posterior nucleus	++	+++	+++
Tuberal nucleus	++	+++	+++
Mesencephalon	++		
Superior colliculus	++	+	+
Inferior colliculus	++	+	+
Metencephalon	−	−	−/+

"−" — no signal; "−/+", "+", "++" and "+++" — arbitrary estimated intensities of KLF8 signal strength.

0.25% acetic anhydride in triethanolamine (pH 8.2). The sections were further dehydrated in ascending series of alcohol (70% EtOH 1 min, 80% EtOH 1 min, 95% EtOH 2 min, 100% EtOH 1 min), kept for 5 min in chloroform, 1 min in 100% EtOH, and 1 min in 95% EtOH.

DNA oligonucleotide probes used were *Klf8* sense: GCA GGG AGA AGA GTC TCT TGA CTT AAA GAG AAG ACG GAT TCA TCA and *Klf8* antisense: CGT CCC TCT TCT CAG AGA ACT GAA TTT CTC TTC TGC CTA AGT AGT. The probe ^{35}S-dATP was labeled on ice. The labeling reaction mixture (50 μl per slide) was incubated for 30 min at 37 °C, and stopped by adding 180 μl Tris-base EDTA buffer (10 mM Tris, 1 mM EDTA, pH = 8) and 4 μl tRNA (25 μg/μl). The labeled probe precipitated by adding 15 μl of 4 M NaCl and 660 μl of 100% EtOH, after which it was incubated for 1 h on dry ice and centrifuged for 30 min (13,000 *g*) at 4 °C. The sediment was dissolved in 50 μl Tris-base EDTA buffer with 0.5 M dithiothreitol. The radioactivity was measured by adding 1 μl of probe to 500 μl of scintillation liquid and 10 μl of supernatant to 500 μl of scintillation liquid and counted by a beta-counter. The proportion of labeling success and the number of conjugated ^{35}S-dATP per oligo were calculated.

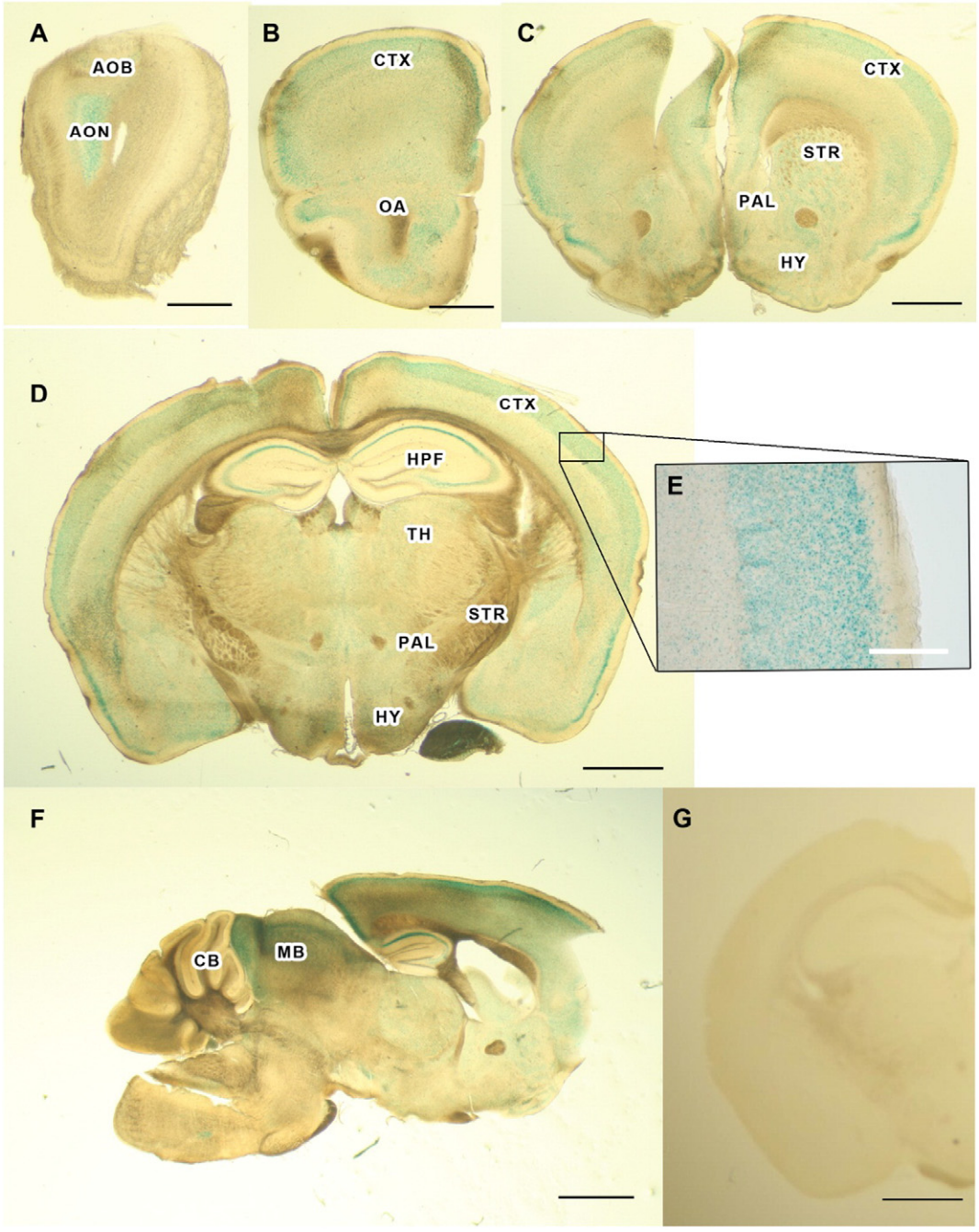


Fig. 1. β-galactosidase activity as a marker of *Klf8* expression in the mouse brain. X-gal histochemical staining resulted in a blue precipitate shown in representative 100 μm thick coronal (A–E, G) and sagittal (F) heterozygous mouse brain cryosections. The stained structures include lateral part of the anterior olfactory nucleus — AON, the accessory olfactory bulb — AOB, olfactory area — OA, cortex — CTX, striatum — ST, pallidum — PA, hypotalamus — HY, CA1, CA2, CA3 fields of the amons horn of the hippocampal formation — HPF, thalamus — TH, striatal regions — STR (striatal dorsal region, caudoputamen, striatal ventral region and nucleus accumbens), pallidum — PAL (pallidum medial region, medial septal complex, medial septal nucleus, substantia innominata and caudal region of the pallidum) and midbrain — MB. Non-stained structure cerebellum — CB (F). X-gal blue precipitate present in the laminae II, III and IV (E). Absence of specific β-galactosidase activity signal of wild type mouse brain cryosections (G). Scale bar represents 1 mm (black), 0.1 mm (white).

Hybridization was performed overnight at 40 °C in the hybridization buffer supplemented with 100 mg/ml dextran sulfate, 10 μl/ml dithiothreitol and ^{35}S labeled oligonucleotide probe (600,000–800,000 cpm/50 μl). The next day the sections were prewashed three times with 5× saline sodium citrate buffer with 100 μl 1 M dithiothreitol at 52 °C and washed four times with 5× saline sodium citrate buffer for 15 min at 58 °C after which they were dipped twice in 70% EtOH and air-dried.

The dried slides were positioned with a ^{14}C-microscale into a labeled autoradiogram cassette, covered in the dark under red save light with Kodak BioMaxMR film and stored in a dark place for 15 days. The film was developed under red save light: bathing for 90 s in developer, washing shortly in water, bathing for 5 min in fixation solution, rinsing for 5 to 10 min under a stream of water, and air-dried. The visible signal was digitized using a Flatbed Epson perfection 4870 photo scanner (Epson America, Inc., Long Beach, CA, USA), with a resolution of 4800 dpi.

2.4. Immunohistochemistry

The mice were anesthetized with intraperitoneal injection of 2.5% Avertin (Sigma Aldrich, St. Louis, MO, USA) and transcardially perfused with PBS, followed by PBS buffered 4% paraformaldehyde. Mouse brains were carefully dissected and postfixed overnight in the same fixative at 4 °C, rinsed in PBS, and transferred to 10% sucrose followed by 30% sucrose in PBS at 4 °C for cryoprotection. After the brains sunk in sucrose, approximately 3 days at 4 °C, the brains were embedded in Tissue-Tek (O.C.T. compound, Sakura, Torrance, CA, USA), cut into 35 μm-thick coronal or sagittal sections using cryostat (Leica CM3000, Leica Instruments GmbH, Germany), and stored till use at −20 °C.

For brightfield immunohistochemistry, 35 μm mouse brain slices were rinsed in PBS 4 times for 5 min. PBS buffered 18% H_2O_2 was put on slides for 30 min at 4 °C. Slices were blocked with 5% goat serum and 1% Triton X-100 in PBS for 1 h on 4 °C. Primary antibody against KLF8 (rabbit polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, USA) was diluted 1:50 in PBS containing 5% goat serum and 1% Triton X-100 and incubated overnight at 4 °C. Control slices were incubated in a buffer not containing primary antibody. Slices were rinsed with PBS containing 0.25% Triton X-100 4 times for 5 min. Secondary antibody was anti-mouse Fc specific (Jackson ImmunoResearch Europe Ltd., Suffolk, UK) diluted 1:500 in PBS containing 5% goat serum and 1% Triton X-100, incubated for 4 h at 4 °C. Slices were rinsed 2 times for 15 min with PBS. Tertiary complex (VectaStain ABC anti-mouse kit Standard) was prepared in PBS containing 5% goat serum, incubated for 2 h at 4 °C, and then rinsed 2 times for 15 min with PBS. SIGMAFAST™ DAB (3,3′-diaminobenzidine tetrahydrochloride) was diluted in demineralized water and incubated for 20 s. Brain slices were rinsed twice with PBS and coverslipped with HistoMount (Invitrogen). After drying, the slices were analyzed under bright field using light microscope (Olympus Provis AX70, Tokyo, Japan).

For immunofluorescence, 35 μm thick coronal cryostat sections were rinsed in PBS 4 times for 5 min. The sections were blocked for 30 min in PBS containing 10% goat or donkey serum (depending on the secondary antibody) and 0.25% Triton X-100. Primary antibodies used were against KLF8 (rabbit polyclonal, diluted 1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, USA), β-gal (Chicken polyclonal, diluted 1:200, Abcam, Cambridge, UK), NeuN (Mouse monoclonal, diluted 1:300, Millipore, Billerica, MA, USA), GFAP (Chicken polyclonal, diluted 1:100, Abcam, Cambridge, UK), Iba1 (Goat polyclonal, diluted 1:50, Abcam, Cambridge, UK), Map2 (Chicken polyclonal, diluted 1:750, Abcam, Cambridge, UK), and Olig4 (Mouse monoclonal, diluted 1:300, Millipore, Billerica, MA, USA). Incubation with primary antibody was performed overnight at room temperature in PBS containing 1% goat serum (or donkey serum depending on the secondary antibody) and 0.25% Triton X-100 (both Sigma Aldrich). Control slices were incubated in a buffer not containing primary antibody. Slices were rinsed with PBS containing 0.25% Triton X-100 4 times for 5 min. The secondary antibodies were Alexa Fluor 488 goat anti rabbit (Invitrogen), Alexa Fluor 488 donkey anti-rabbit (Invitrogen, A11056), Alexa Fluor 546 goat anti-chicken (Invitrogen), Alexa Fluor 546 goat anti-mouse (Invitrogen), and Alexa Fluor 546 donkey anti-goat (Invitrogen). All secondary antibodies were diluted at a concentration of 1:500, and incubation was performed for 2 h at room temperature. After incubation, slides were washed with PBS containing Triton X-100, mounted with Fluoromount Aqueous Mounting Medium (Sigma Aldrich), coverslipped and left overnight to dry. Brain sections

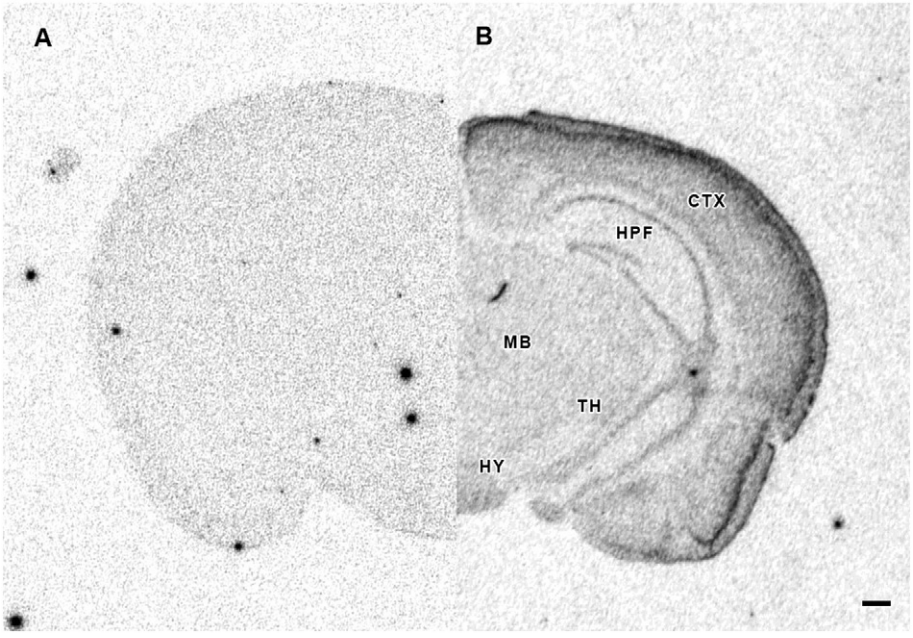


Fig. 2. Representative autoradiographic images depicting the distribution *Klf8* mRNA in the murine brain by in situ hybridization. *Klf8* mRNA expression on coronal wild type mice brain cryosections confirms the expression pattern obtained by X-gal staining (right — antisense (AS)). Note the absence of specific hybridization signal in all regions when sections were hybridized with the sense (control) probe (left — sense (S)). Abbreviations: CTX — cortex, HPF — hippocampal formation, MB — midbrain, TH — thalamus, HY — hypothalamus. Scale bar represents 1 mm.

were examined under a confocal microscope (Zeiss LSM 510 Meta, Carl Zeiss Microscopy GmbH).

3. Results

3.1. β-galactosidase activity was present in different regions of the gray matter in the brain as analyzed by X-gal staining of Klf8^{Gt1Gaj} transgenic mouse

The present study used a specific transgenic *Klf8*^{Gt1Gaj} mouse line in which *lacZ* transcription was driven by endogenous promoter of *Klf8* gene. In order to visualize the expression pattern of *Klf8* gene histochemical detection of β-galactosidase activity via its substrate X-gal was used. X-gal staining as a blue precipitate was present throughout the gray matter of the brain, in the olfactory bulbs, cerebral cortex, hippocampus, hypothalamus, pallidum and striatum (Table 1, Fig. 1). β-galactosidase activity was not present in the cerebellum (Fig. 1F).

3.1.1. Telencephalon

The highest density of X-gal staining was observed in the cerebral cortex and hippocampus (Fig. 1D). Interestingly, the distribution of the staining varied among layers of the cortex. The most intense X-gal staining was in the pyramidal layer of the piriform area (Fig. 1D) and in laminae II, III, and IV of the temporal, motor, somatosensory, parietal and visual cortex (Fig. 1D, E). In contrast to these laminae, a weaker staining was present in laminae I, V, and VI. The weakest staining was in the ecthorhinal, perirhinal and entorhinal cortices (Fig. 1D). Strongly labeled were also the CA1, CA2, and CA3 fields of the hippocampal formation. The granular layer of dentate gyrus did not show a β-galactosidase activity (Fig. 1D). In the striatum, the staining was also seen scattered through the striatal dorsal region, caudate nucleus,

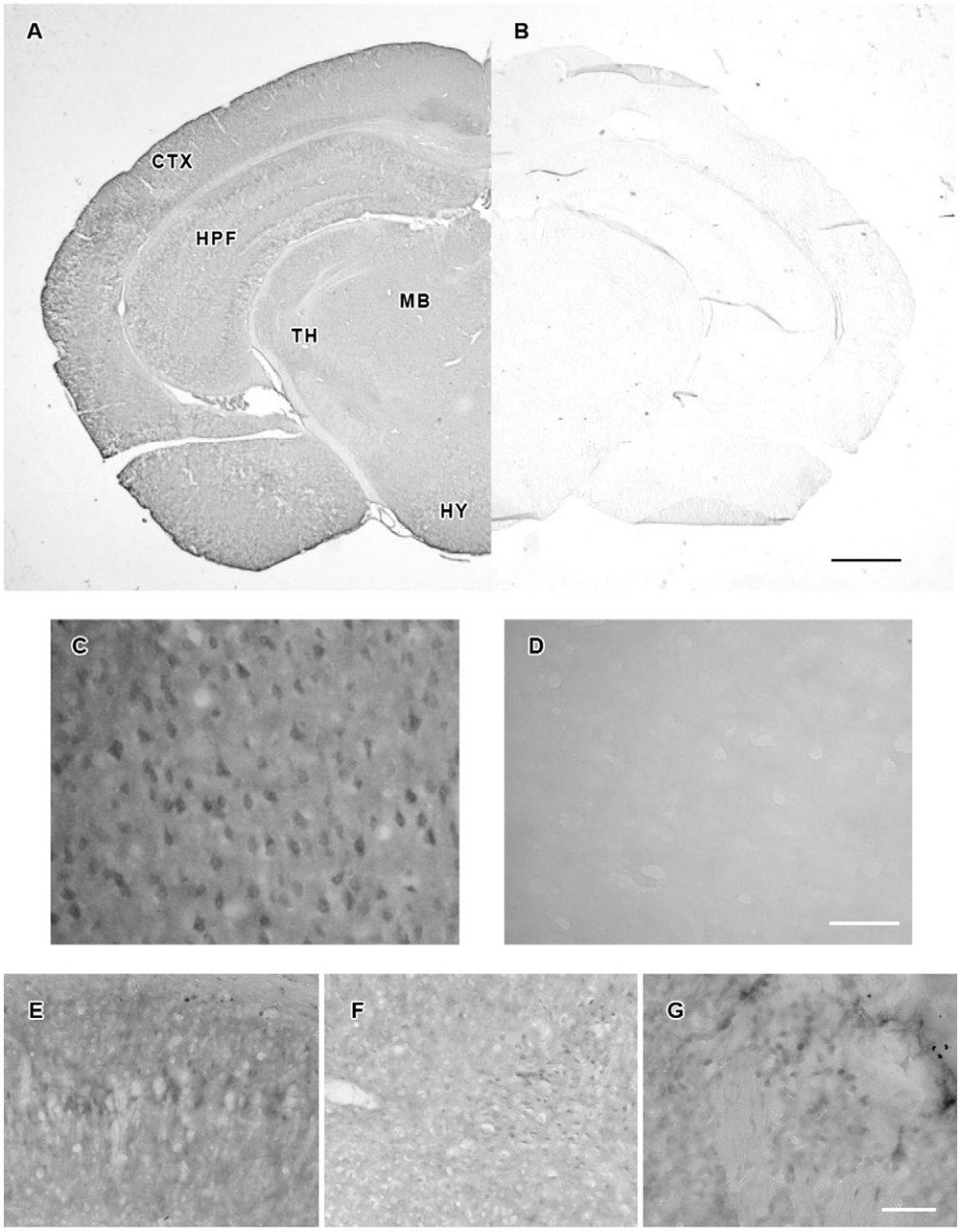


Fig. 3. Immunolocalization of KLF8 in coronal wild type mice brain cryosections. Immunohistochemical staining shows positive KLF8 expression in the cortex — CTX, hippocampal formation — HPF, thalamus — TH, midbrain — MB and hypothalamus — HY (A). High magnification of KLF8 immunolocalization in the cortex (C), CA1 field of the hippocampal formation (E), hypotalamus (F) and median habenula (G), versus negative control without primary antibody (D). Scale bar represents black 100 μm, white 10 μm.

putamen, striatal ventral region and nucleus accumbens (Fig. 1C). Similarly, the staining was present in the medial septal complex, medial septal nucleus, substantia innominata, and medial and caudal regions of the pallidum (Fig. 1C).

3.1.2. Diencephalon

In the diencephalon, weak X-gal staining was observed in many thalamic regions (Fig. 1D) including the paraventricular, intermediodorsal, centrolateral, mediodorsal, lateral dorsal and lateral posterior thalamic nuclei. The staining was also visible in the medial habenula region of the epithalamus. In the hypothalamus, most of the X-gal staining was distributed in the dorsomedial, arcuate, posterior hypothalamic and tuberal nuclei.

3.1.3. Mesencephalon

In the midbrain, most of the X-gal blue staining was detected throughout the superior and inferior colliculus (Fig. 1F).

3.1.4. Metencephalon

In the cerebellum β-galactosidase activity was not present (Fig. 1F).

3.2. Klf8 expression determined by in situ RNA hybridization corresponded to β-galactosidase activity in gene trap mutant

In order to confirm that the observed β-galactosidase activity pattern indeed reflected endogenous *Klf8* expression, the in situ hybridization was performed with ^{35}S-labeled probe for *Klf8* mRNA in the adult mouse brain. *Klf8* mRNA was shown throughout the rostral–caudal extent of the brain, with labeled cells concentrated in the cerebral cortex, hippocampus, thalamus and hypothalamus (Fig. 2B). The relative intensity of hybridization signal (grains over cells) and the density of labeled cells in different regions of the brain were similar to those of X-gal staining (Table 1).

3.3. Immunohistochemistry showed KLF8 presence in the brain and in β-galactosidase positive cells

To visualize KLF8 protein the brain coronal cryosections were labeled with anti-KLF8 antibody. Brightfield immunohistochemistry evaluation demonstrated KLF8 protein distribution similar to the observed β-galactosidase activity pattern. Evaluation of the stained sections also showed the rostro-caudal distribution of KLF8 protein in the brain (Table 1). The relative density of labeled perikarya was the highest in the cerebral cortex, hippocampus, hypothalamus and thalamus (Fig. 3A, C, E, F, G).

In order to verify whether β-galactosidase distribution reflects those of KLF8, representative coronal heterozygous mouse brain cryosections were double labeled with anti-β-galactosidase and anti-KLF8 antibody. Strong fluorescence signals of both β-galactosidase and KLF8 were present in the same brain cells (Fig. 4). The overlap of the observed fluorescent signals suggested that the X-gal staining reliably displayed *Klf8* expressing cells.

Still the overlap of the three methods used, X-gal staining, in situ RNA hybridization, and immunohistochemistry was not present overall. The most differences include the intensity of the staining, but in some instances the differences were more pronounced. The most prominent example was dentate gyrus of the hypocampal formation (Table 1).

3.4. Double staining immunohistochemistry showed that KLF8 was active in the neuronal nuclei

To identify cells of the brain, which expressed KLF8, mouse brain cryosections were double-labeled with anti-KLF8 antibody and different cell specific markers: neuron cytoskeletal-specific marker (Map2), neuron nuclear-specific marker (NeuN), markers of astrocytes (GFAP), microglia/macrophages (Iba1) and oligodendrocytes (Olig4). KLF8-positive cells of three distinct regions of the brain (cortex, hippocampus and hypothalamus) co-stained with the neuronal markers NeuN and Map2, indicating that KLF8-expressing cells were neurons (Fig. 5A–D). Fluorescent KLF8 signal was clearly present in the neuronal nuclei marked with DAPI (Fig. 5A–C, H–J). Fluorescent double labeling of KLF8/GFAP, KLF8/Iba1 and KLF8/Olig4 showed that KLF8-was not present in the astrocytes, microglia or oligodendrocytes (Fig. 5E–G).

4. Discussion

The results of the present study were first to show *Klf8* expression pattern in the adult mouse brain. X-gal histochemistry of the *lacZ*-tagged *Klf8* gene overlapped with the signal observed by in situ RNA hybridization and immunohistochemistry. The slight difference in detection levels of the three methods used was expected, since it is shown that X-gal histochemistry can underestimate the extent of gene expression (Couegnas et al., 2007; Pereira et al., 2006). *Klf8* expression was detected in the gray matter, the most prominent regions being cortex and hippocampus, but as well in the olfactory bulbs, thalamus, hypothalamus, pallidum and striatum, although not in the cerebellum.

Strong KLF8 signal was found in the nuclei of neurons, which corresponded to KLF8 function as transcription factor. This finding confirmed previously reported KLF8 nuclear localization in HEK293 and NIH3T3 cells (Eaton et al., 2008; Mehta et al., 2009). The

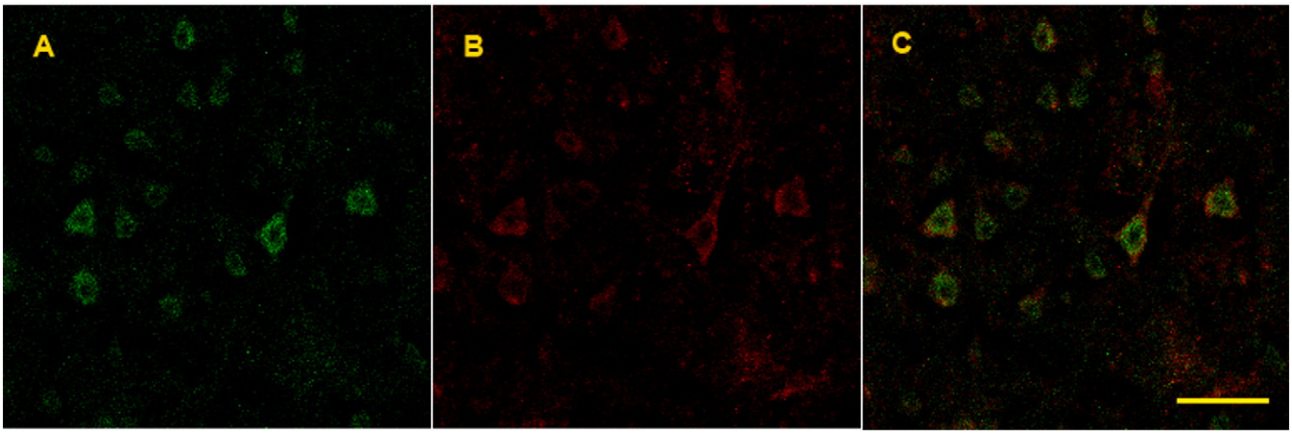
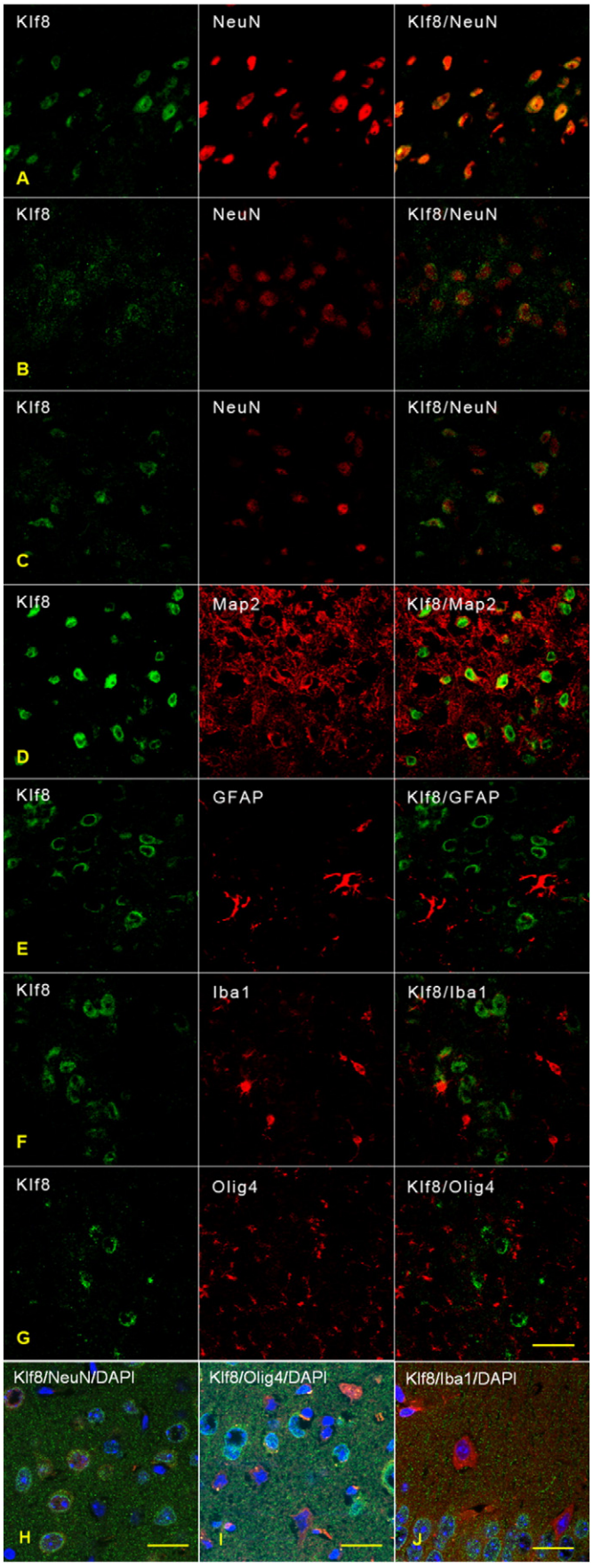


Fig. 4. Confocal photomicrographs showing colocalization of KLF8 (green, A) and β-galactosidase (red, B) in the brain of heterozygous mice. Overlap in distribution of KLF8 protein and β-galactosidase confirmed the *lacZ* insertion in the *Klf8* gene. Scale bar represents 10 μm.



cooperation between two functional nuclear localization signals (NLSs) together with the first two C_2/H_2 zinc fingers are considered to play an essential role in both nuclear localization and DNA binding of KLF8 (Mehta et al., 2009).

The currently observed *Klf8* expression in the adult brain complemented the previously observed expression in the developing central nervous system (Ćurlin et al., 2002). *Klf8* is also well expressed in different types of human cancer (Zhang et al., 2005; Lahiri and Zhao, 2012) and in the brain affected by Alzheimer's disease (Yi et al., 2014). Still the observed expression pattern in the mouse did not completely match those in the zebra fish, where *Klf8* is required for maintenance of *pft1a*-expressing neuronal progenitors originating from the ventricular zone, which are essential for the development of Purkinje cells during normal cerebellar development (Tsai et al., 2014). In relation to the nervous system, other KLF family members are expressed as well in the developing and adult rodent and human brain. *Klf5* mRNA and the corresponding protein were shown in neurons of human brain gray matter with suggested involvement in glutamatergic neurotransmission modulation (Yanagi et al., 2008). KLF6 protein was found in neurons and endothelial cells of rodent forebrain with suggested involvement in the regulation of cell differentiation or phenotypic neurons maintenance (Jeong et al., 2009). Furthermore, loss of function of *Klf7* showed deficient neurite growth in the cerebral cortex and hippocampus (Laub et al., 2005).

Recent findings support the important roles of KLF8 playing in malignant cell transformation and tumor growth of several different non-CNS tumors including breast cancer, ovarian cancer, hepatocellular carcinoma and renal carcinoma (Wang et al., 2007, 2008; Wei et al., 2006). *Klf8* also emerged as an oncogenic transcription factor with a high expression and functional impact in primary brain tumors (Schnell et al., 2012). Epithelial–mesenchymal transition is acquired for invasive tumor phenotypes, which can be mediated through Focal Adhesion Kinase regulation of KLF8 expression and cyclin D1 expression activation (Wang et al., 2008; Ding et al., 2005; Wei et al., 2006; Guarino et al., 2007). Our study addressed healthy brain structures showing KLF8 presence in neurons, but not in glia cells. The neurons are postmitotic cells; hence we had not shown KLF8 in cells capable for mitotic division. Whether the eventual ectopic expression of *Klf8* in mitotic cells in the brain could contribute to the tumorigenesis is to be verified in the future. Moreover, in cells expressing *Klf* family members' posttranslational modifications such as acetylation, sumoylation, and phosphorylation can modify the activating or repressor functions of KLF by changing their binding partners or altering affinities for specific target promoters.

The KLF8 strong presence in the neurons as post-mitotic cells is rather controversial due to its suggested involvement in the cell cycle regulation. FAK regulates cell cycle progression in normal cells by enhancing KLF8 binding activity and subsequent transcriptional control of the cyclin D1 promoter in NIH3T3 cells (Zhao et al., 2001, 2003). At the other hand several other members of the KLF family were suggested to stimulate cell differentiation and inhibition of proliferation in different cell types (Bieker, 2001; Perkins, 1999). For example, expression of KLF4 and KLF8 are critical for the restrain of growth and differentiation of epithelial cells by repression of cyclin D1 promoter (Dang et al., 2000; Okano et al., 2000; Shie et al., 2000a,b; Shields et al., 1996; Laub et al., 2001). Taken together, these data suggest that different members

Fig. 5. KLF8 was localized exclusively in the neuronal nuclei. Confocal photomicrographs showing fluorescent signal overlap of KLF8 (green) and NeuN neuron-specific marker (red) in the cortex (A), cornu ammonis 2 (CA2, B) and hypothalamus (C) of coronal wild type mice brain cryosections. KLF8 (green) neuronal localization was confirmed by costaining with Map2 (red) specific marker for neurons (D). KLF8 (green) fluorescence signals did not overlap with fluorescence signals (red) specific for glial cells (GFAP, E), microglia/macrophage (Iba1, F) or oligodendrocytes (Olig4, G). Specific neuronal subcellular nuclear localization of Klf8 (green) was confirmed by costaining with DAPI (blue), and NeuN, Olig4, and Iba 1 (all red) showing clear green/blue overlap wherever Klf8 was expressed (H, I, J). Scale bar represents 10 μm.

of the KLF family may have antagonistic effects on cell cycle regulation. In this context one of the possible functions of KLF8 in the brain could be its involvement in the regulation of neuronal differentiation and phenotypic maintenance. To address in the future whether KLF8 expression or activity will change with aging can clarify its function and eventual involvement in brain diseases.

In conclusion, the present study provided for the first time spatial and cell type distribution of KLF8 in the adult mouse brain. *Klf8* was expressed in different gray matter regions, and its expression was specific for the neurons. Given the involvement of KLF8 in malignant cell transformation and progression (van Vliet et al., 2006; Wang et al., 2007, 2008; Wang and Zhao, 2007; Schnell et al., 2012), non-syndromic X linked mental retardation (Lossi et al., 2002), development of Alzheimer's disease (Tsai et al., 2014) and rodent central nervous system development (Ćurlin et al., 2002), it seems that KLF8 could have an important function in the control of neurons and brain homeostasis.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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