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Expression pattern of delta-like 1 homolog in developing sympathetic neurons and chromaffin cells

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A B S T R A C T

Delta-like 1 homolog (DLK1) is a member of the epidermal growth factor (EGF)-like family and an atypical notch ligand that is widely expressed during early mammalian development with putative functions in the regulation of cell differentiation and proliferation. During later stages of development, DLK1 is downregulated and becomes increasingly restricted to specific cell types, including several types of endocrine cells. DLK1 has been linked to various tumors and associated with tumor stem cell features. Sympathoadrenal precursors are neural crest derived cells that give rise to either sympathetic neurons of the autonomic nervous system or the endocrine chromaffin cells located in the adrenal medulla or extraadrenal positions. As these cells are the putative cellular origin of neuroblastoma, one of the most common malignant tumors in early childhood, their molecular characterization is of high clinical importance. In this study we have examined the precise spatiotemporal expression of DLK1 in developing sympathoadrenal cells. We show that DLK1 mRNA is highly expressed in early sympathetic neuron progenitors and that its expression depends on the presence of Phox2B. DLK1 expression becomes quickly restricted to a small subpopulation of cells in sympathetic ganglia, while virtually all chromaffin cells in the adrenal medulla and the Organ of Zuckerkandl still express high levels of DLK1 at late gestational stages.

1. Introduction

Sympathetic neurons of the autonomic nervous system and the endocrine chromaffin cells are neural crest derived cells that share many characteristics, including the expression of the catecholaminergic pathway enzymes tyrosine-hydroxylase (TH) and dopamin-β-hydroxylase (DBH). The later constitute the adrenal medulla and release catecholamines into the blood stream in response to stimulation by preganglionic sympathetic nerve fibers. During development chromaffin cells are also located in extra-adrenal positions, such as the organ of Zuckerkandl (OZ), which is believed to be the major source of catecholamines during fetal life (West et al., 1953). In mouse embryos it is located on the anterior surface of the aorta at the level of the renal pelvis and can be identified by the presence of TH-immunofluorescence and the absence of neurofilament (NF) expression (Schober et al., 2013). Originally, it was postulated that sympathetic neurons and chromaffin cells originate from a common bipotential sympathoadrenal precursor (Anderson and Axel, 1986), but Furlan and colleagues reported recently that at least a major subpopulation of chromaffin cells develops indirectly from neural crest cells via Schwann cell precursors (Furlan et al., 2017). However, due to the strong similarities between the precursors of sympathetic neurons and chromaffin cells with regard to their molecular profile and developmental transcription factor dependence (Huber, 2006, 2015) the term “sympathoadrenal (SA)” is still used here. The development of SA cells is governed by a transregulatory transcription factor network (Chan et al., 2018; Huber, 2006), which among others comprises the homeodomain transcription factor Phox2B (Huber et al., 2005; Pattyn et al., 1999) and the basic helix loop helix transcription factors Mash1 (Huber et al., 2002; Pattyn et al., 2006). A precise knowledge of the molecular players and pathways that operate during SA development is of high significance in a clinical context, as these precursor cells are the cellular origin of neuroblastoma, a malignant early childhood tumor derived from embryonic tissue (for a recent review see Tsubota and Kadomatsu, 2018).

Delta Like-1 homolog (DLK1), also known as preadipocyte factor 1 (Pref-1) and pG2 (Lee et al., 1995), is a paternally imprinted gene located on human chromosome 14q32 (Gubina et al., 1999) and mouse chromosome 12 (Gubina et al., 2000). It encodes a transmembrane epidermal growth factor (EGF)-like protein containing six tandem EGF-like repeats (Smas and Sul, 1993; Smas et al., 1994). DLK1 is a non-

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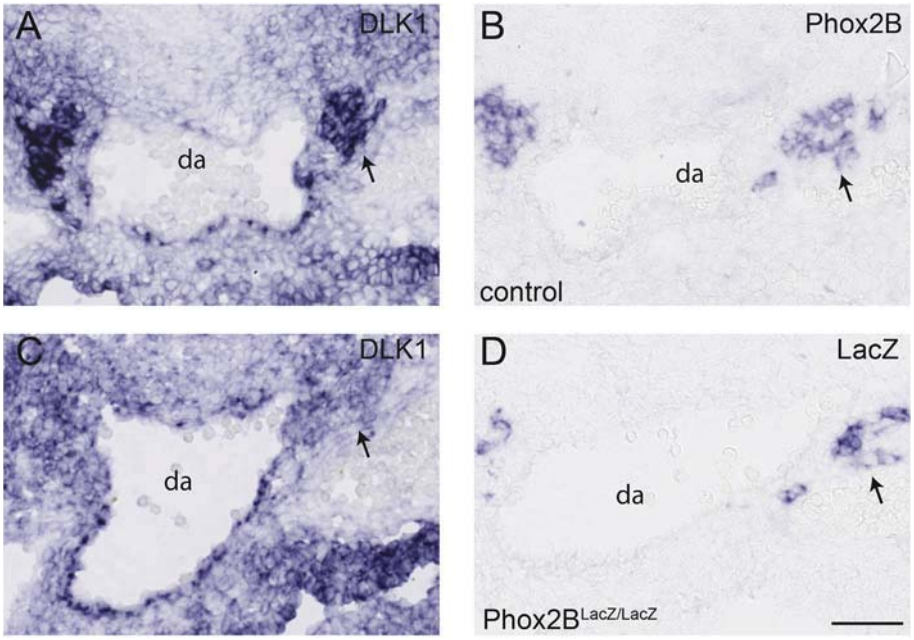

Fig. 1. (A) DLK1 is expressed at high levels in primary sympathetic ganglia (arrows) of E10.5 control mouse embryos. (C) In Phox2B$^{LacZ/LacZ}$ mice the strong signal at the sites of primary sympathetic ganglia is lacking. Photomicrographs show cross sections through thoracic sympathetic ganglia (arrows) of E10.5 (A,B) control and (C,D) Phox2B$^{LacZ/LacZ}$ mouse embryos. In-situ-hybridizations for (A,C) DLK1, (B) Phox2B and (D) LacZ were performed in near adjacent sections. (da) dorsal aorta. Bar: 50 µm.

canonical notch ligand interacting with the Delta-Notch signaling pathway, which is involved in cell fate decisions, progenitor maintenance and cell differentiation (Bray, 2006; Fiúza and Arias, 2007; D'Souza et al., 2010). DLK1 is widely expressed during embryonic development of mammals (Falix et al., 2013), but in the adult its expression is downregulated and highly restricted to certain organs, including adrenal chromaffin cells (Jensen et al., 1993; Larsen et al., 1996; Hedlund et al., 2003). Despite its widespread expression during embryonic development, mice carrying deletions of DLK1 display relatively mild deficits, including a partially penetrant neonatal lethality, growth retardation, skeletal deficits and accelerated adiposity (Moon et al., 2002; Appelbe et al., 2013). Yet, the functions of DLK1 are only partially understood (Appelbe et al., 2013).

DLK1 has also been linked to tumor biology and associated with cancer stem-cell features (Yin et al., 2006; Kim et al., 2009; Xu et al., 2012; Cai et al., 2016). Its expression has been detected in a variety of tumor cells, including certain types of neuroblastoma cells (van Limpt et al., 2003; Kim et al., 2009; Begum et al., 2012). Here we report the spatiotemporal expression pattern of DLK1 in sympathoadrenal cells in the course of their development to link the normal molecular properties of these cells to their tumor biology.

2. Materials and methods

2.1. Experimental animals

Phox2B^{Lacz} mice (Pattyn et al., 1999) were described previously. Pregnant C57BL/6J or Phox2B^{Lacz} mice were sacrificed by cervical dislocation and embryos were removed at embryonic day (E)10.5, E11.5, E13.5 or E18.5. The day of vaginal plug identification was designated E0.5. The study was carried out in strict accordance with the German Federal Animal Welfare Law and care of animals was in accordance with institutional guidelines.

2.2. Histology

Embryos were fixed in 4% paraformaldehyde (PFA) overnight. Tissues were then rinsed 3 times with PBS and transferred into 30% sucrose in PBS for cryoprotection. After immersion in sucrose overnight the tissue was coated with OCT™ compound (Tissue Tek), frozen on dry ice, and stored at −80 °C until further processing. Tissues were then cut into 10 µm serial sections, mounted on Superfrost™ slides, and air dried for 30 min, before performing in situ hybridization or immunocytochemistry as indicated below.

2.3. Immunohistochemistry

For immunohistochemistry, slides were pretreated with 3% hydrogen peroxide in PBS for 15 min. After incubation with sheep polyclonal anti-TH (1:500; AB1542, Merck-Millipore, Darmstadt, Germany) or rabbit anti-Phox2B (1:400; kindly provided by Dr. Christio Goridis, 'École Normale Supérieure, Inserm, Paris, France) diluted in PBS, sections were incubated with the appropriate biotinylated secondary antibody, rinsed with PBS and incubated for 1 h with avidin and biotinylated horseradish-peroxidase-macromolecular complex (Vector: Elite ABC reagent) according to the manufacturer's instructions. Sections were then rinsed with PBS and stained with 3-amino-9-ethylcarbazol (Sigma; red staining) according to the manufacturer's instructions. After being rinsed with PBS, sections were mounted with Kaiser's glycerol gelatine (Merck).

2.4. In situ hybridization

In situ hybridization (ISH) on cryosections and preparation of digoxigenin-labelled riboprobes for mouse Phox2B (Pattyn et al., 1997), SF-1 (Gut et al., 2005), neurofilament (NF) 68 (Huber et al., 2002), and LacZ (Huber et al., 2005) were carried out by using a modification of the protocol of D. Henrique (IRFDBU, Oxford, UK) as previously described (Ernsberger et al., 1997). Mouse DLK1 (gene bank accession number: NM_010052 number; 236bp −741 bp) was cloned by RT-PCR using a pGEM-T vector system (Promega) following the manufacturer's instructions. The plasmids were linearized with SacII and transcribed with SP6. The specificity of the probes was tested using appropriate sense controls. If required, immunohistochemistry was carried out following in situ hybridization.

3. Results

We investigated the expression of DLK1 by in-situ-hybridization in the area of sympathetic ganglia, the adrenal gland, and the OZ in mouse embryos of different developmental stages starting at E10.5. At this age DLK1 is expressed in a variety of tissues and particular strong signals

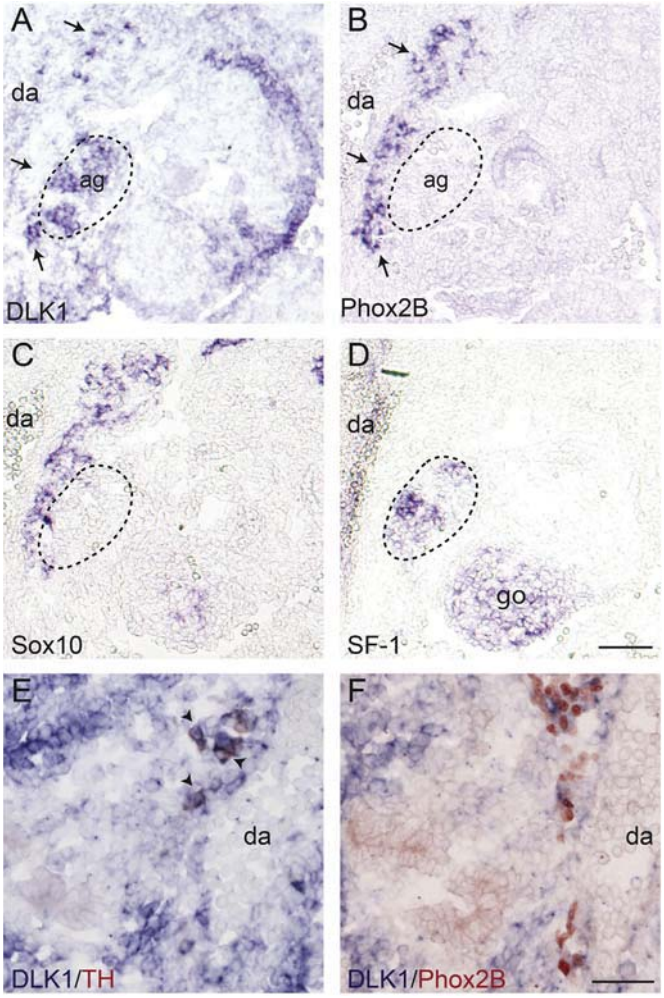

Fig. 2. DLK1 is expressed at the site of the developing adrenal cortex. Cross sections through E10.5 mouse embryos at the level of the developing adrenal gland. In-situ-hybridizations for (A) DLK1, (B) Phox2B, (C) Sox10 and (D) SF-1, a marker for the developing adrenal cortex, were performed in near adjacent sections. Note that in the area of Phox2B and Sox10 expression only few cells appear positive for DLK1 (arrows). (E,F) In-situ-Hybridizations for DLK1 (blue) followed by immunostainings for TH (E: red cytoplasmatic stain) and Phox2B (F: red nuclear stain). Note that all TH immunoreactive cells (arrowheads) are positive for DLK1, while only some of Phox2B immunoreactive cells co-express DLK1. (ag) adrenal gland; (da) dorsal aorta; (go) gonad. Bars: A–D: 100 μm; E,F: 50 μm.

were observed in the developing liver and at the sites of the primary sympathetic ganglia adjacent to the dorsal aorta (Fig. 1A). To identify the position of the primary sympathetic ganglia, a near adjacent section was labelled using a probe for Phox2B (Fig. 1B). DLK1-ISH combined with TH-immunostaining revealed that virtually all TH-positive cells in the thoracic primary ganglia express DLK1 (Fig. 4A) at this developmental stage.

The differentiation of sympathoadrenal cells from neural crest cells is controlled by a complex transregulatory transcription factor network, whose activation essentially depends on the homeodomain transcription factor Phox2B (Huber et al., 2005; Pattyn et al., 1999). To investigate, whether DLK1 expression in the SA cells is downstream of Phox2B, we analyzed DLK1 expression in Phox2B deficient ($Phox2B^{Lacz/Lacz}$) mouse embryos. As shown in Fig. 1C the strong DLK1 signal that was detected in control mice was not observed at the site of LacZ positive cells accumulating adjacent to the dorsal aorta in E10.5 $Phox2B^{LacZ/LacZ}$ embryos (Fig. 1D), indicating that Phox2B is directly or indirectly implicated in the upregulation of DLK1 in SA cells.

At more caudal regions, in the area of the developing adrenal cortex, identified by the expression of steroidogenic factor 1 (SF-1), a strong DLK1 signal matching the area of SF-1 expression was observed (Fig. 2 A,D). A stream of cells expressing Phox2B and Sox10, a marker for neural crest cells, glial cells, and early SA precursors, was identified in close proximity to the developing adrenal cortex (Fig. 2B and C). These cells most likely represent chromaffin cell precursors migrating to the adrenal Anlage. Interestingly, in the area of Phox2B/Sox10 expression only few cells were positive for DLK1. At this developmental stage and axial level not all SA precursors have undergone catecholaminergic differentiation as indicated by the greater number of Phox2B than TH-immunoreactive cells in this region (Fig. 2E and F). DLK1-ISH in combination with either TH or Phox2B immunostaining revealed that TH-immunoreactive cell co-express DLK1, while only a subpopulation of Phox2B positive cells are positive for DLK1. This finding suggests that at least some of the more immature Phox2B positive/TH negative precursors lack DLK1 expression, indicating that DLK1 is upregulated during early SA cell differentiation most likely between the onset of Phox2B and TH expression.

At E11.5 TH-positive cells have invaded the adrenal gland. At this age DLK1-expression in the adrenal gland appears diffuse and only partially overlaps with TH-immunoreactivity (Fig. 3A–C). The OZ at E11.5 was identified by the presence of TH-immmunoreactivity and Phox2B-expression and the absence of neurofilament-68 expression, which distinguishes it from prevertebral ganglia (Schober et al., 2013). A strong signal for DLK1 was detected in the area of the OZ (Fig. 3D–E).

In sympathetic ganglia from E11.5 onwards throughout embryonic development the expression of DLK1 is restricted to a small subpopulation of cells, while the majority of TH positive cells are negative for DLK1. In Fig. 4 DLK1 expression and TH-immunoreactivity in

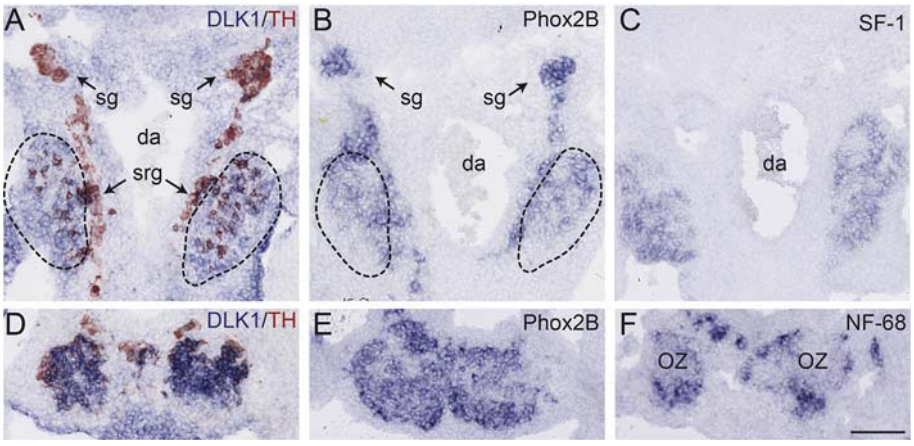

Fig. 3. Expression of DLK1 in (A) the adrenal gland and (D) the organ of Zuckerkandl at E11.5. Photomicrographs show cross sections of E11.5 mouse embryos at the level of the (A–C) adrenal gland and (D–F) the OZ. (A, D) In-situ-Hybridizations for DLK1 (blue) followed by immunostainings for TH (red) were carried out. Near adjacent sections were labelled with (B,E) Phox2B, (C) SF-1, a marker for the adrenal cortex, or (F) neurofilament-68. (da) dorsal aorta; (OZ) organ of Zuckerkandl; (sg) sympathetic ganglion; (srg) suprarenal ganglion; bar: 100 μm.

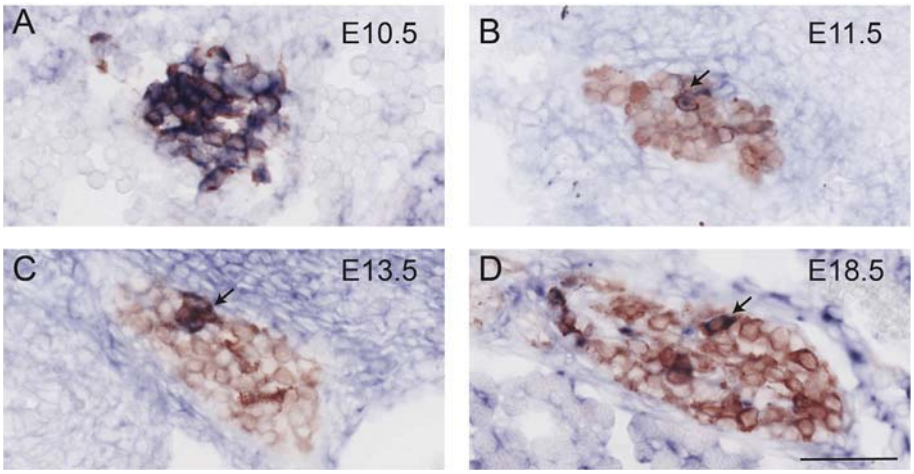

Fig. 4. Expression of DLK1 in thoracic sympathetic ganglia at E10.5, E11.5 E13.5 and E18.5. In-situ-hybridizations for DLK1 (blue) followed by immunostainings for TH (red cytoplasmatic stain) were carried out. Note that DLK1 expression is restricted to a subpopulation of TH-immunoreactive cells (arrows) from E11.5 onwards. Bar: 50 µm.

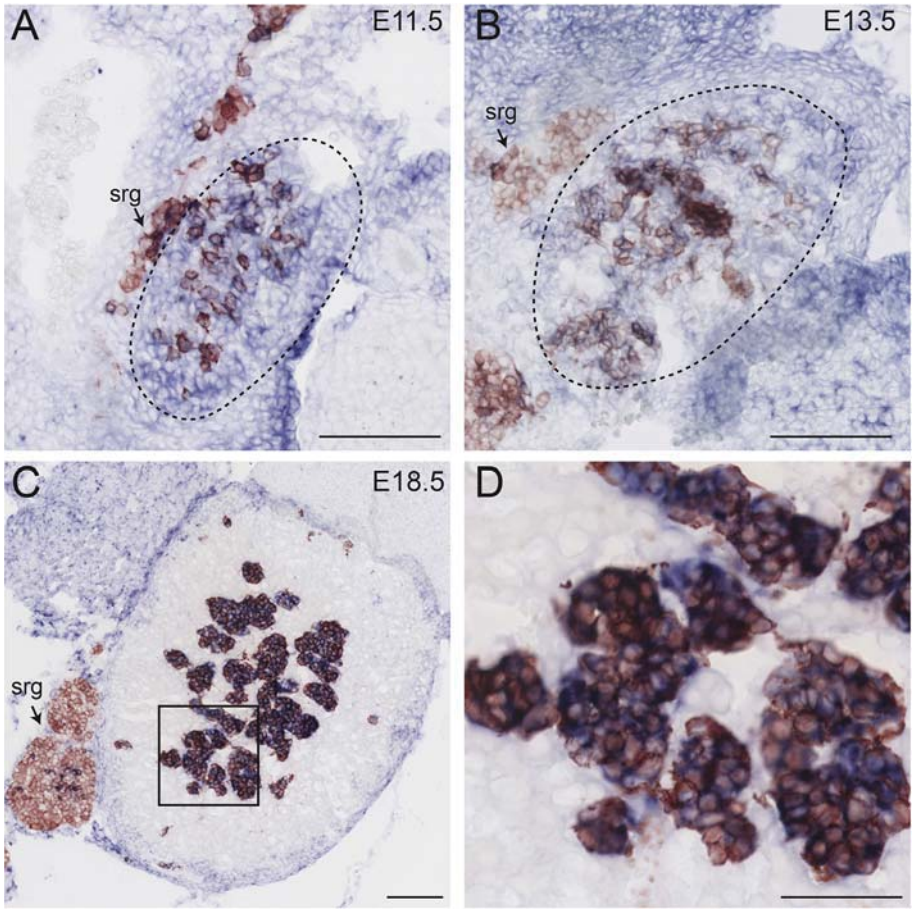

Fig. 5. Expression of DLK1 in the developing adrenal gland at (A) E11.5, (B) E13.5, and (C) E18.5. (D) higher magnification of inset in (C). In-situ-hybridizations for DLK1 (blue) followed by immunostainings for TH (red cytoplasmatic stain) were carried out. (srg) suprarenal ganglion; Bars: (A–C) 100 µm, (D) 50 µm.

thoracic sympathetic ganglia of E10.5, E11.5, E13.5 and E18.5 mouse embryos are shown. A Similar staining pattern was observed in paravertebral sympathetic ganglia of other axial levels and in prevertebral ganglia like the suprarenal ganglion (Figs. 3A, Fig. 5A–C) and the celiac-superior mesenteric ganglion complex (not shown), with a slight temporal shift depending on the axial level. In contrast to this, in the adrenal gland the expression of DLK1 becomes progressively confined to chromaffin cells, with all TH-immunoreactive cells exhibiting a strong signal for DLK1 at E18.5 (Fig. 5). Moderate expression of DLK1 was also observed in the periphery of the adrenal cortex. Similarly, the TH-positive cells in the organ of Zuckerkandl maintain DLK1 expression throughout embryonic development (Fig. 6).

4. Discussion

We show that DLK1 is transiently expressed in early sympathetic neuron progenitors at E10.5. and that its expression is downstream of Phox2B. This suggests that DLK1 is upregulated in the course of early SA differentiation, which is essentially controlled by Phox2B. However, as Phox2B is required for all aspects of SA differentiation and initiates a large set of other transcription factors (Huber, 2006), the requirement of Phox2B may well be indirect. It has been reported that hypoxia

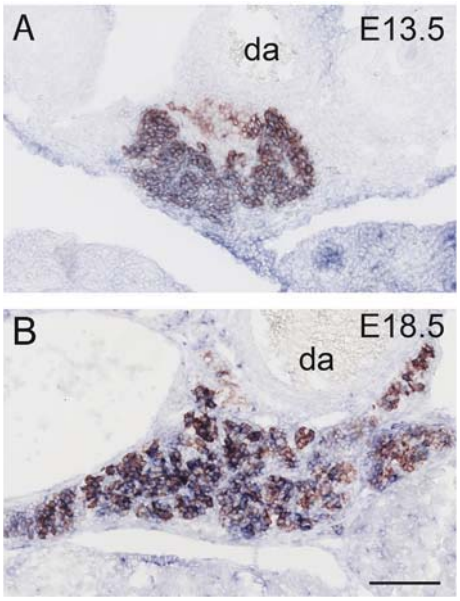

Fig. 6. Expression of DLK1 in the organ of Zuckerkandl at (A) E13.5 and (B) E18.5. In-situ-hybridizations for DLK1 (blue) followed by immunostainings for TH (red cytoplasmatic stain) were carried out. (da) dorsal aorta; bar: 100 µm.

upregulates DLK1 expression in neuroblastoma cells, mediated by hypoxia-inducible factors (HIFs) 1 and 2 (Kim et al., 2009). Interestingly, HIF-2a shows a similar spatiotemporal expression pattern in developing sympathetic ganglia as DLK1 and it is also absent in primary sympathetic ganglia of mice lacking Phox2B (El Faitwri, unpublished data).

From E11.5 onwards the expression of DLK1 mRNA is restricted to a small subpopulation of TH-positive cells in sympathetic ganglia, which may represent immature progenitor-like cells. This is conceivable, as neurogenesis from neural crest derived progenitors is going on for several days in sympathetic ganglia (Tsarovina et al., 2008). Alternatively, DLK1-expressing cells in sympathetic ganglia may represent small-intensively fluorescent cells, which resemble chromaffin cells (Eränkö, 1978). As shown here and by others, chromaffin cells, as well as some other endocrine cells, maintain DLK1 throughout prenatal and postnatal life (Jensen et al., 1993; Larsen et al., 1996). Together, this expression pattern suggests a role of DLK1 in early SA differentiation as well as in endocrine differentiation and/or function.

DLK1 is believed to exert its functions primarily during development and regeneration of various cells and tissues such as preadipocytes (Smas and Sul, 1993), the hematopoietic system (Li et al., 2005; Mirshekar-Syahkal et al., 2013) and the liver (Zhu et al., 2012). Furthermore, it plays an important role in tumor biology. In neuroblastoma high DLK1 expression was attributed to poorly differentiated cells and linked to increased tumor growth and tumorigenicity (Kim et al., 2009; Begum et al., 2012). Interestingly, another report associated high DLK1 expression in neuroblastoma with a cell type exhibiting traits of differentiated chromaffin cells (van Limpt et al., 2003). These contradictory findings, however, correlate well with the normal spatiotemporal expression pattern of DLK1, with high DLK1 expression in immature sympathetic neuron progenitors and chromaffin cells of later developmental stages.

The narrow time frame of DLK1 expression in sympathetic ganglia suggests a function during the early development of sympathetic neuron progenitors. Studies in neuroblastoma have suggested that DLK1 suppresses neuronal differentiation, inhibits neurite outgrowth, and promotes progenitor maintenance (Kim et al., 2009; Kim, 2010; Begum et al., 2012). Thus, possible roles of DLK1 in developing sympathetic ganglia may include the regulation of progenitor maintenance and the timing of differentiation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found

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