Development/Plasticity/Repair

Neuroblastoma Phox2b Variants Stimulate Proliferation and Dedifferentiation of Immature Sympathetic Neurons

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Neuroblastoma is a pediatric tumor that is thought to arise from autonomic precursors in the neural crest. Mutations in the *PHOX2B* gene have been observed in familial and sporadic forms of neuroblastoma and represent the first defined genetic predisposition for neuroblastoma. Here, we address the mechanisms that may underlie this predisposition, comparing the function of wild-type and mutant Phox2b proteins ectopically expressed in proliferating, embryonic sympathetic neurons. Phox2b displays a strong antiproliferative effect, which is lost in all Phox2b neuroblastoma variants analyzed. In contrast, an increase in sympathetic neuron proliferation is elicited by Phox2b variants with mutations in the homeodomain when endogenous Phox2b levels are lowered by siRNA-mediated knockdown to mimic the situation of heterozygous *PHOX2B* mutations in neuroblastoma. The increased proliferation is blocked by Hand2 knockdown and the antiproliferative Phox2b effects are rescued by Hand2 overexpression, implying Hand2 in Phox2b-mediated proliferation control. A Phox2b variant with a nonsense mutation in the homeodomain elicits, in addition, a decreased expression of characteristic marker genes. Together, these results suggest that *PHOX2B* mutations predispose to neuroblastoma by increasing proliferation and promoting dedifferentiation of cells in the sympathoadrenergic lineage.

Introduction

Neuroblastoma is a tumor of the developing autonomic nervous system that shows a variety of phenotypes, from undifferentiated tumors in patients with poor outcome to those containing differentiated cell types which, depending on the degree of differentiation, show a better prognosis (Maris et al., 2007). The correlation between the degree of differentiation and tumor growth properties suggests that different tumor stages maintain molecular mechanisms characteristic for particular stages of autonomic neuron development (Edsjö et al., 2007). A detailed knowledge of mechanisms controlling proliferation and differentiation during embryonic development may thus lead to a better understanding of neuroblastoma and eventually also to novel tumor treatment approaches.

Autonomic neuron development is controlled by a network of transcription factors which includes Phox2b, Phox2a, Ascl1, Hand2 and Gata2/3 (Goridis and Rohrer, 2002; Howard, 2005; Huber, 2006). Phox2b is essential for the initial differentiation of autonomic neurons and, together with Hand2 and Gata3, regulates noradrenergic gene expression. Hand2 additionally controls proliferation of sympathetic progenitors and immature neurons

(Hendershot et al., 2008; Schmidt et al., 2009). Whereas our understanding of autonomic neuron differentiation has considerably increased in the last few years, the molecular control of neurogenesis in autonomic ganglia is not well known. In sympathetic ganglia, as opposed to parasympathetic and sensory ganglia, both progenitors and immature neurons proliferate. The major increase in sympathetic neuron number during neurogenesis is due to neuronal proliferation rather than to progenitor proliferation and subsequent differentiation (Rohrer and Thoenen, 1987; Tsarovina et al., 2008) and it is unclear how proliferation of sympathetic neurons is terminated.

The finding that Phox2b overexpression inhibits the proliferation of CNS motoneuron progenitors (Dubreuil et al., 2000) together with antiproliferative effects of Phox2a/b overexpression in several cell lines (Paris et al., 2006; Raabe et al., 2008) suggested Phox2b as a molecular link between sympathetic neuron differentiation and proliferation and a tumor-suppressor role for Phox2a/b. It is, however, unclear whether the tumor predisposition caused by mutations in the *PHOX2B* gene in familial (Mossé et al., 2004; Trochet et al., 2004) and sporadic (van Limpt et al., 2004; McConville et al., 2006) cases of neuroblastoma, is due to the loss of tumor-suppressor function. On the contrary, tumors in familial cases of neuroblastoma showed neither mutations of the second allele nor loss of heterozygosity, suggesting that gain-of-function or dominant-negative effects may account for the oncogenic effects (Bourdeaut et al., 2005). The absence of tumors in response to a heterozygous deletion of the PHOX2B locus (Benailly et al., 2003), also argues against a loss-of-function effect in neuroblastoma predisposition.

Here, we analyzed the effects of Phox2b neuroblastoma mutations in potential tumor founder cells in the sympathoadrenergic

lineage, using cultures of immature sympathetic neurons from chick and mouse embryos. The demonstration that neuroblastoma mutations lead to gain-of-function and dominant-negative Phox2b variants that increase sympathetic neuron proliferation and dedifferentiation provides a molecular explanation for neuroblastoma predisposition.

Materials and Methods

Construction of plasmids

Phox2b mutants were based on the murine isoform of Phox2b and created via mutagenesis of *pCAGGS-Phox2b* (site directed mutagenesis kit, Stratagene). Primers used for the mutagenesis PCR are as follows: *Phox2b*[R100L], 5′-CGCAAGC-AGCGGCTCATCCGCACCACC-3′, 5′-GG-TGGTGCGGATGAGCCGCTGCTTGCG-3′; *Phox2b*[R141G], 5′-CCTCACAGAGGCGGGAG-TCCAGGTGTGG-3′, 5′-CCACACCTGGA-CTCCCGCCTCTGTGAGG-3′; *Phox2b*[676delG], 5′-GCGCTCCGGGGCGGCGGGCCC-3′, 5′-GGGCCCGCCGCCCAGGAGCGC-3′; and *Phox2b*[K155X], 5′-GCTAGCACCACCAT-GTATAAAATGGAATATTCTTACCTC-3′, 5′-TTATCGATCTAGCGAAACTTAGCC-CG-3′.
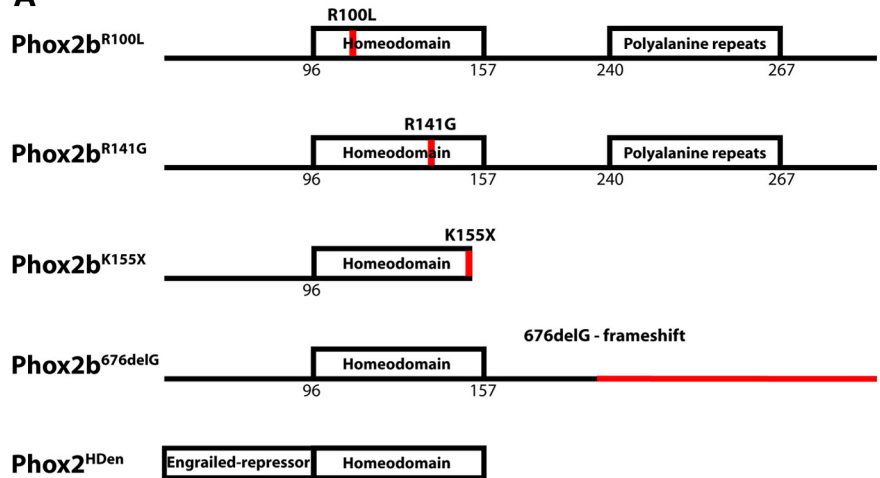
siRNA

The siRNA against chick *Phox2b* targets the following sequence: 5′-CAAGAACGGAGCGGC-CGGCAA-3′ (Qiagen, *siPhox2b*). SiPhox2b does not target the murine isoform of *Phox2b* due to nucleotide mismatches. The siRNA against *Hand2* binds to the sequence 5′-CACAGTT-AGCAGCAGCGATAA-3′ (Qiagen, *siHand2*) and results in selective Hand2 knockdown (Schmidt et al., 2009). The control siRNA, *siLuc*, recognizes the firefly luciferase (Qiagen): 5′-CACCTAGATCCTTTTAAAT-3′.

Primary sympathetic neuron culture preparation and transfection

Chick sympathetic neuron culture. Paravertebral lumbosacral sympathetic chain ganglia were harvested from embryonic day 7 (E7) chicken embryos. Chemical and mechanical dissociation was performed as described before (Rohrer and Thoenen, 1987; Zackenfels et al., 1995). An Amaxa Nucleofector II was used to electroporate 200,000 cells according to Amaxa's protocol for primary chicken neurons (Program G-13, transfection efficiency: ≈50%). One microgram of *pCAGGS-GFP* was mixed with either 2 µg of *empty vector/siLuc* for controls or 2 µg of the appropriate *pCAGGS*-plasmid containing *Phox2b*-variants, *Hand2*, *p27*[kip1], *NMYC* and *Id2* or 2 µg of *siPhox2b* and *siHand2*. For the Phox2b rescue experiment cells were electroporated with 0.25 µg of *Phox2b* and 2 µg of *Hand2 pCAGGS*-plasmid. Transfected cells were plated on 4-well culture dishes coated with poly-DL-ornithine (Sigma) and laminin (Invitrogen). The cells were incubated at 5% CO_2 and 37°C in MEM containing 10% horse serum, 5% FCS, 1% glutamine, 1% penicillin, and streptomycin. For proliferation analysis, bromodeoxyuridine/5-ethynyl-2′-deoxyuridine (BrdU/EdU) was added to the culture medium either 24 or 3 h before fixation (1:1000, Roche). Plasmids containing chick *Id2*, chick *Hand2*, human *NMYC* and mouse *p27*[kip 1] were generously provided by C. Goridis (École Normale Supérieure, Paris, France), M. Howard (University of Toledo, Toledo, Spain), C. Beltinger (University Clinic Ulm, Ulm, Germany), and P. Politis (Biomedical Research Foundation, Athens, Greece), respectively.

A

B

Figure 1. Schematic representation of Phox2b[wt] and mutant expression constructs (***A***) and cellular localization (***B***) of Phox2[wt], Phox2b[R100L], Phox2b[R141G], Phox2b[K155X], Phox2b[676delG] and Phox2[HDen]. ***A***, Scheme illustrating the Phox2b structure, the location of the homeodomain and polyalanine repeats (plain boxes) and of Phox2b mutations (red). The fusion construct between the homeodomain and the engrailed repressor (Phox2[HDen]) is also shown schematically. ***B***, Phox2b expression plasmids were transfected in DF1-cells and the expression of Phox2b[wt] and Phox2b variants was analyzed by RT-PCR and immunostaining, using an antibody directed against the Phox2 HD. All Phox2b variants were detected at RNA and protein level. Scale bar, 10 µm.

Mouse sympathetic neuron cultures. Conditions for culture and proliferation analysis of embryonic mouse sympathetic neuroblasts were modified from Wyatt et al. (1999). Superior cervical ganglia (SCG) and stellate ganglia were dissected from E12 mouse embryos (C57BL/6J) staged according to the method of Theiler (1989). Single cell suspensions were obtained by enzymatic (0.1% collagenase/trypsin) and mechanical dissociation. For each *Phox2b* construct 30.000 cells were electroporated with either *Phox2b pCAGGS*-plasmids (0.6 µg) and *GFP pCAGGS*-plasmid (0.3 µg) or an empty vector (0.6 µg) and *GFP pCAGGS*-plasmid (0.3 µg) as control using an Amaxa Nucleofector II (program: SCN 5). Electroporated cells were plated in a defined serum-free medium (DMEM/F12 1:1, 1% N2-Supplement (Invitrogen), 0.1% BSA, 50 U/ml penicillin, 50 µg of streptomycin and 0.2 mg/ml L-glutamine) on poly-L-ornithine/collagen-coated glass coverslips (diameter 14 mm). NGF at a concentration of 50 ng/ml was added to the medium. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Protein and mRNA assays. Dissection and dissociation were performed as described previously (Rohrer and Thoenen, 1987; Zackenfels et al., 1995). E7 chick sympathetic ganglion cells (400,000) were electroporated with 3 µg of *pCAGGS-GFP/siLuc* for controls or 3 µg of the appropriate *plasmid/siRNA*. Transfected cells were then plated on 35 mm culture

dishes coated with poly-DL-ornithine and laminin. Cells were harvested by scraping and pelleted by centrifuging ($150 \times g$, 10 min). The pellet was either used for mRNA-isolation (Qiagen, mRNEasy Mini Kit) according to Qiagen's protocol or for protein isolation. To isolate cytoplasmatic and nuclear protein, cells were lysed in cold lysis buffer (containing, in mM: 50 Tris-HCl, pH 7.4, 150 NaCl, 40 NaF, 5 EDTA, 5 EGTA, 1 Na_3VO_4, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 1 PMSF, 10 μg/ml aprotinin) for 10 min on ice. After short centrifugation ($10,000 \times g$, 10 min, 4°C) the supernatant was collected and protein content determined with the DC protein assay (Bio-Rad).

Protein blotting

Protein lysates were analyzed by SDS-PAGE and wet blotted on a PVDF-membrane (Bio-Rad). The membrane was blocked with 5% instant milk powder in TBST (containing, in mM: 10 Tris-HCl, pH 8, 150 NaCl, 0.05% Tween 20) for 1 h before antibody incubation. Phox2b (Santa Cruz Biotechnology, SC 13224, 1:1000) and the actin antibodies (Dianova, MS-1295, 1:5000) were diluted in blocking solution and incubated overnight at 4°C. After washing 3 times with TBST, the appropriate HRP-coupled secondary antibody (1:5000 in TBST) was incubated for 1 h at room temperature and washed off twice (TBS). Blots were developed using standard ECL according to manufacturer's instructions (Pierce, Super-Signal). For quantification of protein bands the one-dimensional gel analysis software (GE Healthcare, ImageQuant TL v2005) was used.

Coimmunoprecipitation. SY5Y neuroblastoma cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (containing, in mM: 25 Tris-HCl, pH 7.6, 150 NaCl, 0.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with complete protease inhibitor mix (Roche). Lysates were centrifuged for 10 min at $10,000 \times g$ and 4°C to remove cell debris and afterward precleared with 30 μl of Protein G-agarose beads (Sigma-Aldrich) for 30 min under constant rotation. Precleared SY5Y lysates were incubated with anti-Hand2 or anti-GFP antibodies (Santa Cruz Biotechnology, SC 22818, 1:1000; Invitrogen, 1:1000) overnight at 4°C under constant rotation. Protein G-agarose beads were added for 4 h at 4°C with rotation. After extensive washes in RIPA buffer, the immunoprecipitates were separated by SDS-PAGE and analyzed by Western blot using a rabbit Phox2b antibody (kind gift from Christo Goridis, Paris, France, 1:1000).

GST-pulldown. EcoRI and XbaI restriction sites were added to the 5′ and 3′ ends, respectively, of Phox2b[wt] and Phox2b mutant variants and subcloned into the corresponding site in pGEX_5x_1 bacterial expression vector (GE Healthcare Biosciences). To the 5′ and 3′ ends of Hand2 sequence BamHI and HindIII restriction sites were added and subcloned into the pRSET-A bacterial expression vector (Invitrogen) which adds a C-terminal His Tag. The proteins were expressed in *Escherichia coli* BL21 (DE3) (Merck). Expression was induced by 0.2 mM isopropyl-1-thio-β-D-galactopyranoside at 25°C for 20 h. The molecular weight of GST-fusion proteins Phox2b[wt], Phox2b[R100L], Phox2b[R141G] and the truncated Phox2b[K155X] corresponds to the expected molecular weight of the proteins fused with GST, 58 kDa and 42 kDa, respectively (Trochet et al., 2009). The frameshift mutant Phox2b[676delG] displays a lower molecular weight, which might be due to impaired translation in the bacterial system. GST-Phox2b fusion proteins (500 μl) were incubated with 40 μl of glutathione Sepharose beads for 2 h and washed 3 times with PBS. The matrix was incubated with His-tagged Hand2 protein lysate with 0.1% Triton X-100 to prevent unspecific binding for 2 h at 4°C and washed 3 times with PBS 0.5% Triton X-100. After washing, the GST-beads were eluted twice for 5 min at 48°C with 25 mM Tris-HCl pH 7.5, 192 μm glycine, 0.1% SDS and the supernatants were analyzed by SDS-PAGE and Western blotting. GST-beads alone and GST-protein lysates without GST-beads incubated with His-tagged Hand2 protein lysates served as negative controls for specific protein interaction. GST-fusion proteins were detected by anti-GST-HRP antibodies (Abcam) (1:40.000, TBS-T with 5% low fat milk powder) and Hand2 was visualized by anti-His antibodies (Abcam) (1:5000, TBST with 5% milk powder) and anti-rabbit-HRP-coupled secondary antibodies (Dianova) (1:5.000, TBS-T with 5% milk powder). Hand2 bacterial lysate was used as positive control for the Hand2 Western blot.
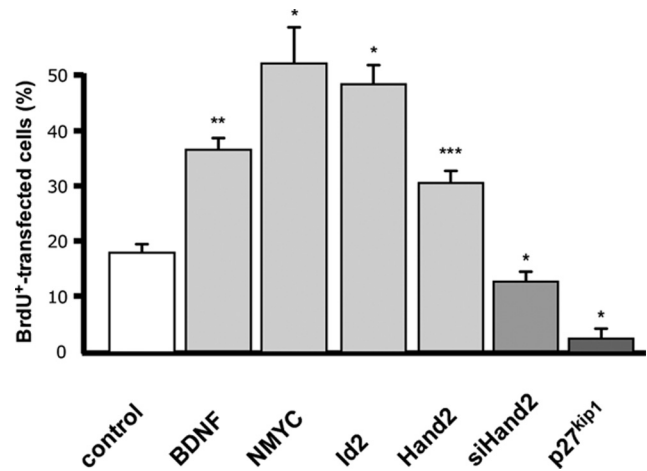

Figure 2. Sympathetic neuron proliferation is increased by BDNF, NMYC, Id2 and Hand2 and reduced by Hand2 knockdown and p27[kip1]. E7 sympathetic neurons, transfected with expression vectors for GFP, or cotransfected with *NMYC, Id2, Hand2, p27[kip1]* or *siHand2* were analyzed for proportion of GFP-positive neurons labeled by BrdU as in Figure 3. BDNF was administered in a concentration of 50 μM. Data are the mean ± SEM ($n = 3$ independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from control.

Semiquanitative reverse transcription PCR

cDNA synthesis on total RNA from mRNA-isolations of sympathetic neuron cultures was performed using the SuperScript III reverse transcriptase kit (Invitrogen). The following primer pairs were used for PCR: *trkA* forward, 5′-CCCCTCATCATGGTCTTCGA-3′ *trkA* reverse, 5′-TGTGGATGTCCTGGATGCGC-3′; *tyrosine hydroxylase* (*Th*) forward, 5′-GCGGCTACAATGAGAACAACATC-3′, *Th* reverse, 5′-GGCTCG-TATTTCACTGAGAAGGG-3′; *Dbh* forward, 5′-GCACAACCGAGT-CTGTCAACATC-3′, *Dbh* reverse, 5′-CATCGTGGGAACGCAGTTTG-3′; *p27[kip]* forward, 5′-AAGGGAGGGGGAGATGTCAAAC-3′, *p27[kip]* reverse, 5′-CATCGTGGGAACGCAGTTTG-3; *Tlx3* forward, 5′-CTTC-CAGAAGTCGCTCAACG-3′, *Tlx3* reverse, 5′-TCAGACGAGGGAG-GTGAC-3′; *p75* forward, 5′-TGGGGCTCCAAGGAAAAGTG-3′, *p75* reverse, 5′-ACCTCGTTCTCTTCGCAGATGG-3′; and *Gapdh* forward, 5′-AAGGGTGGTGCTAAGCGT-3′, *Gapdh* reverse, 5′-GCAGGGGC-TCCAACAAAG-3′.

All PCRs were performed within the linear range that had been determined empirically for each pair of primer and cell preparation. The PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide. Quantification was done with the ScionImage Software version 4.03 (Scion) and the appropriate band intensity of amplified marker cDNAs was normalized to *Gapdh*. All results are given as the percentage ± SEM of control transfection of at least three independent experiments and were statistically analyzed using paired two-tailed Student's *t* test.

Immunostaining

BrdU/EdU. Cells were washed with PBS and fixed using 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 15 min. After washing with PBS, DNA was denatured with 2N HCl in case of BrdU staining. Cells were stained with anti-BrdU or EdU labeling kit according to the manufacturer's protocol (1:100, mouse, Roche, BrdU labeling kit) (Invitrogen, Click-It EdU Imaging Kit 594). GFP-expression was analyzed by staining with anti-GFP antibody (1:500, rabbit, Invitrogen). To detect BrdU-antibodies, biotinylated anti-mouse antibodies (1:500 in PBS, 0.5% Triton X-100 and 5% FCS, Jackson ImmunoResearch) followed by Cy3-coupled Streptavidin were used (Millipore Bioscience Research Reagents). Alexa488 anti-rabbit was used as secondary antibody to anti-GFP. Nuclei were stained with DAPI (Sanofi Aventis). Stainings were covered with Aqua Polymount (Polysciences) and analyzed at room temperature with a Zeiss Axiophot 2 microscope (40× objective, 0.75 Ph2). Data were raised by analyzing GFP-positive neurons with neurites for BrdU/EdU staining in at least 20 visual fields per culture dish at a magnification of 40×. A Visitron Systems Spot RT3 camera was used for image acquisition

and MetaVue (7.1.3.0) for digital image processing (adjustment of brightness and contrast). All results are given as mean ± SEM of at least three independent experiments and statistically analyzed with paired two-tailed Student's *t* test.

Th, phospho-histone-3, and Phox2b. Cells were fixed and washed as described before. Primary antibodies were diluted 1:500 in staining buffer and incubated for 1 h [mouse, Th, rabbit, phospho-histone-3 (pH^3); Abcam]; at room temperature or overnight (goat, Phox2b, Santa Cruz Biotechnology). After washing with PBS, appropriate secondary antibodies were used (1:500, Alexa 594, Invitrogen). Cells were washed again with PBS and mounted as described before. Transfected cells were identified by GFP-fluorescence. Stainings were analyzed as described before.

Luciferase assays. DF1 cells were grown on 24-well plates in DMEM with 10% FCS until achieving 80% confluency and transfected with Effectene according to manufacturer's protocol (Qiagen). Fifty nanograms of the different Phox2b-constructs were combined with 300 ng of DBH promotor Luciferase reporter-plasmid (Trochet et al., 2005a) and 30 ng of pRL-CMV *Renilla* reporter-plasmid as internal control. After 48 h luciferase and renilla activities were assayed with the Dual-Glo Luciferase assay system (Promega) and measured with a Glomax Luminometer (Turner Biosystems). Luciferase activity results were normalized to the internal *Renilla* controls and analyzed with Microsoft Excel. Reporter assays were repeated for at least 4 times in duplicate.

Results

Phox2b^{wt} displays an antiproliferative effect, which is lost in neuroblastoma variants

Mutations in *PHOX2B* have been linked to several congenital human diseases, including congenital central hypoventilation syndrome (CCHS), Hirschsprung's disease and neuroblastoma (Amiel et al., 2003; Weese-Mayer et al., 2003; Mossé et al., 2004; Trochet et al., 2004). Whereas CCHS is caused by heterozygous *PHOX2B* mutations that selectively affect the length of the polyalanine repeats (Fig. 1*A*) (Amiel et al., 2003; Weese-Mayer et al., 2003; Trochet et al., 2004, 2005a), there is no obvious site selectivity of mutations that predispose to neuroblastoma. Mutations in neuroblastoma are either missense alterations in highly conserved regions, or frameshift mutations that lead to an altered or truncated protein. Here we investigated the functional properties of a subset of neuroblastoma Phox2b variants with point mutations in the homeodomain (R100L, Phox2b^{R100L}; R141G, Phox2b^{R141G}) (Trochet et al., 2004, 2005a,b), the K155X mutation (Phox2b^{K155X}) (Weese-Mayer et al., 2003) that results in a C-terminally truncated Phox2b protein, and a frameshift mutation (676delG, Phox2b^{676delG}) (Mossé et al., 2004) (Fig. 1*A*). The analysis also included wild-type mouse Phox2b (Phox2b^{wt}; amino acid sequence identity mouse vs human Phox2b is 100%) and a dominant-negative variant of Phox2 proteins, composed of the Phox2 homeodomain (identical in Phox2b and Phox2a) fused to the engrailed repressor domain (Phox2^{HDen}) (Dubreuil et al., 2002) (Fig. 1*A*).
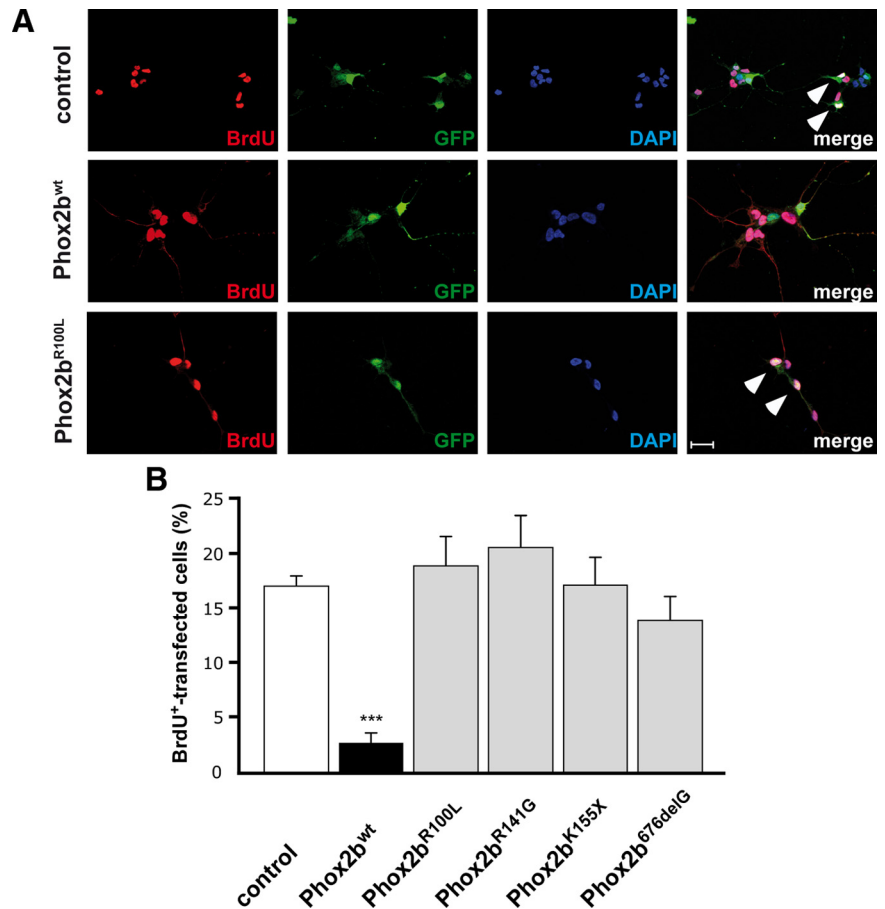

Figure 3. Phox2b^{wt} overexpression inhibits the proliferation of E7 sympathetic neurons, whereas Phox2b mutants Phox2b^{R100L}, Phox2b^{R141G}, Phox2b^{K155X}, Phox2b^{676delG} show no antiproliferative effect. Sympathetic neurons, transfected before plating with expression vectors for GFP, or cotransfected with Phox2b^{wt} or Phox2b^{R100L}, Phox2b^{R141G}, Phox2b^{K155X} and Phox2b^{676delG}, were labeled and immunostained for BrdU (red), GFP (green) and cell nuclei (DAPI) after 2 d in culture. Expression of Phox2b^{wt}, but not of Phox2b variants caused a decrease in the proportion of BrdU-labeled, GFP-positive transfected neurons. *A*, Effect of Phox2b^{wt} and Phox2b^{R100L} is shown. BrdU-labeled transfected neurons are indicated by arrowheads. Scale bar, 20 μm. *B*, The proportion of transfected GFP-positive neurons labeled by BrdU was determined after transfection with GFP only (control) or after cotransfection with Phox2b^{wt} or the Phox2b mutants indicated. Data are the mean ± SEM ($n = 3$, independent experiments). ***$p < 0.001$, significantly reduced compared with control.

After transfection of DF-1 chick fibroblast cells, expression of Phox2b^{wt} and all variants was observed by reverse transcription (RT)-PCR (Fig. 1*B*; data not shown). Immunohistological analysis of transfected fibroblasts (Fig. 1) and neurons (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) revealed nuclear localization for Phox2b^{wt}, Phox2b^{R100L}, Phox2b^{R141G}, Phox2b^{K155X} and Phox2^{HDen}. Phox2b^{676delG} was also detected in the nucleus, albeit at lower levels (Fig. 1*B*).

To investigate the effects of Phox2b on proliferation in the sympathoadrenergic lineage we used sympathetic neuron cultures from E7 chick embryos. These cultures have previously been shown to contain proliferating cells that display neuronal morphology and express characteristic pan-neuronal and adrenergic markers (Rohrer and Thoenen, 1987; Zackenfels et al., 1995). They express trkB and proliferation is stimulated by BDNF (Straub et al., 2007) and forced expression of NMYC, Id2 and Hand2 (Fig. 2). TrkB signaling, NMYC and Id2 are implied in the altered proliferation control in neuroblastoma (Brodeur et al., 1984; Nakagawara et al., 1994; Jögi et al., 2002). The culture conditions closely mimic neurogenesis *in vivo* as neuron pro-
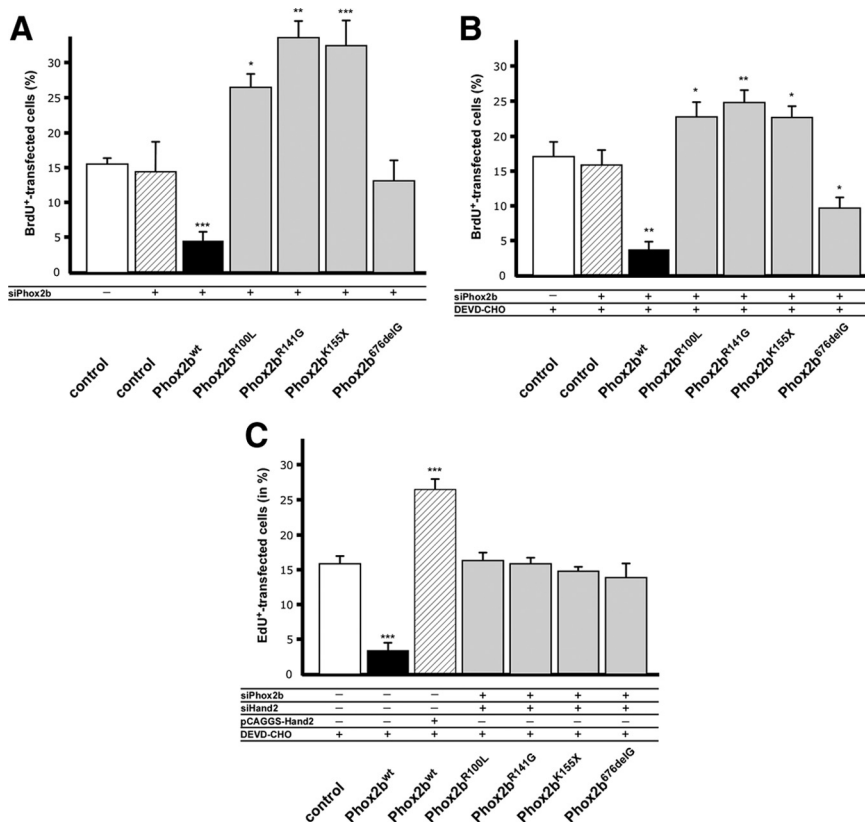
Figure 4. Phox2b mutants Phox2b^{R100L}, Phox2b^{R141G}, Phox2b^{K155X} increase sympathetic neuron proliferation when endogenous Phox2b levels are reduced by siRNA knockdown. ***A***, E7 sympathetic neurons were transfected before plating with either pCAGGS-GFP (control), with pCAGGS-GFP and *siPhox2b* directed against endogenous chick Phox2b (Phox2b knockdown) or cotransfected with pCAGGS-GFP, *siPhox2b* plus pCAGGS vectors for Phox2b^{wt} and the Phox2b mutants indicated. The proportion of BrdU-labeled transfected neurons was determined after 2 d in culture. BrdU-positive cells were reduced by Phox2b^{wt} and increased by Phox2b^{R100L}, Phox2b^{R141G}, Phox2b^{K155X}. Data are the mean ± SEM ($n = 3$ independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from control. ***B***, Effect of Phox2b mutants Phox2b^{R100L}, Phox2b^{R141G}, Phox2b^{K155X} in the presence of apoptosis block and Phox2b knockdown. Phox2b^{R100L}, Phox2b^{R141G}, Phox2b^{K155X} elicit increased sympathetic neuron proliferation also in the presence of the apoptosis inhibitor DEVD-CHO (5 μM). Data are the mean ± SEM ($n = 3$ independent experiments). *$p < 0.05$, **$p < 0.01$, significantly different from control. ***C***, Effect of Phox2b^{wt}, combined expression of Phox2b^{wt} and Hand2, and Phox2b mutants Phox2b^{R100L}, Phox2b^{R141G}, Phox2b^{K155X} and Phox2b^{676delG} in the presence of apoptosis block and Phox2b/Hand2 knockdown conditions. Increased Hand2 levels rescue the antiproliferative effect of Phox2b^{wt}. siRNA-mediated Hand2 knockdown abolishes the proliferation stimulating effect of Phox2b mutants (compare ***B*** and ***C***). Data are the mean ± SEM ($n = 4$ independent experiments). ***$p < 0.001$, significantly different from control.

liferation is controlled by Hand2 (Hendershot et al., 2008; Schmidt et al., 2009) (Fig. 2) and IGFs (Zackenfels et al., 1995). Here, the effects of Phox2b^{wt} and Phox2b variants were analyzed by cotransfecting sympathetic ganglion cells with Phox2b and GFP expression plasmids (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) and determining the proportion of BrdU-labeled GFP-positive cells (Fig. 3).

Overexpression of Phox2b^{wt} resulted in a strong reduction in the number of BrdU-labeled cells, an effect not observed for the neuroblastoma Phox2b variants analyzed (Fig. 3*A,B*). Virtually identical results were obtained using cultures of embryonic (E12) mouse sympathetic neuron cultures (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The loss of antiproliferative function in Phox2b mutants is in agreement with the notion of a tumor suppressor role for Phox2b. However, the effect of ectopically expressed Phox2b variants may be restricted by the presence of the normal complement of endogenous Phox2b in sympathetic neurons, whereas in neuroblastoma, heterozygous *PHOX2B* mutations may be more effective due to

the reduction of endogenous PHOX2B^{wt}. To address the question of how neuroblastoma Phox2b variants affect sympathetic neuron proliferation in cells with lower Phox2b levels, we have used chick sympathetic neuron cultures and a siRNA approach to reduce endogenous Phox2b expression.

Phox2b^{R100L}, Phox2b^{R141G} and Phox2b^{K155X} stimulate sympathetic neuron proliferation after knockdown of endogenous *Phox2b*

Phox2b knockdown was achieved by nucleoporation of *Phox2b*-siRNAs and compared with *Luciferase*-siRNA (*siLuc*) as controls. *Phox2b* siRNA was designed to selectively interfere with endogenous chick *Phox2b* but not with ectopically expressed mouse *Phox2b* variants. *Phox2b* siRNA (*siPhox2b*) reduced the proportion of Phox2b-immunoreactive (Phox2b-IR) sympathetic neurons to 26 ± 9% compared with 100% Phox2b-IR neurons after transfection with control siRNA or pCAGGS (supplemental Fig. 3*A*, available at www. jneurosci.org as supplemental material). Western-blot analysis confirmed this result and showed a strong reduction (90%; $n = 2$) of Phox2b protein expression by *siPhox2b* (supplemental Fig. 3*B,C*, available at www. jneurosci.org as supplemental material).

For the analysis of sympathetic neuron proliferation after Phox2b knockdown, expression vectors for *GFP* and *Phox2b* variants were cotransfected with siRNAs and analyzed for BrdU-incorporation. Interestingly, the neuroblastoma Phox2b variants with mutations in the homeodomain, i.e., Phox2b^{R100L}, Phox2b^{R141G}, and Phox2b^{K155X}, elicited a strong increase in sympathetic neuron proliferation when endogenous Phox2b was reduced (Fig. 4*A*). This is, to our knowledge, the first demonstration that neuroblastoma Phox2b mutations result in a gain-of-function phenotype. Basal proliferation of sympathetic neurons and the response to Phox2b^{wt} and Phox2b^{676delG} were not affected by Phox2b knockdown (compare Figs. 3*B* and 4*A*).

The interpretation of experiments with Phox2b knockdown are complicated by the fact that sympathetic neuron survival, determined by the number of GFP-positive transfected neurons, is impaired by reducing endogenous Phox2b levels (38% of control without *siPhox2b*). However, this effect is largely compensated by the coexpression of Phox2b^{wt} and Phox2b neuroblastoma variants (Phox2b^{wt}, Phox2b^{R100L}, Phox2b^{R141G}, and Phox2b^{K155X} 87.3 ± 1.6%, 107 ± 3%, 102 ± 2%, and 107 ± 4% of control), arguing against the possibility that increased proliferation is compromised by effects on survival. To further support this notion, the effects of combined Phox2b knockdown and overexpression of Phox2b variants were analyzed under conditions where apoptotic cell death was blocked by the addition of the apoptosis inhibitor DEVD-CHO (Fig. 4*B*). The sympathetic neuron survival in *siPhox2b*-treated cultures was maintained at 91 ±

8% of controls by the addition of DEVD-CHO, compared with $38 \pm 1\%$ after Phox2b knockdown in the absence of the apoptosis inhibitor. The proliferation increase in response to $Phox2b^{R100L}$, $Phox2b^{R141G}$ and $Phox2b^{K155X}$ was also observed under these conditions (Fig. 4B). The frameshift mutant $Phox2b^{676delG}$ showed no effect which is most likely due to the inefficient transfer to the nucleus. Together, these findings demonstrate that the increased proportion of BrdU-labeled cells in response to $Phox2b^{R100L}$, $Phox2b^{R141G}$ and $Phox2b^{K155X}$ is caused by increased proliferation rather than by increased survival of proliferating cells. In agreement with this conclusion, proliferation effects by $Phox2b^{wt}$, $Phox2b^{R100L}$, $Phox2b^{R141G}$ and $Phox2b^{K155X}$ were also observed after short-term (3 h) BrdU-labeling (data not shown) and by phospho-histone-3 staining (supplemental Fig. 4, available at www.jneurosci.org as supplemental material), which determines the number of cells in M phase.

Proliferation effects of Phox2b and Phox2b variants involve Hand2

Hand2 is essential for the proliferation of immature sympathetic neurons *in vivo* (Hendershot et al., 2008; Schmidt et al., 2009) and sufficient to increase sympathetic neuron proliferation *in vitro* (Fig. 2). As Hand2 was previously shown to synergistically interact with Phox2a in the control of noradrenergic differentiation (Rychlik et al., 2003; Xu et al., 2003) we investigated the functional interaction of Hand2 and Phox2b in the control of sympathetic neuron proliferation by coexpression experiments. The antiproliferative effect of $Phox2b^{wt}$ could be completely compensated by coexpression of Hand2 (Fig. 4C) and was enforced upon siRNA-mediated Hand2 knockdown (supplemental Figs. 5, 6, available at www.jneurosci.org as supplemental material), which suggests that the $Phox2b^{wt}$ effect is mediated by interfering with Hand2. Interestingly, also the proliferation increase elicited by the Phox2b variants depends on Hand2, as this effect could be blocked by knockdown of Hand2 (Fig. 4C; supplemental Fig. 6, available at www.jneurosci.org as supplemental material). However, Phox2b variants display a positive functional interaction with Hand2, in contrast to $Phox2b^{wt}$.

The antagonistic and stimulatory interactions between Phox2b/Phox2b variants and Hand2 could be explained by mechanisms shown to act during noradrenergic neuron specification and differentiation, i.e., effects of Phox2b/Phox2b variants on Hand2 expression (Hendershot et al., 2008) or to a direct interaction of Phox2b/Phox2b variants and Hand2 proteins. Interestingly, Phox2b overexpression in proliferating sympathetic neurons resulted in a reduction of Hand2 mRNA levels to $59.9 \pm 15\%$ of control transfections ($p < 0.02$), whereas siRNA-mediated Phox2b knockdown increased Hand2 mRNA levels to $172 \pm 22\%$ of controls ($p < 0.05$). Notably, Hand2 expression is negatively regulated by Phox2b in sympathetic neurons, in contrast to the Phox2b-dependent Hand2 expression during initial development of sympathetic progenitors (Hendershot et al.,
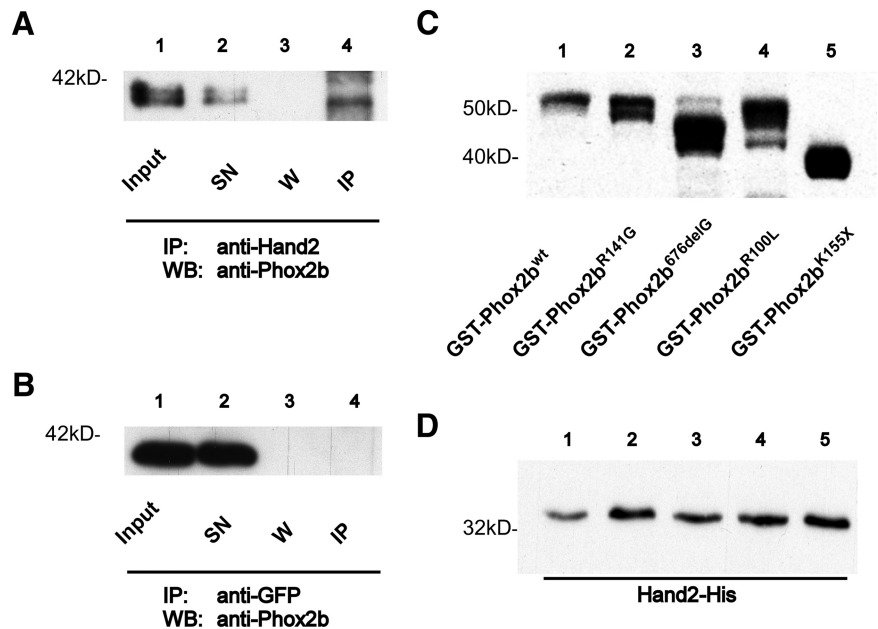

Figure 5. Protein–protein interaction between Phox2b and Hand2. *A*, *B*, Coimmunoprecipitation of Phox2b and Hand2 from SY5Y neuroblastoma cells. Western blots with antibodies specific for Phox2b after immunoprecipitation with anti-Hand2 (*A*) or anti-GFP (*B*) antibodies. Input, Input control; SN, supernatant after agarose bead and Hand2 (*A*) or GFP (*B*) antibody incubation; W, supernatant after washing immunopellet with lysis buffer; IP, immunopellet. Anti-GFP antibody (*B*) and agarose beads alone (data not shown) served as negative controls. Phox2b bands with different apparent molecular weights most likely represent differentially phosphorylated forms (Adachi and Lewis, 2002). *C*, *D*, Analysis of Phox2b–Hand2 interaction by pulldown experiments. Bacterial lysates of GST-Phox2b fusion proteins were first incubated with GST-beads and subsequently with Hand2 bacterial lysate. *C*, Western blot analysis of GST-Phox2b fusion proteins that were used for the pulldown of Hand2 visualized by GST-HRP-coupled antibodies. *D*, Hand2-His was detected by antibodies against the His-tag and is present in every lane after GST-pulldown with GST-Phox2b fusion proteins. GST-beads alone and GST-Protein served as negative controls whereas Hand2 protein lysate served as positive control for specific protein–protein interaction (data not shown).

2008). In addition, we now demonstrate the interaction between Hand2 and Phox2b by coimmunoprecipitation (Fig. 5A) and provide evidence for direct protein–protein binding between Hand2, $Phox2b^{wt}$ and Phox2b variants by pulldown experiments (Fig. 5C,D). Neither protein-G agarose beads coated with anti-GFP antibody (Fig. 5B) nor agarose beads alone (data not shown) showed Phox2b signal after immunoprecipitation. To study protein–protein interactions between Hand2 and Phox2b, glutathione-agarose beads containing bacterially expressed GST-$Phox2b^{wt}$ or GST-Phox2b variants were incubated with lysates of Hand2-His-expressing bacteria. Hand2 did not interact with the glutathione-agarose beads or GST protein alone (data not shown), but when GST-Phox2b-bound beads were added, strong signals were observed (Fig. 5D), demonstrating protein–protein interaction between $Phox2b^{wt}$, Phox2b variants and Hand2. As both $Phox2b^{wt}$ and Phox2b variants bind to Hand2 the homeodomain mutations may elicit a change from the antagonistic function of $Phox2b^{wt}$ to a synergistic interaction of Phox2b variants with Hand2. Alternatively, Phox2b variants may deplete antiproliferative $Phox2b^{wt}$ from the Hand2 interaction partner. Together, these results suggest that the proliferation effects of Phox2b are mediated, at least in part, by Hand2 and imply both transcriptional and posttranscriptional mechanism in this interaction.

Sympathetic neuron cell cycle withdrawal is not strictly linked to $p27^{kip1}$ increase

Cyclin-dependent kinase (Cdk) inhibitors (CKIs) are important regulators of cell cycle exit during development. The Cip/Kip family of inhibitors ($p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$) inactivate
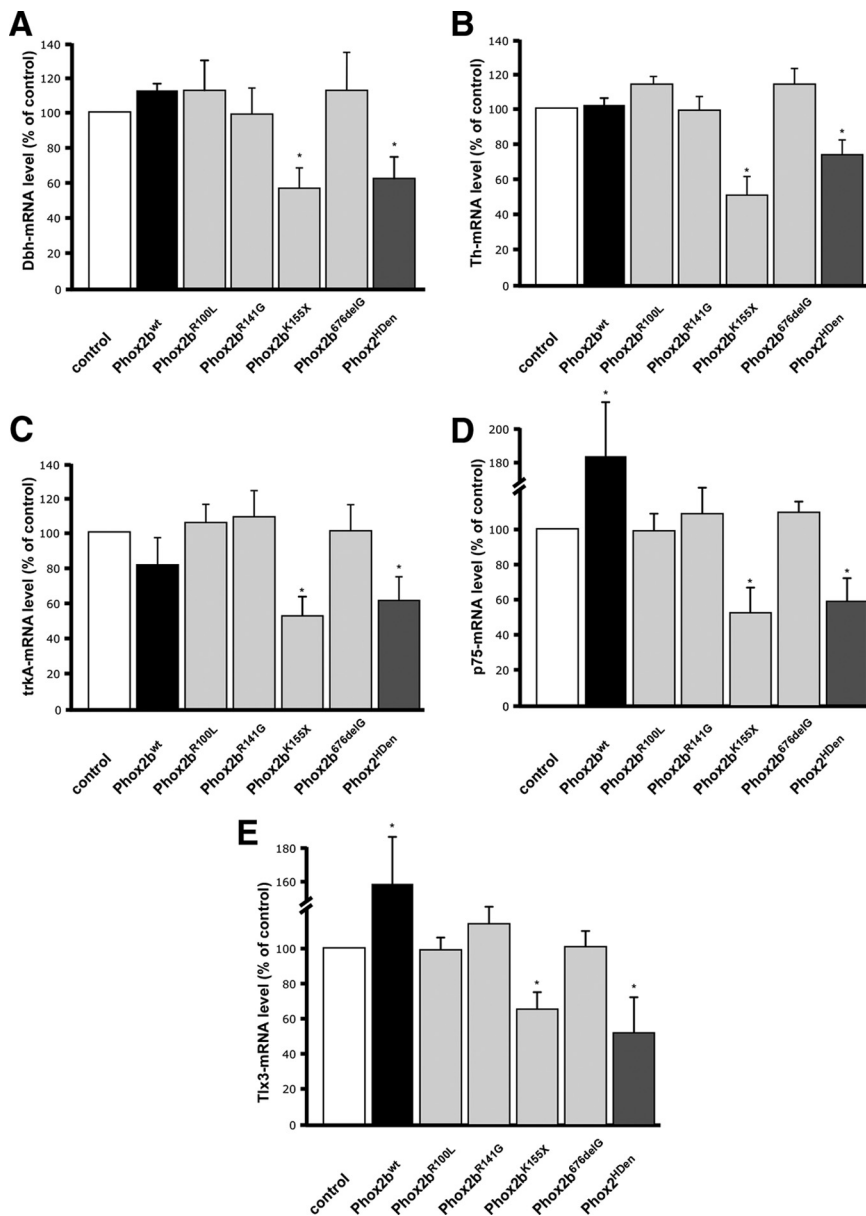
Figure 6. Effects of Phox2b[wt] and Phox2b mutants on *Dbh*, *trkA*, *Th*, *Tlx3* and *p75* mRNA levels in E7 sympathetic neuron cultures. Sympathetic neurons were transfected before plating with pCAGGS-GFP (control) or with pCAGGS-expression vector for Phox2b[R100L], Phox2b[R141G], Phox2b[K155X], Phox2b[676delG] or Phox2[HDen]. Cells were harvested after 2 d in culture, and expression of *Dbh*, *trkA*, *Th*, *Tlx3* and *p75* was analyzed by semiquantitative RT-PCR. Expression levels are normalized to *Gapdh* and referred to control transfections. Data are the mean ± SEM (*n* = 3 independent experiments). *$p < 0.05$, significantly different from control.

sion, whereas increased proliferation by Hand2 overexpression resulted in lower *p27*[kip1] expression levels (supplemental Fig. 7, available at www.jneurosci.org as supplemental material). However, no correlation between sympathetic neuron proliferation and *p27*[kip1] was observed after Phox2b and Hand2 knockdown (see Fig. 2 and supplemental Fig. 7, available at www. jneurosci.org as supplemental material). Thus, cell cycle exit of sympathetic neurons is not exclusively linked to an increase in p27[kip1]. Rather, additional mechanisms of action for Phox2 and Hand2 proteins in cell cycle control are implied.

In cultured proliferating sympathetic neurons Phox2a expression was not detectable by immunostaining and RT-PCR (40 cycles) (data not shown). Together with the observation that Phox2b but not Phox2a is essential for sympathetic neuron development (Coppola et al., 2005) our findings argue against an involvement of Phox2a in sympathetic neuron proliferation.

Phox2b[K155X] elicits a decrease in the expression of characteristic marker genes and all neuroblastoma Phox2b variants have lost the ability to induce *p75* and *Tlx3*

One of the best understood functions of Phox2b is its role in the initial expression of *Dbh* and *Th* in sympathetic neuron progenitors (Zellmer et al., 1995; Yang et al., 1998; Pattyn et al., 1999; Adachi et al., 2000). Here, we addressed the question whether *Th* and *Dbh* expression is affected by Phox2b[wt] and Phox2b neuroblastoma variants in E7 chick sympathetic neurons, i.e., sympathoadrenergic cells that already have acquired noradrenergic neuron properties. In addition, effects on the neurotrophin receptors *trkA*, *p75* and the transcription factor *Tlx3* were investigated. The analysis of *trkA* expression and *p75* was of particular interest as elevated *trkA* and *p75* levels correlate with good prognosis in neuroblastoma patients (Kogner et al., 1993; Nakagawara et al., 1993). Effects of Phox2b on *Tlx3* expression were studied since *Tlx3* is the chick ortholog of *Tlx2*, a direct Phox2 target gene (Borghini et al., 2006). Sympathetic neurons were transfected with *Phox2b[wt]* and *Phox2b* mutant expression vectors and analyzed by RT-PCR for *Dbh*, *Th*, *trkA*, *p75* and *Tlx3* expression. *Phox2b[wt]* increased the expression of *Tlx3* and *p75* but did not affect *Dbh*, *Th* and *trkA* (Fig. 6). This suggests that the endogenous Phox2b levels are saturating in these cells for *Dbh*, *Th* and *trkA* expression, but not for *Tlx3* and *p75*. Interestingly, Phox2b[K155X] elicited a significant decrease in the expression of all genes investigated. Considering the transfection efficiency of ~50%, effects are substantially stronger if only transfected cells were taken into account. Indeed, when the effect on TH expression was analyzed by TH-immunostaining of identified transfected cells, the proportion of TH-expressing cells was

Cdk2 and Cdk1 and play a central role as negative regulators of cell cycle progression in the developing nervous system (Dyer and Cepko, 2001; Gui et al., 2007). Individual CKIs are required for cell cycle exit in specific subpopulations of cells in retina and spinal cord, suggesting that different cell populations employ a distinct molecular equipment to control cell cycle exit (Dyer and Cepko, 2001; Gui et al., 2007). A previous study implied p27[kip1] downstream of Phox2a in cell cycle withdrawal of the noradrenergic CAD cell line (Paris et al., 2006). In cultured sympathetic neurons, p27[kip1] overexpression results in a virtually complete proliferation block (Fig. 2). To further address the role of p27[kip1] in cultured sympathetic neurons, *p27*[kip1] levels were determined by RT-PCR under various conditions that increase or decrease sympathetic neuron proliferation. Phox2b overexpression reduced proliferation and significantly increased *p27*[kip1] expres-

reduced to 24 ± 4% (Fig. 7). The comparable decrease in *Dbh, Th, trkA, p75* and *Tlx3* expression in response to the Phox2b^{K155X} and Phox2^{HDen} (Figs. 6, 7) suggests that the K155X nonsense mutation results in a dominant-negative function of this Phox2b variant. This notion was confirmed using the *DBH* promotor luciferase reporter system (supplemental Fig. 8, available at www.jneurosci.org as supplemental material). Phox2b^{K155X} and Phox2^{HDen} completely repress the Phox2b^{wt}-induced transcription whereas other Phox2b variants with mutations in the homeodomain were unable to antagonize the Phox2b^{wt} effect (Phox2b^{R141G} is shown as representative example). The transactivating function of Phox2b^{wt} at the *DBH* pro-

Figure 7. Effects of Phox2b^{wt} and Phox2b mutants on the expression of TH-protein in E7 sympathetic neuron cultures. Sympathetic neurons, transfected with pCAGGS-GFP (for controls), or cotransfected with Phox2b^{wt} or Phox2b mutants were labeled and immunostained for TH (red) after 2 d in culture. *A*, Control GFP and Phox2^{HDen} transfections are shown. Scale bar represents 20 μm. *B*, Quantitative analysis of TH-immunoreactive GFP-positive transfected neurons. Data are the mean ± SEM (*n* = 3 independent experiments). *$p < 0.05$, significantly reduced compared with control.

motor is strongly reduced in all Phox2b neuroblastoma variants analyzed using *DBH* luciferase reporter assays (supplemental Fig. 8, available at www.jneurosci.org as supplemental material; data not shown), which is in agreement with previous results (Trochet et al., 2005a, 2009; Raabe et al., 2008).

In conclusion, all Phox2b neuroblastoma mutations investigated abolished the transactivating properties of Phox2b^{wt}. The K155X mutation, in addition, gives rise to a Phox2b variant with novel, dominant-negative functions that lead to dedifferentiation of sympathetic neurons.

Discussion

Heterozygous mutations in the transcription factor *PHOX2B*, described in familial and sporadic forms of neuroblastoma represent the first defined mutations predisposing to this childhood tumor. Here, we demonstrate that Phox2b mutations lead to both loss- and detrimental gain-of-function variants, affecting proliferation and differentiation in the sympathoadrenergic lineage. These results provide an explanation for the tumor predisposing role of *PHOX2B* neuroblastoma mutations.

Proliferation effects of Phox2b^{wt} and Phox2b variants
The generation of differentiated neurons in the developing nervous system involves proliferation of progenitors, followed by cell cycle exit and onset of neuron differentiation. Phox2b coordinates cell cycle exit and neuronal differentiation in hindbrain motoneurons (Dubreuil et al., 2000, 2002). In the sympathoadrenergic lineage, Phox2b is also essential for neuron differentiation (Pattyn et al., 1999) but cell cycle exit and differentiation are not coupled in sympathoadrenal cells (Rohrer and Thoenen, 1987), and Phox2b is expressed in proliferating progenitors and proliferating sympathetic neurons. Thus, it was unclear whether cell cycle withdrawal involves Phox2b.

The present findings reveal that forced expression of Phox2b reduces sympathetic neuron proliferation and that the neuroblastoma Phox2b variants have lost their antiproliferative function. A recent comparison of the effects of Phox2b and neuroblastoma variants in neuroblastoma cell lines also showed impaired antiproliferative effects of Phox2b variants (Raabe et al., 2008). However, the importance of this loss-of-function for neuroblastoma predisposition is unclear, as Hirschsprung's disease and hypotonia but not neuroblastoma were reported in a patient with a heterozygous deletion of the entire Phox2b locus (Benailly et al., 2003). The phenotype in heterozygous Phox2b mice is even weaker and only includes atrophy of ciliary ganglia and a defec-
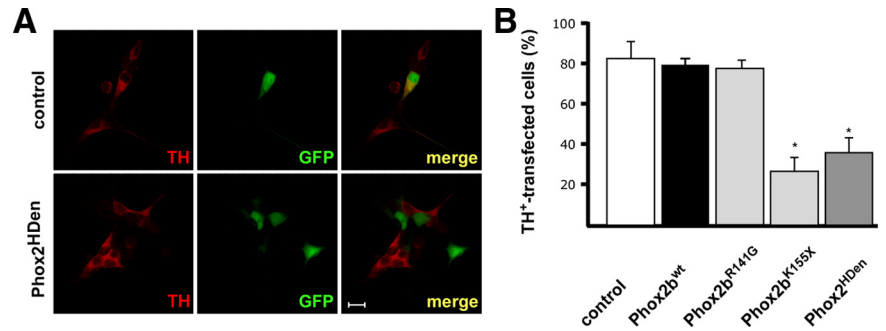
tive response to hypercapnia (Dauger et al., 2003; Cross et al., 2004). We now provide the first demonstration that neuroblastoma Phox2b mutations give rise to gain-of-function variants that stimulate proliferation of potential tumor founder cells of the sympathoadrenergic lineage. Thus, predisposition to tumor formation may rather rely on the acquisition of novel, proliferation-stimulating properties rather than on the loss of antiproliferative function. Increased sympathetic neuron proliferation in response to neuroblastoma variants is revealed when the levels of endogenous Phox2b are reduced by siRNA-mediated knockdown. This can be explained by the fact that the effects of ectopically expressed Phox2b variants are occluded in the presence of the normal complement of endogenous Phox2b. The Phox2b knockdown resembles the situation in neuroblastoma, where patients with PHOX2B mutations are thought to have a 50% reduction of PHOX2B^{wt} levels.

Why is the reduction of endogenous Phox2b levels by siRNA-mediated knockdown not sufficient to increase sympathetic neuron proliferation? This may be explained by an indirect action of Phox2b, interfering with the function of a proliferation regulating protein upon overexpression but unable to relieve its control. An alternative possibility would be that the knockdown of Phox2b does not sufficiently reduce endogenous Phox2b levels to overcome the antiproliferative effect of Phox2b. It should also be noted that the transactivation activity and protein interaction of Phox2a/b proteins is negatively regulated by phosphorylation (Adachi and Lewis, 2002) and that Phox2b overexpression may lead to an excess of active, unphosphorylated protein, whereas the knockdown effect may be compensated by dephosphorylation.

Effects of Phox2b and Phox2b variants on sympathetic neuron gene expression
The major role of Phox2b during development is the initiation of autonomic neuron differentiation. In the present study we have compared the effects of Phox2b^{wt} and Phox2b variants on the expression of the Phox2b target genes *Dbh, Th*, and *Tlx3* (Zellmer et al., 1995; Yang et al., 1998; Borghini et al., 2006), and *trkA, p75*, which are of interest in the context of neuroblastoma (Kogner et al., 1993; Nakagawara et al., 1993). Forced Phox2b expression does not affect *Th, Dbh* and *trkA* but leads to increased *Tlx3* and *p75* expression. This suggests that the endogenous Phox2b levels are saturating for *Th, Dbh* and *trkA* expression but not for *Tlx3* and *p75*. All Phox2b variants analyzed have lost the ability to induce *Tlx3* and *p75* expression. Notably, Phox2b^{K155X} decreased the expression of all genes investigated. As a similar decrease was

observed in response to the dominant-negative Phox2 ^{HDen} variant and both Phox2b ^{K155X} and Phox2 ^{HDen} display a dominant-negative function at the *DBH* promotor, we conclude that Phox2b ^{K155X} interferes with the function of endogenous Phox2b and causes dedifferentiation. The strong dedifferentiation of sympathetic neurons observed here is in agreement with massive disturbance in the affected patients, suffering from CCHS, Hirschsprungs's disease and neuroblastoma (Weese-Mayer et al., 2003). Thus, impaired differentiation and/or dedifferentiation represent additional mechanisms underlying neuroblastoma predisposition.

Structure/function correlations of Phox2b and Phox2b variants

Phox2b and Phox2a are transcriptional activators that interact with homeodomain binding sites in the promotor region and directly stimulate the transcriptional activity of genes like *Dbh* (Yang et al., 1998; Adachi et al., 2000), *Phox2b* (Cargnin et al., 2005) and *Tlx2* (Borghini et al., 2006). The effects of Phox2a/b are blocked by the dominant-negative chimera Phox2 ^{HDen} (Dubreuil et al., 2000, 2002). Thus, the repression of *Dbh, Th, trkA, p75* and *Tlx3* by both Phox2 ^{HDen} and Phox2b ^{K155X} may be explained by a dominant-negative function at homeodomain-binding promotor regions. However, as the homeodomain mutation in Phox2b ^{K155X} not only reduces promotor binding (Trochet et al., 2009) but may also affect protein–protein interaction (Kasahara et al., 2001), dominant-negative effects may be caused by heterodimerization with endogenous transcriptional regulators like Phox2b (Adachi et al., 2000) and Hand2, resulting in inactive transcription complexes. All Phox2b mutations analyzed have lost the ability to induce *Tlx3* and *p75* expression. This was expected from the lack of transactivation activity of Phox2b ^{R100L}, Phox2b ^{R141G}, Phox2b ^{676delG} and Phox2b ^{K155X} at the *Phox2b* and *DBH* promotor (Trochet et al., 2005a, 2009; Raabe et al., 2008). The loss-of-function is explained for the homeodomain mutations by impaired promotor interaction, for the frameshift mutation by the formation of oligomers with reduced nuclear transfer (Trochet et al., 2009).

Whereas the effects of Phox2b mutations on neuron differentiation are explained by altered functions of a transcriptional activator, the mechanisms involved in the proliferation effects seem to be more complex. Increased $p27^{kip1}$ expression during cell cycle exit elicited by Phox2b ^{wt} in cultured sympathetic neurons, together with the $p27^{kip1}$-induced proliferation stop suggests that $p27^{kip1}$ may mediate the effect of Phox2b on the cell cycle. The correlation between increased proliferation and reduced $p27^{kip1}$ expression is also observed upon Hand2 overexpression, but was not maintained upon Phox2b and Hand2 knockdown. Thus, $p27^{kip1}$ levels are not strictly linked to cell cycle exit in sympathetic neurons, arguing against a general role of $p27^{kip1}$ in the termination of sympathetic neuron proliferation.

An attractive mechanism would be that the proliferation effects of Phox2b ^{wt} and Phox2b variants are mediated through Hand2. This notion is supported by four kinds of observations: (1) both antiproliferative effects of Phox2b ^{wt} and proliferation stimulatory effects of Phox2b variants depend on Hand2; (2) Hand2 is essential for the proliferation of sympathetic neurons *in vivo* and *in vitro*; (3) Phox2b ^{wt} and Phox2b variants directly interact with Hand2; and (4) Phox2b ^{wt} negatively regulates the expression of Hand2 in sympathetic neurons. The proposed mechanism involving Phox2b–Hand2 interactions would imply that the homeodomain mutations

lead to a change from an antagonistic to a synergistic interaction. An alternative explanation would be that Phox2b mutants displace Phox2b ^{wt} from the putative direct interaction with Hand2 and thus abrogate the antiproliferative effects of Phox2b ^{wt} by a dominant-negative mechanism. The N-terminal region of Phox2b which contains the transactivation domain of Phox2b, the CBP-interaction domain (Adachi et al., 2000) and a number of highly conserved phosphorylation sites (Trochet et al., 2009) is inferred in the proliferation stimulatory interaction of the Phox2b variants as proliferation is increased by the C-terminally truncated Phox2b ^{K155X}.

An essential role of Hand2 mediating the tumor predisposition of Phox2b mutations would also explain the restriction of the Phox2b mutation effects to sympathoadrenal cells as Hand2, in contrast to Phox2b, is not expressed in parasympathetic neurons (Müller and Rohrer, 2002).

Phox2b mutations and predisposition to neuroblastoma
The appearance of neuroblastoma in childhood or *in utero* indicates that early disruption of normal developmental processes plays an important role in tumor initiation. Familial forms of neuroblastoma, representing 1–2% of cases, show autosomal dominant inheritance with incomplete penetrance. Genetic analyses have identified several predisposition loci, which suggests that initiation of tumorigenesis could require multiple alterations (Maris et al., 2007). In addition to *PHOX2B* (Mossé et al., 2004; Trochet et al., 2004), the orphan tyrosine kinase receptor *ALK* has recently been identified as a second gene where mutations predispose to neuroblastoma (Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mossé et al., 2008). Mutations in Phox2b and Alk increase proliferation and thus may share a similar predisposition mechanism by prolonging the period of neurogenesis and expanding the time window for tumor-initiating events, i.e., (an) additional somatic mutation(s) (Knudson, 2001). A second consequence of Phox2b mutations is dedifferentiation, reflected by a decreased expression of characteristic differentiation genes. Terminal differentiation is a critical barrier to malignant transformation (Brodeur, 2003) and the abrogation of the differentiation process by the Phox2b ^{K155X} variant may increase the susceptibility to additional tumor-initiating events. In conclusion, our data suggest that neuroblastoma Phox2b variants may predispose to this disease by increasing neurogenesis and interfering with the differentiation in the sympathoadrenal lineage.

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