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Ongoing roles of Phox2 homeodomain transcription factors during neuronal differentiation

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SUMMARY

Transcriptional determinants of neuronal identity often stay expressed after their downstream genetic program is launched. Whether this maintenance of expression plays a role is for the most part unknown. Here, we address this question for the paralogous paired-like homeobox genes *Phox2a* and *Phox2b*, which specify several classes of visceral neurons at the progenitor stage in the central and peripheral nervous systems. By temporally controlled inactivation of *Phox2b*, we find that the gene, which is required in ventral neural progenitors of the hindbrain for the production of branchio-visceral motoneuronal precursors, is also required in these post-mitotic precursors to maintain their molecular signature – including downstream transcription factors – and allow their tangential migration and the histogenesis of the corresponding nuclei. Similarly, maintenance of noradrenergic differentiation during embryogenesis requires ongoing expression of *Phox2b* in sympathetic ganglia, and of *Phox2a* in the main noradrenergic center, the locus coeruleus. These data illustrate cases where the neuronal differentiation program does not unfold as a transcriptional 'cascade' whereby downstream events are irreversibly triggered by an upstream regulator, but instead require continuous transcriptional input from it.

KEY WORDS: Cranial motoneuron, Neuronal differentiation, Sympathetic ganglion, Transcription factor, Mouse

INTRODUCTION

During the proliferation of neuroblasts and the differentiation of neurons, many developmental transcription factors (TFs) are expressed in defined time-windows, the functional significance of which is largely unknown. Indeed, simple knockouts – from which, most often, we infer the actions of developmental TFs – cannot reveal the phases of the expression window during which the inferred actions are carried out. In case of cell death or fate switch, they altogether obscure later actions. Documented cases where a neuronal TF, first required in progenitors or early postmitotic precursors, also carries later roles, are few and far between. They include: *Nkx6.1*, the forced maintenance of which in *Nkx6.1*^{OFF} somatic motoneurons endows them with the axonal projections of their *Nkx6.1*^{ON} counterparts (De Marco Garcia and Jessell, 2008), indirectly arguing for a post-mitotic role executed in the latter; *Nkx2.1*, the conditional inactivation of which shows that, after determining the fate of progenitors in the medial ganglionic eminence (Sussel et al., 1999), it guides the migration of some of their post-mitotic progeny (Nobrega-Pereira et al., 2008); *Isl1*, which has unique early and late embryonic roles in primary sensory neurons (Sun et al., 2008); *Nurr1*, the inactivation of which in the adult causes a loss of dopaminergic neurons (Kadkhodaei et al., 2009); and *Gata3*, which keeps an anti-apoptotic role in sympathetic ganglionic cells throughout embryonic and adult life (Tsarovina et al., 2010).

The homeobox gene *Phox2b* and its paralog *Phox2a* are switched on in most classes of visceral neurons, starting either at the progenitor stage or soon after exit from the cell cycle (Brunet and Pattyn, 2002), and stay expressed in differentiating precursors for various lengths of time, depending on the cell type. The constitutive knockout of *Phox2b* leads to a rapid demise of most *Phox2b*-positive neuronal precursors, and that of *Phox2a* to the agenesis of the locus coeruleus (LC) and of oculomotor and trochlear motoneurons (Brunet and Pattyn, 2002), thus precluding assessment of all but their earliest roles. These roles, in the branchiomotor, visceromotor (BM/VM) and noradrenergic neurons (the focus of the present study), can be summarized as follows.

In the domain of the hindbrain ventricular zone (VZ), which gives rise to BM/VM neurons, *Phox2b* is expressed during the production of these neurons, then downregulated, which allows the production of serotonergic (5-HT) neurons (Pattyn et al., 2003a). In progenitors, *Phox2b* blocks the 5-HT fate while specifying the BM/VM fate (Pattyn et al., 2000b; Pattyn et al., 2003a), and promotes the exit of neuroblasts from the cell cycle by acting like a proneural gene (Dubreuil et al., 2000; Pattyn et al., 2004). Thereafter, *Phox2b* stays on in post-mitotic BM/VM precursors for periods that range from a couple of days (e.g. in trigeminal motoneurons, E.C. and J.-F.B., unpublished) to the lifetime of the animal [e.g. in vagal motoneurons (Kang et al., 2007) for rat] and nothing is known of the potential roles of this second phase of expression. *Phox2a* is induced in early postmitotic BM/VM neurons but its knockout has no phenotype in these cells.

In the precursors of sympathetic neurons, *Phox2b* is expressed as soon as they aggregate at the dorsal aorta and before all sympathoadrenergic markers, including the transcription factors *Hand2*, *Gata2/3*, *Insm1* and *Phox2a*, and the effector genes tyrosine hydroxylase (*Th*), dopamine-β-hydroxylase (*Dbh*), *Scg10* (*Stmn2* – Mouse Genome Informatics), peripherin (*Prph*) and β-III tubulin (Pattyn et al., 2006; Wildner et al., 2008). The knockout of *Phox2b* prevents sympathoblasts from switching on any of these markers and kills them, leading to agenesis of the sympathetic chain (Pattyn et al., 1999). Again, the significance of the continued expression of *Phox2b* in sympathetic ganglia, which persists at least up to late embryonic stages (Hendershot et al., 2008) (E.C.,

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C. Goridis and J.-F.B., unpublished data), is unknown. *Phox2a* is also expressed at least throughout embryogenesis, but its knockout has only a weakly penetrant morphological phenotype in the superior cervical ganglion (Morin et al., 1997).

Finally, in the main noradrenergic center of the hindbrain, the LC, *Phox2a* is expressed first and required for the expression of *Phox2b*, which in turn is required for the expression of *Dbh*, before being sharply downregulated, while *Phox2a* stays on for the lifetime of the animal (Pattyn et al., 2000a). Again, nothing is known of the role of the ongoing expression of *Phox2a*.

We set out to determine whether the persistent expression of Phox2 genes has any function in noradrenergic and BM/VM neurons by inactivating the genes after their onset of expression. We show that this leads in all cell types to the downregulation of a whole set of differentiation markers after an initial spike of expression, and that cell behaviors such as caudal migration of the facial motor neurons are severely disrupted. In contrast to what is observed in the constitutive knockouts, in noradrenergic cells, delayed inactivation of one of the two paralogs can be partially (sympathetic chain) or fully (LC) compensated by the persistent expression of the other – the full phenotype being revealed by the conditional inactivation of both genes. Together, the results show a requirement for Phox2 genes beyond the initial phase of neuronal specification previously revealed by the simple knockouts.

MATERIALS AND METHODS

Animals

The mutant lines used in this study are the conditional *Phox2a* (*Phox2a*$^{lox/lox}$) and *Phox2b* (*Phox2b*$^{lox/lox}$) lines (Coppola et al., 2010; Dubreuil et al., 2009), and the *Isl1*^{Cre} (Srinivas et al., 2001) and the *CMV-βactin-Cre-ERT2* lines (Santagati et al., 2005) (hereafter designated as *CreERT2*) lines. Mice were mated overnight and noon of the day of the vaginal plug was considered to be E0.5. Embryos were dissected out in PBS and fixed at 4°C in 4% paraformaldehyde in PBS. All animal studies were carried out in accordance with the guidelines issued by the French Ministry of Agriculture and have been approved by the Direction départementale des services vétérinaires de Paris.

Tamoxifen treatment

Tamoxifen (Sigma, USA) was dissolved (20 mg/ml) in pre-warmed corn oil (Sigma) and stored at 4°C. It was administered to pregnant females by intraperitoneal injections. Females were treated for 2 consecutive days (E13.5 and E14.5), twice a day, using 2 mg of tamoxifen in the morning and 1 mg in the afternoon. The embryos were collected at gestational day (E) 18.5.

BrdU incorporation

BrdU (Sigma, USA) was injected intraperitoneally into pregnant mice (100 μg/g body weight) 1 hour before dissection.

Immunostaining, in situ hybridization and TUNEL assay

Fixed embryos or embryo tissues were cryoprotected overnight in 20% sucrose in PBS and embedded in OCT. Sections (12 μm) were cut in the transversal or sagittal planes. The methods for single in situ hybridization or immunostaining or the combination of both have been described previously (Tiveron et al., 1996). All probes were synthesized using the DIG RNA labeling kit (Roche) as specified by the manufacturer. The primary antibodies used were as following: rabbit anti-Phox2a (1/500) (Tiveron et al., 1996), rabbit anti-Phox2b (1/500) (Pattyn et al., 1997), guinea pig anti-Phox2b (1/500) (Dubreuil et al., 2009), mouse anti-Isl1/2 (1/100) (40.2D6 and 39.4D5, Developmental Study Hybridoma Bank, Iowa city, USA), rabbit anti-Th (1/200) (Chemicon, USA), mouse anti-βIII tubulin (1/200) (Sigma, USA) and rat anti-BrdU (AbCam, Cambridge, UK). The primary antibodies were revealed for bright field observation by biotin-labeled secondary antibodies using the Vectastain ABC kit, or for fluorescent observation using secondary antibodies of the appropriate specificity labeled with Cy3 or Cy5 (Jackson ImmunoResearch Laboratories, West Grove, USA) or Alexa 488 (Invitrogen, Oregon, USA). For whole-mount in situ hybridization, fixed embryos were dehydrated in ethanol in PBS-0.1% Tween (from 25% up to 100%) after fixation in 4% PFA. The method was as described by Tiveron et al. (Tiveron et al., 1996).

Apoptotic cells were detected on cryosections by using the Apoptag in situ apoptosis detection kit (Millipore).

Retrograde carbocyanine dye labeling

Single crystals of the carbocyanine tracer dyes DiI (1,1′-dioctade-cyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) or DiA [4-(4-dihexadecylaminostyryl)-*N*-methylpyridinium iodide (Molecular Probes, Invitrogen, Oregon, USA)] were applied in the first or second branchial arch of E11.5 embryos previously fixed in 4% buffered paraformaldehyde. The dye was left to diffuse for 1 week at room temperature in 4% paraformaldehyde, then the embryos were dissected and the hindbrains photographed under fluorescent light.

Quantitative analysis

Facial BM neurons

Isl1/2-positive cells were counted in rhombomere 4 (r4) on one section out of five on each side of three mutant and wild-type embryos.

Locus coeruleus

To evaluate the effect of the different genotypes on the LC, the number of Th-immunoreactive cells, as well as the number of those co-immunoreactive for Phox2a or Phox2b, were counted. Measurements were made on one 12 μm transverse section out of four, throughout the LC. Both sides of three control, three *Phox2a/b*$^{CreERT2\text{-}KO}$, and two *Phox2a*$^{CreERT2\text{-}KO}$ fetuses were analyzed.

Stellate ganglion

Quantitative analysis of the stellate ganglion was carried out by measuring the area enclosed in the outline of the βIII-tubulin in situ hybridization signal corresponding to the ganglion, on one section out of four throughout the ganglion, using the Image J software. Both ganglia of six controls (12 ganglia), three *Phox2b*$^{Islet\text{-}CKO}$ (six ganglia) and three *Phox2a/b*$^{Islet\text{-}CKO}$ mutants (six ganglia) were measured. For BrdU analysis, BrdU-positive cells were counted on one section out of four throughout one ganglion in three embryos for each genotype (wild-type and *Phox2a/b*$^{Islet\text{-}CKO}$ mutants), within the limits defined by the βIII-tubulin immunofluorescence signal, and the number divided by the corresponding surfaces.

Statistical analyses

Means were compared using Student's *t*-test. Equality of variances was analyzed with an F-test and Welch's correction was employed when variances of populations was significantly different.

RESULTS

Continued expression of *Phox2b* is required for the formation of branchiomotor nuclei

To uncover a possible role for *Phox2b* after progenitor specification in BM/VM motoneurons, we conditionally inactivated the gene by partnering a floxed *Phox2b* allele (Dubreuil et al., 2009) with a Cre recombinase driven by the promoter of *Isl1* (Srinivas et al., 2001), a homeobox gene switched on after cell-cycle exit in BM/VM motoneuron precursors (Ericson et al., 1992; Varela-Echevarría et al., 1996). In *Isl1*^{Cre}*; Phox2b*$^{lox/lox}$ embryos (hereafter designated as *Phox2b*$^{Islet\text{-}CKO}$), at embryonic day (E) 16.5, in situ hybridization for peripherin, an intermediate filament gene, on transverse sections of the hindbrain revealed only a few scattered cells at the place where the facial (nVII) and trigeminal (nV) motor nuclei are found in the wild type (Fig. 1). Thus, continued expression of *Phox2b* past the progenitor stage is required for the development of the facial and trigeminal motor nuclei.

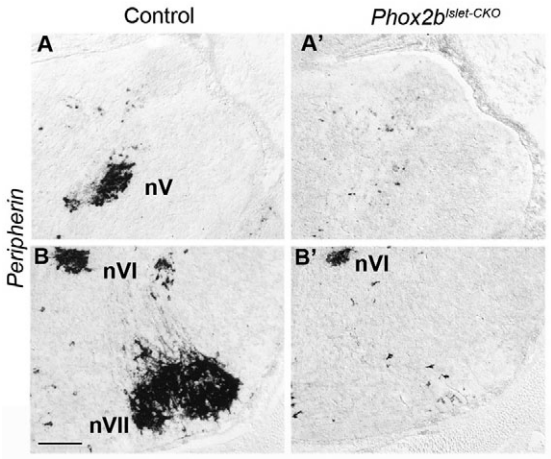

Fig. 1. Atrophy of the trigeminal and facial motor nuclei in *Phox2b*$^{Islet\text{-}CKO}$ mutants. (**A-B′**) Transverse sections through the pons (A,A′) and medulla (B,B′) of E16.5 control (A,B) and *Phox2b*$^{Islet\text{-}CKO}$ (A′,B′) embryos hybridized with a peripherin probe that labels all motoneurons. Both the trigeminal (A′) and facial (B′) motor nuclei are massively atrophic in *Phox2b*$^{Islet\text{-}CKO}$ mutants compared with controls. nV, nVI and nVII: trigeminal, abducens and facial motor nucleus, respectively. Scale bar: 200 μm.

Post-mitotic expression of *Phox2b* maintains the identity of BM/VM precursors

To elucidate the mechanism for the abnormal development of facial and trigeminal motor nuclei, we examined earlier stages of ontogeny. For most purposes, we focused our study on facial motoneurons, which are easier to track, owing to their abundance and protracted period of generation [E10-E13 (Taber Pierce, 1973)] from the ventral-most progenitor domain of the fourth rhombomere (r4) (Pattyn et al., 2000b). At E11.5, in r4 of *Phox2b*$^{Islet\text{-}CKO}$ embryos, Phox2b protein was present in facial BM progenitors of the VZ – as expected, because they have not expressed Isl1 yet – and in the post-mitotic Isl1/2^{+} (hereafter Isl1^{+}) precursors closest to VZ (presumably the youngest, which have just switched on *Isl1*). However, it was lost from the Isl1^{+} precursors further away from the VZ (Fig. 2A,A′), which were presumably born earlier. This rapid and complete disappearance of the Phox2b protein after expression of the *Isl1*^{Cre} transgene attests the efficacy of action of the Cre recombinase, and the short half-life of both Phox2b protein and mRNA in that cell type.

We then assessed the consequence of the secondary loss of the Phox2b protein on the expression of a battery of markers for facial BM neuronal precursors: the TFs *Tbx20* (Dufour et al., 2006), *Tbx2* (Song et al., 2006), *Ebf1* (Garel et al., 2000), *Nkx6.1* (Pattyn et al., 2003b), the intermediate filament peripherin and the pan-neuronal microtubule protein βIII-tubulin. These markers fell into two categories according to their expression in the mutants: those whose expression was either abrogated (*Tbx2*) or, after an initial spike in early post-mitotic precursors, abruptly downregulated, alongside the disappearance of Phox2b protein (*Ebf1*, *Tbx20*) (Fig. 2B-D′); and those that were maintained in the mutant at their wild-type levels – *Nkx6.1*, βIII-tubulin and peripherin (Fig. 2E-F′; not shown), and Isl1 (Fig. 2A,D′). Thus, there is a transcriptional program downstream of *Phox2b* that requires continuous input from *Phox2b* for its implementation.

Among the potential downstream genes of *Phox2b* is *Phox2b* itself, which might even be a direct transcriptional target owing to three auto-regulatory sites validated by transient transfection assays (Cargnin et al., 2005). To test whether ongoing expression of *Phox2b* relies on an auto-activation loop, we used a *Phox2b* RNA probe, which covers the first exon and should thus detect both the native and recombined alleles. In *Phox2b*$^{Islet\text{-}CKO}$, a *Phox2b* mRNA was detectable in facial motoneuronal precursors at E11.5 (Fig. 3A-B′) and still at E13.5 (Fig. 3C-D′, arrowheads), albeit at a lower level than in wild type. The signal is unlikely to come from a long-lived pre-recombination full-length mRNA, as the protein that such an intact mRNA would produce disappears within hours of *Isl1*^{Cre} expression. Therefore, the maintenance of expression of *Phox2b* does not entirely depend on auto-activation in BM/VM neurons during the time scale investigated.

Finally, we checked whether premature extinction of *Phox2b* redirected BM/VM post-mitotic precursors towards the fate of 5-HT neurons, as it does for the dividing progenitors of the p3 neuroepithelial domain, common to BM/VM and 5-HT neurons (Pattyn et al., 2003a). The expression of *Pet1*, a determinant of 5-HT differentiation, was unchanged in the mutants (see Fig. S1 in the supplementary material). Thus, the 5-HT fate, which is accessible to BM/VM progenitors if Phox2b is removed (Pattyn et al., 2003a), is no longer available to post-mitotic BM/VM precursors.

Post-mitotic expression of *Phox2b* is required for the migration of VM neurons

Two non-mutually exclusive mechanisms could account for the massive atrophy of the facial nucleus in *Phox2b*$^{Islet\text{-}CKO}$ embryos at E16.5: depletion by cell death and abnormal migration. Indeed, soon after their birth, facial BM precursors migrate caudally to r6 along the edge of the floor plate, then radially in r6 to form the facial nucleus close to the pial surface (Auclair et al., 1996). This migration is disrupted, to various extents, in a number of mutant backgrounds, including the knockouts of the transcription factors *Tbx20* and *Ebf1* (Garel et al., 2000), which both happen to be downregulated in *Phox2b*$^{Islet\text{-}CKO}$ embryos (Fig. 2). To follow the migration of FBM precursors in *Phox2b*$^{Islet\text{-}CKO}$ embryos, we first used immunofluorescence against Isl1 proteins on serial transverse sections of the hindbrain. At E11.5, in wild-type embryos, many facial BM precursors had colonized r5 and some had reached r6, commencing their radial migration towards the pia. In *Phox2b*$^{Islet\text{-}CKO}$, more neurons than in the wild type were found in r4 (Fig. 4A,A′,F), fewer neurons had reached r5 (Fig. 4B,B′) and practically none were radially migrating in r6 (Fig. 4C,C′). We confirmed this migration block on flatmounted hindbrains retrogradely labeled from the main branch of the facial nerve by DiI (Fig. 4D,D′). Simultaneous labeling of the trigeminal nerve with DiA showed that the migration of trigeminal BM precursors, which proceeds dorsally in r2, was slowed down (Fig. 4D,D′). To rule out the possibility that these migration defects are a mere delay that is later compensated for, we hybridized a whole-mount preparation of the hindbrain at E13.5 with a peripherin probe. Although the majority of nVII precursors had reached their final destination in the wild-type embryos, their bulk was still stuck in r4 and r5 in *Phox2b*$^{Islet\text{-}CKO}$ embryos. The anlage of the trigeminal nucleus appeared smaller and scattered along the migratory path (Fig. 4E,E′). Thus, the caudal migration of facial BM precursors and dorsal migration of trigeminal BM precursors is disrupted in *Phox2b*$^{Islet\text{-}CKO}$ mutants. Although this defective migration is sufficient to explain the agenesis of the respective nuclei, it does

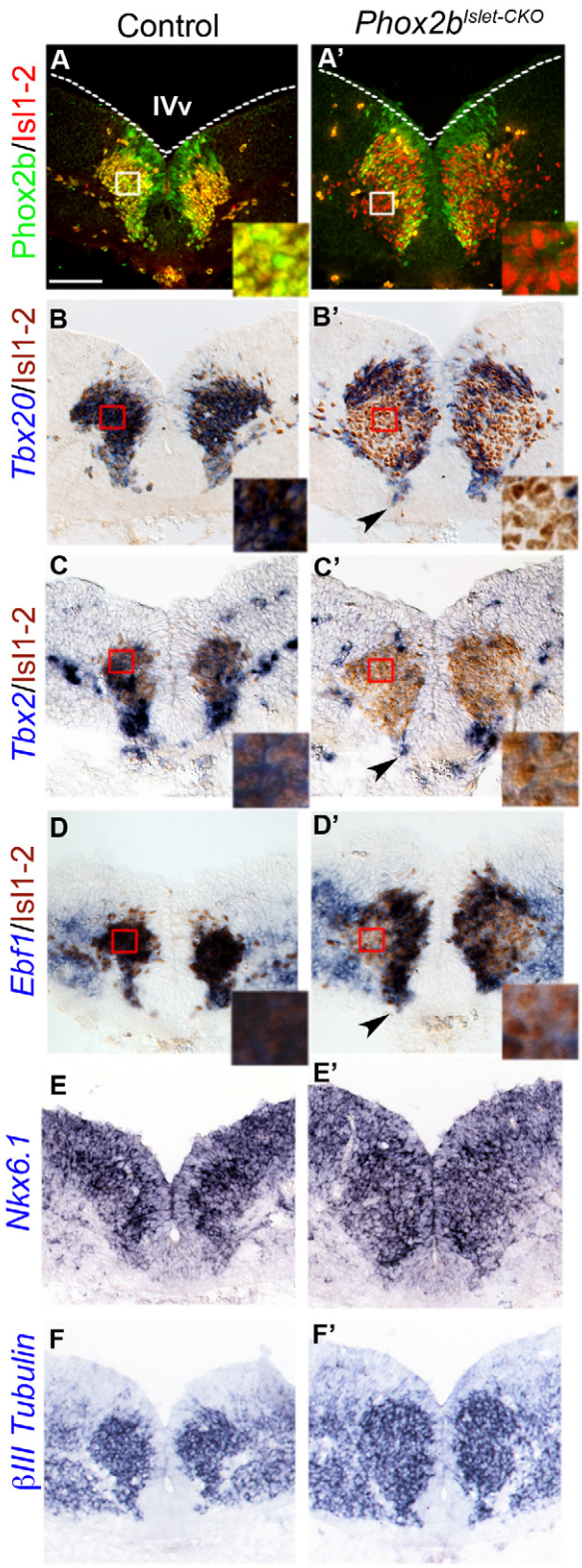

Fig. 2. Molecular profile of facial motoneurons in *Phox2b*$^{Islet\text{-}CKO}$ mutants. Transverse sections at the level of r4 in E11.5 control (A-F) and *Phox2b*$^{Islet\text{-}CKO}$ embryos (A′-F′) labeled with the antibodies or probes indicated on the left. (**A**,**A′**) Double immunofluorescence with anti-Phox2b and anti-Isl1/2 antibodies, showing the disappearance of Phox2b protein concomitantly with the onset of Isl1/2 expression. Insets are higher magnifications of the boxed areas. (**B-D′**) In situ hybridization with the indicated probes (blue), followed by Isl1/2 immunohistochemistry (orange), showing the downregulation of *Tbx20*, *Tbx2* and *Ebf1* in the mutants. The complete abrogation of *Tbx2* expression most probably stems from the fact that *Tbx2* lies downstream of *Tbx20* (Song et al., 2006), which might not have the time to trigger *Tbx2* expression before being downregulated. A small ventral population retains expression of the three genes (arrowhead) and corresponds to inner ear efferents, on the basis of *Gata3* expression (not shown). Insets are higher magnifications of the boxed areas. (**E-F′**) In situ hybridization with *Nkx6.1* and βIII-tubulin, showing intact expression in *Phox2b*$^{Islet\text{-}CKO}$ embryos compared with controls. IVv: fourth ventricle. The dotted lines in A and A′ mark the ventricular surface of the neuroectoderm. Scale bar: 100 μm.

not rule out the concomitant occurrence of cell death. The lack of proper nucleogenesis made it difficult to count the cells. Staining for apoptotic cells by the TUNEL method did not reveal excess cell death in the area occupied by facial precursors, although their lack of histological coherence could make it difficult to detect.

Continued expression of Phox2 genes is required in sympathetic ganglia

Another *Phox2b*-dependent structure that was altered in *Isl1*^{Cre}*; Phox2b*$^{lox/lox}$ embryos was the sympathetic chain, in which *Isl1* is switched on concomitantly with Phox2a (see Fig. S2 in the supplementary material) and noradrenergic differentiation. In E16.5 *Phox2b*$^{Islet\text{-}CKO}$ embryos, the sympathetic chain, the outline of which could be detected by in situ hybridization for βIII-tubulin, was 64% atrophic at the level of the stellate ganglion (Fig. 5A,B,D). Together with *Phox2b*, sympathetic ganglion cells express its paralogous gene, *Phox2a*, which has an identical homeodomain. We found that *Phox2a*, although strictly under the control of *Phox2b* for its onset of expression (Pattyn et al., 1997), is maintained at a normal level in *Phox2b*$^{Islet\text{-}CKO}$ at E13.5 (see Fig. S3 in the supplementary material), showing that *Phox2a* secondarily escapes dependence on *Phox2b*, whether through auto-activation, or another mechanism. We thus tested the possibility that *Phox2a* could partially compensate for the deletion of *Phox2b* by conditionally inactivating both genes. [No such compensation was expected for facial or trigeminal BM motoneurons, since they normally downregulate *Phox2a* soon after its onset of expression (see Fig. S3 in the supplementary material).] In *Isl1*^{Cre}*; Phox2a*$^{lox/lox}$*; Phox2b*$^{lox/lox}$ (hereafter designated as *Phox2a/b*$^{Islet\text{-}CKO}$) embryos, the atrophy of the stellate ganglion was significantly more pronounced than in *Phox2b*$^{Islet\text{-}CKO}$ embryos [~77% (Fig. 5A,C,D)]. This shows that the Phox2a protein, although unable to replace Phox2b at the onset of sympathoadrenergic differentiation in vivo (Coppola et al., 2005) can compensate for the absence of *Phox2b* to a moderate extent, at later stages of sympathetic development. To be sure to evaluate the full extent of late Phox2 function, we mostly restricted our subsequent study to double *Phox2a/b*$^{Islet\text{-}CKO}$ mutants.

The atrophy of the sympathetic chain could be due to apoptosis or decreased cell proliferation. To assess these possibilities, we analyzed *Phox2a/b*$^{Islet\text{-}CKO}$ embryos at E13.5, a time point intermediate between the onset of ganglion formation and the hypoplasia observed at E16.5. We could not detect any apoptotic cell in the anlage of the stellate ganglion of E13.5 *Phox2a/b*$^{Islet\text{-}CKO}$ embryos (see Fig. S4 in the supplementary material). This was unexpected, given that sympathetic precursors die in large numbers

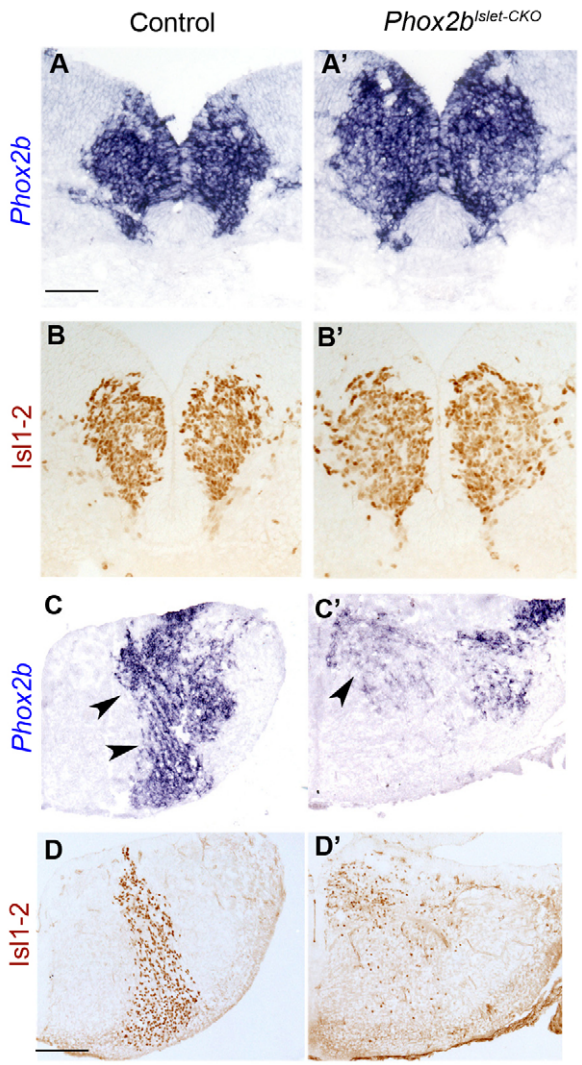

Fig. 3. Transcription of *Phox2b* is partially independent of Phox2b in branchiomotor neurons. (**A-D′**) Transverse sections through r4 at E11.5 (A-B′) and through the medulla at E13.5 (C-D′) of control (A-D) and *Phox2b*$^{Islet\text{-}CKO}$ (A′-D′) embryos stained by in situ hybridization with a *Phox2b* probe (blue) (A,A′,C,C′) or immunohistochemistry for Isl1/2 (brown) (B,B′,D,D′). Phox2b transcripts are detectable in the facial motoneurons at E11.5 (A,A′) and are still present, albeit at a weaker level at E13.5 (C,C′, arrowheads). Scale bars: in A, 100 μm in A-B′; in D, 200 μm in C-D′.

at around E11.5 in *Phox2b* constitutive knockouts (Pattyn et al., 1999). This shows that after an initial spike of activity, *Phox2b* becomes dispensable for cell survival. However, BrdU incorporation was decreased by 44% in the sympathetic ganglia of *Phox2a/b*$^{Islet\text{-}CKO}$ embryos (Fig. 5E-G), which is likely to account for the eventual atrophy. Thus, *Phox2b* expression promotes sympathoblast proliferation during embryogenesis.

Continued expression of Phox2 genes maintains noradrenergic neuron differentiation

Sympathoblasts derive from neural crest cells, and form a chain by aggregating alongside the dorsal aorta. Soon after aggregation, ganglion cells switch on *Phox2b*. In *Phox2a/b*$^{Islet\text{-}CKO}$ embryos at E10.5, *Phox2b* was, as expected, normally expressed at E10.5, and every Phox2b-positive ganglionic cell had switched on a whole battery of markers downstream of it (see Introduction) at a normal level: Phox2a, Isl1, *Hand2*, *Gata3*, *Insm1*, βIII-tubulin and the effectors of the noradrenergic phenotype *Dbh* and *Th* (Fig. 6; data not shown). Thus, in *Phox2a/b*$^{Islet\text{-}CKO}$, the full program of cell-type and generic neural differentiation is triggered in sympathetic progenitors. At E11.5, the Phox2b protein was absent from most sympathoblasts, showing that, as in BM/VM neurons, the recombination of the floxed *Phox2b* locus by *Isl1*^{Cre} and the disappearance of the corresponding protein and mRNA takes 1 day at most (see Fig. S5A,A′ in the supplementary material). Unexpectedly, Phox2a protein was still detected (see Fig. S5B,B′ in the supplementary material), owing either to its longer half-life or to the lesser accessibility of the locus to recombination. Other markers were still expressed at levels only slightly diminished compared with wild type, including *Hand2*, *Gata3*, *Dbh* or *Th* (see Fig. S5C-F′ in the supplementary material) and the *Phox2b* locus itself (see Fig. S6 in the supplementary material).

By contrast, at E16.5 (at which stage Phox2a protein was still detectable but only in a minority of neurons, not shown), most markers tested were downregulated to undetectable levels in the vast majority of ganglion cells, including *Gata3*, *Dbh* and peripherin, with the notable exception of βIII-tubulin (Fig. 5), *Hand2* and *Ret* (Fig. 7) which stayed on in most ganglionic cells. In single *Phox2b*$^{Islet\text{-}CKO}$ embryos, noradrenergic marker expression was only marginally higher than in the double knockout (see Fig. S7 in the supplementary material), showing that *Phox2a* has only a modest capacity to enforce the maintenance of the noradrenergic phenotype. The sparse expression of vasoactive intestinal polypeptide (*Vip*) and *VAChT* (*Slc18a3* – Mouse Genome Informatics) in the stellate ganglion was unchanged by the *Phox2a/b*$^{Islet\text{-}CKO}$ mutation (see Fig. S8 in the supplementary material). The *Phox2b* locus was expressed but markedly weaker than in the wild type at E13.5 (see Fig. S6 in the supplementary material). Altogether, these data show that part of the differentiation program of sympathetic ganglionic cells requires continuous input from Phox2 TFs for maintenance.

Another group of noradrenergic cells that differentiate under the control of Phox2 genes is the LC (Pattyn et al., 2000a). Here, the dynamic of expression and the roles of Phox2 genes diverge from those in sympathoblasts (see Introduction): the expression of *Phox2a* both precedes and outlasts that of *Phox2b*, which is switched on by *Phox2a*, then downregulated. We therefore asked whether, in this case, *Phox2a* instead of *Phox2b* maintains the noradrenergic phenotype during late gestation. We inactivated *Phox2a* while circumventing its early requirement in LC development, by crossing the *Phox2a*$^{lox/lox}$ allele in a *CreERT2* background (Santagati et al., 2005), producing a *Phox2a*$^{CreERT2\text{-}KO}$ progeny, and examined the brains of E18.5 pups whose mother was treated with tamoxifen from E13.5 to E14.5. A LC of normal size and location was present in *Phox2a*$^{CreERT2\text{-}KO}$ embryos on the basis of *Dbh* (Fig. 8A,A′) and *Th* expression (Fig. 8B,B′), despite the fact that, as expected, Phox2a was lost from 67% of LC cells (Fig. 8C). The fact that some LC cells had kept detectable levels of Phox2a is probably due to the mosaic action of the Cre recombinase, evident in other parts of the brain (not shown) – another, non mutually exclusive explanation being a long half-life of the Phox2a protein or mRNA. Unexpectedly, Phox2b, which, by this time, was completely extinguished from the LC of wild-type embryos (Fig. 8B, inset), was maintained in the LC of the mutants, specifically in those cells that had lost *Phox2a* expression (Fig. 8B′, inset). This shows that the normal extinction of *Phox2b* at

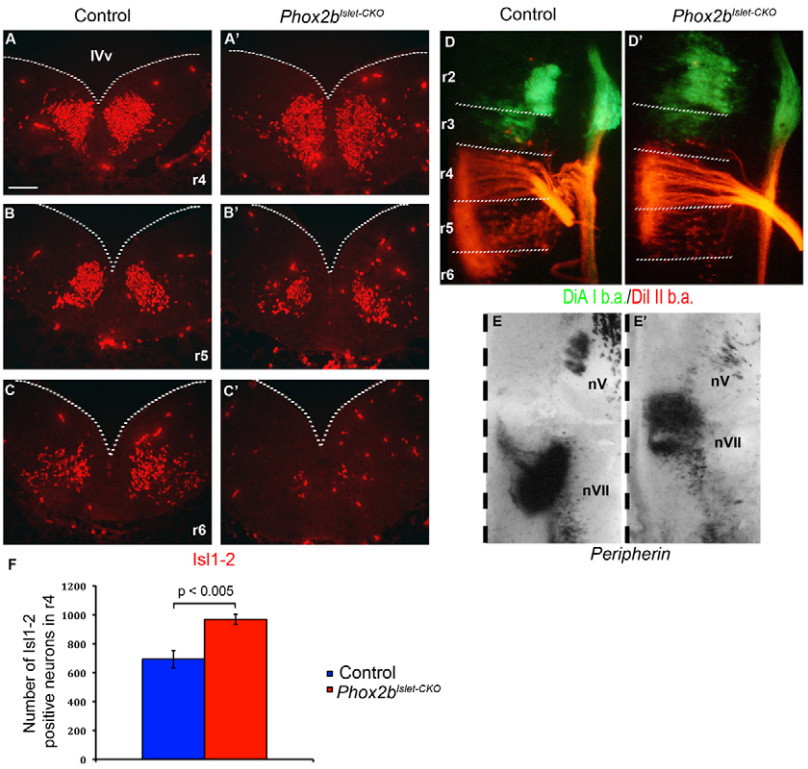

Fig. 4. Stalled or slower migration of branchiomotor neurons in *Phox2b*$^{Islet\text{-}CKO}$ embryos. (**A-C′**) Transverse sections through r4 (A,A′), r5 (B,B′), r6 (C,C′) of E11.5 control (A-C) and *Phox2b*$^{Islet\text{-}CKO}$ (A′-C′) embryos stained by immunofluorescence for Isl1/2. The facial motoneurons in *Phox2b*$^{Islet\text{-}CKO}$ embryos are more numerous in r4 (A), fewer in r5 (A′) and do not reach r6 (C′). (**D**,**D′**) Flat-mounted E11.5 hindbrains, retrogradely labeled with DiA (green) and DiI (red) placed in the first and second branchial arc, respectively. The trigeminal motor nucleus (green) is less condensed in the mutant, and the facial motoneurons (red) have not migrated beyond r5. (**E**,**E′**) Flat-mounted hindbrains hybridized with a peripherin probe, in E13.5 control and *Phox2b*$^{Islet\text{-}CKO}$ embryos. (**F**) Quantification of post-mitotic facial precursors in r4, showing the more pronounced accumulation (+39%) of precursors in the mutant relative to wild type. r2, r3, r4, r5 and r6: rhombomeres 2, 3, 4, 5 and 6; IVv: fourth ventricle; nV and nVII: trigeminal and facial motor nuclei. The dotted lines indicate the ventricular surface of the neuroectoderm in A-C′ and the boundaries between rhombomeres in D,D′. The dashed lines indicate the ventral midline in E,E′. Scale bar: 100 μm.

E13.5 (Pattyn et al., 2000a) results from an active repression by *Phox2a*, which is relieved in inducible *Phox2a* mutants. To circumvent the possibility that this upregulation of *Phox2b* compensates for the loss of *Phox2a*, we examined the fate of the LC in a *CreERT2; Phox2a*$^{lox/lox}$*; Phox2b*$^{lox/lox}$ (hereafter designated as *Phox2a/b*$^{CreERT2\text{-}KO}$) background. In contrast to *Phox2a*$^{CreERT2\text{-}KO}$, in *Phox2a/b*$^{CreERT2\text{-}KO}$ embryos treated with tamoxifen at E13.5-E14.5 and examined at E18.5, the LC was markedly atrophic, as judged from *Dbh* (Fig. 8A″) and Th expression (Fig. 8B″), the number of Th+ cells being reduced by 58% (Fig. 8D). All Th-expressing cell spared in *Phox2a/b*$^{CreERT2\text{-}KO}$ embryos were *Phox2a* positive and *Phox2b* negative

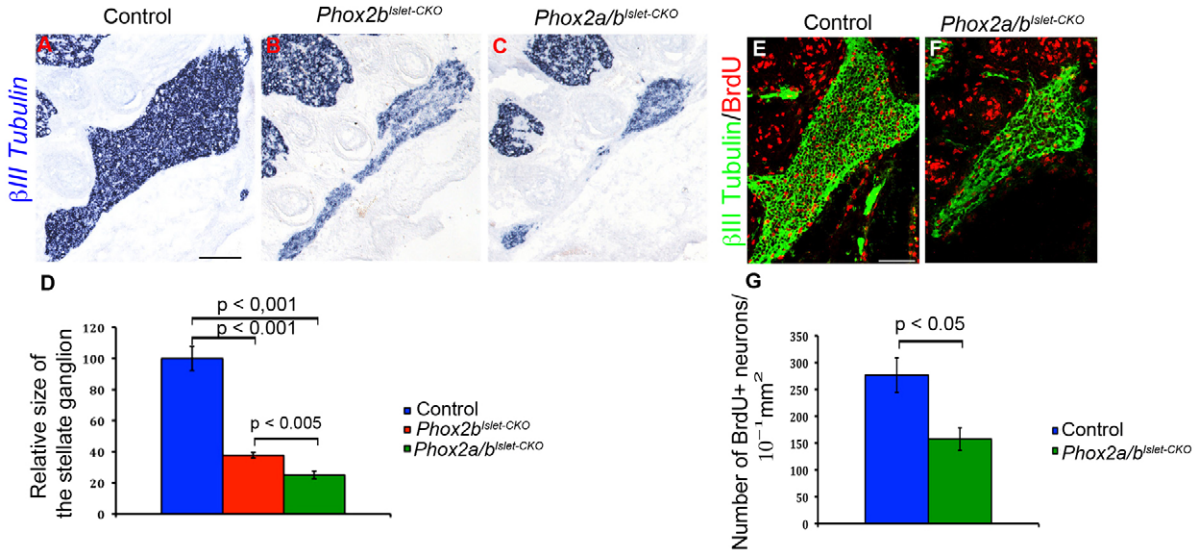

Fig. 5. Marked atrophy of the stellate ganglion in *Phox2b*$^{Islet\text{-}CKO}$ and *Phox2a/b*$^{Islet\text{-}CKO}$ mutants, and decreased sympathoblast proliferation rate. (**A-C**) Parasagittal sections of E16.5 control (A), *Phox2b*$^{Islet\text{-}CKO}$ (B) and *Phox2a/b*$^{Islet\text{-}CKO}$ (C) embryos through the stellate ganglion stained by in situ hybridization with βIII-tubulin, showing the atrophy of this neuronal population when Phox2b is removed alone (B) or in combination with Phox2a (C). (**D**) Quantification of the stellate ganglion, visualized by in situ hybridization for βIII-tubulin. The size of the ganglion in *Phox2b*$^{Islet\text{-}CKO}$ and *Phox2a/b*$^{Islet\text{-}CKO}$ mutants is expressed as a percentage of the surface occupied by the control ganglion (100%, *n*=12 for control ganglia; 37.7±1.8%, n=6 for *Phox2b*$^{Islet\text{-}CKO}$ ganglia and 25.1±2.4%, *n*=6 for *Phox2a/b*$^{Islet\text{-}CKO}$ ganglia). (**E**,**F**) Parasagittal sections of E13.5 control (E) and *Phox2a/b*$^{Islet\text{-}CKO}$ (F) embryos stained by immunofluorescence for βIII-tubulin (green) and BrdU incorporation (red). (**G**) Quantification of BrdU-positive cells per surface of ganglionic tissue. Scale bars: 200 μm in A-C; 100 μm in E.

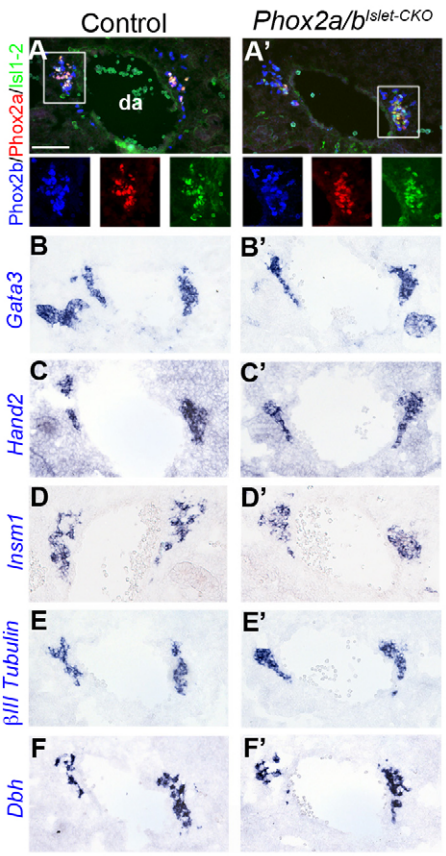

Fig. 6. Sympathetic neurons specification is normal in *Phox2a/b*$^{Islet\text{-}CKO}$ mutants. (**A-F′**) Transverse sections at forelimb levels of control (A-F) and *Phox2a/b*$^{Islet\text{-}CKO}$ (A′-F′) embryos at E10.5 through the anlagen of the sympathetic chain. (A,A′) Upper panels: merged pictures of triple immunofluorescence detecting Phox2b (blue), Phox2a (red) and Isl1/2 (green). Lower panels: higher magnifications of one ganglion anlage. (B-F′) In situ hybridization with the probes indicated on the left. *Gata3*, *Hand2*, *Insm1*, βIII-tubulin and *Dbh* are normally expressed in *Phox2a/b*$^{Islet\text{-}CKO}$ mutants at this stage. da, dorsal aorta. Scale bar: 100 μm.

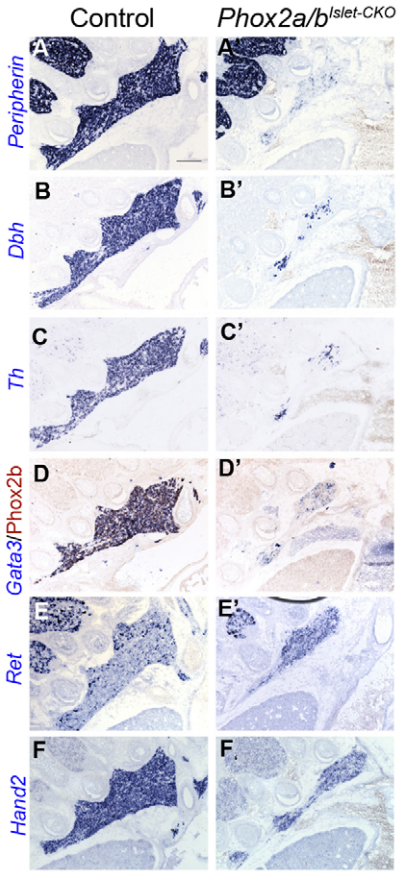

Fig. 7. Sympathetic differentiation traits are secondarily lost in *Phox2a/b*$^{Islet\text{-}CKO}$ mutants. Parasagittal sections of E16.5 control and *Phox2a/b*$^{Islet\text{-}CKO}$ embryos, through the stellate ganglion stained by in situ hybridization with the markers indicated on the left. (**A-D′**) Peripherin, *Dbh*, *Th* and *Gata3* are downregulated in *Phox2a/b*$^{Islet\text{-}CKO}$ mutants. After in situ hybridization for *Gata3* (D,D′), the sections were stained with an anti-Phox2b antibody to monitor its disappearance. (**E-F′**) Expression of *Ret* and *Hand2* is only marginally altered. Scale bar: 200 μm.

(Fig. 8B″, inset). The most likely explanation is that, in *Phox2a/b*$^{CreERT2\text{-}KO}$ mutants, recombination is mosaic and LC cells are either recombined for both genes (and lose Th and *Dbh* expression or die) or recombined for neither, in which case *Phox2b* is repressed by *Phox2a*, as in the wild type. Owing to a lack of additional markers for LC cells, which are embedded in neuronal tissue, we could not determine whether the depletion of Th^{+} cells reflects the loss of *Th* expression or loss of the cells themselves. Altogether, these data show that ongoing expression of a Phox2 gene is required for the survival of LC cells and/or the maintenance of their noradrenergic phenotype, at least until late gestation. *Phox2a* normally plays that role, but *Phox2b* can compensate for its loss.

DISCUSSION

In this study we have investigated whether the homeodomain proteins Phox2b and Phox2a were still required once they have launched the program of neuronal differentiation of BM/VM neurons and noradrenergic neurons. We find that many, but not all, of the genes that lie downstream of *Phox2b* in these cell types (based on gain and loss-of-function studies), including transcription factors and effector genes, are downregulated if *Phox2b* and *Phox2a* are prematurely silenced by time-controlled recombination. These results illustrate a case where the differentiation program downstream of a neuronal fate determinant is not readily locked in an ‘on’ mode, but requires continuous transcriptional input. The source of this input has been conceptualized (Hobert, 2008) under the term ‘terminal selector gene’, of which *Phox2b* might be an example in vertebrates.

Transcriptional maintenance of the motoneuronal phenotype

The constitutive knockout of *Phox2b* had revealed that the BM neuronal fate depends on *Phox2b*, in the sense that BM precursors are not born from *Phox2b*^{KO} ventral progenitors. However, the transcriptional architecture of the BM differentiation program is not known. The conditional inactivation of *Phox2b* soon after exit from the cell cycle reveals two subsets of BM precursor markers with respect to *Phox2b*-dependence: one that has an ongoing requirement for *Phox2b* (including *Tbx20*, *Tbx2*, *Ebf*) and one that

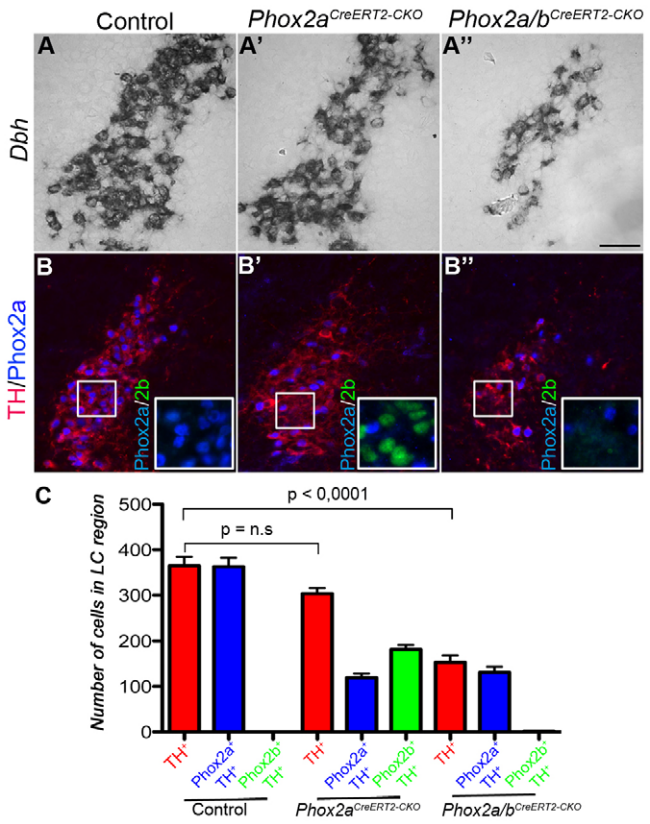

Fig. 8. Maintenance of noradrenergic phenotype in the locus coeruleus requires the expression of either Phox2a or Phox2b. (**A-B″**) Transverse sections of E18.5 control (A,B), *Phox2a*$^{CreERT2\text{-}KO}$ (A′,B′) and *Phox2a/b*$^{CreERT2\text{-}KO}$ (A″,B″) embryos stained by in situ hybridization for *Dbh* (A-A″) or triple stained with anti-Th (red), anti-Phox2a (blue) and anti-Phox2b (green) antibodies (B-B″ and insets). The loss of Phox2a observed in *Phox2a*$^{CreERT2\text{-}KO}$ mutants (B′) following tamoxifen administration did not affect the LC, as assessed by *Dbh* (A′) and *Th* (B′) expression, and induced an abnormal maintenance of Phox2b expression (B′, inset). Late removal of both Phox2a and Phox2b in *Phox2a/b*$^{CreERT2\text{-}KO}$ embryos dramatically reduced the number of *Dbh* (A″) and *Th* (B″) positive cells. (**C**) Quantification of Th, Phox2a- and Phox2b-positive LC neurons in the indicated genetic backgrounds. Scale bar: 50 μm.

has not (*Nkx6.1*, Isl1, peripherin and βIII-tubulin). Genes of the second subset could either never depend on *Phox2b* in the first place, or emancipate themselves from this dependence after cell cycle exit. The first hypothesis is likely for *Nkx6.1*, the expression of which in progenitors is not affected in *Phox2b* constitutive knockouts (Pattyn et al., 2000b). As to the other genes that are maintained after *Phox2b* inactivation, they tend to be less specific than those that are downregulated, expressed as they are in all neurons (βIII-tubulin), all motoneurons (Isl1) or all neurons that project to or are situated in the periphery (peripherin).

Consistent with the downregulation of *Tbx20*, the migration of motoneurons is blocked in a manner comparable with that of *Tbx20* knockouts – the slightly less dramatic phenotype of the *Phox2b*$^{Islet\text{-}CKO}$ embryos being attributable to a transient expression of *Tbx20*. This shows that the machinery for facial motoneuronal migration – which mobilizes the planar cell polarity pathway (Carreira-Barbosa et al., 2003; Jessen et al., 2002; Vivancos et al., 2009), and involves Sema3A (Schwarz et al., 2004) and the chemokine receptor CXCR4 (Cubedo et al., 2009), but is still largely elusive – requires continuous input from *Phox2b*.

Transcriptional maintenance of the noradrenergic phenotype

Downstream of *Phox2b*, several TFs [among which are *Gata3*, *Hand2* (reviewed by Apostolova and Dechant, 2009) and, more recently, *Insm1* (Wildner et al., 2008)] have been implicated in the noradrenergic differentiation of sympathetic ganglion neurons by constitutive knockout. Among those, *Hand2* was recently found to be required also for the maintenance of noradrenergic traits (Schmidt et al., 2009): knockdown of *Hand2* in cultured post-mitotic sympathetic neurons, which express both *Th* and *Dbh*, led to the downregulation of both genes – which require *Hand2* also for their onset of expression (Hendershot et al., 2008; Lucas et al., 2006; Morikawa et al., 2007). Moreover, in vivo inactivation of *Hand2* with a *Dbh::Cre*, i.e. after the onset of noradrenergic differentiation, resulted in the secondary extinction of *Th*, demonstrating a role for *Hand2* in maintaining *Th* expression, already suggested from a study on parasympathetic ganglia (Muller and Rohrer, 2002). Our results establish that, like *Hand2*, the ongoing expression of *Phox2b* is required for noradrenergic differentiation to be maintained. They also indicate that *Hand2* is not sufficient for the maintenance of the noradrenergic phenotype, as the expression of *Hand2* after dual conditional inactivation of *Phox2a* and *Phox2b*, largely exceeded that of noradrenergic differentiation traits. The simplest scenario is that, in order to maintain *Th* and *Dbh* expression, *Phox2b* functions in tandem with *Hand2*, which was switched on by *Phox2b* in the first place (Brunet, 2008; Goridis and Brunet, 1999), in a ‘feedforward’ transcriptional loop (Shen-Orr et al., 2002). The proposed interactions of the *Dbh* promoter with both Phox2 and Hand2 proteins (Rychlik et al., 2005; Seo et al., 2002; Xu et al., 2003), is compatible with such a transcriptional logic. Similarly, the decrease in cell division that we report in *Phox2a/b*$^{Islet\text{-}CKO}$ implies that the proliferative role documented for *Hand2* (Hendershot et al., 2008) is also exerted in tandem with *Phox2b*. The same logic might apply to *Gata3*, which is switched on by *Phox2b*, is required for *Dbh* expression (Apostolova and Dechant, 2009; Goridis and Brunet, 1999) and associates with the promoter of that gene, albeit indirectly (Hong et al., 2008). Finally, this type of molecular partnership might also underlie the dual ongoing requirement for *Gata3* (Tsarovina et al., 2010) and *Phox2b* (this study) for sympathoblast proliferation.

The comparison between wild type, *Phox2b*$^{Islet\text{-}CKO}$ and *Phox2a/b*$^{Islet\text{-}CKO}$ reveals that *Phox2b* is required for the maintenance of *Dbh* expression at their wild-type levels in sympathoblasts, and that *Phox2a* can only poorly compensate for *Phox2b* in this role (see Fig. S7 in the supplementary material). This stands in contrast with the LC in which *Phox2b*, although essential for the onset of *Dbh* expression, is later downregulated (Card et al., 2010; Pattyn et al., 2000a) while noradrenergic traits are maintained throughout life. *Phox2a* is the only Phox2 gene expressed at later stages in the LC and, as we show here in *Phox2a/b*$^{CreERT2\text{-}KO}$, it ensures the maintenance of the noradrenergic phenotype. This situation might also prevail in the other noradrenergic centers of the hindbrain, A1-A5 (Card et al., 2010) (and data not shown). This differential capacity of Phox2a to maintain noradrenergic differentiation in sympathetic versus LC neurons possibly reflects a fundamental difference between the central and peripheral noradrenergic neurons: in central neurons,

noradrenergic differentiation is strictly post-mitotic, whereas in sympathoblasts, it occurs concurrently with cell division (Rothman et al., 1980). Maintenance of gene expression is likely to have different requirements in a post-mitotic cell than across cell division, making it conceivable that, in the same way as *Phox2b* (but not *Phox2a*) can trigger *Dbh* expression in vivo (Coppola et al., 2005), *Phox2b* (but not *Phox2a*) can ensure the reset of expression after each cell cycle. The same type of explanation could apply to peripherin, the levels of which drop sharply in sympathoblasts after dual inactivation of *Phox2a* and *Phox2b*, but are maintained in post-mitotic BM precursors at least until E16.5 (Fig. 4E and not shown) in the absence of both genes.

Transcriptional maintenance of Phox2 genes

Although we show that proper differentiation of BM and sympathetic ganglion neurons requires ongoing expression of *Phox2b*, we do not know how this ongoing expression itself is implemented. An auto-activatory loop is an intuitive solution for maintaining the expression of TFs beyond the initial conditions that switch them on in the first place. The requirement for such loops, proposed as a key feature of 'terminal selector genes' (Hobert, 2008), has rarely been directly demonstrated in vivo (e.g. Baumgardt et al., 2007; Kadkhodaei et al., 2009; Way and Chalfie, 1989), and most often inferred from gain of function experiments. It seemed an attractive possibility for *Phox2b*, owing to the existence of binding sites for the protein, which are active in vitro (Cargnin et al., 2005) and the fact that forced expression of mouse *Phox2b* in chicken embryos induces the endogenous gene (Dubreuil et al., 2002). Accordingly, in the sympathetic chain and BM neurons the levels of *Phox2b* mRNA are markedly reduced at around E13.5 and beyond. However, they remain detectable, and earlier on (at E11.5), the levels of *Phox2b* mRNA corresponding to the transcription of the newly invalidated locus are comparable with those of the wild type in the absence of Phox2 proteins, pointing to an unexpected degree of independence from auto-activation.

Like many TFs, *Phox2a* and *Phox2b* are eventually downregulated in several classes of neurons, including BM/VM neurons. This feature departs from the proposed property of 'terminal selector genes' (largely inferred from invertebrate examples) of being expressed throughout the life of the neurons (Hobert, 2008), and might be specific to vertebrates. Once again, nothing is known about the physiological importance of this phenomenon. In BM/VM neurons, downregulation occurs as early as E11 in trigeminal motoneurons and during the first postnatal weeks in the facial motor nucleus. In the LC, where *Phox2b* is abruptly extinguished at E13.5, we have shown that Phox2a is required for this extinction, possibly via the autoregulatory sites in the promoter of *Phox2b* (Cargnin et al., 2005): Phox2a and Phox2b have identical DNA-binding domains. Such a discrete mechanism suggests developmental relevance. For example, a specific amount of Phox2 protein might be crucial for LC maintenance, and insured by repression of *Phox2b* by *Phox2a*. Alternatively, *Phox2b* might have different properties than Phox2a, deleterious to the maintenance of LC cells. Abnormally maintaining *Phox2b* expression [as occurs in $Phox2a^{CreERT2\text{-}KO}$ (this study) or in $Phox2a^{KIPhox2b}$(Coppola et al., 2005)] rescues LC cells, which rules out gross toxicity, but subtle or later anomalies in their projections or physiology remain a possibility. More generally, monitoring the consequences of the maintenance of developmental TFs beyond their normal window of expression might reveal important aspects of the transcriptional control of neuronal differentiation.

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The authors declare no competing financial interests.

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