Reciprocal gene replacements reveal unique functions for *Phox2* genes during neural differentiation

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The paralogous paired-like homeobox genes *Phox2a* and *Phox2b* are involved in the development of specific neural subtypes in the central and peripheral nervous systems. The different phenotypes of *Phox2* knockout mutants, together with their asynchronous onset of expression, prompted us to generate two knock-in mutant mice, in which *Phox2a* is replaced by the *Phox2b* coding sequence, and *vice versa*. Our results indicate that *Phox2a* and *Phox2b* are not functionally equivalent, as only *Phox2b* can fulfill the role of *Phox2a* in the structures that depend on both genes. Furthermore, we demonstrate unique roles of *Phox2* genes in the differentiation of specific motor neurons. Whereas the oculomotor and the trochlear neurons require *Phox2a* for their proper development, the migration of the facial branchiomotor neurons depends on *Phox2b*. Therefore, our analysis strongly indicates that biochemical differences between the proteins rather than temporal regulation of their expression account for the specific function of each paralogue.

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Introduction

Gene duplication has been a key mechanism for increasing the gene pool and generating diversity during evolution. For example, transcription factors important for vertebrate development are often expressed as paralogous pairs. Such pairs of closely related factors include many homeodomain (HD) proteins such as *Otx1* and *Otx2* (Acampora *et al*, 1998, 1999), *En1* and *En2* (Hanks *et al*, 1995) and *Nkx6.1* and *Nkx6.2* (Vallstedt *et al*, 2001). They have similar expression patterns and bind to the same target sequences. The question

thus arises whether duplicate copies have been conserved during evolution. One possibility is that their coding regions are functionally interchangeable but differ in their spatio-temporal expression patterns. Alternatively, one or both proteins may have acquired novel biochemical functions and fulfill different roles in the same cells.

One striking example of a paralogous pair of HD transcription factors with very similar expression patterns is provided by *Phox2a* and *Phox2b*. Found in all vertebrates, they are expressed in a limited number of developing neuronal types and are required for their proper development. In the peripheral nervous system, *Phox2* genes are expressed in all autonomic ganglia (sympathetic, parasympathetic and enteric) and in the three epibranchial-placode-derived distal ganglia of the facial (VII), glossopharyngeal (IX) and vagus (X) cranial nerves. Expression in the central nervous system includes the noradrenergic centers, the hindbrain branchio-viscero- (bm/vm) motor neurons, the nucleus of the solitary tract and the associated area postrema, as well as the nuclei of the IIIrd (oculomotor) and IVth (trochlear) cranial nerves (Tiveron *et al*, 1996; Pattyn *et al*, 1997). *Phox2a* and *Phox2b* are mostly coexpressed in the same lineages but in distinct temporal order. One gene is switched on earlier and is necessary for initiating the expression of the second one (Pattyn *et al*, 1997). These expression sequences may account in part for the phenotype observed in *Phox2a* and *Phox2b* null mutant mice. Absence of *Phox2a* results in the loss or atrophy of the structures, in which *Phox2a* precedes *Phox2b*, such as the oculomotor (nIII) and trochlear (nIV) neurons, the locus coeruleus (LC) and the three cranial ganglia. Parasympathetic ganglia of the head are also missing in *Phox2a* mutants, but the order of *Phox2* gene expression has not been elucidated in these structures. The other cell types that normally express *Phox2a* are unaffected, which has been attributed to a redundant function of *Phox2b* in the same structures. On the other hand, inactivation of *Phox2b* leads to loss of the following structures, in which *Phox2b* precedes *Phox2a*: the hindbrain bm/vm neurons, the enteric nervous system, the sympathetic ganglia and the parasympathetic ganglia of the trunk. In addition, proper development of the LC, the cranial sensory ganglia and the parasympathetic ganglia of the head requires not only *Phox2a* but also *Phox2b* (Pattyn *et al*, 1999, 2000a, b). Hence, with the exception of the nIII and nIV neurons, absence of *Phox2b* affects all structures that normally express it. This suggests, but does not prove, that *Phox2a* cannot compensate for the absence of *Phox2b*.

Analysis of the loss-of-function phenotypes has provided the groundwork for understanding the *in vivo* function of the *Phox2* genes. However, it is still not clear to which extent *Phox2a* and *Phox2b* are functionally equivalent, and thus whether the differences between the two paralogues are mainly due to their asynchronous onset of expression or rather to unique properties of the two proteins. To address

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these issues, we have used a knock-in (KI) approach, replacing the coding region of *Phox2a* by the coding region of *Phox2b* and *vice versa*. In this way, *Phox2a* should be expressed like *Phox2b* (*Phox2b*^{KI2a}), and *Phox2b* like *Phox2a* (*Phox2a*^{KI2b}).

Here, we show that *Phox2* genes are not functionally equivalent, and that *Phox2b* plays a predominant role in the development of several structures coexpressing the two transcription factors. Moreover, in some cases, replacing one *Phox2* gene by the other overcame the early arrest in differentiation described in knockout embryos, giving us access for the first time to later functions of the *Phox2* factors.

Results

Generation of *Phox2a*^{KI2b} knock-in mice by replacement of *Phox2a* by *Phox2b*

In order to investigate the functional similarities and differences between these two paralogous transcription factors, we replaced *Phox2a* by *Phox2b* and *vice versa*. To generate the *Phox2a*^{KI2b} strain, we engineered a targeting vector that, upon homologous recombination, results in the insertion of the coding sequence of *Phox2b* into the first exon of *Phox2a* (Figure 1A). The fusion protein generated was as efficient as the wild-type protein in activating the dopamine beta-hydroxylase (DBH) promoter, a known transcriptional target of *Phox2a* and *Phox2b* (Adachi *et al*, 2000; Seo *et al*, 2002). This shows that *Phox2b* expressed from the *Phox2a* locus has basically the same biochemical properties as wild-type

Phox2b (Supplementary Figure 1). The correct homologous recombination event and offspring genotype were identified by Southern blotting (Figure 1B) and polymerase chain reaction (PCR), respectively (Figure 1C). We also obtained mice without the neomycin gene whose phenotype was indistinguishable from the one described below (data not shown).

To confirm that *Phox2b* is expressed from the *Phox2a* locus in the same way as *Phox2a*, we analyzed its expression at the mid-hindbrain border of E10.5 wild-type (*Phox2a*^{+/+}) and homozygous (*Phox2a*^{KI2b}) embryos (Figure 1D–F). In the nIII and nIV lineages, *Phox2a* is normally expressed in cycling progenitors in the ventricular layer and remains in the postmitotic neurons in the mantle layer (Figure 1D), whereas *Phox2b* is only present in postmitotic neurons (Figure 1E) (Pattyn *et al*, 1997). In *Phox2a*^{KI2b} embryos, *Phox2b* was now found in the ventricular layer where nIII and nIV neurons are born (Figure 1F). Together with the maintenance of *Phox2b* expression in the LC (see below; Figure 2D and D'), these results indicate that in *Phox2a*^{KI2b} mutants, *Phox2b* protein is correctly processed and localized, and that the *Phox2a*^{KI2b} allele is expressed as the endogenous *Phox2a* gene.

Phox2b can rescue the lethality of *Phox2a* null mice and compensate for the absence of *Phox2a* in sensory and autonomic ganglia and in the locus coeruleus

Mice homozygous for the initial *Phox2a* null mutation die at birth (Morin *et al*, 1997); this has also been verified for

Figure 1 Targeting strategy to introduce the *Phox2b* cDNA into the *Phox2a* locus. (A) Schematic diagram of *Phox2a* genomic locus (top), the targeting vector (middle) and the expected recombinant allele (bottom). The restriction enzymes, the probes and the size of the resulting wild-type and recombinant restriction fragments are indicated, together with the primers (red arrows). (B) Southern blot analysis of DNA derived from mouse ES cells. (C) PCR genotyping of the recombinant mouse line using primers recognizing specifically the KI and wild-type alleles. (D–F) Immunofluorescence on transverse sections through the midbrain of E10.5 wild-type (*Phox2a*^{+/+}) and homozygous (*Phox2a*^{KI2b}) embryos using antibodies directed against *Phox2a* and *Phox2b* proteins. The asterisk in panel F indicates the appropriate misexpression of *Phox2a*^{KI2b} allele according to the endogenous *Phox2a* gene in homozygous mutants. IIIv: third ventricle. Dashed lines indicate the ventricular (next to IIIv) and pial surfaces of the neuroectoderm.

Figure 2 *Phox2b* fully rescues the development of the LC and parasymphetic and cranial sensory ganglia. (A–D') Sagittal and (E–H') parasagittal sections of *Phox2a*^{+/+} (A–H) and *Phox2a*^{K12b} (A'–H') embryos at E13.5 (A, A') and E18.5 (B–H'), labeled with the antibodies or RNA probes shown on the left. (A–B') DBH expression shows that differentiation of the LC is completely restored in *Phox2a*^{K12b} embryos both at E13.5 and E18.5. (C–D') In *Phox2a*^{K12b} embryos, staining for Phox2a is lost, but is replaced by Phox2b staining (arrow in D'), which is normally extinguished at this stage (D). The sphenopalatine ganglia (spg) are normal, as shown by the expression of *peripherin* (E–E'). The nodose-petrose (IX–X) cranial sensory ganglia are fully rescued, as assessed by the presence of Phox2b (F–F') and by the expression of *peripherin* and *Ret* (G–H'). (I) Quantification of the ganglionic size visualized by *peripherin* staining and expressed as a percentage of the area occupied by the wild-type ganglion (100 ± 15%, n = 4 for wild type and 88.4 ± 9.5%, n = 6 for *Phox2a*^{K12b} ganglia). Note that the difference is not statistically significant; *P* = 0.19. LSC: locus subcoeruleus.

another null allele (*Phox2a*^{tauLacZ}) (Jacob *et al*, 2000) (unpublished observations). Mendelian proportions of *Phox2a*^{K12b} homozygous mice were obtained from intercrosses of *Phox2a*^{K12b/+} heterozygotes and gave rise to fertile adults. Thus, the ectopic expression of *Phox2b* from the *Phox2a* locus is sufficient to rescue the lethality of *Phox2a*-deficient mice.

We then asked whether *Phox2b* could prevent all the neural defects caused by the absence of *Phox2a*. We first analyzed the LC, whose development and survival rely on both *Phox2* genes (Morin *et al*, 1997; Pattyn *et al*, 2000a). In particular, DBH, the last enzyme of noradrenaline (NA) synthesis, is never expressed in LC neurons of *Phox2a*-deficient embryos (Morin *et al*, 1997). Strikingly,

in *Phox2a*^{K12b} embryos, the LC was completely rescued both at earlier (E13.5) and later (E18.5) stages, as assessed by DBH expression (Figure 2A–B'). *Phox2b* expression was maintained in the LC of *Phox2a*^{K12b} mutants, differently from the wild type, in which *Phox2b*, but not *Phox2a*, expression is already switched off at this stage (Figure 2D and D'). Finally, we analyzed the sphenopalatine ganglion, a parasymphetic ganglion, and the cranial sensory ganglia, which are, respectively, absent and severely affected in *Phox2a* embryos (Morin *et al*, 1997). In *Phox2a*^{K12b} embryos, the sphenopalatine and the nodose-petrose (IX–X) ganglionic complex were completely rescued, as assessed by expression of *Phox2b* (Figure 2F and F'; data not shown), *peripherin*

(Figure 2E, E', G and G') and *Ret* (Figure 2H and H') and confirmed by direct measurement of the relative size of the IX–X ganglionic complex (Figure 2I). Taken together, these data demonstrate that *Phox2b* expressed from the *Phox2a* locus fully compensates for the loss of *Phox2a* during differentiation of the LC and of the IX, X and sphenopalatine ganglia.

***Phox2b* can only partially compensate for the loss of *Phox2a* function in oculomotor and trochlear neurons**

We next asked whether *Phox2b* was also able to supply *Phox2a* function in nIII and nIV neurons, which never form in *Phox2a*^{−/−} mutants (Pattyn *et al*, 1997). In E18.5 *Phox2a*^{K12b} embryos, these neurons were detectable, although their number was markedly reduced (Figure 3A–D'). In particular, the nIII was hardly detectable (Figure 3A', C' and K), the number of *Phox2b*-positive cells being reduced by 87%. The nIV was better preserved, albeit also reduced by 50% (Figure 3B', D' and K). The reduction in the number of differentiated motor neurons was already evident at E13.5 (Figure 3E–H'). The remaining cells, however, expressed *Isl-1* (*Isl1*) (Figure 3G–H'). Moreover, nIII precursors that normally migrate to the contralateral side (Puelles, 1978) were preserved in *Phox2a*^{K12b} embryos (Figure 3E' and G'), indicating that the rescued neurons were correctly specified. Neuronal projections, however, were affected. The trochlear nerve was shorter in *Phox2a*^{K12b} embryos (arrowhead in Figure 3L'), whereas the oculomotor nerve was not detectable (asterisk in Figure 3L'). Either the IIIrd nerve is not formed or the greatly reduced numbers of rescued neurons preclude the visualization of the residual axons. Indeed, at E10.5, the number of *Isl*-positive precursors was drastically reduced in *Phox2a*^{K12b} embryos compared with control animals (Figure 3I–J'). To assess whether the reduced number of motor neurons was due to insufficient proliferation or abnormal cell death, we performed BrdU assays on wild-type and *Phox2a*^{K12b} embryos at E10.5 when most nIII and nIV neurons are being born and TUNEL assays at E10.5 and E11.5. We detected neither a defect in cell proliferation nor an increase in apoptotic cells (Supplementary Figure 2), suggesting that the defect might be due to insufficient specification of the initial precursor population. Thus, *Phox2b* can only partially replace *Phox2a* during the specification of nIII and nIV precursors.

The full rescue of the LC, cranial sensory ganglia and sphenopalatine ganglia, and the partial rescue of the nIII and nIV neurons in *Phox2a*^{K12b} embryos indicate different requirements for *Phox2a* in *Phox2a*-dependent neuronal populations. *Phox2b* is able to replace *Phox2a* in the structures that depend on both genes, whereas only *Phox2a* can drive the full differentiation of the motor neurons that innervate extraocular muscles.

***Phox2a* produced from the *Phox2b* locus does not rescue the embryonic lethality of *Phox2b* null mice**

We then asked whether the reciprocal mutation (the *Phox2b* coding sequence replaced by that of *Phox2a*) could result in a similar rescue of the structures affected by the absence of *Phox2b* (Pattyn *et al*, 1999, 2000a,b). We thus generated *Phox2b*^{K12a} mutant mice, using a strategy similar to the one described for *Phox2a*^{K12b} mice. Again, we confirmed that in cultured cells, the resulting fusion protein maintained the

same *trans*-activating properties as wild-type *Phox2a* (Supplementary Figure 1). As we electroporated the targeting vector into PC3 embryonic stem (ES) cells, which have the Cre recombinase gene under the control of the protamine promoter (O'Gorman *et al*, 1997), the heterozygous mice were devoid of the neo cassette (Figure 4D).

To assess the correct expression of *Phox2a* from the *Phox2b* locus, we analyzed E10.5 wild-type (*Phox2b*^{+/+}) and homozygous (*Phox2b*^{K12a}) hindbrains. In wild-type embryos, *Phox2b*-positive cells are found in three distinct stripes in the hindbrain: a ventral one, which contains bm/vm neuron precursors (Pattyn *et al*, 1997), a lateral one, which presumably gives rise to ventral medullary neurons, and a dorsal one, which contains nTS precursors (Dauger *et al*, 2003). The lateral stripes do not normally express *Phox2a* (Figure 4E and F), but they expressed in *Phox2b*^{K12a} embryos (Figure 4G), indicating that the KI allele is expressed according to the endogenous *Phox2b* gene. In addition, ectopic *Phox2a* expression from the *Phox2b* locus was also found in the bm/vm progenitor domain, in which normally only *Phox2b* is present (see below; Figure 6A').

Homozygous *Phox2b*^{K12a} embryos die around E13.5 owing to a deficit in NA synthesis as *Phox2b* null mice do (Pattyn *et al*, 2000a). Like the *Phox2b* null mutants, they could be rescued up to birth by treating the mothers with NA agonists (Thomas *et al*, 1995). Thus, substituting *Phox2b* by *Phox2a* does not prevent the noradrenergic deficit leading to embryonic death due to the loss of *Phox2b*.

We analyzed the fate of sympathetic neurons, the major source of NA in the embryo. Their phenotype was very similar, if not identical to that seen in *Phox2b*^{−/−} embryos. Likewise, the enteric nervous system and the parasympathetic ganglia were absent in *Phox2b*^{K12a} embryos (Supplementary Figure 3, and data not shown).

***Phox2a* does not fully compensate for the loss of *Phox2b* in the locus coeruleus and in cranial sensory ganglia**

We next asked whether *Phox2a* could replace *Phox2b* in the development of the LC, the major source of central noradrenergic innervation. In E13.5 control embryos, the LC is clearly visible by expression of *Phox2a* and *DBH* (arrows in Figure 5A and B). At this stage, LC neurons were undetectable in *Phox2b*^{K12a} embryos (Figure 5A' and B'). However, an initial and partial rescue of the noradrenergic phenotype was observed at earlier stages (Figure 5C'–E'). At E10.5, *Phox2a*-positive cells accumulated at the most dorsolateral position of the LC anlage in *Phox2b*^{K12a} embryos, similar to what was described in *Phox2b*^{−/−} embryos (Figure 5C and C') (Pattyn *et al*, 2000a). In contrast to the *Phox2b*^{−/−} mutants, where no *DBH* expression was ever detected in the presumptive LC, a few *DBH*-positive cells were present in E10.5 *Phox2b*^{K12a} embryos (Figure 5D and D'). However, whereas in control embryos, almost all *Phox2a*-expressing cells were also *DBH* positive (Figure 5E and inset), only a small subset of *Phox2a*-positive LC precursors coexpressed *DBH* (Figure 5E' and inset). Hence, *Phox2a* can trigger differentiation of a subpopulation of LC neurons in the absence of *Phox2b*, which, however, is not maintained.

Also in cranial sensory ganglia, *Phox2a* could not fully substitute for *Phox2b* function. In *Phox2b* null mutants, the cranial sensory ganglia become atrophic as early as

Figure 3 *Phox2b* partially rescues the development of oculomotor and trochlear neurons. Coronal (A–H') and transverse (I–J') sections of *Phox2a*^{+/+} (A–J) and *Phox2a*^{KI2b} (A'–J') embryos at E18.5 (A–D'), E13.5 (E–H') and E10.5 (I–J'). Strong reduction in the size of nIII and nIV in the KI mutant embryos as shown by the decrease of *Phox2b*- (A–B', E–F'), *peripherin*- (C–D') and *Isl1*- (G–H') positive neurons. The asterisks in panels E, E', G and G' indicate the contralaterally migrating nIII neurons. (I–J') Double immunofluorescence with anti-*Phox2b* (green) and anti-*Isl1* (red) at E10.5 shows a marked reduction of double-labeled cells (arrows in I', J') in *Phox2a*^{KI2b} embryos. (K) Quantification of the number of *Phox2b*-positive cells in nIII and nIV nuclei. In control embryos, oculomotor neurons were 99.2 ± 3.5 per section ($n=9$), whereas in *Phox2a*^{KI2b} mutants, they were 23.8 ± 3.1 ($n=9$). Control trochlear neurons were 51.5 ± 7 per section ($n=4$), whereas *Phox2a*^{KI2b} neurons were 26.7 ± 1 ($n=4$). (L, L') Whole-mount immunohistochemistry with anti-neurofilament antibody shows a reduced trochlear (III) nerve and absence of the oculomotor (IV) nerve. IIIv: third ventricle.

E13.5 and virtually disappeared by E16.5 (Dauger *et al*, 2003). By contrast, in E16.5 *Phox2b*^{KI2a} embryos, *Phox2a*-, *peripherin*- and *Ret*-positive cells were still present in the IX/X complex, albeit they were less densely packed than in the wild type (Figure 5F–I'). The atrophy of the IX/X complex was estimated to be 72% by surface area measurement (Figure 5J).

***Phox2b* is necessary for proper migration of facial branchiomotor neurons**

Phox2b controls hindbrain bm/vm motor neuron generation by driving cycling progenitors to exit the cell cycle and to initiate a generic and type-specific differentiation program (Dubreuil *et al*, 2000, 2002). Consequently, none of the hindbrain nuclei develops in *Phox2b* null embryos and

Figure 4 Targeting strategy to introduce *Phox2a* cDNA into the *Phox2b* locus. (A) Schematic diagram of the *Phox2b* genomic locus (top), the targeting vector (middle) and the expected recombinant allele (bottom). The restriction enzymes, the probes and the sizes of the resulting wild-type and recombinant restriction fragments are indicated, together with the primers (red arrows). (B) Southern blot analysis of genomic DNA derived from mouse ES cells. (C) PCR genotyping of the recombinant mouse line using primers recognizing specifically the KI and wild-type allele. (D) PCR strategy to recognize the neomycin excision event following germline transmission. (E–G) Ventricular views of flat-mount wild-type (*Phox2b*^{+/+}) and homozygous (*Phox2b*^{KI2a}) E10.5 hindbrains hybridized with RNA probes for *Phox2b* (E) and *Phox2a* (F, G). In the *Phox2b*^{+/+} embryos, the lateral stripes are identified by the expression of *Phox2b* (asterisk in E); however, they do not express *Phox2a* (asterisk in F). In *Phox2b*^{KI2a} embryos, the lateral stripes are now positive for *Phox2a* expression (asterisk in G), indicating that in *Phox2b*^{KI2a}, *Phox2a* is expressed according to *Phox2b*.

there is an early arrest of bm/vm differentiation (Pattyn *et al*, 2000b). By contrast, *Phox2a* is switched on only in postmitotic precursors and its absence has no effect on bm/vm development (Pattyn *et al*, 1997).

We first investigated whether at E10.5, *Phox2a* could substitute for *Phox2b* in bm/vm precursors. In rhombomere (r) 4, *Phox2a* is normally expressed only in postmitotic precursors in the mantle layer (Figure 6A), but in *Phox2b*^{KI2a} embryos, its expression extends into the ventricular layer (asterisk in Figure 6A'), confirming expression of *Phox2a* in the appropriate *Phox2b* pattern. Furthermore, *Isl1*-positive cells, which are completely absent in the *Phox2b* null embryos, were found in *Phox2b*^{KI2a} mutants, indicating that *Phox2a* was able to support motoneuronal differentiation in the absence of *Phox2b* (Figure 6B'). The rescue, however, was incomplete, particularly at caudal hindbrain levels (Supplementary Figure 4). Consistently, at later stages, the trigeminal and the facial nuclei were still detectable, whereas the dmX and the nA were absent (Figure 6F–J'). In E11.5 homozygous *Phox2b*^{KI2a} embryos, the trigeminal nucleus was smaller (see Figure 6C'), in accordance with the reduced

number of *Isl1*-positive neurons generated at E10.5 (Supplementary Figure 4).

Next, we analyzed the behavior of differentiated fbm neurons. Most hindbrain bm/vm neurons migrate dorsally within the rhombomere in which they are generated (Cordes, 2001). However, fbm neurons born in r4 migrate first caudally to r6, where they turn dorsally toward the pial surface (Auclair *et al*, 1996). Strikingly, fbm neurons accumulated in r4 and in the rostralmost part of r5, where they adopted a dorsal migratory path (Figure 6C'). At E13.0, a subpopulation of r4 neurons started to condense in dorsal r4 in *Phox2b*^{KI2a} embryos, although most fbm neurons were still stalling in ventral r4/r5 (Figure 6D'). At later stages, the facial nucleus of *Phox2b*^{KI2a} mutants was smaller and mislocated in more anterior positions (Figure 6F' and H'). Finally, we crossed heterozygous KI mice with *Nkx6.2*^{tauLacZ} mice (Vallstedt *et al*, 2001), to visualize the peripheral projections of bm/vm neurons by expression of β -galactosidase. We did not observe striking anomalies of the initial axonal trajectories in *Phox2b*^{KI2a} embryos, but the thickness of the axonal tracts was reduced (Figure 6E').

Figure 5 *Phox2a* partially rescues the differentiation of the LC and of cranial sensory ganglia. (A–B') Sagittal sections of heterozygous and homozygous *Phox2b*^{KI2a} embryos at E13.5, stained with the markers indicated on the left. The asterisks in panels A' and B' indicate lack of LC cells. (C–D') Flat-mounted preparations of the met-mesencephalic region of E10.5 heterozygous and homozygous *Phox2b*^{KI2a} embryos hybridized with *Phox2a* (C, C') or *DBH* (D, D') probes. The arrows indicate an abnormal distribution of *Phox2a*-positive cells (C') and partial rescue of *DBH* expression (D'). (E, E') Transverse sections through the isthmus of E10.5 heterozygous and homozygous *Phox2b*^{KI2a} embryos stained by combined *DBH in situ* hybridization (dark blue) and anti-*Phox2a* immunohistochemistry (brown). The arrowheads in panel E' indicate that only a subpopulation of LC neurons is double positive for *Phox2a* and *DBH* in *Phox2b*^{KI2a} embryos; in control embryos, all the cells are double labeled (E). Outlined areas in panels E and E' are magnified in the insets. (F, F') Lateral views of *Phox2b*^{+/+} and *Phox2b*^{KI2a} embryos hybridized with *Phox2a* showing normal appearance of the cranial ganglia at E10.5. (G–I') Parasagittal sections of E16.5 *Phox2b*^{+/+} and *Phox2b*^{KI2a} embryos immunostained with anti-*Phox2a* and hybridized with *peripherin* and *Ret* probes. (J) Quantification of the ganglionic size visualized by *peripherin* staining and expressed as a percentage of the area occupied by the wild-type ganglion (100 ± 8.8%, *n* = 9 for wild type and 37.8 ± 5.1%, *n* = 4 for mutant ganglia).

This was particularly striking for the Xth nerve, in line with the fact that the caudal vm nuclei are much less preserved in the mutants.

These results uncover a novel function of *Phox2b*: beyond being required for the early steps of neuronal differentiation, it is also involved in the caudal migration of fbm precursors.

Figure 6 *Phox2a* can partially substitute for *Phox2b* during differentiation of hindbrain branchio-visceral-motor neurons, but cannot drive proper fbm neuron migration. (A–B') *In situ* hybridization with *Phox2a* and *Isl1* probes on transverse sections through r4 of *Phox2b*^{+/+} (A, B) and *Phox2b*^{K12a} (A', B') E10.5 embryos. The asterisk in panel A' indicates *Phox2a* misexpression in the r4 ventricular zone in *Phox2b*^{K12a} embryos. (B, B') *In situ* hybridization with *Isl1* probe reveals a mild reduction of *Isl1*-expressing bm neurons in r4 in *Phox2b*^{K12a} embryos. Ventricular (C, C') and pial (D, D') views of flat-mounted hindbrains hybridized with *Tbx20*, a specific marker of bm/vm neurons (Kraus *et al*, 2001; unpublished results), in *Phox2b*^{+/+} and *Phox2b*^{K12a} embryos at E11.5 and E13. (C') In *Phox2b*^{K12a} embryos, trigeminal neurons migrate to their appropriate position (asterisk in C'), but fbm neurons fail to undergo a caudal migration (arrowhead in C'). At E13, in *Phox2b*^{K12a} mutants, most fbm neurons are still stalling in r4 (arrowhead in D') and a facial-like nucleus is forming in dorsal r4 (arrow in D'). (E, E') Lateral views of E11.5 *Phox2b*^{+/+}/*Nkx6.2*^{tauLacZ} (E) and *Phox2b*^{K12a}/*Nkx6.2*^{tauLacZ} (E') embryos stained for β -galactosidase to label the bm/vm projections. Note that in *Phox2b*^{K12a} embryos, all the axonal tracts are thinner compared to control embryos (arrowheads in E'). (F–J') Transverse sections through the brainstem of E16.5 *Phox2b*^{+/+} and *Phox2b*^{K12a} embryos hybridized with a *peripherin* probe. Sections are shown in an anterior (upwards) to posterior (downwards) direction. The mutant facial nucleus is located more anteriorly, whereas in wild-type embryos, it extends from the level of the abducens nucleus (G) to more posterior locations (H). nVI and nXII: nuclei of the abducens and hypoglossal nerves, respectively; MesV: mesencephalic nucleus of the trigeminal nerve.

Phox2a expressed from the *Phox2b* locus is able to overcome most of the early differentiation block of fbm precursors imposed by the absence of *Phox2b*, but is unable to promote their characteristic caudal migration.

Discussion

Predominant role of *Phox2b* function in the locus coeruleus, in cranial sensory ganglia and in sympathetic and enteric neurons

Noradrenergic differentiation of LC neurons and formation of the LC depend on both genes (Morin *et al*, 1997; Pattyn *et al*, 2000a). During LC development, *Phox2a* and *Phox2b* act sequentially in a linear pathway, so that in the absence

of *Phox2a*, neither *Phox2b* nor *DBH* is expressed, and when *Phox2b* is deleted, *DBH* expression is abrogated, although *Phox2a* is unaffected. One possible interpretation of these data is that in LC cells, *Phox2b* acts as downstream mediator of *Phox2a*. However, *Phox2b*, but not *Phox2a*, expression in the LC is transient, and it is equally plausible that *Phox2a* is required in combination with *Phox2b* for *DBH* induction or has later functions in maintaining the differentiated phenotype. Here, we show that in *Phox2a*^{K12b} embryos, the LC is fully rescued and the structure is maintained at later stages. By contrast, in *Phox2b*^{K12a} embryos, the LC degenerates and noradrenergic differentiation is deficient. A plausible interpretation of these data is that the main function of *Phox2a* in LC precursors is to switch on *Phox2b* expression, which is

necessary and sufficient for initiation and maintenance of their differentiation. Hence, in this case, *Phox2a* can be considered a surrogate promoter of *Phox2b*. However, we cannot formally exclude an early role of *Phox2a* in a process preceding expression of endogenous *Phox2b*, a role that can be taken over by *Phox2b* in the KI mice. That *Phox2a* is not entirely devoid of LC specification function is suggested by the weak and transient rescue of *DBH* expression in *Phox2b*^{KI2a} embryos, that is, in the presence of four doses of *Phox2a*. A similar situation is present during development of the *Phox2*-expressing cranial sensory ganglia, where also *Phox2a* precedes *Phox2b* and are fully rescued in *Phox2a*^{KI2b} mutants, but only partially in *Phox2b*^{KI2a} embryos.

The sympathetic ganglia provide another example of the preeminent role of *Phox2b* in establishing the noradrenergic phenotype. In this lineage, *Phox2b* precedes *Phox2a*, and only *Phox2b* is essential for sympathetic development (Pattyn *et al*, 1997, 1999). In *Phox2a* null mutants, sympathetic development is grossly normal, indicating that *Phox2b* alone can ensure the proper differentiation of this neuronal population (Morin *et al*, 1997). This suggests either that *Phox2a* has no function in specifying the sympathetic phenotype or that proper development along the sympatho-adrenal lineage depends on a 'Phox2' generic function, which may be supplied by either *Phox2a* or *Phox2b*. If the latter hypothesis was true, we should have rescued sympathetic neuronal development in *Phox2b*^{KI2a} embryos, which, however, was not observed.

We could rule out the trivial explanation that *Phox2a* expressed as a fusion protein from the *Phox2b* locus might have biochemical properties different from the native *Phox2a* protein, as wild-type and fusion proteins did not differ in their capacity to transactivate the *DBH* promoter. However, *Phox2a* might not be expressed as efficiently from the *Phox2b* locus as is *Phox2b* from its own locus. We deem this extremely unlikely because at places where only the *Phox2b* gene is active, *Phox2a* expression in *Phox2b*^{KI2a} embryos appears as strong as the normal *Phox2b* expression in wild-type embryos and also because in heterozygous *Phox2a* mutants, likely to have half of normal *Phox2a* protein, noradrenergic differentiation is not impaired (Morin *et al*, 1997).

Taken together, these results strongly suggest that *Phox2b* is the main regulator of the noradrenergic phenotype, a function that *Phox2a* is unable to ensure.

Specific requirements of *Phox2* genes in the differentiation of cranial motor neurons

Phox2 genes are expressed in all developing cranial motor neurons except for the abducens and hypoglossal neurons, which are bona fide somatic motor neurons. NIII and nIV neurons are generally considered as somatic motor neurons, but probably represent a class apart (Pattyn *et al*, 1997). *Phox2* genes are differentially expressed in the cycling progenitors of these neurons: in the nIII and nIV lineages, *Phox2a* is switched on first and is the only *Phox2* protein expressed in the progenitors before cell cycle exit, whereas in the bm/vm lineage, the situation is reversed. The role of *Phox2* function in motor neuron development has been studied most extensively during bm/vm development. In this lineage, *Phox2b* provides proneural activity, promoting both cell cycle exit and acquisition of generic neuronal properties, and is required for all aspects of type-specific differentiation (Dubreuil *et al*, 2000). *Phox2a* may play an analogous role in

nIII and nIV precursors, which are absent in *Phox2a*^{-/-} embryos; however, this has not been formally proven.

In contrast to the predominant role of *Phox2b* in the differentiation of the LC, cranial sensory ganglia and enteric and sympathetic neurons, nIII and nIV neurons rely mainly on *Phox2a* for their proper development. The reverse situation is encountered in bm/vm neurons: in *Phox2b*^{KI2a} embryos, *Phox2a* can substitute in part, but not completely, for *Phox2b*. Therefore, in both KI models, the swapping of *Phox2* genes is able to overcome the early differentiation block seen in the null mutants and to initiate a correct motoneuronal specification program. However, fewer post-mitotic cells are generated, although in the correct place. How can this phenomenon be explained? One could postulate a stochastic process where four doses of one factor would suffice to reach a threshold for correct specification in some, but not in other, cells of the same tissue. Alternatively, subpopulations with distinct requirements for one protein versus the other may exist in an apparently homogeneous population of cells. Finally, we noticed that the extent of rescue differs along the rostro-caudal axis. The atrophy of the oculomotor nucleus in *Phox2a*^{KI2b} embryos is thus much more pronounced than that of the trochlear nucleus, and there is substantial rescue of the trigeminal and facial neurons but hardly any of the dnmX and nA nuclei. At present, we cannot explain the rostro-caudal differences in motor neuron rescue. One unifying feature underlying the structures, in which *Phox2b* cannot be replaced by *Phox2a*, is that they are part of the medullary visceral reflex circuits and regulate vegetative functions (VIIth, IXth and Xth distal sensory ganglia, the dnmX and nA, the parasympathetic, sympathetic and enteric ganglia).

***Phox2b* is required in fbm neuronal migration**

All bm/vm neurons migrate dorsally from their birthplace and form their corresponding nuclei at distinct dorsolateral locations. In most vertebrate species, fbm neurons are an exception in that they first migrate caudally to r6, where they undergo their dorsal migration. Although the dorsal migration of the trigeminal neurons within r2 was preserved in *Phox2b*^{KI2a} embryos, the caudal migration of fbm neurons was severely perturbed. Most fbm neurons migrated dorsally already within r4. Therefore, *Phox2a* cannot provide all the information needed for the correct migration of fbm neurons.

Mutations in several transcription factor genes have been found to cause fbm migratory phenotypes. In *Hoxb1*^{-/-} embryos, r4-derived motoneurons migrate dorsally within r4 (Studer *et al*, 1996; Davenne *et al*, 1999), but still express *Phox2b* (Gavalas *et al*, 2003). This phenotype has been attributed to a switch of rhombomeric identity (r4 becoming an r2-like territory) rather than to an intrinsic problem in neural differentiation and migration. In *Ebf1* mutants, a population of late-born facial motoneurons migrates dorsally already within r5 (Garel *et al*, 2000) and, in *Nkx6.1* mutants, facial motoneurons never reach r6, but instead migrate dorsally within r4 and r5 (Muller *et al*, 2003; Pattyn *et al*, 2003). In both *Ebf1* and *Nkx6.1* mutants, *Phox2b* expression is not altered, making it unlikely that there is a causal relationship. Our results identify a novel role for *Phox2b* in fbm migration, which cannot be assumed by *Phox2a*. We checked whether we could find changes in molecules expressed during or involved in fbm migration (such as *neuropilin-1* (Schwarz *et al*, 2004), *Ebf1* and *Ret* (Garel

et al, 2000)) but we could not detect any abnormalities (data not shown). The precise role of *Phox2b* in facial motor neuron migration thus needs further investigation.

The two *Phox2* genes evolved differently

During evolution, *Phox2a* and *Phox2b* arose by gene duplication from a unique ancestral gene found in invertebrates (Pujol *et al*, 2000; unpublished observations). This duplication event appears to have occurred at the onset of vertebrate evolution, as all vertebrates examined so far have both a *Phox2a* and a *Phox2b* orthologue. The HDs of *Phox2a* and *Phox2b* are identical, implying that the two proteins bind the same DNA sequences. With one exception (Hong *et al*, 2001), the two paralogous factors have also been found to have very similar transactivating properties in cultured cells (Adachi *et al*, 2000; Seo *et al*, 2002) and both are capable of inducing a noradrenergic phenotype in neural crest derivatives upon ectopic expression *in ovo* (Stanke *et al*, 1999, 2004). This contrasts with our results showing that each protein has specific functions that the other one is unable to assume and, in particular, that only *Phox2b* efficiently activates the *DBH* gene *in vivo*. The fact that *Phox2a* is able to activate *Phox2b*, observed in gain-of-function experiments (Flora *et al*, 2001; Hong *et al*, 2001; Dubreuil *et al*, 2002), may explain these divergent results. Indeed, the C-terminal domains of the two proteins are very different, and it is plausible that they create different interfaces for protein-protein interactions. Therefore, differential binding of still unknown cofactors specific for individual *Phox2* proteins may confer specificity to *Phox2a* and *Phox2b* activity.

The present study shows that, after the initial duplication of the ancestral *Phox2* genes, *Phox2a* and *Phox2b* have diverged extensively in their biochemical properties. This seems also true at the promoter level, because we did not find significant stretches of homology between the two promoter regions by ClustalW and Blast analysis of 5 kb upstream sequences (data not shown). For the most part, in each gene, the promoter and coding sequences might have co-evolved, in the sense that the *Phox2* gene switched on first is also the one that ensures differentiation of the structure, while the other has lost—or never acquired—the capacity to be expressed without transactivation by the first. There are two intriguing exceptions to this rule: the cranial sensory ganglia and the LC, which depend on both genes. There, *Phox2a* is expressed first and is required for the expression of *Phox2b*, which in turn is required to correctly specify the cells. Our studies show that in both cases, the first gene (*Phox2a*) cannot replace the second one (*Phox2b*) but that *Phox2b* can fully replace *Phox2a*, suggesting that the main function of the first might be to transactivate the second. Within the framework of the duplication-degeneration-complementation model (Force *et al*, 1999), we propose the following: after the initial duplication of the ancestral *Phox2* gene, *Phox2b*, but not *Phox2a*, lost the capacity of being activated at certain sites (e.g. in the epibranchial placodes, from which arise the *Phox2*-expressing cranial ganglia) where *Phox2a* became *de facto* its surrogate promoter. This situation in turn set the *Phox2a* protein free to lose many of its functions and to evolve new functions (e.g. the specification of the nIII and nIV neurons), the sole constraint being that it could still transactivate *Phox2b*, a function probably derived from the auto-activating properties of the ancestral gene.

Eventually, the respective functions of the two genes would have been entirely segregated, both at the promoter and protein levels. This contrasts with the situation encountered in other paralogous transcription factor genes, where all (as in the case of *paired*, *gooseberry* and *gooseberry neuro*; Li and Noll, 1994) or most (such as for *Otx1/Otx2* (Acampora *et al*, 1998, 1999), *En1/En2* (Hanks *et al*, 1995, 1998), *Pax2* and *Pax5* (Bouchard *et al*, 2000)) of the functional differences can be explained by divergent expression patterns.

Materials and methods

Generation of *Phox2a*^{K12b} and of *Phox2b*^{K12a} targeted mice

The *Phox2a* genomic clone (pg8SX) (Jacob *et al*, 2000) was cut at the *NcoI* site, located 44 bases downstream of the translation start (coding for the first 15 amino acids of *Phox2a*). *Phox2b* cDNA in pBluescript (Pattyn *et al*, 1997) was digested with *NdeI*, which cuts 88 bases downstream of the translation start site (removing the first 29 amino acids of *Phox2b*), and the remaining *Phox2b* sequence inserted into the *NcoI*-cut pg8SX, after filling in both sites. A cassette coding for the diphtheria toxin A (DTA) chain was inserted into a *Sall* site provided by pg8SX and located 1 kb upstream of the *Phox2a* translation start site. A *loxP*-flanked cassette coding for a PGK-driven neomycin resistance gene (*neo*) was cloned downstream of the *Phox2b* sequence. The targeting vector was linearized by *NotI* and electroporated into TBV2 ES cells according to standard protocols. Homologous recombination events were identified by Southern blotting using *EcoRV* to digest the genomic DNA and a 900 bp *NcoI*-*XhoI* fragment from pg8SX clone as internal probe. Recombinant ES cell clones were injected into blastocysts from C57/Bl6 females and gave germline transmissions of the *Phox2a*^{K12b} mutation. All the results reported in this study have been obtained using embryos having retained the neomycin resistance gene (*neo*). We have also generated mice in which the floxed *neo* sequence was removed by crossing the *Phox2a*^{K12b} mice with the pCX-NLS-Cre line (obtained from A Nagy).

The *Phox2b* genomic clone (H1) used to generate the *Phox2b*^{K12a} targeting vector (Pattyn *et al*, 1999) was digested at the *NdeI* site, located 88 bases downstream of the start site of translation (coding for the first 29 amino acids of *Phox2b*), and the *Phox2a* cDNA sequence (Valarché *et al*, 1993) at the *NcoI* site, located 44 bases downstream of the transcription start site, removing the first 15 amino acids of *Phox2a*. After blunting both ends, the *Phox2a* cDNA was inserted into the H1 clone. A cassette encoding EGFP (Clontech) and a *loxP*-flanked *neo* cassette were inserted one after the other downstream of the *Phox2a* sequence. The targeting vector was linearized with *NotI* and electroporated into PrmCre (PC3) ES cells according to standard protocols. Homologous recombination events were identified by Southern blotting using *EcoRI* to digest the genomic DNA and a 700 bp *BamHI*-*HindIII* fragment from C1-Xba clone as 3' external probe. Recombinant ES cell clones were injected into blastocysts from C57/Bl6 females and gave germline transmissions of the *Phox2b*^{K12a} mutation. For unknown reasons, the EGFP cassette was not functional. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Cardarelli Hospital, Naples, Italy. The detailed genotyping strategy is described in Supplementary data.

RNA *in situ* hybridization, immunohistochemistry, β -galactosidase staining and quantitative analysis

Midday of the day of the vaginal plug was considered E0.5. *In situ* hybridization and combined *in situ* hybridization/immunostaining on cryosections or whole-mount preparations of embryos were carried out as previously described using rabbit anti-*Phox2a* (Tiveron *et al*, 1996) or rabbit anti-*Phox2b* (Pattyn *et al*, 1997), or monoclonal mouse anti-Islet1/2 (Developmental Studies Hybridoma Bank) antibodies. Antisense RNA probes for *peripherin* (Escurat *et al*, 1990), *Ist1* (Karlsson *et al*, 1990), *Phox2a* (Tiveron *et al*, 1996), *Phox2b* (Pattyn *et al*, 1997), *Tbx20* (Kraus *et al*, 2001), *Ret* (Pachnis *et al*, 1993), *TH* (Pattyn *et al*, 1999), *DBH* (Tiveron *et al*, 1996), *Mash1* (Guillemot and Joyner, 1993), *Ebf1* (Garel *et al*, 1997), *Neuropilin-1* (He and Tessier-Lavigne, 1997) and *Sox10* (Kuhlbrodt *et al*, 1998) were labeled using a DIG-RNA labeling kit (Roche). Double immunofluorescence was performed using the following

fluorescent secondary antibodies (Alexafluor 488 anti-rabbit, Alexafluor 594 anti-rabbit, Alexafluor 594 anti-mouse; Molecular Probes; 1:400) for 1 h at room temperature. β -Galactosidase staining (Knittel *et al*, 1995) and whole-mount immunohistochemistry using the 2H3 mouse anti-neurofilament antibody (Developmental Studies Hybridoma Bank) were as in Maina *et al* (1998). Quantitative analysis of cranial sensory ganglia was carried out by calculating the surface of ganglia stained with *peripherin* with the help of the Image J program. Quantitative analysis of nIII and nIV neurons in wild-type and *Phox2a*^{K12b} embryos was performed by counting Phox2b-positive cells in all sections. All data were analyzed using Microsoft Excel software. All error bars represent the standard error of the mean (s.e.m.). Statistical significance was determined using two-tailed Student's *t*-test. **P* < 0.05, ***P* < 0.01.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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