Nkx6-1 controls the identity and fate of red nucleus and oculomotor neurons in the mouse midbrain

Nilima Prakash1,†, Eduardo Puelles2, Kristine Freude3, Dietrich Trümbach1, Daniela Omodei4, Michela Di Salvio4, Lori Sussel5, Johan Ericson6, Maike Sander3,*, Antonio Simeone4,† and Wolfgang Wurst1,7,†

Little is known about the cues controlling the generation of motoneuron populations in the mammalian ventral midbrain. We show that Otx2 provides the crucial anterior-posterior positional information for the generation of red nucleus neurons in the murine midbrain. Moreover, the homeodomain transcription factor Nkx6-1 controls the proper development of the red nucleus and of the oculomotor and trochlear nucleus neurons. Nkx6-1 is expressed in ventral midbrain progenitors and acts as a fate determinant of the Brn3a+ (also known as Pou4f1) red nucleus neurons. These progenitors are partially dorsalized in the absence of Nkx6-1, and a fraction of their postmitotic offspring adopts an alternative cell fate, as revealed by the activation of Dbx1 and Otx2 in these cells. Nkx6-1 is also expressed in postmitotic Isl1+ oculomotor and trochlear neurons. Similar to hindbrain visceral (branchio-)motoneurons, Nkx6-1 controls the proper migration and axon outgrowth of these neurons by regulating the expression of at least three axon guidance/neuronal migration molecules. Based on these findings, we provide additional evidence that the developmental mechanism of the oculomotor and trochlear neurons exhibits more similarity with that of special visceral motoneurons than with that controlling the generation of somatic motoneurons located in the murine caudal hindbrain and spinal cord.

KEY WORDS: Red nucleus, Oculomotor/trochlear nucleus, Nkx6-1, Nkx6-2, Otx2, Dbx1, Mouse

INTRODUCTION
During mouse development, distinct neuronal populations organized in discrete nuclei arise in the ventral midbrain (VM). The best characterized are the substantia nigra and ventral tegmental area dopaminergic (mDA) neurons due to their clinical relevance, but less is known about the cues controlling the development of other nuclei in the VM or rostral hindbrain. These include the oculomotor nucleus (OM), the trochlear nucleus (TN) and the red nucleus (RN). The OM and TN give rise to the third (nIII) and fourth (nIV) cranial nerve, respectively. The nIII innervates the ipsilateral extraocular muscles and ciliary ganglion, thereby controlling most eye movements, eye accommodation and pupil contraction, and the nIV regulates the movements of the contralateral superior oblique muscle of the eye. The RN is located in close vicinity to the OM and mDA area (Evinger, 1988) and contains both excitatory glutamate- and inhibitory γ-aminobutyric acid (GABA)-synthesizing neurons, which project to the cerebellum, brainstem and spinal cord (Keifer and Houk, 1994). Together with the corticospinal tract, the rubrospinal tract plays a fundamental role in the control of limb movements (Kennedy, 1990).

Whereas little is known about the molecular mechanism and factors controlling the specification of the RN, similar cues for the motoneuron (MN) and interneuron (IN) populations arising in the vertebrate hindbrain and spinal cord appear to act during the development of the OM and TN (Eisen, 1999; Jessell, 2000; Jurata et al., 2000). In particular, the POU homeodomain (HD) transcription factor (TF) Pou4f1 (also known as Brn3a) is expressed in postmitotic RN neurons and is required for their survival (Agarwala and Ragsdale, 2002; Fedtsova and Turner, 1995; Turner et al., 1994; McEvilly et al., 1996; Xiang et al., 1996). A similar RN phenotype was reported in mouse mutants lacking the homeobox gene Emx2 (Agarwala and Ragsdale, 2002). The LIM HD TF islet1 (Isl1), a generic MN marker, is expressed in the OM/TN (Agarwala and Ragsdale, 2002; Ericson et al., 1992) and, although Isl1 is necessary for survival of spinal MNs (Pfaff et al., 1996), its function in OM/TN development remains unknown. The paired-like homeobox gene Phox2a is expressed in OM/TN progenitors and, together with its paralog Phox2b, in their postmitotic offspring (Pattyn et al., 1997). The OM and TN are lost in Phox2a−/− but not in Phox2b−/− mice, indicating that Phox2a is a crucial fate determinant in OM/TN precursors (Coppola et al., 2005; Pattyn et al., 2000; Pattyn et al., 1997).

The induction of fate-determining TFs by extrinsic and intrinsic signals along the dorsoventral (D/V) and anterior-posterior (A/P) axis of the neural tube is a crucial process that is likely to precede the differentiation of RN and OM/TN neurons in the VM/rostral hindbrain (Agarwala and Ragsdale, 2002; Fedtsova and Turner, 2001). Relevant studies support a general model in which the glycoprotein sonic hedgehog (Shh), secreted from the ventral midline of the neural tube (the floor plate, FP), is essential for D/V patterning by controlling the expression of different sets of HD TFs.
(Dessaud et al., 2008; Jessell, 2000). Among these, the HD TFs Nkx6-1 and Nkx6-2 are transcriptional repressors induced by Shh signaling in the ventral hindbrain and spinal cord (Briscoe et al., 2000; Cheesman et al., 2004; Muhr et al., 2001; Qiu et al., 1998), and are required for the specification of hindbrain and spinal somatic MNs (sMNs) and INs (Brohier et al., 2004; Hutchinson et al., 2007; Sander et al., 2000a; Vallstedt et al., 2001), as well as for proper muscle nerve formation and innervation patterns (De Marco Garcia and Jessell, 2008). Moreover, Nkx6-1 and Nkx6-2 are also required for the proper migration and axon pathfinding of hindbrain visceral (branchio-) MNs (vMNs), but not for their initial specification (Müller et al., 2003; Pattyn et al., 2003). Ablation of Nkx6 function leads to the derepression of the Shh-repressed HD TFs developing brain homeobox 1 and 2 (Dbx1 and Dbx2) in the ventral spinal cord, and to a fate-switch of MNs and ventral (V2) INs into more dorsal (V0) INs (Sander et al., 2000a; Vallstedt et al., 2001). Notably, most of these studies have been focussed on spinal cord and hindbrain, thus leaving largely unknown the role of Nkx6-1 and Nkx6-2 in the development of VM neuronal populations (Cai et al., 2001; Puellas et al., 2004; Puelles et al., 2001).

The mammalian HD protein Otx2 controls fore- and mid-brain patterning (Acampora et al., 2005). Conditional inactivation of Otx2 in the mid-/hind-brain region of the mouse results in a complete loss of the RN and hypoplasia of the OM (Puelles et al., 2004). Here we studied the functional involvement of Otx2 and Nkx6-1 in specification of identity and fate of RN and OM/TN neuronal populations. We show that Otx2 is necessary and sufficient for the maintenance and ectopic induction of Nkx6-1 in the VM/rostral hindbrain of mutant mice. Moreover, Nkx6-1 is required for the generation of Pou4f1+ RN neurons and for the proper migration and axon outgrowth of OM/TN neurons, thus revealing intriguing similarities between the midbrain phenotype of Nkx6-1−/− mice and the sMN and vMN defects previously described in the same mutants. These findings suggest that functional aspects of the mechanism controlling hindbrain and spinal cord MN development have been conserved and recruited to a more rostral position in the mouse brain.

MATERIALS AND METHODS

Mutant mice

Generation and genotyping of En1Cre;Otx2fl/fl;En1Cre;Otx2fl/fl;Nkx2-2−/−;Nkx2-2−/−;En1Cre;Otx2fl/fl;En1Cre;Otx2fl/fl;Nkx2-2−/−, Nkx2-2−/−;Nkx2-2−/−, Nkx2-2−/−;En1Cre;Otx2fl/fl;En1Cre;Otx2fl/fl;Nkx2-2−/− and Nkx2-2−/−;Nkx2-2−/−;Nkx2-2−/− mice has previously been described (Panhuysen et al., 2004; Prakash et al., 2006; Puelles et al., 2004; Sander et al., 2000b; Sussel et al., 1998; Vallstedt et al., 2001). Animal treatment was conducted under federal guidelines for the use and care of laboratory animals and was approved by the HMGU Institutional Animal Care and Use Committee.

Radioactive in situ hybridization (ISH) and immunohistochemistry

Mouse embryo paraffin sections (8 μm) or cryosections (16 μm) were processed for radioactive ISH or for immunohistochemistry (Fischer et al., 2007; Prakash et al., 2006). Details on riboprobes, antibodies and image acquisition are available upon request. The whole-mount neurofilament staining procedure was performed as described in Huber et al. (Huber et al., 2005).

BrdU treatments

BrdU treatments were performed as described in Omodei et al. and Puellas et al. (Omodei et al., 2008; Puelles et al., 2006).

Unbiased stereology and cell counting

Pou4f1+ and Th+ cells were evaluated by the optical fractionator method and phospho-Histone H3+ cells were counted using the Stereo Investigator 5.05.4 software (MBF Bioscience, Williston, VT, USA) on serial coronal midbrain cryosections or paraffin sections from E18.5 or E11.0 embryos.

Averaged cell numbers for each genotype were subjected to a Student’s t-test for estimation of statistical significance. Counting of Nkx6-1−/−, Nkx6-2−/−, Pou4f1+ and Isl1+ single- and double-labeled cells was performed on z-stack confocal image series (1 μm intervals) recorded from VM sections of E12.5 embryos.

Bioinformatics prediction of Nkx6-1 target genes

All gene symbols, promoter and transcript identifiers in Table S1 (in the supplementary material) were derived from the promoter sequence retrieval database ElDorado (Genomatix, Munich, Germany). A genome-wide scan for conserved orthologous promoters with Nkx6-1 binding sites (BSs) was performed with the Gene2Promoter program (Genomatix). The selection of putative Nkx6-1 target genes was based on the Nkx6-1−/− midbrain phenotype and on their implication in neural development and/or axon guidance. Promoter sequences from up to seven different mammalian species were aligned with the DiAlign TF program (Cartharius et al., 2005) in the Genomatix software suite GEMS Launcher to evaluate overall promoter similarity and to identify conserved Nkx6-1 BSs. The promoter regions were defined as ~900 bp upstream, including the proximal region, and 100 bp downstream of the transcription start site (TSS). Position weight matrices were used according to Matrix Family Library Version 7.0 (October 2007) for promoter analyses. BSs were considered as ‘conserved BSs’ only if the promoter sequences for all given orthologs could be aligned in the region of the Nkx6-1 BS with the help of the DiAlign TF program (using default settings).

RESULTS

Midbrain basal plate progenitors and postmitotic OM/TN neurons express Nkx6-1

To establish the function of Nkx6 TFs in the development of VM progenitors and postmitotic neurons, we first performed a detailed expression analysis for Nkx6-1 and Nkx6-2 and a related TF, Nkx2-2, in the embryonic mouse midbrain at mid-gestational stages. From the earliest stage analyzed (E9.0), Nkx6-1 is expressed in the midbrain basal plate (BP) initially overlapping with the adjacent Nkx6-2+ territory in the ventrolateral midbrain, but becoming mutually exclusive at E9.5 (Fig. 1A,B). The medial FP expresses Nkx6-1 at E9.0 but not at later stages (Fig. 1A,B). At E11.0, an intermediate zone of weaker Nkx6-1 expression is apparent between the Nkx6-1+ ventricular/subventricular zone (VZ/SVZ) and mantle zone (MZ) (Fig. 1D). This subdivision into an Nkx6-1+ domain within the VZ/SVZ and one within the MZ is clearly discernible at E12.5 (Fig. 1I). Although the Nkx6-1+ and Nkx6-2+ domains appear to be mutually exclusive, some cells expressing Nkx6-2 are detected within the Nkx6-1+ MZ from E10.5 onwards (Fig. 1C,D). At E12.5, ~63% of the Nkx6-1+ cells in the BP MZ coexpress Nkx6-2. Nkx6-1 is also expressed within the dorsal Nkx6-2+ domain in the VZ/SVZ of the lateral midbrain at this stage (Fig. 1I). Expression of Nkx2-2 overlaps with the Nkx6-2+ domain in the lateral midbrain and initially (at E9.0-9.5) extends further ventrally into the BP (Fig. 1E,F). From E10.5 onwards, Nkx2-2 expression is confined to a narrow transverse stripe coinciding with the ventrolateral sulcus of the midbrain (His, 1904), and overlapping with the Nkx6-2+ domain (Fig. 1G,H,M). In contrast to Nkx6-1 and Nkx6-2, Nkx2-2 expression extends throughout the neuroepithelium including the VZ/SVZ and MZ at E12.5 (Fig. 1M). At this stage, Nkx2-2 expression also expands ventrally into the Nkx6-1+ VZ/SVZ (Fig. 1L,M).

Together, the combined expression of these TFs defines three adjacent domains along the D/V axis of the midbrain: the ventral-most expressing only Nkx6-1 in the BP; the intermediate coexpressing Nkx6-2 and Nkx2-2; and the dorsal-most expressing Nkx6-2 in the lateral midbrain. It is noteworthy that Nkx6-1 expression also initiates in more dorsal progenitors at late midgestation. Next, we examined the identity of the Nkx6-1−/− and Nkx6-
2+ cells in the VM MZ by analyzing the coexpression of tyrosine hydroxylase (Th), Pou4f1 and Isl1, which label mDA, RN and OM neurons in the midbrain, respectively. Nkx6-1 or Nkx6-2 are not expressed in Th+ mDA neurons arising from the FP (Fig. 1J,N) (Bonilla et al., 2008; Kittappa et al., 2007; Ono et al., 2007); some Pou4f1+ RN neurons coexpress Nkx6-1 (~9%) or Nkx6-2 (~15%) (Fig. 1K,O); and a considerable number of Isl1 + OM neurons (~69%) are Nkx6-1 + but not Nkx6-2 + at E12.5 (Fig. 1L,P). The E9.0-E12.5 rostral hindbrain displays a similar spatial pattern of Nkx6-1, Nkx6-2, Nkx2-2 and Isl1 expression, although Nkx6-2 expression within the ventral MZ is not detected at this brain level (not shown).

We concluded that Pou4f1+ RN and Isl1+ OM neurons might derive from Nkx6-1+ progenitors and designated the Nkx6-1+ VZ/SVZ in the midbrain BP as ‘ventral Nkx6-1+ progenitor domain’, in contrast to the Nkx6-1+ VZ/SVZ in the lateral midbrain, which we designated as ‘dorsal Nkx6-1+ progenitor domain’. Moreover, our findings suggest that, with the exception of a few Pou4f1+ RN neurons, Nkx6-2 labels a distinct and as yet unidentified cell population in the mouse VM.

Otx2 is required for maintenance of the VM Nkx6-1+ progenitor domain

We next investigated which signals apart from Shh control the expression of Nkx6 TFs in the murine VM. As reported before, inactivation of Otx2 in the mid-/hind-brain region, including the VM, of En1+/Cre; Otx2fl/fl/lox mice is not rescued in En1+/Cre; Otx2fl/fl/lox; Nkx2-2+/− (D,H,L,P,T,X) mutants. (F,H) There is a slight reduction of Isl1+ OM neurons in both Otx2 mutants. (I,L,N,P) The E9.0-E12.5 rostral hindbrain displays a similar spatial pattern of Nkx6-1, Nkx6-2, Nkx2-2 and Isl1 expression, although Nkx6-2 expression within the ventral MZ is not detected at this brain level (not shown).

We concluded that Pou4f1+ RN and Isl1+ OM neurons might derive from Nkx6-1+ progenitors and designated the Nkx6-1+ VZ/SVZ in the midbrain BP as ‘ventral Nkx6-1+ progenitor domain’, in contrast to the Nkx6-1+ VZ/SVZ in the lateral midbrain, which we designated as ‘dorsal Nkx6-1+ progenitor domain’. Moreover, our findings suggest that, with the exception of a few Pou4f1+ RN neurons, Nkx6-2 labels a distinct and as yet unidentified cell population in the mouse VM.
Moreover, the ventral Nkx6-1+ progenitor domain was still lost and Otx2 in green) in the triple mutant midbrain (Fig. 2D,H,L). The Isl1+ OM might only be generation of RN neurons in the VM. To assess if the ectopic relationship between Otx2 and Nkx6-1 expression and the preceding loss-of-function (LOF) data suggested a causal gene

expression of Otx2 leads to the ectopic induction of Nkx6-1, and concomitantly to the ectopic generation of Pou4f1+ RN neurons, we used a previously characterized mouse model in which ectopic expression of Wnt1 under the control of the En1 promoter (En1Wnt1 knock-in mice) induces ectopic expression of Otx2 in the Shh+ rostral hindbrain FP (Prakash et al., 2006). In this mutant, ectopic expression of Otx2 in the hindbrain FP was first detected at E9.5, concomitant with ectopic Nkx6-1+ cells, some of which coexpressed Otx2 (Fig. 3A,G) (Prakash et al., 2006). At E12.5, the ectopic Nkx6-1+ cells were located in the MZ lateral to the Otx2+ and Shh+ domains (Fig. 3H-J). This result indicated that Otx2 is sufficient for the ectopic induction of Nkx6-1, although a certain level of Shh signaling might be required in addition. Notably, ectopic Pou4f1+, but not Isl1+, cells were detected at E12.5 in the MZ at this caudal level of the En1Wnt1 rostral hindbrain FP (Fig. 3K,L; see Fig. S2 in the supplementary material). These cells probably arose from the ectopic Otx2+ and Nkx6-1+ domain, thus suggesting that Otx2 and Nkx6-1 might be sufficient for the generation of Pou4f1+ RN neurons but not of Isl1+ OM neurons.

**Nkx6-1 is required for the generation of Pou4f1+ RN neurons**

Our previous results strengthened the hypothesis that Nkx6-1 is necessary for the generation of Pou4f1+ RN neurons in the VM. To test this, we analyzed the midbrain phenotype of an Nkx6-1 null mutant mouse (Sander et al., 2000b). Although gross morphological defects were not apparent in the Nkx6-1−/− midbrain, a drastic reduction of the VM Pou4f1+ domain was already discernible at E12.5 (Fig. 4A-D), 1 day after Pou4f1+ cells were first detected in the midbrain tegmentum (Fedtschenko and Turner, 1995). The loss of Pou4f1 expression was most pronounced in the rostral and caudal third of the corresponding wild-type (WT) domain (Fig. 4A-D). At E18.5, the Pou4f1+ RN was strongly reduced in the mutants (Fig. 4E-H). RN neurons express the homeobox gene Hb9 (also known as Mnx1) at this stage (Fig. 4I). The Hb9+ RN domain was also strongly reduced at E18.5, and the corresponding Nissl-stained sections confirmed the hypoplasia of the RN and revealed the absence of the OM in the Nkx6-1−/− midbrain (Fig. 4I-L). Quantification of Pou4f1+ RN neurons at E18.5 showed that only 27% of these neurons remained in the mutants (Fig. 4M). The loss of Pou4f1+ RN neurons could be due to reduced progenitor proliferation, increased apoptosis or defective neurogenesis in the Nkx6-1−/− VM. In this context, the number of phosphorylated

---

**Fig. 3. Otx2 is sufficient for the ectopic induction of Nkx6-1 and Pou4f1+ RN neurons.** (A-L) Representative coronal sections from the rostral hindbrain of E9.5 (A,G) and E12.5 (B-F,H-L) WT (A-F) and En1Wnt1 (G-L) embryos. (A,G) At E9.5, ectopic expression of Otx2 induces Nkx6-1 ectopically in the FP of the En1Wnt1 rostral hindbrain. (D,J) Ectopic Nkx6-1+ cells are located lateral to the Otx2+ domain (inset in J; Nkx6-1 in red, Otx2 in green) in the En1Wnt1 rostral hindbrain FP at E12.5. (E,K) Ectopic Pou4f1+ cells arise from the Otx2+/Shh−/Nkx6-1− mutant FP. (F,L) Isl1+ cells are not detected at this caudal level of the WT and mutant brain. Plane of sectioning in B-F and H-L is more horizontal than in the inset in J.

**Otx2 is sufficient for the ectopic induction of Nkx6-1 and Pou4f1+ RN neurons**

The preceding loss-of-function (LOF) data suggested a causal relationship between Otx2 and Nkx6-1 expression and the generation of RN neurons in the VM. To assess if the ectopic
Histone H3 (pH3)+ mitotic cells and cleaved caspase 3 (cCasp3)+ apoptotic cells was not significantly altered in the Nkx6-1−/− VM between E9.0 and E12.5 (see Fig. S3 in the supplementary material; data not shown), indicating that the reduction of Pou4f1+ RN neurons is not due to impairments in proliferation or survival. Furthermore, the normal expression of the two proneural genes neurogenin 1 and 2 (Neurog1 and Neurog2) in the Nkx6-1−/− VM (see Fig. S3 in the supplementary material) suggests that neurogenesis was not impaired in the mutants. Altogether, these data suggest that Nkx6-1 is intrinsically required for the generation and/or identity of VM Pou4f1+ RN neurons.

Midbrain BP progenitors acquire a partial dorsal fate in the absence of Nkx6-1

Based on the previous conclusion, the loss of Pou4f1+ RN neurons might be due to an altered cell fate of the mutant progeny. To test this, we analyzed the expression of a number of markers to assess abnormalities in D/V patterning, as well as progenitor and postmitotic identity, in the Nkx6-1−/− midbrain.

In the absence of Nkx6-1, the transcription of Nkx6-2 was upregulated and dorsally expanded in the VZ/SVZ corresponding to the dorsal Nkx6-1 progenitor domain in the E12.5 WT (Fig. 5A-D'). Nkx6-2 upregulation was already evident at earlier embryonic stages (E9.5-E11.0, data not shown), when Nkx6-1 is not expressed in this region (Fig. 1), suggesting that Nkx6-1 initially regulates Nkx6-2 expression non-cell-autonomously in the lateral midbrain. By contrast, the expression of Nkx6-2 in postmitotic cells or the expression of Nkx2-2 was not altered in the Nkx6-1−/− VM (Fig. 5C-D'; see Fig. S5 in the supplementary material). However, Dbx1 and Otx2 were induced ectopically in the SVZ/MZ of the mutant VM at the same caudal level where Pou4f1 expression was strongly reduced (Fig. 5E-J'). Ectopic induction of Dbx1 was first detected at E10.5 within the Phox2a+ domain of the Nkx6-1−/− VM (Fig. 1; see Fig. S4 in the supplementary material). The Dbx1 paralog Dmbx2 was not expressed in the WT midbrain at E9.5-E10.5, and Dmbx2 was not induced ectopically in the mutants (see Fig. S4 in the supplementary material). At E12.5, Dbx1 was expressed ectopically in cells other than Isl1+ OM neurons (Fig. 5K,M). Otx2, by contrast, was expressed at low levels in Isl1+ OM neurons of the WT embryo and might be slightly upregulated in these neurons in the mutants (Fig. 5L,N). Nevertheless, ectopic Otx2+ cells that did not coexpress Isl1 were intermingled with OM neurons in the mutant VM (Fig. 5N). Some ectopic Otx2+ cells coexpressed Nkx6-2 (see Fig. S9 in the supplementary material), indicating that Nkx6-2 was not sufficient to repress Otx2 in these cells. Although the fate of the ectopic Otx2+ cells was difficult to assess at later stages due to Otx2 expression in other midbrain neurons, ectopic Dbx1+ cells were found adjacent to mDA neurons in the Nkx6-1−/− VM at E18.5 (data not shown).

The ectopic expression of Dbx1 suggested a partial dorsalization of the Nkx6-1−/− VM. To support this conclusion, we analyzed the expression of Pax7 in Nkx6-1−/− embryos. At E12.5, Pax7 was ectopically activated in the VZ/SVZ of the mutant BP corresponding to the ventral Nkx6-1−/− progenitor domain of WT embryos, but Pax7 expression was also strongly upregulated within and around the Isl1+ OM (Fig. 5O-R). The transcription of Shh, Foxa2, Wnt1, Wnt5a, Lmx1a, Lmx1b and Emx2, however, was unchanged in the mutant VM (see Fig. S5 in the supplementary material; data not shown). Next, we studied whether the glutamatergic phenotype of Pou4f1+ cells and the GABAergic identity of Helt-derived neurons (Nakatani et al., 2007) were abnormal in Nkx6-1−/− embryos. Expression of the vesicular glutamate transporter 2 (Vglut2, also known as Slc17a6), as well as that of glutamic acid decarboxylase 1 (Gad1) and Helt, were apparently not affected (see Fig. S6 in the supplementary material), and mDA neurons developed normally until E18.5 in the mutants (not shown). Thus, the neurotransmitter and general identity of the ectopic Dbx1+ and Otx2+ cells in the mutant VM remains to be established.

We concluded that in the absence of Nkx6-1, midbrain BP progenitors acquired a partial dorsal (Pax7+) identity and probably generated ectopic Dbx1+ and Otx2+ neurons. In this context, Nkx6-1 might be cell autonomously required to repress Pax7+ in progenitors and/or Dbx1 and Otx2 in postmitotic neurons.

Nkx6-1 controls the proper migration and axon outgrowth of OM and TN neurons

Some of the reported alterations in the Nkx6-1−/− VM might also affect the Isl1+ OM and TN neurons, as Nkx6-1 is expressed in a significant proportion of these neurons (Fig. 1) (Müller et al., 2003), and the OM was not distinguishable on Nissl-stained sections at
E18.5 (Fig. 4). The Phox2a+ OM/TN progenitor domain, however, was established correctly in Nkx6-1−/− embryos at E9.5 (see Fig. S4 in the supplementary material), and Phox2a expression persisted in postmitotic OM/TN neurons until E12.5 (see Fig. S7 in the supplementary material). These neurons also expressed Isl1, but the appearance of the Isl1+ OM/TN was less compact in Nkx6-1−/− embryos, and several Isl1+ cells were misplaced and dispersed throughout the domain that would normally be occupied by the Pou4f1 RN neurons (Fig. 6A–P; see Fig. S7 in the supplementary material). Furthermore, expression of the vesicular acetylcholine transporter Vacht (also known as Slc18a3), a marker for these cholinergic MNs, was reduced markedly throughout development (Fig. 6Q–X; see Fig. S7 in the supplementary material). Therefore, these findings indicate that Nkx6-1 is also required for the proper migration/positioning of the Isl1+ OM/TN neurons in the VM/rostral hindbrain, and prompted us to examine other aspects of OM/TN development in Nkx6-1−/− embryos.

Neurofilament staining at E12.5 revealed that the nIII (oculomotor nerve) failed to grow out properly and stalled after a distance from its central nervous system (CNS) exit point, and that the nIV (trochlear nerve) was entirely missing in the mutants (Fig. 7A–D). Before stalling, the mutant nIII displayed a characteristic rostromedial deflection instead of its normal ventral turning towards the eye, growing back in the direction of the neural tube (Fig. 7C). Defasciculation of the nIII was evident before most of its axons stopped to grow, although single axons were seen to grow beyond the stalling point into different directions (Fig. 7C). In one out of five Nkx6-1−/− embryos analyzed at E12.5, the left nIII displayed an initially normal trajectory but became progressively thinner and the remaining axons stalled at a distance (Fig. 7D; data not shown). In the same mutant embryo, a few axons exited the ventral neural tube close to the mid-/hind-brain boundary (MHB) and projected in a similar manner as the nIV before stalling on their trajectory towards the eye (data not shown), and some axons projected dorsally within the neural tube at a position that might correspond to the nIV (Fig. 7D). No difference in the aberrant projection of the nIII was observed between the left and right side in all other mutant embryos, and several Isl1+ cells were misplaced and dispersed throughout the domain that would normally be occupied by the Isl1+ OM neurons (K,M), but ectopic Otx2+ cells intermingle with these neurons (L,N). (O–R) Pax7 is induced ectopically (white arrowheads in R) in the VZ/SVZ and upregulated in the MZ of the mutant BP (A*,J,O–R). Pseudo-colored overlays of consecutive sections hybridized with probes for Pou4f1 (light blue) and Nkx6-1 (A*,B*), Nkx6-2 (C*,D*), Dbx1 (E*,F*) or Otx2 (I*,J*) (red); with overlapping expression domains in dark blue, and for Pax7 (red, O–R) and Nkx2-2 (O,Q) or Isl1 (P,R) (green); with overlapping expression domains in yellow. G* and H* are enlarged darkfield images of brightfield images in G and H.

**Fig. 5.** Midbrain BP progenitors acquire a partial dorsal fate in the absence of Nkx6-1. (A–J, O–R) Representative coronal sections from the caudal midbrain of E12.5 WT (A,A’,C,C’,E,E’,G,G’,I’,O,P) and Nkx6-1−/− (B,B’,D,D’,F,F’,H,H’,J,J’,Q,R) embryos. (A–B’) Nkx6-1 is not expressed in Nkx6-1−/− mutants. (C–D’) Nkx6-2 expression is upregulated (arrows in D) in the Nkx6-1−/− lateral midbrain. There is ectopic induction of Dbx1 (E–F’; white arrowheads in F’) and of Otx2 (G–H’; white arrowheads in H’) in the BP of the mutant midbrain. (I–J’) Ventral Pou4f1 expression is strongly reduced (black arrowheads in I) at this level of the mutant midbrain. (K–N) Close-up views of the OM (red boxed area in G’,H’) in caudal midbrain sections of E12.5 WT (K,L) and Nkx6-1−/− (M,N) embryos. The ectopic Dbx1+ cells do not coincide with Isl1+ OM neurons (K,M), but ectopic Otx2+ cells intermingle with these neurons (L,N).
its transcription was also prematurely and ectopically initiated at E9.5 (Fig. 7I-L). Second, expression of the roundabout receptor Robo1, which normally overlaps only partly with the Isl1+ OM/TN domain, was upregulated in these regions in Nkx6-1–/– embryos (Fig. 7M-P). Third, transcription of the secreted molecule Slit2, a ligand of Robo receptors, was reduced or even abolished in the MZ of the midbrain/rostral hindbrain BP, but remained unaffected in the FP of the mutant embryos (Fig. 7Q-T). Expression of the Ntn1 receptor deleted in colorectal carcinoma (Dcc), the Plexin A semaphorin receptor family (Plxna1-4), their coreceptor neuropilin2 (Nrp2), and the repulsive guidance molecule receptor neogenin (Neo1), by contrast, did not appear abnormal in the Nkx6-1–/– VM/rostral hindbrain (not shown). We concluded that in Nkx6-1–/– mutants, the aberrant migration and axon pathfinding defects of the OM/TN neurons are a consequence of the misexpression of at least three cell migration/axon guidance molecules in the OM/TN neurons themselves, or in their close vicinity.

Nkx6-2 is not required for RN and OM/TN neuron development

Finally, we reasoned that Nkx6-2 might also contribute to the patterning of the VM and to the proper development of RN neurons, as ~63% of the postmitotic Nkx6-1+ cells and ~15% of the Pou4f1+
RN neurons coexpressed Nkx6-2 (Fig. 1). Thus, we analyzed the midbrain phenotype of single Nkx6-2 (Nkx6-2^{+/+}), hereafter termed Nkx6-2^{+/--}, and double Nkx6 (Nkx6-1^--; Nkx6-2^{+/--}) null mutants (Vallstedt et al., 2001). The generation of Pou4f1^{+/+} RN and Isl1^{+} OM/TN neurons was not affected in Nkx6-2^{+/--} embryos (see Fig. S8 in the supplementary material). Moreover, the RN and OM/TN phenotype of Nkx6-1^{--}; Nkx6-2^{+/--} double mutants did not differ from that of Nkx6-1^{--} single mutants (see Fig. S8 in the supplementary material). In line with this finding, Otx2 and Dbx1 were induced ectopically in the VM of Nkx6-1^{--}; Nkx6-2^{+/--} double, but not of Nkx6-2^{+/--} single, mutants (see Fig. S9 in the supplementary material). Notably, the dorsal Dbx1 domain expanded ventrally in both Nkx6-2^{+/--} single and Nkx6-1^{1--}; Nkx6-2^{+/--} double mutants (see Fig. S9 in the supplementary material), indicating that Nkx6-2 is required for the repression of Dbx1. These data demonstrated that in the midbrain, Nkx6-2 is dispensable for the normal development of Pou4f1^{+} RN and Isl1^{+} OM/TN neurons but is necessary for the dorsal antagonism of Dbx1.

**DISCUSSION**

We identified the HD TF Nkx6-1 as a key factor controlling the development of the rostral MNs and related neurons in the mouse midbrain/rostral hindbrain. Nkx6-1 is required for the generation of proper numbers of Pou4f1^{+} RN neurons, for the correct migration of Isl1^{+} OM/TN neurons in the VM/rostral hindbrain and for the proper outgrowth of nIII and nIV towards their extraocular target muscles. Nkx6-1 thus exhibits functional similarity in the VM/rostral hindbrain and in the caudal hindbrain/spinal cord. Therefore, these data reinforce the notion that the ontogenetic profile of the OM/TN neurons has more similarities to special (branchio-) VMNs than to the sMNIs located in the caudal hindbrain/spinal cord. We also identified the HD TF Otx2 as necessary and sufficient for the maintenance of Nkx6-1 expression and Pou4f1^{+} RN neurons in the VM, and for their ectopic induction in the rostral hindbrain.

**Otx2 confers positional information for the generation of RN neurons**

Although earlier reports showed that Shh is both necessary and sufficient for the generation of Pou4f1^{+} and Isl1^{+}Phox2a^{+} neurons in the ventral and dorsal midbrain, respectively (Agarwala and Ragsdale, 2002; Bayly et al., 2007; Blaess et al., 2006; Fedtsova and Turner, 2001; Fogel et al., 2008; Watanabe and Nakamura, 2000), our study indicates that Shh alone is not sufficient to maintain Nkx6-1 expression and to instruct VM progenitors with the Pou4f1^{+} RN neuron fate. Otx2 is additionally required for the maintenance of the VM Nkx6-1 expression domain and subsequent generation of postmitotic Pou4f1^{+} neurons. In the hindbrain, A/P positional information is conferred by the Hox factors for the generation of the different cranial motor nuclei (Cordes, 2001; Gauf et al., 2003; Jungbluth et al., 1999). In the En1^{--}; Otx2^{flox/tau} mutant midbrain, only the most ventral progenitors of Th/Pitx3^{+} mDA and Pou4f1^{+} RN neurons adopted a rostral hindbrain identity, whereas the overall VM identity remained unchanged as judged by the fact that this territory in the mutants is rostral to the FgII^{8} domain at the MHB, expresses Otx1 and exhibits normal development of OM/TN neurons (Puelles et al., 2004). Removal of the ectopic Nkx2-2 in the compound En1^{--}; Otx2^{flox/tau}; Nkx2-2^{+/--} mutants restored the VM identity of mDA progenitors (Prakash et al., 2006), but notably this rescue did not include the VM Nkx6-1^{--} progenitor domain and Pou4f1^{+} RN neurons. Apart from imparting midbrain identity, Otx2 is thus required cell autonomously for the maintenance of Nkx6-1 expression either directly (direct activation) or indirectly (inactivation of another repressor) in BP progenitors, and subsequently for the generation of Pou4f1^{+} RN neurons. Consistent with this finding, the ectopic activation of Otx2 in the Shh^{+} rostral hindbrain FP of En1^{+/+};Wnt1 mice was sufficient to induce Nkx6-1^{--} expression and the ectopic generation of Pou4f1^{+} neurons. Shh and Otx2 might thus act synergistically for the induction and maintenance of the ventral Nkx6-1 expression domain in the developing mouse midbrain (Fig. 8). However, the fact that Shh gain-of-function (GOF) or LOF did not affect Otx2 transcription (Fogel et al., 2008; Watanabe and Nakamura, 2000), but the expression of Shh was altered in Otx2 GOF or LOF mutants.
(Prakash et al., 2006; Puelles et al., 2003; Puelles et al., 2004), suggests that Otx2 might even be upstream of Shh in this genetic cascade as a factor controlling the positioning of Shh expression.

**Nkx6-1 directs BP progenitors to ventral neuronal fates in the midbrain**

Nkx6-1 expression is confined mostly to a domain within the midbrain BP from where Pou4f1+ RN and Isl1+ OM neurons arise during development. The loss of ~73% Pou4f1+ RN neurons and the developmental deficits of the Isl1+ OM neurons (reduced Vacht expression, faulty migration and defective nLII outgrowth) in Nkx6-1−/− embryos indicate that Nkx6-1 is indeed required in BP progenitors for the proper specification of these two VM neuronal populations. The function of Nkx6-1 in these progenitors can be either permissive (by repressing alternative neural fates) or instructive (by initiating the corresponding developmental program). Our findings show that Pax7, a marker of dorsal progenitors, is ectopically induced in BP progenitors of the Nkx6-1−/− midbrain. This is indicative of a partial dorsalization as several other ventral genes were still expressed normally in these progenitors, and suggests a permissive function of Nkx6-1. The ectopic expression of Dbx1, Otx2 and Pax7 in postmitotic cells of the Nkx6-1−/− VM suggests that these cells also acquired dorsal features. In this context, the ectopic Dbx1+ and some of the ectopic Otx2+ cells represented a separate subpopulation that did not overlap with Isl1+ OM neurons, and/or their precursors might also have contributed to their developmental defects. Although an instructive role of Nkx6-1 in the generation of RN and OM/TN neurons remains to be established, the loss of only ~73% Pou4f1+ RN neurons suggests that Nkx6-1 should not be the only fate determinant for these neurons. In this regard, a participation of Nkx6-2 in the specification of Pou4f1+ RN and Isl1+ OM/TN neuron development has been conserved in the midbrain BP and in the hindbrain/spinal cord V2, sMN and vMN/V3 domains (Pattyn et al., 2003; Vallstedt et al., 2001), and Nkx6-1 represses Nkx6-2 in the lateral midbrain and in the pMN/p2 domains of the spinal cord (Vallstedt et al., 2001). Based on these conserved features, we suggest that the developmental profile of the OM/TN neurons bears significantly more resemblance to special (branchio-) vMNs, as compared with sMNs located in the caudal hindbrain/spinal cord. In support of this, it has previously been reported that OM/TN neurons share additional features with vMNs rather than sMNs, such as the requirement of the presence of a Phox2 gene for their generation (Coppola et al., 2005; Pattyn et al., 2000; Pattyn et al., 1997), the absence of Hb9 expression (Thaler et al., 1999), and the dorsal CNS exit point and repulsion by Slit1 and Slit2 in the case of the nIV and VMN axons (Hammond et al., 2005).

**Postmitotic control of OM/TN neuron migration and axon guidance by Nkx6-1**

Apart from the BP progenitors, Nkx6-1 is expressed in a significant proportion of Isl1+ OM/TN neurons, the migration and axon outgrowth of which was strongly affected in Nkx6-1−/− embryos, suggesting that Nkx6-1 also plays a role in the terminal differentiation of these neurons. Nkx6-1 is a transcriptional repressor (Mirmira et al., 2000; Muhr et al., 2001), and most of our data also point towards such a function of Nkx6-1 in the VM. Indeed, ectopic expression of Dbx1 and Otx2 in Nkx6-1−/− postmitotic neurons is consistent with a repression of these genes by Nkx6-1 (Fig. 8). Supporting this possibility is the fact that Nkx6-1 binding sites (BSs) in the Dbx1 and Otx2 promoters are particularly conserved among mammalian species (see Table S1 in the supplementary material). A further abnormality was the activation or derepression of two genes involved in migration and axon guidance, the Nn11 receptor Unc5c and the Slit receptor Robo1, in the mutant OM/TN neurons. It is noteworthy that Unc5c mediates nIV axon guidance and overexpression of Unc5 may cause a short- and long-range repulsion of axons (Burgess et al., 2006; Keleman and Dickson, 2001), but Slit1/2 may also have a repulsive effect on TN axons (Hammond et al., 2005).

**A conserved function of Nkx6-1 in RN/OM and MN development**

Taken together, our data indicate that several aspects of Nkx6-1 function in hindbrain/spinal cord sMN and vMN development have been conserved in the midbrain/rostral hindbrain, despite obvious spatiotemporal, molecular and anatomical differences (Fig. 8). Findings from this and previous studies indicate that Nkx6-1 is required for the generation of most Pou4f1+ RN neurons and spinal sMNs (Sander et al., 2000a; Vallstedt et al., 2001), as well as for the correct migration and axon outgrowth of Isl1+ OM/TN and hindbrain (branchio-) vMNs (Müller et al., 2003; Pattyn et al., 2003). Moreover, Nkx6-1 represses Dbx1 in the midbrain BP and in the hindbrain/spinal cord V2, sMN and vMN/V3 domains (Pattyn et al., 2003; Vallstedt et al., 2001), and Nkx6-1 represses Nkx6-2 in the lateral midbrain and in the pMN/p2 domains of the spinal cord (Vallstedt et al., 2001). Based on these conserved features, we suggest that the developmental profile of the OM/TN neurons bears significantly more resemblance to special (branchio-) vMNs, as compared with sMNs located in the caudal hindbrain/spinal cord. In support of this, it has previously been reported that OM/TN neurons share additional features with vMNs rather than sMNs, such as the requirement of the presence of a Phox2 gene for their generation (Coppola et al., 2005; Pattyn et al., 2000; Pattyn et al., 1997), the absence of Hb9 expression (Thaler et al., 1999), and the dorsal CNS exit point and repulsion by Slit1 and Slit2 in the case of the nIV and VMN axons (Hammond et al., 2005).

**Acknowledgements**

We thank S. Laass for excellent technical assistance; A. Pierani for the Dbx1 antibody; F. Giebert, S. Goetz, J. Guimera, A. Huber-Brösamle for riboprobes; A. Huber-Brösamle and R. Koester for help with confocal laser scanning microscopy; and K. Pfueger and S. Steininger for initial participation in this work. This work was supported by the ‘Ramon y Cajal’ Program and Micinn (BFU2008-03708) to E.P.; a CIRM postdoctoral fellowship (Training Grant No T1-00008) to K.F.; NIH/NIDDK-1R01-DK8471-01 and NIH/NIDDK-1U19-DK072495-01 grants to M.S.; the Italian Association for Cancer Research (AIRC); the FP6 EuTRACC Integrated Project (LSHG-CT-2007-037445) and ‘Fondazione Cassa di Risparmio’ of Rome to A.S.; by the Initiative and Networking Fund in the framework of the Helmholtz Alliance on Systems Biology, Bayerischer Forschungsverbund ‘ForNeuroCell’ (F2-F2410-1020697), the European Union (EuTRACC LSHTM-CT-2006-037445) and the Deutsche Forschungsgemeinschaft (DFG) WU 164/3-2 and WU 164/4-1 to W.W. Deposited in PMC for release after 12 months.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/15/2545/DC1

**References**


<table>
<thead>
<tr>
<th>E12.5</th>
<th>rostral r1</th>
<th>caudal r1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>En1&lt;sup&gt;+&lt;/sup&gt;/Wnt1</td>
</tr>
<tr>
<td></td>
<td><img src="image1" alt="Image A" /></td>
<td><img src="image2" alt="Image B" /></td>
</tr>
<tr>
<td></td>
<td><img src="image5" alt="Image E" /></td>
<td><img src="image6" alt="Image F" /></td>
</tr>
<tr>
<td></td>
<td><img src="image9" alt="Image I" /></td>
<td><img src="image10" alt="Image J" /></td>
</tr>
<tr>
<td></td>
<td><img src="image13" alt="Image M" /></td>
<td><img src="image14" alt="Image N" /></td>
</tr>
</tbody>
</table>

**Legend:**
- A: ![Image A](image1)
- B: ![Image B](image2)
- C: ![Image C](image3)
- D: ![Image D](image4)
- E: ![Image E](image5)
- F: ![Image F](image6)
- G: ![Image G](image7)
- H: ![Image H](image8)
- I: ![Image I](image9)
- J: ![Image J](image10)
- K: ![Image K](image11)
- L: ![Image L](image12)
- M: ![Image M](image13)
- N: ![Image N](image14)
- O: ![Image O](image15)
- P: ![Image P](image16)
Prakash_Fig. S3

**Table:**

<table>
<thead>
<tr>
<th>pH3</th>
<th>wt</th>
<th>Nkx6-1 +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>E9.0</td>
<td>E11.0</td>
</tr>
<tr>
<td>B</td>
<td>E11.0</td>
<td>E11.0</td>
</tr>
<tr>
<td>C</td>
<td>E9.5</td>
<td>E11.0</td>
</tr>
<tr>
<td>D</td>
<td>E11.0</td>
<td>E11.0</td>
</tr>
<tr>
<td>E</td>
<td>E10.5</td>
<td>E11.0</td>
</tr>
<tr>
<td>F</td>
<td>E11.0</td>
<td>E11.0</td>
</tr>
<tr>
<td>G</td>
<td>E11.0</td>
<td>E11.0</td>
</tr>
<tr>
<td>H</td>
<td>E10.0</td>
<td>E11.0</td>
</tr>
</tbody>
</table>

**Diagram M:**

**phospho-Histone H3** cells in E11.0 ventral midbrain

**Graph:**

Number of pH3 cells/μm²

- **wt**
- **Nkx6-1 +/-**

**Genotype**

- **wt**
- **Nkx6-1 +/-**

**Timepoints:**

- E9.0
- E10.5
- E11.0
- E12.5
E12.5

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>Nkx6-1 -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dbx1</td>
<td><img src="A" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
</tr>
<tr>
<td>Vglut2</td>
<td><img src="C" alt="Image" /></td>
<td><img src="D" alt="Image" /></td>
</tr>
<tr>
<td>Helt/Mgn</td>
<td><img src="E" alt="Image" /></td>
<td><img src="F" alt="Image" /></td>
</tr>
<tr>
<td>Gad1</td>
<td><img src="G" alt="Image" /></td>
<td><img src="H" alt="Image" /></td>
</tr>
</tbody>
</table>
### Table S1. Bioinformatics prediction of Nkx6-1 target genes

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Promoter ID</th>
<th>Transcript ID</th>
<th>Nkx6-1 B5 in mouse promoter</th>
<th>Promoter sequences with Nkx6-1 B5</th>
<th>Nkx6-1 activity on promoter§</th>
<th>Nkx6-1–/- phenotype</th>
<th>Ortholog†</th>
<th>Conserved BS</th>
<th>Number</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otx2</td>
<td>GXP_236948</td>
<td>NM_144841</td>
<td>6 -518 to -175 bp</td>
<td>7 Hs, Mm, Rn, Cl, Bt, Ec, Pt</td>
<td>4 Ectopic induction</td>
<td>Repression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dbx1</td>
<td>GXP_206557</td>
<td>NM_001005232</td>
<td>1 -329 bp</td>
<td>7 Hs, Mm, Rn, Cl, Bt, Ec, Pt</td>
<td>1 Ectopic induction</td>
<td>Repression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helt</td>
<td>GXP_157998</td>
<td>NM_173789</td>
<td>4 -379 to +24 bp</td>
<td>5 Hs, Mm, Rn, Ec, Pt</td>
<td>4 Not changed</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gad1</td>
<td>GXP_815397</td>
<td>NM_008077</td>
<td>1 -765 bp</td>
<td>5 Mm, Rn, Cl, Bt, Ec</td>
<td>1 Not changed</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nrp2</td>
<td>GXP_1241740</td>
<td></td>
<td>1 No TSS defined</td>
<td>5 Hs, Mm, Rn, Cl, Pt</td>
<td>1 Not changed</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slit2</td>
<td>GXP_1246669</td>
<td>XM_001513206</td>
<td>2 -121 to -37 bp</td>
<td>4 Hs, Mm, Rn, Pt</td>
<td>1 Lost expression</td>
<td>Activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unc5c</td>
<td>GXP_430782</td>
<td>AK138987</td>
<td>1 -420 bp</td>
<td>4 Hs, Mm, Bt, Pt</td>
<td>- Ectopic induction</td>
<td>Repression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Robo1</td>
<td>GXP_1239247</td>
<td></td>
<td>1 No TSS defined</td>
<td>4 Hs, Mm, Cl, Pt</td>
<td>1 Ectopic induction</td>
<td>Repression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nkx6-2</td>
<td>GXP_70860</td>
<td>NM_183248</td>
<td>1 -267 bp</td>
<td>3 Mm, Rn, Pt</td>
<td>1 Ectopic induction</td>
<td>Indirect repression²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pax7</td>
<td>GXP_105702</td>
<td>NM_011039</td>
<td>1 -926 bp</td>
<td>2 Mm, Rn</td>
<td>1 Ectopic induction</td>
<td>Repression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pou4f1</td>
<td>GXP_1238014</td>
<td>XM_001473425</td>
<td>7 -476 to -335 bp</td>
<td>2 Hs, Mm</td>
<td>1 Lost expression</td>
<td>Activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. The genes in the first column were ranked by the degree of conservation of predicted Nkx6-1 B5s across seven mammalian species. The higher the amount of conserved sites in an increasing number of orthologous promoter sequences, the higher the rank of the corresponding gene listed from top to bottom in the table.

*Position relative to transcription start site (TSS).

†Ortholog: Hs, Homo sapiens (human); Mm, Mus musculus (mouse); Rn, Rattus norvegicus (rat); Cl, Canis lupus (dog); Bt, Bos taurus (cow); Ec, Equus caballus (horse); Pt, Pan troglodytes (chimpanzee).

‡No transcript assigned, predicted promoter from orthologs.

§Presumed activity based on Nkx6-1–/- midbrain phenotype.

¶Nkx6-1 and Nkx6-2 are not coexpressed at the relevant stages.