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Expression patterns of homeobox genes in the mouse vomeronasal organ at postnatal stages

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ABSTRACT

Homeodomain proteins are encoded by homeobox genes and regulate development and differentiation in many neuronal systems. The mouse vomeronasal organ (VNO) generates *in situ* mature chemosensory neurons from stem cells. The roles of homeodomain proteins in neuronal differentiation in the VNO are poorly understood. Here we have characterized the expression patterns of 28 homeobox genes in the VNO of C57BL/6 mice at postnatal stages using multicolor fluorescent *in situ* hybridization. We identified 11 homeobox genes (*Dlx3, Dlx4, Emx2, Lhx2, Meis1, Pbx3, Pknox2, Pou6f1, Tshz2, Zhx1, Zhx3)* that were expressed exclusively in neurons; 4 homeobox genes (*Pax6, Six1, Tgif1, Zfhx3)* that were expressed in all non-neuronal cell populations, with *Pax6, Six1* and *Tgif1* also expressed in some neuronal progenitors and precursors; 12 homeobox genes (*Adnp, Cux1, Dlx5, Dlx6, Meis2, Pbx2, Pknox1, Pou2f1, Satb1, Tshz1, Tshz3, Zhx2*) with expression in both neuronal and non-neuronal cell populations; and one homeobox gene *(Hopx)* that was exclusively expressed in the non-sensory epithelium. We studied further in detail the expression of *Emx2*, *Lhx2*, *Meis1*, and *Meis2*. We found that expression of *Emx2* and *Lhx2* initiated between neuronal progenitor and neuronal precursor stages. As far as the sensory neurons of the VNO are concerned, *Meis1* and *Meis2* were only expressed in the apical layer, together with *Gnai2*, but not in the basal layer.

1. Introduction

The mouse relies mainly on its vomeronasal organ (VNO) to detect pheromones and kairomones. Behavioral responses towards conspecifics or allospecifics are essential for the survival of the species (Tirindelli et al., 2009; Chamero et al., 2012). Vomeronasal sensory neurons (VSNs) reside within the medial, concave surface of the cavity of the VNO (Døving and Trotier, 1998). These chemosensory neurons express vomeronasal receptor (VR) genes or formyl-peptide receptor (FPR) genes (Liberles et al., 2009; Rivière et al., 2009), which belong to the G protein-coupled receptor superfamily. There are two families of VR genes: the *V1r* family (Dulac and Axel, 1995) and the *V2r* family (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). These two families are expressed in two distinct VSN populations: apical neurons coexpress *V1r* genes with the G-protein subunit *Gnai2* (also known as *Gαi2*), and basal neurons coexpress *V2r* genes with the G-protein subunit *Gnao1* (also known as *Gαo*) (Dulac and Axel, 1995; Berghard and Buck, 1996; Jia and Halpern, 1996; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Saito et al., 1998). Apical neurons project their axons to multiple small glomeruli in the anterior accessory olfactory bulb, and basal neurons project their axons to glomeruli in the posterior accessory olfactory bulb (Jia and Halpern, 1996; Belluscio et al., 1999; Rodriguez et al., 1999; Del Punta et al., 2002). Other cell populations are present in the VNO: progenitors, precursors and immature neurons residing at the margins of the VNO and in the basal layer (Weiler and Benali, 2005), sustentacular cells residing at the most apical part of the medial epithelium (Adams, 1992), and cells of the lateral non-sensory epithelium (Tarozzo et al., 1998). The VNO maintains neurogenesis at adult age, from cells located at the edges for growth of the organ and from basal cells located in the center for neuronal replacement (Farbman, 1990; Brann and Firestein, 2014), but little is known about the mechanisms that regulate differentiation of a stem cell to a mature neuron.

Homeobox genes (McGinnis et al., 1984; Scott and Weiner, 1984)

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encode homeodomain transcription factors, and are critical in development from embryogenesis to cell differentiation, including neuronal differentiation (Bürglin, 2011; Prochiantz and Di Nardo, 2015). The homeodomain consists typically of 60 amino acids forming three alpha helices, but other homeodomain structures have been described (Bürglin and Affolter, 2016). Little is known about homeobox gene expression in the mouse VNO after birth. *Meis2* is expressed in apical VSNs at postnatal day (PD)0 and PD14 (Enomoto et al., 2011), and *Lhx2* is expressed in VSNs at PD1 (Berghard et al., 2012). Here, we describe and classify the expression patterns of 28 homeobox genes in the VNO at postnatal stages.

2. Results and discussion

2.1. Gene expression patterns of 9 markers in the VNO of C57BL/6 mice at PD21

We used several reference genes as markers in order to define various cell populations within the VNO. Their expression patterns and the localization of the different cell populations are shown in Fig. 1. For the screening of homeobox genes presented in Subsection 2.2, we used markers *Gap43* for immature neurons, *Omp* for mature neurons and *Cbr2* for sustentacular cells (Yu et al., 2005). *Cbr2* also labels cells of the non-sensory epithelium but this population is not taken into account here, except for *Pax6, Six1, Tgif1, Zfhx3*, and *Hopx*. In order to characterize the layer-specific expression of homeobox genes *Meis1* and *Meis2* in Subsection 2.4, *Gnai2* and *Gnao1* were used, as they separate the neuronal population into, respectively, an apical layer where neurons express *V1r* family genes, and a basal layer where neurons express *V2r* family genes (Dulac and Axel, 1995; Berghard and Buck, 1996; Jia and Halpern, 1996; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Saito et al., 1998). Finally, to determine at which stages of cell differentiation homeobox genes *Emx2, Lhx2, Meis1* and *Meis2* were expressed, markers of cell differentiation were used in Subsections 2.3 and 2.4. In addition to *Gap43* for immature neurons and *Omp* for mature neurons, we used as additional markers: *Ascl1* for neuronal progenitors, *Neurog1* for neuronal precursors, *Neurod1* for differentiating cells/postmitotic neurons (Cau et al., 1997) and *Stmn2* as another marker for immature neurons (Camoletto et al., 2001; Pellier-Monnin et al., 2001; McIntyre et al., 2010).

2.2. Expression patterns of 28 homeobox genes in the VNO of C57BL/6 mice at PD21

We have previously reported the expression patterns of 49 homeobox genes in the mouse MOE (Parrilla et al., 2016). Using the same ISH probes, a first ISH screening in the VNO of C57BL/6 males at PD21 eliminated 21 genes (*Alx1, Alx3, Barx1, Barx2, Dmbx1, Emx1, En2, Isl1, Meis3, Msx1, Msx2, Otx1, Pax3, Pbx1, Phtf1, Pitx1, Pou2f2, Pou2f3, Prrx1, Six4, Zfhx4)* because no consistent signal could be observed (data not shown). The remaining 28 genes (*Adnp, Cux1, Dlx3, Dlx4, Dlx5, Dlx6, Emx2, Hopx, Lhx2, Meis1, Meis2, Pax6, Pbx2, Pbx3, Pknox1, Pknox2, Pou2f1, Pou6f1, Satb1, Six1, Tgif1, Tshz1, Tshz2, Tshz3, Zfhx3, Zhx1, Zhx2, Zhx3)* were considered as potentially expressed in the VNO and studied further.

A recent high-throughput RNA-seq analysis revealed the expression of 140 homeobox genes in adult female VNO (Oboti et al., 2015). These 140 genes include the 28 genes that we chose for further study, but also the 21 genes that we had eliminated in our first ISH screening. Several reasons could explain the absence of labeling for the set of 21 genes: they could be expressed too lowly on a per-cell basis to be detected by ISH, they could be expressed at the adult ages studied in the RNA-seq analysis but not at PD21, they could be expressed specifically in female mice, and/or the ISH probes did not result in clear staining in the VNO under our ISH conditions. Thus, the 28 homeobox genes studied here represent 20% of the homeobox genes expressed in the female adult VNO, according to Oboti et al., 2015.

In the VNO of C57BL/6 mice at PD21, we performed multi-color

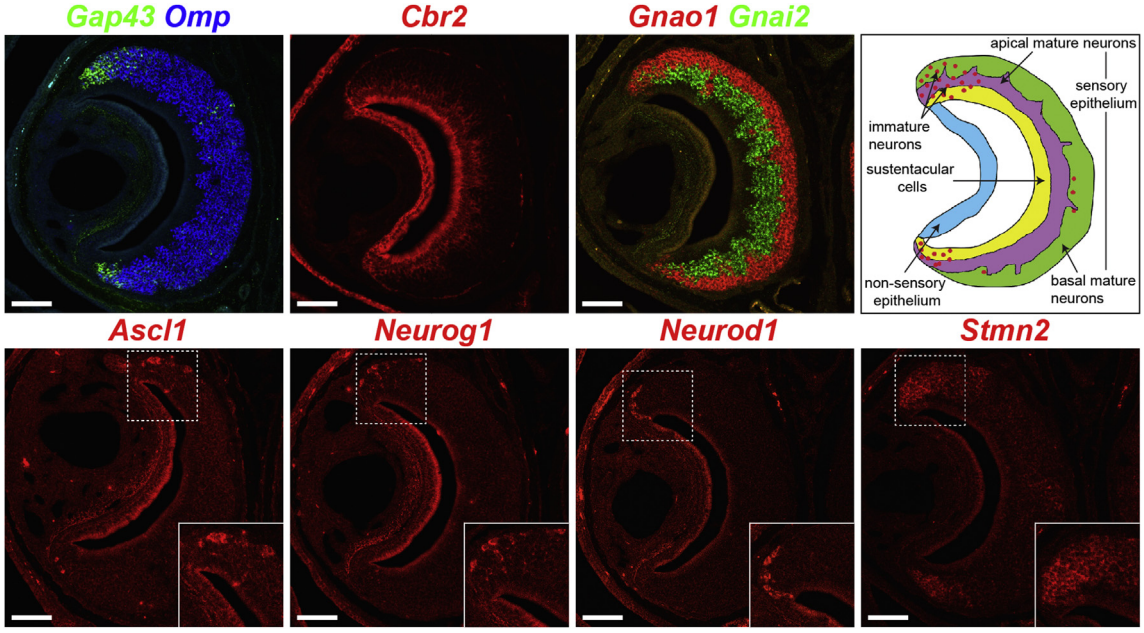

Fig. 1. Expression patterns of markers for cell subpopulations in the VNO of C57BL/6 mice at PD21, by fluorescent *in situ* hybridization. *Gap43* is a marker for immature neurons and *Omp* for mature neurons. *Cbr2* marks sustentacular cells and the non-sensory epithelium. *Gnao1* and *Gnai2* mark mature neurons that reside within, respectively, the basal and apical layer. The diagram shows the location of these cell subpopulations within the VNO. *Ascl1* is a marker of neuronal progenitors, *Neurog1* of neuronal precursors, *Neurod1* of differentiating cells/postmitotic neurons, and *Stmn2* is another marker for immature neurons. Insets (1.5× magnification) detail the dorsal margin, where the positive cells are located. Scale bars: 100 μm.

in situ hybridization (ISH) with specific riboprobes for these 28 homeobox genes in combination with markers *Gap43*, *Omp* and *Cbr2* (described in Subsection 2.1, Fig. 1). We identified four categories of patterns of homeobox gene expression in the VNO: neuronal, non-neuronal, both neuronal and non-neuronal, and other.

The neuronal category comprises 11 homeobox genes: *Dlx3, Dlx4, Emx2, Lhx2, Meis1, Pbx3, Pknox2, Pou6f1, Tshz2, Zhx1*, and *Zhx3*. These genes were coexpressed with *Gap43* and *Omp* but not with *Cbr2* (Fig. 2). *Dlx3, Dlx4, Emx2, Lhx2, Pbx3*, *Tshz2*, and *Zhx3* showed a particularly strong expression in all sensory neurons. *Meis1* expression was moderate and specific for a layer of sensory neurons. *Pknox2*, *Pou6f1,* and *Zhx1* showed a weak expression among sensory neurons.

The non-neuronal category consists of 4 homeobox genes: *Pax6, Six1, Tgif1, and Zfhx3*. These genes were coexpressed with *Cbr2* but

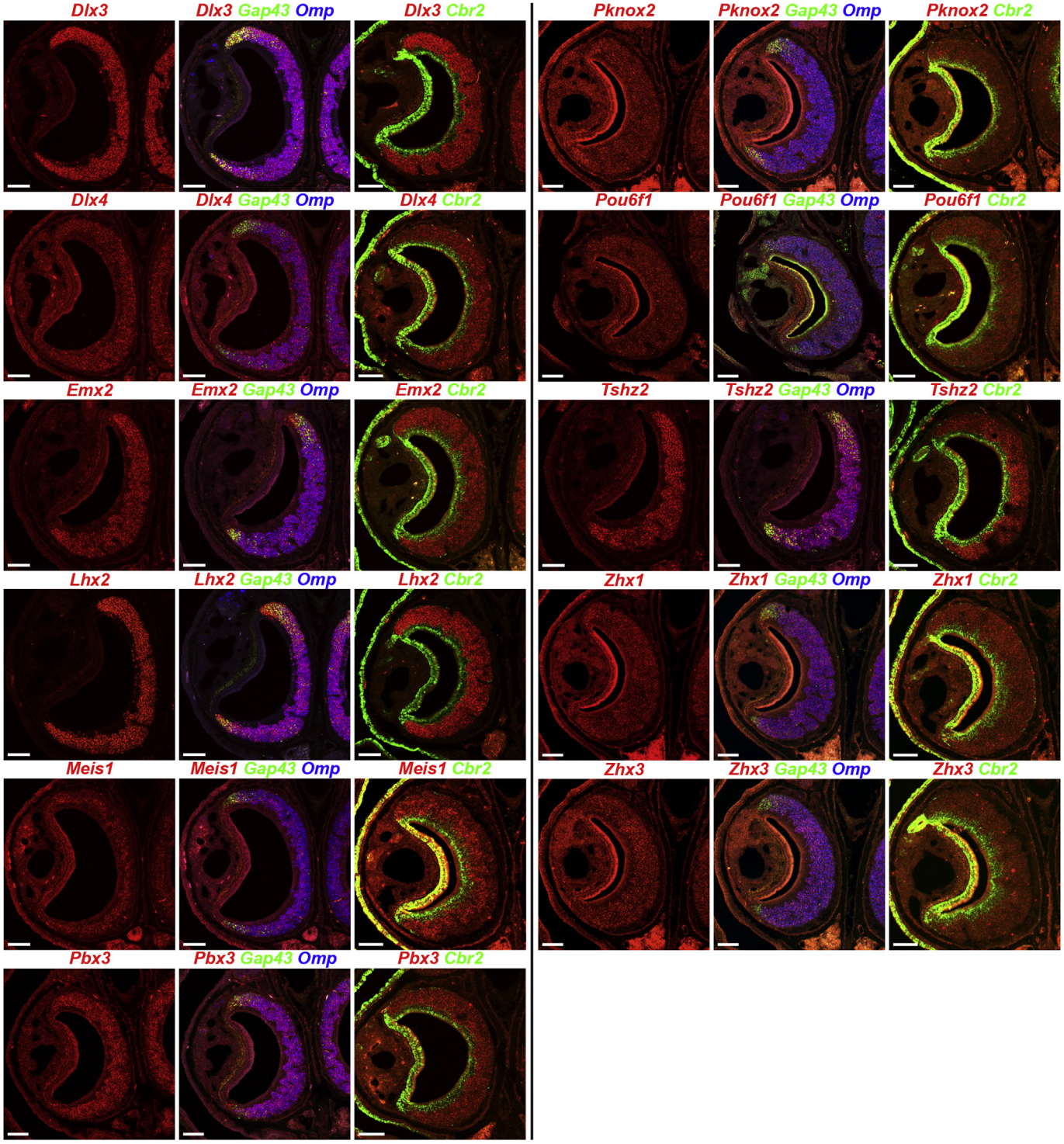

Fig. 2. Expression patterns of 11 homeobox genes expressed in neuronal populations in the VNO of C57BL/6 mice at PD21, by fluorescent *in situ* hybridization. The genes *Dlx3, Dlx4, Emx2, Lhx2, Meis1, Pbx3, Pknox2, Pou6f1, Tshz2, Zhx1*, and *Zhx3* were coexpressed with *Gap43* and *Omp*, but not with *Cbr2*. Scale bars: 100 μm.

not with *Gap43* or *Omp* (Fig. 3). Notably, they were expressed not only in the sustentacular cells, but also in the non-sensory epithelium. *Pax6, Six1* and *Tgif1* were, furthermore, expressed in some neuronal progenitors and precursors; nonetheless, we classified them as non-neuronal because these genes were not expressed in immature and mature neurons. Interestingly, *Pax6* and *Six1* have in the MOE at PD21 a similar expression pattern as in the VNO: they are expressed in neuronal progenitors and sustentacular cells (Guo et al., 2010; Ikeda et al., 2010; Parrilla et al., 2016).

The 12 homeobox genes *Adnp, Cux1, Dlx5, Dlx6, Meis2, Pbx2, Pknox1, Pou2f1, Satb1, Tshz1, Tshz3, and Zhx2*, were coexpressed with *Gap43, Omp* and *Cbr2* (Fig. 4). *Cux1, Pknox1, Pou2f1, Satb1*, and *Zhx2* showed a relatively homogenous expression within the VNO. *Dlx5* was highly expressed in sensory neurons, in particular in immature neurons. *Dlx6* was weakly expressed in sustentacular cells and in mature sensory neurons but strongly expressed at the margins of the VNO, where progenitors, precursors and immature neurons are located. A study of *Dlx6* expression from PD0 to PD56 (data not

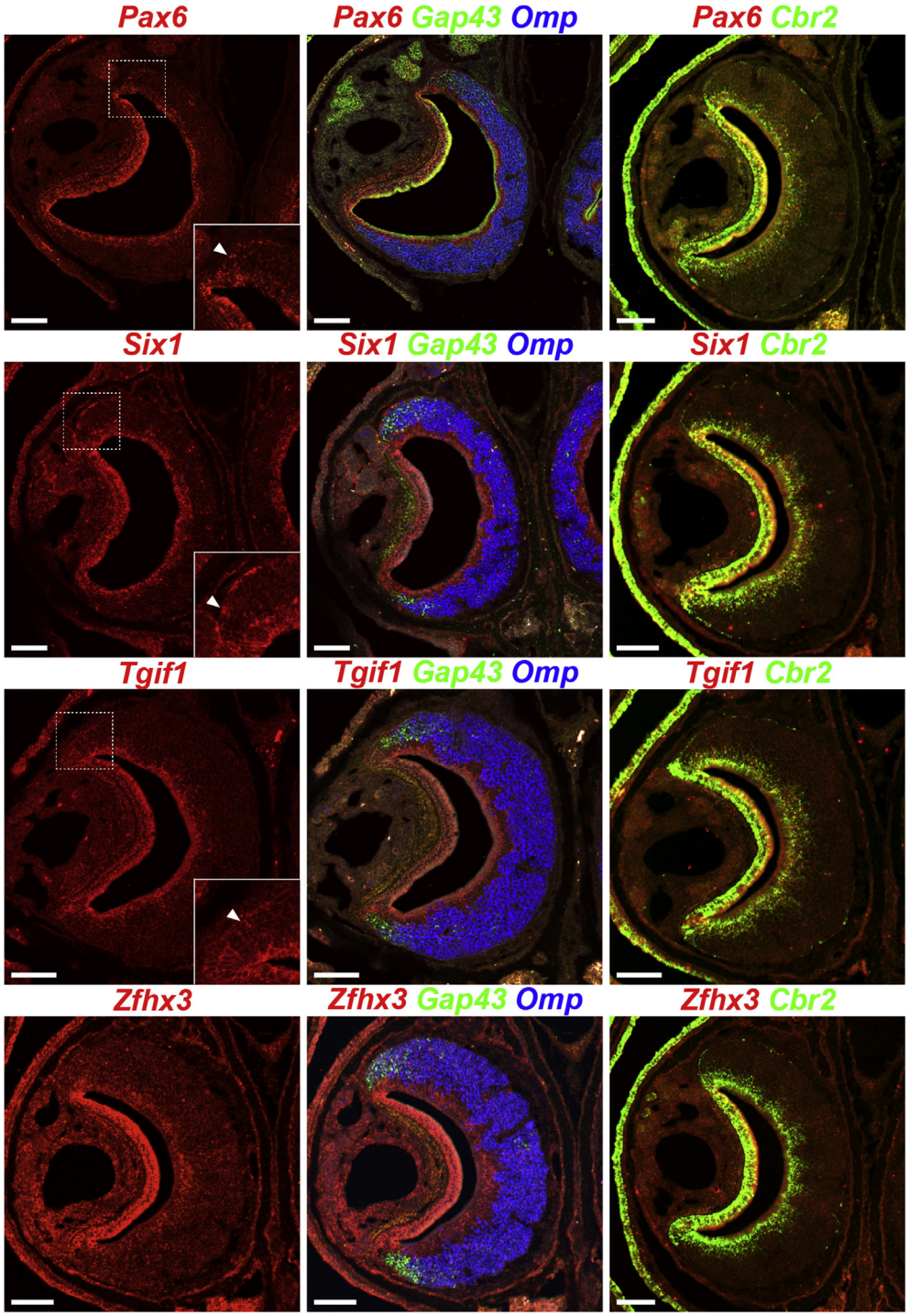

Fig. 3. Expression patterns of 4 homeobox genes expressed in non-neuronal populations in the VNO of C57BL/6 mice at PD21, by fluorescent *in situ* hybridization. The genes *Pax6, Six1, Tgif1*, and *Zfhx3* were coexpressed with *Cbr2*, but not with *Gap43* and *Omp*. The genes *Pax6, Six1* and *Tgif1* were furthermore expressed in progenitors and precursors. Insets (2× magnification) detail the dorsal margin of the VNO where arrowheads indicate the progenitors and precursors. Scale bars: 100 μm.

shown) showed that its expression pattern followed the same pattern in the sensory epithelium as *Bcl11b/Ctip2*, a gene encoding a transcription factor known to regulate the choice between *V1r* and *V2r* genes: like *Bcl11b/Ctip2, Dlx6* was highly expressed in the whole sensory epithelium at PD0, and progressively its high expression became restricted to the margins of the VNO while its expression in mature neurons got weaker (Enomoto et al., 2011). *Adnp, Tshz1,* and *Tshz3* showed at PD21 the same pattern as *Dlx6*, but with a moderate expression in mature sensory neurons. *Meis2* showed a strong layer-specific expression in the sensory neurons. *Pbx2* was expressed in all cell populations. This broad expression is also found in the MOE, where *Pbx2* is expressed in globose basal cells, olfactory sensory neurons (OSNs) and sustentacular cells (Parrilla et al., 2016).

Not belonging to any of the three previous categories is the gene *Hopx*. This homeobox gene was not expressed in either VSNs or

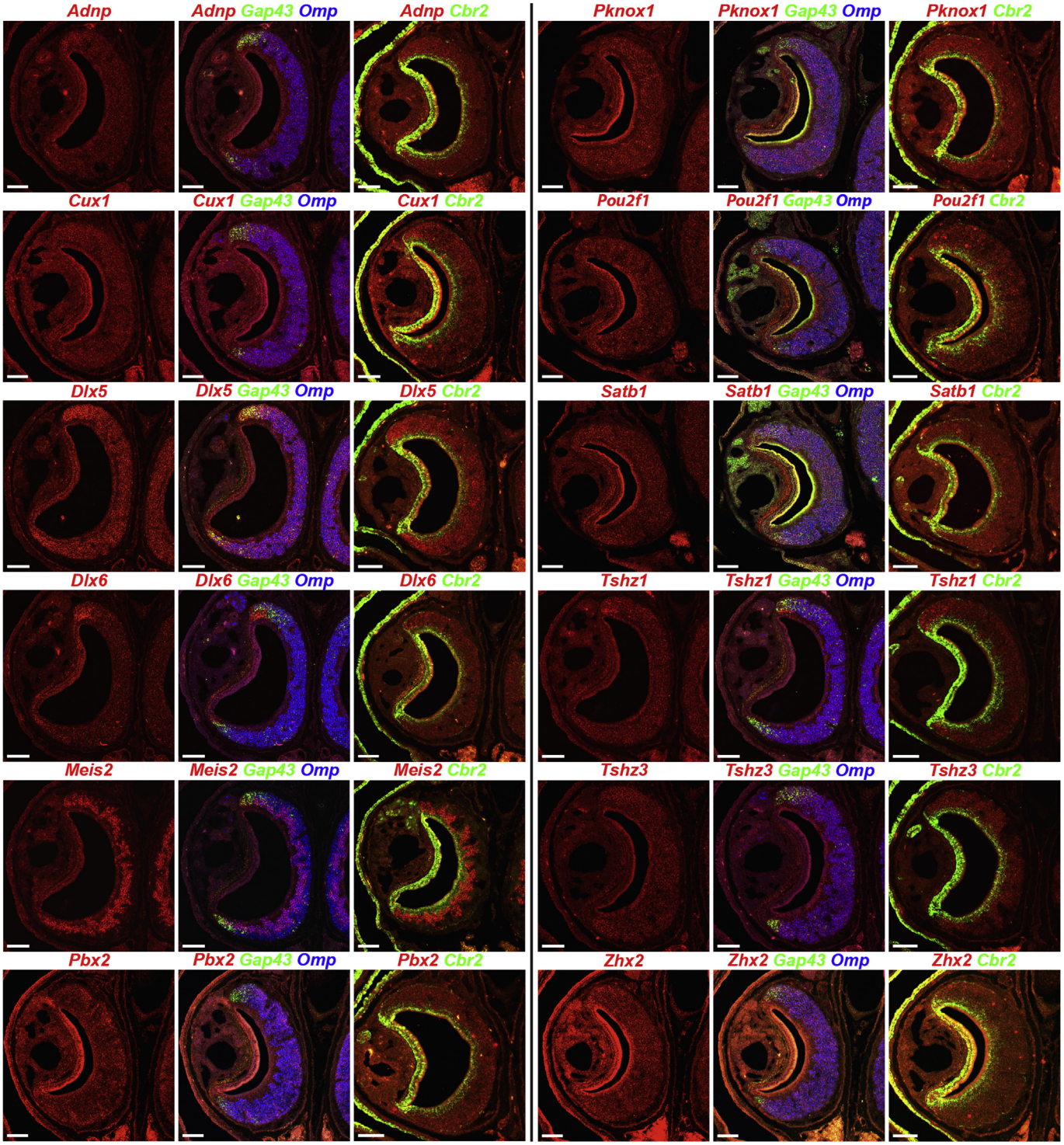

Fig. 4. Expression patterns of 12 homeobox genes expressed in both neuronal and non-neuronal populations in the VNO of C57BL/6 mice at PD21, by fluorescent *in situ* hybridization. The genes *Adnp, Cux1, Dlx5, Dlx6, Meis2, Pbx2, Pknox1, Pou2f1, Satb1, Tshz1, Tshz3,* and *Zhx2* were coexpressed with *Gap43*, *Omp* and *Cbr2*. Scale bars: 100 μm.

sustentacular cells, but exclusively expressed in the non-sensory epithelium (Fig. 5). In the MOE, *Hopx* has a different expression pattern: it is expressed only in OSNs (Parrilla et al., 2016). *Hopx* is thus a new marker for the non-sensory epithelium of the VNO.

In the end, we have here categorized the expression patterns of 28 homeobox genes in the VNO of C57BL/6 mice at PD21 (Table 1). Some homeobox genes (*Adnp, Cux1, Emx2, Lhx2, Pax6, Pbx2, Pbx3, Pknox2, Pou2f1, Pou6f1, Satb1, Six1, Tshz2, Zhx2*) have the same expression pattern in the VNO as in the MOE, whereas others (*Dlx3, Dlx5, Dlx6, Hopx, Meis1, Meis2, Pknox1, Tgif1, Tshz1, Tshz3, Zfhx3, Zhx1, Zhx3*) have different expression patterns (Parrilla et al., 2016). These similarities and differences show how broad the possible roles of homeobox genes can be, even in closely related tissues such as the olfactory and the vomeronasal epithelium.

Since *Emx2* and *Lhx2* have a high and exclusive expression in VSNs, and that they are known to affect the differentiation of OSNs and the odorant receptor gene expression in the MOE (Hirota and Mombaerts, 2004; Kolterud et al., 2004; Hirota et al., 2007; McIntyre et al., 2008; Berghard et al., 2012), we selected them for further study. We also selected *Meis1* and *Meis2*, because of their layer-specific expression in the VSNs.

2.3. Characterization of expression of Emx2 and Lhx2

In order to determine how *Emx2* and *Lhx2* are expressed postnatally, we performed single-color chromogenic ISH in C57BL/6 mouse VNOs at ages PD0, PD7, PD14, PD21 and PD56. We found that both genes were expressed across sensory neurons from PD0 to PD56, with *Lhx2* giving a stronger signal than *Emx2* at all ages (Fig. 6). It appeared that the *Lhx2* signal was stronger in the apical than in the basal layer at PD21 and PD56. In addition, both *Emx2* and *Lhx2* showed from PD7 to PD56 a stronger signal at the margins, where the immature neurons of the VNO are located, compared to the rest of the epithelium.

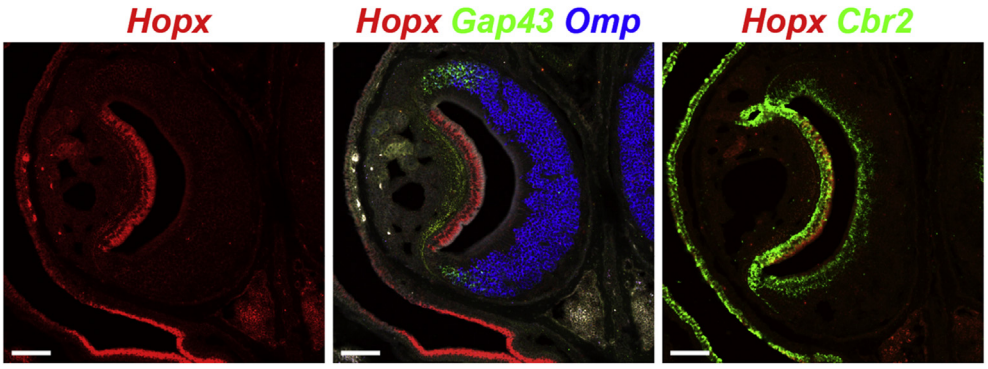

Fig. 5. Expression pattern of the *Hopx* gene, expressed exclusively in the non-sensory epithelium in the VNO of C57BL/6 mice at PD21, by fluorescent *in situ* hybridization. *Hopx* was coexpressed with *Cbr2* in the non-sensory epithelium but not in the sustentacular cells. It was not coexpressed with *Gap43* or *Omp*. Scale bars: 100 μm.

Table 1
Classification of the expression patterns of 28 homeobox genes in the vomeronasal organ of C57BL/6 mice at PD21. VSNs: vomeronasal sensory neurons, NSE: non-sensory epithelium.

Category	Gene	Expression in
Neuronal	*Dlx3*	VSNs
	Dlx4	VSNs
	Emx2	VSNs
	Lhx2	VSNs
	Meis1	VSNs in apical layer
	Pbx3	VSNs
	Pknox2	VSNs (weak)
	Pou6f1	VSNs (weak)
	Tshz2	VSNs
	Zhx1	VSNs (weak)
	Zhx3	VSNs
Non-neuronal	*Pax6*	sustentacular cells and NSE + some progenitors/precursors
	Six1	sustentacular cells and NSE + some progenitors/precursors
	Tgif1	sustentacular cells and NSE + some progenitors/precursors
	Zfhx3	sustentacular cells and NSE
Neuronal and non-neuronal	*Adnp*	VSNs (stronger in immature) and sustentacular cells
	Cux1	VSNs and sustentacular cells
	Dlx5	VSNs (strong in mature, very strong in immature) and sustentacular cells
	Dlx6	VSNs (weak in mature, strong in progenitors/precursors and immature) and sustentacular cells
	Meis2	VSNs in apical layer and sustentacular cells
	Pbx2	VSNs (stronger in basal layer) and sustentacular cells
	Pknox1	VSNs and sustentacular cells
	Pou2f1	VSNs and sustentacular cells
	Satb1	VSNs and sustentacular cells
	Tshz1	VSNs (stronger in immature) and sustentacular cells
	Tshz3	VSNs (stronger in immature) and sustentacular cells
	Zhx2	VSNs and sustentacular cells
Other	*Hopx*	non-sensory epithelium

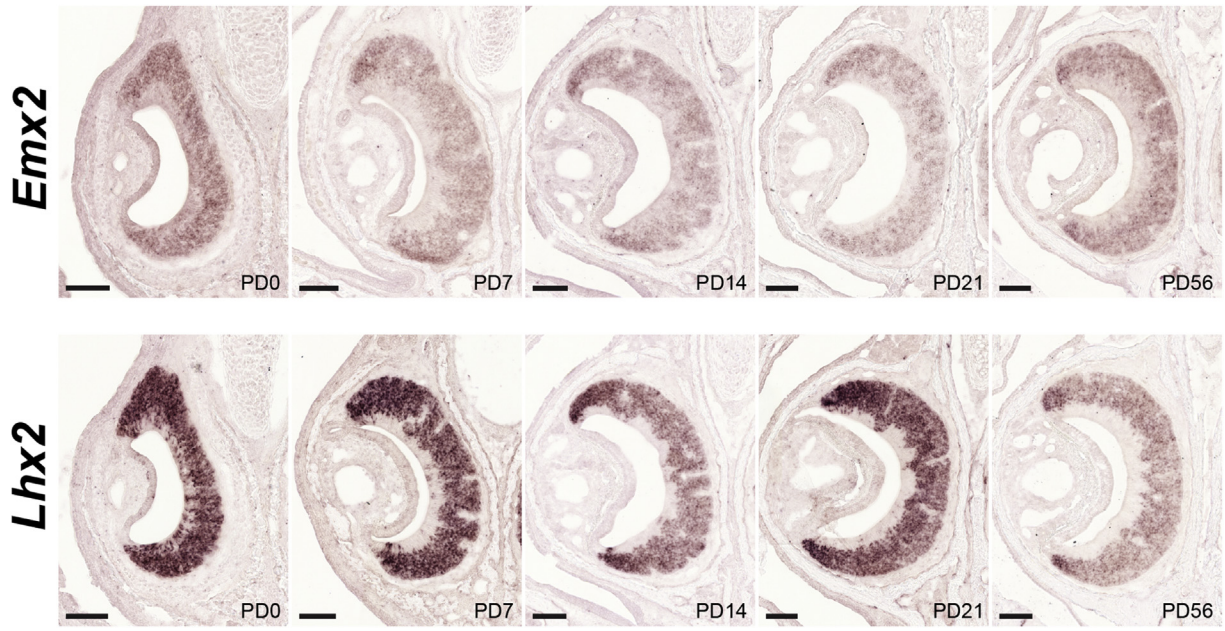

Fig. 6. Expression patterns of *Emx2* and *Lhx2* in the VNO of C57BL/6 mice at ages PD0, PD7, PD14, PD21 and PD56, by chromogenic *in situ* hybridization. *Emx2* and *Lhx2* were expressed across sensory neurons from PD0 to PD56, with a stronger signal in immature than mature neurons from PD7 to PD56. The *Lhx2* signal was stronger in apical than basal layer at PD21 and PD56. Scale bars: 100 μm.

Next we determined, at PD21, the stage of cell differentiation at which expression of *Emx2* and *Lhx2* initiated, by carrying out multi-color fluorescent ISH with markers *Ascl1, Neurog1, Neurod1, Stmn2, Gap43* and *Omp* (described in Subsection 2.1, Fig. 1). We found coexpression of *Emx2* in some but not all *Ascl1*-expressing cells. All cells expressing *Neurog1, Neurod1, Stmn2, Gap43*, or *Omp* coexpressed *Emx2*. Results were similar for *Lhx2*. We found coexpression of *Lhx2* in some but not all *Ascl1*-expressing cells. All cells expressing *Neurog1, Neurod1, Stmn2, Gap43*, or *Omp* coexpressed *Lhx2* (Fig. 7). It thus appeared that the expression of *Emx2* and *Lhx2* initiated after *Ascl1* expression and before *Neurog1* expression.

In the MOE at PD21, *Emx2* and *Lhx2* are expressed in progenitors and in mature and immature OSNs (Parrilla et al., 2016). Their expression pattern is thus similar as in the VNO, where we found them to be expressed in mature and immature VSNs, and also in progenitors and precursors. As *Emx2* and *Lhx2* have effects on the differentiation of OSNs and on OR gene expression (Hirota and Mombaerts, 2004; Kolterud et al., 2004; Hirota et al., 2007; McIntyre et al., 2008; Berghard et al., 2012), it would be interesting to determine if they also affect the differentiation of VSNs and the expression of vomeronasal receptor genes.

2.4. Characterization of expression of Meis1 and Meis2

To determine the expression pattern of the closely-related genes *Meis1* and *Meis2* (Steelman et al., 1997) in the postnal VNO, we performed single-color chromogenic ISH in C57BL/6 mouse VNOs at ages PD0, PD7, PD14, PD21 and PD56. We found that both genes were expressed in sensory neurons from PD0 to PD56 (Fig. 8). The *Meis1* signal was much weaker than the *Meis2* signal in chromogenic ISH. A layer-specific expression of *Meis2* was clearly visible from PD0 to PD56. In the case of *Meis1*, due to the weakness of the signal, we could not confirm a layer-specific expression at all ages, but a layer-specific expression was detectable at PD21 and PD56.

In order to confirm the expression of *Meis1* and *Meis2* in the apical layer of the VNO at PD21, we performed fluorescent ISH with *Gnao1* and *Gnai2* (described in Subsection 2.1, Fig. 1). The expression pattern of *Meis1* and *Meis2* matched that of *Gnai2* and was complementary to that of *Gnao1* (Fig. 9). Thus, *Meis1* and *Meis2* were expressed in the sensory neurons of the apical layer, which express *V1r* family genes (Dulac and Axel, 1995; Saito et al., 1998; Enomoto et al., 2011).

Our next aim was to determine, at PD21, the stage of cell differentiation at which expression of *Meis1* and *Meis2* initiated, by carrying out multi-color fluorescent ISH with markers *Ascl1, Neurog1, Neurod1, Stmn2, Gap43* and *Omp* (described in Subsection 2.1, Fig. 1). For each of the six markers, we found coexpression of *Meis1* or *Meis2* in some but not all cells (Fig. 10). Because *Meis1* and *Meis2* are only expressed in the apical layer together with *Gnai2*, our results did not allow us to determine at which stage of differentiation these genes begin to be expressed. We have two hypotheses to explain the presence of cells expressing the markers but not *Meis1* or *Meis2*: these cells could be basal, *Gnao1*-expressing cells, or *Meis1* and *Meis2* could initiate their expression at later stages of differentiation.

Thus, in the VNO at PD21, *Meis1* and *Meis2* were expressed selectively in cells of the apical layer. In the MOE at PD21, *Meis1* and *Meis2* have a different expression pattern: *Meis1* is expressed in globose basal cells, OSNs and sustentacular cells in the ventrolateral MOE, and *Meis2* only in sustentacular cells (Parrilla et al., 2016). These results might indicate a different role of *Meis1* and *Meis2* in the VNO than in the MOE.

3. Experimental procedures

3.1. Mouse husbandry

All mice were wild-type C57BL/6 mice. For the first ISH screening aiming to eliminate the homeobox genes not expressed in the VNO, males at PD21 were used. For the study of the 28 homeobox genes, both males and females were used for ages from PD0 to PD21, and males for age PD56. Mice were maintained in specified pathogen-free conditions in individually ventilated cages of the Tecniplast green line. They received *ad libitum* gamma-

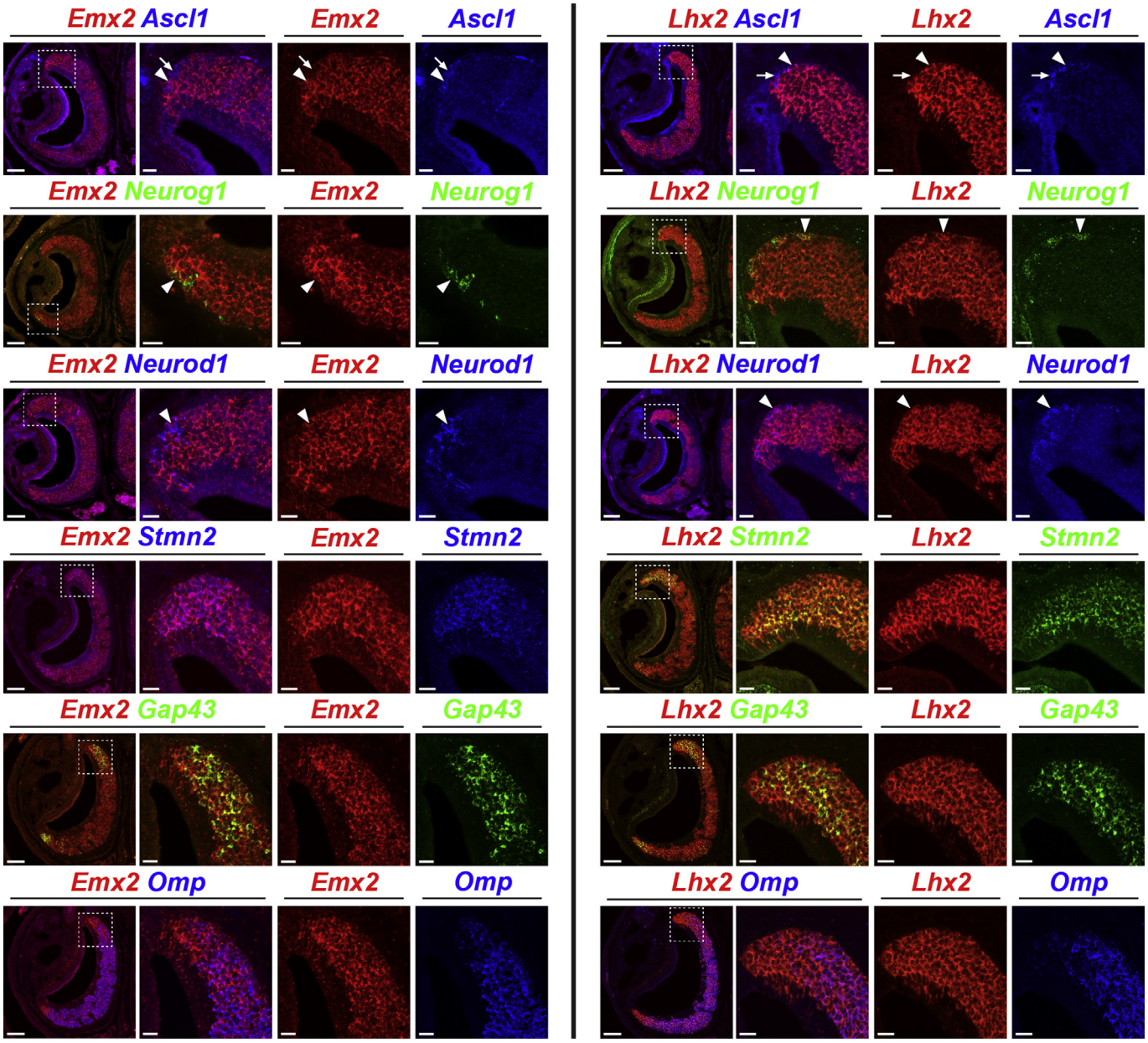

Fig. 7. Expression of *Emx2* and *Lhx2* with regard to the stage of cell differentiation in the VNO of C57BL/6 mice at PD21, by fluorescent *in situ* hybridization. *Lhx2* and *Emx2* were expressed in some but not all cells expressing *Ascl1*. All cells expressing *Neurog1*, *Neurod1*, *Stmn2*, *Gap43* and *Omp* coexpressed *Lhx2* and *Emx2*. Arrowheads indicate coexpression; arrows indicate no coexpression. Scale bars: first and fifth columns, 100 μm; all other columns, 20 μm.

irradiated ssniff V1124-727 (ssniff, Soest, Germany). Nesting, bedding and enrichment were provided as nestpak, Datesand Grade 6 (Datesand, Manchester, UK). Mouse experiments were carried out in accordance with the German Animal Welfare Act, the European Communities Council Directive 2010/63/EU and the institutional ethical and animal welfare guidelines of the Max Planck Institute of Biophysics and the Max Planck Research Unit for Neurogenetics. Approval came from the *Regierungspräsidium* of Darmstadt and the *Veterinäramt* of Frankfurt.

3.2. Sample preparation

Mice were anaesthetized by injection of ketamine HCl and xylazine, respectively at 210 mg/kg and 10 mg/kg of body weight. Mice were then perfused intracardially with an ice-cold solution of 4% paraformaldehyde (PFA) in 1x phosphate-buffered saline (PBS). After perfusion, the head was dissected and post-fixed overnight in 4% PFA at 4 °C and on shaker. It was then decalcified for ages PD7 to PD56 in 0.45 M EDTA pH 8.0 in 1x PBS at 4 °C and on shaker, for 24–48 h. The head was then cryoprotected by consecutive immersions of 24 h in 15% sucrose then 30% sucrose in 1x PBS on a shaker at 4 °C. After cryoprotection, the sample was trimmed and embedded in O.C.T. compound (Tissue Tek, Torrance, CA) on dry ice. Coronal sections with a thickness of 12 μm were generated using a Leica CM3050S cryostat.

3.3. RNA probe preparation

Whole olfactory mucosa from C57BL/6 mice at PD21 was dissected, and RNA was extracted using RNeasy Mini Kit (QIAGEN,

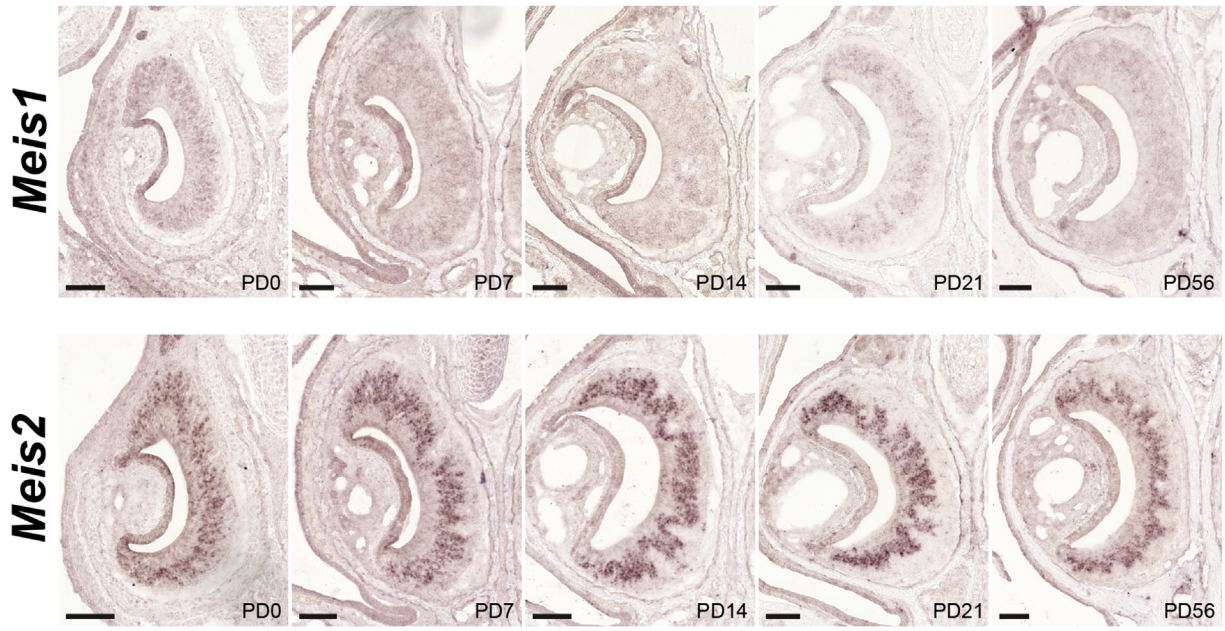

Fig. 8. Expression patterns of *Meis1* and *Meis2* in the VNO of C57BL/6 mice at ages PD0, PD7, PD14, PD21 and PD56, by chromogenic *in situ* hybridization. *Meis2* had a clear layer-specific expression from PD0 to PD56. The *Meis1* signal was too weak to determine a layer-specific expression at all ages, but it had a detectable layer-specific expression at PD21 and PD56. Scale bars: 100 μm.

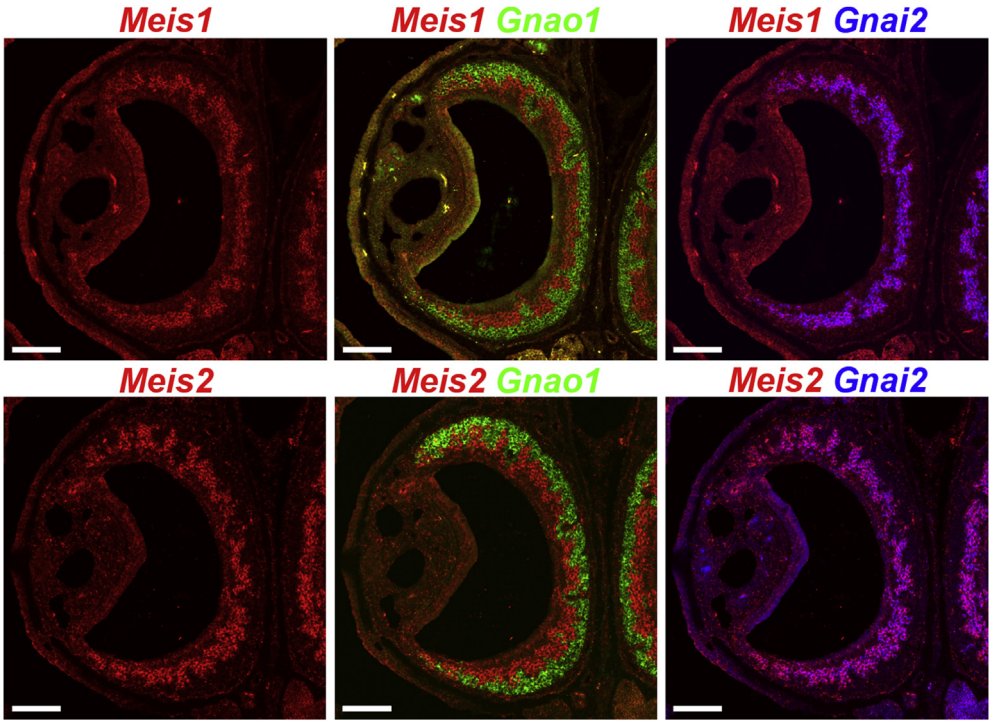

Fig. 9. Expression of *Meis1* and *Meis2* with *Gnao1* and *Gnai2* in the VNO of C57BL/6 mice at PD21, by fluorescent *in situ* hybridization. Cells expressing *Gnai2* (apical layer) coexpressed *Meis1* and *Meis2*, whereas cells expressing *Gnao1* (basal layer) did not coexpress *Meis1* and *Meis2*. Scale bars: 100 μm.

Hilden, Germany). RNA was reverse-transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) (Khan et al., 2011). A 50 ng sample of this cDNA was used as template to obtain PCR products with PCR conditions of an initial denaturation for 5 min at 94 °C, then 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C and 1 min extension at 72 °C, and in the end 10 min extension at 72 °C. PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI), cloned into pGEM-T Easy Vectors (Promega) and transformed into competent DH5 alpha *E. coli* bacteria. Plasmids were purified with QIAprep Spin Miniprep Kit (QIAGEN) or, after expansion, with QIAprep Plasmid Plus Midi Kit (QIAGEN) and used to prepare RNA probes. Plasmids were linearized using restriction enzymes (New England Biolabs, Frankfurt am Main, Germany) and

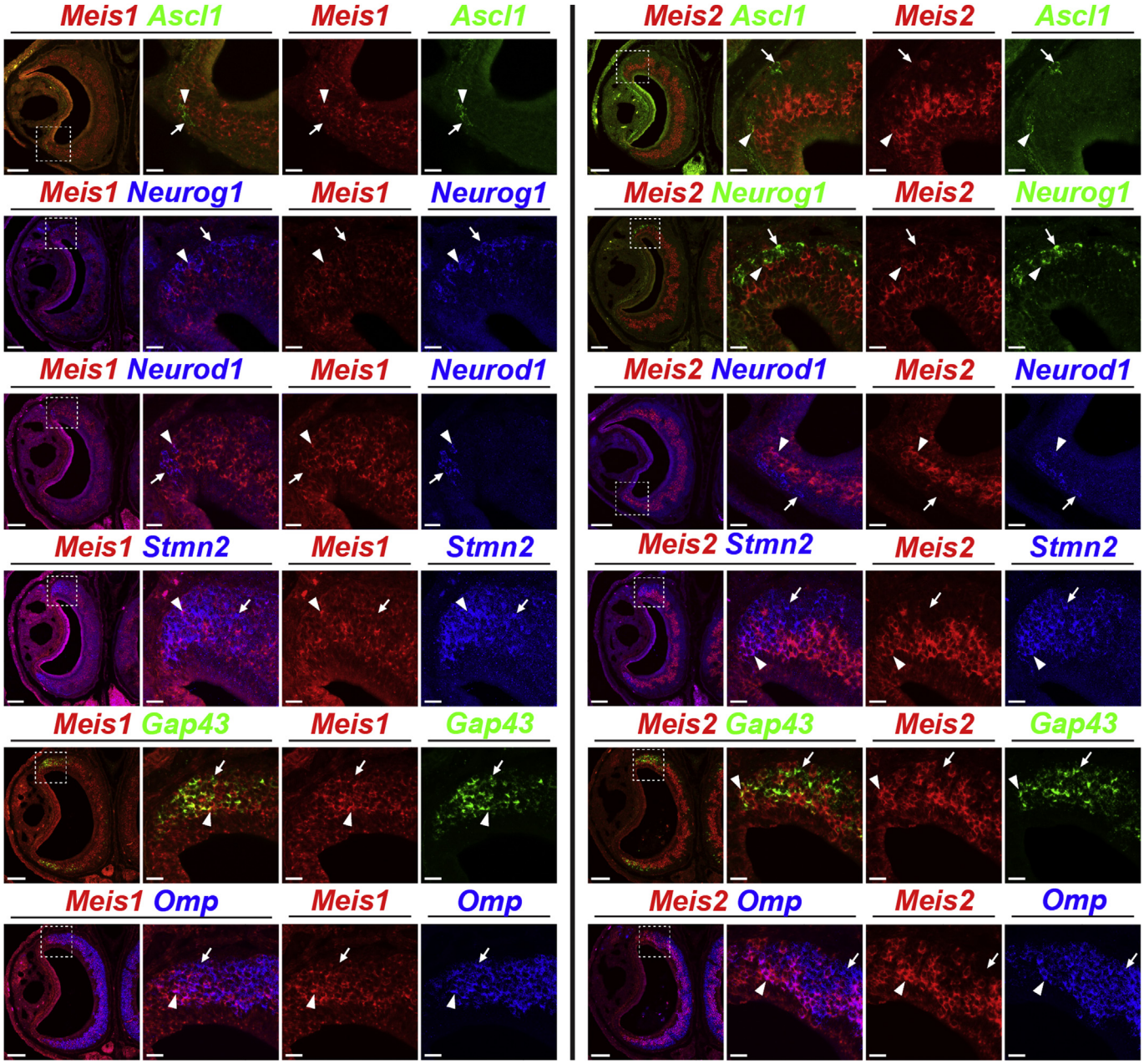

Fig. 10. Expression of *Meis1* and *Meis2* with regard to the stage of cell differentiation in the VNO of C57BL/6 mice at PD21, by fluorescent *in situ* hybridization. Some cells expressing *Ascl1, Neurog1, Neurod1, Stmn2, Gap43*, or *Omp* coexpressed *Meis1* or *Meis2* and some did not. Arrowheads indicate coexpression; arrows indicate no coexpression. Scale bars: first and fifth columns, 100 μm; all other columns, 20 μm.

purified with Wizard SV Gel and PCR Clean-Up System (Promega). Finally, linear plasmids were transcribed into RNA probes labeled with digoxigenin (DIG) (Roche, Basel, Switzerland), fluorescein (FLU) (Roche) or 2,4-dinitrophenyl (DNP) (Perkin Elmer, Waltham, MA). RNA probes were prepared for 28 homeobox genes and 9 genes used as markers. Plasmids for these riboprobes are available from Addgene (Table 2).

3.4. *In situ hybridization*

Each ISH result was obtained in at least three mice. ISH was performed as described in Ishii et al., 2004. Two types of experiments were done: chromogenic (single-color) ISH and fluorescent (1-, 2- or 3-color) ISH. In chromogenic ISH, DIG-labeled RNA probes were applied, and using an anti-digoxigenin-alkaline phosphatase (anti-DIG-AP) (Roche), signal was revealed through reaction of the alkaline phosphatase with 5-bromo-4-chloro-3-indolylphsphate (BCIP) (Roche) and nitro blue tetrazolium (NBT) (Roche). In fluorescent ISH, anti-DIG-AP (Roche) was detected by HNPP/Fast Red (Roche), anti-FLU-HRP (Perkin Elmer) was amplified with TSA Plus Fluorescein System (Perkin Elmer) and anti-DNP-KLH from Rabbit IgG fraction (Thermo Fisher Scientific, Waltham, MA) was detected by Alexa Fluor 647-conjugated Donkey anti-Rabbit IgG (H + L) (Jackson ImmunoResearch, West Grove, PA).

3.5. *Image processing*

Images from chromogenic ISH were taken with a Pannoramic

Table 2
List of ISH probes and their Addgene references.

Category	Probe	Gene full name	Link	Reference
Homeobox genes	pISH-Adnp	activity-dependent neuroprotective protein	http://www.addgene.org/74340	Parrilla et al., 2016
	pISH-Cux1	cut-like homeobox 1	http://www.addgene.org/74341	Parrilla et al., 2016
	pISH-Dlx3	distal-less homeobox 3	http://www.addgene.org/74342	Parrilla et al., 2016
	pISH-Dlx4	distal-less homeobox 4	http://www.addgene.org/74343	Parrilla et al., 2016
	pISH-Dlx5	distal-less homeobox 5	http://www.addgene.org/74344	Parrilla et al., 2016
	pISH-Dlx6	distal-less homeobox 6	http://www.addgene.org/74345	Parrilla et al., 2016
	pISH-Emx2	empty spiracles homeobox 2	http://www.addgene.org/74346	Parrilla et al., 2016
	pISH-Hopx	HOP homeobox	http://www.addgene.org/74347	Parrilla et al., 2016
	pISH-Lhx2	LIM homeobox protein 2	http://www.addgene.org/74337	Hirota and Mombaerts, 2004
	pISH-Meis1	Meis homeobox 1	http://www.addgene.org/74348	Parrilla et al., 2016
	pISH-Meis2	Meis homeobox 2	http://www.addgene.org/74349	Parrilla et al., 2016
	pISH-Pax6	paired box 6	http://www.addgene.org/74350	Parrilla et al., 2016
	pISH-Pbx2	pre B cell leukemia homeobox 2	http://www.addgene.org/74351	Parrilla et al., 2016
	pISH-Pbx3	pre B cell leukemia homeobox 3	http://www.addgene.org/74352	Parrilla et al., 2016
	pISH-Pknox1	Pbx/knotted 1 homeobox	http://www.addgene.org/74353	Parrilla et al., 2016
	pISH-Pknox2	Pbx/knotted 1 homeobox 2	http://www.addgene.org/74354	Parrilla et al., 2016
	pISH-Pou2f1	POU domain, class 2, transcription factor 1	http://www.addgene.org/74355	Parrilla et al., 2016
	pISH-Pou6f1	POU domain, class 6, transcription factor 1	http://www.addgene.org/74356	Parrilla et al., 2016
	pISH-Satb1	special AT-rich sequence binding protein 1	http://www.addgene.org/74357	Parrilla et al., 2016
	pISH-Six1	sine oculis-related homeobox 1	http://www.addgene.org/74358	Parrilla et al., 2016
	pISH-Tgif1	TGFB-induced factor homeobox 1	http://www.addgene.org/74359	Parrilla et al., 2016
	pISH-Tshz1	teashirt zinc finger family member 1	http://www.addgene.org/74360	Parrilla et al., 2016
	pISH-Tshz2	teashirt zinc finger family member 2	http://www.addgene.org/74361	Parrilla et al., 2016
	pISH-Tshz3	teashirt zinc finger family member 3	http://www.addgene.org/74362	Parrilla et al., 2016
	pISH-Zfhx3	zinc finger homeobox 3	http://www.addgene.org/74363	Parrilla et al., 2016
	pISH-Zhx1	zinc fingers and homeoboxes 1	http://www.addgene.org/74364	Parrilla et al., 2016
	pISH-Zhx2	zinc fingers and homeoboxes 2	http://www.addgene.org/74365	Parrilla et al., 2016
	pISH-Zhx3	zinc fingers and homeoboxes 3	http://www.addgene.org/74366	Parrilla et al., 2016
Markers	pISH-Ascl1	achaete-scute family bHLH transcription factor 1	http://www.addgene.org/74338	Hirota and Mombaerts, 2004
	pISH-Cbr2	carbonyl reductase 2	http://www.addgene.org/74339	Ishii et al., 2004
	pISH-Gap43	growth associated protein 43	http://www.addgene.org/74336	Hirota and Mombaerts, 2004
	pISH-Gnai2	guanine nucleotide binding protein (G protein), alpha inhibiting 2	http://www.addgene.org/15929	Rodriguez et al., 2002
	pISH-Gnao1	guanine nucleotide binding protein, alpha O	http://www.addgene.org/15930	Rodriguez et al., 2002
	pISH-Neurod1	neurogenic differentiation 1	http://www.addgene.org/74396	–
	pISH-Neurog1	neurogenin 1	http://www.addgene.org/74395	–
	pISH-Omp	olfactory marker protein	http://www.addgene.org/15928	Rodriguez et al., 2002
	pISH-Stmn2	stathmin-like 2	http://www.addgene.org/74397	–

MIDI Slide Scanner from 3D HISTECH. Images from fluorescent ISH were taken using a Zeiss LSM510 or LSM710 confocal microscope. Brightness and contrast of the images were adjusted using the software ZEN 2009 Light edition from Zeiss. Figures were prepared using Adobe Illustrator CS5.1.

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