Zinc-finger gene *Fez* in the olfactory sensory neurons regulates development of the olfactory bulb non-cell-autonomously

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Fez is a zinc-finger gene encoding a transcriptional repressor that is expressed in the olfactory epithelium, hypothalamus, ventrolateral pallium and prethalamus at mid-gestation. To reveal its function, we generated *Fez*-deficient mice. The *Fez*-deficient mice showed several abnormalities in the olfactory system: (1) impaired axonal projection of the olfactory sensory neurons; (2) reduced size of the olfactory bulb; (3) abnormal layer formation in the olfactory bulb; and (4) aberrant rostral migration of the interneuron progenitors. *Fez* was not expressed in the projection neurons, interneurons or interneuron progenitors. Transgene-mediated expression of *Fez* in olfactory sensory neurons significantly rescued the abnormalities in olfactory axon projection and in the morphogenesis of the olfactory bulb in *Fez*-knockout mice. Thus, *Fez* is cell-autonomously required for the axon termination of olfactory sensory neurons, and *Fez* non-cell-autonomously controls layer formation and interneuron development in the olfactory bulb. These findings suggest that signals from olfactory sensory neurons contribute to the proper formation of the olfactory bulb.

KEY WORDS: *Fez*, Zinc finger, Olfactory sensory neuron, Olfactory bulb, Axon guidance, Mitral cell, Interneuron, Rostral migratory stream, Mouse

INTRODUCTION

The olfactory system is one of the most basic sensory systems in vertebrates (Mori et al., 2000; Mori and Yoshihara, 1995; Yoshihara and Mori, 1997). Olfactory sensory neurons (OSNs) reside in the olfactory epithelium (OE) and vomeronasal organs, where they express seven transmembrane-type odorant or pheromone receptors, respectively. Their axons extend through the lamina cribrosa and reach the olfactory bulbs (OBs), which protrude from the anterior telencephalon. The OB is a laminar structure that contains limited types of neurons and glia. The outermost layer is the olfactory sensory nerve layer (ONL), which consists of olfactory afferent axons and ensheathing glia. Beneath the ONL, OSNs converge and synapse onto the dendrites of glutamatergic projection neurons (mitral and tufted cells) in structures called glomeruli [the glomerular layer (GL)]. Between the glomeruli in the GL, local circuit interneurons (periglomerular cells) send their dendrites into the glomeruli. Below the GL, the external plexiform layer (EPL) contains the cell bodies of the tufted cells, and the secondary dendrites of the mitral and tufted cells. Beneath the EPL is the mitral cell layer (MCL), which contains the mitral cell bodies. The projection neurons send their axons into the fibrous plexiform layer (IPL). Below the IPL lie the cell bodies of granule cells, which are interneurons [granule cell layer (GCL)]. Beneath the GCL,

interneuron progenitor cells are localized to the subventricular zone (SVZ), which continuously generates the interneurons, periglomerular cells and granule cells from the embryonic stage to adult (Goldman and Luskin, 1998; Hinds, 1968a; Hinds, 1968b; Lois and Alvarez-Buylla, 1994; Luskin, 1993).

The OB projection neurons and interneurons are born at distinct time periods under different kinds of genetic control. The projection neurons are born earlier than the interneurons (Hinds, 1968a; Hinds, 1968b). They have a pallial origin, whereas the interneurons originate in the subpallium. The development of the OB can be divided into two steps. First, evagination of the primordial OB from the telencephalon takes place on embryonic day (E) 12-E13 in mouse. Second, at E14-E18 in mouse, the interneuron progenitors arise from the SVZ of the lateral ganglionic eminence (LGE) (Luskin, 1998; Wichterle et al., 2001). The interneurons are continuously generated in the SVZ of the LGE and, thereafter, travel through the rostral migratory stream (RMS) throughout the life of the animal (Goldman and Luskin, 1998; Lois and Alvarez-Buylla, 1994; Luskin, 1993).

Analyses of genetically modified mice have revealed that the homeobox Dlx and *Arx* genes function cell-autonomously to generate interneurons, and that these genes are required for layer formation in the OB and the axon projection of OSNs to the OB (Bulfone et al., 1998; Levi et al., 2003; Long et al., 2003; Qiu et al., 1995; Yoshihara et al., 2005). As *Arx* is not expressed in the OE or in mitral cells, *Arx*-dependent instructive signals from the OB are required for proper innervation of the OB by OSNs, and *Arx* non-cell-autonomously controls the MCL formation in the OB. By contrast, there is no direct molecular or genetic evidence that signals from the OSNs are involved in OB formation, although primary olfactory axons are reported to alter the cell cycle of neurons within the OB primordium (Gong and Shipley, 1995).

Fez (forebrain embryonic zinc-finger) is a zinc-finger gene that was originally isolated as a forebrain- and OSN-specific gene in *Xenopus* (Matsuo-Takasaki et al., 2000). Identification of a homolog

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(*Fez-like*), and of orthologs in zebrafish, mouse and human, revealed that there are two highly related genes, *Fez* and *Fez-like* (*Fezl*), in all of these animal genomes (Hashimoto et al., 2000) (see Fig. S1 in the supplementary material). *Fezl*, which is more similar to zebrafish *fezl* than to *Xenopus Fez*, is expressed in the prospective forebrain region at E8.5 in mouse; later, it is expressed in the vomeronasal organ, hypothalamus, pallium and prethalamus (Hirata et al., 2004). Recent reports show that *Fezl* is involved in the development of neural tissues: the zebrafish *fezl* mutant *too few* displays lost or reduced monoaminergic neurons in the hypothalamus (Levkowitz et al., 2003); *Fezl*-deficient mice show abnormalities in the formation of subplate neurons and thalamocortical axons, and loss of the fornix/fimbria system (Hirata et al., 2004). It has also been recently reported that *Fezl* is required for the development of subcerebral projection neurons in the neocortex (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). However, nothing is known about the function of *Fez* in the development of the forebrain and olfactory system in any animal species.

In this study, we examined the expression of *Fez* during mouse development and generated *Fez*-deficient mice. *Fez* was expressed in the OE and in forebrain regions, where it partially overlapped with *Fezl* expression (Hirata et al., 2004), but it was not expressed in the OB projection neurons, interneurons or interneuron progenitors. *Fez*-deficient mice showed impaired projection of the OSN axons, abnormal OB layer formation and aberrant rostral migration of the interneuron progenitors. We found that *Fez* expressed in OSNs cell-autonomously controlled the axon targeting of the OSNs, and non-cell-autonomously regulated layer formation in the OB and the migration of interneurons. Our data provide genetic evidence that signals from the OSNs contribute to the formation of the OB.

MATERIALS AND METHODS

Isolation of *Fez* and generation of *Fez*-deficient mice

The sequence of mouse *Fez* cDNA (*Zfp312-like* – Mouse Genome Informatics) was previously isolated from the RIKEN Fantom Project (Okazaki et al., 2002) and deposited in the Mouse Genome Informatics Database under the accession number AK014242. Mouse *Fez* cDNA was isolated by polymerase chain reaction (PCR) from a mouse E12.5 cDNA library and subcloned into pBluescript II SK+ (pBLSK *Fez*). To make a riboprobe of *Fez*, the 5′ region of the cDNA was inserted into the *Eco*RI and *Bam*HI sites of pBluescript II SK+ (pBLSK *Fez*ISH). The full-length cDNA fragment of zebrafish *fez* was isolated by 5′ and 3′ rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (BD Biosciences, Clontech), based on the sequence information deposited in the Database (AI722599) (Matsuo-Takasaki et al., 2000). The sequence of zebrafish *fez* was deposited in the DDBJ databank under the accession number AB207804. The mouse gene (AI325906) originally reported as the mouse ortholog of *Xenopus Fez* was discovered to be more similar to zebrafish *fezl* (Hashimoto et al., 2000) than to zebrafish *fez* (see Fig. S1 in the supplementary material), and is referred to as mouse *Fezl* (full-length sequence, AB042399) in this report and in our previous publications (Hashimoto et al., 2000; Hirata et al., 2004). We previously reported the expression of *Fezl* in mouse and the phenotypes of *Fezl*-deficient mice (Hirata et al., 2004).

In this study, we investigated the mouse *Fez* gene (AK014242), which displays a stronger sequence similarity to zebrafish *fez* than to zebrafish *fezl*. The mouse genomic DNA fragment of *Fez* was obtained from the mouse 129sv strain genomic BAC library. The exon-intron structure was determined from the published mouse genome sequence and by restriction enzyme site mapping and sequencing. A targeting vector was constructed by replacing the region encoding amino acids 1-350 with a cassette composed of a phosphoglycerate kinase I promoter-linked neomycin-resistance gene (PGK-NEO) and the herpes simplex virus thymidine kinase gene ligated to the 3′ end. Transfected R1 and EB3 embryonic stem cell (ES) colonies that survived selection with G418 and ganciclovir were subcloned,

and the homologous recombination events determined by PCR and Southern blot hybridization. Targeted ES cells were injected into C57BL/6 blastulas to create chimeric male founders. Chimeric offspring were mated to C57BL/6 mice to generate F1 heterozygous progeny, and the heterozygotes were maintained by crossing with C57BL/6. Homozygous embryos were obtained by natural mating or by in vitro fertilization, using sperm and oocytes from the heterozygotes. The genotypes of embryos were identified by routine PCR. The primers used for the genotyping were: 5′-CTA-ATTCAGGGGCAAATGCCAGGCTATA-3′ and 5′-CTTGATTCAGGGA-ATGAGCACCACCTCC-3′ (the common primer) for the wild-type allele, and 5′-CAGCAGCCTCTGTTCCACATACACTTCAT-3′ and the common primer for the targeted allele. *Arx*-deficient mice have been described previously (Yoshihara et al., 2005). Mice were housed in an environmentally controlled room in the Animal Facility of the Center for Developmental Biology (CDB), RIKEN, under the guidelines of the CDB for animal experiments.

Nissl and Hematoxylin-Eosin (HE) staining

Brains or embryos were fixed with Carnoy's fixative solution at room temperature overnight. Specimens were dehydrated and embedded in paraffin wax. Serial sections (12 μm) were prepared and stained with 0.1% Cresyl Violet. For HE staining, embryos were fixed overnight with 4% paraformaldehyde, dehydrated, and embedded in paraffin wax. Serial sections (12 μm) were prepared and stained with Mayer's Hematoxylin and 1% Eosin (Muto Pure Chemicals). Pictures of Nissl and HE staining, in situ hybridization and immunohistochemistry were taken using an AxioPlan2 microscope and AxioCam CCD camera (Zeiss). The figures were assembled using Adobe PhotoShop version 7.

RNA probes and in situ hybridization

In situ hybridization was performed as described previously (Hirata et al., 2004). Single-stranded digoxigenin-UTP-labeled (Roche Applied Science) riboprobes were used and signals were detected with an anti-digoxigenin antibody and BM purple substrate (Roche Applied Science) for most of the figures, except Fig. 8I-L. For Fig. 8I-L, NBT and BCIP (Roche Applied Science) were used for staining. The probes used in this study were as follows: reelin (D'Arcangelo et al., 1995), *Dlx1* (Bulfone et al., 1993), *Gad67* (Condie et al., 1997), tyrosine hydroxylase (*Th*, a PCR product), *Slit1* (Yuan et al., 1999) and *Arx* (Miura et al., 1997). A *Fez* riboprobe was generated from *Bam*HI-digested pBLSK *Fez*ISH using T7 RNA polymerase (Promega).

Immunohistochemistry

Immunohistochemistry was performed as described previously (Hirata et al., 2004). Immune complexes were visualized using an ABC kit (Vector) and FAST 3,3′-diaminobenzidine tablet sets (Sigma). For immunofluorescence detection (Fig. 5M,N, Fig. 8A-D), Alex488- and Alex568-conjugated antibodies (Invitrogen/Molecular Probes) were used. The primary antibodies used in this study were anti-NCAM (Chemicon, AB5032), anti-GAP43 (Chemicon, AB5220), anti-OMP (Keller and Margolis, 1975) (a gift from F. L. Margolis), anti-GFP (MBL), anti-reelin (Chemicon, MAB5364), and anti-TBX21 and anti-ARX antibodies (Yoshihara et al., 2005).

BrdU labeling

BrdU labeling was performed as described previously (Hirata et al., 2004). Pregnant female mice were injected intraperitoneally with a single pulse (50 mg/kg body weight) of 5-bromo-2′-deoxyuridine (BrdU) and sacrificed on the indicated day (sacrificed at 2 hours after injection at E14.5 or E16.5 for Fig. 6; injected at E14.5 and sacrificed at E18.5 for Fig. 7A,B). For proliferation analysis, 12-μm serial coronal sections were incubated with monoclonal anti-BrdU antibodies (Pharmingen). The signals were detected using the ABC kit (Vector) and FAST 3,3′-diaminobenzidine tablet sets. In the experiments shown in Fig. 6, sections were counter-stained with Hematoxylin.

Generation and genotyping of transgenic mice

To rescue *Fez* expression in *Fez*-mutant mice, a transgene plasmid was constructed by inserting *Fez* cDNA into the OSN-specific transgene vector between the #123 promoter and the IRES-GapVenus-polyadenylation signal

(#123*p-Fez-IRES-GapVenus*). The #123 promoter drives transgene expression specifically in OSNs from E11 (Y.Y., unpublished). Transgenic mice were generated by injecting the purified *#123p-Fez-IRES-GapVenus* DNA cassette into wild-type C57BL/6 oocytes, which were fertilized with $Fez^{+/-}$ sperm in vitro. Transgene-derived *Fez* expression was monitored by the expression of membrane-tagged Venus (GAP43-Venus), observed under a dissection microscope (Leica FLIII), and detected by immunohistochemistry with anti-GFP antibodies. Genotyping of transgenic mouse lines was carried out by PCR using the primers: 5′-ACTTT-ACACACGAAGGGTCTGG-3′ and 5′-AACGTATTTAGCCGAAAGG-AAT-3′ (200-bp PCR products were generated). Three independent *#123p-Fez-IRES-GapVenus* transgenic lines were established and used in this study.

RESULTS
Expression of *Fez* and the generation of *Fez*-deficient mice

We found that the mouse cDNA deposited in the Mouse Genome Informatics Database (AK014242) displayed a stronger sequence similarity to *Xenopus* and zebrafish *fez* (Matsuo-Takasaki et al., 2000) (M.H., unpublished, for zebrafish *fez*), than to mouse *Fezl* cDNA (Hashimoto et al., 2000) (see Fig. S1 in the supplementary material). We also found orthologs of the *fez* and *fezl* genes in the pufferfish and human genomes (data not shown). These data indicate that the teleost and mammalian genomes contain only two genes belonging to the *fez/fezl* family, and that the mouse cDNA AK014242 is the mouse ortholog of *fez*.

Fez expression was first detected in the prospective forebrain region at E8.0 (Fig. 1A). The expression domain of *Fez* was slightly wider than that of *Fezl* at E8.5 (Hirata et al., 2004). *Fez* was continuously expressed in the forebrain region during early embryogenesis (Fig. 1B-D). At E12.5, *Fez* transcripts were detected in the OE, septum, roof of the telencephalon, amygdala, prethalamus (also called ventral thalamus) and hypothalamus (Fig. 1E-I). *Fez* expression was barely detected in the vomeronasal organs at E12.5 (Fig. 1I), and was detected weakly in the OE, amygdala and hypothalamus at E15.5 (Fig. 1J; data not shown). *Fez* expression was not detected in the OB or in the ganglionic eminences (Fig. 1F,K), where the interneuron progenitors of the OB are generated.

To reveal the role of *Fez* in mouse development, we disrupted the *Fez* gene in embryonic stem cells by replacing the region corresponding to amino acids 1-350 of FEZ with a neomycin-resistance gene transcriptional unit (PGK-NEO) (Fig. 2). The heterozygous ($Fez^{+/-}$) mice appeared normal; the homozygous ($Fez^{-/-}$) mice were born alive but most of them died within one day for unknown reasons. The OBs of $Fez^{-/-}$ mice were smaller than those of their wild-type littermates at E18.5 and at postnatal day 0 (P0; Fig. 3). Nissl staining showed disorganization of the Nissl-positive layer, which corresponds to the MCL at E18.5, in the $Fez^{-/-}$ mice. However, the $Fez^{-/-}$ mice did not show abnormalities in the morphology of the telencephalon or diencephalon at E14.5 or E18.5 (Fig. 3).

Fez is required for the proper termination of olfactory sensory neurons

In wild-type mice, OSNs extended their axons, which stain with anti-NCAM and anti-GAP43 antibodies (Terkelsen et al., 1989; Verhaagen et al., 1989), through the lamina cribrosa, and innervated
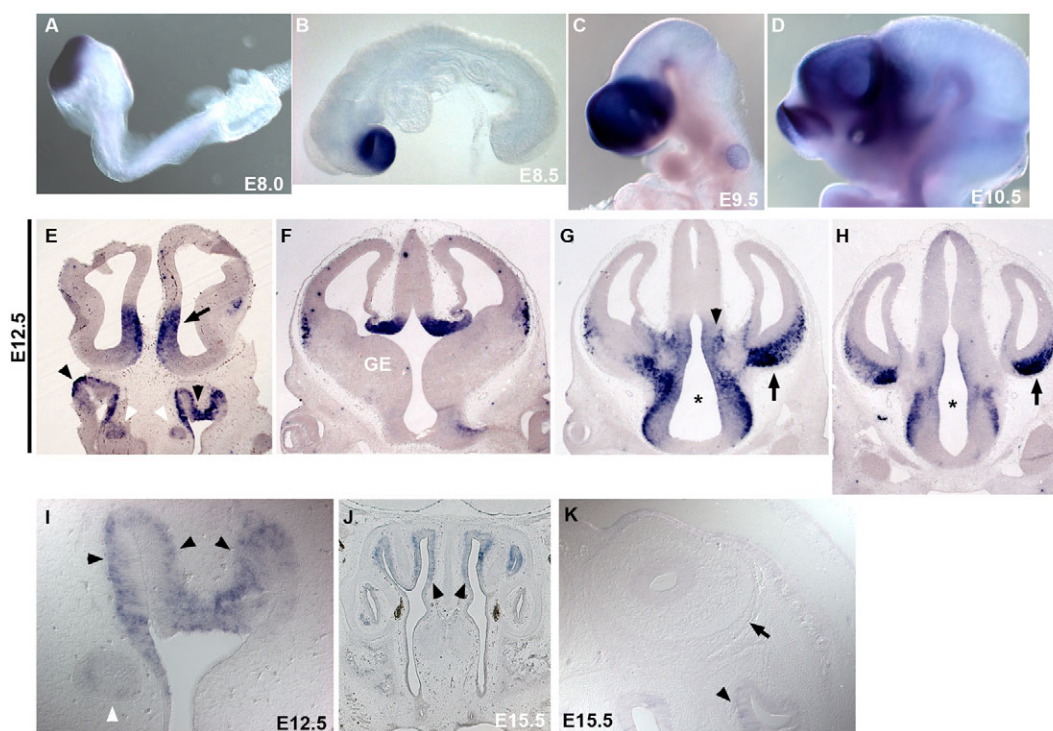

Fig. 1. Expression of *Fez* during mouse development. (**A-K**) Expression of *Fez* at E8.0 (A), E8.5 (B), E9.5 (C), E10.5 (D), E12.5 (E-I) and E15.5 (J,K). (A-D) Whole-mount in situ hybridization, lateral views with anterior to the left. (E-K) In situ hybridization of coronal sections from the anterior (E) to the posterior (H), and coronal sections of the olfactory epithelium (I-K) and the olfactory bulb (K). *Fez* transcripts were detected in the olfactory epithelium (black arrowheads; E,I,J,K), septum (black arrow, E), roof of the telencephalon (F), hypothalamus (asterisks; G,H), prethalamus (black arrowhead, G) and amygdala (black arrows; G,H), but not in the olfactory bulb (black arrows in K). (I) High-magnification view of the olfactory epithelium. *Fez* expression was detected in the olfactory epithelium, but was scarce in the vomeronasal organs (VNO, white arrowheads in E,I).
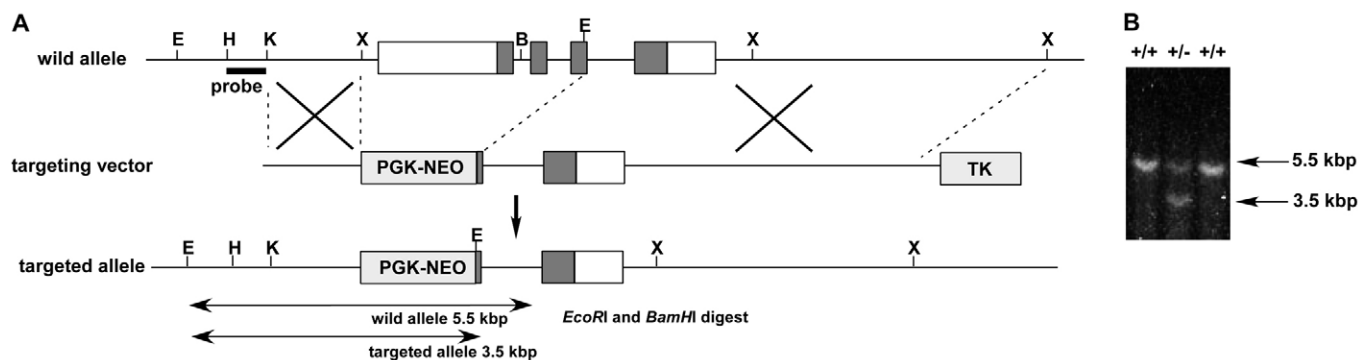
Fig. 2. Generation of *Fez*-deficient mice. (**A**) Schematic diagram of the *Fez* gene locus (wild-type allele), targeting vector and targeted allele. Exons containing the zinc-finger domains are indicated by gray boxes. The targeting vector contains a phosphoglycerate kinase I promoter-linked neomycin-resistance gene (PGK-NEO) in place of part of the coding region (amino acids 1-350), and a thymidine kinase gene (TK) at the end of the 3′ arm. E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; X, *Xba*I; B, *Bam*HI. (**B**) Southern blot analysis of the targeted ES clones. *Eco*RI- and *Bam*HI-digested DNA from wild-type ES clones (+/+) and targeted clones (+/–) was hybridized with the probe shown in A (*Hin*dIII-*Kpn*I fragment). The wild-type and targeted alleles showed 5.5- and 3.5-kb bands, respectively.

the OB at E12.5 (Fig. 4A,C,E). In the *Fez*$^{-/-}$ mice, the NCAM- and GAP43-positive olfactory axons passed through the lamina cribrosa, but they stopped before the OB at E12.5 and never reached it (Fig. 4B,D,F). These data indicate that *Fez* is required for the axons of the OSNs to target the OB. *Fez*$^{-/-}$ mice showed GAP43, NCAM and OMP expression in OSNs that was comparable to that of their wild-type littermates (Fig. 5A-H), indicating that the differentiation of the OSNs was not significantly affected in the *Fez*$^{-/-}$ mice.

Fez is required for the formation of the olfactory bulb layers

We next examined the laminar organization of the OB at E18.5 with various molecular markers. In the outermost layer, ONL, the OB was completely covered by the GAP43- and OMP-positive axons of the OSNs in wild-type mice (Fig. 5A,C). However, the *Fez*$^{-/-}$ mice showed no GAP43- or OMP-positive domains, indicating a complete lack of the ONL. Instead, the axons of the OSNs formed a tangled structure outside of the OB, called a fibrocellular mass (FCM), in *Fez*$^{-/-}$ mice (Fig. 5B,D). Consistently, in *Fez*$^{-/-}$ mice, the expression of S100, which marks the ensheathing cells, was detected not in the outermost layer of the OB but in the FCM (data not shown). Reelin and *Tbx21* are specifically expressed in the mitral cells in the OB (Bulfone et al., 1998; Faedo et al., 2002) (Fig. 5I,K,M). The reelin-positive MCL of *Fez*$^{-/-}$ mice was thicker than that of wild-type mice and was located in the outermost region of the OB (Fig. 5J). In wild-type mice, the expression patterns of the reelin transcripts and proteins indicated the polarity of the mitral cells, whose dendrites were oriented in the apical direction (relative to TBX21, which labels the nuclei; Fig. 5K,M). By contrast, the polarity of the mitral cells was disorganized in *Fez*$^{-/-}$ mice (Fig. 5L,N). In wild-type mice, *Dlx1* is expressed in interneurons (periglomerular cells and granule cells) in the GL and GCL, and in interneuron progenitors in the SVZ (Bulfone et al., 1998) (Fig. 5O), and *Gad67* and *Th* mark GABAergic and dopaminergic neurons in the OB, and are expressed in interneurons in the GL and GCL (Gall et al., 1987; Kosaka et al., 1995) (Fig. 5Q,S). In *Fez*$^{-/-}$ mice, the expression domains of *Dlx1*-, *Gad67*- and *Th*-expressing cells were disorganized, and the GL did not form (Fig. 5P,R,T), indicating that the *Fez*$^{-/-}$ mice had defects in the development of interneurons. The lateral olfactory tracts (LOT) of the OB

projection neurons, a part of which stain with anti-neuropilin1 and lot1 antibodies (Kawakami et al., 1996; Sato et al., 1998; Sugisaki et al., 1996), were not significantly affected in *Fez*$^{-/-}$ mice (data not shown). These data indicate that *Fez* is required for the layer formation of the OB and for the development of interneurons.

Fez is required for the rostral stream migration of interneuron progenitors

Because the development of the OB interneurons was impaired in *Fez*$^{-/-}$ mice, we next examined the proliferation and apoptosis of cells in the SVZ of the OB. We labeled proliferating cells with BrdU (by peritoneal injection into the mother mice) at E14.5 and E16.5 for 2 hours, and detected them with anti-BrdU antibodies. At E14.5, the number of BrdU-positive cells in the SVZ, which correspond to the interneuron progenitor cells, was not significantly different between the wild-type and *Fez*$^{-/-}$ mice (Fig. 6A,B). However, at E16.5, the number of BrdU-positive cells was greatly reduced in the *Fez*$^{-/-}$ mice when compared with the wild-type mice (55.5±7.6%; Fig. 6C,D). We did not detect a significant difference in the apoptosis of cells in the OB between wild-type and *Fez*$^{-/-}$ mice by TUNEL assay (data not shown). When we labeled proliferating cells with BrdU at E14.5 and examined the position of the BrdU-positive cells at 18.5 (Fig. 7A,B), the number of BrdU-positive interneurons and their progenitors in *Fez*$^{-/-}$ mice was comparable to that in their wild-type littermates. However, the *Fez*$^{-/-}$ mice had no BrdU-positive cells in the GL (Fig. 7B), suggesting that the migration of OB interneurons to the GL is impaired.

Next, we investigated the RMS, the route through which the OB interneuron progenitors migrate from the lateral ganglionic eminence to the OB (Lois and Alvarez-Buylla, 1994; Luskin, 1993), by examining *Dlx1* expression. In *Fez*$^{-/-}$ mice, the RMS was wider and shorter at E15.5 and E18.5 than in wild type, and the anterior tip of the RMS was disorganized at E18.5 (Fig. 7C-F). SLIT1 and SLIT2 are repulsive factors that are reported to be involved in formation of the RMS; they are expressed in the choroid plexus and septum (Nguyen-Ba-Charvet et al., 2004; Wu et al., 1999), where *Fez* is expressed. However, the expression of *Slit1* and *Slit2* in these regions in *Fez*$^{-/-}$ mice appeared to be normal (Fig. 7G,H, data not shown for *Slit2*). The data indicate that *Fez* is required for proper rostral migration of the interneuron progenitors, but that SLIT1 and SLIT2 may not be responsible for the abnormal RMS in the *Fez*$^{-/-}$ mice.

The phenotypes of *Fez*$^{-/-}$ mice are similar to those observed in *Arx*-deficient mice (Yoshihara et al., 2005), including the abnormal projection of the OSNs and the abnormal layer formation of the OBs. Although *Fez* and *Arx* are expressed in different regions of the olfactory system, the data suggest that *Fez* and *Arx* function in the same genetic cascade. In an attempt to address this issue, we examined ARX/*Arx* expression in *Fez*$^{-/-}$ mice and *Fez* expression in *Arx*-deficient mice (Fig. 8). We could not, however, detect any significant difference in ARX/*Arx* expression between control and *Fez*$^{-/-}$ mice (Fig. 8A-H), or any difference in *Fez* expression between

control and *Arx*-deficient mice (Fig. 8I-L). The results indicate that *Fez* and *Arx* do not regulate the expression of one another in the formation of the OEs and OBs.

Fez in olfactory sensory neurons plays a role in olfactory axon projection and olfactory bulb formation

Fez was not expressed in OB projection neurons, OB interneurons or interneuron progenitors. Thus, *Fez* in the OSNs might non-cell-autonomously control the morphogenesis of the OB. We examined
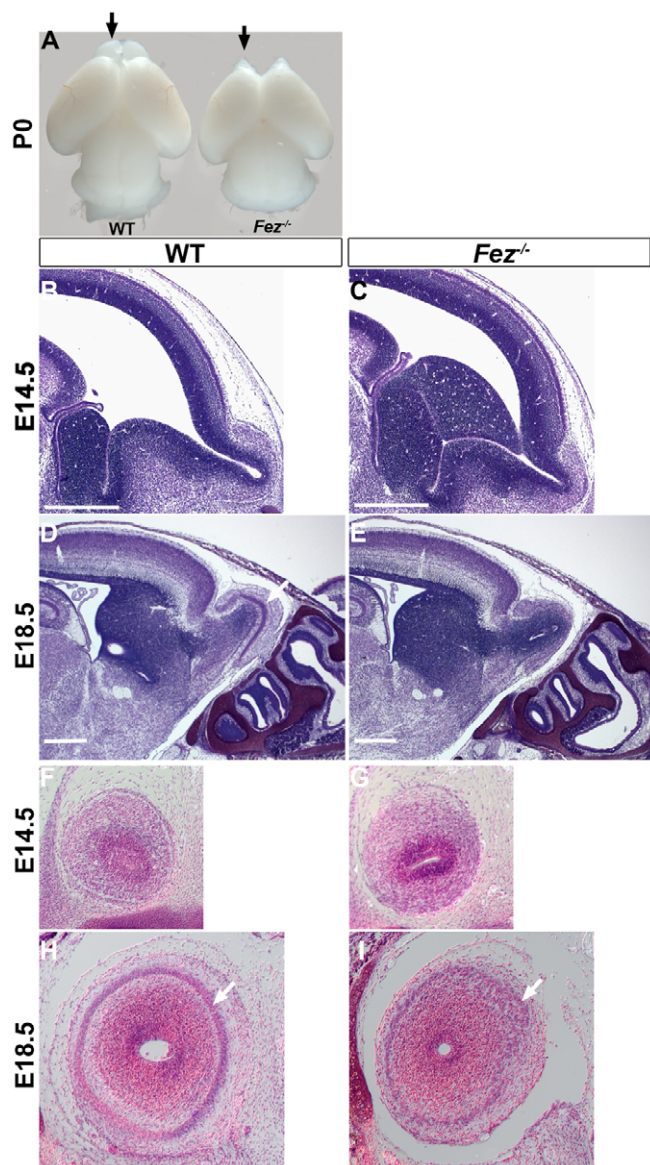

Fig. 3. Abnormal morphology of the olfactory bulb in *Fez*-deficient mice. (**A**) The olfactory bulbs were smaller and farther apart in *Fez*-deficient embryos (right) than in wild-type embryos (WT, left), at P0. (**B-E**) Nissl staining of sagittal sections of the head region of E14.5 (B,C) or E18.5 (D,E) wild-type (B,D) and *Fez*-deficient (C,E) embryos; anterior to the right. Scale bars: 0.5 mm. (**F-I**) Nissl staining of coronal sections of olfactory bulbs from E14.5 (F,G) or E18.5 (H,I) wild-type (F,H) and *Fez*-deficient (G,I) mice; medial to the right. The olfactory bulb was smaller in *Fez*-deficient embryos than in wild-type embryos at E18.5 and P0. An irregular formation of the mitral cell layer (arrows, D,H,I) was detected in *Fez*-deficient embryos at E18.5.
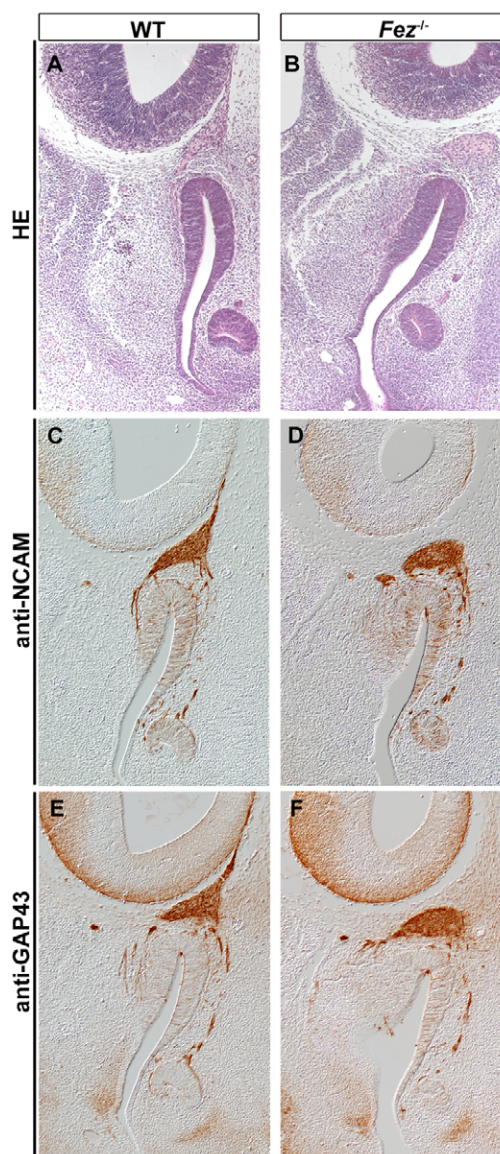

Fig. 4. Defects in the axonal tracts of olfactory sensory neurons in *Fez*-deficient mice. (**A-F**) Coronal sections of the frontal region, including the nasal epithelium and anterior telencephalon, of E12.5 wild-type (WT; A,C,E) and *Fez*-deficient (*Fez*$^{-/-}$; B,D,F) embryos were stained with Hematoxylin and Eosin (HE; A,B), and analyzed by immunohistochemistry with anti-NCAM (C,D) or anti-GAP43 (E,F) antibodies. Immune complexes were visualized by FAST 3,3'-diaminobenzidine (DAB); medial to the right. The axons of OSNs, which express NCAM and GAP43, reached the olfactory bulb in wild-type embryos, but did not extend and generated a fibrocellular mass in *Fez*-deficient embryos.
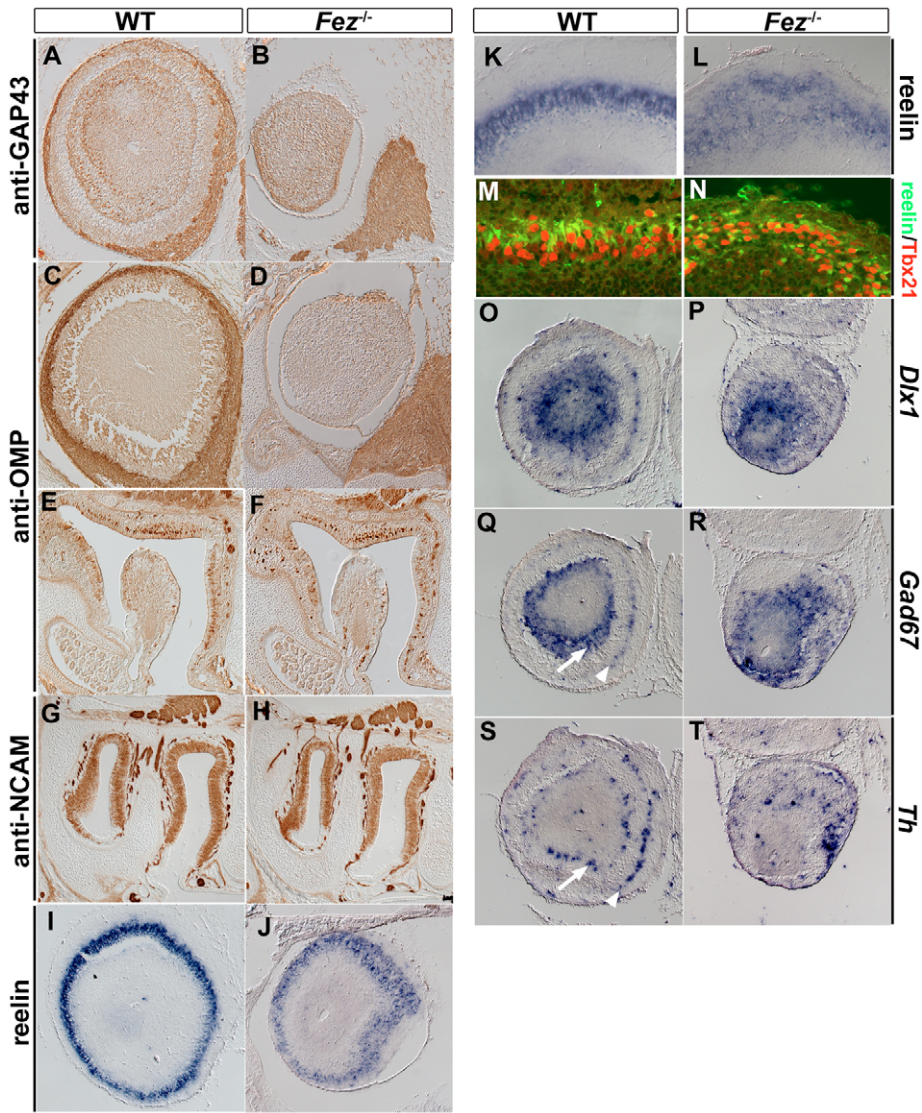
Fig. 5. Abnormal layer formation of the olfactory bulb in *Fez*-deficient mice. (A-T) Coronal sections of the olfactory bulb (A-D,I-T) or olfactory epithelium (E-H) from E18.5 wild-type (WT) and *Fez*-deficient (*Fez*−/−) embryos were analyzed by immunohistochemistry with anti-GAP43, anti-OMP, anti-NCAM, or a combination of anti-reelin and anti-TBX21 antibodies, or by in situ hybridization with reelin, *Dlx1*, *Gad67* and tyrosine hydroxylase (*Th*) probes. Medial is to the right. Immune complexes were visualized with DAB (A-H), or Alexa488 (anti-reelin) and Alexa568 (anti-TBX21, M,N). In situ signals were stained with BM purple substrate. GAP43 and OMP were expressed in OSNs and in the olfactory nerve layer (ONL) in the wild-type bulb. (A-F) GAP43- and OMP-positive OSNs did not extend after the lamina cribrosa and formed the FCM in *Fez*-deficient embryos. The expression of GAP43 and OMP in the nasal epithelium was not affected in *Fez*-deficient embryos. *Fez*-deficient embryos showed an aberrantly wide mitral cell layer, which expresses reelin and TBX21 (K-N), and displayed a reduced number local circuit neurons, which express *Gad67* and *Th* (Q-T), with aberrant positioning. Periglomerular cells and granule cells are marked by arrowheads and arrows, respectively (Q,S). (O,P) Progenitors for local circuit neurons were located in the ventricular/subventricular zone (VZ/SVZ) and expressed *Dlx1*; the number of *Dlx1*-expressing cells was reduced in the olfactory bulb of *Fez*-deficient embryos.
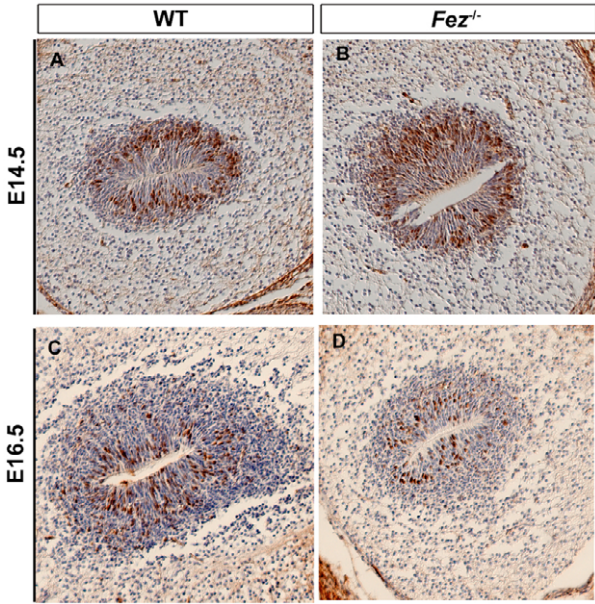

Fig. 6. Reduction of interneurons in the olfactory bulb of *Fez*-deficient mice. (A-D) Neurons born at E14.5 (A,B) or E16.5 (C,D) in wild-type (WT, A,C) and *Fez*-deficient (*Fez*−/−, B,D) embryos were labeled with bromodeoxyuridine (BrdU) for two hours, and the proliferating neuronal cells analyzed the same day. Coronal sections of the olfactory bulb were stained with anti-BrdU antibody and DAB, and counter-stained with Hematoxylin; medial is to the right. The BrdU-positive local-circuit progenitors in the VZ/SVZ of the olfactory bulb appeared normal in *Fez*-deficient embryos. The number of neurons born at E16.5 was reduced in *Fez*-deficient embryos compared with wild type (55.5±7.6%). BrdU-positive cells from five sections were counted (*n*=3).

this possibility by rescuing *Fez* expression in the OE of *Fez*$^{-/-}$ mice. We constructed a *Fez* rescue plasmid, in which *Fez* cDNA was expressed under the #123 gene promoter [*Tg(#123p-Fez)*; Fig. 9, Table 1]. The *#123* gene encodes a novel OSN-specific molecule, and its 3-kb promoter drives the expression of the transgene in the OSNs from as early as E11 (Y.Y., unpublished). We monitored exogenous *Fez* expression with a green fluorescence protein variant, Venus (IRES-GapVenus) (Nagai et al., 2002), and confirmed transgene-mediated expression in the OE but not the OB at E14.5 (Fig. 9J). We generated three different *Tg(#123p-Fez)* transgenic lines that were all heterozygous for the *Fez*-mutant allele [*Fez*$^{+/-}$,*Tg(#123p-Fez)1-3*], and intercrossed them or crossed them with *Fez*$^{+/-}$ mice to generate *Fez*$^{-/-}$ mice that expressed *Fez* only in the OE (Table 1, Fig. 9). We then examined the layer formation of the OB at P0 by immunohistochemistry with anti-

OMP (for the ONL), and by in situ hybridization with reelin (MCL) and *Gad67* (GL and GCL) (Fig. 9A,D,G). As described above, most of the *Fez*$^{-/-}$ mice showed abnormal layer formation of the OB bilaterally (with the exception of a small percentage that showed unilateral abnormalities; Table 1, Fig. 9B,E,H). The transgene-mediated expression of *Fez* in the OSNs of *Fez*$^{-/-}$ mice partly or completely rescued the layer formation of the OB at a
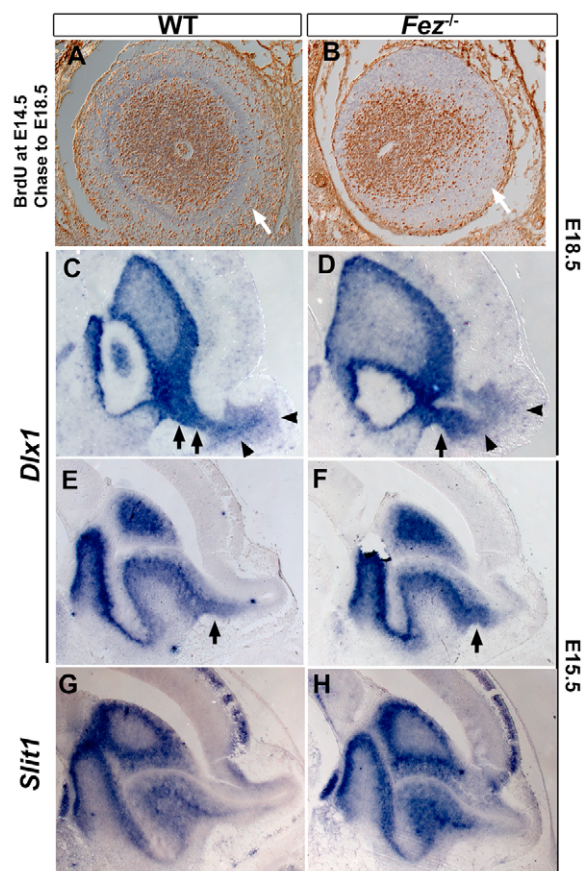

Fig. 7. Defects in the rostral migratory stream (RMS) in *Fez*-deficient mice. (**A**,**B**) Neurons born at E14.5 in control (WT) and *Fez*-deficient (*Fez*$^{-/-}$) embryos were labeled with BrdU and their positions were determined at E18.5; coronal sections, medial to the right. The total number of BrdU-positive neurons in *Fez*-deficient embryos was slightly reduced, and they were not localized to the glomerular layer but were located mainly on the medial side of the granule cell layer. (**C-F**) Expression of *Dlx1* at E18.5 (C,D) and E15.5 (E,F); sagittal sections, with anterior to the right. *Dlx1* is expressed in interneuron progenitors of the OB and marks the rostral migratory stream (RMS, arrows). The RMS from the ganglionic eminence to the olfactory bulb was shorter in *Fez*-deficient embryos than in wild type at E15.5 and E18.5. The distribution of *Dlx1*-expressing cells was affected in the *Fez*$^{-/-}$ olfactory bulb (arrowheads, C,D). (**G**,**H**) The expression of *Slit1* in the septum and ganglionic eminence was not significantly affected in *Fez*-deficient embryos (H), compared with wild type (G).
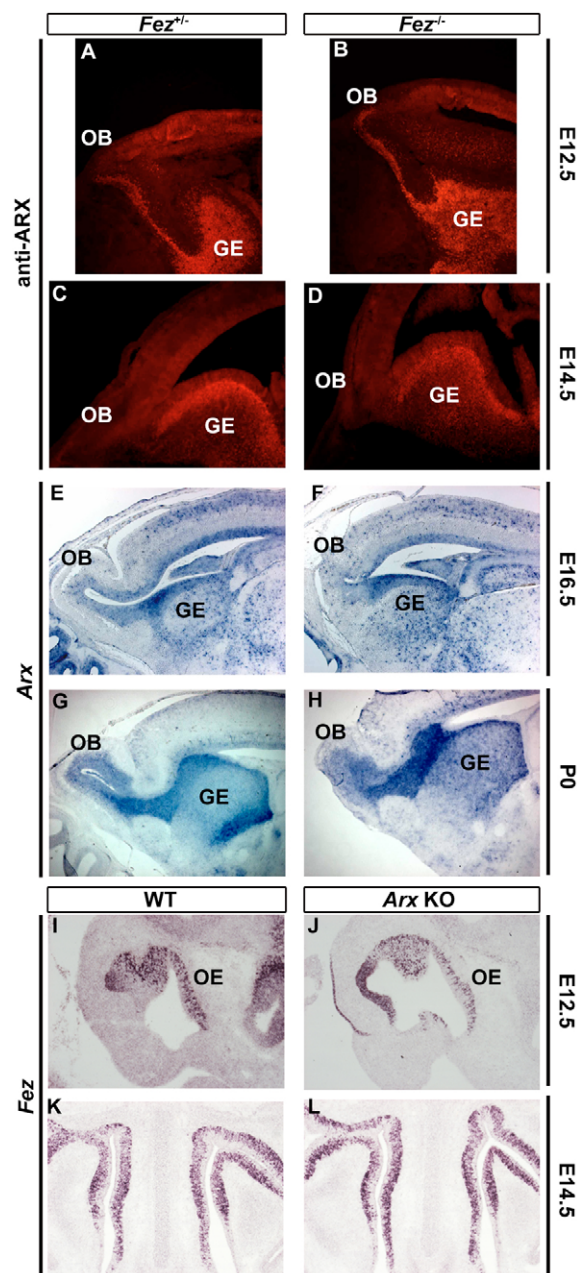

Fig. 8. Expression of *Fez* and *Arx* is mutually independent. (**A-D**) Expression of ARX in *Fez*$^{+/-}$ (A,C) and *Fez*$^{-/-}$ embryos (B,D) at E12.5 (A,B) and E14.5 (C,D) was detected by immunofluorescence staining. ARX proteins are detected in the ganglionic eminence and the RMS to the olfactory bulb (OB). (**E-H**) Expression of *Arx* in *Fez*$^{+/-}$ (E,G) and *Fez*$^{-/-}$ embryos (F,H) at E16.5 (E,F) and P0 (G,H). Expression of ARX and *Arx* was not affected at the developmental periods examined. (**I-L**) Expression of *Fez* in wild-type control (WT, I,K), and *Arx*-deficient (*Arx* KO, J,L) embryos at E12.5 (I,J) and E14.5 (K,L). Expression of *Fez* in the olfactory epithelium (OE) is not affected in *Arx*-deficient embryos.
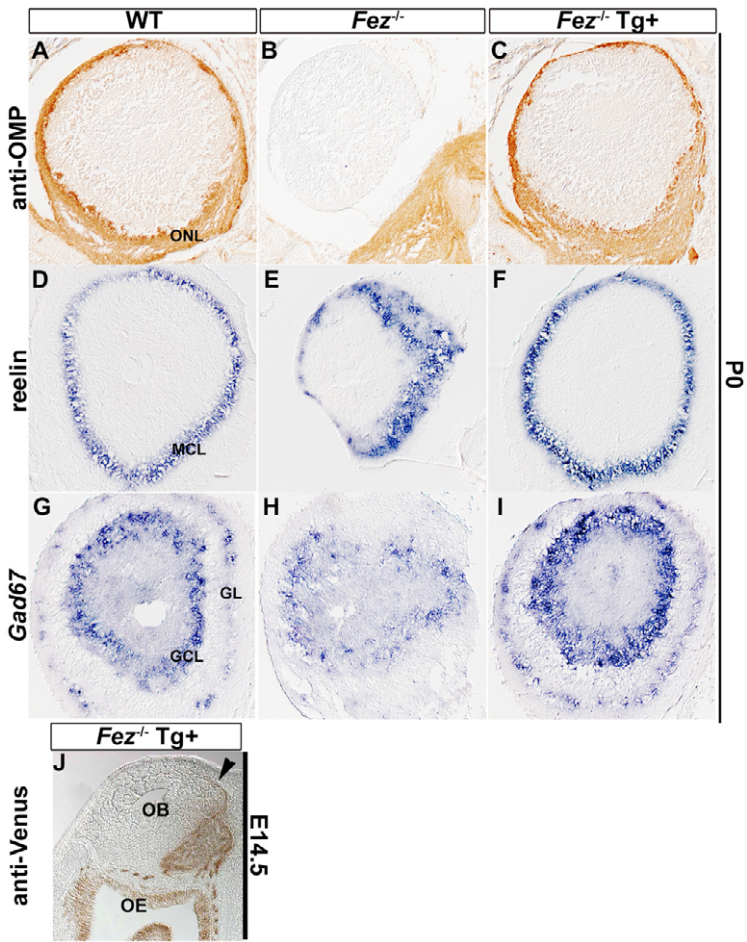
DEVELOPMENT

Fig. 9. *Fez* is required in olfactory sensory neurons for axon targeting and OB layer formation. (A-I) The OMP protein (A-C), and the reelin (D-F) and *Gad67* (G-I) transcripts in control (WT) and *Fez*-deficient (*Fez*$^{-/-}$) mice, and in *Fez*-deficient mice in which *Fez* expression was rescued under the control of the olfactory sensory neuron-specific #123 promoter (*Fez*$^{-/-}$, Tg+), at postnatal day 0 (P0) were detected by immunohistochemistry or in situ hybridization. *Fez*$^{+/-}$ mice containing a transgene composed of #123 promoter-linked *Fez-IRES GapVenus* expression units [*Fez*$^{+/-}$,*Tg(#123p-Fez)*] were crossed with *Fez*$^{+/-}$ mice to generate control mice (WT), *Fez*-deficient mice (*Fez*$^{-/-}$) and rescued *Fez*-deficient mice (*Fez*$^{-/-}$, Tg+). In some of the rescued *Fez*-deficient embryos, formation of the ONL (OMP-positive, C), the mitral/tufted cell layer (MCL, reelin-positive, F), and the layer of local circuit neurons (glomerular layer, GL; granule cell layer, GCL) was rescued in one or both bulbs. Three different transgenic alleles were used. The numbers of rescued and non-rescued embryos are shown in Table 1. (J) Transgene-mediated *Fez* expression in the olfactory epithelium (OE), but not the olfactory bulbs (OB), of *Fez*$^{-/-}$ mice partially rescued axon projection (arrowhead) of OSNs at E14.5. The OSNs expressing the transgene were monitored by immunohistochemistry with an anti-GFP (anti-Venus) antibody.

relatively high penetrance (Table 1). In some of the rescued mutant embryos, the axons of the OSNs innervated the OB, and layer formation was indistinguishable from that seen in wild-type mice (Fig. 9C,F,I), indicating that the expression of *Fez* in the OE was able to suppress the *Fez*-mutant phenotypes associated with OSN and OB development. The data suggest that *Fez* expression is required in OSNs to cell-autonomously regulate OSN axon projections and non-cell-autonomously control the layer formation of the OB.

DISCUSSION
Fez is involved in the axonal projection of olfactory sensory neurons
Here, we have shown that *Fez* is required for the axon termination of OSNs. In normal development, the olfactory axons exit the OE, coalesce to form fascicles, and grow toward the OB. Upon reaching the OB, the olfactory axons defasciculate, sort into smaller subsets, and then make synaptic connections in the target glomeruli with the dendrites of the OB projection neurons. In *Fez*$^{-/-}$ mice, OMP and

Table 1. Rescue of OB formation by *Fez* expression in OSNs

Genotype of parents	Genotype	Both bulbs normal	Partial rescue‡	One bulb normal†	Abnormal bulbs*
Fez$^{+/-}$×*Fez*$^{+/-}$	–/–	0	0	3	>100
Fez$^{+/-}$,*Tg(#123p-Fez)1*×*Fez*$^{+/-}$	–/–	0	0	0	4
	–/–, Tg+	0	2	1	2
Fez$^{+/-}$,*Tg(#123p-Fez)2*×*Fez*$^{+/-}$,*Tg(#123p-Fez)2*	–/–	0	0	0	1
	–/–, Tg+	1	0	0	0
Fez$^{+/-}$,*Tg(#123p-Fez)2*×*Fez*$^{+/-}$	–/–	0	0	0	1
	–/–, Tg+	1	0	0	1
Fez$^{+/-}$,*Tg(#123p-Fez)3*×*Fez*$^{+/-}$	–/–, Tg+	1	0	0	0

Fez$^{+/-}$ mice and *Fez*$^{+/-}$ transgenic mice containing a transgene composed of olfactory-sensory-specific #123 promoter-linked *Fez* expression units [*Fez*$^{+/-}$,*Tg(#123p-Fez)*] were crossed as indicated, and mice with the indicated genotypes were obtained. Three different transgenic alleles [*Tg(#123p-Fez)1, Tg(#123p-:Fez)2*, etc.] were used. Mice were sacrificed on postnatal day one (P0) and formation of the olfactory bulb was examined by morphological inspection, immunostaining and in situ hybridization, as described in Fig. 9.
*Mice that showed typical *Fez*$^{-/-}$ phenotypes in the olfactory bulbs.
†Mice in which one bulb, but not both, showed normal layer formation of the ONL, MCL, and GL+GCL.
‡Mice in which the layer formation was rescued in part(s) of both bulbs.

NCAM expression was observed in OSNs, and the olfactory axons formed fascicles and grew through the lamina cribrosa, but their axons did not reach the OB and, instead, formed a tangled structure, an FCM (Fig. 5). These data suggest that the differentiation of OSNs was not significantly affected, but that axonal projection was impaired in $Fez^{-/-}$ mice. The expression of *Fez* in the OSNs of $Fez^{-/-}$ mice rescued the innervation of the OB by OSN axons (Fig. 9, Table 1), which strongly suggests that *Fez* controls OSN axon projection cell-autonomously.

Several molecules have been shown to be involved in the axon sorting and targeting of OSNs. They include odorant receptors, semaphorin 3A/neuropilin 1, ephrin As and Slit/Robo. Odorant receptors, neuropilin 1, ephrin As and Robos are expressed in OSNs (Cutforth et al., 2003; Feinstein et al., 2004; Feinstein and Mombaerts, 2004; Miyasaka et al., 2005; Mombaerts et al., 1996; Schwarting et al., 2000; Taniguchi et al., 2003; Wang et al., 1998; Yuan et al., 1999), and the expression of the genes encoding these molecules may be controlled by *Fez*. However, FEZ contains an engrailed homology 1 (EH1) repressor motif (see Fig. S1 in the supplementary material), which interacts with the Groucho/TLE-family transcriptional co-repressors (Muhr et al., 2001), and FEZL, the homolog of FEZ, has been shown to function as a transcriptional repressor (Levkowitz et al., 2003). Therefore, regulation of the genes involved in the axonal termination by FEZ is likely to be indirect and may be mediated by another transcriptional regulator. Intriguingly, the axon termination phenotype is similar to that observed in *Arx*-deficient embryos (Yoshihara et al., 2005). *Arx* is expressed in interneurons and radial glia in the OB, but not in the OE, and *Arx* is proposed to regulate the expression of putative instructive signals for the proper innervation of OSN axons (Yoshihara et al., 2005). We have found that the expression of *Fez* and *Arx* was mutually independent (Fig. 8), indicating that the phenotypes of *Fez* and *Arx*-deficient mice are not due to the reduced expression of *Arx* and *Fez*, respectively. It is tempting to speculate that *Arx* and *Fez* coordinately control the expression of guidance molecules and their receptors. Future studies isolating the genes that function downstream of FEZ and ARX will clarify the mechanisms underlying the bidirectional interaction between the developing OE and OB.

In $Fez^{-/-}$ mice, axonal growth of OSNs was perturbed after the axons passed through the lamina cribrosa, which is located on the boundary between the OE and OB. The responsiveness of axonal growth cones is known to be controlled spatially and temporary by intrinsic factors (Tessier-Lavigne and Goodman, 1996). In zebrafish, inhibition of PKA in the OSNs affects axonal growth in the olfactory placodes, whereas the activation of PKA affects it only in the OB, suggesting that PKA activity in OSNs is differently controlled in the OE and OB, and that the regulation of PKA may confer changes in the responsiveness of OSNs to guidance molecules (Yoshida et al., 2002). Such a change in the responsiveness to guidance molecules may play a role in the long-distance axon projection through the intermediate checkpoints. It is an intriguing possibility that *Fez* cell-autonomously controls the expression of molecules that are involved in the responsiveness to guidance molecules.

Non-cell-autonomous roles for *Fez* in OB layer formation and interneuron development

In addition to the defects in the axonal contribution to the OB layers (i.e. lack of the ONL), we also found abnormal layer formation of the OB, and abnormal development and migration of the interneurons and their progenitors in $Fez^{-/-}$ mice. There are several reports showing a role for the olfactory placode and/or OE in the formation of the OB. Removal of the olfactory placode in *Xenopus*

influences the development of the OB, for example, by reducing the number of OB projection neurons (Byrd and Burd, 1993; Graziadei and Monti-Graziadei, 1992). In rats, the arrival of pioneer olfactory axons to the OB is linked to cell-cycle control of the OB primordium (Gong and Shipley, 1995). Here, we have demonstrated that *Fez* expressed in the OSNs non-cell-autonomously controls OB layer formation and the development of interneurons.

Among cells in the olfactory system, at E12.5 we detected *Fez* expression in only the OSNs, and not in the other cell types that contribute to the formation of the OB. *Fez* was not expressed in the ganglionic eminence, where the interneuron progenitors are generated, supporting a non-cell-autonomous role for *Fez* in the formation of the OB layers, and in the migration and development of the interneurons and their progenitors. However, we cannot completely exclude the possibility that *Fez* is transiently expressed in the precursors of the OB projection neurons and interneuron progenitors, as *Fez* is globally expressed in the forebrain at E8.5-E9.5. To address this issue, Cre-loxP-mediated cell-fate mapping of the *Fez*-expressing cells (Zinyk et al., 1998) will be required. However, when evagination of the OB from the telencephalon and migration of the interneuron progenitors take place, *Fez* is not expressed in the projection neurons, the interneurons or interneuron progenitors. More importantly, transgenic *Fez* expression in the OSNs of $Fez^{-/-}$ mice rescued the formation of all of the OB layers. Taken together, these data indicate that *Fez* expressed in the OSNs non-cell-autonomously controls the formation of the OB. Our data provide genetic evidence that signals from the OSNs are required for the proper formation of the OB.

In addition to the OSNs, *Fez* was expressed in the ventrolateral pallium, septum, roof of the telencephalon, hypothalamus and prethalamus. We did not find significant abnormalities in the development of these regions in the forebrain, but it is possible that their functions contribute to OB layer formation and the migration/development of interneurons. *Slit1* and *Slit2* are expressed in the septum and are reported to be involved in migration of interneuron progenitors through the RMS (Nguyen-Ba-Charvet et al., 2004). However, the expression of *Slit1* and *Slit2* was not affected in $Fez^{-/-}$ mice (Fig. 7, data not shown), suggesting that SLIT1 and SLIT2 are not responsible for the abnormal RMS formation in these mice. We do not rule out the possibility that *Fez* expressed in regions other than the OE functions redundantly with *Fezl* in the formation of the OB. *Fezl* is expressed in overlapping regions of the telencephalon and hypothalamus (Hirata et al., 2004), and mice deficient in both the *Fez* and *Fezl* genes showed a much greater size reduction of the OB (T.H., M.N., O. Muraoka, R. Nakayama, Y. Suda and M.H., unpublished) than do *Fez*-deficient mice [*Fezl*-deficient mice do not show abnormalities in OB development (Hirata et al., 2004)]. These data suggest that *Fez* has functions other than in the OE for the development of the OB. A study involving region-specific disruption of the *Fez* and *Fezl* genes in the forebrain will clarify this issue.

Functional interactions between OSNs and the OB

How do signals from OSNs control OB layer formation and interneuron development? The abnormal OB layer formation and RMS phenotypes in $Fez^{-/-}$ mice were similar to those observed in *Dlx5*- and *Arx*-deficient mice; these mutants also show absence of the ONL, a disorganized MCL, a reduction in the number of interneurons, and aberrant RMS (Levi et al., 2003; Long et al., 2003; Yoshihara et al., 2005). Both *Dlx5* and *Arx* are expressed in interneurons and their progenitors, and they cell-autonomously control the RMS and interneuron development, and non-cell-

autonomously control MCL formation (Levi et al., 2003; Long et al., 2003; Yoshihara et al., 2005). Therefore, the abnormal development of the MCL can be explained, at least in part, by the abnormal migration of the interneuron progenitors from the lateral ganglionic eminence. Because axons of the OSNs do not directly contact the interneuron progenitors at the beginning of OB development, the axon terminals of the OSNs could provide diffusible guidance molecules that control the RMS and the migration of interneurons from the SVZ to the GL in the OB. Alternatively, the absence of olfactory sensory input into the OB could disrupt the formation of glomeruli and lead to the disorganized layer formation of the OB projection neurons (MCL), which may secondarily affect the development of the interneurons. *Tbr1*-mutant mice, which lack projection neurons, show aberrant layer formation of the GL and GCL, and reduced size of the OB (Bulfone et al., 1998), implying a role for projection neurons in the development of interneurons. The abnormal layer formation of the projection neurons may contribute to the impaired development of the interneurons. It is less likely, but also possible, that the tangled structure of the olfactory sensory axons in *Fez*$^{-/-}$ mice places a physical pressure on the OB primordium, which interferes with the formation of the RMS and the OB layer. We favor a hypothesis combining the first two possibilities: that signals from OSNs independently control the development of the OB projection neurons and interneurons/interneuron progenitors, and that these two cell populations functionally interact with each other later to form the correct layers in the OB.

In summary, we found that *Fez* is an essential factor that cell-autonomously controls the axonal projection of the OSNs, and non-cell-autonomously regulates layer formation of the OB and the development of the interneurons. Our data provide evidence that signals from the OSNs control OB development.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/8/1433/DC1

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