Cellular/Molecular

Hierarchical Regulation of Odorant Receptor Gene Choice and Subsequent Axonal Projection of Olfactory Sensory Neurons in Zebrafish

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In both *Drosophila* and mice, olfactory sensory neurons (OSNs) expressing a given odorant receptor (OR) project axons to specific glomeruli in the antennal lobe or olfactory bulb (OB), developing a topographic odor map. To gain insights into the modes of OR expression and axonal projection in zebrafish, we generated a bacterial artificial chromosome transgenic line carrying an OR gene cluster in which two OR-coding sequences, *OR111-7* and *OR103-1*, were replaced with yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP), respectively. In the transgenic embryos, YFP and CFP signals appear in small populations of OSNs at an early stage of development when OR expression is first observed. Time-lapse imaging of living embryos revealed that both YFP- and CFP-expressing OSNs project axons to the medial portion of the OB. This pattern of axonal projection is maintained in the adult transgenic fish, in which fluorescently labeled OSN axons target a topographically fixed cluster of glomeruli in the medial OB. Because the OR-coding sequences were replaced with fluorescent reporter genes, we examined which OR genes are expressed in YFP/CFP-expressing OSNs and found that the OR choice is mostly restricted to OR members within the same subfamily of the cluster. Furthermore, we found that the one receptor–one neuron rule is not always applicable to zebrafish OSNs and that multiple receptors–one neuron is true for a subpopulation of OSNs in both wild-type and transgenic fish. These data demonstrate two distinct modes of OR expression and suggest a model of the hierarchical regulation of OR gene choice and subsequent axonal projection in the zebrafish olfactory system.

Key words: olfactory bulb; glomerulus; odor map; axon guidance; teleost olfactory system; fluorescent protein

Introduction

Individual olfactory sensory neurons (OSNs) in mice express only one conventional odorant receptor (OR) gene of a repertoire of ~1300 possibilities (Chess et al., 1994; Malnic et al., 1999), which defines the functional identity of each OSN. OSNs expressing a given OR converge their axons onto a few defined glomeruli within the olfactory bulb (OB) (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996), providing a topographic odor map in the brain. In addition, the OR protein is responsible for axonal guidance of OSNs and the selective expression of OR genes (Wang et al., 1998; Serizawa et al., 2003; Feinstein and Mombaerts, 2004; Lewcock and Reed, 2004; Shykind et al., 2004). Thus, the choice of one functional OR in an OSN and its axonal projection to a specific glomerulus are fundamental bases for the organization and functions of the olfactory system.

Zebrafish provide an attractive vertebrate model system in which to investigate molecular and cellular mechanisms underlying the development and functions of the olfactory system. The zebrafish olfactory system contains molecular constituents, cell types, and overall organization that are all very similar to those of mice. Functional imaging of OB glomeruli in zebrafish demonstrated that different odorants elicit defined patterns of glomerular activity (Friedrich and Korsching, 1997, 1998), indicating the existence of a topographic odor map also in fish. Besides these organizational and functional similarities, the numbers of OR genes and OB glomeruli in zebrafish are smaller than those in mice by an order of magnitude (Baier and Korsching, 1994; Friedrich and Korsching, 1997; Alioto and Ngai, 2005; Niimura and Nei, 2005), enabling us to reduce the complexity of questions on OR choice and glomerular targeting mechanisms. The fish olfactory epithelium (OE) contains two major types of OSNs, ciliated and microvillous neurons. These two types of OSNs express distinct types of chemosensory receptors, project their axons to different regions of the OB, and probably mediate different types of behaviors (Cao et al., 1998; Morita and Finger, 1998; Speca et al., 1999; Hamdani et al., 2001; Hamdani and Døving, 2002; Hansen et al., 2003, 2004; Weltzien et al., 2003; Sato et al., 2005). *In situ* hybridization analyses revealed that individual ORs are typically expressed in a small population of OSNs (Ngai et al., 1993; Barth et al., 1997; Speca et al., 1999; Sato et al., 2005).

However, the modes of OR choice and axonal projection of OSNs expressing a given OR are not well understood in fish.

Here, we generated a bacterial artificial chromosome (BAC) transgenic zebrafish line carrying an OR gene cluster in which two OR-coding sequences, *OR111-7* and *OR103-1*, were replaced with yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP), respectively. In the transgenic fish, fluorescently labeled OSNs expressed limited members of OR genes and projected their axons to spatially invariant glomeruli within the OB. These observations suggest a model of the hierarchical regulation of OR gene choice and subsequent axonal projection in the zebrafish olfactory system.

Materials and Methods

Animals. Zebrafish, *Danio rerio*, were maintained in our laboratory as described previously (Westerfield, 1995). Embryos were incubated at 28.5°C and staged according to hours postfertilization and morphological criteria (Kimmel et al., 1995).

BAC transgene construction. The BAC Zebra Fish Library (Genome Systems, St. Louis, MO) was screened by PCR to obtain a clone containing the zebrafish OR gene cluster previously characterized (Barth et al., 1997; Dugas and Ngai, 2001; Alioto and Ngai, 2005). The 100G14 BAC clone was used to construct a BAC–YC transgene by two steps of targeted BAC modifications as follows.

The first BAC modification was the replacement of the *OR111-7* coding sequence with *gap–YFP* cDNA. A couple of homology arms containing 0.8 kb fragments of 5′ and 3′ flanking regions of the *OR111-7* coding sequence were amplified from the BAC DNA by PCR and subcloned. The *gap–YFP* cDNA (pEYFP-Mem; BD Biosciences Clontech, Palo Alto, CA) was flanked by the homology arms and inserted into the *Sal*I site of pKOV–KanF vector (Lalioti and Heath, 2001) to create a shuttle vector. Using the shuttle vector and the pDF25–Tet plasmid, homologous recombination of BAC DNA was performed as described previously (Lalioti and Heath, 2001) to generate a modified BAC clone BAC[*OR111-7:gap–YFP*].

The second modification was the replacement of the *OR103-1* coding sequence with *gap–CFP* cDNA. For a shuttle vector, the *gap–CFP* cDNA (pECFP-Mem; BD Biosciences Clontech) was flanked by a couple of 0.5 kb homology arms for the *OR103-1* coding sequence and inserted into the *Sal*I site of pSV1.*RecA* vector (Yang et al., 1997). Targeted BAC modification was performed with the clone BAC[*OR111-7:gap–YFP*] as described previously (Yang et al., 1997) to obtain a construct BAC[*OR111-7:gap–YFP/OR103-1:gap–CFP*] (abbreviated as BAC–YC construct), in which the coding sequences of *OR111-7* and *OR103-1* were replaced with *gap–YFP* and *gap–CFP*, respectively.

All of the PCR fragments used in the construction were subjected to DNA sequencing, and several modified BAC clones isolated in individual steps of the modification were screened by Southern blot analysis.

Generation of transgenic zebrafish. The modified BAC transgene was purified using QIAfilter Plasmid Maxi kit (Qiagen, Hilden, Germany) and diluted to 120 ng/μl in distilled water containing 0.1% phenol red. The DNA solution was injected into cytoplasm of one-cell stage embryos, and the injected embryos with fluorescence in OSNs were raised to sexual maturity. Germ line-transmitting founders were screened by PCR with genomic DNA extracted from their progeny using a pair of primers for the green fluorescent protein (*GFP*) gene. One line of transgenic fish was generated and termed Tg (BAC[*OR111-7:gap–YFP/OR103-1:gap–CFP*])[rw039] (abbreviated as BAC–YC line).

The transgenic zebrafish lines Tg(OMP[2k]:gap–YFP)[rw032a] (OMP: YFP) and Tg(OMP[2k]:lyn–mRFP)[rw035a] (OMP:RFP) were described previously (Miyasaka et al., 2005; Sato et al., 2005). All of the transgenic zebrafish lines including Tg (BAC[*OR111-7:gap–YFP/OR103-1:gap–CFP*])[rw039a] can be obtained from us under the support of Zebrafish National BioResource Project of Japan (www.shigen.nig.ac.jp/zebra/index_en.html).

Time-lapse imaging of OSN projection in zebrafish embryos. Time-lapse imaging of OSN projection in zebrafish embryos was performed as described previously (Sato et al., 2005).

Immunohistochemistry. Seven- to 13-month-old adult fish (~3.5 cm body length) were anesthetized with 0.016% ethyl-*m*-aminobenzoate methanesulphonate (tricaine) (Nacalai Tesque, Kyoto, Japan), and tissues were dissected out.

For immunostaining of OE sections, cryostat slices were prepared as described previously (Sato et al., 2005). OE sections (14 μm thickness) from adult BAC–YC fish were incubated with rat anti-GFP monoclonal antibody (GF090R, rat IgG2a, 1:1000; Nacalai Tesque) and then incubated with biotin-conjugated goat anti-rat IgG (1:500; Jackson ImmunoResearch, West Grove, PA), followed by staining with a Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA). Signals were visualized with the Ni^{2+}-intensified diaminobenzidine (DAB)/peroxide reaction. OE sections (10 μm thickness) from adult BAC–YC;OMP:RFP double-transgenic fish were incubated with rat anti-GFP monoclonal antibody (1:1000; Nacalai Tesque) and subsequently incubated with AlexaFluor488-conjugated goat anti-rat IgG (1:300; Invitrogen, Carlsbad, CA). Fluorescent signals of AlexaFluor488 and red fluorescent protein (RFP) were imaged by a confocal laser scanning microscope (FV500; Olympus Optical, Tokyo, Japan).

Whole-mount immunohistochemistry was performed essentially as described previously (Macdonald, 1999; Miyasaka et al., 2005) with some modifications. Briefly, telencephalic hemispheres together with olfactory epithelia were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. After permeabilization with acetone, tissues were incubated with 0.3% H_2O_2 to quench endogenous peroxidase activities, blocked with 10% normal goat serum in PBS containing 1% dimethylsulfoxide and 0.1% Tween-20 (PBDT), and incubated overnight at 4°C with rabbit polyclonal anti-GFP antibody (1:500; a kind gift from Dr. N. Tamamaki, Kumamoto University, Kumamoto, Japan) diluted in PBDT. The tissues were then incubated with biotin-conjugated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch), followed by staining with a Vectastain ABC elite kit. Signals were visualized with the DAB/peroxide reaction. The tissues were postfixed with 4% PFA overnight at 4°C, washed with PBS, and examined with a light microscope (MVX10; Olympus Optical). Images at 10 μm steps were acquired and stacked for composition of in-focus images using Dynamic Eye software (Mitani, Fukui, Japan).

Immunostaining of OB sections from adult BAC–YC;OMP:RFP double-transgenic fish was performed as described previously (Sato et al., 2005).

Analysis of OR gene expression. DNA fragments of zebrafish OR genes were amplified by PCR from the zebrafish genomic DNA and the 100G14 BAC DNA using specific primers for each OR gene. PCR primers were designed on the basis of predicted coding sequences for zebrafish OR genes (Niimura and Nei, 2005) and the DNA sequence of the 100G14 BAC clone (supplemental Table S1, available at www.jneurosci.org as supplemental material). PCR products containing full or partial length of OR-coding sequences were subcloned into pGEM-T Easy vector (Promega, Madison, WI) and used as templates for cRNA probes. *In situ* hybridization was performed essentially as described previously (Inaki et al., 2004; Sato et al., 2005).

For analysis of coexpression of the GFP variants with OR genes in adult BAC–YC fish, OE sections (10–14 μm thickness) were subjected to fluorescent *in situ* hybridization using 2-hydroxy-3-naphtoic acid-2-phenylanilide phosphate (HNPP)/Fast Red (Roche, Mannheim, Germany) and subsequently immunostained with rabbit anti-GFP polyclonal antibody (1:1000; BD Biosciences Clontech). At least 50 GFP-immunoreactive OSNs derived from several individuals were examined for each OR probe, and the percentages of the OR-positive cells among GFP-immunoreactive cells were calculated.

For double-color *in situ* hybridization, cRNA probes were labeled with digoxigenin or fluorescein and used for hybridization to OE sections (10 μm thickness). After hybridization, sections were washed, treated with blocking reagent (Roche), and incubated overnight at room temperature (RT) with a mixture of alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1000; Roche) and horseradish peroxidase-conjugated anti-fluorescein antibody (1:100; Roche). After washing in 100 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20 (TNT), the sections were incubated with biotinyl tyramide (1:50, TSA Biotin System; PerkinElmer, Wellesley, MA) at RT for 10 min, washed in

TNT, and incubated with streptavidin-conjugated AlexaFluor488 (1:300; Invitrogen) at RT for 30 min. For detection of alkaline phosphatase, the sections were incubated with HNPP/Fast Red (Roche).

To estimate the numbers of cells hybridizing to individual OR probes in an entire olfactory rosette, OE sections (10 μm thickness) from adult wild-type fish were examined by either single or double *in situ* hybridization with digoxigenin- and/or fluorescein-labeled cRNA probes. Detection was accomplished via the use of alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1000; Roche) followed by HNPP/Fast Red (Roche) or the use of peroxidase-conjugated anti-digoxigenin (1:100; Roche) or anti-fluorescein (1:100; Roche) antibody followed by biotinyl tyramide (PerkinElmer) and streptavidin-conjugated AlexaFluor488 (1:300; Invitrogen). For each OR probe, every four slices from serial OE sections (10 μm thickness) were hybridized, and the total number of OR probe-positive cells was counted. Different individual rosettes (*n* = 3–7) were examined for each OR probe.

Nomenclature and genomic distribution of zebrafish OR genes. The organization of an OR gene cluster on chromosome 15 was based on the array of OR genes that was described previously (Dugas and Ngai, 2001). The new nomenclature for zebrafish ORs (Alioto and Ngai, 2005) was adopted in this manuscript. The OR nomenclature equivalents were provided in supplemental Table S1 (available at www.jneurosci.org as supplemental material). Chromosomal localization of individual ORs followed the data from Niimura and Nei (2005).

Results

Construction of a BAC OR transgene tagged with two fluorescent reporters

Among ~150 members of the zebrafish OR gene family, a total of 22 functional OR genes belonging to five subfamilies (*OR102*, *OR103*, *OR111*, *OR107*, and *OR119*) are clustered within a 140 kb region on the zebrafish chromosome 15 (Dugas and Ngai, 2001; Alioto and Ngai, 2005) (Fig. 1*A*). We screened a zebrafish BAC library and isolated a clone containing an array of 16 functional OR genes belonging to *OR102*, *OR103*, and *OR111* subfamilies within a 95 kb stretch of genomic DNA (Fig. 1*B*). To visualize functional subsets of OSNs with distinct fluorescent proteins, the BAC clone was modified by replacement of the coding sequences of *OR111-7* and *OR103-1* with membrane-targeted fluorescent reporters, *gap–YFP* and *gap–CFP*, respectively (Fig. 1*C*), and used to generate a transgenic zebrafish line BAC–YC (*OR111-7:YFP/OR103-1:CFP*). *OR111-7* and *OR103-1* were chosen for replacement because of early onsets of expression during development and relatively large numbers of OSNs expressing these two ORs (Barth et al., 1996, 1997). We have shown previously that *OR111-7* and *OR103-1* are expressed in a subset of ciliated OSNs (Sato et al., 2005).

BAC transgene expression in small populations of OSNs during development

Zebrafish is an excellent model organism for studies in developmental neurobiology because of its external fertilization, rapid development, and transparency of embryos. These advantageous properties in combination with transgenic expression of fluorescent proteins enable us to perform *in vivo* time-lapse observation of axon growth dynamics in living zebrafish embryos. We established a transgenic zebrafish line, OMP:RFP, in which membrane-targeted RFP is expressed in almost all ciliated OSNs under the control of zebrafish olfactory marker protein (OMP) promoter (Sato et al., 2005). To examine the expression patterns
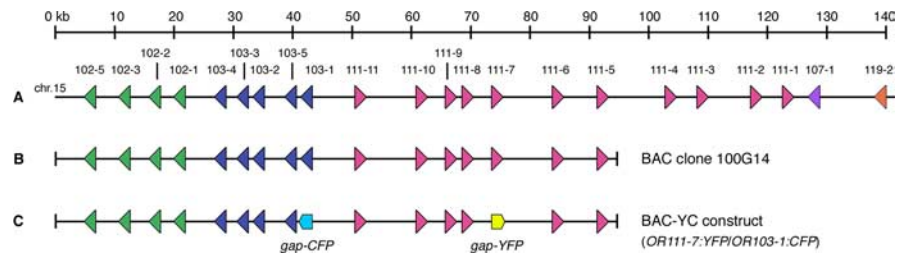

Figure 1. Organization of a zebrafish OR gene cluster and a BAC transgene construct. *A*, An OR gene cluster located on the zebrafish chromosome 15 contains 22 functional OR genes within a 140 kb region. The positions and transcriptional orientations of OR genes are indicated by arrowheads. Members belonging to individual gene subfamilies are shown in the same colors. *B*, A relative location of the insert contained in the BAC clone 100G14. *C*, In a BAC–YC construct, the coding sequences of *OR111-7* and *OR103-1* were replaced with *gap–YFP* and *gap–CFP* cDNAs (yellow and light-blue pentagons), respectively. Note that all OR genes and two fluorescent reporter genes are ~1 kb in size and are not drawn to scale.

of YFP and CFP in the BAC–YC transgenic fish, we obtained double-transgenic fish by crossing the BAC–YC line with the OMP:RFP line. In BAC–YC;OMP:RFP double-transgenic embryos, fluorescent signals of YFP and CFP were detected by 1.5 d postfertilization (dpf) when several endogenous OR genes begin to be expressed (Barth et al., 1997) (Fig. 2*A,D,G*). At this stage, YFP and CFP were expressed in small numbers of OSNs, and these two populations appeared to be primarily overlapping. Outgrowing axons of YFP- and CFP-labeled OSNs extended toward the forebrain along the preceding RFP-labeled axons (Fig. 2*A,D,G*). YFP- and CFP-labeled OSNs greatly increased in number by 2–3 dpf, and the two differentially labeled OSN populations gradually segregated in the OE (Fig. 2*B,E,H*). In the presumptive OB, RFP-labeled axons projected to form many protoglomerular structures, whereas YFP- and CFP-labeled axons shaped a specific cluster of protoglomeruli in the medial portion (Fig. 2*B,E,H*). Such topographically fixed axonal targeting of YFP- and CFP-labeled OSNs to the medial glomeruli in the OB was clearly observed at 5 dpf when glomerular structures become evident (Fig. 2*C,F,I*). Although YFP- and CFP-labeled OSN populations were distinct in the OE, their axons targeted the same cluster of glomeruli (Fig. 2*H,I*).

Selective innervation of BAC transgene-expressing OSN axons to a medial glomerular cluster in the adult OB

In BAC–YC embryos, YFP- and CFP-labeled OSNs projected their axons to a specific cluster of protoglomeruli in the medial region of the OB (Fig. 2). We next examined whether this pattern of axonal projection persists in adult BAC–YC fish. Fluorescence of neither YFP nor CFP could be detected directly in the OE and OB from adult BAC–YC fish. For this reason, we adopted whole-mount OE/OB immunostaining with the anti-GFP antibody detecting both YFP and CFP, although we could not discriminate two fluorescent proteins. In the OMP:YFP line as a control, YFP-expressing ciliated OSNs projected their axons to a large number of glomeruli distributed widely in the dorsal and medial regions of the OB (Fig. 3*A–C*). In contrast, only a small cluster of glomeruli located in the antero-medial portion of the OB was targeted by GFP-immunoreactive YFP/CFP-expressing OSNs in the BAC–YC line (Fig. 3*D–F*).

We also investigated a series of horizontal sections through the OB of adult BAC–YC;OMP:RFP double-transgenic fish. GFP-immunoreactive OSN axons approached and entered the OB from the ventral side (Fig. 4*D,E*, arrowheads) and converged onto a cluster of glomeruli in the medial portion of the OB at the intermediate level along the dorsoventral axis (Fig. 4*B,C*). Merged images of anti-GFP and anti-RFP labeling revealed that GFP-immunoreactive glomeruli were completely included in RFP-
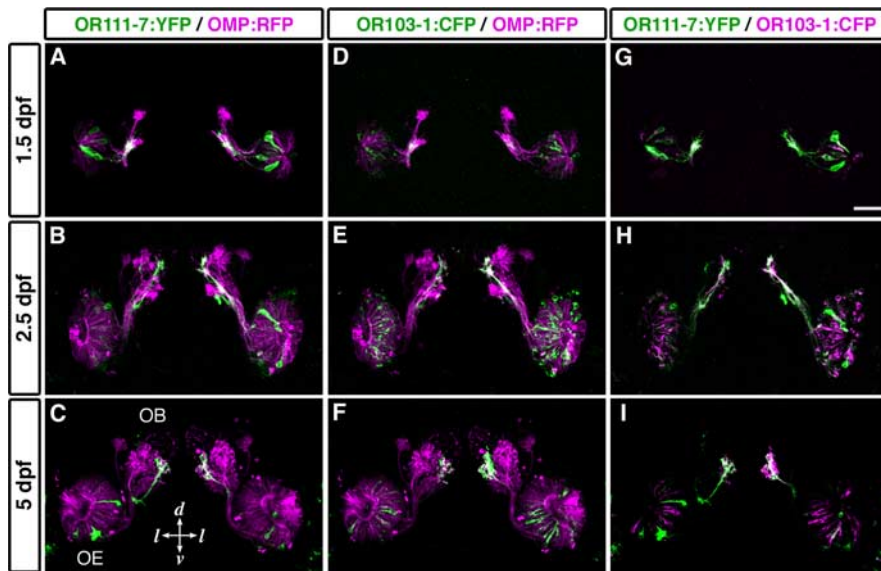
Figure 2. Expression of YFP and CFP in subpopulations of OSNs in a living embryo. Labeled OSNs in a representative BAC–YC; OMP:RFP double-transgenic embryo were observed at 1.5 dpf (***A***, ***D***, ***G***), 2.5 dpf (***B***, ***E***, ***H***), and 5 dpf (***C***, ***F***, ***I***). YFP-labeled (green in ***A–C***, ***G–I***), CFP-labeled (green in ***D–F***; magenta in ***G–I***), and RFP-labeled (magenta in ***A–F***) OSNs are shown. All photographs represent stacked images of optical sections. YFP- and CFP-labeled OSNs project their axons to a specific cluster of glomeruli in the medial portion of the OB. d, Dorsal; v, ventral; l, lateral. Scale bar, 50 μm.
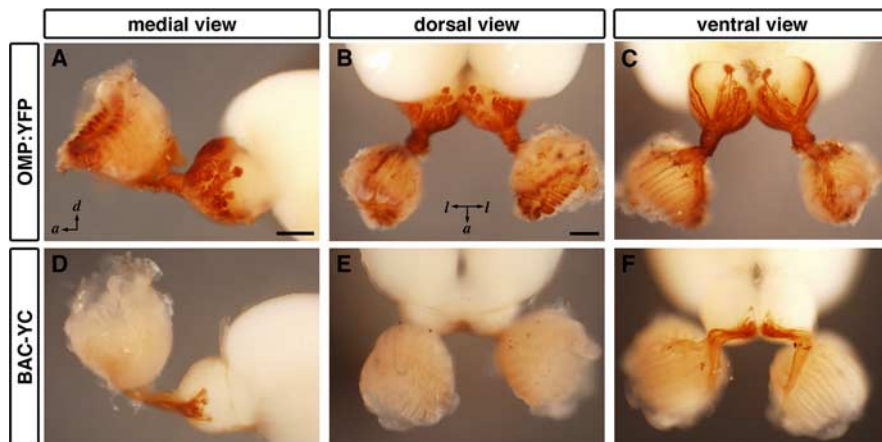

Figure 3. Projection of YFP/CFP-expressing OSN axons to the medial region of the OB in adult BAC–YC fish. Whole-mount OBs together with OE were stained with anti-GFP antibody. ***A–C***, In adult OMP:YFP fish, YFP-labeled OSNs project their axons widely to a number of glomeruli in the dorsal and ventromedial OB. ***D–F***, In adult BAC–YC fish, axonal projection of GFP-immunoreactive YFP/CFP-expressing OSNs is observed only in a small portion of the medial OB. ***A***, ***D***, Medial views; ***B***, ***E***, dorsal views; ***C***, ***F***, ventral views. a, Anterior; d, dorsal; l, lateral. Scale bars: (in ***A***), ***A***, ***D***, 200 μm; (in ***B***), ***B***, ***C***, ***E***, ***F***, 200 μm.

labeled glomeruli (Fig. 4*F–J*), suggesting that YFP and CFP are expressed in a subset of RFP-expressing ciliated OSNs. Counterstaining with the antibody against a synaptic vesicle protein SV2 for definition of glomerular structures demonstrated that GFP-immunoreactive axons definitely innervated a small number of glomeruli (Fig. 4*L–N*). The patterns of axonal targeting from GFP-immunoreactive OSNs in the BAC–YC line were almost same between the right and left OBs and among different individuals tested (data not shown).

Expression of YFP/CFP in a subpopulation of ciliated OSNs in the adult OE
The axonal trajectory of GFP-immunoreactive OSNs in BAC–YC fish was completely included in that of ciliated OSNs (Figs. 3, 4), suggesting that YFP and CFP are expressed in a subset of ciliated

OSNs. This idea is likely, because the replaced ORs, *OR111-7* and *OR103-1*, are expressed exclusively in ciliated OSNs (Sato et al., 2005). Ciliated and microvillous OSNs in fish can be distinguished by their different morphologies and relative positions in the OE. The ciliated OSNs with long dendrites are situated in the deep layer of the OE, whereas microvillous OSNs with short dendrites are located in the superficial layer (Morita and Finger, 1998; Hansen et al., 2003, 2004). To confirm that YFP/CFP was actually expressed in ciliated OSNs, OE sections from adult BAC–YC fish were immunostained with the antibody against GFP. GFP immunoreactivity was observed in a subpopulation of cells that were distributed sparsely within the sensory area of the OE (Fig. 5*A*). Almost all labeled OSNs possessed long dendrites, and their somata were located in the deep layer of the OE (Fig. 5*B*), representing the features of ciliated OSNs. In addition, double-fluorescent labeling of OE sections from BAC–YC;OMP:RFP double-transgenic fish revealed that all of the GFP-immunoreactive cells were included in the population of RFP-labeled ciliated OSNs (Fig. 5*C*). These findings indicate that YFP and CFP inserted into the two OR gene loci within the BAC–YC transgene are allowed to be expressed exclusively in ciliated OSNs as well as endogenous ORs.

Expression of OR genes belonging to limited subfamilies in YFP/CFP-expressing OSNs
Although we succeeded in visualization of a specific subset of OSNs by using BAC transgenesis, there still remains an important open question as to which OR genes are expressed in YFP/CFP-expressing OSNs. Because we failed to induce bicistronic expression of the OR-coding sequence and the fluorescent reporter gene using an internal ribosome entry site in zebrafish (data not shown), the coding sequences of *OR111-7* and *OR103-1* were replaced with fluorescent reporter genes in the BAC–YC construct (*OR111-7:YFP/OR103-1:CFP*). Previous studies with transgenic mice showed that OSNs expressing transgenes with an OR pseudogene or a truncated OR-coding sequence transcribe an additional OR gene, whereas OSNs expressing a functional OR do not (Serizawa et al., 2003; Lewcock and Reed, 2004). It has been also reported that OSNs initially expressing a mutant OR switch to express alternate ORs at high frequencies (Shykind et al., 2004). These findings suggested that the expression of a functional OR elicits a feedback signal that terminates OR switching and abrogates expression of additional OR genes. If a similar regulation mechanism of OR gene expression exists in the zebrafish olfactory system, OSNs that made the first expression choice on the fluorescent reporters would make the second choice on additional OR genes.

To determine which OR genes are expressed in YFP/CFP-expressing OSNs, OE sections from adult BAC–YC fish were hybridized with cRNA probes for OR genes and immunostained with the antibody against GFP. First, we investigated whether the YFP/CFP-expressing OSNs chose the replaced OR, *OR111-7* or *OR103-1*, from the endogenous alleles. A careful examination of double-labeled OE sections clarified that only a small population of GFP-immunoreactive OSNs expressed *OR111-7* (5.8%) or *OR103-1* (7.1%) (Fig. 6*A*,*B*), raising a possibility that the YFP/CFP-expressing OSNs might express some OR genes other than *OR111-7* and *OR103-1*. Then we extended the double-labeling experiment for various OR genes located in the same cluster on chromosome 15. Individual OR members belonging to the *OR111* subfamily (*OR111-1*, *OR111-2*, *OR111-3*, *OR111-5*, and *OR111-10*) were also expressed in small populations of GFP-immunoreactive OSNs (1.8–10.5%). *OR103-2* and/or *OR103-5* belonging to the same subfamily as *OR103-1* were expressed in a large population of GFP-immunoreactive OSNs (93.5%). In contrast, we could not detect any GFP-immunoreactive OSNs that expressed *OR102–1*, *OR107-1*, or *OR119-2* despite their localization in the same OR gene cluster as the *OR111* and *OR103* subfamilies (Fig. 6*A*,*B*). The majority of zebrafish OR genes are distributed in five large clusters on chromosomes 10, 15, and 21 in the zebrafish genome (Alioto and Ngai, 2005; Niimura and Nei, 2005). When we examined expression of OR genes contained in other OR gene clusters, none of the tested OR genes was expressed in GFP-immunoreactive OSNs, except for *OR106-11* (7.9%) (Fig. 6*A*,*B*).

To confirm that the OR gene choice in GFP-immunoreactive OSNs in BAC–YC fish is not random but strictly regulated, we analyzed the expression rates of individual OR genes by estimating the numbers of OR probe-positive cells in an entire olfactory rosette of wild-type fish. Individual OR genes were expressed in disparate numbers of OSNs in the adult OE (Fig. 6*C*), and the profile of OR gene expression in GFP-immunoreactive OSNs in BAC–YC fish did not reflect the expression rate of individual OR genes in the OE at all. These data indicate that OR genes expressed in YFP/CFP-expressing OSNs in BAC–YC fish are not randomly chosen according to the frequency of OR expression but are restricted within members of the *OR111* and *OR103* subfamilies to which the replaced ORs (*OR111-7* and *OR103-1*) belong.

A subpopulation of zebrafish OSNs express multiple OR genes

In the analysis of OR gene expression in YFP/CFP-expressing OSNs in BAC–YC fish, summation of all of the percentages for
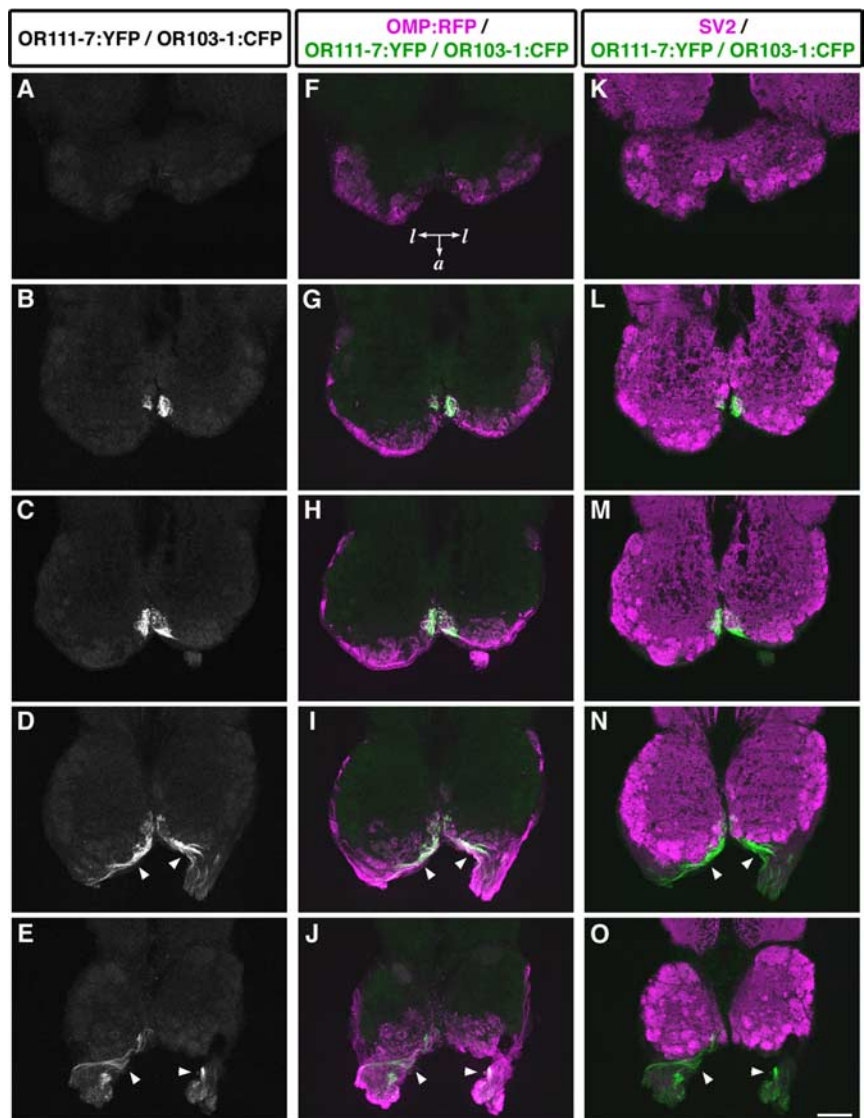

Figure 4. Targeting of YFP/CFP-expressing OSNs to specific glomeruli in the medial OB of adult BAC–YC fish. Horizontal sections through the OB of adult BAC–YC;OMP:RFP double-transgenic fish were triple labeled with anti-GFP, anti-RFP, and anti-SV2 antibodies. **A–E**, GFP immunoreactivities to YFP and CFP. **F–J**, Merged images of GFP (green) and RFP (magenta) immunoreactivities. **K–O**, Merged images of GFP (green) and SV2 (magenta) immunoreactivities. Five slices of 20-μm-thick OB sections are selected from a series of horizontal sections at 60 μm intervals and shown in the order from dorsalmost (top) to ventralmost (bottom). All photographs represent stacked images of optical sections. GFP-immunoreactive axons innervate a small population of RFP-labeled glomeruli in the medial OB. Arrowheads in **D,E**, **I,J**, and **N,O** indicate OSN axons entering the OB. a, Anterior; l, lateral. Scale bar, 100 μm.

OR-positive cells among GFP-immunoreactive OSNs reached 150%, significantly exceeding 100% (Fig. 6*B*). This result suggests that there may be a substantial population of OSNs expressing multiple OR genes. To examine the possibility of multiple OR gene expression in single OSNs, we performed double *in situ* hybridization analysis for various combinations of OR members belonging to the *OR111* and *OR103* subfamilies (Fig. 7). In both wild-type and BAC–YC fish, all pairwise combinations of the *OR111* subfamily members tested were expressed in nonoverlapping subsets of OSNs (Fig. 7*A*,*B*,*E*,*F*) (supplemental Table S2, available at www.jneurosci.org as supplemental material). These results are essentially consistent with the previous report showing that the *OR111* subfamily members are expressed individually in different OSNs (Barth et al., 1997). To investigate whether OR members of the *OR111* and *OR103* subfamilies were coexpressed
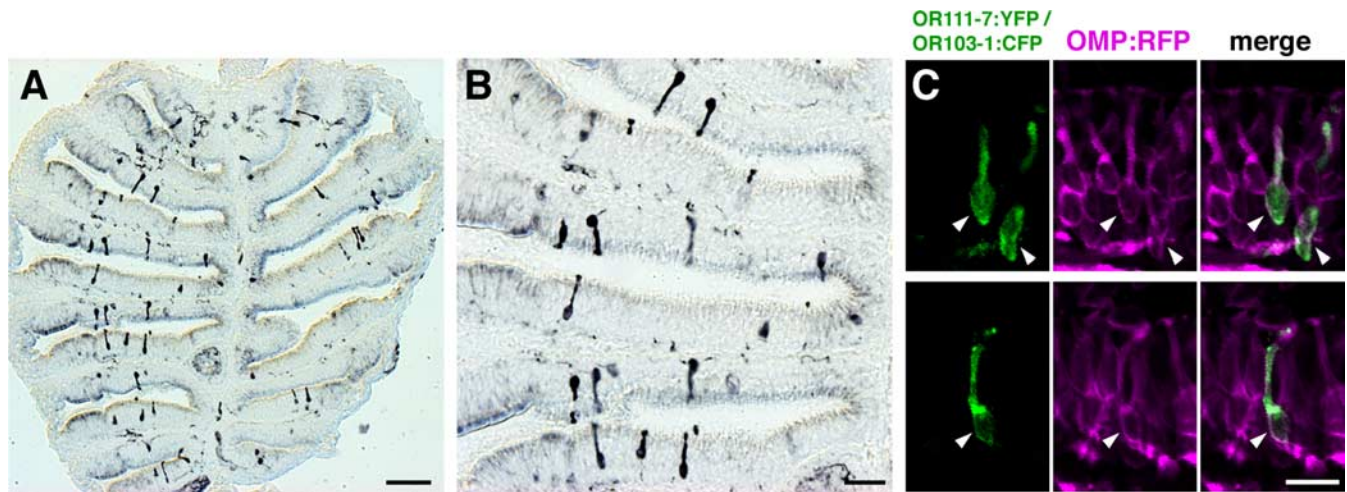
Figure 5. Expression of YFP/CFP in ciliated OSNs of the OE from adult BAC–YC fish. **A**, Immunostaining of YFP and CFP with anti-GFP antibody was performed on horizontal sections of the OE from adult BAC–YC fish. Scale bar, 50 μm. **B**, A high-power view of **A**. GFP immunoreactivity is detected in OSNs with long dendrites. Scale bar, 20 μm. **C**, GFP immunoreactivity and RFP fluorescence on OE sections from adult BAC–YC;OMP:RFP double-transgenic fish. GFP immunoreactivity is detected in RFP-positive ciliated OSNs (arrowheads). Scale bar, 10 μm.

in the same OSN or not, double *in situ* hybridization was performed using mixed probes for the respective subfamilies. In wild-type fish, a cell population expressing the *OR111* members is completely discrete from that expressing the *OR103* members (Fig. 7C). In BAC–YC fish, however, coexpression of the *OR111* and *OR103* subfamily members was detected in 19 OSNs among 345 *OR111*-positive and 156 *OR103*-positive cells (Fig. 7G, arrowheads). We next examined coexpression of OR members within the *OR103* subfamily. Surprisingly, OSNs labeled with the *OR103-1* probe were always positive for a mixture of the *OR103-2* and *OR103-5* probes in both wild-type and BAC–YC fish (Fig. 7D,H). Because, in our hybridization experiments, cross-hybridization did not occur even when cRNA probes with high sequence identities were used together (*OR111-1* vs *OR111-7*, 81%; *OR111-7* vs *OR111-3*, 80%), there would be much less possibility of cross-hybridization between *OR103-1* and *OR103-2/OR103-5* (67% identity). Colocalization of signals for *OR103-1* and *OR103-2/OR103-5*, therefore, directly indicates that all *OR103-1*-positive OSNs simultaneously coexpress *OR103-2* and/or *OR103-5*. Expression of multiple OR genes in a subpopulation of OSNs could provide an account for the excess in a sum of the percentages for OR-positive cells.

Discussion
In this study, we generated BAC transgenic zebrafish carrying an OR gene cluster in which the coding sequences of two OR-type receptors, *OR111-7* and *OR103-1*, were replaced with YFP and CFP, respectively. We found that the choice of OR genes in YFP/CFP-expressing OSNs was limited to OR members belonging to the *OR111* and *OR103* subfamilies and that the YFP/CFP-expressing OSNs expressing OR members of the *OR111* and *OR103* subfamilies converged their axons onto a topographically fixed glomerular cluster in the medial OB. These results demonstrate the hierarchical regulation of OR gene choice and the subsequent axonal convergence to defined glomeruli in the zebrafish olfactory system.

Hierarchical regulation of OR gene choice in zebrafish OSNs
In BAC–YC (*OR111-7:YFP/OR103-1:CFP*) transgenic fish, OR genes expressed in YFP/CFP-expressing OSNs were not ran-

domly distributed on various chromosomal loci but were mostly restricted within members of the *OR111* and *OR103* subfamilies (Fig. 6). Not only OR genes contained in BAC–YC transgene (*OR111-5*, *OR111-10*, and *OR103-2/OR103-5*) but also other OR genes located only in endogenous alleles (*OR111-1*, *OR111-2*, *OR111-3*, *OR111-7*, and *OR103-1*) were expressed in GFP-immunoreactive OSNs, in which either YFP or CFP gene within BAC–YC transgene was also activated for expression. These findings indicate that all OR members of the *OR111* and *OR103* subfamilies had a potential for expression in the YFP/CFP-expressing OSNs, regardless of their location either within BAC–YC transgene or on the endogenous allele. Thus, the genomic organization of OR subfamilies might provide a basis for regulation of OR expression in the zebrafish olfactory system.

Whereas OR choice in YFP/CFP-expressing OSNs was mostly restricted within the *OR111* and *OR103* subfamily members, *OR106-11* mRNA was exceptionally detected in YFP/CFP-expressing OSNs among the tested OR genes belonging to different subfamilies. Although the chromosomal location of the *OR106* subfamily (chromosome 10) is different from that of the *OR111* and *OR103* subfamilies (chromosome 15), the *OR106* and *OR111* subfamilies are classified into the same or closely related OR groups based on the phylogenic analysis (Alioto and Ngai, 2005; Niimura and Nei, 2005). Expression of the *OR111* and *OR106* subfamily members may be mediated by a common regulatory mechanism.

Individual *OR111* subfamily members were expressed in non-overlapping subsets of OSNs (Fig. 7), suggesting that OSNs, which are allowed to express *OR111* subfamily members, would choose only one OR among the subfamily. One attractive mechanistic model to ensure the "one receptor–one neuron" rule emerged from several lines of evidence with transgenic mice (Serizawa et al., 2003; Lewcock and Reed, 2004; Shykind et al., 2004). Once a functional OR is expressed, the OR will prevent expression of additional OR genes through putative feedback signals. If the negative feedback regulation demonstrated in mice is also applicable to zebrafish, OSNs that initially express the YFP gene in BAC–YC fish would fail to produce a feedback signal and subsequently choose alternate ORs within the *OR111* subfamily.

This notion leads to a two-step model of the hierarchical regulation of OR gene choice in the zebrafish olfactory system (Fig. 8): (1) individual OSNs would initially choose one or a few OR subfamilies for expression, or, alternatively, the choice of OR subfamilies may be predetermined for individual OSNs according to their cell lineage or spatial localization in the OE; (2) the OSNs subsequently choose a single OR gene among the limited subfamily members.

Similarities in OR gene choice mechanisms between zebrafish and mice

Our hypothetical model of hierarchical OR gene choice in zebrafish might be similar to the mode of OR gene choice in mice within the meaning that individual OSNs choose a single OR gene among limited OR members. In mice, ORs are approximately classified into four groups according to their expression zones in the OE (Ressler et al., 1993; Vassar et al., 1993; Sullivan et al., 1996), although two recent papers reported that patterns of OR gene expression are arranged not in conventional zones but rather in continuous and overlapping domains (Iwema et al., 2004; Miyamichi et al., 2005). Switching of OR gene choice in OSNs expressing a mutant OR is permitted within the repertoire of OR genes in the same zone (Serizawa et al., 2003; Shykind et al., 2004). In a given zone, structurally related ORs belonging to same subfamilies are generally expressed, which may underlie the zonal difference in reception of odors with distinct qualities (Mori et al., 2000). Some zebrafish ORs also show concentric expression patterns within the OE (Weth et al., 1996). If zonal patterns of OR expression are related to the classification of OR subfamilies in zebrafish, it is likely that a common hierarchical mechanism of OR gene choice is used between zebrafish and mice.

A recent report demonstrated that an enhancer element, *H* region, can mediate the stochastic, interchromosomal *trans*-activation of a single OR gene locus in mouse OSNs (Lomvardas et al., 2006). The presence of a *trans*-acting enhancer element homologous to the mouse *H* region has not been proved in zebrafish. However, a similar enhancer-mediated transcriptional mechanism may be used also in zebrafish in the process of a single OR gene choice among limited OR subfamily members.

Expression of multiple ORs in single OSNs

A double-labeling experiment for YFP/CFP and OR mRNA expression in BAC–YC fish yielded a curious but interesting result: a sum of the percentages for OR-positive cells among YFP/CFP-
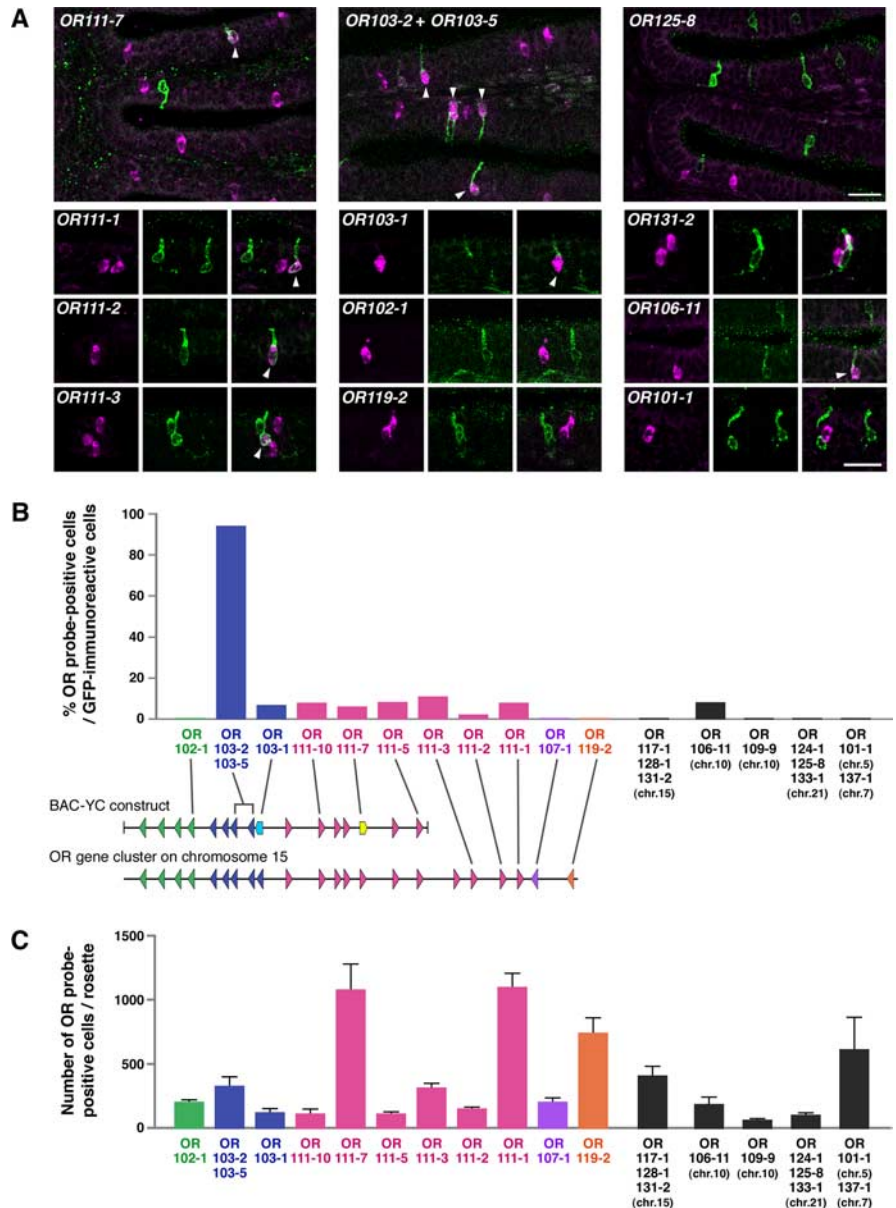

Figure 6. Expression of OR genes belonging to restricted subfamilies in YFP/CFP-expressing OSNs. *A*, OE sections from adult BAC–YC fish were hybridized with cRNA probes for various OR genes (magenta) and subsequently immunostained with anti-GFP antibody (green). The arrowheads indicate GFP-immunoreactive OSNs that are labeled with the OR gene probe tested. Scale bars, 20 μm. *B*, Percentages of the OR probe-positive cells among GFP-immunoreactive cells are shown in relation to an array of OR genes in the cluster on chromosome 15. OR genes expressed in GFP-immunoreactive OSNs are mostly restricted within the *OR111* and *OR103* subfamilies. *C*, Numbers of OSNs labeled with individual OR probes in an entire olfactory rosette from adult wild-type zebrafish. To estimate the numbers of OSNs expressing individual OR genes in an entire olfactory rosette, *in situ* hybridization was performed on OE sections from adult wild-type fish. For each OR probe, the total number of OR probe-positive cells on every four slices from serial sections is quadruplicated and shown as the estimated number of OR probe-positive cells per an olfactory rosette. Data represent means ± SEM (n = 3–7). Individual OR genes are expressed in different numbers of OSNs in the wild-type OE with no relation to the OR choice in YFP/CFP-expressing OSNs in BAC–YC fish. Mixed OR probes were used as follows: *OR103-2* and *OR103-5*; *OR117-1*, *OR128-1*, and *OR131-2* on chromosome 15; *OR124-1*, *OR125-8*, and *OR133-1* on chromosome 21; *OR101-1* on chromosome 5; and *OR137-1* on chromosome 7.

expressing OSNs reached 150% (Fig. 6*B*). This finding prompted us to examine a possibility that a subpopulation of OSNs may express multiple OR genes. In BAC–YC fish, double *in situ* hybridization analysis demonstrated the coexpression of ORs in a small but significant population of OSNs: (1) *OR103-1*-expressing OSNs were always positive for *OR103-2* and/or *OR103-5* (Fig. 7*H*); (2) coexpression of the *OR111* and *OR103* subfamily members was observed in a small population of OSNs
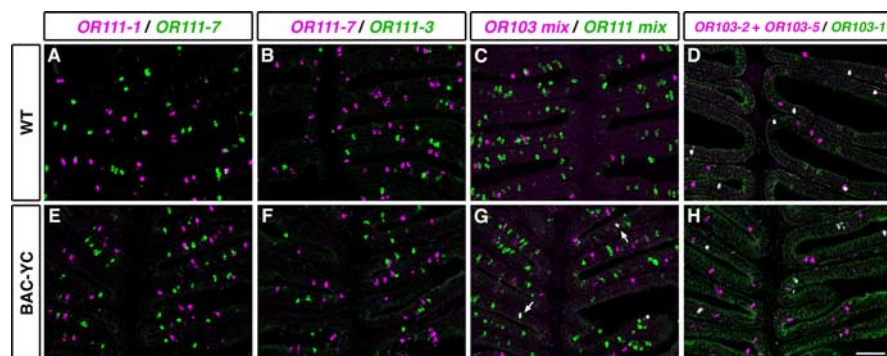
Figure 7. Coexpression of multiple OR receptors in single OSNs in adult wild-type (WT) and BAC–YC (*OR111-7:YFP/OR103-1: CFP*) fish. Double *in situ* hybridization was performed on OE sections from wild-type (*A–D*) and BAC–YC (*E–H*) fish with various combinations of OR members belonging to the *OR111* and *OR103* subfamilies. *A*, *E*, Nonoverlapping expression of *OR111-1* (magenta) and *OR111-7* (green) in wild-type (*A*) and BAC–YC (*E*) fish. *B*, *F*, Nonoverlapping expression of *OR111-7* (magenta) and *OR111-3* (green) in wild-type (*B*) and BAC–YC (*F*) fish. *C*, *G*, Expression of ORs belonging to the *OR103* (magenta) and *OR111* (green) subfamilies. Mixed probes for the *OR103* subfamily contains probes for *OR103-1*, *OR103-2*, and *OR103-5*. Mixed probes for the *OR111* subfamily contains probes for *OR111-1*, *OR111-2*, *OR111-3*, *OR111-5*, *OR111-7*, and *OR111-10*. No overlapping signal is observed in wild-type fish (*C*), whereas overlapping signals are detected at low frequency in BAC–YC fish (arrows in *G*). *D*, *H*, Coexpression of *OR103-1* (green) with *OR103-2* and/or *OR103-5* (magenta) in both wild-type (*D*) and BAC–YC (*H*) fish. All OSNs labeled with the *OR103-1* probe are also positive for a mixture of the *OR103-2* and *OR103-5* probes (shown in white). All photographs represent stacked images of optical sections. Scale bar, 50 μm.
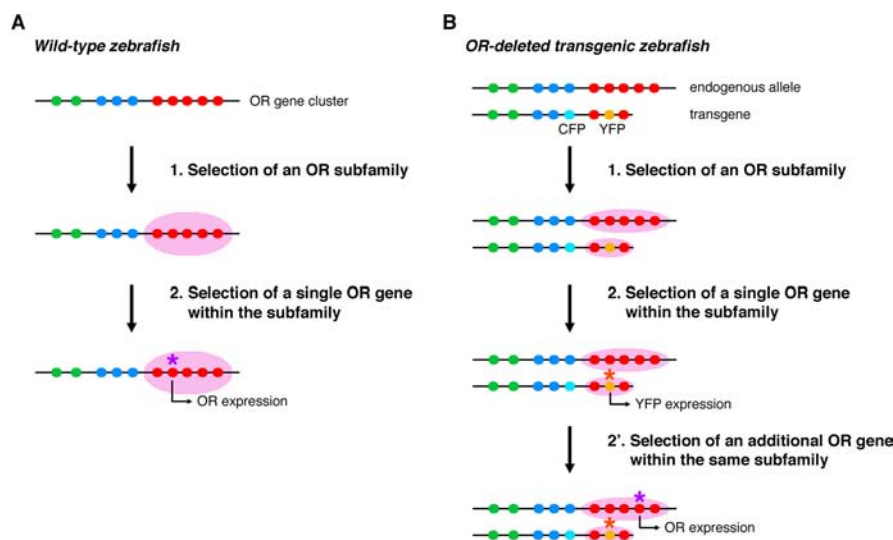

Figure 8. A model of hierarchical regulation of zebrafish OR gene choice. *A*, A single OR gene for expression is determined by the two-step selection in OSNs. For individual OSNs, the OR gene choice is initially confined to one or a few OR subfamilies (pink circles). The OSNs subsequently choose a single OR gene (a purple asterisk) among the limited subfamily members. *B*, The OR gene choice in transgenic zebrafish in which OR-coding sequences are replaced with reporter genes. After the selection of an OR subfamily (pink circles), if OSNs choose a reporter gene (an orange asterisk) and fail to express a functional OR, the OSNs choose an additional OR gene (a purple asterisk) among the limited subfamily members. OR genes belonging to individual OR subfamilies are represented by small circles of the same colors.

(Fig. 7*G*). These results indicate that the one receptor–one neuron rule is not always the case for a subpopulation of OSNs in the BAC–YC transgenic zebrafish. Because the coding sequence of *OR103-1* is replaced with CFP in the BAC–YC transgene, CFP-positive OSNs should simultaneously express *OR103-2* and/or *OR103-5*. This notion, together with the dominant number of CFP-expressing cells over YFP-expressing cells, may explain why a large population (93.5%) of GFP-immunoreactive OSNs expressed *OR103-2* and/or *OR103-5* in BAC–YC fish.

Intriguingly, also in wild-type fish, *OR103-1*-positive OSNs simultaneously expressed *OR103-2* and/or *OR103-5* (Fig. 7*D*).

This finding clearly demonstrates that the one receptor–one neuron rule is not necessarily applicable to zebrafish OSNs and that the "multiple receptors–one neuron" rule is true at least for the *OR103* subfamily members. Coexpression of functional chemosensory receptors in single neurons has been also reported in *Drosophila* and *Caenorhabditis elegans* (Troemel et al., 1995; Goldman et al., 2005). For example, the AWC neuron in *C. elegans* expresses multiple ORs, responds to various odorants such as aldehydes, ketones, alcohols, and thiazoles without discrimination, and mediates attractive behavior to all of these odorants (Bargmann et al., 1993; Troemel et al., 1995). By analogy, it may be unnecessary for zebrafish to discriminate odorant molecules received by the individual *OR103* subfamily members. These OSNs expressing multiple *OR103* members thus may integrate odor information at the most peripheral level, leading to a particular behavioral or hormonal response.

Glomerular targeting of OSN axons in zebrafish

Previous studies using molecular biological and genetic techniques demonstrated that, in *Drosophila* and mice, OSNs expressing a given OR project their axons onto one or a few topographically fixed glomeruli in the antennal lobe or the OB (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Gao et al., 2000; Vosshall et al., 2000). This principle of axonal convergence to target glomeruli underlies the development of odor maps in the brain (Mori et al., 1999). In zebrafish, the existence of an odor map was demonstrated by neural activity imaging of OSN axon terminals and OB projection neurons (Friedrich and Korsching, 1997, 1998; Li et al., 2005). Different classes of odor molecules (e.g., amino acids, nucleotides, bile acids, steroids, and prostaglandins) elicit distinct patterns of glomerular activity in the zebrafish OB. However, no direct evidence has been reported, to date, for axonal convergence of OSNs expressing the same type of OR genes onto specific glomeruli in zebrafish.

In this study, we analyzed in detail the relationship between OR gene choice and glomerular innervation of YFP/CFP-expressing OSNs in BAC–YC fish. The YFP/CFP-expressing OSNs expressed limited members of OR genes belonging mostly to the *OR111* and *OR103* subfamilies and projected their axons to a specific cluster of glomeruli in the medial portion of the OB. Such OR gene choice profiles and patterns of OSN axon projection were indistinguishable among different individuals. These findings indicate the axonal convergence of OSNs expressing OR genes belonging to particular subfamilies onto a topographically fixed glomerular cluster in the OB. Thus, the principle of "axonal

convergence of like OSNs" seems to be also applicable to zebrafish, although the "one OR–one glomerulus" relationship remains to be elucidated.

In the fish olfactory system, two major types of OSNs, ciliated and microvillous OSNs, are contained in different layers of the OE. Previous studies have proposed that ciliated OSNs project their axons to mainly medial regions of the OB and mediate avoidance behaviors, whereas microvillous OSNs target lateral regions of the OB and mediate feeding behaviors (Hamdani et al., 2001; Hamdani and Døving, 2002; Hansen et al., 2003; Weltzien et al., 2003; Sato et al., 2005). An anteromedial portion of the zebrafish OB is activated by bile acids (Friedrich and Korsching, 1998), suggesting that a cluster of glomeruli targeted by YFP/CFP-expressing OSNs in BAC–YC fish would also respond to bile acids. Biological function of bile acids is not clear in zebrafish, but, in sea lampreys, it has been reported that bile acids function as migratory attractants (Li et al., 2002; Sorensen et al., 2005). Additional experiments will clarify whether ORs belonging to the *OR111* and *OR103* subfamilies are actually the receptors specific for bile acids or not.

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