Expression of *sall4* in Taste Buds of Zebrafish

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Received 18 December 2012; revised 22 February 2013; accepted 25 February 2013

ABSTRACT: **We characterized the expression of *sall4*, a gene encoding a zinc finger transcription factor involved in the maintenance of embryonic stem cells, in taste buds of zebrafish (*Danio rerio*). Using an enhancer trap line (ET5), we detected enhanced green fluorescent protein (EGFP) in developing and adult transgenic zebrafish in regions containing taste buds: the lips, branchial arches, and the nasal and maxillary barbels. Localization of EGFP to taste cells of the branchial arches and lips was confirmed by co-immunolabeling with antibodies against calretinin and serotonin, and a zebrafish-derived neuronal marker (zn-12). Transgenic insertion of the ET construct into the zebrafish genome was evaluated and mapped to chromosome 23 in proximity (i.e. 23 kb) to the *sall4* gene. *In situ* hybridization and expression analysis between 24 and 96 h post-fertilization (hpf) demonstrated that transgenic *egfp* expression in ET5 zebrafish was correlated with the spatial and temporal pattern of expression of *sall4* in the wild-type. Expression was first observed in the central nervous system and branchial arches at 24 hpf. At 48 hpf, *sall4* and *egfp* expression was observed in taste bud primordia surrounding the mouth and branchial arches. At 72 and 96 hpf, expression was detected in the upper and lower lips and branchial arches. Double fluorescence *in situ* hybridization at 3 and 10 dpf confirmed colocalization of *sall4* and *egfp* in the lips and branchial arches. These studies reveal *sall4* expression in chemosensory cells and implicate this transcription factor in the development and renewal of taste epithelia in zebrafish.** Develop Neurobiol 00: 000–000, 2013

Keywords: **chemoreceptor; taste; development; *sall4*; zebrafish**

INTRODUCTION

Taste buds in fish are conspicuous organs found throughout the oropharyngeal epithelium, on the oral aspect of the branchial arches, and in some species on the head and other regions of the body. As in other vertebrates, the taste buds of fish allow discrimination between palatable and noxious particles (Reutter and Witt, 1993; Finger, 1997; Finger and Simon, 2000; Hansen et al., 2002). Each taste bud is composed of a heterogeneous population of cells. Elongate taste cells extend apical processes, where transduction of taste stimuli occurs, to an opening in the epithelium, called the taste receptor area (equivalent to the mammalian taste pore). Located deeper within the taste buds are Merkel-like cells, which reside atop a basal lamina and form synaptic contacts with both taste cells and primary afferent fibers that innervate them (Toyoshima et al., 1984; Jakubowski

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Contract grant sponsor: World Class Institute/National Research Foundation of Korea; contract grant number: WCI 2009-003.

Contract grant sponsor: NIH; contract grant number: DC005259-39.

Contract grant sponsors: Natural Sciences and Engineering Research Council of Canada, Canadian Foundation for Innovation, Canadian Institutes of Health Research, the Nova Scotia Health Research Foundation.

Published online 00 Month 2013 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/dneu.22079

and Whitear, 1990; Reutter and Witt, 1993; Zachar and Jonz, 2012).

In zebrafish, taste cells are divided into three major types, which appear consecutively during taste bud development: dark cells, light cells, and finally cells with a brush-like apical ending (Hansen et al., 2002). Taste primordia on the lips first appear at 3 dpf and complete taste buds are usually formed by 4 or 5 dpf (Hansen et al., 2002). It is well established that taste bud cells arise from the local epithelium in amphibians (Barlow and Northcutt, 1995) and mammals (Stone et al., 1995) and have a limited life span (Finger and Simon, 2000; Miura et al., 2006). In mammals, taste buds are maintained, or renewed, by the continuous proliferation of epithelial stem and progenitor cells. However, the identities of these cell types, and the mechanisms of taste bud renewal, remain controversial (Stone et al., 2002; Miura et al., 2006; Sullivan et al., 2010; Miura and Barlow, 2010).

This study characterized a zebrafish transgenic line, in which enhanced green fluorescent protein (EGFP) is expressed in the oropharyngeal epithelium and branchial arches. Using a *Tol2* transposon mediated enhancer trap approach, Parinov et al. (2004) produced several lines of transgenic zebrafish in which EGFP was expressed in live embryos. Of these we selected the ET5 line (ZETRAP 2.0) for further analysis to characterize potential expression patterns in taste cells. We examined the development of the taste buds and demonstrate, using *in situ* hybridization and immunohistochemistry, that EGFP labels taste cells in ET5 zebrafish. Furthermore, using the sequence information of the insertion site, we found that the transgene is located in the vicinity of the *sall4* gene, which encodes a transcription factor involved in regulating embryonic stem cell pluripotency and self renewal (Zhang et al., 2006; Yang et al., 2008). *sall4* has also been implicated in reprogramming of differentiated cells in amphibian limb regeneration (Neff et al., 2011). Expression analysis confirmed the colocalization of *sall4* and *egfp* in the ET5 line, indicating that *sall4* is expressed in taste cells. Characterization of this early marker in taste bud development may permit identification of taste bud progenitors or stem cells in zebrafish.

EXPERIMENTAL PROCEDURES

Animals and Tissue Preparation

ET5 transgenic zebrafish (*Danio rerio*) from the Zebrafish Enhancer Trap Lines Database (ZETRAP 2.0; Choo et al., 2006) were produced and kindly provided by Dr. Vladimir Korzh. Production of enhancer trap (ET) transgenics is described in Parinov et al. (2004). Briefly, the ET5 transgenic fish was produced using a *Tol2* transposon-mediated random insertion of the *egfp* gene into the zebrafish genome driven by a 460 bp minimal promoter from the zebrafish *keratin 8* (*krt8*) gene. We selected the ET5 line based on its expression of enhanced green fluorescent protein (EGFP) in tissue consistent with the presence of chemosensory cells, such as the lips, mouth cavity, and nares. Wild-type zebrafish were obtained from a commercial supplier (Mirdo Importations, Montréal, QC, Canada). All animals were maintained in a closed recirculated system at 28.5°C on a 14/10 h light-dark cycle (Westerfield, 2000) and fed commercial flake food and brine shrimp, *Artemia salina*. All handling and care was conducted in accordance with the guidelines established by the Canadian Council for Animal Care.

Embryos were collected from breeding tanks and transferred to Petri dishes containing E3 medium and kept in an incubator at 28.5°C. E3 medium contained 5 m*M* NaCl, 0.17 m*M* KCl, 0.33 m*M* $CaCl_2$, 0.33 m*M* $MgSO_4$, pH 7.8. At 1-day post-fertilization (dpf), ET5 embryos were screened for EGFP expression using a fluorescence stereomicroscope (SMZ1500, Nikon, Tokyo, Japan). Selected larvae were kept in beakers filled with dechlorinated system water maintained at 28.5°C. When necessary, ET5 larvae were transferred to the main nursery system at 10–15 dpf and reared to adulthood (90–120 dpf). Zebrafish embryos and larvae were euthanized with 1 mg mL^{-1} MS 222 (tricaine methanesulfonate; Syndel Laboratories, Vancouver, BC, Canada) dissolved in system water. Animals were prepared as whole mounts for immunohistochemistry and *in situ* hybridization.

Immunohistochemistry

Previously established procedures for immunohistochemistry were employed (Jonz and Nurse, 2003; Zachar and Jonz, 2012). Embryos and larvae were washed in phosphate-buffered solution (PBS) containing 137 m*M* NaCl, 15.2 m*M* Na_2HPO_4, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, at pH 7.8 (Bradford et al., 1994) and then fixed by immersion in 4% paraformaldehyde in PBS at 4°C overnight. Tissue was then rinsed three times (3 min each) in PBS and permeabilized for 48 h at 4°C. The permeabilizing solution (PBS-TX) contained 1% fetal calf serum (FCS) and 2% Triton X-100 in PBS (pH 7.8).

Tissue was then incubated in primary antibodies diluted with PBS-TX (Table 1) for 24 h at 4°C.

Table 1 Details of Primary and Secondary Antibodies Used for Immunohistochemistry

Antibody	Dilution	Antigen	Host	Source	Cat. No.	Secondary
Primary						
Calretinin	1:250	Calretinin	Mouse	Swant (monoclonal)	6B3	Alexa 594
GFP	1:100	GFP	Rabbit	Invitrogen (polyclonal)	A-11122	FITC
5-HT	1:100	Serotonin	Rabbit	Immunostar (polyclonal)	20080	Cy5
zn-12[a]	1:100	Neuron surface	Mouse	DSHB[b] (monoclonal)	zn-12	Alexa 594
Secondary						
Alexa 594	1:100	Mouse	Goat	Invitrogen	A11005	–
FITC[c]	1:50	Rabbit	Goat	Cedar Lane	111-095-003	–
Cy5	1:50	Rabbit	Donkey	Jackson Immunoresearch	711-175-152	–

[a]Zebrafish-derived neuronal antibody.
[b]Developmental Studies Hybridoma Bank, University of Iowa.
[c]Fluorescein isothiocyanate.

Primary antibodies were used with other antibodies or endogenous EGFP fluorescence (as indicated in the figure legends). After incubation in primary antibodies, tissue was rinsed in PBS and then incubated minimally for 1 h in secondary antibodies (Table 1) in PBS-TX in a dark chamber. Following another rinse in PBS, the tissue was mounted in Vectashield (Vector Laboratories, Burlingame, CA) on glass slides. The head and branchial arches were typically examined in both larvae and adults. Particular attention was given to the lips of larvae, since this is a site in which taste bud primordia are first observed (Hansen et al., 2002). For some specimens, heads of embryos or larvae were dissected and adhered to a cavity slide using poly-L-lysine (Sigma) or submersed in Permafluor (Thermo Fisher Scientific, Ottawa, ON, Canada) for precise orientation of tissue. Over 100 ET5 and wild-type zebrafish were examined in these experiments.

Antibody Characterization

The antibodies used in the present study are listed in Table 1. Briefly, taste cells were identified with a monoclonal antibody against anti-calretinin (Swant, Bellinzona, Switzerland), which was produced from human calretinin-22k. This antibody recognizes a 29 kDa protein in zebrafish (Zimmermann and Schwaller, 2002; manufacturer specifications) and has previously been shown to label taste cells in the same species (Germanà et al., 2007; Zachar and Jonz, 2012). A rabbit polyclonal anti-GFP antibody (Invitrogen, Burlington, ON, Canada) was used in some preparations to enhance the fluorescence signal produced by EGFP. Merkel-like basal cells were also labeled to help identify taste buds using antibodies directed against serotonin (5-HT; Immunostar, Hudson, WI, USA), as previously described in zebrafish (Zachar and Jonz, 2012). In addition, the neural innervation of taste cells was identified using an antibody (zn-12, Developmental Studies Hybridoma Bank, University of Iowa, IA, USA) against a zebrafish-derived neuron-specific antigen that is expressed on the surface of nerve fibers (Trevarrow et al., 1990). Monoclonal zn-12 was raised in mouse against membrane fractions from adult zebrafish CNS and recognizes an HNK-1-like epitope (human natural killer-1, a carbohydrate expressed on certain neural cell adhesion molecules; manufacturer specifications). Western blot analysis has indicated that both zn-12 and HNK-1 antibodies label similar bands ranging in molecular weight from 60–248 kDa (see Metcalfe et al., 1990). All primary antibodies were labeled with secondary antisera conjugated with fluorophores, as indicated in Table 1.

Location of Insertion Sites in the Zebrafish Genome

The DNA sequences for insertion of the ET construct were obtained from the ZETRAP database (Choo et al., 2006). The Basic Local Alignment Search Tool (BLAST) was used to search for sites of insertion within the zebrafish genome using the Ensembl genome database (Zv9, release 62; European Bioinformatics Institute and Wellcome Trust Sanger Institute, Cambridge, UK). To select the best sequence alignment, we considered both alignment score and percent identities.

In Situ Hybridization

A 1,554 bp fragment of the zebrafish *sall4* cDNA corresponding to the region previously described by Harvey and Logan (2006) was cloned by RT-PCR

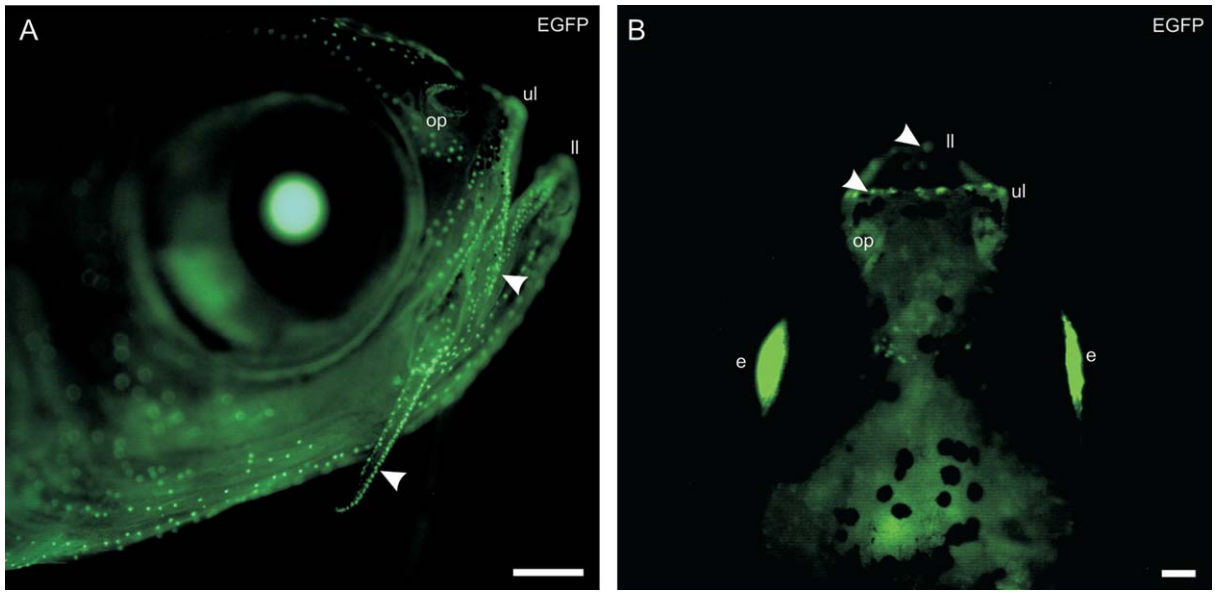

Figure 1 *In vivo* imaging of EGFP-positive cells on the head of ET5 transgenic zebrafish. A: In the adult (120 days post-fertilization, dpf), EGFP-positive cells were found across the head with primary concentrations on the nasal and maxillary barbels (arrowheads), upper and lower lips (ul, ll), the jaw, and the olfactory pits (op). Scale bar 200 μm. B: EGFP-positive cells were also found on the upper and lower lips (arrowheads) in developing ET5 transgenic zebrafish, as shown in a 6 dpf larva in dorsal view with the rostral aspect oriented toward the top of the figure. e, eye. Scale bar 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

from a cDNA library of 24 hpf zebrafish embryos with forward primer 5'-CTTACTATTCGCCCCT-GATGGTTGTA-3' and reverse primer 5'-AGAA-GAAATCGATGCACCATCACTG-3'. *In vitro* transcription of the anti-sense digoxigenin (DIG)-labeled RNA probe (1,554 bases) from the *sall4* cDNA fragment and whole-mount *in situ* hybridization with wild-type embryos were performed as previously described (Thisse and Thisse, 2008). The anti sense DIG-labeled RNA probe for *egfp* (663 bases) was prepared as previously described in MacDonald et al. (2010), and *in situ* hybridization was performed with ET-5 transgenic embryos. Briefly, embryos were hybridized with the anti-sense DIG-labeled RNA probes at 70°C overnight (~16 h). Following washes, the embryos were incubated with anti-DIG antibody conjugated with alkaline phosphatase (Roche, cat. no. 11093274910) overnight at 4°C. Finally, after

Figure 2 Labial taste cells of ET5 transgenic zebrafish are EGFP-positive. A–E: Double immunolabeling with anti-calretinin and anti-5-HT combined with endogenous EGFP fluorescence in the lips of ET5 larvae at 10 dpf. All panels are dorsal views of a whole-mounted larva oriented with the rostral aspect toward the top of the figure (see schematic). F–H: Double immunolabeling of a single taste bud from the lip of an adult ET5 with anti-calretinin and anti-GFP. A: EGFP fluorescence of taste cells (arrowheads) of the upper lip (ul). Cells of the olfactory epithelium (oe) are also weakly labeled. B: Image in A showing localization of anti-calretinin in taste cells. C: The merged image shows colocalization of anti-calretinin and EGFP. D: In the same tissue, immunolocalization of Merkel-like cells (arrowheads) of the basal aspect of taste buds is shown with anti-5-HT. Labeling of the forebrain (fb) is also visible. E: Merged image of EGFP, anti-calretinin and anti-5-HT indicates that EGFP-positive cells are taste cells. F, G: The taste bud marked with an arrowhead in E is shown at higher magnification with only EGFP and 5-HT labeling (F), and EGFP and anti-calretinin/5-HT labeling (G). H, I: The labeling of taste cells in adults with anti-calretinin matches the labeling pattern with anti-GFP. J: The merged image demonstrates colocalization within the taste bud. A large apical microvillus (arrowhead) and several taste cell somata (arrows) are visible. Scale bar in A is 20 μm and applies to B–E. Scale bar in F is 10 μm and applies to G. Scale bars in H–J, 5 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

washes, embryos were stained with Nitro Blue Tetrazolium (NBT) and 5-Bromo 4-chloro 3-indolyl phosphate (BCIP). 25 individuals were examined for *sall4* and *egfp* expression at each developmental stage between 24 hpf and 4 dpf.

Fluorescence *In Situ* Hybridization

In vitro transcription and whole-mount double fluorescence *in situ* hybridization (FISH) were carried out following the protocol described online (Zebrafish

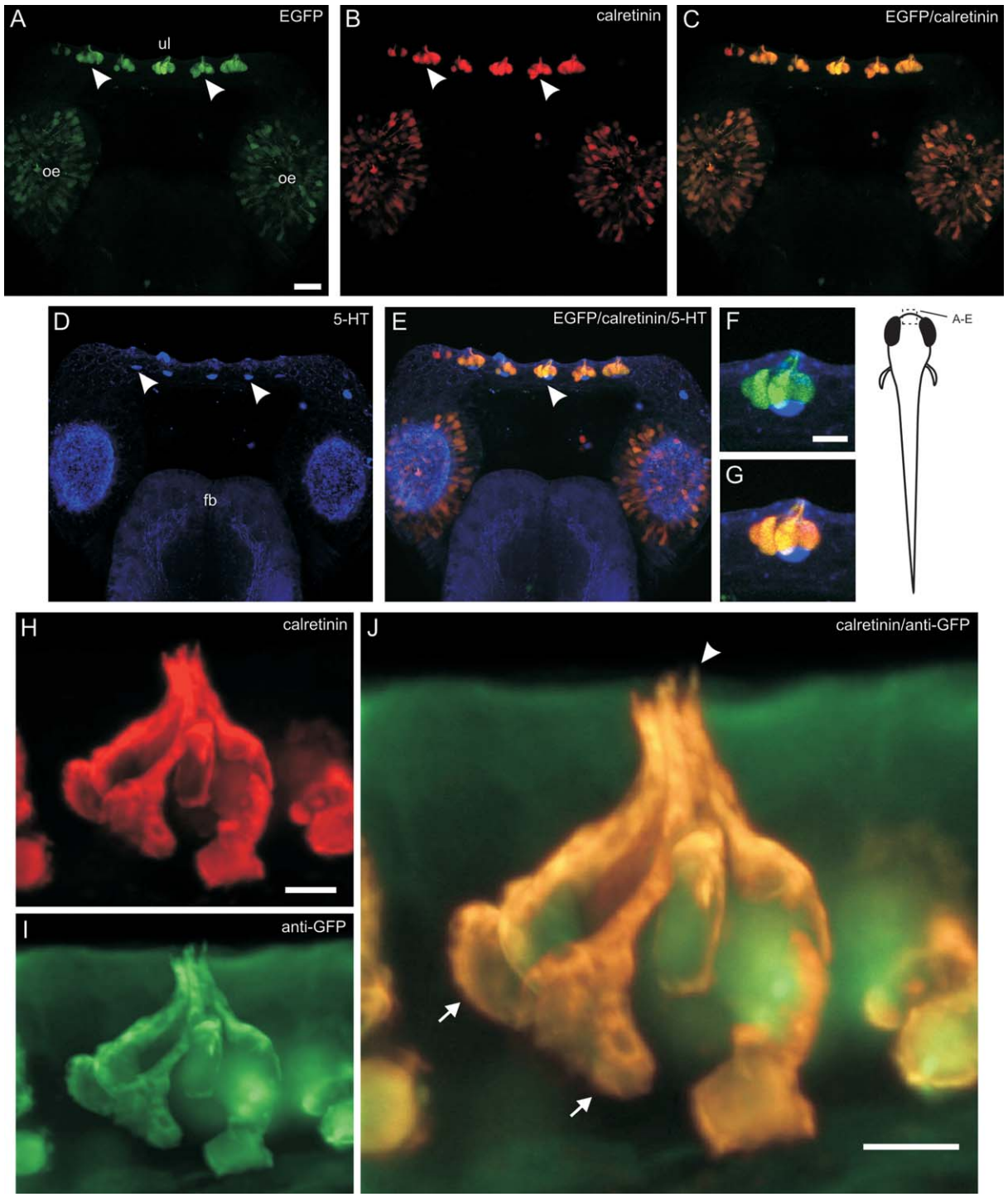

Figure 2

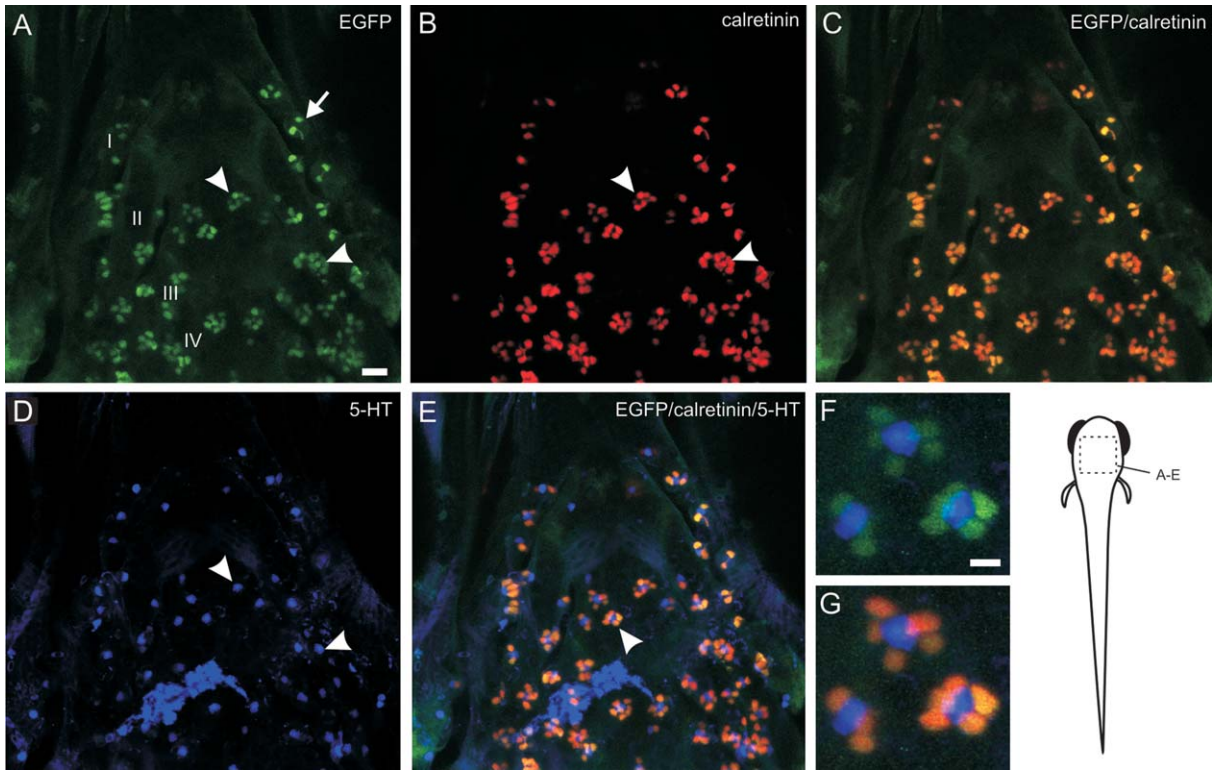

Figure 3 Taste cells in the branchial arches of ET5 transgenic zebrafish are EGFP-positive. Double immunolabeling with anti-calretinin and anti-5-HT combined with endogenous EGFP fluorescence in a 10 dpf larva oriented in ventral view with the rostral aspect facing the top of the figure (see schematic at lower right). A: EGFP fluorescence in taste cells (arrowheads) of the branchial arches and gill rakers. The location of branchial arches I–IV on one side is indicated. Arrow indicates a taste bud with only two cells labeled. B: Image in A showing localization of anti-calretinin in taste cells. C: Merged image shows colocalization of anti-calretinin and EGFP. D: In the same tissue, localization of Merkel-like cells (arrowhead) of the basal aspect of taste buds is shown with anti-5-HT. E: Merged image of EGFP, anti-calretinin and anti-5-HT indicates that EGFP-positive cells are taste cells. F, G: The group of three taste buds marked with an arrowhead in E is shown at higher magnification with only EGFP and 5-HT labeling (F), and EGFP and anti-calretinin/5-HT labeling (G). Scale bar in A is 20 μm and applies to B-E. Scale bar in F is 10 μm and applies to G. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Information Network) and based on previous studies (Jowett and Yan, 1996; Lopez et al., 2006; Welton et al., 2006; Manfroid et al., 2007). Briefly, embryos were hybridized at 70°C overnight (~16 h) with a dinitrophenol (DNP)-labeled *sall4* RNA probe (1,554 bases) and a digoxigenin (DIG)-labeled *egfp* RNA probe (663 bases). The DNP probe was incubated with anti-DNP-horseradish peroxidase (HRP). The signal was amplified with a tyramide signal amplification (TSA) system (NEL747A001KT, Perkin Elmer, Waltham, MA, USA) and revealed by tyr-Cy3 (NEL753001KT, Perkin Elmer). The DIG probe was subsequently incubated with anti-DIG-HRP. This signal was then amplified with the TSA system and revealed by tyr-fluorescein (NEL753001KT, Perkin Elmer). Twenty-five individuals were examined for *sall4* and *egfp* expression at 3 and 10 dpf. For imaging of *egfp* and *sall4* expression surrounding the mouth, heads of larvae were removed and oriented in frontal view immediately before confocal imaging. For 10 dpf larvae, individual gill arches were removed with fine forceps and mounted.

Microscopy and Image Analysis

In some experiments (i.e., Fig. 1), imaging of anesthetized ET5 larvae and adults was performed using a fluorescence microscope (Axiophot, Zeiss, Jena, Germany). Larvae were anesthetized with 0.06 mg mL^{-1} MS 222 prepared in E3 medium and immobilized in depression slides coated with Sylgard (Dow Corning Corporation, Midland, MI). Some specimens were additionally immobilized with 0.05% low-melting Agarose (A4018, Sigma, Oakville, ON, Canada), which helped to reduce small movements during imaging. Adults were anesthetized with 0.1 mg mL^{-1} MS 222 dissolved in dechlorinated water.

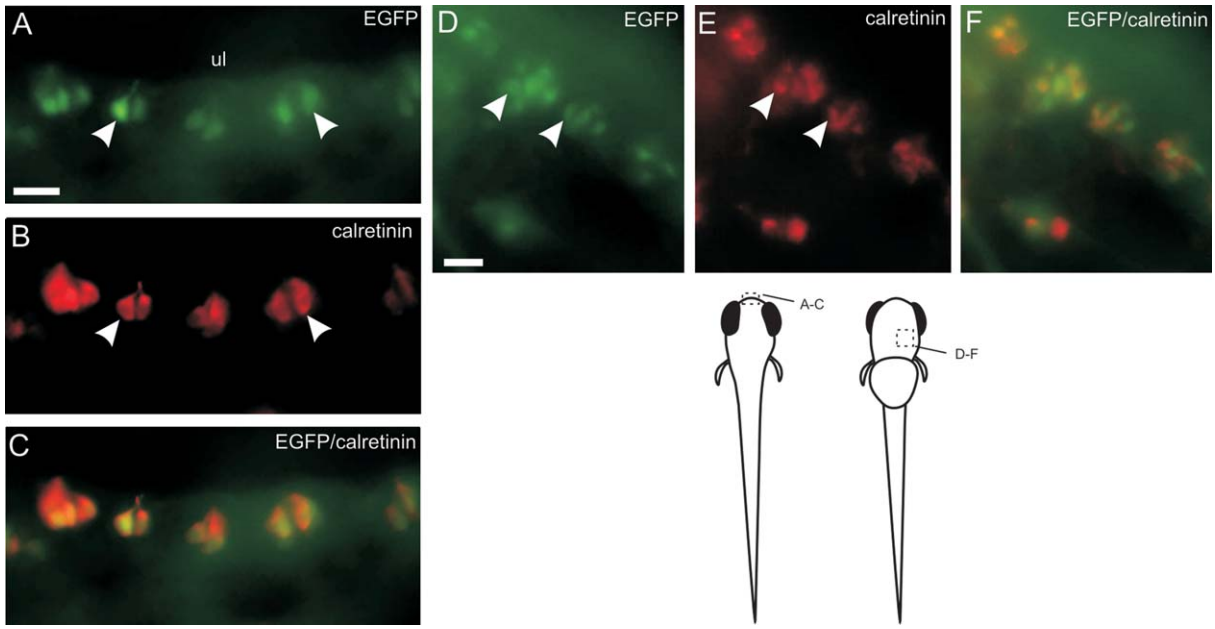

Figure 4 Taste cells in the lips and branchial arches of ET5 transgenic zebrafish at 4 days post-fertilization (dpf) are EGFP positive. Immunolabeling with anti-calretinin combined with endogenous EGFP fluorescence in a 4 dpf larva oriented in dorsal (A–C) or ventral (D–F) view with the rostral aspect facing the top of the figure (see schematic at lower right). A: EGFP fluorescence of taste cells (arrowheads) of the upper lip (ul). B: Image in A showing localization of anti-calretinin in taste cells. C: The merged image shows co-localization of anti-calretinin and EGFP. D: EGFP fluorescence of taste cells (arrowheads) in a branchial arch. E: Image in A showing localization of anti-calretinin in taste cells. F: Merged image shows colocalization of anti-calretinin and EGFP. Scale bars in A and D are 20 μm and apply to B–C and E–F, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Specimens labeled with EGFP, antibodies, or by FISH were examined using an epifluorescence microscope (Axiophot, Zeiss) or a confocal scanning system (LSM 510 META, Zeiss). For all samples imaged on the confocal system, a composite projection of optical sections was produced and digital images were prepared using Image J (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij) and CorelDraw 10 (Corel Corp., Ottawa, ON, Canada). Images from *in situ* hybridization were collected with a stereomicroscope (Leica, Wetzlar, Germany) equipped with a color video camera (3CCD Sony Corp., Tokyo, Japan). Images were captured with Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON, Canada).

RESULTS

EGFP Expression in ET5 Zebrafish

In anesthetized adult and larval ET5 transgenic zebrafish, EGFP expression was found in cells across the body and head, in the lens of the eye, the olfactory pits, and in the branchial arches. As shown in Figure 1(A), EGPF-positive cells were observed on the head of adult fish but were particularly concentrated on the upper and lower lips, the nasal and maxillary barbels, and the jaw. Similarly, we identified EGFP in cells in larvae concentrated on the upper and lower lips [Fig. 1(B)]. Both larval and adult wild-type zebrafish were observed under the same conditions; these specimens lacked detectable fluorescence.

Colocalization of EGFP with Immunohistochemical Markers of Taste Cells

In order to establish the identity of EGFP-positive cells in ET5 transgenic zebrafish, we utilized anti-calretinin, a known intracellular marker of taste cells in fish (Germanà et al., 2007; Zachar and Jonz, 2012). Fortunately, EGFP fluorescence in ET5 zebrafish was not attenuated in larvae by processing for immunohistochemistry (e.g., fixation and permeabilization), thus enabling us to counterstain fixed tissue from transgenic zebrafish.

In ET5 larvae, immunolabeling with anti-calretinin displayed colocalization with EGFP in labial taste cells [Fig. 2(A–C)]. EGFP-positive taste cells were also closely apposed to Merkel-like cells of taste buds labeled with anti-5-HT (Fig. 2D-G). In addition, cells of the olfactory epithelium, which were also

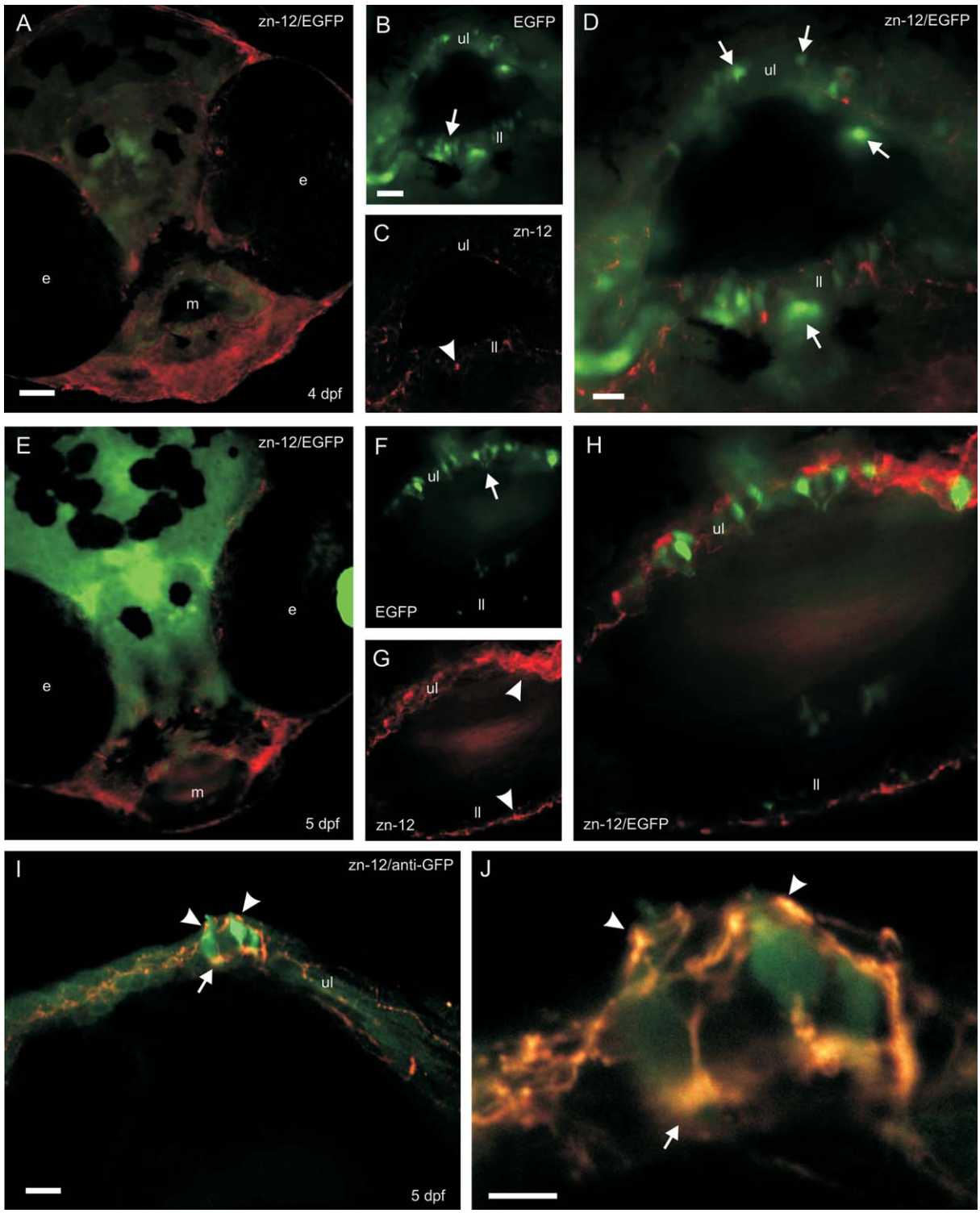

Figure 5

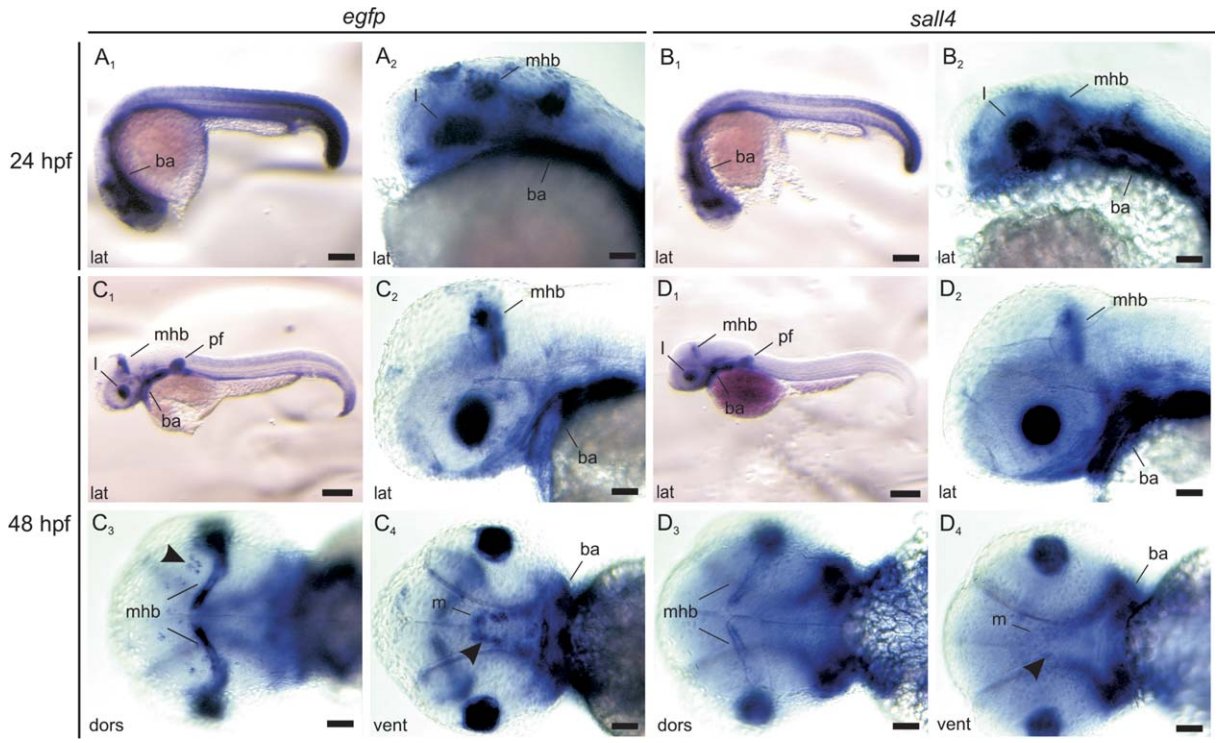

Figure 6 Whole-mount *in situ* hybridization showing expression of *sall4* in wild-type zebrafish embryos and *egfp* in ET5 transgenics. *egfp* (A) and *sall4* (B) expression at 24 h post-fertilization (hpf). *egfp* (C) and *sall4* (D) expression at 48 hpf. Arrowhead in C_3 indicates unidentified cells. Arrowheads in C_4 and D_4 indicate *egfp* and *sall4* labeling surrounding the mouth. Specimens are oriented in either lateral (lat), dorsal (dors) or ventral (vent) view. Abbreviations: branchial arches (ba), lens of the eye (l), midbrain-hindbrain boundary (mhb), mouth (m), pectoral fin bud (pf). Scale bars are 50 μm in A_1, B_1; 25 μm in A_2, B_2, C_{2-4}, D_{2-4}; 100 μm in C_1, D_1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

calretinin-immunoreactive, displayed relatively weak EGFP fluorescence [Fig. 2(A–C,E)]. In adult tissue, anti-calretinin and anti-GFP (used to amplify the endogenous EGFP signal in adult tissue) were co-localized in labial taste cells [Fig. 2(H–J)]. GFP labeling in the taste buds extended from taste cell bodies to apical microvilli that penetrated the taste receptor area [Fig. 2(J)]. In some cases, a single, large

Figure 5 The neural innervation of EGFP-positive taste cells increases between 4 and 5 days post-fertilization (dpf). A–D: ET5 larvae a 4 dpf. E-J: ET5 larva at 5 dpf. A: Combined endogenous EGFP fluorescence with zn-12 immunolabeling shows the location of nerve fibers and endings relative to EGFP-positive taste cells in a 4 dpf larva. e, eye; m, mouth. B, C: EGFP and zn-12 labeling separated indicates taste cells (arrow) and nerve fibers (arrowhead) in the lower lip (ll). ul, upper lip. D: Merged images from B and C at higher magnification illustrate that at this stage many EGFP-positive labial taste cells are not innervated (arrows). E: Combined EGFP fluorescence with zn-12 immunolabeling shows the distribution of nerve fibers relative to EGFP-positive taste cells in a 5 dpf larva. e, eye; m, mouth. F, G: EGFP and zn-12 labeling separated indicates taste cells (arrow) and nerve fibers (arrowheads) in the upper and lower lip. H: Merged images from F and G at higher magnification illustrate that at 5 dpf there are many more nerve fibers present in the lips surrounding taste cells. I, J: A lip preparation similar to that shown in H illustrates zn-12-immunoreactive nerve fibers extending to the taste receptor area (arrowheads) of a taste bud containing EGFP-positive taste cells. Innervation at the basal aspect of a taste cell is also shown (arrow). Scale bar in A, 50 µm (applies to E). Scale bar in B, 10 µm (applies to C, F and G). Scale bar in D, 10 µm (applies to H). Scale bar in I, 10 µm. Scale bar in J, 5 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

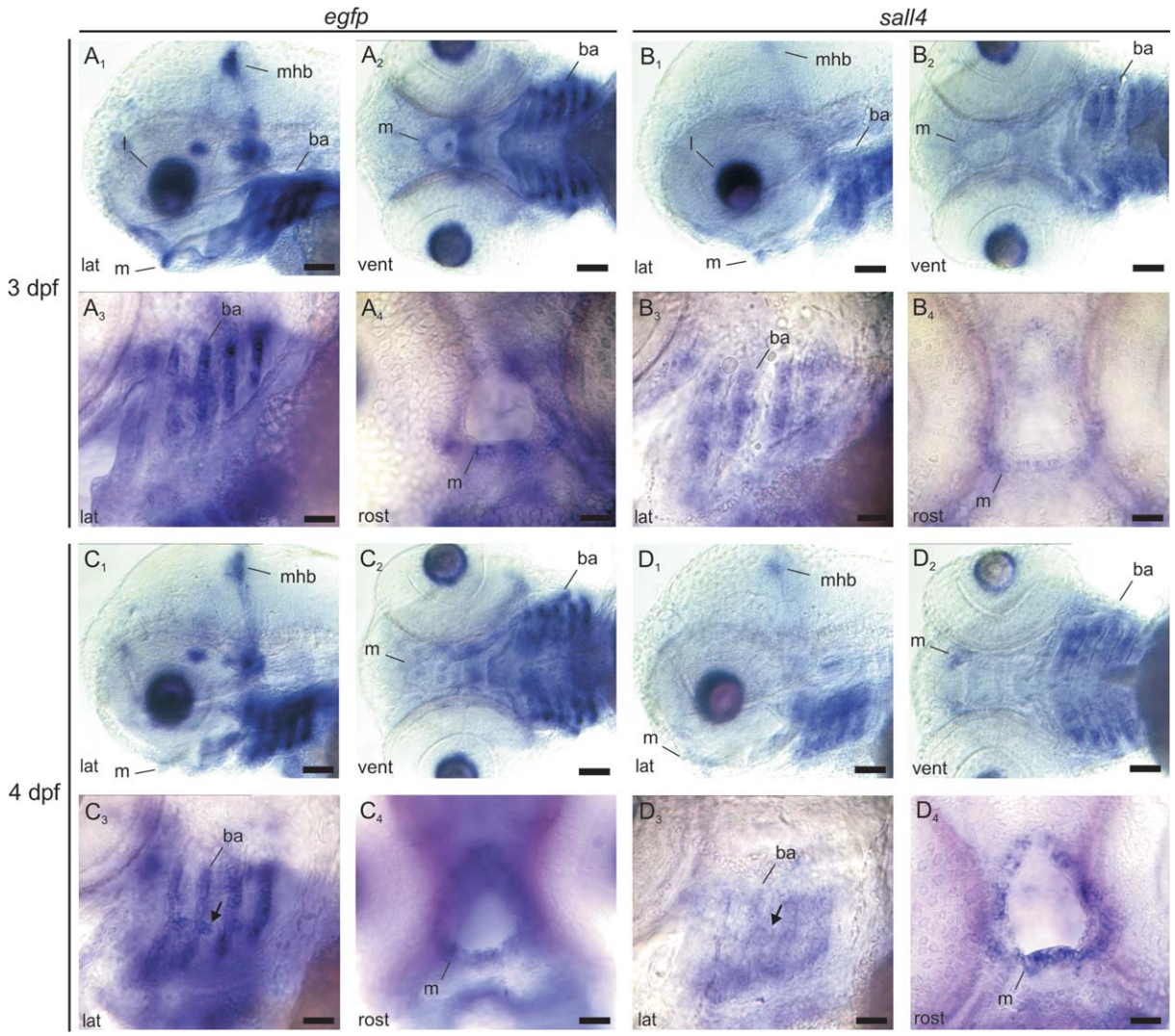

Figure 7 Whole-mount *in situ* hybridization showing expression of *sall4* in wild-type zebrafish larvae and *egfp* expression in ET5 transgenics. *egfp* (A) and *sall4* (B) expression at 3 days post-fertilization (dpf). *egfp* (C) and *sall4* (D) expression at 4 dpf. Arrows in C_3 and D_3 indicate developing gill filaments. Specimens are oriented in either lateral (lat), ventral (vent) or rostral (rost) view. Abbreviations: branchial arches (ba), lens of the eye (l), midbrain-hindbrain boundary (mhb), mouth (m), pectoral fin bud (pf). Scale bars are 50 μm in A_{1-2}, B_{1-2}, C_{1-2}, D_{1-2}; 25 μm in A_{3-4}, B_{3-4}, C_{3-4}, D_{3-4}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

microvillus of taste cells was observed in the taste receptor area [Fig. 2(J), arrowhead], which may be indicative of a gustatory light cell (Hansen et al., 2002). As in the lips, immunolabeling with anti-calretinin and anti-5-HT combined with EGFP fluorescence showed that EGFP labeled taste cells in the gill rakers and branchial arches in larvae (Fig. 3). Although in some cases only two or three taste cells within a taste bud were labeled in the branchial arches [e.g., arrow in Fig. 3(A)], we observed colocalization of EGFP and anti-calretinin in this tissue. Similar colocalization of EGFP and anti-calretinin was observed in the lips and branchial arches in earlier larvae at 4 dpf (Fig. 4).

Innervation of EGFP-Positive Taste Cells

At approximately the time that labial taste cells in zebrafish differentiate (4–5 dpf), nerve fibers penetrate developing taste buds to form a nerve plexus (Hansen et al., 2002). These nerve fibers will eventually innervate taste cells (Hansen et al., 2002; Zachar

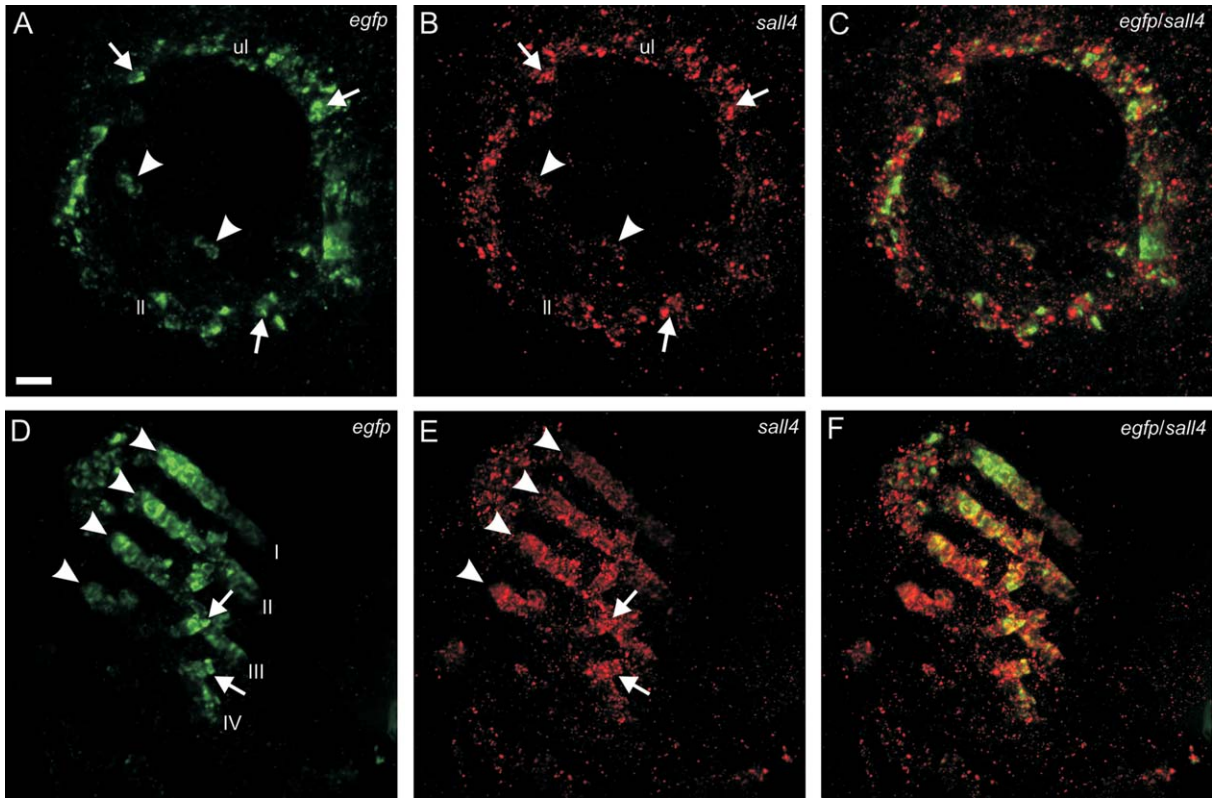

Figure 8 Confocal imaging of whole-mount fluorescence *in situ* hybridization showing expression of *egfp* and *sall4* in the lips (A–C) and branchial arches (D–F) of ET5 transgenic zebrafish at 3 days post-fertilization (dpf). A: Frontal view of *egfp* expression (arrows) in the upper lip (ul) and lower lip (ll) surrounding the mouth cavity. Note also expression in taste cells within the mouth cavity (arrowheads). The larva was oriented as in Figure 5. B: Image in A showing *sall4* expression in the same cells around the mouth. C: The merged image demonstrates colocalization of *egfp* and *sall4*. D: Ventral view of *egfp* expression in the branchial arches (indicated by arrowheads and numbered I-IV). Note also expression within gill filament primordia (2 filaments are indicated by arrows). The larva was oriented as in Figure 3 and the branchial arches on the left side are shown. E: Image in D showing *sall4* expression in the branchial arches and gill filaments. F: The merged image demonstrates colocalization of *egfp* and *sall4*. Scale bar in A is 20 μm and applies to all panels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and Jonz, 2012). To confirm that EGFP-positive taste cells received neural innervation, we observed EGFP fluorescence in taste buds during early development via immunolabeling of nerve fibers with zn-12 (Fig. 5). We found that taste cells were only sparsely innervated at 4 dpf, and most taste cells did not make contact with nerve fibers [Fig. 5(A–D)]. However, after 5 dpf there was a dramatic increase in nerve fibers surrounding taste cells in the lip epithelium, at which time this innervation appeared to target fully formed taste buds [Fig. 5(E–H)]. Further observation indicated that at 5 dpf nerve fibers appeared to make contact with EGFP-positive taste cells at the basal region of taste buds [Fig. 5(H–J)]. Moreover, nerve fibers extended to the taste receptor area, where they surrounded apical microvilli of taste cells [Fig. 5(I,J)].

Expression of *egfp* and *sall4* Are Colocalized in Developing Zebrafish

To further examine the control of EGFP expression in ET5 transgenic zebrafish, we evaluated insertion of the enhancer trap (ET) construct into the zebrafish genome to predict a candidate gene. Flanking sequences of insertion sites for ET5 (Choo et al., 2006) were analyzed using Ensembl (Zv9). Inserts were mapped onto chromosome 23 in proximity (~23 kb, but on a different contig) to the *sall4* gene. *sall4* is the closest gene to the insertion and encodes a zinc finger transcription factor (Jürgens, 1988; Reuter et al., 1989).

On the basis of our analysis of the ET5 insertion site, we evaluated transgenic expression of *egfp* in ET5 zebrafish, and expression of *sall4* in wild-type

zebrafish, using *in situ* hybridization (Figs. 6 and 7). In 24 hpf embryos, both *egfp* and *sall4* expression were observed in the developing branchial arches, the midbrain-hindbrain boundary, the lens of the eye, and in the tail [Fig. 6(A,B)]. At 48 hpf, *egfp* and *sall4* expression were additionally observed around the developing mouth and pectoral fin buds [Fig. 6(C,D)]. In 3 and 4 dpf larvae, the branchial arches and mouth were further developed. At these stages, both *egfp* and *sall4* expression were clearly visible in the lips surrounding the mouth and in individual branchial arches [Fig. 7(A–D)]. Overall, *sall4* expression in the wild-type zebrafish was strongly correlated with *egfp* expression in ET5 transgenic fish at the developmental stages analyzed. It is noteworthy that the expression of *egfp* appeared to be relatively stronger than that of *sall4*, and this may have resulted in some heterogeneity in expression patterns between *egfp* and *sall4* (e.g., Fig. $6A2_2$ vs. B_2 and C_3 vs. D_3). This may have been due to the presence of several tandem copies of the transgene, or to the differential strengths of the probes used in these experiments. In addition, while we observed EGFP activity in cells of the olfactory epithelium [Fig. 2(A)], *sall4* expression was not detectable in this tissue.

We confirmed colocalization of *egfp* and *sall4* expression in ET5 larvae using double fluorescence *in situ* hybridization and confocal imaging. At 3 dpf, *egfp* and *sall4* expression overlapped or occurred together within 1–2 μm in both the lips and branchial arches [Fig. 8(A–F)]. This suggests that *egfp* and *sall4* were both expressed within the same cells. At 10 dpf, we observed colocalization of *egfp* and *sall4* in the lips and branchial arches [Fig. 9(A–G)], though with a relatively weaker fluorescence signal when compared with 3 dpf. Additionally, we observed colocalization of *egfp* and *sall4* in gill filament primordia at both stages [Figs. 8(D–F) and 9(E–G)]. These are sites of respiratory chemoreceptors in zebrafish (Jonz and Nurse, 2005).

DISCUSSION

EGFP and Expression of *sall4* in Taste Chemoreceptors of Zebrafish

In this study, we investigated the development of taste buds in a zebrafish transgenic line and demonstrated that EGFP-positive cells surrounding the mouth, and in the branchial arches, were taste cells. We confirmed the identity of taste cells by combining EGFP fluorescence with immunohistochemistry. Taste cells were labeled with antibodies against calretinin, they resided near serotonergic Merkel-like cells within the taste bud, and they were first associated with zn-12-positive nerve fibers between 4 and 5 dpf. In addition, expression of *egfp* in ET5 transgenic embryos and larvae was co-localized with expression of *sall4*, a gene encoding a zinc finger transcription factor involved in the maintenance of stem cell pluripotency and self renewal (Zhang et al., 2006; Yang et al., 2008). This is the first report of *sall4* expression in chemoreceptors.

The enhancer activating EGFP expression was not previously identified for ET5 transgenic zebrafish by Parinov et al. (2004); however, this group has recently noted that the site of transgenic insertion was within the intergenic region upstream of *sall4* (ZETRAP 2.0). Our analysis of insertion sites in the zebrafish genome confirmed that a possible candidate for EGFP control was an enhancer of the *sall4* gene, based on its proximity to the site of insertion of the EGFP construct. Sall4 belongs to the Spalt family of transcription factors originally described in *Drosophila* (Jürgens, 1988; Reuter et al., 1989). In zebrafish, Sall4 has been implicated in pectoral fin development (Harvey and Logan, 2006), although expression of *sall4* in zebrafish up to 20 hpf also occurs in the brain, spinal cord, branchial arches and lens of the eye (Thisse et al., 2001; Harvey and Logan, 2006). Sall4 is a regulator of cell pluripotency and self-renewal in mouse embryonic stem cells, and interacts with a network of other transcription factors (Zhang et al., 2006; Yang et al., 2008). It has also been shown to be upregulated following limb amputation in amphibians at a stage corresponding to cell dedifferentiation and reprogramming, and to participate in blastema formation (Neff et al., 2011).

In this study we analyzed 24 hpf to 4 dpf zebrafish, a time frame corresponding to developmental stages when taste buds are forming. We found that transgenic expression of *egfp* in ET5 zebrafish was strongly correlated with expression of *sall4* in the wild-type fish at all stages tested. This suggests that an enhancer of *sall4* may promote EGFP expression in ET5 zebrafish. Further to previous studies that have investigated *sall4* expression in zebrafish (Thisse et al., 2001; Harvey and Logan, 2006), we show that *egfp* and *sall4* are also expressed in regions containing taste buds, such as around the mouth and in the branchial arches. Using double fluorescence *in situ* hybridization, we demonstrate that *egfp* and *sall4* expression were colocalized in these regions.

A Possible Role for *sall4* in the Taste Bud

Cells of taste buds have a limited life span and are maintained by continuous proliferation of epithelial

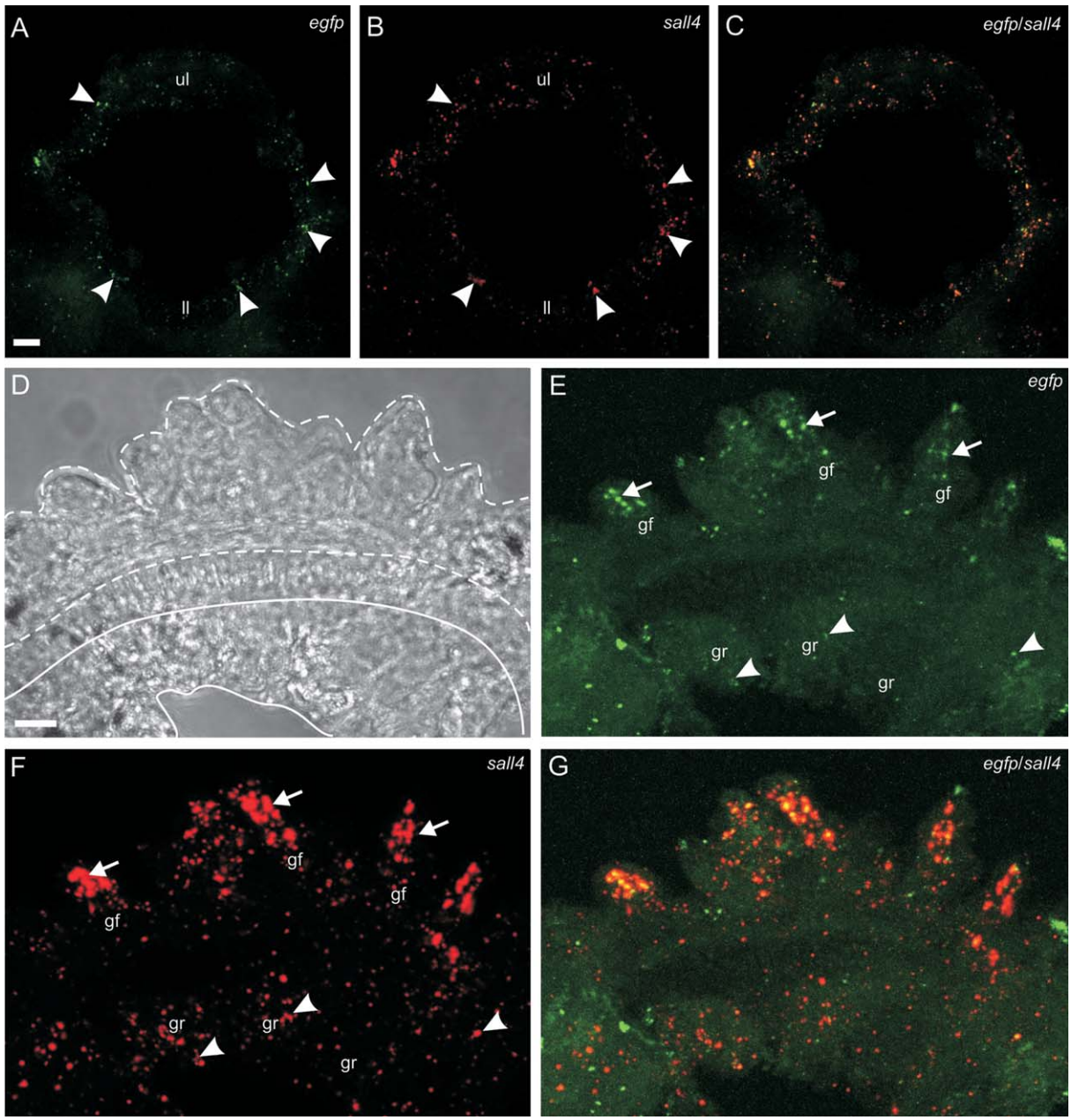

Figure 9 Confocal imaging of whole-mount fluorescence *in situ* hybridization showing expression of *egfp* and *sall4* in the lips (A–C) and an isolated branchial arch (D–G) of ET5 transgenic zebrafish at 10 days post-fertilization (dpf). A: Frontal view of *egfp* expression (arrowheads) in the upper lip (ul) and lower lip (ll) surrounding the mouth cavity. The larva was oriented as in Figures 5 and 8(A–C). B: Image in A showing *sall4* expression in the same cells around the mouth. C: The merged image demonstrates colocalization of *egfp* and *sall4*. D: Bright-field image of an isolated branchial arch. The region containing taste cells (i.e., the gill rakers) is indicated between by solid lines. Gill filament primordia are indicated between dashed lines. E: *egfp* expression in the same tissue shown in D. Note the strong expression in the gill filaments (arrows) but only weak expression in the gill rakers and branchial arches. gf, gill filaments; gr, gill rakers. F: Image in E showing *sall4* expression in filament primordia and gill rakers. G: The merged image demonstrates colocalization of *egfp* and *sall4*. Scale bar in A is 10 μm and applies to B and C; Scale bar in D is 20 μm and applies to E–G. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

stem and progenitor cells (Finger and Simon, 2000; Miura et al., 2006; Sullivan et al., 2010). The average turnover rate of taste bud cells in catfish, for example, is once every 12 days at 30°C (Raderman-Little, 1979). Although the "marginal cells" (not strictly part of the taste bud) may be regenerative or stem cells of taste buds in fish (Reutter and Witt, 1993; Northcutt, 2004), the identity of stem and progenitor cells, and the mechanisms of taste bud renewal, remain controversial (Stone et al., 2002; Miura et al., 2006; Sullivan et al., 2010; Miura and Barlow, 2010). We observed *sall4* and *egfp* expression in labial taste bud primordia at 48 hpf, a time that precedes complete formation of the taste buds (Hansen et al., 2002). In addition, EGFP was continuously expressed in taste cells of larvae and adults that were colabeled with calretinin. These cells were associated with nerve fibers and, in some cases, had large microvilli suggestive of gustatory light cells (Hansen et al., 2002). These results suggest that the Sall4 transcription factor may regulate stem cell populations in the taste buds of zebrafish, and that multiple populations of stem or progenitor cells may exist within the taste bud, as has been proposed by other authors (Stone et al., 2002; Miura et al., 2006; Sullivan et al., 2010). Cells within the taste bud are generally presumed to be postmitotic and express cell cycle inhibiting proteins (Miura and Barlow, 2010). However, *sall4* expression in zebrafish suggests that taste cells are capable of re-entry into the cell cycle, or dedifferentiation. In mouse, taste cells previously thought to be postmitotic appear to re-enter the cell cycle and undergo rapid cell division (Sullivan et al., 2010), and mitotic cells within the taste bud of rabbit have been observed at the ultrastructural level (Toyoshima and Tandler, 1986). Thus, while the role of *sall4* in taste cells in zebrafish is presently unclear, it may be involved in taste cell dedifferentiate to promote subsequent cell renewal or regeneration.

It was recently demonstrated that taste buds of the maxillary barbels in zebrafish undergo complete regeneration after amputation (LeClair and Topczewski, 2010). One potential mechanism may involve the transcription factor, Sox2, which interacts with Sall4 (Zhang et al., 2006; Yang et al., 2008; Neff et al., 2011). Sox2 has been implicated in embryonic taste bud development (Okubo et al., 2006), and is expressed in taste cells and progenitor cells surrounding taste buds of mice (Suzuki, 2008; Okubo et al., 2009) and in taste cells of zebrafish (Germanà et al., 2009). Furthermore, there is evidence for hair cell dedifferentiation in the avian inner ear (Warchol, 2011), and the dedifferentiation potential of postmitotic supporting cells into otic stem cells is correlated with methylation of *Sox2* enhancers in mammals (Waldhaus et al., 2012). Collectively, these observations suggest a combined role for Sall4 and Sox2 in taste bud development and/or regeneration. Alternatively, Merkel-like basal cells may regulate taste organ morphogenesis, as they do in amphibians (Toyoshima et al., 1999). However, we did not find *sall4* expression in Merkel-like cells, and these cells were not immunoreactive for Sox2 in zebrafish (Germanà et al., 2009).

Our results from *in situ* hybridization also demonstrated that *sall4* and *egfp* expression were colocalized within the gill filaments as early as 3 dpf and up to 10 dpf. In zebrafish, the gill filaments are structures that support the respiratory lamellae (where gas exchange occurs) and are sites of respiratory chemoreceptors that first appear during development at 3 dpf (Jonz and Nurse, 2005). Future studies may reveal whether *sall4* also plays a role in the development of respiratory chemoreceptors.

CONCLUSIONS

The present study has demonstrated the expression of *sall4* in taste cells of zebrafish, and that EGFP fluorescence in the ET5 transgenic line may serve as a marker of *sall4* expression in taste buds *in vivo*. An understanding of the role of Sall4 in taste buds, and its potential link with other transcription factors that may regulate stem cells, awaits further study. The most powerful application of the ET5 line may be to observe and characterize putative stem or progenitor cell populations in embryonic and adult zebrafish so as to further understand the mechanisms of taste bud development and maintenance.

The authors thank Dr. Vladimir Korzh for providing the ET5 transgenic line. zn-12 was developed by Trevarrow et al. (1990) and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, 52242.

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