Development 138, 3473-3484 (2011) doi:10.1242/dev.058669
© 2011. Published by The Company of Biologists Ltd

Fgf signaling controls pharyngeal taste bud formation through miR-200 and Delta-Notch activity

Marika Kapsimali[1,2,3,*], Anna-Lila Kaushik[1,2,3], Guillaume Gibon[1,2,3], Lara Dirian[1,2,3], Sylvain Ernest[1,2,3] and Frederic M. Rosa[1,2,3,*]

SUMMARY

Taste buds, the taste sensory organs, are conserved in vertebrates and composed of distinct cell types, including taste receptor, basal/presynaptic and support cells. Here, we characterize zebrafish taste bud development and show that compromised Fgf signaling in the larva results in taste bud reduction and disorganization. We determine that Fgf activity is required within pharyngeal endoderm for formation of Calb2b$^+$ cells and reveal miR-200 and Delta-Notch signaling as key factors in this process. miR-200 knock down shows that miR-200 activity is required for taste bud formation and in particular for Calb2b$^+$ cell formation. Compromised *delta* activity in *mib*$^{-/-}$ dramatically reduces the number of Calb2b$^+$ cells and increases the number of 5HT$^+$ cells. Conversely, larvae with increased Notch activity and *ascl1a*$^{-/-}$ mutants are devoid of 5HT$^+$ cells, but have maintained and increased Calb2b$^+$ cells, respectively. These results show that Delta-Notch signaling is required for intact taste bud organ formation. Consistent with this, Notch activity restores Calb2b$^+$ cell formation in pharyngeal endoderm with compromised Fgf signaling, but fails to restore the formation of these cells after miR-200 knock down. Altogether, this study provides genetic evidence that supports a novel model where Fgf regulates Delta-Notch signaling, and subsequently miR-200 activity, in order to promote taste bud cell type differentiation.

KEY WORDS: *ascl1a*, *sox2*, Calbindin 2, Serotonin, Pharyngeal epithelium, Mindbomb, Zebrafish

INTRODUCTION

Taste buds are the vertebrate sensory organs of taste located in a patterned manner in the oropharyngeal cavity. A typical differentiated taste bud is an onion-shaped structure composed of support (type I, glial-like), taste receptor (type II, sensing bitter, sweet, umami or salt, mainly communicating with the sensory neurons via ATP) and presynaptic (mammalian type III, forming synapses with sensory afferents and sensing sour) and/or basal Merkel-like (in fish and amphibia) cells (Chandrashekar et al., 2010; Chaudhari and Roper, 2010; Delay et al., 1997; Delay et al., 1993; Finger et al., 2005; Hansen et al., 2002; Krimm, 2007; Miura et al., 2006; Northcutt, 2004; Roper, 2007; Zachar and Jonz, 2011). Taste buds are induced in epithelial sites, the placodes, which, in mammals, generate papillae: tongue structures that can contain several taste buds. Although principally considered to be similar in cell type content and function, taste buds have dual origin in vertebrates. Taste papillae/buds derive from local epithelium. Rostral and caudal mammalian taste buds derive from ectoderm and endoderm, respectively, and endodermal signals may influence taste bud induction in adjacent ectoderm (Barlow, 2000; Barlow and Northcutt, 1995; Stone et al., 1995). Given the diversity of taste bud localization in the vertebrate oropharynx (i.e. mammalian tongue versus teleost pharyngeal arch epithelium), defining the tissue of origin of a particular taste bud population is prerequisite for dissecting the molecular mechanisms that underlie the formation of these sensory organs.

[1]Ecole Normale Supérieure, Institut de Biologie, 75005 Paris, France. [2]INSERM U1024, 75005 Paris, France. [3]CNRS UMR 8197, 75005 Paris, France.

*Authors for correspondence (kapsimal@biologie.ens.fr; rosa@biologie.ens.fr)

Accepted 14 June 2011

Molecular studies have almost exclusively focused on mammalian rostral (fungiform) taste papillae (for a review, see Chaudhari and Roper, 2010; Krimm, 2007). Shh-expressing cells in rostral taste placodes generate support and receptor cells (Thirumangalathu et al., 2009). Shh, Egf and Bmp signaling regulate taste bud patterning on the rostral tongue (Hall et al., 2003; Liu et al., 2008; Mistretta et al., 2003; Zhou et al., 2006). For example, follistatin-null mice form ectopic differentiated taste buds on the tongue (Beites et al., 2009). The effects of Shh and Bmp signaling on rostral taste bud formation are at least partially mediated by Wnt/β-catenin activity. Abrogation of β-catenin signaling leads to severe reduction of rostral papillae; conversely, Wnt/β-catenin activity increases their number and size (Iwatsuki et al., 2007; Liu et al., 2007; Mistretta et al., 2003; Zhou et al., 2006). A downstream target of Wnt/β-catenin signaling is Sox2, a transcription factor expressed in taste bud cells and required for the maintenance of both fungiform and palate papillae (Okubo et al., 2008; Okubo et al., 2006). Therefore, the early molecular interactions of taste placode and bud induction are rather well studied in the rostral tongue.

By contrast, little is known about the development of caudal (mammalian circumvallate) taste buds. Surprisingly, inactivation of key signals such as follistatin or Wnt/β-catenin, which are necessary for proper rostral taste bud development, leaves the caudal organs unaffected (Beites et al., 2009; Liu et al., 2007). This suggests that other signals are involved in this process. Several pieces of evidence support the idea that Notch activity is required for mammalian caudal taste bud formation. Notch ligands (Dll1, Jag1 and Jag2) and receptors (Notch1, Notch2 and Notch 3) are broadly expressed in mouse embryonic posterior papillae (Miura et al., 2006; Seta et al., 2003). Abrogation of *hes1*, a Notch signaling intracellular repressor, results in taste receptor cell increase in posterior papillae (Ota et al., 2009), but the underlying cellular mechanism of this defect remains unknown. *ascl1* is expressed in

DEVELOPMENT

immature developing taste bud cells, which could give rise to taste receptor and presynaptic cells (Miura et al., 2006), or exclusively in differentiated presynaptic cells (Seta et al., 2006). However, the role of *ascl1* in taste bud formation has not been functionally explored. Therefore, how, subsequent to initial organ induction, distinct cell types – support, receptor and presynaptic – differentiate to form a functional taste bud remains unclear.

Fgf signaling, although crucial for the development of several sensory organs, has not been examined in the context of taste bud formation (Hayashi et al., 2008; Schimmang, 2007; Schneider-Maunoury and Pujades, 2007). Fgf signaling is activated when two heparan sulfate-connected Fgf ligands bind to extracellular domains of Fgf receptors (Eswarakumar et al., 2005). Several Fgf receptors are expressed in the vertebrate oropharynx around the timing of taste bud formation. For example, mouse *Fgfr2b* and *Fgfr1c* are expressed in the tongue at E11 to E13 (Nie, 2005). Zebrafish *fgfr1*, *fgfrl1a*, *fgfrl1b* and *fgfr2* are expressed in pharyngeal arch epithelium (Hall et al., 2006; Thisse and Thisse, 2005; Tonou-Fujimori et al., 2002) during the second day post fertilization (dpf), i.e. after the formation of pharyngeal pouches (Crump et al., 2004) and prior to taste bud differentiation, raising the possibility that Fgf signaling plays a role in taste bud formation.

Finally, among factors that promote cell differentiation, the miR-200 family (miR-200) induces epithelial differentiation by inhibiting the expression of stem cell transcription factors such as *sox2* (Wellner et al., 2009). In addition, miR-200 are required for late steps of olfactory epithelium differentiation (Choi et al., 2008). miR-200 is divided into two subfamilies defined by their seed regions: miR-200a and miR-141; and miR-200b, miR-200c and miR-429 (Flynt et al., 2009). miR-200 family members are expressed in the taste buds (Kapsimali et al., 2007; Wienholds et al., 2005). However, whether they play a role in taste bud cell differentiation remains to be established.

In this work, we identify molecular interactions that guide taste bud formation and, in particular, cell type differentiation in the posterior taste buds in zebrafish. We chose zebrafish as a model, as posterior, pharyngeal, taste buds are numerous, easily accessible and not assembled into papillae, allowing us to decipher the cellular and molecular events involved in taste bud generation per se. We first reveal that Fgf signaling is required for taste bud formation. Second, we show that miR-200 activity is necessary for taste bud formation and, in particular, differentiation of Calb2b$^+$ cells. Then we examine whether Notch signaling is implicated in zebrafish taste bud formation as in mammals. Finally, by manipulating gene expression within the pharyngeal endoderm, we address whether and how Fgf, miR-200 and Delta-Notch signals interact to regulate taste bud development.

MATERIALS AND METHODS

Fish strains

Embryos were obtained from natural spawning of wild-type (*AB, TL), *ace*^{ti282a} (Reifers et al., 1998), *ascl1a*$^{t25215/t25215}$ (Pogoda et al., 2006), *mib*$^{ta52b/ta52b}$ (Itoh et al., 2003), *Tg(hsp70l:dnfgfr1-EGFP)pd1/+* (Lee et al., 2005) and *Tg(hsp70l:Gal4)1.5kca4/+;Tg(UAS:myc-notch1a-intra)kca3/+* (Scheer and Campos-Ortega, 1999) zebrafish lines.

Construction of *Tg(tph1b:egfp)* zebrafish

A 5 kb fragment just upstream of the zebrafish *tph1b* gene was PCR amplified and cloned into the Tol2pT2KHGpGATA2. To generate transgenic fish, one-cell embryos were injected with pT2KHG-Promoter *tph1b* and transposase RNA, then screened at 72 hours post fertilization (hpf) for eGFP fluorescence in taste buds. eGFP expression was found similar to 5HT expression in the developing taste buds (see Fig. S1M-O in the supplementary material, data not shown).

Microinjection, transplantation and heatshock experiments

Synthetic mRNAs were transcribed using mMessage mMachineTM (Ambion). For transplantation experiments, donor embryos [wild type or *Tg(hsp70l:dnfgfr1-EGFP)pd1/+* (*Hsdnfgfr1*), or *Tg(hsp70l:Gal4)1.5kca4/+;Tg(UAS:myc-notch1a-intra)kca3/+* (*HsNicd*) or *Tg(hsp70l:dnfgfr1-EGFP)pd1/+; Tg(hsp70l:Gal4)1.5kca4/+;Tg(UAS:myc-notch1a-intra)kca3/+* (*Hsdnfgfr1;HsNicd*)] were injected at the four-cell stage with *gfp, mcherry* (70-100 pg) RNA or miniruby (Invitrogen) as a lineage tracer and zebrafish *tar** RNA (2 pg) or *cas* RNA (100 pg) (Ai et al., 2007; Dickmeis et al., 2001; Kikuchi et al., 2001). Five to ten cells from sphere stage donors were transplanted into the marginal zone of sphere stage host embryos as described previously (David and Rosa, 2001). In these grafts, the total size of the pharyngeal endoderm clone is comparable between transgene and tracer-expressing embryos.

Heatshocks were performed as follows: 38-42 hpf *Hsdnfgfr1* embryos at 37-38.5°C for 2 hours; 52-54 hpf *HsNicd* embryos at 40°C for 1 hour; 52-54 hpf wild-type embryos transplanted with cells from *Hsdnfgfr1* donors at 38.5°C for 2 hours; or from *HsNicd* donors at 40°C for 2 hours; or from *Hsdnfgfr1;HsNicd* donors at 40°C for 2 hours.

When necessary, to ensure constant activation of the transgene, successive heatshocks were performed every 12 hpf (38.5 or 40°C for 30-60 minutes) until fixation. The overall development of the larvae was not affected by the heatshock itself; however, heat-shocked embryos were delayed for about 2 hours compared with embryos without heatshock. Wild-type embryos after heatshock have lower taste bud cell numbers than non heatshocked embryos, and they are comparable with wild-type embryos staged ~1-2 hours earlier (n=10, P<0.01). As a result, we considered that in whole-mount and graft experiments, the comparison of taste bud cells is reliable among heatshocked embryos (transgene+ or tracer+).

Morpholinos against miR-200a, miR-200b and miR-429 are as published (Choi et al., 2008): anti-miR-200a (5′-AACATCGTTACCAGACAGTGTTAGA-3′), anti-miR-200b (5′-GTCATCATTACCAGGCAGTATTA-3′), anti-miR-429 (5′-ACGGCA-TTACCAGACAGTATTA-3′) (Genetools). Control morpholino was 5′-CCTCTTACCTCAGTTACAATTTATA-3′. Stock solutions were diluted at 2 mM in Danieau. In single MO injections, maximal injected quantity was 1 ng for MOmiR-200a and 1.3 ng for MOmiR-200b, MOmiR-429 or MOcontrol. In the triple MOmiR-200 injection, 0.3 ng MOmiR-200a, 0.45 ng MOmiR-200b and 0.45 ng MOmiR-429 were co-injected.

SU5402 treatment

SU5402 [Calbiochem (Mohammadi et al., 1997)], was diluted to final concentration of 20 μM in embryo medium. Control embryos were treated with the same amount of DMSO.

In situ hybridization and immunohistochemistry

In situ hybridization and whole-mount immunohistochemistry were carried out as described previously (Hauptmann and Gerster, 2000; Kapsimali et al., 2007). Probes used were for: *ascl1a* (Li et al., 1994); *dlb* (Haddon et al., 1998); *fgf8* (Reifers et al., 1998); *fgfr4* (Thisse et al., 1995); *fgfrl1a* (Hall et al., 2006); *foxa2/axial* (Strahle et al., 1993); miR-200a, miR-200b/c and miR-429 (Wienholds et al., 2005); *notch1a* (Bierkamp and Campos-Ortega, 1993); *pea3* (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001); and *sox2* (Cunliffe and Casaccia-Bonnefil, 2006). Primary antibodies were rabbit anti-GFP (1/1000, Torrey-Pines), rat anti-GFP (1/500, NacalaiTesque), rabbit anti-5HT (1/1000, Sigma), rabbit or mouse anti-Calb2b (1/1000, Swant), rabbit anti-active caspase 3 (BD Biosciences), mouse anti-Prox1 (1/500, Millipore) and mouse anti-Myc (1/200, Sigma). Secondary antibodies were conjugated with Alexa fluorochromes 350, 488, 568 or 647 (Invitrogen).

Microscopy and image analysis

For bright-field photography, embryos were photographed on a Nikon SMZ1500 stereoscope or a Leica upright microscope using a Nikon camera. Fluorescent images were obtained by Leica TCS-SP2-AOBS or

DEVELOPMENT

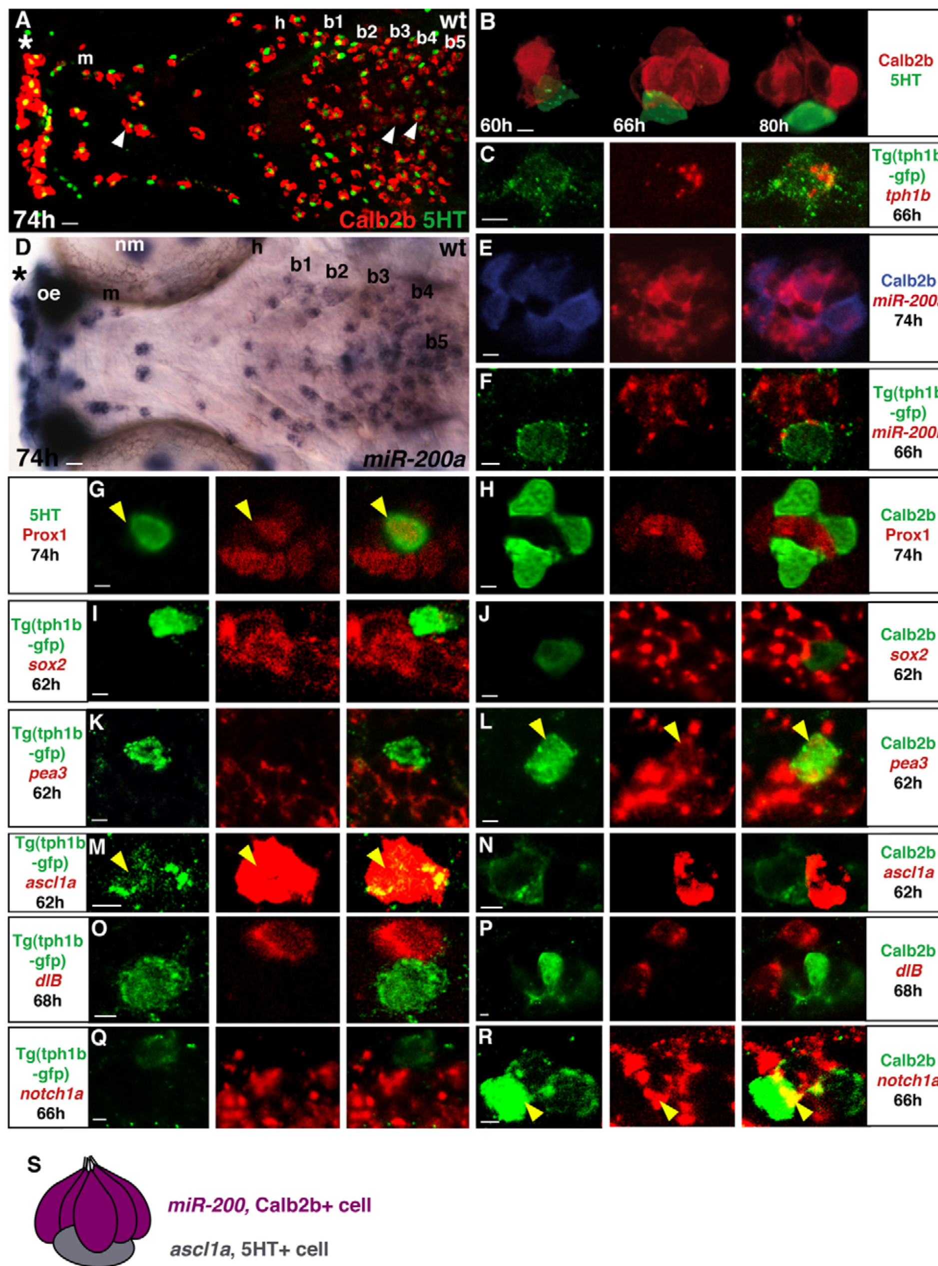

Fig. 1. Taste buds are patterned in the zebrafish oropharynx and composed of distinct cell types. Marker expression in developing taste buds. Experimental conditions are indicated in the top right-hand corner, embryonic stage and scale bar are in the bottom left-hand corner and marker expression is in the bottom right-hand corner or on the left or right. Anterior is towards the left. (**A**,**D**) Ventral views of whole-mount zebrafish larvae heads showing taste bud cells expressing Calb2b (A, red), 5HT (A, green) or miR-200a (D, blue). Asterisks indicate the row of taste buds localized in the lips. Arrowheads in A indicate palate taste buds. Scale bar: 10 μm. (**B**) Confocal images showing the shape of the developing taste bud organ. (**C**) Optical section (1 μm) showing co-expression (yellow) of *tph1b* mRNA and GFP expression in *Tg(tph1b:egfp)* larva. (**E-R**) Optical sections (0.5-1.5 μm) showing the relationship between Calb2b, 5HT or *Tg(tph1b:egfp)* and other taste bud marker expression. Yellow arrowheads indicate marker co-expression in a single cell. (E) Most miR-200a (red) cells express Calb2b (blue). Scale bars: 2 μm. (**S**) The main markers discriminating Calb2b$^+$ (purple) and 5HT$^+$ (gray) cells. b1-b5, branchial arches 1-5; h, hyoid arch; m, mandibular arch; nm, neuromast; oe, olfactory epithelium.

SP5 confocal microscopes using 25×/40×/63× oil immersion objectives. Series of images were acquired at 0.4-2.5 μm intervals. ImageJ (NIH), Adobe Photoshop and Illustrator were used to analyze stacks, adjust brightness/contrast and mount images. Three-dimensional reconstructions and cell counting were carried out using Imaris (Bitplane).

RESULTS

Zebrafish oropharyngeal taste buds are composed of distinct cell types

To analyze taste bud development in zebrafish, we first analyzed expression of taste bud markers already characterized in jawed vertebrates. Serotonin (5HT) is expressed in basal Merkel-like taste bud cells in fish (Zachar and Jonz, 2011), amphibia (Barlow and Northcutt, 1995; Delay et al., 1993) and mammalian presynaptic cells (Dvoryanchikov et al., 2007). Calretinin (Calb2b), a teleost taste bud marker (Diaz-Regueira et al., 2005; LeClair and Topczewski, 2010; Yamamoto et al., 2009) is expressed in mammalian taste receptor cells (Rebello et al., 2011). Calb2b has been reported as one of the earliest markers of taste buds in teleosts (Northcutt, 2005) compared with other taste transduction molecules expressed in 5 dpf larvae and adult, when the taste sense is already functional (e.g. Aihara et al., 2007). Therefore, we focused on 5HT and Calb2b expression to search for taste buds in the early developing zebrafish larva. Consistently, we found two taste bud cell populations in zebrafish oropharynx: one that was basal, oval-shaped, Merkel-like and 5HT$^+$ (Fig. 1A,B; or *Tg(tph1b:egfp)* expressing, Fig. 1C; see Fig. S1M-O in the supplementary material); the other pear-shaped, Calb2b$^+$ and later acquiring the characteristic taste receptor cherry shape (Fig. 1A,B, see Fig. S1A-C,G in the supplementary material). As physiological data that precisely characterize 5HT$^+$ and Calb2b$^+$ taste bud cells are unavailable in teleosts, and co-expression of 5HT and Calb2b was never observed in taste bud single cells (60 hpf to 6 dpf, Fig. 1B, see Fig. S1G in the supplementary material, data not shown), we

Table 1. Number of 5HT+ and Calb2b+ cells in the developing zebrafish oropharynx at 60-74 hpf

Wild type (60-61 hpf)	5HT+ cells	Calb2b+ cells
Lips	8	8
Pharyngeal arches	53	40
Palate	22	7
Total	83±7	55±4
Wild type (62-63 hpf)		
Lips	12	14
Pharyngeal arches	75	54
Palate	29	35
Total	116±9	103±3
Wild type (66-68 hpf)		
Lips	15	46
Pharyngeal arches	82	155
Palate	39	109
Total	136±9	310±12
Wild type (69-70 hpf)		
Lips	16	48
Pharyngeal arches	94	193
Palate	42	131
Total	152±6	372±14
Wild type (71-74 hpf)		
Lips	18	52
Pharyngeal arches	121	275
Palate	64	157
Total	203±7	484±14

Data are mean±s.d.; *n*=9.

refer for clarity and precision to these two distinct taste bud cell populations as 5HT+ and Calb2b+ cell types. Isolated oropharyngeal 5HT+ cells were observed from 59 to 60 hpf in the lips, pharyngeal arch (mandibular, hyoid and branchial arches 1-5) and palate epithelium (see Fig. S1A,G in the supplementary material). Slightly later, Calb2b+ cells became obvious, adjacent to 5HT+ cells with their number progressively increased during the third developmental day (Fig. 1A,B, see Fig. S1A-C in the supplementary material, Table 1). At that time, the patterned distribution of taste buds in zebrafish oropharynx is also evident (Fig. 1A, see Fig. S1A-C in the supplementary material). These results reveal that differentiating taste bud cells (5HT+ and Calb2b+) are obvious in zebrafish as early as 60 hpf, well before it was previously indicated (Aihara et al., 2007; Hansen et al., 2002).

To obtain insight into the formation of 5HT+ and Calb2b+ cell types, we analyzed a combination of early and late markers. miR-200 family members miR-200a, miR-200b and miR-429 are expressed in the taste bud region from 60 hpf onwards (Fig. 1D, Fig. 2D, data not shown). Most miR-200-expressing cells co-express Calb2b but not *Tg(tph1b:egfp)*, indicating that miR-200 expression is characteristic of Calb2b+ cells (Fig. 1E,F). By contrast, from 60 hpf, cells that express *ascl1a* are devoid of Calb2b but many of them express *Tg(tph1b:egfp)*, indicating that *ascl1a* is an early marker of 5HT+ cells (Fig. 1M,N). Prox1 is generally expressed from 60 hpf in taste bud and surrounding epithelial cells. 5HT+ cells express Prox1, whereas Calb2b+ cells have low or are devoid of Prox1 expression (Fig. 1G,H). Finally, *sox2* is expressed along the entire oropharyngeal epithelium (see Fig. S1D in the supplementary material). Calb2b+ and 5HT+ cells have low levels or are devoid of *sox2* expression, although adjacent epithelial cells express high levels of *sox2* (Fig. 1I,J). Altogether, these data show that at least two distinct cell populations characterized by miR-200 and Calb2b, and by *ascl1a* and 5HT expression can easily be recognized in the 2.5-3 dpf zebrafish larvae (Fig. 1S).

Pharyngeal Calb2b and 5HT expressing cells originate from pharyngeal endoderm

To dissect the molecular mechanisms that underlie the formation of posterior (pharyngeal) taste buds, experimental manipulation of their tissue of origin is required. In other vertebrates, posterior taste buds derive from pharyngeal endoderm. To examine the origin of zebrafish posterior taste buds, two fate mapping approaches were used: transplants of endoderm-fated cells in wild-type oropharynx and Kaede photoconversion in a limited number of pharyngeal pouch cells, respectively. We found that pharyngeal endoderm contributes to Calb2b+ and 5HT+ cell types in pharyngeal (posterior) but not lip (anterior) taste buds, as in other vertebrates (see Fig. S2 in the supplementary material) (Northcutt, 2004).

Fgf signaling is required for intact pharyngeal taste bud formation

Fgfs, their receptors (Fgfrs) and transcriptional mediators are expressed in the developing pharyngeal epithelium before and/or during the period of taste bud formation (see Fig. S1J-L in the supplementary material, data not shown). *pea3* is expressed in taste bud Calb2b+ and surrounding cells, but not *Tg(tph1b:egfp)*-expressing cells (Fig. 1K,L), prompting us to ask whether Fgf signaling is involved in taste bud formation. When Fgfr signaling was compromised, by heatshock activation of the Fgfr1 dominant-negative form (Dnfgfr1) in *Hsdnfgfr1* larvae, from 38-42 hpf onwards, general reduction and disorganization of 5HT+, Calb2b+ and miR-200-expressing cells was observed (Fig. 2A-E, total 5HT+cells: wild type, 125±4 and *Hsdnfgfr1*, 15±4; total Calb2b+ cells: wild type, 264±33 and *Hsdnfgfr1*, 38±19; heatshock at 38 hpf, fixed at 68 hpf, *n*=5, $P<0.001$, and data not shown). Similar results were obtained from SU5402 treatments and in *fgf8*$^{-/-}$ (*ace*) mutants, showing that Fgf8 is one of the ligands contributing to pharyngeal taste bud formation (Fig. 2F-I, see Fig. S3A-D in the supplementary material, data not shown). Altogether, these results show that Fgf signaling is required for oropharyngeal taste bud development.

Fgf signaling is necessary within the pharyngeal endoderm for Calb2b+ cell formation

General Fgf signaling abrogation provokes additional defects in oropharyngeal structures [e.g. cartilage (Crump et al., 2004), see Fig. S3E-H in the supplementary material, data not shown], and taste bud defects could be an indirect consequence. To rule out this possibility, we blocked Fgfr signaling within the pharyngeal epithelium by grafting endoderm from donor *Hsdnfgfr1* into wild-type embryos and applying heatshock at 52-54 hpf to avoid interference with cartilage defects (see Fig. S4A-D in the supplementary material, *n*=11). Within the pharyngeal arch epithelium, Calb2b+ or miR-200-expressing cells were severely reduced in number or absent in *Hsdnfgfr1* sites compared with control (GFP+ or wild type, Fig. 3A-H,M-R, see Fig. S4E-J in the supplementary material, Table 2, data not shown). Therefore, Fgf signaling is required within pharyngeal arch endoderm for miR-200-expressing Calb2b+ cell formation.

Next, the effect of *Hsdnfgfr1* expression on palate taste buds was analyzed. The number of Calb2b+ cells was not significantly reduced within the palate *Hsdnfgfr1* graft compared with the control (Table 2). In addition, the number of 5HT+ cells was not

DEVELOPMENT

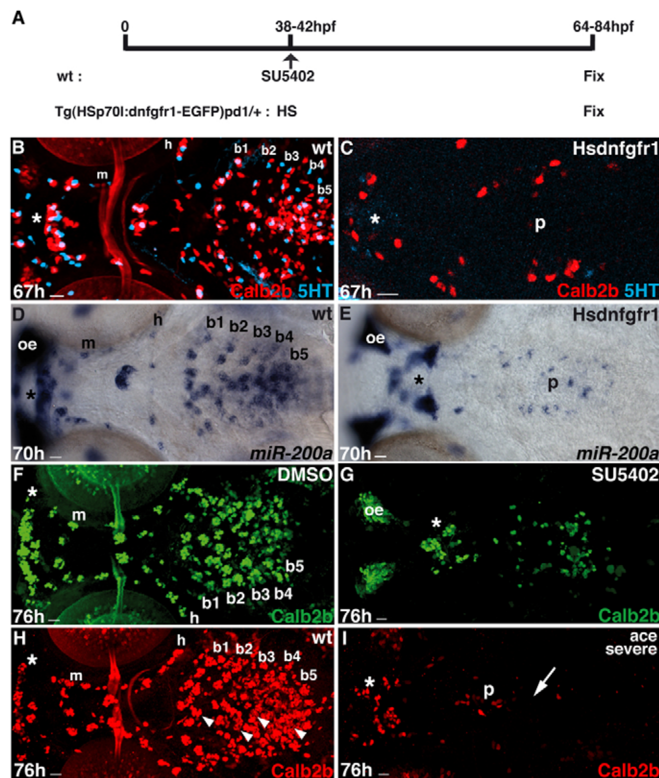

Fig. 2. Taste bud development requires Fgf signaling. (**A**) Heatshock and SU5402 treatments in *tg(hsp70l:dnfgfr1-EGFP)pd1/+* and wild-type embryos, respectively. (**B-I**) Ventral views of whole-mounted larvae heads. Asterisks indicate taste buds in the lips. Experimental conditions are indicated in the top right-hand corner, embryonic stage and scale bar are in the bottom left-hand corner and marker expression is in the bottom right-hand corner. Anterior is towards the left. (**B-E**) Calb2b (B,C, red), 5HT (B,C, cyan) and miR-200*a* (D,E, blue) expression in control (B,D) and *Hsdnfgfr1*+ (C,E) larvae after heatshock. (**F**,**G**) Wild-type immunostained for Calb2b after DMSO (F) and SU5402 (G) treatment, respectively. (**H**,**I**) Wild-type and *ace*$^{-/-}$ siblings (severe phenotype, for mild see Fig. S3A-D in the supplementary material) immunostained for Calb2b with strong reduction (arrow) of taste bud cells. Arrowheads in H indicate the palate (p) taste buds. Scale bars: 10 μm. b1-b5, branchial arches 1-5; h, hyoid arch; m, mandibular arch; oe, olfactory epithelium.

significantly affected in *Hsdnfgfr1* grafts in pharyngeal arch and palate epithelium (Fig. 4G-L, ratio of 5HT$^+$ tracer+ cells to tracer+ cells in wild-type and *Hsdnfgfr1* grafts: 0.08±0.01 and 0.09±0.04, n=7, P>0.05). Given, the dramatic reduction of Calb2b$^+$ and 5HT$^+$ cells when Fgf activity is abrogated in entire *Hsdnfgfr1* embryos from earlier stages (Fig. 2A-E), these results suggest that Fgf signaling is required earlier or indirectly for 5HT$^+$ and palate Calb2b$^+$ cell formation.

Altogether, the Fgf signaling loss-of-function experiments helped us define a tissue, the pharyngeal arch epithelium, where Fgf signaling is required at a specific time for Calb2b$^+$ cell formation. To further dissect the mechanism through which Fgf affects taste bud formation, we first examined cell proliferation. The number of phospho-histone H3 (pH3) cells in *Hsdnfgfr1* grafts was comparable with the control (ratio of pH3+tracer+cells/tracer+cells in wild-type and *Hsdnfgfr1* grafts: 0.09±0.02 and 0.1±0.03, n=5, P>0.05). An alternative possibility

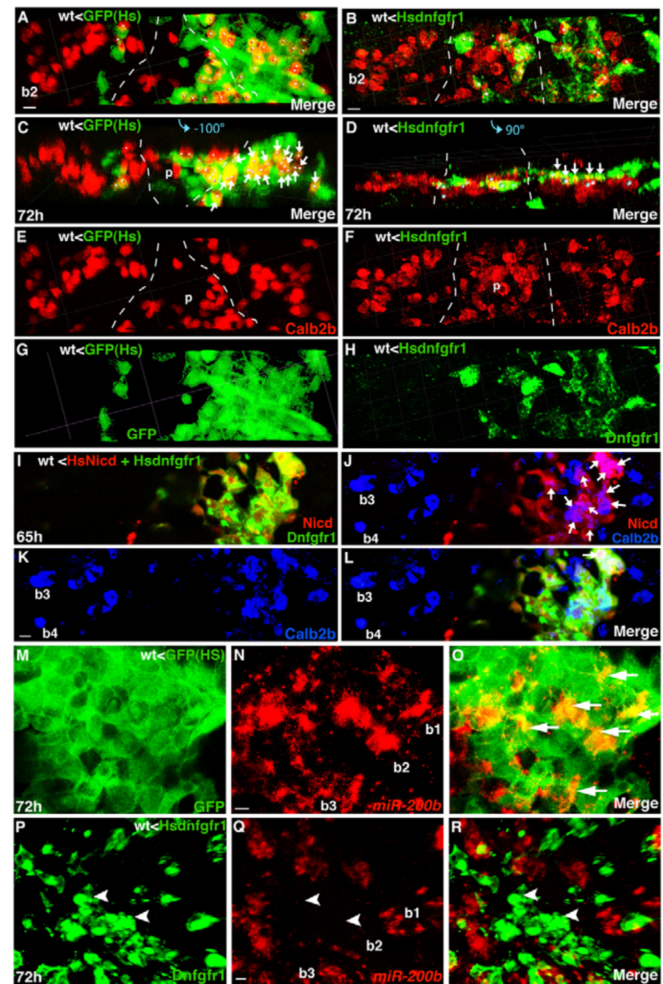

Fig. 3. Fgf signaling is required within the pharyngeal endoderm for Calb2b$^+$ cell formation. Experimental conditions are indicated in the top left- or right-hand corner, embryonic stage and scale bar are in the bottom left-hand corner and marker expression is in the bottom right-hand corner. Anterior is towards the top. Arrows indicate colocalization, arrowheads indicate exclusive marker expression. (**A-L**) Embryos from the same experiment. Scale bars: 5 μm. (A-H) Overlays (A-D) and single-color projections (E-H) of pharyngeal endodermal (Tar*) grafted cells (in wild type) that express GFP or *Hsdnfgfr1* after heatshock. Broken lines mark the palate (p) cells. (C,D) Rotated 3D reconstructions of the views shown in A,B (–100°, +90°, respectively). In A-D, white dots are landmarks of individual cells with potential colocalization of tracer/*Hsdnfgfr1* (green) and Calb2b (red). In A,C, almost all cells (17/20 dots) in branchial arch 2 (b2), co-express GFP and Calb2b (arrows, orange/yellow). By contrast, in B,D, only 5/15 cells (arrows, yellow) co-express Dnfgfr1 and Calb2b in b2. See also another example in Fig. S4E-G in the supplementary material. (I-L) Activation of Notch signaling restores Calb2b$^+$ cell formation in pharyngeal epithelium with compromised Fgfr signaling. Confocal projection of pharyngeal endodermal (Tar*) grafted cells (in wild type) that express *HsNicd* (I,J,L, red/orange), *Hsdnfgfr1* (I,L, green/orange) and Calb2b (J-L, blue/purple/cyan). (L) Superimposition of I,K. Arrows in J indicate cells with *HsNicd* and Calb2b co-expression. b3-b4, branchial arches 3-4. (**M-O**) Optical sections (2.5 μm); (**P-R**) confocal projection through pharyngeal endodermal (Tar*) grafted cells (in wild type) that express GFP (M-O, green) or Dnfgfr1 (P-R, green) and miR-200b (red) after heatshock. (O,R) Superimposition of M,N (O) and P,Q (R). GFP and miR-200b are co-expressed in many cells of several taste buds (arrows, yellow/orange), whereas miR-200b is absent from most Dnfgfr1$^+$ cells (arrowheads) in b2. Scale bars: 4 μm. See also Fig. S4H-J in the supplementary material.

Table 2. Number of Calb2b$^+$ taste bud cells in the oropharynx of wild-type embryos that received endodermal grafts

Pharyngeal arches

	Calb2b+ cells	Tracer+ cells	Calb2b+ tracer+ cells	Ratio of Calb2b+ tracer+ to tracer+ cells
WT<tracer (*n*=5)	195±14	120±27	42±16	0.34±0.07
WT<tracer+HS (*n*=8)	139±12	104±36	36±14	0.34±0.06
WT<HSdnfgfr1 (*n*=8)	100±15	117±35	6±2	0.05±0.01
WT<Hsdnfgfr1+HsNicd (*n*=6)	138±7	119±13	25±6	0.21±0.06
WT<HsNicd (*n*=6)	137±27	107±37	27±10	0.25±0.05
	***P* values**			
WT<tracer, WT<tracer+HS	**	0.9	0.78	0.73
WT<tracer+HS, WT<HSdnfgfr1	**	0.5	***	***
WT<tracer+HS, WT<HSNicd	0.8	0.8	0.23	0.06
WT<tracer+HS, WT<HSdnfgfr1+HSNicd	0.5	0.6	0.11	*
WT<HSdnfgfr1, WT<HSdnfgfr1+HSNicd	**	0.3	***	***

Palate

	Calb2b+ cells	Tracer+ cells	Calb2b+ tracer+ cells	Ratio of Calb2b+ tracer+ to tracer+ cells
WT<tracer (*n*=5)	135±7	139±33	53±15	0.39±0.09
WT<tracer+HS (*n*=8)	100±17	90±13	33±8	0.36±0.06
WT<HSdnfgfr1 (*n*=8)	97±16	76±23	25±5	0.34±0.09
	***P* values**			
WT<tracer, WT<tracer+HS	0.053	0.06	0.12	0.90
WT<tracer+HS, WT<HSdnfgfr1	0.75	0.19	0.07	0.06

Pharyngeal arches and palate

	Calb2b+ cells	Tracer+ cells	Calb2b+ tracer+ cells	Ratio of Calb2b+ tracer+ to tracer+ cells
WT<tracer (*n*=5)	330±16	258±42	94±25	0.37±0.08
WT<tracer+HS (*n*=8)	238±26	194±35	68±17	0.35±0.04
WT<HSdnfgfr1 (*n*=8)	197±26	198±23	25±8	0.13±0.04
	***P* values**			
WT<tracer, WT<tracer+HS	**	0.15	0.2	0.90
WT<tracer+HS, WT<HSdnfgfr1	*	0.83	***	***

Grafts expressed: tracer without heatshock (WT<tracer); tracer after heatshock (WT<tracer+heatshock); transgenes activated after heatshock (*Hsdnfgfr1*+, *HsNicd*+ or both). In each embryo, Calb2b$^+$ and grafted cells were counted in specific areas (pharyngeal arches except mandibular and palate) and then added together. WT<tracer+heatshock embryos have lower total number of Calb2b$^+$ cells compared with WT<tracer embryos. This is consistent with other morphological characteristics (mouth position and head-body angle), indicating that heat-shocked embryos were slightly delayed in their development compared with non heat-shocked ones. Differences in Calb2b$^+$ cell number are more significant between pharyngeal arches of WT<Hsdnfgfr1 and WT<tracer+heatshock grafts; therefore, statistical analysis of *HsNicd*+ grafts was limited to pharyngeal arches.
Values are mean±s.d.; *$P<0.05$; **$P<0.01$; ***$P<0.001$ (*t*-test).

is that Calb2b$^+$ cell reduction in *Hsdnfgfr1* grafts is due to general epithelial cell reduction. However, *sox2* expression and the number of active caspase 3-expressing cells were comparable between *Hsdnfgfr1* and control grafts (ratio of Sox2$^+$ tracer+ cells to tracer+ cells in wild-type and *Hsdnfgfr1* grafts, 0.16±0.03 and 0.21±0.04, *n*=5, $P>0.05$; ratio of Casp3$^+$ tracer+ cells to tracer+ cells in wild-type and *Hsdnfgfr1* grafts, 0.07±0.02 and 0.1±0.02, *n*=5, $P>0.05$). Therefore, we searched for molecules that are expressed in the pharyngeal epithelium and potentially involved in taste bud formation around the period of Fgf activity.

miR-200 family members are necessary for taste bud cell formation

miR-200 expression was dramatically reduced in embryos with compromised Fgf signaling (Fig. 2D,E, Fig. 3M-R). Calb2b$^+$ cells expressed miR-200 [in contrast to 5HT$^+$ cells (Fig. 1E,F)]. Furthermore, *sox2* 3′UTR is a conserved target of miR-200b and miR-429 (Wellner et al., 2009) and Calb2b$^+$ or 5HT$^+$ cells were devoid or had lower *sox2* expression compared with their neighboring epithelial cells (Fig. 1I,J). These observations led us to examine the role of miR-200 in taste bud formation using a knockdown approach.

Single morpholino injections for miR-200a, miR-200b or miR429 did not noticeably alter taste bud development, although the expression of the corresponding miRNA was downregulated (see Fig. S5H-J in the supplementary material, Fig. 5A-B, Table 3, data not shown). In sharp contrast, combined injections of the three MOs (triple MOmiR-200) resulted in oropharyngeal Calb2b$^+$ cell reduction. The number of 5HT$^+$ cells was less dramatically but significantly reduced (Fig. 5C,D, see Fig. S5A-G in the supplementary material, Table 3, data not shown). Furthermore, *sox2* expression was upregulated in the triple MOmiR-200 pharyngeal epithelium compared with the control (Fig. 5E-H, *n*=23), suggesting that miR-200 members repress Sox2 expression within the pharyngeal epithelium to promote taste bud cell differentiation.

The miR-200 knockdown experiments show that miR-200 activity is required within the (presumptive) Calb2b$^+$ cells to regulate their differentiation. Together with the specific reduction of miR-200-expressing cells in the pharyngeal epithelium with compromised Fgf signaling, these results show that the effect of Fgf signaling on Calb2b$^+$ cell formation is mediated by miR-200 activity. By contrast,

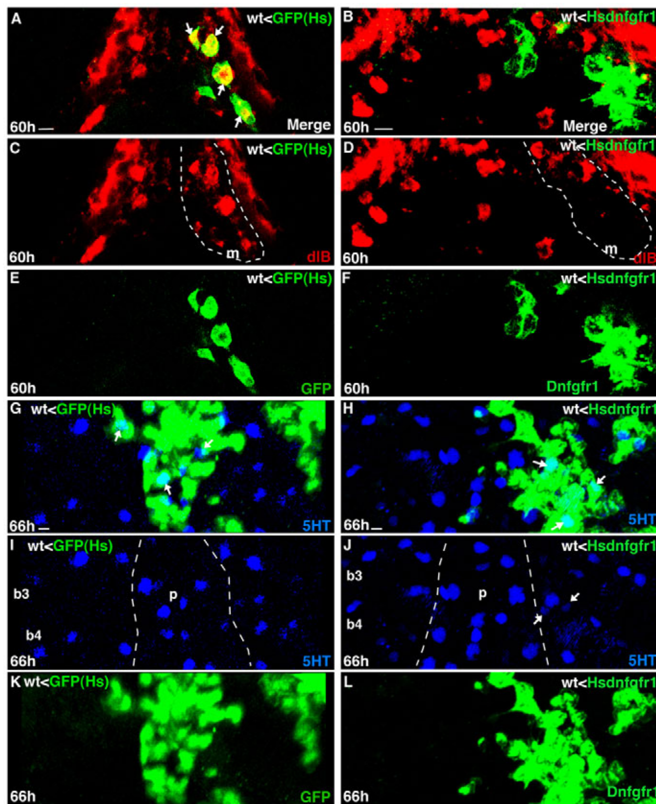

Fig. 4. Fgf signaling is necessary within the pharyngeal endoderm for *dlb* expression, but is not required for 5HT expression in taste buds. Experimental conditions are indicated in the top right-hand corner, embryonic stage and scale bar are in the bottom left-hand corner and marker expression is in the bottom right-hand corner. Ventral views, anterior is towards the top. Arrows indicate marker co-expression in a single cell. Scale bars: 5 μm. (**A-E**) Optical sections (A,C,E) (2 μm) and asymmetric confocal projections (B,D,F) through pharyngeal endodermal (Tar*) grafted cells (in wild type) that express *dlb* (red) and GFP or Dnfgfr1 (green), after heatshock. *dlb* taste bud-related expression in the mandibular arch (m) is within the broken lines. Most GFP cells in the control graft co-express *dlb* (A, arrows); by contrast, most Dnfgfr1 cells are devoid of *dlb* expression (B, green). (**G-L**) Confocal projections through pharyngeal endodermal (Tar*) grafted cells (in wild type) that express 5HT (blue) and GFP or Dnfgfr1 (green) after heatshock. Broken lines mark the palate (p).

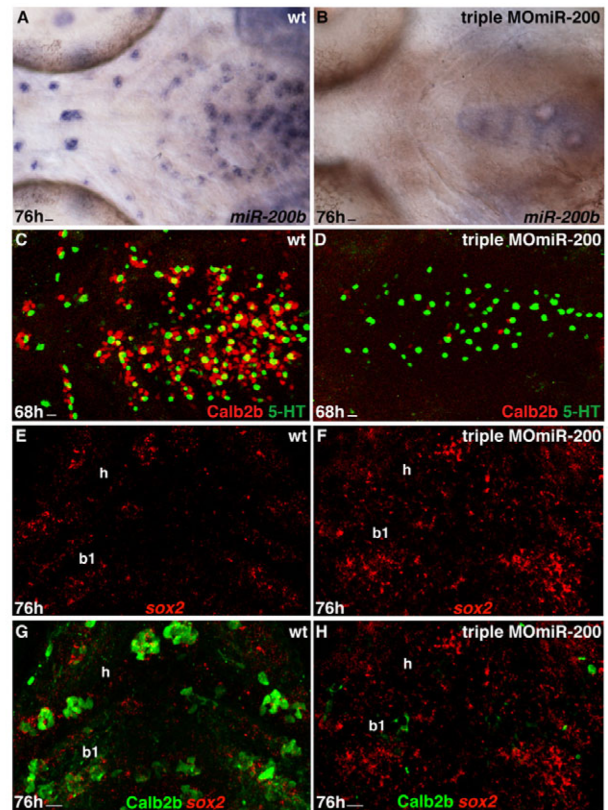

Fig. 5. miR-200 knockdown results in reduction of taste bud cells. Experimental conditions are indicated in the top right-hand corner, embryonic stage and scale bar are in the bottom left-hand corner and marker expression is in the bottom right-hand corner. Anterior is towards the left. Scale bars: 8 μm. (**A**,**B**) Whole-mount ventral views of wild-type and triple MOmiR-200-injected embryo labeled with miR-200b probe. miR-200b expression is absent from the pharyngeal epithelium of the morpholino-injected embryo. (**C**,**D**) Whole-mount ventral views of taste buds of wild-type and triple MOmiR-200-injected embryo labeled with anti-Calb2b (red) and anti-5HT (green). Note the strong and mild reduction in the numbers of Calb2b^{+} and 5HT^{+} cells, respectively in the triple MOmiR-200 injected embryo (D) compared with the wild-type control. (**E-H**) Ventral view, anterior towards the top. Confocal projection of hyoid (h) and first branchial arch (b1) epithelium of a wild-type and a triple MOmiR-200 injected embryo labeled with the *sox2* probe (E-H) and anti-Calb2b (G,H). Calb2b expression is severely decreased but *sox2* expression is increased in the triple MOmiR-200 injected embryo (F,H) compared with the control (E,G).

the absence of miR-200 expression in 5HT^{+} cells and the mild reduction of 5HT^{+} cells in the miR-200 knockdown experiments suggest an indirect miR-200 role on 5HT^{+} cell formation.

Intact Ascl1a-Delta-Notch signaling is required for proper formation of zebrafish taste bud cell types

Among the known zebrafish Notch receptors and ligands, only *notch1a* and *dlb* had an obvious taste bud-related expression profile. By 62 hpf, *dlb* expression is restricted to one or two cells per taste bud, always devoid of Calb2b and 5HT expression (Fig. 1O-P). However, earlier, at 54-56hpf, *dlb* expression is evident in lips, pharyngeal arch and palate epithelium in a patched multicellular manner (see Fig. S1F,I in the supplementary material, data not shown). *notch1a* expression is widespread in cells of the lips, palate and pharyngeal arch epithelium, and other pharyngeal tissues, from early stages (e.g. 55 hpf, see Fig. S1E,H in the supplementary material). Later on, some of the Calb2b^{+} but not *Tg(tph1b:egp)+* cells, express *notch1a* (Fig. 1Q-R). Thus, before taste bud differentiation, multicellular *notch1a* and *dlb* expression patterns in the oropharyngeal epithelium are reminiscent of early Notch ligand/receptor expression in the prosensory epithelium of other sensory organs [e.g. inner ear (Adam et al., 1998)], where Notch signaling maintains the cells in a prosensory state making them competent to differentiate subsequently in distinct cell types (e.g. Daudet et al., 2007).

mib$^{-/-}$ is a mutant with non-functional E3 ubiquitin-protein ligase necessary for internalization and therefore signaling of Notch ligands. In this mutant, Delta ligands are overexpressed (Itoh et al., 2003). In the 60 hpf *mib*$^{-/-}$ oropharynx, *dlb* was overexpressed in continuous rows of cells and not restricted to patches as in wild-

DEVELOPMENT

Table 3. Number of Calb2b+ and 5HT+ (oropharyngeal taste bud) cells and pH3+ cells in 3 dpf wild type and embryos injected with the highest non-toxic dose of miR-200 morpholinos

	5HT+ cells	Calb2b+ cells	pH3+ cells
Wild type (*n*=11)	188±7	406±14	133±11
Control MO (*n*=11)	164±9	393±8	140±14
MO-miR-200a (*n*=11)	198±6	423±13	
MO-miR-200b (*n*=11)	176±6	402±15	
MO-miR-429 (*n*=11)	172±11	445±10	
Triple MO200 (*n*=11)	115±9	43±11	112±17
		***P* values**	
Wild type, control MO	0.51	0.6	0.5
Wild type, MO-miR-200a	0.65	0.5	
Wild type, MO-miR-200b	0.47	0.52	
Wild type, MO-miR-429	0.7	0.3	
Wild type, TripleMO200	***	***	0.07

Data are mean±s.d.; ***P<0,001 (t-test).
See also Fig. S5 in the supplementary material.

type siblings (Fig. 6A,B). 5HT+ cells and *ascl1a* expression were dramatically increased in *mib*−/− oropharynx (Fig. 6C,D,G,H, 5HT+ cells in wild type and *mib*−/−: 162±3 and 361±7, *n*=11, *P*<0.001). This is reminiscent of *mib*−/− nervous system where progenitors prematurely develop neuronal fate and therefore other (later occurring) cell type differentiation is prevented.

ascl1a is expressed in 5HT+ basal Merkel-like cells (Fig. 1M) suggesting that *ascl1a* is required for their formation. Consistently, *ascl1a*−/− oropharyngeal taste buds were devoid of 5HT+ cells (Fig. 6M,N, 5HT+ cells in wild type and *ascl1a*−/−: 181±6 and 0, *n*=11, *P*<0.001). Complementary to these results, activation of Notch signaling in *HsNicd* embryos by heatshock at 52-54 hpf entirely blocked oropharyngeal 5HT+ cell formation (Fig. 7A-C, 5HT+ cells in wild type and *HsNicd*: 140±7 and 0, *n*=8, *P*<0.001). Therefore, *ascl1a* activity is required for 5HT cell formation and conversely, Notch activation a few hours before taste bud differentiation inhibits the formation of 5HT+ cells.

Strikingly, the number of miR-200 and Calb2b+ cells was severely reduced in *mib*−/− oropharynx (Fig. 6C-F, Calb2b+ cells in wild type and *mib*−/−: 385±14 and 99±5, *n*=11, *P*<0.001). Given the role of Fgf signaling in Calb2b+ cell formation, Calb2b+ cell reduction in *mib*−/− pharyngeal epithelium could be due to altered Fgf activity. *pea3* was expressed in *mib*−/− pharyngeal arch epithelium and even (rostral) expanded in the palate epithelium, supporting the idea that taste bud defects in *mib*−/− are not due to compromised Fgf signaling (Fig. 6I-L). When Notch signaling was activated in *HsNicd* embryos by heatshock at 52-54 hpf, Calb2b+ and miR-200 cells were maintained but disorganized (Fig. 7D-G, Calb2b+ cells in wild type and *HsNicd*: 122±24 and 96±35, *n*=8, *P*>0.05). Furthermore, *ascl1a*−/− oropharyngeal taste buds had a higher number of Calb2b+ cells (Fig. 6M,N, Calb2b+cells in wild type and *ascl1a*−/−: 413±11 and 525±12, *n*=11, *P*<0.001). Altogether, these results show that Delta-Notch signaling is necessary for Calb2b+ cell formation and/or maintenance, whereas *ascl1a* activity, at least partially, inhibits Calb2b+ cell identity.

Fgf signaling is necessary for intact *dlb* expression in the pharyngeal epithelium

The reduction of Calb2b+ cells in embryos with compromised Delta signaling (*mib*−/−) led us to examine whether Fgf activity is required for taste bud related *dlb* expression. Heatshock-induced inhibition of Fgf signaling in *Hsdnfgfr1* grafts resulted in severe

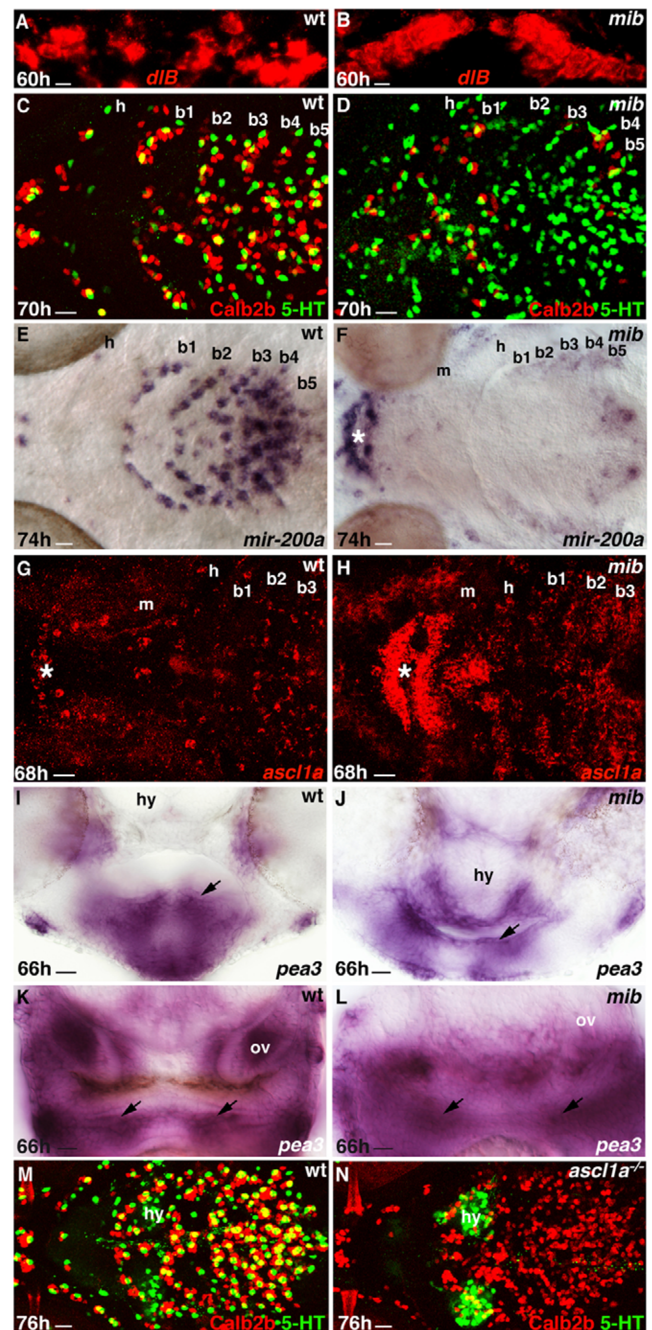

Fig. 6. Delta and *ascl1a* activity are necessary for intact taste bud organ formation. Genotypes are indicated in the top right-hand corner, embryonic stage and scale bar are in the bottom left-hand corner and marker expression is in the bottom right-hand corner. (**A**,**B**) Anterior is towards the top. Ventral confocal projections of the first branchial arch showing *dlb* overexpression in *mib*−/− (B) compared with the wild-type sibling (A). (**C-H**,**M**,**N**) Anterior is towards the left. Ventral views. Asterisks in F-H indicate taste buds in the lips. 5HT+ and *ascl1a*-expressing cells are increased in number (D,H) and Calb2b+ and miR-200a-expressing cells reduced in number (D,F) in *mib*−/− compared with the wild type (C,G and C,E, respectively). By contrast, 5HT+ (green) cells are absent and Calb2b+ (red) cells disorganized in the *ascl1a*−/− pharynx (N) compared with the wild type (M). (**I-L**) Transverse sections of wild-type (I,K) and *mib*−/− siblings (J,L) labeled with the *pea3* probe. Arrows indicate *pea3* expression in the pharyngeal arch epithelium (I,J, hyoid; K,L, b4). Scale bars: 15 µm. b1-b5, branchial arches 1-5; h, hyoid arch; m, mandibular arch; ov, otic vesicle; hy, hypothalamus.

DEVELOPMENT

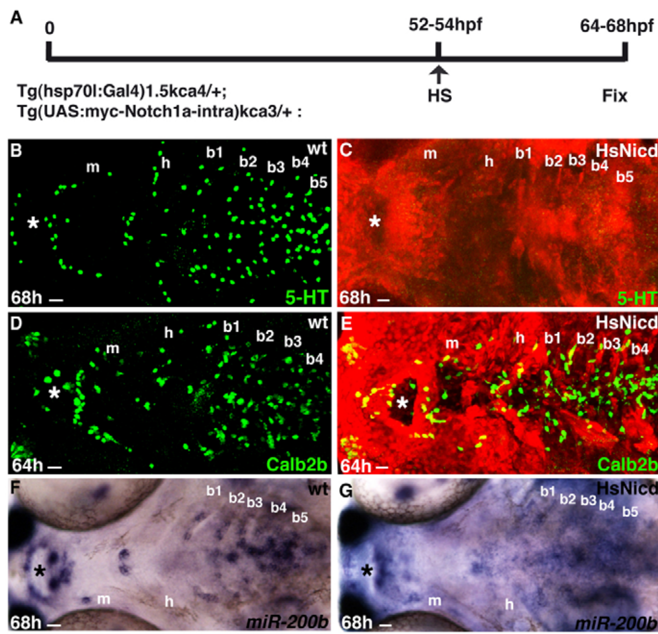

Fig. 7. Activation of Notch signaling maintains taste bud Calb2b$^+$ but not 5HT$^+$ cells. (**A**) Heatshock treatment in *HsNicd* siblings. (**B-G**) Embryonic stage and scale bar are in the bottom left-hand corner and marker expression is in the bottom right-hand corner. Anterior is towards the left. Ventral views of whole-mounted larvae heads. Asterisks indicate taste buds in the lips. Note the absence of 5HT (C) and maintenance of Calb2b (E) and miR-200b (G) expression in the Hsnicd-expressing embryos compared with wild-type (heatshocked) siblings. Hsnicd embryos were identified by anti-myc (red, C,E) immunohistochemistry or genotyped after in situ hybridization (G). Scale bars: 12 μm. b1-b5, branchial arches 1-5; e, eye; h, hyoid arch; m, mandibular arch; nm, neuromast; oe, olfactory epithelium.

reduction of *dlb*-expressing cells compared with the control (at 60 hpf, Fig. 4A-F, ratio of *dlb*+ tracer+ cells to tracer+ cells in wild-type and *Hsdnfgfr1* grafts, 0.2±0.02 and 0.04±0.03, n=5, P<0.001). This result shows that intact Fgf signaling is required for proper *dlb* expression in the pharyngeal epithelium before and/or during Calb2b$^+$ cell differentiation, and suggests that Fgf signaling is necessary for Delta-Notch activity in the pharyngeal epithelium. A prediction from this model is that activation of Notch signaling should restore the Calb2b$^+$ cell reduction observed in the *Hsdnfgfr1* grafts. Strikingly, when Fgf and Notch signaling were simultaneously inhibited and activated, respectively, by heatshock at 52 hpf of wild-type embryos grafted with *Hsdnfgfr1;HsNicd* cells, Calb2b$^+$ cells were maintained (Fig. 3I-L, Table 2). Altogether, these results show that Fgf and Notch signaling interact genetically to promote formation of Calb2b$^+$ cells within the pharyngeal epithelium, and Fgf signaling acts upstream of Delta-Notch activity in this process.

Notch signaling is required upstream of miR-200 activity to regulate taste bud cell formation

miR-200 expression is detected later (60 hpf, data not shown) than *dlb/notch1a* (54-56 hpf, see Fig. S1E-F in the supplementary material), is severely reduced in *mib*$^{-/-}$ pharyngeal epithelium (Fig. 6E,F) and is maintained in Hsnicd embryos (Fig. 7F,G), supporting the idea that Notch signaling acts upstream of miR-200. To further assess this, we focused on pharyngeal arch Calb2b$^+$ cells. When triple MOmiR-200-expressing cells were introduced to wild-type pharyngeal endoderm by grafting, the number of Calb2b$^+$ cells was reduced within the graft compared with the control (Fig. 8A,D,G, Table 4). Simultaneous heatshock activation of Notch signaling (*HsNicd*) and blockage of miR-200 activity (triple MOmiR-200) in endodermal cells grafted to wild-type pharynx failed to restore Calb2b$^+$ cell formation in pharyngeal arch epithelium with blocked miR-200 activity (Fig. 8A-I, Table 4), showing that Notch signaling acts upstream of miR-200.

DISCUSSION

Through loss-of-function and rescue approaches, we provide a novel model of molecular interactions necessary for taste bud development (Fig. 9). First, Fgf is necessary for intact taste bud organ formation. Fgf is required for the formation of Calb2b$^+$ cells within the pharyngeal epithelium. This Fgf requirement is mediated: first, through miR-200 activity and miR-200 downregulation, which results in Calb2b$^+$ (miR-200+) cell reduction without downregulation of *sox2* expression; second, through *delta*

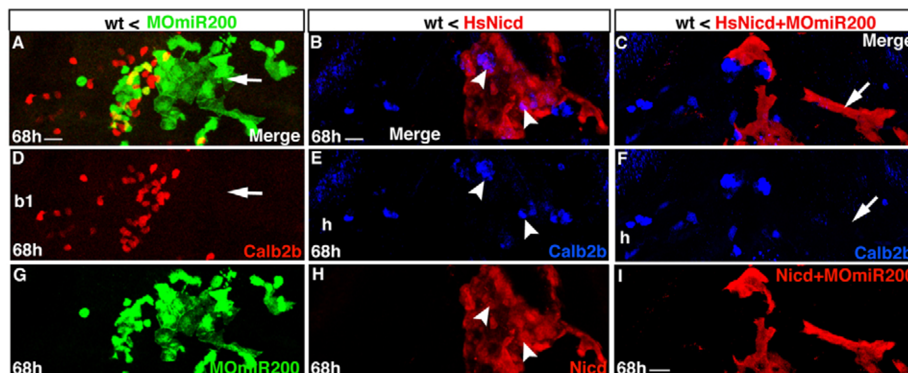

Fig. 8. Activation of Notch signaling does not restore Calb2b$^+$ cell formation in pharyngeal epithelium after miR-200 knock-down. Experimental conditions are indicated in the top right-hand corner, embryonic stage and scale bar are in the bottom left-hand corner and marker expression is in the bottom right-hand corner or in attached insets. Anterior is towards the top. Ventral views. Scale bars: 10 μm. (**A**,**D**,**G**) Confocal projections of grafted endodermal (Tar*) cells (in wild type) expressing triple MOmiR-200 (green) and Calb2b (red/yellow). The arrow indicates a severe reduction in Calb2b$^+$ cell number in the triple MOmiR-200-expressing branchial arch epithelium (b1). (**B**,**E**,**H**) Confocal projections of grafted endodermal (Tar*) cells (in wild type) expressing *HsNicd* (red) and Calb2b (blue/purple). Arrowheads indicate Calb2b$^+$ cells in the *HsNicd* hyoid epithelium (h). (**C**,**F**,**I**) Confocal projections of grafted endodermal (Tar*) cells (in wild type) co-expressing *HsNicd*, triple MOmiR-200 (red) and Calb2b (blue). The arrow indicates the absence of Calb2b$^+$ cells in the *HsNicd* and triple MOmiR-200-expressing (red) hyoid epithelium (h).

DEVELOPMENT

Table 4. Number of Calb2b+ taste bud cells in anterior pharyngeal arches (hyoid and branchial arches 1 and 2) of wild-type embryos that received endodermal (Tar*) grafts

	Tracer+ cells	Calb2b+ tracer+ cells	Ratio of Calb2b+tracer+ to tracer+ cells
WT<Tracer+HS (*n*=6)	63±13	21±5	0.34±0.06
WT<MO200+HS (*n*=6)	59±12	5±1	0.08±0.02
WT<MO200+HsNicd (*n*=6)	53±9	4±2	0.08±0.03
WT<HsNicd (*n*=6)	58±11	16±5	0.28±0.05
		***P* values**	
WT<Tracer+HS, WT<MO200+HS	0.5	***	***
WT<MO200+HS, WT<MO200+HsNicd	0.8	0.7	0.8
WT<Tracer+HS, WT<HsNicd	0.6	0.06	0.1
WT<MO200+HsNicd, WT<HsNicd	0.3	***	***

After heatshock, grafts expressed: tracer (WT<Tracer+Hs), triple MOmiR-200 (WT<MO200+heatshock), triple MOmiR-200 and *HsNicd* (WT<MO200+HsNicd) or *HsNicd* (WT< HsNicd).
Data are mean±s.d.; ***$P<0.001$ (*t*-test).

(potentially *dlb*) activity and compromised *delta* signaling (*mib*$^{-/-}$), which results in miR-200 expression and Calb2b$^+$ cell reduction. Notch activity is required at least for the maintenance of the Calb2b$^+$ pool of cells in wild type or in larvae with compromised Fgf signaling, but Notch activation cannot compensate the Calb2b$^+$ cell loss when miR-200 are knocked down. In agreement, compromised *delta* and activated Notch signaling result in reduced and maintained miR-200-expressing cells, respectively. Finally, Fgf and miR-200 contribute rather indirectly, and perhaps at different timing to 5HT$^+$ cell formation. By contrast, *ascl1a* activity and timely regulated inhibition of Notch activity are required for 5HT$^+$ cell development.

Fgf signaling, a novel key player in taste bud formation

Fgf signaling was already known to regulate patterning and/or differentiation in other sensory organs (e.g. Hayashi et al., 2008; Maier et al., 2010; Nechiporuk and Raible, 2008) or pharyngeal structures (Jackman et al., 2004). Here, we provide evidence for its role in taste bud development. First, compromised Fgf signaling in zebrafish larvae results in severe loss of both Calb2b$^+$ and 5HT$^+$ cells. By targeted inactivation of Fgf signaling within the pharyngeal arch epithelium, we demonstrate that Fgf signaling is crucially required within this tissue for differentiation of a taste bud cell type by regulating early and late gene expression (miR-200, Calb2b$^+$). *pea3* expression in Calb2b$^+$ cells suggests that the Fgf signaling requirement in Calb2b$^+$ taste bud cells is direct. Based on the targeted inhibition of Fgf signaling within the pharyngeal arch epithelium, we propose that Fgf signaling specifically regulates Calb2b$^+$ cell formation in this tissue from 52-54 hpf onwards. As compromised Fgf signaling from 38 hpf severely reduces Calb2b$^+$ cell formation in the endoderm-derived palate epithelium and the ectoderm-derived skin of the lips, it is likely that the Fgf requirement in Calb2b$^+$ cell formation is time dependent in different oropharyngeal areas. This is in agreement with mammalian studies supporting the observation that other signaling pathways are required at different periods for rostral and caudal taste bud development (Iwatsuki et al., 2007).

5HT$^+$ cells behave differently than Calb2b$^+$ cells when Fgf signaling is compromised. Whereas abrogated Fgf signaling leads to 5HT$^+$ cell reduction, pharyngeal endoderm-restricted Fgf inactivation has no significant effect. One possibility is that as 5HT$^+$ cells appear earlier than Calb2b$^+$ cells in the oropharyngeal taste buds, Fgf signaling is also required earlier for 5HT$^+$ cell formation. Alternatively, Fgf signaling could affect the 5HT$^+$ cells through a different cellular/molecular mechanism than Calb2b$^+$ cells, a hypothesis consistent with our results on the role of miR-200 and Delta-Notch signaling in taste bud formation.

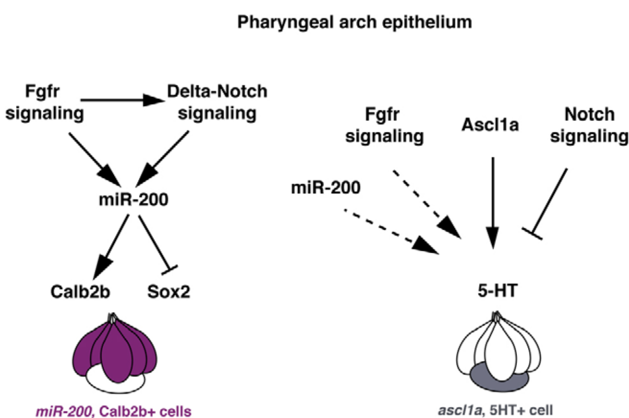

Fig. 9. Fgf, Delta-Notch and miR-200 interactions that regulate Calb2b$^+$ and 5HT$^+$ cell formation. See text for details.

The essential role of miR-200 family members in taste bud cell formation

miR-200 are expressed in Calb2b$^+$ cells and, strikingly, knock down of their activity within the pharyngeal epithelium results in a severe reduction of this cell population. There is a precedent for the regulation of the late steps of differentiation of another sensory organ, the olfactory epithelium, by the same miRNAs (Choi et al., 2008). We face a similar situation for zebrafish Calb2b$^+$ cells and, more generally, the role of miR-200 in cell differentiation may be common in all sensory organs in which they are expressed [i.e. mechanosensory cells (Kapsimali et al., 2007; Wienholds et al., 2005)].

One issue regarding this result is through which mechanism do miRNAs control taste bud cell formation. This study provides evidence to link miR-200 activity with *sox2*, a transcription factor extensively studied for its role in mammalian taste bud development (Okubo et al., 2008; Okubo et al., 2006). Recently it has been shown that *sox2* 3′UTR is a miR-200 conserved target (Wellner et al., 2009). We analyzed *sox2* expression in triple MOmiR-200-injected embryos and, as expected for a miRNA target mRNA (Fabian et al., 2010), *sox2* expression is maintained and upregulated in the pharyngeal epithelium. We also found that differentiating (Calb2b$^+$ or 5HT$^+$) taste bud cells have low, or are devoid of, *sox2* mRNA levels compared with the adjacent cells that express high levels of *sox2*. So far, it has been reported that *sox2* is expressed in a similar manner in progenitor and differentiated taste bud cells (Okubo et al., 2008; Okubo et al., 2006). Based on our results and on previous studies, we suggest that *sox2* is

DEVELOPMENT

necessary for the initial steps of taste bud induction, but that *sox2* expression is downregulated as taste bud cell differentiation proceeds, and this is ensured by miR-200.

In contrast to Calb2b$^+$ cells, miR-200 expression was undetectable in 5HT$^+$ cells and knocking down miR-200 activity reduced mildly the number of these cells. This result suggests that miR-200 and/or Calb2b$^+$ cells are important for maintenance of 5HT$^+$ cells rather for their formation. Furthermore, pharyngeal epithelium-restricted inhibition of Fgf signaling at 54 hpf inhibits the formation of miR-200/Calb2b$^+$ but not of the 5HT$^+$ cell population. Taken together, these results reveal, for the first time, a combination of signals that differentially affects the development of two taste bud cell types: Calb2b$^+$ versus 5HT$^+$ cells.

Delta-Notch signaling interacts with Fgf and miR-200 during taste bud formation

The role of Fgf and miR-200 in taste bud formation has not been examined so far and therefore we aimed to examine how these two signals interact with other already studied pathways in posterior taste bud development. Our data support Delta-Notch as an additional key signal in the mechanism of distinct taste bud cell type formation as in the case of mammals (Ota et al., 2009). The dynamic *dlb* and *notch1a* expression patterns suggest multiple functions for Notch signaling during taste bud formation, and the functional approaches used here further support them. First, zebrafish *HsNicd* or *ascl1a*$^{-/-}$ oropharynx is devoid of 5HT$^+$ cells. Thus, Notch signaling must be inhibited at some developmental point, and *ascl1a* activity promotes 5HT$^+$ cell differentiation as in lateral inhibition cases in the nervous system (e.g. Daudet and Lewis, 2005; Louvi and Artavanis-Tsakonas, 2006; Nieto et al., 2001; Pierfelice et al., 2011).

Second, Delta-Notch signaling is crucially required for Calb2b$^+$ cell formation as miR-200-expressing and Calb2b$^+$ cells are severely reduced in *mib*$^{-/-}$, but their number is maintained when Notch is activated at 52 hpf and this Notch activation is sufficient to maintain Calb2b$^+$ cells in epithelium with compromised Fgf signaling. However, whether Notch activity controls the formation/maintenance of taste bud prosensory domains (lateral induction), as in the case of other sensory organs that eventually will differentiate to Calb2b$^+$ cells, or is instructive towards Calb2b$^+$ cell fate, as for example in gliogenesis (Cau and Blader, 2009; Daudet et al., 2007; Daudet and Lewis, 2005; Furukawa et al., 2000; Louvi and Artavanis-Tsakonas, 2006; Pierfelice et al., 2011; Scheer et al., 2001), remains to be established. To dissect precisely the functions of Notch signaling in zebrafish taste bud formation, specific markers of the progenitor taste bud state should be identified (Miura and Barlow, 2010). In addition, in vivo cell-specific and timely restricted approaches should be developed because the process of taste bud organ differentiation is relatively short (within few hours) compared with other sensory organs and species (Daudet and Lewis, 2005) and adjacent tissues are sensitive to Notch signaling (Kikuchi et al., 2004; Zuniga et al., 2010).

In conclusion, this study reveals key signaling interactions that contribute to the formation of two distinct cell types within the taste bud organ. Based on genetic evidence, we show that Fgf signaling within the pharyngeal epithelium, through Delta-Notch and miR-200 activity regulates the differentiation of Calb2b$^+$ cells. By contrast, Fgf and miR-200 have a rather indirect role in 5HT$^+$ cell formation and Notch activity must be inhibited with appropriate timing to allow the formation of this cell type. In zebrafish, 5HT$^+$ cells are Merkel-like cells and Calb2b$^+$ cells may, as in mammals, correspond to a taste receptor cell pool. It will be of interest to analyze how additional cell types do form and assemble in taste bud organs in zebrafish and whether these mechanisms are conserved in other vertebrates.

Acknowledgements
We thank Brigitte Boyer, Nicolas David, INSERM-U1024 and Nirupa Chaudhari for helpful discussions, Benjamin Mathieu for imaging expertise and the zebrafish community for reagents. We are indebted to Linda Barlow for critical reading and sharing data before publication. This work was funded by FP6-ZF MODELS, ANR06-BLAN-0321 and ANR-09-BLAN-077.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.058669/-/DC1

References

Adam, J., Myat, A., Le Roux, I., Eddison, M., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1998). Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with *Drosophila* sense-organ development. *Development* **125**, 4645-4654.
Ai, H. W., Shaner, N. C., Cheng, Z., Tsien, R. Y. and Campbell, R. E. (2007). Exploration of new chromophore structures leads to the identification of improved blue fluorescent proteins. *Biochemistry* **46**, 5904-5910.
Aihara, Y., Yasuoka, A., Yoshida, Y., Ohmoto, M., Shimizu-Ibuka, A., Misaka, T., Furutani-Seiki, M., Matsumoto, I. and Abe, K. (2007). Transgenic labeling of taste receptor cells in model fish under the control of the 5′-upstream region of medaka phospholipase C-beta 2 gene. *Gene Expr. Patterns* **7**, 149-157.
Barlow, L. A. (2000). Taste buds in ectoderm are induced by endoderm:implications for mechanisms governing taste bud development. In *Regulatory Processes in Development: The Legacy of Sven Horstadius. Proceedings of the Wenner-Gren International symposium* (ed. L. Olsson and C.-O. Jacobson), pp. 185-190. London, UK: Portland Press.
Barlow, L. A. and Northcutt, R. G. (1995). Embryonic origin of amphibian taste buds. *Dev. Biol.* **169**, 273-285.
Beites, C. L., Hollenbeck, P. L., Kim, J., Lovell-Badge, R., Lander, A. D. and Calof, A. L. (2009). Follistatin modulates a BMP autoregulatory loop to control the size and patterning of sensory domains in the developing tongue. *Development* **136**, 2187-2197.
Bierkamp, C. and Campos-Ortega, J. A. (1993). A zebrafish homologue of the *Drosophila* neurogenic gene Notch and its pattern of transcription during early embryogenesis. *Mech. Dev.* **43**, 87-100.
Cau, E. and Blader, P. (2009). Notch activity in the nervous system: to switch or not switch? *Neural Dev.* **4**, 36.
Chandrashekar, J., Kuhn, C., Oka, Y., Yarmolinsky, D. A., Hummler, E., Ryba, N. J. and Zuker, C. S. (2010). The cells and peripheral representation of sodium taste in mice. *Nature* **464**, 297-301.
Chaudhari, N. and Roper, S. D. (2010). The cell biology of taste. *J. Cell Biol.* **190**, 285-296.
Choi, P. S., Zakhary, L., Choi, W. Y., Caron, S., Alvarez-Saavedra, E., Miska, E. A., McManus, M., Harfe, B., Giraldez, A. J., Horvitz, H. R. et al. (2008). Members of the miRNA-200 family regulate olfactory neurogenesis. *Neuron* **57**, 41-55.
Crump, J. G., Maves, L., Lawson, N. D., Weinstein, B. M. and Kimmel, C. B. (2004). An essential role for Fgfs in endodermal pouch formation influences later craniofacial skeletal patterning. *Development* **131**, 5703-5716.
Cunliffe, V. T. and Casaccia-Bonnefil, P. (2006). Histone deacetylase 1 is essential for oligodendrocyte specification in the zebrafish CNS. *Mech. Dev.* **123**, 24-30.
Daudet, N. and Lewis, J. (2005). Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. *Development* **132**, 541-551.
Daudet, N., Ariza-McNaughton, L. and Lewis, J. (2007). Notch signalling is needed to maintain, but not to initiate, the formation of prosensory patches in the chick inner ear. *Development* **134**, 2369-2378.
David, N. B. and Rosa, F. M. (2001). Cell autonomous commitment to an endodermal fate and behaviour by activation of Nodal signalling. *Development* **128**, 3937-3947.
Delay, R. J., Taylor, R. and Roper, S. D. (1993). Merkel-like basal cells in Necturus taste buds contain serotonin. *J. Comp. Neurol.* **335**, 606-613.
Delay, R. J., Kinnamon, S. C. and Roper, S. D. (1997). Serotonin modulates voltage-dependent calcium current in Necturus taste cells. *J. Neurophysiol.* **77**, 2515-2524.
Diaz-Regueira, S. M., Lamas, I. and Anadon, R. (2005). Calretinin immunoreactivity in taste buds and afferent fibers of the grey mullet Chelon labrosus. *Brain Res.* **1031**, 297-301.
Dickmeis, T., Mourrain, P., Saint-Etienne, L., Fischer, N., Aanstad, P., Clark, M., Strahle, U. and Rosa, F. (2001). A crucial component of the endoderm formation pathway, CASANOVA, is encoded by a novel sox-related gene. *Genes Dev.* **15**, 1487-1492.
Dvoryanchikov, G., Tomchik, S. M. and Chaudhari, N. (2007). Biogenic amine synthesis and uptake in rodent taste buds. *J. Comp. Neurol.* **505**, 302-313.
Eswarakumar, V. P., Lax, I. and Schlessinger, J. (2005). Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.* **16**, 139-149.
Fabian, M. R., Sonenberg, N. and Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.* **79**, 351-379.

Finger, T. E., Danilova, V., Barrows, J., Bartel, D. L., Vigers, A. J., Stone, L., Hellekant, G. and Kinnamon, S. C. (2005). ATP signaling is crucial for communication from taste buds to gustatory nerves. *Science* **310**, 1495-1499.

Flynt, A. S., Thatcher, E. J., Burkewitz, K., Li, N., Liu, Y. and Patton, J. G. (2009). miR-8 microRNAs regulate the response to osmotic regulate the response to osmotic stress in zebrafish embryos. *J. Cell Biol.* **185**, 115-127.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M. and Cepko, C. L. (2000). rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. *Neuron* **26**, 383-394.

Haddon, C., Jiang, Y. J., Smithers, L. and Lewis, J. (1998). Delta-Notch signalling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant. *Development* **125**, 4637-4644.

Hall, C., Flores, M. V., Murison, G., Crosier, K. and Crosier, P. (2006). An essential role for zebrafish Fgfrl1 during gill cartilage development. *Mech. Dev.* **123**, 925-940.

Hall, J. M., Bell, M. L. and Finger, T. E. (2003). Disruption of sonic hedgehog signaling alters growth and patterning of lingual taste papillae. *Dev. Biol.* **255**, 263-277.

Hansen, A., Reutter, K. and Zeiske, E. (2002). Taste bud development in the zebrafish, *Danio rerio*. *Dev. Dyn.* **223**, 483-496.

Hauptmann, G. and Gerster, T. (2000). Multicolor whole-mount in situ hybridization. *Methods Mol. Biol.* **137**, 139-148.

Hayashi, T., Ray, C. A. and Bermingham-McDonogh, O. (2008). Fgf20 is required for sensory epithelial specification in the developing cochlea. *J. Neurosci.* **28**, 5991-5999.

Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4**, 67-82.

Iwatsuki, K., Liu, H. X., Gronder, A., Singer, M. A., Lane, T. F., Grosschedl, R., Mistretta, C. M. and Margolskee, R. F. (2007). Wnt signaling interacts with Shh to regulate taste papilla development. *Proc. Natl. Acad. Sci. USA* **104**, 2253-2258.

Jackman, W. R., Draper, B. W. and Stock, D. W. (2004). Fgf signaling is required for zebrafish tooth development. *Dev. Biol.* **274**, 139-157.

Kapsimali, M., Kloosterman, W. P., de Bruijn, E., Rosa, F., Plasterk, R. H. and Wilson, S. W. (2007). MicroRNAs show a wide diversity of expression profiles in the developing and mature central nervous system. *Genome Biol.* **8**, R173.

Kikuchi, Y., Agathon, A., Alexander, J., Thisse, C., Waldron, S., Yelon, D., Thisse, B. and Stainier, D. Y. (2001). casanova encodes a novel Sox-related protein necessary and sufficient for early endoderm formation in zebrafish. *Genes Dev.* **15**, 1493-1505.

Kikuchi, Y., Verkade, H., Reiter, J. F., Kim, C. H., Chitnis, A. B., Kuroiwa, A. and Stainier, D. Y. (2004). Notch signaling can regulate endoderm formation in zebrafish. *Dev. Dyn.* **229**, 756-762.

Krimm, R. F. (2007). Factors that regulate embryonic gustatory development. *BMC Neurosci.* **8 Suppl. 3**, S4.

LeClair, E. E. and Topczewski, J. (2010). Development and regeneration of the zebrafish maxillary barbel: a novel study system for vertebrate tissue growth and repair. *PLoS ONE* **5**, e8737.

Lee, Y., Grill, S., Sanchez, A., Murphy-Ryan, M. and Poss, K. D. (2005). Fgf signaling instructs position-dependent growth rate during zebrafish fin regeneration. *Development* **132**, 5173-5183.

Li, Y., Allende, M. L., Finkelstein, R. and Weinberg, E. S. (1994). Expression of two zebrafish orthodenticle-related genes in the embryonic brain. *Mech. Dev.* **48**, 229-244.

Liu, F., Thirumangalathu, S., Gallant, N. M., Yang, S. H., Stoick-Cooper, C. L., Reddy, S. T., Andl, T., Taketo, M. M., Dlugosz, A. A., Moon, R. T. et al. (2007). Wnt-beta-catenin signaling initiates taste papilla development. *Nat. Genet.* **39**, 106-112.

Liu, H. X., Henson, B. S., Zhou, Y., D'Silva, N. J. and Mistretta, C. M. (2008). Fungiform papilla pattern: EGF regulates inter-papilla lingual epithelium and decreases papilla number by means of PI3K/Akt, MEK/ERK, and p38 MAPK signaling. *Dev. Dyn.* **237**, 2378-2393.

Louvi, A. and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat. Rev. Neurosci.* **7**, 93-102.

Maier, E., von Hofsten, J., Nord, H., Fernandes, M., Paek, H., Hebert, J. M. and Gunhaga, L. (2010). Opposing Fgf and Bmp activities regulate the specification of olfactory sensory and respiratory epithelial cell fates. *Development* **137**, 1601-1611.

Mistretta, C. M., Liu, H. X., Gaffield, W. and MacCallum, D. K. (2003). Cyclopamine and jervine in embryonic rat tongue cultures demonstrate a role for Shh signaling in taste papilla development and patterning: fungiform papillae double in number and form in novel locations in dorsal lingual epithelium. *Dev. Biol.* **254**, 1-18.

Miura, H. and Barlow, L. A. (2010). Taste bud regeneration and the search for taste progenitor cells. *Arch. Ital. Biol.* **148**, 107-118.

Miura, H., Kusakabe, Y. and Harada, S. (2006). Cell lineage and differentiation in taste buds. *Arch. Histol. Cytol.* **69**, 209-225.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.

Nechiporuk, A. and Raible, D. W. (2008). FGF-dependent mechanosensory organ patterning in zebrafish. *Science* **320**, 1774-1777.

Nie, X. (2005). Apoptosis, proliferation and gene expression patterns in mouse developing tongue. *Anat. Embryol.* **210**, 125-132.

Nieto, M., Schuurmans, C., Britz, O. and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. *Neuron* **29**, 401-413.

Northcutt, R. G. (2004). Taste buds: development and evolution. *Brain Behav. Evol.* **64**, 198-206.

Northcutt, R. G. (2005). Taste bud development in the channel catfish. *J. Comp. Neurol.* **482**, 1-16.

Okubo, T., Pevny, L. H. and Hogan, B. L. (2006). Sox2 is required for development of taste bud sensory cells. *Genes Dev.* **20**, 2654-2659.

Okubo, T., Clark, C. and Hogan, B. L. (2008). Cell lineage mapping of taste bud cells and keratinocytes in the mouse tongue and soft palate. *Stem Cells* **27**, 442-450.

Ota, M. S., Kaneko, Y., Kondo, K., Ogishima, S., Tanaka, H., Eto, K. and Kondo, T. (2009). Combined in silico and in vivo analyses reveal role of Hes1 in taste cell differentiation. *PLoS Genet.* **5**, e1000443.

Pierfelice, T., Alberi, L. and Gaiano, N. (2011). Notch in the vertebrate nervous system: an old dog with new tricks. *Neuron* **69**, 840-855.

Pogoda, H. M., von der Hardt, S., Herzog, W., Kramer, C., Schwarz, H. and Hammerschmidt, M. (2006). The proneural gene ascl1a is required for endocrine differentiation and cell survival in the zebrafish adenohypophysis. *Development* **133**, 1079-1089.

Raible, F. and Brand, M. (2001). Tight transcriptional control of the ETS domain factors Erm and Pea3 by Fgf signaling during early zebrafish development. *Mech. Dev.* **107**, 105-117.

Rebello, M. R., Aktas, A. and Medler, K. F. (2011). Expression of calcium binding proteins in mouse type II taste cells. *J. Histochem. Cytochem.* **59**, 530-539.

Reifers, F., Bohli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y. and Brand, M. (1998). Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* **125**, 2381-2395.

Roehl, H. and Nusslein-Volhard, C. (2001). Zebrafish pea3 and erm are general targets of FGF8 signaling. *Curr. Biol.* **11**, 503-507.

Roper, S. D. (2007). Signal transduction and information processing in mammalian taste buds. *Pflugers Arch.* **454**, 759-776.

Scheer, N. and Campos-Ortega, J. A. (1999). Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. *Mech. Dev.* **80**, 153-158.

Scheer, N., Groth, A., Hans, S. and Campos-Ortega, J. A. (2001). An instructive function for Notch in promoting gliogenesis in the zebrafish retina. *Development* **128**, 1099-1107.

Schimmang, T. (2007). Expression and functions of FGF ligands during early otic development. *Int. J. Dev. Biol.* **51**, 473-481.

Schneider-Maunoury, S. and Pujades, C. (2007). Hindbrain signals in otic regionalization: walk on the wild side. *Int. J. Dev. Biol.* **51**, 495-506.

Seta, Y., Seta, C. and Barlow, L. A. (2003). Notch-associated gene expression in embryonic and adult taste papillae and taste buds suggests a role in taste cell lineage decisions. *J. Comp. Neurol.* **464**, 49-61.

Seta, Y., Stoick-Cooper, C. L., Toyono, T., Kataoka, S., Toyoshima, K. and Barlow, L. A. (2006). The bHLH transcription factors, Hes6 and Mash1, are expressed in distinct subsets of cells within adult mouse taste buds. *Arch. Histol. Cytol.* **69**, 189-198.

Stone, L. M., Finger, T. E., Tam, P. P. and Tan, S. S. (1995). Taste receptor cells arise from local epithelium, not neurogenic ectoderm. *Proc. Natl. Acad. Sci. USA* **92**, 1916-1920.

Strahle, U., Blader, P., Henrique, D. and Ingham, P. (1993). Axial, a zebrafish gene expressed along the developing body axis, shows altered expression in cyclops mutant embryos. *Genes Dev.* **7**, 1436-1446.

Thirumangalathu, S., Harlow, D. E., Driskell, A. L., Krimm, R. F. and Barlow, L. A. (2009). Fate mapping of mammalian embryonic taste bud progenitors. *Development* **136**, 1519-1528.

Thisse, B., Thisse, C. and Weston, J. A. (1995). Novel FGF receptor (Z-FGFR4) is dynamically expressed in mesoderm and neurectoderm during early zebrafish embryogenesis. *Dev. Dyn.* **203**, 377-391.

Thisse, C. and Thisse, B. (2005). High throughput expression analysis of ZF-models consortium clones. In *ZFIN Direct Data Submission (http://zfin.org)*.

Tonou-Fujimori, N., Takahashi, M., Onodera, H., Kikuta, H., Koshida, S., Takeda, H. and Yamasu, K. (2002). Expression of the FGF receptor 2 gene (fgfr2) during embryogenesis in the zebrafish *Danio rerio*. *Mech. Dev.* **119 Suppl. 1**, S173-S178.

Wellner, U., Schubert, J., Burk, U. C., Schmalhofer, O., Zhu, F., Sonntag, A., Waldvogel, B., Vannier, C., Darling, D., zur Hausen, A. et al. (2009). The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat. Cell Biol.* **11**, 1487-1495.

Wienholds, E., Kloosterman, W. P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H. R., Kauppinen, S. and Plasterk, R. H. (2005). MicroRNA expression in zebrafish embryonic development. *Science* **309**, 310-311.

Yamamoto, Y., Byerly, M. S., Jackman, W. R. and Jeffery, W. R. (2009). Pleiotropic functions of embryonic sonic hedgehog expression link jaw and taste bud amplification with eye loss during cavefish evolution. *Dev. Biol.* **330**, 200-211.

Zachar, P. C. and Jonz, M. G. (2011). Confocal imaging of Merkel-like basal cells in the taste buds of zebrafish. *Acta Histochem.* (in press).

Zhou, Y., Liu, H. X. and Mistretta, C. M. (2006). Bone morphogenetic proteins and noggin: inhibiting and inducing fungiform taste papilla development. *Dev. Biol.* **297**, 198-213.

Zuniga, E., Stellabotte, F. and Crump, J. G. (2010). Jagged-Notch signaling ensures dorsal skeletal identity in the vertebrate face. *Development* **137**, 1843-1852.