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

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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.

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, Efg 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). A downstream target of Wnt/β-catenin signaling activity 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 (DI1, Jig1 and Jig2) 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...
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*, *fgfr1a*, *fgfr1b* and *fgfr2* are expressed in pharyngeal arch epithelium (Hall et al., 2006; Thiese and Thiese, 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**2525a** (Reifers et al., 1998), ascl1a**122521** (Pogoda et al., 2006), miR**b2525b** (Itoh et al., 2003), Tg(*hsp70:dnfgfr1-EGFP*)pa1/+ (Lee et al., 2005) and Tg(*hsp70:Gal41.5kca4/+;Tg(UAS:myc-notch1a-intra)kca3/+*) (Scheer and Campos-Ortega, 1999) zebrafish lines.

**Construction of Tg(*phlb:egfp*) zebrafish**

A 5 kb fragment just upstream of the zebrafish *phlb* gene was PCR amplified and cloned into the Tol2TKHGPgata2A. To generate transgenic fish, one-cell embryos were injected with pT2KHG-Promoter *phlb* and transposase RNA, then screened at 72 hours post fertilization (hpf) for eGFP fluorescence in taste buds. eGFP expression was found similar to SH expression in the developing taste buds (see Fig. SIM-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(*hsp70:dnfgfr1-EGFP*)pa1/+; *Hsdnfgfr1*), or Tg(*hsp70:Gal41.5kca4/+;Tg(UAS:myc-notch1a-intra)kca3/+*) and Tg(*hsp70:dnfgfr1-EGFP*)pa1/+; Tg(*hsp70:Gal41.5kca4/+;Tg(UAS:myc-notch1a-intra)kca3/+*) (HsNicd, HsNicd) were injected at the four-cell stage with gfp, mcherry (70-100 pg) RNA or miniruby (Invitrogen) as a lineage tracer and zebrafish tau**+** 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'-AACACTCGTACCAGACAGTGTTAGA-3'), anti-miR-200b (5'-GTCATCTACCCAGGCACAGTTATTA-3'), anti-miR-429 (5'-ACCGGCA- TTACCACTAGATTA-3') (GeneTools). Control morpholino was 5'-CCCTTACTCTCTGACTATATTATA-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); db (Haddon et al., 1998); fgf8 (Reifers et al., 1998); fgfr4 (Thiese et al., 1995); fgfr1a (Hall et al., 2006); foxa2/axial (Stabell 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 (Cunilffe and Casaccia-Bonnefond, 2006). Primary antibodies were rabbit anti-GFP (1/1000, Torrey-Pines), rat anti-GFP (1/500, NacalaiTesque), rabbit anti-SHT (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
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 nasal 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
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

Fgf8, its 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). Fgf8 is expressed in taste bud Calb2b+ and surrounding cells, but not in Calb2b+ and 5HT+ (Fig. 1I,J). Altogether, these results show that Fgf signaling is involved in taste bud formation. When Fgf 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 Fgf 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 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).

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

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<td></td>
<td>SHT+ cells</td>
<td>Calb2b+ cells</td>
<td>SHT+ cells</td>
<td>Calb2b+ cells</td>
<td>SHT+ cells</td>
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<tr>
<td>Lips</td>
<td>8</td>
<td>8</td>
<td>12</td>
<td>14</td>
<td></td>
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<tr>
<td>Pharyngeal arches</td>
<td>53</td>
<td>40</td>
<td>75</td>
<td>54</td>
<td></td>
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<td>Palate</td>
<td>22</td>
<td>7</td>
<td>29</td>
<td>35</td>
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<tr>
<td>Total</td>
<td>83±7</td>
<td>55±4</td>
<td>116±9</td>
<td>103±3</td>
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<td>372±14</td>
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Data are means±s.d.; n=9.
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. 2. Taste bud development requires Fgf signaling. (A) Heatshock and SU5402 treatments in tg(hsp70: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-200a (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,J) 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.

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). 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 Fgf 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 (I-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 endoderm (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.
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Table 2. Number of Calb2b⁺ taste bud cells in the oropharynx of wild-type embryos that received endodermal grafts

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<tr>
<th>Pharyngeal arches</th>
<th>Calb2b+ cells</th>
<th>Tracer+ cells</th>
<th>Calb2b+ tracer+ cells</th>
<th>Ratio of Calb2b+ tracer+ to tracer+ cells</th>
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<tr>
<td>WT&lt;tracer (n=5)</td>
<td>195±14</td>
<td>120±27</td>
<td>42±16</td>
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<td>WT&lt;tracer+HS (n=8)</td>
<td>139±12</td>
<td>104±36</td>
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<td>WT&lt;Hsdnfgfr1 (n=8)</td>
<td>100±15</td>
<td>117±35</td>
<td>6±2</td>
<td>0.05±0.01</td>
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<td>WT&lt;Hsdnfgfr1+HsNicd (n=6)</td>
<td>138±7</td>
<td>119±13</td>
<td>25±6</td>
<td>0.21±0.06</td>
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<td>WT&lt;HsNicd (n=6)</td>
<td>137±27</td>
<td>107±37</td>
<td>27±10</td>
<td>0.25±0.05</td>
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<th>Palate</th>
<th>Calb2b+ cells</th>
<th>Tracer+ cells</th>
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<th>Ratio of Calb2b+ tracer+ to tracer+ cells</th>
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<tr>
<td>WT&lt;tracer (n=5)</td>
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<td>WT&lt;Hsdnfgfr1 (n=8)</td>
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<th>Pharyngeal arches and palate</th>
<th>Calb2b+ cells</th>
<th>Tracer+ cells</th>
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<th>Ratio of Calb2b+ tracer+ to tracer+ cells</th>
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<td>WT&lt;Hsdnfgfr1 (n=8)</td>
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Grafts expressed: tracer without heat shock (WT<tracer); tracer after heat shock (WT<tracer+heatshock); transgenes activated after heat shock (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 means±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. 1LJ). 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, 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,
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 dll had an obvious taste bud-related expression profile. By 62 hpf, dll expression is restricted to one or two cells per taste bud, always devoid of Calb2b and 5HT expression (Fig. 10-P). However, earlier, at 54-56hpf, dll 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(php1b:egfp)+ cells, express notch1a (Fig. 1Q-R). Thus, before taste bud differentiation, multicellular notch1a and dll 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, dll was overexpressed in continuous rows of cells and not restricted to patches as in wild-
This is reminiscent of Delta signaling (inhibition of Fgf signaling in required for taste bud related ascl1a–/– suggesting that reduced in mib–/– maintenance, whereas premature develop neuronal fate and therefore other (later occurring) cell type differentiation is prevented.

The reduction of Calb2b+ cells in embryos with compromised expression in the pharyngeal epithelium (Fig. 6M,N, Calb2b+ 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). Complementary to these results, activation of Notch signaling in HsNicd embryos by heat shock 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.

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

<table>
<thead>
<tr>
<th></th>
<th>5HT+ cells</th>
<th>Calb2b+ cells</th>
<th>pH3+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n=11)</td>
<td>188±7</td>
<td>406±14</td>
<td>133±11</td>
</tr>
<tr>
<td>Control MO (n=11)</td>
<td>164±9</td>
<td>393±8</td>
<td>140±14</td>
</tr>
<tr>
<td>MO-miR-200a (n=11)</td>
<td>198±6</td>
<td>423±13</td>
<td>148±11</td>
</tr>
<tr>
<td>MO-miR-200b (n=11)</td>
<td>176±6</td>
<td>402±15</td>
<td>133±11</td>
</tr>
<tr>
<td>MO-miR-429 (n=11)</td>
<td>172±11</td>
<td>445±10</td>
<td>133±11</td>
</tr>
<tr>
<td>Triple MO200 (n=11)</td>
<td>115±9</td>
<td>43±11</td>
<td>112±17</td>
</tr>
</tbody>
</table>

Data are means±s.d.; ***P<0.001 (t-test).
See also Fig. 55 in the supplementary material.

Fgf signaling is necessary for intact dlb expression in the pharyngeal epithelid

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. Heat shock-induced inhibition of Fgf signaling in HsdNFgfr1 grafts resulted in severe 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 heat shock 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.

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,H) 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 (J,L, 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.
reduction of dlbb-expressing cells compared with the control (at 60 hpf, Fig. 4A-F, ratio of dlbb+ 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 dlbb 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 dlbb/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 M0miR-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 M0miR-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...
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 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.

**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
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 signaling during taste bud formation, and the functional approaches other sensory organs that eventually will differentiate to Calb2b+ cells, of taste bud prosensory domains (lateral induction), 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 signaling during taste bud formation, and the functional approaches other sensory organs that eventually will differentiate to Calb2b+ cells, of taste bud prosensory domains (lateral induction), 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 signaling during taste bud formation, and the functional approaches other sensory organs that eventually will differentiate to Calb2b+ cells, of taste bud prosensory domains (lateral induction), 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.

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, 2005; Daudet and Lewis, 2005; Barlow and Northcutt, 1995). Embryonic origin of amphibian taste buds. Dev. Biol. 169, 237-239.

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