



Multiple Shh signaling centers participate in fungiform papilla and taste bud formation and maintenance



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ABSTRACT

The adult fungiform taste papilla is a complex of specialized cell types residing in the stratified squamous tongue epithelium. This unique sensory organ includes taste buds, papilla epithelium and lateral walls that extend into underlying connective tissue to surround a core of lamina propria cells. Fungiform papillae must contain long-lived, sustaining or stem cells and short-lived, maintaining or transit amplifying cells that support the papilla and specialized taste buds. Shh signaling has established roles in supporting fungiform induction, development and patterning. However, for a full understanding of how Shh transduced signals act in tongue, papilla and taste bud formation and maintenance, it is necessary to know where and when the Shh ligand and pathway components are positioned. We used immunostaining, *in situ* hybridization and mouse reporter strains for *Shh*, *Ptch1*, *Gli1* and *Gli2*-expression and proliferation markers to identify cells that participate in hedgehog signaling. Whereas there is a progressive restriction in location of Shh ligand-expressing cells, from placode and apical papilla cells to taste bud cells only, a surrounding population of *Ptch1* and *Gli1* responding cells is maintained in signaling centers throughout papilla and taste bud development and differentiation. The Shh signaling targets are in regions of active cell proliferation. Using genetic-inducible lineage tracing for *Gli1*-expression, we found that *Shh*-responding cells contribute not only to maintenance of filiform and fungiform papillae, but also to taste buds. A requirement for normal Shh signaling in fungiform papilla, taste bud and filiform papilla maintenance was shown by *Gli2* constitutive activation. We identified proliferation niches where Shh signaling is active and suggest that epithelial and mesenchymal compartments harbor potential stem and/or progenitor cell zones. In all, we report a set of *hedgehog* signaling centers that regulate development and maintenance of taste organs, the fungiform papilla and taste bud, and surrounding lingual cells. Shh signaling has roles in forming and maintaining fungiform papillae and taste buds, most likely via stage-specific autocrine and/or paracrine mechanisms, and by engaging epithelial/mesenchymal interactions.

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Introduction

The adult fungiform papilla is a complex set of tissues and cell types that reside in the specialized multilayered epithelium of the tongue. This unique sensory organ includes: taste buds; a layered and keratinized surface epithelium; and, lateral epithelial walls that extend into the underlying connective tissue to surround a core of lamina propria cells (Fig. 1). The core is engorged with blood vessels and bundles of nerve fibers from geniculate and

trigeminal sensory ganglia. Furthermore, the core itself is specialized into apical and more basal regions. Surrounding the fungiform papillae are other specializations of the lingual epithelium, the filiform papillae. The filiform structures do not contain taste buds, but have sharp, heavily keratinized, apical spines. Thus, to develop and maintain the fungiform papillae, many regulatory steps must be orchestrated that require close interactions between the tongue epithelium and connective tissue core.

Placement of fungiform papillae also is tightly regulated, on the anterior two thirds of the tongue (Mistretta, 1991; Mbiene et al., 1997). These patterned papillae, accessible for observation and manipulation, are an important but often over-looked model for the development of ectodermal specializations, and for understanding fundamentals of sensory cell differentiation.

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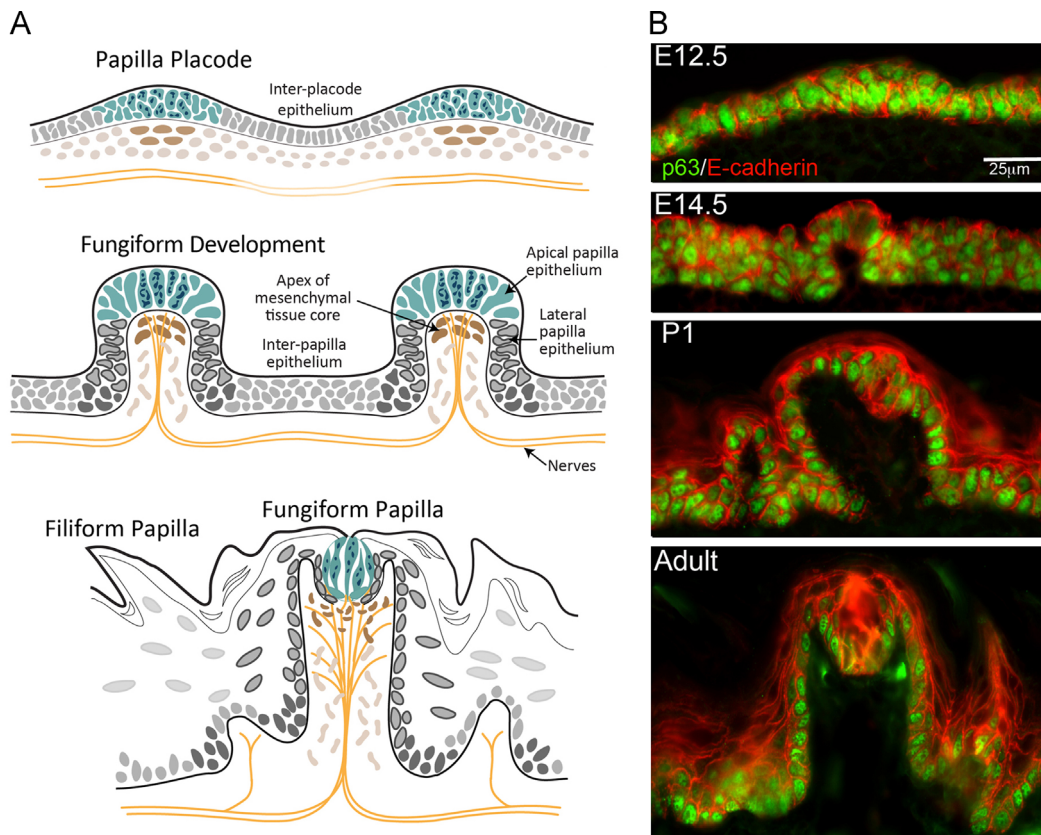


Fig. 1. Papillae and taste buds develop in a complex epithelium. (A) Schema for fungiform papilla development. The Papilla Placode (about E12.5 to E13.5) develops as a collection of epithelial cells over mesenchymal cells, with an inter-placode epithelium that is a single layer of cells. Nerves are within the tongue but not yet approaching the epithelium. With Papilla Development (about E14.5 to E17.5) cell compartments become distinctive. The fungiform papilla epithelium is differentiated at the apex and forms lateral papilla walls that delimit the papilla and extend around a papilla core of mesenchymal cells. From within the core, nerve fiber bundles distribute to the epithelium. The inter-papilla epithelium brackets fungiform papillae. In the adult, the Fungiform Papilla has a differentiated, apical collection of cells, the taste bud, in the apical epithelium. The inter-papilla epithelium has differentiated into non-gustatory, Filiform Papilla structures. In the papilla core a set of apical connective tissue, lamina propria cells are distinguished from other cells in the core. Nerves within the papilla branch to taste buds and surround, and innervate the taste bud cells. (B) Cell layers and basal cells are apparent with immunoreactions for p63 and E-cadherin. The papilla placode at E12.5 is a collection of cells within an epithelium between placodes that is essentially a single layer. Proliferating cells (p63-positive) are throughout the epithelium. At E14.5 the developing papilla has basal (p63-positive) and apical (E-cadherin-positive) cell layers. Apical papilla cells are distinguished from lateral walls. The P1 papilla has multiple cell layers (E-cadherin) over a basal cell layer (p63) and a distinct apical cell collection that is the early taste bud. Filiform papillae are seen in inter-papilla epithelium. The Adult papilla has a taste bud in the apex and interruptions in the basal cell layer under the taste bud are apparent, where nerves enter to innervate the taste bud cells. Filiform papillae surround the fungiform papillae.

After embryonic formation and development, the taste papillae and non-gustatory filiform papillae in the postnatal tongue grow, differentiate and turn over in cell renewal cycles (Mistretta, 1991; Mistretta and Hill, 1995, 2003). Importantly, the taste buds make a perinatal emergence and continue to differentiate and turn over postnatally, as taste bud cells are renewed throughout the life span (Beidler and Smallman, 1965; Hendricks et al., 2004; Krimm and Hill, 2000). Increased extent and complexity of taste bud innervation coincides with development of taste function and papilla and taste bud cell turn over.

Among the growing cast of identified molecules that regulate the fungiform papillae, Shh is a key player (reviewed in Mistretta and Liu, 2006). Shh signaling has roles in fungiform induction, development and patterning (Hall et al., 2003; Liu et al., 2004; Mistretta et al., 2003) and is proposed to function in taste bud formation (Thirumangalathu et al., 2009). However, there is no deep grasp of where the Shh signal originates and where signaling targets are positioned in the embryonic, postnatal and adult papilla and taste bud. Whereas there is demonstrated tight control of hedgehog signals that talk across epithelium and mesenchyme in formation of hair follicle, tooth and intestinal villus (Chuong et al., 2000; Dassule et al., 2000; St Jacques et al., 1998; Walton et al., 2012), we have scarce data about locales and timing for expression of *Hh* signaling components in papilla development.

The Hh pathway is well defined. When Hh ligands bind to the Patched (Ptch) transmembrane receptor, Ptch repression of a second transmembrane protein, Smoothened (Smo), is relieved (Robbins et al., 2012). Smo then initiates intracellular signaling, ultimately activating Gli transcription factors. This leads to induction of Shh target genes. Effectors of Hh signaling in vertebrates are Gli proteins, Gli1, Gli2, Gli3. Diffusible morphogens such as Shh can be strong activators at short range and continue activation at longer range, of 200 μm or more (Saha and Schaffer, 2006). At the same time, a surrounding zone of lateral inhibition can act to pattern tissues in coordination with other pathways (Liu et al., 2004; Zhou et al., 2006).

To understand how Shh signals in tongue, papilla and taste bud formation and maintenance, it is necessary to know where and when Shh ligand and pathway components are positioned. We identified Shh signaling centers in the context of defined cell and tissue compartments in fungiform papillae with reporter mouse lines. By mapping expression of the hedgehog targets *Ptch1* and *Gli1*, which provide a direct indication of *Shh* responsiveness (Ahn and Joyner, 2004; Marigo et al., 1996), spatial and temporal changes in signaling centers were demonstrated and responding cells shown to bracket the restricted location of Shh protein and message. With lineage tracing for *Gli1* we found that Shh-responding cells contribute progeny not only for maintenance of

filiform and fungiform papillae, but also for taste buds. A requirement for normal Shh signaling in fungiform papilla, taste bud and filiform papilla maintenance was shown by *Gli2* constitutive activation. We identified proliferation niches where Shh signaling is active and suggest that epithelial and connective tissue compartments harbor proposed stem and/or progenitor cell zones. In all, we report a set of hedgehog signaling centers that regulate development and maintenance of the taste organ, fungiform papilla and taste bud, and the lingual surround.

Methods

Animals

Animal maintenance and use were in compliance with institutional animal care protocols and in accordance with National Institutes of Health Guidelines for care and use of animals in research. All dissections of E12.5–18.5 embryos were between 9:00 AM and 12:00 PM for consistency across litters (Mbiene et al., 1997). Noon of the day of vaginal plug detection was designated embryonic day 0.5 (E0.5). Embryos were staged by vaginal plug detection and confirmed by Thielier staging for development of multiple organs. P1 was the day when pups were born.

Mouse lines and tissue collection

Timed, pregnant C57BL/6 mice (E12.5, E14.5 and E18.5), postnatal mice (1–12 months) were from Charles River breeders.

Mouse lines that carry the bacterial β -galactosidase (*lacZ*) gene driven by *Sonic hedgehog* (*Shh-lacZ*, Jeong et al., 2004), *Patched 1* (*Ptch-lacZ*, Goodrich et al., 1997; available Jackson Laboratory Strain 008211), *Gli1* (*Gli1-lacZ*, Bai et al., 2002; available Jackson Laboratory Strain 003081), or *Gli2* (*Gli2-lacZ*, Bai and Joyner, 2001; Jackson Laboratory Strain 007922) were used to monitor the expression of hedgehog signaling components. The reporter gene positive litters were genotyped by PCR and X-Gal staining, and wild type littermates were used for comparison.

Gli1-CreERT2;R26R bitransgenic mice were used for lineage tracing with conditional activation of *lacZ* reporter upon inducible Cre activation driven by the *Gli1* promoter. Tamoxifen chow (0.4 mg per g diet) was fed for 4 weeks to induce *lacZ* gene expression in *Gli1*-expressing cells, which is maintained in their progeny. Tongues were analyzed from 1 day to 3 months after the termination of tamoxifen treatment, with X-Gal staining in whole tongue followed by cryostat sectioning.

*K5-rtTA;TRE-GLI2** mice (Diamond et al., 2000; Gratchchouk et al., 2011) were used for functional analysis with a doxycycline-inducible, constitutively active truncation mutant of human *GLI2* controlled by a *Keratin 5* (*K5*) promoter-driven reverse tet transactivator (rtTA), so that the hedgehog pathway was activated in a ligand-independent manner. Doxycycline was administered at a dose of 200 μ g/ml in 5% sucrose in drinking water, and doxycycline-containing chow (1000 mg/kg, Bio-Serve, Frenchtown NJ). Mice received doxycycline in chow and drinking water *ad lib* for 3 days, after which the mice were maintained on doxycycline chow for up to 7 or 12 days.

Mice were euthanized by an intraperitoneal injection of urethane (60 mg/g body weight) or a slow stream of CO₂. Embryonic or postnatal tongue on mandibles were removed into cold, sterile phosphate buffered saline (PBS) and tissues were dissected and processed for different analyses.

Scanning electron microscopy

Tongues were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde (PFA) in 0.1 M cacodylate buffer (pH 7.3) at 4 °C, post-fixed in a sequence of aqueous 1% O₅O₄, 1% tannic acid, 1% O₅O₄, for 1 h each on ice, and processed as described (Mbiene et al., 1997). Tissues were mounted, sputter coated with gold/palladium, and analyzed with SEM. Digital images were acquired at $\times 25$, $\times 40$, $\times 100$, $\times 1000$ original magnification and assembled using Photoshop (Adobe Systems, Mountain View, CA).

Histology

Mouse tongues were dissected and fixed in 4% PFA in 0.1 M PBS, pH 7.4, at 4 °C for overnight, then dissected into pieces. Anterior half tongues were transferred to 70% ethanol, embedded in paraffin and serially sectioned in sagittal plane at 6–8 μ m for hematoxylin and eosin staining. To compare lingual tissues between control and transgenic tongues, serial sections were examined to ensure evaluation of the complete anterior tongue. Photomicroscopy presented comparative tongue and papilla phenotypes.

Immunohistochemistry

Antibodies

Primary antibodies and dilutions were: β III-tubulin (T2200, 1:1000, Sigma Aldrich, St Louis MO); BrdU (G3G4, 1:400, Developmental Studies Hybridoma Bank, University of Iowa); E-cadherin (AF748, 1:500, R&D Systems, Minneapolis MN); Gli2 (AF3635, 1:1000, R&D Systems); Keratin 5 (PVB-160P, 1:5000, Covance, Emeryville CA); Keratin 8 (TROMA-1, 1:200–1000, Developmental Studies Hybridoma Bank); Ki67 (M7249, 1:400, DakoCytomation, DK); Laminin (L9393, 1:500, Sigma Aldrich, St Louis MO.); Neurofilament-L, M, H (NB300-131, -133, -135, 1:3000, Novus Biologicals, Littleton CO); p63 (sc-8431, 1:100, Santa Cruz Biotechnology, Santa Cruz CA); Shh (AF464, 0.1 μ g/ml, R&D Systems).

Tissue section immunohistochemistry

Dissected embryonic and postnatal tongues were fixed in 4% PFA in 0.1 M PBS, pH 7.4, at 4 °C for 2–5 h, then transferred to 30% sucrose in PBS at 4 °C for 24 h. Tissues were frozen in O.C.T. Serial sagittal sections were cut at 10 μ m, thaw-mounted onto Superfrost[®] Plus slides (12-550-15, Fisher Scientific, Pittsburgh PA) and reacted as described (Liu et al., 2004; Mistretta et al., 2003). Sections were blocked with 10% donkey serum for 1 h and incubated with primary antibody at 4 °C for overnight. Following incubation with Alexa fluor[®] 488 or 568 conjugated secondary antibodies for 1–2 h (1:500, Invitrogen/Molecular Probes, Grand Island NY). Slides treated with no primary antibody or with the same concentration of normal IgG were used as controls. For Shh, Ki67 and p63 immunoreactions, sections underwent antigen retrieval in citrate buffer (pH 6.0) at 90–95 °C for 5 min.

X-Gal staining

Tongue or tongue sections from *lacZ* reporter mice were fixed in 4% PFA on ice for 15 min, washed in PBS containing 2.0 mM MgCl₂, transferred into freshly prepared X-Gal solution (1 mg/ml X-Gal in 0.1 M PBS with 2.0 mM MgCl₂/0.01% sodium deoxycholate/0.2% Nonidet P-40/5 mM potassium ferricyanide/5 mM potassium ferrocyanide), and incubated at 37 °C for 1–5 h. Sections were counterstained with neutral red, dehydrated in serial ethanol solution, cleared in xylenes and mounted with Permount medium (SP-15, Fisher Scientific, Pittsburgh PA). Stained tissues

were photographed as whole mounts and then cryo-sectioned for light microscopy.

In situ hybridization

The following probe templates were used for RT-PCR: *Shh* (NM_009170 nucleotides 1–1700), *Ptch1* (NM_008957 nucleotides 300–1489), *Gli1* (NM_010296.2 nucleotides 1072–3058). The PCR products were cloned into pCR4 TOPO vectors (Invitrogen/Molecular Probes, Grand Island NY). Probes were labeled using a DIG RNA labeling kit (SPR/P7, 11 175 025 910, Roche Applied Science, Indianapolis IN). The sense probes were used as controls in parallel at every stage.

All tissues were fixed in 4% PFA at 4 °C overnight and equilibrated in 30% sucrose for at least 24 h at 4 °C. Cryostat sections (10 μm) were treated with 10–50 μg/ml proteinase K in PBS for 15 min. The slides were post-fixed in 4% PFA for 10 min, followed by acetylation in acetic anhydride (pH 8.0) for 10 min. Sections were hybridized at 65 °C overnight with the denatured probes in 50% deionized formamide, 1x SSC, 1x Denhardt's solution, 10% dextran sulfate, 0.2 mg/ml yeast tRNA and 0.5 mg/ml herring sperm DNA. After rinses, the slides were incubated with anti-digoxigenin-AP, Fab fragments (11 093 274 910, 1:1000, Roche Science) for overnight at 4 °C. Following rinses, the slides were equilibrated in the staining buffer (100 mM Tris · HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂) at room temperature for 30 min. Color detection was performed in the dark in staining buffer supplemented with 100 mg/ml NBT (11 383 213 001, Roche Applied Science), 100 mg/ml BCIP (11 383 221 001, Roche Applied Science), 0.3% Tween-20 for 5–24 h. After thorough rinses, slides were mounted with 70% glycerol in PBS and photographed for light microscopy.

Tissue analysis

Quantification for Shh-positive cells in placode and papilla development

Numbers of Shh-positive cells in placodes or papillae, were counted at E12.5, E14.5 and P1 stages. Two or three tongues were studied per stage, in serial sagittal sections of the anterior one third of the tongue, immunostained for Shh expression. Two or three non-consecutive slides were selected, excluding the median furrow, and in 8 to 10 sections per slide, numbers of Shh-positive cells was counted in 11 placodes or papillae. Cell numbers were quantified at 40X with light microscopy. Data are presented as range and median values.

At E14.5 using the same procedures, *Ptch1*-expressing cells were counted in one *Ptch1* reporter tongue that had been immunostained for Shh. Eleven papillae were analyzed.

Papilla and taste bud quantification in transgenic mice

Fungiform papillae and taste buds were quantified in one *Gli1-CreERT2;R26R* mouse tongue at 3 months after termination of tamoxifen administration and in one *K5-rtTA;TRE-GLI2*⁺ tongue after 12 days of doxycycline treatment. Serial sagittal sections from half of the tongue were reacted with X-Gal staining (*Gli1-CreERT2;R26R*) or hematoxylin and eosin staining (*K5-rtTA;TRE-GLI2*⁺) and sections in about 1.0 mm tissue width (100 sections, from the median furrow toward lateral border) were examined with light microscopy. For reference, in the control mouse, each fungiform papilla contains one taste bud in the papilla apex. Each taste bud has a taste pore where microvilli from taste bud cells extend, in an opening (pore) in the taste bud surface. The taste pores can be identified with SEM. Within a single taste bud there are about 60 to 80 cells.

In *Gli1-CreERT2;R26R* mouse tongue sections, taste buds and papillae were divided into four groups or patterns (described in Results) based on distribution of intensely (most cells are X-Gal positive) or sparsely (most cells are X-Gal negative) labeled cells in the taste bud, and labeled cells in the perigemmal region, lateral papilla walls, and underlying mesenchyme. The percentage of patterns for each group was calculated as papilla number with the particular pattern divided by the total number of examined papillae in the half tongue ($n=37$).

In the *K5-rtTA;TRE-GLI2*⁺ mouse, serial tongue sections, 50 fungiform papillae were examined in the half tongue. Papillae were scored for presence or absence of a taste bud, and, taste buds were counted as typical or aberrant (thin and without a taste pore). The numbers of fungiform papillae without a taste bud, with a typical taste bud, or with an abnormal bud were quantified.

Results

Cell compartments of the developing and adult fungiform papilla, taste bud and surrounding epithelium, and mesenchyme or lamina propria

Fungiform placode and papilla have defined epithelial and connective tissue compartments

Specialized cell compartments can be identified in developmental stages from papilla placode to embryonic papilla, through the adult stage when a single taste bud is differentiated in each rodent fungiform papilla (Fig. 1). These compartments provide the context for demonstrating Shh signaling centers.

The papilla placode is a differentiated cluster of epithelial cells and underlying mesenchyme. Within the placode, a central set of cells is further differentiated (Fig. 1A, Papilla Placode). Inter-papilla lingual tissue is less differentiated. Nerves in the mesenchyme are near but not yet within the placode epithelium. As the embryonic fungiform papilla develops (Fig. 1A, Fungiform Development), epithelial layers extend into the mesenchyme and early lamina propria to encompass a core with gustatory innervation (Mbiene and Mistretta, 1997). With further growth, as epithelial layers increase and grow into the tongue, the core expands with some extension of the papilla above the lingual epithelium. The apical papilla epithelium is molecularly and cellularly distinct from lateral papilla walls.

At later postnatal and adult stages the fungiform papilla contains a well-developed, apical taste bud, in a stratified squamous epithelium with proliferation niches at the base of the papilla (Fig. 1A, Fungiform Papilla). The papilla core has an extensive innervation and contains an apically specialized lamina propria that is rather dense under the taste bud. Well-developed filiform papillae surround gustatory fungiform organs (Fig. 1A, Filiform Papilla).

These defined compartments provide a basis for interpreting Shh signaling centers in papilla development and maintenance.

Acquisition of a stratified epithelium and basal cells

We used immunoreactions for p63 to label early basal cells and for the transmembrane protein, E-cadherin to mark cell membranes. There is progressive acquisition of a multi-layered lingual and papilla epithelium. At placode formation, clusters of about 25 bilayered cells, all positive for p63 and E-cadherin, are in the lingual epithelium whereas the inter-placode epithelium remains as an essentially single layer of columnar cells with a periderm covering (Fig. 1B, E12.5). The embryonic papilla already has distinctive apical epithelial cells that are not p63-positive (Fig. 1B, E14.5) and the suprabasal inter-papilla epithelium is multilayered.

The postnatal day 1 (P1) papilla and lingual epithelium have a defined basal cell layer, positive for p63, and the large fungiform

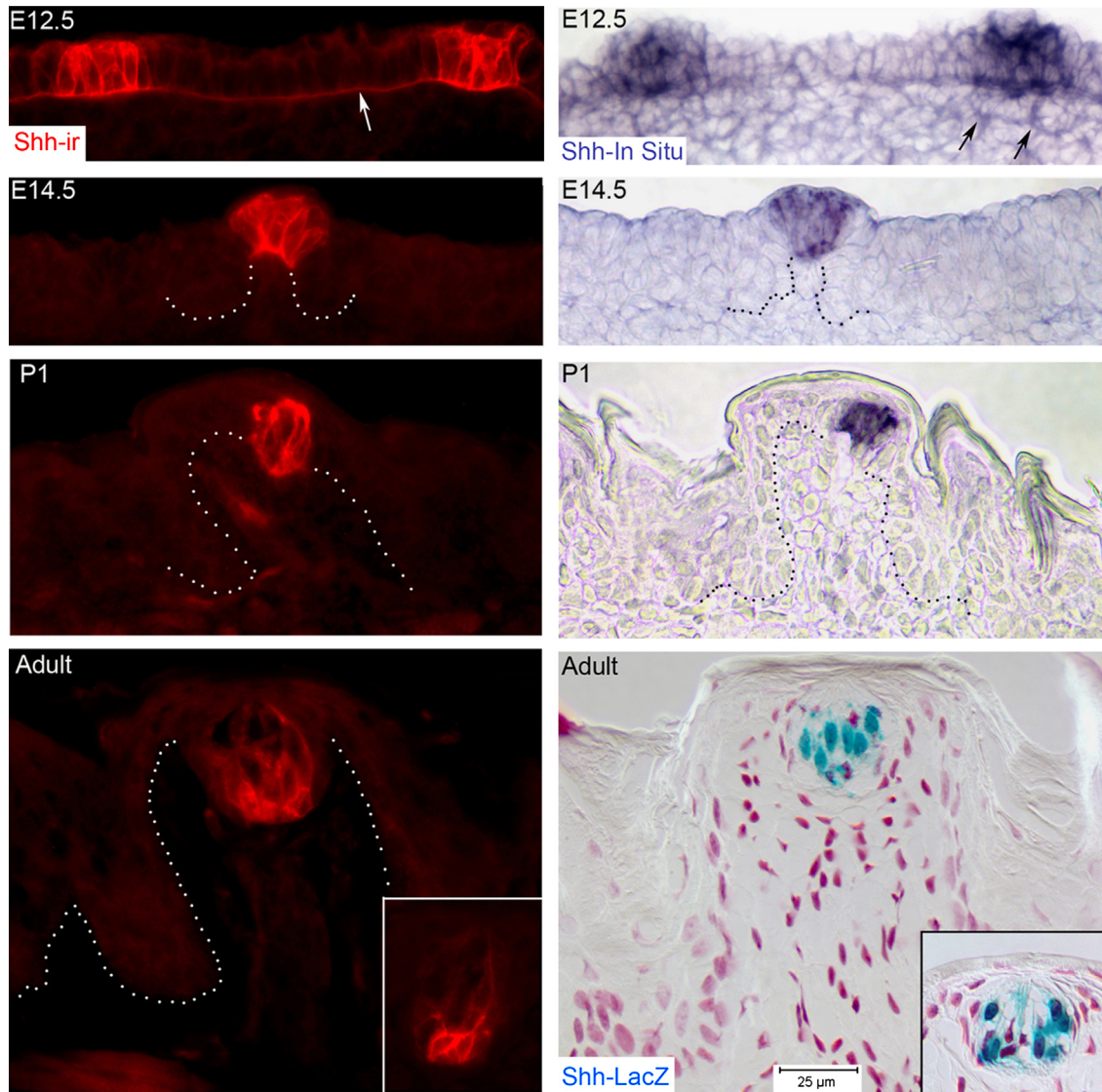


Fig. 2. Shh ligand and message are spatially and temporally restricted in papilla development and maintained in adult taste bud. With Shh immunoreaction (Shh-ir) and in situ hybridization (Shh-in situ), and in reporter mice (Shh-LacZ), an early and continuing restriction of Shh is apparent. At E12.5, Shh is within a cluster of about 9 epithelial cells within the papilla placode, and some specialization of mesenchymal cells is apparent (arrows, in situ). Notably Shh is located in the basal lamina of the placode and between placode epithelium (white arrow, Shh-ir). By E14.5 the emerging fungiform papilla can be distinguished (dotted outline) and the central apical epithelium is positive for Shh (ir and in situ). Shh, tightly restricted in the early postnatal, structurally differentiated papilla, P1, is confined to the early taste bud. Some Shh-positive, presumed endothelial cells are seen in the papilla core. In the Adult papilla, Shh positive cells are within the taste bud, and in serial sections a few basally placed cells are apparent (see insets).

papilla encompasses a core of lamina propria, connective tissue cells (Fig. 1B, P1). Layers of cells, outlined by E-cadherin positive membranes, are in fungiform papilla and surround epithelium. Incipient filiform papillae are found throughout the tongue. The adult mouse fungiform papilla is distinctive in its apical taste bud, composed of dozens of E-cadherin-positive cells with surrounding p63-positive cells (Fig. 1B, Adult). p63-positive basal cells also delimit the fungiform papilla and taste bud and there are multiple cell layers on the papilla apex and inter-papilla tissue. Thus, differentiation of taste papillae, taste buds and non-gustatory papillae, progress in the context of stratification of the tongue epithelium.

The Shh ligand is temporally and spatially restricted in developing and mature papillae and taste buds

Progressive restriction of Shh ligand location

To determine with spatial and temporal accuracy where Shh is localized during fungiform and lingual epithelial development and maintenance, we used independent assays: *immunostaining, in situ*

hybridization and LacZ reporter mice. The emerging placodes and developing papillae are strongly positive for the hedgehog signaling ligand, Shh.

At E12.5 Shh protein and message are intensely expressed in cells of the fungiform papilla placode, with some evidence of graded mRNA expression (Fig. 2, E12.5). There is a range of 7–11 (median=9) Shh-positive cells per central placode region. Notably, Shh protein also is throughout the basal lamina zone of papilla and in the between-papilla tongue regions (Fig. 2, E12.5, Shh-ir, arrow). The intense distribution of Shh in the basal lamina of E12.5 lingual epithelium is important because components of the basal lamina are in a natural position to concentrate or sequester molecular regulators of epithelial development. Some specialization of mesenchyme cells just under the placodes is apparent with in situ hybridization (Fig. 2, E12.5, *in situ*, arrows).

As the fungiform papilla epithelium extends into the tongue, to surround a core of mesenchyme and developing lamina propria, the Shh protein and message are restricted in cells of the apex of the developing papilla (Fig. 2, E14.5). A range of 6–7 (median=7)

Shh-positive cells is confined in the central, apical epithelium of each papilla. This matches the distinctive apical papilla region seen in Fig. 1B, E14.5. Association of Shh with the basal lamina of the inter-papilla epithelium no longer is apparent.

The *postnatal* fungiform papilla has differentiated into a large structure, with basal cell and suprabasal epithelial layers at the papilla apex and in lateral walls (Fig. 2, P1). Well-developed filiform papillae bracket the fungiform. However, Shh protein and mRNA are strictly confined to a subset of apical cells in the fungiform apical epithelium (range=5–7; median=6), within the cells of the early taste bud.

In the adult mouse tongue, Shh-positive cells are apparent only within the well differentiated taste bud, seen in immunoreactions (range=3–7 cells; median=5) and *LacZ* reporter mice (Fig. 2, Adult). Intensely immunoreactive, basally positioned, Shh-positive cells are frequently observed in the adult taste bud (Fig. 2, Adult, insets). Numbers of Shh-positive cells are variable and it is important to view expressing cells within the center of the bud if comparing among papillae. In sections at the taste bud center, intense Shh immunostaining or gene expression using *Shh-lacZ* reporter mouse tongues is generally detected in a few cells. Differences in numbers of Shh-expressing cells in taste buds detected by immunostaining and *LacZ* reporter mice could relate to differences in detection sensitivity, retention of β -Gal activity, or post-transcriptional regulation of Shh expression.

Thus, Shh ligand and mRNA are strong distinctive markers of the developing fungiform placode, papilla and eventually, taste bud. The restricted, intensely staining Shh protein and mRNA expression from placode through adult taste bud stages indicate areas of potential high hedgehog activity.

We also demonstrated Shh in a restricted taste bud cell localization in mice at 12 months of age (Supplementary Fig. S1). Shh-positive cells are in basal locations of the taste bud. As positive controls for Shh immunoreaction reliability, data from hair follicle illustrate Shh-positive cells in the follicle matrix (Supplementary Fig. S1).

Shh positive cells are epithelial only and partially overlap with cytokeratin 8, a taste bud cell marker in adult

To confirm that the Shh-positive cells are epithelial only in embryonic papilla development, we used double labeling with Shh and laminin antibody to delineate the basal lamina. A strict epithelial localization of Shh-positive cells is obvious in placodes, embryonic and postnatal papillae (Fig. 3, Shh and Laminin, E12.5; E14.5; P1). In adult fungiform papilla, the taste bud is circumscribed by a laminin-positive basal lamina and there is a close association of some Shh-positive taste bud cells with the basal lamina (Fig. 3, Shh and Laminin, Adult). Laminin-positive reactivity also labels vascular, endothelial cell lining in the papilla core.

To determine timing for potential differentiation of taste bud precursor cells, in association with Shh protein restriction, we used an antibody to cytokeratin 8 (K8), a taste bud cell marker (Knapp et al., 1995), in double immunoreactions with Shh (Fig. 3, Shh and K8). In epithelial differentiation, K8 appears initially as a marker of “simple” epithelium (Bagutti et al., 1996). At E12.5 the Shh-positive papilla placodes are within an epithelium characterized by K8 immunostaining that is distributed along the apex of periderm cells of the entire lingual epithelium, with no co-localization with Shh (Fig. 3, Shh and K8, E12.5). Shh label is seen in the between-papilla basal lamina at E12.5. In the embryonic papilla, K8 is detected throughout the epithelium (Fig. 3, Shh and K8, E14.5). There is some double labeling for K8 and Shh in apical epithelial cells of the early fungiform papilla, but there is a clear predominance of Shh-positive cells in the papilla apex.

At P1 there is considerable overlap in Shh-positive and K8-positive cells, demonstrating gustatory differentiation in the early

taste bud cells and loss of K8 expression in the lingual surround (Fig. 3, Shh and K8, P1). Thus any simple epithelial cell signature of tongue epithelium is lost whereas K8 now exclusively labels some taste bud cells. In the adult taste bud, molecular markers have shifted in proportion and numerous taste bud cells are positive for the K8 marker, whereas far fewer cells are positive for Shh (Fig. 3, Shh and K8, Adult; and, illustrated in confocal images, Supplementary Fig. S2). Furthermore, Shh-positive only cells remain in the taste bud, demonstrating that not all Shh-expressing intra-bud cells have differentiated to K8-positive cells.

Overall, these data demonstrate that Shh expressing cells of the papilla placode and early papilla are not sustained in large proportion in the adult taste organ, during the multiple cell turnover cycles of postnatal taste bud differentiation and maintenance in the adult. In all, the *Shh* signaling ligand is intense in about 6–10 epithelial cells throughout placode and papilla development but with a progressive tight restriction to cells of the taste bud only. This restriction to some intensely reactive taste bud cells is maintained in older mice, at 12 months. Within the taste bud, there is an emerging predominance of K8-positive cells from P1 to Adult, with proportionately fewer Shh-positive cells retained. In addition, there is an early intense association of Shh with basal lamina components of the lingual epithelium, and then papilla and taste bud epithelium, which demonstrates potential for signaling regulation between epithelium and connective tissue elements.

Shh positive cells are surrounded by proliferating cells from placode to taste bud stages

Shh is a known mitogen with roles in promoting cell proliferation in a range of tissue and organ types (Agathocleous et al., 2007; Mao et al., 2010; Rowitch et al., 1999). We used double labeling of Shh and Ki67, a mitotic activity marker for proliferating cells, to determine proliferation compartments in tongue and papilla and relation to Shh ligand. During papilla placode formation, Ki67-positive cells bracket the Shh-positive cells in placodes and are in the inter-placode epithelium, and in clusters of mesenchyme cells (Fig. 3, E12.5, Shh and Ki67). Shh-positive cells, differentiated and well within the placode center, are apparently no longer dividing. In the early papilla there are dividing cells that bracket Shh-positive apical papilla cells (Fig. 3, E14.5, Shh and Ki67). Proliferating cells surround the taste bud at P1 and in the adult (Fig. 3, P1, Shh and Ki67).

Proliferating cells are also clustered in a particular basal region of the fungiform papilla. The early papilla has epithelial clusters of Ki67 positive cells at the papilla base (Fig. 3, E14.5, Shh and Ki67). In the well developed, postnatal and adult fungiform papilla, clusters of proliferating cells remain at the fungiform papilla base (Fig. 3, Shh and Ki67, P1; Adult, arrows).

Thus proliferation zones bracket Shh ligand during apical papilla and taste bud development and maintenance. Proliferating cell clusters also characterize the base of the papilla.

Location of Shh responding cells

We located Shh responding cells with immunostaining, *in situ* hybridization and *LacZ* reporter mice to identify hedgehog targets, the *Ptch* receptor and *Gli1* transcription factor. *Ptch1* and *Gli1* are transcriptional targets of Shh signaling and thus reflect Shh activity (Ahn and Joyner, 2004; Marigo et al., 1996).

In the embryonic papilla (E14.5) the *Ptch1* receptor is in cells that bracket or surround Shh-positive cells, in the early papilla apex (Fig. 4, *Ptch*, E14.5, three images and inset). From 8–12 cells (median=10) are in the cell cluster that includes Shh-positive centrally positioned cells, and *Ptch1*-positive surround cells. An overlap of labeled Shh and *Ptch1* cells is seen in some cells of

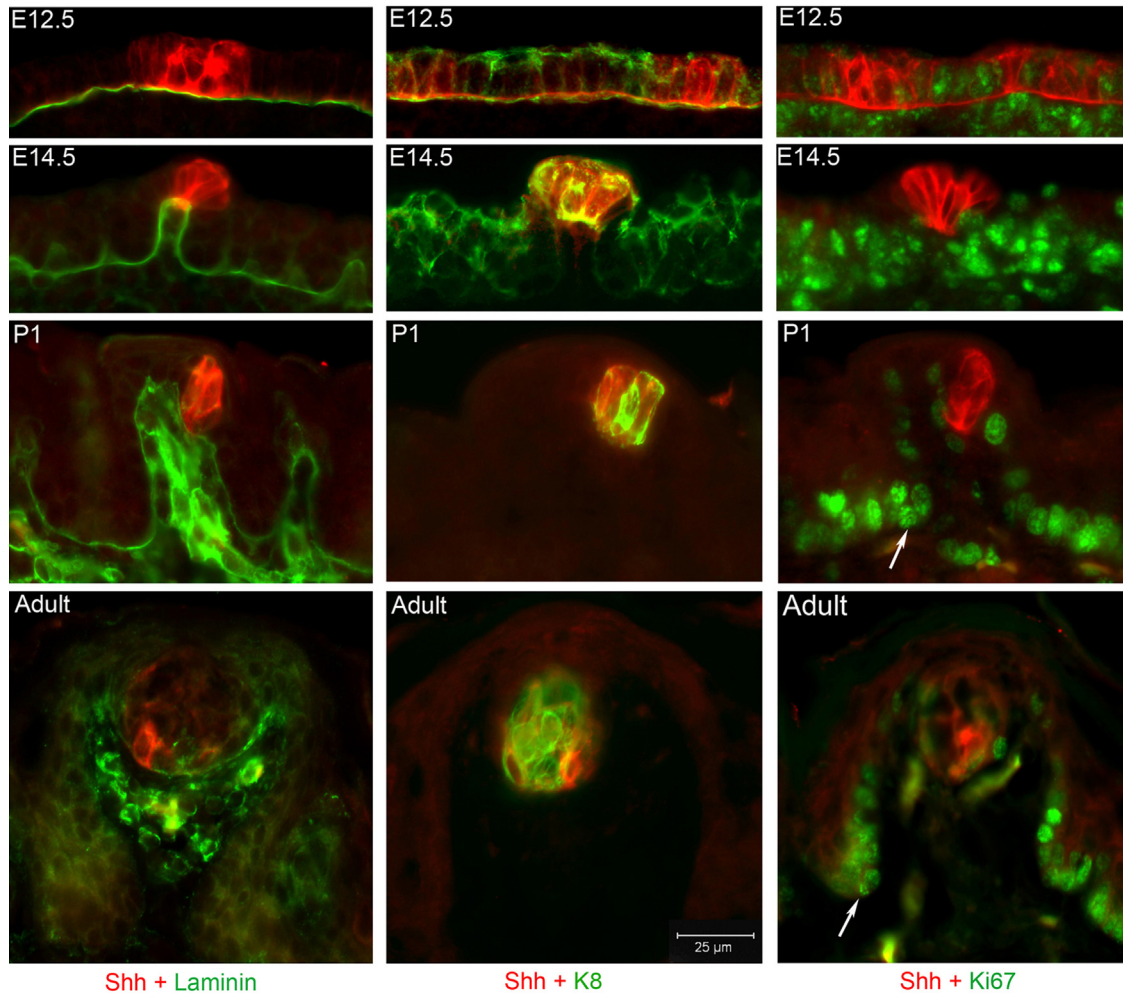


Fig. 3. Shh positive cells are epithelial only, have progressive overlap with taste bud cells, and are surrounded by proliferating cells. In immunoreactions for Shh and Laminin, the basal lamina is delineated from placode, E12.5, through Adult papilla stages, and demonstrates that Shh is epithelial only. Laminin also marks basal lamina in endothelial cells of the papilla core at P1 and Adult. With immunostaining for Shh and K8, the taste bud cell marker, K8 is apparent as an early marker of the single layered epithelium at E12.5. By E14.5, K8 still marks the lingual epithelium and also some cells within the apical papilla epithelium. At P1 Shh-positive cells predominate within the early taste bud whereas in the Adult several cells of the taste bud are K8-positive and fewer are Shh-positive or overlapping with Shh. In Shh and Ki67 immunoreactions, proliferating cells, Ki67-positive, surround the placodal cells in epithelium and mesenchyme at E12.5, although a few remain within the placode epithelium. At E14.5 proliferating cells surround Shh-positive, apical cells of the early papilla, and are within papilla walls and inter-papilla epithelium. In the P1 papilla, proliferating cells surround the early taste bud, are evident as basal cells of the papilla wall, and there are clusters of proliferation at the papilla base (arrow). Clusters of Ki67-positive cells surround the adult taste bud and also are seen at the papilla base in the Adult fungiform (arrow). Ki67-positive cells in the papilla core are most probably marking basal endothelial cells.

the papilla apex. *Ptch1* protein and mRNA also are in mesenchyme cells of the developing papilla core, positioned to receive *Shh* signals from the epithelium.

In the P1 fungiform papilla, *Ptch1* mRNA surrounds a region of weak expression in the apical epithelium (Fig. 4, *Ptch1*, P1). In *lacZ* reporter mice, *Ptch1* expression surrounds the adult taste bud cells, is expressed in basal cells of the papilla wall, in lamina propria just under the taste bud, and is in possible co-localization with papilla innervation (Fig. 4, *Ptch1*, Adult).

Gli1 mRNA in the P1 papilla brackets cells of the early taste bud and is within basal cells of the lateral papilla epithelium (Fig. 4, *Gli1*, P1), similar to the *Ptch1* P1 expression. In *Gli1* reporter mice, the adult papilla has *Shh* signaling activity, or *Gli1* expression, in: cells surrounding the taste bud; lamina propria cells collected just under the taste bud; and, in basal cells of the fungiform papilla wall (Fig. 4, *Gli1*, Adult).

These results place the Hh responding cells, expressing *Ptch1* and *Gli1*, in positions that bracket *Shh*-positive cells, that is: in the apex of early fungiform papillae; intensely in cells surrounding the early taste bud; intensely in cells around the adult taste bud; and in the lamina propria just under taste buds. Thus, responding cells

for *Shh* signaling activity are well positioned to participate in a paracrine signaling process in placode and papilla formation, as well as in papilla and taste bud differentiation and maintenance. Responding cells and ligand define signaling centers of *Shh* activity in development of placodes and the apex of fungiform papillae; in maintaining fungiform papilla lateral walls and apical epithelium; and, in interactions with underlying mesenchyme or lamina propria. The similar *Ptch1* and *Gli1* labeling in lamina propria cells of the apical papilla core, in a basket-like distribution under the taste bud cells suggests co-localization with neural elements. Furthermore, the *Shh* signaling targets are positioned in regions of active cell proliferation shown in Fig. 3, located to receive *Shh* signals, trigger proliferation and contribute cells to the developing and maintained fungiform papilla and taste buds.

Shh signaling cells maintain multiple lineages of the fungiform papillae and taste bud, and filiform papillae

In the postnatal tongue and fungiform papilla, organ development continues and the taste bud continues to differentiate, to acquire a full cell complement and mature innervation (Krimm

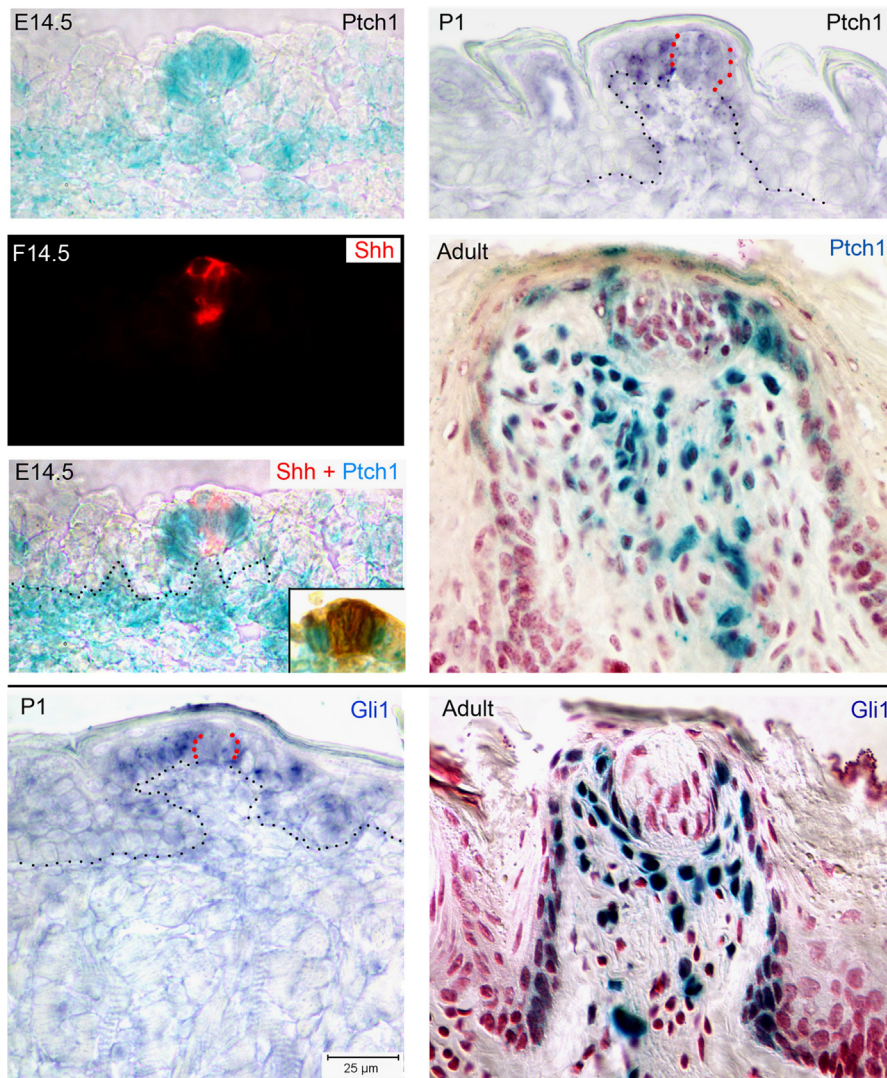


Fig. 4. Shh responding cells, Ptch1- and Gli1-positive, bracket the Shh ligand, in epithelium and mesenchyme. Locations of Shh signaling were identified with reporter mice and in situ hybridization. At E14.5, in reporter mice Ptch1 is intense in cells that surround Shh-positive cells of the early papilla apex. Ptch1 also is within mesenchyme just under the epithelium. At P1, Ptch1 surrounds the early taste bud cells (red outline; black outline for papilla), and in the Adult papilla, Ptch1 positive cells are bracketing the taste bud, in cells of the lateral papilla wall and in lamina propria under the taste bud. Gli1 is similarly located, in taste bud surround at P1 (in situ, red dots outlining taste bud cells) and in Adult, surrounding taste bud cells, in lateral papilla walls and in lamina propria cells around and under the taste bud, but not within the taste bud.

and Hill, 2000; Hendricks et al., 2004). In the *adult* tongue, papilla and mature taste bud, cell turnover maintains a stratified squamous papilla epithelium and specialized sensory cells. We used lineage tracing to test the hypothesis that Shh signaling cells contribute to sustained maintenance of papillae and taste bud cells. Because Gli1 is positioned in key surround locations of the Shh ligand and provides a known indicator of Shh signaling (Ahn and Joyner, 2004), we used a Gli1-CreER mouse line with conditional activation of a *lacZ* reporter to identify the specific tongue and papilla zones where Shh signaling is active in adult Gli1-CreER; R26R mice. The reporter irreversibly activates the *lacZ* gene in Shh-responding, Gli1-expressing cells at the time of tamoxifen induction through genetic recombination, and thereby permits tracing to determine subsequent locations of progeny of these cells. Thus, we could identify genetically labeled Gli1 expressing cells and analyze the distribution of progeny in tissues of the tongue, fungiform papillae and taste buds at different times after gene activation.

Gli1 expressing cells are in fungiform papillae throughout anterior tongue locations at 1 week to 3 months after administration of

tamoxifen for 4 weeks (Fig. 5A, whole tongues). Within one week after concluding the tamoxifen induction of *lacZ* gene expression, large clusters or clones of labeled cells are seen at the papilla base and in lateral papilla walls (Fig. 5B, arrows). Gli1 expressing cells also are within the taste bud and in the lamina propria, just under the taste bud and in the papilla core. The Gli1 progeny are in papilla epithelium sites where proliferating cells are identified with Ki67 immunoreactions (Fig. 5B, Ki67, arrow; and compare Fig. 3, Shh and Ki67, Adult).

In tongue sections at various stages post tamoxifen treatment, we confirmed the presence of Gli1 expressing cells in: the basal cells at the fungiform papilla base and lateral papilla wall; in cells surrounding the taste bud; in cells within taste buds; and in lamina propria cells of the papilla core (Supplemental Fig. S3A and B). To demonstrate that Gli1 labeled cells in the taste bud indeed are taste bud cells, we used the K8 pan taste cell marker to compare with Gli1 progeny (Supplemental Fig. S3C and D).

To learn if Gli1 labeled cells are long-lived progenitors for fungiform papilla and taste bud cells over several weeks of multiple taste cell turnover cycles, we studied the Gli1 lineage-labeled

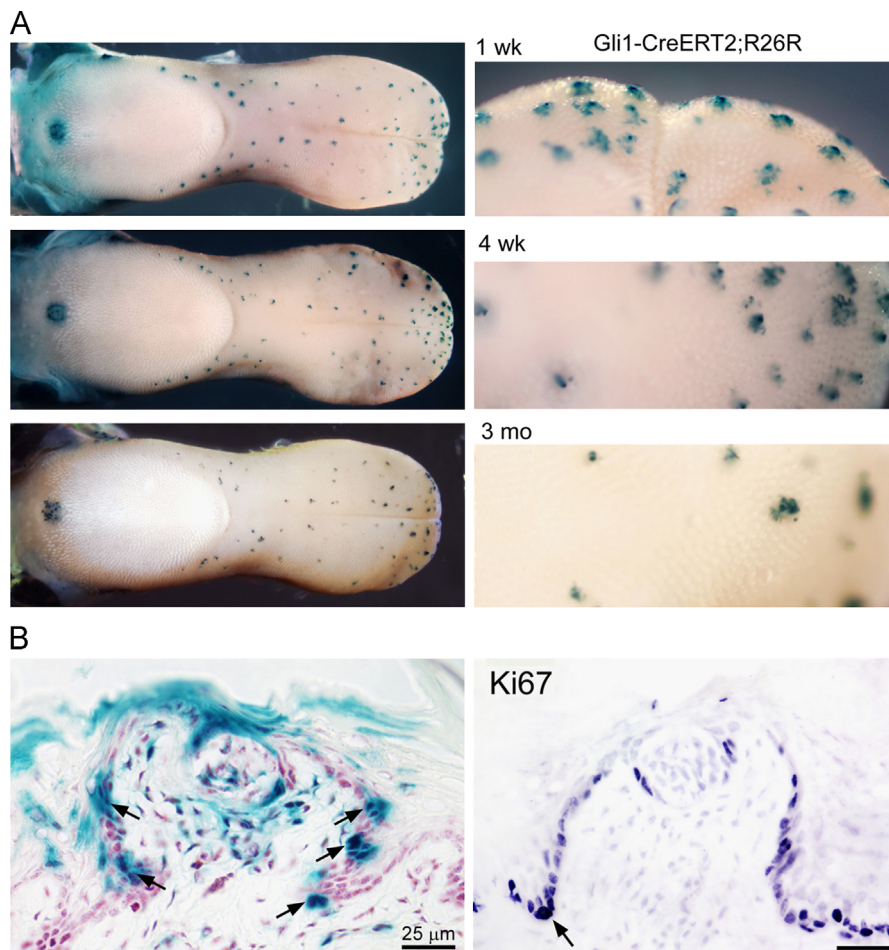


Fig. 5. *Gli1* expressing, derived cells are within epithelium and lamina propria of the adult fungiform papilla and in taste buds. With lineage tracing in *Gli1-CreERT2;R26R* mice, we identified *Gli1*-expressing, derived cell progeny in fungiform papilla locations. (A) In whole tongues at 1 and 4 weeks and 3 months after tamoxifen induction, fungiform papilla labeling is obvious, and in the single circumvallate papilla on posterior tongue. (B) Tongue sections within the first week show large clusters of *Gli1-CreER* labeled cells, at papilla base and lateral walls (arrows, section at left). With Ki67 label, it is apparent that *Gli1*-expressing progeny are in proliferation zones of the papilla (for example, arrow at papilla base, section at right).

mouse tongues at 3 months post activation in detail. We analyzed serial sections that included 37 taste buds and fungiform papillae and characterized four “patterns” of *Gli1* lineage distributions (Fig. 6A: 1,2,3,4). The patterns and proportion of pattern representation were: (1) with extensive label of the taste bud, perigemmal cells, basal cells at papilla base and lateral walls, and cells in the lamina propria core (22%); (2) with extensive label of the taste bud, perigemmal label, little or no label of papilla basal cells, and extensive label in the lamina propria core (11%); (3) no taste bud label, extensive label of lateral papilla basal cells and base of papilla, label in basal cells of filiform papilla, and extensive label of lamina propria cells (24%); (4) little or no taste bud label, perigemmal cell label, label of basal cells at papilla base only, extensive label in the core (43%). These patterns have similar characteristics to those reported in *K14* lineage tracing (Okubo et al., 2009). In sum, the progeny of *Gli1*-lineage marked cells persist in the taste bud, perigemmal cells and cells of the papilla wall and base through at least 3 months or at least 9 taste cell renewal cycles. Thus some of the cells that expressed *Gli1* during the tamoxifen labeling period function as taste bud progenitor cells. Other papilla examples illustrate variations of the patterns and the apparent ‘clonal’ nature of *Gli1*-marked cell distribution within taste buds (Fig. 6B).

Through identification of descendants of cells that originally express *Gli1*, the patterns demonstrate that *Shh*-responsive cells and their progeny contribute to: fungiform papilla maintenance in

the epithelial cell base, walls and apex, and in the connective tissue, lamina propria core; cells of the taste bud and perigemmal cells; and, filiform papilla maintenance. Thus, cells that transduce *Shh* signals contribute to multiple lineages of the epithelium and mesenchyme of filiform and fungiform papillae and to the taste buds, over many cycles of papilla and taste bud maintenance and renewal.

Sustained postnatal activation of the Shh signaling transcription factor, Gli2, alters fungiform papillae and taste bud maintenance, and integrity of filiform papillae and lingual epithelium

We have shown that *Gli1* labeled cells or progeny of *Gli1* expressing cells, indicators of *Shh* signaling activity, contribute to multiple life span cycles of cells of the fungiform papilla and taste bud cells, and of filiform papillae. *Gli2* is the principal transcriptional activator of hedgehog signaling (Bai et al., 2002) and we proposed that *Gli2* acts in maintaining taste organs and tongue epithelium.

Shh, Gli1 and Gli2—distinct ligand and transcription factor locations compared in adult tongue

With *Gli2 LacZ* reporter mice, we found that *Gli2* expressing cells are in the basal cells throughout the lingual epithelium (Fig. 7A, *Gli2*). This contrasts with location of *Gli1* expressing cells, restricted to cells surrounding the taste bud and the basal cells of

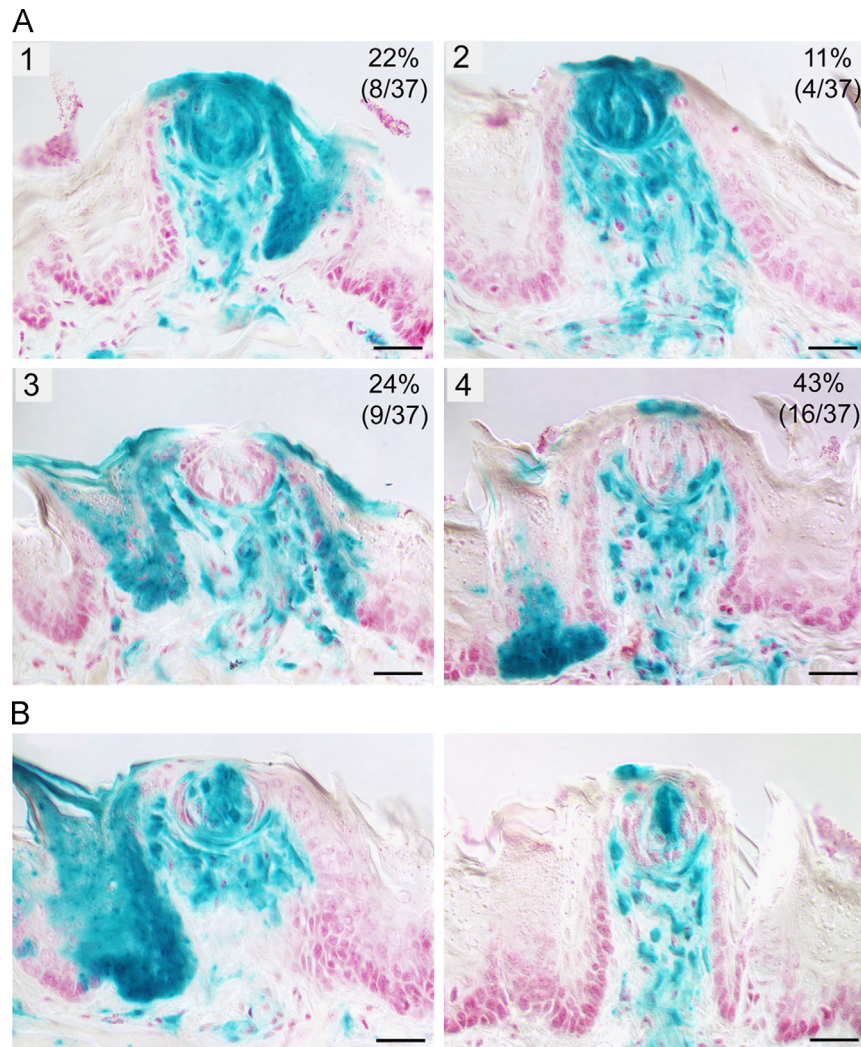


Fig. 6. Gli1 labeled progenitors are retained in fungiform papilla, epithelium and connective tissue, and in taste buds over several weeks of multiple cell turnover cycles. (A) At three months post-activation, Gli1-expressing, derived cell progenitors were traced in serial sections and four patterns of lineage distributions were identified (numbered 1,2,3,4 and described in text). The patterns vary in overall representation (the % and number of papillae, out of 37 traced, are at top right in each pattern), and in the extent of Gli1 progeny within papilla walls, perigemmal cells, taste buds, and papilla lamina propria cells. (B) Additional examples of Patterns 3 and 4 illustrate the apparent clonal nature of Gli1 progeny distribution within taste buds. Scale bars=25μm.

fungiform papillae only, *not inter-papilla epithelium*, and with *Shh* expressing cells, restricted to the taste buds in adult tongue (Fig. 7A, *Gli1*, *Shh*). Similar to *Gli1*, the *Gli2* expressing cells are in epithelium surrounding the taste bud, in basal cells of the fungiform papillae, and in apical papilla lamina propria cells (Fig. 7A), placing hedgehog transcription factors in positions to receive *Shh* signals from the taste bud. Scale bars = 25 μm.

Activating *GLI2* in adult tongue: tongue, papilla and taste bud effects

In a functional test of *Shh* signaling actions we asked whether activation of the pathway, by autonomously over-expressing the *Gli2* transcription factor in postnatal mice, would alter papilla and/or taste bud maintenance. We used *K5rtTA;TRE-GLI2*⁺ mice, with doxycycline-inducible activation of *GLI2*⁺ in tongue epithelium basal cells. After 7 or 12 days postnatal doxycycline treatment, transgene activation was confirmed by immunostaining for MYC-tagged *GLI2*⁺ in the tongue epithelium (Supplementary Fig. S4).

In hematoxylin and eosin stained sections at 24–32 h after doxycycline administration to activate *GLI2*⁺, fungiform papillae with taste buds are observed across anterior tongue of bitransgenic mice, similar to control tongues (data not shown). Therefore, one

day of *GLI2*⁺ activation is not sufficient to alter papilla and taste bud morphology. However, scanning electron micrographs (SEM) of control littermate or *GLI2*⁺ mice demonstrate a profound effect after 7 or 12 days of *GLI2*⁺ expression (Fig. 7B). Although size of the tongue is not substantially different, the surface architecture is much altered (Fig. 7B). In SEM, fungiform papillae do not have an obvious taste pore (Fig. 7B, arrowheads). Filiform papillae have not retained spinous keratin extensions. Quantification from serial histological sections after 12 days activation indicates that taste buds are not maintained in 80% of papillae (Fig. 7B, H and E). In the remaining 20% of papillae, taste buds are “aberrant”, with size reduction and disoriented cells (shown in Fig. 8). Filiform papilla spines are lost although the keratinized surface remains.

In hematoxylin and eosin sections, the typical fungiform papilla characteristics of a highly structured, single basal cell layer outlining the papilla and multilayered epithelium, with surrounding, spinous filiform papillae, are compromised in the *GLI2*⁺ tongue (Fig. 8A and B). Furthermore, taste buds that remain in fungiform papillae do not retain typical size and structure in the *GLI2*⁺ tongue (Fig. 8A and B, taste buds are outlined and indicated with an arrow). Ki67 immunostaining demonstrates that the basal cell layer is disrupted with *GLI2*⁺ activation and suprabasal proliferation is

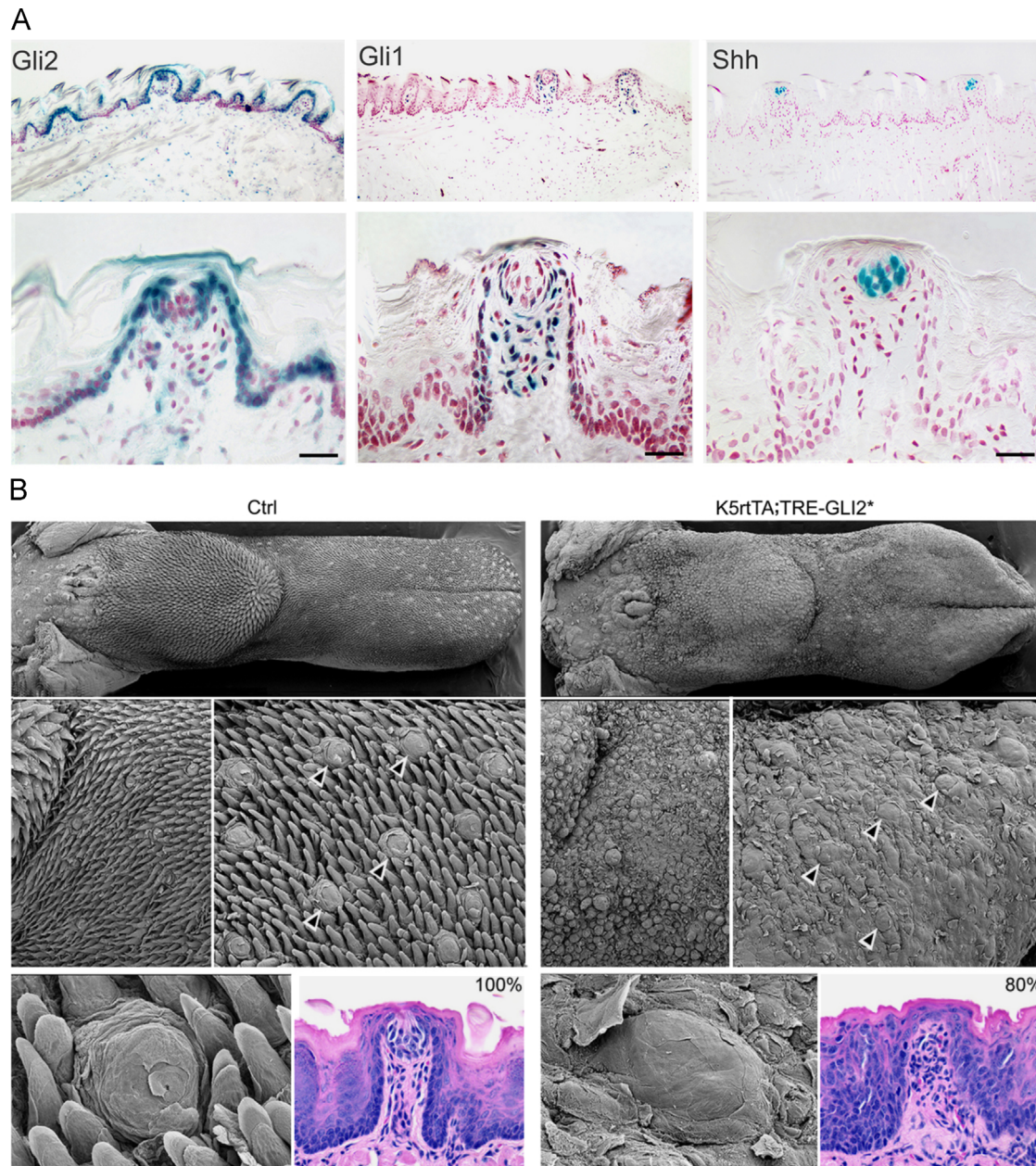


Fig. 7. Gli2 is in basal cells of papilla walls and lamina propria, surrounds Shh expression in the taste bud, and overlaps with, and extends beyond, Gli1 expression; activation of Gli2 for 7–12 days disrupts integrity of fungiform and filiform papillae and taste buds. (A) In reporter mice, for Gli2, Gli1 and Shh expression, Gli2 expressing cells are throughout basal cells of the tongue epithelium and fungiform papilla, in perigemmal cells around the Shh-positive taste bud, and in apical papilla lamina propria. This overlaps Gli1 expression, but Gli2 extends further, in inter-papilla basal cells. Low power tongue tissue and select fungiform papillae at higher power are shown. (B) In the bottom panel, scanning electron micrographs (SEM) illustrate surface topography for Control (Ctrl) and $GLI2^*$ activated tongues (K5-rtTA;TRE- $GLI2^*$). Fungiform papillae are seen in $GLI2^*$ tongues but distinct taste pores are not obvious compared to Control (arrowheads). Characteristic, spinous, filiform papillae are not seen. In hematoxylin and eosin sections, 80% of fungiform papillae in $GLI2^*$ tongues did not have an observed taste bud. Scale bars=25 μ m.

seen throughout the epithelium (Fig. 8C and D, arrows in D). Intense Ki67 label within the taste bud suggests possible increased intra-bud cell proliferation (Fig. 8D, taste buds are outlined). However, based on immunostaining for the taste bud cell marker, K8, remaining taste buds are thinner but *do* contain labeled taste cells (Fig. 8E and F). We quantified diameter at the widest point for 9 taste buds in Control and 6 clearly identified taste buds in $GLI2^*$ tongue after 7 days transgene activation. Mean diameters (and standard deviations) were $36.6 \pm 3.5 \mu\text{m}$ in control taste buds compared to $20.2 \pm 5.3 \mu\text{m}$ in the $GLI2^*$ tongue. The thin, remaining taste buds retain innervation, demonstrated with neurofilament immunoreactions (Fig. 8G and H).

Not only is fungiform papilla and taste bud maintenance disrupted in the $GLI2^*$ mouse tongue, but also the filiform papillae

have lost their characteristic, sharp, spinous extensions (Fig. 8I and J). Some surface keratin and a general orientation of the papillae are retained. From Ki67 immunoreactions, suprabasal proliferation in filiform papilla regions is apparent (Fig. 8K and L arrows).

With $GLI2^*$ activation, therefore, there is a rapid loss of fungiform papillae and taste buds; taste buds that remain are thinner but retain taste cells and innervation; spines are eliminated from filiform papillae; and, there is atypical cell proliferation in supra-basal layers of the epithelium. Thus, when the balance of hedgehog signaling is disrupted, lingual epithelial integrity is lost and differentiation of fungiform papillae and taste buds, and filiform papillae, is no longer sustained. The basal cell compartment is expanded and suprabasal cell proliferation is observed.

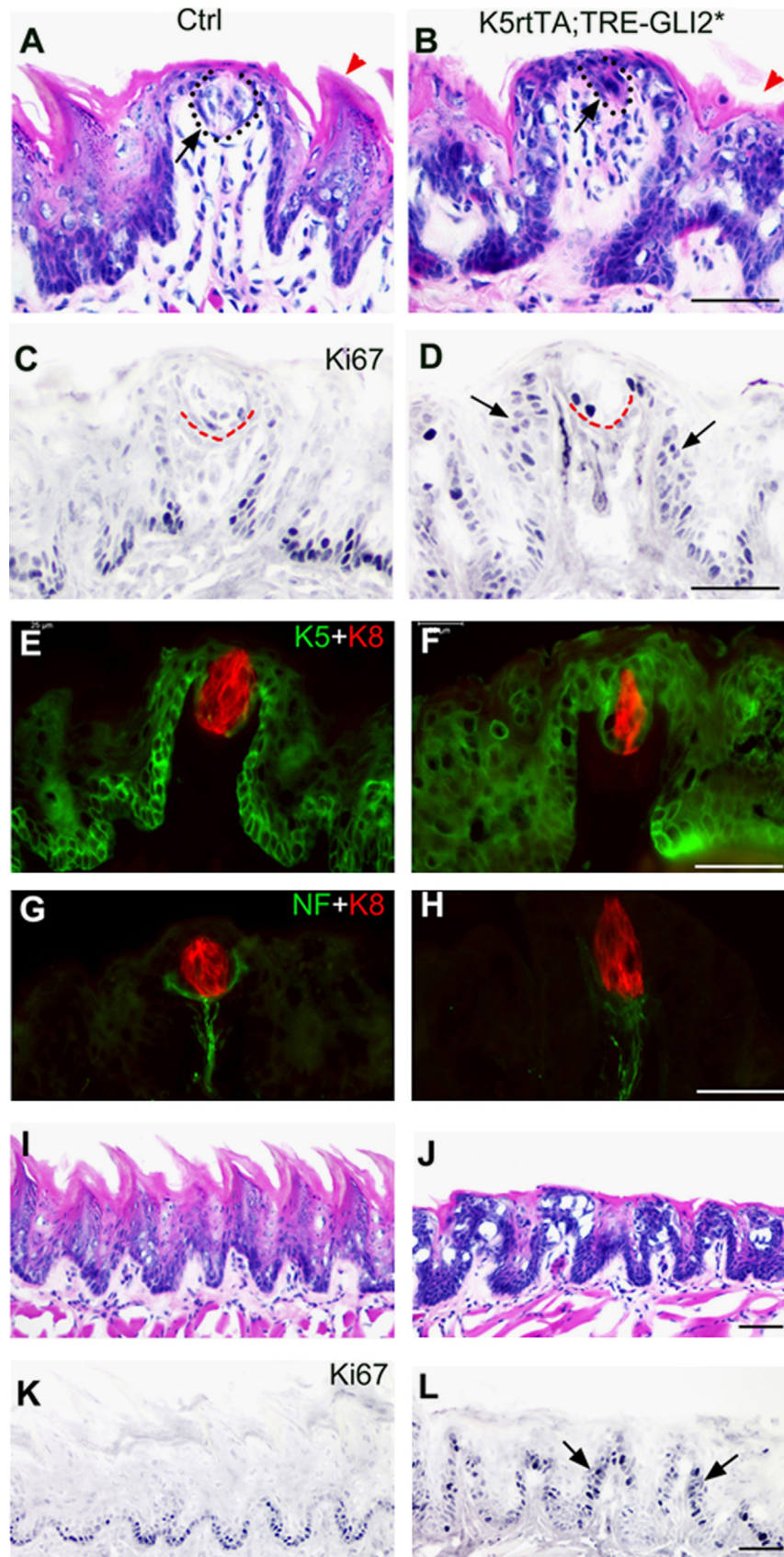


Fig. 8. With Gli2* activation, fungiform and filiform papilla epithelium is hyperproliferative, remaining taste buds are thin, but K8-positive and innervated. (A) and (B) Hematoxylin and eosin sections illustrate that compared to the Control (Ctrl) papilla, fungiform papillae in the GLI2* tongue (K5rtTA;TRE-GLI2*) are misshapen. Clusters of cells are observed in some papillae that presumably represent thin, taste bud remnants (arrow points to dotted cell outline in B; compare to A). Red arrowheads in A and B point to filiform papillae. (C) and (D) With Ki67 immunoreactions, cell proliferation is observed in suprabasal cell layers, where not seen normally, in the GLI2* tongue (arrows in D). A red outline indicates a taste bud (C) or cell cluster (D). (E) and (F) K8 is used as a taste bud cell marker and demonstrates a much reduced set of taste bud cells in the GLI2* tongue. Also with K5, a basal cell cyokeratin, the suprabasal proliferation is obvious. (G) and (H) Nerve fibers innervate the taste bud in Control and GLI2* tongues (neurofilament immunoreaction, NF). (I) and (J) Hematoxylin and eosin stain, and (K) and (L) Ki67 immunoreactions, demonstrate loss of spinous filiform papillae (J) and suprabasal proliferation in GLI2* tongue epithelium (arrows in L). Scale bars=50 μ m.

These effects match predicted outcomes from localization of *Shh*, *Gli1* and *Gli2* expression, and from *Gli1* lineage tracing in lingual epithelium, lamina propria and taste organs.

Discussion

We have located *Shh* ligand in progressively restricted placode, papilla and ultimately taste bud cell locations, surrounded by responding cells in developing and adult gustatory organs. Because *Shh* can signal up to a few hundred microns in distance, not only cells in the immediate vicinity but also responding cells further afield can participate in *Shh* signaling centers. The responding cells overlap with zones of active epithelial cell proliferation and lamina propria regions of stromal cell clusters where nerve nets and vasculature abut the basal lamina.

For development and organ maintenance, therefore, the fungiform papilla is defined by signaling compartments, centers or niches, within: the placode; the forming papilla apex and base; and, the adult papilla with taste bud. The multiple, stage-specific centers render the taste organ vulnerable to imbalance of the powerful *Shh* morphogen and mitogen, in several cell compartments. Indeed progeny of *Gli1*-expressing, *Shh*-responding cells contribute to multiple cell types in taste buds and taste papilla epithelial and connective tissues. Signal disruption in epithelial or lamina propria cell compartments can alter stem and progenitor cell cycling and lead to hyperproliferation and accompanying loss of differentiation. Our data identify *Shh* signaling centers in fungiform placode and papilla development, and demonstrate an essential reliance of the adult fungiform papilla and taste bud on properly regulated *Shh* signaling homeostasis.

The developing taste placode and papilla incorporate Shh signaling centers

A prior report that taste placodes do not contribute to papillae, but rather to taste buds only, and with no mesenchymal contribution, relies on a definition of placode that includes *Shh*-expressing cells only (Thirumangalathu et al., 2009). We show that the entire placode is composed of the *Shh*-expressing centrally positioned cells and the *Shh*-responding cells in surrounding epithelium and mesenchyme, to thereby contribute to progressive formation of the full papilla and taste buds. In fact stage-specific, functional roles for *Shh* signaling in placode induction and papilla development have been demonstrated (Liu et al., 2004). In short, the placode is more than a *Shh*-positive cell region but is an early *Shh* signaling center.

Shh is positioned to signal in postnatal and adult tongue, papilla and taste bud maintenance

Fungiform papillae must contain long-lived, sustaining or stem cells and short-lived, maintaining or transit amplifying cells that support the lateral and apical papilla epithelium and the specialized taste buds. The continuously renewing tongue epithelium and taste organs are extremely complex and therefore require balanced molecular signaling to retain structure and function. In the postnatal and adult fungiform papilla, the highly restricted *Shh* expression in the taste bud is well positioned to signal to receptors and transcription factors in surrounding perigemmal epithelium, abutting stromal cells in lamina propria, and in cells of lateral papilla walls. In short, *Shh* is tightly localized but, because it is a diffusible molecule, can regulate progenitor cell formation, turnover and maintenance in surrounding regions to sustain papilla- and taste bud-contributing cells.

Shh lineage tracing in fungiform papillae demonstrated that progeny of *Shh*-expressing progenitors are in two taste bud cell types, basal taste bud cells and 'edge' cells in P0 mice (Thirumangalathu et al., 2009). However the *Shh*-expressing progeny began to disappear prior to weaning and were absent in adults at 4 months. Although it was proposed that the depletion and loss of *Shh*-expressing progenitors might relate to loss of *lacZ* transcription, it was concluded that *Shh*-expressing, placode-derived cells are lost from mature taste buds. If correct, loss of *Shh*-expressing progeny from adult taste bud cells would suggest that the *Shh*-positive cells that we observe in taste bud cells, through 12 months of age in mouse, have differentiated from *Shh*-negative surround cells that migrate into the taste bud. Rather, we propose that maintained *Shh*-positive taste bud cells could derive also from internal, long-lived progenitors.

Miura and colleagues also reported limited *Shh* expression, in basal cells of taste buds of the circumvallate papilla, and a *Ptch*-expressing region around each taste bud (Miura et al., 2001). *Ptch* expressing cells were not seen in connective tissue under the circumvallate epithelium. In our data, the clear distribution of *Ptch*, *Gli1* and *Gli2* expressing cells in apical papilla lamina propria suggests opportunity for direct interaction between epithelium and connective tissue in fungiform papilla and taste bud regulation, as seen in other organ systems of intestinal crypt and villus (Mao et al., 2010; Walton et al., 2012), hair follicle (St Jacques et al., 1998) and tooth (Dassule et al., 2000). In demonstrations of neural crest-derived cell contributions to taste buds, roles for surrounding epithelium and underlying stroma in taste bud maintenance also are indicated (Liu et al., 2012). Furthermore, there are demonstrated roles for *Shh* signaling in neural crest cells in the mesenchyme of facial primordia in facial patterning and growth, and mesenchyme cells in the developing tongue that express *Ptch1* contain a high density of cranial neural crest cells (Jeong et al., 2004). Certainly, the lamina propria cannot be ignored in papilla and taste bud differentiation and maintenance.

Gli1 lineage tracing and Shh-responding papilla and taste cell progenitors

With *Gli1* lineage tracing we have shown that *Gli1* expressing, *Shh* responding, cells contribute daughters to epithelium and lamina propria of the fungiform papilla and to nearby filiform papillae. Whereas the taste bud cells themselves do not express *Gli1*, the progeny of *Gli1* expressing, progenitor cells come to reside in the taste bud. This is the first demonstration that *Shh*-signaling cells contribute progeny to cells of the taste bud, fungiform and filiform papillae.

We find at least three regions of *Gli1* expressing progenitor cells that contribute to taste bud and fungiform papilla maintenance: (1) a highly proliferative zone at the base of the fungiform papilla; (2) a highly proliferative zone around the taste bud (perigemmal); (3) a zone in the apical connective tissue core of the papilla. The observed patterns of *Gli1* expressing cells are compatible with the idea from classic and current literature that self-renewing or "stem" cells are at the base of the fungiform papilla, and from there the transit amplifying progeny migrate along lateral papilla walls to contribute to perigemmal and taste bud cells, or along filiform papilla walls to contribute to maintenance of non-gustatory papillae (Hume and Potten, 1976; Okubo et al., 2009). Contributions to taste bud cells both from progenitors at the papilla base and from perigemmal cells are shown in K14 lineage tracing experiments (Okubo et al., 2009). In small intestine development, stem cells reside at the base of crypts and proliferate continuously; transit amplifying cell progeny migrate from the base up along the crypt walls and divide as they migrate (Itzkovitz et al., 2012). In hair follicle lineage tracing, large clones of multi-potent progenitor,

transit amplifying cells migrate within the renewing follicle during the hair cycle (Zhang et al., 2009). The large clones of *Gli1* labeled basal cells that we observe at the papilla base and in lateral papilla walls suggest that these *Gli1* progeny are clusters of rapidly cycling, transit amplifying cells.

The importance of lamina propria, connective tissue contributions is noted again in these experiments, where *Gli1*-expressing, stromal cells of the apical papilla core are positioned for epithelial/connective tissue interactions, papilla/taste bud and nerve interactions, and maintenance of papilla structure. *Gli1*-expressing cells maintain the dermal papilla of the hair follicle and these target cells receive Shh from surrounding cells in the papilla (Brownell et al., 2011). In hair follicle dermal papilla, *Shh*-expressing, innervating neurons from the dorsal root ganglion are an additional signaling source for responding cells in the follicle. We propose that some of the *Ptch1* and *Gli1* expressing cells in the region of the nerve “net” just under the taste bud could be on the perineural sheath and receive Shh signals from the taste bud cells.

Overall, our *Gli1* lineage data provide a new demonstration of Shh-responding cell contribution to fungiform papillae and taste bud cells. The *Gli1* progeny persist through several cycles of tongue epithelial and taste bud cell turnover. Therefore at least some of the cells that contribute to taste bud cell maintenance are *Gli1* expressors. The *Gli1*-Cre label-retaining cells are maintained over three months in mosaic patterns, and show label of: potential “stem” like cells, transit amplifying progeny, and differentiated cells. The results place *Shh*-signaling in a central position for taste bud and papilla maintenance.

Shh expressing and responding cells and taste bud cell turnover

The proposal that Shh expressing cells within the taste bud also contribute to taste bud cell renewal (Miura et al., 2006) is not excluded by our data. Mitotically active, potential progenitor and stem cell compartments were identified in mouse taste buds (Sullivan et al., 2010). Basal taste bud cells included rapidly cycling, progenitor cells and label retaining, slow cycling, stem cell-like cells. The data suggested that slow-cycling, candidate stem-like cells are principally within basal cells of the taste bud. Although the average taste cell life span is about 10 days, taste cell life span reportedly can range from 2 days to more than three weeks and longer-lived, stem-like taste bud cells include those labeled with taste cell specific markers (Beidler and Smallman, 1965; Hamamichi et al., 2006).

We propose that the Shh-positive taste bud cells in fungiform papillae, in a basal position, may be slowly cycling cells retained in the taste bud whereas the large proportion of K8-positive cells presumably derive from perigemmal cells that migrate into the taste bud and differentiate. The concept of multiple taste cell progenitors is reinforced by our observation that within the taste bud a subset of Shh-positive cells do not co-express K8, which marks simple epithelium (Bagutti et al., 1996) and taste bud cells (Knapp et al., 1995; Okubo et al., 2009). Shh-positive cells might be activated to proliferate more actively in injury or regeneration and/or might contribute to one particular type of taste bud cell.

Functional effects for activated *Shh* signaling in adult tongue and taste organs

By autonomously activating the transcription factor *GLI2*, the principal effector for hedgehog signaling (Bai et al., 2002), we tested functional effects of over-activating *Shh* signaling. In this model the activated hedgehog signaling is continuous (Grachtchouk et al., 2011). Constitutive activation of hedgehog signaling contributes to various basal cell and hair follicle tumors

in skin (Grachtchouk et al., 2011) and is a logical potential regulator of tongue epithelial integrity.

We found that deregulating *GLI2* for 7–12 days resulted in lost or aberrant filiform and fungiform papillae and taste buds. Taste bud cells have a turnover cycle ranging from 2 to at least 21 days (Hamamichi et al., 2006) and turnover cycles for lingual epithelial cells are on average a few days (Cameron, 1966). Therefore, rapid effects of this profound disruption in a major signaling pathway are compatible with effects on cell turnover.

The filiform papillae, fungiform papillae and taste buds are maintained in the lingual epithelium in the adult tongue, and the fungiform papilla depends also on a connective core that houses nerves and vessels essential to epithelial and taste bud integrity. Disruption of any lingual tissues will in turn disturb papilla and taste bud maintenance. Thus, in tongue and taste organs, uncontrolled *Shh* signaling can drive proliferation of epithelial and connective tissue cells at the expense of sustained differentiation programs that regulate turnover in fungiform papilla, fungiform taste bud cells and filiform papillae. The *Shh* signaling centers of ligand and responding cells can function in a set of epithelial and stromal cell microenvironments that regulate papilla and taste bud development and maintenance; when out of balance, in uncontrolled activation, there is a dysplastic phenotype.

In *GLI2*⁺ tongues, suprabasal proliferation was observed in fungiform and filiform papilla epithelium, and the basal cell compartment was hyperproliferative. Our results are similar to aspects of carcinoma in stratified squamous epithelia, for example basal cell carcinoma in which hedgehog signaling is continuously activated and independent of ligand binding (Grachtchouk et al., 2011; Saran, 2010). In skin, oncogenic Hh signaling can disrupt the hair follicle growth cycle and epithelial cell proliferation is sustained, in contrast to ligand-dependent, cyclic physiological *Shh* signaling. When *Shh* signaling is deregulated in tongue, also, cell proliferation via upregulated self-renewal of progenitors takes over at the expense of differentiation.

Niches in fungiform papilla and taste bud regulation

We suggest that the fungiform papilla and taste bud encompass a developmental series of stem cell niches and propose that the identified *Shh* signaling centers are potential niches. A stem cell niche is recognized in several organ systems as a specialized, restricted microenvironment with tissue, matrix, cell and molecular attributes to support the stem cells, cell fate commitment and differentiation (Greco and Guo, 2010; Chen et al., 2013; Lander et al., 2012). The concept of the stem cell niche has not been developed in the taste field but consideration of possible niches can contribute to identification of stem cells that sustain papillae and taste buds.

Possible niches include the early fungiform papilla apex where a bracketing set of proliferative epithelial cells and mesenchymal cells express *Ptch* and *Gli1*, responding targets for the *Shh* signal in the central papilla apex. The Shh-positive epithelial cells are seated on a basal lamina, part of the niche, to provide matrix support and a venue for epithelial and mesenchymal molecular sequestration and exchange (Chen et al., 2013). Within the taste bud of the adult fungiform papilla, there is a potential basal cell niche of a few Shh-positive cells that are bracketed by proliferative perigemmal cells that express *Ptch*, *Gli1*, *Gli2* and *p63*. Furthermore, these cells are seated on a basal lamina; are in direct proximity to apical lamina propria cells that also express *Ptch*, *Gli1* and *Gli2*; are surrounded by a neural net of finely branched axons; and, are near the vasculature of the papilla core.

Another potential stem cell niche is identified at the base of the fungiform papilla, the lowest extension of papilla epithelium into underlying connective tissue, where basal cells are highly

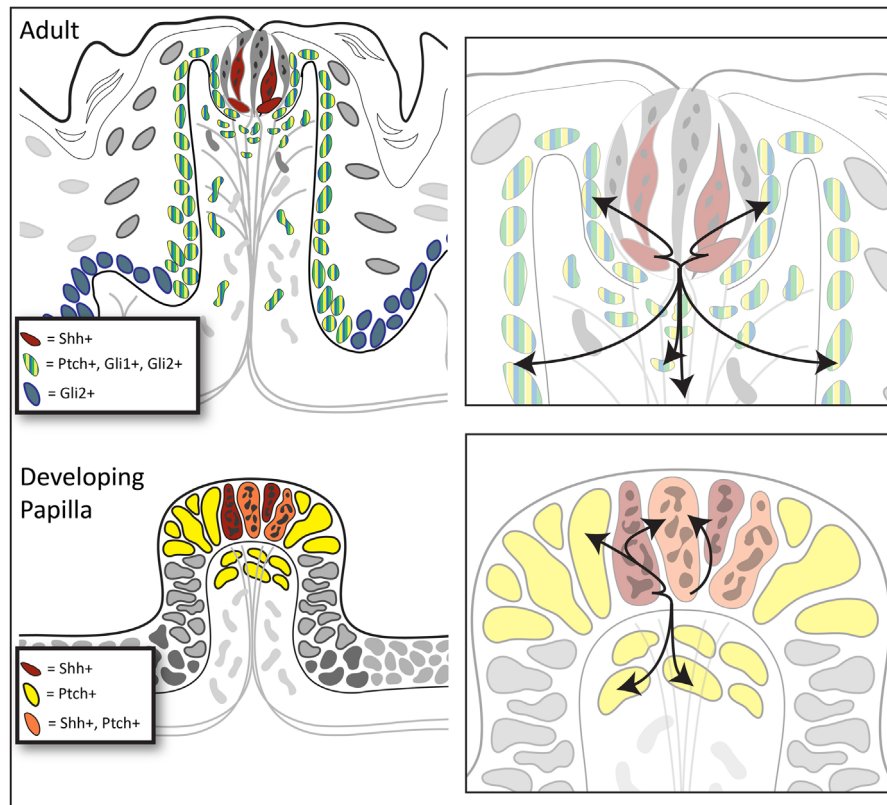


Fig. 9. Locations of Shh responding cells demonstrate potential microenvironments or niches for Shh signaling and a stage-specific autocrine and/or paracrine signaling mode. **Adult Papilla:** Summary Diagram on the left places Shh only within the taste bud and *Ptch1*, *Gli1* and *Gli2* responding targets in perigemmal cells, in basal cells of the lateral papilla walls, and in lamina propria, connective tissue cells of the fungiform core. In addition, *Gli2* expression extends to basal cells throughout the lingual epithelium. In the Expanded Diagram, arrows indicate *paracrine signaling* from taste bud cells to regulate perigemmal cells, lateral papilla basal cells, and lamina propria cells. **Developing Papilla:** Summary Diagram on the left places Shh within apical papilla epithelium and *Ptch1* responding targets overlapping with some of the Shh-expressing cells, in epithelial cells surrounding the centrally placed Shh-positive cells, and in mesenchymal cells of the early papilla core and subepithelial tissue. In the Expanded Diagram on the right, arrows indicate *autocrine signaling* within the papilla apical epithelium and *paracrine signaling* to epithelial and mesenchymal cell surround.

proliferative and on the basal lamina over a lingual stroma environment. This niche might well be the origin for cells that migrate along the lateral papilla wall and eventually reach perigemmal regions, to contribute to taste bud cells, as proposed from *Gli1* lineage tracing results.

Multiple centers for Shh signaling in a paracrine mode

Hh signaling is necessary for various cell processes in organ formation and maintenance, and regulates cell renewal and differentiation in a concentration-dependent manner (Robbins et al., 2012). Thus, deregulated Hh signaling can lead to cell cycle effects and uncontrolled proliferation. With knowledge about where the Shh ligand and responding cells are located throughout papilla and taste bud development, we can predict and explain effects of Hh signaling in fungiform papilla and taste bud formation and maintenance.

In developing and adult fungiform papilla and taste bud our data have positioned the Shh ligand to signal in various microenvironments in a paracrine mode (Fig. 9). The Shh ligand, restricted to taste bud cells in the adult, can signal to responding and transcribing cells: in the perigemmal basal cell compartment, in the basal cell region of the lateral papilla walls, and in the apical connective tissue core of the papilla (Fig. 9, Adult Papilla). During the early postnatal period there is an active increase in taste bud volume (Hendricks et al., 2004), in which taste bud cell number matches with innervation density (Krimm and Hill, 2000). An accelerated period of cell production is proposed as a potential mechanism for the increase in taste bud volume, shown to be

associated with increased numbers of differentiated taste bud cells that have migrated into the taste bud (Hendricks et al., 2004). At this time the perigemmal and basal cell regions of lateral papilla walls are zones of high proliferation and paracrine Shh signaling to the taste bud surround can regulate proliferation.

We propose that Shh maintains the adult fungiform papilla and resident taste bud by regulating progenitor cells and, thereby, cell proliferation in papilla epithelium, stromal cells, and taste bud cells. With concentration-dependence, Shh signals nearby and at a distance to the papilla base and apex, to the lamina propria of the apical core, to the taste bud surrounding cells. Within each niche, responding cells in various cell and tissue contexts will interact with distinct levels of the Shh signal.

In the embryonic, early fungiform papilla, Shh-responding cells in epithelium and mesenchyme bracket a central Shh-expressing region in the epithelium, suggesting a paracrine mode of signaling as in the postnatal and adult papilla and taste bud (Fig. 9, Developing Papilla). However, because *Ptch1*-expressing cells overlap with some of the Shh-expressing cells, an autocrine signaling mode also is likely during placode and papilla development (Fig. 9, Developing Papilla).

Conclusions

In conclusion, our data define specific locations for Shh expression and Shh signaling; bring forward new demonstrations of Shh roles in tongue, papilla and taste bud development and maintenance; show for the first time a direct contribution of

hedgehog responding, *Gli1* labeled progeny to both taste bud cells and to cells of the papilla epithelium; and demonstrate a requirement for Shh signaling with the distinctive effects of activating Shh transcription factors in adult tongue. *Shh* signaling has roles in forming and maintaining fungiform papillae and taste buds, most likely via stage-specific autocrine and/or paracrine mechanisms, and by engaging epithelial/mesenchymal or connective tissue interactions.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2013.07.022>.

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