© 2015. Published by The Company of Biologists Ltd | Development (2015) 142, 4309-4317 doi:10.1242/dev.121012

RESEARCH ARTICLE

β-Catenin signaling regulates temporally discrete phases of anterior taste bud development

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ABSTRACT

The sense of taste is mediated by multicellular taste buds located within taste papillae on the tongue. In mice, individual taste buds reside in fungiform papillae, which develop at mid-gestation as epithelial placodes in the anterior tongue. Taste placodes comprise taste bud precursor cells, which express the secreted factor sonic hedgehog (*Shh*) and give rise to taste bud cells that differentiate around birth. We showed previously that epithelial activation of β-catenin is the primary inductive signal for taste placode formation, followed by taste papilla morphogenesis and taste bud differentiation, but the degree to which these later elements were direct or indirect consequences of β-catenin signaling was not explored. Here, we define discrete spatiotemporal functions of β-catenin in fungiform taste bud development. Specifically, we show that early epithelial activation of β-catenin, before taste placodes form, diverts lingual epithelial cells from a taste bud fate. By contrast, β-catenin activation a day later within *Shh*$^+$ placodes, expands taste bud precursors directly, but enlarges papillae indirectly. Further, placodal activation of β-catenin drives precocious differentiation of Type I glial-like taste cells, but not other taste cell types. Later activation of β-catenin within *Shh*$^+$ precursors during papilla morphogenesis also expands taste bud precursors and accelerates Type I cell differentiation, but papilla size is no longer enhanced. Finally, although Shh regulates taste placode patterning, we find that it is dispensable for the accelerated Type I cell differentiation induced by β-catenin.

KEY WORDS: Taste placode, Taste bud precursor cell, Fungiform taste papilla, β-Catenin, Sonic hedgehog (Shh), Glial-like taste cells, Cell lineage, Cre-lox, Tamoxifen

INTRODUCTION

The sense of taste is mediated by multicellular taste buds in the oral cavity, which transmit chemical information via sensory nerves to the brain. In mammals, lingual taste buds are housed within epithelial-mesenchymal specializations named taste papillae. In rodents, fungiform taste papillae, each with a single taste bud, form a stereotypical array on the anterior tongue (Mistretta, 1972). Taste bud development begins at embryonic day (E) 12 in mice, as bilateral rows of columnar epithelial thickenings, i.e. taste placodes, appear in the lingual epithelium (Hall et al., 1999). Taste placodes undergo morphogenesis starting at E14.5; the edges of placodal epithelium invaginate into the underlying mesenchyme to form mushroom-shaped, i.e. fungiform, taste papillae (Mistretta and Liu, 2006). Immature taste buds are evident in papillae by E17.5, and then differentiate into each of the three taste receptor cell types (I, II and III) found in mature taste buds following birth (Barlow and Klein, 2015; Kapsimali and Barlow, 2013).

The morphogen sonic hedgehog (*Shh*) is expressed specifically by taste placodes as they form, and *Shh* expression persists in the apical papilla epithelium during morphogenesis (Bitgood and McMahon, 1995; Hall et al., 1999; Jung et al., 1999). Through genetic lineage tracing, we showed that *Shh*$^+$ placodes give rise only to taste bud cells, and not to the papillae housing them (Thirumangalathu et al., 2009). Thus, *Shh*$^+$ placodes are taste bud precursor cells, TBpcs (Thirumangalathu et al., 2009), which are distinct from the surrounding taste papilla epithelium and are specified well before papilla morphogenesis.

In addition to *Shh*, Wnt/β-catenin activity is evident throughout taste bud and papilla development (Iwatsuki et al., 2007; Liu et al., 2007): (1) as taste placodes are specified, Wnt reporter activity, i.e. TopGAL, is high in placodal epithelium and low in interplacodal non-taste epithelium; (2) during papilla morphogenesis, TopGAL reporting consolidates to apical TBpcs; and (3) remains restricted to the TBpc population as taste cell differentiation commences around birth (Iwatsuki et al., 2007; Liu et al., 2007). In sum, the pattern of TOPGAL activity suggests that canonical Wnt signaling has spatially and temporally distinct functions in fungiform taste bud development.

To date, only the earliest function of Wnt/β-catenin has been defined (Iwatsuki et al., 2007; Liu et al., 2007). Epithelial stabilization of β-catenin concomitant with taste placode specification drives taste bud fate, such that immediately prior to birth expanded *Shh*$^+$ TBpcs are housed in enlarged fungiform papillae that entirely replace the non-taste epithelium (Liu et al., 2007). Additionally, within expanded fungiform papillae, we found occasional, precociously differentiated taste bud cells. Thus, activation of β-catenin promotes taste placode specification and *Shh* expression, but the degree to which subsequent elements of the phenotype, i.e. expanded papillae and accelerated taste cell differentiation, are direct or indirect consequences of elevated β-catenin, and/or involve the *Shh* pathway, have not been examined.

In the present study, we sought to delineate the spatiotemporal role(s) of β-catenin in taste bud development using a Shh-driven, tamoxifen-inducible Cre recombinase (*Shh*^{CreERT2}; Harfe et al., 2004) to stabilize β-catenin (*Ctnnb1*$^{(ex3)fl}$; Harada et al., 1999) during specific phases of taste bud development. Our results demonstrate a progressive and spatially restricted role for β-catenin. First, we show that β-catenin activation within lingual epithelium before taste placode specification perturbs acquisition of taste fate, in contrast to β-catenin's established pro-taste fate role during taste placode specification (Liu et al., 2007). Second, we demonstrate that β-catenin functions placode-autonomously to expand the *Shh*$^+$ TBpc population, but drives enlargement of surrounding taste papillae indirectly. Third, we show that placode-autonomous

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Received 16 December 2014; Accepted 22 October 2015

Development (2015) 142, 4309-4317 doi:10.1242/dev.121012

β-catenin causes precocious differentiation of taste cells before birth, and these are exclusively Type I glial-like taste cells. Finally, we show via genetic analysis, that Shh signaling is dispensable for this impact of stabilized β-catenin on Type I cell differentiation.

RESULTS

Stabilization of β-catenin before taste placode formation perturbs taste development

Previously, we showed that epithelial stabilization of β-catenin as taste placodes are specified (E12.5) converts the entire tongue surface into a carpet of enlarged and ectopic fungiform taste buds (Liu et al., 2007). Here, we investigated whether β-catenin promotes taste fate prior to the establishment of taste placodes. As *Shh* is expressed throughout the epithelium of the tongue primordium at E11.5 (Hall et al., 1999; Jung et al., 1999), we first treated pregnant females with tamoxifen at E11.5 to activate Cre broadly in the embryonic lingual epithelium of $Shh^{CreERT2}$*;ROSAtdTomato* embryos. By E12.5, tdTomato was expressed by many, but not all, epithelial cells in the anterior tongue (Fig. S1A); by E14.5, most epithelial cells were $tdTomato^{+}$ (Fig. S1B). Thus, tamoxifen treatment at E11.5 broadly activates reporter gene expression in tongue epithelium in a mosaic pattern that expands progressively.

Thus, we next treated $Shh^{CreERT2}$*;*$Ctnnb1^{(ex3)fl}$*;TopGAL* embryos with tamoxifen at E11.5 to both manipulate [$Ctnnb1^{(ex3)fl}$] and monitor (TopGAL) β-catenin signaling prior to taste placode specification. In control tongues at E14.5 or E18.5, TopGAL activity is restricted to apical TBpcs within bilateral rows of fungiform papillae with well-defined cores (Fig. 1A,C,E, arrowheads) (Iwatsuki et al., 2007; Liu et al., 2007). By contrast,

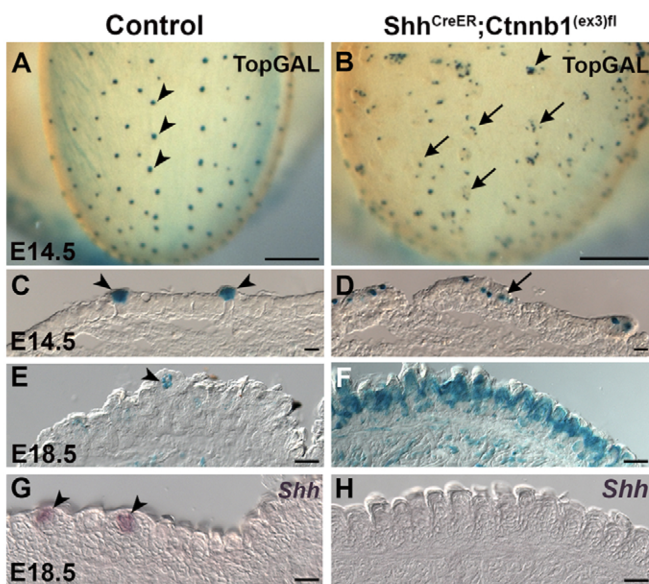

Fig. 1. Epithelial stabilization of β-catenin prior to taste placode formation interrupts taste bud development. (A-D) In control E14.5 tongues, TopGAL activity is high in taste papillae (A,C, arrowheads) whereas in mutant tongues (*ShhCre*^{ERT2}*;Ctnnb1*$^{(ex3)fl}$, tamoxifen at E11.5), TopGAL pattern is perturbed, appearing as sparse cells in overgrown papillae in the anterior tongue (B,D, arrows), with occasional normal-appearing fungiform papillae (B, arrowhead). (E,F) In controls at E18.5, TopGAL is restricted primarily to immature taste bud cells (E, arrowhead), but in mutants, TopGAL is active throughout the lingual epithelium (F). TopGAL is also evident in some lingual mesenchyme cells in both controls and mutants at E18.5 (E,F). (G,H) At E18.5, *Shh* is expressed by TBpcs in control tongues as expected (G, arrowheads), whereas in mutant tongues, *Shh* expression is lost (H). Dorsal surface of the tongue is up in C-H. Scale bars: 250 µm (A,B); 10 µm (C,D); 20 µm (E-H).

in mutant tongues at E14.5, fungiform papillae were not obvious, and $TopGAL^{+}$ cells formed disorganized puncta within irregular epithelial outgrowths (Fig. 1B,D, arrows). Occasional fungiform papillae with more normal TopGAL expression were evident (Fig. 1B, arrowhead); this was probably due to mosaic Cre induction (Fig. S1). In mutants at E18.5, $TopGAL^{+}$ cells were widespread in the tongue surface, which was composed primarily of non-taste filiform papillae (Fig. 1F). In sum, these observations suggested that, in contrast to later activation where excess β-catenin promotes taste fate (Liu et al., 2007), epithelial β-catenin activation prior to placode specification perturbs taste bud development.

To test this hypothesis explicitly, we examined the expression of specific markers of developing taste buds in control and mutant embryos treated with tamoxifen at E11.5. At E12.5, *Shh* expression consolidates to taste placodes (Hall et al., 1999), and these TBpcs express *Shh* throughout development, producing differentiated taste bud cells postnatally (Thirumangalathu et al., 2009). As expected, in control tongues, Shh^{+} cells reside apically in taste papillae at E18.5 (Fig. 1G, arrowheads). By contrast, Shh^{+} cells were absent in anterior mutant tongues (Fig. 1H). Additionally, we compared the expression of cytokeratin 8 (K8; Krt8 – Mouse Genome Informatics), a marker of immature taste bud cells (Mbiene and Roberts, 2003; Zhang et al., 1995). At E18.5, $K8^{+}$ cells are evident in fungiform papillae in controls (24 $K8^{+}$ buds in the anterior tongue of each of two embryos), whereas in mutant tongues, $K8^{+}$ cells are rare (four and six taste buds in each of two tongues). The fact that some taste buds developed in mutants with β-catenin stabilization again was probably due to mosaic Cre induction (Fig. S1). In sum, these data indicate that stabilization of β-catenin prior to specification of taste placodes generally diverts lingual epithelium from developing taste buds, in contrast to the induction of taste fate when β-catenin is activated in lingual epithelium as taste placodes are established (Liu et al., 2007).

Stabilization of β-catenin in taste placodes increases TBpcs directly and taste papilla size indirectly

When β-catenin was stabilized in $K14^{+}$ (Krt14 – Mouse Genome Informatics) embryonic lingual epithelium, the tongue surface was covered by enlarged taste papillae with expanded Shh^{+} TBpcs (Liu et al., 2007). These studies demonstrated a widespread competency of tongue epithelium to generate taste bud-replete papillae in response to activation of β-catenin, but left open the question of β-catenin's taste placode-autonomous role. Thus, we next activated β-catenin in Shh^{+} taste placodes once these structures were specified ($Shh^{CreERT2}$*;*$Ctnnb1^{(ex3)fl}$, tamoxifen administered at E12.5). At E14.5, taste placodes are not readily visible in intact wild-type tongues (Fig. 2A), although they are evident in histological sections (Fig. 2C, arrow). By contrast, β-catenin activation results in enlarged taste placodes that are readily visible in both intact (Fig. 2B, arrowheads) and histological sections of (Fig. 2D, asterisks) mutant tongues. Taste papillae begin morphogenesis at E14.5, as the epithelium invaginates to surround a mesenchymal core (Mistretta and Liu, 2006); the epithelium is identified as a bilayer composed of $K14^{+}$ cuboidal basal cells with a superficial squamous layer of $K14^{-}$ cells (Byrne et al., 1994; Fuchs, 1993). Using K14 immunostaining to distinguish epithelium from mesenchyme, we found that excess growth of mutant taste papillae at this stage was due largely to an expanded epithelial compartment (Fig. 2E,F, arrows).

In enlarged mutant placodes, stabilized β-catenin in Shh^{+} cells drives TopGAL reporting (Fig. 2G, compare with Fig. 1A,H,I) and increases nuclear β-catenin-immunoreactivity (ir) in apically situated cells (Fig. 2K, arrowheads) compared with controls, in

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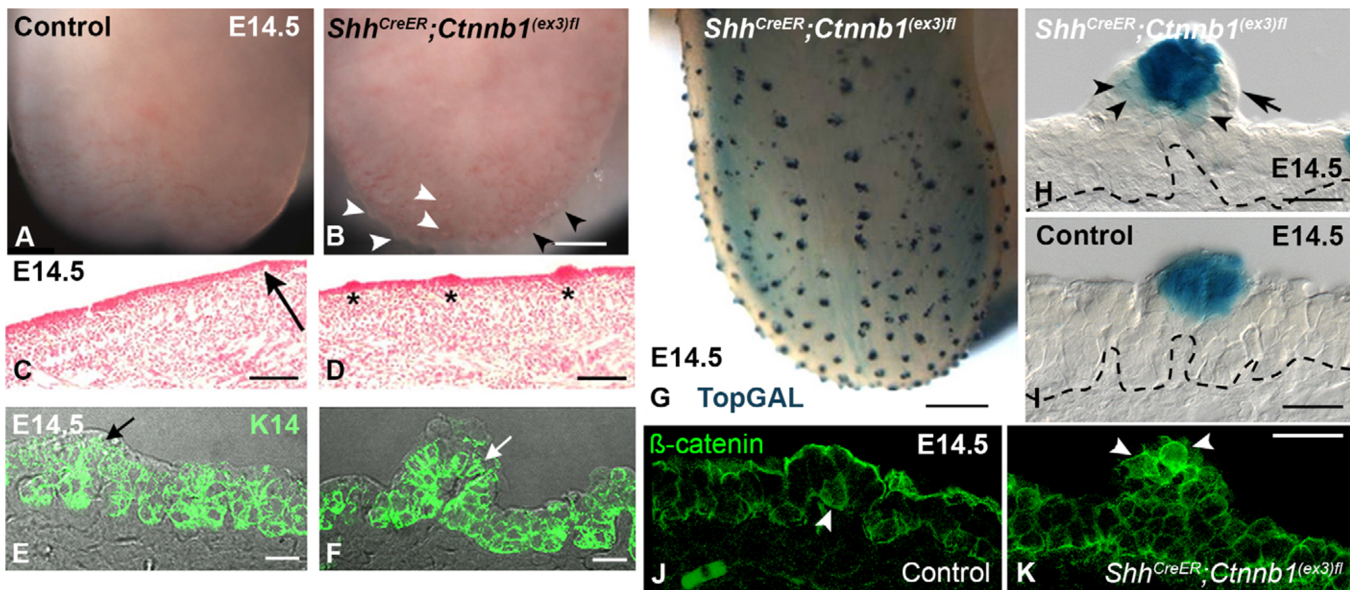

Fig. 2. Activation of β-catenin in *Shh*$^+$ taste placodes expands fungiform papillae. (A-D) In controls at E14.5, fungiform taste papillae are present (C), but are difficult to discern in intact tongues (A). In mutant tongues (*ShhCre*^{ERT2}*;Ctnnb1*$^{(ex3)fl}$, tamoxifen at E12.5), fungiform papillae are enlarged and readily visible (B, black and white arrowheads). In histological sections stained with Neutral Red, control papillae are small with recognizable mesenchymal cores (C, arrow), whereas mutant papillae are large and primarily epithelial (D, asterisks). (E,F) K14-ir (green) reveals that the epithelial component of mutant taste papillae is greatly expanded compared with controls (E, black arrow; F, white arrow). (G-I) In control tongues at E14.5, TopGAL$^+$ cells are restricted to apical cells in organized rows of fungiform papillae (I; Fig. 1A). In mutants induced at E12.5, fungiform papilla pattern is roughly normal, but papillae are expanded (G). Expanded papillae comprise apically situated cells with both high and low (H, arrowheads) levels of TopGAL whereas most taste papilla epithelium is TopGAL-negative (H, arrow). (J,K) In mutants and controls, β-catenin-ir is primarily membrane associated (J,K, green). In mutants, many nuclear β-catenin-ir cells are in the apices of enlarged papillae, whereas in controls, nuclear β-catenin-ir is detected in occasional basal cells within fungiform papillae (J,K, arrowheads). Dashed line in H and I indicates basement membrane. Dorsal surface of the tongue is up in C-F,H-K. Scale bars: 250 µm (A,B,G); 50 µm (C,D); 10 µm (E,F,H,I); 20 µm (J,K).

which fungiform placodes house occasional basal epithelial cells with nuclear β-catenin-ir (Fig. 2J, arrowhead). In both controls and mutants, all epithelial cells exhibit membrane-associated localization of β-catenin-ir. These data suggested that stabilized β-catenin functions within *Shh*$^+$ cells to increase the TBpc population, whereas expansion of adjacent papilla epithelium is indirect, i.e. induced by signals other than canonical Wnt ligands.

To test this idea, we next assessed whether the expanded taste papilla epithelium represents an increase in *Shh*$^+$ TBpcs and/or taste papilla epithelial cells. In control tongues, *Shh* is expressed in apical cells, whereas adjacent taste papilla epithelium is *Shh* negative (Hall et al., 1999) (Fig. 3A,C, asterisk). *Shh* expression persists in enlarged mutant papillae (Fig. 3B, arrows; Fig. 3D, asterisk), with additional apical epithelial cells also expressing *Shh*, albeit at lower levels (Fig. 3D, arrows). In control tongues, the Shh receptor gene *Ptch1* is expressed broadly in both lingual epithelium and mesenchyme; however, expression intensifies in epithelial and mesenchymal cells surrounding *Shh*$^+$ TBpcs (Fig. 3E, arrowheads) (Hall et al., 1999). Consistent with the increase in *Shh* expression in mutants, *Ptch1* expression is expanded in mutant papillae (Fig. 3F, arrowheads) around TBpcs with low *Ptch1* expression (Fig. 3F, asterisks).

In addition to *Shh*$^+$, TBpcs express the mammalian *prospero* homolog Prox1 (Nakayama et al., 2015, 2008). In controls, consistent with published reports, we found Prox1$^+$ cells in fungiform papillae at E14.5, whereas many more Prox1$^+$ cells were present in enlarged taste papillae in mutant tongues (Fig. 3G-J). Thus, the increase in taste papilla size driven by stabilized β-catenin is associated with increases in taste bud precursors (*Shh*$^+$, Prox1$^+$) and taste papilla epithelium (*Ptch1*$^+$).

During normal development, taste placodes are mitotically quiescent, whereas the adjacent taste papilla epithelium is highly proliferative (Farbman and Mbiene, 1991; Liu et al., 2013; Mbiene and Roberts, 2003; Okubo et al., 2006). Hence, we next addressed whether β-catenin activation expands embryonic taste papillae in part by triggering proliferation of mitotically silent placodes cell-autonomously. To do this, we employed lineage tracing to compare the effect of β-catenin stabilization on proliferation of *Shh*$^+$ descendent cells in control versus mutant tongues. *Shh*^{CreERT2} males were mated with females possessing *R26R-EYFP* and *Ctnnb1*$^{(ex3)fl}$, so that tamoxifen induction at E12.5 resulted in lineage labeling of and stabilization of β-catenin in *Shh*$^+$ placodes of mutant embryos. As expected, in control E14.5 tongues (*Shh*^{CreERT2}*; R26R-EYFP*, tamoxifen administered at E12.5), Ki67 (Mki67 – Mouse Genome Informatics; a marker of proliferating cells) and phosphoHistone3 (PH3; a marker of cells in M phase) signals were evident only in non-lineage-labeled cells (YFP$^-$), whereas YFP$^+$ *Shh*-descendent placode cells were non-proliferative and therefore Ki67$^-$ PH3$^-$ (Fig. 3K,M). In mutant tongues, most proliferating epithelial cells were YFP$^-$, and most YFP$^+$ *Shh*-descendent cells were not dividing (Fig. 3L,N); however, occasional proliferating *Shh*-descendent cells were also evident (Fig. 3L,N, arrows). In general, cells undergoing mitosis appeared to be increased in mutant papilla epithelium compared with controls (Fig. 3M,N, arrowheads). Thus, our data suggest that cell proliferation underlies the expansion of taste papilla epithelium and, to a lesser extent, of TBpcs.

Stabilization of β-catenin within *Shh*$^+$ taste placodes accelerates differentiation of a single taste cell type

In mice, taste buds contain a heterogeneous collection of ~60 receptor cells, including Type I glial cells, Type II sweet, bitter and umami receptor cells, and Type III sour-sensing cells, each uniquely identified by expression of specific markers (Chaudhari and Roper,

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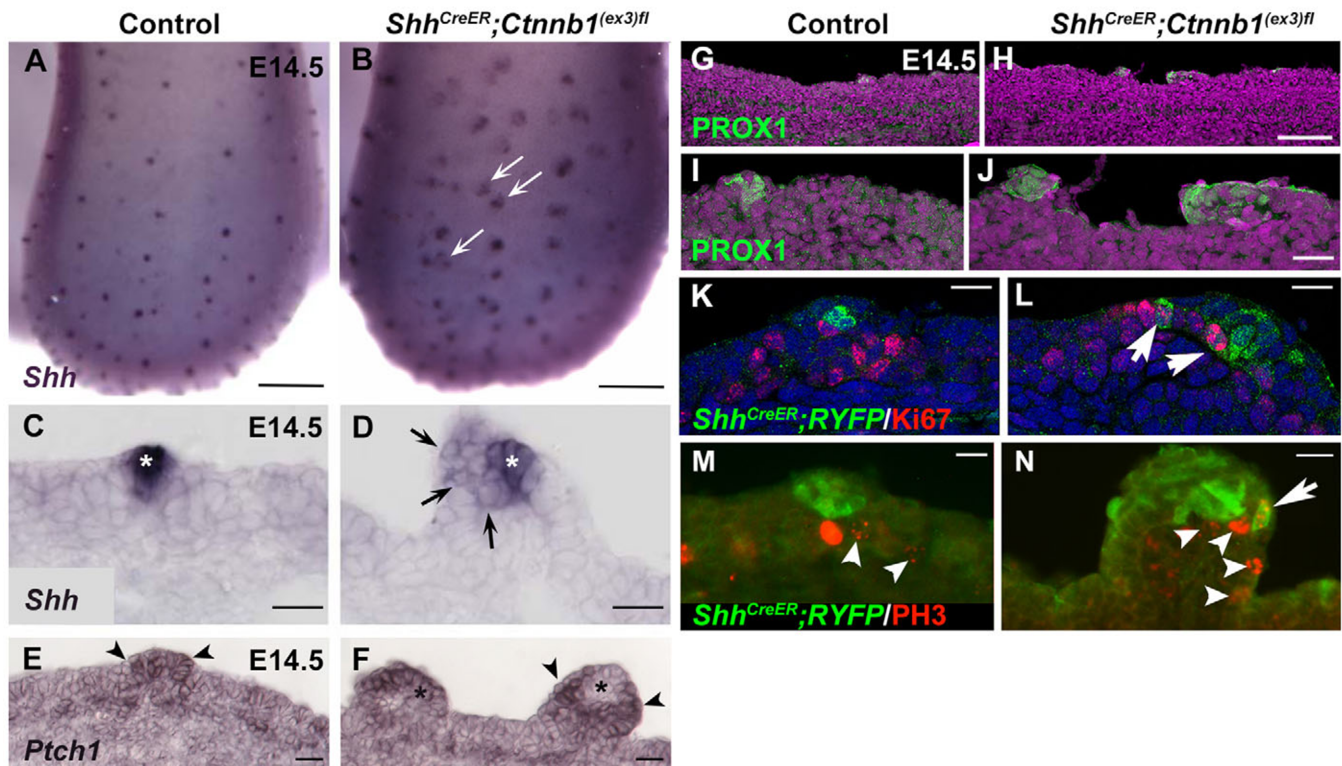

Fig. 3. Stabilized β-catenin within *Shh*$^+$ placodes drives expansion of TBpcs directly and enlargement of taste papillae indirectly. (A-D) In controls, *Shh* is expressed by small clusters of cells in fungiform papillae at E14.5 (A,C, asterisk). In mutants (*ShhCre^{ERT2};Ctnnb1$^{(ex3)fl}$*, tamoxifen at E12.5), *Shh* is expanded within enlarged fungiform papillae (B), with high *Shh*-expressing cells (D, asterisk) surrounded by low *Shh*-expressing cells (D, arrows). (E,F) In controls at E14.5, *Ptch1* is expressed broadly in lingual epithelium and mesenchyme, and more highly in fungiform papillae (E, arrowheads). In mutants, *Ptch1* expression is increased in enlarged papillae (F, arrowheads), but not in apical epithelium (F, asterisks). (G,I) At E14.5, control papillae house several Prox1$^+$ TBpcs (green). (H,J) In mutant tongues, Prox1$^+$ cells are increased. (K-N) Lineage tracing of Shh$^+$ placodes in controls (*ShhCre^{ERT2};R26R-EYFP*, tamoxifen at E12.5) reveals that Shh-descendent cells (green) are Ki67$^-$ (K, red) and pH3$^-$ (M, red), and that most cycling (K) and mitosing (M, arrowheads) cells are EYFP$^-$. In mutants (*ShhCre^{ERT2};Ctnnb1$^{(ex3)fl}$;R26R-EYFP*, tamoxifen at E12.5), some Shh-descendent cells (green) are proliferating (L,N, arrows). In G-L nuclei are counterstained with Draq5. Dorsal surface of the tongue is up in C-N. Scale bars: 250 µm (A,B); 10 µm (C-F,K,L); 100 µm (G,H); 20 µm (I,J).

2010). Although immature taste buds are detectable in embryonic taste papillae by K8-ir (Ito and Nosrat, 2009; Thirumangalathu et al., 2009), most fungiform taste buds do not express markers of specific taste cell types until the first postnatal week (Kapsimali and Barlow, 2013).

Previously, we found that activation of β-catenin in K14$^+$ embryonic epithelium caused precocious differentiation of a small number of Type II taste cells (Liu et al., 2007). Thus, we first examined whether this was also the case when β-catenin was activated within Shh$^+$ taste placodes. Using antisera against PLCβ2, a broad marker for all Type II cells (Clapp et al., 2004), we found infrequent Type II taste cells in control fungiform papillae at E18.5 (Fig. S2A), consistent with a report of sparse Type II cells present at birth (Zhang et al., 2007). In support of our previous report (Liu et al., 2007), we found slightly more differentiated PLCβ2$^+$ Type II cells in tongues of *Shh^{CreERT2};Ctnnb1$^{(ex3)fl}$* mutant embryos at E18.5 (Fig. S2B); however, these occurred in numbers not significantly different from controls (controls: 3.67±1.45 versus mutants: 6±1.53 PLCβ2$^+$ fungiform papillae; n=3 mutants, 3 controls; Student's t-test P=0.33). We next examined Type III cells, detectable by neural cell adhesion molecule (NCAM)-ir (Nelson and Finger, 1993) or Snap25-ir (Yang et al., 2000). Type III cells were not detected in control or mutant tongues, although NCAM- and Snap25-ir neurites were present in mutant and control fungiform papillae (data not shown). Finally, we examined differentiation of Type I glial cells using NTPDase2 (Entpd2 – Mouse Genome Informatics) antiserum (Bartel et al., 2006). In controls, although most immature taste buds expressing K8 were NTPDase2-immunonegative (Fig. 4A), over a third of control K8$^+$ buds were NTPdase2$^+$ (Fig. 4B). By contrast, in mutant tongues, 100% of K8$^+$ taste buds were also NTPDase2$^+$ (38± 7.9% controls versus 100±0% mutants; Student's t-test, P=0.0014; N=3 controls, 3 mutants, n=8-35 control K8$^+$ taste buds versus 32-40 mutant K8$^+$ buds) (Fig. 4C). Thus, stabilized β-catenin accelerates differentiation of Type I cells, but not Type II or III cells; Type II cells begin differentiation in both mutants and control tongues prior to birth, whereas Type III cells are not evident in taste buds of either group.

Finally, we investigated whether the expansion of *Shh*$^+$/Prox1$^+$ taste bud precursors and taste papillae observed at E14.5 in response to β-catenin activation at E12.5 (see Fig. 3) led to a larger complement of differentiating taste cells at E18.5. In fact, the diameters of K8$^+$ cell clusters (Fig. 4, yellow dotted arrows), NTPDase2$^+$ cell clusters (Fig. 4, turquoise dotted arrows) and fungiform papillae (Fig. 4, white dashed lines) were all significantly greater in mutants than in controls (Fig. 4E-G).

Activation of β-catenin within *Shh*$^+$ TBpcs during papilla morphogenesis accelerates taste cell differentiation and expands taste buds but not papillae

As stabilized β-catenin in *Shh*$^+$ placodes at E12.5 drives taste bud expansion and precocious Type I cell differentiation directly and increases taste papillae size indirectly, we investigated whether

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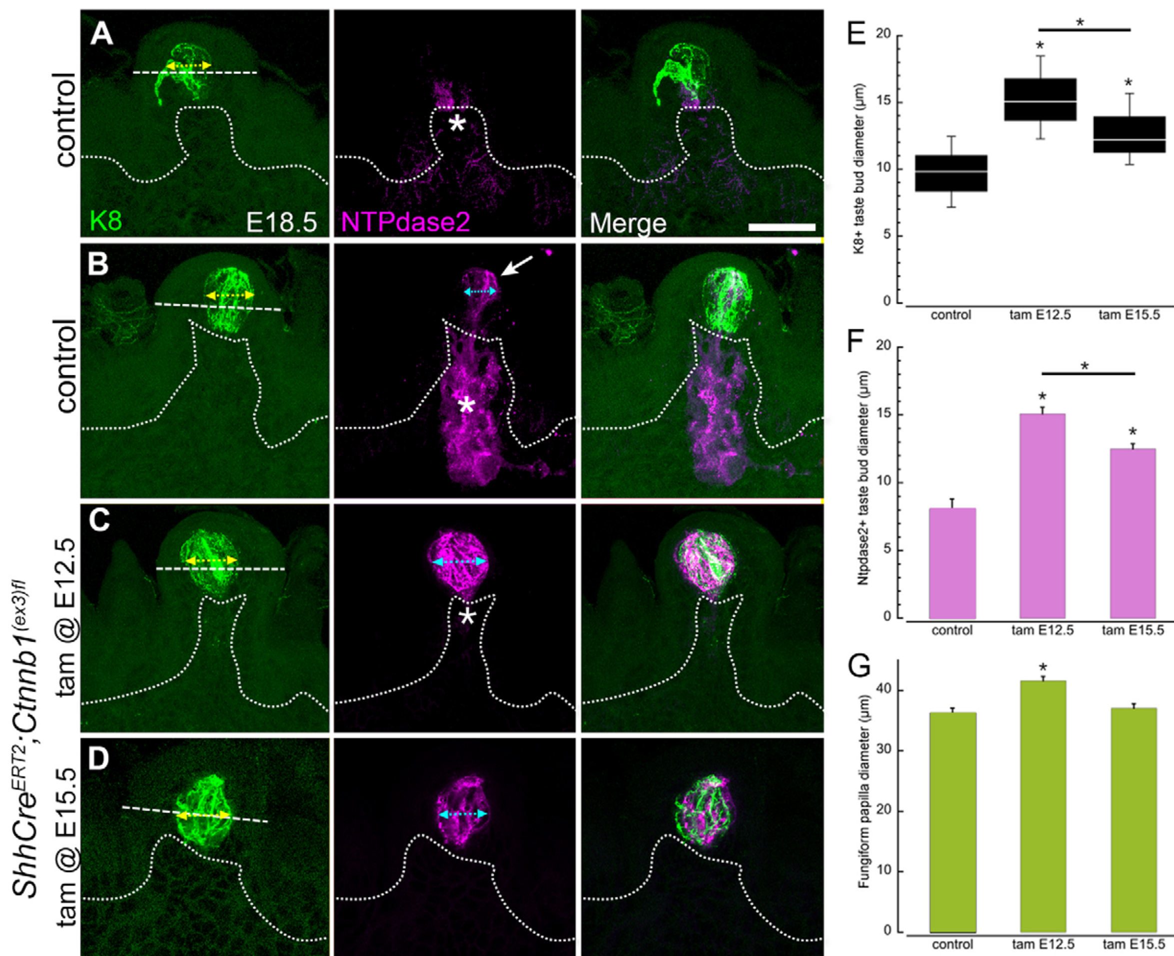

Fig. 4. Stabilized β-catenin within taste placodes promotes precocious differentiation of Type I taste cells. (A) In most control fungiform papillae at E18.5, K8$^+$ taste cells (green) are evident but NTPdase2$^+$ Type I taste cells are not present; mesenchymal cells associated with nerve fibers within the papilla core express NTPdase2 at lower levels (magenta, asterisk). (B) In 38% of control papillae, a subset of K8$^+$ taste cells is also NTPdase2$^+$ (arrow), and mesenchymal cells are NTPdase2$^+$ (asterisk). There was no correspondence between the presence or absence of NTPdase2$^+$ mesenchyme with the presence or absence of NTPdase2$^+$ Type I cells. (C) In E18.5 mutant tongues (*ShhCre^{ERT2};Ctnnb1$^{(ex3)fl}$*, tamoxifen at E12.5), K8$^+$/NTPdase2$^+$ cells are present in enlarged fungiform papillae, in addition to dim NTPdase2$^+$ mesenchymal staining (asterisk). (D) K8$^+$/NTPdase2$^+$ cells are also present in papillae of mutants induced at E15.5. (E) In mutants with stabilized β-catenin at E12.5 or E15.5, the diameter of immature K8$^+$ taste buds (yellow dotted arrows in A-D) are significantly larger than those of controls [Kruskal–Wallis non-parametric ANOVA on ranks *$P<0.05$ (asterisks over error bars) N=3 mice per condition, n=79-110 K8$^+$ immature taste buds per condition], whereas the diameter of K8$^+$ buds in mutants induced at E12.5 is significantly greater than that of mutants induced at E15.5 [*$P<0.05$ (asterisk over black bar)]. (F) The diameter of NTPdase2$^+$ taste bud cells (turquoise dotted arrows in A-D) is significantly greater in mutants induced at either E12.5 or E15.5 compared with controls [ANOVA, Tukey post-hoc, *$P<0.05$ (asterisks over error bars), N=3 mice per condition, n=24-107 Ntpdase2$^+$ taste buds per condition], and the diameter of Ntpdase2$^+$ buds in mutants induced at E12.5 is significantly greater than that of mutants induced at E15.5 [*$P<0.05$ (asterisk over black bar)]. (G) Fungiform papilla diameter (white dashed lines in A-D) is significantly greater in mutants induced at E12.5 than mutants induced at E15.5 or controls (ANOVA, Tukey post hoc, *$P<0.05$, N=3 mice per condition, n=79-110 fungiform papillae per condition). E is a percentile plot: black box=second and third quartiles, white bar=median, upper and lower error bars indicate maximum and minimum values, respectively. Bars in F and G represent mean±s.e.m. of the diameter of Ntpdase2$^+$ taste bud and fungiform papillae, respectively. Dorsal surface of the tongue is up in A-D. Dotted white lines indicate the basement membrane in all panels. Scale bar: in A for A-D, 25 μm.

these elements of the phenotype were temporally separable. Hence, we activated β-catenin in *Shh^{CreERT2};Ctnnb1$^{(ex3)fl}$* embryos at E15.5 and assayed tongues at E18.5. Similar to β-catenin activation within taste placodes at E12.5, β-catenin activation at E15.5 resulted in larger K8$^+$ cell clusters than in controls, although these were significantly smaller than K8$^+$ buds in mutants induced at E12.5 (Fig. 4A-D,E). Activation of β-catenin at E15.5 also caused robust differentiation of NTPDase2$^+$ Type I cells (Fig. 4B-D), and the diameter of NTPdase2$^+$ buds was significantly larger than controls, yet smaller than that of mutants induced at E12.5 (Fig. 4F). However, E15.5 activation of β-catenin did not result in expanded papillae; rather the size of mutant papillae was comparable to those of controls and smaller than papillae enlarged by β-catenin activation at E12.5 (Fig. 4A-D,G).

Shh is dispensable for Type I cell differentiation induced by stabilized β-catenin

Although activated β-catenin within taste placodes at E12.5 expands *Shh* expression at E14.5 (see Fig. 3A-D), at E18.5 *Shh* mRNA was lost in mutant fungiform papillae (Fig. 5A,B); this was accompanied by diminished *Ptch1* expression in the papilla epithelium and mesenchyme (Fig. 5E,F). Similarly, β-catenin activation within *Shh*$^+$ cells during papilla morphogenesis at E15.5 caused loss of *Shh* and *Ptch1* expression in mutant fungiform

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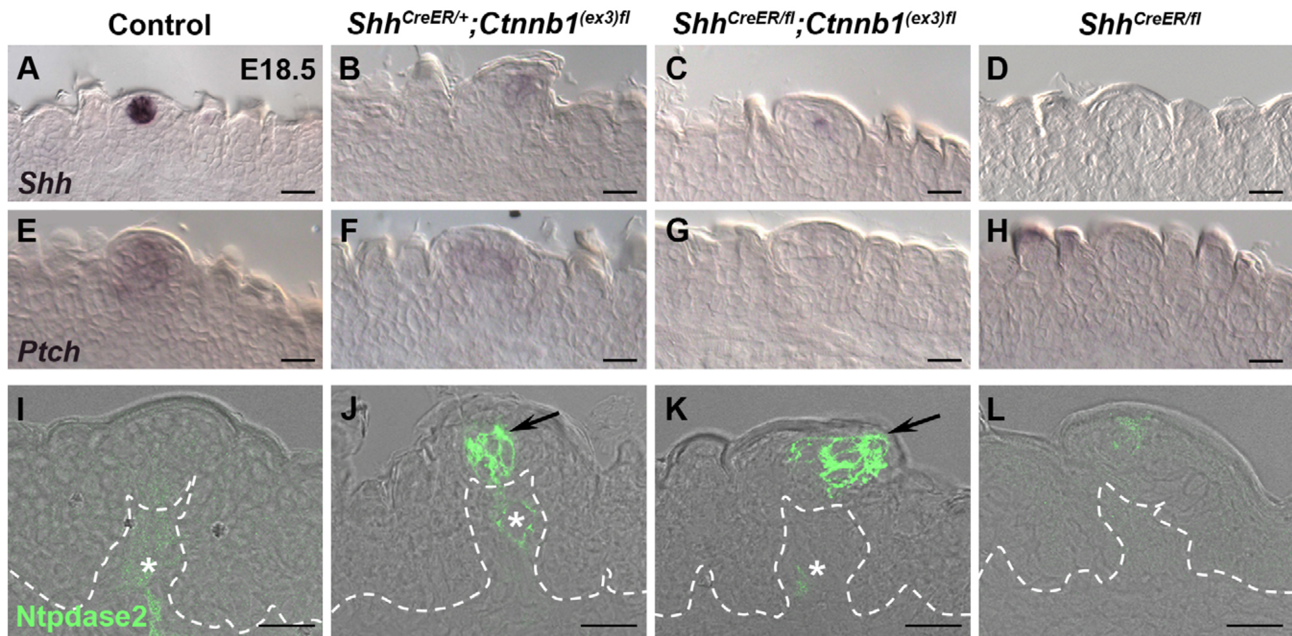

Fig. 5. Shh is dispensable for precocious differentiation of Type I cells induced by stabilized β-catenin. (A-H) Tongue sections from E18.5 embryos probed for *Shh* (A-D) and *Ptch1* (E-H) by *in situ* hybridization show loss of *Shh* and *Ptch1* in $ShhCre^{ERT2}$;$Ctnnb1^{(ex3)fl}$ (B,F), $ShhCre^{ERT2/fl}$;$Ctnnb1^{(ex3)fl}$ (C,G) and $ShhCre^{ERT2/fl}$ (D,H) mutants compared with controls (A,E). (I-L) NTPdase2-ir at E18.5 reveals NTPdase2^{+} cells in taste papillae of $ShhCre^{ERT2}$;$Ctnnb1^{(ex3)fl}$ (J, arrow), $ShhCre^{ERT2/fl}$;$Ctnnb1^{(ex3)fl}$ (K, arrow), but not in $ShhCre^{ERT2/fl}$ (L) or control (I) fungiform papillae. Asterisks in I-K indicate NTPDase2^{+} cells in taste papilla mesenchyme. Dashed line in I-K delimits basement membrane. Dorsal surface of the tongue is up in all panels. Scale bars: 10 µm (A-H); 20 µm (I-L).

papillae at E18.5 (data not shown). Under both temporal protocols, Type I cells differentiated precociously (Fig. 4; Fig. 5J). Paradoxically, we showed previously that *Shh* expression was robust and persistent at E18.5 in enlarged fungiform taste papillae that were induced by K14Cre stabilization of β-catenin (Liu et al., 2007). We therefore reasoned that although Shh signaling is required for earlier elements of the stabilized β-catenin phenotype, it might be dispensable for accelerated Type I cell differentiation. Thus, we next performed genetic experiments in order to understand the relationship of Shh and β-catenin at distinct stages of taste bud development.

Male $Shh^{CreERT2}$ mice were crossed with $Ctnnb1^{(ex3)fl}$;$Shh^{flox/flox}$ females; pregnant mice were treated with tamoxifen at E12.5, and tongues harvested at E18.5. Strikingly, the phenotype of fungiform papillae with *Shh* deletion and activated β-catenin ($Shh^{CreERT2/flox}$; $Ctnnb1^{(ex3)fl}$) (Fig. 5C,G,K) was identical to that of activated β-catenin alone ($Shh^{CreERT2}$;$Ctnnb1^{(ex3)fl}$) (Fig. 5B,F,J); papillae were enlarged, devoid of *Shh* and *Ptch1* expression, and exhibited precociously differentiated Type I cells. Although fungiform papillae appeared enlarged in $Shh^{CreERT2/flox}$ embryos, with *Shh* conditionally deleted in Shh^{+} cells, no Type I taste cells were detected (Fig. 5D,H,L). These data, combined with our prior results (Liu et al., 2007), indicate that loss of *Shh* is neither sufficient nor required for Type I taste cell differentiation in response to stabilized β-catenin.

DISCUSSION

Taste is one of our five primary senses, yet relatively little is known of the molecular and cellular mechanisms governing development of taste buds. Previously, we and others have demonstrated that Wnt/β-catenin signaling is sufficient and required for the initiation of taste bud and papilla development (Iwatsuki et al., 2007; Liu et al., 2007; Zhu et al., 2014). Specifically, stabilized β-catenin within K14^{+} lingual keratinocytes is sufficient to induce widespread formation of enlarged clusters of Shh^{+} TBpcs within expanded taste papillae (Liu et al., 2007). In this study, we used conditional molecular genetic approaches to dissect both spatially and temporally the roles of β-catenin in taste placode specification, taste papilla morphogenesis and taste cell differentiation. First, we show that broad activation of β-catenin within the lingual epithelium prior to taste placode specification perturbs taste bud development. Second, we find that β-catenin signals directly within taste placodes to expand the embryonic Shh^{+} TBpc population, whereas the enlargement of the surrounding fungiform papillae is an indirect result of β-catenin stabilization in TBpcs; further, these direct and indirect effects are temporally separable. Third, stabilized placodal β-catenin drives precocious differentiation of Type I, but not Type II and III, taste cells, and, finally, that this is independent of Shh function.

Epithelium-wide stabilization of β-catenin prior to placode formation prevents fungiform development

Although epithelial β-catenin stabilization during placode specification at E12.5 broadly promotes fungiform development (Liu et al., 2007), we found that broad epithelial stabilization of β-catenin at E11.5 blocks taste development. Recently, another group reported that early stabilization of β-catenin in lingual epithelium using Shh^{GFPCre} promotes fungiform papilla development, and can rescue loss of papillae owing to deletion of epithelial Wnt ligand secretion (Zhu et al., 2014). Like Zhu and colleagues, we observed expanded epithelial protrusions in mutant tongues, but we found that expression of markers of TBpcs (*Shh*) and immature taste buds (K8) were greatly reduced in these irregular papilla-like structures. Thus, we favor the conclusion that excess β-catenin in lingual epithelium prior to placode formation drives excess epithelial growth, but does not promote taste bud development.

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Stabilization of β-catenin within TBpcs drives taste progenitor expansion directly and papilla expansion indirectly

Elevated β-catenin has a markedly different function at E12.5 when placodes are specified; both the TBpc population and surrounding papillae are enlarged. In control tongues, *Shh*$^+$ TBpcs are mitotically quiescent throughout gestation, whereas the fungiform papilla epithelium is highly proliferative (Farbman and Mbiene, 1991; Liu et al., 2013; Mbiene and Roberts, 2003). By contrast, in stabilized β-catenin mutant tongues, some normally static taste bud precursor cells proliferate. Extrapolating from TOPGAL reporting only in the apical fungiform cells as well as direct lineage tracing, we found that β-catenin directly promotes mitoses of *Shh*$^+$ TBpcs, whereas any impact on the taste papilla epithelium is indirect. One component of the signal downstream of β-catenin might be downregulation of Shh. Initially, *Shh*$^+$ TBpcs and adjacent *Ptch1* expression are augmented by stabilized β-catenin, but by E18.5, *Shh* and *Ptch1* expression are lost from fungiform taste buds (compare Fig. 3A-F with Fig. 5). Consistent with *Shh* conditional deletion results (Fig. 5D,H,L), pharmacological inhibition of Shh signaling in cultured embryonic tongues increases placodal Wnt reporter activity, results in more *Shh*$^+$ TBpcs, and drives formation of enlarged taste papillae (Hall et al., 2003; Iwatsuki et al., 2007; Mistretta et al., 2003). By contrast, excess Shh ligand negatively regulates Wnt/β-catenin function during placode specification (Iwatsuki et al., 2007). Thus, our data support a model in which the interplay between β-catenin positive regulation of Shh, which in turn feeds back negatively on Wnt/β-catenin, is crucial for development of normally sized fungiform taste buds and papillae (Iwatsuki et al., 2007).

Stabilization of β-catenin within *Shh*$^+$ taste precursor cells accelerates taste cell differentiation, and biases taste cell fate

In mice, taste buds are assemblies of ∼60 elongate cells, categorized as three morphological types: glial-like Type I cells, which make up 50% of the cells per bud; Type II cells are receptor cells for sweet, bitter and umami (amino acid) tastants and represent ∼20% of cells; and Type III cells are sour detectors and are the least common, making up ∼10% of cells (Chaudhari and Roper, 2010; Miura et al., 2014). In fungiform papillae, small numbers of Type II and III cells are present at birth, with the majority of taste cells differentiating postnatally (Ohtubo et al., 2012; Zhang et al., 2006, 2007). Although we did not detect Type III cells at E18.5, a small proportion of fungiform papillae contained Type II cells and at least a third possessed Type I cells, indicating that taste cell differentiation begins late in embryonic development. Following β-catenin activation, 100% of K8$^+$ taste buds were NTPdase2$^+$ by E18.5. Although more differentiated Type II cells were also present in mutant taste buds, these numbers were not significantly greater than controls. Finally, we found no induction of Snap25$^+$ or NCAM$^+$ Type III cells. Thus, we conclude that the primary impact of activated β-catenin in *Shh*$^+$ TBpcs is a dramatic increase in precociously differentiated Type I cells. These data are consistent with our recent report of β-catenin function in adult taste bud renewal (Gaillard et al., 2015); activation of β-catenin drives adult taste bud progenitors to produce almost exclusively Type I taste cells, promotes Type II cell differentiation in a small subset of cells, and has no effect on Type III cells.

Precocious Type I cell differentiation by β-catenin activation is independent of Shh function. Type I cell differentiation is accelerated when β-catenin is activated solely in taste placodes or broadly in K14$^+$ epithelium, but this occurs in the genetic absence of *Shh* in the former (Fig. 5), and in the presence of *Shh* expression in the latter (data not shown). Significantly, loss of Shh combined with activation of β-catenin in taste placodes phenocopies β-catenin activation alone, i.e. precocious differentiation of Type I cells; whereas Type I cells are not evident in the fungiform papillae in which only Shh is deleted. In renewing adult taste buds, *Shh* expression ceases as *Shh*$^+$ taste precursors differentiate into each of the three taste cell types (Miura et al., 2006, 2014), yet Type I, II and III cells will differentiate in ectopically located adult taste buds despite persistent *Shh* expression (Castillo et al., 2014). Consistent with these data, cells within immature, mature and ectopic taste buds are not Shh responsive, as evidenced by their lack of expression of Shh reporter genes, *Gli1* and *Ptch1* (Castillo et al., 2014; Hall et al., 1999; Liu et al., 2013; Miura et al., 2001). Thus, we conclude that once taste placodes are specified, Shh is dispensable for taste cell differentiation.

We showed previously that *Shh*$^+$ placodes do not contribute to taste papillae, but rather differentiate into the first set of adult taste buds (Thirumangalathu et al., 2009). However, embryonic Shh-descendent taste cells are lost within a few months of birth, indicating that placodal cells are not, nor do they produce, stem cells responsible for continuous renewal of taste cells in adults. Rather, genetic fate tracing in adults has shown that K14$^+$ basal keratinocytes outside of taste buds give rise to both taste bud cells and lingual epithelium (Okubo et al., 2009).

Additionally, activation of β-catenin within the K14$^+$ lineage revealed a broad competency of non-taste epithelium to undergo the taste bud developmental program in response to increased β-catenin (Liu et al., 2007). In fact, manipulation of several signaling pathways results in excess and ectopic taste placodes, including inhibition of Shh, activation of either bone morphogenetic protein or epidermal growth factor signaling *in vitro* (Hall et al., 2003; Liu et al., 2004; Mistretta et al., 2003), as well as genetic loss of follistatin (Beites et al., 2009). Because taste bud precursor patterning, although initiated by β-catenin function, is subsequently regulated by a host of signals acting on a broadly competent epithelium, small changes in their relative signal strengths would be expected to alter taste bud pattern. In fact, in rodents and humans, the number of fungiform taste buds and papillae is highly variable (Bartoshuk et al., 1996; Fischer et al., 2013; Miller, 1988; Miller and Reedy, 1990), and this variability might be functionally important, as taste papilla number may be correlated with differences in taste responses and ultimately influence taste behavior and diet (Bartoshuk et al., 1996; Tordoff et al., 2008).

MATERIALS AND METHODS

Animals

Mice used include: *Shh*^{CreERT2} (Harfe et al., 2004) from Cliff Tabin, Harvard University, MA, USA; *Shh*^{flox} (Lewis et al., 2004) *(Jax 004293); Ctnnb1*$^{(Ex3)fl}$ (Harada et al., 1999) from T. Carroll, UT Southwestern Medical Center, TX, USA; *TopGAL* (DasGupta and Fuchs, 1999) and *ROSALacZ* (Soriano, 1999) from T. Williams, University of Colorado School of Dental Medicine, CO, USA; *ROSAEYFP* (Srinivas et al., 2001) (Jax 006148); and ROSAtdTomato (Jax 007914). *Shh*^{CreERT2} mice are maintained on a C57BL/6 background. All other lines are mixed. Mice and embryos were genotyped as described previously and maintained and sacrificed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver School of Medicine.

Tamoxifen induction of Cre recombination

Shh^{CreERT2} males were crossed with *Ctnnb1*$^{(Ex3)fl/fl}$, *ROSALacz*, *ROSAEYFP* or *ROSAtdTomato* females and noon on the day of a vaginal plug was

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designated E0.5. Pregnant dams were dosed intraperitoneally once between E11.5 and E15.5 with 4-5 mg tamoxifen (10 mg/ml in mineral oil, T-5648, Sigma), which was prepared and administered as described (Thirumangalathu et al., 2009). Three control and three mutant embryos for each experiment were recovered from at least two pregnant females and staged according to Kaufman (1999).

In situ hybridization, immunofluorescence and β-galactosidase histochemistry

For *Shh* mRNA *in situ* hybridization of whole tongues, tissue was fixed in 4% paraformaldehyde (PFA) overnight at 4°C, and processed as described (Hall et al., 1999) with hybridization of a *Shh* probe (640 bp; Echelard et al., 1993) and stringency washes at 62°C. For *in situ* hybridization of tissue sections, tongues were fixed in 0.2% PFA overnight, cryoprotected and sectioned as described (Miura et al., 2001). Antisense RNA probes were: *Shh* (340-2668: Genbank AKO77688; Echelard et al., 1993) and *Ptch1* (1318-2362: Genbank MMU 46155). For whole-mount X-gal reactions, E14.5 tongues were fixed in 0.25% glutaraldehyde (15 min at 4°C) and stained for X-gal (Thirumangalathu et al., 2009). E18.5 tongues were fixed in 0.2% PFA overnight and 12 µm cryosections were cut and stained for X-gal. Immunofluorescence staining of sections followed (Thirumangalathu et al., 2009). Primary antisera were: rabbit anti-Prox-1 (Millipore, AB5475; 1:1000), rabbit anti-β-catenin (Sigma, C2206; 1:1000), guinea pig anti-K14 (gift from Dr Maranke Koster, University of Colorado School of Medicine, CO, USA; 1:1000), rabbit anti-K14 (Covance, PRB-155P; 1:1000), rabbit anti-PH3 (Millipore, 06-570; 1:1000), rabbit anti-Ki67 (Thermo Scientific, RM-9106S, 1:250), rabbit anti-NTPdase2 (Centre de Recherche du CHUL, Quebec, Canada; http://ectonucleotidases-ab.com; 1:1000), rabbit anti-PLCβ2 (Santa Cruz Biotechnology, SC-206; 1:1000), rabbit anti-SNAP25 (Sigma, S9684; 1:8000), rabbit anti-N-CAM (Millipore, AB5032; 1:1000), mouse anti-keratin 8 (Developmental Studies Hybridoma Bank, TROMA1; 1:250) Primary antibodies were detected with species-appropriate secondary antisera conjugated with Alexa488 or Alexa546 (Molecular Probes; 1:500). Sections were nuclear counterstained with DRAQ5 (Abcam, ab108410, 1:7500) or DAPI (Invitrogen, D3571, 1:10,000).

Microscopy and image acquisition

Bright-field and fluorescence images were acquired with an Axioplan fluorescence microscope, Axiocam CCD camera with Axiovision software, or a Retiga 4000R camera and Q-Capture Pro-7 software. *z*-stack confocal images were acquired at 0.75 µm optical sections with a laser-scanning Olympus Fluoview or Leica SP5 confocal microscope. Images were saved as TIFFs, contrast adjusted and cropped, and figures were compiled using Adobe Photoshop.

Quantitative analysis

For E14.5 tongues, analysis was limited to the first 1 mm of the tongue; 20 12 µm sections spanning the first 1.0 mm of each E14.5 tongue were tallied in the analysis, and TBpcs/papillae in every fourth section (4×12 µm=48 µm intervals) were measured to avoid double counting. K8 and NTPDase2-ir E18.5 tongue sections of controls (n=3), mutants induced at E12.5 (n=3), or mutants induced at E15.5 (n=3) were used to compare $K8^+$ taste bud diameter, $NTPdase2^+$ diameter, and fungiform papilla diameter. Fungiform papillae were identified via the presence of $K8^+$ cells. Thirty-four 12 µm sections spanning the first 2.5 mm of each E18.5 tongue were tallied in the analysis, and taste buds/papillae in every sixth section (6×12 µm=72 µm intervals) were measured to avoid double counting. Fungiform papilla diameter was taken to include the resident taste bud and spanned the entire papilla epithelium (see Fig. 4A-D, white dashed lines). $K8^+$ and $Ntpdase2^+$ taste bud measurements were made at the widest midpoint along the apical-basal axis of each bud (Fig. 4A-D, yellow and turquoise dotted arrows, respectively). Diameters of $K8^+$ and $NTPDase2^+$ immature taste buds were used because neither marker allows for identification of individual cells in tightly packed buds; K8 is an intermediate filament associated with the cytoskeleton (Bragulla and Homberger, 2009), and NTPDase2 is a membrane associated ecto-ATPase (Bartel et al., 2006). All measurements were made blind to treatment.

Statistical analysis

Data in Fig. 4E did not meet a test of normality and were compared using a Kruskal–Wallis ranks test, followed by Dunn's method of post-hoc pairwise multiple comparisons. Data in Fig. 4F,G comprise normal distributions and were compared using ANOVA with post-hoc Tukey pairwise comparisons. Comparisons between mutants and controls at the same time points post-Cre induction were made using Student's *t*-tests and significance taken at $P<0.05$.

Acknowledgements
We thank Dr Maranke Koster for the K14 antiserum; the Rocky Mountain Taste and Smell Center for collegial, animal and imaging support; Alon Bajayo and Dany Gaillard for imaging and statistical expertise; Jennifer Scott for valuable help with *in situ* hybridization experiments; and Brendan Ross, Lauren Gross, Kelly Zaccone and Brooke Baxter for expert technical assistance.

Competing interests
The authors declare no competing or financial interests.

Author contributions
S.T. and L.A.B. designed experiments, and S.T. generated genetic crosses. S.T. and L.A.B. harvested processed and imaged all tissue. S.T. and L.A.B. performed quantitative and statistical analyses, and wrote the manuscript. Both authors edited drafts of the manuscript.

Funding
This work was supported by The National Institutes of Health National Institute on Deafness and Other Communication Disorders (NIDCD) [R01 DC008373 and R01 DC63004568 to L.A.B.; R03 DCO1141 to S.T.; and P30 DC004657 to Dr Diego Restrepo]. Deposited in PMC for release after 12 months.

Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.121012/-/DC1

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