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Wnt-β-catenin signaling initiates taste papilla development

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Fungiform taste papillae form a regular array on the dorsal tongue. Taste buds arise from papilla epithelium[1] and, unusually for epithelial derivatives, synapse with neurons, release neurotransmitters and generate receptor and action potentials[2,3]. Despite the importance of taste as one of our five senses, genetic analyses of taste papilla and bud development are lacking. We demonstrate that Wnt-β-catenin signaling is activated in developing fungiform placodes and taste bud cells. A dominant stabilizing mutation of epithelial β-catenin causes massive overproduction of enlarged fungiform papillae and taste buds. Likewise, genetic deletion of epithelial β-catenin or inhibition of Wnt-β-catenin signaling by ectopic dickkopf1 (*Dkk1*)[4–6] blocks initiation of fungiform papilla morphogenesis. Ectopic papillae are innervated in the stabilizing β-catenin mutant, whereas ectopic *Dkk1* causes absence of lingual epithelial innervation. Thus, Wnt-β-catenin signaling is critical for fungiform papilla and taste bud development. Altered regulation of this pathway may underlie evolutionary changes in taste papilla patterning.

Lingual taste buds develop within several different types of tongue papillae: fungiform, foliate and circumvallate. In mouse embryos, roughly 100 fungiform papillae form in a stereotypical array on the anterior two-thirds of the dorsal lingual surface. The number and pattern of papillae vary between species: the human tongue contains approximately 200 fungiform papillae, for instance. Taste papillae appear first at embryonic day (E) 12.5 of mouse development as localized epithelial thickenings, termed 'placodes', that show specifically elevated expression of the signaling molecules *Shh* and *Bmp4* (refs. 7,8). By E14.5, placodes begin to evaginate, forming raised papillae with mesenchymal cores. Taste buds are first morphologically evident at approximately E18 as onion-shaped aggregates of elongated cells within apical papillary epithelium[1,9]. Each taste bud contains 50–150 specialized fusiform cells, a subset of which transduces gustatory stimuli into neural signals. Nerves reach the basal lamina of developing taste papillae between E13.5 and E14.5 and densely innervate the papillary epithelium by E16 (ref. 7).

Wnts form a large family of secreted ligands that activate several receptor-mediated pathways[10]. In the Wnt-β-catenin pathway, binding of Wnt ligands to Frizzled (Fz) receptors and low-density lipoprotein receptor–related protein (LRP) family coreceptors causes accumulation of cytoplasmic β-catenin and its translocation to the nucleus, resulting in transcriptional activation by complexes of β-catenin and Lef or Tcf transcription factors[10]. Wnt-β-catenin signaling is critical for development of a broad range of epithelial appendages[5,6,11–13]. Here, we use genetic approaches to investigate whether this pathway has a central role in the formation of tongue papillae.

Consistent with this hypothesis, several genes encoding Wnt ligands are expressed in embryonic tongue epithelium[14] (**Fig. 1a–c** and **Supplementary Fig. 1** online). In mice carrying a *Tg(Fos-lacZ)34Efu* (also known as *TOPGAL*) *lacZ* Wnt-β-catenin reporter gene[15], β-galactosidase activity was broadly activated in anterior tongue at E12.0 and subsequently extended posteriorly, becoming upregulated specifically at sites of epithelial fungiform placode development starting at E12.5 (**Fig. 1d–j**). At E17–E18.5, we detected *Tg(Fos-lacZ)34Efu* expression in apical balls of fungiform papilla cells, which we identified as developing taste bud cells by their elongated morphology (**Fig. 1k–n**). Recent analysis of an independent Wnt-β-catenin reporter transgenic line produced similar results[14].

To test functionally whether Wnt-β-catenin signaling is important for fungiform papilla formation, we first asked whether LiCl, which activates Wnt-β-catenin signaling downstream of the receptor[16], promotes placode initiation in cultured tongue primordia. Three hours of LiCl treatment resulted in expansion of epithelial *Tg(Fos-lacZ)34Efu* activity in E12.0 tongues and the appearance of larger and ectopic clusters of β-galactosidase–positive cells (**Fig. 2a–d**). We

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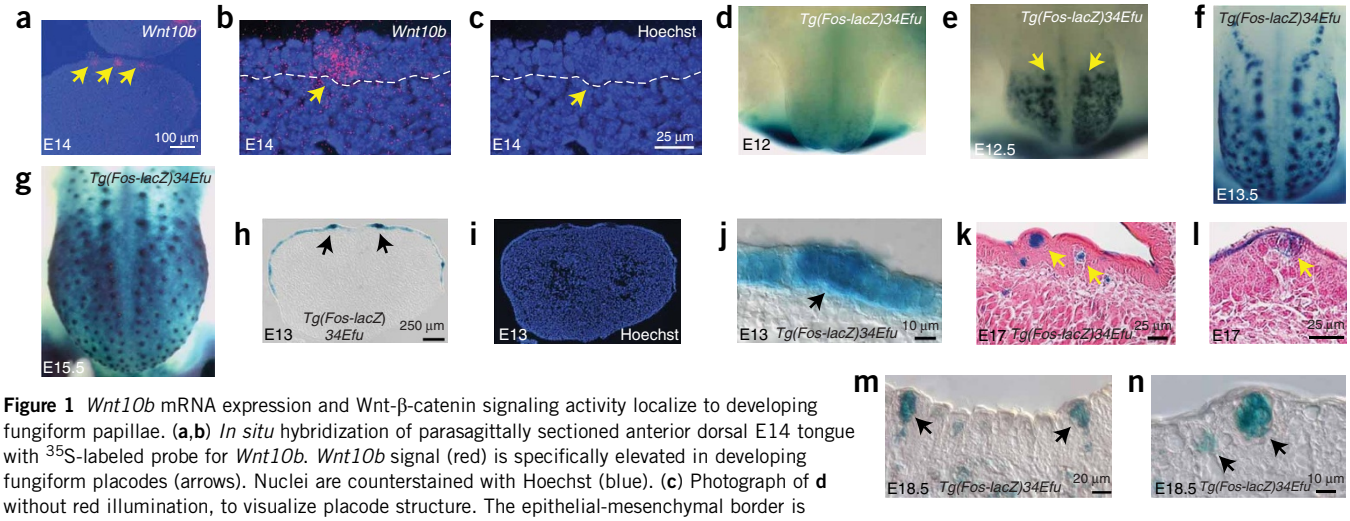

Figure 1 *Wnt10b* mRNA expression and Wnt-β-catenin signaling activity localize to developing fungiform papillae. (**a**,**b**) *In situ* hybridization of parasagittally sectioned anterior dorsal E14 tongue with ^{35}S-labeled probe for *Wnt10b*. *Wnt10b* signal (red) is specifically elevated in developing fungiform placodes (arrows). Nuclei are counterstained with Hoechst (blue). (**c**) Photograph of **d** without red illumination, to visualize placode structure. The epithelial-mesenchymal border is indicated by dashed white lines in **b** and **c**. (**d**–**g**) X gal–stained whole-mounted *Tg(Fos-lacZ)34Efu* tongues at E12 (**d**), E12.5 (**e**), E13.5 (**f**) and E15.5 (**g**). *Tg(Fos-lacZ)34Efu* is expressed in anterior dorsal tongue at E12 and by E12.5 expands posteriorly and begins to localize in a stereotypical fashion, first in anterior regions and immediately to either side of the ventral midline (arrows in **e**) and then in posterior and lateral areas. Smaller *Tg(Fos-lacZ)34Efu*-positive spots subsequently appear interspersed between parasaggital and lateral placodes (**f**). (**h**,**i**) Bright-field (**h**) and Hoechst-counterstained (**i**) images of a mid-posterior coronal section of E13 X gal–stained *Tg(Fos-lacZ)34Efu* tongue showing epithelial *Tg(Fos-lacZ)34Efu* activity concentrating in developing placodes to each side of the midline (arrows). (**j**) Higher-magnification view of an E13 X gal–stained *Tg(Fos-lacZ)34Efu* tongue section showing *Tg(Fos-lacZ)34Efu* expression in the epithelium. (**k**–**n**) X gal–stained *Tg(Fos-lacZ)34Efu* tongue sections at E17 (**k**,**l**) and E18.5 (**m**,**n**). Sections in **k**,**m** and **n** are coronal at the level of posterior tongue; section in **l** is sagittal and from the more developed anterior region. *Tg(Fos-lacZ)34Efu* activity localizes to developing taste bud cells (arrows) within fungiform papillae. Section in **l** is understained with X-gal to show cell morphology. Sections in **k**,**l** are counterstained with eosin. Size bar in **h** applies to **h**,**i**.

used whole-mount *in situ* hybridization for the fungiform placode marker *Shh* to examine placode development in tongues explanted at E11.5 and subjected to 3 h of LiCl treatment and 3 d of culture, compared with tongues cultured for 3 d without LiCl treatment (**Fig. 2e**,**f**). *Shh*-expressing placodes were thicker in LiCl-treated tongues than in control tongues (**Fig. 2g**,**h**). LiCl-treated tongues also showed statistically significant increases in the number ($P < 0.017$) and mean diameter ($P < 3.21 \times 10^{-13}$) of *Shh*-expressing cell clusters compared with controls cultured without LiCl treatment (**Fig. 2i**,**j**; $n = 3$ control explants, $n = 5$ explants treated with LiCl for 1 h, $n = 3$ explants treated with LiCl for 3 h). The increased mean diameter resulted from a shift in the size distribution of

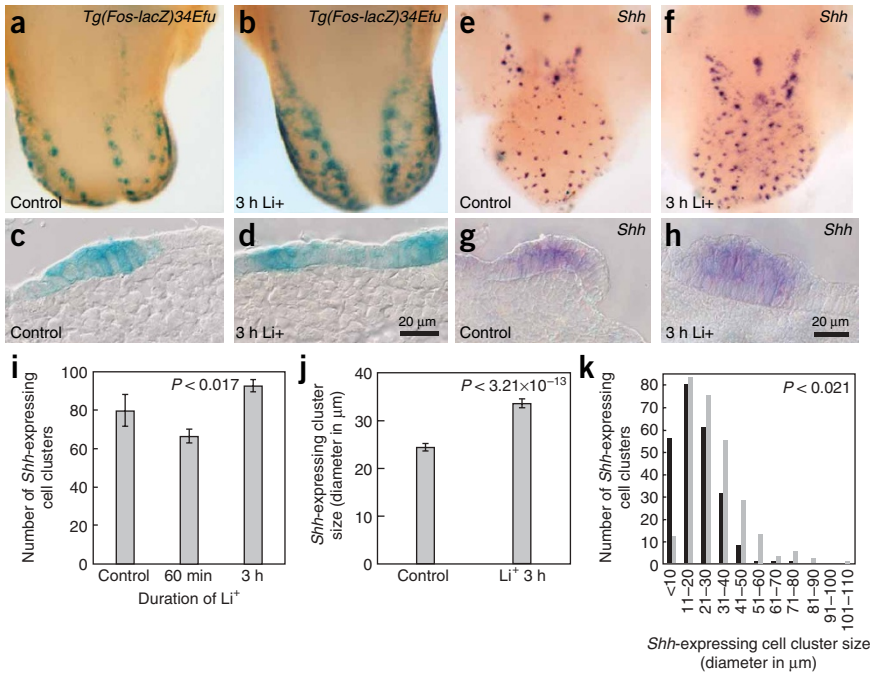

Figure 2 Stimulation of Wnt-β-catenin signaling *ex vivo* promotes fungiform placode fate. (**a**,**b**) X gal–stained *Tg(Fos-lacZ)34Efu* tongues dissected at E12 and cultured for 3 h with (**b**) or without (**a**) 25 mM LiCl. Note expanded X-gal staining and increased number and size of *Tg(Fos-lacZ)34Efu*-positive cell clusters in the LiCl-treated sample. (**c**,**d**) Coronal sections of mid-anterior control and LiCl-treated tongues, showing expanded *Tg(Fos-lacZ)34Efu* activity in dorsal LiCl-treated tongue. X-gal staining is confined to the epithelium in both control and LiCl-treated samples. (**e**,**f**) Tongues dissected at E11.5, cultured for 3 d with (**f**) or without (**e**) 3 h of 25 mM LiCl treatment on day 1 and hybridized with digoxygenin-labeled probe for *Shh*. Note the increased number and sizes of *Shh*-positive cell clusters in the LiCl-treated sample. (**g**,**h**) Coronal sections of mid-anterior control and LiCl-treated tongues photographed in **e**,**f**, showing expanded *Shh* expression and increased placode diameter and thickness in dorsal LiCl-treated tongue. (**i**) Quantification of the number of *Shh*-expressing cell clusters in control tongues ($n = 3$) and in tongues subjected to 1 h ($n = 5$) or 3 h ($n = 3$) of LiCl exposure on day 1 of culture. (**j**,**k**) Quantification of the mean diameter (**j**) and distribution of the diameters (**k**) of *Shh*-expressing cell clusters in control tongues ($n = 3$) and in tongues subjected to 3 h of LiCl exposure on the first day of culture ($n = 3$). Error bars in **i**,**j** represent s.e.m. In **k**, black bars represent data from control explants, and gray bars represent data from LiCl-treated explants.

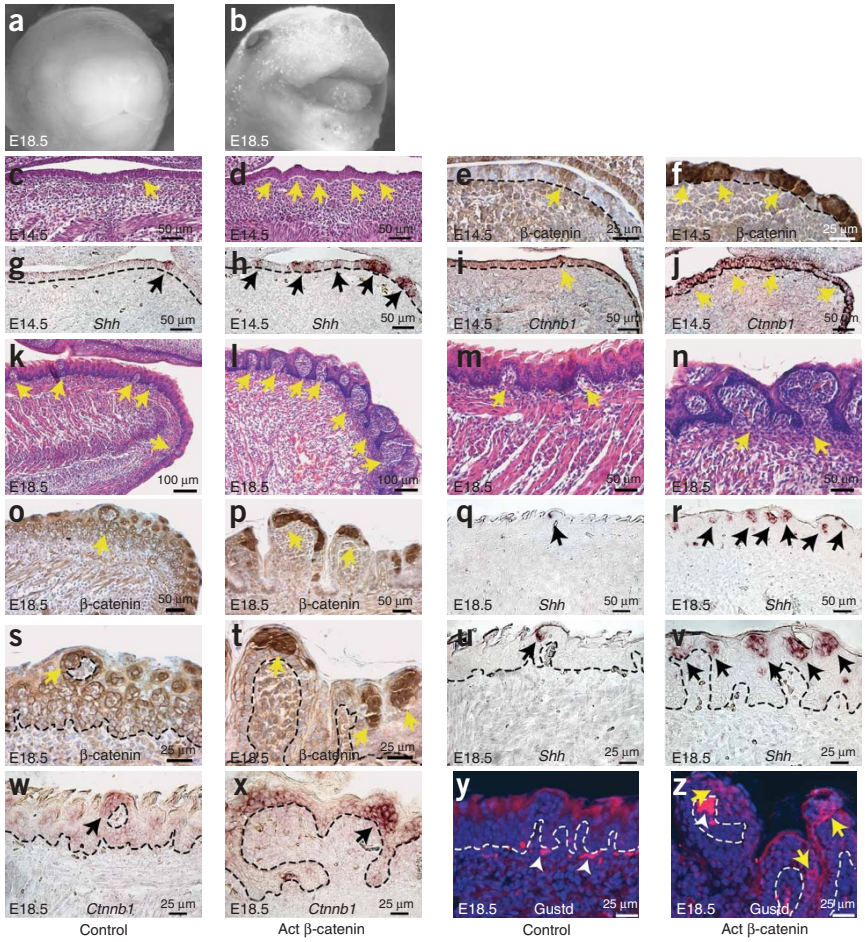

Figure 3 Mutation of epithelial β-catenin to a stabilized form promotes fungiform papilla and taste bud development *in vivo*. (**a**,**b**) Control littermate (**a**) and *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ ('act β-catenin'; **b**) embryo heads at E18.5. Note protruding tongue and tongue blebs in the mutant. (**c**–**j**) Coronal sections of mid-posterior dorsal E14.5 control littermate (**c**,**e**,**g**,**i**) and *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ (**d**,**f**,**h**,**j**) tongues at E14.5 subjected to hematoxylin and eosin staining (**c**,**d**), immunohistochemistry for β-catenin (brown) (**e**,**f**) and *in situ* hybridization with digoxygenin-labeled probes for *Shh* (**g**,**h**) or *Ctnnb1* (**i**,**j**) (purple-brown signals). Note increased placode numbers and evaginations, broader epithelial regions showing nuclear and cytoplasmic-localized β-catenin protein and *Shh* expression and elevated *Ctnnb1* mRNA in the mutant. (**k**–**z**) Parasagittal sections of control littermate (**k**,**m**,**o**,**q**,**s**,**u**,**w**,**y**) and *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ (**l**,**n**,**p**,**r**,**t**,**v**,**x**,**z**) E18.5 tongues subjected to hematoxylin and eosin staining (**k**–**n**); anti-β-catenin immunohistochemistry (**o**,**p**,**s**,**t**); *in situ* hybridization with digoxygenin-labeled probe for *Shh* (**q**,**r**,**u**,**v**) or *Ctnnb1* (**w**,**x**) (purple-brown signal); immunofluorescence (red) for Gustducin (**y**,**z**). **k**–**p**,**s**,**t**,**w**–**z** show anterior dorsal tongue; **q**,**r**,**u**,**v** show mid-dorsal regions. Fungiform papillae are increased in density and size in the mutant. Mutant apical epithelial cell clusters show increased size and higher amounts of β-catenin protein, *Ctnnb1* mRNA and *Shh* mRNA. Gustducin is not expressed in control fungiform papillae at E18.5 (**y**) but shows weak expression in mutant apical epithelial cell clusters (yellow arrows in **z**). Nuclei are counterstained with hematoxylin (pale blue) in **e**,**f**,**o**,**p**,**s**,**t** and with DAPI in **y**,**z**. White arrowheads in **y**,**z** indicate autofluorescence. Dashed lines in **e**–**j** and **s**–**z** indicate epithelial-mesenchymal boundaries. **m**,**n**,**s**,**t**,**u**,**v** show higher-magnification photographs of the samples in **k**,**l**,**o**,**p**,**q**,**r**, respectively. The two panels in each panel pair were photographed at the same magnification.

Shh-expressing cell clusters (**Fig. 2k**; $P < 0.021$). To determine whether ectopic placodes induced by β-catenin signaling could develop into fungiform papillae, we stimulated Wnt-β-catenin signaling *in vivo* by mutating the endogenous β-catenin gene (*Ctnnb1*, also known as *Catnb*) to a dominant active form in epithelial cells (**Fig. 3**). We mated *Ctnnb1*$^{(Ex3)fl}$ mice, in which exon 3 of *Ctnnb1* (encoding serine and threonine residues that are key targets of phosphorylation required for degradation of β-catenin) is flanked by *loxP* sequences that serve as targets for Cre-mediated recombination[17], to *KRT14-Cre* line 43 transgenic mice[18] expressing Cre recombinase under the control of a keratin 14 promoter[19]. We observed *KRT14-Cre*–mediated recombination of a *ROSA26R* Cre reporter gene[20] in tongue epithelium by E12.5 (**Supplementary Fig. 2** online).

KRT14-Cre Ctnnb1$^{(Ex3)fl/+}$ mice died at birth and had protruding tongues with blebs on their dorsal surfaces (**Fig. 3a**,**b**). Histological examination at E14.5 showed higher fungiform placode density in mutant tongues compared with control tongues (**Fig. 3c**,**d**). We detected cytoplasmic and nuclear-localized β-catenin in control fungiform placodes (**Fig. 3e**), consistent with Wnt-β-catenin pathway activation at these sites. In E14.5 *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ mutants, epithelial β-catenin protein amounts were elevated, and epithelial regions showing cytoplasmic and nuclear β-catenin localization and *Shh* expression were greatly expanded (**Fig. 3e**–**h**). *Ctnnb1* mRNA levels were markedly higher in large stretches of mutant epithelium than in control epithelium (**Fig. 3i**,**j**), indicating that dominant active β-catenin positively regulates expression or stability of its own mRNA. β-catenin protein expression was not uniform in mutant epithelium at E14.5 (**Fig. 3f**), possibly owing to mosaic deletion of the *Ctnnb1*$^{(Ex3)fl}$ allele at this stage and/or modulation of positive feedback regulation by inhibitory factors acting downstream of β-catenin signaling.

Histological examination at E18.5 demonstrated that tongue bleb-like structures showed clear morphological similarity to fungiform papillae, with cores of mesenchymal cells evaginating into the epithelium and characteristic mushroom shapes (**Fig. 3k**–**n**). Consistent with increased density of fungiform placodes at E14.5, these structures were more numerous than littermate control tongue fungiform papillae (17 ± 2 (mean ± s.d.) in mutant and 4 ± 1 in control per 40× field; we counted five fields each for mutant and control; $P = 0.00000323$). In addition, mutant fungiform papillae were larger than in controls (75 ± 25 μm wide in mutants and 31 ± 5 μm wide in controls ($P = 0.000293$); 91 ± 17 μm high in mutant; 40 ± 8 μm high in controls ($P = 0.00000136$); $n = 10$ mutant and $n = 10$ control anterior papillae measured) (**Fig. 3k**–**n**). In some cases, adjacent mutant fungiform papillae appeared to have fused (**Fig. 3x**). *Bmp4* expression was expanded in mutant fungiform papilla epithelium compared with controls (**Supplementary Fig. 3** online). We observed preferential localization of cytoplasmic and nuclear β-catenin protein and *Ctnnb1* mRNA in epithelial cell clusters in apical and apical-lateral regions of the enlarged fungiform papillae (**Fig. 3o**,**p**,**s**,**t**,**w**,**x**). These cell clusters expressed *Shh*, which localizes to basal cells in mature taste

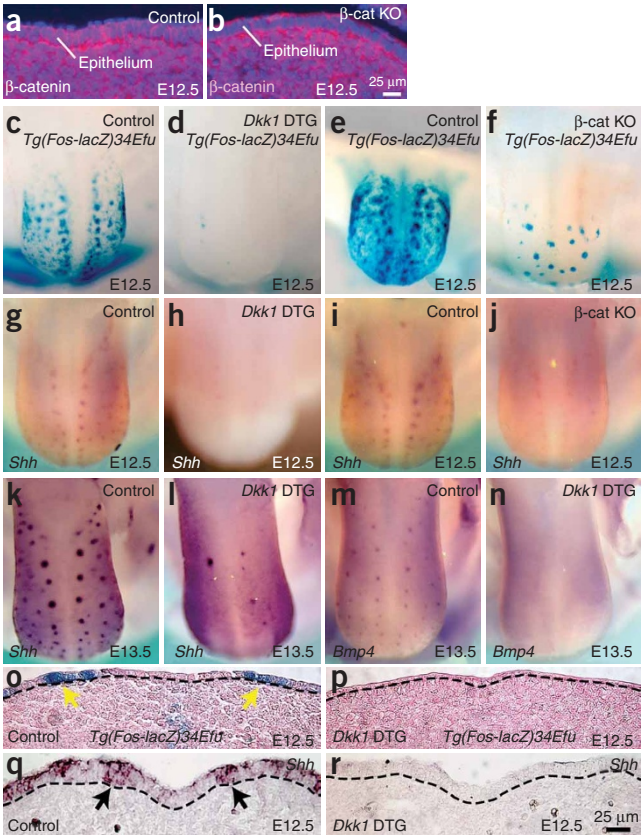

Figure 4 Wnt-β-catenin signaling is required for expression of fungiform placode markers. (**a**,**b**) Parasaggital sections of anterior E12.5 tongues from control littermate (**a**) and *KRT14-Cre Ctnnb1*$^{fl/fl}$ (β-cat KO) (**b**) mice subjected to immunofluorescence for β-catenin (red) and counterstained with DAPI (blue). Note loss of β-catenin from mutant epithelial cells. (**c**,**d**) X gal–stained E12.5 tongues from control *Tg(Fos-lacZ)34Efu* (**c**) or *KRT5-rtTA tetO-Dkk1* (*Dkk1* DTG) *Tg(Fos-lacZ)34Efu* (**d**) littermates treated with doxycycline from E0.5. (**g**,**h**,**k**–**n**) Tongues from control (**g**,**k**,**m**) or *KRT5-rtTA tetO-Dkk1* (*Dkk1* DTG; **h**,**l**,**n**) littermates treated with doxycycline from E0.5, harvested at E12.5 (**g**,**h**) or E13.5 (**k**–**n**) and subjected to *in situ* hybridization with digoxygenin-labeled probes for *Shh* (**g**,**h**,**k**,**l**) or *Bmp4* (**m**,**n**). Note absence or marked reduction of *Tg(Fos-lacZ)34Efu* activity or localized expression of *Shh* and *Bmp4* in *Dkk1*-expressing tongues. (**e**,**f**) X gal–stained E12.5 littermate control *Tg(Fos-lacZ)34Efu* (**e**) or *KRT14-Cre Ctnnb1*$^{fl/fl}$ (β-cat KO) *Tg(Fos-lacZ)34Efu* (**f**) tongues. Note the reduced number of *Tg(Fos-lacZ)34Efu*-expressing cell clusters in the mutant. (**i**,**j**) Control (**i**) and *KRT14-Cre Ctnnb1*$^{fl/fl}$ (**j**) E12.5 tongues subjected to *in situ* hybridization for *Shh*. Note the marked reduction in localized *Shh*-positive cell clusters in the mutant. (**o**,**p**) Anterior coronal paraffin sections of tongues photographed in **c**,**d**, respectively, counterstained with eosin. Note absence of *Tg(Fos-lacZ)34Efu* activity in *Dkk1*-expressing epithelium and absence of thickened placodal structures. (**q**,**r**) *In situ* hybridization for *Shh* (purple) with digoxygenin-labeled probe and posterior coronal paraffin sections of E12.5 tongues from control (**q**) and *KRT5-rtTA tetO-Dkk1* (*Dkk1* DTG) (**r**) littermates treated with doxycycline from E0.5. Note *Shh* expression in placodes in the control and absence of *Shh* expression and placodes in *Dkk1*-expressing tongue.

buds[21], suggesting that the cell clusters are taste bud precursors (**Fig. 3q**,**r**,**u**,**v**). Gustducin, a specific marker for one class of taste bud cells, is not normally expressed in fungiform papillae before birth[22] (**Fig. 3y**). However, we detected weak immunofluorescence for gustducin in apical cell clusters in mutant fungiform papillae (**Fig. 3z**), further identifying these as developing taste buds and suggesting that taste bud formation is accelerated in the mutant. Specific localization of cytoplasmic and nuclear β-catenin protein in mutant taste buds suggests that additional levels of regulation are important in patterning the fungiform papillae. These data indicate that enhanced stimulation of Wnt-β-catenin signaling promotes the development of fungiform papillae and taste buds.

To determine whether Wnt-β-catenin signaling is required for initiation of fungiform papilla morphogenesis, we used inducible transgenic *KRT5-rtTA tetO-Dkk1* mice, in which expression of *Dkk1*, a secreted Wnt antagonist capable of blocking signaling via LRP coreceptors and β-catenin[4], can be induced by doxycycline in cells in which the *KRT5* promoter is active[6] (**Fig. 4**). Sites of *KRT5* and *KRT14* promoter activity are essentially identical[6,19] (**Supplementary Fig. 2**). Wnt-β-catenin signaling activity, assayed by *Tg(Fos-lacZ)34Efu* expression, was completely or almost completely blocked in *Dkk1*-expressing tongues at E12.5 ($n = 8$ *Dkk1*-expressing transgenics, $n = 9$ controls) (**Fig. 4c**,**d**) and E13.5 ($n = 8$ *Dkk1*-expressing transgenics, $n = 8$ controls) (**Supplementary Fig. 4** online). Sectioning of control and *Dkk1*-expressing *Tg(Fos-lacZ)34Efu* tongues demonstrated an absence of thickened placode structures in *Dkk1*-expressing samples

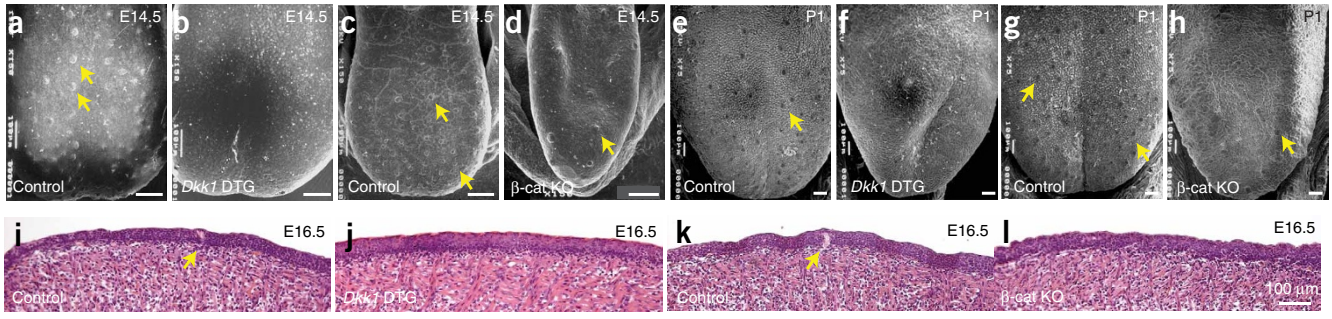

Figure 5 Wnt-β-catenin signaling is required for the development of fungiform papillae. (**a**,**b**,**e**,**f**) Scanning electron microscopic images of tongues from control littermate (**a**,**e**) or *KRT5-rtTA tetO-Dkk1* (*Dkk1* double transgenic (DTG)) (**b**,**f**) embryos treated with doxycycline from E0.5 and sacrificed at E14.5 (**a**,**b**) or birth (**e**,**f**). Note absence of fungiform papillae (arrows) in samples from *Dkk1*-expressing embryos. (**c**,**d**,**g**,**h**) Scanning electron microscopic images of tongues from control littermate (**c**,**g**) or *KRT14-Cre Ctnnb1*$^{fl/fl}$ (β-cat KO) (**d**,**h**) mice at E14.5 (**c**,**d**) and birth (**g**,**h**). Note the marked reduction in numbers of fungiform papillae (arrows) at E14.5 and birth in the mutants. (**i**,**j**) Hematoxylin and eosin–stained parasagittal tongue sections from E16.5 control (**i**) and *KRT5-rtTA tetO-Dkk1* (**j**) embryos treated with doxycycline from E0.5 showing absence of fungiform papillae (arrow) in the mutant. (**k**,**l**) Hematoxylin and eosin–stained parasagittal tongue sections from E16.5 control (**k**) and *KRT14-Cre Ctnnb1*$^{fl/fl}$ (**l**) embryos showing absence of fungiform papillae (arrow) in the mutant. Size bars in **a**–**h** represent 100 μm; size bar in **l** applies to **i**–**l**.

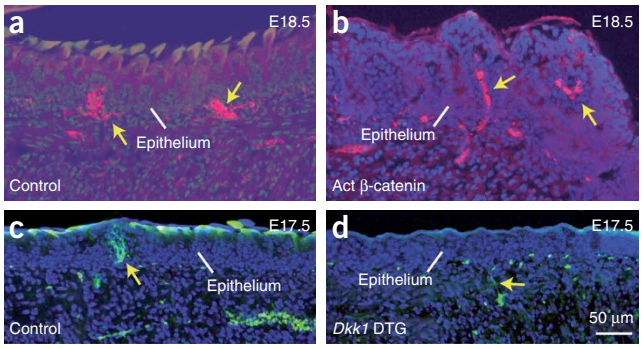

Figure 6 Wnt-β-catenin signaling controls tongue epithelial innervation. (**a**,**b**) Parasagittal sections of anterior E18.5 control littermate (**a**) and *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ (act β-catenin) (**b**) tongues subjected to immunofluorescence with anti-PGP9.5 (red). Note nerve fibers in control and mutant fungiform papillae (arrows). (**c**,**d**) Parasagittal sections of anterior E17.5 tongues from littermate control (**c**) and *KRT5-rtTA tetO-Dkk1* (**d**) embryos treated with doxycycline from E0.5 and subjected to immunofluorescence with anti-PGP9.5 (green). Note innervation within the epithelium in the control but not the *Dkk1*-expressing sample. Superficial staining in the control is background immunofluorescence from keratinized filiform papillae. Nuclei are counterstained with DAPI (blue). Size bar in **d** applies to **a**–**d**.

(**Fig. 4o**,**p**). Expression of the placode markers *Shh* and *Bmp4* was inhibited in *Dkk1*-expressing tongues ($n = 7$ controls, $n = 6$ *Dkk1*-expressing transgenics probed for *Shh* at E12.5; $n = 7$ controls and $n = 7$ *Dkk1*-expressing transgenics probed for *Shh* at E13.5; $n = 4$ controls and $n = 4$ *Dkk1*-expressing transgenics probed for *Bmp4* at E13.5) (**Fig. 4g**,**h**,**k**–**n**,**q**,**r** and **Supplementary Fig. 4**). Expression of the Shh receptor PTCH1 in placodal and underlying mesenchymal cells was downregulated in *Dkk1*-expressing samples at E13.5 and E14.5 (**Supplementary Fig. 4** and data not shown). Thus, Wnt-β-catenin signaling is required for expression of molecular markers of fungiform placode development.

Tg(Fos-lacZ)34Efu expression patterns (**Fig. 1**) suggested that Wnt-β-catenin signaling activity is confined to the epithelium during fungiform papilla development. To determine whether Wnt-β-catenin signaling is required specifically within epithelial cells, we used *KRT14* promoter-driven expression of Cre recombinase to delete the cell-autonomous Wnt effector gene β-catenin in tongue epithelium (**Fig. 4**). We crossed *KRT14-Cre* line 43 mice[18] to mice carrying a conditional allele of β-catenin (*Ctnnb1*^{fl}) that, when recombined, produces a nonfunctional allele[23]. Immunofluorescence for β-catenin showed that in *KRT14-Cre Ctnnb1*$^{fl/fl}$ embryos, β-catenin was deleted in large stretches of tongue epithelium at E12.5 but was not entirely absent, suggesting mosaic deletion at this stage (**Fig. 4a**,**b** and data not shown). *Tg(Fos-lacZ)34Efu* activity was markedly reduced in *KRT14-Cre Ctnnb1*$^{fl/fl}$ *Tg(Fos-lacZ)34Efu* compared with control *Tg(Fos-lacZ)34Efu* littermate tongues ($n = 7$ controls and $n = 4$ mutants at E12.5; $n = 8$ controls and $n = 5$ mutants at E13.5) (**Fig. 4e**,**f** and **Supplementary Fig. 4**). Fewer *Shh*-expressing cell clusters were present in mutant tongues, and the hybridization intensity of those remaining was reduced compared with controls ($n = 6$ controls and $n = 4$ mutants hybridized with *Shh* at E12.5; $n = 6$ controls and $n = 5$ mutants hybridized with *Shh* at E13.5) (**Fig. 4i**,**j** and data not shown). Thus, β-catenin is required within the epithelium for initiation of fungiform placode marker expression. The partial phenotypes are consistent with observed mosaic β-catenin deletion at E12.5.

Absent or reduced expression of molecular markers for fungiform placodes in epithelium ectopically expressing *Dkk1* or lacking β-catenin suggested that fungiform papilla morphogenesis would be disrupted in these mutants. In support of this, scanning electron microscopy and histological analysis carried out between E14.5 and birth demonstrated an absence or marked reduction of fungiform papilla morphogenesis in both types of mutants (**Fig. 5a–l**).

These data indicate a major role for Wnt-β-catenin signaling in initiating fungiform papilla morphogenesis and are consistent with reports that fungiform papilla patterning does not require epithelial innervation and is established before epithelial innervation[24–26]. Circumstantial evidence suggests that taste epithelium may direct its own innervation, as nerves that innervate taste papillae appear to grow directly to the papillae[24,25]. Consistent with this, ectopic and enlarged fungiform papillae in stabilized β-catenin mutant tongues were innervated (**Fig. 6a**,**b**), resulting in increased density of epithelial innervation in mutant tongue (21 ± 1 (mean ± s.d.) *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ taste papillae per parasagittal tongue section compared with 4.0 ± 0.8 control taste papillae per parasagittal section showing positive staining for the pan-neural marker PGP9.5; $n = 4$ independent sections analyzed each for control and mutant; $P = 0.00000347$). Conversely, epithelial innervation was absent in *Dkk1*-expressing tongue epithelium at E17.5 ($n = 5$ parasagittal sections analyzed each for control and mutant) (**Fig. 6c**,**d**). As β-catenin acts cell autonomously, increased innervation in *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ tongue epithelium indicates that Wnt-β-catenin pathway activation influences epithelial innervation indirectly. Therefore, our data suggest that localized Wnt-β-catenin signaling induces subsequent signals that emanate from the developing papillae and taste buds and are necessary for initiating or maintaining epithelial innervation.

Although taste papillae possess characteristics of both epithelial and neural cells, we demonstrate here that their formation uses a signaling pathway that has key roles in the induction of other epidermal appendages. Initial broad activation of Wnt signaling is consistent with an early role in placode induction[27] and suggests importance of broadly expressed Wnt ligands at early developmental stages. Wnt activity subsequently becomes upregulated in fungiform placodes and then in taste buds within fungiform papillae. Wnt signaling is downregulated in adjacent epithelium and is required for expression of *Bmp4*, a secreted placode inhibitory factor[8]. These observations suggest a model for fungiform placode development similar to those proposed for feather bud and hair follicle morphogenesis[5,13,27], in which secreted positive and inhibitory factors compete to establish an array of fungiform placode and interplacode regions. In this model, stimulation of Wnt pathway activity by LiCl or stabilized β-catenin tips the balance of placode-promoting and inhibitory factors towards placode fate, whereas its suppression allows adoption of a default interplacode fate. Wnt-β-catenin signaling appears to be confined to the epithelium throughout early stages of fungiform papilla morphogenesis and taste bud formation, consistent with data demonstrating epithelium intrinsic regulation of taste bud development in amphibians[28,29]. Our data further suggest that, during evolution, taste organ number and distribution could be driven by altered regulation of Wnt-β-catenin pathway activity.

METHODS

Mouse lines and genotyping. For mutation of the β-catenin gene to a stabilized form in epithelial cells, *Ctnnb1*$^{(Ex3)fl/+}$ mice[17] were crossed to *KRT14-Cre* line 43 transgenic mice[18]. To determine the efficiency of *KRT14-Cre*–mediated deletion in tongue epithelium, *KRT14-Cre* mice were crossed with *ROSA26R* Cre reporter mice[20] (The Jackson Laboratory). For generation of *Dkk1*-expressing and control littermates, heterozygous *tetO-Dkk1* transgenic mice were crossed with heterozygous *KRT5-rtTA* mice, and pregnant females

were placed on chow formulated with 1 g doxycycline per kg body weight (BioServ) immediately after observation of a copulation plug[6]. For generation of an epidermal-specific β-catenin knockout, *Ctnnb1*$^{fl/fl}$ mice[23] (The Jackson Laboratory) were crossed to *KRT14-Cre* line 43 transgenic mice[18]. *Tg(Fos-lacZ)34Efu* Wnt reporter mice[15] were a gift from E. Fuchs (Rockefeller University) and were also obtained from The Jackson Laboratory. To assess the efficiency of Wnt-β-catenin pathway inhibition in *Dkk1*-expressing mice and β-catenin mutants, *Tg(Fos-lacZ)34Efu* mice were crossed with *KRT5-rtTA tetO-Dkk1* mice or *KRT14-Cre Ctnnb1*$^{fl/+}$ mice over several generations to obtain *KRT5-rtTA tetO-Dkk1 Tg(Fos-lacZ)34Efu* mice and control littermates and *KRT14-Cre Ctnnb1*$^{fl/fl}$ *Tg(Fos-lacZ)34Efu* mice and control littermates. Mice were genotyped by PCR analysis of tail biopsy DNA using primers listed in **Supplementary Table 1** online. All experiments were performed with approved animal protocols according to the institutional guidelines established by the University of Pennsylvania, the University of Colorado Health Sciences Center and the University of Washington Institutional Animal Care and Use Committees. *Ctnnb1*$^{(Ex3)fl}$ mice were obtained under a Material Transfer agreement between Kyoto University and the University of Pennsylvania.

Analysis of *Tg(Fos-lacZ)34Efu* expression. *Tg(Fos-lacZ)34Efu* mice[15] were maintained on an FVB/N background. Noon of the day of vaginal plug appearance was designated E0.5. Embryonic stages were confirmed by analysis of limb and tongue morphology. Embryonic tongues were dissected, fixed in 4% paraformaldehyde, stained with X-gal[6], photographed, and frozen or paraffin-embedded, sectioned and counterstained with eosin or Hoechst 33258 (Molecular Probes). Tongues from E18.5 embryos were first sectioned and then reacted with X-gal.

Scanning electron microscopy. Tongues were dissected from E14.5 embryos and newborn mice and fixed overnight at 4 °C in 4% paraformaldehyde in PBS. Tissue was washed in distilled water, dehydrated in graded ethanols, transferred to freon overnight and air dried. Tongues were sputter coated with gold to approximately 100-Å thickness and viewed using a JEOL 330 scanning electron microscope.

Histology, immunofluorescence and *in situ* hybridization. Tissue preparation and histology were as described previously[18]. For immunofluorescence staining and immunohistochemistry, paraffin or cryostat sections or whole-mounted tongues were incubated with the following antibodies: primary monoclonal antibody that recognizes β-catenin encoded by both wild-type and exon 3–deleted alleles[30] (Sigma) as described previously[18]; rabbit anti-gustducin (Santa Cruz) (1:1,000); anti-Ptch1 (Santa Cruz) (1:100); and the pan-neural antibody to ubiquitin carboxyl terminal hydrolase (anti-PGP9.5) (Abcam) (1:25). Sections were mounted with Vectashield containing 4,6-diamidino-2-phenylindole (DAPI) or Hoechst dye (Vector Laboratories) or were counterstained with hematoxylin. We performed whole-mount and on-slide *in situ* hybridizations with digoxygenin-labeled or ^{35}S-labeled probes as previously described[5,6]. *Wnt10a*, *Wnt10b*, *Wnt4*, *Bmp4*, *Ctnnb1* and *Shh* probes were as described previously[5,6].

Organ culture experiments. Tongue primordia were dissected from E11.5–E12.0 embryos in L15 culture medium. Each explant was placed in a drop of medium on a Nucleopore filter (8 μm) and incubated in DMEM/10% FBS at the liquid-air interface in an airtight chamber filled with 5% CO_2/3% O_2. Cultures were maintained for 3 h or 3 d with daily changes of medium and gas, followed by fixation and X-gal staining or whole-mount *in situ* hybridization. Some explants were exposed to 25 mM LiCl for 1 or 3 h on day 1 of culture to hyperactivate the Wnt-β-catenin pathway[16].

Statistical analyses. We used a two-tailed Student's *t*-test to calculate statistical significance for measurements of mean fungiform placode diameter in *ex vivo* experiments, number and dimensions of fungiform papillae, and density of PGP9.5-positive nerve fibers. Significance of altered numbers of *Shh*-positive cell clusters in *ex vivo* experiments was calculated by ANOVA. Significance of the altered size class distribution of *Shh*-expressing cell clusters was determined using a nonparametric Sign test.

Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

The study was designed by F.L., S.T., N.M.G., L.A.B. and S.E.M. *In situ* hybridizations were carried out by F.L., S.T. and S.T.R. Immunofluorescence staining was carried out by F.L., S.H.Y. and S.T. X-gal staining was performed by F.L., S.T. and C.L.S.-C. Organ culture experiments were performed by S.T. *Ctnnb1*$^{(Ex3)fl}$ mice were provided by M.M.T. *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ mice were generated by F.L., S.T., S.H.Y. and A.A.D. *KRT5-rtTA tetO-Dkk1* mice were generated by F.L. and T.A. *KRT14-Cre Ctnnb1*$^{fl/fl}$ mice were generated by N.M.G. and F.L. F.L. and N.M.G. performed scanning electron microscopy analyses. R.T.M. was involved in study design and discussion of results. The manuscript was written by F.L., R.T.M., L.A.B. and S.E.M.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

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