Patched1 is required in neural crest cells for the prevention of orofacial clefts

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Defects such as cleft lip with or without cleft palate (CL/P) are among the most common craniofacial birth defects in humans. In many cases, the underlying molecular and cellular mechanisms that result in these debilitating anomalies remain largely unknown. Perturbed hedgehog (HH) signalling plays a major role in craniofacial development, and mutations in a number of pathway constituents underlie craniofacial disease. In particular, mutations in the gene encoding the major HH receptor and negative regulator, patched1 (PTCH1), are associated with both sporadic and familial forms of clefting, yet relatively little is known about how PTCH1 functions during craniofacial morphogenesis. To address this, we analysed the consequences of conditional loss of Ptch1 in mouse neural crest cell-derived facial mesenchyme. Using scanning electron microscopy (SEM) and live imaging of explanted facial primordia, we captured defective nasal pit invagination and CL in mouse embryos conditionally lacking Ptch1. Our analysis demonstrates interactions between HH and FGF signalling in the development of the upper lip, and reveals cell-autonomous and non-autonomous roles mediated by Ptch1. In particular, we show that deletion of Ptch1 in the facial mesenchyme alters cell morphology, specifically in the invaginating nasal pit epithelium. These findings highlight a critical link between the neural crest cells and olfactory epithelium in directing the morphogenesis of the mammalian lip and nose primordia. Importantly, these interactions are critically dependent on Ptch1 function for the prevention of orofacial clefts.

INTRODUCTION

The craniofacial complex is one of the most commonly affected structures in human congenital disease (1). Abnormal development of the head and neck can result in some of the most disfiguring and debilitating defects, and are a significant cause of infant mortality (2). Cleft lip and/or palate (CL/P) is among the most commonly observed craniofacial deformity in humans, with an estimated frequency of 1/700 live births. CL/P can occur sporadically as an isolated entity with complex underlying genetics, or as part of up to 500 distinct genetic syndromes (3). Despite this prevalence in human disease, the signalling pathways and downstream cellular mechanisms that co-ordinate the complex morphogenic events dictating lip and palate development remain poorly understood.

Hedgehog (HH) signalling plays a key role in the development of the craniofacial complex and is implicated in a number of craniofacial disorders (4). Mutations in the gene encoding the HH receptor and major negative regulator patched1 (PTCH1) cause Gorlin’s (or nevoid basal cell carcinoma) syndrome (5,6), which is characterized by predisposition to a range of tumours, in addition to developmental defects. These can include craniofacial anomalies such as macrocephaly, midfacial hypertelorism, broad nasal bridge and a high-arch secondary palate (7–9). A number of studies have also reported an increased incidence of CL/P in Gorlin’s syndrome patients (7,10), and variation at the PTCH1 locus has also been associated with sporadic CL/P (11). These findings highlight the importance of PTCH1 in orofacial disease, yet to date no previous study has directly addressed the role of Ptch1 in regulating lip and/or palate formation.

At the molecular level, Ptch1 functions in the negative regulation of HH signalling (12). In the absence of ligand, PTCH1 inhibits the transmembrane protein smoothened (SMO), the obligatory signalling molecule of the HH pathway (13). In this state, the bipotential GLI transcription factors are proteolytically 

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cleaved to form GLI repressors that inhibit target gene transcription (14), a process that is intricately regulated at the primary cilium (15,16). The major ligand of the HH pathway expressed in the developing craniofacial primordia is sonic hedgehog (SHH) (17). Upon binding of SHH to PTCH1, constitutive inhibition of SMO is relieved (13,18). This results in the formation of GLI transcriptional activators that direct the transcription of HH target genes in the nucleus. Two universal transcriptional targets of the HH pathway are Glil, a positive regulator of the pathway, and Ptch1 itself (12), providing important negative feedback mechanisms that help to buffer physiological levels of HH signalling. Thus, when Ptch1 function is lost, appropriate inhibition of SMO is no longer maintained, leading to constitutive activation of the HH pathway in a ligand-independent manner. Ubiquitous inactivation of Ptch1 in the mouse results in early embryonic lethality at ~9.5 days post coitus (dpc), reiterating the profound requirement for Ptch1 during embryogenesis (12,19).

To further understand the basis of craniofacial anomalies in Gorlin’s syndrome, we conditionally inactivated mouse Ptch1 in the major cell type that responds to HH signalling during facial development—the neural crest cells (NCCs) (17). Originating in the dorsal part of the neural tube, NCCs are a highly multipotent and migratory population of cells that ultimately contribute to the vast majority of craniofacial mesenchyme (20). Using the Wnt1Cre transgenic mouse line (21), Ptch1 inactivation in the facial mesenchyme resulted in a profound defect in nasal pit invagination, mid-facial widening and CL. While the Wnt1Cre;Ptch1c/c mutant embryos died prior to formation of the secondary palate, the defects observed in the primary palate are very likely to preclude correct development of this structure. Our molecular analysis suggests a negative interplay between HH signalling and the fibroblast growth factor (FGF) signalling pathway, which is known to regulate nasal pit invagination (22,23). Furthermore, we present evidence that PTCH1 in the underlying facial mesenchyme functions non-cell-autonomously in the regulation of epithelial cell shape, specifically in the invaginating nasal pit epithelium (NPE). We identify a number of defects in the cellular architecture of the Wnt1Cre;Ptch1c/c mutant mesenchyme and NPE that help to explain the cellular basis of clefting phenotypes.

**RESULTS**

**Loss of Ptch1 up-regulates HH signalling and causes craniofacial abnormalities**

To examine the requirement for Ptch1 specifically during facial development, Wnt1Cre mice were bred with a Ptch1 conditional mouse line (19) to obtain homozygous Wnt1Cre;Ptch1c/c mutant embryos. Resulting mutants were embryonic lethal at 12.0 dpc, after primary but before secondary palate development (Supplementary Material, Fig. S1). Heterozygous animals (Wnt1Cre;Ptch1+/c) were indistinguishable from controls that lack the Cre transgene.

HH pathway activity was examined by wholemount (Fig. 1A and B) and section (Fig. 1C and D) in situ hybridisation (ISH) for the universal downstream target gene, Glil. Relative to control embryos, homozygous Wnt1Cre;Ptch1c/c embryos displayed expanded Glil expression throughout the facial primordia at 10.5 dpc, indicative of ectopic HH signalling (Fig. 1B and D, black arrowheads). This expansion in signalling correlated with the presence of Cre-deleted NCCs, as indicated by lacZ lineage tracing, in both Wnt1Cre;Ptch1c/c;R26R (E) and homozygous Wnt1Cre;Ptch1c/c;R26R (F) embryos at 10.5 dpc confirms the presence of Cre-expressing cells in the facial primordia. md, mandible; mx, maxilla; pa2, second pharyngeal arch. Scale bars: 500 μm.

![Figure 1](https://academic.oup.com/hmg/article-abstract/22/24/5026/569096/5027?slug=fig1)
highlights grossly abnormal nasal pit invagination in the Wnt1Cre;Ptch1c/c mutant, and links defective invagination to the pathogenesis of CL.

Defective invagination observed at the placodal surface using whole explant live imaging

Having identified a nasal pit morphogenesis defect in the Wnt1Cre;Ptch1c/c embryos, we sought to determine the basis of the defective invagination. We took advantage of the accessibility of the nasal placodes to image the onset of invagination using a novel, ex vivo time-lapse imaging approach. In these experiments, the nasal placodes and adjacent facial prominences were isolated prior to invagination (10.0 dpc), and incubated with fluorescently conjugated wheat germ agglutinin (WGA) to visualize invagination (Supplementary Material, Fig. S2). In control explants, the nasal placodes rapidly began to invaginate (Supplementary Material, Movie S1). Extensive remodelling was observed at each control placode, as the surface reorganized and displayed cellular aggregates that appeared to be cells extruded from the epithelial layer. Consistent with these findings, previous SEM analysis of the chick lens, otic and nasal placodes during invagination revealed debris accumulation in the lumina (27). In sharp contrast, face explants from Wnt1Cre;Ptch1c/c embryos demonstrated reduced remodelling at the placodal surface and defective invagination (Supplementary Material, Movie S2). These findings suggest that the reorganization of the placodes in wild-type embryos coincides with the onset of nasal pit invagination, and likely contributes to the invagination process.

Wnt1Cre;Ptch1c/c embryos display defects in FGF signalling in the nasal processes

We next sought to determine the molecular and cellular mechanisms resulting in perturbed invagination in the Wnt1Cre;Ptch1c/c mutant nasal pit. Live imaging analyses suggested that the placodal epithelium plays an important role in the invagination process. Previous studies have shown that this specialized epithelium is a source of FGF signalling that is important for nasal pit invagination, with epithelial-specific deletion of Fgf8 or Fgfr1/2 with Foxg1Cre resulting in defective nasal pit invagination in mouse (22, 23). We, therefore, examined whether FGF signalling was altered in the Wnt1Cre;Ptch1c/c mutant. Wholemount ISH analysis of 10.5 dpc embryos confirmed that, relative to control NPE (Fig. 3A, red arrowhead), Fgf8 expression appeared reduced in Wnt1Cre;Ptch1c/c mutants (Fig. 3B, red arrowhead). Furthermore, the downstream FGF target Spry1 was also reduced in the mutant nasal pit and facial prominences when compared with control embryos.
PTCH1, the major negative regulator of HH signalling, plays an essential role in regulating signalling crosstalk between the HH and FGF pathways in the developing nasal pit, with effects in both the mesenchyme and overlying NPE.

**Wnt1Cre;Ptch1<sup>1/2c</sup> mutants display defects in mesenchymal cell density and epithelial cell death**

We next addressed whether cell proliferation and/or death contributed to the phenotype in the *Wnt1Cre;Ptch1<sup>1/2c</sup>* mutant embryos. To assess proliferation, we performed BrdU labelling of the frontonasal mesenchyme (FNM), the primordium that will give rise to the MNPs and LNP<sub>s following nasal pit invagination. We performed this analysis at 10.0 dpc because, unlike the differences observed in MNPs and LNP<sub>s at 10.5 dpc (Fig. 2F and G), the morphology of the mutant and control FNM at 10.0 dpc appears indistinguishable (Fig. 4A and B). This allows a more reliable comparison between control and *Wnt1Cre;Ptch1<sup>1/2c</sup>* mutant tissues just prior to the onset of the nasal pit phenotype. A relatively even distribution of proliferating cells was detected throughout the FNM in both control and *Wnt1Cre;Ptch1<sup>1/2c</sup>* embryos (Fig. 4A and B). Quantification of the percentage of proliferating cells indicated no significant difference between control and mutant sections (Fig. 4C), within a defined region of FNM (Fig. 4A and B, boxed region). However, we did detect a reduction in total cell number in this defined region in *Wnt1Cre;Ptch1<sup>1/2c</sup>* samples relative to controls (Fig. 4D), suggesting that cells were either individually larger or less closely packed in the mutant tissue. Consistent with the latter, histological analysis slightly later, at 10.5 dpc, revealed that, unlike control tissue which consisted of tightly packed mesenchymal cells surrounding the invaginated nasal pit (Fig. 4E, inset), mutant tissue sections displayed a looser network of mesenchymal cells throughout the MNP and LNP mesenchyme (Fig. 4F, inset). To determine whether ectopic apoptosis in the mutant mesenchyme contributed to the change in cell density observed, we performed cleaved caspase-3 (CASP-3) immunofluorescence (IF). While no detectable difference in the frequency or distribution of apoptotic cells was found in the mesenchyme at 9.5 or 10.5 dpc (data not shown), altered apoptosis was found in the overlying NPE in both wholemount (Fig. 3G and H) and sections (Fig. 3I and J) analysis. Unlike control samples, where a restricted band of apoptotic cells lined the fusing epithelia between the LNP and MNP (Fig. 4G and H, white arrowheads), mutant embryo samples demonstrated an ectopic domain of apoptotic cells extending throughout the NPE (Fig. 4H, white arrowheads), particularly evident in sections through posterior regions of the nasal pit (Fig. 4J, yellow arrowhead).

The data presented thus far suggest that *Wnt1Cre;Ptch1<sup>1/2c</sup>* mutants display altered signalling interactions that affect cell properties in both the NCC-derived facial mesenchyme and the overlying NPE. To further confirm that Wnt1Cre-mediated excision of *Ptch1* was in fact restricted to the NCC-derived facial mesenchyme, and not unexpectedly active in the NPE, we lineage traced *Wnt1Cre* expressing cells in 10.5 dpc *Wnt1Cre;Ptch1<sup>1/2c</sup>/R26<i>R</i> embryos (Fig. 5A–D). LacZ staining showed that, consistent with previous studies (30), *Wnt1Cre* expressing cells were almost exclusively associated with the NCC-derived mesenchyme at 10.5 dpc, in both anterior...
(Fig. 5A and B) and posterior (Fig. 5C and D) regions of the NPE. This confirms that the inactivation of \( Ptch1 \) is essentially confined to the mesenchyme, yet results in non-cell-autonomous effects on signalling and cell survival in the NPE.

**\( Ptch1 \) regulates cell shape in the NPE**

Based on the interplay between mesenchyme and NPE described so far, and on previous studies showing that the invagination of the otic placode (31) and optic cup (32) requires dynamic epithelial cell shape changes, we hypothesized that a similar mechanism was likely to occur during nasal pit invagination. Using confocal imaging of tissue sections, we examined a number of cell adhesion and cytoskeletal proteins in the NPE during invagination. As we had already confirmed that ectopic cell death was particularly evident in sections through the posterior NPE (Fig. 4J), anterior NPE sections were used for this analysis. While the general morphology of epithelial cells outside of the NPE (the region beyond the limits of \( Fgf8 \) expression) was similar between control and mutant sections (data not shown), a number of striking defects in epithelial cells were found specifically within the mutant NPE when compared with the invaginating NPE in controls (Fig. 5E–R). Z-stack maximum intensity projections of control tissue sections revealed the tightly condensed apical surface of the invaginating NPE stained with actin (Fig. 5E), whereas in sections from \( Wnt1Cre;Ptch1^{l/c} \) embryos, actin staining highlighted the broad and disorganized apical surface of the NPE (Fig. 5F). Single confocal slices through NPE sections counterstained with DAPI also revealed smaller, rounder nuclei in the \( Wnt1Cre;Ptch1^{l/c} \) NPE, compared with the elongated nuclei in sections from control embryos (Fig. 5G and H). Further IF analysis of p120 catenin (p120ctn), a critical regulator of adherens junctions (33), revealed staining tightly associated with the cell periphery in control NPE and highlighted the columnar morphology of these cells (Fig. 5I, K and M). In \( Wnt1Cre;Ptch1^{l/c} \) mutant NPE, p120ctn staining was more weak and diffuse, suggesting that junctional contacts were improperly formed in the mutant cells (Fig. 5J).

![Figure 4. Cell proliferation and death in the Wnt1Cre;Ptch1^{l/c} nasal processes.](https://academic.oup.com/hmg/article-abstract/22/24/5026/569096/5030)

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To determine whether the NPE retained epithelial identity and functional cell–cell junctions, we next examined E-Cadherin (E-CAD), which is bound by p120ctn (34). Compared with control E-CAD antibody staining at cell–cell junctions, staining within mutant NPE was disorganized at the junctions, as observed in Z-stack projections (Fig. 5O and P, compare white arrowheads). Co-labelling with the tight junction marker zonula occludens-1 (ZO1) revealed staining associated with the apical/luminal surface of the nasal pit in both control and \( Wnt1Cre;Ptch1^{l/c} \) embryos (Fig. 5O and P), and at the basal side of the tissue, laminin IF staining revealed that \( Wnt1Cre;Ptch1^{l/c} \) mutants maintained deposition of this basement membrane protein (Supplementary Material, Fig. S3).

These data indicate defects in cell adhesion and cell elongation within the mutant NPE. Likewise during both optic cup and otic placode morphogenesis, cell shape changes are essential for invagination to occur, and are dependent on the molecular motor protein myosin II (31,35). Consistent with this, analysis of the \( Wnt1Cre;Ptch1^{l/c} \) mutant revealed that, unlike in control epithelium, myosin IIa was detected throughout the NPE and failed to concentrate to the cell periphery (Fig. 5Q and R). Our findings demonstrate a pronounced defect in epithelial cell...
shape, and support a role for myosin IIa-driven contractile machinery in the invagination of the NPE.

DISCUSSION

It is well established that perturbed HH signalling can result in a spectrum of craniofacial anomalies in humans (36), many of which affect mid-facial width (25,26). By conditionally inactivating Ptc1 in the craniofacial mesenchyme, our study provides the first direct model of Ptc1 in the pathogenesis of craniofacial anomalies associated with Gorlin’s syndrome, an inherited disorder resulting from mutations in PTCH1 (5,6). Similar to findings in humans (7), the disregulation of Ptc1 in mouse causes mid-facial expansion and an early defect in the nasal pit that culminates in CL (Fig.2C and E). In addition, abnormalities of the secondary palate such as a high arched palate (9) and cleft palate (CP) (10) are also part of the clinical spectrum of Gorlin’s syndrome. Were the Wnt1Cre;Ptc1c/c embryos to survive until later in embryogenesis, it is extremely likely that they would also display defective palatogenesis, given the severity of the craniofacial phenotype observed just prior to secondary palate development (Fig. 2B), and the fact that CL is often a precursor to CP (37).

The present study focuses on the morphogenesis of the upper jaw primordia, to understand how facial clefts of the lip and nose arise. Our analysis of the Wnt1Cre;Ptc1c/c mutant demonstrates that strictly controlled Ptc1-mediated suppression of HH signalling in the facial mesenchyme is essential for correct invagination of the nasal pit. In mammals, this invagination ensures the proper formation of the primitive nose and upper lip, as it promotes the formation of the paired MN and LN processes that surround the nasal pit. In addition, these craniofacial primordia must also fuse with each other and the Mx, in the right time and space (Fig. 2D), as inappropriate growth and/or union can result in an array of clefting phenotypes. Despite the frequency of orofacial clefts in the human population, the morphological processes that contribute to the formation of the lip and nose, and indeed how these processes are regulated at a molecular level, have remained largely undescribed. Thus, the Wnt1Cre;Ptc1c/c mutant presented in this study offers a rare opportunity to examine the molecular and cellular mechanisms that give rise to defects in the lip and nose primordia that parallel anomalies underlying human orofacial clefting disease.
In addition, the Wnt1Cre;Ptc1c/c model provides one of the few, ligand-independent HH signalling mouse models for craniofacial disease. Other HH activation models have been described, but these predominantly result from manipulation of positive regulators of the pathway such as Shh (38) or Smo (17). While the resulting phenotypes show some overlap with the Wnt1Cre;Ptc1c/c mutant in terms of defects in mid-facial width, each of these models displays differences in overall morphology, and are generally characterized by less severe phenotypes than the Wnt1Cre;Ptc1c/c mutant. These differences clearly demonstrate that the way in which the HH pathway is manipulated, either through loss of negative inhibition or enhancement of positive regulators, critically impacts on the resulting craniofacial phenotype. Differences among these HH models are likely a reflection of subtle yet important changes in the timing, duration and level of HH activity across different genetic models, changes which are known to influence overall craniofacial morphology (26,39). It is therefore clear that a number of complex, regulatory mechanisms are required to fine-tune the level of HH signalling during normal craniofacial development, in a spatio-temporal manner. One such mechanism is through the presence of Ptc1, which actively restricts the HH signal and is indispensible for facial development both in humans (7,9) and in mouse (this study).

At the molecular level, our analysis further demonstrated that altered HH signalling results in downstream effects on FGF signalling during nasal pit development (Fig. 3B, D and F). These data are consistent with a previous link between reduced FGF signalling and impaired nasal pit invagination, with conditional loss of Fgfr1/2 (23) or Fgf8 from the NPE resulting in defective invagination (22,23). Similar to our findings, exogenous application of SHH beads in chick results in a down-regulation of Fgf8 in the NPE, and malformed nasal pits (40). In the current study, we present evidence for altered signalling downstream of FGF in the mesenchyme (Fig. 3D, F and H). This may be directly mediated through signalling events in the mesenchyme or via FGF signals emanating from the NPE, as previously demonstrated in chick (28). Although we cannot distinguish between these possibilities, our work suggests that molecular conservation exists between mouse and chick in the regulation of nasal pit morphogenesis, and places the HH pathway upstream of FGF in this context. Importantly, as with Ptc1 (11), genetic variation at the FGFRI, FGFR2 and FGF8 loci is each associated with sporadic CL/P in humans (41). Our work therefore highlights an important molecular link between HH and FGF, two major developmental signalling pathways that are implicated in the pathogenesis of orofacial clefts.

In addition to the signalling alterations in the Wnt1Cre;Ptc1c/c mutant, we also observed defects in cellular organization, in both the facial mesenchyme and overlying NPE. The mesenchyme was characterized by changes in cell packing (Fig. 4, compare E and F), resulting in a more loosely packed cellular network that may have contributed to the wider mid-facial morphology observed in mutant embryos (Fig. 2B and C). In addition, we found that these mesenchymal effects could not be explained by alterations in cell death or proliferation (Fig. 4C). In the limb (42) and lung (43), it has recently been demonstrated that the plane of cellular division can contribute to the morphogenic events required to shape an organ. In the lung, this is regulated by ERK1/2 signalling, in the absence of altered cell number, shape or size (43). Future studies should investigate any association between mitotic spindle orientation and nasal pit morphogenesis, especially in light of the changes in ERK signalling observed in the Wnt1Cre;Ptc1c/c mutant (Fig. 3H).

In addition to the cell-autonomous requirements for Ptc1 in the NCC-derived mesenchyme, both for the maintenance of cell signalling and cell packing, our work uncovered a novel, non-cell-autonomous requirement for Ptc1 in the maintenance of epithelial cell shape in the invaginating NPE. Our data suggest that the invagination process is dependent on cell elongation, and that Myosin IIa-driven contractility appears to be at least one mechanism driving this dynamic process. Indeed, live imaging analyses performed in this study revealed the dynamic behaviour of the control placodal epithelium, which appears to extrude cells during invagination, in contrast to the more static behaviour and defective invagination in the mutant tissue. These findings support previous observations in chick by SEM that suggested invaginating lens, otic and indeed nasal placodes each feature the presence of cellular debris at the placodal surface (27). Furthermore, they are fitting with an already known role for Myosin II in the process of cell extrusion in Drosophila (44).

Extensive mammalian cell culture studies have also shown that Myosin IIa-driven cell contractility is regulated by E-CAD (45). In agreement with this, our studies indicate a defect in E-CAD localization in the Wnt1Cre;Ptc1c/c mutant NPE, suggesting that a similar interaction between E-CAD and Myosin IIa exists in vivo during nasal pit invagination. Intriguingly, the defects in cell shape were specifically confined to the epithelium that fails to invaginate, rather than appearing as a global phenomenon across the ectodermal surface of Wnt1Cre;Ptc1c/c mutant face. As this invaginating epithelium is demarcated by Fgf8 expression in control embryos (Fig. 3A), and FGF signalling can regulate myosin-dependent cell shape changes during otic invagination (31), we reasoned that exogenous application of recombinant FGF8 protein to Wnt1Cre;Ptc1c/c mutant explant cultures may rescue the invagination defect. However, mutant face explants exposed to FGF8 as part of this study displayed only partial phenotypic rescue at very low frequencies (3/16; data not shown). It is likely that more complete, consistent rescue in these experiments would be dependent on the precise timing and dosage of FGF8 delivered to the NPE, possibly in combination with other FGFs expressed in the nasal processes (46). In addition, rescue would require resulting downstream signalling and cell biological changes to take effect within a very rapid timeframe, as the nasal placode begins invagination within hours of ex vivo growth in our experimental system. It is also possible that additional mechanisms may act in concert with FGF signalling to regulate the complex remodelling events that take place during nasal pit invagination. In Drosophila, mesoderm invagination can be triggered by mechanical induction, resulting in a redistribution of myosin II and the apical constriction of cells (47). Given the dependency of the NPE on the NCC-derived mesenchyme shown in this study, it is possible that the mesenchyme may provide additional mechanical cues that together with biochemical cues, help coordinate the invagination of the nasal pit.

In summary, our findings suggest that co-ordinated interaction between the facial mesenchyme and overlying epithelium is crucial to the process of nasal pit invagination. We show for the first time that loss of Ptc1 in mouse neural crest cells...
causes orofacial clefting, and illustrate the role of nasal pit invagination in the pathogenesis of CL. These data highlight the interconnected signalling networks and downstream cellular processes driving morphogenesis of the upper jaw, and potentially link changes in cell shape and cytoskeletal organization to clefting phenotypes in the human population.

MATERIALS AND METHODS

Mouse strains

Ptch1c/c mice (19) on a C57BL6/SV129 background were crossed to Wnt1Cre mice (21) on a C57BL6 background to obtain Wnt1Cre;Ptch1c/c mutants. For lineage tracing, Wnt1Cre;Ptch1c/c,R26R mutants were obtained by crossing the Ptch1c/c line onto the Rosa26R (R26R) lacZ reporter line (48), maintained on a C57BL6 background, and the Wnt1Cre line. PCR genotyping of mice was performed as previously described (19, 24, 48). All animal work was approved by the relevant University of Queensland animal ethics committee.

ISH analyses

Wholemount (49) and section (50) ISH was performed as previously described using stage-matched embryos. Gli1, Fgf8 and Spry1 probes are as described previously (24). The Pax7 probe (AK133693) encompassed nucleotides 1234–1770. Wholemount and section ISH was performed on a minimum of three control and three mutant embryos.

LacZ staining

Wholemount lacZ staining to detect Cre activity was performed as previously described (51) on three heterozygous and three homozygous mutants. After staining, embryos were fixed in 4% paraformaldehyde (PFA) in PBS, dehydrated and processed into paraffin wax; 8 μm sections of the whole head were collected and counterstained with eosin.

BrdU analysis

Proliferating cells were detected after 1 h labelling with bromo-2-deoxyuridine:5-fluoro-2-deoxyuridine (BrdU; Invitrogen) as previously described (24). To standardize the region analysed, a box was placed on images in approximately the same mesenchymal region in each section. Cells from a total of 15 mutant and 14 control sections were counted from stage-matched mutant (n = 3) and control (n = 3) nasal processes. The percentage of proliferating cells was determined by dividing the number of BrdU positive cells by the total number of haematoxylin-stained nuclei. The mean value for each control and mutant biological replicate was graphed showing the standard error of the mean (SEM). Statistical significance was assessed using Student’s unpaired t-test in Prism 5 (GraphPad).

IHC and IF

IHC was performed as previously described on three mutant and three control littermate, stage-matched embryos using the phospho-p44/42 MAPK (Erk1/2) antibody (Cell Signaling, #9101) (29). Section (52) and wholemount (53) IF was performed as previously described, on two control and mutant embryos for laminin and on a minimum of three control and mutant embryos for all other antibodies. Antibodies used for IF were as follows: CASP-3 (Cell Signaling); actin (Millipore); p120ctn (Becton Dickinson) E-CAD (34), ZO1 (Invitrogen) myosin Ila (Sigma) and laminin (Sigma). Rabbit or mouse Alexa Fluor 488, 594 and 647 were used as secondary antibodies. CASP-3 wholemount staining was captured on a ZEISS Axio Examiner Z1 LSM 710 laser scanning confocal microscope. All other IF images were captured on a Zeiss Axio Examiner Z1 LSM 710 laser scanning confocal microscope. For myosin Ila and ZO1, iso-surface rendering was performed in Imaris.

Scanning electron microscopy

SEM of embryonic heads was performed as previously described (53) on three control and mutant embryos at 10.5 and 11.5 dpc.

Quantification of mid-facial width

Heads from control (n = 6) and mutant (n = 6) embryos fixed in 4% PFA and stored in PBS at 4°C were imaged using bright-field microscopy in the frontal view. The distance between the nasal pits was measured using ImageJ software. Data were graphed and an unpaired t-test was performed using Prism 5 (GraphPad). Error bars represent the standard deviation.

Live imaging

The nasal processes and maxillae were isolated from three tail somite (ts) control (n = 3) and Wnt1Cre;Ptch1c/c (n = 3) embryos (Supplementary Material, Fig. S3A) and cultured as explants for 2–3 h (Supplementary Material, Fig. S3B) as previously described (54). Ts were counted from the posterior edge of the hindlimb to the end of the tail. For imaging, explants were rinsed in PBS and placed face down in a mattek dish immersed in CO2-independent medium (Invitrogen, number 18 045) supplemented with 1 mg/ml WGA conjugated to Alexa Fluor 647 (Invitrogen), 10% foetal bovine serum (Invitrogen), 25 units/ml of penicillin, 25 μg/ml streptomycin and 0.5 mM glutamine (Supplementary Material, Fig. S3C). Explants were placed in a 37°C light-protective chamber fitted to a Zeiss Axio Observer Z1 Inverted LSM 710 confocal microscope and imaged for ~10 h (×10 objective). Z-stacks were collected at 10-minute intervals, reconstructed into MIPs using Imaris and resulting movies were presented at 6 frames per second.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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REFERENCES


