Fuz Mutant Mice Reveal Shared Mechanisms between Ciliopathies and FGF-Related Syndromes

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SUMMARY

Ciliopathies are a broad class of human disorders with craniofacial dysmorphology as a common feature. Among these is high arched palate, a condition that affects speech and quality of life. Using the ciliopathic Fuz mutant mouse, we find that high arched palate does not, as commonly suggested, arise from mid-face hypoplasia. Rather, increased neural crest expands the maxillary primordia. In Fuz mutants, this phenotype stems from dysregulated Gli processing, which in turn results in excessive craniofacial Fgf8 gene expression. Accordingly, genetic reduction of Fgf8 ameliorates the maxillary phenotypes. Similar phenotypes result from mutation of oral-facial-digital syndrome 1 (Ofd1), suggesting that aberrant transcription of Fgf8 is a common feature of ciliopathies. High arched palate is also a prevalent feature of fibroblast growth factor (FGF) hyperactivation syndromes. Thus, our findings elucidate the etiology for a common craniofacial anomaly and identify links between two classes of human disease: FGF-hyperactivation syndromes and ciliopathies.

INTRODUCTION

Craniofacial dysmorphology is a common component of the ciliopathy disease spectrum, but while defective neural crest (NC) cell migration has been implicated in Bardet-Biedl Syndrome (BBS) (Tobin et al., 2008), little else is known about the underlying developmental processes in ciliopathy-associated craniofacial defects. Among the more consistent craniofacial phenotypes in ciliopathies is the presence of a high arched palate (Beales et al., 1999). In this condition, the palate is characterized by a pronounced median groove, but the roof of the mouth remains intact across the midline. This condition is often referred to as a “pseudo-cleft.” Later in life, a high arched palate is also associated with secondary dental anomalies, such as postnatal gingival swelling and crowding of the molars. These defects can impair speech and complicate intubation, a major concern for craniofacial patients who frequently require multiple surgeries during childhood. Thus, high arched palate has a significant impact on patients’ quality of life.

The etiology of high arched palate remains obscure. One long-held hypothesis proposes that the arch arises from a midface hypoplasia causing insufficient maxillary growth and subsequent compression of the upper dental arch (Hennekam et al., 2010; Kreiborg and Cohen, 1992; Slaney et al., 1996). However, recent morphometric analyses suggest that this may not be true (Martinez-Abadias et al., 2010). Although a chemically induced rat model has existed for several decades, the embryological progression of the phenotype is unknown (Lorente et al., 1981). Surprisingly, no genetic model of high arched palate has yet been reported, so the underlying cellular and anatomical causes remain unknown and developmental hypotheses are untested.

Clinical observations also complicate our picture of high arched palate, as this defect is associated with a variety of seemingly unrelated syndromes (Hayward et al., 2004; Hennekam et al., 2010; Kreiborg and Cohen, 1992; Vadiati Saberi and Shakoopour, 2011). For example, though rarely mentioned in the literature, high arched palate is a central feature in many ciliopathies, including BBS, oral-facial-digital syndromes (OFD types I and IV), Joubert syndrome, and Sensenbrenner syndrome (Beales et al., 1999; Hennekam et al., 2010; Moore et al., 2005; Prattichizzo et al., 2008; Sensenbrenner et al., 1975; Somlo et al., 1993). In fact, 88% of BBS patients present with high arched palate, and many also display associated dental crowding and soft tissue swellings (Beales et al., 1999). High arched palate is also consistently observed in an array of syndromes characterized by dysregulation of fibroblast growth factor (FGF) signaling or downstream components such as the Ras kinase (Goodwin et al., 2012). Examples include Apert, Crouzon, Muenke, and cardio-facio-cutaneous syndromes (Agochukwu et al., 2012).
et al., 2012; Berkowitz, 1971; Hennekam et al., 2010; Kreiborg and Cohen, 1992; Letra et al., 2007; Rynearson, 2000; Vadiati Saberi and Shakoorpour, 2011). This finding is curious, because while FGF signals have been implicated in controlling cillum length (Hong and Dawid, 2009; Neugebauer et al., 2009), there has been no evidence to date suggesting a link between FGF dysregulation syndromes and the ciliopathies.

To characterize ciliopathic craniofacial defects, we examined mice with a mutation in the gene encoding Fuzzy (Fuz), which has recently emerged as a key regulator of ciliogenesis (Gray et al., 2009; Park et al., 2006). Initially described as a Drosophila planar cell polarity effector gene (Collier and Gubb, 1997), studies in both Xenopus and mice identified Fuz as a central regulator of vertebrate ciliogenesis (Brooks and Wallingford, 2012; Gray et al., 2009; Park et al., 2006). Consistent with the key role of cilia in Hedgehog (Hh) signaling, disruption of Fuz affects Hedgehog-dependent patterning events in both frogs and mice (Gray et al., 2009; Park et al., 2006), and in mice, loss of Fuz has been shown directly to disrupt the processing of Gli3 (Heydeck et al., 2009). Recently, live imaging analyses revealed that Fuz is essential for normal trafficking of the retrograde intraflagellar transport (IFT) machinery in vertebrate cilia; when Fuz is depleted, localization of IFT-A proteins, such as IFT43, is disrupted (Brooks and Wallingford, 2012). As a consequence, IFT trains become stalled, leading to shortened cilia and impaired signal transduction. Finally, Fuz is of particular interest because it is mutated in human patients with birth defects (Seo et al., 2011) and because Fuz mutant mice display a variety of craniofacial phenotypes (Zhang et al., 2011).

Here, we present the Fuz mutant mouse as a useful genetic model for the study of high arched palate. Although current hypotheses suggest that high arched palate arises from constriction of the upper jaw (Hennekam et al., 2010), our data demonstrate that the primary cause of ciliopathic high arched palate is instead excessive NC producing an enlarged first branchial arch (BA1) and maxillary hyperplasia early in embryogenesis. We have also discovered a surprising mechanistic basis for this phenotype, as we observed a dramatic increase in FGF signaling due to increased cranial Fgf8 gene expression. Genetic reduction of Fgf8 rescues the maxillary defects in Fuz mutant mice. Finally, we observed a similar maxillary expansion and upregulation of Fgf8 expression in another ciliopathy mouse model, OFD-1 (Ferrante et al., 2006). Thus, we have identified dysregulation of FGF function as the cause of facial defects in ciliopathic mutant mice, demonstrating etiological commonalities between two broad categories of human congenital anomalies: the ciliopathies and the FGF-related syndromes.

RESULTS

Fuz Mutant Mice Are a Model of High Arched Palate
Fuz mutant mice appear to have a cleft secondary palate (Figures 1A and 1B); however, frontal sections revealed that the palatal shelves were, in fact, not clefted (Figures 1C–1D). Instead, in all mutants analyzed, the palatine bones displayed the classic inverted-V shape typical of a high arched palate (Figures 1D–1D). Palatal narrowing and palatine bone defects were observed throughout the anterior-posterior extent of the secondary palate in mutants (Figure S1 available online). We also observed expanded mesenchyme within the oral cavity in Fuz mutant mice (Figure 1D, “P” in light blue area; Figure 1D’, gray area). Thus, the maxillary phenotypes of Fuz mutant mice bear a striking similarity to the high arched palate reported for human ciliopathy patients (Beales et al., 1999; Hennekam et al., 2010; Tagliani et al., 2010).
The embryological events leading to high arched palate have not been previously described, so we compared maxillary development in a staged series of wild-type and Fuz<sup>-/-</sup> littermates. In controls, the palatal shelves developed bilaterally, growing and extending ventrally into the oral cavity at E13.5 (Figures 1E and 1E’). The palatal primordia were evident at the appropriate stage in Fuz mutants but were displaced medially compared to littermates and did not extend ventrally (Figures 1H and 1H’, outlined in gray). At E14.5, palatal condensations in controls remained bilateral and were fully extended, flanking the tongue (Figures 1F–1F’), with the palate fusing by E16.5 (Figures 1G and 1G’). In mutants, however, the palatal condensations did not extend and instead appeared as one contiguous domain (Figures 1I–1J). This medial shift of the palatal shelves was also evident from Patched1 (Ptc1) expression, which spans the midline (Figure S1A and S1B). Likewise, ossification of palatine bones was apparent at E17.5 in controls, and in Fuz mutants the palatine bones were displaced medially (Figures 1D and 1D’). Furthermore, analysis by microcomputed tomography (μCT) revealed that the majority of midfacial bones are present and ossifying. Though small and constrained, the mutant palatine bones are roughly normal in shape (Figures S1M–S1M’). These data raised the intriguing possibility that a recognizable palate can form despite an initial failure of shelf outgrowth (Figures 1H and 1H’).

**Fuz Mutant Mice Displayed Enlarged Maxillary Processes**

When examined at earlier stages, we found that a larger maxillary process was evident in Fuz mutants as early as E9.0 (Figures 2A and 2B); by E9.5 the maxillary domain is substantially larger (compare Figures 2C and 2E to Figures 2D and 2F). Cell numbers were significantly increased in the maxilla, but there was no coincident increase in mitotic cells or change in apoptosis (Figures 2G and 2H; data not shown). Overall, this developmental progression is strikingly divergent from that underlying traditional cleft palatates (Chai and Maxson, 2006), suggesting that the high arched palate, though commonly referred to as a “pseudo-cleft,” is unrelated to cleft palate and arises by a distinct developmental mechanism.

**Disruption of Fuz Leads to Excessive NC**

We next sought to understand the molecular basis of the maxillary hyperplasia in Fuz mutant mice. Fuz controls ciliogenesis, and cilia are central to proteolytic cleavage of Gli effector proteins that transduce Hedgehog signals (Goetz and Anderson, 2010; Liu et al., 2005; Singla and Reiter, 2006). Indeed, loss of Fuz leads to defective Hedgehog signaling in the spinal cord and limb (Gray et al., 2009; Heydeck et al., 2009; Park et al., 2006), and Heydeck et al. showed that, during patterning of the limb, Fuz mutation leads to aberrant proteolytic processing of Gli3 (Heydeck et al., 2009).

However, changes in palatal Hh signaling could not account for the observed craniofacial phenotypes, as the Hh target gene Ptc1 continues to be expressed in Fuz mutant palatal primordia at E14.5 (Figures S1A–S1B’), and unlike the Fuz mutation, loss or gain of Hh activation in the palate leads to a true cleft rather than an arched palate (Cobourne et al., 2009; Gritti-Linde et al., 2007; Hu and Helms, 1999; Lan and Jiang, 2009; Mo et al., 1997; Rice et al., 2004). Furthermore, Gli3 mutation also leads to sporadic cleft palate due to obstruction by the tongue (Huang et al., 2008). These data suggest that, if a Gli processing defect is involved, it must occur prior to palate formation.

**Loss of Fuz Leads to Disorganized NC Migration**

In addition to the significant increase in NC cell numbers, the migration of the NC was disrupted in Fuz mutant mice. For example, we frequently observed a large proportion of rostral crest cells collecting ectopically in the optic cup regions (Figure 2B, arrowhead). To examine this at a cellular level, we utilized membrane-bound GFP in Wnt1-cre; R26<sup>mt/mG</sup> embryos. Strikingly, three-dimensional confocal imaging revealed that the depth of the migratory streams was far greater in Fuz mutants (79 ± 16 μm deep in mutants versus 41 ± 20 μm deep in wild-type embryos; Figures 2K and 2L). This was surprising, as mesencephalic NC cells should remain adjacent to the epidermal ectoderm, avoiding the underlying mesenchyme (Noden, 1975). Furthermore, we noted cells rostral to the trigeminal ganglion in a region that should ordinarily be clear of NC cells (compare Figure 2I to Figure 2J’).

We had previously shown that morpholino oligonucleotides (MOs) were an effective means to block Fuz function in Xenopus (Park et al., 2006). When we examined expression of Twist, a migratory NC marker, following Fuz knockdown, we observed aberrant migration of the anterior cranial crest (Figures 2M and 2N, arrowheads), consistent with the observed craniofacial defects (Park et al., 2008). In many cases, we noted increased and ectopic migration of the anterior NC into the eye field (Figure 2N, black arrowhead and inset). Together, these data suggest that Fuz plays an evolutionarily conserved role in controlling NC cell contributions to BA1.

**Disruption of Cranial Hedgehog Signaling at Early Stages in Fuz Mutant Mice**

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Figure 2. Increase in NC in Maxillary Compartment

Wnt1-cre-driven LacZ (blue) or GFP (green) marks NC contributions.
(A and B) Lateral views of E9.25 embryos. BA1 and BA2 NC streams are wider compared to controls (B compared to A, yellow bracket). Increased NC disrupts the optic cup (OC) in Fuz mutants compared to controls (B compared to A, arrowhead). BA2 NC stream is also increased in size and has failed to migrate as far as BA2 control NC. Mx, maxillary compartment of BA1. OV, optic vesicle.
(A’ and B’) Dorsal views. Maxillary BA1 is enlarged compared to controls (B compared to A, top arrowhead). BA2 has failed to migrate sufficiently compared to controls (bottom arrowhead).
(C and D) PH3 staining (green) and DAPI (blue) of E9.0, maxillary compartment. Mutant maxilla is enlarged (white dotted line). Epithelial membrane-Tomato (red) highlights all other tissue derivatives. Mutant maxillae are larger compared to controls. Enlarged maxillae comprise NC-derived mesenchyme.
(G) Quantification of DAPI-positive cells from representative sections of E9.5 and 10.5 embryos. Note increase in cell number in mutant (red) maxilla compared to wild-type (blue) (p < 0.004). Error bars indicate SD.
(H) Quantification of E9.5 maxillary PH3-positive cells compared to total cell number. A significant decrease in the percentage of PH3-positive cells is observed in mutant maxillae (red) compared to controls (blue) (p < 0.01). Error bars indicate SD.
(I and J) Single confocal z-sections of Wnt1-cre; R26RmT/mG;Fuz+/+ or Wnt1-cre; R26RmT/mG;Fuz−/− E9.5 embryos. Wnt1-cre-driven membrane-GFP (green) marks NC contributions.
(I’–J’) Magnified maximum projections of (I) and (J), indicated by a white dotted box. In controls, chains of Wnt1-cre-driven membrane-GFP-positive NC are observed (yellow arrowheads), with few isolated cells between brain and maxillary compartment (white arrowheads). Isolated NC cells are observed in mutant embryos, indicated by a white arrowhead in (J’), and NC chains are disorganized.

(legend continued on next page)
As it happens, Gli3 plays a crucial role in patterning the tissues that give rise to NC destined for BA1 (Blaess et al., 2006). Therefore, we assessed Hh signaling in the cranial regions of E9.0 embryos. We found a dramatic increase of full-length Gli3 in our mutants and a concurrent decrease in the short repressor form of Gli3 (Figure 3A). We also observed a reduction of Hh target gene expression in cranial tissues (Figure 3B), which may contribute to the observed NC defect.

**Expanded FGF Expression in Fuz Mutants**

We then considered molecular changes downstream of the early Gli processing defects in Fuz−/− mice. One possibility was that

(K and L) Z-projections of (I′) and (J′). The thickness of NC streams anterior to the prospective trigeminal ganglion is increased in mutants (WT = 41 ± 20 μm thick; mutant = 79 ± 16 μm deep). The immediately underlying membrane-Tomato-positive mesenchymal cells are shown in red, at the bottom of the image. The overlying epithelium is not included. Scale bar, 100 μm. This doubling in thickness is consistent with increased NC invasion into BA1.

(M and N) Twist in situ hybridization of stage 22 embryos injected unilaterally with Fuz MO. In controls, Twist is expressed in three streams, where the anterior NC stream surrounds the optic placode, indicated in (M) by open arrowhead and inset (bracket). Ectopic Twist expression is increased in mutant maxillae (yellow arrowhead).

(G and H) Lateral views of Erm1 mRNA expression in E9.5 embryos. Erm1 is expressed in the MHB, frontonasal prominence (FNP), and BA1. In mutants, expression of Erm1 is expanded ventrally from the MHB (yellow asterisk) and in BA1 (yellow bracket).

(Figure S2)
loss of Fuz leads to upregulation of Wnt target genes (e.g., Zhang et al., 2011); however, we found no changes in levels of activated β-catenin at early stages (e9.0) in Fuz−/− heads (data not shown). Next, as mentioned, high arched palate is common in FGF hyperactivation syndromes (Hajihosseini et al., 2001; Ioth and Ornitz, 2011; Wilkie et al., 1995). While there is no link between cilia and FGF signal transduction per se, loss of Gli3 can lead to increased Fgf8 gene transcription, most obviously in the telencephalon (Aoto et al., 2002; Blaess et al., 2006; Cordero et al., 2004; Kuschel et al., 2003; Okada et al., 2008; Rash and Grove, 2007; Thell et al., 1999; Ueta et al., 2008). Indeed, a recent report suggests that loss of cilia-dependent signaling can similarly result in Fgf8 expansion during development of the corpus callosum (Benadiba et al., 2012).

Therefore, we examined Fgf8 levels in Fuz mutants. Strikingly, at E9.5, Fgf8 expression was significantly expanded in Fuz mutants (Figures 3D and 3F), though there was minimal difference in mRNA levels prior to e9.0 (Figure S2). Fgf8 is normally expressed in the midhindbrain boundary, the frontal process, and at low levels within the lateral epithelium of the maxillo-mandibular cleft (Figures 3C and 3C′). In Fuz mutants, Fgf8 expression was expanded in all of these domains, with an anterior expansion from the midhindbrain domain (Figure 3D, bracket) and a mediolateral expansion within the mandibular and maxillary prominences (Figures 3D and 3D′). This expansion was maintained in mutant maxilla at E10.5 (Figure 3F). In addition, we found that Fuz knockdown in Xenopus also resulted in broader Fgf8 gene expression, most strikingly in the domain abutting the migratory NC and in the frontal midline (Figure S3).

Key transcriptional targets of FGF signaling, Erm1 and Pea3 (Firnberg and Neubüser, 2002; Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001), were strongly upregulated in Fuz mutants (Figures 3I–3J) including a clear rostral expansion of both messenger RNAs (mRNAs) surrounding the mesencephalon (Figures 3G–3J, asterisk). Furthermore, both genes were robustly expressed throughout the expanded maxillary primordia, in contrast to the wild-type littermates (Figures 5H and 5J, arrowhead). Together, these data identify a surprising role for Fuz in the regulation of Fgf8 gene expression, and suggest that an aberrant increase in FGF signaling underlies the craniofacial anomalies in Fuz mutant mice.

**Fgf8 Heterozygosity Rescues Maxillary Hyperplasia and Palate Defects in Fuz Mutant Mice**

Our analysis of Fuz mutants suggested that excessive FGF signals drive the maxillary hyperplasia that underlies the observed palate defects. To test this model directly, we asked if decreasing the Fgf8 gene dose might ameliorate the craniofacial defects in Fuz mutants. Using a null allele of Fgf8, in which the coding region is replaced with the lacZ gene (Iliagan et al., 2006), we halved the dose of Fgf8 in Fuz−/− mice. We observed substantial rescue of maxillary hyperplasia in Fuz−/−; Fgf8lacZ/− compound mutants (compare Fuz mutants in Figures 4B and 4B′ to rescued embryos in Figures 4C and 4C′). Heterozygosity of Fgf8 also rescued the brain overgrowth and ocular phenotypes observed in Fuz mutants (compare Figure 4E to Figure 4F). Most strikingly, loss of one allele of Fgf8 restored the e16.5 Fuz−/− palate to a normal width (Figures 4H–4K; Figure S3), confirming that the early maxillary phenotype is due in large part to an increase in FGF.

Because the Fgf8 and NC phenotypes we observed seemed localized to the cranial structures, we hypothesized that phenotypic rescue after decreasing Fgf8 dose should be specific to the head. Indeed, we found that Fgf8 heterozygosity had little effect on digit development phenotypes in Fuz mutant mice (Figure S3). Together, these data suggest that the proximate cause for craniofacial defects in Fuz mutant mice is expanded Fgf8 expression, while the polydactyly is independent of Fgf8, resulting directly from aberrant cilia-mediated Gli3 regulation (Heydeck et al., 2009).

**Conditional Disruption of Fuz in the NC Does Not Result in High Arched Palate**

Our data so far lead us to propose a model in which early dysregulation of cranial Gli processing in Fuz mutant mice leads to excessive expression of Fgf8, which in turn causes an excess of NC. We further propose that it is this early excess of NC, rather than attendant crest migration defects, that results in maxillary hyperplasia and high arched palate. To test this model, we used the NC-specific Wnt1-cre driver to conditionally delete the Fuz gene. Notably, specific deletion of Fuz in NC cells did not elicit early hyperplasia of the maxillary process or high arched palate (Figure 5; data not shown). Instead, these animals had a true cleft, indicating that, in the NC, Fuz is required only much later for palatal shelf elevation or depression of the tongue. These data suggest that the early maxillary phenotypes in Fuz mutant mice result from Fuz requirements in the neural tissue prior to NC induction (see also the model, Figure 7).

**Maxillary Hyperplasia and FGF8 Upregulation in OFD-1 Mice**

We next asked if Fgf8 misregulation might be a general principle in ciliopathies. We examined the mouse model of OFD syndrome, Ofd-1 (Ferrante et al., 2006). OFD patients frequently present with high arched palate (Figure 6F) (Hennekam et al., 2010; Prattechizzo et al., 2008; Tagliani et al., 2010), and OFD-1 is essential for ciliogenesis and centriole morphology (Ferrante et al., 2006; Singla et al., 2010). Importantly, Ofd-1 mutant animals were strikingly similar to the Fuz−/− animals, with an enormously enlarged maxillary process and a clear expansion of cranial Fgf8 expression (Figures 6A–6D).

**Broad Similarity between Fuz Mutant Mice and FGF Hyperactivation Syndromes**

Finally, the similarity of palatal phenotypes between OFD patients and patients with FGF hyperactivation syndromes such as Apert (Figures 6E–6G) suggest that ciliopathic cranial phenotypes stem from excessive FGF signaling. If this were the case, we hypothesized that Fuz−/− mice might also display craniosynostosis, another common feature of FGF hyperactivation syndromes. Therefore, we examined skull ossification using Alizarin red staining and found complete synostosis of the coronal sutures in Fuz mutant mice, akin to that seen in Apert syndrome (Figures 6I–6I′, arrowhead). Mutants also displayed other hallmarks of FGF syndromes, including fusion of the cervical vertebra and upper airway anomalies (data not
Thus, our data suggest widespread commonalities between ciliopathic craniofacial defects and FGF hyperactivation syndromes.

**DISCUSSION**

Recent evidence reveals significant roles for cilia in human development and disease. First, mutations in genes known to promote cilia biogenesis and IFT have been implicated in a number of human syndromes (Ferkol and Leigh, 2012). Second, analysis of animal models suggests that a variety of developmental disorders, including craniofacial dysmorphology, result from defects in ciliary function (Huber and Cormier-Daire, 2012). Finally, advances in clinical genomics have improved annotation of disease alleles, subsequently identifying numerous, unclassified syndromes as ciliopathies (Brugmann et al., 2010). The challenge now is understanding how these seemingly heterogeneous disorders arise (Novarino et al., 2011), and our data suggest that commonalities in phenotype are likely to reflect shared signaling events, which converge into shared phenotypes. The results reveal an association between ciliopathies...
et al., 2011). We propose that the high arched palate arises due to perturbation of facial development in the Fuz mutant mice. Those reports focus on mandibular outgrowth (Zhang et al., 2012) and likely lead to attenuated Gli3 processing (Figure 3). Subsequently, loss of Gli3R reduces transcriptional inhibition, permitting expansion of hindbrain fates, and Fgf8 expression.

Previous studies in chicken embryos have implicated brain-specific Fgf8 in the control of NC numbers (Creuzet et al., 2004). How does this occur? Dil labeling has shown that, in mouse, anterior NC destined for BA1 arises from the posterior mesencephalon and rhombomere 1 (Osumi-Yamashita et al., 1994). Loss of Hh activity leads to dorsalization or expansion of these structures (Fedtsova and Turner, 2001), concurrently increasing Fgf8 expression and the NC domain. As a consequence, increased numbers of NC cells migrate toward BA1, overfilling it and forming enlarged maxillae (Figures 2 and 3). Subsequently, Fgf8 is also upregulated in the BA1 epithelium, as well as in the frontonasal prominence (Figure 3). Following NC migration, the palatal condensation (Figure 7, red) arises medially, adjacent to Fgf8 expression domains; in mutants, the grossly enlarged maxillary region causes a medial shift in palatal condensations (Figure 7).

These findings are significant for exposing an etiological link between the ciliopathies and FGF hyperactivation disorders. Furthermore, our data suggest that the long-held midface hypoplasia model for high arched palate should be revisited (Hayward et al., 2004; Hennekam et al., 2010). One source of confusion may be the disparity between observations in human patients versus phenotypes associated with mouse models. For example, Snyder-Warwick and colleagues examine palatal development in mice carrying a Crouzon mutation (FGFR2C342Y), which causes increased FGF signaling associated with craniosynostosis (Snyder-Warwick et al., 2010). In these mice, heterozygotes, which should mirror the human genotype, have normal palates. Furthermore, homozygotes have a true cleft, mimicking a loss of FGFR2b (Hosokawa et al., 2009; Rice et al., 2004). This clearly does not model the oral aspects of the human syndrome, as it was noted nearly 40 years ago that true clefts in Crouzon patients were likely to be misdiagnoses (Peterson and Pruzansky, 1974).

Importantly, the relationship between ciliopathy and FGF hyperactivation syndromes is not limited to the palate, as Fuz mutant mice also display other manifestations of the FGFR-associated syndromes, such as craniosynostoses, fusions of the cervical vertebrae, and tracheal cartilaginous sleeve (Figure 7; data not shown). Finally, we note that this relationship is not restricted to animal models, as high arched palate and synostoses also co-occur in the human ciliopathy Sensenbrenner syndrome (Levin et al., 1977; Sensenbrenner et al., 1975). Notably, the cilia defects in Sensenbrenner syndrome are caused by mutations in proteins of the retrograde IFT particle, including IFT43 (Arts et al., 2011), and we recently showed that ciliogenesis defects following disruption of Fuz stem from a failure of IFT43 trafficking (Brooks and Wallingford, 2012).

Thus, in summary, our studies of a high arched palate model have revealed that excessive FGF transcription and increased
NC may be key factors in the poorly understood etiology of ciliopathic craniofacial defects. Furthermore, our report demonstrates that the pathological events underlying this phenotype are surprisingly different from those leading to a traditional cleft palate, raising the possibility that clinical diagnoses and management of high arched palate should also be reconsidered in this developmental and molecular context.

EXPERIMENTAL PROCEDURES

Mouse Lines
The following mouse lines were used: Fuz mutants: Fuz<sup>fl/fl</sup> (Gray et al., 2009); conditional Fuz mutants: Fuz<sup>fl/fl</sup>, which were generated according to standard methods and will be described elsewhere; conditional Ofd-1 mutants: Ofd1tm2.1Bfra (Ferrante et al., 2006); Fgfb mutants: Fgfb<sup>lacZ</sup> (Ilagan et al., 2006); Wnt1-cre driver: Tg(Wnt1-cre)11Rth (Danielian et al., 1998); and reporter lines: R26RmT/mG: GT(Rosa)26Sortm4(ACTB-tdTomato-EGFP)Luo (Muzumdar et al., 2007) and R26RlacZ: Gt(ROSA)26Sortm1Sor (Soriano, 1999). Genotyping was performed as described in the original publications cited earlier. In all phenotypes depicted, at least four animals per genotype were examined. All animal work was performed in accordance with UK Home Office Regulations.

Western Blotting
Tissues rostral to and including BA1 were dissected from E9.0 embryos. Protein preparations and western blotting were carried out according to established protocols. Primary antibodies used were anti-Gli3 (clone H-280, 1:1,000, Santa Cruz Biotechnology no. sc-20688) and anti-alpha tubulin (clone DM1a, 1:5,000, Sigma T6199). Chemiluminescent signal was visualized using a BioRad ChemiDoc.

Real-Time PCR
Tissues rostral to and including BA1 were dissected from E9.0 embryos. Reverse transcription and cDNA synthesis were carried out according to standard protocols. Real-time PCR (RT-PCR) reactions were performed on a Rotorgene Q 2-series using the following gene-specific primer pairs.

- **β-actin** (F: CTAAGGCCAACCGTGAAAG, R: ACCAGAGGCATACAGGGACA)
- **Gli1** (F: CAGGGAAGAGAGCAGACTGAC, R: CGCTGCTGCAAGAGACT)
- **Patched1** (F: AAGCCGACTATCAGGCCAG, R: AAGGGAACTGAGCGTGACTCG)
- **Fgfb** (F: AGGTCTCTACATCTGCATGAAC, R: TGTTCTCCAGCACGATCTCT)
- **Erm** (F: TGCCCACTTCATCGCCTGGAC, R: TAGCGGAGAGAGCGGCTACG)

Staining and Histology
All mRNA in situ hybridization, immunohistochemistry, β-gal activity, and histological staining were performed according to standard protocols. Mouse

(E–G) OFD-1 and Apert syndrome patients exhibit high arched palate. In (E), a ventral view of a normal palate shows a hard palate with shallow, anterior bilateral rugae and smooth posterior palate (after photo by Millicent Odunze: http://plasticsurgery.about.com/od/Cleft-Lip-And-Palate/ss/What-Is-A-Cleft-Palate_3.htm). In (F), a ventral view is shown of the palate from OFD1 (OFD-1) patient (after Tagliani et al., 2010; Figure 2F). (G) shows a ventral view of palate from the Apert syndrome patient (after Rynearson, 2000). Both (F) ciliopathic and (G) FGF-related high arched palates are narrow with a deep medial cleft extending from the anterior hard palate. Rugal-like swellings are more numerous and are extant with the medial cleft. Soft tissue swellings and dental crowding are observed in both groups.

(H–I) Loss of Fuz causes coronal craniosynostosis. E17.5 heads stained with Alizarin red. (H) and (I) show dorsolateral views. (H') and (I') show dorsal views. Alizarin red negative coronal suture (open arrowheads) separates frontal (F) and parietal (P) bones in wild-type embryos. Coronal suture is absent in mutant embryos (closed arrowheads).

Figure 6. Ofd-1 Mutants Also Show Expanded Maxillary Compartments and Cranial FGF8 Expression Domains
(A–D) Lateral views of E9.5 embryos showing cranial Fgf8 mRNA expression domains. The 21 and 23 somite ofd-1 mutants have enlarged maxillae compared to stage-matched control embryos, indicated by yellow brackets in (B) and (D) compared to (A) and (C), respectively. Maxillary Fgf8 expression is expanded in mutants compared to controls [brackets in (B) and (D) compared to (A) and (C), as well as expression in the frononal process, indicated by arrowheads].

β-actin<sup>Cre/+</sup>; Ofd1<sup>+/-</sup> β-actin<sup>Cre/+</sup>; Ofd1<sup>fl</sup>

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Fuz Mutation Links Ciliopathies and FGF Syndromes

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and Xenopus embryos were collected in cold PBS and fixed overnight in 4% paraformaldehyde or MEMFA, respectively. mRNA in situ hybridization was performed as previously described (Sive et al., 2000; Wilkinson et al., 1989). The following mRNA probes were used: mouse Fgf8 (Mahmood et al., 1995), mouse erm (Hippenmeyer et al., 2002), mouse Pea3 (Livet et al., 2002), X. laevis Fgf8 (Monsoro-Burq et al., 2003), and X. laevis twist (Hopwood et al., 1989). Primary antibodies used for immunohistochemistry: anti-rabbit phospho-histone H3 (PH3) (BDH, 1:200) or anti-rabbit β-catenin (Sigma, 1:200). Sections were coverslipped with ProLong Gold antifade reagent with DAPI (Invitrogen, P36931).

Confocal Microscopy and Image Analysis
To analyze NC migration, whole-mount E9.0 embryos were cleared using 70% glycerol/PBS and mounted on slides. Sagittal confocal z-stacks were obtained using a Leica TCS SP5 DM16000. Image sequences were reconstructed using Imaris image analysis software. Thickness of NC streams were determined by measuring the depth of GFP positive NC cells at three points anterior to the trigeminal ganglion, posterior to the maxillary compartment (outlined in Figure 3).

Flow Cytometry of Embryo Heads
Cell purification from embryonic tissues was performed as previously described (Schulz et al., 2012). Heads from Wnt1-cre; R26mT/mG embryos, as depicted in Figure 2E, were digested and passed through a 100 μm cell strainer. Flow cytometry was performed using a BD Biosciences FACSAria II cell sorter. Live cells were identified using side scatter and forward scatter (FSC-A), followed by doublet exclusion using forward scatter width against FSC-A. Populations were identified using endogenous expression of GFP and Tomato. All data were analyzed using FlowJo 9.53 (Celeza GmbH).

SUPPLEMENTAL INFORMATION
Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.05.021.
Developmental Cell

Fuzz Mutation Links Ciliopathies and FGFR Syndromes

LICENSING INFORMATION

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