Fuz Regulates Craniofacial Development through Tissue Specific Responses to Signaling Factors

Zichao Zhang1, Bogdan J. Wlodarczyk2, Karen Niederreither2, Shankar Venugopalan1, Sergio Florez1, Richard H. Finnell2, Brad A. Amendt1*

1 Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, Texas, United States of America, 2 Dell Pediatric Research Institute, University of Texas, Austin, Texas, United States of America

Abstract
The planar cell polarity effector gene Fuz regulates ciliogenesis and Fuz loss of function studies reveal an array of embryonic phenotypes. However, cilia defects can affect many signaling pathways and, in humans, cilia defects underlie several craniofacial anomalies. To address this, we analyzed the craniofacial phenotype and signaling responses of the Fuz−/− mice. We demonstrate a unique role for Fuz in regulating both Hedgehog (Hh) and Wnt/β-catenin signaling during craniofacial development. Fuz expression first appears in the dorsal tissues and later in ventral tissues and craniofacial regions during embryonic development coincident with cilia development. The Fuz−/− mice exhibit severe craniofacial deformities including anopthalmia, agenesis of the tongue and incisors, a hypoplastic mandible, cleft palate, ossification/skeletal defects and hyperplastic malformed Meckel’s cartilage. Hh signaling is down-regulated in the Fuz null mice, while canonical Wnt signaling is up-regulated revealing the antagonistic relationship of these two pathways. Meckel’s cartilage is expanded in the Fuz−/− mice due to increased cell proliferation associated with the up-regulation of Wnt canonical target genes and decreased non-canonical pathway genes. Interestingly, cilia development was decreased in the mandible mesenchyme of Fuz null mice, suggesting that cilia may antagonize Wnt signaling in this tissue. Furthermore, expression of Fuz decreased expression of Wnt pathway genes as well as a Wnt-dependent reporter. Finally, chromatin IP experiments demonstrate that β-catenin/TCF-binding directly regulates Fuz expression. These data demonstrate a new model for coordination of Hh and Wnt signaling and reveal a Fuz-dependent negative feedback loop controlling Wnt/β-catenin signaling.

Introduction

Vertebrate craniofacial development is a complicated process requiring the coordination of multiple signaling pathways and tissue interactions among the three germ layers and neural crest cells in three dimensions. A number of signaling pathways have been implicated in craniofacial development including Hedgehog (Hh), Wnt/β-catenin, TGF-β, Fibroblast Growth Factor (Fgf), Notch, and Planar Cell Polarity (PCP) signaling [1,2,3,4,5]. However, the interactions and components shared among different signaling pathways are not well understood. The recent identification of the PCP effector gene Fuzzy (Fuz) as an important regulator of Hedgehog (Hh) signaling suggests that there may be substantial crosstalk between the different molecular cues. Furthermore, Fuz can coordinate ciliogenesis and secretion, two processes that affect a variety of signaling pathways [6,7]. Our data suggest Fuz plays a pivotal role in the Wnt and Hedgehog pathways [6,7,8]. Furthermore, loss of Fuz leads to dramatic defects in craniofacial development.

PCP signaling, initially discovered in Drosophila, controls a diverse range of polarized cellular behaviors [9]. Recent studies have shown that PCP signaling is also important in regulating cell interactions and tissue movements [10,11,12]. In mice, loss of PCP genes leads to disruption of polarized structures such as the stereociliary bundles in the cochlea [13,14,15,16]. In addition, mutants in PCP genes display an array of morphogenetic defects affecting the neural tube, heart, kidney and other tissues [1]. Thus, the PCP pathway affects a wide range of cell-cell interactions via coordination of morphogenesis, tissue polarity, and potentially growth.

The PCP pathway makes use of the Wnt signaling components Frizzled and Dishevelled, but is β-catenin-independent [17]. Studies of vertebrate planar cell polarity have primarily focused on the “core PCP” pathway. Thus, the function of vertebrate PCP “effectors”, which act downstream of Dishevelled, is unclear. This report focuses on one of the PCP effector proteins, Fuzzy or Fuz, which encodes a transmembrane protein required for tissue polarity. Elegant genetic studies in Drosophila have shown that fuzzy, along with inturned, plays a role in maintaining cytoskeletal integrity in the wing hairs; this role is genetically downstream of dishevelled and frizzled [18,19,20,21].

Recent studies suggest that mutations affecting ciliogenesis lead to congenital human anomalies. Cilia appear to act as mechanical and chemical sensors that interpret extracellular cues via signaling pathways such as Hh, PDGF and Wnt [22]. Because there is no protein synthesis in the cilium, assembly and maintenance of the
ciliation requires intraflagellar transport (IFT). Thus, loss of IFT proteins leads to catastrophic effects on ciliogenesis and cilia function. Human conditions resulting from defects in ciliogenesis or IFT include situs inversus, poly cystic kidney disease, ear defects and craniofacial anomalies [23,24].

Several proteins involved in PCP signaling, including Dishevelled, localize to the base of cilia [25,26,27,28]. This suggests that PCP signaling may be important for cilia function and possibly for coordination of other signaling pathways. In this context, we were particularly interested in the canonical Wnt/β-catenin pathway, as the absence of cilia results in enhanced β-catenin nuclear localization and downstream gene transcription [29,30,31].

In this study, we analyzed the expression, regulation and functional requirements of Fuz during vertebrate craniofacial development. We found that Fuz−/− mice display massive craniofacial defects including anophthalmia, agenesis of the tongue and incisors and a hypoplastic mandible leading to cleft palate. In contrast, we found that Meckel's cartilage, an embryonic structure that develops into the mandible and portions of the inner ear, was hyperplastic and malformed. Some aspects of the craniofacial phenotype, such as missing teeth and cleft palate, could be attributed to decreased Hh signaling. However, the increased growth of Meckel's cartilage was associated with increased Wnt signaling.

To further examine the molecular consequences of Fuz loss of function, we analyzed expression of WNT target genes in Fuz−/− mice. We found that a number of β-catenin/Tcf target genes were up-regulated in our mutants, suggesting that Fuz plays a dual role in Wnt signaling, in both the canonical and non-canonical pathways. Furthermore, we found that the Fuz protein could repress expression of Wnt pathway genes as well as a Wnt-dependent reporter, suggesting a direct role within the canonical pathway. Finally, most interesting, we identified β-catenin/Tcf-binding sites in the Fuz gene, which we confirmed by chromatin immunoprecipitation. Together, these data imply that Fuz may be a critical factor linking the Hh, PCP and Wnt/β-catenin signaling pathways and may function as a switch to balance the activities of these pathways during craniofacial development.

Results

Fuz expression pattern during mouse development

X-gal staining of E9.5 FuzLacZ/+ mouse embryos revealed FuzLacZ expression was restricted to the dorsal tissue and brain (Fig. 1A). At E11.5, FuzLacZ expression was concentrated in the brain, spinal cord and eyes. However, relatively weak expression was also detected in the heart, limbs and craniofacial region (Fig. 1B). At E12.5, FuzLacZ expression in the craniofacial region began to expand from dorsal to the ventral regions (Fig. 1C). At E14.5, FuzLacZ expression became stronger and widespread throughout the craniofacial region (Fig. 1C). Fuz expression at E12.5 in the oral epithelium, mesenchyme and Meckel's Cartilage is shown in sagittal sections (Fig. 1D). FuzLacZ expression at E14.5 in the oral epithelium, mesenchyme, palate (PL), and tongue (TE) has increased from E12.5 (Fig. 1E). Fuz expression in Meckel's cartilage (MC) and the perichondrium (PC) are shown in high magnification sections (Fig. 1F). In addition, we assessed Fuz expression in different cell lines by RT-PCR. Fuz is highly expressed in LS-8 cells (mouse oral epithelium), C3H10T1/2 cells (mouse embryonic fibroblast), HEK 293 FT cells (human embryonic kidney fibroblast) and SW1353 cells (human chondrocyte). It had relatively weak expression in MDPC-23 cells (mouse dental mesenchyme), and no expression in CHO cells (hamster ovary) (Fig. 1G).

Severe craniofacial defects in the Fuz−/− mice

To study Fuz function during craniofacial development, we used the Fuz null mouse created with a gene trap cassette inserted in the second exon of the Fuz gene [7]. The lack of both copies of the Fuz gene in homozygotes (Fuz−/−) was lethal for mice immediately after birth. At E18.5, all null embryos have a hypoplastic mandible and maxilla, and anophthalmia (Fig. 2A). An abnormal bulge in the mandible and a secondary cleft palate are observed in mutant embryos (Fig. 2A,B). Histological analysis on E18.5 embryos revealed further craniofacial defects including a malformed tongue and missing incisors. The ventral bulge of the mutant mandible is due to a hyperplastic and malformed Meckel's cartilage (Fig. 2C,D). The Fuz−/− mice do not form incisor tooth buds and lower and upper incisor tooth initiation did not begin and the tongue muscles appear to be fused with the mandible. The hyperplastic and malformed Meckel's cartilage suggested an increased proliferation of Meckel's cartilage cells.

To examine the developmental progress of craniofacial formation, embryos were harvested at different developmental time points and analyzed. At E12.5, the mandible of the Fuz−/− embryos appeared normal compared with those of their heterozygous littermates. Meckel's cartilage of the Fuz−/− embryos had a normal shape and similar size with heterozygous embryos (Fig. 3A). The normal mouse craniofacial structures such as the chordor zincus (CP) differentiating from the roof of fourth ventricle, the chondroid extension extending into the lateral ventricle (CPL), the corpus striatum mediale (STM), the optic recess of the diencephalon (OR) and the cochlea (CO) are present in the E12.5 Fuz−/− heterozygous mouse (Fig. 3A). These structures are lost in the Fuz null mice at this stage, only the cochlea and corpus striatum are observed and these structures are abnormal (Fig. 3A). In contrast, at E14.5 the Fuz−/− entire mandible is thickened in the dorsal-ventral axis and shortened in the anterior-posterior axis compared with those of heterozygous littermates (Fig. 3B). The trigeminal nerve (TG) appears to replace the pituitary primordium or Rathke's pouch (RP) and many of the brain structures are not developed (Fig. 3B). Meckel's cartilage has begun to elongate in the dorsal-ventral direction instead of the anterior-posterior direction (Fig. 3B). The upper and lower incisor buds have not developed while the tongue had a rudimentary root structure and failed to elongate in the anterior-posterior axis (Fig. 3B). The malformed Meckel's Cartilage not only elongated dorsoventrally but also the dorsal end expanded in the median-lateral axis. An abnormal growth of the palate tissue (PLT) or palate shelf was observed in the E14.5 Fuz null mice compared to the normal palate (PL) seen in the heterozygous mice (Fig. 3B). Coronal sections of E16.5 Fuz−/− mice revealed descending palate shelves (PL) suggesting a delay of elevation, a lack of eyes, however the upper and lower molar (ML) tooth buds have formed (Fig. 3C). Meckel's cartilage has developed in the midline and extends dorsally (Fig. 3C). There were many other defects observed in the nasal cavity, facial bones, cartilage and brain that will not be examined in this report. However, the loss of Fuz affects patterning of much of the craniofacial region with a loss of some structures (incisors, tongue, eyes and bone) and an expansion of other structures (Meckel's cartilage).

Defective craniofacial bone development in Fuz null mice

To determine if bone formation was affected in the Fuz null mice, E16.0 embryos were stained with Alcian Blue/Alizarin Red and compared to their heterozygous littermates (Fig. 4A–D). The blue cartilage stain revealed defective Meckel's cartilage in the Fuz−/− mice and malformed cartilage of the craniofacial region (Fig. 4B,D). The anterior Meckel's cartilage is deformed with an
ascending branch and reduced ossification (red stain) around Meckel’s cartilage. Specifically, membranous ossification of the premaxilla (pmx), maxilla (mx), mandible (md), frontal (fnt) and parietal (par) bones are missing at this stage. Ossification of the sphenoid (sb) and basioccipital (bb) bones are also defective in the Fuz null mice (Fig. 4D). To determine if craniofacial bone development was delayed or reduced in the Fuz null mice we analyzed E18.5 Fuz null mice and found a delayed ossification of the craniofacial region compared to heterozygous littermates (Fig. 4E-H). Further research is ongoing to understand the delayed bone development and in this report we focused on the regulatory mechanisms directing Meckel’s cartilage formation.

**Increased proliferation of Meckel’s cartilage cells in Fuz null embryos**

Cell proliferation of E14.5 Meckel’s cartilage was measured by immunofluorescence with a Ki67 antibody (Fig. 5A). The Ki67 positive cell number to total cell number (DAPI) within Meckel’s cartilage was significantly increased in Fuz null embryos compared to wild type (WT) embryos (Fig. 5B).
cartilage was calculated to estimate the proliferation ratio. Compared with a 75% ratio observed in heterozygous Meckel’s cartilage, the ratio in null samples was significantly increased to 88% (Fig. 5B). These cells are proliferating at a higher rate compared to Fuz heterozygous Meckel’s cartilage.

Fuz regulates cilia development in the mandibular mesenchyme

We asked if cilia formation was affected in the mandible by immunofluorescence with an Arl13b antibody in mandible mesenchyme at E14.5. The amount of primary cilium was significantly decreased in the Fuz^-/- mandible mesenchyme (Fig. 6). The average cilium number per 5000 μm² in the sagittal sections of the mutant mandible mesenchyme was 2.9, compared to 16.5 in the wild type. Because Hh signaling is regulated by the primary cilium components, we hypothesized that the Hh signaling was altered due to the cilium defect in the Fuz null mouse.

Down-regulation of Hedgehog signaling in Fuz^-/- embryos

A loss of primary cilia correlates with an absence of membrane associated Smoothened and failure to activate downstream transcription factors responding to the Hh signal [4,32,33]. We asked if sonic hedgehog (Shh) signaling was altered in the craniofacial region of Fuz mutant embryos. A whole-mount in situ hybridization assay was performed at E9.5 and showed an overall reduction of Patched 1 (Ptc1), and Gli1 expression levels while Shh levels were unchanged (Fig. 7). Given that Ptc1 and Gli1 are indicative of Hh signaling [34,35], these data revealed a general down-regulation of Hh signaling in the Fuz null mouse.

Immunohistochemistry experiments revealed that Ptc1 was highly expressed in the tongue, oral and dental epithelium and mesenchyme, but weakly expressed in Meckel’s cartilage in E14.5 heterozygous embryos (Fig. 8A). In the Fuz null embryos, Ptc1 expression was decreased overall in the oral cavity as well as in Meckel’s cartilage (Fig. 8B). Real-time PCR with mRNA of dissected Meckel’s cartilage and surrounding mesenchyme confirmed the down-regulation of Ptc1 (Fig. 8E). Real-time PCR also revealed a significant decrease in Gli1 transcripts in the Fuz null embryos (Fig. 8E). At E14.5, Gli2 had a similar expression pattern with Ptc1 and Gli2 protein was decreased in the null embryos compared to heterozygotes (Fig. 8C,D), though its transcript level was not significantly changed (Fig. 8E). Shh transcript levels were also reduced in the null embryos suggesting that Fuz was required for the maintenance of Hh signaling. Hh signaling is essential for establishing and maintaining dorsal-ventral patterning and required for incisor development. The disrupted Hh signaling provides a possible explanation for the malformed Meckel’s cartilage and the missing incisors. Hh signaling is also able to stimulate cell proliferation by activating Cyclin D1 (Ccnd1) and Cyclin D2 (Ccnd2) expression [36]. However, the decreased Hh...
signaling is contrary to the increased proliferation of Meckel’s cartilage. We hypothesized that another mechanism must be involved in the enhanced proliferation of Meckel’s cartilage.

Increased Wnt/\(\beta\)-catenin signaling in \(Fuz^{2/2}\) embryos

Wnt/\(\beta\)-catenin signaling is regulated by the basal component of the primary cilium [29,30] and Hh signaling interacts with Wnt/\(\beta\)-catenin signaling during multiple developmental programs [37,38,39]. We asked if Wnt/\(\beta\)-catenin signaling was altered in the \(Fuz^{null}\) embryos, which could affect cell proliferation. Immunofluorescence with a \(\beta\)-catenin antibody was performed using sagittal sections of E14.5 embryos. These experiments revealed that \(\beta\)-catenin was increased in the \(Fuz^{2/2}\) oral epithelium, mesenchyme (Fig. 9A) and Meckel’s cartilage (Fig. 9B), compared to those of heterozygous littermates. Because Lef-1 is a transcription factor whose activity and expression are positively regulated by Wnt/\(\beta\)-catenin signaling, we asked if Lef-1 expression was modulated in the \(Fuz^{null}\) mutants. Lef-1 is normally expressed in the oral and dental epithelium and mesenchyme at E14.5 (Fig. 9C), and further confirmed by Real-time PCR (Fig. 9F). Tcf4 (\(Tcf7l2\)) expression is also regulated by Wnt/\(\beta\)-catenin signaling [40], and immunofluorescence assays revealed increased Tcf4 in \(Fuz^{null}\) Meckel’s cartilage at E14.5, and further confirmed by Real-time PCR (Fig. 9E,F). Inspection of a group of Wnt/\(\beta\)-catenin target genes revealed increased expression of Axin2, Cyclin D1, Cyclin D2 and Runx2 in \(Fuz^{2/2}\) Meckel’s cartilage (Fig. 9F), [41,42,43,44]. On the contrary, non-canonical Wnt signaling including Wnt5a, Ror2, and their downstream target Pkd2 (known as \(Pdpk2\) in Xenopus) [45] were down regulated in \(Fuz^{null}\) Meckel’s cartilage (Fig. 9F). As a
Figure 4. Skeletal defects of *Fuz* null mutant embryos. A, B) Upper panels are Alcian Blue/Alizarin Red staining of cartilage and bone skeletal preparations of E16.0 *Fuz* heterozygous embryos (A) and *Fuz* null littermates (B). Ossification is reduced in the *Fuz* null embryos. Lower panels are higher magnification of the head region. The anterior region of Meckel’s cartilage (MC) is deformed with an ascending branch. E–H) E18.5 head preparations revealing delayed bone formation and defective craniofacial structures in the *Fuz* mutant embryos (arrows denote the malformed and ossified Meckel’s cartilage). Frontal (fnt), parietal (par), premaxilla (pmx), maxilla (mx), sphenoid (sb) and basioccipital (bb) bones and other facial bones are missing. Background red staining was due to soft tissues, which were left intact.
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Figure 5. Enhanced cell proliferation in Meckel’s cartilage of the *Fuz<sup>−/−</sup>* mandible. A) The proliferation of E14.5 Meckel’s cartilage was assessed by immunofluorescence with a Ki67 antibody. B) The proliferation ratio is calculated by dividing the Ki67 positive cell number with DAPI cell number within Meckel’s cartilage. The proliferation of mutant Meckel’s cartilage (88%) is significantly increased compared with wild type (75%). Experiments were repeated three times and *p*-value is shown.
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control real-time PCR demonstrated a lack of Fuz transcripts in the null embryos (Fig. 9F). These data indicate that Fuz acts as a repressor of Wnt/β-catenin signaling during craniofacial development. The increased Cyclin D1 and D2 provide a possible explanation of the enhanced cell proliferation in the Fuz2/2 Meckel's cartilage.

To confirm the repressor role of Fuz on Wnt/β-catenin signaling, Fuz expression plasmid was co-expressed with the 7xTopflash reporter in both HEK 293 FT cells and CHO cells. Co-transfection of Fuz with the 7xTopflash reporter resulted in a 50% decrease in Topflash reporter activity, compared to that with the control vector in both cell lines (Fig. 9G). The result was consistent between 293 cells, which has high endogenous Fuz expression and CHO cells which do not endogenously express Fuz. These results indicate a repressor role of Fuz on Wnt/β-catenin signaling.

β-catenin directly activated the Fuz promoter

Sequence analyses revealed eleven Wnt response elements (Lef/Tcf binding sites) within the murine Fuz 2.4 kb promoter (Fig. 10A). A chromatin immunoprecipitation (ChIP) assay demonstrates

Figure 6. The cillum defect in the Fuz null mouse. A) The primary cilia are shown by immunofluorescence with an Arl13b antibody in mandible mesenchyme at E14.5. B) The amount of cilia in the sagittal sections of Fuz2/2 mandible mesenchyme was quantitated and compared to wild type mandible mesenchyme. Error bars indicate S.E., n = 8, p < 0.01.

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Figure 7. Defective Sonic Hedgehog signaling. Whole-mount in situ hybridization assays with indicated probes were performed with E9.5 embryos. The overall decrease of Pch1, and Gli1 transcript levels are shown.

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endogenous β-catenin associating with the Fuz promoter. Non-transfected LS-8 cells were used as these cells endogenously express Lef-1, β-catenin and Fuz. The Fuz promoter chromatin was amplified by PCR using primers specific for the Fuz promoter flanking the Lef/Tcf binding site (Fig. 10A,B). The primers amplified a 201 bp product from the chromatin input and antibody IP (Fig. 10B, lanes 3 and 5, respectively). The primers did not produce a PCR product from primers only or normal rabbit IgG IP control (Fig. 10B, lanes 2 and 4, respectively). To test whether this association could lead to functional activity, the Fuz 2.4 kb promoter was cloned into a luciferase vector and transfected into LS-8 and CHO cells. Addition of LiCl (10 mM) to the cell culture medium stimulates β-catenin nuclear localization and caused significant increase of Fuz promoter activity in both cell lines (Fig. 10C). These results demonstrate that β-catenin directly targets the Fuz promoter and activates its transcription. Combined with the Fuz repression of Wnt/β-catenin signaling, we conclude that Fuz constitutes a negative feedback loop that controls Wnt/β-catenin signal activity.

Sox9 expression is increased in Fuz<sup>-/-</sup> Meckel’s cartilage

A previous study reported that Wnt/β-catenin signaling repressed Sox9 expression both in vitro and in vivo [46]. Given the increased Wnt/β-catenin signaling in the Fuz null mice, we expected reduced expression of Sox9 in Fuz null Meckel’s cartilage. However, immunofluorescence with Sox9 antibody in E14.5 Meckel’s cartilage revealed that Sox9 expression was increased in the null embryos (Fig. 11A). This increase was validated by Real-time PCR using RNA from E14.5 Meckel’s cartilage (Fig. 11B). Type II Collagen (Col2a1) is a downstream target gene of Sox9 [47]. Col2a1 expression was also increased in Fuz null Meckel’s cartilage measured by Real-time PCR (Fig. 11B). Over-expression of Fuz in SW1353 cells confirmed the repression of Sox9 expression. The human chondrocyte cell line SW1353 was transfected with the Fuz
Figure 9. Wnt/β-catenin signaling is increased in the Fuz null mice. Immunofluorescence on sagittal sections of E14.5 embryos. A) β-catenin expression was increased in the oral epithelium and mesenchyme in mutant (Fuz<sup>−/−</sup>) embryos. The bottom panels are the Fuz<sup>+/+</sup> embryos. B) β-catenin expression was also increased in the Fuz null Meckel’s cartilage (bottom panel), compared to the heterozygote (Fuz<sup>+/−</sup>, top panel) samples. C) Lef-1 expression in the dental epithelium, oral and dental mesenchyme at E14.5. Lef-1 expression increased in the Fuz null oral mesenchyme (bottom panel). D) These are higher magnification of the boxed areas in C. Lef-1 expression was expanded in the Fuz<sup>−/−</sup> mice oral mesenchyme. E) The expression of Tcf4 (Tcf7l2) was increased in Fuz mutant Meckel’s cartilage (bottom panels) at E14.5 compared with wild type samples (top panels). F) Real-time PCR with mRNA from dissected E14.5 Meckel’s cartilage and surrounding mesenchyme. Canonical Wnt target gene expression was increased whereas non-canonical Wnt pathway gene expression was decreased. β-actin served as the reference gene. Experiments were repeated three to five times each from multiple samples. G) Topflash reporter activity was repressed by co-transfection of Fuz in HEK 293FT and CHO cells. The activities are shown as mean fold activation compared to reporter activation co-transfected with pcDNA3.1 empty vector and normalized to SV-40 β-galactosidase activity. Error bars indicate S.E. *: p-values<0.05; **: p-values<0.01.

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expression vector and total RNA was harvested two days after transfection. Real-time PCR revealed that endogenous SOX9 and COL2A1 expression was significantly repressed in the Fuz transfected cells compared to those with control vector. These results indicate that Fuz represses Sox9 and cartilage expansion during craniofacial development. Loss of Fuz leads to increased Sox9 expression in the null mice, which maintains cartilage expansion in the presence of increased Wnt/β-catenin signaling.

**Discussion**

In this study we have analyzed the role of the Fuz gene during craniofacial development. Fuz loss of function analyses revealed a critical function of this gene in the development of multiple craniofacial tissues and structures. Fuz is required for the formation of eyes, bone, tongue and incisors. These affected organs and craniofacial structures show essential roles of Fuz in tissue patterning and cell proliferation.

**Fuz regulates Hh signaling**

In the Fuz null mice, down regulation of Hh signaling has been shown in the neural tube and limb buds [7,8]. In this study we have shown a decrease in Hh downstream gene expression in early and late craniofacial development. Hh signaling is essential for dorsal-ventral patterning and incisor development, but apparently not for molar development, as the molar tooth buds form normally in the Fuz−/− mice. A loss of Hh signaling in the oral epithelium results in a lack of epithelial cell proliferation and tooth bud formation [48]. This would explain the absence of incisor development, but not molar development. We suspect that this is a timing issue as incisors develop earlier than molars, however more experiments are required to understand the defect. Hh signaling is required for mouse brain and craniofacial morphogenesis and loss or gain of Hh function is associated with midline facial anomalies [49,50]. However, the overall craniofacial defects observed in the Fuz null mice do not resemble the other gene defects or Shh mutant mice, with the exception of brain and cleft palate defects. A recent report revealed a role for Fuz in the development of hair follicles [51]. Hair follicle development was impaired due to inhibition of cilia formation and Hh signaling, revealing a direct role for Fuz in regulating Hh signaling through defective cilia formation. Interestingly, Fuz regulated Hh signaling does not appear to regulate Meckel’s cartilage growth but may affect patterning and placement of Meckel’s cartilage in the mandible. Thus, Fuz could regulate Hh signaling which in turn restricts the normal growth and patterning of Meckel’s cartilage by influencing the surrounding tissue formation (such as incisor development) and other craniofacial structures.

**Fuz regulates Wnt signaling**

In the Fuz null mice, the Wnt/β-catenin pathway was up regulated whereas the non-canonical Wnt5a/Ror2 pathway was down regulated. These data suggest that Fuz is a potent repressor of Wnt/β-catenin signaling. The data also indicate that Fuz is critical to maintain the non-canonical Wnt signaling pathway, which is overlapped with PCP signaling. It provides a possible explanation of the neural tube defect (a typical phenotype in non-canonical Wnt deficient mice) previously reported in Fuz null mice [7,32]. The increased Meckel's cartilage growth in the Fuz−/− mice can be attributed to increased Wnt/β-catenin signaling. Thus, PCP, Wnt/β-catenin and Hh signaling pathways may converge to provide cues for and instruct specific cell differentiation, tissue patterning and morphogenesis. Fuz regulation of Wnt/β-catenin signaling may be mediated by primary cilia and interaction of multiple signaling pathways. Previous studies have reported that the basal component of primary cilium could repress Wnt/β-catenin signaling through interaction of Dishevelled protein [29,30]. The loss of Fuz could cause the secondary up-regulation of Wnt/β-catenin signaling. Previous studies have shown that Hh signaling antagonizes Wnt/β-catenin during development of the tongue and cartilage [37,38,53]. The increase in Wnt/β-catenin signaling could be secondary to decreased Hh signaling in the Fuz null mice. Non-canonical Wnt signaling is known to antagonize the canonical Wnt/β-catenin activity [17,45]. In particular, Wnt5a was shown to repress Wnt/β-catenin signaling in the limb buds [54]. An in vitro study revealed that the Ror2 receptor was necessary for Wnt5a repression of Wnt/β-catenin activity [55]. In the Fuz null mice, down-regulation of Wnt5a and Ror2 could attribute to activation of Wnt/β-catenin signaling as well. Whether it is one of those mechanisms or a combination requires further investigation.
Primary cilia defects associated with increased Hh signaling

In contrast to loss of Fuz, mice mutants for cilia intraflagellar transport (IFT) proteins; IFT88/polaris and Kif3a have increased Hh activity [50,56]. Mice with mutations in IFT88 lack cilia on all cells and present with severe neural tube defects, polydactyly, asymmetry defects and ectopic tooth formation [56,57,58]. Kif3a mice mutants display a range of similar developmental defects and a conditional knockout of Kif3a in the neural crest results in an increase in Hh activity associated with truncated cilia [50]. However, other groups have reported that mutations in IFT proteins including Kif3a demonstrate decreased Hh signaling [59,60,61]. These differences could be attributed to the differential tissue expression of the Gli proteins during development [50,56,60,62,63]. Our data reveal Fuz expression is predominantly expressed in the oral and dental epithelium at early stages and in epithelial cell lines. The Fuz+/− mice do not present with a wide facial prominence, which is indicative of increased Hh signaling [50]. Furthermore, as a PCP effector it may play an extended role in regulating signaling pathways and gene expression independent of cilia formation. However, unlike the Wnt and Hh signaling mechanisms, which act at the level of transcription, the PCP pathway controls cell morphology [17,45]. It is not inconceivable that changes in cell morphology induce changes in signaling mechanisms. The Fuz−/− mice have reduced and truncated cilia, but not a complete loss of primary cilia. The presence of truncated cilia in the Fuz null mice may play a limited role in the signaling activities. Fuz interacts with a Rab-similar GTPase (RSG1) to affect trafficking from the cytoplasm to basal bodies and to cilia tips [7]. Fuz may have a bigger role in exocytosis than ciliogenesis and could promote a cell type-specific regulation of signaling mechanisms and gene expression [7,17,64]. Transport and exocytosis facilitated by Fuz maybe be more important and explain the mouse craniofacial phenotypes of the Fuz null mice more than defective cilia.

Craniofacial Bone and Meckel’s Cartilage Defects. Runx2 plays a major role in bone development and specifically intramembranous bone formation during craniofacial development.
Fuz is not in the nucleus but distributed throughout the cytoplasm chromatin-remodeling complex [93]. Recent data reveals that OFD1 is nuclear localized and part of the human TIP60 facial-digital syndromes and is characterized by malformations of oral-cleft palate [80,81]. Sox9 is required for Meckel's cartilage growth and inactivation of Sox9 leads to reduced Meckel's cartilage expression may also contribute to the complex defects in craniofacial structures, delayed bone development and the hyperplastic and malformed Meckel's cartilage.

Materials and Methods

Ethics Statement

All animals were housed at the Institute of Biosciences and Technology under the care of the Program of Animal Resources, and were handled in accordance with the principles and procedure of the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Texas A&M Health Science Center, Institutional Animal Care and Use Committee. Protocol number 09001, mouse models for tooth development.

Animals

The Fuz gene trap mice were generated by the Texas A&M Institute for Genomic Medicine and were described previously [7]. These mice were maintained in the C57BL/6 background. FuzLacZ targeted ES cells corresponding to clone PG00134_Z_E03_2 were purchased from the Knock Out Mouse Project (KOMP) Repository and injected into blastocysts by the Texas A&M Institute for Genomic Medicine. Four chimeras were obtained and mated to wild-type C57BL/6 mice. Two chimeras yielded germ-line transmission. Offspring from both chimeras were used for X-gal staining and exhibited the same expression pattern. Embryos were collected at various time points, considering the day of observation of a vaginal plug to be embryonic day (E) 0.5. Genotyping PCR primers for FuzLacZ and Fuz were described previously [7]. Genotyping PCR primers for FuzLacZ are as below: forward, 5'-CTCTCTGCGCAGCAGTCTCTCC-3'; reverse, 5'-TTCTCTCTACATAGTTGGCAGTG-3'. The resulting PCR product represents the LacZ knockin allele, whereas the wild-type allele does not generate any products. All PCR products were sequenced to confirm their identity.

LacZ staining

Whole embryos of different stages were fixed for 20–40 minutes at room temperature in the fix solution (0.2% glutaraldehyde, 2% formaldehyde, 2 mM MgCl2, 5 mM EDTA pH 8.0 and 100 mM NaH2PO4 pH 7.3) and washed three times in rinse solution (0.2% Nonident P-40 and 0.1% sodium deoxycholate, 100 mM NaH2PO4 pH 7.5 and 2 mM MgCl2). Embryos were stained for 72 hour at 37°C in staining solution (1.65 mg/ml potassium ferricyanide, 1.84 mg/ml potassium ferrocyanide, 2 mM MgCl2, 1 mg/ml X-gal in rinse solution), rinsed in PBS and postfixed in 4% paraformaldehyde. Heads of both E12.5 and E14.5 embryos were dehydrated through alcohol, embedded in paraffin and sectioned at 16 μm thickness.
Histology and immunofluorescence

Samples were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin wax. Sections were cut (7 μm) and stained with Hematoxylin and Eosin. Some embryos were fixed in Bouin’s solution and subjected to Wilson section. Immunofluorescence was done on 7 μm paraffin sections with standard procedure. Antigen retrieval was done by boiling samples in 10 mM Sodium Citrate, pH 6.0. Antibodies were obtained and diluted as follows: Ki67 (Abcam, ab15580-100) 1:500; Ptch1 (Abcam, ab53715) 1:300; Gli2 (Abcam, ab7195) 1:300; β-catenin (Upstate, 06-734) 1:500; Lef1 (Cell Signaling, C12A5) 1:500; Tcf4 (Cell Signaling, C48H11) 1:500; Sox9 (Santa Cruz, sc-20095) 1:500. Secondary antibodies Alexa Fluor 488 goat anti-rabbit HCA were from Invitrogen (A11034) and used at 1:500 dilution. The Arl13b antibody (Abcam, ab83879) for cilia staining was used at 1:500 and visualized using a Zeiss Axiovert 200 confocal microscope. Skeletal defects are shown by using Alcian Blue/Alizarin Red staining of cartilage and bone in mouse (Cold Spring Harb Protoc; 2009).

Real-time PCR analyses

Meckel’s cartilage and surrounding mesenchyme were dissected from E14.5 mouse embryos. The Real-time PCR was performed with different probes listed in the table 1. Experiments were repeated three times each from multiple samples and p-values are shown. Total RNA was reverse transcribed into cDNA by iScript Select cDNA Synthesis kit (BioRad). Real-time PCR was carried out in a total reaction of 25 μl containing 12.5 μl SYBR Green Supermix, 0.1 μM forward primer, 0.1 μM reverse primer, 0.25 μl cDNA template in the MyiQ Singlecolor Real-Time Detection System and analyzed by the MyiQ Optical System Software 2.0 (BioRad). The Real-time PCR was performed with gene specific probes. β-actin served as a reference gene. The thermal cycling profile consisted of 95°C for 4 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec. Samples were run in triplicate. Experiments were repeated three times each from multiple samples and p-values were calculated. No-template control was run in each experiment. Melting curve analyses were performed to confirm amplification specificity of the PCR products. All PCR products were sequenced to confirm their identity.

Expression and reporter constructs

The expression plasmid containing the cytomegalovirus (CMV) promoter linked to the mouse Fuz full length cDNA reverse-transcribed from total RNA of NIH-3T3 cells was constructed into pcDNA3.1 vector (Invitrogen). The 7xTopFlash reporter plasmid was constructed into luciferase vector by inserting seven Lef/Tcf binding sites upstream of the minimal TK promoter [94]. The
mouse Fuz 2.4 kb promoter was constructed into the TK-luciferase vector by replacing the minimal TK promoter [95].

Cell culture, transient transfections, luciferase and β-galactosidase assays
CHO, HEK 293 FT, LS-8 and SW1393 cells (all cells were purchased from the ATCC, except LS-8 cells [96]) were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin and transfected expression vectors. All the plasmids were double-banded CsCl purified. The concentration of 10 mM LiCl was added to the specified cells at a final concentration of 10 mM, 23 h before harvest. The pcDNA3.1 empty vector was added to equalize the total amount of co-transfected expression vectors. All the plasmids were double-banded CsCl purified.

Chromatin Immunoprecipitation (ChIP) analyses
The ChIP analyses were performed as described [97] using the ChIP Assay Kit (Upstate) with the following modifications. LS-8 cells were fed for 24 h, harvested and plated in 60 mm dishes. The pcDNA3.1 empty vector was added to equalize the total amount of co-transfected expression vectors. All the plasmids were double-banded CsCl purified. The detailed robotic procedure can be found at http://empress.har.mrc.ac.uk/browser/ (gene expression section), [Chotteau-Lelie`vre, 2006]. Expression analysis of murine genes using in situ hybridization with radioactive and non-radioactively labeled probes. In: I.A. Darby and T.D. Hewitson, Editors, Methods Mol. Biol. (third ed.), In Situ Hybridization Protocols, Humana Press, Totowa, NJ (2006), pp. 61–87. Chotteau-Lelie`vre et al., 2006, using an Intavis InSitu Pro robot. The detailed robotic procedure can be found at http://empress.har.mrc.ac.uk/browser/ (gene expression section), [Chotteau-Lelie`vre, 2006]. Expression analysis of murine genes using in situ hybridization with radioactive and non-radioactively labeled probes. In: I.A. Darby and T.D. Hewitson, Editors, Methods Mol. Biol. (third ed.), In Situ Hybridization Protocols, Humana Press, Totowa, NJ (2006), pp. 61–87.

Statistics
Statistics were performed by two-sample t-test. P-values less than 0.05 were considered to be significant.

Table 1.

| Mouse | Fuz | 5′ | AAGCTACATCCACTGTCT | 3′ | Runx2 | F | 5′ | AACTCTCTGTGCCGGTGCT | 3′ |
|-------|-----|----|-------------------|----|-------|---|----|---------------------|----|
| Shh   | R   | 5′ | GATGAAATCCACATGAA  | 3′ | R     | 5′ | GCCATCGGTAACCCAGCAAGT | 3′ |
| Ptc1  | R   | 5′ | GGCTGTCTCGGCGATG  | 3′ | R     | 5′ | GAGACGGACATCGATGCAAGC | 3′ |
| Gli1  | R   | 5′ | GAGACCTATCAGCTTGAAG | 3′ | R     | 5′ | CCGACTTCTGCCATGAACCT | 3′ |
| Gli2  | R   | 5′ | GAGACTGTAGCTCTGAG  | 3′ | R     | 5′ | TTCATGAGCTGACAGCTGAGT | 3′ |
| Axin2  | R   | 5′ | ACAGGAACCTCGGCTGCT | 3′ | F     | 5′ | CACTTGAACCTCGGCTGCT | 3′ |
| Ccnd1  | R   | 5′ | GCAGCTGAGTGAACACGCCT | 3′ | R     | 5′ | CACAGGATGAAACGGCTTGG | 3′ |
| Ccnd2  | R   | 5′ | GAGACTGTGCTGCAAGATGC | 3′ | Col2a1 | 5′ | GGCTCCAATGATGGAGTATG | 3′ |
| Lef1   | R   | 5′ | GATGAGGAGCTGATCATT | 3′ | R     | 5′ | ACCACGACGACAGCTG | 3′ |
| Tcf4   | R   | 5′ | AATGGCCACTCAGCTTGCT | 3′ |  

| Human | FUZ | 5′ | GGAATCTAGAGCCGCGCTC CT | 3′ | COL2A1 | F | 5′ | TCTACCCCACATCCAGCAAC | 3′ |
|-------|-----|----|-------------------|----|-------|---|----|---------------------|----|
| SOX9  | R   | 5′ | CTGGTAGACCCTCAGCTTG | 3′ | R     | 5′ | GTGGAGGAGCTGATCATT | 3′ |
| Fuz   | R   | 5′ | TTGGAGATGACGTCGCTG | 3′ |  

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Plots were performed by two-sample t-test. P-values less than 0.05 were considered to be significant.
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Author Contributions

Conceived and designed the experiments: ZZ KN BAA. Performed the experiments: ZZ KN SV SF RHS. Analyzed the data: ZZ KN RHF BAA. Contributed reagents/materials/analysis tools: ZZ BJW KN SV SF RHF.
