Cranial Nerve Development Requires Co-Ordinated Shh and Canonical Wnt Signaling

Hiroshi Kurosaka1,2, Paul A. Trainor1,3*, Margot Leroux-Berger1,4, Angelo Iulianella5*

1 Stowers Institute for Medical Research, Kansas City, MO, United States of America, 2 Department of Orthodontics and Dentofacial Orthopedics, Graduate School of Dentistry, Osaka University, Osaka, Japan, 3 Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS, United States of America, 4 University Pierre and Marie Curie—Paris 6, Paris, France, 5 Department of Medical Neuroscience, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada

* angelo.iulianella@dal.ca (AI); PAT@stowers.org (PAT)

Abstract

Cranial nerves govern sensory and motor information exchange between the brain and tissues of the head and neck. The cranial nerves are derived from two specialized populations of cells, cranial neural crest cells and ectodermal placode cells. Defects in either cell type can result in cranial nerve developmental defects. Although several signaling pathways are known to regulate cranial nerve formation our understanding of how intercellular signaling between neural crest cells and placode cells is coordinated during cranial ganglia morphogenesis is poorly understood. Sonic Hedgehog (Shh) signaling is one key pathway that regulates multiple aspects of craniofacial development, but whether it co-ordinates cranial neural crest cell and placodal cell interactions during cranial ganglia development remains unclear. In this study we examined a new Patched1 (Ptch1) loss-of-function mouse mutant and characterized the role of Ptch1 in regulating Shh signaling during cranial ganglia development. Ptch1Wig/ Wig mutants exhibit elevated Shh signaling in concert with disorganization of the trigeminal and facial nerves. Importantly, we discovered that enhanced Shh signaling suppressed canonical Wnt signaling in the cranial nerve region. This critically affected the survival and migration of cranial neural crest cells and the development of placodal cells as well as the integration between neural crest and placodes. Collectively, our findings highlight a novel and critical role for Shh signaling in cranial nerve development via the cross regulation of canonical Wnt signaling.

Introduction

The cranial nerves are part of the peripheral nervous system that governs various critical functions such as sensing and controlling movement within the craniofacial region. Previous studies in avian embryos have shown that some of the cranial nerves including the trigeminal (V) and facial nerves (VII) originate from both cranial neural crest cells and ectodermal placode cells [1,2]. Cranial neural crest cells arise in the dorsal neuroepithelium, delaminate via an epithelial to mesenchymal transformation, and migrate sub- ectodermally throughout the head...
and neck. In the peripheral nervous system, cranial neural crest cells generate neurons and glia. In contrast, ectodermal placodes comprise thickened regions of surface ectoderm cells, which are distinct from the neuroepithelium. Ectodermal placode cells delaminate from the surface ectoderm to establish the neurogenic core of the cranial nerves [3]. Cellular interactions between neural crest cells and placode cells are essential for proper cranial nerve patterning [4–6], and many signaling pathways influence cranial nerve formation in vertebrates by regulating cranial neural crest and/or ectodermal placode cell development [7]. However, our knowledge of how, and in what cell type or tissue these signals primarily function, and also how these different signaling pathways interact remains limited. This is due in part to the early embryonic lethality of many mutants in key developmental pathways.

In a previous study, we performed an N-ethyl-N-nitrosourea (ENU) mutagenesis screen in mice and identified multiple recessive alleles important for craniofacial development [8]. Here we characterize one of these ENU induced mutants called Wiggable (Wig) that carries a mutation in the Patched1 (Ptch1) gene. Ptch1 encodes a receptor for the Hedgehog family of morphogens which includes Sonic Hedgehog (Shh). Unlike Ptch1 null mutant mice which are lethal at E9.5 [9], Ptch1Wig/Wig mutants survive until E12.0, allowing an analysis of the effects of aberrant Shh signaling on cranial ganglia morphogenesis. In this study, we took advantage of multiple mouse mutants to clarify the role of cross-talk between the Shh and WNT signaling pathways during the formation of the trigeminal and facial nerves. We discovered that elevated Shh signaling restricts canonical Wnt signaling during cranial ganglia development. This affects the survival of migrating neural crest cells, the pattern of placode development and the integration between neural crest cells and placode cells. Our findings describe the importance of cross-talk between Shh and Wnt signaling in regulating tissue interactions during cranial nerve development.

Materials and Methods

Ethics Statement

This study was carried out in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol (2013–0115) was approved by the Institutional Animal Care and Use Committee of The Stowers Institute for Medical Research. Adult mice were euthanized via CO2 and cervical dislocation according to the recommendations of the American Veterinary Medical Association and all efforts were made to minimize any potential suffering.

Mouse Lines

Ptch1Wig, Ptch1LacZ, Wnt1Cre, R26RYFP, TOPgal and HhatCreface mice were maintained as described previously [8–14]. The morning of vaginal plug identification was defined as E0.5 for embryo collection and staging. We designated Ptch1Wig/Wig as Wig homozygous mutants and HhatCreface/Creface;Ptch1Wig/Wig as double-homozygous mice. Either wild-type or heterozygous littermates were used as control mice described in this study. Unless otherwise indicated, we used a minimum of 4 or 5 embryos from multiple distinct litters for each parameter analyzed in this study.

Generation and identification of the Ptch1Wig mouse mutation

The Wiggable (or Wig) mutation was generated in a previously described N-ethyl-N-nitrosourea (ENU) screen for recessive mutations leading to craniofacial and neural tube defects [8]. Briefly, mutagenized fathers from a hybrid C57Bl/6/SV129J background were outcrossed
to FVB females to generate founders. The male founders were subsequently mated to FVB females and the resulting daughters were backcrossed to the founders to identify recessive mutant phenotypes. One phenotype encompassed a kinked and dysmorphogenic neural tube in the craniofacial region and was termed Wiggable to reflect the superficial resemblance to Baroque period English wigs. DNA from mutant and control littermate embryos was collected along with tail biopsies from founder males and subjected to microsatellite and single nucleotide polymorphism (SNP) mapping, which narrowed down the affected region to mouse chromosome 13 (qB2 to qB3). This region is gene dense but contained only a few genes known to regulate embryonic neural development, including Ptch1. We used a candidate approach to sequence the genomic (intron/exon) regions of the genes residing in the minimal interval. Sequencing primers were designed to cover the entire mouse Ptch1 gene (available upon request), which was one of our prioritized gene candidates in the minimal affected region, and we discovered a T to A substitution in the 3’ end of intron 15 which segregated with the mutant phenotype (S1 Fig.).

Validation of the Ptch1\textsuperscript{Wig} mutation by qPCR

RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and quantified using a Bioanalyzer. Primer Express v3.0 was used to make the primers and probes covering the splice junction between exons 15 and 16 of the Ptch1 gene. Amplicon length was 92 base pairs (bp) for the wild type product and 99bp for the Wig mutant product. The following primers were used to generate the amplicon: CAA GCA ACT TCC CCA AAT GTG and ATC CTC CCA GTT TTC CAG TCA. Actin endogenous control probe and primers were ordered as a pre-designed assay from Applied Biosystems (Mouse ACTB (actin, beta) Endogenous Control # 4352933E). For the mutation site, the following dye-coupled sequences were used: Ptch1 wild type probe: 6FAM—TGG CTT CAA GGA CTT C—MGBNFQ; Ptch1\textsuperscript{Wig} mutant probe: VIC—ACT GGC TTC AAG TTT CTA GGA—MGBNFQ. The linear dynamic range of the qPCR assay was determined by making serial dilutions of the RNA and performing the reverse transcription (RT) reaction and subsequent amplification using the wild type, mutant and Actin endogenous control probes and primers. For the reverse transcription (RT) reaction, we utilized the high capacity cDNA reverse transcription kit from Applied Biosystems (Life Technologies, Grand Island, NY), using 100ng of RNA per reaction in a total volume of 20μL. Each sample was then split into 3 reactions where the wild type, mutant and endogenous control primers and probes were added. The cycling conditions for the cDNA amplification were: denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 seconds (sec) and 60°C for 1 minute (min). Each sample was genotyped by amplification of genomic DNA with forward and reverse primers detailed above using the following cycling conditions: 95°C for 2 min then 32 cycles of 95°C/30sec, 60°C/30 sec, and 72°C/30 sec.) The resulting amplicon was sequenced from both directions using the same primers and aligned to the expected sequence to identify the mutation polymorphism (S2 Fig.).

Cloning of the Ptch1\textsuperscript{Wig} mutation and Western blot

The sequencing of Wiggable (Wig) founders and their offspring confirmed the mutation introduced a 7 base pair insertion into the Ptch1 cDNA (S1 Fig.). This in turn was predicted to generate a TGA codon 17 base pairs downstream of Exon 16, producing a premature truncation at position 848 in the mouse PTCH1 protein. To model the Wig mutation in cell culture, we used the QuikChange In Vitro mutagenesis kit (Agilent, Santa Clara, CA) to introduce the 7 bp insertion into the full-length mouse Ptch1 cDNA which was cloned in the expression vector pcDNA3.1 (gift from Dr. Kazushi Aoto). The insertion was confirmed by sequencing. Wild
type Ptch1 or Ptch1Wig was transfected into Hek293T cell lines and after 48 hours culture, protein was extracted using a standard lysis buffer (20mM Tris HCl pH8, 137mM NaCl, 10% glycerol, 1% nonidet P-40, 2mM EDTA, protease inhibitors). Protein was quantified using a BSA curve and 40–60ng of protein was loaded per lane. Blots were transferred onto PVDF membranes (Bio-Rad, Hercules, CA) and blocked with 5% BSA solution. To detect PTCH1 protein, blots were probed with an amino-terminal specific PTCH1 antibody (G-19, Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000).

Complementation of the Ptch1Wig mutation with the Ptch1LacZ null allele
To test whether the Wig mutation was indeed a loss-of-function mutation in Ptch1, we conducted a functional complementation assay with Ptch1LacZ/+ mice (Jackson Laboratory strain 003081). The Ptch1LacZ allele contains a lacZ-neo fusion gene that disrupts endogenous Ptch1 gene function [9]. Ptch1LacZ/LacZ embryos exhibit neural tube abnormalities and are typically lethal at E9.5. Ptch1Wig/+ heterozygotes were interbred with Ptch1LacZ/+ heterozygotes to generate embryos which were genotyped for the presence of both Wig and LacZ alleles. β-galactosidase staining (described below) was evaluated to determine the spatiotemporal expression of the Ptch1 locus in the Ptch1Wig/LacZ mutants.

Immunostaining and cell death detection
Whole-mount immunostaining of mouse embryos was performed as previously described [8,15] using antibodies against Neuronal Class III β-tubulin (TUJ1) (Covance, 1:1000), Neurofilament (Developmental Studies Hybridoma Bank, 2H3, 1:1000) and GFP (Invitrogen, 1:500). For section immunohistochemistry, embryos were fixed in 1% Formaldehyde in PBT overnight at 4°C and were processed through a sucrose gradient, embedded in Tissue-Tek (OCT compound, Sakura), and cut into 10-μm-thick sections. The slides were washed in TBST (TBS with 0.1% Tween) and blocked with 1% BSA in TBST for 1 hour at room temperature. Slides were incubated with anti-SOX10 (R&D systems, 1:50), anti-PAX3 (Developmental Studies Hybridoma Bank, 1:100), anti-TUJ1 (Covance, 1:1000), anti-GFP (Invitrogen, 1:500) with 1% BSA in TBST for overnight at 4°C. After several washes in TBST, the slides were incubated using the appropriate secondary antibody for 1 hour at room temperature and counterstained with DAPI (Sigma-Aldrich) (1:1,000) for 10 minutes at room temperature and mounted with fluorescent mounting medium (DakoCytomation). Apoptosis was visualized using the In Situ Cell Death Detection Kit Fluorescein (Roche) according to the manufacturer’s instructions.

In situ hybridization
Whole-mount in situ hybridization and sectioning was performed as previously described [10].

β-galactosidase staining
TOPgal reporter mice [11] were bred into the Ptch1Wig/+ and HhatCreface/Ptch1Wig/+ mice and embryos were stained for LacZ activity using the β-galactosidase Staining Solution Kit (Chemicon/Millipore) according to the manufacturer’s instructions.

Results
Truncated PTCH1 protein leads to excessive Shh signaling during mouse embryonic development
Through an ENU mutagenesis screen for recessive embryonic phenotypes, we isolated a mutant we named Wiggable (Wig) due to a kinked and overproliferative neural tube that superficially
resembled an English wig (Fig. 1) [8]. Microsatellite and SNP mapping narrowed the candidate region to mouse Chromosome 13 (qB2-qB3). We then focused on candidate genes within the interval known to have embryonic patterning defects. Among them was Patched1 (Ptch1), which encodes a receptor for Hedgehog ligands [9]. Wig ENU mutants segregated with a T to A substitution in the 3’ end of intron 15 of Ptch1, which creates a novel splice acceptor site and produces a 7 base pair insertion in the 5’ end of Exon 16 (Fig. 1A, S1 Fig.). RT-PCR sequencing of cDNAs derived from Wig carrier mouse embryos confirmed the presence of a 7 base pair insertion in the cDNA of Ptch1 (S1 Fig.). This in turn created a premature stop codon 17 base pairs into exon 16 which is predicted to truncate the mouse PTCH1 protein at amino acid position 848. The full-length mouse PTCH1 protein is 1424 amino acids, and the mutation truncates the protein within the 6th extracellular loop, keeping the sterol-sensing domain intact, but abrogating part of the Hedgehog-interacting extracellular domain. Since most of the C-terminal domain is lacking, the Wig ENU mutation is expected to create a Ptch1 loss-of-function allele and abrogate PTCH1 inhibition of Smoothened signaling. In support of this idea, qPCR analysis of Ptch1 expression revealed an approximately 4-fold increase in Ptch1 locus transcription in Ptch1\textsuperscript{Wig/Wig} mutants relative to wild-type controls (S2 Fig.). This reflects the fact that the Ptch1 locus is under the regulatory control of GLI proteins, and also that Ptch1 loss of function mutations result in the hyper-activation of Shh target genes [16].
The Wig allele of Ptch1 was cloned to test whether it would create a truncated protein in transfected Hek293T cells. Using amino (N)-terminal-specific Ptch1 antibodies to probe the resulting Western blot, we confirmed that the Wig allele of Ptch1 did indeed produce a stable truncated form of PTCH1 that migrated at ~90kDa (Fig. 1B). To further evaluate the effect of the Wig mutation on Ptch1 mRNAs levels, we performed a qPCR experiment using mRNA extracted from E10.5 embryos obtained from Ptch1Wig/+ intercrosses (S2 Fig.). Ptch1Wig/Wig mutants (red bars, specimens 216, 218, 219, and 224) expressed only the mutant form of Ptch1, with undetectable levels of wild type Ptch1 mRNA expression (S2 Fig.). Ptch1Wig/+ heterozygotes (Specimens 220–222) showed approximately equal levels of both the wild type and Wig mutant Ptch1 mRNA. No Ptch1Wig mutant mRNA was detected in wild type embryos. Interestingly, in the Ptch1Wig/Wig embryos Ptch1 mRNA levels were highly elevated, consistent with deregulated Shh signaling. This is because the Ptch1 locus is under the direct transcriptional regulation of Shh signaling through the binding of GLI transcription factors [16].

We next performed a genetic complementation assay of the Ptch1Wig allele with the Ptch1LacZ mutation [9]. The Ptch1LacZ mutation creates a null allele that in Ptch1LacZ/LacZ embryos leads to severe patterning defects and lethality at E9.5. In contrast the Ptch1Wig mutation is less severe, with Ptch1Wig/Wig embryos surviving until E11.5–12.5 (Fig. 1C,D), although this could be due in part to strain background differences. The Ptch1LacZ allele can also be used to monitor Ptch1 locus activity via β-galactosidase staining, and is strongly expressed in ventral neural tissues and the endoderm in E9.5 control embryos (Fig. 1E). While Ptch1LacZ/+ and PtchWig/+ heterozygotes are normal (Fig. 1E), Ptch1LacZ/Wig mutants exhibited severe neural tube closure and brain patterning defects together with early embryonic lethality (Fig. 1F). The failure to complement confirmed that the Wig mutation is a novel loss-of-function allele of Ptch1. The fact that β-galactosidase staining was more intense in the Ptch1LacZ/Wig E9.5 embryos than in controls is indicative of elevated Hh signaling and is consistent with the Ptch1 locus being a direct target of activated Shh signaling (Fig. 1F, S2 Fig.).

Ptch1Wig/Wig embryos exhibit disrupted cranial nerve development

The survival of Ptch1Wig/Wig mutants until E11.5–12.5 afforded us the opportunity to examine the role of elevated Shh signaling in the formation and patterning of the cranial nerves. Immunohistochemistry on E9.5 control embryos revealed that the trigeminal (V) and facial nerves (VII) develop as clusters of Neuronal Class III β-tubulin (TUJ1) labeled neurons (Fig. 2A). In contrast, Ptch1Wig/Wig mutant embryos exhibited severely disorganized trigeminal and facial nerves (Fig. 2D). A similar phenotype could be seen via neurofilament (2H3) staining (Fig. 2B and E). By E10.5 the axonal branches of the trigeminal and facial nerves should have begun projecting to the distal facial tissues they eventually innervate as can be seen in control embryos (Fig. 2C). However, in Ptch1Wig/Wig mutant embryos the cranial nerves do not develop properly or project to their appropriate target tissues (Fig. 2F). These results suggest that the Ptch1Wig/Wig mutation perturbs cranial nerve development presumably in association with elevated Shh signaling.

Ptch1Wig/Wig mutation results in cranial neural crest cell death

Cranial nerves are derived from both cranial neural crest cells and ectodermal placodes. The defects in cranial nerve development in Ptch1Wig/Wig mutant embryos could therefore reflect abnormal neural crest formation and migration, and/or aberrant placode cell development. To address the role of the cranial neural crest in the pathogenesis of the Wig phenotype, we crossed Ptch1Wig/+ mice into Wnt1Cre;R26RYFP to genetically label cranial neural crest cells

PLOS ONE | DOI:10.1371/journal.pone.0120821 March 23, 2015 6/19
and their derivatives [13,14]. We did not observe any major defects in the neural crest cells emerging from the anterior hindbrain and migrating into the first pharyngeal arch in Ptch1Wig/Wig embryos (Fig. 3A vs Fig. 3E; red arrowhead). However, there were clearly far fewer migrating neural crest cells within the second pharyngeal arch and facial nerve regions in Ptch1Wig/Wig mutants compared to controls (Fig. 3A vs Fig. 3E; white arrowhead). We used Sox10 staining to further evaluate the effect of the Wig mutation on the migratory cranial neural crest cells. Interestingly, Ptch1Wig/Wig mutant embryos exhibited fewer SOX10 protein (Fig. 3B vs Fig. 3F; white arrow) and Sox10 mRNA (Fig. 3D vs Fig. 3H; red arrowhead) positive neural crest cells specifically within the opV region relative to controls. However in contrast to the situation in the opV region, and taking into account the reduced number of GFP-positive neural crest cells in the facial nerve region, the relative proportion of SOX10/Sox10 positive neural crest cells in Ptch1Wig/Wig mutants embryos was similar to controls (Fig. 3C vs Fig. 3G, Fig. 3D vs Fig. 3H; white arrowhead). Thus there is a consistent diminishment of neural crest cells in both the opV and facial nerve regions in Ptch1Wig/Wig compared to controls.

Ptch1 together with Hh signaling are collectively known to play critical roles in cell proliferation and survival [17–19]. Therefore we hypothesized that the diminished neural crest cell population in the opV and facial nerve regions of Ptch1Wig/Wig embryos may be due to apoptosis of neural crest cells. Indeed, TUNEL staining in combination with GFP labeling of neural crest cells revealed a substantial elevation of cell death specifically in neural crest cells in the opV region of Ptch1Wig/Wig mutants compared to control littermates (Fig. 3I vs Fig. 3M). A similar increase of cell death in GFP-positive neural crest cells was also observed in the facial nerve region of Ptch1Wig/Wig mutants compared to controls (Fig. 3I vs Fig. 3N). Consistent with these results, TUNEL staining together with SOX10 immunohistochemistry also revealed a significant elevation of cell death in cranial neural crest cells in Ptch1Wig/Wig mutants (S3 Fig.). In
contrast, we did not observe any difference in apoptosis of TUJ1 positive neuronal cells either in the opV or facial nerve of control versus Ptch1Wig/Wig embryos (S3 Fig.). These findings demonstrate that aberrant cranial nerve development in Ptch1Wig/Wig embryos is associated with elevated apoptosis of migratory cranial neural crest cells but not of post-mitotic neuronal cells. Since the Wig mutation in Ptch1 results in elevated Shh signaling, our results suggest that the
spatiotemporal activity of this important mitogen has important consequences for the survival of migratory cranial neural crest cells during cranial nerve development.

We recently discovered that Shh signaling restricts the extent of canonical Wnt signaling in the developing craniofacial prominences [10]. Furthermore it has been well established that Wnt signaling is important for neural crest cell survival, as well as development and patterning of the cranial nerves [20–23]. Therefore we investigated the possibility that elevated Shh signaling in the $Ptch1^{Wig/Wig}$ mutants regulates canonical Wnt signaling in the migratory crest and/or placodes, leading to the pathogenesis of cranial nerve defects. Firstly, we performed in situ hybridization for $Wnt1$, which demarcates the dorsal neuroepithelial territory from which neural crest cells are derived and plays an important role in neural crest development and survival [23]. We found a marked down-regulation of $Wnt1$ expression in the neuroepithelium of $Ptch1^{Wig/Wig}$ mutants (Fig. 3O vs Fig. 3K, arrowheads). Secondly, to further characterize the possibility of an association of perturbed canonical Wnt signaling with aberrant cranial nerve development in $Ptch1^{Wig/Wig}$ mutants, we crossed $Ptch1^{Wig/+}$ mice with the $TOPgal$ reporter [11]. We observed a considerable reduction of $TOPgal$ activity in $Ptch1^{Wig/Wig}$ mutant embryos compared to controls (Fig. 3P, vs. Fig. 3L, arrowheads). These observations are consistent with previous work that has shown that $Ctnnb1$ (β-catenin) loss-of-function in migrating neural crest cells leads to neural crest cell death [22]. Collectively, these results illustrate that elevated Shh signaling restricts canonical Wnt signaling in cranial neural crest cells and influences their survival.

$Ptch1^{Wig}$ mutants display defects in cranial placodal formation

In addition to cranial neural crest cells, ectodermal placode cells provide major contributions to the development of the cranial ganglia [24]. We therefore explored whether cranial placode development was altered in association with elevated Shh signaling in $Ptch1^{Wig/Wig}$ mutant embryos. We used PAX3 and SOX10 co-immunostaining to define the placode cells of the trigeminal region [25], and $Ngn1$, $Ngn2$, and $NeuroD$ expression via in situ hybridization as indicators of neuronal maturation in the placode [26]. We observed a marked diminution of PAX3-positive cells in $Ptch1^{Wig/Wig}$ embryos (Fig. 4A vs. Fig. 4D; green arrowhead). This reduction was further confirmed by Pax3 mRNA in situ hybridization (Fig. 4B-C vs Fig. 4E-F). Similarly, $Ngn1$, $Ngn2$ and $NeuroD$ expression were all consistently down-regulated in the trigeminal placode and particularly in the opV branch in $Ptch1^{Wig/Wig}$ mutants compared to controls (Fig. 4G-I vs. Fig. 4J-L, red arrowhead). The downregulation of placodal markers in the $Ptch1^{Wig/Wig}$ mutants could be a result of aberrant development of placodal cells. This likely contributed to the defects in cranial nerve development in the mutants, together with the increased cell death in migrating cranial neural crest cells.

$Ptch1^{Wig/Wig}$ embryos exhibit less integration between cranial neural crest cells and ectodermal placode cells

Cellular and molecular interactions between cranial neural crest cells and ectodermal placodes are essential for proper cranial nerve development [4]. Given that we observed defects in the survival of migrating cranial neural crest cells together with defects in placode development, we carefully examined the interactions between these two cell types during cranial nerve development via TUJ1 immunostaining combined with $Wnt1Cre;R26RYFP$ in control and $Ptch1^{Wig/Wig}$ embryos. Clear cellular integration between neural crest cells and placode cells was observed in the developing trigeminal (Fig. 5A) and facial nerve of control E9.5 control embryos (Fig. 5B). In contrast, $Ptch1^{Wig/Wig}$ mutants exhibited considerably reduced mixing of migrating neural crest cells and placodal cells (Fig. 5C-D). Thus, increased Shh signaling in $Ptch1^{Wig/Wig}$ mutants,
Fig 4. Enhanced Shh signaling in Ptch1Wig/Wig mutants affects cranial placode development. (A and D) Immunostaining of PAX3 (green), SOX10 (red), and DAPI (blue) in wild type (A) and Ptch1Wig/Wig mutants (D). PAX3-positive cells (placodal cells) and PAX3/SOX10 double-positive cells (neural crest cells) are identified by green and white arrowheads respectively. (B, C, E and F) Pax3 mRNA expression in control (B and C) vs. Ptch1Wig/Wig mutants (E and F).
which results in the apoptosis of neural crest cells destined for the trigeminal and facial nerves is associated with the diminished integration between neural crest and placode cells. This contributes to the aberrant cranial nerve development observed in $Ptch^Wig/Wig$ embryos compared to controls.

Reduced Shh signaling restores canonical Wnt signaling and early cranial nerve development

The observation that elevated Shh signaling results in diminished canonical Wnt signaling in association with cranial nerve defects (Fig. 3K and L vs Fig. 3O and P) raised the idea that genetically reducing Shh signaling in $Ptch^Wig/Wig$ embryos might restore Wnt signaling to normal and rescue cranial nerve development. We therefore used the Hedgehog palmitoylase ($Hhat$) loss-of-function mutant ($Hhat^{creface/creface}$) to lower the levels of Shh signaling in $Ptch^Wig/Wig$ mutants by producing compound mutants. The disruption of $Hhat$ diminishes palmitoylation of SHH which perturbs its secretion and long range activity [12,27]. Furthermore, we also bred the $TOPgal$ reporter into $Hhat^{creface/creface};Ptch^Wig/Wig$ compound mutant mice to visualize the spatiotemporal activity of canonical Wnt signaling. In E9.5 $Ptch^Wig/Wig$ embryos, Wnt signaling as measured by $TOPgal$ activity was diminished in the trigeminal (red arrowhead) and facial (white arrowhead) nerve regions (Fig. 6A-B). Strikingly, $TOPgal$ activity was restored to wild-type levels in $Ptch^Wig/Wig;Hhat^{creface/creface}$ compound mutant embryos (Fig. 6C). Furthermore, $Sox10$ in situ hybridization confirmed that while $Ptch^Wig/Wig$ embryos exhibited reduced migrating neural crest cells and hypoplasia of the opV branch of the trigeminal nerve as well as the facial nerve (Fig. 6E), cranial nerve patterning was largely restored in $Ptch^Wig/Wig;Hhat^{creface/creface}$ double mutants (Fig. 6F, compare with Fig. 6D). Collectively, these findings suggest that an appropriate balance between Shh and Wnt signaling is required to ensure the survival of cranial neural crest cells and proper development of the cranial neurogenic placodes which are essential for the orchestrated integration of these two cell populations during cranial nerve development.

Discussion

Proper formation of the cranial nerves relies on dynamic tissue interactions mediated by multiple signaling pathways. In this study, we discovered that the interplay between neural crest cells and placodes cells depends on feedback regulation of Shh and Wnt signaling. We focused on patterning of the trigeminal and facial nerves because of their stereotypical morphology and uncovered a causal link between migratory neural crest cell survival and a balance between Shh signaling and the canonical Wnt pathway.

Using $Ptch^Wig/Wig$ mice to study the role of Shh signaling in early embryogenesis

$Ptch^{LacZ}$ is a null allele of $Ptch1$ that is commonly used to study the distribution, level and effects of perturbed Shh signaling during embryogenesis [9]. However, $Ptch^{LacZ/LacZ}$ homozygous mice are lethal at E9.5 and thus do not survive long enough to examine the effects of elevated Shh signaling on cranial nerve development. In contrast, $Ptch^Wig/Wig$ homozygous mutants
Fig 5. Increased Shh signaling in $\text{Ptch1}^{-\text{Wig}}$ mutants resulted in less cellular interaction between neural crest and placodal cells. (A-D) $\text{Wnt1Cre}; R26\text{RYFP}$ fate mapping in control wild type (A and B) and $\text{Ptch1}^{-\text{Wig}/\text{Wig}}$ mutants (C and D) at E9.5. The YFP fate-mapped cells were identified by GFP immunostaining (green) and neurogenic placode cells were identified by TUJ1 staining (red) in the opthalamic (A and C) and facial nerve (B and D) region. The section planes in A-D are the same as those indicated in Fig. 3B,C,F and G. $\text{Ptch1}^{-\text{Wig}/\text{Wig}}$ mutants (C and D) displayed much less neural crest cell (green) admixture within the ophthalmic and geniculate placodes relative to controls (A and B). Scale bars: 20$\mu$m (A and C); 50$\mu$m (B and D).

doi:10.1371/journal.pone.0120821.g005
survive until around E12.5 (Fig. 1), which makes them amendable to study the effect of elevated Shh signaling on cranial nerve development.

Elevated Shh signaling disrupts cranial nerve development via increased apoptosis of cranial neural crest cells

We observed that Ptc1\textsuperscript{Wig/Wig} embryos exhibited disorganized trigeminal and facial nerve development (Fig. 2). These results are consistent with observations that ectopic SHH administration in chick embryos leads to abnormal trigeminal nerve development [28]. In order to investigate the mechanisms underlying this phenotype, we first explored a role for apoptosis using TUNEL staining together with tissue specific markers and Wnt1Cre;R26RYFP reporter mice. Surprisingly, we detected no differences in cell death between control and Ptc1\textsuperscript{Wig/Wig} embryos in the trigeminal or facial nerves (S3 Fig.). However, Ptc1\textsuperscript{Wig/Wig} embryos exhibited substantially elevated levels of cranial neural crest cell apoptosis (Fig. 3 and S3 Fig.). These results are consistent with other observations that apoptosis of neural crest cells disrupts cranial nerve development in mice [29] and also that ablation of premigratory neural crest cells in chicken embryos results in disorganized trigeminal nerve formation [2,4,30,31]. Collectively, these findings argue that aberrant cranial nerve formation in Ptc1\textsuperscript{Wig/Wig} mutant embryos arises primarily as a consequence of a diminished population of cranial neural crest cells, which are lost via apoptosis.
Increased cell death in cranial neural crest cells in $Ptch1^{Wig}$ embryos associated with reduction of survival factors

Using the $Wnt1Cre;R26RYFP$ reporter line [11,13,14] in combination with $Sox10$ staining, we confirmed elevated apoptosis of cranial neural crest cells in $Ptch1^{Wig/Wig}$ embryos (Fig. 3). We noted the greatest reduction of $SOX10$ positive cells in $Ptch1^{Wig/Wig}$ embryos occurred in the oPv region of the trigeminal nerve and was associated with the apoptotic loss of neural crest cells that typically invade that territory of the neurogenic placode. This prompted us to look for the source of survival signals that act downstream of Shh signaling to promote the survival of neural crest and placodal cells as they interact to form mature cranial nerves.

Elevated Shh signaling had been shown to restrict canonical Wnt signaling in various developmental contexts [10,32,33]. Moreover, Ctnnb1 is thought to function as a survival factor for migrating neural crest cells, and is necessary for activating canonical Wnt signaling in these cells [22,34]. Consistent with these observations, $Ptch1^{Wig/Wig}$ mutants display a reduction in canonical Wnt signaling in migrating cranial neural crest cells together with elevated cell death (Fig. 3). Furthermore, we succeeded in partially restoring cranial nerve development by reducing Shh signaling in $Hhat^{Cre-face/Cre-face}; Ptch1^{Wig/Wig}$ double mutants. In these double mutants, Hh ligands are not palmitoylated and secreted properly to form a long-range signaling gradient. In addition, because the Wig lesion results in a C-terminal truncation of Ptch1, Smoothened is presumably no longer inhibited and is therefore able to activate Shh signaling effectors. The compound $Hhat^{Cre-face/Cre-face}; Ptch1^{Wig/Wig}$ mutants exhibit a restoration of Shh signaling levels to that of normal embryos [10]. Consistent with this result, we observed a restoration of normal cranial nerve development in the $Hhat^{Cre-face/Cre-face}; Ptch1^{Wig/Wig}$ compound mutants, which was accompanied by a restoration of canonical Wnt-dependent survival signaling, resulting in elevated neural crest cell death and consequently cranial nerve developmental defects.

Possible pathway to inhibit trigeminal placode development from elevated Shh signaling

Ectodermal placodes are one of the two principal sources of cells that contribute to the cranial nerves with the other being the neural crest [1,2]. Pax3 is widely used as a marker of trigeminal placodal cells [25] and Pax3 loss-of-function results in defects in trigeminal nerve development [35]. $Ptch1^{Wig/Wig}$ mutant embryos exhibited a significant reduction in Pax3 expressing cells, consistent with a specific effect on trigeminal nerve development (Fig. 4). Interestingly, Wnt signaling [36,37] is required for Pax3 expression in the trigeminal placode, and a recent study demonstrated direct regulation of Pax3 by CTNNB1 [38]. Thus it is possible that the reduction of Pax3 expression in $Ptch1^{Wig/Wig}$ embryos is due to the down-regulation of canonical Wnt-dependent survival signaling, resulting in elevated neural crest cell death and consequently cranial nerve developmental defects.
Possible contribution of poor intermixed cranial neural crest and placodal cells to cranial nerve defects

In this study, we observed relatively poor integration between neural crest and neurogenic cells in the trigeminal and facial nerves of $Ptch1^{Wig/Wig}$ embryos compared to controls (Figs. 5 and 7). Recent studies have shown that neural crest cells play an important role during cranial nerve development by forming “corridors” or “conduits” which guide and surround neuronal cells [5,15]. Thus, any loss of migratory neural crest populations can impact cranial nerve formation by severing the cellular conduit for nascent axons growing out of the placodal cores of the cranial ganglia. In terms of molecular signals that can orchestrate cranial neural crest cell and placodal interactions, several studies have shown that cell adhesion molecules such as $N$-cadherin ($Cdh2$) or $aN$-catenin ($Ctnna2$) are important for cranial nerve integration [6,41]. Excessive $N$-cadherin ($Cdh2$) expression in Xenopus results in abnormal aggregation and migration of neural crest cells [42]. This suggests that inadequate cell adhesion in neural crest cells could affect the integration of neural crest and placodal cells. Furthermore, CTNNB1 can regulate cell adhesion by interacting with Cadherins [43]. Collectively, these studies suggest that the disorganized cranial nerves in $Ptch1^{Wig/Wig}$ embryos may also be due in part to disrupted Wnt signaling-dependent cell adhesion in cranial neural crest or/and placode cells, in addition to the reduced numbers of neural crest cells that is caused by apoptosis.

Elevated Shh signaling results in various well characterized craniofacial anomalies including anencephaly, hypertelorism, and cleft lip and palate [10,44,45] as well as a disease spectrum in humans called congenital cranial dysinnervation disorders, represented by Duane retraction syndrome (DRS) or Moebius syndrome (MBS) [46,47]. MBS is characterized by specific cranial nerve defects including trigeminal, abducens (VI) and facial nerves together with hypertelorism, which is a phenotype known to correlate with elevated Shh signaling [48,49]. Our results

![Diagram](https://www.plosone.org/doi/abs/10.1371/journal.pone.0120821.g007)

**Fig 7. Schematic summary of elevated Shh signaling resulting in cranial nerve defects.** (A) During normal development, neural crest cells migrating from rhombomere 2 (r2) or 4 (r4) interact with placodal cells to develop the cranial nerves. (B) $Ptch1^{Wig/Wig}$ embryos exhibit excessive Shh signaling leading to a reduction in neural crest cells and survival factors, which results in reduced neural crest cell-placode interactions and cranial nerve patterning defects.

<sup>Fig 7. Schematic summary of elevated Shh signaling resulting in cranial nerve defects. (A) During normal development, neural crest cells migrating from rhombomere 2 (r2) or 4 (r4) interact with placodal cells to develop the cranial nerves. (B) $Ptch1^{Wig/Wig}$ embryos exhibit excessive Shh signaling leading to a reduction in neural crest cells and survival factors, which results in reduced neural crest cell-placode interactions and cranial nerve patterning defects.</sup>
indicate that the co-ordinated integration of Shh and Wnt signaling plays a key role in promoting the survival and interaction of neural crest cells and ectodermal placodes during cranial nerve development. Moreover, perturbation of these molecular and cellular interactions may underpin the etiology of some congenital cranial dysinnervation disorders.

In conclusion, we have demonstrated a role for cross-talk between Shh and Wnt signaling in neural crest cells and placodes cells during cranial nerve development. Elevated Shh signaling did not affect the formation and migration of cranial neural crest cells, but did result in selective death of migratory neural crest cells. Elevated Shh signaling also perturbed the development of the neurogenic placodes, particularly the trigeminal placode. Together these events disrupted neural crest and placode cell integration and led to aberrant cranial nerve development. Shh signaling restricts canonical Wnt signaling, and reduced Wnt signaling is associated with neural crest cell death and abnormal cranial nerve development. Using genetic approaches to reduce the levels of elevated Shh signaling, we were able to restore the canonical Wnt signaling in the neural crest and placodal territories of the developing head and rescue cranial ganglia morphogenesis.

**Supporting Information**

**S1 Fig. Schematic of the Ptch1Wig intronic mutation.** The Ptch1Wig mutation creates a novel consensus splice acceptor site at the 3’ end of intron 15 due to A to T substitution. This in turn leads of a 7 base pair insertion (gttcttag) and premature truncation (tga) 17 base pairs downstream of the 5’ end of Exon 16 of the Ptch1 gene. Sequencing of cDNAs derived from the biopsies of Wig carrier mice confirmed the presence of the predicted 7 base pair insertion.

**(TIF)**

**S2 Fig. Quantitative (q) PCR for Ptch1Wig and wild type Ptch1 transcripts in E10.5 embryos.** (A) Bar chart of the qPCR levels in Ptch1Wig/Wig mutants (red), Ptch1Wig/+ heterozygotes or Ptch1+/+ wild type (grey) E10.5 embryos. Embryos derived from Ptch1Wig/+ intercrosses were lysed and subjected to RT-PCR. Primers specific for the Wiggable mutation site in exon 16 were used in qPCR experiments, with values normalized to β-actin transcript levels. Values were plotted as +/- standard deviation. Ptch1Wig/Wig mutants did not display significant levels of wild type Ptch1 transcripts, and show an upregulation of Ptch1Wig levels. This upregulated Ptch1 locus activity was only present in Ptch1Wig mutants and not in heterozygotes. (B) Raw data for the Ptch1 wild type and Wig signal obtained from various embryo specimens.

**(TIF)**

**S3 Fig. Cell death of neuronal cells and neural crest cells in Ptch1Wig/Wig mutant embryos.** (A and F) Whole mount immunostaining of Neuronal Class III β-tubulin (TUJ1) (red) and DAPI (blue). (B-E and G-J) Horizontal sections across the indicated planes in (A) and (F) of the ophthalmic (B, C, G, H) and facial nerve (D, E, I and J) immunostained for TUJ1 (red; B, D, G, H and I) or SOX10 (red; C, H, E and J), along with TUNEL (green) and DAPI (blue). No difference in cell death between control (B and D) and Ptch1Wig mutant embryos (G and I) in the ophthalmic and facial nerve. There was increased cell death in SOX10-positive migratory neural crest cells in Ptch1Wig mutants (H and J; white arrowhead) relative to controls (C and E). (K) No statistically significant difference in neuronal cell death numbers between control and Ptch1Wig/Wig embryos. (L) Ptch1Wig/Wig embryos showed significantly increased number of apoptotic SOX10-positive neural crest cells in the ophthalmic region relative to controls. Scale bars: 100μm (A and F); 20μm (B, C, G and H); 50μm (D, E, I and J). *P < 0.05, Student’s t test. Data are represented as mean ± SEM.

**(TIF)**
Acknowledgments

The authors appreciate members of the Trainor and Iulianella labs for their insights and constructive comments throughout the course of this project and completion of this paper. We are indebted to Melissa Childers for excellent care and maintenance of our mouse colony; Nancy Thomas, Nannette Marsh, and Karen Smith for histological sections; Dr Trevor Williams and Danielle Manning for generating an providing the original AP2-Cre (Creface) mouse line; Dr Jennifer Dennis for identifying and characterizing Creface as an insertional mutation in Hhat (Hhatcreface); and Brian Sanderson for assistance with microsatellite and SNP mapping of the Ptch1Wig mutation.

Author Contributions

Conceived and designed the experiments: HK PAT MLB AI. Performed the experiments: HK PAT MLB AI. Analyzed the data: HK PAT MLB AI. Wrote the paper: HK PAT MLB AI.

References

1. D’Amico-Martel A, Noden D. Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. Am J Anat. 1983; 166: 445–468. PMID: 6858941
2. Hamburger V. Experimental analysis of the dual origin of the trigeminal ganglion in the chick embryo. J Exp Zool. 1961; 148: 91–123. PMID: 13904079
3. Steventon B, Mayor R, Streit A. Neural crest and placode interaction during the development of the cranial sensory system. Dev Biol. 2014; 389: 29–38. doi: 10.1016/j.ydbio.2014.01.021 PMID: 24491819
4. Shiau CE, Lwigale PY, Das RM, Wilson SA, Bronner-Fraser M. Robo2-Slit1 dependent cell-cell interactions mediate assembly of the trigeminal ganglion. Nat Neurosci. 2008; 11: 269–276. doi: 10.1038/nn2051 PMID: 18278043
5. Freter S, Fleenor SJ, Freter R, Liu KJ, Begbie J. Cranial neural crest cells form corridors prefiguring sensory neuroblast migration. Development. 2013; 140: 3595–3600. doi: 10.1242/dev.091033 PMID: 23942515
6. Theveneau E, Steventon B, Scarpa E, Garcia S, Trepat X, Streit A, et al. Chase-and-run between adjacent cell populations promotes directional collective migration. Nat Cell Biol. 2013; 15: 763–772. doi: 10.1038/ncb2772 PMID: 23770678
7. Fleenor SJ, Begbie J. Neural Crest Cell and Placode Interactions in Cranial PNS Development. In: Trainor PA, editor. Neural Crest Cells Evolution, Development and Disease. Academic Press; 2013. pp. 153–165.
8. Sandell LL, Iulianella A, Melton KR, Lynn M, Walker M, Inman KE, et al. A phenotype-driven ENU mutagenesis screen identifies novel alleles with functional roles in early mouse craniofacial development. Genesis 2011; 49: 342–359. doi: 10.1002/dvg.20727 PMID: 21908688
9. Goodrich LV, Millenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. Science 1997; 277: 1109–1113. PMID: 9262482
10. Kurosaka H, Iulianella A, Williams T, Trainor PA. Disrupting hedgehog and WNT signaling interactions promotes cleft lip pathogenesis. J Clin Invest. 2014; 124: 1660–1671. doi: 10.1172/JCI72688 PMID: 24590292
11. DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development 1999; 126: 4557–4568. PMID: 10498690
12. Dennis JF, Kurosaka H, Iulianella A, Pace J, Thomas N, Beckham S, et al. Mutations in Hedgehog Acyltransferase (Hhat) Perturb Hedgehog Signaling, Resulting in Severe Acrania-Holoprosencephaly-Agnathia Craniofacial Defects. PLoS Genet. 2012; 8: e1002927. doi: 10.1371/journal.pgen.1002927 PMID: 23059336
13. Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr Biol. 1998; 8: 1323–1326. PMID: 9843687
14. Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, et al. Cre reporter strains produced by targeted insertion of EFYP and ECFP into the ROSA26 locus. BMC Dev Biol. 2001; 1: 4. PMID: 11299042
15. Sandell LL, Butler Tjaden NE, Barlow AJ, Trainor PA. Cochleovestibular nerve development is integrated with migratory neural crest cells. Dev Biol. 2014; 385: 200–210. doi:10.1016/j.ydbio.2013.11.009 PMID: 24252775

16. Agren M, Kogerman P, Klemans MI, Wessling M, Tofgard R. Expression of the PTCH1 tumor suppressor gene is regulated by alternative promoters and a single functional Gli-binding site. Gene 2004; 330: 101–114. PMID: 15087129

17. Ahlgren SC, Thakur V, Bronner-Fraser M. Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. Proc Natl Acad Sci. 2002; 99: 10476–10481. PMID: 12140368

18. Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. Genes Dev. 2004; 18: 937–951. PMID: 15107405

19. Aoto K, Trainor PA. Coordinated brain and craniofacial development depends upon Patched1/XIAP regulation of cell survival. Hum Mol Genet. 2014; 24(3):698–713. doi:10.1093/hmg/ddu489 PMID: 25292199

20. Hari L, Brault V, Kléber M, Lee HY, Ille F, Leimeroth R, et al. Lineage-specific requirements of beta-catenin in neural crest development. J Cell Biol. 2002; 159: 867–880. PMID: 12473692

21. Lee HY, Kléber M, Hari L, Brault V, Suter U, Taketo MM, et al. Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science 2004; 303: 1020–1023. PMID: 14716020

22. Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch DH, McMahon AP, et al. Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. Development 2001; 128: 1253–1264. PMID: 11262227

23. Ikeya M, Lee SM, Johnson JE, McMahon AP, Takada S. Wnt signalling required for expansion of neural crest and CNS progenitors. Nature 1997; 389: 966–970. PMID: 9353119

24. Schlosser G. Making senses development of vertebrate cranial placodes. Int Rev Cell Mol Biol. 2010; 283: 129–234. doi:10.1016/S1937-6448(10)83004-7 PMID: 20801420

25. Stark MR, Sechrist J, Bronner-Fraser M, Marcelle C. Neural tube-ectoderm interactions are required for trigeminal placode formation. Development 1997; 124: 4287–4296. PMID: 9334277

26. Ma Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ. neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 1998; 20: 469–482. PMID: 9539122

27. Chen MH, Li YJ, Kawakami T, Xu SM, Chuang PT. Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. Genes Dev. 2004; 18: 641–659. PMID: 15075292

28. Fedtsova N, Perris R, Turner EE. Sonic hedgehog regulates the position of the trigeminal ganglia. Dev Biol. 2003; 261: 456–469. PMID: 14499653

29. Coppola E, Rallu M, Richard J, Dubour S, Rietmacher D, Guillemot F, et al. Epibranchial ganglia orchestrate the development of the cranial neurogenic crest. Proc Natl Acad Sci. 2010; 107: 2066–2071. doi: 10.1073/pnas.0910213107 PMID: 2013851

30. Begbie J, Graham A. Integration between the epibranchial placodes and the hindbrain. Science 2001; 294: 595–598. PMID: 11641498

31. Lwigale PY. Embryonic origin of avian corneal sensory nerves. Dev Biol. 2001; 239: 323–337. PMID: 11784038

32. Vacir T, Stubs JL, Lemke G. A novel mechanism for the transcriptional regulation of Wnt signaling in development. Genes Dev. 2012; 26: 414.

33. Lee CS, Buttitta LA, May NR, Kispert A, Fan CM. SHH-N upregulates Sfrp2 to mediate its competitive interaction with WNT1 and WNT4 in the somitic mesoderm. Development 2000; 127: 109–118. PMID: 10654605

34. MacDonald BT, Tamai K, He X. Wnt/beta-Catenin Signaling: Components, Mechanisms, and Diseases. Dev Cell. 2009; 17: 9–26. doi: 10.1016/j.devcel.2009.06.016 PMID: 19619488

35. Dude CM, Kuan CY, Bradshaw JR, Greene ND, Relax F, Stark MR, et al. Activation of Pax3 target genes is necessary but not sufficient for neurogenesis in the ophthalmic trigeminal placode. Dev Biol. 2009; 326: 314–326. doi: 10.1016/j.ydbio.2008.11.032 PMID: 19100251

36. Canning CA, Lee L, Luo SX, Graham A, Jones CM. Neural tube derived Wnt signals cooperate with FGF signaling in the formation and differentiation of the trigeminal placodes. Neural Dev. 2008; 3: 35. doi: 10.1186/1749-8104-3-35 PMID: 19077309

37. Lassiter RN, Dude CM, Reynolds SB, Winters NI, Baker CV, Stark MR. Canonical Wnt signaling is required for ophthalmic trigeminal placode cell fate determination and maintenance. Dev Biol. 2007 308: 392–406. PMID: 17604017
38. Zhao T, Gan Q, Stokes A, Lassiter RN, Wang Y, Chan J, et al. Beta-catenin regulates Pax3 and Cdx2 for caudal neural tube closure and elongation. Development 2014; 141: 148–157. doi: 10.1242/dev.101550 PMID: 24284205

39. Dessaud E, McMahon AP, Briscoe J. Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. Development 2008; 135: 2489–2503. doi: 10.1242/dev.009324 PMID: 18621990

40. Inoue T, Hatayama M, Tohmonda T, Itohara S, Aruga J, Mikoshiba K. Mouse Zic5 deficiency results in neural tube defects and hypoplasia of cephalic neural crest derivatives. Dev Biol. 2004; 270: 146–162. PMID: 15136147

41. Wu CY, Hooper RM, Han K, Taneyhill LA. Migratory neural crest cell alphaN-catenin impacts chick trigeminal ganglia formation. Dev Biol. 2014; 392(2): 295–307. doi: 10.1016/j.ydbio.2014.05.016 PMID: 24882712

42. Kuriyama S, Theveneau E, Benedetto A, Parsons M, Tanaka M, Charras G. In vivo collective cell migration requires an LPAR2-dependent increase in tissue fluidity. J Cell Biol. 2014; 206: 113–127. doi: 10.1083/jcb.201402093 PMID: 25002680

43. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. Science 2004; 303: 1483–1487. PMID: 15001769

44. Murdoch JN, Copp AJ. The relationship between sonic Hedgehog signaling, cilia, and neural tube defects. Birth Defects Res A Clin Mol Teratol. 2010; 88: 633–652. doi: 10.1002/bdra.20686 PMID: 20544799

45. Cobourne MT, Xavier GM, Depew M, Hagan L, Sealby J, Webster Z, et al. Sonic hedgehog signalling inhibits palatogenesis and arrests tooth development in a mouse model of the nevoid basal cell carcinoma syndrome. Dev Biol. 2009; 331: 38–49. doi: 10.1016/j.ydbio.2009.04.021 PMID: 19394325

46. Bosley TM, Abu-Amero KK, Oystreck DT. Congenital cranial dysinnervation disorders: a concept in evolution. Curr Opin Ophthalmol. 2013; 24: 398–406. doi: 10.1097/ICO.0b013e3283645ad6 PMID: 23872818

47. Graeber CP, Hunter DG, Engle EC. The genetic basis of incomitant strabismus: consolidation of the current knowledge of the genetic foundations of disease. Semin Ophthalmol. 2013; 28: 427–437. doi: 10.3109/08820538.2013.825288 PMID: 24138051

48. Verzijl HT, van der Zwaag B, Cruysberg JR, Padberg GW. Mobius syndrome redefined: a syndrome of rhombencephalic maldevelopment. Neurology 2003 61: 327–333. PMID: 12913192

49. Schroder JC, Lassig AK, Galetzka D, Peters A, Castle JC, Diederich S, et al. A boy with homozygous microdeletion of NEUROG1 presents with a congenital cranial dysinnervation disorder [Moebius syndrome variant]. Behav Brain Funct. 2013; 9: 7. doi: 10.1186/1744-9081-9-7 PMID: 23419067