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Functional Analysis of *SPECC1L* in Craniofacial Development and Oblique Facial Cleft Pathogenesis

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Abstract

Background—Oblique facial clefts, also known as Tessier clefts, are severe orofacial clefts, the genetic basis of which is poorly understood. Human genetics studies revealed that disruption in *SPECC1L* resulted in oblique facial clefts, demonstrating that oblique facial cleft malformation has a genetic basis. An important step toward innovation in treatment of oblique facial clefts would be improved understanding of its genetic pathogenesis. The authors exploit the zebrafish model to elucidate the function of *SPECC1L* by studying its homolog, *specc1lb*.

Methods—Gene and protein expression analysis was carried out by reverse-transcriptase polymerase chain reaction and immunohistochemistry staining. Morpholino knockdown, mRNA rescue, lineage tracing and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assays were performed for functional analysis.

Results—Expression of *specc1lb* was detected in epithelia juxtaposed to chondrocytes. Knockdown of *specc1lb* resulted in bilateral clefts between median and lateral elements of the ethmoid plate, structures analogous to the frontonasal process and the paired maxillary processes. Lineage tracing analysis revealed that cranial neural crest cells contributing to the frontonasal prominence failed to integrate with the maxillary prominence populations. Cells contributing to

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lower jaw structures were able to migrate to their destined pharyngeal segment but failed to converge to form mandibular elements.

Conclusions—These results demonstrate that *specc11b* is required for integration of frontonasal and maxillary elements and convergence of mandibular prominences. The authors confirm the role of *SPECCIL* in orofacial cleft pathogenesis in the first animal model of Tessier cleft, providing morphogenetic insight into the mechanisms of normal craniofacial development and oblique facial cleft pathogenesis.

Orofacial clefts are among the most common congenital malformations, with a prevalence of one in 700 to one in 1000 births.^{1,2} Orofacial clefts range from the common cleft lip with or without cleft palate and cleft palate only, to rare and complex oblique facial clefts, also known as Tessier clefts.^{3–6} Although surgical management of cleft lip with or without cleft palate and cleft palate only are routine, and good functional and cosmetic results can be achieved, repair of oblique facial clefts is generally difficult, with more modest surgical results.^{7,8} Affected children require multiple staged surgical procedures in early childhood and need lifelong multidisciplinary treatment with significant financial burden to their families and the health care system.⁹ Better understanding of the genetic basis of palate and cleft formation would aid genetic diagnosis of orofacial clefts and lead to potential advances in the treatment of these common structural malformations.^{10–12}

Many genetic loci associated with cleft lip with or without cleft palate and cleft palate only have been described, such as *MSX1*, *IRF6*, *SUMO1*, and *TGFB*.^{5,13–19} In contrast to the common cleft lip with or without cleft palate, little is known about the genetic basis of oblique facial clefts. For lack of better understanding, clinicians have suggested that oblique facial clefts may be caused by a combination of directly tethered tissue disrupting facial prominence migration (such as amniotic bands) or increased local pressure that causes ischemia.²⁰ It was not until this work that we begin to show that oblique facial clefts, like the more common cleft lip with or without cleft palate and cleft palate only, has a genetic basis, through the discovery of mutations in *SPECCIL* to be causal for oblique facial clefts.²¹

SPECCIL encodes a novel coiled-coil domain containing protein and functionally interacts with both microtubules and the actin cytoskeleton, especially in mitotic spindles.²¹ In a wound-repair assay, *SPECCIL* knockdown led to defective cell adhesion and migration and inability of cells to reorganize their actin cytoskeleton in response to stimuli such as Ca^{2+} and *Wnt5a*. Morpholino-based knockdown of *SPECCIL* homologs in zebra-fish led to a “faceless” phenotype. This work did not describe the craniofacial structures in detail, which precluded evaluation of the mandible and palate. Disruption in *Drosophila* led to cell migration and adhesion defects resulting in a wide range of wing phenotypes.²¹ The wing phenotypes observed in *SPECCIL* knockdown experiments in *Drosophila* had a striking resemblance to mutants in the integrin signaling pathway,²² suggesting that *SPECCIL* may be involved in integrin-mediated cell motility and adhesion. However, the means by which *SPECCIL* participates in integrin and *WNT* pathways during craniofacial development remains undefined. The function of *SPECCIL* in craniofacial morphogenesis is also unknown.

Zebrafish (*Danio rerio*) is a powerful genetic tool for study of the developmental and genetic basis of orofacial clefts. Many features of zebra-fish biology, including small size, rapid and ex vivo embryonic development, and high breeding rates, make this model system very useful. The embryos are optically transparent and genetically tractable, and can be used in large-scale genetic and chemical screens. We and others described the conservation of palatogenic gene expression and function across vertebrates.^{23–26} It was also shown that zebrafish cranial neural crest cells reside in analogous regions of the developing face compared with amniote species, and that the zebrafish palate is analogous to the amniote primary palate (Fig. 1). In addition to structural similarities between zebrafish and mammalian craniofacial development, the gene regulatory network is highly conserved with genes such as *WNT* signaling, sonic hedgehog, fibroblast growth factor 8, transcription factor AP-2a, and *PDGFRA* all playing key roles.^{27–31} Furthermore, we demonstrated that the pathogenesis of orofacial cleft is also conserved between amniotes and zebrafish, where disruption of *wnt9a* and *irf6*, which are important mutations associated with human cleft lip with or without cleft palate, also resulted in zebrafish cleft palate phenotypes.²³ Recent innovations in gene editing and knockout approaches using clustered regularly interspaced short palindromic repeats and transcription activator-like effector nucleases have further advanced zebrafish as a first-line model system of choice to analyze genes of unknown function uncovered from human genetics studies.^{32–35}

This work aimed to elucidate the function of *SPECCIL* through the study of its homolog, *specc1lb*, in the zebrafish model. In zebrafish, we can readily determine the embryonic gene expression of *specc1lb*, assess the functional requirement of *specc1lb* during craniofacial morphogenesis, and study the cellular mechanisms where *specc1lb* acts. This study forms the basis for future functional analysis of *SPECCIL*, such as gene targeting in murine models or to guide future biochemical approaches.

Materials and Methods

Zebrafish Lines and Maintenance

All embryos and fish were raised and cared for using established protocols according to the Subcommittee on Research Animal Care, Massachusetts General Hospital.³⁶

Gene Cloning and Sequence Analysis

SPECCIL homologs were identified in the zebrafish genome using the human *SPECCIL* mRNA sequence and protein sequences, respectively (National Center for Biotechnology Information: NM_001254733.1, NP_056145), in the National Center for Biotechnology Information BLAST tool. Zebrafish genes with the greatest sequence homology are annotated as *specc1la* and *specc1lb* (National Center for Biotechnology Information: *specc1la*, NM_001039816; *specc1lb*, XM_677967). The *specc1la* and *specc1lb* cDNAs were cloned by reverse-transcriptase polymerase chain reaction (Platinum *Taq* DNA Polymerase; Invitrogen, Carlsbad, Calif.) from cDNA prepared from embryos at 48 hours post fertilization and subcloned into pGEM-T Easy Vector (Promega Corp., Madison, Wis.). Sequence analysis and alignment were performed with BioEdit 7.1.9 (Ibis Biosciences,

Carlsbad, Calif.) and Lasergene 9.0 (DNASTAR, Inc., Madison, Wis.) program suites, using the CLUSTAL W alignment algorithm.

Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was isolated from whole embryo lysates and converted to cDNA using iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, Calif.). Primers were designed using the A plasmid Editor sequence editing software (Primer design tool) to amplify from the cDNA library by reverse-transcriptase polymerase chain reaction (Phusion RT PCR kit; Thermo Fisher Scientific, Inc., Waltham, Mass.). *eflα* was used as a positive control. It is ubiquitously expressed across all embryonic time points.³⁷

Immunohistochemistry

Antisera against zebrafish *specc11b* (anti-*specc11b* peptide, 795-RKQDEERGRVYN-806, affinity-purified, Pierce custom services; Thermo Fisher Scientific) was used for immunohistochemical staining. Transverse sections of zebrafish embryos 4 days post fertilization were blocked in 5% normal goat serum, 2% bovine serum albumin in phosphate-buffered saline for 1 hour, and then incubated in serum containing polyclonal antibodies against *specc11b* or preimmunized serum. The serum was diluted 1:200 in phosphate-buffered saline and contained 2% bovine serum albumin to block nonspecific binding. After washing with phosphate-buffered saline containing 0.05% Tween-20, the second antibody, Alexa Fluor 488 Donkey Anti-Rabbit IgG (H+L) (Invitrogen) (diluted 1:1000 in phosphate-buffered saline containing 1% bovine serum albumin) was applied to the samples for 30 minutes. The samples were washed with phosphate-buffered saline, and examined using fluorescence microscopy (Nikon Eclipse 80i; Nikon Instruments, Inc., Melville, N.Y.).

Morpholino and RNA injection

Translation-blocking *specc11a* and *specc11b* morpholinos and mismatch control morpholinos were designed by Gene Tools (Philomath, Ore.) with the following nucleotide sequences: 5'-CCACTGGCCTGCCTGCTTTCTTCAT-3' (for *specc11a* morpholinos), 5'-ACTATGGTTTTGCTTGGAGGCCGTT-3' (for *specc11b* morpholino), 5'-CCACAGCCCAGCCTCCTTTGATCAT-3' (for *specc11a* mismatch control morpholinos), and 5'-ACAATCGTTTTCTTGCACGCCGTT-3' (for *specc11b* mis-match control morpholinos). Mismatch control morpholinos have the same sequence as the real morpholinos, but five nucleotides do not match. This morpholino should not have an effect on the embryo and serves as an optimal control.³⁸ For the morpholinos and mismatch control morpholinos, the 1-mM stock solution was diluted to 0.080 mM with UltraPure DNase/RNase-Free Distilled Water (Life Technologies, Grand Island, N.Y.). Then, 0.5 nl of the solution was injected into one-cell stage zebrafish embryos with borosilicate glass needles (outside diameter, 1.0 mm; inside diameter, 0.50 mm; Sutter Instrument Co., Novato, Calif.) pulled with a Flaming/Brown Micropipette Puller, Model P-97 using a PLI-90A Pico-Liter Injector (Harvard Apparatus, Holliston, Mass.). The phenotype was assessed with Alcian blue staining at 5 days post fertilization.³⁹ The *specc11a* morpholino did not produce a phenotype, even at high concentrations. We screened embryos for toxicity

24 hours post fertilization, which was below 10 percent, and such embryos were excluded from analysis. Screening for neural toxicity was performed because some morpholinos induce non-target-related toxic phenotypes apparent as cell death in the central nervous system.^{40,41} Capped full-length *specc1lb* mRNAs were synthesized from pCS7:*specc1lb* (Addgene, Cambridge, Mass.) using Sp6 mMessage mMachine kit (Life Technologies). For rescue experiments, embryos were injected first with morpholino or mismatch control morpholinos; then, half of these embryos were injected with mRNA (0.5 nl of 125-ng/μl solution).

Cartilage Staining

Alcian blue staining was performed to analyze the craniofacial skeleton, and images were obtained using a Nikon 80i compound microscope.^{42,43}

Lineage Analysis and Imaging

Tg sox10:kaede was used to perform lineage tracing analysis; *sox10* is a neural crest cell marker. Kaede can be photoconverted from green to red by exposure to ultraviolet light, which allows specific groups of cranial neural crest cells to be labeled. Photoconversion was performed unilaterally in *specc1lb* mismatch control morpholinos and *specc1lb* morpholino knockdown *sox10:kaede* embryos at the 10-somite stage using ultraviolet light (404.3 nm). The contralateral side served as an internal control.⁴⁴ Embryos were raised until 4.5 days post fertilization and imaged on the confocal microscope (Nikon A1R Si Confocal Ti series).

Proliferation and Apoptosis Assays

Proliferation was detected using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen). Apoptosis was detected using the Click-iT TUNEL Alexa Fluor 488 Imaging Assay (Invitrogen). Detection was performed in the background of a *sox10:mcherry* transgenic line, so that proliferation of cranial neural crest-derived chondrocytes could be tracked (5-ethynyl-2'-deoxyuridine in green will co-localize with mcherry reporter protein in red).

Results

Conservation and Gene Expression of *SPECC1L* Zebrafish Homologs *specc1la* and *specc1lb*

The zebrafish genome contains two homologs of the human *SPECC1L* gene: *specc1la* (chromosome 8) and *specc1lb* (chromosome 21).⁴⁵ Structurally, human *SPECC1L* protein contains three coiled-coil domains and one calponin homology domain (Fig. 2, *above*). Peptide alignment showed that these domains are highly conserved across vertebrate species, including mouse, chick, *Xenopus*, and zebrafish (Fig. 2, *center* and *below*). Reverse-transcriptase polymerase chain reaction analysis reveals that the *SPECC1L* ortholog *specc1lb* is expressed as early as 6 hours post fertilization and expression persists until 3 to 4 days post fertilization (Fig. 3, *above*). To determine the craniofacial expression of *SPECC1L* homologs, immunohistochemical staining of sections with an antibody specific to a conserved domain shared between *specc1la* and *specc1lb* proteins was carried out,

demonstrating specific localization of *specc1la/b* to the oropharyngeal epithelia and exclusion from chondrocytes of the upper and lower jaw structures (Fig. 3, *center* and *below*). Immunohistochemical staining with preimmunized serum control did not demonstrate specific signal (Fig. 3, *center, right*).

Loss of *specc1lb* Function Results in Upper and Lower Jaw Defects

To assess functional requirements of *specc1la* and *specc1lb* during craniofacial development and cleft pathogenesis, gene disruption was mediated by morpholino knockdown. Although *specc1la* morpholino failed to produce a craniofacial phenotype, *specc1lb* gene disruption produced a distinct craniofacial malformation affecting both the upper and lower jaw elements. (See Video, Supplemental Digital Content 1, which demonstrates cleft development in *specc1lb* morpholino knockdown morphant. Live time-lapse recording of developing ethmoid plate in a *specc1lb* morpholino knockdown Tg *sox10:kaede* zebrafish embryo (ventral view) at 60 hours post fertilization; *sox10* provides lineage restriction of the *kaede* reporter protein to the neural crest. Failure of fusion between median ethmoid plate cells and lateral ethmoid plate cells can be observed. The video was recorded every 30 minutes starting 60 to 72 hours post fertilization, <http://links.lww.com/PRS/B96>.) Further analysis was carried out with *specc1lb* only, as *specc1lb* was found to be the functional ortholog of human *SPECCIL* displaying a knockdown phenotype. The upper jaw phenotype exhibited bilateral clefts between the median and lateral elements of the ethmoid plate, a zebrafish structure analogous to the primary palate in amniotes. Furthermore, the mandible was entirely absent (Fig. 1 and Fig. 4, *center*). The best view to appreciate the absent lower jaw is in Figure 4, *above, center*, which shows a clear palate on lateral view, but no lower jaw. To confirm specificity of the *specc1lb* knockdown phenotype, rescue experiments with full-length *specc1lb* mRNA were performed. Co-injection of nonhomologous *specc1lb* mRNA with *specc1lb* morpholino led to a partial rescue of the phenotype. The partially rescued phenotype consisted of a morphologically complete distal palate without clefts and rescue of lower jaw elements (Meckel's cartilage, palatoquadrate, and hyosymplectic) (Fig. 4, *right*).

The *specc1lb* Phenotype Results from Failure of Integration between the Lateral and Median Primary Palate with Reduced Cell Proliferation

To elucidate cellular and morphogenetic mechanisms underlying the pathogenesis of clefts and absent mandible in *specc1lb* morphants, lineage analysis of migrating cranial neural crest cells was performed. Using the Tg *sox10:kaede* transgenic line, cranial neural crest cells destined for frontonasal, maxillary, and mandibular prominences were labeled in *specc1lb* morphants and mismatch control morpholino-injected controls.⁴⁶ In *specc1lb* mismatch control morpholino, cranial neural crest cells located anterior to the eyes at the 10-somite stage migrated to form the frontonasal prominence, which contributes to the median primary palate (Fig. 5, *above, center*). Cranial neural crest cells located medial to the eyes migrated to form the maxillary prominence, which contributes to the lateral element of the primary palate (Fig. 5, *center, center*). Finally, cranial neural crest cells located posterior to the eyes migrated to form the mandibular prominence (Fig. 5, *below, center*). Lineage tracing labeling experiments of cranial neural crest cells in *specc1lb* morphants showed that migration of cranial neural crest cells contributing to the facial prominences reached only

the proper pharyngeal segment at 48 hours post fertilization, but failed to converge toward the midline and contribute to their respective facial prominence structures. More specifically, the anterior cranial neural crest cell population that normally would contribute to the frontonasal process and the more posterior cranial neural crest cell population that contributes to the maxillary prominence still migrated to their respective pharyngeal domains. However, subsequent morphogenesis of the facial form involving integration of the frontonasal and maxillary prominences with one another failed to occur, leading to clefts between the median and lateral palate elements (Fig. 5, *above, right*, and *center, right*). In addition, cells that normally contribute to the mandible reached the mandibular domain but failed to converge to the midline to form the lower jaw (Fig. 5, *below, right*).

The zebrafish palate forms by a process of convergence and extension to generate the cartilaginous framework, where palate extension is mediated by cell intercalation and proliferation of chondrocytes in the leading edge.⁴⁷ Examination of cell proliferation and apoptosis using 5-ethynyl-2'-deoxyuridine labeling and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay, respectively, reveal that the proliferative front forms but is reduced in *specc1lb* morphants compared with mismatch control morpholino controls (Fig. 6, *above, right*). In contrast, there is no difference in the degree of apoptosis between control and *specc1lb* morphants at 55 to 60 hours post fertilization (Fig. 6, *below, right*).

Discussion

Orofacial clefts represent a diverse malformation spectrum, ranging from the common cleft lip with or without cleft palate and cleft palate only (one in 700 to one in 1000), to the rare and dramatic oblique facial clefts (one in 200,000).^{1,2} Until recent description of the *SPECCIL* mutation resulting in oblique facial clefts, no gene had been implicated in oblique facial clefts, and the pathogenesis of this malformation has been largely speculative.²¹ *SPECCIL* encodes a cytoskeletal protein, which in mammalian cell assays co-localizes with tubulin and actin, and its deficiency results in defective cell adhesion and migration.²¹ However, the role of *SPECCIL* in craniofacial development and oblique facial cleft pathogenesis remained to be elucidated.

This study examined the function of *SPECCIL* by studying its homologs in zebrafish, where *specc1lb* is the ortholog. Interestingly, *specc1lb* expression was found in oropharyngeal epithelia juxtaposed to chondrocytes, suggesting that these genes may be expressed in the endoderm surrounding neural crest cells and not in neural crest cells themselves. The pharyngeal epithelia can induce cartilage in mammals and may also play a patterning role, as it expresses extracellular signaling molecules (e.g., *edn*, *wnt9a*, *fgf8*, *shh*) involved in craniofacial development.^{48–52} *SPECCIL* disruption may alter expression of these molecules and inhibit normal chondrocyte formation.

***specc1lb* Is Required for Upper Jaw Integration and Lower Jaw Formation**

To elucidate the mechanism underlying the *specc1lb* morphant phenotype, lineage-tracing analysis was performed and revealed that in *specc1lb* morphant embryos, cell migration to the corresponding pharyngeal segment occurred. However, cranial neural crest cells

contributing to the frontonasal prominence fail to integrate or “fuse” with the maxillary prominence cranial neural crest cells, leading to clefts between median and lateral elements of the palate. This mechanism explains the pathogenesis of oblique facial cleft development in humans, where failure of fusion between the lateral nasal process (a derivative of frontonasal prominence and analogue of the median ethmoid plate in zebrafish) and maxillary prominence (analogous to the lateral ethmoid plate in zebrafish) occurs.⁵³ In experiments performed by Saadi et al., knockdown in *Drosophila* leads to cell migration and adhesion defects that resemble integrin knockout phenotypes. In zebrafish, integrin knockdown leads to cell-matrix and cell-cell adhesion defects in the basal epidermis.²¹ We posit that *specc11b* may be involved in proper integration and adhesion of chondrocytes in the developing zebrafish palate, interacting with integrin and *specc11b*, which are expressed in the ectoderm.

Cells normally contributing to the Meckel's cartilage were able to migrate to their destined facial prominence domains but failed to converge to form mandibular elements. This defect resembles the same phenotype observed when *wnt9a*, *frzb*, and *fzd7a* are depleted by morpholino, where the lower jaw is grossly absent and the directed migration of cranial neural crest cells to the mandibular arch is intact.^{23,47}

As described by our group and others, the zebrafish palate is analogous to the amniote primary palate, formed by migration of cranial neural crest cells to the proper pharyngeal segment, convergence of facial prominences, and extension of the fused structures.^{23,24} Zebrafish palate morphogenesis can be described by distinct mechanisms: chondrocyte extension, proliferation, and integration, where *wnt9a* is required for palatal extension and proliferation and *irf6* is required for integration of facial prominences along a V-shaped seam.²³ In addition, *frzb* and *fzd7a* are dispensable for directed migration of the bilateral trabeculae, but are required for convergence of the mandibular prominences and for palate extension.⁴⁷ We report failure of full extension of the developing primary palate, reduction of the proliferative front of cranial neural crest cells, and failure of integration or fusion of facial prominences in *specc11b* knockdown morphants; *specc11b* may be involved in this network of genes required for normal convergence of facial prominences and may interact with these genes to ensure correct extension and fusion of the palate. Because the *specc11b* protein structure exhibits features of a scaffolding protein, its function may be to transduce molecular signals to morphologic change, a process important for inter-cellular integration. This function has precedence in β -spectrin, which serves as a scaffolding protein to stabilize transforming growth factor- β receptors and functions in regulating palatogenesis.⁵⁴

Considered together, this analysis suggests that *specc11b* is required for integration of upper jaw palatal elements and convergence of mandibular prominences. Biochemical experiments are underway to elucidate the cellular function and identify protein interactors of *specc11b*. Germline *specc11b* mutants are also being generated using clustered regularly interspaced short palindromic repeats, to serve as the substrate for genetic and chemical screens, to identify signaling pathways regulated by *specc11b*. Moreover, identification of chemical modifiers of *specc11b* mutant phenotype may allow us to find small molecules to mitigate the cleft pathogenesis before its malformation. We present the first animal model of oblique facial clefts and morphogenetic insight into the mechanisms of oblique facial cleft.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Human embryo at 10 weeks (*left*) and zebrafish embryo at 4 days post fertilization (*dpf*) (*right*), at analogous developmental time points. Frontal (*above*) and palatal views (*center* and *below*) of the frontonasal prominence (FNP; *red*) and maxillary prominences (MP; *green*) and their derivative structures demonstrate conservation of normal palate development (*above* and *center*) and cleft pathogenesis (*below*).

Fig. 2.

Schematic diagram of *SPECC1L* protein structure (*above*). *SPECC1L* protein alignment highlights conservation across vertebrates (*center*). Phylogenetic relationship of *SPECC1L* across vertebrate species (*below*).

Fig. 3.

Reverse-transcriptase polymerase chain reaction (*RT-PCR*) analysis shows *specc1la* and *specc1lb* expression up to day 3 and beyond (data not shown); *ef1a* was used as a control (above). Immunohistochemical staining detects *specc1lb* protein in the oropharyngeal epithelia (arrows) but not in the chondrocytes (asterisks) (center, left). Staining with preimmunized serum was used as a negative control (center, right). Diagram of *specc1lb* (green), *frzb* (yellow), *fzd7a* (violet), *wnt9a* (red), and *wls* (red) expression in the oropharyngeal region (below). *hpf*, hours post fertilization.

Fig. 4.

Normal jaw structures are seen in control embryos (*left*). (*Above and second row*) The palate (*p*), mandible (*m*), and eyes (*e*) are surrounded by a *dotted line*. Jaw elements are labeled as the Meckel's cartilage (*mc*), palatoquadrate (*pq*), pterygoid process (*ptp*), ceratohyal (*ch*), and hyosymplectic (*hs*). Knockdown of *specc1lb* led to a shortened primary palate with bilateral clefts between the median and lateral elements of the primary palate (*arrows, dotted line*) and absence of lower jaw (*center*). Co-injection of *specc1lb* mRNA with *specc1lb* morpholino (*MO*) led to a partial rescue of the phenotype consisting of a primary palate without cleft (*dotted line*) and rescue of lower jaw elements (*mc, pq, and hs*) (*below, right*). *MM*, mismatch control morpholinos.

Fig. 5.

Cranial neural crest cells located anterior to the eyes at the 10-somite stage (eyes outlined in *white*) migrate to form the middle part of the primary palate or the frontonasal prominence (*above, left, and center*). Cranial neural crest cells located medial to the eyes migrate to form the lateral part of the primary palate to the maxillary prominence (*center, left, and center*). Cranial neural crest cells located posterior to the eyes migrate to form the mandibular prominence (*below, left, and center*). In *specc1lb* morphants, cranial neural crest cells constituting the frontonasal prominence (*above, right*) (arrows) fail to fuse with cells constituting the maxillary prominence (*center, right*) (arrows). Cells that normally develop into a lower jaw fail to converge toward the midline and instead form ectopic structures beneath the eyes (*below, right*) (arrow).

Fig. 6.

5-Ethynyl-2'-deoxyuridine (*EdU*) staining reveals a green proliferative front (*dotted line*) at the leading edge of the developing primary palate in *specc1lb* mismatch (*MM*) control embryos and in *specc1lb* morphants (*MO*) (*above*). A marked decrease in neural crest cell proliferation is noted (*above, right*). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay for apoptosis shows that *specc1lb* phenotypes are not a result of increased cell death in the ethmoid plate region during extension (*below, right*).