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GENETIC REQUIREMENT OF *TALIN1* FOR PROLIFERATION OF CRANIAL NEURAL CREST CELLS DURING PALATE DEVELOPMENT

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Takashi Okada, MD, PhD†
Eric C. Liao, MD, PhD*‡

**Background:** Craniofacial malformations are among the most common congenital anomalies. Cranial neural crest cells (CNCCs) form craniofacial structures involving multiple cellular processes, perturbations of which contribute to craniofacial malformations. Adhesion of cells to the extracellular matrix mediates bidirectional interactions of the cells with their extracellular environment that plays an important role in craniofacial morphogenesis. Talin (tnl) is crucial in cell-matrix adhesion between cells, but its role in craniofacial morphogenesis is poorly understood.

**Methods:** Talin gene expression was determined by whole mount in situ hybridization. Craniofacial cartilage and muscles were analyzed by Alcian blue in Tg(mylz2:mCherry) and by transmission electron microscopy. Pulse-chase photoversion, 5-ethynyl-2’-deoxyuridine proliferation, migration, and apoptosis assays were performed for functional analysis.

**Results:** Expression of *tnl1* was observed in the craniofacial cartilage structures, including the palate. The Meckel’s cartilage was hypoplastic, the palate was shortened, and the craniofacial muscles were malformed in *tnl1* mutants. Pulse-chase and EdU assays during palate morphogenesis revealed defects in CNCC proliferation in mutants. No defects were observed in CNCC migration and apoptosis.

**Conclusions:** The work shows that *tnl1* is critical for craniofacial morphogenesis in zebrafish. Loss of *tnl1* leads to a shortened palate and Meckel’s cartilage along with disorganized skeletal muscles. Investigations into the cellular processes show that *tnl1* is required for CNCC proliferation during palate morphogenesis. The work will lead to a better understanding of the involvement of cytoskeletal proteins in craniofacial morphogenesis. (Plast Reconstr Surg Glob Open 2018;6:e1633; doi: 10.1097/GOX.0000000000001633; Published online 19 March 2018.)

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sists of multiple matrix molecules such as glycoproteins, fibronectin, proteoglycans, and collagens. Cells within tissues and organs adhere to the ECM scaffold that in turn induces discrete cell surface structures. The cell-matrix adhesions mediate bidirectional interactions of the cell with its extracellular environment and play central roles in embryonic development. Many genes of the cytoskeletal network and the cell-matrix adhesion complex have been implicated in perturbing cellular processes in CNCCs, and thereby result in craniofacial anomalies. For example, caldesmon is an actin modulator, which when mutated, causes severe cranial cartilage defects with aberrant CNCC migration in Xenopus. In zebrafish, knockdown of caldesmon causes defective cardiovascular development. Myosin-X is required for CNCC migration in Xenopus and for cell–cell adhesion in in vitro cell cultures. We and others showed that SPECC1L, 1 of the first genes implicated in Tessier oblique facial cleft, encodes for a cytoskeletal protein known to interact with actin filaments. Mutations in FILAMIN A (FLNA) are known to be associated with craniosynostosis. Loss-of-function (LOF) of capzb, an actin-capping protein causes cleft palate and micrognathia. Stickler syndrome, which includes cleft lip and palate as 1 of its major presentations, is caused by mutations in COL2A1, a gene that encodes collagen, a component of the ECM.

Integrins are the principle cell surface adhesion receptors that regulate interactions between cells and the ECM. Integrin proteins have a large extracellular domain that interacts with ECM proteins, and a small intracellular domain that interacts with various proteins involved in cell signaling, and adapter proteins that provide connections to the cytoskeleton. Talin (tln) is 1 of the key cytoskeletal proteins involved in integrin-mediated cell signaling. Biochemical and cellular studies indicate that Tln activates integrins by binding to the cytoplasmic domain of the integrin β-subunit (Fig. 1, above). In addition to its role in signaling cascades inside the cell, Tln links integrins to the actin cytoskeletal network (Fig. 1, above), which is crucial to transmit force from the actin cytoskeleton to the ECM.

There are 2 Tln isoforms in most vertebrates. In zebrafish, there are 3 tln isoforms: tln1, tln2, and tln2a. Global LOS of tln1 results in embryonic lethality in mice due to defects in cell migration during gastrulation, whereas Tln2-null mice are viable and fertile with only mild dystrophy. These studies suggest that Tln1 plays a critical role during embryonic development. Elucidating the role of Tln1 will provide an important vantage point for understanding craniofacial morphogenesis.

Zebrafish (Danio rerio) is a powerful vertebrate model to study the molecular factors involved in craniofacial morphogenesis. We and others have shown that the zebrafish ethmoid plate (hereafter palate) is analogous to the human primary palate. In addition, many genes involved in human craniofacial morphogenesis are conserved in the zebrafish. Here, we explore the role of tln1 in the development of the craniofacial cartilage and muscle in zebrafish. We also elucidate the role of tln1 in the cellular processes involved in palate development to investigate how forces generated by the cytoskeletal scaffold affect CNCCs. We find that tln1 is required for the formation of the craniofacial complex by regulating CNCC proliferation.

MATERIALS AND METHODS

Zebrafish Lines and Maintenance

The retroviral insertion mutant line Tln1<sup>hi3093Tg/+</sup> (hereafter referred to as Tln1<sup>hi</sup>) was obtained from the Zebrafish International Resource Center. Wildtype (WT), Tg(sox10:kaede), and Tg(sox10:mcherry) were used for various experiments. Embryos were obtained by natural spawning and staged. All embryos and fish were raised and cared for by using established protocols in accordance with the Subcommittee on Research Animal Care, Massachusetts General Hospital.

Total RNA Extraction and cDNA Synthesis

Total RNA extraction and cDNA synthesis was performed as described previously. Forty-eight hours postfertilization (hpf) embryos were used for total RNA synthesis for genera-
tion of tln1 riboprobe. Forty-eight hpf, 72 hpf, and 5 days postfertilization (dpf) embryos were used for quantitative real time polymerase chain reaction (qRT-PCR).

**Whole-mount RNA In Situ Hybridization**

The cDNA template for probe synthesis was generated by RT-PCR (see Fig. 2 for primer sequences). The (digoxin) DIG-labeled riboprobe was synthesized using the mMESSAGE mMACHINE T3 Transcription Kit (Ambion, Foster City, Calif.) according to the manufacturer’s instructions. Whole-mount RNA in situ hybridization (WISH) was performed with staged Tü embryos as previously described.30

**Genotyping of tln1hi3093Tg/+ Retroviral Insertion Line**

Genomic DNA was prepared from adult zebrafish tail-clips and embryos as described previously.11 Briefly, 5 μl genomic DNA was used for separate amplification reaction with primer pairs tln1F-tln1R and tln1F-R1 (see Fig. 2 for primer sequences), respectively. The following conditions were used: 94°C for 3 minutes; 34 cycles of 94°C for 30 seconds, 58°C for 40 seconds, 72°C for 40 seconds; 72°C for 5 minutes; and hold at 8°C. Five hundred eighty base pairs in tln1F-tln1R PCR reaction confirms wild-type (WT), and 256 base pairs in tln1F-R1 PCR reaction confirms presence of the hi3093Tg insertion.

**qRT-PCR**

tln1 Transcripts were quantified in whole tln1-/- and WT embryos via qRT-PCR (see Fig. 2 for primers sequences) and normalized with respect to ef1α expression as described previously.11

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Used for</th>
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<tbody>
<tr>
<td>Fwd</td>
<td>GAATGTGAATGTTGGAAGACGATGCAG</td>
<td>WISH</td>
</tr>
<tr>
<td>Rev.</td>
<td>GGATCCAATTAACCCTCATAAAGGG</td>
<td>WISH</td>
</tr>
<tr>
<td></td>
<td>AGTTGACCTTTGGCTCGATCTC</td>
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<tr>
<td>(the underlined depicts the T3 promoter sequence)</td>
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<tr>
<td>tln1F</td>
<td>TACCCAGCATTTACTCAACAGGAAC</td>
<td>Genotyping</td>
</tr>
<tr>
<td>tln1R</td>
<td>GTAGGCCATCACAACAAATCATT</td>
<td>Genotyping</td>
</tr>
<tr>
<td>R1</td>
<td>CCAACCTACAGGTTGGGTC</td>
<td>Genotyping</td>
</tr>
<tr>
<td>Fwd</td>
<td>ATGTGGTGAAGACGATGCAGTTG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>Rev.</td>
<td>TGGGCTGTTCCCTGTCAGACG</td>
<td>qRT-PCR</td>
</tr>
</tbody>
</table>

**Cartilage Staining**

Alcian blue staining was performed at 4 dpf to analyze the craniofacial cartilage as described previously.31

**mRNA Rescue Injections**

Full-length tln1 (NM_001009560.1) cDNA was synthesized using GeneArt (Thermo Fisher Scientific, Waltham, MA). Eight synonymous substitutions were made to inactivate BamHI sites present in tln1 cDNA (see Table, Supplemental Digital Content 1, http://links.lww.com/PRSGO/A661). The cDNA was cloned into the pCS2+ vector at the BamHI sites in the multiple cloning site by Thermo Fisher Scientific. The pCS2+tln1 plasmid was linearized with NotI (New England Biolabs, Ipswich, MA), and 1 μg of the linearized template was used to synthesize mRNA using SP6 mMESSAGE mMACHINE RNA synthesis kit (Thermo Fisher Scientific) following the manufacturer’s instructions. Approximately 200 pg of RNA was injected into 1-cell stage tln1-/- intercross embryos. Injected embryos were grown to 4.5 days postinjection at 28°C and fixed in 4% PFA. Tails of all embryos were clipped for individual genotyping, and heads were used to perform Alcian blue staining.

**Visualization of Craniofacial Skeletal Muscles**

WT; mylz2:mCherry and Tln1-/-; mylz2:mCherry embryos at 4 dpf were imaged at 10× on the confocal microscope (Nikon A1R Si Confocal Eclipse Ti series).

**Transmission Electron Microscopy**

Transmission electron microscopy (TEM) was performed on WT and tln1-/- embryos at 4.5 dpf, at the Micro-
copy Core at the Program of Membrane Biology at MGH (Mass.) as described previously.11

Cell Migration and Apoptosis Assays
These were performed with WT and mutants in Tg(sox10:kaede) and Tg(sox10:mCherry) transgenic as described previously.11

Cell Proliferation Assays
The pulse-chase photoconversion assay was performed in WT;sox10:kaede and tln1+/−;sox10:kaede embryos at 60 hpf (pulse) and 72 hpf (chase) as described previously.11 5-ethynyl-2′-deoxyuridine (EdU) assay was performed in WT;sox10:mCherry and tln1+/−;sox10:mCherry embryos at 72 dpf with the Click-iT EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific) according to manufacturer’s instructions. The number of CNCCs proliferating at the given time point in WT and tln1+/− was quantified by counting the EdU-positive CNCCs (in yellow CNCCs) in the proliferative, distal most part of the palate (region bound by the white dotted line, Fig. 3, right).

Imaging, Measurements, and Data Processing
Embryos were mounted in 95% glycerol in 1× PBS with Tween 20 and imaged at 10× on Nikon 80i compound microscope (Nikon Instruments, Melville, NY) for WISH and car

\[ \text{Cell proliferation} \]

<table>
<thead>
<tr>
<th>Pulse-chase</th>
<th>EdU</th>
</tr>
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<tbody>
<tr>
<td>sox10:kaede (merge)</td>
<td>sox10: mCherry</td>
</tr>
<tr>
<td>Pulse (60 hpf)</td>
<td>Chase (72 hpf)</td>
</tr>
</tbody>
</table>

Fig. 3. Pulse-chase assay in Tg(sox10:kaede) transgenic to investigate palatal CNCC proliferation (above). Anterior (A) is up in all images. The distal tip of the palate is photoconverted from green to red at 60 hpf (pulse), in WT (left and above) and the mutant (left and below), with the contralateral side as control. Confocal images are taken after chase, at 72 hpf in both WT and tln1 (center). The palate is imaged in the green and the red channels in all instances. Green cells added onto the distal part of the palate during the chase depict the proliferative front, marked with white dotted line (center). Reduced cell proliferation is observed in tln1 mutants, (center and below) when compared with WT (center and above). EdU staining (green) reveals a proliferative front in both the WT and tln1 palate (red), marked by a white dotted rectangle (right). The proliferative cells are in yellow (overlap of green EdU+ and mCherry sox10+ CNCCs), depicted by white arrowheads. Scale bar: 100 μm. A, anterior; P, posterior.

RESULTS

Gene Expression of tln1 in Craniofacial Region of Zebrafish
Examination of gene expression using WISH revealed that tln1 transcripts were detected at the 2-cell stage and strongly expressed in the craniofacial region at 48 hpf (see Figure, Supplemental Digital Content 2, http://links.lww.com/PRSGO/A662). From 60 to 72 hpf, tln1 gene expression was restricted to the palate and the Meckel’s cartilage (Fig. 1, below; see Figure, Supplemental Digital Content 2, http://links.lww.com/PRSGO/A662).

Characterization of tln1 Retroviral Insertional Mutant Line
Analysis of tln1hi3093Tg+/+ intercross revealed morphological anomalies, where genotyping confirmed that the anomalies were exhibited by tln1 homozygotes observing Mendelian ratios (see Table, Supplemental Digital Content 3, http://links.lww.com/PRSGO/A663). The mutant phenotype began to be apparent at 48 hpf when tln1+/− embryos displayed cardiac edema that became more severe by 60 hpf (data not shown). The homozygotes did not survive past 5.5 dpf. qRT-PCR demonstrated a 60% reduction in tln1 mRNA in mutants by 2 dpf, a 70% reduction by 3 dpf, and nearly 75% reduction by 5 dpf, when compared with WT embryos (see Figure, Supplemental Digital Content 4, http://links.lww.com/PRSGO/A664).

Craniofacial Cartilage Defects in tln1 Mutants
Examination of the craniofacial cartilages showed that the tln1+/− mutants exhibited malformed craniofacial structures (Fig. 4, above and center). The Meckel’s cartilage was highly hypoplastic (Fig. 4, left, above, and center). The length/width (L/W) ratio for the Meckel’s cartilage of the tln1+/− mutants was 0.30 ± 0.045, which was significantly smaller than in WT (0.61 ± 0.073; see Figure, Supplemental Digital Content 5, http://links.lww.com/PRSGO/A665). Similarly, the palate was foreshortened with a dysmorphic leading edge (Fig. 4, right, above, and center). The L/W ratio for the mutant palate was 0.59 ± 0.04, which was significantly lower than that in WT (1.10 ± 0.08; see Figure, Supplemental Digital Content 5, http://links.lww.com/PRSGO/A665).
Defects in Craniofacial Skeletal Muscles in \( tln1 \) Mutants

Severe defects were observed in the craniofacial skeletal muscles of the \( tln1 \) mutants (Fig. 5, above and center). Although all the craniofacial muscle elements were present, the overall muscle architecture was malformed. The angles between the 2 interhyoideus (ih) and that of WT was 167.76 ± 3.02 degrees, whereas in the WT, it was 131.29 ± 6.43 degrees, which was higher than that of the \( tln1^- \) mutants (Fig. 5, above and center). The angle between the 2 ih muscles in the mutants (Fig. 5, left, above, and center) was significantly increased in the mutants (Fig. 5, left, above, and center). Similarly, in the EdU experiment, the angle between the 2 ih and hh muscles in the rescued embryos were 79.72 ± 4.26 degrees and 135.90 ± 5.63 degrees, respectively (see Figure, Supplemental Digital Content 5, http://links.lww.com/PRSGO/A665), which were both significantly less than the mutants and closer to the WT measurements. The ultrastructure images of the ih muscle in the rescued mutant revealed improved organization of the sarcomere architecture, with clearly defined A and I-bands and the Z-disk (Fig. 5, right and below).

\( tln1 \) Mutants Exhibit Reduced Proliferation of CNCCs during Palate Formation

CNCCs migration and apoptosis assays did not show any defects in the \( tln1 \) mutants (see Figure, Supplemental Digital Content 7, http://links.lww.com/PRSGO/A667). Proliferation of CNCCs during palatal morphogenesis was examined using 2 methods: pulse-chase experiment using the Tg(sox10:kaede) reporter (Fig. 3, left and center) and by EdU assay (Fig. 3, right). In the pulse-chase assay, green fluorescent cells added onto the distal part of the maxillary process during the chase period, also known as the proliferative front (marked with white dotted line, Fig. 3, center), which was notably deficient in the \( tln1^- \) mutants (Fig. 3, below and center). Similarly, in the EdU experiment, the number of EdU^+ (proliferative) CNCCs at the leading edge of the palate was reduced in the \( tln1^- \) mutants (6.2 ± 1.48) when compared with the WT (11.8 ± 1.3; Fig. 3, right; see Figure, Supplemental Digital Content 8, http://links.lww.com/PRSGO/A668).

DISCUSSION

\( tln1 \) Is Critical during Zebrafish Embryogenesis

Knockout of \( Tln1 \) in mice results in developmental arrest at gastrulation proving that \( tln1 \) has an important role in early mouse embryonic development. We show that zebrafish \( tln1 \) is expressed in very early stages of embryogenesis. At 48 hpf, \( tln1 \) is expressed strongly in the craniofacial region, localizing to the maxillary and mandibular structures by 72 hpf, suggesting that \( tln1 \) is required for development of craniofacial structures.

Zebrafish Model of \( tln1 \) LOF

Reduction of \( tln1 \) transcripts in the homozygous mutants observed by qRT-PCR suggests that the \( tln1^{hs30978} \) is a
null allele. Homozygous tln1 embryos were initially indistinguishable from WT or heterozygous siblings until ~48 hpf, at which point the homozygotes exhibited cardiac edema. Indeed, previous studies in zebrafish showed that tln1 is required for cardiac development.\(^{29}\) Earlier work showed that maternal gene products contribute to early embryonic development in zebrafish.\(^{32,33}\) The maternally showed that maternal gene products contribute to early embryonic development in zebrafish. The maternally encoded TLN1 is required for cardiac development.\(^{20}\) Earlier work showed that TLN1 is required for cardiac development.\(^{20}\) Earlier work showed that the loss of tln1 resulted in significantly underdeveloped Meckel’s cartilage and a shortened palate. Although the craniofacial muscles were all present, the tension between the different muscle structures was increased in the mutants. This is evident by the increased angles between the ih and hh muscle pair in the mutants when compared with the WT. Of note, the angle between the pair of sternohyoideus was not measured as tln1 mutants showed significant cardiac edema that can in turn affect the sternohyoideus muscles located dorsal to the zebrafish heart. The craniofacial skeletal muscles in the mutants were disorganized with disruption of the sarcomere structure. The Z-disk, the A and I-bands were completely absent in the mutants.

**CNCC Proliferation is Affected during Palate Morphogenesis in tln1 Mutants**

The CNCCs primarily undergo migration, proliferation, and control cell death to form craniofacial cartilage, specifically the palate. In the absence of tln1, it was observed that the palate is shortened. The critical cellular processes were studied in the palate to elucidate the role of tln1 during palate morphogenesis. The processes of cell migration and apoptosis were found to be not defective in the tln1 mutants. Pulse-chase experiments suggested that CNCC proliferation is defective in tln1 mutants. Edu assay also confirmed that the palate of tln1 mutants have ~50% less proliferative cells than that of the WT suggesting that defective CNCC proliferation leads to shortened palate in the mutants.

**Perspective in the Clinical Field**

Identification of the genetic requirement of tln1 in craniofacial development is immediately clinically helpful, where detection of human gene variants in Tln1 can now be associated with orofacial cleft anomalies. As next generation sequencing technology continues to move the needle of diagnosis to earlier period in gestation, we will soon be able to better impute clinical significance of human gene sequencing data, where the function of genes important to craniofacial development are assembled.\(^{36}\) Further, the major gap in human genetics is to determine the function of human gene variants, which we are able to assay rapidly in zebrafish rescue assays.\(^{37}\) This tln1 zebrafish mutant can be used as a functional assay to determine the function of human Tln1 gene variants in a injection rescue assay. Lastly, the zebrafish model can be used as a platform for small molecule screens, to discover drugs that one day may mitigate cleft anomalies.\(^{3}\)

**Loss of tln1 Leads to Defects in Craniofacial Cartilage and Skeletal Muscles**

Cartilage staining of tln1 mutants showed that the loss of tln1 resulted in significantly underdeveloped Meckel’s cartilage and a shortened palate. Although the craniofacial muscles were all present, the tension between the different muscle structures was increased in the mutants. This is evident by the increased angles between the ih and hh muscle pair in the mutants when compared with the WT. Of note, the angle between the pair of sternohyoideus was not measured as tln1 mutants showed significant cardiac edema that can in turn affect the sternohyoideus muscles located dorsal to the zebrafish heart. The craniofacial skeletal muscles in the mutants were disorganized with disruption of the sarcomere structure. The Z-disk, the A and I-bands were completely absent in the mutants.
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