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 Genetic Requirement of *talin1* for Proliferation of Cranial Neural Crest Cells during Palate Development

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**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 (*tln*) 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 photoconversion, 5-ethyl-2'-deoxyuridine proliferation, migration, and apoptosis assays were performed for functional analysis.

**Results:** Expression of *tln1* 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 *tln1* 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 *tln1* is critical for craniofacial morphogenesis in zebrafish. Loss of *tln1* leads to a shortened palate and Meckel’s cartilage along with disorganized skeletal muscles. Investigations into the cellular processes show that *tln1* 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 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 COL1A2, 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.

### MATERIALS AND METHODS

#### Zebrafish Lines and Maintenance

The retroviral insertion mutant line Tln1h3093Tg/+ (hereafter referred to as Tln1h+) was obtained from the Zebrafish International Resource Center. Wild-type (WT), Tg(sox10:kaede),27 Tg(sox10:mcherry),28 and Tg(nykl:2:mcherry)29 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-
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. Genotyping of tln1 hi3093Tg/+ Retroviral Insertion Line
Genomic DNA was prepared from adult zebrafish tailclips and embryos as described previously. 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

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

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 inactive 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 10x 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 and 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.

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 carilage staining experiments. The palate and mandible were dissected and flat mounted before imaging them at 40×. Embryos were mounted in 3% methylcellulose and imaged at 20× on the confocal microscope (Nikon A1R Si Confocal Eclipse Ti series) for cell proliferation, migration, and apoptosis assays. All images were processed with NIS-Elements advanced image acquisition and analysis system (Nikon Instruments). Figures were composed with Adobe Photoshop CS6 and Adobe Illustrator CS6 (Adobe, San Jose, CA). All measurements were performed using ImageJ (NIH).

Statistical Analysis
All statistical analyses were performed by two-tailed Student’s t test using the GraphPad software (https://graphpad.com/quickcalcs/). A threshold of P ≤ 0.001 was considered to be statistically significant in all cases. All results are represented as mean ± SD.

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).
The angles between the 2 interhyoideus (ih) muscles of the  
mutants (Fig. 5, above and center), which was notably deficient in the 
mutants (Fig. 4, below; Fig. 5, below). The L/W ratio of both  
the Meckel’s cartilage and the palate were measured in the  
rescued  
tln1-/-  
embryos. The L/W ratio for the Meckel’s cartilage  
of the rescued mutants was 0.59 ± 0.0322, which was  
significantly higher than that of the  
tln1-/-  
muscles and closer to WT ratio (see Figure, Supplemental Digital Content 5, http://  
links.lww.com/PRSGO/A665). 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 6, http://links.lww.com/PRSGO/A666), 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-/-  
mice (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 ar-
rest 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 observed by qRT-PCR suggests that the  
tln1  
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.\(^{26}\) Earlier work showed that maternal gene products contribute to early embryonic development in zebrafish.\(^{22,23}\) The maternally contributed \( tln1 \) protein masked the zygotic requirement of \( tln1 \) at earlier stages of embryonic development till 48 hpf. However, as maternal \( tln1 \) becomes slowly depleted over time, the consequence of \( tln1 \) deficiency manifested in multiple organ systems progressively, as the embryo developed cardiac edema, craniofacial cartilage hypoplasia, and muscle disorganization, leading to eventual embryonic lethality by 5.5 dpf.

**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.

**CNCC Proliferation is Affected during Palate Morphogenesis in \( tln1 \) Mutants**

The CNCCs primarily undergo migration, proliferation, and controlled cell death to form the 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.

\( tln1 \) has been shown to play a key role in integrin-mediated cellular events during the development of kidney, heart, and vascular system.\(^{30,34,35}\) However, the role of \( tln1 \) in craniofacial morphogenesis has not been studied. This study reveals that \( tln1 \) is essential for the formation of craniofacial cartilage and maintenance of the skeletal muscles, and that CNCC proliferation during palate morphogenesis requires function of \( tln1 \). This work provides novel insights into the role of \( tln1 \) in craniofacial development.

**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 a injection 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.\(^{3}\)
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