Published in final edited form as:

Gene Expr Patterns. 2012 ; 12(0): 145–153. doi:10.1016/j.gep.2012.01.002.

***Mustn1* is essential for craniofacial chondrogenesis during *Xenopus* development**

Robert P Gersch^{1,a}, Arif Kirmizitas^{2,a}, Lidia Sobkow¹, Gina Sorrentino³, Gerald H Thomsen², and Michael Hadjiargyrou^{1,*}

¹Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY 11794-5281

²Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY 11794-8575

³Department of Anatomical Sciences, Stony Brook University, Stony Brook, NY 11794-8081

Abstract

Mustn1 is a vertebrate-specific protein that, *in vitro*, was showed to be essential for prechondrocyte function and thus it has the potential to regulate chondrogenesis during embryonic development. We use *Xenopus laevis* as a model to examine *Mustn1* involvement in chondrogenesis. Previous work suggests that *Mustn1* is necessary but not sufficient for chondrogenic proliferation and differentiation, as well as myogenic differentiation *in vitro*. *Mustn1* was quantified and localized in developing *Xenopus* embryos using RT-PCR and whole mount *in situ* hybridization. *Xenopus* embryos were injected with either control morpholinos (Co-MO) or one designed against *Mustn1* (*Mustn1*-MO) at the 4 cell stage. Embryos were scored for morphological defects and Sox9 was visualized via *in situ* hybridization. Finally, *Mustn1*-MO-injected embryos were co-injected with *Mustn1*-MO resistant mRNA to confirm the specificity of the observed phenotype. *Mustn1* is expressed from the mid-neurula stage to the swimming tadpole stages, predominantly in anterior structures including the pharyngeal arches and associated craniofacial tissues, and the developing somites. Targeted knockdown of *Mustn1* in craniofacial and dorsal axial tissues resulted in phenotypes characterized by small or absent eye(s), a shortened body axis, and tail kinks. Further, *Mustn1* knockdown reduced cranial Sox9 mRNA expression and resulted in the loss of differentiated cartilaginous head structures (e.g. ceratohyal and pharyngeal arches). Reintroduction of MO-resistant *Mustn1* mRNA rescued these effects. We conclude that *Mustn1* is necessary for normal craniofacial cartilage development *in vivo*, although the exact molecular mechanism remains unknown.

© 2012 Elsevier B.V. All rights reserved.

*Corresponding author: Robert.Gersch@stonybrook.edu, arifkirmizitas@yahoo.com, lidia_sobkow@yahoo.com, sorrentinogm@gmail.com, gthomsen@notes.cc.sunysb.edu, michael.hadjiargyrou@sunysb.edu (Tel. 631-632-1480; Fax 631-632-3222).

^aThese authors contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Xenopus; Mustn1; chondrogenesis; Sox9; morpholino; knockdown; craniofacial; somite

Background

The development of vertebrate craniofacial structures is an intricate process of differentiation and morphogenesis, predominantly involving and coordinated by endodermal tissues [1]. In particular, the cranial neural crest cells (NCCs), which originate at the interface of anterior neural and non-neural ectoderm, contribute significantly to skeletal and connective components of the vertebrate head [2,3]. Thus, understanding the developmental programs that generate head structures is interesting from developmental and physiological perspectives.

In *Xenopus laevis*, the skeletal structures of the head begin forming in the tailbud tadpole and become visible at swimming tadpole stages [4], but they remain completely cartilaginous until metamorphosis when the action of thyroxine triggers dramatic cartilage remodeling and calcification as osteogenesis proceeds. Knowledge about the molecular and cellular mechanisms of vertebrate chondrogenesis and osteogenesis have been emerging rapidly over the last few years, largely through mammalian model system studies in tissue culture, and *in vivo* studies of embryos and regenerating adult tissues [5, 6]. However the mechanisms underlying head cartilage and bone development are not well understood. The *Xenopus* tadpole has emerged as an excellent system for the study of head skeleton development, and more generally the development of head tissues and organs originating from non-neural ectoderm (e.g. sensory placodes), neural crest (head bones, jaws, gills), and anterior neural plate (brain). Comparative studies of amphibian, fish, chick and mammalian head development have begun to provide significant insight into the evolution of head development and its surprising developmental plasticity [7,8].

Mustn1 is a small, 9.6 kDa nuclear protein that is expressed in the developing and regenerating musculoskeletal system in mammals and is implicated in myogenic and chondrogenic processes [9,10,11,12]. However, little is known about the molecular functions of *Mustn1* and whether it has functional links to known chondrogenic pathways. The biological functions of *Mustn1* have begun to emerge through *in vitro* investigations. For example, the function of *Mustn1* has been evaluated via RNAi gene silencing in the pre-chondrocyte cell line, RCJ3.1 C.5.18 (RCJ), where knockdown of *Mustn1* in these cells reduced both proliferation and differentiation [11]. Moreover, matrix production by hypertrophic chondrocytes was disrupted and accompanied by dramatic decreases in chondrogenic marker (Sox9, Collagen II, and Collagen X) gene expression [11]. These findings indicate that *Mustn1* is correlated with and required for the differentiation of chondrocytes, at least in cell culture, and thus *Mustn1* may serve as a critical regulatory molecule for cartilage development.

To investigate the role of *Mustn1* during cartilage development, we examined its expression and functionally perturbed *Mustn1 in vivo* by gene knockdown in embryos of *X. laevis*, a well-understood model system for vertebrate embryogenesis. *Xenopus* historically has

provided numerous, significant insights into the roles of developmental regulatory genes, by gain of function (by) and loss of function approaches (e.g mRNA injection and oligo-mediated gene knockdown, respectively) [13,14,15]. In the context of cartilage formation, *X. laevis* develops in a manner that is very similar to other vertebrates, including mouse and human. Particularly, craniofacial development in *X. laevis* displays some of the same landmark features observed in mammals, such as branchial, hyoid and mandibular arches, Meckel's Cartilage and the palatoquadrate [16], derived from similar cell lineages (largely cranial neural crest) that expand and mature into homologous structures [17,18]. This homology makes *Xenopus* an attractive model for human craniofacial birth defects.

Several chondrogenesis-related genes that are affected by *Mustn1* silencing in cell culture (e.g. Sox 9, Collagen II, Collagen X), play important roles during *X. laevis* cartilage development, suggesting they potentially function in a developmental gene circuit with *Mustn1*. For example, Sox9 and other Sox transcription factors have been implicated in *Xenopus* neural crest development and chondrogenesis, as in mammalian embryos [19, 20], and Collagen II, a primary target gene of Sox9, is expressed in chondrogenic regions of the *X. laevis* embryo, such as the neurocranium and otic vesicles [21, 22, 23]. The expression patterns of these genes correspond in part to where *Mustn1* is expressed in mid-late stage mouse embryos (E9.5-11.5), which include the limb buds, somites, branchial arches, and frontonasal process [11]. However, the early developmental dynamics of *Mustn1* expression have not been examined in *Xenopus* or mammalian embryos in detail, nor has the developmental function of *Mustn1* been examined by embryonic loss of function. Therefore, we have examined *Mustn1* expression patterns and evaluated its embryonic knockdown phenotypes during *X. laevis* development. Our findings illustrate conserved expression of *Mustn1* in chondrogenic mesoderm, similar to the mouse, and further provide the initial evidence that vertebrate craniofacial chondrogenesis requires *Mustn1*.

Results

Mustn1 is a highly conserved vertebrate-specific protein

Initially, we revisited the issue of whether and to what extent *Mustn1* is conserved among the metazoa. The initial discovery and recent analysis of *Mustn1* sequences suggested that it represents a unique protein family found only among higher vertebrates [9], as reflected in the Universal Protein Resource (UniProt) database (<http://www.uniprot.org/uniprot/?query=family:%22MUSTANG+family%22>). We reevaluated *Mustn1* conservation by performing a new homology comparison, incorporating the most recent database entries for *Mustn1* genes retrieved by BLAST searches. Results indicate that no sequences with significant identity to *Mustn1* could be found outside of the vertebrate clade, but the list of animals with *Mustn1* orthologs encompasses several lower vertebrates in addition to amniotes. The *Mustn1* protein sequence is very conserved among vertebrates, as illustrated by the comparison of mouse (*Mus Musculus*), snake (*Deinagkistrodon acutus*), lizard (*Anolis carolinensis*), frog (*Xenopus tropicalis*, *Xenopus laevis*), and zebrafish (*Danio rerio*) (Fig. 1). Specifically, when compared to mouse, the snake, lizard, frog and zebrafish protein sequences are 79%, 78%, 72%, 70%, and 64% identical, respectively. The only clear motif/domain that can be identified is a nuclear localization signal (NLS) located at residues

10-18, which is very well conserved amongst all species, sharing 6-9 identical aa out of the 10 (Fig. 1, black highlight). Differences in sequences between mouse and the other *Mustn1* sequences are highlighted in grey (Fig. 1).

***Mustn1* expression is temporally and spatially localized to developing craniofacial and somitic mesoderm**

To determine the temporal and spatial expression of *Mustn1* during *X. laevis* development, we evaluated mRNA transcripts by qPCR and whole mount *in situ* hybridization. Temporal analysis of *Mustn1* expression revealed that mRNA transcripts appear initially at stage 18, at the time of anterior neural plate closure, with expression reaching a plateau during early tadpole stages and persisting into swimming/feeding tadpole, stage 35 (Fig. 2A). RT-PCR on dissected pieces of stage 20 and 24 embryos revealed significant *Mustn1* expression in dorsal tissues (Fig. 2B-C), consistent with results of whole mount *in situ* hybridization which showed predominant expression of *Mustn1* in the paraxial mesoderm (Fig. 2D-F). *Mustn1* expression was found to begin at stage 18 and become clearly visible in the paraxial, somitic mesoderm by neurula stage 20. Somites and their derived muscle continue to express *Mustn1* throughout the tadpole stages that were assessed (Fig. 2D-F, black arrows). Focusing on the head, significant *mustn1* expression was detected at late tadpole stage 35, in anterior structures corresponding to mandibular, hyoid, branchial and other head cartilaginous tissues (Fig. 2F,G, black arrow heads), the otic vesicle (Fig. 2F,G, white arrow), and heart (Fig. 2F,G, white arrow head). Sense probe controls with the same stage tadpoles did not show any staining (Figure 2H-J).

***Mustn1* knockdown results in head and body axis defects**

In order to determine if *Mustn1* is necessary for musculoskeletal development, we used morpholino oligos to perturb *Mustn1* levels. Two dorsal blastomeres were injected at the 4-cell stage with either a control morpholino oligonucleotide (Co-MO, Gene Tools scrambled sequence) that does not target known sequences among *X. laevis* ESTs and cDNAs, or antisense *Mustn1* morpholino (*Mustn1*-MO) that targets the start codon of *Mustn1* mRNA. These injections delivered MO into the dorsal and anterior tissues of the developing embryo, including head and anterior paraxial mesoderm, as well as the anterior neural-ectodermal margin from which the cranial neural crest cells (NCCs) originate. Injected embryos were fixed and analyzed for phenotypic changes throughout development, focusing on stages and locations with elevated *Mustn1* expression. Whereas Co-MO-injected embryos showed no significant developmental defects throughout the investigated time course (Fig. 3A, D), the *Mustn1*-MO-injected embryos showed several morphological defects that included, small or absent eye(s), reduced body axis length, and kinks within the body/tail at both stages 37-38 and 40 (Fig. 3B, E), that corresponded to early swimming/feeding tadpoles [4]. When *Mustn1* expression was reintroduced via co-injection of *Mustn1*-MO embryos with modified, MO-resistant *Mustn1* mRNA (Rescue), the frequency and severity of these defects was significantly reduced or ablated completely (Fig. 3C, F). In unilaterally *Mustn1*-MO-injected morphants, we observed the same gross morphological defects on the injected side (Sup. Fig. 1E, F). In contrast, the uninjected contralateral control side of morphants showed reduced or no morphological defects (Sup. Fig. 1A, B) as did the unilateral Co-MO-injected embryos (Sup. Fig. 1C, D).

The morphological defects in *Mustn1* morphants were quantified by scoring specific features of the injected embryos and plotting the data as a function of population percentage. Eye development was scored as an easy indicator of head neuroectodermal development, and 96% of Co-MO-injected embryos (n=176) developed two normal eyes, in comparison to only 32% (n=184) of the *Mustn1*-MO-injected embryos (Fig. 4A). However, in contrast to the latter, 61% (n=158) of Rescue-injected embryos developed normal eyes. Further inspection of the eye phenotype found that 3% of the Co-MO-injected embryos had two small eyes, 0.5% had one eye and 0% had no eyes. In contrast, the *Mustn1*-MO-injected embryos resulted in 27% of tadpole stage embryos with two small eyes, 28% with only one eye, and 13% with no eyes. Finally, the Rescue-Injected group resulted in 22% of embryos with small eyes, 13% with only one eye, and 4% with no eyes (Fig. 4A).

The body axis also displayed gross morphological defects in body length and tail morphology. In the X. laevis stage 40 embryo, the length of the body (snout to posterior edge of endoderm) and tail (endoderm-free portion) are approximately equal (Fig. 3D). In *Mustn1*-MO-injected embryos we observed dorsal axis and total body length truncations that consistently altered this ~1:1 ratio. In the Co-MO-injected group, 90% of embryos developed a normal-length anterior-posterior (A-P) body axis and 10% had a shortened axis (Fig. 4B). In contrast, *Mustn1*-MO-injected embryos resulted in 51% of embryos with normal body axis length and 49% with shortened axis. The Rescue-injected group resulted in 70% and 30% of embryos with normal and shortened body axes, respectively (Fig. 4B).

A number of embryos had tail defects that were classified into three groups: tail development with no defects, mild defects consisting of slight curvature, and severe defects in which tail curvature showed greater than a 90° bend angle with respect to the A-P body axis. In the Co-MO-injected group, 88% of the injected embryos displayed no tail defect, while the remaining 12% showed a mild tail phenotype. In contrast, among *Mustn1*-MO-injected embryos, only 31% showed no tail defect, 45% of embryos displayed mild defects, and the remaining 24% exhibited the severe phenotype. In the Rescue-Injected group, 45% of embryos showed no tail defect, 40% showed mild phenotype, and the remaining 15% of embryos displayed a severe tail bend phenotype (Fig. 4C). Further, we verified that the Co-MO and *Mustn1*-MO-injected embryos were targeted in craniofacial, somite and tail regions by co-injecting embryos with the MOs and FDA (Fluorescent Dextran Amine). In Co-MO-injected embryos, FDA localized to head and body regions, which developed normally (Sup. Fig. 2A, B), but in *Mustn1*-MO-injected embryos FDA staining was associated with areas of morphological defects in the head and body axis (Sup. Fig. 2C-F). We also observe uniform distribution of FDA and mCherry (red fluorescence marker) co-injected with the rescue *Mustn1* mRNA in the Rescue-injected tadpoles (Sup. Fig. 2G-I).

***Mustn1* knockdown inhibits head chondrogenesis**

To determine whether *Mustn1* is essential for chondrogenesis, the anterior tissues of *Xenopus* embryos were targeted with MOs and analyzed for expression Sox9, which marks chondrogenic cells, and for the presence of cartilage by Alcian blue staining. Sox9 expression was examined by *in situ* hybridization in stage 40 tadpoles, and the majority of Co-MO-injected embryos displayed Sox9 staining within the craniofacial region,

particularly anterior and dorsal staining that likely corresponds to neural tube and NCCs along the anterior boundary of the head (Fig. 5A-C, black arrows), in the pharyngeal arches (Fig. 5A-C, white arrows), and ventral and anterior regions near the eyes (Fig. 5A-C, black arrowheads). In contrast, anterior Sox9 expression domains were almost completely ablated in *Mustn1*-MO-injected embryos (Fig. 5B). In embryos rescued with *Mustn1* mRNA injection, Sox9 expression was similar to that of Co-MO-injected embryos, again staining the pharyngeal arches, putative NCCs and cranial structures in the eye periphery (Fig. 5C). These results were further verified using qPCR, where we found significant differences in Sox9 expression between Co-MO-injected and *Mustn1*-MO-injected embryos at stage 34 & 39 (Fig 5, graph). Specifically, the *Mustn1*-MO-injected embryos showed significant reductions in Sox9 expression of ~33.4% (Stage 34) and ~38.7% (Stage 39), respectively, when compared to Co-MO-injected embryos. No significant differences in expression were found in Sox9 expression between Rescue-Injected and Co-MO-injected embryos at any of the three stages evaluated (Fig 5, graph).

Finally, we scored cartilage formation in control and *Mustn1* knockdown embryos and observed a significant and severe loss of Alcian blue-stained tissues in regions of tadpoles injected into one dorsal-anterior blastomere (at the 4-cell stage) with the *Mustn1*-MO (Fig. 6C,D), but not with the Co-MO which looked wild-type with clear cartilage staining of ceratohyal cartilage (Fig. 6A, B, black arrows) and gills (branchial arch derivatives; Fig. 6A, B, black arrows). In contrast, *Mustn1*-MO-injected morphants showed dramatic disruption, and in some cases a complete absence, of cartilaginous structures associated with the eye, as well as the ceratohyal cartilage and gill arches (Fig. 6C,D and Sup. Fig. 3A). In addition, ~93% of the morphants examined with Alcian blues display disrupted cartilage formation (Sup. Fig. 3B).

Discussion

Mustn1 is as a small musculoskeletal specific nuclear protein that is expressed in mesenchymal condensations of limbs, vertebral perichondrium, and mesenchymal cells of the intervertebral discs during skeletal development, as well as periosteal osteoprogenitor cells, osteoblasts and proliferating chondrocytes during bone regeneration [9]. Knockdown of *Mustn1* in pre-chondrocytes *in vitro* resulted in strongly reduced proliferation and differentiation, accompanied by a decrease in matrix production and chondrogenic marker gene expression [11]. We report here that *X. laevis*, *Mustn1* temporal and spatial expression correlates to tissues of musculoskeletal development, particularly the somites and head (at the developmental onset, late neurula and tailbud tadpole, respectively). *Mustn1* expression dynamics and tissue localization is very similar to the patterns observed during mammalian development [9,11]. Further, the *Mustn1* protein sequence is highly conserved across the vertebrate class, but interestingly we have not yet been able to identify *Mustn1* homologs in non-vertebrate metazoans, suggesting *Mustn1* has a vertebrate-specific function in cartilage and skeletal muscle development in vertebrates, or the regulation thereof.

Loss of function experiments reported herein establish *Mustn1* as a gene that is essential for chondrogenesis *in vivo*. When *Mustn1* expression was reduced via antisense morpholino injection, multiple developmental defects were observed compared to Co-MO-injected

embryos (depending on targeting location). These *Mustn1* morphant embryos displayed craniofacial malformations leading to small or absent eye(s), shortened body axis and tail, and curvature or kinks in the tail/body. Reintroducing *Mustn1* into the knockdown embryos greatly reduced the severity of these defects, rescuing embryos to near normalcy and thereby demonstrating the specificity of morpholino targeting. These results were consistent even when multiple stages were examined and when the concentration of morpholino injected was varied from 30-40ng/embryo. However, the mild defects were also observed at lower MO concentrations suggesting that *Mustn1* can function in a graded manner *in vivo*, although this needs to be more fully investigated.

While craniofacial regions were severely affected in *Mustn1* morphants, the presence and size of the cement gland [24], a ventral-anterior ectodermal tissue, remained largely unchanged. This may suggest that the craniofacial defects observed can be attributed to impaired development of dorsal tissues, such as the lack of proper migration of NCCs or differentiation of prechordal or even anterior paraxial mesoderm. However, these effects could be the end result of a number of defective processes such as lack of proliferation of these cells, failure of these lineages to properly differentiate, or failure of neighboring tissues to communicate (such as NCCs and the developing eye placode). Of particular note, we did not observe expression of *Mustn1* in a pattern that would suggest it is expressed in the cranial NCCs *per se*, but we did observe staining patterns in head structures that are very consistent with the location of pre-cartilaginous NCC derivatives, such as the pharyngeal arches.

Our demonstration that *Mustn1* knockdown reduces Sox9 expression and subsequent formation of alcian blue-positive cartilaginous tissues in the tadpole head provides the first direct evidence that *Mustn1* is required for formation of embryonic cartilage. Sox9 is normally expressed within the craniofacial region of swimming tadpoles (after stage 33), corresponding to chondrogenic tissues derived from the cranial NCCs, which originate posterior and dorsal to the craniofacial region and migrate ventrally into the pharyngeal arches and anterior to the eye. Although we did not observe a *Mustn1* expression pattern that mimicked the expression of early (neurula-tailbud) stage NCC markers, such as Slug [25], Sox10 [26], Traf4 [27] or Sox9 [19], *Mustn1* expression at later stages is located where derivatives of the cranial neural crest migrate, and where *Mustn1* knockdown affects late stage Sox9 expression. Precise lineage and expression pattern analyses are needed to determine whether head tissues that express *Mustn1* also express Sox9.

Cartilage loss, the predominant *Mustn1* morphant head phenotype, is congruent with *Xenopus* phenotypes resulting from morpholino knockdown of other genes known to regulate chondrogenesis. For example, when Sox9 was depleted in developing *X. laevis* embryos, eye size and anterior Alcian blue-positive cartilaginous matrix, were reduced [20]. Also, head cartilage formation was almost completely ablated by Runx2 knockdown [28], resulting in craniofacial deformities similar to those we observed in *Mustn1* morphants. Further, knockdown of FoxN3, a subclass N forkhead/winged-helix transcription factor, caused a reduced eye size phenotype, likely caused by a disruption in cranial crest cell migration [18]. While beyond the scope of this report, further determination of the effects of *Mustn1* depletion on critical chondrogenic regulators and differentiation programs is a

promising avenue to pursue and should reveal genetic interactions and molecular interplay among these genes. Our findings with *Xenopus* embryos also are consistent with results from our *in vitro* experiments with the pre-chondrocytic RCJ cell line, which show that *Mustn1* silencing negatively affects proliferation and matrix production, and downregulates chondrogenic marker genes [11]. Taken together, we conclude that *Mustn1* is a vital protein necessary for chondrogenesis *in vitro* and *in vivo*. However, the complete molecular mechanism responsible for *Mustn1* function remains unknown.

Conclusions

Our data demonstrate that *Mustn1* is necessary for the proper patterning and development of head cartilage in the *Xenopus* embryo. Knockdown of *mustn1* caused morphological defects in cartilaginous head structures accompanied by reduced spatial and quantitative expression of the chondrogenic marker, Sox9. That reintroduction of MO-resistant *Mustn1* mRNA partially rescues these morphant phenotypes and validated specificity of the knockdown effects. While the mechanism by which *Mustn1* regulates cartilage formation remains unclear, characterization of this molecule and its genetic network represents an interesting avenue of research to further elucidate the processes of chondrogenesis.

Methods

Antisense morpholino oligonucleotide and rescue plasmid

A *Mustn1* cDNA (Accession# EB473418, corresponding to IMAGE clone ID 854372) was obtained from Open Biosystems and subcloned into the CS2+ plasmid. An antisense morpholino oligonucleotide (*Mustn1*-MO) derived from 25 nucleotides located toward the 3' terminus of the *Mustn1* coding sequence was designed and synthesized by Gene Tools, LLC (Philomath OR), with the sequence 5' GGGAATGTCCCAACCACAGGATGCC 3'. Doses of 30-40ng were injected dorsal of the midline of 4-cell stage embryos. A standard negative control morpholino oligonucleotide (GeneTools LLC) was used as control and injected under identical conditions. Its sequence is 5'-CCTCTTACCTCAGTTACAATTTATA-3', and it corresponds to the antisense complement of the sequence of a human beta globin intron mutation found in beta thalassemia. The *Mustn1* rescue gene was created by modifying the coding sequence of *Mustn1* at the morpholino target sequence, using a site directed mutagenesis Sp6/T7 kit (Roche) to change the sequence to 5'-GGaATtCTcTGGTTaGGcCAcTCaC-3' where lower case nucleotides indicate mutations altering the original sequence. To construct the *Mustn1*-GFP fusion, which was used to verify MO efficacy, the *Mustn1* 5'UTR and coding region was subcloned into pDONR221 Gateway donor vector (Invitrogen Inc) using following primers. *Mustn1* Gateway Forward: 5-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCCTCTGCTTC TCATCAGCGTG-3'. *Mustn1* Gateway Reverse: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCACCAAGACACTTTTTGCCGG-3' After sequence verification, the 5' UTR-mustn1 sequence was moved to the destination vector pCS2-GFP to form an in-frame C-terminal GFP tag on the *Mustn1* protein. Synthetic mRNA was synthesized using an SP6 Txn kit (Ambion) from a *NotI* linearized plasmid, purified and injected together with MOs into 4-cell embryos.

Embryo manipulations

In vitro fertilization and embryo culture were done according to standard protocols. Embryonic stages were determined according to Nieuwkoop and Faber (1967) [4]. Microinjections were done according to routine procedures [27]. Embryos were cultured in 0.1x MMR and were fixed in MEMFA (0.1 M MOPS, pH 7.5, 2.0 mM EGTA, 1.0 mM MgSO₄) for further processing (*in situ* hybridization), observation and photography.

RT-PCR and qPCR analysis

Quantification of gene expression via qPCR was preformed using kits and protocols from either Roche or Qiagen. Briefly, RNA was extracted from either whole wild-type embryos (n=5) or anterior dissections of Co-MO-injected, *Mustn1*-MO-injected, and Rescue-Injected embryos (n=5) via degradation with proteinase K in TNES buffer followed by phenol/chloroform extraction.

For RT-PCR, RNA from whole dissected embryos was used directly for amplification of *Mustn1* (5'-TGCGAACAATCTGGAACAAC-3' and 3'-GTTAGACTCGGCATGGTGTG-5') via a SuperScript III One-Step RT-PCR kit (Invitrogen). Briefly, samples were allowed to reverse transcribe for 30 minutes at 55°C, denatured at 94°C, annealed at 59°C and extended at 68°C. RT-PCR reactions were run for 23 cycles and run on a 1% agarose gel for visualization. For qPCR, RNA from wild-type embryos was reverse transcribed to cDNA (via Invitrogen kit) prior to qPCR for *Mustn1* (using the above primers) using a Lightcycler 480 and one-step qPCR SYBR green kit (Roche). The RNA from MO-injected embryos became template for qPCR of Sox9 (5'-AACAGGAGTTCCATCAATCCCC-3' and 5'-CTTTTGCTAAACCCCGTGTCAC-3') [29] and MyoD (5'-AGCTCCAAGTCTCCGACGGCATGAA-3' and 5'-AGGAGAGAATCCAGTTGATGGAAACA-3') [30] using Lightcycler 2.1 (Roche) and two-step qPCR SYBR green kit (Qiagen). In both cases target gene expression was normalized to the expression level of Ornithine decarboxylase (5'-GCCATTGTGAAGACTCTCTCCATTC-3' and 5'-TTCGGGTGATTCTTGCCAC-3') [30]. cDNA and mRNA were denatured at 94°C, annealed at 55-60°C and extended at 72°C. qPCR reactions were run for 40 cycles and relative levels of expression were determined from log phase amplification values obtained automatically by the software.

Whole mount *in situ* hybridization

Whole mount *in situ* hybridizations were done according to standard procedures [14]. Briefly, embryos were fixed at specific stages with 4% paraformaldehyde for 1 hour and stored in 100% Methanol prior to hybridization. The embryos were re-hydrated in a methanol/PBST gradient and permeabilized with proteinase K for 5 min. Embryos were pre-hybridized prior to incubation overnight in hybridization buffer with 1µg/ml probe at 60°C. Embryos were washed in a SSC gradient before being blocked with MABT + 10% Lamb serum +2% blocking reagent (Roche). Embryos were then incubated in Anti-DIG overnight at 4°C prior to detection with BM Purple (Roche). Full-length *X. laevis Mustn1*, MyoD and Sox9 (Accession number, BC123175, X16106, NM_001090807) sequences were derived and verified by sequencing. Each cDNA was subcloned into pCS2 using XhoI and XbaI

sites. RNAs were transcribed *in vitro* using kits from Ambion on linearized plasmids pCS2-*Mustn1*, pCS2-MyoD, pCS2-Sox9.

Scoring of morphological defects

Criteria were designed for each of the defect categories. For eye defects, embryo eyes were considered normal if they did not demonstrate any of the following: a) small eye(s) showed an observable reduction in eye diameter when compare to average wild type embryos b) 1-eyed embryos showed little to no eye tissue unilaterally; c) embryos with a no eye phenotype did not display any observable eye tissue on either side of the head. For axis defects, an embryo body axis was considered normal if, at stage 40, the body and tail were approximately equal length. If this body: tail length ratio was observably divergent from 1:1, (which always resulted in a truncation of total body length), then the embryo was considered to have a shortened body axis. For tail defects, embryo bodies/tails were considered normal if they contained no kinks. If the embryo showed kinks in the body/tail that caused less than a 90° change in body axis, they were considered mild defects. If the embryo body/tail contained kinks causing a greater than 90° change in body axis, they were considered severe defects.

Cartilage staining

Staining for cartilaginous tissues was done using Alcian blue according to the Kerny et al (2007) [28] modified method of Dingerkus and Uhler (1977) [31]. Briefly, embryos injected with *Mustn1* MO, control MO and uninjected embryos were allowed to develop to stage 45 (swimming tadpole). Animals were fixed overnight, at 4°C, in 4% freshly made PFA. Then samples were subjected to series of PBS-Tween 0.1% washes, followed by equilibration in ethanol solutions: 25, 50, 70 and 100%. 9mg of Alcian blue powder (Alcian blue 8GX, Acros Organics, New Jersey, USA) were dissolved in 60ml of ethanol and 40ml of glacial acetic acid. Staining was carried for 48 hours in 37°C with gentle agitation. After staining was complete samples were gradually rehydrated in 90, 70, 50 and 25% ethanol solution in PBS. To remove excess stain, tissues samples were incubated overnight at 37°C in the digestion solution (1g trypsin+ 30ml of 2% KOH + 70ml water). Then transferred to 25% glycerol/PBS solution with few drops of hydrogen peroxide for another overnight incubation, and followed by series of 50, 70 and 100% glycerol washes. The stained cartilage was manually dissected to remove adherent tissue. Images were taken on a Zeiss Stereomicroscope and AxioVision software.

Statistical analysis

Statistical significance between gene expression levels was determined via Mann-Whitney analysis between Co-MO-injected and *Mustn1*-MO-injected as well as Co-MO-injected and Rescue-Injected embryos. Changes were considered statistically significant when $p < 0.05$ as determined by Mann-Whitney analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

- [1]. Tiedemann H, Asashima M, Grunz H, Knöchel W. Pluripotent cells (stem cells) and their determination and differentiation in early vertebrate embryogenesis. *DevGrowth Differ.* 2001; 43:469–502.
- [2]. Klymkowsky MW, Rossi CC, Artinger KB. Mechanisms driving neural crest induction and migration in the zebrafish and *Xenopus laevis*. *Cell Adh Migr.* 2010; 4:595–608. [PubMed: 20962584]
- [3]. Bronner-Fraser M. Neural crest cell migration in the developing embryo. *TrendsCell Biol.* 1993; 11:392–7.
- [4]. Nieuwkoop, PD.; Faber, J. Normal table of *Xenopus laevis* (Daudin). 2nd Edition. Elsevier/North Holland; Amsterdam: 1967.
- [5]. Karsenty G, Kronenberg HM, Settembre C. Genetic control of bone formation. *Annu Rev Cell Dev Biol.* 2009; 25:629–48. [PubMed: 19575648]
- [6]. Hadjiargyrou M, Lombardo F, Zhao S, Ahrens W, Joo J, Ahn H, Jurman M, White DW, Rubin CT. Transcriptional profiling of bone regeneration: Insight into the Molecular Complexity of Wound Repair. *J Biol Chem.* 2002; 277:30177–30182. [PubMed: 12055193]
- [7]. Alfandari D, Cousin H, Marsden M. Mechanism of *Xenopus* cranial neural crest cell migration. 2010; 4:553–60.
- [8]. Kuratani S. Craniofacial development and the evolution of the vertebrates: the old problems on a new background. *Zoolog Sci.* 2005; 22:1–19. [PubMed: 15684579]
- [9]. Lombardo F, Komatsu D, Hadjiargyrou M. Molecular cloning and characterization of Mustang, a novel nuclear protein expressed during skeletal development and regeneration. *FASEB J.* 2004; 18:52–61. [PubMed: 14718386]
- [10]. Liu C, Hadjiargyrou M. Identification and characterization of the Mustang promoter: regulation by AP-1 during myogenic differentiation. *Bone.* 2006; 39:815–824. [PubMed: 16731063]
- [11]. Gersch RP, Hadjiargyrou M. *Mustn1* is expressed during chondrogenesis and is necessary for chondrocyte proliferation and differentiation in vitro. *Bone.* 2009; 45:330–338. [PubMed: 19410023]
- [12]. Liu C, Gersch RP, Hawke TJ, Hadjiargyrou M. Silencing of *Mustn1* inhibits myogenic fusion and differentiation. *Am J Physiol Cell Physiol.* 2009; 298:C1100–8. [PubMed: 20130207]
- [13]. Kay, BK.; Peng, HB. *Xenopus laevis*: practical uses in cell and molecular biology. Vol. 36. Academic Press; San Diego, California: 1991. *Methods in Cell Biology.*
- [14]. Harland RM. In situ hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol.* 1991; 36:685–95. [PubMed: 1811161]
- [15]. Eisen JS, Smith JC. Controlling morpholino experiments: don't stop making antisense. *Development.* 2008; 135:1735–43. [PubMed: 18403413]
- [16]. Slater BJ, Liu KJ, Kwan MD, Quarto N, Longaker MT. Cranial Osteogenesis and Suture Morphology in *Xenopus laevis*: A Unique Model System for Studying Craniofacial Development. *PLoS ONE.* 2009; 4:e3914. [PubMed: 19156194]
- [17]. Svensson ME, Haas A. Evolutionary innovation in the vertebrate jaw: A derived morphology in anuran tadpoles and its possible developmental origin. *Bioessays.* 2005; 27:526–32. [PubMed: 15832380]
- [18]. Schuff M, Rössner A, Wacker SA, Donow C, Gessert S, Knöchel W. FoxN3 is required for craniofacial and eye development of *Xenopus laevis*. *Dev Dyn.* 2007; 236:226–39. [PubMed: 17089409]
- [19]. O'Donnell M, Hong CS, Huang X, Delnicki RJ, Saint-Jeannet JP. Functional analysis of Sox8 during neural crest development in *Xenopus*. *Development.* 2006; 133:3817–26. [PubMed: 16943273]
- [20]. Spokony RF, Aoki Y, Saint-Germain N, Magner-Fink E, Saint-Jeannet JP. The transcription factor Sox9 is required for cranial neural crest development in *Xenopus*. *Development.* 2002; 129:421–32. [PubMed: 11807034]

- [21]. Su MW, Suzuki HR, Bieker JJ, Solorsh M, Ramirez F. Expression of two nonallelic type II procollagen genes during *Xenopus laevis* embryogenesis is characterized by stage-specific production of alternatively spliced transcripts. *J Cell Biol.* 1991; 115:565–75. [PubMed: 1918153]
- [22]. Bieker JJ, Yazdani-Buicky M. Distribution of type II collagen mRNA in *Xenopus* embryos visualized by whole-mount in situ hybridization. *J Histochem Cytochem.* 1992; 40:1117–20. [PubMed: 1619277]
- [23]. Seufert DW, Hanken J, Klymkowsky MW. Type II collagen distribution during cranial development in *Xenopus laevis*. *Anat Embryol (Berl).* 1994; 189:81–9. [PubMed: 8192239]
- [24]. Van Evercooren A, Picard JJ. Surface changes during development and involution of the cement gland of *Xenopus laevis*. *Cell Tissue Res.* 1978; 194:303–13. [PubMed: 569548]
- [25]. LaBonne C, Bronner-Fraser M. Snail-related transcriptional repressors are required in *Xenopus* for both the induction of the neural crest and its subsequent migration. *Dev Biol.* 2000; 221:195–205. [PubMed: 10772801]
- [26]. Honoré SM, Aybar MJ, Mayor R. Sox10 is required for the early development of the prospective neural crest in *Xenopus* embryos. *Dev Biol.* 2003; 260:79–96. [PubMed: 12885557]
- [27]. Kalkan T, Iwasaki Y, Park CY, Thomsen GH. Tumor necrosis factor-receptor-associated factor-4 is a positive regulator of transforming growth factor-beta signaling that affects neural crest formation. *Mol Biol Cell.* 2009; 14:3436–50. [PubMed: 19458200]
- [28]. Kerney R, Gross JB, Hanken J. Runx2 is essential for larval hyopharyngeal cartilage formation in *Xenopus laevis*. *Dev Dyn.* 2007; 236:1650–62. [PubMed: 17474117]
- [29]. Monsoro-Burq A-H, Fletcher RB, Harland RM. Neural crest induction by paraxial mesoderm in *Xenopus* embryos requires FGF signals. *Development.* 2003; 130:3111–3124. [PubMed: 12783784]
- [30]. Yokota C, Kofron M, Zuck M, Houston DW, Isaacs H, Asashima M, Wylie CC, Heasman J. A novel role for a nodal-related protein; Xnr3 regulates convergent extension movements via the FGF receptor. *Development.* 2003; 130:2199–212. [PubMed: 12668633]
- [31]. Dingerkus G, Uhler LD. Enzyme clearing of Alcian blue stained whole small vertebrates for demonstration of cartilage. *Stain Technol.* 1977; 52:229–232. [PubMed: 71769]

Highlights

- > We use *Xenopus laevis* as a model to examine Mustn1 involvement in chondrogenesis.
- > We examine the effects of Mustn1 downregulation on craniofacial development.
- > Downregulation of Mustn1 results in decrease expression of Sox9 and overall craniofacial cartilage formation.

	1	82 Homology
<i>M. musculus</i>	MSEAGTPECP PIKKKRP VEEDLGARGTLAKNQDIKSKTYQVMRDYEQAGSAAPSVFSRNRGTGTETVFEKPKKEGPAKSVFG*	
<i>D. acutus</i>	MSQ---PA FVKKKRP VEEDLGARGKLSNQ IKSKTYQVMRQCEQ AGSAAPSVFSR ECTG GETVFDKPK EP TKSVFG*	79%
<i>A. carolinensis</i>	MSQ---PA FVKKKRP VEEDLGARGKLSNQ IKSKTYQVMRQCEQ SGAAAPSVFSR DR TGGETVFDKPK EP TKSVFG*	78%
<i>X. tropicalis</i>	MSQ--PQGA FVKKKRP VEEDLGAKHKL LAHQVPIKSKTYQVMQCEQ SGTTAPSVFSQNR TG GETAFD KPK EGPAKSVFG*	72%
<i>X. laevis</i>	MSQ--PQDA FVKKKRP VEEDLGAKNKL LAHQVPIKSKTYQVMQCEQ SGTTAPSVFSQNR TG GETAFD KPK AGPAKSVFG*	70%
<i>D. rerio</i>	MSQ---P FVKKKRP VEEDLGARSKLGLKGEVKS KTYEVM AECE RM CKVAPSVFSQVRS GN ETV IEKPK PPSGSVFGK*	64%

Figure 1. Comparison of *Mustn1* protein and nucleotide sequence between lower vertebrates *Mustn1* protein sequences from mouse (*Mus Musculus*, Accession #NP_852055), snake (*Deinagkistrodon acutus*, Accession #DV559991), lizard (*Anolis carolinensis*, Accession #FG779486), frog (*Xenopus tropicalis*, Accession #EL809374, *Xenopus laevis*, Accession #NP_001165126), and zebrafish (*Danio rerio*, Accession #NP_001183982) are aligned and show percent homology (between mouse and others). Changes in protein sequence are shaded in light grey. Amino acids found in mouse but not other species are denoted by dashes. The Nuclear Localization Sequence (NLS, aa10-18) is indicated by the black highlighted aa. Protein alignment and homology determination was conducted using the NCBI Specialized BLAST Align software program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

Figure 2. *Mustn1* is differentially expressed both temporally and spatially during *X. laevis* development

Agarose gel results of RT-PCR show an increase and plateau of *Mustn1* expression during later neurulation (A). *Mustn1* expression was further elucidated via RT-PCR on Stage 20 (B) and Stage 23 (C) embryos respectively. Total RNA was isolated from these tissues and RT-PCR was used to visualize the relative expression levels of *Mustn1* compared to Whole Body samples. Expression of the metabolic gene Ornithine Decarboxylase (ODC) was used to normalize for RNA recovery, cDNA synthesis and gel loading. *Mustn1* expression was visualized in wild-type embryos and analyzed via anti-sense *in situ* hybridization during development. Dorsal view of representative antisense stained St. 20 embryo (D) and sense control (H) respectively. Lateral view of an antisense stained embryo at St. 25 (E), and St. 39 (F). Lateral view of a sense stained embryo at St. 23 (I), and St. 39 (J). Panel G shows a magnified view of the *Mustn1*-antisense stained stage 39 tadpole head shown in panel F. In panels A, B and C, WB denotes RNA derived from the whole body of the embryo while H denotes tissues dissected from Head, D denotes Dorsal tissues, V denotes Ventral tissues, and P denotes Posterior regions as indicated in Nieuwkoop and Faber (1967) diagrams [4] with dissection planes indicated by dotted lines. In panels D-F, Black arrows denote paraxial mesoderm and somites, and in D-F, and G the black arrowhead denote craniofacial structures (M, mandibular arch; H, hyoid arch; B, branchial arch), the white arrowhead denotes heart (He in panel G), and the white arrow indicates the otic vesicle. All scale bars = 1mm.

Figure 3. Phenotype of injected embryos during embryogenesis

Embryos were injected with morpholinos (35 ng/embryo) dorsal to the midline at the 4-cell stage. Lateral views comparing Co-MO-injected (A), *Mustn1*-MO-injected (B), and Rescue-Injected (C) embryos at stage 37-38. The *Mustn1*-MO-injected embryos have smaller eyes, truncated body axes and/or tail kinks. When the same analysis was completed at stage 40; Co-MO-Injected tadpoles appear normal (D), while *Mustn1*-MO-injected embryos show severe defects - the eyes shrink or disappear and the body axis length is also reduced along with curvature of the tail (E). Rescue-Injected embryos show reduced severity of these defects (F). All micrographs were taken at the same magnification.

Figure 4. Quantification of phenotypes in injected embryos during embryogenesis

Quantification of eye phenotypes in injected embryos, scored at stage 40 (A). Labels correspond to each phenotypic category, for Co-MO-injected, *Mustn1*-MO-injected, and Rescue-Injected embryos. Embryos were categorized as having 2 normal eyes, 2 small eyes, 1 eye, or no eyes. Analysis of body axis length (B); embryos were categorized as having either a normal or shortened body axis. Analysis of Tail Kink phenotype (C); embryos were categorized as having a normal, mild, or severe tail curvature (see Experimental Design for details).

Figure 5. Effects of *Mustn1* depletion on Sox9

Lateral views of embryos subjected to *in situ* whole mount hybridization to detect Sox9 expression. These embryos were from among those scored for phenotypes in Figures 3 and 4. High magnification micrographs were taken of lateral views of the head illustrating Sox9 expression at stage 40 in a Co-MO-injected embryo (A), a *Mustn1*-MO-injected embryo (B) and a Rescue-injected embryo (C). qPCR analysis of Sox9 expression in dorsal tissue dissections of Co-MO-, *Mustn1*-MO-, and Rescue-injected embryos assessed at stages 25, 34, 39 (graph). No significant difference in *Mustn1* mRNA expression was found between Co-MO-injected and Rescue-injected groups at any developmental stage, but a significant difference between Co-MO-injected and *Mustn1*-MO-injected embryos was detected at stages 34 and 39. All bars represent average values of pooled mRNA (n=5 embryos). Error bars indicate standard deviation of PCR runs (n=3). Significance was determined by Mann-Whitney test vs. Co-MO-injected levels, *p<.05. Black arrows indicate staining in cranial cartilage, white arrows indicate pharyngeal arch staining, and black arrowheads denote staining of the anterior structures such as mandibular and nasal processes. Scale bar= 250µm.

Figure 6. Effect of *Mustn1* knockdown on cartilage development

Ventral views of unilaterally injected embryos (stage 45) following Alcian blue staining. Co-MO-injected embryo, with a dotted line indicating the midline (A). The same embryo shown in A after the removal of adherent tissue (B). The same analysis of a representative *Mustn1*-MO-injected embryo shows alteration of midline and loss of symmetrical cartilaginous structures on the contralateral, injected side (C,D respectively). Black arrows indicate the ceratohyal cartilage; and white arrows denote the gill (branchial) arches. Scale bar = 0.5mm.