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Mustn1 is essential for craniofacial chondrogenesis during *Xenopus* development

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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 four 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.

The development of vertebrate craniofacial structures is an intricate process of differentiation and morphogenesis, predominantly involving and coordinated by endodermal tissues (Tiedemann et al., 2001). 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 (Klymkowsky et al., 2010; Bronner-Fraser, 1993). 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 (Nieuwkoop and Faber, 1967), 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 (Karsenty et al., 2009; Hadjiargyrou et al., 2002). 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, and 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 (Alfandari et al., 2010; Kuratani, 2005).

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 (Lombardo et al., 2004; Liu and Hadjiargyrou, 2006; Gersch and Hadjiargyrou, 2009; Liu et al., 2009). However, little is known about the molecular functions of *Mustn1* and whether it has

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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 (Gersch and Hadjiargyrou, 2009). Moreover, matrix production by hypertrophic chondrocytes was disrupted and accompanied by dramatic decreases in chondrogenic marker (Sox9, Collagen II, and Collagen X) gene expression (Gersch and Hadjiargyrou, 2009). 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) (Kay and Peng, 1991; Harland, 1991; Eisen and Smith, 2008). 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 (Slater et al., 2009), derived from similar cell lineages (largely cranial neural crest) that expand and mature into homologous structures (Svensson and Haas, 2005; Schuff et al., 2007). 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, and 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 (O'Donnell et al., 2006; Spokony et al., 2002), 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 (Su et al., 1991; Bieker and Yazdani-Buicky, 1992; Seufert et al., 1994). 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 (Gersch and Hadjiargyrou, 2009). 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*.

1. Results

1.1. 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 (Lombardo et al., 2004), as reflected in the Universal Protein Resource (UniProt) database (http://www.uniprot.org/uniprot/?query = family:%22MUSTANG + family%22). We re-evaluated 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).

1.2. 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 and 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 and G, black arrow heads), the otic vesicle (Fig. 2F and G, white arrow), and heart (Fig. 2F and G, white arrow head). Sense probe controls with the same stage tadpoles did not show any staining (Fig. 2H and J).

1.3. 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. Where as Co-MO-injected embryos showed no significant developmental defects throughout the investigated time course (Fig. 3A and D), the *Mustn1*-MO-injected embryos showed several morphological defects that included, small or