

# Knockdown of hspg2 is associated with mandibular jaw joint fusion and neural crest cell dysfunction in zebrafish

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## Research article

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## Abstract

**Background:** Heparan sulfate proteoglycan 2 (*HSPG2*) encodes for perlecan, a large proteoglycan that plays an important role in cartilage formation, cell adhesion, and basement membrane stability. Mutations in *HSPG2* have been associated with Schwartz-Jampel Syndrome (SJS) and Dyssegmental Dysplasia Silverman-Handmaker Type (DDSH), two disorders characterized by skeletal abnormalities. These data indicate a function for *HSPG2* in cartilage development/maintenance. However, the mechanisms by which *HSPG2* regulates cartilage development are not completely understood. Here, we explored the relationship between this gene and craniofacial development through morpholino-mediated knockdown of *hspg2* using zebrafish.

**Results:** Knockdown of *hspg2* resulted in abnormal development of the mandibular jaw joint at 5 days post fertilization (DPF). We surmised that defects in mandible development were a consequence of neural crest cell (NCC) dysfunction, as these multipotent progenitors produce the cartilage of the head. Early NCC development was normal in morphant animals as measured by distal-less homeobox 2a (*dlx2a*) and SRY-box transcription factor 10 (*sox10*) expression at 1 DPF. However, subsequent analysis at later stages of development (4 DPF) revealed a decrease in the number of *Sox10*<sup>+</sup> and Collagen, type II, alpha 1a (*Col2a1a*)<sup>+</sup> cells within the mandibular jaw joint region of morphants. Concurrently, morphants showed a decreased expression of NK3 homeobox 2 (*nkx3.2*), a jaw joint molecular marker at 4 DPF.

**Conclusions:** Collectively, these data suggest a complex role for *hspg2* in jaw joint formation and late stage NCC differentiation.

## Background

Mutation of the *HSPG2* gene causes Schwartz-Jampel Syndrome (SJS) and Dyssegmental Dysplasia Silverman-Handmaker Type (DDSH) (1–3). SJS is a recessive disorder characterized by muscle stiffness (myotonia) and chondrodysplasia. DDSH is a less frequent but a more severe recessive disorder characterized by reduced joint mobility, severe limb shortening, and short stature (4). Both disorders have common clinical manifestations that include reduced stature, bowing of the long bones, and facial dimorphism (4,5). Interestingly, SJS is generally associated with loss of function mutations in *HSPG2* that vary from missense mutations to splice site mutations. These mutations are spread throughout the *HSPG2* protein, with those located closer to the C-terminus being more readily tolerated (5). Conversely, DDSH is caused by homozygous null mutations (frameshifts and point mutations) wherein there is an absence of functional protein, likely due to degradation (5). Despite mutation heterogeneity underlying SJS and DDSH, craniofacial abnormalities such as micrognathia are very common phenotypes in diagnosed patients (5). These manifestations of chondrodysplasia suggest that *HSPG2* plays a role in regulating craniofacial development.

The function of *HSPG2* during chondrogenesis is not completely understood, but is likely related to the various domains within the *HSPG2* protein and the vast array of interactions between *HSPG2* and the extracellular matrix (ECM) to promote signal transduction and stability (6,7). *HSPG2* encodes for perlecan, a large proteoglycan that consists of five domains, each of which have a unique function (8). Four of these domains (domains II, III, IV, and V) have repeats homologous to low-density lipoprotein receptor (domain II), laminins (domains III and V), and immunoglobulins (domain IV), but domain I remains unique to perlecan (8). The N-terminal domain I possesses three ser-asp-gly motifs that serve as attachment sites for glycosaminoglycan (GAG) side chains like heparan sulfate (HS) and chondroitin sulfate (CS) (8–10). These side chains facilitate interaction with growth factors such as fibroblast growth factor-2 (FGF-2), vascular endothelial growth factors (VEGFs), and bone morphogenetic proteins (BMPs) (6,10,11) upon secretion of perlecan into the ECM. In addition, HS has been shown to specifically interact with ECM proteins such as fibronectin, laminin, and collagens I, II, III, IV, and V, suggesting perlecan and its side chains mediate cell adhesion and basement membrane stability (12).

As it relates to the chondrocyte phenotypes in SJS and DDSH, previous studies, both *in vitro* and *in vivo*, have established that disrupting *HSPG2* expression leads to abnormal chondrocyte proliferation and disorganized columnar arrangement (13,14). This is significant as chondrocytes are the primary cells found in cartilage and are known to secrete a specialized ECM containing glycoproteins and proteoglycans, like perlecan, to maintain structural integrity (15). During craniofacial development, chondrocytes arise from neural crest cells (NCCs), a multipotent progenitor cell population that forms at the dorsal end of the neural tube upon neural tube closure. There are four populations of NCCs, but only cranial NCCs (CNCCs) migrate to the pharyngeal arches and develop into cartilage and bone, making them vital to proper craniofacial development (16–18). These CNCCs are regulated by growth factors like BMP, which are vital for mandibular morphogenesis, and FGFs, which are essential for CNCC differentiation (19–21).

The development of craniofacial bone is due to both endochondral and intramembranous ossification. In endochondral ossification, cells differentiate into chondrocytes and bones develop from a cartilaginous base. This form of ossification necessitates that chondrocytes are present in growth plates where they are arranged into zones of rest, proliferation, and hypertrophy (15,22). Perlecan is present in these cells and additionally in the synovial joints of the body, where it mediates differentiation through regulatory *sox9* expression (23). Synovial joints possess fluid-filled cavities that separate the surface of bones, likely developed by the upregulation of hyaluronic acid (or hyaluronan), which, in large quantities, promotes cellular separation, cavitation, and the formation of a joint (24–26). Studies focused on osteoarthritis have shown that perlecan side chains have the ability to bind to hyaluronan and can potentially mediate joint function through regulating the release of BMP-2 (27,28). Despite this, there are still considerable gaps in the literature on the mechanisms by which perlecan regulates joint development.

To begin to understand these mechanisms, we performed morpholino mediated knockdown using the zebrafish model. Apart from general advantages like a high fecundity rate, extrauterine development, and quick maturation, the zebrafish is an ideal organism to study craniofacial development due to the relative

simplicity of the cartilaginous structures of the head and face, all of which are readily visible with stains like Alcian blue (29,30). Additionally, the zebrafish shares a high degree of genetic similarity to humans and successful studies have been performed using genetically manipulated fish (30). Thus, we performed transient knockdown of zebrafish *hspg2* to determine the CNCC defects present during craniofacial development. Knockdown of *hspg2* resulted in abnormal mandibular jaw joint formation and disrupted late stage differentiation of CNCCs, with little to no effect on early stage CNCC development. Collectively, our results suggest that *hspg2* is essential for joint formation in the developing zebrafish.

## Results

### *Morpholino-induced knockdown of hspg2 is associated with craniofacial phenotypes*

It has previously been reported that *hspg2* mRNA is expressed ubiquitously throughout the head, eyes, and somites of the zebrafish. Additional immunohistochemistry staining revealed positive expression of perlecan in these regions from embryos at 2-3 hours post fertilization to 5 days post fertilization (DPF) (31,32). Based on this expression pattern and previous murine studies establishing that mutation of *Hspg2* results in failure of the chondro-osseous junction of developing bones and craniofacial abnormalities (13), we hypothesized that morpholino mediated knockdown of the zebrafish *hspg2* gene would cause craniofacial abnormalities.

We performed Alcian blue staining to detect craniofacial abnormalities in the developing cartilage. We measured the distance from the top of the ceratohyal to the tip of the Meckel's cartilage as a read out for mandibular truncation as previously described (33). Measurements of the distance between the top of the ceratohyal and Meckel's cartilage at 5 DPF (Figure 1A, 1B, and 1C) showed that the injection of the *hspg2* translation blocking morpholino caused a 7% truncation in the zebrafish mandible when compared to the random control group. The observed truncation was subtle in morphants and did not appear to be the consequence of a malformed Meckel's cartilage or defects in the development of the ceratohyal. However, upon higher magnification, *hspg2* morphants demonstrated an abnormal mandibular jaw joint between the Meckel's cartilage and palatoquadrate (the dorsal component of the mandibular arch), a phenotype that was not present in the random control group (Figure 1A'-B', red arrowheads).

### *nkx3.2 expression is decreased in hspg2 morphants*

NK3 homeobox 2 (*nkx3.2*) was first identified in the *Drosophila melanogaster* model (where it is known as *bapx1*) and is part of the NK family of homeobox genes (34). Homologues of the gene have been found in vertebrates and are expressed predominately in the first pharyngeal arch (a developmental structure that gives rise to the mandible (arrow Figure 2A'), where the gene is essential for proper joint formation (34,35). Knockdown of *nkx3.2* causes abnormal jaw joint phenotypes (primarily fusion phenotypes) in amphibians and zebrafish (34,36). Therefore, we utilized *nkx3.2* expression as a marker of mandibular jaw joint development. *In situ* hybridization performed at 2 DPF (Figures 2A-B) demonstrated decreased expression of *nkx3.2*. *nkx3.2* is primarily expressed in the pharyngeal arches, where we observed high

expression in the 1<sup>st</sup> and 5<sup>th</sup> pharyngeal arches (Figure 2A'-B') (expressed posterior to the eye, annotated by the black arrow) and in the sclerotomal derivatives (36). Knockdown of *hspg2* resulted in decreased expression of *nkx3.2* in the pharyngeal arches when compared with the random control group (Figure 2A'-B'). Subsequent qPCR at 4 DPF confirmed a statistically significant decrease in *nkx3.2* in morphants relative to the random control group (Figure 2C).

### ***Neural crest cells migrate normally in the absence of hspg2***

Because defects in the number of and migration of NCCs are possible mechanisms by which craniofacial deficits may arise (33), we hypothesized that the craniofacial abnormalities present at 5 DPF might be due to early CNCC defects. To determine if *hspg2* affects early CNCCs, we analyzed *Tg(sox10:TagRFP)* embryos at both the 18 somite (aligning with early NCC specification and migration) (37) and Prim-5 (corresponding with NCCs invading the pharyngeal arches) stages. Cells in the NCC lineage express SRY-box transcription factor 10 (*sox10*) at various stages of development where it maintains their survival, specification, and differentiation (38). Previous studies have used *sox10* expression as a valid marker for visualizing NCCs during early developmental stages (33,39). Results revealed no discernable differences in the location or degree of RFP expression in *hspg2* morphants relative to control (Figure 3A-A' and 3B-B').

### ***dlx2a expression in morphants is unaffected***

We next analyzed the expression of distal-less homeobox 2a (*dlx2a*) at the Prim-5 stage in random control and *hspg2* morpholino injected embryos to determine if CNCC specification occurs normally upon knockdown of *hspg2*. This homeobox gene is expressed in cranial neural crest cells migrating to the pharyngeal arches (40) and has been established as a marker of proper CNCC specification (33,39,41). *In situ* hybridization (Figure 4A-B) revealed that there was no significant difference in the expression of *dlx2a* in morphants relative to random control injected. qPCR measurements performed at Prim-5 in both RC and MO groups validated the normal level of *dlx2a* expression in morphant animals (Figure 4C). Collectively, these data suggest that early CNCC development was normal.

### ***hspg2 knockdown affects cell numbers in jaw joint region***

Based on our results, which suggest that the early NCC lineage was not affected, we next hypothesized that *hspg2* was mediating late stage CNCC differentiation and potentially mediating the defects observed at 5 DPF. To test this, we performed analysis of Sox10<sup>+</sup> cells at 3 and 4 DPF using *Tg(sox10:TagRFP)* larvae. Chondrocytes across both random control and *hspg2* morphant groups at 3 and 4 DPF had normal morphology and columnar arrangement. Chondrocytes to the left of the joint were more closely clustered together than those on the right and therefore, only cells from 3 rows to the left of the joint were easily discernable as opposed to 5 rows of identifiable cells on the right of the joint.

At 3 DPF (Figure 5A-B), morphants had a statistically significant increase of Sox10<sup>+</sup> cells at the region of interest (3 rows of chondrocytes to the left of the joint and 5 rows of chondrocytes to the right of the

joint) (Figure 5C-C'). Consistent with these results, qPCR detected an increase in *sox10* expression at 3 DPF (Figure 5D). However, at 4 DPF (Figure 6A-B), the number of Sox10<sup>+</sup> cells were reduced relative to random control injected embryos (Figure 6C-C') and the level of *sox10* expression was approximately 50% of the control according to qPCR (Figure 6D). Subsequent analysis of Col2a1a (collagen, type II, alpha 1a)<sup>+</sup> cells using the *Tg (col2a1a:EGFP)* transgenic reporter in the mandibular jaw joint region revealed similar chondrocyte morphology and arrangement as samples in the *sox10* group. Cell counts (Figure 7A-B) demonstrated a similar decline in numbers of EGFP+ cells in morphant animals at 4 DPF (Figure 7C-C'). Collectively, these data show a progressive loss of differentiated NCCs between 3 and 4 DPF.

## Discussion

Our analysis revealed a 7% mandibular truncation and an abnormal joint phenotype in animals with knockdown of *hspg2*. Deletion of *Hspg2* in mice has been shown to cause truncated snouts, shorter and thicker mandibular structures, and flat faces (13,42,43), but very little has been reported on joint phenotypes and how they pertain to *hspg2* function. Although it is relatively novel, the idea of *hspg2* mediating the mandibular jaw joint region is not unfounded. Similar to other synovial joints, the mandibular jaw joint contains a synovial capsule, which, in previous cell culture work using synovial cells, has been shown to express and require perlecan for proper development (23,44).

The mechanism by which perlecan mediates joint development is currently unknown. However, as discussed in the introduction, perlecan is a multi-domain protein with side chains that interact with various growth factors like BMP, WNT, and FGF; all of which are essential for neural crest development, chondrogenesis, and joint formation (43,44). Deficiencies or abnormalities in the activation or level of such pathways may account for the decreased number of Sox10+ or Col2a1a+ cells at 4 DPF. Perlecan has also been found to bind to *Ihh* (Indian hedgehog) through its HS side chains, which in turn mediates the proliferation of chondrocytes (43). It should be noted however, that many of these effects and pathways have predominately been implicated in the joints of the appendicular skeleton and that development of the mandibular jaw joint could be different.

We further demonstrate that knockdown of *hspg2* is associated with decreased numbers of Col2a1a<sup>+</sup> cells at 4 DPF. These data suggest that *hspg2* has a function regulating CNCC differentiation, a finding that is supported by the number of Sox10<sup>+</sup> cells at an equivalent time point. Our studies are supported by previous analysis in mice (*Hspg2*<sup>-/-</sup>) that demonstrated abnormal arrangement and proliferation of chondrocytes in the appendicular skeleton (13,42). It must be noted however, that although these data support one another, the cells of the appendicular skeleton derive from a different germ layer (the mesoderm) than the cells of the craniofacial skeleton (the ectoderm). Both cell lineages give rise to cartilaginous structures, but the mechanisms by which each population differentiates are likely to be different, prompting further studies. One possible future direction of our work could be to determine the interplay between perlecan and FGF because perlecan binds to FGF-2, which increases the expression of *sox9* *in vitro* (23,45). The protein output of *Sox9* in turn is vital to chondrogenesis because it activates

*Col2a1* expression in mice (46). It is possible however, that there are various mechanisms underlying the function of *hspg2* in joint development because the heparin side chains of perlecan are known to bind to collagen II (12) suggesting a direct function for perlecan in chondrogenesis. Interestingly, we also observed an initial increase in the number of Sox10+ cells at 3 DPF, which at the onset seems to counter the results observed at 4 DPF. However, this increase of cells could be due to a period of proliferation in chondrocytes followed by increased cell death between 3 and 4 DPF. Further studies in this area are warranted.

Knockdown of *hspg2* was also associated with reduced expression of *nkx3.2* at 4 DPF. These results, when understood in the context of the decrease of *Col2a1a*<sup>+</sup> cells found at 4 DPF, are supported by previous results performed in mesenchymal cell culture where *nkx3.2* upregulates *col2a1* by directly binding to the promoter (47). In this situation, diminished expression of *nkx3.2* appears to be directly proportional to a decrease in *Col2a1a*<sup>+</sup> cells and the differentiation of chondrocytes. It is not clear if *hspg2* directly modulates *nkx3.2* expression or if the decreased expression is simply the result of defects in the mandibular jaw joint, but studies performed in the chick have shown that *Nkx3.2* and *Sox9* cooperate to promote chondrogenic differentiation and serve as mediators of Sonic Hedgehog (Shh)-induced chondrogenesis (48). This could be one of the mechanisms by which perlecan indirectly mediates the expression of *nkx3.2* and it would prove to be a novel discovery. Recently, it was shown that *nkx3.2* null animals are viable, making it possible to study this gene in relation to *hspg2* without early lethality (49).

In this paper, we demonstrate zebrafish as an alternative animal model to study the role of *hspg2* during craniofacial development. Induced knockouts in the murine model have resulted in embryonic lethality from mass hemorrhaging in the pericardial cavity and severe chondrodysplasia, both occurrences which can be temporarily circumvented in the developing zebrafish (31). To circumvent these limitations, three additional mouse models have been produced: the first model lacks exon 3, causing loss of the 3 HS side chains (7), the second model is modeled after an SJS patient mutation where there is a G to an A substitution theorized to cause a misfolded protein (42,50), and the third is a model where early lethality is restored via tissue specific expression of *Hspg2* in chondrocytes (51).

While the first two models are viable and can be used to examine adult skeletal phenotypes, the first is centered around exploring the loss of only one domain and the second is mimicking more subtle phenotypes associated with SJS. Our project seeks to understand the role of perlecan in craniofacial development using a null phenotype, a feat not easily performed in a murine model. Zebrafish allow for this type of exploration because unlike mice, they are externally fertilized. This external fertilization enables the study of craniofacial development at early developmental stages, particularly with the use of transgenic fish to target specific genes (52,53). The third model described restores early lethality using a chondrocyte promoter and consequently cannot be utilized to study chondrogenesis or craniofacial development (51). Zebrafish craniofacial development is conserved and the development of the viscerocranum, including the development of the pharyngeal arches, the migration and specification of NCCs, their differentiation, and signaling pathways involved have all been well characterized (29,54).

Additionally, because the zebrafish mandibular joint is a synovial joint which develops in a similar fashion to other vertebrate synovial joints, the mechanisms uncovered from this research could be translational to other areas (55).

Zebrafish are remarkably easy to manipulate genetically and have been used with great success in genetic studies. The zebrafish genome shares a high degree of genetic similarity with humans and thus, provides a manner in which to understand gene function and mechanisms (30). 70% of human genes have one zebrafish ortholog and 82% of the genes associated with disease also have at least one zebrafish ortholog (56). CRISPR mutagenesis has emerged as a manner of genetic manipulation readily adaptable to zebrafish (57) and future studies developing a germline non-sense mutant of *hspg2* are a critical next step.

All work reported here has been completed by use of a single translation-blocking morpholino. While translation-blocking morpholinos are a simple and effective way in which to knockdown genes of interest, they have been associated with off target effects and non-specific cell death. We did, however, utilize a random control morpholino to account for the possibility of morpholino-induced cell death, an endeavor that proved to be rather successful in previous studies (58,59). And while injection of heparin side chains is a potential rescue for the morphant phenotype we observed, there is the possibility that heparin sulfate co-injection would fail to rescue because a domain outside of domain I is essential for regulation of CNCCs. Therefore, such an experiment is unlikely to demonstrate a full rescue. Collectively, this points out a potential caveat to our work in that we study a morpholino derived phenotype. However, our data with one morpholino is supported by previous studies, including those completed using the murine model (13,42). In these studies, numbers of chondrocytes in the lateral skeleton are depleted, chondrocytes congregate abnormally, and mutant mice exhibit craniofacial abnormalities.

Our work contributes an additional angle to the role of *hspg2* in skeletal development by examining the mandibular jaw joint region specifically. We understand that additional morpholinos would help to substantiate our work, but our data is supported by previous studies, suggesting that what we observed is not a consequence of off-target effects. Nevertheless, future studies in a germline zebrafish mutant are required.

## Conclusion

In summary, our results have created novel implications for the role of *hspg2* in the development of the mandibular jaw joint, a region of the craniofacial skeleton for which development has not been well elucidated. Furthermore, connections between *hspg2* and *nkx3.2* have yet to be drawn in literature. Additional data concerning late CNCC differentiation raises pertinent questions about the fate of certain cells within different areas of the developing face. Although future studies to more cohesively understand the role of *hspg2* in craniofacial development are needed, these data lay significant groundwork for future experiments in this field and suggest that zebrafish are an acceptable model to study the function of *hspg2* in craniofacial development.

# Methods

## Animal Care

For all experiments, embryos were obtained by crossing adult *Tg(sox10:tagRFP)*, *Tg(col2a1a:EGFP)*, or AB wildtype fish. Embryos were maintained in E3 embryo medium at 28°C. All zebrafish were maintained at The University of Texas El Paso according to the Institutional Animal Care and Use Committee (IACUC) guidelines protocol 811689-5. All adult fish were obtained from the University of Colorado, Anschutz Medical Campus or the Zebrafish International Resource Center (ZIRC). Adult and larval zebrafish were euthanized and anesthetized according to guidelines from the American Veterinary Medical Association and approved IACUC protocols. For euthanasia, adults beyond the age of peak breeding age (>1.5 years old) were euthanized using a solution of 10g/L buffered solution of pharmaceutical grade MS 222. Fish were emerged in solution for 30 minutes at RT. All euthanized adults underwent secondary euthanasia with a cold ice bath (2-4 degrees C). Cessation of movement was indicative of euthanasia. Embryos (<7 days old) were euthanized using 1-10% sodium hypochlorite solution after being anesthetized in cold ice bath. For any genotyping and before fixation, all fish, adults and larvae, were anesthetized using MS 222 (150mg/L for adults and 300mg/L for embryos). The degree of anesthesia was monitored by operculum movement of adults and cessation of movement for larvae.

## *Antisense oligonucleotide morpholino design and microinjection*

Two antisense oligonucleotide morpholino sequences were designed in conjugation with Gene Tools LLC. The first was a translation blocking morpholino (MO) with the sequence 5'-TATCCTCGCCCCCATTTCTGCCAA-3', created to bind to the *hspg2* translation start site and sterically knockdown perlecan translation in the developing larvae. The second was a random control morpholino with the sequence 5'-AAAAAAAAAAAAAAAAAAAAAAA-3'. This random control morpholino was used to assure that the translation blocking MO microinjections were not causing any form of extraneous cell death as previously described (60).

The initial concentration gradient for the translation blocking morpholino was as follows: 0.10 µM (1.65 ng), 0.30 µM (4.95 ng), 0.50 µM (8.25 ng), and 0.70 µM (11.55 ng). A high mortality rate, not attributed to lack of fertilization, was observed in larvae injected at higher concentrations (0.50 µM and 0.70 µM) when compared to non-injected group at 1 DPF. Morphant larvae at 0.30 µM exhibited growth delays when examined at 1 DPF (verified through somite counts), prohibiting accurate comparison between morphant and control group. Microinjections were then performed at 1.65 ng per embryo at a volume of 0.52 nL per embryo, a concentration and volume where there was minimal mortality and no growth delay. To ensure the final phenotype was not a consequence of global deformities at later stages, larvae were measured for length using Zeiss software and staged according to length at 3 and 4 DPF.

For all experiments, the morphant experimental group is compared to either a random control group or wildtype non-injected. The randomized control morpholino has been shown through previous literature to have no associated phenotypes, indicating that it does not influence final results and therefore is the

appropriate control group for comparison (58,59,61). Thus, for statistical analysis, comparisons were performed using a T-test between the random control group and the morpholino. MOs were injected into embryos at the one cell stage with a stock concentration of 0.10  $\mu$ M (1.65 ng/embryo) and at a volume of 0.52 nL per embryo as explained above. Wildtype -non-injected larvae were used predominately as a baseline for fertilization rates.

### ***Alcian Blue Staining and Imaging***

Zebrafish larvae (aged 5 DPF) were fixed in 2% PFA in PBS, pH 7.5 for 1 hour at room temperature (RT). Samples were washed for 10 minutes with 100mM Tris pH 7.5/10mM MgCl<sub>2</sub>, stained with Alcian blue stain (pH 7.5: 0.4% Alcian blue (Anatech Ltd., MI) in 70% EtOH, Tris pH 7.5 (Fisher, MA), and 1 M MgCl<sub>2</sub> (Fisher, MA)), and incubated overnight at RT. Samples were subsequently destained and rehydrated using an EtOH: Tris pH 7.5 gradient as previously described (39). Embryos were bleached (30% H<sub>2</sub>O<sub>2</sub> (Sigma, St. Louis, MO), 20% KOH (Fisher, MA)) for 10 minutes at RT. Samples were washed twice for 10 minutes per wash in wash buffer (25% glycerol/0.1% KOH (Fisher, MA)) and stored at 4°C in storage buffer (50% glycerol/0.1% KOH (Fisher, MA)) until imaged. The distance between the top of the Meckel's cartilage (the ventral component of the mandibular arch) and the top of the ceratohyal (a pharyngeal arch cartilage) was measured for each embryo as a method to analyze truncation of the Meckel's cartilage, which would be analogous to micrognathia as described previously (33). The final measurement is the distance between the two structures and not the full length of the Meckel's cartilage. Distance measurements were performed using Zeiss software and all distances are in  $\mu$ M. For imaging, a representative sample of the two groups (*hspg2* morphants and random control larvae) were dissected and viscerocranium was mounted on a glass slide with 100% glycerol. A Leica microscope was used to take high-resolution color images of each sample.

### ***Whole mount *in situ* hybridization and quantitative real time polymerase chain reaction (PCR)***

Whole mount *in situ* hybridization was performed as described by Thisse and Thisse (62). Larvae were harvested and dechorionated at the indicated time point and fixed in 4% paraformaldehyde (Electron Microscopy Sciences, PA) overnight at 4°C. Larvae were then dehydrated using a methanol: PBS gradient and stored in 100% methanol overnight at -20°C. Embryos were rehydrated using a PBS:Methanol gradient, washed in PBS with 0.1% Tween 20 and permeabilized with proteinase K (10ug/ml) for the time indicated by Thisse and Thisse (62). Permeabilized larvae were prehybridized for 2 hours in hybridization buffer (HB) (50% deionized formamide (Fisher), 5X SSC (Fisher), 0.1% Tween 20 (Fisher), 50  $\mu$ g/m heparin (Sigma), 500  $\mu$ g/mL of RNase-free tRNA (Sigma), and 1M citric acid (Fisher)). Larvae were then incubated overnight in fresh HB with probe (*dlx2a* and *nkx3.2* at 127 ng) at 70°C. Samples were washed according to protocol, blocked in 2% sheep serum (Sigma) and 2 mg/ml bovine albumin serum (Sigma) for 2 hours at room temperature. Samples were then incubated with anti-DIG Fab fragments (1:10,000)

(Sigma) overnight at 4°C. Samples were developed with BM purple AP substrate (Sigma) and imaged with a Zeiss Discovery Stereo Microscope fitted with Zen Software. Statistical analysis was performed using a Fisher's exact test. For quantitative polymerase chain reaction (qPCR), RNA was isolated from a pool of embryos at the indicated time point using Trizol (Fisher) according to manufacturer's protocol. Reverse transcription was performed using a Verso cDNA Synthesis Kit (Fisher) and total RNA was normalized across all samples. PCR was performed using an Applied Biosystem's StepOne Plus machine with Applied Biosystem's software. Sybr green (Fisher) based primer pairs for each gene analyzed are as follows: *dlx2a* fwd CCTCACGCAAACACAGGTTA, *dlx2a* rev TGTCATTCTCTGGCTGTGC, *nkx3.2* fwd GCAGATTAGCGGACGAGAC, *nkx3.2* rev GCTTCAACCACCAGCGTTAT, *sox10* fwd ACGCTACAGGTCAAGTCAC, *sox10* rev ATGTTGCCATCACGTCTG, *rp/13a* fwd TCCCAGCTGCTCTCAAGATT, and *rp/13a* rev TTCTTCCAATAGCGCAGCTT. Analysis was performed using  $2^{\Delta\Delta ct}$  indicating relative mRNA expression for each gene. For each biological replicate, RNA was isolated from a pool of injected embryos. The morphant pooled group gene expression is expressed relative to the expression from a pooled group of individuals injected with random control morpholinos. Each qPCR analysis was performed in biological replicate, which can be defined as two independent occasions with independent parents and an independent injection. For each biological replicate, the qPCR was performed in technical triplicates, however, the error bars on the graphs depicted are the standard error between biological replicates. Statistical analysis of messenger RNA (mRNA) expression was performed using a Student t-test on biological replicates.

### **Confocal Imaging and Transgenic Cell Counts**

Transgenic larvae (*Tg(sox10:tagRFP)* and *Tg(col2a1a:EGFP)*) were fixed at the stated time points using 4% paraformaldehyde. Fixed larvae were mounted in 0.6% low-melt agar in a glass bottom dish (Fisher). Imaging was performed on a Zeiss LSM 700 at 20X and 40X Oil magnification. Images were restricted to the larval craniofacial region. For each fish, a minimum of 20 to 30 z-stacks were collected. At 3 DPF, micron depth across the *Tg(sox10:TagRFP)* random control group ranged from 60-100 µm while micron depth ranged from 57-84 µm in morphants. Micron depth across the 4 DPF *Tg(sox10:TagRFP)* random control group ranged from 63-100 µm and from 66-93 µm in the morphant group. At 4 DPF *Tg(col2a1a:EGFP)* random control group micron depth ranged from 63-99 µm and from 72-90 µm in the morphant group.

The number of cells per z-stack (20-30 stacks/fish) at both jaw joints were manually counted using the ImageJ cell counter, which marks each individual cell and keeps track of total cell numbers. Region of interest around the jaw joints were selected to be 3 rows of chondrocytes on the left side of the joint and 5 rows on the right. This region was selected because these cells were consistently visible across z-stacks. Statistical significance was obtained by using a Student t-test with random control and morphant data (63).

# Abbreviations

*HSPG2/Hspg2/hsgp2*: heparan sulfate proteoglycan 2; dpf: days-post-fertilization, NCC: neural crest cells; *sox10*: SRY-box transcription factor 10; *nkx3.2*: NK3 homeobox 2; SJS: Schwartz-Jampel Syndrome; DDSH: Dyssegmental Dysplasia Handmaker Type; qPCR: quantitative polymerase chain reaction; *col2a1a*: collagen, type II, alpha 1a; MO: *hsgp2* morphants; NI: non-injected wildtype; RC: random control group

# Declarations

## ***Ethics approval and consent to participate***

All experiments were performed according to protocol 811689-5 approved by The University of Texas El Paso Institutional Animal Care and Use Committee (IACUC).

## ***Consent for publication***

Not applicable.

## ***Availability of data and materials***

Not applicable.

## ***Competing interests***

The authors declare that they have no competing interests.

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National Institute of Neurological Disorders and Stroke 1K01NS099153-01A1 to Anita M. Quintana. This study was designed, performed, and analyzed by the authors. The funding sources provided financial support for the experiments described.

## ***Authors' contributions***

BSC and AMQ synthesized hypothesis, wrote manuscript and BSC performed all experiments described. NGR produced aspects of the manuscript figures. All authors included have read and approved the manuscript being submitted.

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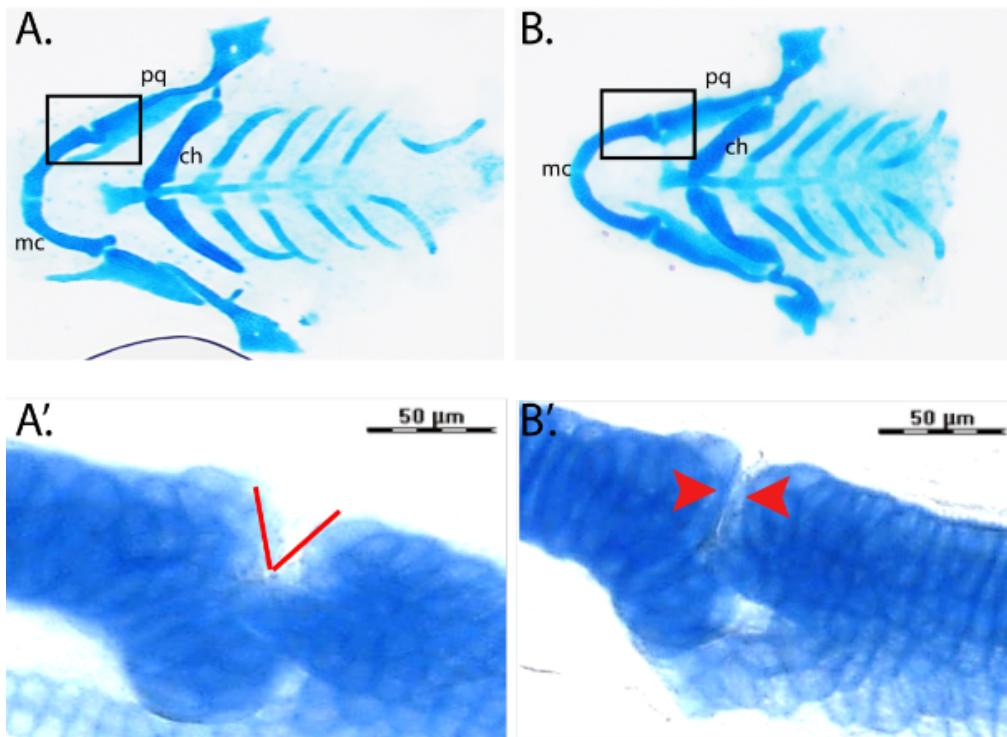
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## Figures

RC

MO



C.

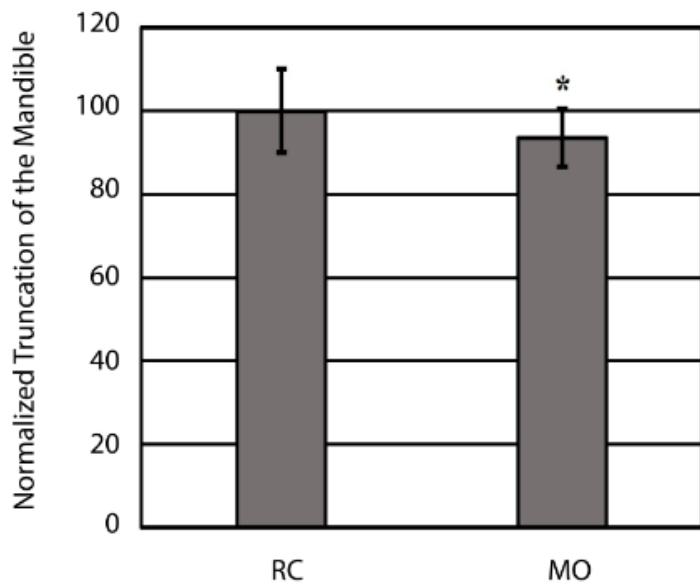


Figure 1

Knockdown of *hspg2* is associated with craniofacial phenotypes. (A-B) Random control (RC) and morphant (MO) groups were stained with Alcian blue at 5 days post fertilization (dpf) ( $N=20$  per group). Ceratohyal, Meckel's cartilage, and the palatoquadrate are labeled as the abbreviations (ch), (mc), and (pq) respectively. The black box on each of the images shows the upper jaw joint. (A'-B') shows the upper mandibular jaw joint enclosed by the black box at 40X magnification. (A') shows a red wedge indicating

the normally developed joint in the mandibular jaw and (B') shows two red arrows which indicate an abnormally tight proximity between the two sides of the joint. (C) The distance between the top of the Meckel's cartilage and top of the ceratohyal was measured across both groups ( $N=20$  per group) as a readout for micrognathia. Mandibular length was normalized to the random control (RC) group. \* $p=0.025$ .

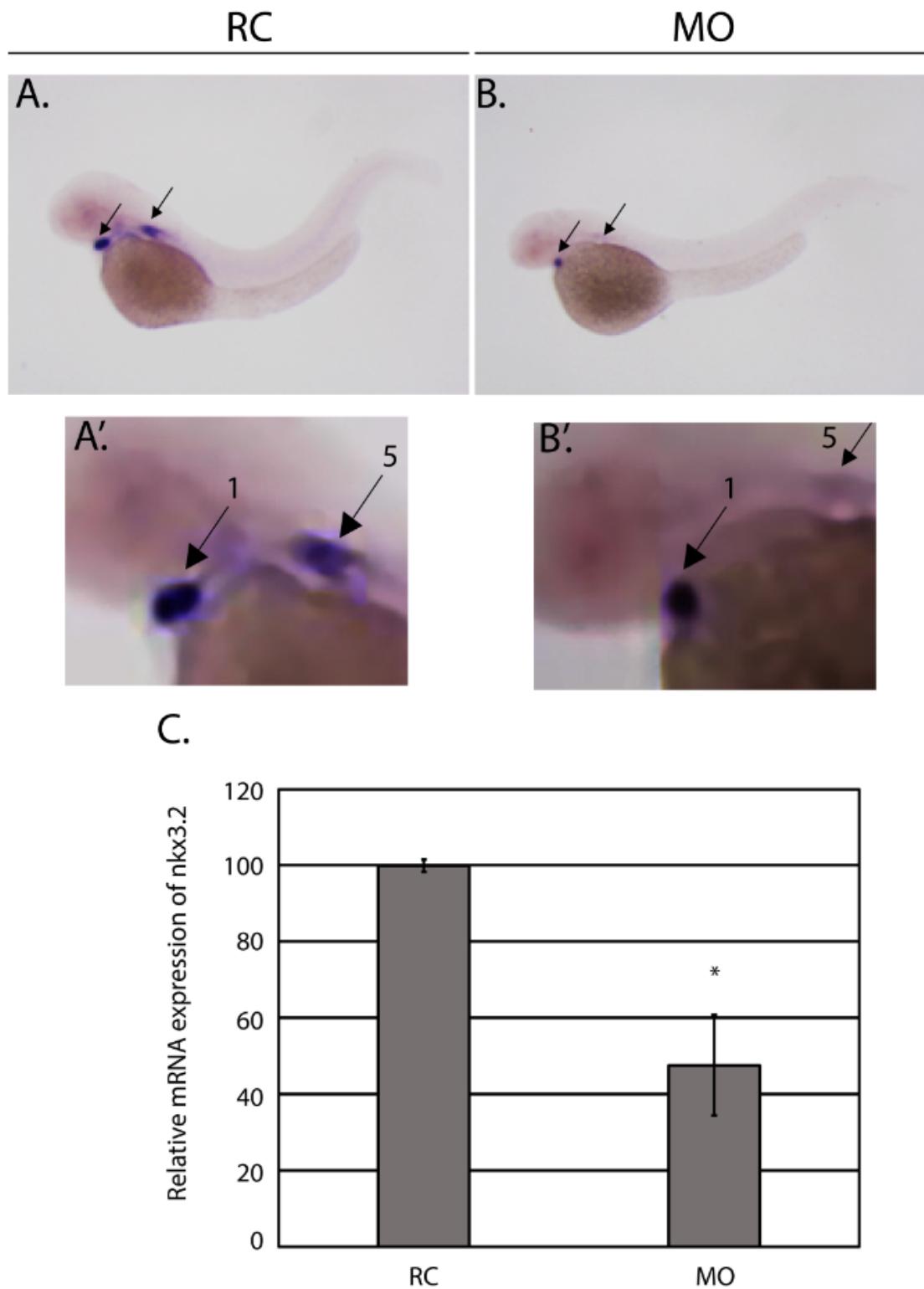
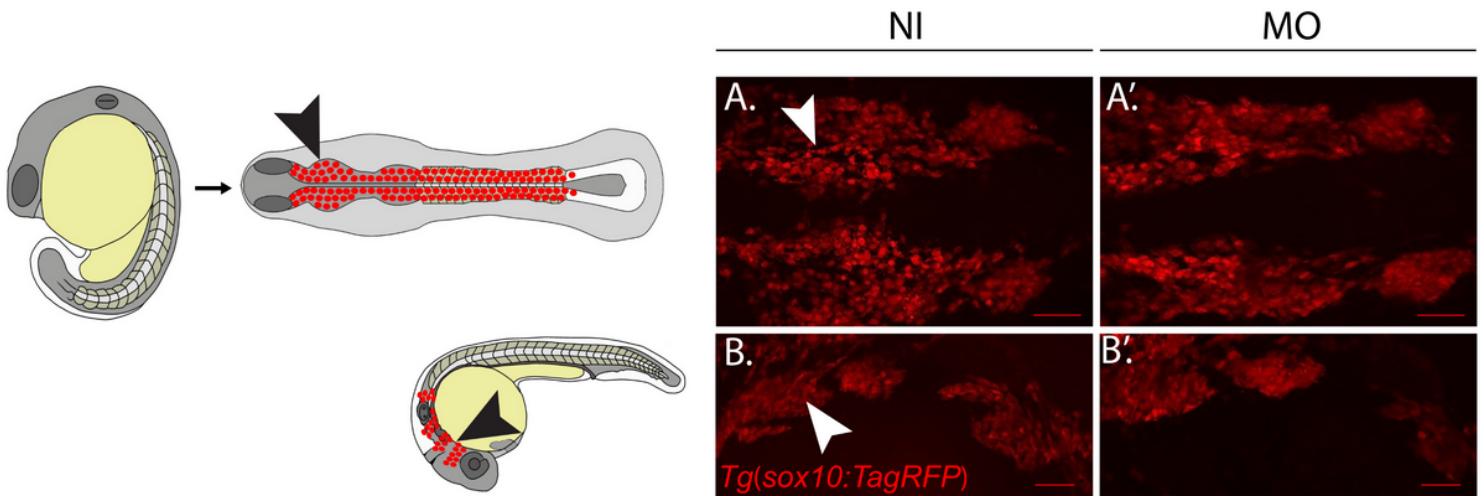


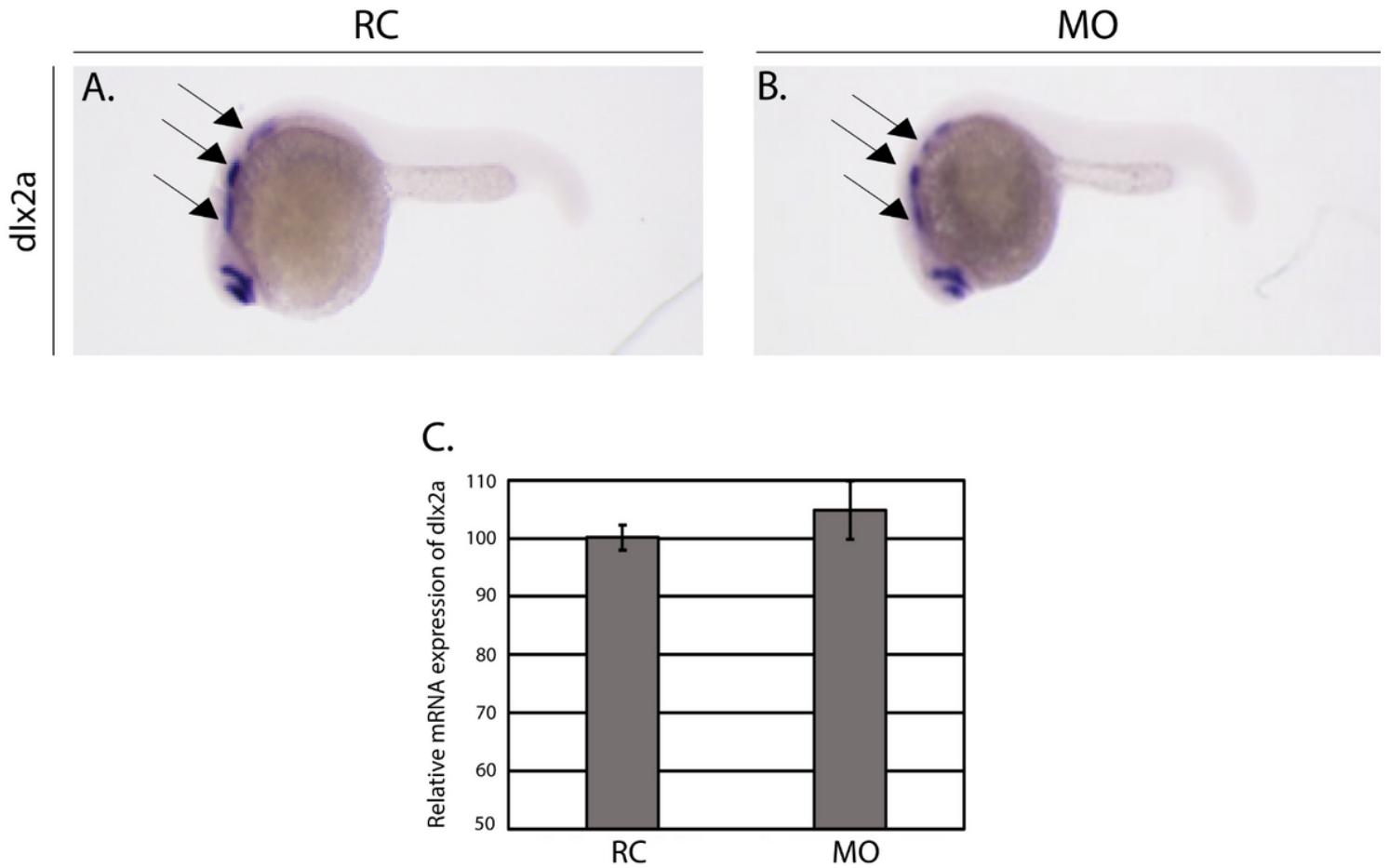
Figure 2

*nkx3.2* expression is decreased in *hspg2* morphants. (A-B) Whole mount *in situ* hybridization (ISH) was performed to detect the expression of *nkx3.2* at the 2 days post fertilization (DPF) stage. Embryos were injected as described in methods section and subjected to ISH to detect *nkx3.2* expression in the developing jaw joint region. Black arrows indicate the expression of *nkx3.2*. There were N=18 in the random control (RC) group and N=14 larvae in the *hspg2* morphants (MO) group. (A'-B') Zoomed region from arrows in A-B demonstrate *nkx3.2* expression. Number one and attached arrow indicate the first and fifth pharyngeal arches. (C) qPCR was performed to detect the expression of *nkx3.2* at 4 DPF. RC and *hspg2* MO samples (N=15 total per group); error bars represent the standard deviation of biological replicates (\*\*p=0.004).



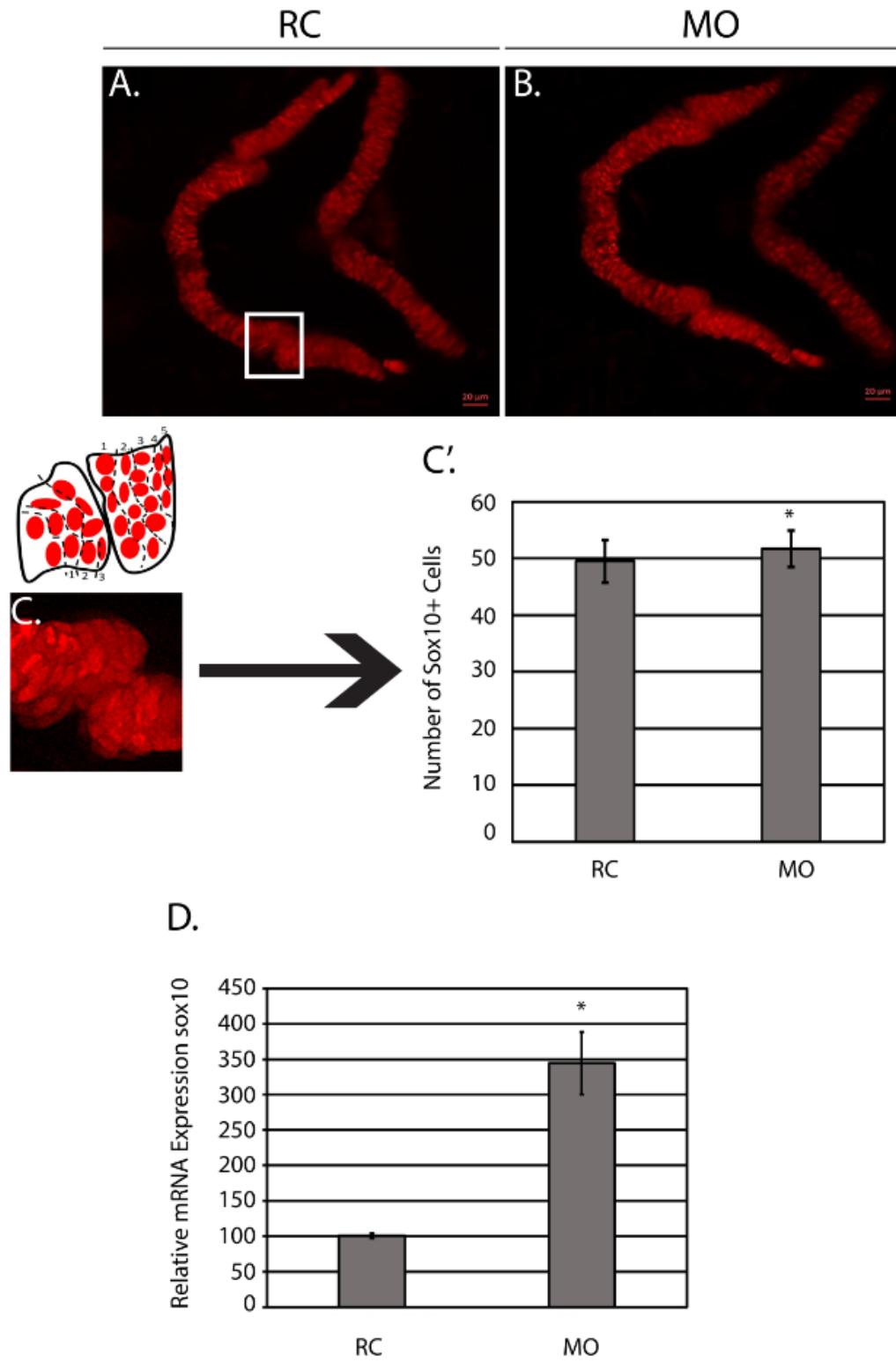
**Figure 3**

Early neural crest cell (NCC) migration and specification are normal in morphants. (A-A') Non-injected (NI) *Tg(sox10:TagRFP)* larvae and *hspg2* morpholino injected *Tg(sox10:TagRFP)* larvae (MO) (N=6 and N=4, respectively) larvae were staged and fixed at the 18-somite stage. Images are scaled at 200  $\mu$ m and demonstrate two lateral streams of Sox10+ migrating NCCs. Cartoon depiction to the left demonstrate an 18 somite embryo with appropriate dorsal view with a black arrowhead to show the equivalent Sox10 stream of cells depicted by the white arrowhead in A'. (B-B') NI and MO larvae (N=8 and N=16 respectively) were staged and fixed at the Prim-5 stage. An appropriate cartoon depiction of an equivalently staged embryo is shown to the left of B, with a black arrow to demonstrate location of pharyngeal arch 1, which is shown in B with a white arrowhead. Images are scaled at 200  $\mu$ m and demonstrate Sox10+ NCCs in the pharyngeal arches. No significant changes were found between the two groups at either timepoint.



**Figure 4**

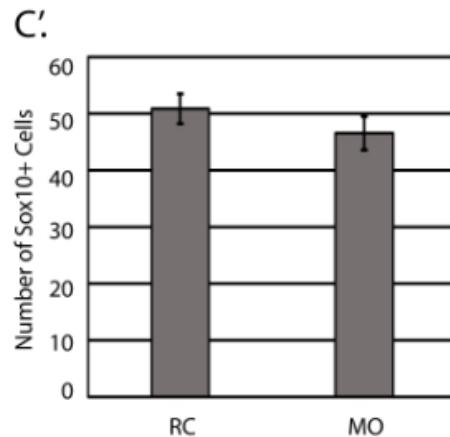
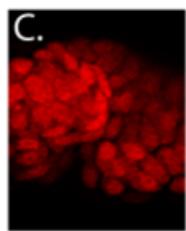
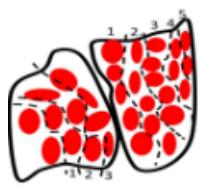
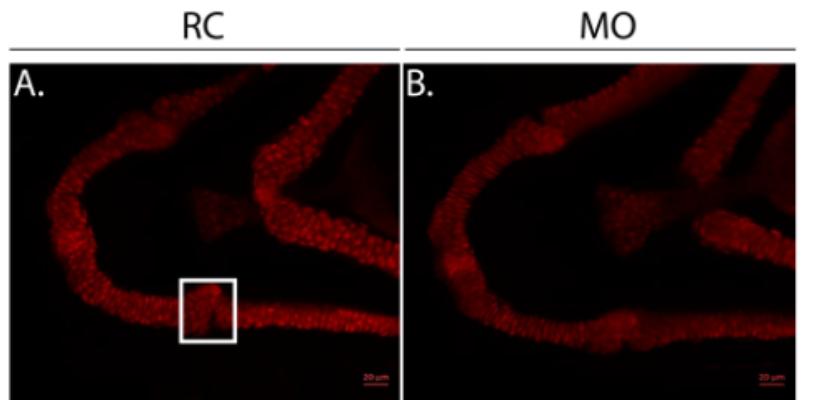
*dlx2a* expression is normal in morphants. (A-B) Whole mount *in situ* hybridization (ISH) was performed to detect the expression of *dlx2a* at the Prim-5 stage (N=12 larvae in random control (RC) group, and N=11 larvae in *hspg2* morphant (MO) group). Embryos were injected as described in the methods section and subjected to ISH to detect *dlx2a* expression in the pharyngeal arches labeled by black arrows. (C) qPCR was performed to detect the expression of *dlx2a*. Total RNA was isolated from RC and *hspg2* MO samples (N=10 per group); error bars represent the standard deviation of biological replicates. Quantification between the two groups was not statistically significant.



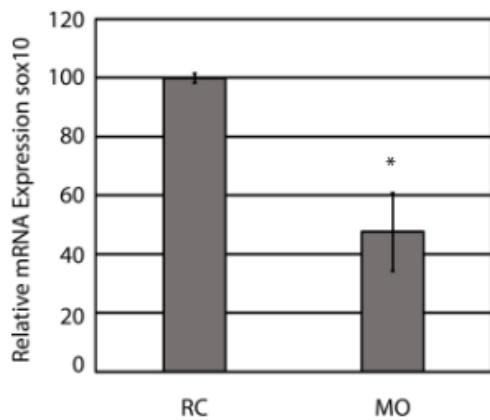
**Figure 5**

The number of Sox10+ cells is increased in morphants at 3 DPF. (A-B) Tg(sox10:TagRFP) random control (RC) and morphant (MO) larvae (N=10 per group) were mounted in agarose and confocal images were taken at 3 days post fertilization (DPF). (C) Depiction of the representative region of cells that were quantified with a corresponding schematic showing the parameters (3 rows left, 5 rows right). (C') Average number of Sox10+ cells counted across both groups (N=10 per group) at 3 DPF. P-value pertains

to the statistically significant difference between the RC group and the MOs (\*p= 0.04). (D) qPCR expression of sox10 (N=24 total) in RC and MO groups at 3 DPF (\*p=0.0005). Error bars represent the standard deviation of biological replicates.



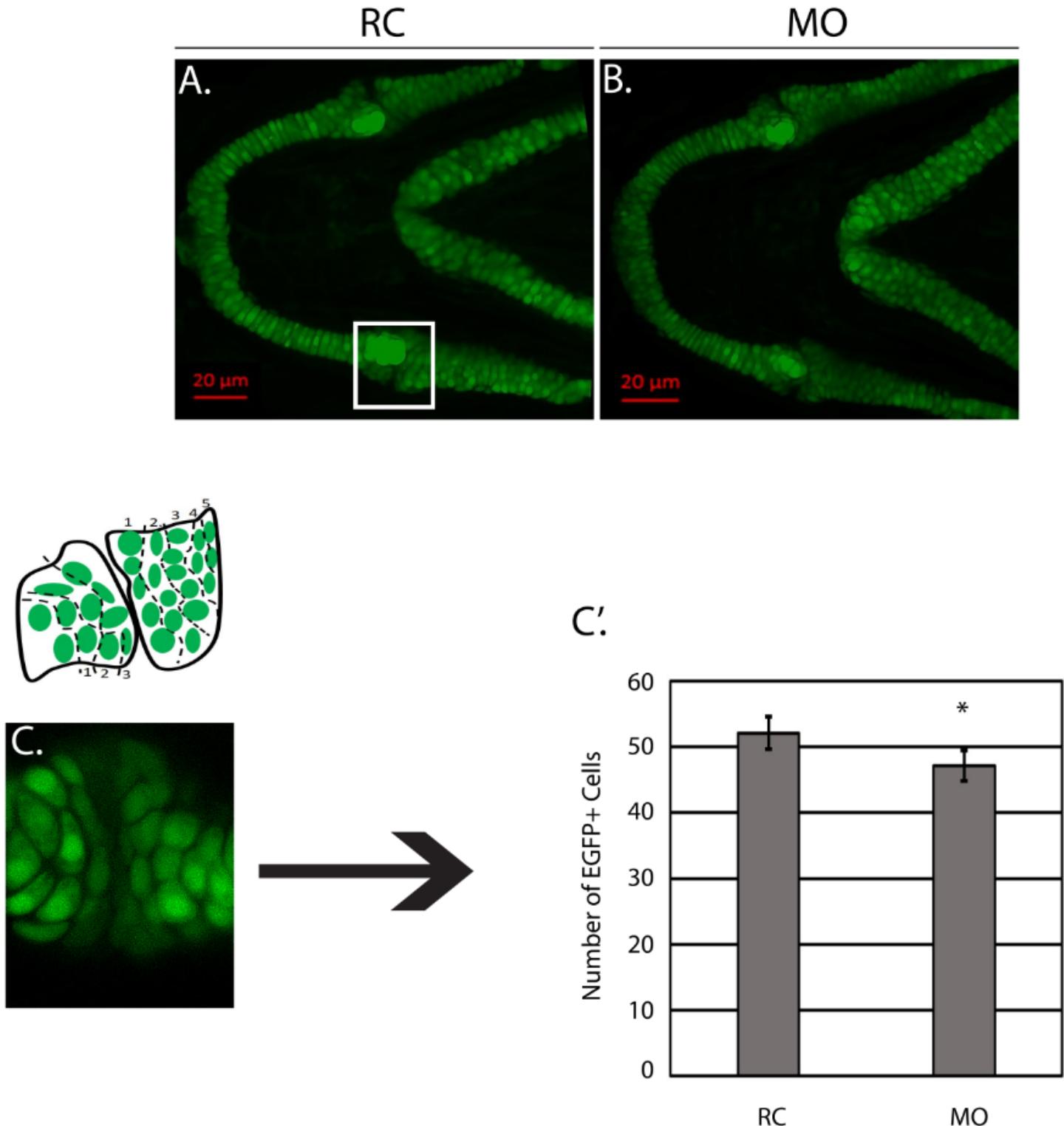
D.



**Figure 6**

The number of Sox10+ cells is decreased in morphants at 4 DPF. (A-B) Tg(sox10:TagRFP) random control (RC) and morphant (MO) larvae (N=10 per group) were mounted in agarose and confocal images were

taken at 4 days post fertilization (DPF). (C) shows the representative region where cells were quantified with a corresponding schematic showing the parameters (3 rows left, 5 rows right). (C') Average number of Sox10+ cells counted across both groups (N=10 per group) at 4 DPF. P-value pertains to the statistically significant difference between the RC group and the MOs (\*p=0.0002). (D) qPCR demonstrating the expression of sox10 (N=15 total) in RC and MO groups at 4 DPF (\*p=0.005). Error bars represent the standard deviation of biological replicates.



**Figure 7**

The number of Col2a1a+ cells is decreased at 4 DPF (A-B) Tg(col2a2a:EGFP) random control (RC) and morphant (MO) larvae (N=10 per group) were mounted in agarose and confocal images were taken at 4 days post fertilization (DPF). (C) shows the representative region where cells were quantified with a corresponding schematic showing the parameters (3 rows left, 5 rows right). (C') Average number of Col2a1a+ cells counted across both groups (N=10 per groups) at 4 DPF. P-value pertains to the statistically significant difference between the RC group and the MOs (\*p= 1.44x-05).