RESEARCH ARTICLE

Cx43-Dependent Skeletal Phenotypes Are Mediated by Interactions between the Hapln1a-ECM and Sema3d during Fin Regeneration

Jayalakshmi Govindan, Kyaw Min Tun, M. Kathryn Iovine*

Department of Biological Sciences, 111 Research Drive, Iacocca B217, Lehigh University, Bethlehem, Pennsylvania, United States of America

* mki3@lehigh.edu

Abstract

Skeletal development is a tightly regulated process and requires proper communication between the cells for efficient exchange of information. Analysis of fin length mutants has revealed that the gap junction protein Connexin43 (Cx43) coordinates cell proliferation (growth) and joint formation (patterning) during zebrafish caudal fin regeneration. Previous studies have shown that the extra cellular matrix (ECM) protein Hyaluronan and Proteoglycan Link Protein1a (Hapln1a) is molecularly and functionally downstream of Cx43, and that hapln1a knockdown leads to reduction of the glycosaminoglycan hyaluronan. Here we find that the proteoglycan aggrecan is similarly reduced following Hapln1a knockdown. Notably, we demonstrate that both hyaluronan and aggrecan are required for growth and patterning. Moreover, we provide evidence that the Hapln1a-ECM stabilizes the secreted growth factor Semaphorin3d (Sema3d), which has been independently shown to mediate Cx43 dependent phenotypes during regeneration. Double knockdown of hapln1a and sema3d reveal synergistic interactions. Further, hapln1a knockdown phenotypes were rescued by Sema3d overexpression. Therefore, Hapln1a maintains the composition of specific components of the ECM, which appears to be required for the stabilization of at least one growth factor, Sema3d. We propose that the Hapln1a dependent ECM provides the required conditions for Sema3d stabilization and function. Interactions between the ECM and signaling molecules are complex and our study demonstrates the requirement for components of the Hapln1a-ECM for Sema3d signal transduction.

Introduction

Vertebrate skeletal morphogenesis is a highly coordinated and tightly regulated process that contributes to the formation of bones of the correct size and shape. The underlying mechanisms that control the regulation of skeletal development are largely unknown. However, it is
clear that proper communication and exchange of information between different cells of the skeletal tissue is crucial for proper bone formation. Gap junction mediated intercellular communication is one such mechanism that is known to contribute to skeletal development [1–3]. Intercellular communication through gap junctions involves the direct exchange of ions, second messengers and small metabolites between the cells and allows for coordinated cellular activity. It is hypothesized that gap junctional intercellular communication (GJIC) facilitates a range of functions including growth, differentiation, morphogenesis, skeletogenesis and developmental signaling [4,5]. Gap junctions are composed of oligomeric integral membrane protein subunits called connexons. Connexons from adjacent cells join to form the continuous gap junction channel that connects their cytoplasmic milieu allowing for direct exchange of low molecular weight (<1000 Da) metabolites [6]. Each connexon in turn is made up of transmembrane protein subunits called connexins. Among the 21 different connexins known to be expressed in humans [7], CONNEXIN43 (CX43) is the major connexin expressed in bone cells [8]. Autosomal dominant missense mutations in human and mouse CX43 results in the syndrome Oculodentodigital dysplasia (ODDD), characterized by craniofacial and limb abnormalities [1,2]. The CX43−/− knockout mouse model exhibits severely delayed appendicular skeletal ossification and hypo-mineralization of craniofacial bones [9]. A similar set of phenotypes was observed in the targeted CX43 knockout chick model [10,11]. Recessive homozygous mutations in zebrafish cx43 results in the short fin (sof) phenotype characterized by short fins, reduced cell proliferation and short bony fin ray segments [3]. Together, these studies reveal that Cx43-dependent GJIC exhibits conserved skeletal functions across vertebrates. The mechanisms by which CX43 dependent mutations result in the observed skeletal phenotypes remain largely unknown.

To study the role of Cx43 during skeletal morphogenesis, we utilize the fin length mutant sofβ123 that expresses reduced levels of cx43 mRNA and Cx43 protein without a lesion in the coding sequence. Three additional alleles of sof are caused by missense mutations in the cx43 coding sequence, which exhibit defects in GJIC using heterologous assays [12]. Based on the sof phenotypes of reduced cell proliferation and short segments (i.e. premature joint formation), we suggest that Cx43 regulates skeletal morphogenesis by coordinating growth (i.e., promoting cell proliferation) and patterning (i.e., inhibiting joint formation) [13,14].

To understand how Cx43-based GJIC influences tangible cellular events like proliferation and differentiation, we completed a microarray analysis to identify candidate genes that may function downstream of cx43 [15]. Two genes identified by the microarray and subsequently validated include semaphorin3d (sema3d), a secreted signaling molecule, and hyaluronan and proteoglycan link protein 1a (hapln1a), an extracellular matrix (ECM) protein [15,16]. Through previous studies, we have shown that Hapln1a and Sema3d are molecularly and functionally downstream of Cx43, mediating Cx43-dependent cell proliferation and joint formation. We have also shown that sema3d and hapln1a are transcriptionally independent of each other, since knockdown of one does not affect the expression of the other [16]. However, the possibility remains that the sema3d and hapln1a gene products functionally interact.

The major function of Hapln1 is to "link" and stabilize the interaction between the glycosaminoglycan (GAG) hyaluronic acid (HA) and proteoglycans (PGs) in the ECM (Hapln1 is also called Cartilage link protein 1 or link protein/LP). HA is an unsulfated GAG composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, and is synthesized in a high molecular weight form, around 10^6–10^7 Da. In cartilage, the majority of PGs are involved in the formation of an aggregated structure built of HA, PGs and LP (Hapln1), that are non-covalently bound [17]. Up to 100 aggrecan molecules may be associated with a single HA molecule in a newly synthesized PG aggregate, each stabilized by Hapln1 [18]. Together HA and PGs confer precise, fixed, and confined charge density imparting specific functional
and structural properties to the tissue, where Hapln1 influences the spacing of the PG monomers along the length of the HA filament [17,19–22]. Other PG members include versican, neurocan and brevican, but only aggrecan and versican have been described in cartilage, and among these aggrecan has been shown to be predominantly present [23]. The mouse knockout for CRTLI (aka HAPLN1) results in perinatal lethality, dwarfism, delayed ossification, short limbs, defects in cartilage and bone development and craniofacial abnormalities [24] showing that CRTLI is both essential and critical for proper skeletal development. In a recent study, we found that hapln1a knockdown in regenerating zebrafish fins causes reduced levels of HA in vivo, suggesting that the ECM becomes partially destabilized when Hapln1a is reduced [16]. However, since Hapln1a is involved in stabilizing the interaction between HA and PGs, it is possible that, in addition to HA, the PG levels could also be affected upon hapln1a knockdown. ECM remodeling is well known to be essential for proper skeletal development and regeneration [25–28]. Mounting evidence suggests that in addition to providing structural stability the ECM also has roles in sequestering growth factors, presenting growth factors to their receptors, and sensing and transducing mechanical signals [29,30]. Therefore, it is of interest to define how reduced Hapln1a causes Cx43-dependent skeletal phenotypes.

This report builds upon our prior studies showing that hapln1a knockdown results in reduced regenerate length, reduced segment length, reduced cell proliferation and reduced HA [16]. Here, we explore the possibilities whether reduced HA is sufficient to cause the observed phenotypes. As a part of these studies, we also found that aggrecan (Acan) levels are reduced following hapln1a knockdown and that reduced Acan also contributes to the hapln1a knockdown phenotypes. Finally, we provide evidence that, while hapln1a and sema3d gene transcription are independently regulated downstream of cx43 [16], the Hapln1a and Sema3d protein products appear to interact functionally. These findings provide important new insights into the role of Hapln1a during fin regeneration and into the requirement for the ECM during Sema3d-based signal transduction.

**Materials and Methods**

**Statement on the ethical treatment of animals**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols used for this manuscript were approved by Lehigh’s Institutional Animal Care and Use Committee (IACUC) (protocol identification #128, approved 11/16/2014). Lehigh University’s Animal Welfare Assurance Number is A-3877-01. All experiments were performed to minimize pain and discomfort.

**Housing and husbandry**

Zebrafish are housed in a re-circulating system built by Aquatic Habitats (now Pentair). Both 3 L tanks (up to 12 fish/tank) and 10 L tanks (up to 30 fish/tank) are used. The fish room has a 14:10 light:dark cycle and room temperature (RT) varies from 27–29°C [31]. Water quality is automatically monitored and dosed to maintain conductivity (400–600 μs) and pH (6.95–7.30). Nitrogen levels are maintained by a bio filter. A 10% water change occurs daily. Recirculating water is filtered sequentially through pad filters, bag filters, and a carbon canister before circulating over UV lights for sterilization. Fish are fed three times daily, once with brine shrimp (hatched from INVE artemia cysts) and twice with flake food (Aquatox AX5) supplemented with 7.5% micropellets (Hikari), 7.5% Golden Pearl (300–500 micron, Brine Shrimp direct), and 5% Cyclo-Peeze (Argent).
Experimental procedures and animals

Wild-type C32 zebrafish (*Danio rerio*) and Tg(hsp70:sema3dgfp) (generously provided by Mary C. Halloran) were used in this study. Fish were anaesthetized in 0.1% tricaine and caudal-fin amputations were performed at 50% level. Fin regeneration was allowed to proceed until the desired time period (3–7 days post amputation [dpa]) depending on the type of experiment. The regenerated fins were harvested at the required time point after anaesthetizing the fish. Whole mount in situ hybridization, immunofluorescence and histochemical staining were performed on regenerating fins. Descriptions of each of these methods follow.

**In situ** hybridization on whole mount fins and cryo-sections

For each gene under study, a minimum of 4–5 fins were used per trial and 3 independent trials were performed. Fins were fixed overnight in 4% paraformaldehyde (PFA) in PBS and dehydrated in 100% methanol and stored at -20°C until use, for in situ hybridization (ISH). RNA probes were generated from PCR amplified linear DNA in which the reverse primer contained the T7 RNA polymerase binding site. The primers used in this study for ISH are summarized in Table 1. DIGoxigenin (DIG) labeled RNA probes were synthesized using DIG labeling mix (Roche) and in situ hybridization on regenerated whole fins was carried out following standard protocols [15,16].

For in situ on cryo-sections, fins were fixed overnight with 4% PFA in PBS after harvest. After a brief methanol wash, fins were dehydrated in 100% methanol and stored at -20°C until use. Before sectioning, fins were sequentially rehydrated in a methanol- PBS series of washes and then were embedded in 1.5% agarose/5% sucrose dissolved in PBS and equilibrated overnight in 30% sucrose. Fins were mounted in OCT and cryo-sectioned (15 μm sections) using a Reicherta® Jung 2800 Frigocut cryostat. Sections were collected on Superfrost Plus slides (Fisher) and allowed to air dry overnight at room temperature. Following this in situ on cryo-sections were performed following standard protocols as described [15,16,32]. Finally sections were mounted in 100% glycerol and examined on a Nikon Eclipse 80i microscope.

| Table 1. Primer Sequences. |
|----------------------------|
| **Gene** | **Primer Sequence for ISH** |
| hapln1a | FP-ggttctccgcttgccgcagcg<br>RT7-taatacgcacactataggggcccctccgtgcctaagacc |
| sema3d | FP-cgaagtgtgtagcatttacgc<br>RT7-taatacgcacactatagggttagggactatgtcct |
| has1 | FP-gaattttgatctctgtgctccc<br>RT7-taatacgcacactataggggaactaagggccg |
| has2 | FP-gttgggacgcactctgctgg<br>RT7-taatacgcacactataggggctgattttgctcagttgccc |
| has3 | FP-ggtgcagctctctcctccac<br>RT7-taatacgcacactataggggcgcattgtgccagttgcc |
| Acana | FP-ctctcagcagacacagttactc<br>RT7-taatacgcacactatagggtacactcgtctggctcagttgcag |
| Acanb | FP-ccctagcagcctgctctgc<br>RT7-taatacgcacactataggccatagcggcagttgcc |

The RNA polymerase T7 binding site in reverse primers is highlighted in bold.

F = Forward primer; RT7 = Reverse primer

doi:10.1371/journal.pone.0148202.t001
Morpholino mediated gene knockdown in regenerating fins

All MOs used in this study were fluorescein tagged, obtained from Gene Tools, LLC and used at a final concentration of 1mM for injection, unless otherwise described. The sequences for MOs used in this study can be found in Table 2. For each gene under study two MOs, one targeting translation initiation and the other targeting splicing were used. The effect of the MO was tested either by histochemistry or by RT-PCR as described below. Injection and electroporation were carried out as described previously [16,33]. Briefly, 3 dpa fish were anesthetized and approximately 50nl of MO (targeting morpholino [MO] or mismatch control morpholino [MM]) or standard control MO 5’ CCTCTTACCTCAGTTACAATTTATA 3’ was injected into either the dorsal or the ventral half of the tail fin. The un-injected half of the fin served as the internal control. Following injection, the entire fin was electroporated using a CUY21 Square Wave electroporator (Protech International Inc). MO-positive fish were selected 24 hpe (hours post electroporation) by examination under a fluorescence microscope. The knockdown fins were evaluated for regenerate length, segment length, histone-3-phosphate (H3P) analysis, alizarin red staining, immuno-fluorescence and histochemical experiments. The procedure for each analysis is described separately. For each MO (i.e., targeting or mismatch) 6–8 fish were used. Reproducibility was confirmed by testing the MO in three independent experiments. Statistical significance was determined using the student’s t-test (P<0.05).

Rescue of hapln1a knockdown phenotypes with sema3d overexpression

We used three groups of fish for these experiments. Groups 1 and 2 were positive for the transgene, Tg(hsp70:sema3d-gfp), while Group 3 fish were negative for the transgene. Knockdown of hapln1a was performed on 3 dpa fins as described above. Four hours after hapln1a knockdown, heat shock was performed for one hour at 37°C (Groups 1 and 3). Alternatively, Group 2 fish were positive for the transgene but were not exposed to heat shock. Induction of transgene expression in Group 1 animals was confirmed after 24 hours of heat shock by screening for GFP-positive fins. For regenerate and segment length analyses the fins were harvested 4 dpe (days post electroporation) and data analysis was performed as described below. For each

Table 2. Morpholino Sequences.

| Gene  | Morpholino                                      |
|-------|------------------------------------------------|
| hapln1a | MO-gccacagaaaaacagagcaatcatct                  |
|        | MM-gccagaaaaacagaccaatgtatct                  |
| sema3d  | MO-tgtccgctccctgcttccttatct                  |
|        | MM-tgtgcctgccccctccacctctcttct               |
| has1    | MO1-tcttaaagactgcctgtcatatc                   |
|        | MO2-gtcacatcctgattgaaaaataata                |
|        | MM-tctaaacagctgcctcatatc                     |
| has2    | MO1-gctgacccctctatcatacctatc                 |
|        | MO2-tgaaagagagacagactacctgta                  |
| Acanb   | MO1-acagccagagccaatatcagacat                  |
|        | MO2-gggctgtatatgaaagtgccttaacct              |

MO1 = ATG-blocking morpholino.
MO2 = splice-blocking morpholino.
MM = Control morpholino with 5 mismatch pairs.

doi:10.1371/journal.pone.0148202.t002
experiment a minimum of 6–8 fins were used per trial and 3 independent trials were performed. Statistical significance was determined using the student’s t-test ($P < 0.05$).

**Fin regenerate length, segment length and H3P analysis**

For each type of analysis, minimum of 6–8 fins were used per trial and 3 independent trials were performed. Statistical significance was determined using the student’s t-test ($P < 0.05$). Images were collected on a Nikon SMZ1500 dissecting microscope using a Nikon DXM1200 digital camera unless otherwise stated. Measurements were performed using Image Pro software.

Fin regenerate length analysis was performed as described [15]. Briefly, fins were harvested at 4 dpe, fixed and stored in methanol until use at -20°C. The measurements were taken from the longest fin ray (i.e. the third ray from either the dorsal or ventral end) that was previously established as a standard [34]. Fin regenerate length was measured using Image Pro Software from the amputation plane (clearly visible in bright field) to the tip of the fin. To evaluate the phenotypic effect of MO based knockdown experiments on fin regenerate length, the targeting MO or control MM injected side of each fin is compared to its un-injected side by the percent similarity method as described [32]. Briefly, the percent similarity for each fin is calculated as ($[\text{length of the injected side in } \mu m / \text{length of the un-injected side in } \mu m] \times 100$). Values close to 100% indicate that the injected MO has no effect on the phenotype whereas a value less than 100% indicate that the MO has an effect on the observed phenotype. The mean of percent similarity for the MO treated experimental group and the corresponding MM treated control group were estimated and compared. Percent similarity of greater than 100% reflects the fact that the experimental side can be measurably larger than the control un-injected side. Statistical significance was determined using the student’s t-test ($P < 0.05$).

Segment length analysis was performed on calcein stained fins. Calcein staining was performed on live fish as described [35]. Briefly, fish were permitted to swim in 0.2% calcein for 10 min, followed by 10 min in fresh water. Fish were anesthetized as described above and fins were imaged at 4X. For segment length, the distance between the first two newly formed joints following amputation was measured in the third fin ray from either the dorsal or ventral end [34]. Segment length was measured using Image Pro Software. To evaluate the phenotypic effect of MO based knockdown experiments on segment length, the percent similarity method was used as described above.

For H3P analysis, the fins were harvested 1 dpe, fixed overnight in 4% paraformaldehyde (PFA) in PBS and dehydrated in 100% methanol and stored at -20°C until use. Cell proliferation analysis was performed by counting number of mitotic cells by H3P immuno-staining as described [15]. H3P positive cells were counted from within the distal most 250 $\mu m$ of each fin above the third fin ray from either the dorsal or ventral end [34]. To evaluate the phenotypic effect of MO based knockdown experiments on cell proliferation, the percent similarity method was used as described above. For cell proliferation, the percent similarity for each fin is calculated as ($[\# \text{H3P positive cells per } 250 \mu m^2 \text{ of injected side} / \# \text{H3P positive cells per } 250 \mu m^2 \text{ of un-injected side}] \times 100$).

**Alizarin red staining**

For alizarin red staining, the fins were harvested 4 dpe, fixed overnight in 4% paraformaldehyde (PFA) in PBS and dehydrated in 100% methanol and stored at -20°C until use. Fins were rehydrated through a decreasing methanol series as described for ISH. Following that fins were bleached for 30 minutes in 0.8% KOH, 0.6% $H_2O_2$ (should be prepared and used within one week). Subsequently, fins were washed twice with water and then washed for 1hr in a saturated...
Alizarin Red solution containing 1% KOH [36] followed by 30 minutes wash in water and mounted in glycerol for imaging. The extent of mineralization was calculated as the ratio of the zone of mineralization (extent of detectable alizarin red staining length) to the total regenerate length. To evaluate the phenotypic effect of MO based knockdown experiments on alizarin red staining, the % similarity method was used as described above. The % similarity for each fin is calculated as (ratio of {regenerate length: alizarin red staining length} of the injected side / ratio of {regenerate length: alizarin red staining length} of un-injected side) X 100. The mean of % similarity for the MO treated experimental group and the corresponding MM treated control group were estimated and compared, and the statistical significance between the groups was determined using two tailed unpaired student’s t-test (P < 0.05). For each experiment minimum of 6–8 fins were used per trial and 3 independent trials were performed.

Cryosectioning for immunofluorescence and histochemistry

Fixed and embedded fins were mounted in OCT and cryosectioned (15 μm sections) using a Reichertâ Jung 2800 Frigocut cryostat. Sections were collected on Superfrost Plus slides (Fisher) and allowed to air dry O/N at RT. Sections can be stored at –20°C for up to a year. The slides were stored at –20°C for at least one day before starting the experiment. For all experiments using cryosections, the slides were brought to RT for one hour. Sections were circled using a marking pen (ImmEdge Pen H-4000; PAP pen, VWR Laboratories). To confirm reproducibility, minimum of 3–5 different fins were used for each experiment and approximately 8–12 sections per fin were analyzed.

Acan Immunofluorescence

For detection of Acan, fins were fixed for 10 min with 2% PFA at RT followed by three 10 min washes with 1X PBS, and then embedded in 1.5% agarose/5% sucrose in PBS and equilibrated in 30% sucrose in PBS and sectioned as described above. Acan immuno-staining was performed as described earlier [37]. Briefly, the sections were rehydrated twice for 10 min in PBS at RT, followed by antigen unmasking by digestion with chondroitinase. First, the slides were incubated with the chondroitinase treatment buffer (50mM Tris, 60mM sodium acetate, 0.02% BSA, pH 8.0) at 37°C for 5 min, followed by deglycosylation using chondroitinase ABC enzyme (Sigma-C2905, final concentration 0.05U in the treatment buffer) for 2 h at 37°C. Following that, slides were washed with block for 10 min at RT. Then, sections were blocked for another one hour at RT, incubated with Mouse anti-aggrecan (BC-3) (Thermo Scientific-MA3-16888) primary antibody O/N at 4°C. Following incubation with primary antibodies, sections were washed three times in block (15 min each), incubated at RT for one hour with secondary antibody goat anti-mouse Alexa-663 (Invitrogen, 1:200) or goat anti-mouse Alexa-568 (Invitrogen, 1:200) and washed again three times in block (15 min each). Sections were next incubated with propidium iodide (final concentration 0.01mg/ml in block) or To-Pro-3-iodide (Life Technologies, 1:1000) for 30 min at RT, followed by a quick wash with distilled water.

Sema3d Immunofluorescence

For detection of Sema3d, fins were fixed for 30 min with 2% PFA at RT followed by three 10 min washes with 1X PBS, and then embedded in 1.5% agarose/5% sucrose in PBS and equilibrated in 30% sucrose in PBS and sectioned as described above. For Sema3d immuno-staining, the sections were rehydrated twice for 10 min in PBS followed by antigen retrieval. Slides were transferred to coplin jars containing 10 mM sodium citrate (pH = 6) and incubated at 99.5°C water bath for 10 minutes. The slides were allowed to cool down to room temperature and again washed in 1X PBS (2X, five minutes each). Following that slides were washed twice with
block (2% BSA, 0.1% TritonX 100 in PBS). Then, sections were blocked for another one hour at RT and then incubated in primary antibody, Rabbit anti-Sema3d (Sigma-SAB1402064, 1:500) or Mouse anti-EGFP (Clonetech, 1:1000) O/N at 4°C. Following incubation with primary antibodies, sections were washed three times in block (15 min each), incubated at RT for one hour with secondary antibody goat anti-rabbit Alexa-663 (Invitrogen, 1:200) or goat anti-mouse Alexa 488 and washed again three times in block (15 min each). Sections were next incubated with propidium iodide (final concentration 0.01mg/ml in block) or DAPI (1:1000) for 30 min at RT, followed by a quick wash with distilled water.

Histo-chemical analysis of HA

For detection of HA, fins were fixed overnight (O/N) with 4% PFA in PBS. After a brief methanol wash, fins were dehydrated in 100% methanol and stored at –20°C until use. Before sectioning, fins were sequentially rehydrated in a methanol-PBS series of washes and then were embedded in 1.5% agarose / 5% sucrose in PBS and equilibrated in 30% sucrose in PBS and then sectioned as described above. HA was detected as described [16]. Briefly the sections were rehydrated twice for 10 min in PBS followed by two washes with block. Then, sections were blocked for another one hour at RT, incubated with biotinylated hyaluronic acid binding protein (bHABP-Calbiochem-385911, 1:100) O/N at 4°C. The sections were washed three times in block (15 min each), incubated at RT for one hour with streptavidin-Alexa 546 conjugate, (Invitrogen, 1:200) and washed again three times in block (15 min each). Sections were next incubated with propidium iodide (final concentration 0.01mg/ml in block) or To-Pro-3-iodide (Life Technologies, 1:1000) for 30 min at RT, followed by a quick wash with distilled water. Then the slides were blotted dry and mounted for imaging as described above.

Confocal microscopy

The slides were blotted dry and mounted with vectashield for imaging. Confocal microscopy was used to image the sections using a 40×/1.3 numerical aperture objective on an inverted microscope (Axiovert 200 M; Carl Zeiss, Jena, Germany) equipped with an LSM510 META scan head (Carl Zeiss). Argon ion and HeNe lasers were used to generate the 488 and 543/633 lines used for excitation, and pinholes were typically set to 1–1.5 Airy units. Images were exported as TIFF files and printed using Photoshop.

Results

Components of Hapln1a-ECM are expressed in the regenerating fin

In a recent study, we showed that hapln1a knockdown results in Cx43 dependent phenotypes: reduced fin regenerate length, reduced segment length and reduced cell proliferation [16]. Since Hapln1a links HA with PGs we were interested in the expression of genes responsible for synthesis of HA and in the expression of PGs. HA is synthesized by hyaluronic acid synthases (Has) and Acan is a major bone associated PGs involved in matrix organization [38]. We performed whole mount in situ hybridization on WT 5 days post amputation (dpa) regenerating fins to determine which of the HA synthesizing enzymes (has1, has2 and has3) and which of the skeletal PGs (i.e, acana and acanb) are expressed. Of the three different HA synthesizing enzymes, has1 and has2 are expressed in the regenerating fin. Of the duplicate copies of acan, only acanb is expressed in the regenerating fin (Fig 1A). To assess in which tissues these genes are expressed, we completed in situ hybridization on cryosections of 5 dpa regenerating fins (Fig 1B). The medial mesenchyme consists of both distally located slowly proliferating cells and proximally located rapidly proliferating cells [39]. The lateral mesenchyme consists mainly
of skeletal precursor cells, which includes the precursors of bone-forming osteoblasts and joint-forming cells [40]. Several epidermal layers surround the mesenchyme, and the basal layer of epidermis resides closest to the mesenchyme. We find that has1 is expressed in the medial and lateral mesenchyme, as well as in the basal layer of the epidermis, while has2 is expressed in the medial mesenchyme (and not in the lateral mesenchyme). The acanb mRNA is expressed throughout the mesenchyme, consistent with the localization of the Acan protein [37].

Reduced HA levels contribute to the Hapln1a mediated phenotypes

Because hapln1a knockdown results in reduced HA [16], we hypothesized that manipulating the HA levels by targeting the HA synthesizing enzymes should recapitulate the hapln1a knockdown phenotypes. Since it is likely that homozygous mutations in genes required for the Hapln1a-ECM will be essential, or will cause serious global skeletal defects, we rely on morpholino (MO) based knockdown for evaluation of gene function in the regenerating fin. For targeted gene knockdown, we use two independent non-overlapping MOs for each gene (one ATG-blocker and one splice blocker). As controls, we use either paired 5 mismatch (MM) non-targeting control MOs or the standard control MO. Further, we also confirm that the target is reduced following knockdown. In order to manipulate the HA levels, we completed MO mediated knockdown of the HA-synthesizing enzymes has1 and has2 in WT regenerating fins. All MOs used in this study are fluorescein tagged, permitting the confirmation of cellular

Fig 1. In situ hybridization showing the expression of Hapln1a-ECM components on 5 dpa regenerating fins. (A) Whole mount in situ hybridization of components of the Hapln1a-ECM. The genes has1, has2, and acanb are expressed during fin regeneration. The amputation plane is indicated by a black line in all the panels. Scale bar represents 100μm. (B) In situ hybridization on a WT 5 dpa cryo-section reveals compartmental expression of has1, has2, and acanb. Blastema (b), mesenchyme (m), and skeletal precursor cells (*). The thick arrow identifies the basal layer of the epidermis, which underlies the epidermis (e). The thin arrow identifies lepidotrichia and the arrowhead identifies the actinotrichia. Scale bar represents 50μm.

doi:10.1371/journal.pone.0148202.g001
uptake. Following MO microinjection and electroporation at 3 dpa, MO positive fish were screened 24 hours post electroporation (hpe) by detection of fluorescein positive cells. We evaluated the effect of the both ATG-blocking and splice-blocking MOs on HA synthesis for the HA synthesizing enzymes (i.e. has1 and has2). MO or MM positive fins were harvested 24 hpe and processed for immuno-histochemical analysis for HA using biotinylated HA binding protein (bHABP). During early regeneration (i.e. 3–5 dpa), HA is predominantly upregulated in epidermis, in the proximal mesenchyme of the regenerate, and in the mesenchyme of the stump tissue [16,37]. Histochemical analysis for HA on has1 and has2 knockdown fins revealed reduced levels of HA compared to the MM treated fins, indicating that HA synthesis has been successfully down regulated upon knockdown of these synthesizing enzymes (Fig 2). We did not evaluate co-localization of the fluorescein-MO and HA since processing the fins for detection with the bHABP limited detection of the fluorescein. However, we have shown previously that the fluorescein MO is found in all tissue compartments [40]. Because the MO is injected
into the mesenchyme, the highest knockdown is typically observed there (although knockdown in the epidermis can sometimes also be detected). The finding that HA is reduced following knockdown demonstrates the specificity of independent MOs targeting has1 and has2 gene function.

We next evaluated the effects of the has1 and has2 knockdowns on Cx43-Hapln1a dependent phenotypes using the ATG-blocking and the splice-blocking targeting MOs, and comparing to control-treated fins. For analysis, either the target MO or the control MM is injected into fin rays in one half of the fin, and the other half is un-injected. Next, the injected side was compared with the respective un-injected side and the percent similarity was determined. This method reduces the effect of fin to fin variation. High similarity between the injected and un-injected side (i.e. close to 100%) indicates little or no effect of the MO treatment, whereas values with low similarity (i.e. less than 100%) indicates that the MO had an effect on treatment side. Fin length was measured at 4 dpe as the distance from the amputation plane to the distal end of the regenerate (Fig 3A). We observed that knockdown of has1 had no effect on regenerate length, whereas has2 knockdown resulted in significant reduction in regenerate length compared to the MM control (Fig 3B). The segment length was measured at 4 dpe by calcein staining as the distance between the first two joints distal to the amputation plane (Fig 3C). Similar to regenerate length, has1 knockdown had no effect on segment length; whereas has2 knockdown resulted in significant reduction in segment length phenotype (Fig 3D). Because has1 is expressed in skeletal precursor cells but has2 is not, this finding suggests that the HA produced from the nearby medial mesenchyme contributes to segment length. The effect of the knockdown of these enzymes on cell proliferation was evaluated by counting the number of mitotic cells following immuno-fluorescence for H3P at 1 dpe (Fig 3E). For cell proliferation we found that has1 knockdown resulted in ~20–30% reduction, whereas has2 knockdown had a reduction of about 35–38%. Therefore, knockdown of both enzymes resulted in significant reduction in cell proliferation compared to the control MM treated fins (Fig 3F), and we note that both has1 and has2 are expressed in the proliferating cells. However, the has2 knockdowns are stronger than the has1 knockdowns for all phenotypes.

Since has1 and has2 knockdown both lead to reduced, but not completely absent levels of HA, it is unclear why has2 knockdown influences regenerate length and segment length and has1 knockdown does not. One possibility is that the HA produced by Has1 and Has2 has sufficiently distinct properties that mediate distinct functions. All Has enzymes synthesize HA of similar composition, but differ in their rate of synthesis, molecular weight of HA, and ease with which HA is released from the cell surface [41]. Moreover, others have shown that neither the Has1 nor the Has3 enzymes can replace the function of Has2 during heart morphogenesis [42], even though all three Has enzymes are expressed [43]. Therefore, it is possible that the HA synthesized by Has2 could be functionally distinct. Alternatively, the effects of the has1 knockdown may be diminished due to potential redundancy with has2. To distinguish these possibilities, we completed double knockdown of Has1 and Has2. Double knockdowns were performed by combining both has1 and has2 ATG-blocking MOs (i.e. 0.5 mM of each MO) and comparing to single knockdowns of has1 and has2 at 0.5 mM MO concentrations. For the double knockdowns and each single knockdown, either the targeting MO or the control MM injected sides were compared with the respective un-injected side and the percent similarity was determined as described earlier (Fig 4). The double knockdown failed to demonstrate synergy of the two MOs, which argues against functional redundancy of the has1 and has2 gene products. Rather, our findings are consistent with a model that the HA produced by has1 and has2 is functionally distinct.

To determine if reduced HA is sufficient to mediate Hapln1a effects, we compared the reduction in regenerate length, segment length and cell proliferation of has2 knockdown with
hapln1a knockdown that we determined previously [16]. The effect of hapln1a knockdown levels are represented as hashed bars in Fig 3B, 3D and 3F. Interestingly, we find that hapln1a knockdown alone resulted in a much stronger effect on the level of cell proliferation than the knockdown of either has1 or has2. Therefore, in addition to HA there could be other Hapln1a-ECM components contributing to Hapln1a-Cx43 dependent phenotypes that account for the stronger hapln1a knockdown phenotype.
Acan contributes to the observed hapln1a knockdown skeletal phenotypes

Acan is a major PG associated with bones [38]. In addition to HA, we hypothesized that the Hapln1a associated Acan may also contribute to Hapln1a mediated skeletal phenotypes. We first determined if Acan protein levels are reduced in hapln1a knockdown fins using immunohistochemistry. In prior studies we found that Acan is initially upregulated in mesenchyme during regeneration (around 3–4 dpa), and later expressed more prominently in the lepidotrichia (around 5 dpa). Upon hapln1a knockdown, we observed that Acan protein levels are

Fig 4. Double knockdown experiments suggest that HA produced by has1 and has2 may be functionally distinct. Double knockdown of has1 and has2 was performed by injecting/electroporating combined 1 mM stocks of each ATG-blocking MO (or of each MM MO), resulting in 0.5 mM concentrations of the each MO. This was compared to single knockdowns of 0.5 mM concentrations for each targeting or MM MO. Bar graphs of percent similarity show that the double knockdown does not result in synergistic defects for regenerate length (A), segment length (B), or cell proliferation (C). Students t-test was performed (p<0.05) to determine significance, and the error bars indicate standard error of the mean. None of the double knockdowns were statistically different from the knockdown of has2 alone (i.e. which is the only single knockdown to show phenotypic effects).

doi:10.1371/journal.pone.0148202.g004
down regulated in hapln1a MO treated fins compared to the control MM treated fins (Fig 5).
Next, we determined whether Acan contributes to Hapln1a-Cx43 dependent skeletal phenotypes. We used two independent non-overlapping MOs (one ATG-blocking and one splice-blocking) to knockdown Acan. The effects of acanb knockdown were confirmed for both MOs by immuno-staining for Acan. Both MOs caused significant reduction of Acan protein levels (Fig 6A). Together, reduced Acan protein levels following knockdown with hapln1a-MO and with two targeting acanb-MOs demonstrates that the Acan antibody detects zebrafish Acan protein, although it does not rule out the possibility that the antibody may also detect related zebrafish proteins. We next evaluated the effect of acanb knockdown on Hapln1a mediated phenotypes. Knockdown of acanb resulted in significant reduction of regenerate length, segment length and cell proliferation (Fig 6B). Similar to has2 knockdown, the effect of acanb knockdown on the level of cell proliferation was not as strong as observed for the hapln1a knockdown (see hashed bars in Fig 6B).

Since Acan is important for bone mineralization, we examined bone calcification at 4 dpe using alizarin red, which stains mature bone matrix. The extent of mineralization was determined by calculating the ratio of the total regenerate length to the zone of mineralization (i.e., the length of detectable alizarin red staining). Either the target ATG-blocking MO or the control MM injected side was compared with the respective un-injected side and the percent similarity was determined. In contrast to the control MM treated fins, acanb MO treated fins showed a much shorter zone of bone calcification (~20% reduced) (Fig 6C and 6D), suggesting that Acan might play a role in regulating bone matrix maturation and joint formation.

Hapln1a and Sema3d gene products interact to mediate Cx43 dependent skeletal phenotypes

Data from our recent study suggested that hapln1a and sema3d are transcriptionally independent of each other, meaning that the expression of either does not depend upon the other [16]. However, these findings do not exclude the possibility that the two proteins work together. Several studies demonstrate that GAGs and the ECM function together to regulate signal

Fig 5. Morpholino mediated knockdown of hapln1a results in reduced Acan protein levels.
Longitudinal section of fin rays treated with hapln1a control morpholino (Hapln1a MM) and hapln1a targeting morpholino (Hapln1a MO). Immuno-staining for Acan (blue) and counterstained for nuclei with Propidium iodide (PI, red). Compared to the control Hapln1a MM treated fins, Hapln1a MO treated fins show reduced staining for Acan. Arrow identifies the basal layer of epithelium; arrow head identifies the bone; m, mesenchyme; e, epithelium. Arrow identifies the basal layer of epithelium; arrow head identifies the bone; m, mesenchyme; e, epithelium. Scale bar represents 20 μm.

doi:10.1371/journal.pone.0148202.g005
transduction pathways. For example, GAGs in the ECM play a role during physiological and pathological remodeling processes, such as regeneration, bone development, cancer metastasis and osteoarthritis [44–46]. Moreover, GAGs have been implicated in regulating signaling pathways of molecules such as FGFs, BMPs, Wnts, Hhs and IGFs (reviewed in [47]). Sema3d is a
signaling molecule that is known to mediate Cx43 dependent phenotypes [15] and Hapln1a is an ECM protein known to mediate Cx43 dependent phenotypes [16]. Therefore, we wished to test the hypothesis that the protein products function together. To test this hypothesis, we looked for evidence of genetic interaction by performing double knockdowns of sema3d and hapln1a and testing for synergistic effects. We first identified sub-threshold concentrations for both the sema3d and hapln1a MOs, where neither single knockdown resulted in a skeletal phenotype. Using MOs validated in our prior studies [16,40], we found that 0.5mM concentration of the hapln1a MO and 0.25mM concentration of the sema3d MO did not result in skeletal phenotypes (S1 Fig). Next, we performed double knockdowns of both hapln1a and sema3d using these sub-threshold concentrations (0.5mM Hapln1a MO + 0.25mM Sema3d MO) and compared with the double MM control at the same concentrations. Either the double MO or the double control MM injected side was compared with the respective un-injected side and the percent similarity was determined as described earlier. Interestingly, we observed that the double knockdown resulted in reduced regenerate length (~11% reduced), reduced segment length (~12% reduced) and reduced cell proliferation (~34% reduced) (Fig 7), revealing that hapln1a and sema3d gene products act synergistically and indeed function in a common molecular pathway.

Hapln1a-ECM stabilizes Sema3d

Based on the genetic interaction data, we hypothesized that Hapln1a-ECM is playing a role in mediating Sema3d dependent signal transduction. It is well known that the ECM can contribute to stabilizing growth factors and thereby influencing growth factor dependent signaling [48]. Hence, it is possible that when Hapln1a protein levels are reduced by hapln1a knockdown, the disruption of Hapln1a-ECM affects either the stabilization of Sema3d or affects the...
presentation of Sema3d to its receptors. Therefore we wished to evaluate Sema3d protein levels. Sema3d protein levels were monitored following Sema3d knockdown to determine if the antibody detected zebrafish Sema3d. Because reduced Sema3d protein levels were observed, this demonstrates that the antibody detects Sema3d (although it does not rule out the possibility that the antibody may also detect related zebrafish proteins) (Fig 8). To determine whether Sema3d stability depends on Hapln1a, we examined the Sema3d protein levels following hapln1a knockdown and compared to control MM treated fins by immuno-staining analysis. We

![Immuno-staining images of Sema3d and Hapln1a knockdown fins.](image-url)

**Fig 8. Morpholino mediated knockdown of hapln1a results in reduced Sema3d protein levels.** (A) Longitudinal section of fin rays treated with control morpholino and sema3d targeting morpholino (Sema3d KD). Immuno-staining for Sema3d (red) and counterstained for nuclei with TOPRO (blue). Compared to the control MM treated fins, Sema3d MO treated fins show reduced staining for Sema3d. (B) Longitudinal section of fin rays treated with hapln1a control morpholino (Hapln1a MM) and hapln1a targeting morpholino (Hapln1a MO). Immuno-staining for Sema3d (blue) and counterstained for nuclei with Propidium Iodide (PI, red). Compared to the control MM treated fins, Hapln1a MO treated fins show reduced staining for Sema3d. Arrow identifies the basal layer of epithelium; arrow head identifies the bone; m, mesenchyme; e, epithelium. Scale bar represents 20 μm.

doi:10.1371/journal.pone.0148202.g008
observed that Sema3d protein is reduced following *hapln1a* knockdown (Fig 8), suggesting that Hapln1a is required for the stability of Sema3d.

To distinguish the possibility of whether Hapln1a is required for stabilization, or required for both stabilization and presentation of Sema3d to its receptors, we asked if Sema3d overexpression could rescue *hapln1a* knockdown phenotypes. We utilized the transgenic line, Tg (*hsp70:sema3d-gfp*) that overexpresses Sema3d-GFP under the control of a heat shock promoter [49]. If Hapln1a is required primarily for the presentation of Sema3d to its receptors, then *hapln1a* knockdown phenotypes will likely not be rescued by overexpression of Sema3d. On the other hand, if Hapln1a dependent ECM is required mainly for Sema3d stabilization, then we expect that *hapln1a* knockdown phenotypes would be rescued by Sema3d overexpression. We first confirmed overexpression of *sema3d-gfp* mRNA and Sema3d-GFP protein in regenerating fins by whole mount ISH for *sema3d* and immuno-staining for Sema3d and GFP on heat shocked versus non-heat shocked Tg(*hsp70:sema3d-gfp*) fins. We observed that both *sema3d* mRNA and Sema3d-GFP protein expression was increased in heat shocked fins compared with fins not treated with heat shock (S2 Fig).

The rescue experiment involved 3 different groups of animals (Fig 9A). Group 1 animals (experimental group) were positive for the transgene. This group was treated for *hapln1a* knockdown and Sema3d-GFP was induced 4 hours after knockdown by a 1 hour heat shock at 37°C. Group 2 animals (control 1) were also positive for transgene. This group was also treated for *hapln1a* knockdown, but did not receive the heat shock (and therefore do not overexpress Sema3d). Group 3 animals (control 2) were transgene negative siblings. This group was treated for *hapln1a* knockdown and received 1 hour of heat shock at 37°C. At 24 hours post *hapln1a* knockdown, all animals were screened for GFP expression. Only Group 1 animals showed positive GFP expression in the fin, since they all carried the transgene and were heat shocked for the overexpression of Sema3d (Fig 8B). Only GFP positive fish were selected from Group 1 for further analysis. All three groups were treated for knockdown at 3 dpa. Four hours post knockdown, Groups 1 and 3 were treated for a 1 hour heat shock at 37°C. Fins were harvested from all groups at 4 dpe and regenerate and segment length analyses were completed using the percent similarity method. Therefore, within each group, the injected side was compared with the respective un-injected side and the percent similarity was determined. We found that Group 1 had a high percent similarity (i.e., close to 100%) between the injected vs un-injected side showing that *hapln1a* knockdown phenotypes were fully rescued by overexpression of Sema3d (Fig 9C). We showed that rescue depended on both the heat shock to induce Sema3d overexpression and on the presence of the transgene since neither animals from Group 2 nor animals from Group 3 exhibited rescue of skeletal phenotypes. Thus, for both control groups regenerate length was reduced by ~20% and for segment length, Group 2 showed ~23% reduction and Group 3 showed ~20% reduction (Fig 9C). Therefore, Sema3d overexpression rescues *hapln1a* knockdown phenotypes. These experiments highlight the importance of the Hapln1a-ECM for stabilization of Sema3d, and for Sema3d-based signal transduction.

**Discussion**

Previously we have shown that *hapln1a* knockdown results in significant reduction in HA levels during fin regeneration [16]. Here we tested whether HA is sufficient to cause the observed Hapln1a dependent phenotypes of reduced regenerate length, reduced cell proliferation, and reduced segment length. We found that in addition to HA, Acan also contributes to these phenotypes. Further, we demonstrate the dependence of Sema3d on the presence of the Hapln1a-ECM. We show that Hapln1a and Sema3d genetically interact and we find that *sema3d* overexpression can rescue *hapln1a* knockdown phenotypes. Based on this current study we propose a
new model for the Cx43 pathway (Fig 10). Even though transcriptionally hapln1a and sema3d have been shown to be independent of each other [16], the current study demonstrates that the Hapln1a-ECM plays a role in stabilizing Sema3d protein. Therefore, it is likely that Sema3d signal transduction requires the Hapln1a-ECM. Our findings indicate that the Sema3d and Hapln1a gene products work together to mediate Cx43 phenotypes during growth and patterning in the regenerating fin.

Independent studies in different model systems provide evidence that components of the Hapln1a-ECM (link protein/Hapln, HA, and Acan) are interdependent. HA plays a central role in organizing the ECM where it serves as a central backbone binding to the PGs Acan via

![Diagram](image)

Fig 9. Sema3d overexpression rescues hapln1a knockdown phenotypes. (A) Experimental design. Three groups of fish were analysed: G1, G2 and G3. Fins from all three groups were amputated at 50% level. On 3 dpa all animals (G1, G2 and G3) were treated for hapln1a knockdown. Four hours post knockdown, G1 and G3 animals were heat shocked for 1hr at 37°C. G2 animals were not treated for heat shock. The following day, 1 dpe, G1 animals were selected for GFP positive fins. For GFP detection, immuno-staining (IS) and in situ hybridization experiments (ISH), fins were harvested 1 dpe and for regenerate and segment length analyses fins were harvested at 4 dpe. (B) Heat shock induces Sema3d-GFP (green) expression in Tg(hsp70:sema3d-gfp). GFP is not detected in the absence of heat shock. The white line indicates the amputation plane. Scale bar represents 50 μm. (C) Following hapln1a knockdown the experimental group (G1) that is positive for both transgene and heat shock alone shows rescue for the phenotypes (i.e., shows high percent similarity compared to the un-injected side) whereas the control groups either negative for heat shock (G2) or negative for transgene (G3) fail to show rescue (i.e., shows reduced percent similarity compared to the un-injected side). Students t-test was performed (p<0.05) to determine significance, and the error bars indicate standard error of mean.

doi:10.1371/journal.pone.0148202.g009
the Hapln1 link protein. Moreover, in addition to reduced HA, Has2 deficiency also resulted in reduced Acan content [50]. Conditional knockout of HAPLN1 (also called CRTL1) in the mouse reveals that the function of link protein is indispensable for stabilizing the HA-PG aggregate [24]. Acan deficient mice and chicks are characterized by dwarf limbs, craniofacial abnormalities, and perinatal lethality [51–53]. We have shown that loss of Hapln1a protein is associated with reduced cell proliferation, reduced bony segment length, reduced HA levels [16], and reduced Acan levels (this study). Therefore, the presence of Hapln1a in zebrafish is similarly required for the integrity and composition of the Hapln1a-ECM. Moreover, we demonstrate a requirement for the Hapln1a-ECM during growth and patterning during of the regenerating fin. Future studies will reveal how particular components of the Hapln1a-ECM contribute to specific cellular functions.

HA is ubiquitously present throughout the body and in all the bones and cartilages of the skeleton. HA fulfills various structural and metabolic functions (Reviewed in [54,55]). For example, HA modulates cell adhesion, cell migration, morphogenesis, tumorigenesis, cell survival, apoptosis, and inflammation. However, these biological effects differ depending on HA size. Endogenous high molecular weight HA has been shown to be anti-inflammatory and antiangiogenic [57], while low molecular weight HA can induce angiogenesis and tumor progression [58]. The three HA synthases Has1, Has2 and Has3 reside on the plasma membrane and extrude HA directly into the extracellular space [59–63]. All three are integral membrane proteins with six putative transmembrane domains [63], and all contain conserved amino acid residues that have both GlcNAc and GlcA transferase activity [64]. The Has enzymes each synthesize HA of similar composition but may differ in the rate of synthesis, chain length and the ease with which HA is released from the cell surface [41], although it is not clear how differences in these activities influence the function of HA. However, of the three enzymes, HAS2 is uniquely essential for life and neither Has1 nor Has3 compensate for the loss of HAS2 [42].

Conditional HAS2 gene knockout in mice causes shortened axial and appendicular skeleton and defects in patterning of digits [50,65], and strikingly, overexpression of Has2 in the developing chick limb bud mesoderm also leads to shortened bones with abnormal morphology and
positioning [56]. Together, these findings suggest a critical need for regulating HA metabolism and highlight the significance of HA produced by Has2. Our findings that has2 knockdown, but not has1 knockdown, influences regenerate length and segment length, also suggests that HA synthesized by Has2 could be functionally distinct, consistent with these prior studies.

During fin regeneration, reduced HA exhibited a stronger effect on cell proliferation than on patterning. Previously, we found that HA is expressed in a conserved pattern during fin regeneration [37]. From 3–5 dpa, HA is strongly upregulated in the distal stump and proximal blastema (i.e., just above and below the amputation plane) compared to the distal end of the regenerate, where it may maintain the proliferative state of the mesenchymal cells by limiting contact inhibition between cells that leads to tissue differentiation [66–70]. Indeed, lower levels of HA resulting from reduced Hapln1a [16], reduced has1, or reduced has2, all reduce the level of cell proliferation.

The ECM is no longer considered to be an inert substance surrounding cells, but rather, can contribute to the regulation of multiple signaling pathways [71]. For example, the ECM can serve as a reservoir for secreted growth factors. Local release of ECM-bound growth factors could influence temporal regulation of signal transduction pathways, and/or the half-life of secreted growth factors. Moreover, the concentration of signaling molecules at a particular location may contribute to the establishment of morphogen gradients that play critical roles during patterning and developmental processes [72,73]. Our findings reveal that the Hapln1a-ECM is crucial for the stability of the secreted growth factor, Sema3d. Further work is required to understand how Sema3d interacts with components of the Hapln1a-ECM, and how these interactions may regulate Sema3d-receptor based signal transduction.

**Supporting Information**

**S1 Fig.** Knockdown of *hapln1a* at 0.5mM concentration and *sema3d* at 0.25mM concentration did not produce significant effects on Cx43 dependent phenotypes. Bar graphs show the effect of *hapln1a* MO at 1mM, 0.75mM and 0.5mM concentrations and *sema3d* MO at 1mM, 0.75mM, 0.5mM and 0.25mM concentrations. Compared to 1mM MM control treated fins, *hapln1a* knockdown at 0.5mM and *sema3d* knockdown at 0.25mM concentration did not have a significant effect on regenerate and segment length. (TIF)

**S2 Fig.** Heat shock induces upregulation of *sema3d* mRNA and Sema3d protein in Tg (*hsp70:sema3d-gfp*). (A) Whole mount in situ hybridization shows increased expression of *sema3d* mRNA in heat shock treated fins compared to the untreated fins. Black line indicates amputation plane. (B) Immuno-staining analysis of longitudinal fin sections reveal increased expression of GFP (green) and Sema3d (Red) in heat shock treated fins compared to the untreated fins. DAPI (blue) is used as the counter stain and stains the nuclei. Arrows indicate basal layer of epithelium and arrow head marks the bone. e, epidermis; m, mesenchyme. Scale bar represents 100μm in both panels. (TIF)

**Acknowledgments**

The authors thank Mary Halloran for providing the Tg(*hsp70:sema3d-gfp*) transgenic line. The authors also thank Rebecca Bowman for maintenance and care of the fish colony, as well as Rajeswari Banerji, Francisca Oniyuke and Harneel Riar for technical assistance, and members of the Iovine lab for critical discussions regarding this manuscript. This research was funded by the NSF (IOS 1145582).
Author Contributions
Conceived and designed the experiments: JG MKI. Performed the experiments: JG KMT. Analyzed the data: JG KMT MKI. Contributed reagents/materials/analysis tools: MKI. Wrote the paper: JG KMT MKI.

References
1. Paznekas WA, Boyadjiev SA, Shapiro RE, Daniels O, Wollnik B, Keegan CE, et al. (2003) Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. Am J Hum Genet 72: 408–418. PMID:12457340
2. Flenniken AM, Osborne LR, Anderson N, Ciliberti N, Fleming C, Gittens JE, et al. (2005) A Gja1 missense mutation in a mouse model of oculodentodigital dysplasia. Development 132: 4375–4386. PMID:16155213
3. Iovine MK, Higgins EP, Hindes A, Coblitz B, Johnson SL (2005) Mutations in connexin43 (GJA1) perturb bone growth in zebrafish fins. Dev Biol 278: 208–219. PMID:15649473
4. Wei CJ, Xu X, Lo CW (2004) Connexins and cell signaling in development and disease. Annu Rev Cell Dev Biol 20: 811–838. PMID:15473861
5. Batra N, Kar R, Jiang JX (2012) Gap junctions and hemichannels in signal transmission, function and development of bone. Biochim Biophys Acta 1818: 1909–1918. doi: 10.1016/j.bbamem.2011.09.018 PMID:21963408
6. Goodenough DA, Goliger JA, Paul DL (1996) Connexins, connexons, and intercellular communication. Annu Rev Biochem 65: 475–502. PMID:8611187
7. Sohl G, Willecke K (2004) Gap junctions and the connexin protein family. Cardiovasc Res 62: 228–232. PMID:15094343
8. Jones SJ, Gray C, Sakamaki H, Arora M, Boyde A, Gourdie R, et al. (1993) The incidence and size of gap junctions between the bone cells in rat calvaria. Anat Embryol (Berl) 187: 343–352.
9. Lecanda F, Warlow PM, Sheikhh S, Furlan F, Steinberg TH, Civitelli R (2000) Connexin43 deficiency causes delayed ossification, craniofacial abnormalities, and osteoblast dysfunction. J Cell Biol 151: 931–944. PMID:11076975
10. McGonnell IM, Green CR, Tickle C, Becker DL (2001) Connexin43 gap junction protein plays an essential role in morphogenesis of the embryonic chick face. Dev Dyn 222: 420–438. PMID:11747077
11. Law LY, Lin JS, Becker DL, Green CR (2002) Knockdown of connexin43-mediated regulation of the zone of polarizing activity in the developing chick limb leads to digit truncation. Dev Growth Differ 44: 537–547. PMID:12492512
12. Hoptak-Solga AD, Klein KA, DeRosa AM, White TW, Iovine MK (2007) Zebrafish short fin mutations in connexin43 lead to aberrant gap junctional intercellular communication. FEBS Lett 581: 3297–3302. PMID:17599838
13. Hoptak-Solga AD, Nielsen S, Jain I, Thummel R, Hyde DR, Iovine MK (2008) Connexin43 (GJA1) is required in the population of dividing cells during fin regeneration. Dev Biol 317: 541–548. PMID:18406403
14. Sims K Jr., Eble DM, Iovine MK (2009) Connexin43 regulates joint location in zebrafish fins. Dev Biol 327: 410–418. doi: 10.1016/j.ydbio.2008.12.027 PMID:19150347
15. Ton QV, Iovine M (2012) Semaphorin 3D mediates Cx43-dependent phenotypes during fin regeneration. Dev Biol 366: 195–203. doi: 10.1016/j.ydbio.2012.03.020 PMID:22524598
16. Govindan J, Iovine MK (2014) Hapln1a is required for connexin43-dependent growth and patterning in the regenerating fin skeleton. PLoS One 9: e88574. doi: 10.1371/journal.pone.0088574 PMID:24533114
17. Perin JP, Bonnet F, Thureau C, Jolles P (1987) Link protein interactions with hyaluronate and proteoglycans. Characterization of two distinct domains in bovine cartilage link proteins. J Biol Chem 262: 13269–13272. PMID:3654411
18. Neame PJ, Barry FP (1993) The link proteins. Experientia 49: 393–402. PMID:8500595
19. Melching LL, Roughley PJ (1985) The role of link protein in mediating the interaction between hyaluronic acid and newly secreted proteoglycan subunits from adult human articular cartilage. J Biol Chem 260: 16279–16285. PMID:4066710
20. Hardingham TE, Muir H (1972) The specific interaction of hyaluronic acid with cartilage proteoglycans. Biochim Biophys Acta 279: 401–405. PMID:4263642
21. Hardingham TE (1979) The role of link-protein in the structure of cartilage proteoglycan aggregates. Biochim J 177: 237–247. PMID:34389
22. Rodriguez E, Roland SK, Plaas A, Roughley PJ (2006) The glycosaminoglycan attachment regions of human aggrecan. J Biol Chem 281: 18444–18450. PMID:16679517

23. Sztrolovics R, White RJ, Roughley PJ, Mort JS (2002) The mechanism of aggrecan release from cartilage differs with tissue origin and the agent used to stimulate catabolism. Biochem J 362: 465–472. PMID:11853556

24. Watanabe H, Yamada Y (1999) Mice lacking link protein develop dwarfism and craniofacial abnormalities. Nat Genet 21: 225–229. PMID:9988279

25. Ghatak S, Misra S, Toole BP (2005) Hyaluronan constitutively regulates ErbB2 phosphorylation and signaling complex formation in carcinoma cells. J Biol Chem 280: 8875–8883. PMID:15632176

26. Jiang D, Liang J, Noble PW (2007) Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 23: 435–461. PMID:17506690

27. Misra S, Toole BP, Ghatak S (2006) Hyaluronan constitutively regulates activation of multiple receptor tyrosine kinases in epithelial and carcinoma cells. J Biol Chem 281: 34936–34941. PMID:16959784

28. Mercer SE, Odelberg SJ, Simon HG (2013) A dynamic spatiotemporal extracellular matrix facilitates epicardial-mediated vertebrate heart regeneration. Dev Biol 382: 457–469. doi:10.1016/j.ydbio.2013.08.002 PMID:23939298

29. Kim SH, Turnbull J, Guimond S (2011) Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. J Endocrinol 209: 139–151. doi:10.1530/JOE-10-0377 PMID:21307119

30. Hynes RO (2014) Stretching the boundaries of extracellular matrix research. Nat Rev Mol Cell Biol 15: 761–763. PMID:23574355

31. Hynes RO (2014) Stretching the boundaries of extracellular matrix research. Nat Rev Mol Cell Biol 15: 761–763. PMID:23574355

32. Bhadra J and Iovine MK (2015) Hsp47 mediates Cx43-dependent skeletal growth and patterning in the regenerating fin. Mech Dev 138: 364–374. doi:10.1016/j.mod.2015.06.004 PMID:26103547

33. Thummel R, Bai S, Sarras MP Jr., Song P, McDermott J, Brewer J, et al. (2006) Inhibition of zebrafish fin regeneration using in vivo electroporation of morpholinos against fgfr1 and msxb. Dev Dyn 235: 336–346. PMID:16273523

34. Iovine MK, Johnson SL (2000) Genetic analysis of isometric growth control mechanisms in the zebrafish caudal Fin. Genetics 155: 1321–1329. PMID:10880491

35. Du SJ, Frenkel V, Kindschi G, Zohar Y (2001) Visualizing normal and defective bone development in zebrafish embryos using the fluorescent chromophore calcein. Dev Biol 238: 239–246. PMID:11784007

36. Munch J, Gonzalez-Rajal A, de la Pompa JL Notch regulates blastema proliferation and prevents differentiation during adult zebrafish fin regeneration. Development 140: 1402–1411. doi:10.1242/dev.08734 PMID:23344707

37. Govindan J, Iovine MK (2015) Dynamic remodeling of the extra cellular matrix during zebrafish fin regeneration. Gene Expr Patterns.

38. Ghokale JRPaB, A. L (2001) The Biochemistry of Bone. Osteoporosis. San Diego: Academic Press. pp. 82.

39. Nechiporuk A, Keating MT (2002) A proliferation gradient between proximal and msxb-expressing distal blastema directs zebrafish fin regeneration. Development 129: 2607–2617. PMID:12015289

40. Ton GV, Iovine MK (2013) Identification of an evx1-dependent joint-formation pathway during FIN regeneration. PLoS One 8: e81240. doi:10.1371/journal.pone.0081240 PMID:24278401

41. Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, Imagawa M, et al. (1999) Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. J Biol Chem 274: 25085–25092. PMID:10455188

42. Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A Jr., et al. (2000) Disruption of hyaluronan synthase-2 abolges normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. J Clin Invest 106: 349–360. PMID:10930438

43. Torronen K, Nikunen K, Karna R, Tammi M, Tammi R, Rilla K (2014) Tissue distribution and subcellular localization of hyaluronan synthase isoenzymes. Histochem Cell Biol 141: 17–31. doi:10.1007/s00418-013-1143-4 PMID:24057227

44. Sallbach J, Rachner TD, Rauner M, Hempel U, Anderegg U, Franz S, et al. (2012) Regenerative potential of glycosaminoglycans for skin and bone. J Mol Med (Berl) 90: 625–635.

45. Toole BP (2009) Hyaluronan-CD44 Interactions in Cancer: Paradoxes and Possibilities. Clin Cancer Res 15: 7462–7468. PMID:20008845
46. Toole BP, Gross J (1971) The extracellular matrix of the regenerating newt limb: synthesis and removal of hyaluronate prior to differentiation. Dev Biol 25: 57–77. PMID: 5557969
47. De Cat B, David G (2001) Developmental roles of the glypicans. Semin Cell Dev Biol 12: 117–125. PMID: 11292377
48. Ruoslahti E, Yamaguchi Y (1991) Proteoglycans as modulators of growth factor activities. Cell 64: 867–869. PMID: 2001586
49. Liu Y, Berndt J, Su F, Tawarayama H, Shoji W, Kuwada JY, et al. (2004) Semaphorin3D guides retinal axons along the dorsoventral axis of the tectum. J Neurosci 24: 310–318. PMID: 14724229
50. Matsumoto K, Li Y, Jakuba C, Sugiyama Y, Sayo T, Okuno M, et al. (2009) Conditional inactivation of Has2 reveals a crucial role for hyaluronan in skeletal growth, patterning, chondrocyte maturation and joint formation in the developing limb. Development 136: 2825–2835. doi: 10.1242/dev.038505 PMID: 19633173
51. Li H, Schwartz NB, Vertel BM (1993) cDNA cloning of chick cartilage chondroitin sulfate (aggrecan) core protein and identification of a stop codon in the aggrecan gene associated with the chondrodystrophy, nanomelia. J Biol Chem 268: 23504–23511. PMID: 8226878
52. Wai AW, Ng LJ, Watanabe H, Yamada Y, Tam PP, Cheah KS (1998) Disrupted expression of matrix genes in the growth plate of the mouse cartilage matrix deficiency (cmd) mutant. Dev Genet 22: 349–358. PMID: 9664867
53. Watanabe H, Kimata K, Line S, Strong D, Gao LY, Kozak CA, et al. (1994) Mouse cartilage matrix deficiency (cmd) caused by a 7 bp deletion in the aggrecan gene. Nat Genet 7: 154–157. PMID: 7920633
54. Bastow ER, Byers S, Golub SB, Clarkin CE, Pitsillides AA, Fosang AJ (2008) Hyaluronan synthesis and degradation in cartilage and bone. Cell Mol Life Sci 65: 395–413. PMID: 17965830
55. Roughley PJ, Alm P (2013) The role of HA and Has2 in the development and function of the skeleton. Extracellular matrix in development: Springer-Verlag Berlin Heidelberg, pp. 219–247.
56. Li XY, Guo X, Wang LX, Geng D, Kang LL, Wang S, et al. (2007) [Serum hyaluronic acid, tumor necrosis factor-alpha, vascular endothelial growth factor, NO, and Se levels in adult patients with Kashin-Beck disease]. Nan Fang Yi Ke Da Xue Xue Bao 27: 941–944. PMID: 17668321
57. Jiang D, Liang J, Noble PW (2011) Hyaluronan as an immune regulator in human diseases. Physiol Rev 91: 221–264. doi: 10.1152/physrev.00052.2009 PMID: 21248167
58. Turley EA, Noble PW, Bourguignon LY (2002) Signaling properties of hyaluronan receptors. J Biol Chem 277: 4589–4592. PMID: 11717317
59. Weigel PH, Hascall VC, Tammi M (1997) Hyaluronan syntheses. J Biol Chem 272: 13997–14000. PMID: 9206724
60. Shyjan AM, Heldin P, Butcher EC, Yoshino T, Briskin MJ (1996) Functional cloning of the cDNA for a human hyaluronan synthase. J Biol Chem 271: 23395–23399. PMID: 8798544
61. Watanabe K, Yamaguchi Y (1996) Molecular identification of a putative human hyaluronan synthase. J Biol Chem 271: 22945–22948. PMID: 8798477
62. Spicer AP, Seldin MF, Olsen AS, Brown N, Wells DE, Doggett NA, et al. (1997) Chromosomal localization of the human and mouse hyaluronan synthase genes. Genomics 41: 493–497. PMID: 9169154
63. Bakkers J, Kramer C, Pothof J, Quaedvlieg NE, Spaink HP, Hammerschmidt M (2004) Has2 is required upstream of Rac1 to govern dorsal migration of lateral cells during zebrafish gastrulation. Development 131: 525–537. PMID: 14729574
64. Yoshida M, Itano N, Yamada Y, Kimata K (2000) In vitro synthesis of hyaluronan by a single protein derived from mouse HAS1 gene and characterization of amino acid residues essential for the activity. J Biol Chem 275: 497–506. PMID: 10617644
65. Moffatt P, Lee ER, St-Jacques B, Matsumoto K, Yamaguchi Y, Roughley PJ (2011) Hyaluronan production by means of Has2 gene expression in chondrocytes is essential for long bone development. Dev Dyn 240: 404–412. doi: 10.1002/dvdy.22529 PMID: 21246657
66. Thorogood PV, Hinchliffe JR (1975) An analysis of the condensation process during chondrogenesis in the embryonic chick hind limb. J Embryol Exp Morphol 33: 581–606. PMID: 1176861
67. Kosher RA, Savage MP, Walker KH (1981) A gradation of hyaluronate accumulation along the proximo-distal axis of the embryonic chick limb bud. J Embryol Exp Morphol 63: 85–98. PMID: 7310297
68. Singley CT, Solursh M (1981) The spatial distribution of hyaluronic acid and mesenchymal condensations in the embryonic chick wing. Dev Biol 84: 102–120. PMID: 7250489
69. Contreras EG, Gaete M, Sanchez N, Carrasco H, Larrain J (2009) Early requirement of Hyaluronan for tail regeneration in Xenopus tadpoles. Development 136: 2987–2996. doi: 10.1242/dev.039501 PMID: 19666825
70. Calve S, Odelberg SJ, Simon HG (2010) A transitional extracellular matrix instructs cell behavior during muscle regeneration. Dev Biol 344: 259–271. doi: 10.1016/j.ydbio.2010.05.007 PMID: 20478295

71. Casini P, Nardi I, Ori M. Hyaluronan is required for cranial neural crest cells migration and craniofacial development. Dev Dyn 241: 294–302. doi: 10.1002/dvdy.23715 PMID: 22184056

72. Kreuger J, Perez L, Giraldez AJ, Cohen SM (2004) Opposing activities of Dally-like glypican at high and low levels of Wingless morphogen activity. Dev Cell 7: 503–512. PMID: 15469839

73. Kirkpatrick CA, Dimitroff BD, Rawson JM, Selleck SB (2004) Spatial regulation of Wingless morphogen distribution and signaling by Dally-like protein. Dev Cell 7: 513–523. PMID: 15469840

74. Ton QV, Kathryn Iovine M (2012) Semaphorin3d mediates Cx43-dependent phenotypes during fin regeneration. Dev Biol 366: 195–203. doi: 10.1016/j.ydbio.2012.03.020 PMID: 22842588