Heparan sulfate proteoglycan expression in the regenerating zebrafish fin

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Numbers: SPP 2084 Bone / KN 1102/2-1, Transregio 67 / 387653785; European Social Fund, Grant/Award Number: LIP2017 / 100316833; H2020 European Research Council, Grant/Award Number: ERC Advanced Grant / Zf-BrainReg; Technische Universität Dresden, Grant/Award Number: Graduiertenakademie / Abschluss-Stipendium

Abstract

Background: Heparan sulfate proteoglycan (HSPG) expression is found in many animal tissues and regulates growth factor signaling such as of Fibroblast growth factors (Fgf), Wingless/Int (Wnt) and Hedgehog (HH). Glypicans, which are GPI (glycosylphosphatidylinositol)-anchored proteins, and transmembrane-anchored syndecans represent two major HSPG protein families whose involvement in development and disease has been demonstrated. Their participation in regenerative processes both of the central nervous system and of regenerating limbs is well documented. However, whether HSPG are expressed in regenerating zebrafish fins, is currently unknown.

Results: Here, we carried out a systematic screen of glypican and syndecan mRNA expression in regenerating zebrafish fins during the outgrowth phase. We find that 8 of the 10 zebrafish glypicans and the three known zebrafish syndecans show specific expression at 3 days post amputation. Expression is found in different domains of the regenerate, including the distal and lateral basal layers of the wound epidermis, the distal most blastema and more proximal blastema regions.

Conclusions: HSPG expression is prevalent in regenerating zebrafish fins. Further research is needed to delineate the function of glypican and syndecan action during zebrafish fin regeneration.

KEYWORDS
extracellular matrix protein, glypican, growth factor, syndecan

1 INTRODUCTION

Heparan sulfate proteoglycan (HSPG) are a class of ubiquitous proteins found on the cell surface and in the ECM of all vertebrate and invertebrate species. They comprise the GPI-anchored glypicans and the transmembrane domain-anchored syndecans, which can be released into the extracellular space by cleavage through a variety of enzymes, and freely diffusible proteins (perlecan, agrin, collagen XVIII). In mammals, 6 glypicans and 4 syndecans have been identified, while 10 glypicans and 3 syndecans are known in zebrafish. HSPG constitute proteoglycans which are complexes consisting of a core protein to which glycosaminoglycan (GAG) side chains are attached. The dominant GAG in HSPG is heparan sulfate (HS), an unbranched polysaccharide consisting of N-acetylgalactosamine and glucuronic acid disaccharide building blocks. A subset of HS sugar residues is
modified in several ways which leads to a great variety of HS chains, associated with different properties and ECM functions. Some organs, tissues, and cell populations produce unique HS compositions, which may differ during embryonic development. Moreover, core protein expression varies depending on the tissue, illustrating the diversity of HSPG.

HSPG are important regulators of extracellular growth factor distribution, especially in the context of morphogen gradient formation. A great number of studies in Drosophila melanogaster have demonstrated their involvement in this process. More recent studies on vertebrate morphogen gradient formation, for example in the mouse epiphyseal growth plate, have added to this concept. HSPG facilitate the attachment of ligands to their specific receptor by acting as co-factors. The highly sulfated HS chains on HSPG bind to a number of growth factors, thereby promoting close contact of the ligands with their respective binding partners. In contrast, they act as ligand scavengers preventing receptor activation after overexpression in the extracellular matrix (ECM). A local change in ligand abundance is induced after modulation of HSPG core protein components or inappropriate HS chain modification. Moreover, cleavage of HSPG from the membrane can cause signaling at more distant sites. Studies from the last two decades revealed that HSPG modulate the activity of members of the Wnt, HH, Fgf, and Bone morphogenic protein (Bmp) signaling pathways during development.

There is growing evidence that HSPG are involved in tissue regeneration. HSPG have the aforementioned ability to interact with a broad array of ligands such as ECM-glycoproteins, growth factors, morphogens, and cytokines that are important regulators of regeneration. For example, Syndecan 1 and Syndecan 4 were shown to be involved in re-epithelialization of the epidermis in mice after damage. Accordingly, the re-epithelialization process is delayed in Syndecan 1-deficient mice. Another example indicates the human ortholog GPC3 to be a negative regulator of liver regeneration and hepatocyte proliferation. Importantly, interference with HS levels through mutation of HS-synthesizing enzymes abolishes pectoral fin formation in zebrafish. Furthermore, differential HS sulfation levels in the axolotl limb are crucial for anterior–posterior patterning during regeneration.

Due to their remarkable ability to regenerate completely and rapidly after amputation, the zebrafish caudal fin is an ideal model to study vertebrate appendage regeneration. Various signaling pathways are involved in the regeneration process, such as Bmp, Insulin-like growth factor (Igf), Wnt/ß-Catenin, Fgf, Notch, and retinoic acid (RA) signaling. While some of the respective pathway components localize to the wound epidermis, which forms shortly after amputation, others are found in the blastema, a mass of proliferative, mostly lineage-restricted cells derived from various stump cell populations. Although ligand, receptor and target gene expression of various signaling pathways have been demonstrated extensively, it is currently unclear whether HSPG contribute to their distribution in the fin regenerate. Here, we investigated the expression pattern of glypicans and syndecans in the 3 days post amputation regenerating fin. We find that most of the glypicans and all syndecans are expressed in partly overlapping regions of the fin regenerate, strongly suggesting that they do contribute to successful appendage regeneration.

## RESULTS

### 2.1 Detection of HSPG via reverse transcription polymerase chain reaction

First, we examined which of the known glypicans and syndecans are expressed in uninjured fin tissue and during zebrafish fin regeneration by using reverse transcription polymerase chain reaction (RT-PCR). We decided to perform HSPG detection during the early outgrowth phase, at a time, at which the blastema has subdivided into a non-proliferative distal and a highly proliferative proximal domain. Both domains are maintained at the growing tip of the fin regenerate until regeneration is complete. As all glypicans and syndecans are expressed in embryos at the Prim-5 stage (24 hpf), we chose this time point as a reference control. We detected marked expression of syndecan 2–4 (sdc2/sdc3/sdc4) and all glypicans in the 3 days post amputation (3 dpa) fin regenerate, except for glypican 3 (gpc3) and glypican 5b (gpc5b), whose expression was very weak (Figure 1A). Expression in uninjured fins was similar but weaker (Figure 1A). This indicated a prevalence of HSPG expression in zebrafish fin tissue and a potential increase in expression during fin regeneration.

### 2.2 Definition of expression domains in the fin regenerate

Different domains can be detected in the 3 dpa fin regenerate; however, the corresponding nomenclature slightly varies according to literature. We therefore decided to provide a definition of the respective domains in this work (Figure 1B). The 3 dpa fin regenerate is covered by a multilayered wound epidermis (WE) which contains a basal layer (BWE) composed of cuboidal cells. The distal...
tip of the outer WE and the BWE are referred to as distal WE and distal BWE, respectively. The WE domains adjoining the distal BWE, which have been reported to express shh and left1,41-44 are termed lateral BWE. For the purpose of this work, the blastema subdomains are denominated as follows: The distalmost blastema (DMB) expressing ald1a2 is found beneath the distal BWE and comprises several cell layers, in agreement to previous reports.28,30,40,45 The proximal blastema cell mass is located below the DMB and the lateral BWE and is subdivided into two regions, the proximal blastema 1 (next to the DMB) and the proximal blastema 2. The lateral domains of the proximal blastema are referred to as the lateral blastema and describe the runx2b + domain in the regenerate.46,47 The lateral blastema is part of proximal blastema 1 and 2. The cells closer to the amputation plane (amp plane) are termed mesenchyme here.

2.3 | Whole mount RNA in situ expression analysis in 3 dpa fin regenerates

We set out to visualize mRNA expression of glypicans and syndecans in 3 dpa zebrafish fin regenerates. Glypican expression results are grouped according to the respective subfamilies, agreeing with our previous phylogenetic analysis.3,48,49 The results for subfamily 1/2/4/6 members gpc1a, gpc1b, gpc2, gpc6a, and gpc6b are shown in Figure 2 and Figure 3, respectively. Expression of the subfamily 3/5 members gpc3, gpc5a, gpc5b, and gpc5c is shown in Figure 4, and syndecan (sdc2, sdc3, sdc4) expression is shown in Figure 5. In situ probe testing was performed on Prim-5 stage or slightly younger embryos (Figure 6) and confirmed previously reported expression patterns.3,36-38

Being exclusively expressed in the lateral BWE, gpc1a has the most confined expression of all investigated HSPG at the examined state (Figure 2A). The second ortholog to the human GPC1, gpc1b, is expressed in the DMB and the adjacent proximal blastema 1 (Figure 2B). gpc2 mRNA is found in the same regions but additionally shows expression in the distal BWE and distal WE (Figure 2C).

gpc4, also known as knypek, was weak but detectable in proximal blastema regions 1 and 2 (Figure 3A). gpc6a expression was mainly localized to proximal blastema 2 but also occurred in proximal blastema 1 (Figure 3B). gpc6b, the second ortholog of GPC6, was detected in both proximal blastema regions as well (Figure 3C).

mRNA of the zebrafish ortholog of human GPC3 (gpc3) was not detected in 3 dpa fin regenerates by whole mount RNA in situ (WMISH) (Figure 4A). In contrast, gpc5a produced a specific staining which was confined to the distal and lateral BWE, the DMB and proximal
blastema 1 (Figure 4B). In accordance with RT-PCR, gpc5b transcripts were not detected by WMISH (Figure 4C). The third ortholog of GPC5, gpc5c, was specifically expressed in proximal blastema 1 and 2, in particular the lateral blastema (Figure 4D).

Syndecan expression was quite broad. In case of sdc2, it occurred in the distal WE (and potentially the BWE), the lateral BWE as well as in the lateral blastema (Figure 5A), where staining was comparatively strong. It was also detected in proximal blastema 1 and 2. sdc3 expression was weakly expressed in the DMB, proximal blastema 1 and proximal blastema 2 (Figure 5B). Faint expression of sdc3 was found in the lateral BWE, however, this might also represent non-specific background staining. The ortholog of human SDC4 (sdc4) showed a unique expression pattern and was detected in proximal blastema 1 and 2, the lateral BWE and individual cells in other parts of the WE (Figure 5C).

3 | DISCUSSION

3.1 | Abundance of HSPG in the regenerating zebrafish fin

Our previous findings emphasized the potential importance of glypicans during zebrafish embryogenesis, since all of them were found to be expressed at the latest at the Prim-5 stage. The wide abundance of HSPG expressed in 3 dpa fin regenerates points toward a potential relevance in the complex process of fin regeneration as well.
In light of the fact that various morphogen signaling pathways are active in the regenerating fin (e.g., Fgf29 and RA31), this is not surprising.

Schematic depiction of HSPG expression (Figure 1B) illustrates the domain specificity versus domain overlap of different HSPG. In each area of the regenerate, members of both glypican subfamilies (subfamily 3/5 and subfamily 1/2/4/6) as well as syndecans are present. The only exception is the outer WE, in which subfamiliy 3/5 is not represented. gpc1a is the only HSPG whose expression is confined to a single domain, i.e. the lateral BWE. All other HSPG are expressed in a minimum of two domains which are generally located adjacent to one another. As an example, gpc2 expression is found both in the distal WE and the underlying distal BWE. gpc6a and gpc6b are both expressed in proximal blastema 1 and 2. Thus, HSPG expression of any given candidate (except gpc1a) appears to be rather broad and does not occur in widely separated domains.

**FIGURE 4** Expression pattern of gpc3, gpc5a, gpc5b, and gpc5c in 3 dpa fin regenerates. Three neighboring fin rays of the same regenerate are displayed on the left (whole mount). Scale bar 100 μm. The longitudinal section view can be seen on the right. Scale bar 50 μm. A, gpc3 expression (n = 5/5 fins), B, gpc5a expression (n = 4/4 fins), C, gpc5b expression (n = 4/4 fins), D, gpc5c expression (n = 6/6 fins). The black dotted line demarcates the amputation plane

**FIGURE 5** Expression pattern of sdc2, sdc3, and sdc4 in 3 dpa fin regenerates. Two to three neighboring fin rays of the same regenerate are depicted on the left (whole mount). Scale bar 100 μm. The longitudinal section view is shown on the right. Scale bar 50 μm. A, sdc2 expression (n = 5/5 fins), B, sdc3 expression (n = 3/3 fins), C, sdc4 expression (n = 5/5 fins). The black dotted line demarcates the amputation plane
Several domains in the regenerate show seemingly redundant HSPG expression. The area with the most diverse HSPG expression is proximal blastema 1 expressing 10 HSPG (gpc1b, gpc2, gpc4, gpc6a, gpc6b, gpc5a, gpc5c, sdc2, sdc3, and sdc4). A similarly high number of seven genes (gpc4, gpc6a, gpc6b, gpc5c, sdc2, sdc3, and sdc4) are expressed in proximal blastema 2. Both domains contain proliferating cells, and have been shown to receive numerous signaling inputs from the overlying DMB and lateral BWE.\textsuperscript{28,44} Notch signaling is one of the active pathways in the proximal blastema,\textsuperscript{30} and strong mitogenic signals are produced in the overlying DMB and BWE.\textsuperscript{50,51} In order to achieve regenerate elongation and appropriate differentiation of bone forming cells in the lateral part of this region, high but not excessive levels of proliferation must be ensured.\textsuperscript{30} HSPG may control propagation of the respective mitogenic signals to do so.

The lateral BWE is yet another region which shows expression of several HSPG—gpc1a, gpc5a, sdc2, sdc3, and sdc4 expression can be detected here. This may have important implications for Fgf and Wnt signaling, which have been shown to regulate expression of genes such as \textit{lef1} and \textit{shh} in this domain.\textsuperscript{44}
The DMB, which is the signaling center organizing outgrowth of the zebrafish fin regenerate is especially interesting in terms of HSPG expression. Notably, there is a rather limited expression of HSPG genes in the DMB, namely of gpc1b, gpc2, gpc5a, and sdc3. gpc2 and gpc5a are also expressed in the overlying distal BWE while gpc1b and sdc3 are detected in the DMB and underlying proximal blastema 1. In the DMB, the four HSPG are good candidates for interaction and retention of Wnt ligands whose (expression) range may need to be limited for correct induction of downstream signaling events. The absence or scarcity of gpc3 and gpc5b in 3 dpa fin regenerates, gpc6a and gpc6b are co-expressed in the blastema. This is in contrast to a somewhat disparate expression pattern of both genes at the embryonic stage. gpc6a and gpc6b are orthologs of the human GPC6 gene. Patients carrying a GPC6 mutation suffer from thanatophoric dysplasia, a genetic condition characterized by shortened limbs, facial dysmorphism, and an overall short stature. In mice, Gpc6 has been shown to stimulate HH signaling by promoting HH’s binding to its receptor Patched1 during long bone development, and its mutation mimics the phenotype observed in patients. Similarly, intestinal elongation and proliferation in mice are dependent on appropriate Gpc6 function. We suggest that, in zebrafish, gpc6a and gpc6b are both involved in regeneration of the fin, possibly by supporting proliferation and elongation of the regenerate. Such a function may be mediated via interaction with Shh, Patched and Wnt ligands, respectively.

3.3 | Expression of GPC6 homologs

While gpc3 is scarcely (or not at all) expressed in fin regenerates, gpc6a and gpc6b are co-expressed in the blastema. This is in contrast to a somewhat disparate expression pattern of both genes at the embryonic stage. gpc6a and gpc6b are orthologs of the human GPC6 gene. Patients carrying a GPC6 mutation suffer from thanatophoric dysplasia, a genetic condition characterized by shortened limbs, facial dysmorphism, and an overall short stature. In mice, Gpc6 has been shown to stimulate HH signaling by promoting HH’s binding to its receptor Patched1 during long bone development, and its mutation mimics the phenotype observed in patients. Similarly, intestinal elongation and proliferation in mice are dependent on appropriate Gpc6 function. We suggest that, in zebrafish, gpc6a and gpc6b are both involved in regeneration of the fin, possibly by supporting proliferation and elongation of the regenerate. Such a function may be mediated via interaction with Shh, Patched and Wnt ligands, respectively.

3.4 | Expression pattern of sdc4

The sdc4 gene showed a notable expression pattern including a strong presence in the lateral blastema and lateral BWE. Furthermore, sdc4 was expressed in the outer WE and particularly in the distal WE, in which only sdc2 was (weakly) co-expressed. In mice, Sdc4 is upregulated in skin dermis after wounding. Mice lacking Sdc4 gene function show delayed wound repair due to impaired dermal fibroblast migration, which relies on Syndecan-Integrin interaction. Sdc4 (along with other syndecans) has also been demonstrated to govern tissue inflammation by regulating leukocyte (trans-) migration and cytokine binding. Broad expression of sdc4 in the regeneration blastema, lateral BWE and spotty expression in the outer WE indicate that sdc4 may have both adhesive and inflammatory functions in the respective tissue domains. In this context, sdc4 expressing cells in the outer WE might either represent epidermal or immune cells dispersed in the WE, regulating inflammation and cell rearrangements in the re-epithelialized appendage.

3.5 | HSPG function in organ regeneration and growth control

Syndecans play a crucial role during neuronal development and regeneration in various species. For example, the Caenorhabditis elegans syndecan ortholog sdn-1 is required for growth cone stabilization during axon...
regeneration. In *Drosophila melanogaster*, Sdc is involved in motor-axon guidance during skeletal muscle formation. Furthermore, correct HS synthesis is needed for appropriate axon sorting during optic tract development in zebrafish. In mice, Sdc3 and Sdc4 mutants fail to regenerate muscle because of dysfunctional satellite cells.

HSPG function is also crucial in vertebrate limb regeneration, such as in salamanders, and might mediate positional identity of cells in particular. Salamanders restore only the missing part of the limb no matter at which level the amputation was carried out. This “awareness” of cells about where they are located or need to locate within a three-dimensional system is also important during development. The biochemical features of positional identity are not fully understood yet but one way of controlling them could be via HSPG, which regulate the spatial and temporal activities of growth factor signaling. A coherence between positional information and its mediation via HS (potentially residing on HSPG) has been demonstrated for limb regeneration in axolotl (*Ambystoma mexicanum*). In line with this, HS rich cells arranged in a grid-like fashion in axolotl skin (similar to what we have observed regarding sdc4 expression) were suggested to be required for pattern formation.

The principle of positional information applies to regenerating zebrafish fins as well, which always grow back to their pre-amputation size. Although HSPG as such have not directly been associated with successful fin regeneration and patterning, such a correlation is likely. Govindan & Iovine (2015) reported the presence of the GAG hyaluronic acid and the chondroitin sulfate proteoglycans Versican and Aggrecan in regenerating fins. Hyaluronidase treatment abolished staining for GAG in the blastema. Moreover, biosynthesis of HS and other GAG is crucial for appropriate zebrafish development and in particular pectoral fin growth. Here, we have detected expression of multiple HSPG in different domains of the regenerate, with most of them in more than one domain. Together with the fact that morphogens known to interact with HSPG (Wnt, Fgf, Bmp, Shh) are involved in fin regeneration, this suggests that HSPG expression in the fin is instructive in terms of ligand distribution and signaling pathway activation.

### 4 | EXPERIMENTAL PROCEDURES

#### 4.1 Animal experiments and fish husbandry

All procedures were approved by and performed in accordance with the animal handling and research regulations of the Landesdirektion Sachsen (Permit numbers AZ DD25-5131/354/87 and DD25-5131/450/4 and amendments). Zebrafish were raised and maintained as described previously. 3 dpa regenerates were obtained from fin-clipped zebrafish. Fin clipping was carried out as previously described. Fish were allowed to regenerate in 28°C water.

#### 4.2 RT-PCR

RNA isolation from uninjured fins, 3 dpa fin regenerates and 24 hpf embryos was carried out using Trizol/Phenol-Chloroform according to the manufacturer’s instructions (Ambion). Subsequently, cDNA was transcribed from the RNA using the SuperScriptIII First-Strand Synthesis system (Invitrogen). cDNA of uninjured fins was transcribed in a separate experiment. Simultaneously, –RT (minus reverse transcriptase) controls were produced replacing the SuperScriptIII RT with water. Primers provided in Table 1 were used to amplify glypicans, syndecans, and the ß-actin control. The PCR reaction was run with DreamTaq DNA Polymerase (Thermo Scientific) with an annealing temperature of 59°C and for 35 cycles for all reactions. Dividing lines within Figure 1A indicate regrouping of gel bands, in cases that gel bands from the same gel were not located next to each other in the original picture (3 dpa and embryo samples). Uninjured RT-PCR samples were run in one separate experiment using the same conditions.

#### 4.3 RNA in situ hybridization

Probes for gpc1a, gpc1b, gpc2, gpc3, gpc4, gpc5a, gpc5b, gpc5c, gpc6a, gpc6b, sdc2, and sdc3 have been previously published. For sdc4 a probe plasmid was generated by amplifying a 822 bp fragment from a cDNA-mix (24 hpf + 3 dpa) as a template using the following primers GACATGTCAGGCTCTGGGTT (fw), AACCACCAC TCCACTGTAGCAC (rev) and cloning it to a pCR-Blunt II-TOPO vector by using the Zero Blunt TOPO PCR cloning Kit (Invitrogen) according to the manufacturer’s instructions. This was then transformed into XLBlue2 competent cells, followed by the selection of several clones and a Miniprep (GeneJET, Thermo Scientific) after culturing. The plasmid was linearized with the restriction enzymes SpeI or PstI for the antisense and sense probe, respectively. For the antisense probe the transcription reaction was carried out using the RNA polymerase T7 and for the sense probe Sp6, followed by RNA precipitation with lithium chloride.
The 3 dpa fin regenerates from the transgenic zebrafish line runx2:GFP<sup>33</sup> served as positive controls by applying a GFP probe within each RNA in situ hybridization experiment (not shown).

The following protocol was used to perform ISH on whole mount fin regenerates (cryosectioning was carried out afterwards) for all glypicans and syndecans except gpc5a and sdc4. Fin regenerates were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), washed twice for 5 minutes each with PBS-0.1% Tween 20 (PBT). The fins were then dehydrated using a methanol series (25% MeOH/75% PBT, 50% MeOH/50% PBT, 75% MeOH/25% PBT, 2x 100% MeOH each for 20 minutes at room temperature (RT)) for long term storage at −20°C. Before use the fins were rehydrated in reverse order and then treated with 5 μg/mL proteinase K (Roche) in PBT for 40 minutes at RT, rinsed once briefly in PBT, washed twice in PBT for 5 minutes each before they were refixed in 4% PFA for 20 minutes at RT. After rinsing the samples once and washing them twice for 5 minutes each in PBT, they were prehybridized at 68°C for 2 hours in prewarmed Hyb + buffer consisting of 50% formamide, 25%, 20x SSC, 0.1% TWEEN 20, 50 μg/mL heparin (Sigma) and 500 μg/mL tRNA yeast RNA (Sigma-Aldrich) (pH to 6.0 with citric acid, filled up with H<sub>2</sub>O DEPC). Afterwards the fins were hybridized at 68°C overnight with digoxigenin (DIG)-labeled antisense RNA probes in Hyb + buffer (varying dilutions 1:20–1:100). The following day probes were taken off and saved for reuse at −20°C and fins were washed at 68°C in prewarmed solutions according to the following order: 1x for 5 minutes in Hyb- buffer (not containing heparin and yeast RNA), 3x for 10 minutes in 25% Hyb- in 2x SSC (0.1% TWEEN 20), 1x 5 minutes in 2x SSC, 2x 30 minutes in 0.2x SSC. This was followed by one wash step for 5 minutes in 50% 0.2x SSC/50% MABT (100 mM maleic acid, 150 mM NaCl, 0.1% TWEEN 20, pH to 7.5 with solid NaOH) and one for 5 minutes in MABT at RT respectively. After blocking for 2 hours at RT in 2% DIG block (dissolved in MABT), fins were incubated overnight at 4°C in anti-DIG antibody solution (Anti-Digoxigenin-AP, Roche, at 1:4000 dilution in DIG block) and washed once briefly as well as 4x for 15 minutes in MABT afterwards. During the last wash step fins were transferred to a 24 well plate and subsequently treated with 500 μL BM purple (Roche) per well for staining in the dark at RT until the desired intensity was obtained. After the reaction was stopped with several PBT washes (1x briefly, 1x 5 minutes, 1x 10 minutes), fins were kept in 50% glycerol in PBT until they were mounted in drops of 80% glycerol in PBT on slides with Plasticine™ spacer and coverslips for imaging. Positive signals were obtained after 4–8 hours of staining. To prepare fins for cryosectioning they were washed again with PBT (2x 5 minutes, 1x 10 minutes), refixed in 4% PFA (in PBS) for 20 minutes at RT, washed with PBS (1x briefly, 2x 5 minutes) and incubated in 0.5 M EDTA overnight at 4°C. For embedding fins were kept 3 hours in 30% sucrose in PBS and 45 minutes in 30% sucrose in PBS/ OCT (Sakura) embedding medium (1:1) at RT, followed by OCT overnight at 4°C. Embedding took place on dry ice, where fins were placed in cylindrical moulds filled with fresh OCT and stored at −80°C until cryosectioning. The frozen blocks were sectioned at

### Table 1: Primer list

| Gene name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| gpc1a<sup>3</sup> | ATGGATCTGACACGGTCGC | TGGATCTAGTACGTGTCAGC |
| gpc1b<sup>3</sup> | ATGGGTTTTGTCTCGCTGGT | GCAATTACCTGCTGTCAGC |
| gpc2 | GGACCAACTCTGGCCAGGA | GAGCTTCGTCCCTGACAGC |
| gpc3<sup>3</sup> | ATGTGCGCTCATCCTTTCAGT | CATACGCAGAGCTCCCTTTC |
| gpc4<sup>3</sup> | CTTGGATCCATGAAGATGATCGTTGCGGT | GTGCTCGAGTATCGTGGAGATG |
| gpc5a<sup>3</sup> | GGATACTGGCTGAAAGCTGGT | TGGTCTCGCTGTCAGC |
| gpc5b<sup>3</sup> | TCTACCTTCAAGGGCATGAT | CTGCATACCTGAGTAC |
| gpc5c | TGAGAGACACACCACCAGACC | GCCTGAGCTCTGCAATAG |
| gpc6a | TTATCGCTTCTGCTGTCGC | GCGGTCTAGATCGTGGAGATT |
| gpc6b<sup>3</sup> | CACCAAAGCTTCAGTCC | CATAAGTCCTGAGGAG |
| sdc2 | CACCAAAGCTTCAGTCC | GCAGAGCATGAAATGAGAG |
| sdc3 | GATCGGGCTGAGGCTAGACT | GCAAGCAGATGAAATGAGAG |
| sdc4 | GCAGAATCTGGAGGTTTGTGCTGCCGGCT | GCGGTCTAGATCGTGGAGATT |
| β-act | TTCCACCAAGCAGGCGGAAGA | TACGCGAGATTCCTACCCA |
12 μm (Microm HM 560, Thermo Scientific) and placed onto SuperFrost plus slides (Thermo Scientific) for imaging.

The protocol used for the ISH against sdc4, gpc5a, and for all embryo samples differed from the rehydration step onwards. Fins were digested with 20 μg/mL proteinase K for 20 minutes and embryos with 2.5 μg/mL for 3 minutes. Specimens were rinsed 2x in PBT to stop the digest, refixed in 4% PFA in PBS for 15 minutes at RT, washed 5x 5 minutes in PBT, prehybridized in prewarmed Hyb + buffer for 2–3 hours at 65°C and hybridized in at least 200 μL prewarmed hybridization mixes (DIG-labeled antisense RNA probes diluted in Hyb + buffer) overnight at 67°C. Probes were recovered and fins washed as follows at 65°C: 1x 20 minutes in Hyb + buffer, 3x 20 minutes in 50% SSCT/50% formamide, 2x 20 minutes in 2x SSCT, 4x 30 minutes in 0,2x SSCT and once 5 minutes in PBT at RT. Blocking was done for ~2–3 hours in blocking buffer consisting of 5% sheep serum and 10 mg/mL bovine serum albumin (BSA) in PBS at RT. The samples were then incubated with anti-DIG antibody solution (Roche, 1:3000 dilution in PBT with 2 mg/mL BSA) overnight at 4°C. After the antibody solution was recovered for reuse the specimens were washed in 2x 5 minutes and 6x 20 minutes in PBT at RT on a shaker with slow agitation, followed by 3x 5 minutes washes with NTMT staining buffer (100 mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20, in H2O DEPC). They were transferred to a 24 well plate during the last wash step and stained with NBT/BCIP (Roche) per ml of NTMT. The reaction was stopped by rinsing twice with PBT and once with STOP solution (0.05 M phosphate buffer pH 5.8, 1 mM EDTA, 0.1% Tween 20) and the samples were kept in STOP solution until ethanol (EtOH) clearing was done (2x rinse with PBT, 2x 15 minutes 100% EtOH, 1x 5 minutes 50% EtOH, all with slow agitation). Preparing for sectioning, embedding as well as sectioning for sdc4 fins was carried out as described above.

ACKNOWLEDGMENTS
We are grateful to Daniel Wehner and Anja Machate for valuable advice concerning the ISH protocol and to Henriette Knopf for proof-reading the manuscript. We thank the Brand and Knopf labs and the CMCB and CRTD technology platforms for technical support. Special thanks goes to Marika Fischer, Jitka Michling, Daniela Mögel and colleagues for their excellent fish care. The work at the TU Dresden is co-financed with tax revenues based on the budget agreed by the Saxonian Landtag.

AUTHOR CONTRIBUTIONS
Sebastian Keil: Formal analysis; investigation; visualization; writing-original draft; writing-review & editing. Mansi Gupta: Investigation; writing-original draft; writing-review & editing. Michael Brand: Conceptualization; funding acquisition; supervision; writing-original draft; writing-review & editing. Franziska Knopf: Conceptualization; funding acquisition; supervision; writing-original draft; writing-review & editing.

DISCLOSURE OF INTEREST
All authors declare no competing financial interest.

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How to cite this article: Keil S, Gupta M, Brand M, Knopf F. Heparan sulfate proteoglycan expression in the regenerating zebrafish fin. *Developmental Dynamics*. 2021;1–13. https://doi.org/10.1002/dvdy.321