University of Huddersfield Repository

Kinnunen, Tarja

Combinatorial Roles of Heparan Sulfate Proteoglycans and Heparan Sulfates in Caenorhabditis elegans Neural Development

Original Citation

Kinnunen, Tarja (2014) Combinatorial Roles of Heparan Sulfate Proteoglycans and Heparan Sulfates in Caenorhabditis elegans Neural Development. PLoS ONE, 9 (7). e102919. ISSN 1932-6203

This version is available at http://eprints.hud.ac.uk/id/eprint/21284/

The University Repository is a digital collection of the research output of the University, available on Open Access. Copyright and Moral Rights for the items on this site are retained by the individual author and/or other copyright owners. Users may access full items free of charge; copies of full text items generally can be reproduced, displayed or performed and given to third parties in any format or medium for personal research or study, educational or not-for-profit purposes without prior permission or charge, provided:

- The authors, title and full bibliographic details is credited in any copy;
- A hyperlink and/or URL is included for the original metadata page; and
- The content is not changed in any way.

For more information, including our policy and submission procedure, please contact the Repository Team at: E.mailbox@hud.ac.uk.

http://eprints.hud.ac.uk/
Combinatorial Roles of Heparan Sulfate Proteoglycans and Heparan Sulfates in Caenorhabditis elegans Neural Development

Tarja K. Kinnunen*

Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom, and Department of Biology, University of Huddersfield, Huddersfield, United Kingdom

Abstract

Heparan sulfate proteoglycans (HSPGs) play critical roles in the development and adult physiology of all metazoan organisms. Most of the known molecular interactions of HSPGs are attributed to the structurally highly complex heparan sulfate (HS) glycans. However, whether a specific HSPG (such as syndecan) contains HS modifications that differ from another HSPG (such as glypican) has remained largely unresolved. Here, a neural model in C. elegans is used to demonstrate for the first time the relationship between specific HSPGs and HS modifications in a defined biological process in vivo. HSPGs are critical for the migration of hermaphrodite specific neurons (HSNs) as genetic elimination of multiple HSPGs leads to 80% defect of HSN migration. The effects of genetic elimination of HSPGs are additive, suggesting that multiple HSPGs, present in the migrating neuron and in the matrix, act in parallel to support neuron migration. Genetic analyses suggest that syndecan/sdn-1 and HS 6-O-sulfotransferase, hst-6, function in a linear signaling pathway and glypican/lon-2 and HS 2-O-sulfotransferase, hst-2, function together in a pathway that is parallel to sdn-1 and hst-6. These results suggest core protein specific HS modifications that are critical for HSN migration. In C. elegans, the core protein specificity of distinct HS modifications may be in part regulated at the level of tissue specific expression of genes encoding for HSPGs and HS modifying enzymes. Genetic analysis reveals that there is a delicate balance of HS modifications and eliminating one HS modifying enzyme in a compromised genetic background leads to significant changes in the overall phenotype. These findings are of importance with the view of HS as a critical regulator of cell signaling in normal development and disease.

Introduction

Heparan sulfate (HS) and HS proteoglycans (HSPGs) critically regulate a range of biological processes in metazoan organisms from early events in gastrulation to adult physiology, disease and host response to pathogens (for reviews see [1,2,3]). The involvement of HS/HSPGs in such a broad range of biological activities is due to their ability to interact with a vast repertoire of proteins such as growth factors, morphogens, enzymes, extracellular matrix and plasma proteins. The molecular interactions of HSPGs are driven and enzymatic reactions do not modify every sugar unit, resulting in huge structural complexity in one of the most heterogeneous molecules in biology.

In vertebrates, most of the HS biosynthetic enzymes are encoded by members of multigene families. Multiple isoenzymes for HS polymerases (EXT/EXTL) [4,5,6], four N-deacetylase/N-sulfotransferases (NDSTs) [7], three glucosaminyl 6-O-sulfotransferases (HS6STs) [8] and seven isoforms of glucosaminyl 3-O-sulfotransferases (HS3STs) [9] have been identified to date. The specific isoenzymes possess differential substrate specificities. Only a single glucuronid C5-epimerase [10] and uronyl 2-O-sulfotransferase (HS2ST; [11]) have been identified to date.

Biochemical analyses have revealed developmental and disease related changes to HS composition. Gene knock-out studies have identified key signaling pathways and developmental processes that require the activities of specific HS biosynthetic enzymes. For example, genetic elimination of mouse Hs2mt leads to complete lack of kidneys and neonatal lethality [11]. Targeted disruption of C5-epimerase in mice leads to renal agensis, lung defects and skeletal malformation [12]. Targeted disruption of HS biosynthetic enzymes is expected to affect all different HSPGs expressed in domains, intermediate sulfate density (NS/NA-domains) and low sulfate density (NA-domains). HS biosynthesis is not template-driven and enzymatic reactions do not modify every sugar unit, resulting in huge structural complexity in one of the most heterogeneous molecules in biology.

In vertebrates, most of the HS biosynthetic enzymes are encoded by members of multigene families. Multiple isoenzymes for HS polymerases (EXT/EXTL) [4,5,6], four N-deacetylase/N-sulfotransferases (NDSTs) [7], three glucosaminyl 6-O-sulfotransferases (HS6STs) [8] and seven isoforms of glucosaminyl 3-O-sulfotransferases (HS3STs) [9] have been identified to date. The specific isoenzymes possess differential substrate specificities. Only a single glucuronid C5-epimerase [10] and uronyl 2-O-sulfotransferase (HS2ST; [11]) have been identified to date.

Biochemical analyses have revealed developmental and disease related changes to HS composition. Gene knock-out studies have identified key signaling pathways and developmental processes that require the activities of specific HS biosynthetic enzymes. For example, genetic elimination of mouse Hs2mt leads to complete lack of kidneys and neonatal lethality [11]. Targeted disruption of C5-epimerase in mice leads to renal agensis, lung defects and skeletal malformation [12]. Targeted disruption of HS biosynthetic enzymes is expected to affect all different HSPGs expressed in...
the targeted tissue or developmental stage. Similarly, most biochemical analyses to structural changes in HS have used whole animal or tissue samples containing a pool of HSPGs. There is limited data on the relationship between HS structures and specific core proteins and whether the core protein influences structural specificity of the HS. Mouse syndecan and glypican HSPGs ectopically expressed in immortalized cell lines are decorated with HS of similar structural composition [13] suggesting that at least in vitro, the core protein context is not critical for HS structure determination. Syndecan and glypican from rat embryonic fibroblasts carry HS chains of different molecular mass, but similar charge and structural composition, yet display differential determination. Syndecan and glypican from rat embryonic fibroblasts are decorated with specificity of the HS. Mouse syndecan and glypican HSPGs bioanalytical approaches to structural changes in HS have used whole targeted tissue or developmental stage. Similarly, most specific HS is more complex

mutations were used: LGI: this study is N2 var. Bristol. The following previously described as described [25] unless otherwise stated. Wild type strain used in this study is N2 var. Bristol. The following previously described mutations were used: LGE: cle-1 (eg120) [22]; LGIII: hse-5 (tm472) [21], rib-2 (tm710) [23]; LGIV: rib-1 (ak556) [24], hst-1 (ok1065); LGX: lon-2 (e678) [18], hst-6 (ok273) [17], sdn-1 (zh20) [26], sdn-1 (ak449) [20], hst-2(ok599) [19,21,26], gpn-1 (ok377) [27], tm734 and tm3006 are deletion alleles of hst-3.1 and hst-3.2, respectively, generated by Shohei Mitani and the Japanese National Bioresource Project. tm734 is a 448 bp deletion and 4 bp insertion eliminating exons 2 and 3 and parts of exons 4 and tm3006 is a 230 bp deletion removing parts of exons two and three. Both mutant alleles were backcrossed at least three times prior to analysis. Both mutants are homozygous viable and fertile and have not overt phenotypic effects as non mutants. The following transgenes were used: zdIs13 [Pthl-1::GFP] [28], mgIs17 [Pthl-1::GFP;rol-6(d)] [29]. Double and triple mutants were generated using standard genetic methods.

Materials and Methods

Strains- C. elegans strains were maintained at 20°C essentially as described [25] unless otherwise stated. Wild type strain used in this study is N2 var. Bristol. The following previously described mutations were used: LGE: cle-1 (eg120) [22]; LGIII: hse-5 (tm472) [21], rib-2 (tm710) [23]; LGIV: rib-1 (ak556) [24], hst-1 (ok1065); LGX: lon-2 (e678) [18], hst-6 (ok273) [17], sdn-1 (zh20) [26], sdn-1 (ak449) [20], hst-2(ok599) [19,21,26], gpn-1 (ok377) [27], tm734 and tm3006 are deletion alleles of hst-3.1 and hst-3.2, respectively, generated by Shohei Mitani and the Japanese National Bioresource Project. tm734 is a 448 bp deletion and 4 bp insertion eliminating exons 2 and 3 and parts of exons 4 and tm3006 is a 230 bp deletion removing parts of exons two and three. Both mutant alleles were backcrossed at least three times prior to analysis. Both mutants are homozygous viable and fertile and display no overt phenotypic effects as non mutants. The following transgenes were used: zdIs13 [Pthl-1::GFP] [28], mgIs17 [Pthl-1::GFP;rol-6(d)] [29]. Double and triple mutants were generated using standard genetic methods.

Reporter constructs

Standard molecular biological techniques were used. The sdn-1::gfp transcriptional reporter contains 1.5 kb of sequence upstream from predicted ATG site cloned into SplI/XbaI sites of pPD95.75. The hst-3.1::gfp and hst-3.2::gfp reporters contain a 2 kb of sequence upstream from corresponding predicted ATG site cloned into SplI/XbaI and SplI/BamHI restriction sites of pPD95.75, respectively. DNA constructs were injected at 30 μg/ml. ptx-3::mCherry or pph-1::mCherry were used as injection markers at 50 μg/ml and Bluescript was used as a filler DNA.

Neuron scoring

HSN neuron migration was scored using cell specific fluorescent reporters that are expressed in the HSNs. Each C. elegans has two HSNs but sometimes it was not possible to see both HSNs due to the position of the animal. Neuron scores are thus presented as number of total neurons scored, rather than number of animals scored, unless otherwise stated. Statistical significance was tested using either Student’s t-test or Fisher’s test.

Microscopy

Microscopy of living C. elegans was performed by mounting the animals on a 4% agarose pad in a drop of M9 buffer containing 30 mM NaN3 as an anesthetic. HSN migration was scored under fluorescent microscopy using GFP expression in all serotonergic neurons including the HSNs. Compound fluorescent images were acquired using Zeiss AxioCam MMRa camera mounted on Zeiss Axioskop2 microscope equipped with x20, x40 and x63 optics. Images were captured using Axiosvision. Confocal images were acquired using Leica AOBS SP2 system and images were captured using Leica software. Microscopy images were further cropped and scaled using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

Results

Parallel HSPG dependent pathways are required for HSN migration

Unlike mammals which have four syndecan and six glypican genes, the genetic redundancy in C. elegans HSPGs more restricted. C. elegans has two orthologues of glypicans, gpn-1 [27] and lon-2 [18] and a single orthologue of syndecan, sdn-1 [20], collagen XVIII, cle-1 [22] and perlecan, une-52 [30]. Mutants in syndecan/sdn-1 display the strongest HSN migration defect. A null allele of sdn-1, sh20, [26] and an in-frame deletion allele ok449 display 52% and 53% HSN migration defects, respectively (Fig. 1). Notably, 12% (sh20; 12 out of 111 neurons) and 17% (ok449; 18 out of 109) of all the HSNs failed to migrate at all in sdn-1 mutants (Fig. 1). In glypican/lon-2 (e678) mutants all the HSNs undergo partial migration but 19% (31 out of 163) fail to reach wild type position (Figure 1), whereas single mutants in the other glypican, gpn-1 (ok377), have wild type HSN migration. Collagen XVIII/cle-1 (eg120) mutants displayed 15% defect in HSN migration (Fig. 1).

To test whether the different HSPGs have redundant functions or whether each HSPG has its own role in HSN migration, HSPG mutants were analyzed in combinations. Mutations in lon-2 (e678) and cle-1 (eg120) enhanced HSN migration defects of sdn-1 (sh20) from 52% (n = 111) to 77% (n = 77; P < 0.001) and 65% (n = 81) respectively (Fig. 2). Similar enhancement of HSN migration defect was observed in combination with the sdn-1 ok449 allele. The neuron migration defect was further enhanced to 82% (47 out of 62 cells) in cle-1 lon-2 sdn-1 triple mutants (Fig. 2), suggesting that CLE-1/collagen XVIII, LON-2/glypican and SDN-1/
syndecan play independent roles in parallel neuron migration pathways. Interestingly however, double mutants in lon-2 (e678) and cle-1 (cg120) did not display significantly enhanced HSN migration defect as compared to either single mutant alone (20% HSN migration defect in cle-1 lon-2 double mutant; n = 117). Mutations in gpn-1 /glypican did not significantly enhance the HSN migration defect of sdn-1 mutants (57%; n = 164). However, gpn-1 (ok377) mutation further enhanced the defects seen in lon-2 sdn-1 double mutants from 77% to 84% in triple mutants. Notably, there was a significant increase in the proportion of neurons that failed to migrate at all in lon-2 sdn-1 gpn-1 triple mutants as compared to lon-2 sdn-1 double mutants (33% compared 9% of all neurons, respectively; P < 0.001).

Role of heparan sulfate biosynthetic enzymes for HSN migration

zh20 is a null allele of sdn-1/syndecan [26], whereas ok449 is an in-frame deletion of the second and third exons [20]. This deletion abolishes two of the three consensus heparan sulfate attachment sites (Ser-Gly) of the SDN-1 protein. Truncated SDN-1 protein is present in ok449 mutants, but it does not contain any HS chains as detected by monoclonal antibodies against HS (Figure S1 and Methods S1). zh20 and ok449 alleles exhibit identical HSN migration defects, suggesting that the role of SDN-1 in HSN migration is predominantly mediated via the HS chains. rib-1 and rib-2 are HS polymerases (EXT/EXTL orthologues) required for the biosynthesis of HS chains [23,24,31]. Homozygous mutations in rib-1 (ok556) are lethal or sterile [24] and although some homozygous rib-2 (tm710) mutants from hetero-

Figure 1. Multiple HSPGs are required for migration of hermaphrodite specific neurons (HSNs). (A) The HSNs visualized using ptp-1::GFP expression (zdIs13) in wild type background, ventral view. Left and right HSNs are labeled. Left HSN (HSNL) is out of focal plane and is marked with an asterisk. The position of the vulva is marked with a dashed line. The HSN cell bodies, which are positioned posterior to the vulval opening, send axons along the left and right ventral nerve cord to the nerve ring in the pharynx. A branch of these axons innervates the vulval muscles. (B) HSN migration defect in glypican/lon-2 (e678) mutant, lateral view. Arrows point to the HSN cell bodies which are both posteriorly misplaced. The position of the vulva is marked with a dashed line. Anterior is to left and posterior is to right in both (A) and (B). (C) Schematic drawing shows ventral view of the HSNs within the C. elegans body plan. The HSNs are born in the tail of the embryo and as the embryo begins to elongate the HSNs migrate through the posterior part of the animal (dashed arrow). The position of HSNs in HSPG mutant background was scored as percentage of neuron migration where 0% is no migration, 100% is wild type neuron position and >100% is over-migration (anterior to vulva). The position of the bubble represents the position of the neurons. The size of the bubble indicates percentage of HSN cell bodies that have migrated to each position; numbers indicate percentage/size of the bubble. All strains carry zdIs13. ncells; number of neurons analyzed for each strain.

doi:10.1371/journal.pone.0102919.g001

Figure 2. Multiple HSPGs are required in parallel guidance pathways for neural migration. Double mutants in HSPG core proteins display more severe neuron migration defects than single mutants and the defects are enhanced in triple mutants. Note that although mutations in gpn-1 (ok377) alone do not result in defects in HSN migration, removing gpn-1 from sdn-1 lon-2 mutant results in significantly more neurons failing to migrate at all. ncells; number of HSNs analyzed for each strain.

doi:10.1371/journal.pone.0102919.g002

zygous mothers become adults their progeny dies shortly after gastrulation [31]. Homozygous rib-2 (tm710) mutants from
heterozygous mothers were analyzed but were found to have no defects in HSN migration (nanimals = 50). The presence of maternal rib-2 mRNA in the embryo at the time of HSN migration may provide enough RIB-2 protein to mask any defects that result from complete lack of RIB-2. hst-1 encodes an orthologue of ndst, heparan N-de-acetylasel-N-sulfotransferase. Deletion mutation in ndst/hst-1 (ok1068) is also homozygous lethal preventing analysis of homozygous offspring from homozygous mutant mothers. Hence the focus on the study was on enzymes modifying HS further in the biosynthetic pathway, mutants of which are all viable. Single mutants in C. elegans heparan C5-epimerase, hse-5 (tm472), and heparan 2-O-sulfotransferases, hst-2 (ok595) have 15% and 30% defects in HSN migration, respectively, whereas single mutants in heparan 6-O-sulfotransferase, hst-6 (ok273), are wild type for HSN migration (Fig. 3 and [19,21,26]. C. elegans has two orthologues of heparan 3-O-sulfotransferases, hst-3.1 and hst-3.2. Single mutants in hst-3.1 (tm734) are wild type for HSN migration, whereas hst-3.2 (tm3006) mutants have 17% defects in HSN migration (Fig. 3).

Removal of HS 3- and 6-O-sulfation enhance HSN migration defects in HS epimerase mutants

Although eliminating either hst-6 or hst-3.1 alone or in combination as a double mutant of hst-3.1; hst-6 do not affect HSN migration, both hst-3.1 (tm734) and hst-6 (ok273) significantly enhance the defects in hse-5 epimerase mutants from 15% to 47% and 36% respectively (Fig. 4; P<0.001). In contrast, hse-5 hst-2 double mutants are not more severely affected than hst-2 single mutants (28% compared to 30% HSN defect; Figs. 3 and 4A).

Heparan 2-O- and 6-O-sulfotransferase mutants have additive defects in HSN migration

Genetic elimination of multiple O-sulfotransferases has very robust effects on HSN migration in C. elegans. Removal of both HS 6- and 2-O-sulfotransferases in hst-6 hst-2 double mutants leads to 83% (n = 86) HSN migration defect compared to 30% defect in hst-2 single mutants (Fig. 4B; P<0.001). In hst-2 single mutants only 2% of the HSNs failed to migrate at all and remained positioned in the tail. In contrast, in hst-6 hst-2 double mutants 17% of all HSNs failed to initiate migration suggesting that removing both HS 2- and 6-O-sulfation severely disrupts molecular interactions required for neuron migration. Intriguingly, further removal of one of the 3-O-sulfotransferases, hst-3.1, from hst-6 hst-2 double mutants suppresses the HSN migration defect (Fig. 4B). In hst-3.1; hst-6 hst-2 triple mutants 53% (n = 176) of the HSNs are posteriorly misplaced, with only 2% failing to migrate at all. Removal of HST-3.1 in the hst-3.1 hst-2 double mutant also weakly suppresses the HSN migration defects of single hst-2 mutants to 21% (n = 83; P<0.05). However, genetic elimination of the other 3-O-sulfotransferase, HST-3.2, from hst-2 mutant slightly increases the HSN migration defects to 35% (n = 268) in the hst-3.2 hst-2 double mutant.

HS modification dependence on HSPG core protein

Specific HS modifications are critical for defined developmental processes in vertebrates. For example, mice lacking heparan 2-O-sulfation have total renal agenesis [11] suggesting that 2-O-sulfates are critical for the growth factors acting in kidney development [32]. HS 2-O-sulfates regulate growth factors involved in the induction and differentiation of kidney mesoteric mesenchyme [33], whereas 6-O-sulfates regulate growth factors required for ureteric bud branching later in kidney development [34]. The HSPGs carrying these HS modifications have not been identified. Neither is it known if the same HSPG undergoes developmental changes in its HS or whether different HSPGs carry the

![Figure 3. Effects of genetic elimination of HS epimerase or HS O-sulfotransferases on HSN migration.](https://doi.org/10.1371/journal.pone.0102919.g003)

![Figure 4. Combinatorial effects of eliminating HS epimerase and O-sulfotransferases for neural migration.](https://doi.org/10.1371/journal.pone.0102919.g004)
structurally different HS chains. Gene knock-out of a HS biosynthetic enzyme such as HS2ST is anticipated to affect all HSPG core proteins.

To gain insight into relationship between HSPG protein cores and specific HS modifications, effects of eliminating HS biosynthetic enzymes in different core protein mutant backgrounds were tested. Eliminating 3- or 6-O-sulfates using hst-3.2 (tm3006) or hst-6 deletion alleles in sdn-1 null background did not alter the severity of the HSN phenotype (53% and 52% defect, respectively) as compared to sdn-1 null mutants alone. In contrast, genetic removal of 2-O-sulfates in the sdn-1 null background (sdn-1 hst-2 double mutants) lead to 87% (n = 119) failure of HSN migration to wild type position, with 37% of HSNs failing to migrate at all (Fig. 5). Similarly, double mutants of hse-5 epimerase and sdn-1 display more severe defects with 61% of HSNs (n = 81) failing to migrate to wild type position and 33% failing to migrate at all (compared with 17% in sdn-1 single mutants; P<0.01). In contrast, additional removal of 2-O-sulfates from glypican/lon-2 and collagen XVIII/cle-1 mutant backgrounds did not significantly alter the neuron defects observed in a single mutant. In lon-2 hst-2 double mutants 37% (n = 91) and in cle-1; hst-2 double mutants 24% of all neurons (n = 129) failed to migrate to wild type position. The pattern of migration defects was not significantly changed either.

**Figure 5.** SDN-1 and HS 2-O-sulfate regulate parallel neuron migration pathways. (A) Removing heparan 2-O-sulfation in sdn-1 mutants significantly enhances HSN migration defects. Note the increased proportion (39%) of HSNs that fail to migrate at all in sdn-1 hst-2 double mutants. Genetic elimination of hst-6 or hst-3.2 in sdn-1 mutant background does not change the HSN migration phenotype. (B–D) Expression profiles of sdn-1, hst-3.2 and hst-3.1 reporter genes. (B) sdn-1 reporter is expressed predominantly in the nervous system, including neurons of the nerve ring (arrow) and in the ventral (VNC; arrowheads) and dorsal nerve cords. (C) hst-3.2 reporter is also expressed in neurons as seen in some neurons in the nerve ring in the pharynx (arrows) and in the tail ganglia and along ventral nerve cord axon tracks (arrowheads). (D) hst-3.1 reporter is expressed in the muscle; both the pharyngeal muscle (arrow) and body wall muscle express hst-3.1 reporter. Scale bars B, D; 50 μm; C, 20 μm. doi:10.1371/journal.pone.0102919.g005

**Tissue specific expression of HS biosynthetic enzymes and HSPGs**

Reporter constructs were used to assess expression patterns of hst-3.1 and hst-3.2 and revealed complementary expression patterns for the two 3-O-sulfotransferases. hst-3.2 reporter is expressed predominantly in neurons (Fig. 5C) whereas hst-3.1 reporter is expressed predominantly in pharyngeal and body wall muscle (Fig. 5D). Similar complementary expression patterns have previously been shown for the other HS biosynthetic enzymes and HSPGs. hst-6 is predominantly expressed by neurons, hst-2 is expressed in the muscle and hypodermis, and hse-5 is expressed in the hypodermis [19,21]. sdn-1 is predominantly expressed in neurons (Fig. 5B; [26], whereas lon-2 and cle-1 are expressed in muscle and hypodermis [18,22].

**Heparan sulfates are required for HSN migration and axon guidance but not process outgrowth**

Deletion mutations in genes encoding for HSPG core proteins and for HS biosynthetic enzyme, led to defects in HSN migration and axon guidance. However, process outgrowth per se was not affected in any of the mutants (Fig. 6). In other words, irrespective of whether the HSN cell body had migrated to wild type or aberrant position or failed to migrate at all, the HSNs extended axonal projections, suggesting that the process of axon outgrowth is independent of HS/HSPGs (Fig. 6). The defects in HSN migration and axon guidance were also independent of each other. HSN cell bodies which had failed to migrate at all often extended axons that appeared projecting correctly (Fig. 6A). Conversely, HSN cell bodies which had migrated to correctly to the vulval proximity frequently projected aberrant axons which failed to reach their appropriate targets. HSN/HSPGs thus play independent roles in HSN neuron migration and in axon guidance.

**Discussion**

Multiple HSPGs were found to play a role in the migration of the HSNs. Glypican/LON-2, collagen XVIII/CLE-1 and syndecan/SDN-1 are required in parallel neuron guidance pathways as genetic elimination of two or all of the HSPGs increases the severity of the HSN migration phenotype. In the absence of all three HSPGs only 20% of HSNs migrate normally. Although mutations in the gpn-1/glypican alone do not lead to defects in HSN migration, genetic elimination of gpn-1 in lon-2 sdn-1 background significantly increased the proportion of neurons that failed to migrate at all suggesting that in the absence of LON-2, GPN-1 plays a redundant role resulting in increased severity of defects when both glypicans are genetically eliminated.

sdn-1 is expressed by predominantly in neurons including the HSNs [26], whereas lon-2 and cle-1 are expressed in the hypodermis and body wall muscle [18,22]. This suggests that SDN-1/syndecan is required in the migrating neuron, the HSNs, whereas LON-2/glypican and CLE-1/collagen XVIII are required in the matrix that supports HSN migration and all three HSPGs act in concert. When LON-2/glypican is present, GPN-1/ glypican is not required for HSN migration. Moreover, SDN-1 HS chains are important for HSN migration as sdn-1 null allele and ‘HS-less’ allele display identical phenotypes.

Simultaneous genetic removal of both hst-2 and hst-6 results in almost complete loss of HSN migration suggesting that heparan O-sulfation is needed for HSN migration. Biochemical HS disaccharide analysis has shown increased N-sulfation in hst-2 hst-6 double mutants [35], however, as suggested by this study, glucosamine N-sulfation is not able to functionally compensate for the lack of O-sulfation. Genetic removal of hst-6 and hst-3.1 in...


Three HSPG core proteins, SDN-1/syndecan, LON-2/glypican and CLE-1/collagen XVIII, are required in parallel for normal HSN migration. The expression of these HSPGs is different and, based on their expression patterns and on ES cell expression analyses, the core proteins are predominantly expressed in the muscle and hypodermis. Combining the normal expression patterns of these HSPGs with the finding that the HSN migration defect in hst-2 single mutants is also rescued by expression of the genes encoding for these HSPGs in ES cells, suggests that the core proteins are engaged in the HS glycosaminoglycan (GAG) signalling network in higher organisms. The core protein is therefore not a carrier per se, but an essential component of the HS network.

In conclusion, a neural development model in C. elegans was used to address relationship between different HSPG core proteins and distinct HS modifications for biological function in vivo. Genetic analysis demonstrated that SDN-1/syndecan, LON-2/glypican and CLE-1/collagen XVIII are required in parallel guidance pathways and genetic elimination of all three HSPGs results in almost complete failure of neural migration. The different HSPG core proteins require distinct HS modifications to mediate in vivo functions. The core protein is thus not a carrier of its HS chains with any random modifications. In C. elegans this core protein-HS specificity may in part be imposed by tissue specific expression of the genes encoding for HSPGs and HS biosynthetic enzymes. This is the first demonstration of relationship between HS modifications and distinct HSPG core proteins in a defined biological process and provides a novel insight into the biological functions of HS and HSPGs. Proteoglycan core protein specificity should be considered when interpreting data or considering therapies relating to distinct HS structures.
Supporting Information

Figure S1 Biochemical analysis of sdn-1 mutants. SDN-1 is absent in both zk(20) (null allele) and ok1449 (in-frame deletion abolishing HS attachment sites) mutants as detected by monoclonal antibodies recognising the HS “stub” as a result of treatment with heparanase III. Proteins were purified using anion-exchange chromatography (DEAE), which enriches for negatively charged HSPGs. SDN-1 core protein is however present in ok1449 mutants as detected by anti-SDN-1 antibodies following immunoprecipitation using anti-SDN-1 antibodies.

Methods S1 HSPG purification and Western blotting. Mixed stage C. elegans were lysed and proteins purified essentially as described [40] with the following modifications. Total protein lysates were either passed through DEAE anion-exchange matrix (GE Healthcare Life Sciences) and after washing the matrix with lysates were either passed through DEAE anion-exchange matrix or purified proteins were treated with heparitinase III (Ibex, Canada) and after washing the matrix with 0.25 M NaCl, bound proteins were eluted with 1.5 M NaCl. Alternatively, SDN-1 was immuno-precipitated with rabbit polyclonal anti-SDN-1 antibodies made against synthetic peptide corresponding to the entire cytoplasmic domain of SDN-1. DEAE purified proteins were treated with heparanase III [Ibex, Canada] as described [27]. Proteins were separated on 10% SDS-PAGE.

Acknowledgments

Some strains used in this study were obtained from the Caenorhabditis Genetics Center, which is supported by the NIH National Center for Research Resources (NCRR). Some of the mutant strains were created by the C. elegans Knockout Consortium and by the Japanese National Bioresource Project.

Author Contributions

Conceived and designed the experiments: TKK. Performed the experiments: TKK. Analyzed the data: TKK. Contributed reagents/materials/analysis tools: TKK. Wrote the paper: TKK.

References

1. Bishop JR, Schukz M, Esko JD (2007) Heparan sulphate proteoglycans fine-tune mammalian physiology. Nature 446: 1030–1037.
2. Bulow HE, Hobert O (2006) The molecular diversity of glycosaminoglycans shapes animal development. Annu Rev Cell Dev Biol 22: 375–407.
3. Kramer KL, Yost HJ (2003) Heparan sulfate core proteins in cell-cell signaling. Annu Rev Biochem 72: 461–494.
4. Kim BT, Kitagawa H, Tamura J, Saito T, Kusche-Gullberg M, et al. (2001) Human tumor suppressor EXT gene family members EXT1 and EXT3 encode alpha 1-4-N-acetylgalactosaminyltransferases that likely are involved in heparan sulphate/beta1,3-galactosylation. Proc Natl Acad Sci U S A 98: 7176–7181.
5. Kitagawa H, Shimakawa H, Sugahara K (1999) The tumor suppressor EXT-like gene EXT2 encodes an alpha1, 4-N-acetylgalactosaminyltransferase that transfers N-acetylgalactosamine and N-acetylglucosamine to the common glycosaminoglycan/ proteoglycan linkage region. The key enzyme for the chain initiation of heparan sulfate. J Biol Chem 274: 13933–13937.
6. Lind T, Tufaro F, McCormick C, Lindahl U, Lidholt K (1998) The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. J Biol Chem 273: 28265–28268.
7. Aikawa J, Grobe K, Tsujimoto M, Esko JD (2001) Multiple isozymes of heparan sulfate/heparin GlcNAc N-deacteylsylase/GlcN N-sulfotransferase. Structure and activity of the fourth member, NDS1. J Biol Chem 276: 5876–5882.
8. Habischi H, Tanaka M, Habuchi O, Yoshida K, Suzuki H, et al. (2000) The occurrence of three isoforms of heparan sulfate 6-O-sulfotransferase having different specificities for hexuronic acid adjacent to the targeted N-sulfoglucosamine. J Biol Chem 275: 2859–2868.
9. Shworak NW, Liu J, Petros LM, Zhang L, Kobayashi M, et al. (1999) Multiple isoforms of heparan sulfate-D-glucosaminid 3-O-sulfotransferase. Isolation, characterization, and expression of human chias and identification of distinct genomic loci. J Biol Chem 274: 5170–5178.
10. Li J, Hagger-McWhirter A, Kijljen I, Palgi J, Jalkanen M, et al. (1997) Biosynthesis of heparin/heparan sulfate. cDNA cloning and expression of D-glucuronyl C5-epimerase from bovine lung. J Biol Chem 272: 28158–28163.
11. Bullock SJ, Fletcher JM, Beddington RS, Wilson VA (1998) Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate glucuronyl C5-epimerase. J Biol Chem 273: 28164–28167.
12. Li JP, Gong F, Hagger-McWhirter A, Fordberg E, Abriuk M, et al. (2003) Targeted disruption of a murine glucuronyl C5-epimerase gene results in heparan sulfate lacking L-iduronic acid and in neonatal lethality. J Biol Chem 28: 28363–28366.
13. Zako M, Dou J, Goldberger O, Berntfield M, Gallagher JT, et al. (2003) Syndecan-1 and -4 synthesized simultaneously by mouse mammary gland epithelial cells bear heparan sulfate chains that are apparently structurally indistinguishable. J Biol Chem 278: 13561–13569.
14. Tumova S, Woods A, Couchman JR (2000) Heparan sulfate chains from glypicans and syndecans bind the Hep II domain of fibronectin similarly despite minor structural differences. J Biol Chem 275: 9410–9417.
15. Kimmunen T, Raalo E, Nolo R, Maccarana M, Lindahl U, et al. (1996) Neurite outgrowth in brain neurons induced by heparin-binding growth-associated molecule (HB-GAM) depends on the specific interaction of HB-GAM with heparan sulfate at the cell surface. J Biol Chem 271: 2243–2248.
32. Merry CL, Bullock SL, Swan DC, Backen AC, Lyon M, et al. (2001) The molecular phenotype of heparan sulfate in the Hs2st−/− mutant mouse. J Biol Chem 276: 35429–35454.
33. Shah MM, Sakurai H, Sweeney DE, Gallegos TF, Bush KT, et al. (2010) Hs2st mediated kidney mesenchyme induction regulates early ureteric bud branching. Dev Biol 339: 354–365.
34. Shah MM, Sakurai H, Gallegos TF, Sweeney DE, Bush KT, et al. (2011) Growth factor-dependent branching of the ureteric bud is modulated by selective 6-O sulfation of heparan sulfate. Dev Biol 356: 19–27.
35. Townley RA, Bulow HE (2011) Genetic analysis of the heparan modification network in Caenorhabditis elegans. J Biol Chem 286: 16824–16831.
36. Casu B, Petitou M, Provasoli M, Sinay P (1988) Conformational flexibility: a new concept for explaining binding and biological properties of iduronic acid-containing glycosaminoglycans. Trends Biochem Sci 13: 221–225.
37. Rudd TR, Skidmore MA, Guimond SE, Cosentino G, Torri G, et al. (2009) Glycosaminoglycan origin and structure revealed by multivariate analysis of NMR and CD spectra. Glycobiology 19: 52–67.
38. Ferro DR, Provasoli A, Ragazzi M, Casu B, Torri G, et al. (1990) Conformer populations of L-iduronic acid residues in glycosaminoglycan sequences. Carbohydr Res 195: 157–167.
39. Holmborn K, Ledin J, Smeds E, Eriksson I, Kusche-Gullberg M, et al. (2004) Heparan sulfate synthesized by mouse embryonic stem cells deficient in Ndst1 and Ndst2 is 6-O-sulfated but contains no N-sulfate groups. J Biol Chem 279: 42355–42358.
40. Polanska UM, Duchesne L, Harries JC, Fernig DG, Kinnunen TK (2009) N-Glycosylation regulates fibroblast growth factor receptor/EGL-15 activity in Caenorhabditis elegans in vivo. J Biol Chem 284: 33030–33039.