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Extracellular sugar modifications provide instructive and cell-specific information for axon guidance choices

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SUMMARY

Heparan sulfates (HS) are extraordinarily complex extracellular sugar molecules that are critical components of multiple signaling systems controlling neuronal development [1-3]. The molecular complexity of HSs arises through a series of specific modifications, including sulfations of sugar residues and epimerizations of their glucuronic acid moieties. The modifications are introduced non-uniformly along protein-attached HS polysaccharide chains by specific enzymes (Fig.1A)[4]. Genetic analysis has demonstrated the importance of specific HS modification patterns for correct neuronal development [2,3]. However, it remains unclear whether HS modifications provide a merely permissive substrate or whether they provide instructive patterning information during development. We show here with single cell resolution that highly stereotyped motor axon projections in *C. elegans* depend on specific modification patterns of HS present in the extracellular environment. We show that by manipulating extracellular HS modification patterns we can cell-specifically re-route axons, indicating that HS modifications are instructive. This axonal re-routing is dependent on the HS core protein *lon-2*/glypican and the axon guidance cue *slt-1*/Slit as well as its receptor *eva-1*. These observations suggest that a changed sugar environment instructs *slt-1*/Slit-dependent signaling via *eva-1* to redirect axons. Our experiments provide genetic *in vivo* evidence for the "HS code" hypothesis which posits that specific combinations of HS modifications provide specific and instructive information to mediate the specificity of ligand/receptor interactions [3,5,6].

RESULTS

Distinct HS modification patterns are required by individual ventral cord motor neurons for correct axon pathfinding

The DA and DB classes of motor neurons are embryonically generated motor neurons (eMNs) in the ventral nerve cord (VNC) of *C. elegans*. Individual DA and DB motor neurons make

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differential, completely invariant choices as to whether to extend circumferentially along the right or left side of the animal (Fig.1B-D). This sidedness does not correlate with the membership of a motor neuron to a specific class (e.g. 2 DA neurons extend along the right side, and 7 DA neurons extend to the left side; Suppl. Table 1), nor position along the a/p axis (Fig.1B; Suppl. Table 1), lineage history (Suppl. Table 1) or any other unique cellular landmark in their environment (Suppl. Table 1). Intriguingly, there are no molecular markers that distinguish individual DA neurons or individual DB neurons from one another (i.e. no genes are known that are expressed in DB2 but not DB3) and no genes have been reported which control the cell-type specificity of the projection patterns. To test whether individual DA/DB motor neurons require distinct HS modifications for correct development, we genetically removed three major HS modifying enzymes, the C5-epimerase encoded by *hse-5*, the 6O-sulfotransferase, encoded by *hst-6* and the 2O-sulfotransferase, encoded by *hst-2* (Fig.1A, E). Since none of these enzymes have paralogs in *C. elegans*, we can assume that animals with null mutations in these genes completely lack C5-epimerization, 6O-sulfation or 2O-sulfation [7]. Removal of C5-epimerization or 2O-sulfation each result in mild defects in the sidedness of motor axon projections [7]. These mild defects show cell-type specificity with DA2, DB3, DB6 and DB7 being most affected by loss of 2O-sulfation (Fig.1E). Generation of double mutants as well as a triple mutant resulted in significantly enhanced mutant phenotypes, revealing cell-type specific redundancies in the requirement of HS modifications for individual motor axons (Fig.1F). For example, the DA2, DB3, and DB6 motor neurons display significantly enhanced defects in the *hst-2 hst-6* and *hse-5; hst-6* double mutant combinations, indicating that *hst-6* function is redundant with both *hse-5* and *hst-2* in these motor neurons. However, in DA6 motor neurons *hst-6* function is redundant with *hse-5* but not with *hst-2* while conversely, in DB5 motor neurons *hst-6* is redundant with *hst-2* but not with *hse-5* (Fig. 1F). The HS modification enzymes *hst-6* and *hst-2/hse-5* appear to act in different tissues during neuronal development with *hst-6* acting in neuronal tissues and, *hse-5/hst-2* in hypodermal tissues [7]. Thus, the observation that *hst-6* and *hse-5/hst-2* serve redundant functions in some neurons may seem non-intuitive given their disparate sites of action. However, HS has been demonstrated to act cell nonautonomously in for example ectodermal/mesodermal interactions [8]. It is therefore conceivable that differentially modified HS on neuronal and hypodermal tissues cooperate in a redundant manner to control axon routing.

In contrast, the defects in *hse-5; hst-2* double mutants are no more severe than the defects in the most severe (*hst-2*) of the single mutants (Fig.1F). This last finding is consistent with analyses in other cellular contexts that demonstrate that *hse-5* and *hst-2* can genetically act in the same pathway [7]. Finally, no further enhancement is observed in the triple mutant combination compared to the *hst-2 hst-6* double null mutant (Fig.1F). We conclude that individual members of the same class of motor neurons (DA or DB) that make apparently identical guidance choices at the ventral midline of *C. elegans* are dependent on different combinations of HS modifications.

Removal of the HS core protein syndecan (*sdn-1*) recapitulates the defects observed in *hse-5; hst-2 hst-6* triple mutants, both in terms of penetrance and cell-type specificity (Fig.1E, F). In contrast, neither null mutations in the two *C. elegans* glypican HS core proteins (*gpn-1* and *lon-2*; [9, 10]) nor mutations in the secreted HS core proteins *unc-52* (perlecan; [11]) or *cle-1* (collagen XVIII; [12]) display significant defects in eMN left/right projection patterns (Suppl. Fig.1A). These results suggest that the majority of HS that controls DA/DB motor axon choices may be attached to the syndecan core protein.

Manipulating HS modification patterns redirect motor axon projections

Our present work with DA/DB motor neurons together with previous work shows that the function of HS requires their extensively modified polysaccharide chains (Fig.1A)(reviewed

in [3,13]). However, it is unclear whether HS modifications constitute a merely permissive environment or whether they are sufficient to guide growing axons. The question of permissiveness versus instructiveness represents one of the key problems in the HS field [3, 13].

To address whether HS modifications are sufficient to guide axons, i.e. serve instructive functions, we manipulated HS modification patterns by inducing ectopic 6O-sulfation in the hypodermal cells that underlie the DA/DB motor neurons (Fig.2A). The 6O-sulfotransferase *hst-6* does not appear to be expressed in hypodermal cells [7](Fig.2A) and as shown above, DA/DB axon choices are unaffected by removal of *hst-6* alone (Fig.1E). To alter HS modification patterns presented by hypodermal cells, we generated multiple transgenic *C. elegans* lines that aberrantly express *hst-6* in an otherwise wild-type background under control of the hypodermis-specific *dpy-7* promoter [14]. Worms expressing this construct show an altered status of HS modification patterns. First, we detect novel staining patterns with an antibody that recognizes HS epitopes comprising specific oligosaccharides rather than individual modifications (Fig.2B) [15]. This antibody labels the anterior intestine and, possibly some neurons and basement membranes in wild-type animals (not shown). However, increased staining is observed consistently in the hypodermis upon misexpression of *hst-6* in this tissue. This increased staining pattern is not observed in non-transgenic but otherwise isogenic controls (Fig.2B). And second, biochemical analyses of HS from transgenic worms expressing *hst-6* in the hypodermis display an increase of 6O-sulfated relative to 2O-sulfated HS disaccharides (Suppl. Fig.2). Each transgenic line shows a significant number of animals in which the projections of individual DA and DB motor neurons are re-routed (Fig.2C, 3A; Suppl. Fig.3). These defects are not the result of apparent changes in cell fate or structure of hypodermal cells misexpressing *hst-6* (Fig.2E) nor are they due to changes in eMN positioning in the VNC (data not shown). Moreover, we cannot detect obvious disruptions of the basement membranes surrounding eMNs and therefore consider it unlikely that ectopic *hst-6* expression grossly disrupts midline structures (Fig.2F).

The axonal misrouting defects we observe are the result of *hst-6* activity as the introduction of a premature stop codon into the *hst-6* expression construct abolishes its ability to induce axon misrouting (Fig.3A). Furthermore, no comparable misrouting defects can be observed upon *dpy-7*^{prom}-driven overexpression of the HS 2O-sulfotransferase *hst-2* (Fig.3A), which is normally expressed in both neuronal and hypodermal tissues [7]. These *hst-2* expressing transgenes are functional: first, the transgenes are able to effectively rescue neuronal *hst-2* loss of function phenotypes (Suppl. Fig.4A); second, using an antibody recognizing specific HS epitopes we detect increased staining in transgenic, *hst-2*-overexpressing strains (Suppl. Fig. 4B); third, biochemical analyses of *hst-2* overexpressing strains display increased 2O-sulfation relative to 6O-sulfation compared to wild type (Suppl. Fig.2). Together, our results argue that the effects of ectopic *hst-6* expression are due to the regiospecific addition of a sulfate moiety to the 6O-hydroxyl group of the glucosamine residue, rather than the unspecific addition of a negative charge.

Examining the axon choices of individual DA and DB motor axons, we find that the defects induced by ectopic 6O-sulfation are cell-type specific with all different transgenic lines displaying qualitatively similar misrouting patterns. Interestingly, there seems to be a graded response of DB neurons to misexpressed *hst-6* with the more posterior neurons being more strongly affected (Fig.3C; Suppl. Fig.3). In contrast, within the DA class of eMNs only DA3 exhibits significant defects. Ectopic hypodermal 6O-sulfation also causes axon guidance defects in a selected number of other neurons in the nervous system. For example, axon midline guidance of the PVQ interneurons is affected (Fig.2D, 3B), while many neurons, such as touch neurons, amphid, phasmid and, RMEV neurons display no apparent defects (Suppl. Table 2). Notably, PVQ interneurons are also affected by loss of endogenous, neuronally acting *hst-6*

[7]. The addition of 6O-sulfation in the wrong location (hypodermis) can therefore have the same detrimental effect for an axon as not having 6O-sulfation in the correct place (neurons). Taken together, manipulating HS modifications in the hypodermis through addition of novel HS modification patterns can redirect axonal projections of individual neurons.

Ectopic 6O-sulfation-induced motor axon misrouting requires Slit/*slt-1* and its co-receptor *eva-1*

Most DA/DB motor neurons that respond to ectopic HS 6O-sulfation require *hst-6* for normal development in the absence of *hst-2* function (compare Fig.1E, F and 3C). The exception is DB7 suggesting that DB7 axonal projections are normally independent of 6O-sulfated HS. However, upon *hst-6* expression in the hypodermis, DB7 is misrouted suggesting that DB7 can be positively instructed by ectopic 6O-sulfated HS. We thus focus our genetic analyses here on the DB7 motor neuron.

We first asked which HS core protein would bear the ectopic 6O-sulfated HS presented by the hypodermis that misdirects the DB7 motor neuron. To this end, we systematically removed the genes coding for four canonical HS core proteins in the *C. elegans* genome, *lon-2*/glypican, *gpn-1*/glypican, *sdn-1*/syndecan, or *unc-52*/perlecan, respectively, and tested the effect on DB7 motor neuron projection patterns in animals expressing *hst-6* in the hypodermis. We find that removal of *lon-2*/glypican but neither of the other HS core proteins suppresses the axonal misrouting of DB7 to a level that is statistically indistinguishable from wild type (Fig. 3E). As *lon-2*/glypican normally acts in the hypodermis [10], our data suggests that the ectopic HS structures in the hypodermis that redirect DB7 may be attached to the *lon-2*/glypican HS core protein.

We next sought to address how alterations in HS modification patterns exert an impact on the DB7 motor axon guidance decision. As HS may play a role in determining ligand/receptor binding specificities *in vitro* (reviewed in [3]), we reasoned that changing HS modification patterns may reprogram the responsiveness of motor axons to defined cues. To address this possibility, we asked whether a specific axon guidance cue is required for the misrouting of individual motor axons induced by ectopic 6O-sulfation. The guidance cue *slt-1*/Slit is known to require different HS modifications in distinct cellular contexts in *C. elegans* [7]. *Slit*-mediated axon growth at the fly midline and, in vertebrate tissue explants are also known to require the presence of HS, albeit of unknown modification status [16-18]. The ligand *slt-1*/Slit signals through the *sax-3*/Robo of the Robo family of guidance receptors [19]. More recently, EVA-1, a conserved transmembrane protein with extracellular domains that display similarity to sugar binding domains has been identified as an additional Slit/SLT-1 receptor that is required for Slit/SLT-1 signaling and physically interacts with SLT-1 [20].

DA/DB motor neurons and in particular DB7 do not require *slt-1* or *eva-1* to make their correct guidance decision at the midline while its canonical receptor *sax-3*/Robo is involved in routing all DA/DB motor neurons (Fig.3D, Suppl. Fig.1B). These findings are consistent with previous findings demonstrating that *sax-3*/Robo has functions that are independent of its canonical ligand *slt-1*/Slit [7,19] and imply that this *slt-1*-independent function of *sax-3*/Robo is likewise independent of *eva-1*. Intriguingly, loss of *slt-1* significantly suppresses the axonal projection defects in DB7 as a result of ectopically expressing *hst-6* in the hypodermis (Fig.3F). This suppression does not result from changes in expression levels of either *slt-1* or its canonical receptor *sax-3* (Suppl. Fig.5). Thus a possible interpretation of our data is that ectopic 6O-sulfated HS attached to *lon-2*/glypican instructs *slt-1* to misroute the DB7 motor axon by possibly promoting the interaction of SLT-1 with a cognate receptor on motor neurons, thereby misrouting an extending axon.

We next asked whether the *slt*-1/Slit receptor *eva-1* could serve as this cognate receptor in the context of DB7 misrouting. Indeed, genetic removal of *eva-1* significantly suppresses the *hst-6*-dependent axonal misrouting of DB7 (Fig.3F). With *eva-1* expressed in eMNs [20], this genetic data is in accord with a scenario where HS of a specific modification status instructs *slt-1* to interact with *eva-1* leading to an axonal misrouting response of DB7. The misrouting of DB7 must be largely *sax-3*-independent because the penetrance of defects in the *hst-6* misexpression strain (10.2%) and *sax-3* loss of function animals (19.8%) are additive in the strain carrying the *hst-6*-misexpressing transgene in conjunction with the *sax-3* null mutation (34.3%)(compare Fig.3D and F). This last finding implies potential *sax-3*-independent functions for *eva-1*.

DISCUSSION

Our analysis of the projection patterns of embryonic motor axons in *C. elegans* reveals that superficially similar neuron types are programmed to extend their axons in distinct directions through diverse mechanisms. In addition to a large number of guidance cues and intracellular signaling molecules, which are involved in patterning motor axon projections at the worm midline (data not shown), HS modifications play a crucial role in determining the projection patterns of individual motor axons. Our findings indicate that HS modifications have specific and instructive functions, likely through mediating specific ligand/receptor interactions (Fig. 4).

This interpretation takes the broadly accepted concept into account that extracellular HS molecules not only promote, but are often essential for the activity of specific ligand/receptor systems [3]. In support of this notion, *in vitro* experiments suggest that differentially modified HS can promote signaling through distinct signaling systems [21,22]. Together with our finding that both the removal of enzymes that modify HS molecules as well as the ectopic addition of HS modifications through misexpression of a HS modifying enzyme causes misrouting defects, we propose that the local modification status of HS in the vicinity of extending motor axons impinges on which ligand/receptor system is engaged (Fig.4). This model is consistent not only with the effects observed upon removal of HS modifying enzymes, but also with the genetic analyses of animals with ectopic expression of *hst-6* in the hypodermis. In this scenario, the introduction of a novel HS modification pattern which is attached to a specific core protein in the hypodermis (LON-2) aberrantly promotes the interaction of the repulsive SLT-1 cue with a receptor molecule, possibly EVA-1, thereby causing a mis-orientation of motor axon pathfinding (Fig.4). This is supported by our genetic data demonstrating that genetic removal of *lon*-2, slt-*1* or *eva-1* suppresses the misrouting defect in DB7 to levels that are statistically indistinguishable from wild type (Fig.3D-F). However, we note that this model is not the only way to interpret the genetic interaction of ectopic *hst-6* with *lon*-2, *slt-1*, or *eva-1*. Indirect effects are conceivable and can not be ruled out by genetic analysis alone.

With this caveat in mind, the finding that ectopic expression of an HS modifying enzyme and the dependence of the resulting phenotype on a specific axon guidance molecule (*slt-1*) and one of its receptors (*eva-1*) provides additional support for a concept in which the enormous molecular complexity embedded with HS molecules may carry not merely permissive but also instructive information content. It has been speculated before that the molecular diversity of HS molecules could provide a code that regulates the regiospecific activity of individual axon patterning systems [3,5-7]. Specifically, in the HS code hypothesis regiospecific HS modification patterns determine the responsiveness of neurons to individual ligand/receptor patterning cues. In accordance with this hypothesis, the removal of individual modifications cause highly specific nervous system patterning defects [7,23-25](this study). Furthermore, we show that the removal of combinations of HS modifications uncovers cell-specific redundancies in the requirements for HS modifications (Fig.2), indicating that the presumptive

HS code may be degenerate. And lastly, as pointed out previously [13], an important additional criterion for the existence of such a code is that HS modifications act instructively, and not merely as permissive co-factors. A genetic test to determine the difference between instructiveness and permissiveness is to ask whether a given gene not only displays a specific loss of function phenotype, but whether a gain-of-function phenotype of the gene upon misexpression can also instruct a novel phenotype [26]. Keeping in mind that these experiments provide genetic rather than biochemical evidence for the action of HS, our results clearly show that we can redirect the DB7 neuron upon misexpression of the HS 6O-sulfotransferase *hst-6*. We can detect the molecular correlate of this *hst-6* misexpression in the hypodermis by using both an HS specific antibody and biochemical analyses of HS disaccharides (Fig.2B, Suppl. Fig.2). Moreover, we find that this novel phenotype is dependent on a specific HS core protein (*lon-2*/glypican) and that aberrant 6O-sulfation appears to activate *slt-1* signaling through the *slt-1* receptor *eva-1* (Fig.3E, F). Further biochemical studies are warranted to corroborate these genetic results and to gain mechanistic insight into how HS can misdirect axons.

EXPERIMENTAL PROCEDURES

Neuroanatomical scoring

All animals were scored at 20°C in a mixed population of mid-larval to adult stages. DA/DB were scored with *unc-129∷gfp (evIs82b)* and PVQ with *sra-6∷gfp (oyIs14)*. GFP expression in the DA/DB neurons of *evIs82b* transgenic animals is variable in DA7, DA8 and DA9; moreover, projection patterns of DA1, DB1 and DB2 are often not easy to follow on the single cell level and we therefore excluded these neuron types from our analysis. Individual motor neurons were identified based on their cell body position and the dorsal projection patterns. In each figure, the error bars denote the standard error of proportion and asterisks indicate statistical significance: * P<0.05, ** P<0.005, *** P<0.0005; n.s. not significant. Statistical significance was calculated using the z-test. If multiple comparisons were made, p-values were subjected to the Bonferroni correction.

Transgenes

For ectopic hypodermal expression of HS modifying enzymes, the complete *hst-6* and *hst-2* loci were fused to the *dpy-7* promoter [14] by PCR fusion [27]. For the *dpy-7*^{prom}*∷hst-6*^{mut} construct, the template for PCR fusion was genomic DNA from OH281 [*mgIs18; lon-2(e678) hst-6(ot17)*], which harbors a premature stop codon in *hst-6* [28]. All constructs were injected at 2.5 ng/μl into *evIs82b* (*Is[unc-129∷gfp]*) using *pceh-22∷gfp* at 50 ng/μl as injection marker and *pBS* at 50 ng/μl as carrier.

Immunohistochemistry

Worms were prepared for immunohistochemistry using a freeze crack protocol [29] followed by 20min fixations in ice cold acetone and methanol. Worms were then incubated for 30min in PBS containing 10% donkey serum and in the same buffer at 4C over night with antibody AO4B08 or HS4C3 (1:5)[15,34]. The immunohistochemical stain was developed using the monoclonal anti-VSV antibody P5D4 (dilution 1:250) and Alexa labeled (555) donkey anti mouse antibodies (dilution 1:250).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Lee JS, Chien CB. When sugars guide axons: insights from heparan sulphate proteoglycan mutants. Nat Rev Genet 2004;5:923–935. [PubMed: 15573124]
2. Van Vactor D, Wall DP, Johnson KG. Heparan sulfate proteoglycans and the emergence of neuronal connectivity. Curr Opin Neurobiol 2006;16:40–51. [PubMed: 16417999]
3. Bülow HE, Hobert O. The Molecular Diversity of Glycosaminoglycans Shapes Animal Development. Annu Rev Cell Dev Biol. 2006
4. Esko JD, Selleck SB. Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annu Rev Biochem 2002;71:435–471. [PubMed: 12045103]
5. Turnbull J, Powell A, Guimond S. Heparan sulfate: decoding a dynamic multifunctional cell regulator. Trends Cell Biol 2001;11:75–82. [PubMed: 11166215]
6. Park PW, Reizes O, Bernfield M. Cell surface heparan sulfate proteoglycans: selective regulators of ligand-receptor encounters. J Biol Chem 2000;275:29923–29926. [PubMed: 10931855]
7. Bülow HE, Hobert O. Differential sulfations and epimerization define heparan sulfate specificity in nervous system development. Neuron 2004;41:723–736. [PubMed: 15003172]
8. Kramer KL, Yost HJ. Ectodermal syndecan-2 mediates left-right axis formation in migrating mesoderm as a cell-nonautonomous Vg1 cofactor. Dev Cell 2002;2:115–124. [PubMed: 11782319]
9. Hudson ML, Kinnunen T, Cinar HN, Chisholm AD. C. elegans Kallmann syndrome protein KAL-1 interacts with syndecan and glypican to regulate neuronal cell migrations. Dev Biol 2006;294:352–365. [PubMed: 16677626]
10. Gumienny TL, MacNeil LT, Wang H, de Bono M, Wrana JL, Padgett RW. Glypican LON-2 is a conserved negative regulator of BMP-like signaling in Caenorhabditis elegans. Curr Biol 2007;17:159–164. [PubMed: 17240342]
11. Rogalski TM, Williams BD, Mullen GP, Moerman DG. Products of the unc-52 gene in Caenorhabditis elegans are homologous to the core protein of the mammalian basement membrane heparan sulfate proteoglycan. Genes Dev 1993;7:1471–1484. [PubMed: 8393416]
12. Ackley BD, Kang SH, Crew JR, Suh C, Jin Y, Kramer JM. The basement membrane components nidogen and type XVIII collagen regulate organization of neuromuscular junctions in Caenorhabditis elegans. J Neurosci 2003;23:3577–3587. [PubMed: 12736328]
13. Holt CE, Dickson BJ. Sugar codes for axons? Neuron 2005;46:169–172. [PubMed: 15848796]
14. Gilleard JS, Barry JD, Johnstone IL. cis regulatory requirements for hypodermal cell-specific expression of the Caenorhabditis elegans cuticle collagen gene dpy-7. Mol Cell Biol 1997;17:2301–2311. [PubMed: 9121480]
15. Kurup S, Wijnhoven TJ, Jenniskens GJ, Kimata K, Habuchi H, Li JP, Lindahl U, van Kuppevelt TH, Spillmann D. Characterization of anti-heparan sulfate phage display antibodies AO4B08 and HS4E4. J Biol Chem 2007;282:21032–21042. [PubMed: 17517889]
16. Johnson KG, Ghose A, Epstein E, Lincecum J, O'Connor MB, Van Vactor D. Axonal heparan sulfate proteoglycans regulate the distribution and efficiency of the repellent slit during midline axon guidance. Curr Biol 2004;14:499–504. [PubMed: 15043815]
17. Steigemann P, Molitor A, Fellert S, Jackle H, Vorbruggen G. Heparan sulfate proteoglycan syndecan promotes axonal and myotube guidance by slit/robo signaling. Curr Biol 2004;14:225–230. [PubMed: 14761655]
18. Hu H. Cell-surface heparan sulfate is involved in the repulsive guidance activities of Slit2 protein. Nat Neurosci 2001;4:695–701. [PubMed: 11426225]

19. Hao JC, Yu TW, Fujisawa K, Culotti JG, Gengyo-Ando K, Mitani S, Moulder G, Barstead R, Tessier-Lavigne M, Bargmann CI. C. elegans Slit Acts in Midline, Dorsal-Ventral, and Anterior-Posterior Guidance via the SAX-3/Robo Receptor. Neuron 2001;32:25–38. [PubMed: 11604136]

20. Fujisawa K, Wrana JL, Culotti JG. The slit receptor EVA-1 coactivates a SAX-3/Robo mediated guidance signal in C. elegans. Science 2007;317:1934–1938. [PubMed: 17901337]

21. Guimond SE, Turnbull JE. Fibroblast growth factor receptor signalling is dictated by specific heparan sulphate saccharides. Curr Biol 1999;9:1343–1346. [PubMed: 10574766]

22. Irie A, Yates EA, Turnbull JE, Holt CE. Specific heparan sulfate structures involved in retinal axon targeting. Development 2002;129:61–70. [PubMed: 11782401]

23. Kinnunen T, Huang Z, Townsend J, Gatdula MM, Brown JR, Esko JD, Turnbull JE. Heparan 2-O-sulfotransferase, hst-2, is essential for normal cell migration in Caenorhabditis elegans. Proc Natl Acad Sci U S A 2005;102:1507–1512. [PubMed: 15671174]

24. Pratt T, Conway CD, Tian NM, Price DJ, Mason JO. Heparan sulphation patterns generated by specific heparan sulfotransferase enzymes direct distinct aspects of retinal axon guidance at the optic chiasm. J Neurosci 2006;26:6911–6923. [PubMed: 16807321]

25. McLaughlin D, Karlsson F, Tian N, Pratt T, Bullock SL, Wilson VA, Price DJ, Mason JO. Specific modification of heparan sulphate is required for normal cerebral cortical development. Mech Dev 2003;120:1481–1488. [PubMed: 14654220]

26. Held, LI. Imaginal Discs: The Genetic and Cellular Logic of Pattern Formation. Cambridge University Press; 2002.

27. Hobert O. PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic C. elegans. Biotechniques 2002;32:728–730. [PubMed: 11962590]

28. Bülow HE, Berry KL, Topper LH, Peles E, Hobert O. Heparan sulfate proteoglycan-dependent induction of axon branching and axon misrouting by the Kallmann syndrome gene kal-1. Proc Natl Acad Sci U S A 2002;99:6346–6351. [PubMed: 11983919]

29. Duerr, JS. T.C.e.R. Community. WormBook. WormBook; Immunohistochemistry.

30. Rhiner C, Gysi S, Frohli E, Hengartner MO, Hajnal A. Syndecan regulates cell migration and axon guidance in C. elegans. Development 2005;132:4621–4633. [PubMed: 16176946]

31. Durbin, RM. Studies on the development and organisation of the nervous system of Caenorhabditis elegans. Cambridge, UK: University of Cambridge; 1987. p. 148

32. Sherwood DR, Butler JA, Kramer JM, Sternberg PW. FOS-1 promotes basement-membrane removal during anchor-cell invasion in C. elegans. Cell 2005;121:951–962. [PubMed: 15960981]

33. Toyoda H, Kinoshita-Toyoda A, Fox B, Selleck SB. Structural analysis of glycosaminoglycans in animals bearing mutations in sugarless, sulfateless, and tout-velu. Drosophila homologues of vertebrate genes encoding glycosaminoglycan biosynthetic enzymes. J Biol Chem 2000;275:21856–21861. [PubMed: 10806213]

34. Ten Dam GB, Kurup S, van de Westerlo EM, Versteeg EM, Lindahl U, Spillmann D, van Kuppevelt TH. 3-O-sulfated oligosaccharide structures are recognized by anti-heparan sulfate antibody HS4C3. J Biol Chem 2006;281:4654–4662. [PubMed: 16373349]

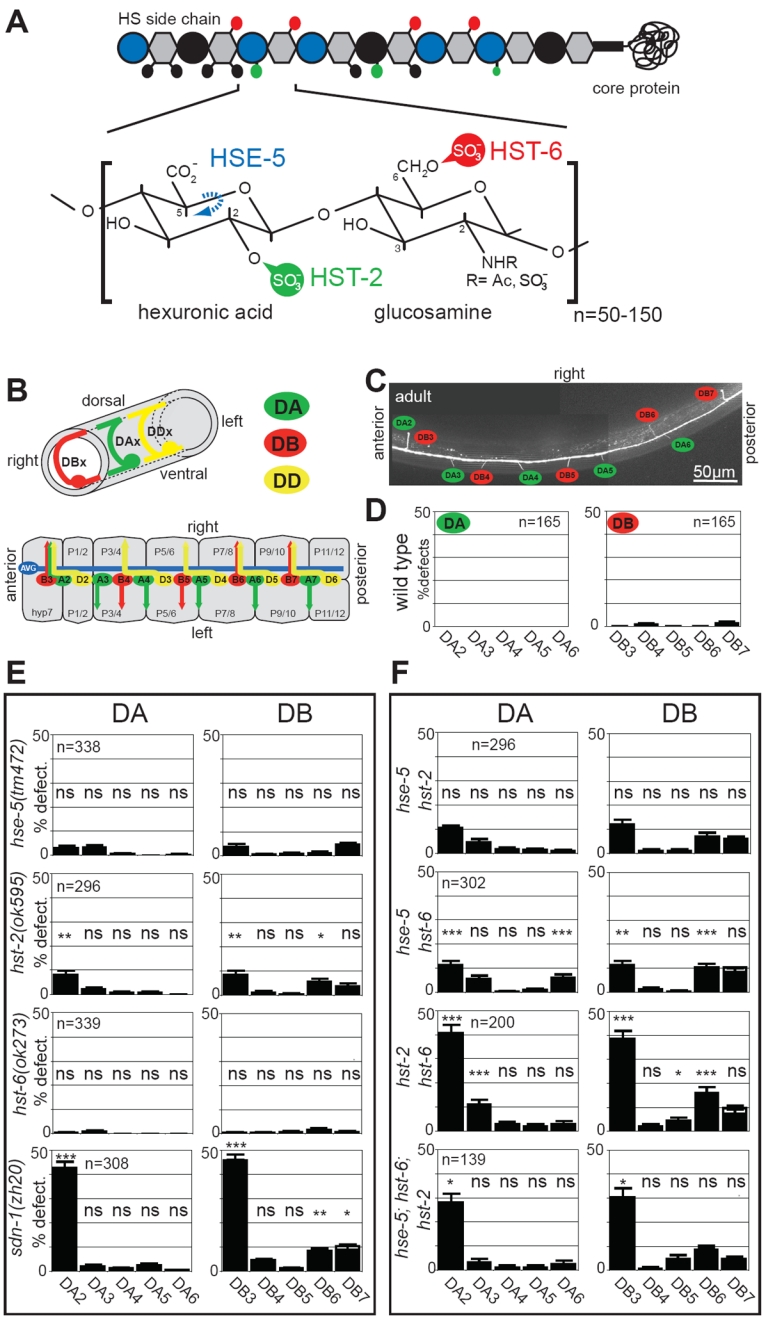

Fig.1. Individual DA/DB motor neurons require distinct combinations of HS modifications

A: Heparan sulfate is a polymer composed of highly modified disaccharide repeats that is attached to a core protein. Shown is a characteristic HS disaccharide repeat unit consisting of a hexuronic acid (circle) and a glucosamine (hexagon) residue and enzymes that modify this structure. Large blue vs. black circles indicate iduronic acid vs. glucuronic acid, respectively. Small circles in black, green and, red indicate modifications as color coded in the disaccharide repeat. HSE-5: HS C5 epimerase; HST-X: HS XO-sulfotransferase (X=2, 6).

B: The upper panel shows the circumferential axonal trajectory of exemplary DA, DB and DD-type eMNs. eMNs are distinguished by their axonal projection patterns and by the neurotransmitter, which they express. The lower panel shows a schematic “open book”

representation of the 2- to 3-fold embryonic stage when eMN axon outgrowth occurs, illustrating that individual DA, DB eMNs show a distinct sidedness of circumferential axonal growth. The eMN cell bodies shown here lie on top of the junctions between bilaterally symmetry P1/2, P3/4, P5/6, P7/8, P9/10 and P11/12 hypodermal cells. More anteriorly and posteriorly positioned eMNs are not shown (see Experimental Procedures). The only other axon that populates the VNC at that stage is the axon of the AVG pioneer neuron. See Supplementary Table 1 for a systematic comparison of individual motor neuron class members.
C: Visualizing the DA and DB motor axons with an *unc-129∴gfp* expressing transgene.
D: The left/right asymmetric, circumferential DA, DB axon projection patterns are highly stereotyped. We focus our scoring on the DA2 to DA6 and DB3 to DB7 motor neurons since those express the *gfp* reporter transgene most reliably. “% defect.” indicates percentage of animals with a defective sidedness of the respective DA or DB axon as indicated.
E: Loss of HS modifications affect DA and DB axon choices in a cell-type specific manner. All mutants used are null mutants [7,30]. Single mutants were statistically compared to wild type (see D.); ns = not significantly different; * $P<0.05$, ** $P<0.005$.
F: Comparison of effects of single, double and, triple HS mutants on specific motor axons. Double mutants were statistically compared to the more severe of two respective single mutants; ns = not significantly different; * $P<0.05$, ** $P<0.005$, *** $P<0.0005$. The defects in both DA2 and DB3 are weakly statistically significant when comparing the triple *hst-2 hst-6 hse-5* mutant and the syndecan null mutant ($p=0.04$ and 0.03, respectively). However, these differences are not significant between the *hst-2 hst-6* double mutant and either the *sdn-1* mutant or the *hst-2 hst-6 hse-5* triple mutant suggesting that the phenotype of the *sdn-1* and the *hst-2 hst-6 hse-5* triple mutant is very similar. See methods for statistics.

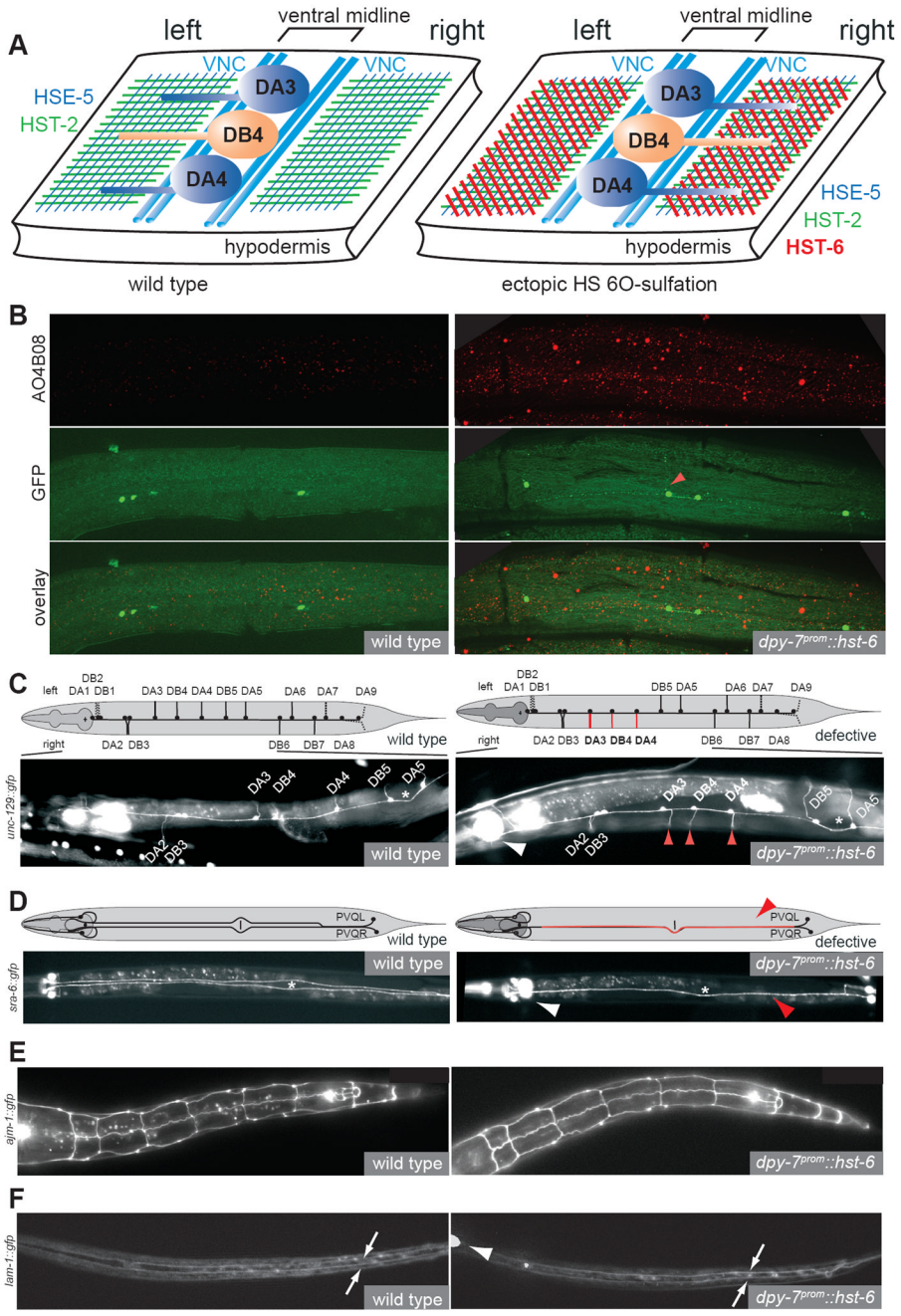

Fig.2. Manipulating HS modification patterns redirects motor axons

A: Schematic view of the ventral cord with HS modifications in the hypodermis indicated by colored hatchings. Note that unlike in larvae, the embryonic midline, defined as a physical barrier between the left and right ventral fascicles, is not constituted by a hypodermal evagination but by motor neuron cell bodies [31].

B: Immunostaining with the AO4B08 HS specific antibody [15] of transgenic animals, which ectopically express the HS modification enzyme *hst-6* in the hypodermis (right panels; *otIs176*). Increased hypodermal staining observed in transgenic animals. This increased staining is not seen in isogenic controls, which do not ectopically express *hst-6* (left panels). Intestinal staining (not shown) serves as an internal staining control as does an unrelated

synthetic antibody (MPB49), which shows no staining at all. Note that the antibody AO4B08 does not recognize individual HS modifications but rather an oligosaccharide, which requires the activities of both, *hst-2* and *hst-6* [15]. Since only *hst-2* but not *hst-6* appears to be expressed in the hypodermis [7], the observed staining of the hypodermis following misexpression of *hst-6* in this tissue are consistent with the known specificities of AO4B08. Note the aberrant projection of DB6 in the *hst-6* misexpressing line (red arrowhead).

C: Axonal misrouting of DA/DB motor neurons in animals with ectopic *hst-6* expression in the hypodermis (right panel, *otEx1711*). Dashed lines indicate neurons that were not scored and, affected neurons are in red. See Fig.3 and Suppl. Fig.3 for quantification of defects.

D: Midline crossover defects of PVQ interneurons in animals with ectopic *hst-6* expression in the hypodermis (right panel, *otEx1711*).

E: Hypodermal cell fate as well as cell morphology, as visualized by normal AJM-1 expression and localization patterns, is not visibly affected by ectopic *hst-6* expression (right panel, *otIs176*).

F: Basement membrane topology, as visualized by a *lam-1::gfp* reporter (kind gift of David Sherwood)[32], is not visibly affected by ectopic *hst-6* expression (right panel, *otIs176*). Arrows point to the basement membrane that ensheathes the ventral midline, and which visually appears most concentrated on each side of the midline as a result of optical sectioning. Left panels represent wild type controls in all cases. White arrowheads point to pharyngeal expression of *gfp* denoting the presence of the hypodermal *hst-6* misexpression array and red arrowheads indicate axonal routing errors. All views are ventral aspects with anterior to the left.

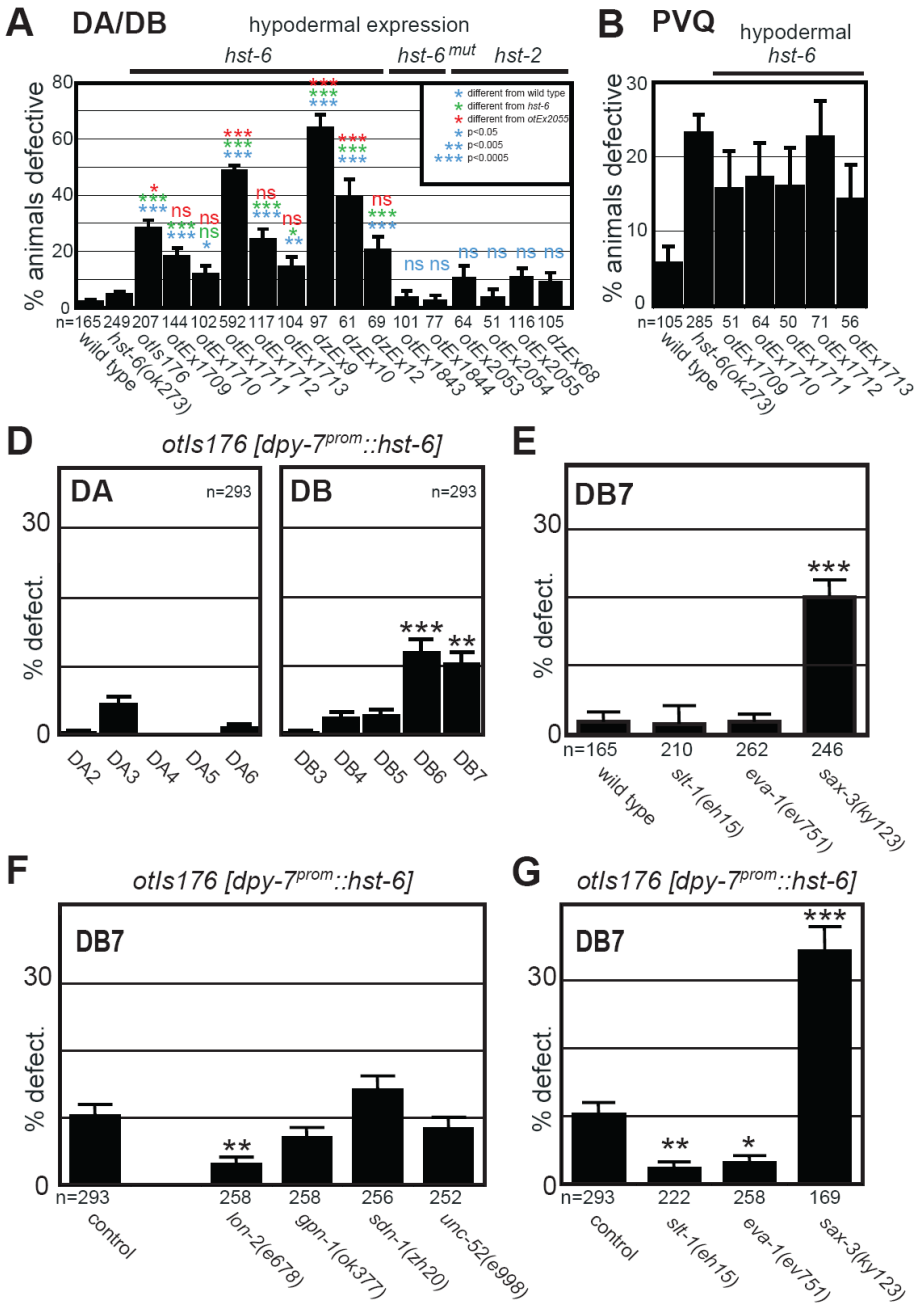

Fig.3. Genetic analyses of *hst-6*-dependent axonal misrouting phenotypes

A: Quantification of DA/DB motor neuron defects in different transgenic strains that express the indicated constructs from the hypodermal specific *dpy-7* promoter. Shown is the percentage of animals with any number (≥1 per animal) of DA or DB axon sidedness defects.

B: Quantification of midline cross over defects in PVQ interneurons in different transgenic strains that express *hst-6* from the hypodermal specific *dpy-7* promoter.

C: Effect of ectopic *hst-6* expression on individual DA and DB neurons. One representative line is shown here, 5 more extrachromosomal lines are shown in Suppl. Fig.2B. Shown is the percentage of animals with defects in individual DA/DB motor axons projections as indicated.

D: Defects in DB7 motor axon projection patterns in different mutants of the Slit/Robo signaling pathway as indicated. Shown is the percentage of animals with defects in DB7 motor axon projections.
E: Effect of genetic removal of HS core proteins on projection errors in DB7 motor axons as a result of ectopic *hst-6* expression. Shown is the percentage of animals with defects in DB7 motor neuron projections. Statistic comparisons are made to the transgenic control. The projection pattern of DB7 in animals that misexpress *hst-6* in the hypodermis and are also mutant for *lon-2*, is statistically indistinguishable from non-transgenic, wild-type animals.
F: Effect of removal of genes in the Slit/Robo signaling pathway on projection errors in DB7 motor axons as a result of ectopic *hst-6* expression. Shown is the percentage of animals with defects in DB7 motor axon projections. Statistic comparisons are made to the transgenic control. The projection pattern of DB7 in animals that misexpress *hst-6* in the hypodermis and are also mutant for *slt-1* or *eva-1*, is statistically indistinguishable from non-transgenic, wild-type animals. Statistical significance is indicated by: ns, not significantly different, * $P<0.05$, ** $P<0.005$, *** $P<0.0005$ in all panels.

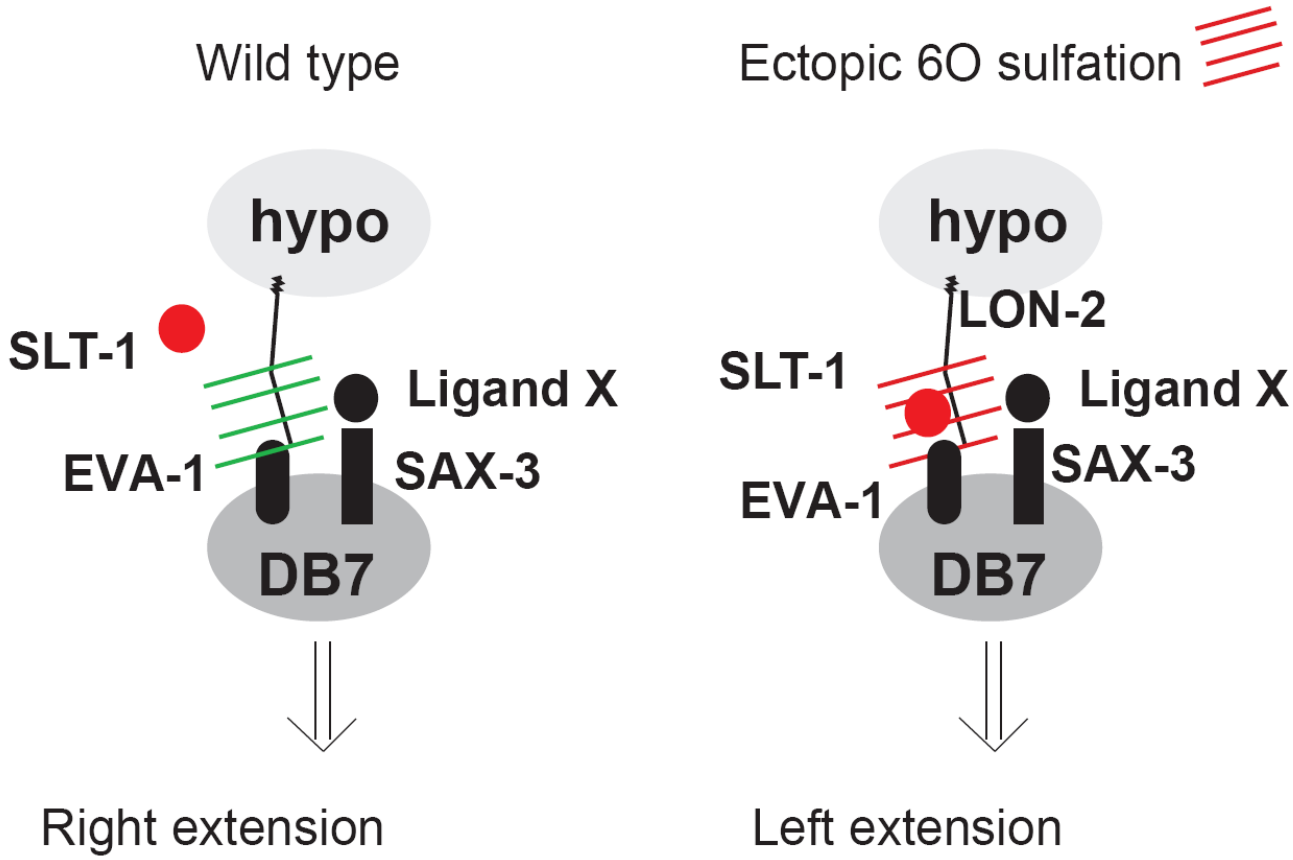

Fig.4. A model for the role of HS in redirecting axonal projections

A possible interpretation of the genetic data presented in Fig.3. In wild-type animals, *slt-1* or *eva-1* is not required for DB7 neuron projections (as *slt-1* and *eva-1* mutants show no axonal defects; Fig.3D), while *sax-3* does normally have a role in DB7 axon extension (Fig.3D). In wild-type animals, SAX-3 may therefore be interacting with an as yet unknown ligand X. Upon introducing ectopic 6O-sulfation patterns (red hatches), an interaction of the SLT-1 protein with EVA-1 may be promoted, which supersedes the effect normally induced by SAX-3/ Ligand X. We emphasize that these conclusions are based on purely genetic arguments and that more indirect mechanistic models can be envisioned which are not shown here.