

The Molecular Diversity of Glycosaminoglycans Shapes Animal Development

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Abstract

Proteoglycans (PGs), molecules in which glycosaminoglycans (GAGs) are covalently linked to a protein core, are components of the extracellular matrix of all multicellular organisms. Sugar moieties in GAGs are often extensively modified, which make these molecules enormously complex. We discuss here the role of PGs during animal development, emphasizing the *in vivo* significance of sugar modifications. We explore a model in which the modification patterns of GAG chains may provide a specific code that contributes to the correct development of a multicellular organism.

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INTRODUCTION

Animal development involves the differentiation of individual cell types and their assembly into specific tissue and organ types. For example, the morphological differentiation of neurons and the generation of specific functional domains in the brain require extensive cell migrations and specific axonal and dendritic growth processes. The extracellular space plays an important role in these processes, as cells have to interact with structures present in the extracellular environment.

The extracellular environment is composed of a complex array of biopolymers, commonly termed extracellular matrices. Ex-

tracellular matrices show several structural specializations. For example, the basement membrane is a specialized, electron-dense extracellular matrix that separates distinct cell types. In addition to key protein components like laminins and collagens, a large portion of extracellular matrices and basement membranes is composed of proteoglycans (PGs), which are proteins with covalently attached glycosaminoglycan (GAG) polysaccharide side chains. These protein-bound GAGs are classified, according to their sugar composition, as keratan sulfate(s) (KS), chondroitin sulfate(s) (CS), dermatan sulfate(s) (DS), and heparan sulfate(s) (HS) (**Figure 1**). The GAGs exhibit complex modification patterns consisting predominantly of sulfations of hydroxyl groups and epimerizations of specific carbon atoms of individual sugar molecules (**Figure 1**). Hyaluronan, a fifth GAG, shows no modifications and is not attached to a protein backbone (Spicer et al. 2002; Toole 2001, 2004). It is therefore not discussed here.

Modifications of sugar residues create enormous molecular diversity, and GAGs have been suggested to be the most information-dense biological molecules (Turnbull et al. 2001). For example, a mere octasaccharide allows for more than one million theoretical combinations of modification patterns. Because the GAG side chains found in vivo are usually 50–150 disaccharide repeat units long, the combinatorial possibilities are rendered nearly boundless (Esko & Lindahl 2001, Esko & Selleck 2002).

A number of articles have recently reviewed the significance of PGs in various experimental systems, with a focus on core proteins and GAG chains, including results from biochemical studies (Esko & Selleck 2002, Häcker et al. 2005, Kramer & Yost 2003, Lee et al. 2004, Lin 2004, Van Vactor et al. 2006). Although GAGs and their core proteins have important physiological and homeostatic roles, e.g. during inflammation and the immune response (Li et al. 2002, Park et al. 2001, Wang et al. 2005), we concentrate

PG: proteoglycan

GAG: glycosaminoglycan

KS: keratan sulfate(s)

CS: chondroitin sulfate(s)

DS: dermatan sulfate(s)

HS: heparan sulfate(s)

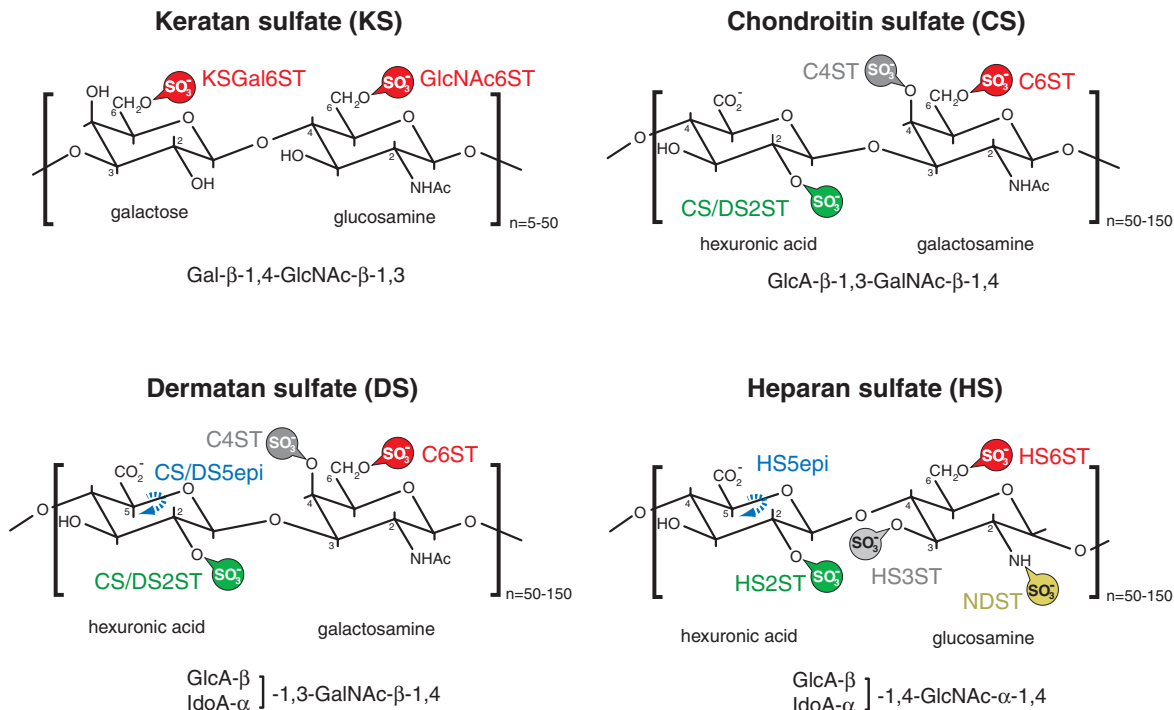


Figure 1

Disaccharide units of glycosaminoglycans (GAGs). Chemical structures of the typical disaccharide repeat units that make up the respective GAG chains. KS, CS, DS, and HS can carry two, three, four, and five modifications on their disaccharide repeat units, respectively. Each modification is shown in a different color and with the enzyme that catalyzes the reaction (see also **Figure 2** and **Figure 3**). In contrast to CS, DS contains iduronic acid, which is epimerized by the CS/DS-C5-epimerase (CS/DS5epi). Abbreviations used: C4ST, CS-4O-sulfotransferase; C6ST, CS-6O-sulfotransferase; CS/DS2ST, CS/DS-2O-sulfotransferase; Gal, galactose; GalNAc, N-acetyl-galactosamine; GlcA, glucuronic acid; GlcNAc, N-acetyl-glucosamine; GlcNAc6ST, N-acetyl-glucosamine-6O-sulfotransferase; HS2ST, HS-2O-sulfotransferase; HS3ST, HS-3O-sulfotransferase; HS6ST, HS-6O-sulfotransferase; IdoA, iduronic acid; KSGal6ST, KS-galactose-6O-sulfotransferase; NDST, N-deacetylase-N-sulfotransferase.

here on the developmental roles of GAGs. We focus on reviewing (*a*) experiments using genetically amenable model organisms and (*b*) results obtained from the analysis of human disease. We first give a brief overview of the *in vivo* role of various core proteins to which GAGs are attached. We then discuss the *in vivo* role of different biosynthetic enzymes involved in GAG synthesis. Finally, we review recent results obtained in different organisms in regard to GAG modifications and discuss the resulting implications. Taken together, the experiments reviewed here demonstrate the importance of GAGs in animal development

and hint at the exciting possibility that distinct modification patterns in GAGs may provide information in the form of a specific code that is required for metazoan development.

CORE PROTEINS

In this section, we review the evidence available for the *in vivo* function of PG core proteins. These studies clearly establish that PG core proteins are indispensable for the correct development of almost all metazoan organisms that have been studied. The classification into core proteins that carry KS, CS, DS, or

Hyaluronan: a glycosaminoglycan consisting of glucuronyl-N-acetylglucosamine disaccharide repeat units.

Metazoan: a multicellular organism

SLRP: small leucine-rich-repeat protein

KSPG: keratan sulfate proteoglycan

CSPG: chondroitin sulfate proteoglycan

DSPG: dermatan sulfate proteoglycan

HS is not always strict because there are a considerable number of proteins that bear more than one kind of GAG side chain.

Keratan Sulfate Core Proteins

The large majority of KS core proteins are members of a family of small leucine-rich-repeat proteins (SLRPs) (Iozzo 1999). Based on sequence, exonic structure, and the presence of a distinctive cysteine-rich cluster in the protein's N-terminal portion, SLRPs can be divided into four subclasses (Iozzo 1999; **Table 1**). Only class II and III SLRPs have been shown to be decorated with KS side chains, whereas for class IV SLRPs, KS decoration is predicted on the basis of conserved attachment sites present in the protein sequence. Leucine-rich repeats are protein-protein interaction domains, and SLRPs are believed to have a characteristic horseshoe-like structure in which the concave surface is involved in binding other proteins, e.g., collagen triple helices (Iozzo 1999). The GAG chain attachment sites seem to be located on the convex side of the horseshoe. Binding of collagen triple helices is important for the regulated assembly of collagen into supramolecular structures (fibrils), whereas the role of the GAG chains in this context is less clear.

Most KS core proteins have now been targeted in mice (**Table 1**). KS is most abundant in the cornea and in cartilage/connective tissues (Funderburgh 2000, 2002), and consistent with KS localization and the function of SLRPs in regulating the assembly of collagen fibrils, mouse mutants have varying defects in collagen-rich tissues such as cornea or connective tissues. Mice lacking mimecan/osteoglycin show mild defects in skin fragility that are associated with ultrastructural changes in collagen fibrils (Chakravarti 2003). Lumican-deficient mice exhibit symptoms of skin fragility and defects in collagen structure, whereas fibromodulin knockout mice show primarily defects in cartilage structure (e.g., tendon stiffness). The phenotypes of mice mutant for any of these

genes are, however, strikingly specific and are both overlapping and nonoverlapping. Double-mutant analyses and expression studies indicate that lumican and fibromodulin have redundant functions in certain tissues while serving exclusive functions in others (for a review, see Chakravarti 2003).

In contrast to the predominant cartilage defects seen in the above-mentioned mutants, loss of function of Keratocan/*KERA*, which is almost exclusively expressed in the cornea, causes eye disease in humans. Mutations in *KERA* have been associated with patients suffering from a disorder called cornea plana type 2 (CNA2, MIM 217 300), in which the cornea is lacking the appropriate convex shape and is unable to correctly refract the light coming in through the lens (Pellegata et al. 2000).

Mutations in the predicted KS carrying the class IV SLRP gene *NYX*/nyctalopin also specifically affect eye development (Bech-Hansen et al. 2000, Pusch et al. 2000). Patients harboring mutations in this gene have X-linked complete stationary night blindness (CSNB1, MIM310500). The disease does not seem to be caused by gross morphological eye defects (Bech-Hansen et al. 2000, Pusch et al. 2000) but rather seems to be due to functional defects, possibly in the neuronal circuitry of the visual system. Again, the significance of KS in the etiology of night blindness is not clear, but together these findings are consistent with important functions of KS and keratan sulfate proteoglycans (KSPGs) in eye development.

Chondroitin/Dermatan Sulfate Core Proteins

Chondroitin and dermatan proteoglycans (CSPGs and DSPGs, respectively) have recently attracted much attention as inhibitors of axon growth and have been shown to be important components of the glial scar that prevents axon regeneration (for a review, see Rhodes & Fawcett 2004). These proteins are a heterogeneous class of proteins consisting of lecticans and several other classes of

Table 1 Proteoglycan core proteins

Core protein	Gene [species] ^a	<i>lof</i> ^b phenotype/syndrome	Reference(s)
KS			
SLRPs			
Class II			
Fibromodulin	Fmod [Mm]	Abnormal collagen structure, no obvious phenotypic consequence	Jepsen et al. 2002, Svensson et al. 1999
Lumican	Lum [Mm]	Skin fragility and corneal opacity	Chakravarti et al. 1998, Jepsen et al. 2002
Keratocan	Kera [Mm] <i>KERA</i> [Hs]	Defects in cornea structure Cornea plana <i>lof</i> not reported <i>lof</i> not reported	Liu et al. 2003 Pellegata et al. 2000
PRELP			
Osteoadherin/ osteomodulin			
Class III			
Mimectan/osteolectin	Ogn [Mm]	Collagen abnormalities <i>lof</i> not reported	Tasheva et al. 2002
Class IV			
Nyctalopin	Nob [Mm] <i>NYX</i> [Hs]	Congenital stationary night blindness Congenital stationary night blindness	Gregg et al. 2003 Bech-Hansen et al. 2000, Pusch et al. 2000
CS/DS			
Aggrecan/CSPG1	Agc1 [Mm] <i>AGC1</i> [Hs]	Perinatal lethality (respiratory failure) Spondyloepiphyseal dysplasia, premature osteoarthritis	Watanabe et al. 1994 Gleghorn et al. 2005
Versican/CSPG2	Cspg2 [Mm] CSPG2	Embryonic lethality, heart defects Wagner Syndrome?	Mjaatvedt et al. 1998
Neurocan/CSPG3	Cspg3 [Mm]	No obvious defects	Rauch et al. 2005, Zhou et al. 2001
NG2/CSPG4	Cspg4 [Mm]	Impaired PDGF signaling	Grako et al. 1999
Neuroglycan C/CALEB/CSPG5	Cspg5 [Mm]	Impaired synaptic function	Jüttner et al. 2005
Bamacan/CSPG6	Cspg6 [Mm]	<i>lof</i> not reported	
Brevican/CSPG7	Bcan [Mm]	Impaired hippocampal long-term potentiation	Brakebusch et al. 2002, Rauch et al. 2005
RPTPbeta/phosphacan	Ptprz1 [Mm]	Impaired remyelination after lesion	Harroch et al. 2002, Harroch et al. 2000
Tenascin-C	Tnc [Mm]	Defects in hematopoiesis, synaptic transmission myelination, mild behavioral defects	Cifuentes-Diaz et al. 1998, Forsberg et al. 1996, Kiernan et al. 1999, Ohta et al. 1998, Saga et al. 1992
Tenascin-R	Tnr [Mm]	Reduced axonal conduction velocities	Weber et al. 1999
SLRPs			
Class I			
Asporin	Aspn [Mm]	<i>lof</i> not reported D (aspartic acid) expansion, osteoarthritis	Kizawa et al. 2005
Biglycan	Bgn [Mm]	Osteoporosis	Xu et al. 1998
Decorin	Dcn [Mm]	Skin fragility	Danielson et al. 1997
Class III			
Epiphycan/ PG-Lb/DSPG3	Dspg3 [Mm]	<i>lof</i> not reported	

(Continued)

Table 1 (Continued)

Core protein	Gene [species] ^a	<i>lof</i> ^b phenotype/syndrome	Reference(s)
HS			
Syndecan	Sdc1 [Mm]	Genetically interacts with Wnt-1	Alexander et al. 2000
	Sdc2 [XI]	Left-right axis formation (dominant-negative experiments)	Kramer & Yost 2002
	Sdc3 [Mm]	Defects in feeding behavior	Reizes et al. 2001
	Sdc4 [Mm]	Delayed wound healing, degeneration of fetal blood vessels in the placenta	Echtermeyer et al. 2001, Ishiguro et al. 2001, Ishiguro et al. 2000a, Ishiguro et al. 2000b
	Sdc4 [XI]	Convergent extension defects (knockdown experiments)	Muñoz et al. 2006
	<i>syndecan (sdc)</i> [Dm]	Axon guidance defects, defects in <i>Slit</i> distribution	Johnson et al. 2004, Steigemann et al. 2004
Glypican	<i>sdm-1</i> [Ce]	Specific neuronal/nonneuronal guidance/migration defects	Minniti et al. 2004, Rhiner et al. 2005
	Gpc1 [Mm]	<i>lof</i> not reported	
	Gpc2 [Mm]	<i>lof</i> not reported	
	Gpc3 [Mm]	Developmental overgrowth	Cano-Gauci et al. 1999
	GPC3 [Hs]	Simpson-Golabi-Behmel syndrome, type 1	Pilia et al. 1996
	Gpc4 [Mm]	<i>lof</i> not reported	
	Gpc5 [Mm]	<i>lof</i> not reported	
	Gpc6 [Mm]	<i>lof</i> not reported	
	<i>knypek (gpl4/gpl6)</i> [Dr]	Gastrulation (convergent extension) defects	Topczewski et al. 2001
	<i>dally (dly)</i> [Dm]	Defective division of neuronal precursor cells, impaired formation of morphogen gradients	Nakato et al. 1995
	<i>dally-like (dlp)</i> [Dm]	Segment polarity defects, axonal guidance defects	Baeg et al. 2001, Rawson et al. 2005
Perlecan (Hspg2)	<i>gpn-1</i> [Ce]	<i>lof</i> not reported	
	<i>lon-2</i> [Ce]	<i>lof</i> not reported	
	Hspg2 [Mm]	Embryonic and perinatal lethality, severe developmental defects	Arikawa-Hirasawa et al. 1999, Costell et al. 1999, Rossi et al. 2003
	<i>HSPG2</i> [Hs]	Schwartz-Jampel Syndrome, Dyssegmental dysplasia, Silverman-Handmaker type (DDSH)	Nicole et al. 2000
	<i>trol</i> [Dm]	Embryonic lethal, defects in neuroblast divisions	Park et al. 2003
Collagen XVIII	<i>unc-52</i> [Ce]	Lethal; muscle defects	Rogalski et al. 1993
	Col18a1 [Mm]	Eye defects, abnormal outgrowth of retinal vasculature	Fukai et al. 2002
	<i>COL18A1</i> [Hs]	Knobloch Syndrome, defects in retina maintenance	Sertie et al. 2000
Agrin	<i>cle-1</i> [Ce]	Axonal and neuronal migration defects	Ackley et al. 2001
	Agrin [Mm]	Defective synaptogenesis	Gautam et al. 1996

^a[Ce], *Caenorhabditis elegans* (nematode); [Dm], *Drosophila melanogaster* (fruit fly); [Dr], *Danio rerio* (zebrafish); [Hs], *Homo sapiens* (human); [Mm], *Mus musculus* (mouse); [XI], *Xenopus laevis* (frog).

^b*lof*, loss of function.

proteins, including, e.g., SLRPs. Lecticans are predominantly secreted proteins and include aggrecan, versican, neurocan, and brevican and their derivatives (reviewed by Yamaguchi 2000). Lectican expression is spatially and temporally regulated. For instance, aggrecan and versican seem to be expressed in all connective tissues and neuronal tissues, whereas neurocan and brevican expression is more prevalent in neuronal tissues. Common to all lecticans is a domain structure predicted to share a rod-like central part with two globular ends at the N and C termini, respectively. Whereas the N-terminal globular domain is believed to mediate interactions with hyaluronan, the C-terminal domain (containing a C-lectin domain—hence the name) is involved in providing interactions with tenascins (Yamaguchi 2000). The central portion of the protein, which exhibits considerable variation through alternative splicing, harbors the GAG attachment sites, which are primarily decorated with CS (Yamaguchi 2000). The CS/DS chains are attached to a conserved serine residue within defined consensus attachment sites (**Figure 2**) (Esko & Zhang 1996). However, several lecticans, including aggrecan and neurocan, have also been shown to contain KS, depending on the tissue source from which they were isolated (Yamaguchi 2000). The functional significance of this diversity in aggrecan remains obscure.

Many CS core proteins have now been investigated through loss-of-function analyses in mice, with surprising results. Whereas two lethal mouse mutants, *cmd* (*cartilage matrix deficiency*) and *hdf* (*heart defect*) have molecular lesions in the aggrecan and versican genes, respectively (Mjaatvedt et al. 1998, Watanabe et al. 1994), many knockouts of CSPGs in mice have only mild, if any, detectable defects (**Table 1**). Lecticans are believed to form a three-dimensional matrix, together with tenascins and hyaluronan (Yamaguchi 2000). This network of GAGs and proteins may exhibit an inherent flexibility and redundancy that allows for the loss of one or more com-

ponents of the network. Alternatively, other compensatory mechanisms may exist. For example, quadruple knockouts of neurocan, brevican, tenascin-C, and tenascin-R are still viable and fertile, possibly owing to compensatory upregulation of ECM molecules such as fibulins (Rauch et al. 2005).

Molecular lesions in class I SLRPs affect primarily collagen and bone structure (**Table 1**). Again, some members of this class may be genetically redundant. Biglycan and decorin double-mutant animals show much more severe defects than do the single mutants, although the extent of the defects is also tissue dependent (Young et al. 2003). It is not clear from these studies whether all defects result from impaired collagen stability or may also be the result of inappropriate signal transduction in, e.g., the transforming growth factor (TGF β) signaling pathway (Young et al. 2003).

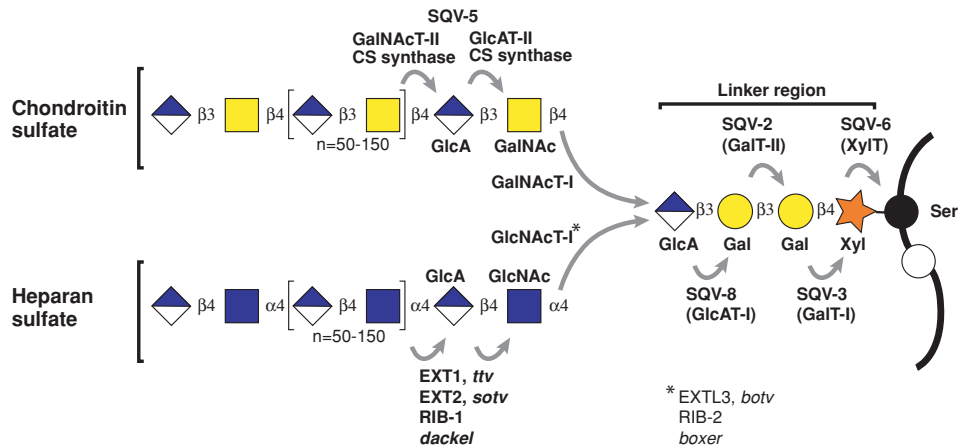
Heparan Sulfate Core Proteins

Heparan sulfate proteoglycans (HSPGs) can be divided in three major classes on the basis of their primary structure. Syndecans are type I transmembrane proteins with an extracellular domain carrying the HS chains near the N terminus and a cytoplasmic tail that typically contains a PDZ-binding domain (**Table 1**) (Bernfield et al. 1999). A second class is composed of glypicans, so called because they are anchored to the membrane via a glycerophosphatidylinositide (GPI). Glypicans share 14 characteristic cysteine residues and are believed to adopt a globular tertiary structure in which GAG chains are attached proximal to the membrane (Bernfield et al. 1999). Both of these membrane-associated HSPGs can be cleaved in the juxtamembrane region to be released from the membrane (Bernfield et al. 1999, Häcker et al. 2005), the biological significance of which is only beginning to emerge (see below). Finally, a third class includes secreted molecules such as perlecan, collagen XVIII, and agrin (**Table 1**),

HSPG: heparan sulfate proteoglycan

Extracellular matrix (ECM): a complex mixture of proteins and proteoglycans that makes up the space between cells

a Step 1: chain synthesis



b Step 2: chain modifications

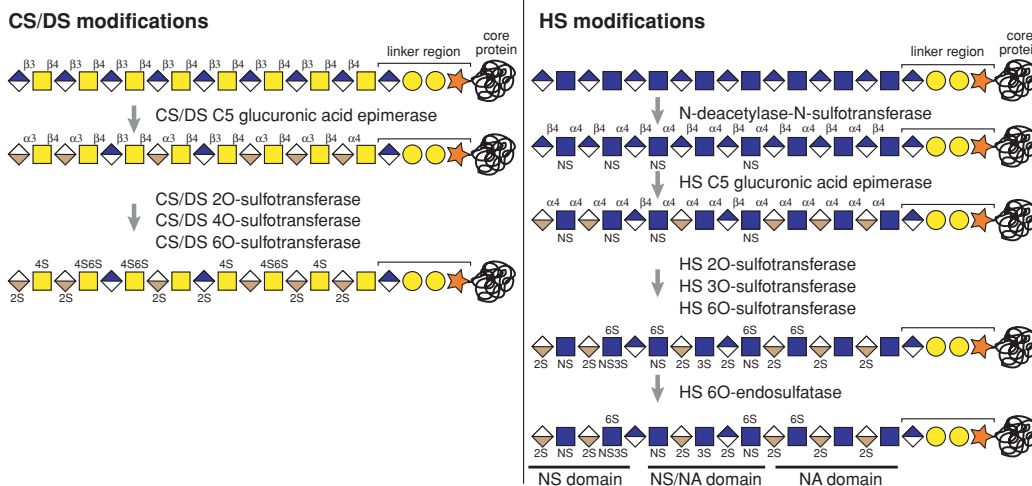


Figure 2

Biosynthesis of heparan, chondroitin, and dermatan sulfates. (a) CS, DS, and HS share the synthesis of the tetrasaccharide linker region that attaches the GAG chains to a serine within the conserved attachment site of the core protein (Esko & Zhang 1996). The activity of a unique galactosyltransferase (GalNAcT-I) or glucosaminyltransferase (GlcNAcT-I) that transfers the first residue on the tetrasaccharide linker commits a growing GAG chain to CS or HS, respectively. The initial attachment step is followed by the activities of specific enzyme complexes that polymerize the GAG chains. Abbreviations used: *ttv*, *tout-velu*; *sotv*, *sister of tout-velu*; *botv*, *brother of tout-velu*. (b) CS and HS chains are modified by a number of Golgi resident epimerases and sulfotransferases that are specific to CS and HS, respectively, creating a remarkable molecular diversity. Modifications in HS occur nonuniformly along the chain, creating distinctly modified domains. Abbreviations used: GalNAcT, galactosaminyltransferase; GalT, galactosyltransferase; GlcAT, glucuronyltransferase; GlcNAcT, glucosaminyltransferase; NA, N-acetylated; NS, N-sulfated; XylT, xylosyltransferase. Individual sugar units and their type of linkage are shown according to convention (Varki et al. 2006).

which are large multidomain proteins (Bernfield et al. 1999).

Vertebrates typically contain four syndecan genes and six glypican genes. In contrast, only one syndecan and two glypicans have been identified in invertebrates (**Table 1**). The HS chains are attached via an invariant tetrasaccharide linker (which HS shares with CS/DS) to a serine within a conserved attachment site (**Figure 2**).

Analyses of loss-of-function alleles of syndecans in vertebrates exhibit surprisingly subtle defects, many of which were discovered only subsequent to initial mis/overexpression experiments (**Table 1**). For example, a physiological role of Sdc3 was uncovered only after a transgenic mouse strain misexpressing Sdc1 in hypothalamic nuclei exhibited abnormal feeding behavior (Reizes et al. 2001), hinting at a functional role for syndecans in the hypothalamus. Subsequent careful analyses of feeding behaviors in mice deficient in Sdc3, the syndecan that is normally expressed in the hypothalamus, confirmed the role of syndecan in regulating feeding behaviors. These experiments and the subtle and tissue-specific defects in different syndecan mutants indicate that syndecan family members may act in a partially redundant manner. In both *Drosophila* and *Caenorhabditis elegans*, mutants in the single syndecan are much more severe and exhibit prominent axonal and cell migration defects (Johnson et al. 2004, Minniti et al. 2004, Rhiner et al. 2005, Steigemann et al. 2004). Genetic experiments suggest that these defects are due at least in part—depending on the cellular context—to impaired signaling of the Slit/Robo axon guidance ligand/receptor cassette, as we discuss further below (Johnson et al. 2004, Rhiner et al. 2005, Steigemann et al. 2004).

Glypican function has also been studied in several invertebrate and vertebrate systems. In zebrafish, *knypek* mutants have molecular lesions in the *gpc4/gpc6* homolog and display cell migration and polarity defects, presumably as a result of impaired Wnt signaling (Topczewski et al. 2001). Studies in *Drosophila*

have shown that both glypicans *dally* and *dally-like* are required to shape morphogen gradients of, e.g., *wingless* and *hedgehog*, although the contribution of the two glypicans for both pathways presumably differ in a tissue-specific manner (Häcker et al. 2005). Adding more complexity to HSPG function, recent studies show that (at least) *dally-like* is shed from the plasma membrane and that this process is required for correct *wingless* signaling. This cleavage is induced by an α/β -hydrolase that is encoded by the *Drosophila* gene *notum*, originally identified as a gene that modulates *wingless* activity (Kirkpatrick et al. 2004, Kreuger et al. 2004).

Mutations in the human *GPC3* gene are associated with Simpson-Golabi-Behmel Syndrome, an overgrowth syndrome characterized by a range of visceral and skeletal abnormalities (Pilia et al. 1996). The molecular mechanisms underlying the disease are still not completely clear but may, at least in part, be caused by inappropriate *Wnt* signaling. In mice, loss of *Gpc3* leads to inhibition of noncanonical *Wnt* signaling and activation of canonical *Wnt* signaling (Song et al. 2005). In cell culture transfection experiments, the HS chains of *Gpc3* seemed to be dispensible for *Gpc3*-mediated activation of noncanonical *Wnt* signaling but could nevertheless potentiate *Gpc3* activity in vivo (Song et al. 2005).

Mutations in the secreted HSPG perlecan have deleterious consequences, and null mutations in all organisms that have been studied are lethal (**Table 1**). In nematodes, *perlecan/unc-52* is required for muscle attachment and growth factor signaling, and complete loss of function leads to mid-embryonic lethality (Merz et al. 2003, Rogalski et al. 1993). In humans, Schwartz-Jampel Syndrome, which is characterized by skeletal defects and developmental eye defects, is likely caused by loss-of-function mutations in the *perlecan* gene (Nicole et al. 2000). In mice, loss of *perlecan* function leads to a variety of defects ranging from severe developmental brain defects to skeletal abnormalities

(Arikawa-Hirasawa et al. 1999). In addition, perlecan is required for proper assembly of functional synaptic basal lamina (Arikawa-Hirasawa et al. 2002). Notably, mice that express a form of perlecan lacking the major HS attachment site are viable and fertile but have small eyes and lenses that progressively degenerate after birth (Rossi et al. 2003). This indicates that some essential functions are associated with the PG core protein alone.

The nonfibrillar collagen XVIII is a multidomain HSPG that is expressed in the basement membrane of nearly all epithelia and endothelia and appears to be part of a higher-order network of integral basement membrane proteins (reviewed in Marneros et al. 2005). Mice homozygous for a null mutation in collagen XVIII have very specific defects in eye development (Fukai et al. 2002) that are similar to the defects observed in human Knobloch Syndrome patients, who harbor a mutation in the human collagen XVIII gene (Sertie et al. 2000). Reminiscent of the situation with CSPGs, there seems to be redundancy between HS-bearing basement membrane proteins, as double mutants between collagen XVIII and HS-deficient perlecan mutants display much more severe eye phenotypes than do the single mutants (Rossi et al. 2003). In *C. elegans* loss of function in the collagen XVIII-like gene *cle-1* leads to multiple defects in axonal development and neuronal migration (Ackley et al. 2001).

Agrin, a multidomain protein, is another major secreted HSPG (for a review, see Bezakova & Ruegg 2003). In mice, agrin is required for correct synaptic development; it regulates the postsynaptic specialization at the neuromuscular junction and in the central nervous system (Bezakova & Ruegg 2003, Gautam et al. 1996). In addition, agrin has also been implicated in the activation of T cells as part of the immunological synapse (Khan et al. 2001). The role of the HS that decorates agrin in these processes is less clear but may involve the formation of multiprotein complexes between agrin and various growth factors (Bezakova & Ruegg 2003).

ENZYMES INVOLVED IN SYNTHESIZING GAG CHAINS

In this section we discuss mutant phenotypes of enzymes that are involved in synthesizing the various GAG chains (**Table 2**). The relevant studies clearly establish that many functions of PGs are mediated by their respective GAG chains rather than by their protein core. In many but not all cases, the latter may be a neutral carrier protein (Bernfield et al. 1999). We include in our discussion enzymes that catalyze reactions involving the sugar units (**Figures 2 and 3**) but omit enzymes and transporters that are involved in providing building components such as the activated monosaccharides or sulfate. Mutations in genes coding for proteins of the latter category are likely to have more pleiotropic consequences, as they may also affect pathways that are unrelated to GAG synthesis. For instance, the enzymes synthesizing PAPS (phosphoadenosylphosphosulfate) as the universal sulfate donor in all sulfation reactions would affect all sulfation reactions, including tyrosine sulfation, which is unrelated to GAG chain synthesis.

Keratan Sulfate Biosynthesis

KS differs from CS, DS, and HS in that it is attached to core proteins via short oligosaccharides rather than the characteristic tetrasaccharide that is common to the latter GAGs. The linkage oligosaccharides may be branched and can be attached to either asparagine (N-linked) or serine/threonine (O-linked) in the respective core protein (**Figure 3a**). On the basis of the structure of these linkage oligosaccharides, KS can be classified into at least three classes (**Figure 3a**) (Funderburgh 2002). Little is known of the specific enzymes synthesizing the oligosaccharides that attach KS to a protein core, but they may be the same enzymes responsible for N/O-glycosylation of proteins with small branched oligosaccharides (Funderburgh 2002).

The KS chain is polymerized onto the oligosaccharides by the alternating actions of

Table 2 Enzymes involved in attachment and polymerization of GAG chains

Enzyme	Gene [species] ^a	<i>lof</i> ^b phenotype/syndrome	Reference(s)
KS attachment/polymerization			
β-1,4-galactosyltransferase-1	Identity unclear	<i>lof</i> not reported	
β-1,3-glucosaminyltransferase	Identity unclear	<i>lof</i> not reported	
CS/DS; HS attachment			
Xylosyltransferase	<i>sqv-6</i> [Ce]	Squashed vulva, embryonic lethality	Hwang et al. 2003a
Galactosyltransferase I	<i>sqv-3</i> [Ce] <i>XGPT1</i> [Hs]	Squashed vulva, embryonic lethality Progeroid Ehlers-Danlos Syndrome	Herman & Horvitz 1999 Okajima et al. 1999, Quentin et al. 1990, Seidler et al. 2006
Galactosyltransferase II	<i>sqv-2</i> [Ce]	Squashed vulva, embryonic lethality	Hwang et al. 2003a
Glucuronyltransferase I	<i>sqv-8</i> [Ce]	Squashed vulva, embryonic lethality	Herman & Horvitz 1999
CS/DS polymerization			
ChSy		<i>lof</i> not reported	
ChSn		<i>lof</i> not reported	
ChSy/ChSn	<i>sqv-5</i> [Ce]	Squashed vulva, embryonic lethality (cytokinesis defects)	Hwang et al. 2003b, Mizuguchi et al. 2003
HS polymerization			
Ext1	Ext1 [Mm] Brain spec. Ext1 [Mm] <i>EXT1</i> [Hs] <i>tout-velu</i> [Dm] <i>rib-1</i> [Ce]	Homozygous lethal (gastrulation defective) Defects in brain morphogenesis (Autosomal-dominant) hereditary multiple exostoses Segment polarity phenotype, defective morphogen gradients <i>lof</i> not reported	Lin et al. 2000, Mitchell et al. 2001 Inatani et al. 2003 Ahn et al. 1995 Bellaiche et al. 1998
Ext2	Ext2 [Mm] <i>EXT2</i> [Hs] <i>dackel (dak)</i> [Dr] <i>sister of tout-velu (sotv)</i> [Dm]	Homozygous: lethal (gastrulation defective); heterozygous: exostoses (Autosomal-dominant) hereditary multiple exostoses Axonal projection defects, fin and branchial arch defects Segment polarity phenotype, defective morphogen gradients	Stickens et al. 2005 Stickens et al. 1996 Lee et al. 2004 Han et al. 2004, Takei et al. 2004
Ext13	<i>boxer (box)</i> [Dr] <i>brother of tout-velu (botv)</i> [Dm] <i>rib-2</i> [Ce]	Axonal projection defects, fin and branchial arch defects Segment polarity phenotype, defective morphogen gradients Embryonic lethality	Lee et al. 2004 Han et al. 2004, Takei et al. 2004 Morio et al. 2003

^a [Ce], *Caenorhabditis elegans* (nematode); [Dm], *Drosophila melanogaster* (fruit fly); [Dr], *Danio rerio* (zebrafish); [Hs], *Homo sapiens* (human); [Mm], *Mus musculus* (mouse); [Xl], *Xenopus laevis* (frog).

^b *lof*, loss of function.

enzymes specific for the individual types of GAGs. Second, the chains are modified by specific sulfotransferases and epimerases (**Figure 2**). It should be noted that, in vivo, polymerization and modifications may occur concomitantly rather than sequentially (Esko & Selleck 2002).

Synthesis of the tetrasaccharide linker region.

In agreement with their structural similarities, CS/DS and HS share the enzymes that synthesize the linker region tethering the sugar chain to a serine residue within the protein backbone (**Figure 2**). In a genetic screen for mutants in *C. elegans* that display an abnormally shaped vulva, mutants in all four genes coding for the enzymes responsible for synthesizing the tetrasaccharide linker region were identified (**Figure 2a**) (Herman & Horvitz 1999, Herman et al. 1999, Hwang et al. 2003a). Mutants in all four genes are maternal-effect lethal; i.e., homozygous animals derived from a heterozygous mother develop to adulthood and display vulval organogenesis defects but then give rise to developmentally arrested embryos. This lethality is likely caused by cytokinesis defects (Hwang & Horvitz 2002). Embryonic phenotypes of certain mutations, e.g., in *sqv-3* and *sqv-8*, are slightly different, which may be due to allelic variability or redundancy

(Herman & Horvitz 1999). A strong maternal contribution of GAG-synthesizing enzymes has also been observed in flies (see below and Perrimon et al. 1996).

In humans, enzymatic defects in galactosyltransferase I, which catalyzes the formation of a β 1,4 glycosidic bond between the first galactose moiety and the xylosyl residue that is attached directly to the serine in the protein, have been associated with progeroid Ehlers-Danlos syndrome (Quentin et al. 1990), a severe condition characterized by mental retardation and connective tissue-related defects. Consistent with this finding, missense mutations have been identified in this gene in patients suffering from the disease. These mutations change highly conserved amino acid residues in the protein, leading to impaired enzymatic activity in vitro (Okajima et al. 1999, Seidler et al. 2006).

Because the linker regions of CS/DS and HS are identical, mutations in the linker-synthesizing enzymes do not distinguish between a requirement for CS/DS or HS synthesis or both. However, they clearly establish that complete lack of CS/DS and HS GAGs is incompatible with metazoan life.

Enzymes that polymerize the GAG chain

CS/DS polymerization. CS/DS GAG synthesis requires the concerted action of three

Figure 3

Structure and biosynthesis of keratan sulfates. (a) Three classes of KS have been described. KSI is N-linked to an asparagine residue in the core protein, whereas KSII and KSIII are O-linked to serine or threonine. KSI is the major corneal KS, yet not exclusive to this tissue. In contrast, KSII is the predominant type of KS in cartilage and is found exclusively linked to aggrecan. KSIII may be a brain-specific version of KS that is attached to serine/threonine via a single mannose. Sulfates are omitted for simplicity. Asn, asparagine; Fuc, fucose; Gal, galactose; GlcNAc, N-acetyl-glucosamine; Man, mannose; NeuAc, neuraminic acid; Ser, serine; Thr, threonine. (b) KS polymerization proceeds by the alternating addition of galactose [by β 4GalT (β 1,4-galactosyltransferase)] and N-acetyl-glucosamine [by β 1,3-GlcNAcT (β 1,3-glucosaminyltransferase)]. Sulfation of N-acetyl-glucosamine [by GlcNAc6ST (N-acetyl-glucosamine 6O-sulfotransferase)] in position 6 appears to occur always on the distal glucosamine, concomitantly with elongation, and may be a prerequisite for polymerization (see text). In contrast, sulfation in position 6 of galactose is catalyzed by KS galactose 6O-sulfotransferase (KSGal6ST). However, not all galactose residues are sulfated, thus creating differentially sulfated domains within the KS chain. In the figure, enzymes are boxed, and sulfotransferases are shown in red. Sulfate modifications are indicated. Individual sugar units and their type of linkage are shown according to convention (Varki et al. 2006).

enzymatic activities that may be encoded by one or more genes. Following synthesis of the tetrasaccharide linker region, a chondroitin N-acetylgalactosaminyltransferase (GalNAcT-I; also known as ChGn) catalyzes attachment of the first galactosamine residue onto the distal glucuronic acid of the linker region (**Figure 2a**) (Sugahara et al. 2003). A chondroitin synthase (ChSy) with GalNAcT-II and GlcAT-II activity then copolymerizes the chondroitin chain by alternately adding glucuronic acid and N-acetylgalactosamine residues (**Figure 2a**). No loss-of-function mutation in either of these enzymes has been reported in vertebrates. The first clues as to the *in vivo* function of chondroitin came again from the squashed vulva screen in *C. elegans* and a concomitant reverse genetic approach (Hwang et al. 2003b, Mizuguchi et al. 2003). *sqv-5* codes for the only ChSy ortholog in *C. elegans* and harbors both ChGn and ChSy activity (**Figure 2a**). *sqv-5*-mutant animals display all the phenotypes of the other *sqv* mutants, thus establishing that chondroitin in *C. elegans* is required for cytokinesis and morphogenesis (Hwang et al. 2003b, Mizuguchi et al. 2003).

HS polymerization. HS is initiated by the addition of GlcNAc to the distal glucuronic acid residue of the linker region (**Figure 2a**). It is this step that commits a growing saccharide chain to either HS or CS/DS. Following initiation, the HS side chain is then synthesized by the alternating addition of glucuronic acid and N-acetylglucosamine, requiring GlcNAcT-II and GlcAT-II transferase activities (**Figure 2a**) (Sugahara & Kitagawa 2002). These activities are provided by enzymes belonging to the exostosis family of proteins (EXTs). EXT genes have been shown to be tumor suppressor genes that upon partial loss of function cause exostoses, a particular type of bone tumors in humans (Ahn et al. 1995, Stickens et al. 1996). In flies, mice, and worms, complete loss of function of the EXT genes causes embryonic lethality, probably owing to inappropriate signaling of

morphogens; this demonstrates that HS is essential for morphogen signaling and viability (Bellaiche et al. 1998, Lin et al. 2000, Morio et al. 2003, Stickens et al. 2005).

More recently, mutations in EXT genes have also been analyzed in nervous system development. By removing EXT1 exclusively in the brain, Inatani and colleagues circumvented the early lethality in the unconditional knockout in mice and showed that brain morphogenesis is severely disrupted in the absence of HS (Inatani et al. 2003). Similarly, mutations in the zebrafish EXT genes *dackel* and *boxer* result in specific defects in axon tract formation in the nervous system (Lee et al. 2004).

ENZYMES INVOLVED IN MODIFYING GAG CHAINS

Following polymerization, the growing GAG chains are subject to a number of modifications that are introduced by resident Golgi enzymes in a nonlinear fashion along the chains (Lindahl et al. 1998). This creates domains that share common basic patterns such as, for example, domains in HS that contain N-sulfated glucosamine, a mix of N-acetylated or -sulfated glucosamine, or merely acetylated domains (**Figure 2b**). Sulfotransferases, epimerases, and sulfatases further modify the GAG chain (**Figure 2b**). In this section we discuss the analysis of loss-of-function mutations in these GAG-modifying enzymes. Obtained in a variety of model systems, recently emerging results provide the best *in vivo* evidence to date that GAG chain modifications play specific roles during development.

Keratan Sulfate Modifications in Development

KS may be modified with a sulfate moiety at position 6O of both the N-acetylglucosamine and the galactose residues (**Figure 3b**). There are several lines of evidence that the glucosamine-6O-sulfotransferase introduces a modification

that is a prerequisite for chain elongation. First, this enzyme can introduce sulfate only on the terminal glucosamine residue. That KS is essentially completely sulfated on its glucosamine residues suggests that sulfation must occur during elongation (see Funderburgh 2002). Second, mutations in this gene have been identified in patients suffering from macular corneal dystrophy (MCD; MIM 217800), an autosomal recessive disorder that leads to opacities in the cornea and eventually blindness (Akama et al. 2000). KS is largely absent in the cornea of patients with the disease, also arguing for the essential role of 6O-sulfation of glucosamine during elongation.

Genetic removal of N-acetylglucosamine-6O-sulfotransferase-1 (also known as Chst2) in mice results in a loss of KS immunostaining in the central nervous system but not in cartilage (Zhang et al. 2006). These animals show reduced glial scar formation in a nerve injury model and increased axonal regeneration (Zhang et al. 2006), demonstrating that not only CS(PGs) but also KS(PGs) may contain anti-regenerative properties. In summary, several N-acetyl-glucosamine-6O-sulfotransferases that are involved in KS synthesis/modification exist in vivo and create tissue-specific modification patterns through nonoverlapping expression patterns.

In contrast, KS-Gal6ST (**Figure 3b**) introduces modifications on the galactose residue in a nonuniform way, thereby creating domains of sulfated and unsulfated galactose. However, no mutational analyses have been conducted for this gene in any organism. In conclusion, KS is an important component of the cornea, and lack of the appropriate amount of KS has deleterious consequences, but more work is required to understand fully the role of keratan sulfation in development.

Chondroitin/Dermatan Sulfate Modifications in Development

CS/DS exhibit complex modification patterns, and in vitro experiments have suggested

functional significance for some of these patterns (for a review, see Sugahara et al. 2003). The modifications are introduced by specific enzymes that transfer sulfate moieties onto defined positions of the sugar residue or convert glucuronic acid to its epimeric iduronic acid (**Figures 1, 2b; Table 3**).

Uchimura et al. (2002) established unambiguously the significance of specific CS modifications in vivo by creating mice deficient in C6ST1 (Chst3), a chondroitin-6O-sulfotransferase (**Figure 2b**). Although mice lacking this enzyme were viable and fertile, they displayed a decreased number of naive T lymphocytes in the spleen. Neuronal anatomy has not been examined in detail in these mouse mutants but is worth testing because enzymatic removal of CS causes axon-targeting defects in the optic system (Chung et al. 2000). Because the mouse genome contains at least two closely related enzymes coding for putative chondroitin-6O-sulfotransferases, these strikingly specific defects may be due to redundancy but nevertheless point to very specific functions of 6O-sulfated CS. As CS is an inhibitor of axon regeneration (Rhodes & Fawcett 2004), it would also be interesting to see whether C6ST-deficient mice display differences in axon regeneration assays, which would make these enzymes interesting as drug targets to promote axonal regeneration.

In contrast to the relatively mild defects in mice, mutations in the human ortholog *CHST3* (C6ST1) are associated with skeletal abnormalities due to severe chondrodysplasia (Thiele et al. 2004). It currently is unclear why the mutation in humans leads to such severe skeletal defects while having only a mild effect on spleen function in mice. One possible explanation may be that requirements for certain modification patterns have diverged between species or, alternatively, that expression patterns between isoenzymes have diverged.

In contrast to the relatively mild defects in a knockout of C6ST1 in mice, a loss-of-function allele of C4ST1, a chondroitin-4O-sulfotransferase, has deleterious effects

Table 3 Enzymes involved in the modification of GAG chains

Enzyme	Gene [species] ^a	<i>lof</i> ^b phenotype/syndrome	Reference(s)
KS			
KS Gal 6O-sulfotransferase	Chst1 (GST-1) [Mm]	<i>lof</i> not reported	
GlcNAc 6O-sulfotransferase	Chst2 (GST-2) [Mm]	Defects in lymphocyte homing, reduced glial scar formation, and enhanced axonal regeneration	Uchimura et al. 2004, Zhang et al. 2006
	<i>CHST6</i> [Hs]	Macular corneal dystrophy	Akama et al. 2000
CS/DS			
CS/DS C5 glucuronyl epimerase	<i>SART2</i> [Hs]	Cloned, but <i>lof</i> not reported	Maccarana et al. 2006
Galactosaminyl uronyl 2O-sulfotransferase	Ust [Mm]	<i>lof</i> not reported	
CS 4O-sulfotransferase	Chst11 (C4st1) [Mm]	Severe chondrodysplasia	Klüppel et al. 2005
	Chst12 (C4st2) [Mm]	<i>lof</i> not reported	
	Chst13 (C4st3) [Mm]	<i>lof</i> not reported	
DS 4O-sulfotransferase	D4st1 [Mm]	<i>lof</i> not reported	
CS 6O-sulfotransferase 1	Chst3 (GST-0) [Mm]	Maintenance of T lymphocytes in the brain	Uchimura et al. 2002
	<i>CHST3</i>	Severe chondrodysplasia	Thiele et al. 2004
CS 6O-sulfotransferase 2	Chst7 (GST-5) [Mm]	<i>lof</i> not reported	
CS 4-sulfate-6O- sulfotransferase	GalNAc4S-6ST	<i>lof</i> not reported	
HS			
N-deacetylase/ N-sulfotransferase	Ndst1 [Mm]	Perinatal lethality due to respiratory failure, forebrain and face abnormalities	Fan et al. 2000, Grobe et al. 2005, Ringvall et al. 2000
	Ndst2 [Mm]	Abnormal mast cells with reduced histamine and protease content	Forsberg et al. 1999, Humphries et al. 1999
	Ndst3 [Mm]	<i>lof</i> not reported	
	Ndst4 [Mm]	<i>lof</i> not reported	
	<i>sulfateless</i> [Dm]	Wingless-dependent segment polarity phenotype	Lin & Perrimon 1999
	<i>hst-1</i> [Ce]	<i>lof</i> not reported	
HS-C5 glucuronyl epimerase	Glce [Mm]	Perinatal death, respiratory failure	Li et al. 2003
	Glce [Dr]	Defects in dorsal/ventral patterning (morpholino-mediated knockdown)	Ghiselli & Farber 2005
	<i>hse-5</i> [Ce]	Specific axon guidance and migration defects	Bülow & Hobert 2004
HS-2O-sulfotransferase	Hs2st [Mm]	Perinatal death; kidney, skeletal, and neuronal defects	Bullock et al. 1998
	<i>pipe</i> [Dm]	Dorsal/ventral patterning defects, likely no HS-modifying enzyme	Sen et al. 1998, Zhu et al. 2005
	CG10234 [Dm]	<i>lof</i> not reported. Likely the bona fide [Dm] Hs2st	Zhu et al. 2005
	<i>hst-2</i> [Ce]	Specific axon guidance and migration defects	Bülow & Hobert 2004, Kinnunen et al. 2005

(Continued)

Table 3 (Continued)

Enzyme	Gene [species] ^a	<i>lof</i> ^b phenotype/syndrome	Reference(s)
HS-3O-sulfotransferase	Hs3st1 [Mm]	Intrauterine growth retardation and lethality in certain genetic backgrounds	HajMohammadi et al. 2003, Shworak et al. 2003
	Hs3st2 [Mm]	<i>lof</i> not reported	
	Hs3st3 [Mm]	<i>lof</i> not reported	
	Hs3st4 [Mm]	<i>lof</i> not reported	
	Hs3st5 [Mm]	<i>lof</i> not reported	
	<i>Hs3st</i> [Dm]	Genetic interaction with <i>Notch</i> (RNAi-mediated knockdown)	Kamimura et al. 2004
<i>bst-3</i> [Ce]	<i>lof</i> not reported		
HS-6O-sulfotransferase	Hs6st1	<i>lof</i> not reported	Bink et al. 2003
	Hs6st2	<i>lof</i> not reported	
	Hs6st3	<i>lof</i> not reported	
	Hs6st [Dr]	Involved in muscle development (morpholino-mediated knockdown)	Kamimura et al. 2001
	<i>bs6st</i> [Dm]	Genetic interaction with <i>breathless</i> (FGFR) (RNAi-mediated knockdown)	
<i>bst-6</i> [Ce]	Specific axon guidance and migration defects	Bülow & Hobert 2004, Bülow et al. 2002	
HS-6O-endosulfatase	Msul1 [Mm]	<i>lof</i> not reported	Dhoot et al. 2001
	Msul2 [Mm]	<i>lof</i> not reported	
	Qsul1 [Cc]	Modulator of Wnt signaling (antisense-mediated knockdown)	

^a[Cc], *Coturnix coturnix* (quail); [Ce], *Caenorhabditis elegans* (nematode); [Dm], *Drosophila melanogaster* (fruit fly); [Dr], *Danio rerio* (zebrafish); [Hs], *Homo sapiens* (human); [Mm], *Mus musculus* (mouse); [Xl], *Xenopus laevis* (frog).

^b*lof*, loss of function.

(Kluppel et al. 2005). Mutant animals show severe skeletal abnormalities and die within hours after birth with severe respiratory problems (Kluppel et al. 2005). The skeletal abnormalities are likely to result from disorganization of the cartilage growth plate, possibly owing to inappropriate TGF β signaling. Like C6ST1 mutants, C4ST1-mutant mice have not been investigated in relation to any neuronal or possible axon regeneration phenotypes.

The substantial range in phenotypes between the C6ST1 and C4ST1 loss-of-function alleles in mice demonstrates the significance of specific modifications in CS/DS. These studies illustrate in vivo that the sulfate residues in CS/DS are not solely negative charges arrayed along the sugar side chains but rather residues that are introduced at defined positions to subserve specific functions.

Although CS has been biochemically identified in *Drosophila* (Toyoda et al. 2000), and orthologs of the CS-synthesizing and -modifying enzymes can be identified in its genome, no mutational analyses have been conducted. In contrast, chondroitin in *C. elegans* does not seem to be sulfated (Toyoda et al. 2000), and consistent with this, no homologous CS sulfotransferase genes can be identified in the *C. elegans* genome (H.E. Bülow, unpublished data).

Heparan Sulfate Modifications in Development

HS contains the largest number of different modifications as compared with KS, CS, and DS (Figure 1). Consequently, HS has the potential to provide a large amount of information content, the significance of which we are only beginning to appreciate (Figure 2b).

The first modification step of the HS chain involves deacetylation and sulfation of the nitrogen atom in N-acetyl-glucosamine (**Figures 1, 2b**) which is catalyzed by the N-deacetylase-N-sulfotransferase (NDST) class of enzymes. This modification seems to be either a prerequisite or a strong facilitator of subsequent modifications (for a review, see Esko & Selleck 2002). N-sulfation occurs nonuniformly along the chain, thereby creating domains from proximal to distal containing nonmodified N-acetyl-glucosamines, stretches in which N-acetyl-glucosamine exists together with N-sulfated glucosamine, and finally domains with nearly exclusively N-sulfated glucosamine (**Figure 2b**; Esko & Selleck 2002). It is not known by what mechanism these domains are formed and what factors may regulate and direct the activity of NDSTs.

The same screen for lethal *Drosophila* mutations that led to the isolation of *tout-velu*, the fly homolog of an HS polymerase (see above), also resulted in the isolation of a gene called *sulfateless*, which codes for the fly homolog of the NDST (Lin & Perrimon 1999). This represents the first in vivo analysis of an HS-modifying enzyme, and the genetic interactions between *sulfateless* and *wingless* establish that sulfation of HS is specifically required for correct growth factor signaling (Lin & Perrimon 1999).

Vertebrates contain four NDST genes, and loss of two of them causes strikingly specific phenotypes (Fan et al. 2000, Forsberg et al. 1999, Grobe et al. 2005, Humphries et al. 1999, Ringvall et al. 2000). Lesions in the NDST2 gene cause severe abnormalities in mast cells (Forsberg et al. 1999, Humphries et al. 1999) that are due to the absence of correctly modified heparin, an intracellular, persulfated version of the extracellular heparan sulfate. In contrast, NDST1 mutants die perinatally of respiratory failure, presumably owing to immature lungs (Fan et al. 2000, Ringvall et al. 2000). These mutants also show low-percentage embryonic lethality and developmental defects of skull and

brain (Ringvall et al. 2000). A closer look at these defects in NDST1-deficient mice established completely penetrant forebrain defects, facial abnormalities, and axon guidance defects probably due to aberrant growth factor signaling (Grobe et al. 2005). These analyses demonstrate that vertebrate development, like fly development, specifically requires sulfation of the GAG chains.

Double mutants of NDST1 and NDST2 knockout mice have more severe phenotypes than do single mutants, suggesting partially redundant roles of the individual NDSTs (Ringvall et al. 2000). Because NDSTs do not display different enzymatic activities, the differences between the single-knockout phenotypes in vertebrates may be a reflection of their nonoverlapping expression patterns.

The HS 2O-sulfotransferase (Hs2st) transfers a sulfate moiety onto the hydroxyl group in position 2 of the hexuronic acid within the HS disaccharides (**Figures 1 and 2b**). Vertebrates and invertebrates have only one obvious Hs2st gene (for a review, see Kusche-Gullberg & Kjellen 2003). Loss-of-function mutations in Hs2st have been studied in mice and worms (Bullock et al. 1998, Bülow & Hobert 2004, Kinnunen et al. 2005). Mice with an enhancer trap mutation in Hs2st display severe developmental kidney defects (Bullock et al. 1998). Additionally, animals show skeletal abnormalities as well as defects in cortical development (Bullock et al. 1998, McLaughlin et al. 2003). Loss of the *C. elegans* Hs2st gene *bst-2* causes axonal guidance defects as well as cell migration defects (Bülow & Hobert 2004, Kinnunen et al. 2005). *Pipe*, a *Drosophila* gene involved in dorsal/ventral patterning, shows similarities to Hs2sts (Sen et al. 1998) yet may not modify HS (Zhu et al. 2005). Indeed, the fly genome contains one gene other than *Pipe* that shows higher similarities to vertebrate and invertebrate Hs2sts (Zhu et al. 2005), but this gene has not been studied functionally.

The epimerase that isomerizes the C5 carbon atom in the hexuronic residue from glucuronic acid to iduronic acid, HS C5

epimerase (HS C5epi) (**Figures 1 and 2b**), is typically only represented by a single copy in both vertebrate and invertebrate genomes. Mice deficient for HS C5epi die perinatally from respiratory failure owing to poorly inflated lungs (Li et al. 2003). Additionally, mutants lack kidneys and have skeletal abnormalities (including polydactyly, twisted tail, and a malformed rib cage). These defects are strongly reminiscent of Hs2st and NDST1 mutants (Bullock et al. 1998, Fan et al. 2000, Ringvall et al. 2000; see above). However, there is at least one important difference: Hs2st mutants seem to have normal lungs, whereas NDST1 mutants have apparently normal kidneys. These data are consistent with the notion that different tissues require specific combinations of HS-modification patterns for their correct development (see below). Zebrafish has two epimerase genes, and knockdown experiments with morpholino analogs lead to defects in dorsal/ventral patterning of the early embryo that are possibly due to impaired bone morphogenetic protein (BMP) signaling (Ghiselli & Farber 2005).

In addition, there are two more known activities that catalyze sulfotransfer reactions onto respective hydroxyl groups in the glucosamine residue: HS 3O-sulfotransferases (Hs3sts) and HS 6O-sulfotransferases (Hs6sts). Unlike in invertebrates, in vertebrates both gene families have expanded; there are at least six Hs3sts and three Hs6sts in mice. Only one Hs3st has been inactivated in mice, resulting in mild defects with intrauterine growth retardation (HajMohammadi et al. 2003, Shworak et al. 2003). Biochemical experiments have shown that HS 3O-sulfation is a crucial component in the antithrombin-binding site of heparin, which mediates antithrombin's anticoagulant activity (for a review, see Esko & Selleck 2002). Although the particular Hs3st enzyme shown biochemically to introduce the modification in the antithrombin-binding site was inactivated in mice, blood coagulation was not affected in these animals

(HajMohammadi et al. 2003, Shworak et al. 2003). Redundancy again may be one possible explanation. Further studies are required to determine which gene(s) code(s) for the enzyme(s) that introduce(s) 3O-sulfate within the antithrombin-binding site of HS in vivo. In flies, Hs3sts have been studied using RNA interference (RNAi)-mediated knockdown techniques, which indicate a role for HS 3O-sulfation in Notch-dependent signaling (Kamimura et al. 2004).

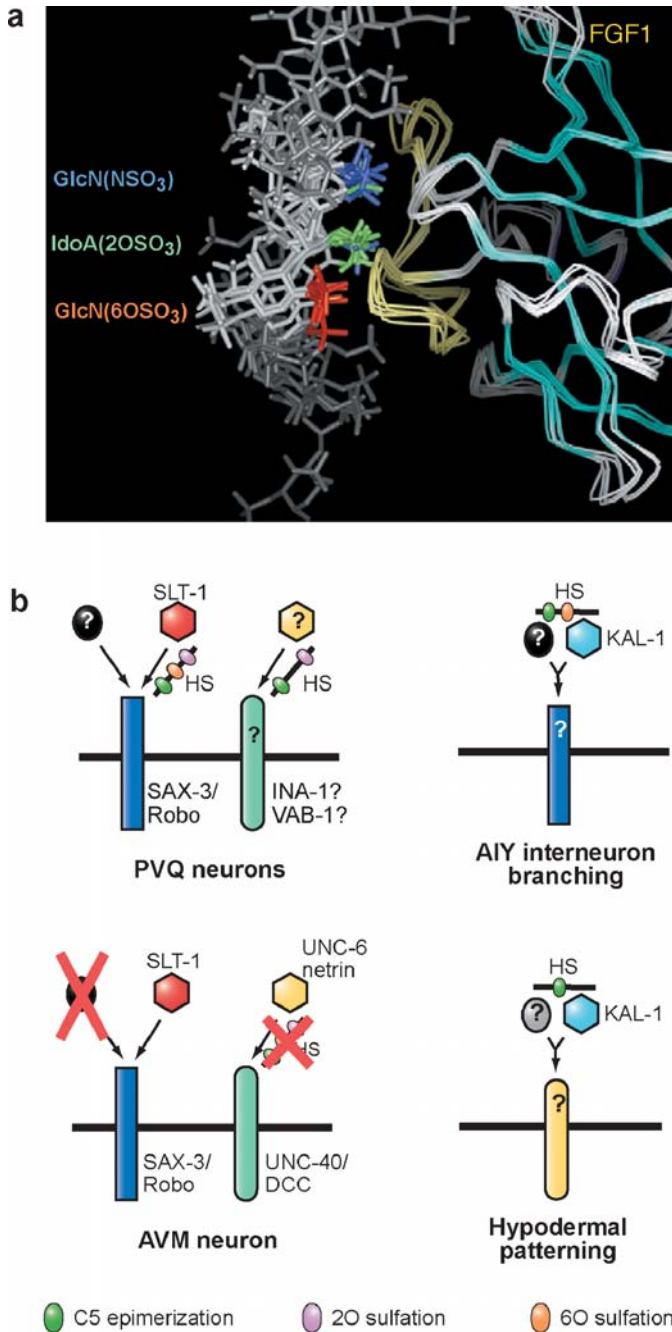
Whereas vertebrates harbor at least three genes coding for Hs6sts, only a single gene is found in flies and worms. Morpholino-mediated knockdown experiments in zebrafish have shown that 6O-sulfation is involved in muscle and vascular development (Bink et al. 2003, Chen et al. 2005), and in flies RNAi-mediated knockdown leads to defects in tracheal development that are very similar to defects observed in the fly mutant *breathless* fibroblast growth factor receptor (FGFR). This result is consistent with the crucial role of 6O-sulfation in fibroblast growth factor (FGF) signaling (Kamimura et al. 2001), as suggested by the crystal structure of FGF/FGFR with heparin (**Figure 4a**) (for a review, see Pellegrini 2001). In *C. elegans*, loss of the Hst6st gene, *hst-6*, leads to cell-specific defects in axon guidance that are similar, yet not identical, to defects caused by loss of function in *hse-5* (the epimerase) or *hst-2* (the HS 2O-sulfotransferase) (Bülow & Hobert 2004). These experiments show that different neurons require different combinations of HS modifications in different cellular contexts. In fact, one class of motor neurons requires distinct HS modifications for different aspects of their development. Longitudinal guidance and fasciculation of these neurons are independent of HS-6O-sulfation yet require *hse-5*- and *hst-2*-mediated HS modifications, whereas dorsal guidance is dependent on all three HS modifications (Bülow & Hobert 2004). These genetic experiments are consistent with combinations of HS modifications serving specific functions during development in a cell-specific manner.

FGFR: fibroblast growth factor receptor

FGF: fibroblast growth factor

All the aforementioned HS modifications are believed to be introduced during HS synthesis in the Golgi apparatus. However, recent work suggests that HS, once it is part of the extracellular matrix, is not static but

may be dynamically modified. This notion is based on the identification of a phylogenetically conserved endosulfatase, *Qsulf1*, which specifically hydrolyzes the 6O-sulfate group of HS (Dhoot et al. 2001). Moreover, antisense experiments show that *Qsulf1* can facilitate Wnt signaling in vitro and may be an antagonist of FGF signaling (for a review, see Lin 2004). Analysis of loss-of-function alleles of *Qsulf* homologs in model organisms will be important in understanding the in vivo significance of this gene.



EMERGING CONCEPTS

Loss of Heparan Sulfate Function Mimics Loss of Function in Signaling Pathways

A common theme throughout all studies is that compromised GAG function, and HS function in particular, leads to remarkably specific defects. Not only are the phenotypes very specific, they are often also

Figure 4

A role for specific HS modifications in signaling. (a) Overlay of all available crystal structures of FGF/heparin complexes. The heparin backbone is colored white where it forms an interface with FGF. In all cases, defined HS modifications make specific contact with FGF. HS modifications are color coded as follows: N-sulfate, blue; 6O-sulfate, red; 2O-sulfate, green. The heparin-binding loop in FGF is shown in yellow. From Pellegrini (2001); copyright by *Current Opinion in Structural Biology*. (b) Different signaling pathways require context-dependent HS modifications, thus providing evidence for the existence of an HS code. Correct pathfinding of the PVQ neurons, mediated by Robo/Slit signaling, requires all three HS modifications indicated, whereas parallel pathways (INA-1, integrin; VAB-1, ephrin receptor) require only two HS modifications. In contrast, axon pathfinding of the AVM neuron is independent of the three HS modifications indicated. KAL-1 also requires different combinations of modifications in a cell-type-specific manner. The receptors/coligands in KAL-1-dependent signaling are unknown. Modified after Bülow & Hobert (2004).

strikingly similar to loss-of-function phenotypes of known developmental signaling pathways (Häcker et al. 2005, Lin 2004).

For example, a screen for genes involved in pattern formation in *Drosophila* not only resulted in the isolation of known mutants in signaling components like *wingless* or *breathless* (FGFR) but also of genes involved in HS biosynthesis or modification. These include *tout-velu* (HS polymerase; see above), *sugarless* (an enzyme required for glucuronic acid biosynthesis and not discussed here), *sulfateless* (HS NDST; see above), *slalom* (a sulfate transporter), and *dally* (a *Drosophila* glypican; see above) (Perrimon et al. 1996). The segment polarity phenotype of these genes is very similar to the phenotype caused by mutations in morphogens like *hedgehog* and *wingless*. Genetic experiments involving the HS mutants show that signaling of the morphogens *wingless* and *hedgehog* are dependent on HS, although, intriguingly, the contribution of HS may be different in different tissues (Häcker et al. 2005, Han et al. 2004).

In addition, HS and HSPGs have a role in shaping the activity gradients of these morphogens, which are believed to regulate target gene expression in a concentration-dependent manner. For example, mutations in the exostosins family of HS polymerases in *Drosophila* show defects in *decapentaplegic* and *wingless* signaling due to changed morphogen gradients (Han et al. 2004, Takei et al. 2004, The et al. 1999). How gradients form is a matter of debate, but they may be the combined result of both passive and active processes including, e.g., diffusion, degradation, and internalization (Kruse et al. 2004, Lander et al. 2002), all of which may be influenced by HS and/or HSPGs. Thus, HS may impinge on growth factor signaling at several levels by modulating signaling as well as by defining the shape of morphogen gradients. It will be interesting to investigate the individual contribution of HS-modification patterns to these processes.

Lastly, a mutation in the HS-modifying enzyme *sulfateless* (NDST) shows defects that

are comparable with those of the FGFR mutants *heartless* and *breathless* (Lin et al. 1999). Indeed, FGFR function in *Drosophila* is dependent on *sulfateless*; that is, it is specifically dependent on sugar modifications of HS (Lin et al. 1999). FGF signaling in *Drosophila* may also be specifically dependent on 6O-sulfation (Kamimura et al. 2001).

During nervous system development, axon guidance by the Slit/Robo signaling cassette is critically dependent on HS, as shown by the fact that mutations in HS-synthesizing enzymes show genetic interactions with Robo mutants in mice and zebrafish (Inatani et al. 2003, Lee et al. 2004). Moreover, Slit/Robo-mediated axon guidance is not only dependent on HS synthesis but on specific HS modifications. In *C. elegans*, loss-of-function mutations in HS-modifying enzymes show characteristic defects in axonal development that are very similar to loss-of-function mutations in the Slit/Robo cassette of guidance cues (Bülow & Hobert 2004). Furthermore, genetic double-mutant analyses suggest that specific combinations of HS are required for correct Slit/Robo signaling (**Figure 4b**; see below) (Bülow & Hobert 2004). This is also consistent with in vitro axon outgrowth assays that show that Slit is dependent on HS for its repulsive activity on axons (Hu 2001).

These studies demonstrate an astonishing degree of complexity of HS function and underscore that HS and, in particular, HS modifications are important components of many signaling pathways. Although HSPGs (e.g., syndecan) may act as bona fide ligands that can induce signaling themselves in both cell-autonomous and -nonautonomous fashions (for reviews, see Bernfield et al. 1999 and Kramer & Yost 2003), HSPGs and HS have more often been described as modulators of signaling rather than as essential factors. This interpretation is based on the observation that both in vitro and in vivo overexpression of a ligand can (at least partially) compensate for loss of HS/HSPG (or of a specific HS modification for that matter).

a

Disaccharide unit	Code	Code	Code
I-HNAc	0	G-HNAc	-0
I-HNS	1	G-HNS	-1
I-HNAc, 3S	2	G-HNAc, 3S	-2
I-HNS, 3S	3	G-HNS, 3S	-3
I-HNAc, 6S	4	G-HNAc, 6S	-4
I-HNS, 6S	5	G-HNS, 6S	-5
I-HNAc, 3S, 6S	6	G-HNAc, 3S, 6S	-6
I-HNS, 3S, 6S	7	G-HNS, 3S, 6S	-7
I2S-HNAc	8	G2S-HNAc	-8
I2S-HNS	9	G2S-HNS	-9
I2S-HNAc, 3S	A	G2S-HNAc, 3S	-A
I2S-HNS, 3S	B	G2S-HNS, 3S	-B
I2S-HNAc, 6S	C	G2S-HNAc, 6S	-C
I2S-HNS, 6S	D	G2S-HNS, 6S	-D
I2S-HNAc, 3S, 6S	E	G2S-HNAc, 3S, 6S	-E
I2S-HNS, 3S, 6S	F	G2S-HNS, 3S, 6S	-F

b

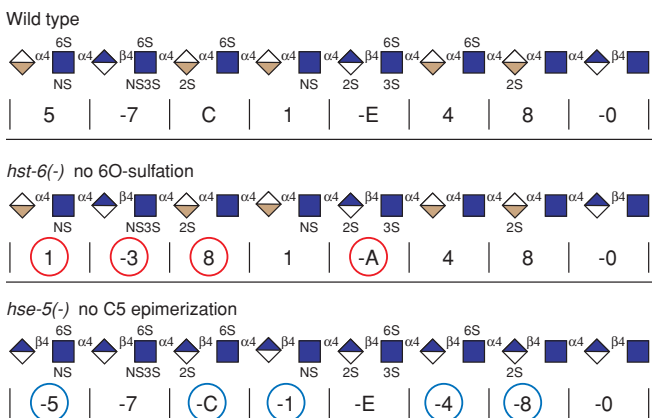


Figure 5

Conceptualizing the HS code hypothesis. (a) A hexadecimal annotation system in which each disaccharide unit is assigned a unique value or code. This value is positive if iduronic acid is contained and negative if the disaccharide contains glucuronic acid (after Venkataraman et al. 1999). For example, I2S-HNAc, 3S designates a disaccharide consisting of iduronic acid 2O-sulfate and N-acetylglucosamine 3O-sulfate and receives the value B, which would become -B if iduronic acid were substituted by glucuronic acid. (b) An imaginary linear HS code is depicted. Sulfations and epimerizations are indicated by different colors. Below the wild-type chain are examples of how the wild-type code could be experimentally manipulated in mutant backgrounds lacking particular HS-modifying enzymes. Resulting changes compared with wild type are circled. *hst-6*, HS-6O-sulfotransferase; *hse-5*, HS-C5-glucuronic acid epimerase.

For instance, overexpression of ligands like *wingless* and *branchless* (FGF) in *Drosophila* can partially rescue the defects in HS mutants (see, for example, Häcker et al. 1997, Lin et al. 1999). Although this suggests that in princi-

ple a ligand may be able to interact molecularly and signal through a receptor independently of HS, genetic studies clearly establish that HS is essential under physiological conditions. In other words, at concentrations normally found in vivo, a ligand may only interact in a biologically meaningful way with its cognate receptor if this interaction is mediated by HS endowed with defined modifications. This interpretation is also consistent with the crystal structure of the FGF:FGFR:heparin complex, which shows that a number of HS modifications form crucial electrostatic bonds with both the ligand and receptor (Figure 4a) (reviewed in Pellegrini 2001).

A Heparan Sulfate Code May Provide Specific and Instructive Information

Given the enormous structural diversity in GAGs and in HS in particular, it is tempting to speculate that this diversity may encode information and perhaps may even constitute an HS code (Figure 5). Specifically, one could envision that specific combinations of HS-modification patterns (Figure 5) may provide a cell-type and/or region-specific “address” that dictates which specific signaling pathways, or combinations thereof, are activated. For example, in one specific region, a defined HS-modification pattern may promote the combinatorial engagement of the Slit and Netrin ligands with their cognate receptors to steer axons in a specific manner, whereas in another region, a different modification pattern prevents efficient binding of these ligands to their receptors and rather promotes binding of, for example, FGF to its receptor. This model provides an intriguing mechanistic basis for the concept that many receptor/ligand systems may act in a combinatorial manner, for example, to guide cellular and axonal migration (Yu & Bargmann 2001). HS molecules are excellent candidates to dictate the combinatorial composition of these ligand/receptor signaling complexes in a cell- and region-type-specific manner.

To be valid, the HS code hypothesis needs to satisfy at least three main criteria. The first criterion is that the information provided by HS modifications (or combinations thereof) is specific. Analyses of mutants in single HS-modifying enzymes in vertebrates demonstrate that specific modifications are important for very distinct aspects of development (Bullock et al. 1998, Fan et al. 2000, Forsberg et al. 1999, Ghiselli & Farber 2005, Humphries et al. 1999, Li et al. 2003, Ringvall et al. 2000, Shworak et al. 2003). One good example are the phenotypes in Hs2st-mutant mice (Bullock et al. 1998), which are complex and do not seem to overlap completely with any one loss-of-function phenotype in signaling genes. For instance, Hs2st mutants display cortical defects that are similar to those of Fgf2 mutants (McLaughlin et al. 2003, Ortega et al. 1998), yet Fgf2 mutants do not have the kidney defects seen in the Hs2st mutant phenotype, and Hs2st mutants do not seem to have the heart defects of Fgf2 mutants (Bullock et al. 1998, Zhou et al. 1998). These experiments suggest that FGF signaling requires HS 2O-sulfation in some but not all cellular contexts and that HS 2O-sulfation regulates different signaling pathways in a cell-specific manner.

Taking this *in vivo* approach one step further, genetic analyses in *C. elegans* showed with single-cell resolution that combinations of HS modifications have cell-specific effects on known cell signaling pathways (Bülow & Hobert 2004). Axon guidance along the ventral nerve cord of *C. elegans* is a well-studied paradigm, and many cues that guide axons at the ventral midline of *C. elegans* have been identified (Hobert & Bülow 2003). For instance, both *sax-3* and *slt-1* (the *C. elegans* genes coding for the Robo/Slit receptor/ligand cassette) are required to guide the axons of one class of interneurons, the PVQs, along the ventral nerve cord (Hao et al. 2001, Zallen et al. 1998). In wild-type animals, PVQ axons never cross the ventral midline but do so in *sax-3*- and *slt-1*-mutant animals. Mutations in *hse-5*, *hst-2*, and *hst-6*, which encode

the HS-modifying enzymes, exhibit strikingly similar phenotypes in PVQ (Bülow & Hobert 2004). Genetic double-mutant analyses between HS-modifying enzymes and Slit/Robo mutants establish that the Robo/Slit signaling cassette requires distinct combinations of HS modifications for correct PVQ development (**Figure 4b**) (Bülow & Hobert 2004). In addition, these studies also show that there are signaling pathways in parallel to the Robo/Slit cassette that may include ephrin receptor and integrin signaling. These parallel pathways require distinct combinations of HS modifications for correct pathfinding of PVQs. Intriguingly, a different neuron (AVM) that requires function of the Robo/Slit cassette for correct ventral guidance (Hao et al. 2001, Zallen et al. 1998) seems completely independent of HS modifications introduced by *hse-5*, *hst-2*, or *hst-6* (Bülow & Hobert 2004). Thus, a given signaling pathway requires distinct combinations of HS modifications in different cellular contexts.

Similar results were obtained by analyzing the *kal-1* signaling pathway (**Figure 4b**). *kal-1* is a cell adhesion molecule defective in Kallmann Syndrome, a human disease characterized by neuronal targeting and migration phenotypes (Rugarli & Ballabio 1993). Misexpression of *kal-1* leads to a variety of defects in *C. elegans*, including axon branching and hypodermal patterning defects (Bülow et al. 2002, Rugarli et al. 2002). These gain-of-function phenotypes are dependent on specific combinations of HS-modifying enzymes, again depending on the cellular context (**Figure 4b**). For example, axon branching induced by *kal-1* requires 6O-sulfation of glucosamine and epimerization of glucuronic acid in HS, whereas the *kal-1*-dependent effects on hypodermal patterning require only epimerization (Bülow & Hobert 2004). The studies involving *sax-3/slt-1* and *kal-1* provide examples of how two signaling pathways require distinct combinations of HS modifications for correct signaling *in vivo*. Vertebrate tissue culture experiments provide further evidence for this notion because

KAL-1: Kallmann Syndrome protein 1

alternative HS structures can elicit distinct FGF-dependent activities (reviewed in Turnbull et al. 2001). Taken together, these results are consistent with the first postulate of an HS code, namely, that HS modifications provide cell-type- and signal-specific information.

The second postulate for the existence of an HS code would be that the code is spatially and presumably temporally regulated. One could imagine that combinations of HS modifications form molecular addresses (or codes; **Figure 5**) that are laid out within an organism to create a three-dimensional molecular map that is read by the cells and the signaling pathways that encounter it. Some available evidence indeed points to this direction. First, biochemical studies demonstrate that HS isolated from the same tissue over the life of an organism displays changes in the modification patterns of disaccharide repeat units (Feyzi et al. 1998), which is consistent with temporal regulation. Second, antibody stainings of different tissues with antibodies that recognize defined HS epitopes also show temporal and spatial regulation of the epitope (Dennissen et al. 2002). Third, the dynamic expression patterns of HS-modifying enzymes in vertebrates and invertebrates (see, for example, Bülow & Hobert 2004, Kinnunen et al. 2005, Sedita et al. 2004) are also consistent with a dynamic extracellular environment in which defined codes may be laid out in a cell-type- and tissue-specific manner. Finally, carbohydrate engagement assays have shown that FGF1 and FGF8 bind to different structures in tissue sections and that this binding is both HS modification dependent and dynamically regulated (Allen & Rapraeger 2003). Taken together, these studies are consistent with spatial and temporal control of HS-modification patterns.

The third postulate of the HS code hypothesis would demand that HS modifications act instructively rather than permissively. Instructiveness of the HS code would predict that changing the code, i.e., intro-

ducing novel modification patterns, would lead to specific defects, possibly through the aberrant engagement of signaling cues. Indeed, misexpression experiments in *C. elegans*, whereby HS enzymes are expressed in tissues in which they may normally not be present, have nonautonomous effects on axon guidance (H.E. Bülow & O. Hobert, manuscript in preparation). Individual motor neurons that in a wild-type situation are independent of HS 6O-sulfation become responsive if spatial modification patterns are altered (i.e., if 6O-sulfation is provided in tissues that usually do not provide this modification). Importantly, these motor neurons do not respond to misexpression of Hs2st, indicating that the observed phenotype is dependent on the regiospecific addition of sulfate in position 6O rather than 2O of N-acetyl-glucosamine (H.E. Bülow & O. Hobert, manuscript in preparation). These studies indicate that HS modifications can act instructively. Moreover, the axon guidance molecule semaphorin 5A can act as a bifunctional cue, eliciting either repulsive or attractive responses (Kantor et al. 2004). Consistent with an instructive role of GAGs, this bifunctionality is determined by the type of GAG, either CS or HS, with which semaphorin 5A interacts (Kantor et al. 2004). These results show that GAGs, and in particular defined modifications, can determine axon guidance and are consistent with the existence of an instructive HS code.

Although the discussed experimental evidence is consistent with the HS code hypothesis, many issues remain to be addressed. Modification patterns of HS molecules need to be determined with sufficient spatial and temporal resolution. How is specificity in the modification patterns of GAG chains controlled? How is the HS code read and interpreted mechanistically? The combination of biochemical methods, such as glycosequencing (Turnbull 2001, Venkataraman et al. 1999), and genetic approaches in model organisms will likely help to address these questions in the future.

SUMMARY POINTS

1. PGs and their attached GAGs are abundant components of the extracellular environment.
2. The functions of PGs are mediated by the core proteins and the GAGs.
3. PGs/GAGs are required for signaling pathways like *wingless*, *hedgehog*, BMP, FGF, Robo, and KAL-1.
4. GAGs such as HS may encode information that is (cell) specific, spatially and temporally regulated, and instructive.

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