Expression of rib-1, a Caenorhabditis elegans Homolog of the Human Tumor Suppressor EXT Genes, Is Indispensable for Heparan Sulfate Synthesis and Embryonic Morphogenesis*  

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Hirosi Kitagawa‡1,2 Tomomi Izumikawa‡1, Souhei Mizuguchi‡1,3, Katsufumi Dejima‡1, Kazuko H. Nomura‡1,4, Noriyuki Egusa‡, Fumiyasu Taniguchi‡, Jun-ichi Tamura‡, Keiko Gengyo-Ando‡4,5, Shohei Mitani‡4,5, Kazuya Nomura‡4, and Kazuyuki Sugahara†‡§¶1,2  

From the ‡Department of Biochemistry, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658-8558, the §Department of Biology, Faculty of Sciences 33, Kyushu University, Fukuoka 812-8581, the ¶Core Research for Evolutional Science and Technology of Japan Science and Technology Agency, Kawaguchi Center Building, 4-1-8, Hon-cho, Kawaguchi, Saitama 332-0012, the †Department of Regional Environment, Faculty of Regional Sciences, Tottori University, Tottori 680-8531, and the **Department of Physiology, Tokyo Women’s Medical University School of Medicine, Tokyo 162-8666, Japan  

The proteins encoded by all of the five cloned human EXT family genes (EXT1, EXT2, EXTL1, EXTL2, and EXTL3), members of the hereditary multiple exostoses gene family of tumor suppressors, are glycosyltransferases required for the biosynthesis of heparan sulfate. In the Caenorhabditis elegans genome, only two genes, rib-1 and rib-2, homologous to the mammalian EXT genes have been identified. Although rib-2 encodes an N-acetylgalcosaminyltransferase involved in initiating the biosynthesis and elongation of heparan sulfate, the involvement of the protein encoded by rib-1 in the biosynthesis of heparan sulfate remains unclear. Here we report that RIB-1 is indispensable for the biosynthesis and for embryonic morphogenesis. Despite little individual glycosyltransferase activity by RIB-1, the polymerization of heparan sulfate chains was demonstrated when RIB-1 was coexpressed with RIB-2 in vitro. In addition, RIB-1 and RIB-2 were demonstrated to interact by pulldown assays. To investigate the functions of RIB-1 in vivo, we depleted the expression of rib-1 by deletion mutagenesis. The null mutant worms showed reduced synthesis of heparan sulfate and embryonic lethality. Notably, the null mutant embryos showed abnormality at the gastrulation cleft formation stage or later and arrested mainly at the 1-fold stage. Nearly 100% of the embryos died before L1 stage, although the differentiation of some of the neurons and muscle cells proceeded normally. Similar phenotypes have been observed in rib-2 null mutant embryos. Thus, RIB-1 in addition to RIB-2 is indispensable for the biosynthesis of heparan sulfate in C. elegans, and the two cooperate to synthesize heparan sulfate in vivo. These findings also show that heparan sulfate is essential for post-gastrulation morphogenetic movement of embryonic cells and is indispensable for ensuring the normal spatial organization of differentiated tissues and organs.  

Heparan sulfate (HS) proteoglycans are found at the surface of most cells and in extracellular matrices of virtually every tissue and play vital roles through their side chains in various biological processes such as cell proliferation, tissue morphogenesis, infection by viruses, and interactions with numerous growth factors, morphogens, and cytokines (for reviews see Refs. 1 and 2). The biosynthesis of HS chains, which governs the expression of these functions, is initiated by the construction of a tetrasaccharide linkage region (GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1–), which is assembled by the stepwise transfer of monosaccharides from respective UDP-sugars to a Ser residue of the core proteins and/or the naked nonreducing terminus through the actions of the respective specific glycosyltransferases. Next, chain elongation, which results in the formation of the repeating disaccharide region of HS, occurs with GlcNAc and GlcUA transferred alternately by the actions of HS copolymerases. Finally, HS chains are modified by GlcNAc N-deacetylase, GlcUA epimerase, and sulfotransferases (for reviews see Refs. 3 and 4). Recently, studies involving cDNA cloning of

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1 To whom correspondence may be addressed: Dept. of Biochemistry, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan. Tel.: 81-78-441-7570; Fax: 81-78-441-7571; E-mail: kitaqawa@kobepharma-u.ac.jp.  
2 Supported by Core Research for Evolutional Science and Technology of the Japan Science and Technology Agency.  
3 To whom correspondence may be addressed: E-mail: smizuscb@mbox.nc.kyushu-u.ac.jp.  
4 To whom correspondence may be addressed: Laboratory of Proteoglycan Signaling and Therapeutics, Graduate School of Life Science, Hokkaido University, Frontier Research Center for Post-Genomic Science and Technology, Nishi-11-chome, Kita-21-jo, Kita-ku, Sapporo, Hokkaido 001-0021, Japan. Tel.: 81-11-706-9054; Fax: 81-11-706-9056; E-mail: k-sugar@sci.hokudai.ac.jp.  
5 The abbreviations used are: HS, heparan sulfate; GFP, green fluorescent protein; GlcAT, glucuronyltransferase; HSN, hermaphrodite-specific neuron; HME, hereditary multiple exostoses; MES, 2-(N-morpholino)ethanesulfonic acid; ECL, enhanced chemiluminescence; Ni-NTA, nickel-nitrilotriacetic acid; NSM, neurosecretory motor; RNAi, RNA interference; DIC, differential interference contrast.
the genes encoding these enzymes and genetic analyses using knock-out mice, Zebrafish, fruit flies, and nematodes have led to unanticipated findings of interesting biological phenotypes (for a review see Ref. 5).

In humans, polymerization of the repeating disaccharide region in HS occurs through the actions of an enzyme complex consisting of EXT1 (6) and EXT2 (7) of the EXT (exostosin) gene family (8–11). These genes were first identified as causative of a genetic bone disorder, hereditary multiple exostoses (HME), and subsequently demonstrated to function as tumor suppressor genes. HME is an autosomal dominant disorder characterized by the formation of a cartilage-capped tumor, caused by mutations in either EXT1 or EXT2 (12). The family of EXT genes has been extended to include three EXT-Like genes, EXTLI, EXTL2, and EXT3, all of which have GlcNAc transferase activities, likely involved in HS synthesis (4, 13, 14).

HS is present in genetically tractable model animals as well (15–17), and EXT genes are well conserved in mammals, Zebrafish (Danio rerio) (ext1, ext2/dackel, and extl3/boxer), Drosophila melanogaster (ttv, sotv, and botv) and Caenorhabditis elegans (rib-1 and rib-2) (18–28). In Zebrafish dackel and boxer mutants, which are defective in ext2 and extl3, respectively, some dorsal retinal ganglion cell axons inappropriately project into the optic tract (19). Even though Dackel and Boxer have not been demonstrated to have glycosyltransferase activities, the amount of HS was drastically reduced in the dackel and boxer mutants, suggesting that both genes are required for the production (19). In addition, Drosophila homologs of EXT genes, tout-velu (ttv), sister of tout-velu (sotv), and brother of tout-velu (botv), which correspond to vertebrate EXT1, EXT2, and EXT3, respectively, are involved in Hh, Wg, and Dpp signaling (20–23). Biochemical and immunohistochemical studies in Drosophila have revealed that HS levels are dramatically reduced in the absence of ttt, sotv, or botv (21–23). We recently demonstrated in vitro that the polymerization is performed by a complex of TTV and SOTV (TTV-SOTV) as an enzyme source (25). However, TTV-SOTV exhibited no GlcNAc transferase-1 (GlcNACT-1) activity required for the initiation of HS, indicating that Bovt, which corresponds to human EXT3 and possesses GlcNACT-1 activity (24), is indispensable for the biosynthesis of HS chains in Drosophila. Thus, all three EXT members in Drosophila, TTV, SOTV, and BOTV, are required for the biosynthesis of a full-length HS.

In C. elegans, rib-1 and rib-2 are most homologous to EXT1 and EXTL3, respectively, among the human EXT genes (26). rib-2 mutants exhibit developmental delay and egg-laying defects, which are most likely caused by a reduction in HS (28). Although rib-2 encodes a GlcNAc transferase involved in initiating the biosynthesis of HS and in the elongation of HS chains (27), the involvement of the protein encoded by rib-1 in HS biosynthesis remains unclear. In this study, we investigated the polymerization of HS using recombinant soluble forms of RIB-1 and RIB-2 to elucidate the mechanism behind the biosynthesis of HS in C. elegans, and we demonstrated that the polymerization was performed by a complex of RIB-1 and RIB-2 as an enzyme source. In addition, to investigate functions of RIB-1 in vivo, we depleted the expression of rib-1 by deletion mutagenesis. Here we demonstrate that in addition to rib-2, rib-1 is indispensable for the biosynthesis of HS and embryonic morphogenesis in C. elegans.

**EXPERIMENTAL PROCEDURES**

**Strains**—Wild type N2 strain and NW1229 strain (evls 111 (F25B3.3;::GFP, dpy-20 (e1362)), a pan-neuronal strain) were obtained from the Caenorhabditis Genetics Center (CGC, Minneapolis, MN). The deletion mutant strains tm516 and tm710 were isolated from pools of worms mutagenized by a combination of treatment with the chemical mutagen trimethylpsoralen and UV light, and they were identified by detecting polymorphisms in the length of the PCR product, as described previously (29). The primers used for the screening and genotyping of the deletion alleles were as follows: For tm516, ExtRev, 5’-AGAGATCCATGAGTGGAGA-3’, ExtFwd, 5’-TTCCAGCTGGACCTCTCCCAT-3’, IntFwd, 5’-CCCATCTTCTCG-TACATCTT-3’, and IntRev, 5’-GTGACACGGAGAACAC- TTC-3’; and for tm710, IntRev, 5’-CGATGAAATGTCGATC- CCCT-3’, ExtRev, 5’-GGCTAGAATCCTCCTCCGA-3’, ExtFwd, 5’-TTGGGACGAGAATTGACTCAT-3’, and IntFwd, 5’-CATTGGTCGCCGAAATGTTG-3’. Because both mutants showed lethal or sterile phenotypes, they were outcrossed four times and balanced with dpy20(e1415) for tm516 and with unc-119(ed3) for tm710.

**Single Worm PCR**—Single animals were picked up with a platinum wire and placed individually in a 10-μl drop of lysis buffer (500 μg/ml proteinase K in 25 mM Tris-HCl, pH 8.5, 50 mM KCl, 0.5% Tween 20, 1 mM EDTA) in a 200-μl PCR tube. The drops were spun down to the bottom of the tube by brief centrifugation, frozen (−80°C, 10 min), and heated (50°C, 30 min followed by 95°C, 20 min). After cooling to room temperature, 1–2 μl of the drops was used as a template for single worm PCR for the tm516 and tm710 deletion alleles with the primers described above.

**Materials**—UDP-[U-14C]GlcUA (285.2 mCi/mmol) and UDP-[3H]GlcNAc (60 Ci/mmol) were purchased from PerkinElmer Life Sciences. Unlabeled UDP-GlcUA and UDP-GlcNAc were obtained from Sigma. Arthrobacter aureus chondroitinase ABC and Flavobacterium hectarinum heparitinases I and II were purchased from Seikagaku Corp. (Tokyo, Japan). N-Acetylheparosan oligosaccharides derived from the capsular polysaccharide of Escherichia coli K5 (30) were gifts from Marion Kusche-Gullberg and Ulf Lindahl, Uppsala University, Uppsala, Sweden. Superdex™ 75 HR10/30 and Superdex™ Peptide HR10/30 columns were supplied by GE Healthcare.

**Construction of Soluble Forms of RIB-1 and RIB-2**—A cDNA fragment of a truncated form of RIB-1, lacking the first 17 amino-terminal amino acids, including the putative cytoplasmic and transmembrane domains, was amplified by reverse transcription-PCR with adult C. elegans total RNA as a template using a 5’-primer (5’-GAAGATCTTGGTCAAGATCATTG-3’) containing a BglII site and a 3’-primer (5’-GGAATCTTAGTCGGATTGAAACCTTATTG-3’) containing a BglII site located 22 bp downstream of the stop codon. In the case of RIB-2, the cDNA fragment encoding a truncated form of RIB-2, lacking the first amino-terminal 38 amino acids of RIB-2 (AB077851), was amplified by reverse transcription-
PCR with adult *C. elegans* total RNA as a template using a 5'-primer (5'-CGGGATCCTCTTTTCCGAGCCTTCTCTG-3') containing a BamHI site and a 3'-primer (5'-CGGGATCCTGAGCACCATTGCA-3') containing a BamHI site located 68 bp downstream of the stop codon. PCR was carried out with KOD polymerase (Toyobo, Osaka, Japan) for 32 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 180 s in 5% (v/v) dimethyl sulfoxide. The PCR fragment was subcloned into the BamHI site of pGIR201protA (31), resulting in the fusion of the insulin signal sequence and the protein A sequence present in the vector as described previously (25). The nucleotide sequence of the site of pGIR201protA (31), resulting in the fusion of the insulin sulfoxide. The PCR fragment was subcloned into the BamHI site of pGIR201protA (31), resulting in the fusion of the insulin signal sequence and the protein A sequence present in the vector as described previously (25). The nucleotide sequence of the amplified cDNA was determined in a 377 DNA sequencer (Applied Biosystems).

**Expression of Soluble Forms of RIB-1 and RIB-2 and Enzyme Assays**—The expression plasmid (6.0 μg) was transfected into COS-1 cells on 100-mm plates using FuGENETM 6 (Roche Applied Science) according to the manufacturer’s instructions. For cotransfection experiments, the RIB-1 and RIB-2 expression plasmids (3.0 μg each) (27) were cotransfected into COS-1 cells on 100-mm plates using FuGENETM 6 as above. After a 2-day culture at 30°C, 1 ml of the culture medium was collected and incubated with 10 μl of IgG-Sepharose (GE Healthcare) for 1 h at 4°C. The beads were recovered by centrifugation, washed with and then resuspended in each of the assay buffers described below, and tested for GlcNAcT-II transforam activity using 10 μg of *N*-acyethyltransferases and GlcNAc, GlcUAβ1-4GlcNAcα1-4GlcUAβ1-4GlcNAcα1-4GlcUAβ1-4GlcNAcα1, and for GlcAT-II activity using 10 μg of GlcNAcα1-4GlcUAβ1-4GlcNAcα1, as an acceptor, respectively, as described previously (25). The assay mixture for GlcNAcT contained 10 μl of the resuspended beads, an acceptor substrate, 0.25 mM UDP-[3H]GlcNAc (8.21 × 10^5 dpm), 100 mM MES buffer, pH 5.8, and 10 mM MnCl2 in a total volume of 20 μl. The assay mixture for GlcAT-II activity contained 10 μl of the resuspended beads, an acceptor substrate, 0.25 mM UDP-[14C]GlcUA (5.66 × 10^5 dpm), 100 mM MES buffer, pH 5.8, and 10 mM MnCl2 in a total volume of 20 μl. A polymerization reaction using 10 μg of *N*-acyethyltransferases and GlcNAc, GlcUAβ1-4GlcNAcα1-4GlcUAβ1-4GlcNAcα1, as an acceptor was conducted in incubation mixtures containing, in a total volume of 20 μl, 0.25 mM UDP-GlcNAc, 0.25 mM UDP-[14C]GlcUA (1.35 × 10^6 dpm), 100 mM MES buffer, pH 5.8, 10 mM MnCl2, and 10 μl of suspended beads. The mixtures were incubated at 26°C overnight, and then radiolabeled products were separated from UDP-[3H]GlcNAc or UDP-[14C]GlcUA by gel filtration chromatography on a column of Superdex™ 75 equilibrated and eluted with 0.2 M NH4HCO3. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

**Western Blot Analysis**—After 3 days of culture at 28°C, the culture medium was collected and incubated with 10 μl of IgG-Sepharose (GE Healthcare) for 1 h at 4°C. The beads recovered by centrifugation were washed with phosphate-buffered saline and then resolved on 7.5% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated for 1 h with mouse IgG antibody. The mouse IgG antibody was diluted 1:1,000 with 25 mM Tris-buffered saline. The bound antibody was detected with anti-mouse IgG conjugated to horseradish peroxidase. Proteins bound to the antibody were visualized with an enhanced chemiluminescence (ECL) advance kit (GE Healthcare).

**Characterization of the Reaction Products**—Products of polymerization reactions on *N*-acyethyltransferases and GlcUAβ1-4GlcNAcα1-4GlcUAβ1-4GlcNAcα1, or GlcUAβ1-3Galβ1-O-C6H4-NH2 were isolated by gel filtration on a column of Superdex™ 75 or Superdex™ Peptide, respectively, with 0.2 M NH4HCO3 as the eluent. The radioactive peak containing the cotransfection reaction product was pooled and evaporated dry. The [14C]GlcUA-labeled oligosaccharide chains were exhaustively digested with 3 ml of papain in a total volume of 20 ml of 50 mM sodium acetate buffer, pH 7.0, containing 2 mM calcium acetate at 37°C overnight. The enzyme digest was analyzed using the same Superdex™ 75 or Superdex™ Peptide column as described above.

**Analysis of Glycosaminoglycans**—Freshly cultured nematode worms were sonicated with a GE-70 ultrasonic processor (Branson Ultrasonics) and freeze-dried. The dried samples (295.2 mg of wild type or 386.3 mg of mutant nematode) were extracted with acetone and then treated with 6 ml of 1.0 M NaBH4, 0.05 M NaOH for 4°C for 20 h. The reaction mixture was neutralized with acetic acid to stop the reaction. The samples were adjusted to 5% trichloroacetic acid and centrifuged. The soluble fraction was extracted with ether. As shown previously (15, 32, 33), the amount of HS in *C. elegans* was so small that 100 μg of shark cartilage chondroitin 6-O-sulfate (Seikagaku Corp.), which contains a negligible proportion of nonsulfated disaccharides, was added as a carrier. The aqueous phase was adjusted to 80% ethanol. The resultant precipitate was dissolved in 50 mM pyridine acetate and subjected to gel filtration on a PD-10 column using 50 mM pyridine acetate as an eluent. The flow-through fraction was collected and evaporated dry. The dried samples were dissolved in water and applied to a column (7 ml) of cat-ion-exchange resin AG 50W-X2 (H+ form; Bio-Rad) pre-equilibrated with water. The unbound fraction containing the liberated O-linked saccharides was neutralized with 1 M NH4HCO3. The purified glycosaminoglycan fraction was digested with chondroitinase ABC or a mixture of heparitinases I and II, and then the digests were derivatized with 2-aminoenzamide and analyzed by high performance liquid chromatography as described previously (34).

**Pulldown Assays**—The cDNA fragment of a truncated form of RIB-2, lacking the first 38 amino-terminal amino acids of RIB-2, was amplified using a 5’-primer (5’-CGGGATCCTCTTTTCCGAGCCTTCTCTG-3’) containing an in-frame BamHI site and a 3’-primer (5’-CGGGATCCTCTTTTCCGAGCCTTCTCTG-3’) containing a BamHI site located 68 bp downstream of the stop codon. The DNA fragment was inserted into the expression vector pcDNA3Ins-His, resulting in the fusion of the protein with the insulin signal sequence and His6 sequence present in the vector. The rib-2 construct and the protein A-tagged RIB-1 expression vector were introduced into COS-1 cells on 100-mm plates using FuGENETM 6 (Roche Applied Science) according to the manufacturer’s instructions. Two days after the cotransfection, 1 ml of the culture medium was collected and incubated with 10 μl of Ni-NTA-agarose (Qiagen) overnight at 4°C. The beads recovered by centrifugation were washed with Tris-buffered saline buffer containing Tween 20...
three times and subjected to SDS-PAGE (7% gel), and proteins were transferred to a polyvinylidene difluoride membrane. The membrane, after blocking in phosphate-buffered saline containing 2% skim milk and 0.1% Tween 20, was incubated with IgG antibody and then treated with anti-mouse IgG conjugated with horseradish peroxidase (GE Healthcare). Proteins bound to the antibody were visualized with an ECL advance kit (GE Healthcare).

Transgenic Construct—The tph-1 (ZK1290.2) reporter gene plasmid was constructed using the vector pFX_DsRedXT (33, 34). The translational fusion construct contained a 3.7-kb genomic fragment, including the 3.1-kb potential promoter region. The fragment was amplified with C. elegans genomic DNA as a template using a 5′-primer (5′-GAGTGAAGACATTAGGGAGT-3′) and a 3′-primer (5′-AGTACGAGTTGTGTAAGAG-3′) located at the start of the 3rd exon. PCR was carried out with Platinum TaqDNA polymerase high fidelity (Invitrogen) for 28 cycles of 94 °C for 30 s, 58 °C for 30 s, and 68 °C for 240 s followed by adenine addition with Taq polymerase at 72 °C for 15 min. The PCR fragment was inserted into pFX_DsRedXT using the TA cloning strategy to fuse the coding sequence region of DsRed.

Phenotypic Analysis—We determined the embryonic phenotypes and extent of lethality of the rib-1/rib-2 mutant worms by monitoring embryonic development with four-dimensional microscopy (a DMRXA full automatic microscope with differential interference contrast and fluorescence optics; Leica) (32) followed by single worm PCR to confirm the genotype. A total of 92 rib-1−/−− worms was monitored from the start of gastrulation until they had reached a terminal phenotype and scored. Phenotypes were also monitored and scored by using a high resolution dissecting microscope (Olympus SZX12) as described previously (36).

Transgenic Rescue Experiment—To rescue the rib-1 deletion mutant tm516, a transgenic rescue construct was created using the C. elegans expression vector (pFX_LVT-R03G5.1) constructed from PFX_venusT cloning vector (35), in which mutant tm516, a transgenic rescue construct was created using marker was used in the experiment.

RESULTS

Coexpression and Characterization of RIB-1—A data base search revealed only two homologs, rib-1 and rib-2, of the mammalian EXT genes in the C. elegans genome (26). Although rib-2 encodes a GlcNACT involved in initiating the biosynthesis and in the elongation of HS (27), the involvement of the protein encoded by rib-1 in HS biosynthesis remains unclear. The RIB-1 protein, composed of 382 amino acids with two potential N-glycosylation sites (Fig. 1A), is about half the size of the RIB-2 protein and the four mammalian EXT family members, EXT1, EXT2, EXT1L, and EXT1L3. Interestingly, the RIB-1 protein shows significant homology to the amino termini of these family members, especially to EXT1 (45% amino acid identity) (Fig. 1A). In fact, a phylogenetic tree of the EXT family members of humans and C. elegans generated based on amino acid sequences showed that RIB-1 and EXT1 are related more closely to one another than to the other members of the family (Fig. 1B). Kyte-Doolittle hydrophathy plot revealed one prominent hydrophobic segment of 18 amino acid residues, which begins at the amino terminus and lacks sufficient length for a membrane-spanning domain. In addition, the hydrophobic segment differs from a membrane anchor in that it contains two cationic residues and is not flanked by cationic residues. Moreover, an analysis using the SOSUI signal system (37) for the prediction of signal peptides and membrane proteins showed that the protein has a signal peptide. These results suggest that RIB-1 appears to be a soluble protein. In this regard, HS d-glucosaminyl 3-O-sulfotransferase is also predicted to be a soluble protein because the protein has only one prominent hydrophobic segment of 18 amino acid residues, which begins at the amino terminus and differs from a membrane anchor in that it contains two glutamine residues and is not flanked by cationic residues (38).

To facilitate the functional analysis of RIB-1, a soluble, protein A-tagged form of the protein was generated by replacing the first 17 amino acids with a cleavable insulin signal sequence and a protein A IgG-binding domain as described under “Experimental Procedures.” Then the soluble protein was expressed in COS-1 cells at 30 °C as a recombinant protein fused with the protein A IgG-binding domain. The fusion protein secreted in the medium was adsorbed onto IgG-Sepharose beads for purification to eliminate endogenous glycosyltransferases, and then the protein-bound beads were used as an enzyme source. Although the bound fusion protein was assayed for glycosyltransferase activity at 26 °C using N-acetylgalactosamino-1-monosaccharides, [GlcUA-GlcNAc]₉, or GlcNAc-[GlcUA-GlcNAc]₉, as a sugar acceptor and either UDP-GlcNAc or UDP-GlcUA as a sugar donor substrate, neither GlcNAcT-II nor GlcAT-II activity was detected (Table 1). However, coexpression of the soluble RIB-1 with the soluble RIB-2 clearly showed GlcAT-II activity, which had not been detected when only RIB-2 was expressed. Notably, these effects of coexpression were not because of differences in the levels of these proteins, as assessed by Western blot analysis (Fig. 2). These results are analogous to the findings previously made for human EXT1 and EXT2 (9). Hence, we examined whether the coexpression of RIB-1 and RIB-2 could result in the expression of polymer-
FIGURE 1. Comparison of *C. elegans* rib-1 with other EXT gene family members and structure of the rib-1 gene. A, alignment of the predicted amino acid sequence of RIB-1 with that of EXT1. Closed boxes indicate that the predicted amino acids in the alignment are identical in the two sequences. Gaps introduced for maximal alignment are indicated by dashes. The putative membrane spanning domain for EXT1 is boxed. Two potential N-glycosylation sites for *C. elegans* RIB-1 are marked with stars. B, phylogenetic tree was generated based on the entire amino acid sequences of RIB-1 and of the EXT proteins reported to date for *Homo sapiens* and *C. elegans*. The GENETYX-MAC (version 10) software was used to produce the sequence alignment and the phylogenetic tree. C, the position of the deletion in the mutant strain (tm516) is indicated (horizontal line).
Heparan Sulfate Biosynthesis in C. elegans

| TABLE 1 | GlcNAcT-II and GlcAT-II activities of the fusion proteins secreted into the culture medium by transfected COS-1 cells |
|---------|-------------------------------------------------------------------------------------------------------------|
| Protein | GlcNAcT-II activity* | GlcAT-II activity* |
|---------|----------------------|--------------------|
| RIB-1   | ND                  | ND                 |
| RIB-2   | 114.2               | ND                 |
| RIB-1/RIB-2 | 172.2           | 6.5               |

* N-Acetylheparosan oligosaccharides, [GlcUA-GlcNAc]n, with the nonreducing terminal GlcUA as an acceptor substrate.
* N-Acetylheparosan oligosaccharides, GlcNAc-[GlcUA-GlcNAc]n, with the nonreducing terminal GlcNAc were used as an acceptor substrate.
* ND, not detected (<0.01 pmol/ml medium/h).
* RIB-1/RIB-2 represents coexpressed RIB-1 and RIB-2.

Interactions between RIB-1 and RIB-2—As shown above, coexpression of RIB-1 and RIB-2 resulted in a marked augmentation of not only glycosyltransferase activities but also polymerase activity. In view of these results, physiological interactions between these molecules were expected. Thus, the interactions of these molecules were evaluated by pulldown assays. For this analysis, a soluble form of RIB-1 fused with protein A at its amino terminus (RIB-1-ProA) and a soluble form of RIB-2 tagged with the His6 epitope at its amino terminus (RIB-2-His) were generated as described under "Experimental Procedures."
mental Procedures.” To evaluate the interactions between RIB-1 and RIB-2, coexpression of RIB-1-ProA with RIB-2-His was carried out. In addition, to ensure specificity, we tried these assays with CPF-1 (PAR2.4; C. elegans chondroitin polymerizing factor), which is expected to interact with SQV-5 (cChSy; C. elegans chondroitin synthase) (32, 33) but not with RIB-1 or RIB-2. Ni-NTA-agarose was added to the culture medium to pull down His-tagged proteins, and the proteins were then subjected to SDS-PAGE followed by Western blotting using an IgG antibody as a primary antibody to protein A-tagged proteins and detected with an ECL advance kit. Lane 1, RIB-1-ProA only; lane 2, RIB-1-ProA and RIB-2-His; lane 3, RIB-1-ProA and PFC-1-His; lane 4, SQV-5 (cChSy)-ProA and PFC-1 (PAR2.4)-His.

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Almost all (99.3% (n = 1622)) of the rib-1−/− fertilized eggs laid by rib-1−/− adult worms showed embryonic lethality and died before the L1 stage. The remaining 0.7% worms hatched and showed abnormal head or tail structures and died just after hatching at L1 (Figs. 5D and 6C). In some cases, axon migration patterns of adult worms hatched from heterozygous worms were severely distorted (Fig. 5F), especially in hermaphroditic-specific neurons (HSNs) (26.5%, n = 34). Two axon neuronal processes from HSNs showed midline crossover defects, whereas in normal N2 worms, they run parallel with each other (Fig. 5G). This phenotype is similar to the HSN abnormality reported on the knock-out of the HS glucuronyl C5-epimerase gene (hse-5) in C. elegans (39). For the Egl phenotype, further detailed study is necessary to know whether the phenotype is related to defects in egg-laying neurons, muscle cell functions, or abnormal vulva structure as found in the protruding vulva.

To determine at what point abnormal development is detected, we monitored 92 rib-1−/− embryos by four-dimensional microscopy. Ingression of E-sister cells seemed to be normal and blastocele was formed. The first abnormality was detected at the “closure of ventral cleft” stage, and worms with an abnormal ventral cleft (80%, n = 10) were observed along with worms with a normal ventral cleft (20%). In severe cases (class I, Fig. 7A), oozing of cells and prominent bulges were observed in various regions of embryos. These phenotypes strongly suggest that normal epidermal enclosure was severely affected (40). All class I embryos were arrested at 1–1.25-fold stages and died (68.4% n = 92). In some cases (class II, Fig. 7B), embryos continued to elongate to the 1.5-fold stage at which point they failed to elongate further and died (21.1%). Oozing of cells from ventral midline region was clearly observed in these embryos. In other cases (class II), embryos developed into 1.5–2.5-fold stages and died (10.5%). Oozing of a few cells was observed. In class I to class III rib-1−/− F1 embryos, active apoptosis was observed after intestinal differentiation (Fig. 6, A and B). Judging from active contractile movement (twisting) in these rib-1−/− embryos (92.4%, n = 92), at least some of the contractile muscle tissues seemed to differentiate normally. In some cases, the differentiation of a pharyngeal bulb-like structure in the head region was observed, and muscles frequently contracted (see Ref. 41 and data not shown). To examine the relative timing of neuronal differentiation and rib-1−/− embryonic death, we crossed rib-1+/− worms with the pan-neuronal GFP reporter strain NW1229 (made and deposited to CGC by J. Culotti), and we examined the differentiation of neuronal cells in rib-1−/− integrated Ex[F25B3.3:gap; dpy-20(+)] and 98.5% of −/− worms developed into the adult stage (n = 404). The successful development of adult worms from rib-1−/− eggs in P0 worms can be attributed to maternal rib-1 mRNA and/or HS proteoglycans from heterozygous worms. The F1 adult worms showed defects in egg-laying (Egl phenotype) (53.5%, n = 143) and accumulated dead embryos in the uterus and were unable to lay eggs from the vulva (Fig. 5, A and B). Among adult worms defective in egg-laying, 65.2% (n = 43) showed a protruding vulva (Pvl) phenotype. In this study, worms having a 10 μm or longer protruding vulva were scored as Pvl worms, and 38.4% of them showed very severe Pvl phenotypes (see Fig. 5C).

 Knocking Out of rib-1 by Deletion Mutagenesis—To understand the functions of the rib-1 gene in vivo, we isolated a deletion mutant of rib-1, tm516, by screening a trimethylpsoralen/ultraviolet-induced deletion mutant library of N2 worms, and we examined the development of the mutant worm by four-dimensional microscopy. The deletion mutant has a 486-bp deletion and a 32-bp insertion (nucleotides 37528/37529 to 38014/38015 in the cosmid F12F6) corresponding to a deletion of most of the 6th intron and 5’ region of the 7th exon of the rib-1 gene (Fig. 1C). The homozygous deletion was lethal or caused sterility, so the mutant worms were maintained as heterozygotes by using a dpy-20(e1415) balancer. Phenotypes were scored by observing and isolating a worm and conducting PCR to determine genotypes of all the worms observed. All rib-1+/− animals were apparently wild type. The rib-1−/− embryos hatched from rib-1+/− worms can develop into adult worms,
embryos. The \( \text{rib-1}^-/^- \) embryos confirmed to be null mutants by single worm PCR with \( \text{rib-1} \) primer pairs showed active neuronal GFP fluorescence (Figs. 5B and 6D). Similar results were obtained in transgenic worms expressing DsRed under the control of the \( \text{tph-1} \) promoter. In the transgenic

N2 worms, differentiated NSM serotonergic neurons were usually stained brightly, and in some worms the differentiated hermaphrodite-specific HSNs were also stained brightly (see Ref. 42 and data not shown). In the transgenic \( \text{rib-1}^-/^- \) worms, bright neuronal fluorescence of possible NSM pair neurons (NSML/NSMR) and neuronal processes were observed in developmentally arrested embryos, suggesting the normal differentiation of these neuronal cells in \( \text{rib-1}^-/^- \) worms (Fig. 6, E and F). In these null mutant embryos, morphogenetic movement stopped in the early stages of elongation and development appeared to be arrested (Fig. 7, A–C). Although some of the neurons and muscle cells seemed to differentiate in the null mutant embryos as described above, further detailed study is necessary to know whether any other cells (including neuronal and muscle cells) show various degrees of defects in the embryos or not.

Similar phenotypes were observed in the \( \text{rib-2}^-/^- \) mutant strain tm710, which was isolated in our laboratory and has a 1306-bp deletion (nucleotide 2128/2129 to 3434/3435) in the cosmid K01G5.6 (Fig. 7, D and E). The tm710 mutant lacks first six exons and five introns and most of the sixth introns. A similar abnormality after gastrulation has been reported for another \( \text{rib-2} \) null mutant (28). Examination of an in situ hybridization data base indicates that \( \text{rib-2} \) mRNA is highly expressed in elongation stage embryos (see NEXTDB on line). These results altogether strongly suggest that HS is indispensable for morphogenesis, including ventral/epidermal enclosure and elongation of embryos. In developmentally arrested embryos of the \( \text{rib-1} \) null mutants, the differentiation of some of the muscles and neurons seemed to proceed normally, but in all abnormal embryos of the null mutants, the spatial organization of these tissues was severely distorted (Fig. 6C). Similar phenotypes (class III) have been observed in \( \text{pps-1} \) (3-phosphoadenosine 5'-phosphosulfate) mutant worms (RNAi) deficient in the sulfation of HS (36) as
large quantities of tures of tm516 worms. Because it was impractical to isolate adult worms (Fig. 5 cued tm516 worms laid eggs, which developed normally into were rescued, and lethal phenotypes were also rescued. Res- rescue of phenotypes was confirmed.

We obtained 20 lines. In all the 20 lines examined, complete plus venus construct driven by elongation factor-1 promoter.

and ventral midline region (arrows) and were arrested at the 1-fold stage with muscular twitching. Prominent bulges in the epidermis were visible (arrowheads). Variable defects in cell move- ments following gastrulation were observed, and some animals resulted in rupture of the embryo possibly because of enclosure failure (data not shown). Images of the same embryo were acquired at intervals of 56 and 152 min, respectively (left to right). B, rib-1 class II animals (~21% of animals) initially appeared normal and started elongation and then internal cells oozed along the ventral midline and were arrested at the 1.5-fold stage. Images of the same embryo were acquired at intervals of 28 and 54 min, respectively (left to right). C, in wild type embryos, ventral enclosure, dorsal intercalation, and epidermal enclosure occurred normally after gastrulation and elongated to the 2-fold stage (430–450-min post-fertilization). No oozing of cells was observed. Images of the same embryo were acquired at intervals of 43 and 49 min, respectively (left to right). D, tm710 rib-2−/− embryo from a rib-2−/− adult worm. Like rib-1 class I animals, cells oozed, and the embryo was arrested at the 1-fold stage. The middle and the right images were acquired at a 38-min interval at one focal plane, and the left image at the different focal plane was acquired 46-min earlier than the middle image. Arrows show oozed cells. E, terminal phenotype of tm710 rib-2−/− embryo. Enclosure failure resulted in rupture of the embryo. Arrows show that internal cells seemed to be ejected through the opening in the epidermis. Scale bar, 20 μm.

as well as pat (paralyzed arrest at 2-fold) mutant worms, including pat-2 (α-integrin) and pat-3 (β-integrin), which are essential for muscle-body wall integration (43), suggesting possible interactions with HS proteoglycans.

Rescue of Null Mutant Phenotypes with Wild Type Gene—To examine whether the above-mentioned phenotypes were because of the lack of rib-1 gene expression, we tried to rescue tm516 worms by introducing wild type rib-1 cDNA. Germ line rescue experiment was performed with full-length rib-1 cDNA plus venus construct driven by elongation factor-1 promoter. We obtained 20 lines. In all the 20 lines examined, complete rescue of phenotypes was confirmed. Egl and Pvl phenotypes were rescued, and lethal phenotypes were also rescued. Rescued tm516 worms laid eggs, which developed normally into adult worms (Fig. 5E).

Reduction of HS Synthesis in rib-1 Knock-out Worms—We next examined the amount of HS and chondroitin in mass cultures of tm516 worms. Because it was impractical to isolate large quantities of rib-1−/− worms because of their lethal con- dition, plate culture samples of tm516 worms possibly containing rib-1−/−, rib-1+/−, and rib-1+/+ worms were used for bio- chemical analysis. The expected ratio of worms containing these genotypes is 1:2:1. Because rib-1−/− worms are expected to die in the later stages of embryogenesis, the rib-1− gene dose in the mass culture is estimated to be 66% that in wild type N2 worms. As shown in Table 2, direct measurements revealed that the amount of HS in the mass culture was reduced to 63% that in the wild type mass culture, whereas the amount of chon- droitin was increased to 143%. Although the change in chon- droitin was unexpected, comparable increases have been

FIGURE 6. Developmental arrest phenotypes in rib-1−/− worms laid by rib-1−/− hermaphrodites. A and B, after intestine differentiation (A), extensive apoptotic cell death (B) was observed in the rib-1−/− embryo. Images of the same embryo were acquired at a 60-min interval. Arrowheads indicate intestinal cells, and arrows indicate apoptotic cells (A and B). C and D, the spatial organization of differentiated tissues and organs was severely distorted in rib-1−/− embryos (C, DIC image). Neuronal differentiation in the null mutant worm was confirmed by neuronal specific fluorescence (D, arrow) in the transgenic worm (see text for details). By using transgenic rib-1−/− worms expressing the DsRed gene under the control of a neuron-specific tph-1 pro- moter, differentiation of NSM neuron pairs (arrows) was detected in dying rib-1−/− embryos (E and F). Scale bar, 20 μm.

FIGURE 7. Arrest of embryonic development after gastrulation stage in rib-1−/− and rib-2−/− null mutants. All subsequent images were collected by four-dimensional microscopy. A, in rib-1 class I animals (~68% of animals, see text), cells oozed from head, tail, and ventral midline region (arrows) and were arrested at the 1-fold stage with muscular twitching. Prominent bulges in the epidermis were visible (arrowheads). Variable defects in cell move- ments following gastrulation were observed, and some animals resulted in rupture of the embryo possibly because of enclosure failure (data not shown). Images of the same embryo were acquired at intervals of 56 and 152 min, respectively (left to right). B, rib-1 class II animals (~21% of animals) initially appeared normal and started elongation and then internal cells oozed along the ventral midline and were arrested at the 1.5-fold stage. Images of the same embryo were acquired at intervals of 28 and 54 min, respectively (left to right). C, in wild type embryos, ventral enclosure, dorsal intercalation, and epidermal enclosure occurred normally after gastrulation and elongated to the 2-fold stage (430–450-min post-fertilization). No oozing of cells was observed. Images of the same embryo were acquired at intervals of 43 and 49 min, respectively (left to right). D, tm710 rib-2−/− embryo from a rib-2−/− adult worm. Like rib-1 class I animals, cells oozed, and the embryo was arrested at the 1-fold stage. The middle and the right images were acquired at a 38-min interval at one focal plane, and the left image at the different focal plane was acquired 46-min earlier than the middle image. Arrows show oozed cells. E, terminal phenotype of tm710 rib-2−/− embryo. Enclosure failure resulted in rupture of the embryo. Arrows show that internal cells seemed to be ejected through the opening in the epidermis. Scale bar, 20 μm.
observed in rib-2 mutants (28), and an analogous increase in HS was observed in sqv-5 (C. elegans) or pfc-1 RNAi-treated worms deficient in chondroitin biosynthesis (32, 33), suggesting levels of HS and chondroitin are interdependent in vivo. In addition, the disaccharide composition of the HS in the mass culture was comparable with that in the wild type mass culture (Table 3). These findings were similar to those obtained for the rib-2 deletion mutant (28) and strongly suggest that the rib-1 mutant is specifically defective in the synthesis of HS and not chondroitin in the nematode C. elegans.

**DISCUSSION**

Recent genetic analyses using the nematode C. elegans together with cell biological and biochemical studies have led to reports exploring the mechanism behind the biosynthesis of glycosaminoglycans and the functions of glycosaminoglycans during development (for a review see Ref. 5). In this study, we demonstrated for the first time that RIB-1 and RIB-2 are indispensable for the biosynthesis of HS chains in vivo. In addition, we showed that in C. elegans, HS is essential for embryonic morphogenesis in the later stages of development.

Although five EXT family genes (EXT1, EXT2, EXT1-like, EXT2-like, and EXT3) have been identified in the human genome, only two homologs, rib-1 and rib-2, have been found in the C. elegans genome. Previously, we revealed that rib-2 encodes GlcNAcT involved in initiating the biosynthesis and in the elongation of HS but not GlcAT-II required for lengthening the HS chains (27). Because HS is found in C. elegans (15, 16), it was predicted that rib-1 encodes the GlcAT-II. However, when a soluble recombinant form of RIB-1 was produced and assayed for GlcAT-II and also GlcNAcT activities, no such glycosyltransferase activities were detected. In this study, despite little glycosyltransferase activity by RIB-1, we showed that the polymerization of HS in C. elegans was achieved by an enzyme complex composed of RIB-1 and RIB-2, which is analogous to the findings made previously for the human EXT1 and EXT2 protein complex (9). In this regard, it should be noted, however, that RIB-1 is unique among members of the EXT family identified to date in that the protein, composed of 382 amino acids, is about half the size of RIB-2 and shows significant homology only to the amino termini of EXT family members, especially to EXT1 (45% amino acid identity, see Fig. 1A). Moreover, RIB-2 is most homologous to human EXT3 (not to EXT2) (see Fig. 1B). In fact, as mentioned above, RIB-2 harbors both GlcNAcT-I and GlcNAcT-II activities involved in the chain initiation and elongation of HS, and its acceptor specificity resembles that of human EXT3 (12, 27), suggesting that rib-2 is a C. elegans EXT3 ortholog (Fig. 1B). These results together indicate that the mechanism of HS biosynthesis in C. elegans is similar but distinct from that in humans and that both RIB-1 and RIB-2 are indispensable for the biosynthesis of HS chains in the nematode.

The human EXT1 and EXT2 genes are involved in HME, an autosomal dominant disorder characterized by the formation of a cartilage-capped tumor, caused by mutations in either EXT1 or EXT2 (12). The family of EXT genes has been extended with the identification of three additional EXT-like genes, EXT1-like, EXT2-like, and EXT3 (3). Although the EXT-like genes are predicted to be involved in the synthesis of HS on the basis of the enzymatic activities of their products in vitro (13, 14), these genes are not linked with HME and have not been associated with any disease pathology, and so their functions in vivo have not been determined. In fact, the polymerization of HS on the linkage analog is achieved by an enzyme complex of EXT1 and EXT2, without the aid of EXT1 proteins (8–10). Because the polymerization of HS was achieved with low efficiency by a complex of RIB-1, the EXT1 counterpart in C. elegans, and RIB-2, the EXT2 counterpart in C. elegans, and the amount of HS in C. elegans was much smaller than that of chondroitin (15, 16) (see Table 2), it is likely that EXT1 and EXT3 can also cooperate to polymerize HS chains albeit with low efficiency in mammals.

**TABLE 2**

**Total amounts of glycosaminoglycan disaccharides**

Values are expressed as picomoles of disaccharide per mg of dried homogenate of the worms. Numbers in parentheses represent percent yields of the chondroitin or HS disaccharides, taking the respective values for disaccharides from the N2 worms (wild type) as 100%. The values are the mean ± S.E. of n determinations.

| Disaccharides | N2 (n = 3) | tm516 (n = 3) |
|---------------|------------|--------------|
|               | pmol/mg (%)| pmol/mg (%)  |
| Chondroitin   | 4477 ± 622 | 6401 ± 830   |
| Heparan sulfate | 6.4 ± 0.2  | 4.0 ± 0.6    |

**TABLE 3**

**Disaccharide composition of C. elegans HS**

For experimental details, see under “Experimental Procedures.” The values are the mean ± S.E. of n determinations.

| Disaccharides     | N2 (n = 4) | tm516 (n = 3) |
|-------------------|------------|--------------|
|                   | mol %      | mol %        |
| HexUAα1–4GlcNac   | 51 ± 2.2   | 52 ± 5.2     |
| HexUAα1–4GlcNac(65) | 11 ± 1.6   | 16 ± 4.4     |
| HexUAα1–4GlcNc(NS) | 26 ± 6.8   | 19 ± 1.4     |
| HexUA(2S)α1–4GlcNc(65) | 8 ± 1.5  | 8 ± 1.8     |
| HexUA(2S)α1–4GlcNc(NS,6S) | 4 ± 2.6  | 5 ± 2.0     |

* ΔHexUA, GlcN, and GlcNac stand for unsaturated hexuronic acid, glucosamine, and N-acetylgalactosamine, whereas 6S, NS, and 2S represent 6-O-sulfate, 2-N-sulfate, and 2-O-sulfate, respectively.*
in the gene trap mouse can interact with EXTL3 and that these two proteins might cooperate to produce short HS chains.

The mechanism for the biosynthesis of HS is reminiscent of that for chondroitin in *C. elegans*. The repeating disaccharide region of chondroitin is synthesized by glycosyltransferases encoded by *sqv-5* (*cChSy*) (32, 48) and *pfc-1* (PAR2.4) (33), which form an enzyme complex (chondroitin polymerase) (see Fig. 3, lane 4). RNAi depletion of *SQV-5* or *PFC-1* in *C. elegans* resulted in a reduction of chondroitin, and the RNAi-treated worms showed similar phenotypes, such as cytokinetic defects in early embryogenesis (32, 33). Thus, it has been suggested that *sqv-5* and *pfc-1* cannot have redundant roles in the biosynthesis of chondroitin, which is similar to the observation that *rib-1* or *rib-2* null mutant worms showed reduced synthesis of HS and similar phenotypes, such as post-gastrulation and egg-laying defects. Therefore, both *rib-1* and *rib-2* have nonredundant functions as do *SQV-5* (*cChSy*) and *PFC-1* (PAR2.4). In addition, these observations suggest that the mechanism for the biosynthesis of HS is similar to that for chondroitin in *C. elegans* and clearly indicate that the functions of HS are different from those of chondroitin in *C. elegans*.

Several recent studies in *C. elegans* have greatly expanded our understanding of how specific modifications to HS can affect different signaling pathways in different tissues (2). In this study and in the related study of Morio *et al.* (28), mutations in *rib-1* and *rib-2*-encoded proteins involved in the polymerization of HS affected post-gastrulation morphogenic movement and the subsequent elongation in F1 embryos as well as egg-laying and other phenotypes in P0 worms. We detected defects in ventral enclosure and epithelial morphogenesis in *rib-1*/*rib-2* F1 worms, and the results suggest the possible involvement of HS in epi-dermal morphogenesis as well. In this respect, Franks *et al.* (41) reported genetic interactions between *pyr-1* and *rib-1*/*rib-2* system. They also showed that the *rib-1* and *rib-2* system play vital roles in body elongation and pharyngeal isthmus morphogenesis. These results strongly indicate the indispensable roles of HS in post-gastrulation and the subsequent morphogenesis.

In contrast, HS-modifying enzymes are not required for viability in *C. elegans*. Bülow and Hobert (39) isolated mutants of the HS 6-O-sulfortransferase, the glucuronyl C5-epimerase, and the HS 2-O-sulfortransferase, and they found each to be viable and fertile, indicating a more specialized function of the modification to HS. Further analyses showed that each of the mutant worms exhibits distinct yet overlapping axonal and cellular guidance defects in specific classes of neurons. Hence, it has been proposed that individual modifications to HS in a given area or on specific neurons give rise to a “sugar code” that regulates axonal guidance by modulating specific receptor-ligand interactions (39). Additional regional specificity in HS modification patterns can be brought about by modifying core proteins, such as syndecan (49–51). However, no studies have yet addressed the fundamental questions of whether signaling molecules interact differently with specifically modified HS sequences and how these sequences affect particular signaling pathways. The development of robust and highly sensitive sequencing methods for HS chains will provide definitive answers to these questions in the future.

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