markedly reduced in the wild-type somites, in concert with formation of skeletal muscle derived from myotomes (Figure 7). Because the myogenic master transcription factor myoD plays essential roles in muscle differentiation [30] and can drive apoptotic pathways as reported by Asakura et al. [37], the perturbed expression of *myod* in the *C4ST-1* morphants may imply an immature developmental state of myotomes and/or disruption of the somitic musculature accompanied by excessive stimulation of the apoptotic cascade, leading to the aberrant morphology in the trunk and tail.

The defects in somites with persistent expression of *myod*, which were evoked by the functional knockdown of *C4ST-1*, have been also reported for zebrafish embryos injected with MOs, which inhibited functions of HS6ST-2, one of the sulfotransferases involved in Hep/HS (heparan sulfate) biosynthesis [38,39]. HS-PGs play roles in various cellular signalling pathways through the high-affinity binding of HS moieties to diverse Hep-binding proteins including growth factors, cytokines and morphogens [40,41]. Recent studies have demonstrated that CS also possesses the capacity to bind various Hep-binding proteins [9,41]. In fact, signalling pathways involving TGFβ (transforming growth factor β) and BMP (bone morphogenetic protein) are dramatically affected in growth plates of *C4ST-1*-deficient mice [19]. Hence, the apparently similar muscular defects in both morphants might be indicative of partially overlapping or compensatory functions of CS and HS in the regulation of local signalling pathways via Hep-binding myogenic morphogens such as hedgehog and Wnt [42,43]. Most recently, Nadanaka et al. [44] reported that the decreased Wnt-3a signalling in sog9 cells lacking *C4ST-1* mRNA is recovered by the introduction of C4ST-1, providing strong support to the notion that the fine structures of CS formed by C4ST-1 are required for efficient signalling inputs mediated by multiple morphogens.

C4ST-1 has been screened as one of the target genes induced by BMP signalling during differentiation of mouse embryonic stem cells [45]. This indicates that C4ST-1 is one of the essential modulators in a sequence of the BMP-dependent cell fate decisions. In zebrafish, BMPs are known to be key regulators of posterior (i.e. trunk and tail) mesoderm patterning, as typified by zebrafish mutants lacking *bmp2b* that do not form a tail [46]. In view of generally mild but definitive phenotypic abnormalities biased to trunk and tail in *C4ST-1* morphants, C4ST-1 might also be one of the downstream targets of the BMP signalling in the posterior half of the body axis and might play important roles in at least several developmental processes induced by BMPs. Therefore elucidation of the transcriptional regulatory mechanism for *C4ST-1* will provide insights into how C4ST-1 participates in body axis formation including muscle development.

Emerging evidence suggests that CS-PGs are crucial environmental modulators in the nervous system of vertebrates. During neuronal development and regeneration, they have apparently contradictory roles as major inhibitors of axonal pathfindings and regeneration and as neuritogenic molecules [2,3]. These functions are exerted mainly through their CS moieties [2,3,9,10]. During zebrafish embryogenesis, CS is abundantly distributed at the interface between the notochord and somites where ventral motor axons located in the middle of each spinal cord hemisegment project into ventral muscle [22]. As reported previously [23], elimination of CS in the trunk by injection with chondroitinase ABC induces axonal projections with abnormal side branches, indicating that CS constrains the outgrowth of the ventral motor nerves through its inhibitory role as a physical barrier or a repulsive cue for axonal growth. In the present study, aberrant axonal outgrowth of ventral motor neurons was also observed in *C4ST-1* morphants (Figure 7). However, the most common abnormality was characterized by truncated axons rather than abnormally branched and misrouted axons (Table 3). One explanation for this discrepancy is that a low but significant level of CS in the trunk of *C4ST-1* morphants supports fasciculation of the ventral motor nerves, preventing the formation of side branches, whereas a nearly complete loss of CS by treatment with chondroitinase ABC does not. Furthermore, in view of the requirement of myotome-derived cues for the migration of motor axons [24,47], we cannot rule out the possibility that defects in muscle development in *C4ST-1* morphants led to additional indirect effects on the axonal pathfindings, because it has been reported that there was no damage in either the notochord or somites in chondroitinase-treated zebrafish embryos [23].

Although the precise molecular basis of the axonal pathfindings of ventral motor nerves involving 4-O-sulfated CS remains unclear, the high incidence of truncated axons, probably representing axons straying from their pathways, in *C4ST-1* morphants suggests potential bifunctional roles of CS not only as a repulsive cue but also as a permissive/attractive guidance cue for specific axons, as described above. In fact, a substrate uniformly precoated with oversulfated CS variants promoted the outgrowth of neurites in rodent embryonic hippocampal neurons, at least in part, by capturing and presenting several Hep-binding growth factors to neurons [2,3,9]. In addition, habenula nucleus axons derived from the developing rat diencephalon were also able to extend over a substrate precoated with a relatively high concentration of CS-PGs [48]. Interestingly, a CS-PG-coated substrate coexisting with sema5A (semaphorin 5A), a bifunctional guidance cue, serves as an inhibitory cue for habenula nucleus axons, resulting from conversion of the attractive property of sema5A into an inhibitory one through its specific interaction with the CS moieties of CS-PGs [48]. Therefore further exploration of CS-interacting molecules in the extracellular matrix and unidentified functional CS receptor(s) is required for a better understanding of the apparent contradictory neuroregulatory functions of CS.

In contrast with *C4ST-1* morphants and chondroitinase-treated embryos, functional knockdown of zygotic *ChSy-1* (chondroitin synthase-1), which encodes one of the glycosyltransferases involved in the biosynthesis of the chondroitin backbone, has been reported to have no significant effects on the pathfinding of the motor axons, although CS immunoreactivity was reduced in the morphants [47]. Recently, Izumikawa et al. [49,50] demonstrated that chondroitin polymerization can be achieved by any two combinations of four ChSy family members including ChSy-1. Therefore, if similar biosynthetic machinery is encoded by the zebrafish genome, a single knockdown of ChSy family members may not always lead to a drastic reduction in the amount of CS, which provokes developmental defects. Consequently, our results in conjunction with earlier studies [18,19,23] strongly suggest the critical functions of C4ST-1 in CS biosynthesis and in zebrafish embryogenesis. Thus further analysis focusing on C4ST-1 will facilitate our understanding of the molecular mechanisms underlying the development and pathology of various diseases involving CS.

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REFERENCES

1 Iozzo, R. V. (1998) Matrix proteoglycans: from molecular design to cellular function. Annu. Rev. Biochem. **67**, 609–652
2 Sugahara, K., Mikami, T., Uyama, T., Mizuguchi, S., Nomura, K. and Kitagawa, H. (2003) Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. Curr. Opin. Struct. Biol. **13**, 612–620
3 Sugahara, K. and Mikami, T. (2007) Chondroitin/dermatan sulfate in the central nervous system. Curr. Opin. Struct. Biol. **17**, 536–545
4 Schwartz, N. B. and Domowicz, M. (2002) Chondrodysplasias due to proteoglycan defects. Glycobiology **12**, 57R–68R
5 Uyama, T., Kitagawa, H. and Sugahara, K. (2007) Biosynthesis of glycosaminoglycans and proteoglycans. In Comprehensive Glycoscience, Volume 3 (Kamerling, J. P., ed.), pp. 79–104, Elsevier, Amsterdam
6 Mark, M. P., Baker, J. R., Kimata, K. and Ruch, J. V. (1990) Regulated changes in chondroitin sulfation during embryogenesis: an immunohistochemical approach. Int. J. Dev. Biol. **34**, 191–204
7 Kitagawa, H., Tsutsumi, K., Tone, Y. and Sugahara, K. (1997) Developmental regulation of the sulfation profile of chondroitin sulfate chains in the chicken embryo development. J. Biol. Chem. **272**, 31377–31381
8 Mitsunaga, C., Mikami, T., Mizumoto, S., Fukuda, J. and Sugahara, K. (2006) Chondroitin sulfate/dermatan sulfate hybrid chains in the development of cerebellum. Spatiotemporal regulation of the expression of critical disulfated disaccharides by specific sulfotransferases. J. Biol. Chem. **281**, 18942–18952
9 Mikami, T. and Sugahara, K. (2006) The biological importance of specific sulfation of chondroitin sulfate/dermatan sulfate in their functional expression. Trends Glycosci. Glycotechnol. **18**, 165–183
10 Gama, C. I., Tully, S. E., Sotogaku, N., Clark, P. M., Rawat, M., Vaidehi, N., Goddard, III, W. A., Nishi, A. and Hsieh-Wilson, L. C. (2006) Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. Nat. Chem. Biol. **2**, 467–473
11 Kusche-Gullberg, M. and Kjellén, L. (2003) Sulfotransferase in glycosaminoglycan biosynthesis. Curr. Opin. Struct. Biol. **13**, 605–611
12 Maccarana, M., Olander, B., Malmström, J., Tiedemann, K., Aebersold, R., Lindahl, U., Li, J. P. and Malmström, A. (2006) Biosynthesis of dermatan sulfate: chondroitin-glucuronate C5-epimerase is identical to SART2. J. Biol. Chem. **281**, 11560–11568
13 Yamauchi, S., Mita, S., Matsubara, T., Fukuta, M., Habuchi, H., Kimata, K. and Habuchi, O. (2000) Molecular cloning and expression of chondroitin 4-sulfotransferase. J. Biol. Chem. **275**, 8975–8981
14 Hiraoka, N., Nakagawa, H., Ong, E., Akama, T. O., Fukuda, M. N. and Fukuda, M. (2000) Molecular cloning and expression of two distinct human chondroitin 4-*O*-sulfotransferases that belong to the HNK-1 sulfotransferase gene family. J. Biol. Chem. **275**, 20188–20196
15 Kang, H.-G., Evers, M. R., Xia, G., Baenziger, J. U. and Schachner, M. (2002) Molecular cloning and characterization of chondroitin-4-*O*-sulfotransferase-3. J. Biol. Chem. **277**, 34766–34772
16 Evers, M. R., Xia, G., Kang, H. G., Schachner, M. and Baenziger, J. U. (2001) Molecular cloning and characterization of a dermatan-specific *N*-acetylgalactosamine 4-*O*-sulfotransferase. J. Biol. Chem. **276**, 36344–36353
17 Mikami, T., Mizumoto, S., Kago, N., Kitagawa, H. and Sugahara, K. (2003) Specificities of three distinct human chondroitin/dermatan *N*-acetylgalactosamine 4-*O*-sulfotransferases demonstrated using partially desulfated dermatan sulfate as an acceptor. J. Biol. Chem. **278**, 36115–36127
18 Uyama, T., Ishida, M., Izumikawa, T., Trybala, E., Tufaro, F., Bergström, T., Sugahara, K. and Kitagawa, H. (2006) Chondroitin 4-*O*-sulfotransferase-1 regulates E disaccharide expression of chondroitin sulfate required for herpes simplex virus infectivity. J. Biol. Chem. **281**, 38668–38674
19 Klüppel, M., Wight, T. N., Chan, C., Hinek, A. and Wrana, J. L. (2005) Maintenance of chondroitin sulfation balance by chondroitin-4-sulfotransferase 1 is required for chondrocyte development and growth factor signaling during cartilage morphogenesis. Development **132**, 3989–4003
20 Nüsslein-Volhard, C., Gilmour, D. T. and Dahm, R. (2002) Introduction: zebrafish as a system to study development and organogenesis. In Zebrafish (Nüsslein-Volhard, C. and Dahm R., eds), pp. 1–5, Oxford University Press, New York
21 Grunwald, D. J. and Eisen, J. S. (2002) Headwaters of the zebrafish – emergence of a new model vertebrate. Nat. Rev. Genet. **3**, 717–724
22 Bernhardt, R. R., Goerlinger, S., Roos, M. and Schachner, M. (1998) Anterior–posterior subdivision of the somite in embryonic zebrafish: implications for motor axon guidance. Dev. Dyn. **213**, 334–347
23 Bernhardt, R. R. and Schachner, M. (2000) Chondroitin sulfates affect the formation of the segmental motor nerves in zebrafish embryos. Dev. Biol. **221**, 206–219
24 Schneider, V. A. and Granato, M. (2003) Motor axon migration: a long way to go. Dev. Biol. **263**, 1–11
25 Stickney, H. L., Barresi, M. J. and Devoto, S. H. (2000) Somite development in zebrafish. Dev. Dyn. **219**, 287–303
26 Inohaya, K., Takano, Y. and Kudo, A. (2007) The teleost intervertebral region acts as a growth center of the centrum: *in vivo* visualization of osteoblasts and their progenitors in transgenic fish. Dev. Dyn. **236**, 3031–3046
27 Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995) Stages of embryonic development of the zebrafish. Dev. Dyn. **203**, 253–310
28 Kitagawa, H., Egusa, N., Tamura, J., Kusche-Gullberg, M., Lindahl, U. and Sugahara, K. (2001) *rib-2*, a *Caenorhabditis elegans* homolog of the human tumor suppressor EXT genes encodes a novel alpha1,4-*N*-acetylglucosaminyltransferase involved in the biosynthetic initiation and elongation of heparan sulfate. J. Biol. Chem. **276**, 4834–4838
29 Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. and Dodd, J. (1994) Floor plate and motor neuron induction by *vhh-1*, a vertebrate homolog of *hedgehog* expressed by the notochord. Cell **76**, 761–775
30 Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J. and Riggleman, B. (1996) Developmental regulation of zebrafish *MyoD* in wild-type, *no tail* and *spadetail* embryos. Development **122**, 271–280
31 Schlte-Merker, S. (2002) Looking at embryos. In Zebrafish (Nüsslein-Volhard, C. and Dahm, R., eds), pp. 39–58, Oxford University Press, New York
32 Nasevicius, A. and Ekker, S. C. (2000) Effective targeted gene 'knockdown' in zebrafish. Nat. Genet. **26**, 216–220
33 Yamada, S., Okada, Y., Ueno, M., Iwata, S., Deepa, S. S., Nishimura, S., Fujita, M., Van Die, I., Hirabayashi, Y. and Sugahara, K. (2002) Determination of the glycosaminoglycan-protein linkage region oligosaccharide structures of proteoglycans from *Drosophila melanogaster* and *Caenorhabditis elegans*. J. Biol. Chem. **277**, 31877–31886
34 Kinoshita, A. and Sugahara, K. (1999) Microanalysis of glycosaminoglycan-derived oligosaccharides labeled with a fluorophore 2-aminobenzamide by high-performance liquid chromatography: application to disaccharide composition analysis and exosequencing of oligosaccharides. Anal. Biochem. **269**, 367–378
35 Souza, A. R., Kozlowski, E. O., Cerqueira, V. R., Castelo-Branco, M. T., Costa, M. L. and Pavão, M. S. (2007) Chondroitin sulfate and keratan sulfate are the major glycosaminoglycans present in the adult zebrafish *Danio rerio* (Chordata-Cyprinidae). Glycoconj. J. **24**, 521–530
36 Stemple, D. L., Solnica-Krezel, L., Zwartkruis, F., Neuhauss, S. C. F., Schier, A. F., Malicki, J., Stainier, D. Y. R., Abdelilah, S., Rangini, Z., Mountcastle-Shah, E. and Driever, W. (1996) Mutations affecting development of the notochord in zebrafish. Development **123**, 117–128
37 Asakura, A., Hirai, H., Kablar, B., Morita, S., Ishibashi, J., Piras, B. A., Christ, A. J., Verma, M., Vineretsky, K. A. and Rudnicki, M. A. (2007) Increased survival of muscle stem cells lacking the *MyoD* gene after transplantation into regenerating skeletal muscle. Proc. Natl. Acad. Sci. U.S.A. **104**, 16552–16557
38 Bink, R. J., Habuchi, H., Lele, Z., Dolk, E., Joore, J., Rauch, G. J., Geisler, R., Wilson, S. W., den Hertog, J., Kimata, K. and Zivkovic, D. (2003) Heparan sulfate 6-*O*-sulfotransferase is essential for muscle development in zebrafish. J. Biol. Chem. **278**, 31118–31127
39 Chen, E., Stringer, S. E., Rusch, M. A., Selleck, S. B. and Ekker, S. C. (2005) A unique role for 6-*O* sulfation modification in zebrafish vascular development. Dev. Biol. **284**, 364–376
40 Esko, J. D. and Selleck, S. B. (2002) Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annu. Rev. Biochem. **71**, 435–471
41 Raman, R., Sasisekharan, V. and Sasisekharan, R. (2005) Structural insights into biological roles of protein–glycosaminoglycan interactions. Chem. Biol. **12**, 267–277
42 Feng, X., Adiarte, E. G. and Devoto, S. H. (2006) Hedgehog acts directly on the zebrafish dermomyotome to promote myogenic differentiation. Dev. Biol. **300**, 736–746
43 Pownall, M. E., Gustafsson, M. K. and Emerson, Jr, C. P. (2002) Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu. Rev. Cell Dev. Biol. **18**, 747–783

44 Nadanaka, S., Ishida, M., Ikegami, M. and Kitagawa, H. (2008) Chondroitin 4-*O*-sulfotransferase-1 modulates Wnt-3a signaling through control of E disaccharide expression of chondroitin sulfate. J. Biol. Chem. **283**, 27333–27343

45 Klüppel, M., Vallis, K. A. and Wrana, J. L. (2002) A high-throughput induction gene trap approach defines C4ST as a target of BMP signaling. Mech. Dev. **118**, 77–89

46 Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C. P. et al. (1996) Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. Development **123**, 81–93

47 Zhang, I., Lefebvre, J. L., Zhao, S. and Granato, M. (2004) Zebrafish *unplugged* reveals a role for muscle-specific kinase homologs in axonal pathway choice. Nat. Neurosci. **7**, 1303–1309

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48 Kantor, D. B., Chivatakarn, O., Peer, K. L., Oster, S. F., Inatani, M., Hansen, M. J., Flanagan, J. G., Yamaguchi, Y., Sretavan, D. W., Giger, R. J. and Kolodkin, A. L. (2004) Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. Neuron **44**, 961–975

49 Izumikawa, T., Uyama, T., Okuura, Y., Sugahara, K. and Kitagawa, H. (2007) Involvement of chondroitin sulfate synthase-3 (chondroitin synthase-2) in chondroitin polymerization through its interaction with chondroitin synthase-1 or chondroitin-polymerizing factor. Biochem. J. **403**, 545–552

50 Izumikawa, T., Koike, T., Shiozawa, S., Sugahara, K., Tamura, J. and Kitagawa, H. (2008) Identification of chondroitin sulfate glucuronyltransferase as chondroitin synthase-3 involved in chondroitin polymerization: chondroitin polymerization is achieved by multiple enzyme complexes consisting of chondroitin synthase family members. J. Biol. Chem. **283**, 11396–11406