Differential roles for 3-OSTs in the regulation of cilia length and motility

Judith M. Neugebauer*, Adam B. Cadwallader*‡, Jeffrey D. Amack§, Brent W. Bisgrove and H. Joseph Yost¶

SUMMARY
As cells integrate molecular signals from their environment, cell surface receptors require modified proteoglycans for the robust activation of signaling pathways. Heparan sulfate proteoglycans (HSPGs) have long unbranched chains of repetitive disaccharide units that can be sulfated at specific positions by heparan sulfate O-sulfotransferase (OST) families. Here, we show that two members of the 3-OST family are required in distinct signaling pathways to control left-right (LR) patterning through control of Kupffer’s vesicle (KV) cilia length and motility. 3-OST-5 functions in the fibroblast growth factor pathway to control cilia length via the ciliogenic of the 3-OST family are required in distinct signaling pathways to control left-right (LR) patterning through control of Kupffer’s vesicle (KV) cilia length and motility. 3-OST-5 functions in the fibroblast growth factor pathway to control cilia length via the ciliogenic transduction factors FoxJ1a and Rfx2. By contrast, a second 3-OST family member, 3-OST-6, does not regulate cilia length, but regulates cilia motility via kinesin motor molecule (Kif3b) expression and cilia arm dynein assembly. Thus, two 3-OST family members cell-autonomously control LR patterning through distinct pathways that regulate KV fluid flow. We propose that individual 3-OST isozymes create distinct modified domains or ‘glycocodes’ on cell surface proteoglycans, which in turn regulate the response to diverse cell signaling pathways.

KEY WORDS: Heparan sulfate proteoglycans, O-Sulfotransferase, OST, 3-OST, HSPG, Fibroblast growth factors, FGF, Cilia, Fluid flow, Left-right development, Heart asymmetry, Gut asymmetry, Zebrafish, Glycocode

INTRODUCTION
Molecular interactions in the extracellular matrix and at the cell surface are important for dynamic regulation of cell-cell signaling. At the cell surface, heparan sulfate proteoglycans (HSPGs) are known to modulate interactions between receptors and ligands (Rapraeger et al., 1991; Yayon et al., 1991; Bellaiche et al., 1998; Tsuda et al., 1999; Bellenkaya et al., 2004; Häcker et al., 2005). Heparan sulfate (HS) chains attached to a core protein consist of alternating glucuronic acid and N-acetylgalcosamine residues, which undergo a series of modifications, including sulfation, to produce mature long, unbranched HS chains. HSPG core proteins are either inserted into the cell membrane, attached to the extracellular surface of the cell membrane, or in the extracellular matrix. In vivo, loss of core proteins or HS chains indicate that HSPGs regulate multiple developmental processes, including gastrulation and left-right (LR) patterning (Alexander et al., 2000; Topczewski et al., 2001; Kramer et al., 2002; Kramer and Yost, 2002; Marjoram and Wright, 2011). Much less is known about specific rare modifications to HS chains and their specialized function in development (HajMohammadi et al., 2003; Kamimura et al., 2004; Tecle et al., 2013).

The addition of sulfates to HS chains is mediated by three distinct families of heparan sulfate O-sulfotransferases (OSTs): 2-OSTs, 3-OSTs and 6-OSTs. Members of these families have dynamic spatial and temporal regulation during vertebrate development (Shworak et al., 1999; Habuchi et al., 2000; Xia et al., 2002; Nogami et al., 2004; Xu et al., 2005; Cadwallader and Yost, 2006b; Cadwallader and Yost, 2006a). Within these multigene families in the HS chain biosynthetic pathway, the 3-OST family is the most isozyme-rich, but is responsible for only 0.5% of all HS chain sulfation (Colliec-Jouault et al., 1994; Shworak et al., 1999). Considering that each 3-OST family member performs the same biochemical function, sulfation of the hydroxyl group at the 3-carbon on select glucosamine residues in the HS chain (Colliec-Jouault et al., 1994; Shworak et al., 1999), it is intriguing that there are so many 3-OST family members in vertebrates (Cadwallader and Yost, 2013). Here, we explore the hypothesis that each 3-OST family member has a distinct context-dependent, non-redundant function in development.

In all vertebrates, the heart, brain and gut develop left-right asymmetries that are crucial for normal organ function (Yost, 2001; Yamada et al., 2002; Bisgrove et al., 2003; Levin, 2005; López-Gracia and Ros, 2007). These organ asymmetries are dependent on a conserved cascade of asymmetric expression in left lateral plate mesoderm (LPM) of the nodal, lefty and pitx2 gene families. Several upstream components contribute to normal asymmetric LPM gene expression, including (1) cilia-dependent fluid flow in a ‘ciliated organ of asymmetry’ at the posterior of the embryonic midline, the node in mouse (Zhou et al., 1993), Kupffer’s vesicle (KV) in zebrafish (Essner et al., 2005) and medaka (Hojo et al., 2007; Soroldoni et al., 2007), and the gastrocoel roof plate in Xenopus laevis (Schweickert et al., 2007), (2) an intact embryonic midline (Danos and Yost, 1995; Danos and Yost, 1996; Chen et al., 1997; Meno et al., 1998; Yamamoto et al., 2003), and (3) in Xenopus at least, molecular asymmetries in cleavage and gastrula stages (Danos and Yost, 1995; Kramer and Yost, 2002; Yamamoto et al., 2003; Adams et al., 2006) that precede the appearance of cilia (Essner et al., 2002). Several important signaling pathways have been implicated in the establishment of LR asymmetries, including Shh, fibroblast growth factor (FGF), bone morphogenetic protein (BMP) and Wnt pathways (Albertson and Yelick, 2005; Levin, 2005; Hong and Dawid, 2009; Neugebauer et al., 2009). Understanding how the signals are mediated by the developing embryo will be crucial to our understanding of LR asymmetry.
In zebrafish, at least five 3-OST family members are normally expressed in and around the ciliated cells of the KV (Cadwallader and Yost, 2006a). To determine whether individual members of the 3-OST family have distinct cellular and developmental functions, we knocked down each family member by injection of morpholinos (MOs; antisense oligonucleotides) and assessed KV cilia and downstream LR patterning in zebrafish. Strikingly, two of the five family members expressed in KV ciliated cells, 3-OST-5 (gene name: hs3st1l2) and 3-OST-6 (gene name: hs3st5l), had distinct roles in regulating motile KV cilia, indicating non-redundant functions within the 3-OST family.

We propose that specific sulfation patterns on HS chains create ‘glycocodes’ that are developmentally regulated, cell-type specific, and ultimately confer specificity to most major cell signaling pathways at the cell surface (Cadwallader and Yost, 2013). Perhaps small subsets of the vast number of possible combinations of HS chain modifications are utilized as gatekeepers for specific molecular interactions, such as ligand-receptor interactions. If so, uncovering the bioinformation potentially embedded in the sequences of HSPG chain modifications will enjoin a substantial leap in our understanding of cell signaling. The first postulate of the glycocode hypothesis is that each member of an OST family performs a distinct function: the same catalytic reaction but in a context-dependent fashion, generating distinct sequences of modification in the same cells during development.

MATERIALS AND METHODS

Maintenance of zebrafish stocks and embryo culture

Danio rerio were maintained at 28.5°C on a 14-hour light/10-hour dark cycle. Embryos were collected from natural spawns, cultured and staged as previously described (Westerfield, 2000). Heart looping was determined in wild-type (WT) embryos by visualization in living embryos, by in situ hybridization or by fluorescence microscopy of GFP expression in cardiomyocytes in Tg(cmlc2:GFP) (Huang et al., 2003). 3-OST-5/Fgf8 epistasis experiments were conducted with the Fgf8 hypomorphic allele fgf8a<sup>tm2426</sup>, which is predicted to generate a truncated Fgf8 protein (Reifers et al., 1998).

Embryo injections

Antisense morpholinos (MOs) were obtained from Gene Tools. To knock down gene expression in all cells, MO was injected between the one- and four-cell stages as previously described (Nasevicius and Ekker, 2000). For one-four cell injections, 1 nl MO was injected into embryos; MO concentrations used were as listed in Table 1. To deliver MOs specifically to dorsal forerunner cells (DFCs), the precursors of KV ciliated cells, a fluorescein-labeled control MO was co-injected with a gene-specific MO in a 1:1 ratio into the yolk cell between the 500- and 1000-cell stages; embryos with fluorescent DFCs were selected for analysis, as described (Amack and Yost, 2004). The sub-threshold dose of GTACGTATTGTGATCATAGT for fgf8/ace experiments was injected at a concentration of 0.175 mM.

mRNA synthesis

The 3-OST-6 ΔTM construct was subcloned from a construct containing the full-length open reading frame (ORF) of 3-OST-6 (Cadwallader and Yost, 2006a) by PCR using the following primers: 5'-CCCATTGGA-CTTCCTCGAAAAGTCCCAAGGCA-3' and 5'-CTCTTAGGTTCATTGCAT-CAGCAGTCATAC-3'. The full-length ORF of 3-OST-5 and 3-OST-6, as well as 3-OST-6 ΔTM were subcloned into pcDNA3-NMCS. mRNA for injection was synthesized using the Message Machine Kit (Ambion) or the AmpliCap (Epitome) utilizing the SP6 polymerase. Newly synthesized mRNA was purified by ammonium acetate precipitation.

RT-PCR

RT-PCR was used to determine the effect of MO injections on mRNA splicing (primers listed in supplementary material Figs S1, S2). Total RNA was extracted from embryos using TRIzol Reagent (Invitrogen) and cDNA was synthesized by MMLV-RT primed by random decamers (Ambion Retroscript kit). PfuUltra (Stratagene) was used for amplification of cDNA using 33 cycles of PCR. Semi-quantitative analysis was performed to calculate the percentage decrease of spliced mRNA using ImageQuant TL (GE Healthcare).

Whole-mount in situ hybridization

Zebrafish embryos were fixed in PBS containing 4% paraformaldehyde (PFA) plus sucrose, rinsed in 1×PBS, dehydrated in methanol series and stored at −20°C. To generate antisense RNA probes, linear DNA templates were transcribed using either T3 or T7 polymerase in the presence of digoxigenin labeling mix (Roche) and free nucleotides were removed using LiCl precipitation. In situ hybridizations were carried out as previously described (Essner et al., 2000) using a Bioline HT1 in situ machine (Huller and Hutten AG). Embryos were cleared in 70% glycerol in PBST (0.1% Tween-20 in PBS) and photographed using either a Leica MZ12 or a Nikon SMZ1000 dissecting microscope. Digital images were processed using Adobe Photoshop and ACD Systems Canvas. cDNA templates used include spaw (Long et al., 2003), ssh (Essner et al., 2005), nt1 (Amack and Yost, 2004), foxl7 (Amack and Yost, 2004), left1 (Essner et al., 2005), dnah9 (Essner et al., 2005), foxJ1a (Tian et al., 2009), rfx2 (Bisgrove et al., 2012), ki3b (B.W.B., unpublished), cmlc2 (mvl7) (Yelon et al., 1999), 3-OST-5 (Cadwallader and Yost, 2006a), 3-OST-6 (Cadwallader and Yost, 2006a), sox (il17rd) (Tsang et al., 2002) and fkd2 (fox3) (Odenthal and Nüsslein-Volhard, 1998).

Immunohistochemistry

Embryos were fixed overnight in 4% PFA at 4°C and dehydrated stepwise into methanol for storage at −20°C. After stepwise rehydration, embryos were rehydrated stepwise in 70% ethanol and 95% ethanol and stored at 20°C. To generate antisense RNA probes, linear DNA templates were transcribed using T7 polymerase in the presence of digoxigenin labeling mix (Roche) and free nucleotides were removed using LiCl precipitation. In situ hybridizations were carried out as previously described (Essner et al., 2000) using a Bioline HT1 in situ machine (Huller and Hutten AG). Embryos were cleared in 70% glycerol in PBST (0.1% Tween-20 in PBS) and photographed using either a Leica MZ12 or a Nikon SMZ1000 dissecting microscope. Digital images were processed using Adobe Photoshop and ACD Systems Canvas. cDNA templates used include spaw (Long et al., 2003), ssh (Essner et al., 2005), nt1 (Amack and Yost, 2004), foxl7 (Amack and Yost, 2004), left1 (Essner et al., 2005), dnah9 (Essner et al., 2005), foxJ1a (Tian et al., 2009), rfx2 (Bisgrove et al., 2012), ki3b (B.W.B., unpublished), cmlc2 (mvl7) (Yelon et al., 1999), 3-OST-5 (Cadwallader and Yost, 2006a), 3-OST-6 (Cadwallader and Yost, 2006a), sox (il17rd) (Tsang et al., 2002) and fkd2 (fox3) (Odenthal and Nüsslein-Volhard, 1998).

Table 1. Morpholino sequence and injection amounts

<table>
<thead>
<tr>
<th>Protein</th>
<th>MO name</th>
<th>MO sequence (5'-3')</th>
<th>MO target type</th>
<th>One-cell injection concentration</th>
<th>DFC-targeted concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-OST-5</td>
<td>3-OST-5 MO1</td>
<td>GTCCATGCGTAAGGGCAGTCAA</td>
<td>Translational start site</td>
<td>0.35 mM</td>
<td>0.15 mM</td>
</tr>
<tr>
<td>3-OST-5</td>
<td>3-OST-5 MO2</td>
<td>CGGTCCTGGACACAGAGGAATT</td>
<td>Intron 1/exon 2</td>
<td>0.1 mM</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>3-OST-6</td>
<td>3-OST-6 MO1</td>
<td>CGTTGAGGATCTCCTGGATGTCGCC</td>
<td>Translational start site</td>
<td>0.2 mM</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>3-OST-6</td>
<td>3-OST-6 MO2</td>
<td>GTAATGTCATTGACATCCAGTGCT</td>
<td>Exon 1/intron 1</td>
<td>0.27 mM</td>
<td>0.27 mM</td>
</tr>
<tr>
<td>3-OST-7</td>
<td>3-OST-7 MO1</td>
<td>CATAAGCTCAGAGGATTGCCCAGT</td>
<td>Exon 1/intron 1</td>
<td>0.27 mM</td>
<td>0.27 mM</td>
</tr>
<tr>
<td>Non-specific</td>
<td>Control MO</td>
<td>CCTTCTACCTCAGTACATATTATA</td>
<td>Translational start site</td>
<td>0.6 mM</td>
<td>0.6 mM</td>
</tr>
<tr>
<td>(fluorescein tagged)</td>
<td></td>
<td></td>
<td>Non-specific</td>
<td>0.4 mM</td>
<td>0.3 mM</td>
</tr>
</tbody>
</table>
laser confocal microscope with a 60× objective and images were processed using ImageJ and Metamorph software.

** Fluorescent bead injections**

KV fluid flow was assayed as previously described (Essner et al., 2005). Embryos were removed from their chorions at 6-8 somite stage (SS) and mounted in 1% low melt agarose on glass slides. Fluorescent beads (0.5-2 μm; Polysciences) were injected into KV, and beads were imaged on a Leica DMRA compound microscope using a 40× Plan Apo objective. Movies were generated using a CoolSnap HQ digital camera (Photometrics) and Metamorph (Universal Imaging Corp.) and Quicktime (Apple) software. Beads were tracked using Metamorph.

**Electron microscopy**

Electron microscopy protocol was modified from a previously published protocol to examine cilia in zebrafish KV (Kreiling et al., 2007). Live SS embryos were dechorionated by hand and then fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C. Embryos were washed three times (15 minutes per wash) in 0.1 M sodium cacodylate buffer and incubated for 1 hour on ice in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. After osmium treatment, embryos were washed three times (15 minutes per wash) in 0.1 M sodium cacodylate buffer. Embryos were embedded in 1% agarose (in H2O) to allow orientation during sectioning. Embryos embedded in agarose were dehydrated in an EtOH series ending in acetone washes. Embryos were then embedded in Epon through washes with 3:1, 1:1 and 1:3 Acetone:Epon for 2 hours each at room temperature. Finally, the embryos were placed in 100% Epon with at least two changes (2 hours per wash) before placing them at 60°C overnight to solidify epoxy. Embedded samples were sectioned and stained with uranyl acetate and lead citrate. Electron micrographs were taken using a FEI Tecnai T12 at 80 kV. Images were de-identified and blindly scored by three individuals for presence or absence of inner and outer dynein arms.

**High resolution melt analysis**

After confocal analysis, embryos were removed from microscope slides and rinsed in 1× PBST with one change of buffer over 2 hours. Embryos were placed at 55°C overnight in DNA extraction buffer containing 20 mM Tris pH 8.0, 50 mM KCl, 1.5 mM MgCl2, 0.3% Tween-20, 0.3% NP-40 and 15 μg/ml of ProteinaseK. After overnight incubation, ProteinaseK was deactivated by 10 minutes at 95°C. A PCR reaction was set up in 96-well plates (Bio-Rad, HSP9665) using 1 μl DNA in a 10 μl reaction and included LCGreen (Idaho Technology). PCR primers were designed to create a 73-base fragment flanking the G→A transition mutation causing the acetabellar fragment (forward, 5′-TGTATGACAGGGGGGA-3′; reverse, 5′-CATTTAAAAGTACAAAAAGTG-3′). The following PCR conditions were used: 95°C for 10 seconds; 40 cycles of 95°C for 8 seconds, 52°C for 8 seconds, 95°C for 8 seconds; 40 cycles of 95°C for 5 seconds, 45°C for 10 seconds. PCR samples were analyzed for different melting curves on a Lightscanner (Idaho Technology) over a 65-90°C range as previously described (Parant et al., 2009).

**Statistical analysis**

Each experiment was repeated at least three times in order to assess statistical significance. Means, standard deviations and standard errors were calculated using R Statistical Software platform. Student’s t-test was performed for analysis of cilia length, and heart and gut looping. Fisher’s exact test was used to analyze changes in in situ expression categories. A one-way ANOVA followed by a Tukey analysis was performed for fluorescent bead velocity. A multi-way ANOVA followed by a Tukey analysis was performed for cilia length analysis in gg/uce embryos injected with 3-OST-5 MO1. P values below 0.05 were considered significant.

**RESULTS**

**3-OST-5 is required for LR development**

Injection of a translation-blocking MO specific to 3-OST-5 (3-OST-5 MO1) resulted in embryos with a curved body axis at 48 hours post-fertilization (hpf) (Fig. 1A,B) and altered LR patterning, with high frequencies of reversed heart (39%, n=120) and gut (41%, n=120) looping (Fig. 1C-E; supplementary material Fig. S1A). Injection of a second 3-OST-5 MO (3-OST-5 MO2), designed to inhibit 3-OST-5 mRNA splicing, resulted in similar LR defects, including frequent heart (29%, n=121) and gut (29%, n=121) reversals (Fig. 1E). 3-OST-5 MO2 morphants had increased accumulation of unspliced RNA and corresponding reduction of mRNA (supplementary material Fig. S1B,C).

To test MO specificity, we performed rescue experiments by co-injecting 3-OST-5 MO1 with a 3-OST-5 MO-resistant ‘rescue’ mRNA, specifically engineered to prevent 3-OST-5 MO1 from binding injected mRNA. Co-injection of 3-OST-5 MO-resistant ‘rescue’ mRNA resulted in a statistically significant (P<0.001) rescue of 3-OST-5 MO1 induced heart (17%, n=137) and gut (18%, n=137) looping defects (Fig. 1E). Injection of 3-OST-5 MO-resistant ‘rescue’ mRNA alone did not alter heart and gut orientation (4% and 5%, respectively, n=171; Fig. 1E). The similar phenotype seen with two distinct morpholinos and the ability to rescue the phenotype indicate that these effects are specific for 3-OST-5, revealing its function in zebrafish LR development.

As an important control for functional specificity within the 3-OST family, we knocked down three other family members and found that none had roles in LR development. 3-OST-3Z (gene name: hs3st3b1b), a closely related family member concomitantly expressed with 3-OST-5 in early development (Cadwallader and Yost, 2006a), was targeted for knockdown by injection of a highly efficient splice-blocking MO resulting in reduction of 3-OST-3Z mRNA (supplementary material Fig. S1D) but normal LR patterning, with normal heart (95%, n=172) and gut (94%, n=172) orientation (Fig. 1E). This 3-OST-3Z MO was used as a parallel control morpholino throughout this study. Similarly, knockdown of another family member, 3-OST-7 (gene name: hs3st3l1), although expressed in overlapping patterns with 3-OST-5, resulted in normal LR development (100%, n=24; compared with 96% uninjected embryos, n=46). Although this 3-OST-7 MO did not alter LR patterning, it resulted in a distinct phenotype, cardiac ventricular non-contraction at 48 hpf, that could be partially rescued by transgene expression (S. C. Samson, T. Ferrer, C. J. Jou, F. B. Sachse, S. S. Shankaran, R. M. Shaw, N. C. Chi, M. Tristani-Firouzi and H. J. Y., unpublished), indicating the efficacy and specificity of this MO. Together, these results indicate that the LR patterning defects seen in embryos injected with 3-OST-5 MO are not observed with reduction of at least two other 3-OST family members.

To assess where 3-OST-5 functions in the LR pathway, we analyzed the expression of southpaw (spaw; a zebrafish nodal homolog) by in situ hybridization. spaw is expressed in the left LPM directly downstream of the molecular cascade that initiates LR axis formation (Fig. 1F) (Long et al., 2003). In 3-OST-5 morphants, spaw expression was altered, resulting in high incidence of bilateral spaw expression (Fig. 1F). Control 3-OST-3Z morphants had normal spaw expression (Fig. 1F), consistent with normal organ LR orientation (Fig. 1E).

Two transient embryonic structures, the embryonic midline (floor plate and notochord) and KV, are required for normal asymmetric gene expression in the LPM (Danos and Yost, 1996; Amack and Yost, 2004; Essner et al., 2005). 3-OST-5 is expressed throughout the embryo at 70-90% epiboly, including midline cells and precursors of KV cells, dorsal forerunner cells (DFCs; supplementary material Fig. S1E). At the 7-10 somite stage (SS), when signals are being transferred from KV to the LPM (Essner et al., 2005), 3-OST-5 is expressed in KV, LPM and intervening taibud tissues (supplementary material Fig. S1F). Thus, the
ubiquitous expression patterns do not help define the location of 3-OST-5 function in the LR pathway.

Considering that the embryonic midline is essential for LR development (Danos and Yost, 1995; Danos and Yost, 1996; Chen et al., 1997; Meno et al., 1998; Bisgrove et al., 1999; Yamamoto et al., 2003), we assessed whether 3-OST-5 morphants had intact midline structures. At 16 SS, in situ RNA expression of lefty1, ntl and shh in 3-OST-5 MO1-injected embryos showed normal midline and floor plate development (lefty1, present in 83%, n=82; ntl, present in 100%, n=122; shh, present in 100%, n=122; supplementary material Fig. S1G-J). These results indicate that LR patterning defects in 3-OST-5 morphants are not due to gross perturbations of midline development.

DFCs appeared normal in 3-OST-5 MO1-injected embryos, as assessed by whole-mount in situ analysis of sox17 expression (present in 95%, n=136). KV was analyzed in live embryos using stereo microscopy at 6 SS and appeared to form normally in 3-OST-5 MO1-injected embryos (present in 88%, n=94). Together, these results suggest that LR defects in 3-OST-5 morphants are not due to defects in midline structure, DFC specification or KV morphogenesis.

3-OST-5 is required cell-autonomously in KV cells for LR development

To test directly whether 3-OST-5 functions cell-autonomously in the DFC/KV cell lineage, we utilized a technique we developed to target MOs to DFC/KV cells by injection into the yolk cell at the 500-cell stage (Amack and Yost, 2004). This creates chimeric embryos in which targeted gene function is knocked down in DFC/KV cells, but remains wild type in all other embryonic cells. In embryos with targeted knockdown of 3-OST-5 in DFC/KV cells (DFC3-OST-5MO1), aberrant spaw expression was predominantly left-sided (Fig. 1F). This phenotype is similar to the spaw expression profile seen in global knockdown of 3-OST-5 in mutants or morphants that have perturbed KV shape (Amack et al., 2003), we assessed whether 3-OST-5 morphants had intact midline structures. At 16 SS, in situ RNA expression of lefty1, ntl and shh in 3-OST-5 MO1-injected embryos showed normal midline and floor plate development (lefty1, present in 83%, n=82; ntl, present in 100%, n=122; shh, present in 100%, n=122; supplementary material Fig. S1G-J). These results indicate that LR patterning defects in 3-OST-5 morphants are not due to gross perturbations of midline development.

DFCs appeared normal in 3-OST-5 MO1-injected embryos, as assessed by whole-mount in situ analysis of sox17 expression (present in 95%, n=136). KV was analyzed in live embryos using stereo microscopy at 6 SS and appeared to form normally in 3-OST-5 MO1-injected embryos (present in 88%, n=94). Together, these results suggest that LR defects in 3-OST-5 morphants are not due to defects in midline structure, DFC specification or KV morphogenesis.

3-OST-5 is required cell-autonomously in KV cells for LR development

To test directly whether 3-OST-5 functions cell-autonomously in the DFC/KV cell lineage, we utilized a technique we developed to target MOs to DFC/KV cells by injection into the yolk cell at the 500-cell stage (Amack and Yost, 2004). This creates chimeric embryos in which targeted gene function is knocked down in DFC/KV cells, but remains wild type in all other embryonic cells. In embryos with targeted knockdown of 3-OST-5 in DFC/KV cells (DFC3-OST-5MO1), aberrant spaw expression was predominantly left-sided (Fig. 1F). This phenotype is similar to the spaw expression profile seen in global knockdown of 3-OST-5 in mutants or morphants that have perturbed KV shape (Amack et al., 2003), we assessed whether 3-OST-5 morphants had intact midline structures. At 16 SS, in situ RNA expression of lefty1, ntl and shh in 3-OST-5 MO1-injected embryos showed normal midline and floor plate development (lefty1, present in 83%, n=82; ntl, present in 100%, n=122; shh, present in 100%, n=122; supplementary material Fig. S1G-J). These results indicate that LR patterning defects in 3-OST-5 morphants are not due to gross perturbations of midline development.

DFCs appeared normal in 3-OST-5 MO1-injected embryos, as assessed by whole-mount in situ analysis of sox17 expression (present in 95%, n=136). KV was analyzed in live embryos using stereo microscopy at 6 SS and appeared to form normally in 3-OST-5 MO1-injected embryos (present in 88%, n=94). Together, these results suggest that LR defects in 3-OST-5 morphants are not due to defects in midline structure, DFC specification or KV morphogenesis.
SS when comparing 3-OST-5 morphants (49.27±14.90, n=28, P<0.001; Fig. 3C-F; Fig. 4A). Embryos injected with either uninjected (eight embryos, five beads/embryo) or 3-OST-3Z MO1 (three embryos, five beads/embryo) showed a characteristic clockwise flow of fluorescent beads in 3-OST-5 morphants (Fig. 2D; supplementary material Fig. S1K,L; Movies 1 and 2). Each color represents a single tracked bead. (F) Average velocity of fluorescent beads was significantly slower in 3-OST-5 MO1 morphants (five embryos, five beads/embryo; P<6.19e–10) compared with either uninjected (eight embryos, five beads/embryo) or 3-OST-3Z MO1 (three embryos, five beads/embryo) controls. Error bars represent s.e.m.

**Foxj1a and Rfx2 ciliogenic transcription factors are dependent on 3-OST-5**

We next focused on several markers known to be involved in cilia formation and function. In 3-OST-5 morphants, expression of **dnah9** mRNA, a dynein subunit, was expressed normally in DFCs in >89% of embryos (Fig. 3A,B; Fig. 5H). This concurred with our mRNA, a dynein subunit, was expressed normally in DFCs in >89%

**3-OST-5 and FGF signaling interact to control normal length KV cilia**

Our previous work has shown that two FGF ligands, Fgf8 and Fgf24, and the FGF receptor 1 (Fgfr1), regulate a ciliogenic transcription factor network controlling cilia length (Neugebauer et al., 2009). Interestingly, the Fgfr8 hypomorphic mutant *acebellar* (ace) does not have a shortened cilia phenotype. However, further reduction of FGF ligands by Fgf24 MO into ace mutants, or other combinations of Fgf8 and Fgf24 mutants and morphants, results in a shortened cilia phenotype (Neugebauer et al., 2009), indicating that cilia length determination is very sensitive to FGF ligand dosage. In order to determine whether 3-OST-5 works with the FGF pathway to control cilia length, we injected 3-OST-5 MO1 at a sub-threshold dose that does not cause a cilia length phenotype by itself, into embryos generated by mating ace heterozygotes (Fig. 3I). This produces embryos with sub-threshold knockdown of 3-OST-5 in the context of three distinct doses of Fgf8 ligand (homozygous wild type, heterozygous and homozygous mutant; Fig. 3J). If HS modifications incurred by 3-OST-5 gene function are required to modulate FGF signaling, then the partial loss of both Fgf8 and 3-OST-5 should be synergistic and lead to a shortened cilia phenotype.

Embryos were individually assessed for KV structure, cilia length and cilia number, blinded to Fgf8 genotype. Each individual embryo was then genotyped using high resolution melt analysis (HRMA) (Parant et al., 2009), and identified as WT, ace heterozygous or ace mutant in both the 3-OST-5 MO1-injected and uninjected classes (Fig. 3I). Together, the results indicate (1) that the sub-threshold dose of 3-OST-5 MO1 did not produce a shortened cilia phenotype and (2) that combining sub-threshold reduction of 3-OST-5 and sub-threshold reduction of FGF ligand produced a shortened cilia phenotype (Fig. 3J). These results show that 3-OST-5 and FGF signaling work together to control cilia length through ciliogenic transcription factors (Fig. 3K).

**3-OST-6 controls LR development through a distinct KV cell-autonomous pathway**

Substantiating the glycocode hypothesis that individual 3-OST family members have distinct functions (Cadwallader and Yost, 2013), we found that 3-OST-6 controls LR patterning through a mechanism that is strikingly different from that of 3-OST-5. In contrast to the curved body axis defects in 3-OST-5 morphants, the gross morphology of 3-OST-6 morphants appeared similar to uninjected control embryos (Fig. 4A). Embryos injected with either a translation-blocking MO targeting 3-OST-6 (3-OST-6 MO1) or a splice-blocking MO (3-OST-6 MO2) displayed a high frequency of reversed heart (MO1 45%, n=172; MO2 22%, n=89) and gut (MO1 45%, n=172; MO2 23%, n=89) orientations (Fig. 4B). The injection of 3-OST-6 MO2 effectively reduced accumulation of 3-OST-6
mRNA (supplementary material Fig. S2A,B). The 3-OST-6 MO1 was significantly rescued by injection of MO-resistant 3-OST-6 mRNA, which lacked the N-terminus and transmembrane coding regions (TM) and could not bind MO1 (3-OST-6ΔTM; Fig. 4B).

Expression of 3-OST-6ΔTM alone did not alter heart (3% abnormal, n=140) or gut (3%, n=140) orientation (Fig. 4B). As with 3-OST-5, the observation that two distinct morpholinos share a similar LR phenotype, and that the phenotype can be rescued by mRNA co-injection, indicates that 3-OST-6 has a role in LR patterning.

Upstream of organ laterality, we found that spaw expression was altered in 3-OST-6 morphants (Fig. 4C). Interestingly, the alteration in spaw expression pattern was different from that of 3-OST-5 morphants. 3-OST-5 morphants showed a high incidence of bilateral spaw expression (Fig. 1F), whereas 3-OST-6 morphants had a more randomized spaw expression profile (Fig. 4C). When comparing the classes of spaw, we found a statistically significant difference (Fisher’s Exact Test P<0.001) between 3-OST-5 MO2 morphants and 3-OST-6 MO2 morphants, with embryos derived from injections of sibling embryos. Similar to 3-OST-5, DFC3-OST-6 MO1 embryos had aberrant spaw expression that was predominantly bilateral (Fig. 1F; Fig. 4C). As with the controls in the 3-OST-5 analysis, DFCcontrol MO, YolkControl MO and Yolk3-OST-6 MO embryos did not alter left-sided spaw expression (Fig. 4C). Together, these results demonstrate that 3-OST-6 functions cell-autonomously in the DFC/KV cell lineage to regulate LR development.

Similar to 3-OST-5, 3-OST-6 is expressed ubiquitously in the early embryo, both at 70% epiboly and 7-10 SS (supplementary material Fig. S2C,D). In addition, it appears that early embryonic structures important for LR development are also intact in 3-OST-6 morphants, including normal DFC development and migration (98% normal sox17 whole-mount in situ expression patterns, n=138) and normal KV differentiation and morphogenesis (98% normal by live stereo microscopy, n=90; Fig. 4D, aPKC confocal imaging). At 16 SS, in situ RNA expression of lefty1, ntl and shh in 3-OST-6 MO1-injected embryos indicated normal floor plate and midline development (lefty1, expressed in 87.8%, n=49; ntl, expressed in 100%, n=108; shh, expressed in 100%, n=109;
supplementary material Fig. S2E,F). These results indicate that LR patterning defects in 3-OST-6 morphants are not due to perturbations of DFC migration, KV formation or midline development. Cilia motility, but not cilia length, is dependent on 3-OST-6

Despite having altered LR development, 3-OST-6 morphants had normal KV cilia length (Fig. 4D,E) and normal KV cilia number (51.1±10.47, n=18, P<0.99). This is in striking contrast to the short cilia phenotype in 3-OST-5 morphants (Fig. 2A-C). However, bead tracking in 3-OST-6 morphants showed very little bead movement, and average bead velocity was significantly reduced (Fig. 4F,G; supplementary material Fig. S2G; Movie 4), indicating that the normal length cilia were immotile in 3-OST-6 morphants. We further analyzed the images by drawing two concentric circles centered at the start of each bead track and scored whether each bead exited one or both circles. The inner circle had a 12.5 μm radius and the outer circle had a 25 μm radius (see example in Fig. 4H). In control and 3-OST-3Z morphants, beads exited both circles with 100% frequency (control, n=45 beads; 3-OST-3Z MO, n=15 beads). Consistent with reduced flow but in line with some residual directionality, beads exited the circles with reduced frequency in 3-OST-5 morphants, with only 50% of beads exiting the small circle and <20% exiting the large circle (n=32 beads; P<0.001 compared with controls). In 3-OST-6 morphants, only 12% of beads exited the small circle and <8% exited the large circle (n=25 beads; P<0.001 compared with controls), indicating that the normal length cilia in 3-OST-6 morphants were immotile. This further indicates a statistical difference between bead behaviors in 3-OST-6 morphants compared with 3-OST-5 morphants (P<0.01).
HS 3-OSTs regulate cilia function in zebrafish

**DISCUSSION**

Here, we present the first *in vivo* evidence in vertebrates, to our knowledge, that two closely related enzymes in the HS biosynthetic pathway, 3-OST-5 and 3-OST-6, have distinct cellular and developmental functions in the same cells. Significantly, at least two other members, 3-OST-3Z and 3-OST-7, are expressed in these cells, but are unable to compensate for these functions, and knockdown of these two 3-OSTs did not result in LR patterning defects. It is striking that knockdown of two members of the 3-OST family result in a shared developmental end-point phenotype, altered organ LR asymmetry, through cell-autonomous functions in ciliated KV cells. However, knockdown of each of these two 3-OSTs generates this convergent LR phenotype by apparently discrete cellular and molecular mechanisms (Fig. 6). 3-OST-5 regulates cilia length through FGF-dependent ciliogenic transcription factor expression, and 3-OST-6 regulates cilia motility through kinesin expression and cilia dynein arm morphology.

3-OST-5 controls cilia length through a pathway that controls the expression of two ciliogenic transcription factor genes in KV, *foxj1a* and *rfx2*, and the FGF response gene *sef*. *Fgfr1* and 3-OST-5 have similar functions: they regulate DFC/KV expression of *foxj1a* and *rfx2*, cilia length and asymmetric fluid flow in KV, and are cell-autonomously required in KV cells for LR development (Neugebauer et al., 2009). In addition, they have similar phenotypes, such as curved body axis, that are common in morphants or mutants of ciliogenic genes such as *polaris* (*ift88*) and *foxj1a* (Bisgrove et al., 2005; Yu et al., 2008; Tian et al., 2009; Hellman et al., 2010). Thus, it is likely that 3-OST-5 and Fgfr1 signaling function in the same ciliogenic pathway to regulate LR development; this is further substantiated by dosage experiments with 3-OST-5 and the Fgfr8 ligand (Fig. 3). We propose that 3-OST-5 in DFC/KV cells generates a glycocode necessary for FGF signaling at the cell surface, which then maintains the downstream ciliogenic pathway to control cilia length (Fig. 6). Interestingly, involvement of 3-OST-5 in FGF signaling appears to be tissue specific. For example, 3-OST-5 has otolith defects, but not the pronephric duct defects observed in *fgfr1* mutants.

**3-OST-6 is required for kinesin motor molecule expression and ciliary dynein arms**

Consistent with normal length cilia, and in striking contrast to 3-OST-5 morphants, the ciliogenic transcription factor genes *rfx2* and *foxj1a* had normal expression patterns in a majority of 3-OST-6 morphants (P<0.05 and P<0.001, respectively; Fig. 5C,D). To explore further the cause of cilia immotility, we assessed other motor molecules. Kinesin 3b, *kif3b*, is expressed in DFCs from 30% epiboly to tailbud stages. *kif3b* was reduced in the DFCs of 3-OST-6 morphants but not in the DFCs of 3-OST-5 morphants or controls (P<0.001 3-OST-6 MO1 compared with WT and 3-OST5 MO1; Fig. 5E-H). This suggests that the loss of cilia motility in 3-OST-6 morphants is due to a novel regulatory pathway that controls kinesin expression in KV ciliated cells.

Electron microscopy analysis of KV cilia revealed another important distinction between 3-OST-5 and 3-OST-6 morphants. Cilia were scored for the presence or absence of dynein arms (inner and outer); if a single dynein arm was present in a cilium, the entire cilium was scored as having dynein arms. Under this criterion, most cilia in WT uninjected and 3-OST-5 MO embryos had dynein arms (seven out of ten cilia from three WT embryos had detectable dynein arms; nine out of 12 cilia from four 3-OST-5 MO1 embryos had detectable dynein arms; Fig. 5I,J). However, in 3-OST-6 MO1 embryos, only 10% of cilia had detectable dynein arms (one out of 11 cilia from four 3-OST-6 MO embryos had detectable dynein arms; Fig. 5K), which was significantly different (P<0.0018) from WT and 3-OST-5 MO-injected embryos. This suggests that the non-motile cilia, loss of KV fluid flow and LR patterning defects in 3-OST-6 morphants are due to an absence of dynein arms in KV cilia.
Fig. 6. Glycocode model: 3-OST-5 and 3-OST-6 regulate distinct cell signaling pathways for ciliogenesis and cilia motility. Schematic of glycocode regulation of cilia length and cilia motility. 3-OST-5 and FGF pathway perturbations have the same downstream molecular and short cilia phenotypes, and display dose-dependent genetic interactions. Therefore, we propose that 3-OST-5 generates a glycocode (blue) on HS chains on proteoglycans (indicated by HSPGs) that modulates interactions of FGF ligands (for example, Fgf8 and Fgf24) and Fgfr1 at the cell surface. It is important to note that the modifications can be on the same HSPG core protein (as shown HSPG for simplicity) or multiple different HSPGs in the same cell. This interaction activates downstream ciliogenic transcription factors including foxj1a and rfx2 for normal length KV cilia. By contrast, 3-OST-6 generates a different glycocode (green) that modulates a distinct pathway regulating kif3b expression, dynein arms and cilia motility.

morphants (Neugebauer et al., 2009) (data not shown). Downregulation of FGF pathway targets in 3-OST-5 morphants is unique to the DFCs during early embryonic development (Fig. 3G,H), suggesting that other glycocode combinations participate in FGF signaling in other tissues. Preliminary examination of 3-OST-5 putative mutants did not reveal an embryonic phenotype (data not shown). Given the maternally contributed stores of several family members in embryos (Cadwallader and Yost, 2006a), further analysis of maternal-zygotic mutants of this and other family members, perhaps in different strain backgrounds, will be required to fully understand the functional relationships within the 3-OST family, and will allow analysis of later phenotypes that are beyond the reach of morpholino technology.

In both fgfr1 MO- and 3-OST-5 MO-treated embryos, it was somewhat surprising that foxj1a is diminished but dnah9 expression is relatively normal (Neugebauer et al., 2009), in contrast to other reports linking dnah9 expression to foxj1a (Yu et al., 2008; Hellman et al., 2010; Caron et al., 2012). foxj1a expression turns on early, during 30% epiboly (Aamår and Dawid, 2008), and is initiated by the Wnt/β-catenin pathway (Caron et al., 2012). We suggest that FGF signaling, and now 3-OST-5, are required to maintain foxj1a expression. Loss of this pathway results in a reduction of foxj1a mRNA expression, but perhaps a sufficient amount of early Foxj1a protein is available to initiate dnah9 expression. It is important to note that in both 3-OST-5 and Fgfr1 knockdown, cilia are motile even in the face of diminished foxj1a expression.

In contrast to 3-OST-5, 3-OST-6 controls cilia motility without affecting cilia length, through an apparently separate pathway that is necessary for normal kif3b expression and normal LR axis formation (Fig. 6). Thus, 3-OST-6 is a novel and specific cell regulator of cilia motility that controls the organization or maintenance of dynein arms within cilia by generating a distinct glycocode from that generated by 3-OST-5. 3-OST-6 morphants have immotile but normal length cilia, consistent with their relatively normal expression of foxj1a and rfx2 ciliogenic transcription factor genes. Other non-motile cilia mutants have similar phenotypes to 3-OST-6 morphants, such as the LR defects exhibited by seahorse mutants (Serluca et al., 2009), and the axonomal dynein arm defects, exhibited by ktu mutants (Omran et al., 2008; Mitchison et al., 2012). The observation that knockdown of 3-OST-6 in whole embryos and knockdown in DFC/KV have different spaw expression patterns suggests that, in addition to its cell-autonomous role in DFC/KV, 3-OST-6 might also have roles in LR patterning in other cell lineages. It will be interesting to pursue the possibility that 3-OST-6 modulates a distinct signaling pathway to control kif3b transcription and/or assembly of dynein arms in cilia through cargo sorting. In addition, although 3-OST6 morphants have a very distinct phenotype from modulations of the Fgfr8/Fgfr24/Fgfr1 pathway, we have not excluded the possibility that 3-OST-6 modulates other components of FGF signaling in DFC/KV or other tissues.

We have demonstrated that distinct cell signaling pathways are regulated by distinct 3-OST functions in ciliated cells. These findings suggest that each member of the 3-OST family generates a distinct component of the HSPG glycocode at the cell surface of ciliated cells, perhaps by placing sulfation in the specific context of other regional modifications on the HS chain (Cadwallader and Yost, 2013). Previous reports have demonstrated that different members of the 3-OST family can generate the same sulfated disaccharides in vitro (Shworak et al., 1999; Xia et al., 2002; Xu et al., 2005). Unfortunately, current HS sequencing technology has a limit of ~6-14 disaccharides from a homogeneous in vitro source (Stringer et al., 2003; Zaia and Costello, 2003; Thanawiroon et al., 2004; Saad and Leary, 2005; Volpi and Linhardt, 2010). In vivo, HS chains range in size from 40 to 160 disaccharides with a heterogeneous composition (Esko and Selleck, 2002). Technical advances in HS structural analysis will be necessary to determine experimentally the sequences of distinct glycocodes, and ultimately the mechanisms by which different members of the 3-OST family generate functionally distinct HS glycocodes. We predict that each cell type will utilize different combinations of OST family members to build distinct combinations of glycocodes at the cell surface in order to discriminate among the myriad of cell-cell signaling pathways available during vertebrate development.

Acknowledgements

We thank Bradley Demarest, Todd Townsend, Nick Trede, Manju Karthikeyan, Megan Smith and Elaine Martini for technical assistance; Maureen Condic, Jonathon Hill and Brad Olwin for comments on the manuscript; M. Condic and Yuko Sajioh for blind scoring of cilia TEMs; and Nancy Chandler for outstanding assistance with electron microscopy.

Funding

This work was supported by grants from the National Heart, Lung, and Blood Institute [HL075472 to H.J.Y.]; and the Primary Children’s Medical Foundation. Deposited in PMC for release after 12 months.
and regional differences in heparan sulfate structure in chick limb buds. J. Biol. Chem. 279, 8219-8229.


