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Core fucosylation is required for midline patterning during zebrafish development

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Abstract

Complex carbohydrates represent one of the most polymorphic classes of macromolecules, but their functions during embryonic development remain poorly defined. Herein, we show that knockdown of FucT8, the fucosyltransferase responsible for adding an α 1,6 fucosyl residue to the core region of *N*-linked oligosaccharides, results in defective midline patterning during zebrafish development. Reduced FucT8 expression leads to mild cyclopia, small forebrains, U-shaped somites, among other midline patterning defects. One of the principal FucT8 substrates was identified as Apolipoprotein B (ApoB), the major scaffold protein that is responsible for assembly and secretion of lipoprotein particles in vertebrates. In *Drosophila*, lipoprotein particles are thought to facilitate cell signaling by serving as a transport vehicle for lipid-modified cell signaling proteins, such as hedgehog. In this regard, knockdown of ApoB expression in zebrafish embryos leads to similar midline patterning defects as those seen in FucT8 morphant embryos. Furthermore, preliminary studies suggest that ApoB facilitates Sonic hedgehog signaling during zebrafish development, analogous to the function of lipoprotein particles during hedgehog signaling in *Drosophila*.

Keywords

zebrafish; fucosylation; apolipoprotein B; sonic hedgehog

Introduction

It has long been appreciated that the carbohydrate chains that decorate glycoproteins, glycolipids and other complex carbohydrates play critical roles during development. However, a precise understanding of their function has been hampered by their enormous heterogeneity and by the fact that nearly 300 distinct glycosyltransferases are responsible for their synthesis. Nevertheless, it is now clear that specific glycosylations can influence a variety of developmental events, including the trafficking of soluble morphogens in the embryo, the ability of glycoprotein ligands to bind their receptors, as well as the ability of receptors to elicit intracellular signal cascades (Ma et al., 2006; Varki, 1993; Zhao et al., 2008)

A variety of biochemical and molecular studies have implicated terminal fucose residues, in particular, as playing critical roles in a variety of cellular interactions (Ma et al., 2006). The

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fucose residues that decorate carbohydrate chains on glycolipids and glycoproteins are synthesized by four fucosyltransferase (FucT) families (Ma et al., 2006). The α 1,2 and α 1,3/4 FucTs add fucose to peripheral positions of carbohydrate chains, or antennae, whereas the α 1,6 FucT adds fucose to the innermost, or core, *N*-acetylglucosamine residue that is attached to the polypeptide backbone (Fig. 1A). Unlike the α 1,2 and α 1,3/4 FucTs, there is only one α 1,6 FucT transcript in mammals, encoding FucT8, the sequence of which has been highly conserved during evolution. The fourth family member, O-FucT, fucosylates serine/threonine residues within EGF repeats of selective glycoprotein substrates, such as Notch (Ma et al., 2006).

The α 1,2 and α 1,3/4 FucTs have been the subject of much study, and participate in the synthesis of blood group antigens and carbohydrate epitopes required for selectin-based recognition between immune cells (Ma et al., 2006). FucT8 has received less attention, but is essential for viability as FucT8-null mice show severe growth retardation and neonatal lethality (Wang et al., 2005). In vitro studies suggest that core fucosylation of transmembrane receptors is required for their ability to elicit intracellular signal transduction cascades, including the TGF-1, EGF, and LRP-1 receptors, as well as the α 3 β 1 integrins (Li et al., 2006; Wang et al., 2006a; Wang et al., 2006b; Zhao et al., 2006). However, the consequences of the FucT8 deletion on embryonic patterning or development have not yet been investigated.

Zebrafish are an excellent experimental system to explore glycoconjugate function during development due to the ability to reduce, or knockdown, specific glycosyltransferase activities and examine the consequences on morphogenesis. Therefore, in this study we explored the function of FucT8 during zebrafish development. Knocking down FucT8 activity leads to defective midline patterning due to an apparent lack of functional Apolipoprotein B100 (ApoB), the homolog of which has been implicated in long-range hedgehog signaling in *Drosophila* (Panakova et al., 2005). These results suggest that core fucosylation is required for ApoB function, and that lipoprotein particles may facilitate cell signaling in vertebrates.

Results

Zebrafish fucosyltransferases

The zebrafish database was analyzed for sequences homologous to the mammalian FucTs families. As reported by others, the zebrafish database does not contain α 1,2 FucT sequences (Kageyama et al., 1999). On the other hand, there appear to be five zebrafish α 1,3/4 FucTs, similar to the presence of multiple α 1,3/4 FucTs in other species, and only one α 1,6 FucT, as is the case in mammals (Ma et al., 2006) (Fig. 1B). As noted above, the loss of FucT8 leads to growth retardation and neonatal lethality in mice (Wang et al., 2005), although the consequences on embryonic development and patterning have not been addressed. Therefore, in this study we focused on the role of α 1,6 FucT (FucT8) during zebrafish development; a study that is facilitated by the apparent lack of redundant family members. The zebrafish *fuct8* gene is 1746bp long with 9 exons and predicts a protein with 78% identity to mammalian FucT8 (Fig.1C).

FucT8 morphants resemble midline pathway mutants

To determine the possible roles of FucT8 during zebrafish development, 1-2 cell stage embryos were injected with antisense morpholino oligonucleotides (MO) designed against a FucT8 splice donor site. RT-PCR analysis confirmed the efficacy of the splice blocking MO to prevent FucT8 splicing (Fig. S1). Parallel studies using a translation blocker antisense MO produced similar phenotypes to that seen with splice blocker MO. For controls,

embryos were injected with identical levels of control oligonucleotides as indicated in the Experimental Procedures.

Out of more than 500 embryos injected with FucT8 MOs, 85-90% showed a similar morphant phenotype. At 24 hours post fertilization (hpf), the FucT8 MO-injected embryos have smaller eyes as compared to controls. This phenotype is more apparent at 48 hpf when the embryos show mild cyclopia (i.e., close set eyes), small forebrains, and a hindbrain 'bump' that appears to reflect a swollen hindbrain ventricle (Fig. 2A-C). Furthermore, FucT8 MO-injected embryos have U-shaped somites and a highly curved body axis (Fig. 2B). Most of these phenotypes, including mild cyclopia, U-shaped somites and a curved body axis, are similar to those seen in previously described zebrafish midline pathway mutants (Brand et al., 1996). These observations suggest that FucT8 might be required for midline signaling.

We confirmed that the MO injection reduced FucT8 activity by staining embryos with AAL, a lectin that specifically recognizes α 1,6-linked fucose moieties. Control-injected embryos retain strong AAL labeling, which is reduced to background levels following MO injection, thus confirming the loss of FucT8 activity (eye sections shown in Fig. 2D).

Loss of differentiated retinal cells in FucT8 morphants

Since eye development appeared to be a clear target of FucT8 MO injection, we characterized the extent of retinal cell differentiation in control and FucT8 MO-injected embryos. DAPI staining shows that although the FucT8 morphant eye is much smaller than controls (Fig. 3A), the retina contains numerous cells that are loosely stratified into layers, similar to that seen in some patterning mutants, such as *smu* (Masai et al., 2005). Retinal ganglion cells (RGCs), Müller glial cells and photoreceptors were identified within the retina using zn-5, anti-carbonic anhydrase II (CA-II), and *zpr-1* antibodies, respectively. RGCs are the first cells to differentiate in the retina and appear around 28 hpf (Laessing and Stuermer, 1996). Even by 48 hpf, FucT8 morphants have few to no RGCs (Fig. 3B). As a result, the optic nerve and optic chiasm are not seen in morphant embryos (Fig. 3C). Müller glia and photoreceptor cells are also extremely reduced in the morphants (Fig. 3D,E). The reduced eye size in FucT8 morphants is not a consequence of decreased cell proliferation as assayed by BrdU incorporation (Fig. 3F); rather, BrdU incorporation appears higher in FucT8 morphant eyes, similar to that reported in *smu* mutant eyes (Masai et al., 2005). Furthermore, FucT8 morphants show a slight overall increase in apoptotic cells throughout the embryo as assayed by Acridine Orange (Fig. S2), similar to that reported in the *smo* mutant (Chen et al., 2001), and which might contribute to the overall reduced size of the FucT8 morphant embryos, relative to controls. Regardless, the absence of multiple cell types suggests that core fucosylation may play some role in patterning of the retina.

Earlier patterning defects in the eye

To determine if the lack of differentiated cell types in the retina results from defective patterning, we analyzed the expression of well-established patterning markers by in situ hybridization. *Islet-1* is a lim homeodomain transcription factor that is expressed in the neural retina and elsewhere in the embryo (Inoue et al., 1994). Although present in other domains, *islet-1* expression is lost in the developing eye of FucT8 morphants (27 hpf, Fig. 4A). *ath5* is a homolog of mammalian *atonal* and is expressed in RGC precursors (Masai et al., 2000). *ath5* expression is lost in FucT8 morphants, consistent with the loss of RGCs seen at 48 hpf (Fig. 4B). Finally, *pax 2.1* labels the astrocytes in the optic stalk (Krauss et al., 1991), and at 27 hpf is mis-expressed at the midline of FucT8 morphants, which illustrate a shorter, or reduced, optic stalk and expanded *pax 2.1* expression in eyes (Fig. 4C).

Collectively, these results implicate FucT8 as being essential for proper specification of the retina.

Other cell types and tissues affected in FucT8 morphants

As discussed above, FucT8 morphant embryos display patterning defects similar to those seen in midline pathway mutants. We therefore examined for the presence of midline patterning defects by assessing the expression of various markers. In the spinal cord, *islet-1* labels the primary motor neurons at 24 hpf and all motor neurons by 48 hpf. When stained with *islet-1* at 48 hours, the motor neurons in FucT8 morphants appear disorganized, being scattered along the dorsal-ventral axis (Fig. 5A). This is accompanied by a loss of axonal projections from motor neurons, as viewed by anti-acetylated tubulin antibody (Fig. 5B). Dorsal root ganglion cells are also significantly reduced in the morphants and lack any axonal projections as judged by zn-5 labeling (Fig. 5C).

The spinal cord defects seen in FucT8 morphants are associated with U-shaped somites that have fewer and shorter muscle fibers than control somites, as assessed by anti-myosin heavy chain (F-59) antibody (Fig. 5D). Similarly, the ventral migration of neural crest cells into the somitic mesoderm is greatly reduced (Fig. 5E), as are the jaw cartilages that derive from neural crest precursors (Fig. 5F). Other midline defects include a lack of hindbrain commissures (Fig. 5G). In summary, FucT8 morphants show a broad range of midline patterning defects indicating loss of proper midline signaling in the absence of α 1,6 fucosylation.

Identification of the FucT8 substrate

In order to identify the target proteins that are α 1,6 fucosylated, we first compared the AAL-reactive glycoproteins in lysates of control and FucT8 MO-injected embryos by SDS-PAGE and lectin blotting. AAL specifically recognizes α 1,6 fucosyl residues, and as expected, lectin staining was inhibited by an excess of free fucose (bracket, Fig. 6A). FucT8 morphants have greatly reduced AAL reactivity in the high molecular weight area, suggesting that one of the major FucT8 target proteins is ≥ 200 kDa. The overall protein profile showed no difference in overall staining intensity (data not shown), indicating that the reduced AAL staining was not a result of reduced protein load for morpholino-injected embryos.

AAL-conjugated agarose beads were used to precipitate the target protein from control and morphant lysates. Similar to what was found by AAL lectin blotting, AAL lectin precipitated a complex of two-three large molecular weight glycoproteins in control lysates that was absent in FucT8 morphants (bracket, Fig. 6B). The corresponding AAL-reactive bands were excised from Coomassie-stained gels and sequenced by Mass Spectrometry (MS). Of the 21 candidate matches, the “hypothetical” protein LOC321166, molecular weight of 412 kDa, was represented by 96 distinct peptide sequences (score = 5385), whereas all others were represented by four or fewer peptides (scores range from 31-149). BLAST searches reveal that LOC321166 has high homology to human and mouse Apolipoprotein B 100 (ApoB), and is predicted to be zebrafish ApoB.

In retrospect, it is not surprising that the AAL-reactive proteins migrated on SDS-polyacrylamide gels as a complex of large molecular mass proteins, since others have reported that ApoB 100 migrates as a family of related polypeptides, some of which are constitutively seen when resolved on SDS-PAGE, with others being the result of posttranscriptional editing of the ApoB mRNA (Chen et al., 1986; Young et al., 1986). Nevertheless, sequence analysis verified that each of the individual high molecular weight AAL-reactive bands were derived from the ApoB polypeptide. Furthermore, when AAL-

reactive proteins were collected by AAL-agarose beads and resolved by SDS-PAGE, LOC321166 (zfApoB) was again identified by MS sequence analysis.

ApoB is α 1,6 fucosylated

Human ApoB is a large molecular weight glycoprotein with a well-defined role in lipid metabolism and transport of cholesterol (Fisher and Ginsberg, 2002). To confirm that ApoB is indeed α 1,6 fucosylated, we used the human hepatocyte cell line, HepG2, since there are no known antibodies that recognize zebrafish ApoB. HepG2 lysates were incubated with either AAL-agarose to identify α 1,6 fucosylated glycoproteins, or with anti-ApoB antibodies; the precipitates were collected, washed and resolved by SDS-PAGE. The proteins were transferred to PVDF and stained with anti-ApoB antibody. Anti-ApoB antibody detected a high molecular weight polypeptide in Hep2G lysates that was of similar size to the ApoB-reactive polypeptide precipitated by AAL beads (Fig. 6C). As expected, the immunoprecipitated ApoB was reactive with AAL by lectin blot analysis (data not shown). These results confirm that ApoB is α 1,6 fucosylated, and are consistent with the loss of AAL reactivity following FucT8 morpholino injection.

ApoB morpholino injections phenocopy FucT8 morphant phenotypes

If the loss of ApoB core fucosylation is indeed one of the primary targets of FucT8 morpholinos, then ApoB knockdown should phenocopy the FucT8 morphants. 1-2 cell stage embryos were injected with 5-8 ng of either one of two ApoB translation blocking morpholinos and probed with markers that were used to characterize the FucT8 morphants. Both ApoB morpholino oligonucleotides produced similar phenotypes (~ 400 embryos injected), at ~90% penetrance. Consistent with ApoB being a target of FucT8 morpholino knockdown, ApoB morphants display similar or more severe phenotypes as the FucT8 morphant embryos. For example, *islet-1* expression in the eye is greatly reduced at both 27 and 48 hpf (arrowheads, Fig. 7A,B), similar to that seen in FucT8 morphants (Fig. 4A). Similarly, the occurrence of RGC precursors, as assayed by *ath-5* expression at 27 hpf, is greatly reduced in both ApoB (arrowheads, Fig. 7C) and FucT8 morphants (Fig. 4B). The optic stalk is also reduced, or absent, in both ApoB (arrow, Fig. 7D) and FucT8 morphants (Fig. 4C), as assayed by *pax 2.1* expression. Finally, immunolabeling with zn-5 antibodies illustrates the loss of RGCs (arrowheads) and optic chiasma (arrow) in 48 hpf ApoB morphants, similar to that seen in FucT8 morphants (Fig. 3C). In summary, the ApoB and FucT8 morphants display similar phenotypes as assessed by these markers, and further suggest that the lack of ApoB α 1,6 fucosylation leads to early midline patterning defects.

Discussion

Results presented here suggest that FucT8 activity is required for midline patterning and eye development during early zebrafish development. In mice, loss of FucT8 leads to neonatal lethality and emphysema-like changes in lungs (Wang et al., 2006b). Subsequent studies have shown that core fucosylation is required for the proper functioning of a number of transmembrane signaling receptors, which partly explains the retarded growth and neonatal lethality of FucT8-null pups (Wang et al., 2006a; Lee et al., 2006; Zhao et al., 2006). Although FucT8 appears critical for full viability, its role during embryonic development has not been previously investigated.

Knockdown of FucT8 leads to defective midline patterning

Knocking down FucT8 during zebrafish development results in phenotypes that are very similar to those seen in midline patterning mutants, in general, and in Shh pathway mutants and morphants, in particular. For example, FucT8 morphants have reduced or disorganized motor neurons and lack axonal projections from motor neurons and dorsal root ganglia,

similar to Shh pathway mutants *sonic you (syu)*, *smoothened (smo)* and *detour (dtr)* (Lewis and Eisen, 2001; Ungos et al., 2003). Shh signaling is also required for slow muscle formation and proper somite architecture, as evidenced by the *smu* and *yot* mutations, and which are also sensitive to FucT8 knockdown (Barresi et al., 2000; Lewis et al., 1999). Similarly, neural crest migration is disrupted in FucT8 morphants, leading to jaw defects and other anomalies, as seen in the *syu* and *smu* mutants (Barresi et al., 2000; Honjo and Eisen, 2005). Shh pathway mutants and morphants are also characterized by severely reduced numbers of RGCs, Müller glia and photoreceptors in the developing retina, which is seen in FucT8 morphants as well (Shkumatava et al., 2004). Similarly, the increased cell proliferation seen in FucT8 morphant eyes is similar to that reported by Masai et al. (2005) in *smu* mutants, consistent with their suggestion that Shh signaling is required for exit from the cell cycle. All these results strongly indicate that core fucosylation is necessary for proper eye development and midline patterning during zebrafish development. Initial attempts to rescue the FucT8 morphant phenotypes by co-injection of FucT8 mRNA were unsuccessful, but we were unable to determine if the RNA was properly translated and/or stable. Nevertheless, the lack of AAL reactivity clearly indicates the loss of FucT8 activity in FucT8 morphant embryos.

Apolipoprotein B function and core fucosylation

Analysis of the core fucosylated glycoproteins during zebrafish development suggests that ApoB serves as one of the major FucT8 substrates, and that failure of ApoB core fucosylation leads to defective midline patterning. This is supported by, among other results, the ability of ApoB morpholinos to phenocopy the FucT8 morphant phenotypes.

ApoB forms the protein component of low-density lipoprotein particles (LDL), which transport plasma lipids and carry free or esterified cholesterol. In mice, the loss of ApoB leads to hypercholesterolemia in heterozygous animals, whereas most ApoB homozygous-null mice die during embryonic development between days 9-11.5 and show severe neurological defects, including exencephalus. Heterozygous mice survive further into development, and are characterized by incomplete neural tube closure and hydrocephalus (Homanics et al., 1993; Huang et al., 1995; Farese et al., 1995). Similarly, functional ApoB appears necessary for viability in humans, as the only known ApoB mutations are single point substitutions in the receptor-binding domain that are maintained in heterozygous form and lead to hypobetalipoproteinemia and hypercholesterolemia (Soutar and Naoumova, 2007). All of these findings suggest that ApoB plays critical roles during early development in both mice and humans.

Although ApoB is heavily glycosylated, possessing at least 16 *N*-linked oligosaccharide chains, the function of ApoB glycans is not yet fully understood. It is known that *N*-linked chains are required for ApoB secretion and assembly into LDL particles, since tunicamycin treatment or mutagenesis of the glycosylation motifs leads to greatly reduced ApoB secretion and LDL assembly, as well as increased intracellular degradation of ApoB (Taniguchi et al., 1989; Liao and Chan, 2001; Vukmirica et al., 2002; Harazono et al., 2005). Interestingly, seven of the 16 *N*-glycosylation sites are located within the ApoB domain responsible for LDL-receptor binding as well as interactions with heparan sulfate proteoglycans. However, it is not yet clear how *N*-linked glycans influence receptor binding and/or interactions with the extracellular matrix (Yang et al., 1989; Vukmirica et al., 2002; Harazono et al., 2005). Nevertheless, these studies indicate that ApoB activity is greatly influenced by the extent and quality of its *N*-glycosylation. Results presented here support and extend this view, in that the loss of a terminal fucosyl residue, rather than the global loss of all *N*-linked chains, also leads to a loss of ApoB activity.

Studies from other model systems indicate that core fucosylation of cell surface receptors influences ligand binding and/or ligand induced intracellular signaling (Li et al., 2006; Wang et al., 2006a; Wang et al., 2006b; Zhao et al., 2006). In this light, core fucosylation of ApoB *N*-linked chains that reside within the LDL receptor-binding motif may influence ApoB binding to its receptor, such as megalin (Lpl-2), and/or its ability to elicit LDL receptor signaling. Alternatively, the loss of core fucosylation may prevent ApoB secretion, as is the case when the entire *N*-linked chains are eliminated (Taniguchi et al., 1989; Liao and Chan, 2001; Vukmirica et al., 2002; Harazono et al., 2005). In this regard, FucT8 morphants appear to have somewhat reduced levels of ApoB protein as judged by protein-stained gels (Fig. 6D). Nevertheless, further studies in better defined model systems are required to distinguish between these, as well as other, possible modes of action. In any event, the loss of core fucosylation leads to a loss of ApoB activity and a consequent reduction in midline signaling.

Sonic hedgehog signaling in FucT8 and ApoB morphant embryos

Since most of the phenotypes observed in the FucT8 and ApoB morphants are similar to those seen in Shh pathway mutants and Shh morphant embryos (Barresi et al., 2000; Honjo and Eisen, 2005; Lewis and Eisen, 2001; Shkumatava et al., 2004; Ungos et al., 2003), we are obviously interested in whether Shh signaling is altered in these morphant embryos. As an initial approach to this possibility, we examined the expression of the Shh receptor, *patched-1* (*ptc-1*), a well-established marker of Shh levels (Chen and Struhl, 1996). Interestingly, *ptc-1* expression is upregulated around the notochord and neural tube in 24 hpf FucT8 and ApoB morphant embryos (Fig. S3). The increased expression of *ptc-1* close to the source of Shh release suggests that Shh may not be transported appropriately and accumulates around its source in the absence of FucT8/ApoB leading to increased *ptc-1* expression. Although these results are preliminary and there are likely other factors contributing to the morphant phenotypes, these data are consistent with the possibility that loss of core fucosylation leads to a nonfunctional ApoB that is normally required for Shh transport and long-range signaling.

Recent studies in model organisms show that lipoproteins can serve as direct carriers of signaling proteins, such as Shh and Wnt, thus establishing and/or maintaining the morphogen gradient (Panakova et al., 2005; Willnow et al., 2007). Shh is one of the most extensively studied signaling molecules in vertebrate development, the activity of which requires post-translational processing, including N-terminal palmitoylation and the addition of a cholesterol moiety at the C-terminus (Jeong and McMahon, 2002; Guerrero and Chiang, 2007). The cholesterol component has been implicated in both long-range Shh signaling as well as restricting the spread of Shh (Lewis et al., 2001; Li et al., 2006). Similarly, the precise role of the palmitate modification is undefined, but recent studies suggest that it is required for the production of large molecular weight hedgehog signaling complexes and long-range signaling (Chen et al., 2004). In this light, it is particularly interesting that the loss of functional ApoB, resulting from FucT8 knockdown, leads to phenotypes similar to those seen in loss of Shh signaling, consistent with the view that ApoB-containing lipoproteins facilitate Shh signaling in vertebrates, as they do in lower organisms (Panakova et al., 2005).

In summary, we show here for the first time that core fucosylation of ApoB is essential for proper midline patterning, presumably by facilitating the transport of lipid-modified signaling molecules, such as Shh. Further studies are required to explore this possibility in more detail.

Experimental Procedures

Fish husbandry—Zebrafish were maintained at 28.5°C in the animal facility of Emory University. The wild type strain AB was used for all experiments.

Cell culture—HepG2 (ATCC) cells were maintained at 37°C in ATCC recommended MEM medium with 10% Fetal Bovine Serum. Cells were split 1:4 when confluent.

Cladogram—Fucosyltransferase sequences were obtained from the NCBI database and Sanger database for zebrafish sequencing. The sequences were compared to mouse and human sequences using SDSC Biology workbench (<http://workbench.sdsc.edu>).

Morpholino microinjections—One-two cell staged embryos were microinjected with 8-10 ng of a splice blocking anti-sense morpholino synthesized against zebrafish *fucosyltransferase 8* (5'-CGCCTACTGCTGCCCTCCCCTTTC-3', GeneTools). Morpholino efficacy was confirmed by RT-PCR analysis. Parallel studies using a translation blocking anti-sense morpholino (5'-AACATCCCAGGACCTGAACCTCCAT-3') produced similar results, but all data presented here were derived from the splice blocking morpholino. ApoB was knocked down by injecting 1-2 cell embryos with one of two translation blocking anti-sense morpholinos against zebrafish *apolipoprotein B* (8 ng of 5'-CCATGATGGGTTTCAGGTAAGCTCGT-3'; or 5ng of 5'-AGTCAGTAGCTTATTCCAGGGAGAT-3'). For controls, a similar amount of a standard control morpholino (5'-CCTCTTACCTCAGTTACAATTATA-3') was injected. All injections occurred within 30 minutes of fertilization, and the stages are presented as hpf (hours post fertilization) throughout.

RT-PCR—RNA was extracted from 24 hpf dechorionated embryos using the Trizol Reagent (Sigma). cDNA was generated and DNA was amplified according to the manufacturer's protocol using Superscript one step RT-PCR (Invitrogen). The following *fut8* gene-specific primers were used to amplify the cDNA: a) FT-8RT.1Fw 5'-CCATTTCTTGGTTCGGCTG-3', b) FT-8RT.2Rv 5'-CTGAACAATAGGCCCCCCC-3' and c) FT-8RT.3Rv 5'-GTCGCTGTTGCGTGGTTG-3'.

In situ hybridizations—Embryos were maintained at 28.5°C and staged as described previously (Kimmel et al., 1995; Westerfield, 1993). For stages later than 24 hours, embryos were grown in 0.003% PTU to prevent melanin synthesis. Embryos were fixed in 4% paraformaldehyde for 2 hours at room temperature or overnight at 4°C for in situ staining and immunolabeling. In situ labeling was performed as described using anti-sense probes for *pax2.1*, *islet-1*, and *ath5* (Karlstrom et al., 1999).

Immunohistochemistry—Immunolabeling for whole mounts and cryosections were performed as previously described (Devoto et al., 1996). Antibodies used were anti-acetylated tubulin (Sigma); anti-slow muscle heavy chain (F-59) (Santa Cruz, SC-32732); anti-neuroilin (zn-5) (ZIRC); anti-zpr-1, a photoreceptor marker (ZIRC); and anti-carbonic anhydrase (CAII) (gift from Dr. Paul Linser). Lectin staining was performed similar to antibody staining.

BrdU and Acridine Orange staining—For BrdU staining, dechorionated embryos (28 hpf) were incubated in embryo medium containing 10 mM Bromodeoxyuridine (Sigma) for 2 hours at 28.5°C. Embryos were then washed three times with embryo medium, followed by fixation in 4% paraformaldehyde. Embryos were then sectioned using a cryostat. Anti-BrdU antibody (Developmental Studies Hybridoma Bank) was used to perform immunostaining on sections. For Acridine orange staining, 28 hpf embryos were

dechorionated and incubated in 5 µg/ml solution of acridine orange dye for 15 minutes in the dark. Embryos were then washed three times, five minutes each, in embryo medium. Live embryos were mounted in 2% methylcellulose and images were taken using a fluorescent microscope.

Western and lectin blots—Duplicate protein samples were resolved on 5% 7.5% or 10% SDS-PAGE gels, one of which was stained using Coomassie based stains (Gel Code Blue or Imperial stain, Pierce Biotechnologies). The other gel was transferred overnight to PVDF membrane and used for western or lectin blots. The following antibodies and lectins were used: anti-ApoB, 1:1000 (Biodesign International, k45253G), and biotinylated AAL, 1 µg/ml (Vector labs).

Protein lysate—48 hour-old embryos were dechorionated and anesthetized using tricaine. Embryo heads were cut using 50 µ thick tungsten wire tools, just anterior to the yolk sac to avoid any yolk contamination in the protein lysate. Dissected heads were rinsed twice with ice-cold egg rinsing medium and 100 heads were placed in 250 µl lysis buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM CaCl₂, 50 mM Tris, pH 8.0) (Schlegel and Stainier, 2006). The tissue was sonicated for 6-7 seconds on ice and placed on a nutator for 1 hour at room temperature prior to use for lectin or antibody pull-down experiments. Alternatively, 24 hour-old embryos were dechorionated and deyolked as described previously (Link et al., 2006). The cell pellet was placed in lysis buffer and treated similarly as described above. For cell lysates, cells were rinsed with PBS 4 times, 2 minutes each, placed in lysis buffer and incubated for 1 hour at room temperature.

Lectin-pull down—Agarose-conjugated lectins were used to pull down specific glycoproteins from tissue or cell lysates. 100 µl of Aleuria aurantia lectin (AAL) beads (Vector labs) were washed 3 times, 20 minutes each, in lysis buffer at 4°C. Beads were then incubated overnight at 4°C with protein lysate on a nutator. For controls, beads were incubated with lysate in the presence of 200 mM fucose, the competing sugar. The following morning, beads were collected and stored at 4°C, and the remaining lysate was incubated again with a batch of fresh washed beads to increase the yield of bound protein. After three rounds of incubation, beads were pooled together and rinsed three times with lysis buffer. Proteins were eluted by placing the beads in 1X sample buffer with 500 mM fucose and boiled for 5 minutes. The depleted lysate was saved for SDS-PAGE. For antibody pull down, the lysate was incubated with 3-5 µg of primary antibody at 4°C overnight. The lysate was then incubated with Protein A or G beads for 3 hours at 4°C. Protein A or G beads were washed 3 times, 5 minutes each in lysis buffer, and proteins were eluted by boiling beads for 5 minutes in 1X sample buffer.

Sectioning—Fixed embryos were washed and incubated in PBS with 0.1% Tween (PTw) for 3-4 hours. Embryos were then embedded in 1.5% agarose and dehydrated in a 30% sucrose solution overnight, as described (Devoto et al., 1996). Ten µ thick sections were cut and stained with antibody or biotinylated lectin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Barresi MJ, Stickney HL, Devoto SH. The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity. *Development*. 2000; 127:2189–2199. [PubMed: 10769242]
- Brand M, Heisenberg CP, Warga RM, Pelegri F, Karlstrom RO, Beuchle D, Picker A, Jiang YJ, Furutani-Seiki M, van Eeden FJ, Granato M, Haffter P, Hammerschmidt M, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Nusslein-Volhard C. Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development*. 1996; 123:129–142. [PubMed: 9007235]
- Chen MH, Li YJ, Kawakami T, Xu SM, Chuang PT. Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. *Genes Dev*. 2004; 18:641–659. [PubMed: 15075292]
- Chen SH, Yang CY, Chen PF, Setzer D, Tanimura M, Li WH, Gotto AM, Chan L. The complete cDNA and amino acid sequence of human apolipoprotein B-100. *J Biol Chem*. 1986; 261:12918–12921. [PubMed: 3759943]
- Chen Y, Struhl G. Dual roles for patched in sequestering and transducing hedgehog. *Cell*. 1996; 87:553–563. [PubMed: 8898207]
- Chen W, Burgess S, Hopkins N. Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity. *Development*. 2001; 128:2385–2396. [PubMed: 11493557]
- Devoto SH, Melancon E, Eisen JS, Westerfield M. Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development*. 1996; 122:3371–3380. [PubMed: 8951054]
- Farese RV Jr, Ruland SL, Flynn LM, Stokowski RP, Young SG. Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc Natl Acad Sci U S A*. 1995; 92:1774–1778. [PubMed: 7878058]
- Guerrero I, Chiang C. A conserved mechanism of Hedgehog gradient formation by lipid modifications. *Trends Cell Biol*. 2007; 17:1–5. [PubMed: 17126548]
- Harazono A, Kawasaki N, Kawanishi T, Hayakawa T. Site-specific glycosylation analysis of human apolipoprotein B100 using LC/ESI MS/MS. *Glycobiology*. 2005; 15:447–462. [PubMed: 15616123]
- Huang LS, Voyiatzakis E, Markenson DF, Sokol KA, Hayek T, Breslow JL. apo B gene knockout in mice results in embryonic lethality in homozygotes and neural tube defects, male infertility, and reduced HDL cholesterol ester and apo A-I transport rates in heterozygotes. *J Clin Invest*. 1995; 96:2152–2161. [PubMed: 7593600]
- Homanics GE, Smith TJ, Zhang SH, Lee D, Young SG, Maeda N. Targeted modification of the apolipoprotein B gene results in hypobetalipoproteinemia and developmental abnormalities in mice. *Proc Natl Acad Sci U S A*. 1993; 90:2389–2393. [PubMed: 8460149]
- Honjo Y, Eisen JS. Slow muscle regulates the pattern of trunk neural crest migration in zebrafish. *Development*. 2005; 132:4461–4470. [PubMed: 16162652]
- Inoue A, Takahashi M, Hatta K, Hotta Y, Okamoto H. Developmental regulation of islet-1 mRNA expression during neuronal differentiation in embryonic zebrafish. *Dev Dyn*. 1994; 199:1–11. [PubMed: 8167375]
- Jeong J, McMahon AP. Cholesterol modification of Hedgehog family proteins. *J Clin Invest*. 2002; 110:591–596. [PubMed: 12208857]
- Kageyama N, Natsuka S, Hase S. Molecular cloning and characterization of two zebrafish alpha(1,3)fucosyltransferase genes developmentally regulated in embryogenesis. *J Biochem (Tokyo)*. 1999; 125:838–845. [PubMed: 10101300]
- Karlstrom RO, Talbot WS, Schier AF. Comparative synteny cloning of zebrafish you-too: mutations in the Hedgehog target gli2 affect ventral forebrain patterning. *Genes Dev*. 1999; 13:388–393. [PubMed: 10049354]

- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn*. 1995; 203:253–310. [PubMed: 8589427]
- Krauss S, Johansen T, Korzh V, Fjose A. Expression of the zebrafish paired box gene *pax[zf-b]* during early neurogenesis. *Development*. 1991; 113:1193–1206. [PubMed: 1811936]
- Laessing U, Stuermer CA. Spatiotemporal pattern of retinal ganglion cell differentiation revealed by the expression of *neurolin* in embryonic zebrafish. *J Neurobiol*. 1996; 29:65–74. [PubMed: 8748372]
- Lee SH, Takahashi M, Honke K, Miyoshi E, Osumi D, Sakiyama H, Ekuni A, Wang X, Inoue S, Gu J, Kadomatsu K, Taniguchi N. Loss of core fucosylation of low-density lipoprotein receptor-related protein-1 impairs its function, leading to the upregulation of serum levels of insulin-like growth factor-binding protein 3 in *Fut8*^{-/-} mice. *J Biochem*. 2006; 139:391–398. [PubMed: 16567404]
- Lewis KE, Eisen JS. Hedgehog signaling is required for primary motoneuron induction in zebrafish. *Development*. 2001; 128:3485–3495. [PubMed: 11566854]
- Lewis KE, Currie PD, Roy S, Schauerte H, Haffter P, Ingham PW. Control of muscle cell-type specification in the zebrafish embryo by Hedgehog signalling. *Dev Biol*. 1999; 216:469–480. [PubMed: 10642786]
- Li W, Nakagawa T, Koyama N, Wang X, Jin J, Mizuno-Horikawa Y, Gu J, Miyoshi E, Kato I, Honke K, Taniguchi N, Kondo A. Down-regulation of trypsinogen expression is associated with growth retardation in α 1,6-fucosyltransferase-deficient mice: attenuation of proteinase-activated receptor 2 activity. *Glycobiology*. 2006; 16:1007–1019. [PubMed: 16861703]
- Link V, Shevchenko A, Heisenberg CP. Proteomics of early zebrafish embryos. *BMC Dev Biol*. 2006; 6:1. [PubMed: 16412219]
- Ma B, Simala-Grant JL, Taylor DE. Fucosylation in prokaryotes and eukaryotes. *Glycobiology*. 2006; 16:158R–184R.
- Masai I, Yamaguchi M, Tonou-Fujimori N, Komori A, Okamoto H. The hedgehog-PKA pathway regulates two distinct steps of the differentiation of retinal ganglion cells: the cell-cycle exit of retinoblasts and their neuronal maturation. *Development*. 2005; 132:1539–1553. [PubMed: 15728672]
- Masai I, Stemple DL, Okamoto H, Wilson SW. Midline signals regulate retinal neurogenesis in zebrafish. *Neuron*. 2000; 27:251–263. [PubMed: 10985346]
- Panakova D, Sprong H, Marois E, Thiele C, Eaton S. Lipoprotein particles are required for Hedgehog and Wntless signalling. *Nature*. 2005; 435:58–65. [PubMed: 15875013]
- Schlegel A, Stainier DY. Microsomal Triglyceride Transfer Protein Is Required for Yolk Lipid Utilization and Absorption of Dietary Lipids in Zebrafish Larvae. *Biochemistry*. 2006; 45:15179–15187. [PubMed: 17176039]
- Shkumatava A, Fischer S, Muller F, Strahle U, Neumann CJ. Sonic hedgehog, secreted by amacrine cells, acts as a short-range signal to direct differentiation and lamination in the zebrafish retina. *Development*. 2004; 131:3849–3858. [PubMed: 15253932]
- Soutar AK, Naoumova RP. Mechanisms of disease: genetic causes of familial hypercholesterolemia. *Nat Clin Pract Cardiovasc Med*. 2007; 4:214–225. [PubMed: 17380167]
- Taniguchi T, Ishikawa Y, Tsunemitsu M, Fukuzaki H. The structures of the asparagine-linked sugar chains of human apolipoprotein B-100. *Arch Biochem Biophys*. 1989; 273:197–205. [PubMed: 2757392]
- Ungos JM, Karlstrom RO, Raible DW. Hedgehog signaling is directly required for the development of zebrafish dorsal root ganglia neurons. *Development*. 2003; 130:5351–5362. [PubMed: 13129844]
- Varki A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*. 1993; 3:97–130. [PubMed: 8490246]
- Vukmirica J, Nishimaki-Mogami T, Tran K, Shan J, McLeod RS, Yuan J, Yao Z. The N-linked oligosaccharides at the amino terminus of human apoB are important for the assembly and secretion of VLDL. *J Lipid Res*. 2002; 43:1496–1507. [PubMed: 12235182]
- Wang X, Gu J, Ihara H, Miyoshi E, Honke K, Taniguchi N. Core fucosylation regulates epidermal growth factor receptor-mediated intracellular signaling. *J Biol Chem*. 2006a; 281:2572–2577. [PubMed: 16316986]

- Wang X, Gu J, Miyoshi E, Honke K, Taniguchi N. Phenotype changes of Fut8 knockout mouse: core fucosylation is crucial for the function of growth factor receptor(s). *Methods Enzymol.* 2006b; 417:11–22. [PubMed: 17132494]
- Wang X, Inoue S, Gu J, Miyoshi E, Noda K, Li W, Mizuno-Horikawa Y, Nakan M, Asahi M, Takahashi M, Uozumi N, Ihara S, Lee SH, Ikeda Y, Yamaguchi Y, Aze Y, Tomiyama Y, Fujii J, Suzuki K, Kondo A, Shapiro SD, Lopez-Otin C, Kuwaki T, Okabe M, Honke K, Taniguchi N. Dysregulation of TGF-beta1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. *Proc Natl Acad Sci U S A.* 2005; 102:15791–15796. [PubMed: 16236725]
- Westerfield, M. *The Zebrafish Book*. University of Oregon Press; Eugene, Oregon: 1993.
- Willnow TE, Hammes A, Eaton S. Lipoproteins and their receptors in embryonic development: more than cholesterol clearance. *Development.* 2007; 134:3239–3249. [PubMed: 17720693]
- Yang CY, Gu ZW, Weng SA, Kim TW, Chen SH, Pownall HJ, Sharp PM, Liu SW, Li WH, Gotto AM Jr. Structure of apolipoprotein B-100 of human low density lipoproteins. *Arteriosclerosis.* 1989; 9:96–108. [PubMed: 2912424]
- Young SG, Bertics SJ, Scott TM, Dubois BW, Curtiss LK, Witztum JL. Parallel expression of the MB19 genetic polymorphism in apoprotein B-100 and apoprotein B-48. Evidence that both apoproteins are products of the same gene. *J Biol Chem.* 1986; 261:2995–2998. [PubMed: 3949756]
- Zhao Y, Itoh S, Wang X, Isaji T, Miyoshi E, Kariya Y, Miyazaki K, Kawasaki N, Taniguchi N, Gu J. Deletion of core fucosylation on alpha3beta1 integrin down-regulates its functions. *J Biol Chem.* 2006; 281:38343–38350. [PubMed: 17043354]
- Zhao YY, Takahashi M, Gu JG, Miyoshi E, Matsumoto A, Kitazume S, Taniguchi N. Functional roles of N-glycans in cell signaling and cell adhesion in cancer. *Cancer Sci.* 2008; 99:1304–1310. [PubMed: 18492092]

Fig 1. Cladogram and sequence homology of zfFucT8

(A) Schematic showing the potential fucosylation sites on *N*-linked oligosaccharide chains by different FucTs. Whereas α 1,2 FucT and α 1,3/4 FucT add fucose moieties to terminal galactose (●) or *N*-acetylglucosamine (■, GlcNAc) residues, α 1,6 FucT adds fucose to the core GlcNAc. (●) mannose, (◆) *N*-acetylneuraminic acid. (B) Phylogenetic tree showing divergence of mouse (m) and zebrafish (zf) FucTs. α 1,6 FucTs are in red, α 1,3/4 FucTs are in blue and α 1,2 FucTs are in black. (C) Sequence alignment of zebrafish and mouse FucT8. Identical sequences are in blue, highly conserved sequences in green, and non-conserved sequences in black.

Fig 2. Knockdown of zfFucT8 leads to early midline defects

(A) Ventral views of 48 hpf morphant embryos showing mild cyclopia (i.e., close-set eyes), and a smaller forebrain (arrow). (B) 48 hpf FucT8 morphants have a curved body axis with U-shaped somites (inset), as well as (C) 'swelling' in the hindbrain ventricle (arrowhead). (D) AAL lectin stains α 1,6 fucosyl residues in the eye, which is reduced to background levels in morpholino-injected embryos as compared to control-injected, or no lectin, controls. More than 500 embryos were injected with FucT8 MOs, of which 85-90% demonstrated the mutant phenotype.

Fig 3. Defects in retinal cell differentiation in FucT8 morphant embryos

(A) DAPI staining of eye cross-sections from 5 day-old control and FucT8 MO-injected embryos. Morphant embryos have a smaller retina, and although cell layers seem to be present, they are not as distinct as in control embryos. (B,C) zn-5 immunostaining at 48 hpf identifies the RGCs (B, arrowhead) and optic chiasma (C, arrow) in control embryos, which are absent in morphant sections. (100% of 20 injected embryos showed this phenotype). (D) At 4 days, anti-CA-II antibody shows a lack of Müller glia (arrowhead) in the morphant retina. (85% of 40 injected embryos showed this phenotype). (E) zpr-1 positive photoreceptor cells are also severely reduced in FucT8 morphant eyes. (~85% of 40 injected embryos showed this phenotype). (F) BrdU incorporation illustrates increased cell

proliferation in 28 hpf FucT8 morphant eyes. (82% of 22 injected embryos showed this phenotype).

Fig 4. Patterning defects in the eye and forebrain of 27 hpf FucT8 morphant embryos

(A) Ventral view of a FucT8 morphant embryo showing severe loss of *islet-1* expressing retinal precursors (arrow). (90% of 20 injected embryos showed this phenotype). (B) Whole eyes showing a reduced number of *ath-5* positive RGC precursors (arrow) in FucT8 morphant embryos. (~85% of 40 injected embryos showed this phenotype). (C) In situ hybridization using *pax 2.1* shows that morphant embryos have a shorter and thicker optic stalk, relative to controls (arrow), and increased *pax 2.1* expression in the eye field. (~85% of 40 injected embryos showed this phenotype).

Fig 5. Defects in the trunk, spinal cord and other regions of 48 hpf FucT8 morphants
(A-E) Lateral views, anterior to the left. **(A)** *islet-1* expression at 48 hours in control and morphant embryos. In FucT8 morphants, motor neurons are disorganized and expanded in the dorsoventral plane (arrowhead). (90% of 20 injected embryos showed this phenotype). **(B)** Immunostaining with anti-acetylated tubulin (Ac-tubulin) antibody shows a lack of motor neuron projections (arrowhead in Control MO) in the spinal cord of morphant embryos. (~85% of 40 injected embryos showed this phenotype). **(C)** zn-5 antibody labeling shows a loss of projections from the DRG (arrowhead) in FucT8 morphants. (90% of 20 injected embryos showed this phenotype). **(D)** FucT8 morphants have U-shaped somites with fewer slow muscle fibers, as shown by F-59 antibody. (90% of 20 injected embryos showed this phenotype). **(E)** In situ hybridization with *crestin* shows that neural crest cell migration is delayed in FucT8 morphants, remaining near their site of origin at the dorsal aspect of the neural tube (arrowhead). (~85% of 40 injected embryos showed this phenotype). **(F)** Ventral view of alcian blue stained embryos show a lack of jaw cartilage (arrowheads) in FucT8 morpholino-injected embryos. (100% of 4 injected embryos illustrate a similar phenotype). **(G)** Dorsal views, anterior to the left. zn-5 staining shows that hindbrain commissures (arrowheads in Control MO) are significantly reduced in FucT8 morphants. (90% of 20 injected embryos showed this phenotype).

Fig 6. Identification of the major FucT8 substrates

(A) AAL lectin blot of control and FucT8 morphant embryo lysates resolved by SDS-PAGE. The morphant lysate has reduced AAL positive proteins in the high molecular weight region (brackets). AAL lectin binding to these high molecular weight polypeptides is inhibited by excess fucose in both control and morphants samples, whereas the lower two bands are not and represent non-specific bands (indicated by the *). (B) Control and FucT8 morphant lysates were incubated with AAL-conjugated beads to precipitate the core fucosylated proteins, which were subsequently resolved by SDS-PAGE and subjected to AAL lectin blotting. AAL beads precipitate high molecular weight AAL-reactive glycoproteins that are not present in similarly treated morphant lysates (brackets). The same non-specific protein band seen in panel A is indicated by the *. (C) AAL-conjugated beads were used to pull down core fucosylated proteins from a HepG2 cell lysate, which were resolved by SDS-PAGE and probed with ApoB antibody. The anti-ApoB detects a large molecular mass polypeptide in HepG2 lysates that migrates above the 250 kDa marker, and anti-ApoB antibodies were able to immunoprecipitate a polypeptide of similar size. AAL beads were also able to precipitate an ApoB-reactive polypeptide of similar molecular weight, although not all ApoB was precipitated by AAL, since ApoB-reactive material remained in the AAL bead supernatant. (D) Coomassie blue stained gel of high molecular weight polypeptides, which correspond to the ApoB-containing, AAL-reactive bands in control embryos. The amount of high molecular weight ApoB polypeptides (brackets) appears to be somewhat reduced in FucT8 morphants.

Fig 7. Knockdown of zfApoB phenocopies FucT8 morphants

(A,B) *islet-1* expression in the optic field is severely reduced (arrowheads) in ApoB morphants, similar to that seen in FucT8 morphants (see Fig. 4A). (27 hpf: 86% of 50 injected embryos showed this phenotype; 48 hpf: 85% of 40 injected embryos showed this phenotype). (C) Whole eyes, dissected out from *ath-5* labeled embryos, shows a reduced number of *ath-5* expressing cells (arrowheads) in 27 hpf ApoB morphants, as seen in FucT8 morphants (see Fig. 4B). (86% of 43 injected embryos showed this phenotype). (D) *pax 2.1* expression in 27 hpf ApoB morphants illustrates a shorter and thicker optic stalk, relative to controls (arrow), and parallels what is seen in FucT8 morphants (see Fig. 4C). (82% of 49 injected embryos showed this phenotype). (E) Ventral views, anterior to the top. Immunostaining with zn-5 antibody shows loss of RGCs (arrowheads) and optic chiasma (arrow) in 48 hpf ApoB morphant embryos phenocopying that seen in FucT8 morphants (see Fig. 3C). (98% of 47 injected embryos showed this phenotype).