Neuroepithelial cells require fucosylated glycans to guide the migration of vagus motor neuron progenitors in the developing zebrafish hindbrain

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The molecular mechanisms by which neurons migrate and accumulate to form the neural layers and nuclei remain unclear. The formation of vagus motor nuclei in zebrafish embryos is an ideal model system in which to address this issue because of the transparency of the embryos and the availability of established genetic and molecular biological techniques. To determine the genes required for the formation of the vagus motor nuclei, we performed N-ethyl-N-nitrosourea-based mutant screening using a zebrafish line that expresses green fluorescent protein in the motor neurons. In wild-type embryos, the vagus motor neuron progenitors are born in the ventral ventricular zone, then migrate tangentially in the dorsolateral direction, forming the nuclei. However, in towhead (twdw685) mutant embryos, the vagus motor neuron progenitors stray medially away from the normal migratory pathway and fail to stop in the right location. The twdw685 mutant has a defect in the GDP-mannose 4,6 dehydratase (gmds) gene, which encodes a key enzyme in the fucosylation pathway. Levels of fucosylated glycans were markedly and specifically reduced in twdw685 mutant embryos. Cell transplantation analysis revealed that GMDS is not essential in the vagus motor neuron progenitors for correct formation of the vagus motor nuclei, but is required in the neuroepithelial cells that surround the progenitors. Together, these findings suggest that fucosylated glycans expressed in neuroepithelial cells are required to guide the migration of vagus motor neuron progenitors.

KEY WORDS: Fucosylation, GMDS, Neuroepithelial cells, Neural migration, Vagus motor neurons, Zebrafish

INTRODUCTION

During development of the nervous system, neural migration plays a crucial role in expanding the size of the brain and ensuring correct arrangement of the neural layers and nuclei. The final location of neurons is especially crucial, because neural function depends on precise connections made by neurons and their targets. Defects in neural migration result in neurological diseases; for example, lissencephaly with cerebellar hypoplasia is caused by disruption of the glycoprotein Reelin (Gresslin, 2006).

Many studies have identified molecules involved in the control of neural migration. These studies have revealed that proper neural migration requires various genes that function not only in migrating neurons, but also in the cells that support them. For example, we have reported that the planar cell polarity genes frizzled3a and celsr2 function in neuroepithelial cells to guide the caudal migration of facial motor neurons (Wada et al., 2006). Despite this, there is still very little known about the molecular mechanisms that regulate termination of neural migration and the accumulation of neural progenitors to form the proper layers or nuclei.

The vagus nerve is the tenth cranial nerve, located in rhombomere (r) 8 in the caudal hindbrain. It controls various body functions, including heart beat and gastrointestinal movement (Gilland and Baker, 2005; Taylor et al., 1999). Correct arrangement of vagus motor nuclei following neural migration and accumulation may be crucial for the normal functioning of vagus motor neurons. For example, although sudden infant death syndrome (SIDS), the most common cause of postnatal infant death, is a complex and multifactorial disorder (Moon et al., 2007), it has been suggested that the reduced number of neurons in vagus motor nuclei in victims of SIDS is due to a failure of neuronal migration (Macchi et al., 2002). Thus, there is a need to understand how vagus motor nuclei are formed by neural migration, and the molecular mechanisms that govern this process.

In the present study, we used vagus motor nuclei as a new model system in which to study the molecular mechanisms underlying neural migration. Time-lapse observations of hindbrain explants from the transgenic zebrafish line Tg(CM-isl1:GFP)rw0, hereafter referred to as isl1:GFP, which expresses green fluorescent protein (GFP) in motor neurons, including vagus motor neurons (Higashijima et al., 2000), demonstrated that vagus motor neuron progenitors are born near the ventral midline, migrate in a dorsolateral direction and accumulate to form the vagus motor nuclei. An N-ethyl-N-nitrosourea (ENU)-based mutant screen using the isl1:GFP transgenic zebrafish isolated towhead (twdw685) mutant embryos, in which the vagus motor neuron progenitors migrate beyond their proper position to the dorsal roof of the hindbrain. The twdw685 locus encodes GMDS, a key enzyme in the fucosylation pathway (Staudacher et al., 1999).
Fucosylated glycans are involved in a variety of biological and pathological processes, including cell migration (Ma et al., 2006). For example, sialyl Lewis X (sLeX), a fucosylated carbohydrate structure, is the core recognition epitope that mediates lymphocyte homing and initial leukocyte-endothelial cell adhesion (Ma et al., 2006). The donor substrate for fucosylation, GDP-fucose, is synthesized in the cytoplasm mainly through de novo synthesis pathway, which includes an enzymatic reaction catalyzed by GDP-mannose 4,6-dehydrase (GMDS) (Fig. 1). Subsequently, a GDP-fucose transporter at the Golgi membrane transports GDP-fucose from the cytoplasm to the Golgi lumen, and fucosyltransferases (FUTs) transfer GDP-fucose to the acceptor molecules. Leukocyte adhesion deficiency II (LAD II), also known as congenital disorder of glycosylation IIc (CDG IIc), results in mental retardation, short limbs and stature, and a flat face with a broad, depressed nasal bridge (Freeze, 2001). These features are caused by a defect in the Golgi-localized GDP-fucose transporter, resulting in impaired expression of the fucosylated glycans (Lubke et al., 2001; Lubke et al., 1999; Luhn et al., 2001). Although individuals with LAD II/CDG IIc exhibit neurological abnormalities, how fucosylated glycans regulate neural development is not yet understood. The findings of the present study reveal a novel molecular mechanism in the establishment of the structure of the vagus motor nuclei following neural migration, whereby fucosylation of the substrate plays a key role in preventing vagus motor neuron progenitors from migrating along an aberrant pathway.

MATERIALS AND METHODS
Zebrafish strains, mutagenesis, and mapping of mutant loci
Maintenance of zebrafish, ENU-based mutagenesis and genetic mapping of mutant loci were performed as described previously (Tanaka et al., 2007; Wada et al., 2005; Wada et al., 2006; Westerfield, 2007). The simple sequence length polymorphism (SSLP) marker BXS10653-1 was generated based on the sequence of the eighth intron of the gmds gene. The isl1::GFP fish and the twd\(^{w685}\) fish are available from the National BioResource Project of Japan (http://www.shigen.nig.ac.jp/zebra/index_en.html).

Time-lapse imaging of hindbrain explant culture and cell transplantation
The procedures for time-lapse imaging of the hindbrain were essentially the same as those described previously (Tanaka et al., 2007). The hindbrain explant in the culture chamber was observed under a confocal microscope (LSM510; Carl Zeiss) every 15 minutes from 24 to 48 hours post-fertilization (hpf). Labeling of wild-type cells by rhodamine-dextran and transplantation were performed according to standard protocols (Westerfield, 2007).

Molecular biology and phylogenetic comparison
CDNA cloning, reverse transcription-polymerase chain reaction (RT-PCR) and synthesis of sense-capped mRNAs were performed as described previously (Wada et al., 2006). Details of primers and the antisense morpholino oligonucleotides (MOs; Gene Tools) used in this study can be provided on request. For the injection of mRNA or MO solution, approximately 1 nl of the solution at the concentrations indicated in Table 1 was injected into one-cell stage embryos. The GenBank Accession Numbers for zebrafish L-gmds and S-gmds are AB290320 and AB290319, respectively. Amino acid sequence similarity was analyzed using the CLASTAL W program. The following amino acid sequences of GMDS were used for sequence comparisons: Homo sapiens (GenBank accession number NM 001500), Mus musculus (BC093502), Xenopus laevis (BC111472), Drosophila melanogaster (NM_135044), Caenorhabditis elegans (NM_069162) and Escherichia coli (NZ_AAJT01000120). Embryo genotypes were identified by direct sequencing of the mutation site.

Histochemistry and protein chemistry
Embryos were fixed at the indicated developmental stage with 4% paraformaldehyde (Wako) at 4°C overnight. In situ hybridization, whole-mount staining, cryosection staining and retrograde labeling of the reticulospinal neurons were performed essentially as described previously (Aizawa et al., 2007; Shepard et al., 2004; Wada et al., 2005; Westerfield, 2007). Details of primary antibodies and biotinylated lectins used in the present study can be provided on request. These probes were detected by fluorescein-conjugated secondary antibodies (Invitrogen; 1:500 dilution for each) or avidin (Chemicon; 1:500 dilution). For immunoblotting, 25 embryos of wild-type and the twd\(^{w685}\) mutants were pooled and lysed in Laemmli sample buffer. The remaining steps in the immunoblotting procedure were as described previously (Ohata et al., 2009).  

RESULTS
Time-lapse imaging of the migration and accumulation of vagus motor neuron progenitors using isl1::GFP transgenic zebrafish
The isl1::GFP transgenic fish expresses GFP in several cranial and spinal motor neurons, including the vagus neurons (Higashijima et al., 2000). Like mammals (Gilland and Baker, 2005; Taylor et al., 1999), zebrafish form two vagus motor nuclei in r8, the most caudal part of the hindbrain. One is the dorsolateral motor nucleus of the vagus (dlX), whereas the other is the medial motor nucleus of the vagus (mmX) (Fig. 2A, parts a,b). In the present study, we used time-lapse imaging of hindbrain explants to observe the process of vagus motor nuclei formation (Fig. 2B). The vagus motor neuron progenitors were born near the ventral midline from 24 to 48 hpf (Fig. 2B; see Movie 1 in the supplementary material). The dlX was formed first by the dorsolateral migration and accumulation of vagus motor neuron progenitors from 24 to 36 hpf (Fig. 2B). Then, mmX was formed in the ventromedial region of r8 by 48 hpf (Fig. 2B).

Isolation of the twd\(^{w685}\) mutants showing defects in the cessation of migration of vagus motor neuron progenitors
To dissect genetically the process of the formation of vagus motor nuclei, we performed ENU-based mutant screening and isolated the twd\(^{w685}\) mutant. In the twd\(^{w685}\) embryos, the vagus motor nuclei were fused across the midline (Fig. 3A). In addition, the distribution of anterior and posterior trigeminal (Va and Vp) and facial (VII) motor neurons was perturbed in the twd\(^{w685}\) mutant (33/33 (100%) (Fig. 3A), presumably because of defects in the lateral migration of the trigeminal motor neurons and the posterior migration of the facial...
motor neurons. The morphology of the twd<sup>w685</sup> embryos appeared to be normal at 2 days post-fertilization (dpf), except for their ‘curled up’ tails (Fig. 3B). The twd<sup>w685</sup> mutants died between 6 and 7 dpf.

In the twd<sup>w685</sup> embryos, ectopic neurons labeled by isl1:GFP are visible as distinctive nuclear structures at 36 hpf. They are widely distributed throughout the dorsomedial hindbrain region between the areas where the dlX is normally formed. By 48 hpf, these ectopic neurons are seen to reach the dorsal roof of the hindbrain (‘overshooting’) (Fig. 3C; see Movie 2 in the supplementary material).

To distinguish between dlX and mmX neurons, we took advantage of the differences in time when these neurons are born. Specifically, wild-type and twd<sup>w685</sup> embryos were labeled with BrdU for 30 minutes at 38 hpf and were fixed at 3 dpf for staining with anti-BrdU and anti-GFP antibodies. In wild-type embryos, most dlX neurons were BrdU negative (Fig. 3D, part a), whereas all mmX neurons were BrdU positive. Most of the ectopic neurons in twd<sup>w685</sup> embryos were also BrdU negative (Fig. 3D, part b), indicating a common origin with dlX neurons. In addition, most BrdU and GFP double-positive cells from isl1:GFP transgenic embryos were localized ectopically in twd<sup>w685</sup> mutants. This indicates that the twd<sup>w685</sup> mutation may affect the migration of both dlX and mmX neurons.

To clarify the effect of the twd<sup>w685</sup> mutation on the migration of dlX progenitors, we observed the embryos using time-lapse imaging. In wild-type embryos, the dlX progenitors migrate in the dorsolateral direction soon after they are born (Fig. 3E; see Movie 1 in the supplementary material). However, in twd<sup>w685</sup> embryos, the dlX progenitors migrate in more dorsal directions, straying away from the normal migratory pathway (Fig. 3E; see Movie 2 in the supplementary material).

**Effects of the twd<sup>w685</sup> mutation are specific to neural migration**

Various markers were used in twd<sup>w685</sup> mutants to determine the effects of the twd<sup>w685</sup> mutation. The zn-5 antibody specifically labels segmentally repeated commissural axons in the zebrafish hindbrain (Trevorrow et al., 1990). The formation of zn-5-immunoreactive axons appeared normal in twd<sup>w685</sup> embryos (Fig. 2A; see Movie 3 in the supplementary material).
4A,B). Furthermore, labeling of reticulospinal neurons by injection of a tracer dye into the spinal cord revealed that the anterior-posterior patterning of the reticulospinal neurons in twdrw685 embryos was identical to that in wild-type embryos (Fig. 4C,D). The LIM homeodomain proteins Lhx2 and Lhx9, as well as glutamate decarboxylases Gad1 and Gad2, are segmentally expressed in the hindbrain (Ando et al., 2005; Sassa et al., 2007). In the present study, the location of both Lhx2/9- and Gad1/2-immunoreactive neurons was almost normal in twdrw685 embryos (Fig. 4E-H). These results suggest that the overall patterning and differentiation of neurons other than the vagus motor neurons in the posterior hindbrain were unaffected by the twdrw685 mutation.

To investigate neuroepithelial apicobasal polarity and tight junctions in twdrw685 embryos, the twdrw685 mutants were stained with anti-atypical protein kinase C (aPKC) and anti-zonula occludens-1 (ZO-1) antibodies (Fig. 4I-L). The localization of both aPKC and ZO-1 was normal, indicating that neuroepithelial apicobasal polarity and tight junctions were unaffected in twdrw685 embryos.

**The twdrw685 locus encodes GMDS**

The twdrw685 locus was mapped between SSLP markers z14995 and z14614 on linkage group 20 (LG20) and no recombination was detected at the gene encoding GMDS in 702 meioses (Fig. 5A). The gmds gene consists of 12 exons and 11 introns (Fig. 5B). cDNA cloning of wild-type embryos identified two splicing variant mRNAs, depending on whether the fourth exon, which consists of 21 bases, is included (L-gmds) or skipped (S-gmds) in the mature mRNA. These two variants share an identical amino acid sequence, except for the seven residues encoded by the fourth exon (Fig. 5B,C). A mutation from T to A was detected in the seventh exon of the gmds gene from twdrw685 embryos (Fig. 5B-D), resulting in amino acid substitution from Trp193 in L-GMDS (or Trp186 in S-GMDS) to Arg. Trp193 (or Trp186 in S-GMDS) is conserved in the GMDS from various species, ranging from human to bacteria (Fig. 5C).

To confirm that gmds is the gene responsible for the twdrw685 phenotype, we performed loss-of-function and gain-of-function analyses of gmds. Injection of an MO designed to inhibit splicing of premature gmds mRNA into one-cell stage wild-type embryos effectively blocked the maturation of gmds mRNA (Fig. 6A). Although injection of the 5-mis-pair control MO had no effect on motor neurons in morphants (0/113; 0%) (Fig. 6B, part a), injection of the splice-blocking MO against gmds produced a fused vagus motor nuclei phenotype (130/133; 98%) (Fig. 6B, part b) that was identical to that observed in the twdrw685 mutant (Fig. 3A). In addition, the distribution of trigeminal and facial motor neurons was perturbed in gmds morphant embryos (41/41; 100%) (Fig. 6Bb), as in the twdrw685 mutant embryos (Fig. 3A).

Further experiments were performed in which the effects of injection of either of the in vitro-synthesized sense-capped S- or L-gmds mRNAs (40 or 80 μg/ml) into one-cell stage embryos derived from heterozygous twdrw685 parents were investigated. Of the twdrw685 embryos injected with 80 μg/ml L-gmds mRNA, 88% (21/24) exhibited a completely rescued phenotype (Table 1; Fig. 6B, part c) and 13% exhibited a partially rescued phenotype, in which some, but not all, vagus motor neurons were found in abnormal locations (Fig. 6B, part c’, arrowheads). When the lower concentration of L-gmds mRNA (40 μg/ml) was injected, there was a concomitant decrease in the number of twdrw685 embryos rescued [14/27 (52%) and 8/27 (30%) embryos completely and partially rescued, respectively] and 19% (5/27) of mutant embryos still showed the twdrw685 phenotype (Table 1). Injection of 80 μg/ml L-gmds mRNA rescued twdrw685 embryos with a similar efficiency as that seen following injection of the same concentration of L-gmds mRNA [22/30 (73%) and 8/30 (27%) embryos completely and partially rescued, respectively] (Table 1; Fig. 6B, part d). By contrast, injection of the mutant-type mRNA did not result in the rescue of any twdrw685 mutants (Table 1). Overexpression of L- or S-gmds mRNA in wild-type embryos had no effect on the differentiation and migration of vagus motor neurons (Fig. 6B, part e).
In addition, the migration and accumulation of dI progenitors was investigated using time-lapse imaging. In twdrw685 embryos injected with 80 μg/ml S- and L-gmds mRNA, the dI progenitors migrated dorsolaterally and formed the dI as seen in wild-type embryos (Fig. 6C; see Movie 3 in the supplementary material).

Reduced levels of fucosylated glycans in twdrw685 embryos are rescued by injection of gmds mRNA at the one-cell stage

The expression profile of fucosylated glycans in the hindbrain of zebrafish embryos was examined in the present study using biotinylated lectins, because GMDS is a key enzyme in the fucosylation pathway (Sullivan et al., 1998). In wild-type embryos, N-linked fucosylated glycans recognized by Aleuria aurantia lectin (AAL) and Lens culinaris agglutinin (LCA) were expressed ubiquitously throughout the hindbrain (Fig. 7A; see Fig. S1A in the supplementary material) (Varki et al., 1999). However, in twdrw685 embryos, glycan levels were markedly reduced (Fig. 7B; see Fig. S1B in the supplementary material).

Repression of FUT7-9, FT1, FT2, POFUT1 and POFUT2 does not phenocopy twdrw685 mutant

FUTs are classified according to the site of fucose addition as follows: α1,2 (encoded by FUT1 and FUT2); α1,3/4 (encoded by FUT3); α1,3 (encoded by FUT4-7 and FT1-2); and α1,6 (encoded by FUT8) (Kageyama et al., 1999; Ma et al., 2006; Mollicone et al., 2008). Protein O-fucosyl transferases (POFUTs)
are encoded by *pofut1* and *pofut2*. Of the FUTs, *pofut1*, *pofut2* and *FUT8* have already been cloned in zebrafish. In mammals, these genes encode the enzymes catalyzing O-fucosylation in the consensus sequence of the epidermal growth factor (EGF)-like repeat (POFUT1) (Fig. 8A, part a), O-fucosylation in the consensus sequence of the thrombospondin type-1 repeat (POFUT2) (Fig. 8A, part b) and α1,6-fucosylation to the innermost N-acetylglucosamine (GlcNAc) moiety of the core N-linked glycans (FUT8; core α1,6-fucosylation) (Fig. 8A, part c). Therefore, we knocked down these genes to investigate the importance of O- and core α1,6-fucosylation in the formation of the vagus motor nuclei. Although MOs against *pofut1*, *pofut2* and *FUT8* effectively knocked down the translation of EYFP-tagged mRNAs (Fig. 8B, parts a-f), these MOs did not induce fusion of the bilateral vagus motor nuclei (Fig. 8C, parts a-c). Instead, some of the morphants showed aberrant positioning of the vagus motor neurons lateral to the dIX (Fig. 8C, parts a-c), whereas embryos injected with the standard control MO were identical to the wild-type embryos (data not shown). These results indicate that O- and core α1,6-fucosylation do not contribute to the cessation of migration of vagus motor neurons.

α1,3-fucosylation consists of core α1,3-fucosylation to the innermost GlcNAc moiety of the core N-linked glycans and terminal fucosylation, where GDP-fucose is linked to the oligosaccharide chains as a terminal modification that is not elongated further (Fig. 8A, part c) (Ma et al., 2006). Of the terminal α1,3-FUTs, α1,3FUT7 and α1,3FUT9 have been identified. However, knocking down those genes did not induce fusion of the bilateral vagus motor nuclei. Therefore, we knocked down *FUT10* effectively knocked down the translation of EYFP-tagged mRNA (Fig. 8B, parts g,h), and caused fusion of the bilateral vagus motor nuclei (22/35; 63%), (Fig. 8C, part d). However, unlike *twdrw685* mutants, FUT10 morphants did not show the overshooting of the vagus motor neurons, but showed disruption of neuroepithelial apicobasal polarity and adherens junctions (data not shown). These results indicate that α1,3-fucosylation catalyzed by FUT10 affects neural development in a different manner from that of GMDS.

**Notch activity is not reduced in twdrw685 embryos**

Fucosylation has been shown to regulate Notch signaling, and genetic inactivation of the Notch activator γ-Secretase causes mis-migration of the facial motor neurons (Ishikawa et al., 2005; Louvi et al., 2004; Ma et al., 2006; Sasamura et al., 2007). Although O-fucosylation has an important role in Notch signaling, the expression of the Notch target gene her4 (Takke et al., 1999) was not affected in *twdrw685* mutants in the present study (Fig. 9A-C). Because Notch signaling has an antineurogenic effect on neural stem cells, we observed primary motor neurons and Rohan-Beard sensory neurons in the spinal cord (Inoue et al., 1994; Tokumoto et al., 1995). There were no significant differences detected in the spinal cord between wild-type and *twdrw685* mutant embryos (Fig. 9D-G). Because Notch activation is also required for the segregation of the rhombomere boundary (Cheng et al., 2004), we examined the expression of genetic markers for hindbrain segmentation. The expression pattern of *radical fringe* and *wnt1* in *twdrw685* mutant embryos was identical to that in wild-type embryos (Fig. 9H-K), indicating that there is no significant defect in Notch activity in *twdrw685* mutant embryos.

**Surrounding neuroepithelial cells regulate the migration and accumulation of vagus motor neurons**

To determine which cells require *gmds* for the correct formation of vagus motor nuclei, we performed mosaic analysis by transplanting rhodamine-dextran-labeled wild-type cells at the blastoderm stage into *gmds* morpant host embryos at the shield stage. The progeny of the donor cells that differentiated into the motor neurons could be identified by their GFP and rhodamine signals. The wild-type dlX progenitors in the hindbrain of wild-type localized normally (Fig. 10A, part a), but those in the hindbrain of *gmds* morpant embryos did not stop at the correct position and were positioned ectopically, close to the midline, similar to the positioning of dlX progenitors in *twdrw685* mutants (Fig. 10A, parts b,c; C, parts a,b) (n=3). Similar results were obtained following transplantation of wild-type dlX progenitors into *twdrw685* mutants (data not shown; n=3). These results indicate that *gmds* expression in vagus motor neuron progenitors is not essential for correct migration of these progenitor cells.

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**Fig. 6. Confirmation of the gmds gene as the twdrw685 locus by knock-down and rescue experiments.** (A) The efficacy of the splice-blocking MO against *gmds* was confirmed by RT-PCR at 32 hpf. Total RNA of *gmds* morphants and 5-mis-pair control morphants was incubated with (RT+) or without (RT−) reverse transcriptase. Samples were amplified with primers in which the target region of the MO is covered. Therefore, a band is not detected if maturation of *gmds* mRNA is inhibited by the MO. *ef1α* loading control. (B) Dorsal views of the *gmds* morphant and *twdrw685* mutant embryos injected with GMDS mRNAs at 50 hpf (rostral towards the top). Wild-type embryos injected with the 5-mis-pair control MO (a), the *gmds* MO (b) and 5-GMDS mRNA (e). The *twdrw685* mutants injected with L-gmds mRNA (c,c′) and S-gmds mRNA (d). Completely rescued (c,d) and partially rescued (c′) mutants. Arrowheads indicate ectopic vagus motor neurons. (C) Time-lapse observation of the migration of vagus motor neuron progenitors in *twdrw685* embryos injected with L-gmds mRNA. The broken line shows the outline of the hindbrain. Dorsal is towards the top.
Neuroepithelial cells are likely to be regulators of the migration of vagus motor neuron progenitors, because they have been shown to support the caudal migration of facial motor neuron progenitors by preventing the integration of migrating facial motor neuron progenitors into the neuroepithelial layer (Wada et al., 2006) and $gmds$ mRNA was expressed ubiquitously (data not shown). In the present study, when wild-type neuroepithelial cells were placed into the dorsomedial region of the hindbrain of $gmds$ morphants, the vagus motor progenitors were found to migrate to normal positions (line 2 in Fig. 10B, parts a,f; C, part c) ($n=5$). Of note, morphant vagus motor neuron progenitors entered the dorsomedial region and positioned more dorsally if wild-type cells were not distributed in the dorsomedial region of the hindbrain (line 1 in Fig. 10B, parts a,e). These results suggest that the recovery of fucosylation in neuroepithelial cells in the dorsomedial region of the hindbrain is sufficient to restore normal migration of mutant vagus motor progenitors.

**DISCUSSION**
The $twd^{w685}$ mutation may affect the oligomerization of GMDs, which results in severe impairment of the de novo synthesis of GDP-fucose

The fucosylation donor substrate GDP-fucose is generated through a de novo synthesis pathway that converts GDP-mannose to GDP-fucose, and a salvage pathway that converts free cytosolic fucose to GDP-fucose (Fig. 1). In HeLa cells, more than 90% of GDP-fucose is derived from the de novo synthesis pathway (Ma et al., 2006). The $twd^{w685}$ mutation occurs in the $gmds$ gene, which encodes a key enzyme in the de novo synthesis pathway (Figs 1 and 5). As a result, expression of fucosylated glycans recognized by AAL and LCA (core-fucosylation), as well as the anti-sLeX antibody (terminal fucosylation), was markedly and specifically reduced. Injection of normal $gmds$ mRNA was able to restore the expression of these.

![Fig. 7. The expression of fucosylated glycans is reduced in $twd^{w685}$ embryos.](image)
glycans (Fig. 7; see Fig. S1 in the supplementary material). These results indicate that core and terminal fucosylation greatly depend on the de novo synthesis pathway.

The structure of GMDS is highly conserved among organisms; in bacteria and Arabidopsis thaliana, GMDS works as an oligomer (Mulichak et al., 2002; Somoza et al., 2000; Webb et al., 2004). The twd<sup>665</sup> mutation changes the conserved hydrophobic residue Trp<sup>193</sup> in L-GMDS (Trp<sup>186</sup> in S-GMDS) into a basic residue, Arg, in the region involved in oligomer formation (Fig. 5C). Therefore, this mutation may disrupt oligomerization of GMDS, which may be crucial for its enzymatic activity. However, we cannot rule out the possibility that the twd<sup>665</sup> mutation affects the enzymatic activity directly and/or the stability of the GMDS protein.

**How does the fucosylation pathway regulate neural migration and accumulation?**

In the present study, we proposed a model in which fucosylated glycans of surrounding neuroepithelial cells prevent overshooting in the tangential migration of vagus motor neuron progenitors (Fig. 10D). There are at least two ways in which fucosylated glycans may guide migration. First, fucosylated glycans may function to maintain...
Fucosylation regulates neural migration

The Notch signaling pathway is critical for regulating cell fate decisions and morphogenesis in many developmental processes, including somitogenesis, vasculogenesis, and neurogenesis (Ma et al., 2006). The Notch signaling pathway involves the interaction of Notch receptors with ligands, such as Delta and Serrate, which are expressed in a spatially and temporally regulated manner. This interaction triggers a series of biochemical events, including proteolysis of the Notch receptor, cleavage and release of the intracellular domain, and translocation to the nucleus, where it interacts with the CSL (CBF1, Su(H), and Lag-1) complex to activate transcription of target genes.

In zebrafish, the Notch signaling pathway is involved in the guidance of vagus motor neuron progenitors. These progenitors migrate from the ventral midline to the dorsolateral region of the hindbrain, where they give rise to the vagus motor nuclei. The Notch signaling pathway is activated by the binding of Delta and Serrate ligands to their respective Notch receptors on the surface of migrating cells. This binding triggers the proteolytic cleavage of Notch, which results in the release of the intracellular domain of Notch. The intracellular domain then translocates to the nucleus, where it interacts with the CSL complex to activate the transcription of target genes.

In the present study, we have demonstrated that fucosylation is involved in the regulation of Notch signaling. Fucosylation is a post-translational modification of carbohydrates on glycoproteins and glycolipids. It is a critical step in the formation of terminal fucosylation, which is required for the future study. Terminal fucosylation is involved in the formation of fucosylated glycans, which are important for cell adhesion and signaling.

The Notch signaling pathway has been shown to be a key regulator of the development of many tissues and organs, including the nervous system. The Notch signaling pathway is involved in the regulation of cell fate decisions, cell proliferation, cell death, and cell migration. The Notch signaling pathway is also involved in the regulation of vascular development, including vasculogenesis and vasculature remodeling. In the present study, we have shown that fucosylation is involved in the regulation of Notch signaling, and that this regulation is critical for the development of the nervous system and the vasculature.
like repeats in Notch. However, there was no prominent decrease in Notch signaling observed in twd<sup>1662</sup> mutants, as determined by the expression level of the Notch target gene her<sup>4</sup>, hindbrain segmentation and the number of neurons (Fig. 9). These results suggest that O-fucosylation is less sensitive to the loss of GMDS compared with core and terminal fucosylation, which are significantly reduced in the twd<sup>1662</sup> mutant (Fig. 7; see Fig. S1 in the supplementary material). This difference in sensitivity may be due to a smaller quantitative demand for O-fucosylation or to differences in the properties of POFUT1 and POFUT2 compared with other FUTs.

One possible reason why the Notch signal was not affected in twd<sup>1662</sup> mutants is that the maternally supplied GMDS is sufficient for normal activation of Notch during the earliest stages of neurogenesis. In a preliminary study, we found that injection of an MO that inhibits the translation of maternal/zygotic gmds mRNA causes severe embryonic malformations and cell death at 24 hpf. Therefore, maternally supplied GMDS may have a crucial role in early development. Another possibility is that the GDP-fucose produced by the salvage pathway is sufficient for the O-fucosylation of Notch. In mammals, this pathway uses free cytosolic fucose as a substrate, which is derived from an extracellular source or from lysosomal degradation, converting it to GDP-fucose via fucokinase and GDP-fucopyrophosphorylase (Fig. 1). Conversely, there are no sequence-encoding enzymes involved in the salvage pathway in the Drosophila genome (Roos et al., 2002). Although it is unclear whether the teleost contains the salvage pathway, a candidate gene that encodes zebrafish fucokinase is registered in GenBank (XM_001344236) (Fig. 1). Therefore, zebrafish may have the salvage pathway, which could account for the O-fucosylation of the Notch EGF-like repeat in the twd<sup>1662</sup> mutant. However, in our preliminary study, the twd<sup>1662</sup> mutants were not rescued after the injection of fucose. This suggests that even if the salvage pathway does exist in zebrafish, it is not sufficient to rescue the twd<sup>1662</sup> mutant because less than 10% of GDP-fucose is derived from this salvage pathway (Ma et al., 2006).

**Human congenital disorders of fucosylation**

The molecular basis of the immunodeficiency in LAD II/CDG IIc can be explained by the function of fucosylated glycans in cell adhesion, mediated by an interaction between fucosylated oligosaccharides, sLe<sup>a</sup> and the Selectin family (Becker and Lowe, 2003; Ma et al., 2006). However, it remains unclear why individuals with LAD II/CDG IIc exhibit other defects, including mental retardation. The mouse and fly knockout strains with different developmental deletions of the fucosylation is embryonic lethal and shows reduced expression of sialyl Lewis<sup>x</sup> (Ishikawa et al., 2005; Smith et al., 2002). The FX knockout mouse and the GDP-fucose deficient mutant because less than 10% of GDP-fucose is derived from this salvage pathway (Ma et al., 2006).

**References**


peptide motifs and phylogeny of two new alpha 1,3-fucosyltransferase families (FUT10 and FUT11). J. Biol. Chem. 284, 4723-4738.


