

Characterization of *Fut10* and *Fut11*, Putative α 1-3/4 Fucosyltransferase Genes Important for Vertebrate Development

Santosh K. Patnaik

Department of Cell Biology, Albert Einstein College of Medicine, New York, NY 10461, USA

Current address: Plot 2612, Leelakunj, Santoshi Ma Mandir Road, Patrapada, Bhubaneswar, Orissa 751019, India; phone: 9238100181; e-mail: snt.pat@gmail.com

Keywords: Development, fucosyltransferase, GDP-fucose hydrolysis, morpholino, zebrafish

Running title: Characterization of Fuc-TX and Fuc-TXI

Two new, putative α 1-3/4 fucosyltransferases (α 1-3/4 Fuc-Ts), Fuc-TX and Fuc-TXI, were identified in the vertebrate genome and transcriptome sequence databases through sequence homology-based queries. These proteins have a significant sequence similarity to only α 1-3/4 Fuc-Ts, and possess peptide motifs that are evolutionarily conserved among the known vertebrate α 1-3/4 Fuc-Ts. However, Fuc-TX and Fuc-TXI lack the HH[R/W][D/E] sequence that determines the specificity for type 1 or 2 substrates among the known vertebrate enzymes, and Fuc-TXI proteins do not possess a transmembrane domain. The *Fut10* and *Fut11* genes that encode these proteins are expressed ubiquitously in the adult mouse and in the mouse embryo throughout development. Though a Fuc-T activity of the mouse proteins could not be detected, Fuc-TXI, but not Fuc-TX, was found to hydrolyze GDP-fucose. The interaction of Fuc-TXI with GDP-fucose was also confirmed by its binding to GDP-hexanolamine. In zebrafish, *Fut11* transcripts could be detected during early embryonic development. A knock-down of Fuc-TXI in zebrafish embryos with *Fut11*-specific antisense morpholino oligonucleotides resulted in malformations of the posterior trunk and tail.

Alpha-1-3/4 fucosyltransferases (α 1-3/4 Fuc-Ts) catalyze the transfer of fucose from GDP-fucose to *N*-acetyl glucosamine (GlcNAc) of glycans in an α 1-3 or α 1-4 glycosidic linkage. Glycan moieties with α 1-3/4-linked fucose, such as Lewis X (Le^x ; Gal β 1-4[Fuc α 1-3]GlcNAc), α 2-3-sialyl Le^x (s Le^x) and Le^a (Gal β 1-3[Fuc α 1-4]GlcNAc), are important biological determinants. Such fucosylated glycans on lipid and protein glycoconjugates participate in normal physiological processes such as selectin-the trafficking of leukocytes (1,2) and compaction of early embryos (3,4), and are markers of certain malignancies (e.g., 5), stem cells (e.g., 6) and allergens (e.g., 7). They also mediate pathogenic processes, like the adhesion of *Helicobacter pylori* to gastric epithelial lining (8) and of *Anaplasma phagocytophilum* to neutrophils (9), and tumor metastasis (10).

Six families of mammalian α 1-3/4 Fuc-Ts, Fuc-TIII, -TIV, -TV, -TVI, -TVII and -TIX, encoded by the *Fut3*, *Fut4*, *Fut5*, *Fut6*, *Fut7* and *Fut9* genes have been identified and characterized. These genes were discovered by population genetics (11), expression cloning using genomic or cDNA libraries (12,13), or sequence similarity-based cloning by amplification (14,15). All six genes are present in humans whereas only *Fut4*, *Fut7* and *Fut9* exist in the mouse. Surprisingly, given the importance of α 1-3/4 fucosylated glycans, *Fut9*^{-/-} mice appear to

be normal (16), and *Fut4*^{-/-}, *Fut7*^{-/-} and *Fut4*^{-/-}/*Fut7*^{-/-} mice have only subtle, immunological phenotypes (17,18). This has raised the possibility for the existence of unknown α 1-3/4 Fuc-Ts that can compensate for those inactivated in these mice. Furthermore, characterization of novel α 1-3/4 Fuc-Ts can elucidate biological roles of fucosylated glycans and their regulatory mechanisms.

Genome and transcriptome sequence databases have made it possible to identify new members of protein families through sequence homology-based database searches. The α 1-3/4 Fuc-T proteins, including those from non-vertebrates, have homologous sequences with conserved α 1-3/4 Fuc-T motifs (19). This was used to identify two novel, putative α 1-3/4 Fuc-T proteins in this laboratory a while ago, while the human and mouse genome sequencing projects were under way. Since then, others have described the *Fut10* and *Fut11* genes that encode these proteins (20). The two genes and their products, the Fuc-TX and Fuc-TXI proteins, are characterized here.

MATERIALS AND METHODS

Antibodies, chemicals and molecular biology reagents - Horseradish peroxidase (HRP)-conjugated mouse or goat antibodies against human IgG1, mouse IgG or rabbit IgG were purchased from Zymed Laboratories[®]. A monoclonal antibody against mouse β -tubulin was obtained from Dr. E. Richard Stanley, Albert Einstein College of Medicine (AECOM). A rabbit polyclonal antibody against protein disulfide isomerase (PDI) was purchased from Stressgen[®]. GDP-fucose, GDP-mannose, UDP-galactose and UDP-GlcNAc were from Calbiochem[®] or Oxford Glycosystems[®], and their ³H- or ¹⁴C-labeled forms were from Calbiochem[®] or Perkin Elmer[®]. GDP-hexanamine sepharose beads were a gift of Dr. T. Beyer, Duke University. ATP, *E. coli* tRNA, GlcNAc, L-fucose, heparin, levamisol, proteinase K and yeast RNA were from Sigma[®]. Oligonucleotide primers for polymerase chain reaction (PCR) were ordered from Invitrogen[®].

Cell culture and transfection - CHO-Tag Chinese hamster ovary cells expressing the polyoma T antigen (21) and human embryonic kidney 293T cells were grown adherent at 37 °C in complete alpha-modified Eagle's medium (α +MEM) supplemented with 10% fetal bovine serum. Penicillin (100 U/ml) and streptomycin (100 μ g/ml) were sometimes added. The cell culture reagents were obtained from Invitrogen[®] and Gemini[®]. Cells were transfected with DNA using the calcium phosphate precipitation method (22), or Fugene6[™] (Roche[®]) or Lipofectamine 2000[™] (Invitrogen[®]) as per protocols suggested by the manufacturers.

Reverse transcription (RT) and PCR - RNA was prepared using TRIzol[™] reagent (Invitrogen[®]) as per the manufacturer's guidelines. Total RNA (0.5-5 μ g) was reverse transcribed at 45-48 °C using 1 μ g oligo-dT and 100 ng random hexamers with Superscript II[™] (Invitrogen[®]) reverse transcriptase in 25 μ l. Products were treated with RNase H (Invitrogen[®]) for 15 min at 37 °C. For PCR, using Taq, Pfx or Pfu polymerases (Invitrogen[®]), 1-5 ng plasmid DNA or 1-5 μ l cDNA was used as template. Products were resolved by electrophoresis on agarose gels and detected by ethidium bromide staining. Primers used for amplifying zebrafish *lunatic fringe* were 662 (5'ATG TTC TGC AGG GTG ACG GAG CTG CAG TCT CGA GAT GCA T) and 623 (5'CCC AAG CTT TGG AGG GAG GTT TAG ATA GCT GTG), and for zebrafish *fut11* were 776

(5'GCC CTT GTT CTC TAT TAA TAT TCA TAG) and 777 (5'CTT GTG ACT AGA CAG GAT GAG TGG).

Mouse Fut10 expression constructs - Mouse *Fut10* coding region was amplified from brain cDNA with primers PS711 (5'ATG CAT GCT AGC CCA CCA TGG TGC GGT TTC AGA GGA GGA AGC) and PS685 (5'ATG CAT CTC GAG CTA GGA ATA GAG CCC TGT GGC AGG CAA CC; has XhoI restriction site). After amplification with Platinum™ Pfx polymerase (Invitrogen®), 3' A-overhangs were added to the PCR product with Taq polymerase for TA cloning into pCR3.1 vector (Invitrogen®). Sequence of *Fut10* in thus cloned pCR3.1-Fut10 plasmid was confirmed by sequencing. To generate an expression construct for secreted mouse Fuc-TX fused at N-terminus to human IgG1 Fc fragment (CH2 and CH3 regions), plasmid pSecFc was prepared first. The Fc fragment was amplified from pLunIg plasmid (obtained from Dr. Thomas Vogt, Merck Research Laboratories) with primers 642 (5'TAC GAA GCT TGG ACA TGC CCA CCG TGC CCA GCA CCT G; has HindIII restriction site) and 643 (5'TGG AGT GGA TCC GTT TAC CCG GAG ACA GGG AGA GGC TC; has BamHI restriction site) and cloned between HindIII and BamHI restriction sites of pSecTag2C-hygromycin vector (Invitrogen®). Thus generated plasmid pSecFc was digested with NotI and XhoI restriction enzymes to receive a mouse *Fut10* insert. *Fut10* coding region missing the first 30 codons was amplified using primers 683 (5'ATG CAT GCG CGG CCG CTC GAG CTT GGG AAA TTT GAA AGG AAG AAG C; has NotI restriction site) and 685 (5'ATG CAT CTC GAG CTA GGA ATA GAG CCC TGT GGC AGG CAA CC; has XhoI restriction site). NotI/XhoI-digested PCR product was ligated with NotI/XhoI-digested pSecFc to generate pSecFc-Fut11 that encodes a 782 aa-long fusion protein consisting of 21, 223 and 406 residues of mouse Igk leader sequence, Fc fragment, and Fuc-TX respectively.

Mouse Fut11 expression constructs - Plasmid pCR3.1-Fut11 was generated like pCR3.1-Fut10. Mouse *Fut11* coding region was amplified by PCR from an IMAGE consortium kidney cDNA clone (GenBank accession number AI956528). Primers used were 562 (5'CCA CCA TGG CTG CTC GTT GTA CCG AGG C) and 563 (5'GCA GTT AGA GAT TTT TAT TCC GTT TCA TGA AGA TCT C). For plasmid pSecFc-Fut11, encoding a secreted Fuc-TXI with an N-terminus human IgG1 Fc fragment, the *Fut11* coding region without the first 30 codons was amplified from placenta cDNA using primers 609 (5'CCC GCG GCC GCT CGA GGC AGA GAG GGA GGA GCC GTG GGA TG; has NotI restriction site) and 631 (5'ATC CTC GAG TTA GAG ATT TTT ATT CCG TTT CAT GAA GAT CT; has XhoI site). This plasmid encodes an 845 aa-long fusion protein consisting of 21, 223 and 459 residues of mouse Igk leader sequence, Fc fragment, and Fuc-TXI respectively.

Analysis of Fut10 and Fut11 gene expression by Northern analysis - RNA blots were purchased from Seegene®, South Korea. Total RNA (20 µg) from mouse embryos at different stages of development, and from various adult mouse organs was electrophoresed on a 1% agarose-formaldehyde gel and transferred and UV-crosslinked on a nylon membrane. Source tissues representing E4.5-E6.5 embryos included extra-embryonic and maternal uterine material, while those for E7.5-E9.5 embryos were from conceptuses (embryo and extra-embryonic tissue). The supplier provided photographs of ethidium bromide-stained gels. Blots were pre-hybridized overnight at 42 °C in UltraHyb™ buffer (Ambion®) containing sheared salmon sperm DNA at 0.2 mg/ml. Hybridization was similarly carried out after adding DNA probes labeled with

[$\alpha^{32}\text{P}$]dCTP using the PrimeIt RmT™ kit (Stratagene®). Finally, blots were washed for 10 min each in 2x SSC buffer (1x SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7) containing 0.1% SDS twice at room temperature (RT) and then twice at 42 °C. Final wash was done with 0.1x SSC/0.1% SDS at 68 °C. Blots were then exposed to Biomax™ MR films (Kodak®) at -80 °C. The *Fut10* probe was obtained by amplifying the entire *Fut10* coding region (~1.3 kb) in pCR3.1-Fut10 with primers 709 (5'AGC TTA AAG ACT CCA ATG TGC AAG) and 710 (5'ATA CAA CCC ATC GTC ATA ATC CAG). The *Fut11* probe was an ~0.7 kb *SacI* fragment of pCR3.1-Fut11 containing the first 680 b of mouse *Fut11* coding region.

Preparation of Fut10 and Fut11 RNA probes - RNA probes incorporating digoxigenin-UTP were prepared from linearized plasmid DNA by run-off transcription using the DIG RNA labeling kit (Roche®). Mouse *Fut10* coding region from position 116 to 1,018 was amplified from pCR3.1-Fut10 with primers 709 and 710 (above), and TA-cloned in pCR2.1 vector (Invitrogen®). The pCR2.1-Fut10 and pCR3.1-Fut11 plasmids, with inserts in both orientations, were digested with *XhoI* and *XbaI* restriction enzymes respectively. The digestions linearize the plasmid by cutting a few bases downstream of the insert. One μg of linearized DNA, purified using the phenol-chloroform method, was used in the transcription/labeling reaction as per the manufacturer's protocol. Labeled RNA was electrophoresed on a formaldehyde-agarose RNA gel and its concentration was assessed by comparison with a control RNA sample provided in the kit. As the *Fut11* probes were larger than 1 kb, they were hydrolyzed with 40 mM Na_2CO_3 and 60 mM NaHCO_3 at 60 °C to a size of ~500 b as checked on an agarose gel. Finally, the RNA probes were purified on a MicroSpin™ S-400 HR column (Amersham®) and stored at -80 °C.

Whole mount mouse embryo in situ hybridization - Normal appearing, and thus wild type or *Pofut1*^{+/-} 8.5-11-day old embryos were obtained by crossing *Pofut1*^{+/-} mice that were being used in another study in the laboratory (23). Embryos collected in diethyl-propyl carbonate (DEPC)-treated PBS or α +MEM/10% fetal bovine serum were fixed in 4% paraformaldehyde (PFA) for 1 h at RT, washed thrice for 5 min each in DEPC-treated PBS and then twice for 5 min each in DEPC-treated PBS/0.1% Tween-20 (PTW), dehydrated through a graded series of methanol in PTW (25%, 50%, 75% and 100% v/v), and stored in methanol at -20 °C. Embryos rehydrated through a similar graded series but in reverse were washed twice with PTW, and treated with 10 $\mu\text{g}/\text{ml}$ proteinase K in PTW for different durations depending on their age: 8.5 d, 10 min; 9.5 d, 15 min; 10.5-11 d, 20 min. After two washes with PTW, they were fixed in 4% PFA for 30 min at RT. The embryos were then washed twice with PTW, once with 1:1 PTW/hybridization buffer (Hyb; 50% formamide, 1.3x SSC, pH 5.0, 5 mM EDTA pH 8.0, 50 $\mu\text{g}/\text{ml}$ yeast RNA, 50 $\mu\text{g}/\text{ml}$ *E. coli* tRNA, 0.5% CHAPS [3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate] and 100 $\mu\text{g}/\text{ml}$ heparin) and once with Hyb. Five to ten embryos were then left in 2 ml Hyb at 65 °C for 3-4 h for pre-hybridization. They were then moved into 2 ml of pre-warmed Hyb containing 0.2-2 μg of DIG-labeled RNA probe for hybridization overnight at 65 °C. Next day, they were rinsed twice in pre-warmed Hyb, washed twice with it for 30 min each, and then washed again for 10 min with 1:1 Hyb/MABT (100 mM maleic acid, 150 mM NaCl, 0.1 Tween 20, pH 7.5). Embryos were then rinsed twice and washed once for 15 min in MABT at RT. They were then incubated at RT in MABT with 2% Blocking Reagent (Roche®) and in MABT with 2% Blocking Reagent and 20% sheep serum (Invitrogen®) (1 h each). Finally, they were incubated with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche®) at RT for 4 h or overnight at 4 °C. The antibody was pre-absorbed with a powder of 13-14-day old mouse

embryos made by acetone precipitation of embryo homogenates in PBS. Three mg of the powder was mixed at 4 °C for 2 h in 0.5 ml MABT containing 1-2 µl of the antibody. After centrifugation at 14,000 rpm for a minute, the supernatant was diluted to 2 ml with MABT with 2% Blocking Reagent and 20% sheep serum. Embryos were then rinsed thrice in 2 ml MABT, washed five times for 12 min each with 2 ml MABT, and then twice for 10 min each with 2 ml NTMT (1 mM levamisole, 1% Tween 20, 50 mM MgCl₂, 100 mM NaCl, 0.1 M Tris HCl, pH 9). Embryos were then moved into a solution of nitroblue tetrazolium chloride and bromo-chloro-indolyl phosphate (NBT/BCIP, Roche®) in NTMT (0.5 ml of stock solution in 2 ml). Incubation was done at RT, or at 37 °C or 4 °C for faster or slower color development. After the color had developed to a desirable extent, embryos were rinsed in PTW and fixed in 4% PFA/PTW for 2 h at RT. They were then washed with PTW and stored at 4 °C in PTW/0.1% sodium azide. Photographs were taken on a Leica® Wild™ M3Z dissection microscope with a Canon® PowerShot™ S4 digital camera. All incubations at RT were done on a gently rocking platform.

Protein gel electrophoresis and Western analysis - Protein concentrations were determined by the Dc™ assay (Bio-Rad®). For electrophoresis, samples were diluted in Laemmli loading buffer (24) containing β-mercaptoethanol (final concentration 0.1 M), boiled for 5 min, and electrophoresed under reducing conditions on denaturing Tris-glycine SDS-polyacrylamide gels. Gels (7.5% or 10%) were either cast using Mini-Protean™ apparatus (Bio-Rad®) or were purchased from Bio-Rad®. Benchmark™ or MagicMark™ molecular weight markers (Invitrogen®) were loaded in separate lanes. When required, protein gels were stained with Coomassie blue. For immunoblotting, proteins were electro-transferred overnight to a Polyscreen™ PVDF membrane (Perkin Elmer®) in Tris-glycine buffer containing 8-12% methanol. To assess transfer and protein loading, membranes were sometimes reversibly stained with 0.5% Ponceau S (Sigma®) in 1% acetic acid. Subsequent incubations were for 1-2 h at RT or overnight at 4 °C. Membranes were blocked in 5% non-fat dry milk (Nestle®) in TBS (150 mM NaCl, 10 mM Tris, pH 7.4) with 0.05% Tween 20 or NP-40 detergent before addition of primary antibodies. Secondary antibodies were used at 1:5,000-10,000 dilutions. Chemiluminescent detection was performed with SuperWest Pico™ (Pierce®) reagents and exposure to Biomax™ MR X-ray films.

Generation of antisera against mouse Fuc-TXI - 100-200 mg of peptides 102 (CSRDRRARADPRT) and 185 (CYLRRPAPPPRERAE), representing mouse Fuc-TXI aa from positions 102 to 113 and from 185 to 198 respectively, were synthesized and purified with high pressure liquid chromatography (HPLC) by the Laboratory for Macromolecular Analysis, AECOM. Both peptides had C-terminal cysteine and N-terminal amide residues. Mass spectrometry was used to verify purity. Peptides were conjugated to maleimide-activated mariculture key limpet hemagglutinin (KLH; Pierce®) as follows: ~3 mg (~1.5 µmole) peptide was dissolved in PBS containing calcium and magnesium. DMSO to 5% v/v concentration was added to aid in the dissolution of peptide 185. The pH of the solutions was adjusted to 7 using 2 M NaOH and 2 M HCl. Two mg of KLH was resuspended in 200 µl PBS and added to the peptide solution. The mixtures were left at RT for 3 h and then at 4 °C for 4 h. The peptide-KLH conjugation reactions were then transferred to a Slide-a-lyzer™ (Pierce®) unit (3.5 kDa cut-off) for dialysis against 800 ml PBS, pH 7.4 at 4 °C for 14 h. The dialyzed KLH-peptide conjugates were frozen on dry ice and shipped to Covance Research Products® for immunization of New Zealand White (NZW) female rabbits. One animal per KLH-peptide was immunized and bled as

per this protocol: day 0, pre-bleed (~5 ml serum) and primary immunization - 0.3 mg immunogen injected intradermally with Freund's complete adjuvant; days 14, 28, 42, 56 and 70, secondary immunization - 0.1 mg (0.3 mg on day 14) immunogen injected subcutaneously with Freund's incomplete adjuvant; days 38 and 52, test bleeds (~5 ml serum each); days 66, 80 and 94, production bleeds (~20 ml serum each); and, terminal bleed (~50 ml serum) 1-2 weeks later. Sera were shipped on dry ice and kept frozen at -20 °C.

Purification of polyclonal antibodies against mouse Fuc-TXI - Two grams of epoxy-activated, 4%-beaded agarose (Sigma[®]) was hydrated with 50 ml water by gentle mixing at RT for 20 min. After washes with water on a sintered glass filter, about 5 ml swollen agarose was obtained. Approximately 15 mg each of peptides 102 and 185 was dissolved separately in 3 ml Na₃PO₄ (50 mM, pH 7.2). Half of the activated agarose was added to each solution and the slurry, with agitation, was incubated at RT for 20 h and then for another 20 h at 4 °C. This peptide-agarose conjugate was poured into an empty glass chromatography column and slowly rinsed with 2 M ethanolamine, pH 10 at RT for 4 h. The column was moved to 4 °C and sequentially washed (2x10 ml) with PBS, 0.1 M sodium acetate, pH 4, 0.1 M sodium bicarbonate, pH 8.5 and, finally, PBS. Eight ml of serum from the terminal bleeds was loaded on the washed column. Flow-rate was set to ~1 ml/min. The flow-through was reloaded twice. The column was then washed five times with 10 ml of PBS. Bound antibodies were eluted with 10 ml of 0.1 M glycine-HCl, pH 3. Halfway through the elution, 0.3 ml 1 M Tris, pH 9 was added to the eluate to neutralize its low pH. Eluates were concentrated in a CentriPrep[™]-30 tube (Millipore[®]) by centrifugation at 3,000 g for 3 h at 4 °C. Retentates (200-400 µl) were made to 50% glycerol and stored at -20 °C.

Analysis of Fuc-TXI protein levels in mouse by Western blotting - Blots representing adult mouse organs well as embryos at different development stages were purchased from RNWay[®], South Korea. Whole lysates containing 30 µg of protein from various mouse tissues were electrophoresed on a denaturing and reducing 10% SDS-PAGE gel, and transferred to Hybond[™] polyvinylidene fluoride (PVDF) membrane (Amersham[®]) by the supplier. Source tissues for E4-E7 embryos included maternal uterine material, and E8-E10 embryos were conceptuses. Western analysis was done using the two purified anti-Fuc-TXI antibodies at 1:1200-1500 dilution. Quality and relative quantity of samples was assessed by immunoblotting with anti-tubulin (undiluted) and anti-PDI (1:5,000 dilution) antibodies. Membranes were thus stripped and reused three times.

Preparation of Fc-Fuc-TX and Fc-Fuc-TXI sepharose beads - 293T cells were transiently transfected with pSecFc, pSecFc-Fut10 or pSecFc-Fut11 using the calcium phosphate precipitation method. After 2-3 days, cells from a 10 cm dish were washed and resuspended in ~150 µl of 1.5% Triton-X 100 in TBS containing 1x EDTA-free Complete[™] protease inhibitor cocktail (Roche[®]) and put on ice for 10 min. After a few seconds of vigorous vortexing, the lysate was centrifuged at RT for 2-3 min at 4-6,000 g. Proportionately, to 100 µl supernatant, 200-300 µl TBS with 1x EDTA-free Complete[™] protease inhibitor cocktail and 20-40 µl Protein A/G Plus[™] sepharose beads (Santa Cruz Biotechnology[®]) were added. After incubation with constant agitation at 4 °C for 12-24 h, beads were pelleted, washed twice with TBS, once with 500 mM NaCl/10 mM Tris, pH 7.4, and once with 0.2 M sodium cacodylate, pH 6.5. Beads were finally resuspended to a 20-30% slurry with 0.2 M sodium cacodylate, pH 6.5, and stored at 4 °C.

Fc-Fut11 beads thus prepared and stored retained GDP-fucose hydrolase activity for at least 4-6 weeks.

Binding of Fuc-TXI to GDP-hexanolamine - GDP-hexanolamine (2 μ M) sepharose beads (75 μ l) was added with 600 μ l incubation buffer (1x EDTA-free Complete™ protease inhibitor cocktail, 150 mM NaCl, 10 mM MnCl₂, 25 mM sodium cacodylate, pH 6.5) to 200 μ l cell lysate obtained from pSecFc or pSecFc-Fut11 transfectants of 293T cells as described above. After overnight incubation at 4 °C, the beads were pelleted by centrifugation at 100-200 g and washed thrice with 1 ml incubation buffer containing 500 mM NaCl. Fuc-TXI protein bound to the beads was detected by Western blotting using an HRP-conjugated anti-human IgG1 antibody.

Enzyme assays – Nucleotide sugar hydrolysis assays were typically done in 50 μ l volume in 50 mM sodium cacodylate, pH 6.5 containing 10 mM MnCl₂, 5 mM ATP, 10 mM *L*-fucose and 6 nmole GDP-[¹⁴C]fucose or -[¹⁴C]mannose, or UDP-[³H]galactose or -[³H]GlcNAc (20-200 x10³ cpm). After incubation at 37 °C for 1-3 h with continuous shaking, reactions were stopped by adding 1 ml cold water. Reaction products were separated on a small AG-1X4 (Bio-Rad®) anion exchange column as described previously (25). Eluates were mixed with Ecolume™ (ICN Biochemicals®) and measured for radioactivity in a scintillation counter (Beckman Coulter®). β 1-4 galactosyltransferase activity was assayed using GlcNAc as acceptor as described elsewhere (26).

Microinjection of zebrafish embryos with morpholino oligonucleotides - Morpholinos were synthesized by GeneTools®, and dissolved in Danieau's injection buffer (116 mM NaCl, 1.4 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgSO₄ and 10 mM HEPES, pH 7.6) to prepare a 10 mM stock solution. Further dilutions were made in the injection buffer. The morpholinos zf1 (5'CGC CAT AGT TGA CTT GAG AAA AGC A) and zf2 (5'ACA TAA TTC AAG TAT TTA CAC TGT A) are respectively complementary to the putative zebrafish *fut11* transcript from nucleotide position 4 to 28 (coding region) and -39 to -63 (5' untranslated region). A control morpholino (5'ATA TCC ATC ACA CTG GCG GC) complementary to a segment of the multiple cloning site of pcDNA3 vector (Invitrogen®) was kindly gifted by Dr. Yuko Miyana (AECOM). Wild type zebrafish of AB/Tübingen strain were bred and maintained as per standard protocols in the laboratory of Dr. Todd Evans (AECOM). Freshly fertilized eggs were collected and placed on wedge-shaped troughs made of 1.5% agarose for injection. Glass needles were drawn from Nanoject™ II 204-G/X (Drummond®) flared tubes with a micropipette puller (Sutter Instrument®) and their tips were beveled on a horizontal grinder (Narishige®) to a tip of 40-60 μ m outer diameter. A Nanoject™ II-controlled Drummond® electrical micro-injector was used to inject a volume of 2.3 nl in embryos at one- or two-cell stage of development. Embryos were grown in fish-system water in a 28 °C incubator.

Sequence analyses - Nucleic acid and protein sequences were analyzed on the internet using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>), ClustalW 1.8 (<http://www.ch.embnet.org/software/ClustalW.html>), and Transmembrane HMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Alignment of protein sequences was done using the BLOSUM62 matrix (27) with a word-size of 3, and gap opening and extension penalties of 11 and 1, respectively. GenBank and the Ensembl version 39 annotated sequence databases for chicken (*Gallus gallus*; genome assembly version 1, March 2004), cow (*Bos taurus*; genome

assembly version 2, March 2005), dog (*Canis familiaris*; genome assembly version 1, July 2004), human (genome assembly version 36, October 2005), frog (*Xenopus (Silurana) tropicalis*; genome assembly version 4.1, April 2005), Japanese medaka fish (*Oryzias latipes*; genome assembly version 1, October 2005), tiger pufferfish (*Takifugu rubripes*; genome assembly version 4, June 2005), mouse (*Mus musculus*; genome assembly version 36, December 2005), rat (*Rattus norvegicus*; genome assembly version 3.4, December 2004), spotted puffer fish (*Tetraodon nigroviridis*; genome assembly version 7, April 2003), and zebrafish (*Danio rerio*; genome assembly version 6, July 2005) were queried respectively at <http://ncbi.nlm.nih.gov/GenBank> and <http://www.ensembl.org>. GenBank accession numbers of some of the protein sequences mentioned here are: for Fuc-TX – chicken, NP_989761; cow, NP_892032; dog, NP_00101266569; frog, NP_001002903; human, NP_116053; mouse, NP_598922; rat, NP_001012360; zebrafish, NP_001012374, XP_697342; and for Fuc-TXI – chicken, NP_989759; cow, XP_586457; dog, XP_852401; frog, NP_001002904; human, NP_775811; tiger pufferfish, NP_001027930; medaka, CAI52077; mouse, NP_082704; rat, NP_775430; rhesus macaque (*Macaca mulatta*), XP_001100876; spotted pufferfish, CAH04181; zebrafish, XP_697432. For designing peptides to generate antibodies, the antigenicity, hydrophilicity and surface accessibility of mouse Fuc-TXI residues was analyzed using the Lasergene™ software (DNASar®).

RESULTS

Identification of Fut10 and Fut11 genes - Prior to early 2000, queries of the dbEST expressed sequence tag (EST) database (28) in GenBank using human and mouse α 1-3/4 Fuc-T peptide sequences failed in identifying any EST representing a novel α 1-3/4 Fuc-T. However, using the entire amino acid sequence of a zebrafish Fuc-TIX ortholog, zFT1 (29), an EST (Genbank accession number AW233458) from an adult zebrafish olfactory epithelium cDNA library was identified; it had a putative translation product with ~50% sequence similarity to the C-terminal region of zFT1. The EST sequence was used to query the mouse EST collection to identify two ESTs (GenBank accession numbers AI509025 and BB072523) that did not represent any known mouse α 1-3/4 Fuc-T. The cDNA clones for the ESTs were sequenced, and the new sequence information was used to re-query dbEST. After more cDNA sequencings, a contig was assembled and its sequence was used to design primers to amplify and clone the coding region of the putative α 1-3/4 Fuc-T gene from mouse brain and lung cDNA. Using the sequence of the coding region in homology-based searches of data in the mouse genome sequencing project, a second putative gene was identified. The coding region of this gene was also amplified and cloned from mouse lung and brain cDNA. Orthologous sequences in other genomes were identified by homology-based searches of sequence databases. The human orthologs were also identified by Roos, et al. by homology-based searches of sequence databases and named *Fut10* and *Fut11* by them (20). The two mouse genes were also previously identified by such searches (30).

Fut10 and *Fut11* orthologs can be identified in the sequenced genomes of the frogs *Xenopus (Silurana) tropicalis* and *Xenopus laevis* as well as in that of zebrafish. That the genes are transcribed is suggested by dbEST sequences representing the *Fut10* and *Fut11* genes of *X. tropicalis* (8 and 31 respectively) and of zebrafish (1 and 7 respectively). The *X. tropicalis* Fuc-TX and Fuc-TXI proteins each are 63% identical to the mouse orthologs over stretches of 408 and 458 aa respectively; for the zebrafish proteins the values are 62% and 57% over stretches of

351 and 454 aa respectively. In the three other sequenced teleostian genomes that were analyzed (*Tetraodon nigroviridis*, *Takifugu rubripes* and *Oryzias latipes*), only Fuc-TXI orthologs could be identified. These genomes are less than half in size than that of zebrafish.

Analysis of Fuc-TX and Fuc-TXI sequences - As has been noted for the human and mouse sequences (20,30), peptide motifs characteristic of α 1-3/4 Fuc-Ts are present in the various Fuc-TX and Fuc-TXI family members. However, the sequences of Fuc-TX and Fuc-TXI proteins differ from those of known α 1-3/4 Fuc-Ts in some important respects.

Compared to well-characterized α 1-3/4 Fuc-Ts, the Fuc-TX and Fuc-TXI proteins are larger, with most of the extra residues contributing to a longer C-terminal region. E.g., in humans, they have 428 and 492 aa, respectively, as against 359-405 in the other α 1-3/4 Fuc-Ts. The mouse Fuc-TX (436 aa) and Fuc-TXI (489 aa) sequences are most similar to that of mouse Fuc-TIX – 27% identity and 45% similarity over a stretch of 281 aa, and 27% identity and 45% similarity over a stretch of 255 aa, respectively. For comparison, in mouse, Fuc-TIX is most homologous to Fuc-TIIV with 42% identity and 56% similarity over a stretch of 292 aa. A hidden Markov model-based hydrophobicity analysis of one avian, one amphibian, one piscian and five mammalian Fuc-TX orthologs using TMHMM (31) suggests that all the Fuc-TX proteins are type II transmembrane proteins with a single N-terminal transmembrane domain and short (8-11 aa) cytoplasmic regions. All known mammalian α 1-3/4 Fuc-T proteins have this topology. On the other hand, a transmembrane region was not noticed in any of the one avian, one amphibian, three piscian and six mammalian Fuc-TXI orthologs that were analyzed.

Like most glycosyltransferases, all Fuc-TX and Fuc-TXI orthologs have a conserved DXD motif, but it is placed more C-terminally, by about 60 residues, than in the known α 1-3/4 Fuc-Ts. This motif is represented by the sequence DRD in all the Fuc-TX and Fuc-TXI orthologs (Fig. 1). Three cysteine residues that are positionally conserved among the known α 1-3/4 Fuc-Ts are similarly present in the Fuc-TX and Fuc-TXI orthologs; however, all the orthologs have four extra positionally-conserved cysteine residues that are missing in the known α 1-3/4 Fuc-Ts. One of them is present between the conserved LAFENS and DYITEK α 1-3/4 Fuc-T peptide motifs (Fig. 1).

The HH[R/W][D/E] sequence in the hypervariable stem portion of mammalian α 1-3/4 Fuc-Ts that has been implicated in determining their type 1 or type 2 acceptor specificity (32,33) is entirely missing in all Fuc-TX and Fuc-TXI orthologs, being replaced with a completely conserved [L/I]FYGTD sequence (Fig. 1). Besides the LFYGTD motif, there are other motifs that are present in all Fuc-TX and Fuc-TXI family members but not in the known α 1-3/4 Fuc-Ts. An example is the WGVXD motif, with X being glutamine among all Fuc-TX, but asparagine among the Fuc-TXI orthologs (Fig. 1).

Expression of the Fut10 and Fut11 genes in the adult mouse - Presence of *Fut10* and *Fut11* transcripts in various organs of the adult mouse was examined by Northern analysis using DNA probes against the coding regions. A *Fut10* transcript of ~3.9 kb was detected in all the 13 organs that were examined, with highest expression in liver, heart, testis and placenta, and lowest in stomach, uterus and skeletal muscle (Fig. 2A). Another transcript of >10 kb was also detected; except in testis and placenta, its expression was weaker than that of the shorter transcript. For *Fut11*, a transcript of ~2.7 kb was detected in all the 14 organs that were examined, with highest expression in placenta and lung, and lowest in brain, liver and testis (Fig. 2B). The staining of

18S and 28S RNA with ethidium bromide was used to confirm the approximate equal loading of the total RNA samples in the experiment.

Expression of the Fut10 and Fut11 genes during mouse development - The same coding region probes were also used in Northern analyses to assess the expression of *Fut10* and *Fut11* transcripts during the development of the mouse embryo. All of the total RNA samples from embryos at various days of development (E4.5 to E18.5) showed the ~3.9 kb *Fut10* transcript that was also seen in adult mouse organs (Fig. 3A). The >10 kb transcript was also detected in all the samples, with its levels weaker but correlating well with those of the smaller transcript. *Fut10* expression appears to weaken gradually from day 4.5 to 9.5 only to strengthen suddenly to the highest level on day 10.5, and again weakening gradually from that level thereafter. For *Fut11*, the ~2.7 kb transcript of the adult mouse is also present in the developing embryos. Its expression is dramatically strong from day 7.5 to 9.5 (Fig. 3B). During this period, an intense expression of transcripts of many other sizes (3-20 kb) is also seen. The transcript levels fall abruptly in the next two days, reaching their lowest values, and then rise and remain at an intermediate value till parturition.

Spatial expression of the *Fut10* and *Fut11* genes in mouse embryos in mid-gestation was studied by RNA:RNA in situ hybridization using probes against the coding regions. As shown in Fig. 4A, both *Fut10* and *Fut11* transcripts are ubiquitous in 9- and 10.5-day old embryos. Consistent with findings of Northern analyses, *Fut10* expression is much stronger in d10.5 embryos than d9 or d9.5 ones whereas *Fut11* expression in d9 and d10.5 embryos appears equivalent (Fig. 4B). Compared to that in d9 and d9.5 embryos, *Fut10* expression in d10.5 embryos is stronger in the head relative to rest of the body. In d10.5 and d11 embryos, *Fut11* is expressed more in ventral portion of the head, and both upper and lower limb buds.

Generation of anti-mouse Fuc-TXI polyclonal antibodies and Fuc-TXI expression in the mouse - Two synthetic mouse Fuc-TXI peptides (aa 102-113 and 185-198) conjugated to key limpet hemagglutinin were separately injected in rabbit to generate polyclonal antibodies against the mouse protein. Strong immune responses, as suggested by high antibody titers in antisera, were observed. Anti Fuc-TXI antibodies in the antisera were purified by affinity chromatography on columns of peptide-conjugated agarose beads. The purified polyclonal antibodies were tested for sensitivity by Western blotting using lysates of CHO cells transfected with a mouse *Fut11* cDNA (data not shown).

To examine the expression of Fuc-TXI protein in adult mouse organs and during development of mouse embryos, immunoblotting was performed on pre-made, commercially available blots. Mouse Fuc-TXI protein is predicted to have a native molecular weight of 58 kDa. Using either antibody (ab 102 or 185), proteins of 58 kDa and 49 kDa were detected in detergent extracts of the mouse tissues (Fig. 5). The 58 kDa signal was stronger than the 49 kDa one in all samples with either antibody, except those from skeletal muscle and from 14-18-day old embryos in which it was weaker when ab 102 was used. As expected from the *Fut11* Northern analysis (Figs. 2B and 3B), Fuc-TXI protein is thus ubiquitously expressed, with relatively high levels in the heart, lung and placenta of adult mouse (Fig. 5A). For the spleen and thymus, protein levels are lower than that expected from RNA levels. Mouse Fuc-TXI protein expression is higher during days 5 to 10 of embryonic development than during days 14 to 18 (Fig. 5B). The same blots were stripped and re-probed with antibodies against protein disulfide isomerase or β -tubulin. As can be seen in Fig. 5, there was wide variability in the levels of the

two housekeeping proteins on the blots. It should therefore be noted that, contrary to the blot supplier's claims, the samples on the blots were not of similar quantity or quality.

Hydrolysis of GDP-fucose by Fuc-TXI - Various biochemical assays that were performed to identify Fuc-T activities of the Fuc-TX and Fuc-TXI proteins were unsuccessful. However, hydrolysis of the GDP- β -L-fucose sugar donor was found to be enhanced in the presence of Fuc-TXI (but not Fuc-TX). Data from such an assay is shown in Fig. 6A. Triton-X 100 detergent extracts of CHO-Tag cells transiently transfected with expression constructs for mouse *Fut10* or *Fut11* coding regions, or neither, were assayed for GDP-fucose hydrolysis at pH 6.5 and 37 °C in the presence of 10 mM MnCl₂, 5 mM ATP and 10 mM L-fucose. Extracts of *Fut11* transfectants had ~10x higher specific activity for hydrolysis compared to *Fut10* or empty vector transfectants. Equivalent quality and quantity of lysates was confirmed by a β 1-4 galactosyltransferase assay.

Size exclusion and anion exchange chromatography showed that free fucose and not phosphorylated fucose was a product of the hydrolysis reaction (data not shown). GDP-fucose hydrolysis was also observed with lysates of human *Fut11*, but not *Fut10*, transfectants (data not shown). A purified recombinant mouse Fuc-TXI protein also showed GDP-fucose hydrolase activity. Fig. 6B shows that the hydrolase activity was seen with protein A/G sepharose beads that had mouse Fuc-TXI (aa 31-489) C-terminally tagged to the Fc fragment of human IgG1 (Fc Fuc-TXI), but not with those that had only the Fc fragment. Enhanced hydrolysis of three other nucleotide sugars, GDP-mannose, UDP-galactose and UDP-GlcNAc was not observed (Fig. 6B). Hydrolysis of GDP-fucose by Fuc-TXI was dose-dependent, higher at pH 5.5 than pH 6.5, independent of ATP or fucose, and inhibited by *N*-ethyl-maleimide, GDP or guanidine monophosphate (data not shown). Fucosidase activity in mouse Fuc-TXI was not detected in assays using *p*-nitrophenol- α -L-fucose as substrate (data not shown).

The interaction of Fuc-TXI with GDP-fucose that is suggested by Fuc-TXI's GDP-fucose hydrolase activity was indirectly confirmed by the binding of GDP-hexanolamine to mouse Fuc-TXI. Fuc-T proteins such as Fuc-TVII (34) and POFUT1 (35) have been shown to bind GDP-hexanolamine. As shown in Fig. 6C, GDP-hexanolamine-conjugated sepharose beads could pull down Fc Fuc-TXI, but not Fc, from lysates of 293T transfectant cells.

Expression of fut11 gene in the developing zebrafish - RNA:RNA in situ hybridization of whole zebrafish embryos to study the temporo-spatial expression of *fut11* gene was attempted. While transcripts of certain other genes could be detected by the technique, those of *fut11*, however, could not be detected. This is expected and observed for genes (e.g., *manic fringe* (36)) whose transcripts are present at a low level. The expression of *fut11* during zebrafish development was therefore studied by RT-PCR. As shown in Fig. 7A, the entire coding region of *fut11* could be detected in total RNA isolated from zebrafish embryos at 6, 12, 18 and 30 hours after fertilization. At these time-points, the embryos are respectively at the shield, 5-9 somites, 20-25 somites and prim-15 stages of development (37). As expected, transcripts of a control gene, *lunatic fringe*, could be detected at all the four development stages (36).

Requirement of fut11 during early zebrafish development - To identify possible roles of Fuc-TXI protein in vertebrate development, the zebrafish *fut11* gene was knocked down using antisense morpholino oligonucleotides (38). Morpholinos have been shown to be highly efficient and specific in inhibiting translation of transcripts in zebrafish embryos (39). Two morpholinos with non-overlapping sequences, *zf1* and *zf2*, that targeted the *fut11* transcript close to the

translational start codon were synthesized (positions -63 to -29, and 4 to 28). Maximal effects of morpholinos can be achieved by targeting this region as this sterically hinders the assembly of the translation initiation complex. Analysis of zebrafish sequence databases shows that the morpholinos are complimentary to only the *fut11* sequence. It should be noted that a mismatch at just 5 positions with no matches at 15 contiguous positions is enough to prevent a 25-mer morpholino from binding to its target (information communicated by the manufacturer).

Various amounts of *zf1* or *zf2*, or of a control, irrelevant 25-mer morpholino (complimentary to the cloning site of pcDNA3 vector), were injected into embryos at 1-2 cell stage. The developing embryos were then observed along with uninjected embryos for upto the next 5-6 days. Embryonic lethality in the first 5-6 hours because of the injection procedure was 10-20% for all the morpholinos. Embryos injected with the irrelevant morpholino developed like wild-type embryos (Table I).

However, a reduced rate of survival was readily noticed in the *zf1* and *zf2* morphants, a large proportion of which showed abnormalities of the posterior trunk (Table I and Fig. 7C). In mildly affected embryos, the posterior trunk curved dorsally and/or laterally, while in those severely affected there was additional shortening as well as twisting of the body axis with truncation of the tail (Fig. 7B and 7C). Developmental abnormalities in the form of an irregularly shaped tail-tip could be discerned at about the 19-somite stage (20 h). The embryos were edematous and pericardial edema was visible in many (Fig. 7C). The moderately to severely affected animals generally died within 4-5 days. That *zf1* and *zf2* resulted in similar abnormalities (Fig. 7C) indicates that the morpholinos indeed targeted only the *fut11* transcripts.

DISCUSSION

Based on a significant sequence homology to known α 1-3/4 Fuc-Ts, and no other protein families, including other fucosyltransferases, and the presence of conserved α 1-3/4 Fuc-T motifs, the Fuc-TX and Fuc-TXI proteins are speculated to be α 1-3/4 Fuc-Ts (20,30). CHO and COS-7 cells, which do not possess any α 1-2/3/4 Fuc-T activity (40,41), were engineered to express the human or mouse Fuc-TX or Fuc-TXI proteins to detect any α 1-3/4 Fuc-T activity of the proteins. However, an α 1-3/4 Fuc-T activity could not be discerned in the transfectants, including those transfected with both *Fut10* and *Fut11* of mouse (data not shown). The assays included those for the binding of antibodies against the Lewis antigens Le^x , sLe^x , Le^a or sLe^a , and of α -linked fucose-specific *Ulex europaeus* agglutinin (UEA)-I and *Aleuria aurantia* lectin (AAL) to the cell surface, and for in vitro GDP- β -L-fucose fucosyltransferase activity against common α 1-3/4 Fuc-T acceptors such as lactosamine (LacNAc), α 2-3- or α 2-6-sialyl LacNAc and lacto-*N*-biose. It has similarly been reported that COS cells transfected with human *Fut10* or mouse *Fut11* cDNA failed to express Le^x , sLe^x or Le^a on their cell surface, and their extracts did not have a fucosyltransferase activity against LacNAc, α 2,3-sialyl LacNAc or lacto-*N*-biose (30).

This shows that *Fut10* and *Fut11*, evolutionarily distinct from other fucosyltransferase genes as suggested by phylogenetic analysis (20), do not encode typical α 1-3/4 Fuc-Ts. This is also suggested by the analyses of Fuc-TX and Fuc-TXI sequences, which, in spite of the high sequence homology to α 1-3/4 Fuc-Ts and the presence of α 1-3/4 Fuc-T motifs and conserved cysteines, nevertheless have unusual features, such as the lack of the HH[R/W][D/E] motif and the presence of extra, highly conserved cysteines, including one between the LAFENS and DYITEK α 1-3/4 Fuc-T motifs (Fig. 1). It is, however, worth noting that bacterial α 1-3/4 Fuc-Ts

and the CEFT-1 α 1-3 Fuc-T of *Caenorhabditis elegans* that can act on LacNAc (42) also lack the HH[R/W][D/E] motif; for *H. pylori* α 1-3/4 Fuc-Ts, type 1 or type 2 acceptor specificity is instead determined by C-terminal peptide sequences (43). Similarly, CEFT-1, as well as the *Drosophila melanogaster* FucTA (44) and *C. elegans* FUT-1 (45) that fucosylate the chitobiose core of *N*-glycans in α 1-3 linkage have a cysteine between the LAFENS and DYITEK motifs.

Mouse *Fut10* and *Fut11* expression constructs were also singly or jointly transfected into CHO and the Lec8 galactosylation mutant, defective in the Golgi UDP-galactose transporter (46), to identify any core α 1-3 Fuc-T activity in Fuc-TX and Fuc-TXI. However, an increased binding of core α 1-3 fucose-recognizing anti-HRP or anti-bee venom antibodies (47,48) was not seen (data not shown). Other studies have not found evidence for core α 1-3 Fuc-T activity in the *D. melanogaster* FucTB whose sequence is most similar to those of Fuc-TX and Fuc-TXI of vertebrates (44,49).

A change in the global glycan profile of CHO resulting from the exogenous expression of Fuc-TX or Fuc-TXI that may adumbrate the nature of the proteins was also partially assessed for (data not shown). Neutral *N*-glycans of CHO cells transfected with human or mouse *Fut10* or *Fut11*, or an empty vector, were released with peptide *N*-glycanase A for matrix-assisted laser desorption and de-ionization mass spectrometry, but a difference could not be discerned, and CHO transfectants of mouse or human *Fut11* had similar sensitivity to concavilin A, leukocyte phytohemagglutinin, pea lectin, ricin and wheat germ agglutinin in plating tests. It should be noted that transcripts of endogenous, hamster *Fut10* and *Fut11* orthologs could be detected by RT-PCR in CHO cells (data not shown).

The ubiquitous expression of *Fut10* and *Fut11* in the adult mouse (Fig. 2) also sets these genes apart from the known α 1-3/4 Fuc-T genes that have a restricted expression pattern (12,50,51). Similar ubiquitous expression was also observed by Baboval and Smith (30). While they too observe the \sim 3.9 kb *Fut10* transcript shown in Fig. 2A, the *Fut11* transcripts noted in their study are of length \sim 1.6 and \sim 4.5 kb, and not \sim 2.7 kb (Fig. 2B). This discrepancy is difficult to explain, especially since detailed information about the probe used by them is unavailable. It should be noted that the same \sim 2.7 kb transcript is also present in the mouse embryos (Fig. 3B).

Fut10 and *Fut11* transcripts are also ubiquitous in the mouse embryo during its development (Fig. 4). Although they are present throughout development, there are remarkable changes in their levels during E7.5 to E9.5 (Fig. 3). The level of *Fut10* transcripts falls to its lowest while that of *Fut11* rises to the highest. This points to an important role for the products of the genes during mammalian development.

Fut11 transcripts are also expressed from early on during development in the zebrafish (Fig. 7A). Knock-down of Fuc-TXI in zebrafish embryos using antisense morpholinos targeted to *Fut11* transcripts results in developmental abnormalities (Figs. 7B and 7C, and Table I), and shows that Fuc-TXI is required for vertebrate development. Further characterization of the morphants to identify the affected genetic pathways was not undertaken as the observed phenotypes did not point towards a specific, molecularly-characterized development process that might have been disturbed. However, the posterior trunk and tail malformations seen in the more severely affected morphants resemble those of zebrafish embryos with perturbed metabolism of chitin oligosaccharides. Such embryos have been generated by injection of an antibody against the zebrafish DG42 chitin synthase (52), of the NodZ chitin α 1,6-fucosyltransferase of *Rhizobia* (52), of a NodZ expression construct (53), of a chitinase from *Streptomyces* (54), and of the chitinase-inhibiting allosamidin pseudo-trisaccharide (54). It is thus possible that Fuc-TX and Fuc-TXI are involved in the metabolism of chitin sugars.

The mouse Fuc-TXI protein, but not Fuc-TX, was found to specifically hydrolyze GDP-fucose (Fig. 6A and 6B). The interaction of the GDP-sugar with the protein was also affirmed by the binding of GDP-hexanolamine (Fig. 6C). Catalysis by fucosyltransferase enzymes are believed to proceed in an ordered, sequential two-step mechanism in which the glycosidic bond linking terminal phosphate of GDP to fucose is hydrolyzed before nucleophilic attack by the acceptor substrate on the anomeric carbon (C1) of fucose (55). Thus, hydrolysis of GDP-fucose can be indicative of a fucosyltransferase activity. Recombinant human Fuc-TV (56), and A and B ABO glycosyltransferases (57) have been shown to have nucleotide sugar hydrolyzing activities in the absence of an acceptor *in vitro*.

The predicted lack of a transmembrane domain in the Fuc-TXI proteins is consistent with the observation that mouse Fuc-TXI is localized in the cytoplasm and not the ER-Golgi compartment of transfected cells (30). The GDP-fucose hydrolase activity observed of Fuc-TXI (Figs. 6A and 6B) may play a role in the intracellular homeostasis of fucosylated glycoconjugates by affecting the levels of the nucleotide sugar in the cytoplasm. Changes in GDP-fucose hydrolase activity in rat organs during physiological processes has been described (58,59).

The availability of large-scale genome and transcriptome sequence data in recent years has made it very facile to identify novel homologous proteins by the sequence similarity-based *in silico* approach. However, an assignment of activity based on homology needs to be used with extreme caution. Specific functions involve fewer residues than structural folds and motifs, and the power of sequence in predicting protein function rapidly falls off at lower homologies, even more so for enzymes. It is estimated that a 60% pairwise sequence identity is needed to 'homology transfer' the function of a novel putative enzyme with 70% accuracy; a 90% accuracy requires a 75% similarity (60). Fuc-TX and Fuc-TXI have pairwise sequence similarities of <50% with Fuc-TIX, the most homologous protein of known function. It is then, perhaps, not surprising that the identification of their predicted fucosyltransferase activity remains elusive. Such an activity, unlike those of the known α 1-3/4 Fuc-Ts, may require unusual substrates or cofactors, or may be restricted to specific substrate-bearing proteins.

ACKNOWLEDGEMENTS

The work described here was carried out in the laboratory of Pamela Stanley at AECOM under her mentorship. This work was supported by grants from the National Institutes of Health to her and the AECOM Cancer Center. The author thanks Sunny Gupta for maintaining zebrafish.

ABBREVIATIONS

α 1-3/4 Fuc-T, α 1-3/4 fucosyltransferase; aa, amino acid; CHO, Chinese hamster ovary; GlcNAc, *N*-acetyl glucosamine; Le^X, Lewis X; PBS, phosphate-buffered saline; RT, room temperature; TBS, Tris-buffered saline.

REFERENCES

1. Phillips, M. L., Nudelman, E., Gaeta, F. C., Perez, M., Singhal, A. K., Hakomori, S., and Paulson, J. C. (1990) *Science* **250**(4984), 1130-1132

2. Lowe, J. B., Stoolman, L. M., Nair, R. P., Larsen, R. D., Berhend, T. L., and Marks, R. M. (1990) *Cell* **63**(3), 475-484
3. Bird, J. M., and Kimber, S. J. (1984) *Dev Biol* **104**(2), 449-460
4. Fenderson, B. A., Zehavi, U., and Hakomori, S. (1984) *J Exp Med* **160**(5), 1591-1596
5. Kim, Y. S., Itzkowitz, S. H., Yuan, M., Chung, Y., Satake, K., Umeyama, K., and Hakomori, S. (1988) *Cancer Res* **48**(2), 475-482
6. Capela, A., and Temple, S. (2002) *Neuron* **35**(5), 865-875
7. van Ree, R. (2002) *Int Arch Allergy Immunol* **129**(3), 189-197
8. Ilver, D., Arnqvist, A., Ogren, J., Frick, I. M., Kersulyte, D., Incecik, E. T., Berg, D. E., Covacci, A., Engstrand, L., and Boren, T. (1998) *Science* **279**(5349), 373-377
9. Goodman, J. L., Nelson, C. M., Klein, M. B., Hayes, S. F., and Weston, B. W. (1999) *J Clin Invest* **103**(3), 407-412
10. Qian, F., Hanahan, D., and Weissman, I. L. (2001) *Proc Natl Acad Sci U S A* **98**(7), 3976-3981
11. Weitkamp, L. R., Johnston, E., and Guttormsen, S. A. (1974) *Cytogenet Cell Genet* **13**(1), 183-184
12. Kudo, T., Ikehara, Y., Togayachi, A., Kaneko, M., Hiraga, T., Sasaki, K., and Narimatsu, H. (1998) *J Biol Chem* **273**(41), 26729-26738
13. Goelz, S. E., Hession, C., Goff, D., Griffiths, B., Tizard, R., Newman, B., Chi-Rosso, G., and Lobb, R. (1990) *Cell* **63**(6), 1349-1356
14. Weston, B. W., Nair, R. P., Larsen, R. D., and Lowe, J. B. (1992) *J Biol Chem* **267**(6), 4152-4160
15. Weston, B. W., Smith, P. L., Kelly, R. J., and Lowe, J. B. (1992) *J Biol Chem* **267**(34), 24575-24584
16. Kudo, T., Kaneko, M., Iwasaki, H., Togayachi, A., Nishihara, S., Abe, K., and Narimatsu, H. (2004) *Mol Cell Biol* **24**(10), 4221-4228
17. Bartunkova, J., Maly, P., Smetana, K., Jr., Sediva, A., Klubal, R., Mayerova, D., Sedlacek, A., and Splichalova, V. (2000) *Apmis* **108**(6), 409-416
18. Weninger, W., Ulfman, L. H., Cheng, G., Souckova, N., Quackenbush, E. J., Lowe, J. B., and von Andrian, U. H. (2000) *Immunity* **12**(6), 665-676
19. Oriol, R., Mollicone, R., Cailleau, A., Balanzino, L., and Breton, C. (1999) *Glycobiology* **9**(4), 323-334
20. Roos, C., Kolmer, M., Mattila, P., and Renkonen, R. (2002) *J Biol Chem* **277**(5), 3168-3175
21. Smith, P. L., and Lowe, J. B. (1994) *J Biol Chem* **269**(21), 15162-15171
22. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., and Chasin, L. (1979) *Proc Natl Acad Sci U S A* **76**(3), 1373-1376
23. Shi, S., and Stanley, P. (2003) *Proc Natl Acad Sci U S A* **100**(9), 5234-5239
24. Laemmli, U. K. (1970) *Nature* **227**(259), 680-685
25. Potvin, B., Kumar, R., Howard, D. R., and Stanley, P. (1990) *J Biol Chem* **265**(3), 1615-1622
26. Chaney, W., Sundaram, S., Friedman, N., and Stanley, P. (1989) *J Cell Biol* **109**(5), 2089-2096
27. Henikoff, S., and Henikoff, J. G. (1992) *Proc Natl Acad Sci U S A* **89**(22), 10915-10919
28. Adams, M. D., Soares, M. B., Kerlavage, A. R., Fields, C., and Venter, J. C. (1993) *Nat Genet* **4**(4), 373-380
29. Kageyama, N., Natsuka, S., and Hase, S. (1999) *J Biochem (Tokyo)* **125**(4), 838-845
30. Baboval, T., and Smith, F. I. (2002) *Mamm Genome* **13**(9), 538-541

31. Sonnhammer, E. L., von Heijne, G., and Krogh, A. (1998) *Proceedings / ... International Conference on Intelligent Systems for Molecular Biology ; ISMB* **6**, 175-182
32. Dupuy, F., Petit, J. M., Mollicone, R., Oriol, R., Julien, R., and Maftah, A. (1999) *J Biol Chem* **274**(18), 12257-12262
33. Sherwood, A. L., Upchurch, D. A., Stroud, M. R., Davis, W. C., and Holmes, E. H. (2002) *Glycobiology* **12**(10), 599-606
34. de Vries, T., Storm, J., Rotteveel, F., Verdonk, G., van Duin, M., van den Eijnden, D. H., Joziassse, D. H., and Bunschoten, H. (2001) *Glycobiology* **11**(9), 711-717
35. Wang, Y., and Spellman, M. W. (1998) *J Biol Chem* **273**(14), 8112-8118
36. Qiu, X., Xu, H., Haddon, C., Lewis, J., and Jiang, Y. J. (2004) *Dev Dyn* **231**(3), 621-630
37. Westerfield, M. (1995) *The zebrafish book : a guide for the laboratory use of zebrafish (Danio rerio)*, Ed. 3. Ed., M. Westerfield, Eugene, OR
38. Summerton, J., and Weller, D. (1997) *Antisense & nucleic acid drug development* **7**(3), 187-195
39. Nasevicius, A., and Ekker, S. C. (2000) *Nat Genet* **26**(2), 216-220
40. Oulmouden, A., Wierinckx, A., Petit, J. M., Costache, M., Palcic, M. M., Mollicone, R., Oriol, R., and Julien, R. (1997) *J Biol Chem* **272**(13), 8764-8773
41. Howard, D. R., Fukuda, M., Fukuda, M. N., and Stanley, P. (1987) *J Biol Chem* **262**(35), 16830-16837
42. DeBose-Boyd, R. A., Nyame, A. K., and Cummings, R. D. (1998) *Glycobiology* **8**(9), 905-917
43. Ma, B., Wang, G., Palcic, M. M., Hazes, B., and Taylor, D. E. (2003) *J Biol Chem* **278**(24), 21893-21900
44. Rendic, D., Linder, A., Paschinger, K., Borth, N., Wilson, I. B., and Fabini, G. (2006) *J Biol Chem* **281**(6), 3343-3353
45. Paschinger, K., Rendic, D., Lochnit, G., Jantsch, V., and Wilson, I. B. (2004) *J Biol Chem* **279**(48), 49588-49598
46. Deutscher, S. L., and Hirschberg, C. B. (1986) *J Biol Chem* **261**(1), 96-100
47. Wilson, I. B., Harthill, J. E., Mullin, N. P., Ashford, D. A., and Altmann, F. (1998) *Glycobiology* **8**(7), 651-661
48. Prenner, C., Mach, L., Glossl, J., and Marz, L. (1992) *Biochem J* **284** (Pt 2), 377-380
49. Fabini, G., Freilinger, A., Altmann, F., and Wilson, I. B. (2001) *J Biol Chem* **276**(30), 28058-28067
50. Gersten, K. M., Natsuka, S., Trinchera, M., Petryniak, B., Kelly, R. J., Hiraiwa, N., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., and Lowe, J. B. (1995) *J Biol Chem* **270**(42), 25047-25056
51. Smith, P. L., Gersten, K. M., Petryniak, B., Kelly, R. J., Rogers, C., Natsuka, Y., Alford, J. A., 3rd, Scheidegger, E. P., Natsuka, S., and Lowe, J. B. (1996) *J Biol Chem* **271**(14), 8250-8259
52. Bakkers, J., Semino, C. E., Stroband, H., Kijne, J. W., Robbins, P. W., and Spaink, H. P. (1997) *Proc Natl Acad Sci U S A* **94**(15), 7982-7986
53. Semino, C. E., Allende, M. L., Bakkers, J., Spaink, H. P., and Robbins, P. P. (1998) *Ann N Y Acad Sci* **842**, 49-54
54. Natunen, J., Aitio, O., Helin, J., Maaheimo, H., Niemela, R., Heikkinen, S., and Renkonen, O. (2001) *Glycobiology* **11**(3), 209-216
55. Murray, B. W., Wittmann, V., Burkart, M. D., Hung, S. C., and Wong, C. H. (1997) *Biochemistry* **36**(4), 823-831

56. Murray, B. W., Takayama, S., Schultz, J., and Wong, C. H. (1996) *Biochemistry* **35**(34), 11183-11195
57. Patenaude, S. I., Seto, N. O., Borisova, S. N., Szpacenko, A., Marcus, S. L., Palcic, M. M., and Evans, S. V. (2002) *Nat Struct Biol* **9**(9), 685-690
58. Biol, M. C., Lenoir, D., Greco, S., Galvain, D., Hugueny, I., and Louisot, P. (1998) *Am J Physiol* **275**(5 Pt 1), G936-942
59. Lenoir, D., Ruggiero-Lopez, D., Louisot, P., and Biol, M. C. (1995) *Biochim Biophys Acta* **1234**(1), 29-36
60. Rost, B., Liu, J., Nair, R., Wrzeszczynski, K. O., and Ofran, Y. (2003) *Cell Mol Life Sci* **60**(12), 2637-2650

TABLE I*Morphogenesis defects in zebrafish injected with anti-Fut11 morpholino oligonucleotides*

Morpholino	Dose	No. of experiments	Percent displaying phenotype (No. affected/Total)
Control	2 ng	1	0% (0/18)
	6 ng	3	0% (0/29)
	20 ng	2	0% (0/28)
zf1	1 ng	1	23% (3/13)
	2 ng	1	18% (2/17)
	3 ng	1	25% (4/16)
	6 ng	2	84% (43/51)
	10 ng	1	58% (11/19)
	20 ng	1	57% (4/7)
	zf2	2 ng	2
	6 ng	2	56% (18/32)
	20 ng	1	88% (14/16)

Zebrafish embryos at 1-2 cell stage were injected with an irrelevant, control morpholino or the zf1 or zf2 morpholinos designed to block translation of *Fut11* transcripts. Live animals were evaluated 48-72 hours later for posterior trunk and tail malformations.

FIGURE LEGENDS

Figure 1. Characteristics of Fuc-TX and Fuc-TXI sequences. Sequences of Fuc-TX and Fuc-TXI of various species (six mammalian, four piscian, and one each avian and amphibian), and known human α 1-3 Fuc-Ts (Fuc-TIII-VII and Fuc-TIX), were aligned using the ClustalW 1.8 algorithm. Sections of the alignment are shown to illustrate certain characteristics of the Fuc-TX and Fuc-TXI family members. The HH[R/W][D/E] motif of the known Fuc-Ts is replaced by the [L/I]FYGTD motif (*LFYGTD*) in the Fuc-TX and Fuc-TXI families. The two families have a conserved DXD motif (DRD) characteristic of most glycosyltransferases, the LAFENS and DYITEK α 1-3/4 Fuc-T motifs, and an exclusive WGV[Q/N]D motif (*WGVXD*). Two positionally-conserved cysteines (C) exclusive to the two families, and two others conserved among all the known α 1-3/4 Fuc-Ts are also shown. Identical and similar residues are shown with black or grey shading respectively. GenBank accession numbers for the Fuc-TX and Fuc-TXI sequences are noted in the *Material and methods* section.

Figure 2. Expression of *Fut10* and *Fut11* transcripts in adult mouse tissues. *Fut10*, **A**, and *Fut11*, **B**, mRNA was detected by Northern blotting and hybridization with radiolabeled DNA probes (~1.3 kb and ~0.7 kb, respectively) that are specific to the coding regions. Each lane has 20 μ g total RNA from the indicated tissues. Ethidium bromide (*EtBr*) staining of the RNA gels before blotting shows similar loading and quality of the RNA samples (*bottom panels*).

Figure 3. Expression of *Fut10* and *Fut11* transcripts during mouse development. *Fut10*, **A**, and *Fut11*, **B**, mRNA was detected by Northern blotting and hybridization with radiolabeled DNA probes (~1.3 kb and ~0.7 kb, respectively) that are specific to the coding regions. Each lane has 20 μ g total RNA isolated from mouse embryos with different days of development as indicated. Ethidium bromide (*EtBr*) staining of the RNA gels before blotting shows similar loading and quality of the RNA samples (*bottom panels*).

Figure 4. Mouse *Fut10* and *Fut11* transcripts are detected by whole embryo RNA:RNA in situ hybridization. Expression of *Fut10* or *Fut11* RNA in mouse embryos with various days (*d*) of development was analyzed by in situ hybridization as described under *Material and methods*. **A**, d9.5 and d10.5 embryos were probed with digoxigenin-labeled antisense (*as*) or sense (*s*; negative control) RNA probes against the *Fut10* or *Fut11* coding regions. **B**, a series of embryos with increasing days of development probed with antisense *Fut10* or *Fut11* RNA. Hybridized probes were detected colorimetrically using an alkaline phosphatase-conjugated anti-digoxigenin antibody fragment. Embryos shown here represent cohorts of at least two and an average of four.

Figure 5. Fuc-TXI protein is ubiquitous in the adult mouse and present during mouse development. Western blots of detergent extracts from various adult mouse tissues, **A**, and mouse embryos at different stages of development, **B**, were serially probed with two anti Fuc-TXI polyclonal antibodies (ab 102 and 185). The blots were again stripped and re-probed with antibodies against protein disulfide isomerase (PDI; 58 kDa) and β -tubulin (44 kDa).

Figure 6. A, GDP-fucose hydrolase activity in *Fut11* transfectants. Triton-X 100 detergent extracts were prepared from CHO-Tag cells transiently transfected with expression constructs for the mouse *Fut10* or *Fut11* coding regions or just the empty vector (*vector*). The extracts were

assayed for GDP-fucose hydrolysis activity and β 1-4 galactosyltransferase (*Gal-T*) activity with GlcNAc as the acceptor. **B, GDP-fucose-specific hydrolase activity of Fuc-TXI.** Hydrolysis of different radiolabeled nucleotide sugars in the presence of protein A/G-conjugated sepharose beads carrying a human IgG1 Fc fragment (*Fc*) or Fc-tagged mouse Fuc-TXI (*Fc Fuc-TXI*) was assayed. Radioactivity (*cpm*) in released sugars was measured after separation by anion exchange chromatography. Beads carrying neither of the two proteins (*none*) were used in second control reactions. The *cpm* values include radioactivity from sugars released by chemical hydrolysis of the nucleotide sugars. **C, Fuc-TXI binds GDP-hexanolamine.** Lysates of 293T cells transiently transfected with expression constructs for Fc or Fc Fuc-TXI were incubated overnight with GDP-hexanolamine-conjugated sepharose beads at pH 6.5 with 10 mM MnCl₂ at 4 °C. An anti-Fc antibody was used to detect by immunoblotting the recombinant proteins in the fraction bound to the beads (*B*) after washing with 0.5 M NaCl and that left unbound and precipitated with trichloro-acetic acid (*S*).

Figure 7. A, Expression of *fut11* during zebrafish development. Transcripts of *fut11* in developing zebrafish at various hours post-fertilization (*hpf*) were detected by RT-PCR. Reverse transcription of 0.5 μ g total RNA was carried out in presence (+) or absence (-) of reverse transcriptase (*RT*). A fifth of the reaction was subjected to 45 cycles of amplification. Transcripts of *lunatic fringe* (*lfn*) were similarly detected, but with 42 cycles. **B, Morphogenesis defects in zebrafish *fut11* morphants.** Zebrafish embryos at 1-2 cell stage were injected with 6 ng of an irrelevant morpholino (*control*) or the *zfl* morpholino designed to block the translation of *fut11* transcripts. Animals shown are at 52 hpf. **C, Examples of phenotypes observed in the *fut11* morphants.** Zebrafish were injected at 1-2 cell stage with indicated doses of *zfl* or *zfl2* morpholinos that block the translation of *fut11* transcripts. Animals representing observed phenotypes were photographed at 60 or 100 hpf. .

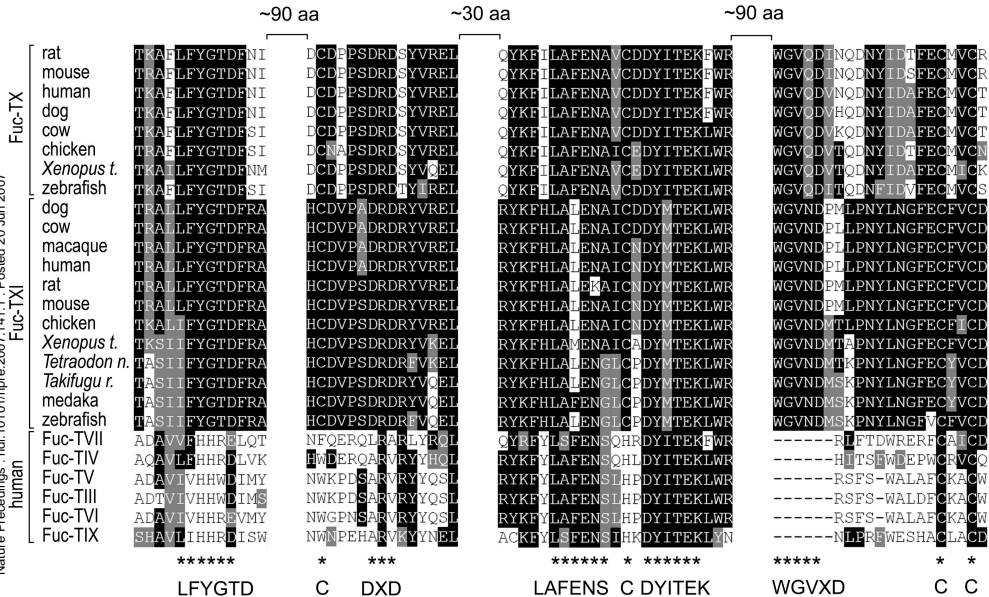


Fig. 1

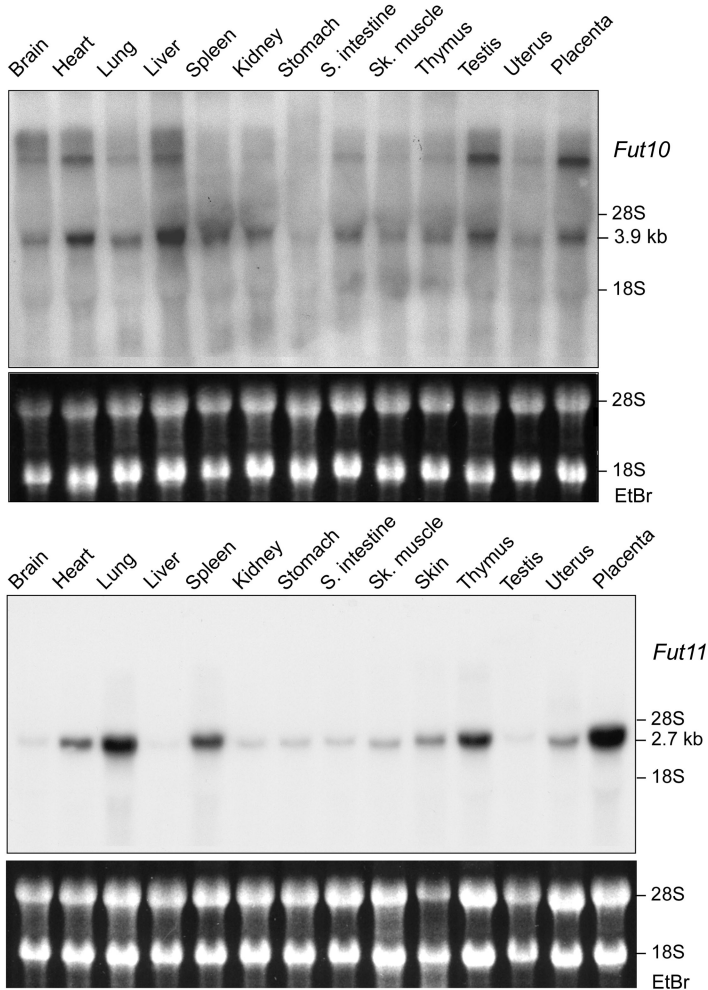
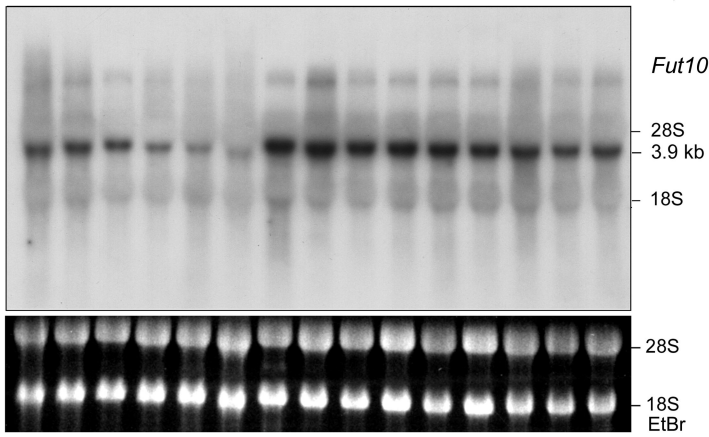


Fig. 2

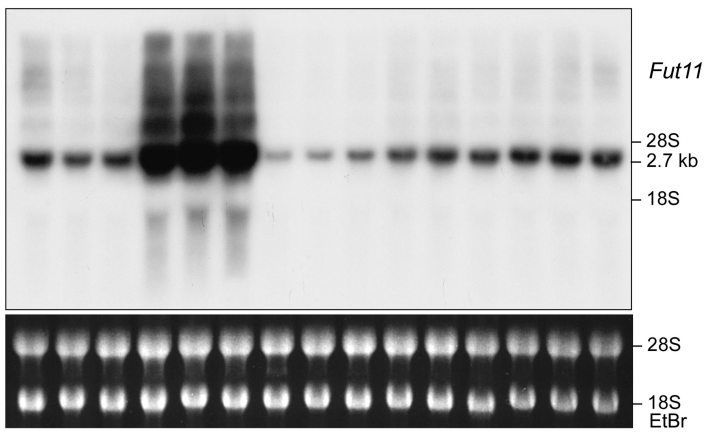
Embryonic day

4.5 5.5 6.5 7.5 8.5 9.5 10.5 11.5 12.5 13.5 14.5 15.5 16.5 17.5 18.5

A



B



Nature Precedings : hdl:10101/npre.2007.041.1 : Posted 20 Jun 2007

Fig. 3

A

Fut10

Fut11

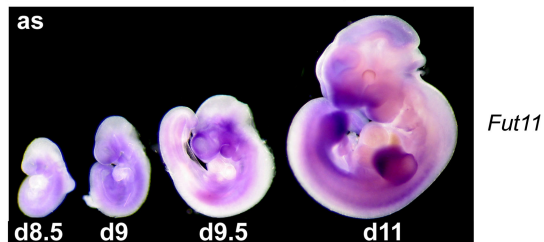
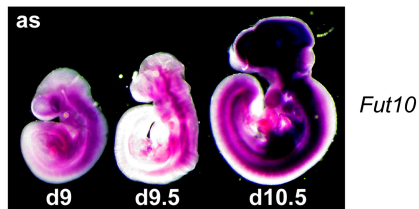
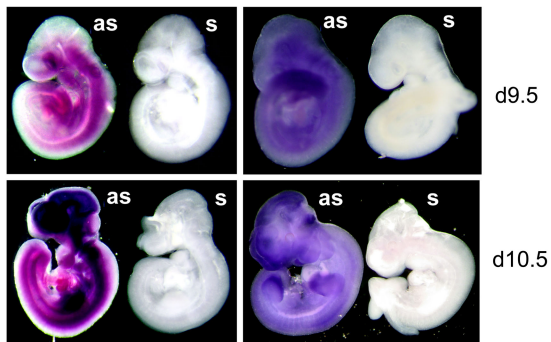
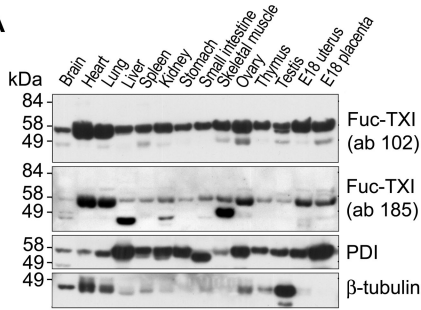
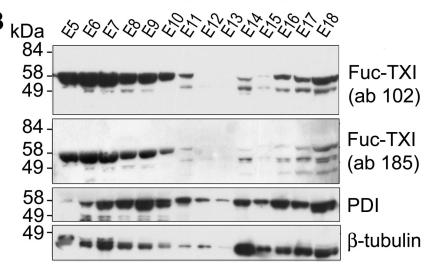


Fig. 4

A**B**

Nature Precedings : hdl:101/01/npre.2007.141.1 : Posted 20 Jun 2007

Fig. 5

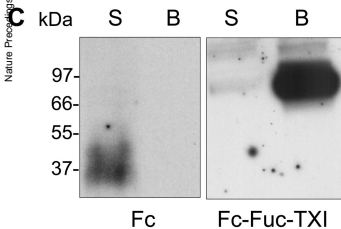
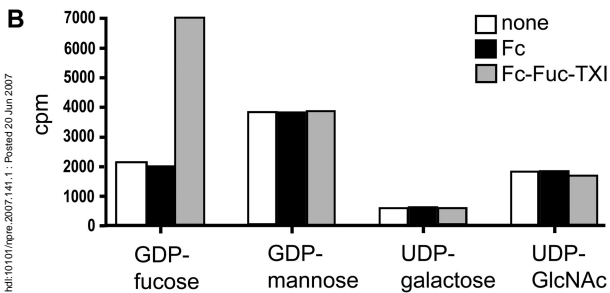
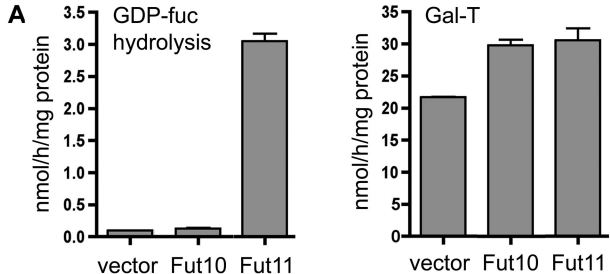
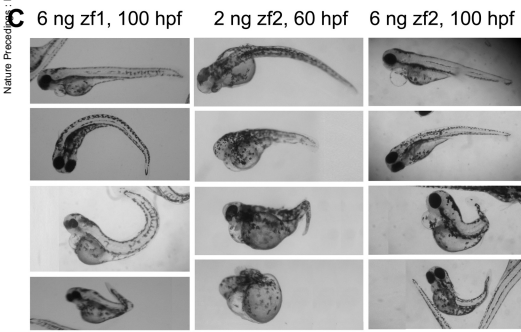
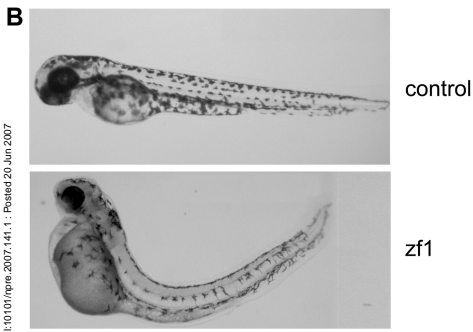
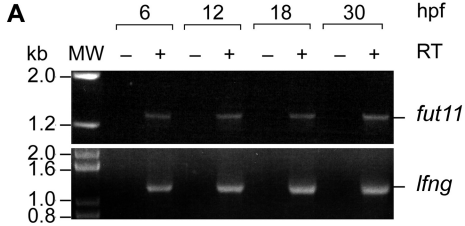


Fig. 6



Nature Precedings : doi:10.1016/npre.2007.141.1 : Posted 20 Jun 2007

Fig. 7