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Supporting Online Material for

In Vivo Imaging of Membrane-Associated Glycans in Developing Zebrafish

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This PDF file includes:

Materials and Methods
SOM Text
Figs. S1 to S14
References

Other Supporting Online Material for this manuscript includes the following: (available at www.sciencemag.org/cgi/content/full/320/5876/664/DC1)

Movies S1 to S9

Supporting Online Material for:

In Vivo Imaging of Membrane-Associated Glycans in Developing Zebrafish

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Materials and Methods

General materials and methods. All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification. With the exception of reactions performed in aqueous media, all reaction vessels were flame-dried prior to use. Reactions were performed in a N_2 atmosphere, except in the case of reactions performed in aqueous media, and liquid reagents were added with a syringe unless otherwise noted. Reversed phase high-performance liquid chromatography (HPLC) was performed using a Rainin Dynamax SD-200 HPLC system with 210 nm detection on a Microsorb C18 analytical or preparative column. FT-ICR mass spectra were obtained at the Howard Hughes Medical Institute Mass Spectrometry Facility at UC Berkeley. Cell disruption was performed with a Misonix Sonicator 3000 equipped with a microprobe. Flow cytometry analysis was performed on a BD FACSCalibur flow cytometer using a 488 nm argon laser. At least 5×10^4 cells were analyzed for each sample. Cell viability was ascertained by gating the samples on the basis of forward scatter (to sort by size) and side scatter (to sort by granularity). Multidimensional protein identification technology (MUDPIT) analysis was performed at the UC Berkeley Cancer Research Laboratory Proteomics/Mass Spectrometry Facility. Fluorescence and brightfield images were acquired on either a Zeiss 200M epifluorescence microscope (Fig. 2 and Fig. S6) or a Zeiss LSM 510 META laser scanning confocal microscope (all other imaging figures). All images were analyzed using Slidebook 4.2 (Intelligent Imaging Innovations); further, images acquired on the Zeiss 200M epifluorescence microscope were deconvolved using the nearest neighbor deconvolution algorithm in Slidebook 4.2. Laser-scanning confocal

microscopy was performed at the UC Berkeley Cancer Research Laboratory Molecular Imaging Center.

HRP-conjugated anti-β-tubulin was obtained from Abcam. Zebrafish embryonic fibroblast (ZF4) cells were obtained from the American Type Culture Collection. Bis-Tris polyacrylamide gels and the detergent-compatible (DC) protein assay kit were obtained from Bio-Rad. Fetal bovine serum (FBS) was obtained from HyClone Laboratory. Wide-bore Pasteur pipets (borosilicate glass disposable serological pipets with wide tip) were purchased from Fisher Scientific. Alexa Fluor cadaverines (488, 555, 647), Dulbecco's phosphate-buffered saline (PBS), Dulbecco's modified eagle's media (DMEM), Ham's F12 (F12) media, and both HiMark and SeeBlue Plus2 pre-stained protein molecular weight markers were obtained from Invitrogen Life Technologies, Inc. HRP-conjugated anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories. Amicon Ultra centrifugal filter devices and reversed phase C18 ZipTips were obtained from Millipore. Peptide N-glycosidase F (PNGase F) was obtained from New England Biolabs. Restore Western blot stripping buffer and SuperSignal West Pico chemiluminescent substrate were obtained from Pierce Biotechnology. Complete mini protease inhibitor cocktail tablets with ethylene diamine tetraacetic acid (EDTA) were obtained from Roche Applied Science. Trypsin Gold (mass spectrometry grade) was obtained from Promega Corporation. Ni-NTA agarose was obtained from Qiagen. Anti-chondroitin sulfate ΔDi-Os was obtained from Seikagaku Corporation. Anti-Flag M2 agarose, bovine serum albumin (BSA), chondroitinase ABC from *Proteus vulgaris*, HRP-conjugated anti-Flag M2, Nonidet P 40 Substitute, *N*-phenyl thiourea (PTU), Ponceau S, pronase (protease, Type XIV, from *Streptomyces griseus*), tricaine (ethyl 3-

aminobenzoate methanesulfonate), and tris(2-carboxyethyl)phosphine (TCEP) hydrochloride were obtained from Sigma-Aldrich. HRP-conjugated concanavalin A was obtained from United States Biological.

General cell culture conditions. ZF4 cells were maintained in a 5% CO_2, water-saturated atmosphere at 28 °C, and grown in 1:1 DMEM:F12 media supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (0.1 mg/mL). Cell densities were maintained between 1.25 x 10^5 and 1.6 x 10^6 cells/mL.

Zebrafish stocks and husbandry. Adult fish strains AB and ABC (wild type) were kept at 28.5 °C on a 14-h light/10-h dark cycle. Embryos were obtained from natural spawnings and were maintained in embryo medium (150 mM NaCl, 0.5 mM KCl, 1.0 mM $CaCl_2$, 0.37 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 2.0 mM $MgSO_4$, 0.71 mM $NaHCO_3$ in deionized (18.2 MΩ·cm) H_2O, pH 7.4). Embryos and larvae were developmentally staged according to Kimmel and coworkers(*11*).

Synthetic Procedures. Phosphine-Flag(*16*), phosphine-Flag-His_6(*16*), Ac_4GalNAz(*16*), Ac_4GalNAc(*23*), DIFO-647(*10*), DIFO-488(*10*), and DIFO pentafluorophenyl ester(*10*) were synthesized as previously described, and all final compounds were purified by reversed phase HPLC prior to use.

DIFO-555. To a solution of Alexa Fluor 555 cadaverine (2.0 mg, 0.0021 mmol) in DMF (1.5 mL) was added a solution of DIFO pentafluorophenyl ester (2.5 mg, 0.0059

mmol) and then *N,N*-diisopropylethylamine (3.7 µL, 0.021 mmol). The solution was stirred at rt overnight in the dark, and then the solvent was removed on a rotary evaporator. The residue was dissolved in water, purified by reversed phase HPLC using a gradient of 0% to 35% acetonitrile in water over 30 min, with the product eluting at ~20–25 min, and lyophilized to a fine powder (1.1 mg, 47%). FT-ICR-MS: Found: 1116.3349.

Cell surface labeling of ZF4 cells with DIFO-488. ZF4 cells were incubated for 3 d in media containing 0–100 µM Ac_4GalNAz. The cells were lifted with a 1 mM EDTA solution in PBS lacking calcium and magnesium. The cells were then distributed into a 96-well V-bottom tissue culture plate, pelleted by centrifugation (2500 x *g*, 3 min, 4 °C), and washed twice with 200 µL of labeling buffer (PBS, pH 7.4 containing 1% FBS). Cells were then incubated with 10 µM DIFO-488 in labeling buffer for 1 h at rt. After incubation, cells were pelleted by centrifugation (2500 x *g*, 3 min, 4 °C), washed three times with labeling buffer, and then diluted to a volume of 400 µL for flow cytometry analysis. The average fluorescence intensity was calculated from each of three replicate experiments to obtain a representative value in arbitrary units.

Immunoblot analysis of ZF4 cells incubated with Ac_4GalNAz and Ac_4GalNAc. ZF4 cells were incubated for 3 d in media containing 100 µM Ac_4GalNAz or Ac_4GalNAc. The cells were lifted with a 1 mM EDTA solution in PBS lacking calcium and magnesium, pelleted, and resuspended in ~200 µL of ice-cold lysis buffer

(150 mM NaCl, 20 mM Tris, 1% Nonidet P 40 Substitute, pH 7.4, one tablet of complete mini protease inhibitor cocktail with EDTA per 10 mL of lysis buffer). The cells were disrupted by sonication on ice with 30-s, ~9-Watt pulses three times, and then insoluble debris was removed by centrifugation (16,000 x *g*, 10 min, 4 °C). The protein concentration of each sample was measured using the detergent-compatible (DC) protein assay kit. Twenty micrograms of protein was reacted with 10 μM DIFO-Flag overnight (~12 h) at rt. The samples were then analyzed by standard immunoblotting techniques, probing the blot with a 1:1000 dilution of HRP-conjugated anti-Flag M2. To control for equal protein loading, the blot was stained with Ponceau S.

Glycoproteomic analysis of Ac_4GalNAz-labeled species in ZF4 cells. The glycoproteomic analysis of the species labeled with azides upon metabolic incorporation of GalNAz into ZF4 cell glycoproteins was carried out essentially as previously described(*16*). Briefly, one hundred 15-cm tissue culture plates of ZF4 cells were incubated for 3 d in media containing 100 μM Ac_4GalNAz. After the third day, the cells were rinsed three times with 25 mL of PBS and then lifted by addition of 2.5 mL of a 1 mM solution of EDTA in PBS without calcium and magnesium for approximately 15 min at 28 °C. The cells were combined into one 15-mL conical tube, pelleted by centrifugation (2000 x *g*, 10 min, 4 °C), and the supernatant was discarded. The cell pellet was resuspended in ~10 mL of ice-cold lysis buffer (150 mM NaCl, 20 mM Tris, 1% Nonidet P 40 Substitute, pH 7.4, one tablet of complete mini protease inhibitor cocktail with EDTA per 10 mL of lysis buffer) and disrupted by sonication on ice with 30-s, ~9-Watt pulses three times. The insoluble debris was

removed by centrifugation (3500 x *g*, 30 min, 4 °C), and phosphine-Flag-His_6 was added as a solid directly to the supernatant at a final concentration of 500 μM. The Staudinger ligation was run at rt for 24 h under an argon atmosphere, and the unreacted phosphine-Flag-His_6 was removed by two sequential Bio-Rad P-10 size-exclusion columns. The combined flow-through fractions were concentrated using an Amicon Ultra centrifugal filter device to approximately 10 mL and then incubated with anti-Flag M2 agarose beads for 12 h at 4 °C. The beads were washed with 10 column volumes of the following solutions: 50 mM Tris (pH 7.4), 300 mM NaCl, 1% triton X-100; 50 mM Tris (pH 7.4), 1.3 M NaCl, 1% triton X-100; 50 mM Tris (pH 7.4), 300 mM NaCl, 1% triton X-100, 1 M urea. The bound species were eluted with one column volume of 8 M urea (pH 8.0), 0.1 M Na_2HPO_4, 10 mM Tris. The eluate was then incubated with Ni-NTA agarose beads for 12 h at 4 °C, washed with 10 column volumes of each of the following solutions: 8 M urea, 0.1 M Na_2HPO_4, 10 mM Tris (pH 8.0); and 8 M urea, 0.1 M Na_2HPO_4, 10 mM Tris (pH 8.0), 15 mM imidazole. The bound species were eluted with one column volume of a solution of 8 M urea, 0.1 M Na_2HPO_4, 10 mM Tris (pH 8.0), and 250 mM imidazole. The eluate was diluted to a concentration of urea of 2 M, and the purified azide-labeled glycoproteins were proteolytically digested with mass spectrometry grade trypsin for 12 h at 37 °C. The peptide solution was then treated sequentially with 5 mM dithiothreitol for 30 min and 10 mM iodoacetamide for 30 min to reduce disulfide bonds and alkylate free cysteine residues. The solution was then concentrated under vacuum, desalted by reversed phase C18 ZipTip, and submitted for MUDPIT analysis.

General protocol for in vivo metabolic labeling of developing zebrafish with Ac_4GalNAz or Ac_4GalNAc. Strain AB or ABC zebrafish embryos were manually cleaned and rinsed ten times with 25 mL of embryo medium prior to reaching 3 hpf. At approximately 2.5 hpf, the embryos were enzymatically dechorionated by incubation in a 1 mg/mL solution of pronase in embryo medium at ~22 °C and for approximately 5 min. The embryos were gently drawn into a fire-polished wide-bore Pasteur pipet and expelled to facilitate chorion removal. When >75% of the chorions had been removed, the embryos were very gently rinsed ten times with approximately 25 mL to remove excess pronase. Then, using a fire-polished wide-bore Pasteur pipet, the embryos were gently transferred into 1% agarose-coated 10-cm petri dishes containing 2.5 mL of a 5 mM Ac_4GalNAz or 5 mM Ac_4GalNAc solution in embryo medium, in as little volume as possible (< 10 µL), using the following procedure. The embryos were allowed to enter the solution by inserting the pipet into the new solution but not expelling solution; i.e., the embryos were allowed to fall from the pipet directly into the Ac_4GalNAz- or Ac_4GalNAc-containing media by gravity. The embryos were transferred to fresh Ac_4GalNAz- or Ac_4GalNAc-containing media and agar-coated petri dishes at 12 hpf, to fresh Ac_4GalNAz- or Ac_4GalNAc-containing media in non-agar coated dishes at 24 hpf, and subsequently to fresh Ac_4GalNAz- or Ac_4GalNAc-containing media in non-agar coated dishes at 72 hpf. When embryos were to be used for imaging, 131 µM *N*-phenylthiourea (PTU) was included in all media starting at 12 hpf to inhibit melanin production.

Immunoblot analysis of zebrafish embryos incubated with Ac_4GalNAz and Ac_4GalNAc. An equal number of 120 hpf zebrafish embryos metabolically labeled

with $Ac_4GalNAz$ or $Ac_4GalNAc$ were removed from $Ac_4GalNAz$ or $Ac_4GalNAc$ and put into 25 mL of embryo medium in a 10-cm petri dish. After gentle agitation of the plate for ~10 s, the zebrafish were transferred into an identical plate containing fresh media. This was repeated once more for a total of three washes, and the fish were then transferred to 1.5 mL eppendorf tubes and euthanized by incubation in an ice bath for 1 h. The majority of the embryo medium was removed, leaving the zebrafish larvae in ~20 μL of embryo medium. The volume was brought to ~200 μL by addition of ice-cold lysis buffer (150 mM NaCl, 20 mM Tris, 1% Nonidet P 40 Substitute, pH 7.4, one tablet of complete mini protease inhibitor cocktail with EDTA per 10 mL of lysis buffer). The embryos were disrupted by sonication on ice with 30-s, ~12-Watt pulses three times, and then insoluble debris was removed by centrifugation (16,000 x *g*, 10 min, 4 °C). The protein concentration of each sample was measured using the detergent-compatible (DC) protein assay kit. Twenty micrograms of protein was reacted with 250 μM phosphine-Flag overnight (~12 h) at rt by addition of an equal volume of a 500 μM phosphine-Flag stock solution in PBS. The samples were then analyzed by standard immunoblotting techniques, probing the blot with a 1:1000 dilution of HRP-conjugated anti-Flag M2. To control for equal protein loading, the nitrocellulose was stripped of the anti-Flag antibody with the Restore Western blot stripping buffer using the manufacturer's suggested protocol and re-probed with a 1:1000 dilution of HRP-conjugated anti-β-tubulin.

Reaction of lysates from metabolically labeled 120 hpf zebrafish with PNGase F. Lysates were generated from 120 hpf zebrafish embryos metabolically labeled with $Ac_4GalNAz$ or $Ac_4GalNAc$ as described above. Twenty micrograms of total protein

was reacted at 37 °C for 12 h with 1 U of either PNGase F or PNGase F that had been heat-killed for 1 h at 100 °C. After the reaction had been completed, an equal volume of a 500 μM solution of phosphine-Flag in PBS was added to yield a final concentration of 250 μM. This reaction proceeded at rt for 12 h. The completed reaction was analyzed by standard immunoblotting techniques, either probing the blot with a 1:1000 dilution of HRP-conjugated anti-Flag M2 or, to control for enzyme activity, with a 1:1000 dilution of HRP-conjugated concanavalin A, which recognizes most N-linked glycans and should yield chemiluminescence signal in the heat-killed but not active PNGase F reactions. To control for equal protein loading, each nitrocellulose blot was stripped of the anti-Flag antibody or concanavalin A lectin with the Restore Western blot stripping buffer using the manufacturer's suggested protocol and re-probed with a 1:1000 dilution of HRP-conjugated anti-β-tubulin.

Reaction of lysates from metabolically labeled 120 hpf zebrafish with chondroitinase ABC. Lysates were generated from 120 hpf zebrafish embryos metabolically labeled with $Ac_4GalNAz$ or $Ac_4GalNAc$ as described above. Twenty micrograms of total protein was reacted at 37 °C for 12 h with ~100 mU of either chondroitinase ABC from *Proteus vulgaris* or chondroitinase ABC that had been heat-killed for 1 h at 100 °C. After the reaction had been completed, an equal volume of a 500 μM solution of phosphine-Flag in PBS was added to yield a final concentration of 250 μM. This reaction proceeded at rt for 12 h. The completed reaction was analyzed by standard immunoblotting techniques, either probing the blot with a 1:1000 dilution of HRP-conjugated anti-Flag M2 or, to control for enzyme activity, with a 1:1000 dilution of anti-chondroitin sulfate ΔDi-Os, an antibody that

recognizes only the remnants of chondroitin sulfate left behind after chondroitinase ABC treatment(*24*), followed by an HRP-conjugated anti-mouse IgG. To control for equal protein loading, each nitrocellulose blot was stripped of the anti-Flag or anti-chondroitin sulfate antibodies with the Restore Western blot stripping buffer using the manufacturer's suggested protocol and re-probed with a 1:1000 dilution of HRP-conjugated anti-β-tubulin.

Reaction of metabolically labeled zebrafish embryos with DIFO-647. Zebrafish embryos were metabolically labeled with Ac_4GalNAz or Ac_4GalNAc using the general procedure. Embryos were then removed from Ac_4GalNAz or Ac_4GalNAc into 25 mL of embryo medium in a 10-cm petri dish. After gentle agitation of the plate for ~10 s, the fish were transferred into an identical plate containing fresh media. This was repeated once more for a total of three washes, and the fish were transferred into a 96-well plate in 50 µL of embryo medium. Fifty microliters of a 200 µM DIFO-647 solution in embryo medium was added, for a final concentration of 100 µM, and the plate was gently agitated prior to incubation for 1 h in the dark at 28 °C. Upon completion of the reaction, 200 µL of fresh embryo medium was added to the well, and the embryos were transferred to 25 mL of embryo medium in a 10-cm petri dish. After gentle agitation of the plate for ~10 s, the fish were transferred into an identical plate containing fresh media. This washing protocol was repeated four more times for a total of six washes. The embryos were then transferred into a solution of 2.6 µM tricaine (ethyl 3-aminobenzoate methanesulfonate) in embryo medium prior to mounting in 0.6% low melting point agarose and optical imaging.

Timecourse of reaction of DIFO-647 with 72 hpf embryos metabolically labeled with Ac_4GalNAz or Ac_4GalNAc. Zebrafish embryos were metabolically labeled with Ac_4GalNAz or Ac_4GalNAc using the general procedure. Embryos were then removed from Ac_4GalNAz or Ac_4GalNAc into 25 mL of embryo medium in a 10-cm petri dish. After gentle agitation of the plate for ~10 s, the fish were transferred into an identical plate containing fresh media. This was repeated once more for a total of three washes, and the fish were transferred into a 96-well plate in 50 μL of embryo medium. Fifty microliters of a 200 μM DIFO-647 solution in embryo medium was added, for a final concentration of 100 μM, and the plate was gently agitated prior to incubation in the dark for 1 min at rt or 15, 30, or 60 min at 28 °C. The labeled embryos were then diluted in fresh embryo medium and quickly transferred to 25 mL of embryo medium in a 10-cm petri dish. After gentle agitation of the plate for ~10 s, the fish were transferred into an identical plate containing fresh media. This was repeated four more times for a total of six washes. The embryos were then transferred to 2.6 μM tricaine and mounted for imaging in 0.6% low melting point agarose.

Developmental timecourse of metabolic labeling with Ac_4GalNAz or Ac_4GalNAc and reaction with DIFO-647. Zebrafish embryos were metabolically labeled with Ac_4GalNAz or Ac_4GalNAc using the general procedure. Embryos were then removed from Ac_4GalNAz or Ac_4GalNAc into 25 mL of embryo medium in a 10-cm petri dish at 24, 36, 48, 60, 72, 96, or 120 hpf. After gentle agitation of the plate for ~10 s, the fish were transferred into an identical plate containing fresh media. This was repeated once more for a total of three washes, and the fish were transferred into a 96-well plate in 50 μL of embryo medium. Fifty microliters of a 200 μM DIFO-647 solution

in embryo medium was added, for a final concentration of 100 μM, and the plate was gently agitated prior to incubation in the dark for 1 h at 28 °C. The labeled embryos were then diluted in fresh embryo medium and quickly transferred to 25 mL of embryo medium in a 10-cm petri dish. After gentle agitation of the plate for ~10 s, the fish were transferred into an identical plate containing fresh media. This was repeated four more times for a total of six washes. The embryos were then transferred to 2.6 μM tricaine and mounted for imaging in 0.6% low melting point agarose.

Reaction of metabolically labeled zebrafish embryos with multiple DIFO-fluorophores. Zebrafish embryos were metabolically labeled with $Ac_4GalNAz$ or $Ac_4GalNAc$ using the general procedure and, at 60 hpf, reacted with DIFO-647 (100 μM, 1 h) as described above. Immediately after the completion of the washes performed to remove excess DIFO-647, the embryos were treated with 50 mM TCEP (pH 7.4) in embryo medium for 10 min. Upon completion of the 10-min TCEP reaction, the fish were immediately transferred to 25 mL of embryo medium in a 10-cm petri dish. After gentle agitation of the plate for ~10 s, the fish were transferred into an identical plate containing fresh media. This was repeated four more times for a total of six washes. For multicolor labeling, we used the following three protocols:

1. Two-color, 2-h time-resolution: The embryos were immediately labeled with DIFO-488 (100 μM, 1 h) using the protocol described for single-color labeling with DIFO-647 and imaged by laser scanning confocal microscopy.
2. Two-color, 3-h time-resolution: The embryos were transferred to a 10-cm petri dish containing embryo medium supplemented with 131 μM PTU and the appropriate sugar (5 mM $Ac_4GalNAz$ or 5 mM $Ac_4GalNAc$) and incubated at

28 °C for 1 h. The embryos were then reacted with DIFO-488 (100 µM, 1 h) using the protocol described for single-color labeling with DIFO-647 and imaged by laser scanning confocal microscopy.

3. Three-color, 12-h time resolution: The embryos were dually reacted with DIFO-647, followed by DIFO-488, as described above in part 2. Immediately after the completion of the washes performed to remove excess DIFO-488, the embryos were treated with 50 mM TCEP (pH 7.4) in embryo medium for 10 min. Upon completion of the 10-min TCEP reaction, the fish were immediately transferred to 25 mL of embryo medium in a 10-cm petri dish. After gentle agitation of the plate for ~10 s, the fish were transferred into an identical plate containing fresh media. This was repeated four more times for a total of six washes. The embryos were transferred to a 3-cm petri dish containing embryo medium supplemented with 131 µM PTU and the appropriate sugar (5 mM $Ac_4GalNAz$ or 5 mM $Ac_4GalNAc$) and incubated at 28 °C for 9 h. The embryos were then reacted with DIFO-555 (100 µM, 1 h) using the protocol described for single-color labeling with DIFO-647 and imaged by laser scanning confocal microscopy.

Supporting Online Materials Text

Evaluation of toxicity of in vivo imaging reagents. In all of the in vivo labeling experiments described above, embryos were allowed to live until 120 hpf. At no point during these experiments did we observe any toxicity or morphological abnormalities, including treatment with some or all of the following reagents: (a) Ac_4GalNAc or Ac_4GalNAz (5 mM, 3–120 hpf), (b) DIFO-488, DIFO-555, and DIFO-647 (100 µM, 1 h), TCEP (50 mM, 10 min). We further performed two experiments to assess the long-term viability of embryos treated with these reagents. Here, we incubated embryos with (a) Ac_4GalNAz (5 mM, 3–120 hpf) or (b) Ac_4GalNAz (5 mM, 3–72 hpf), DIFO-488 (100 µM, 72–73 hpf), and embryo medium (73–120 hpf). At 120 hpf, all of the fish had developed swim bladders. We allowed the larvae to further mature and observed their ability to feed on brine shrimp thereafter. We did not observe any developmental abnormalities during these experiments.

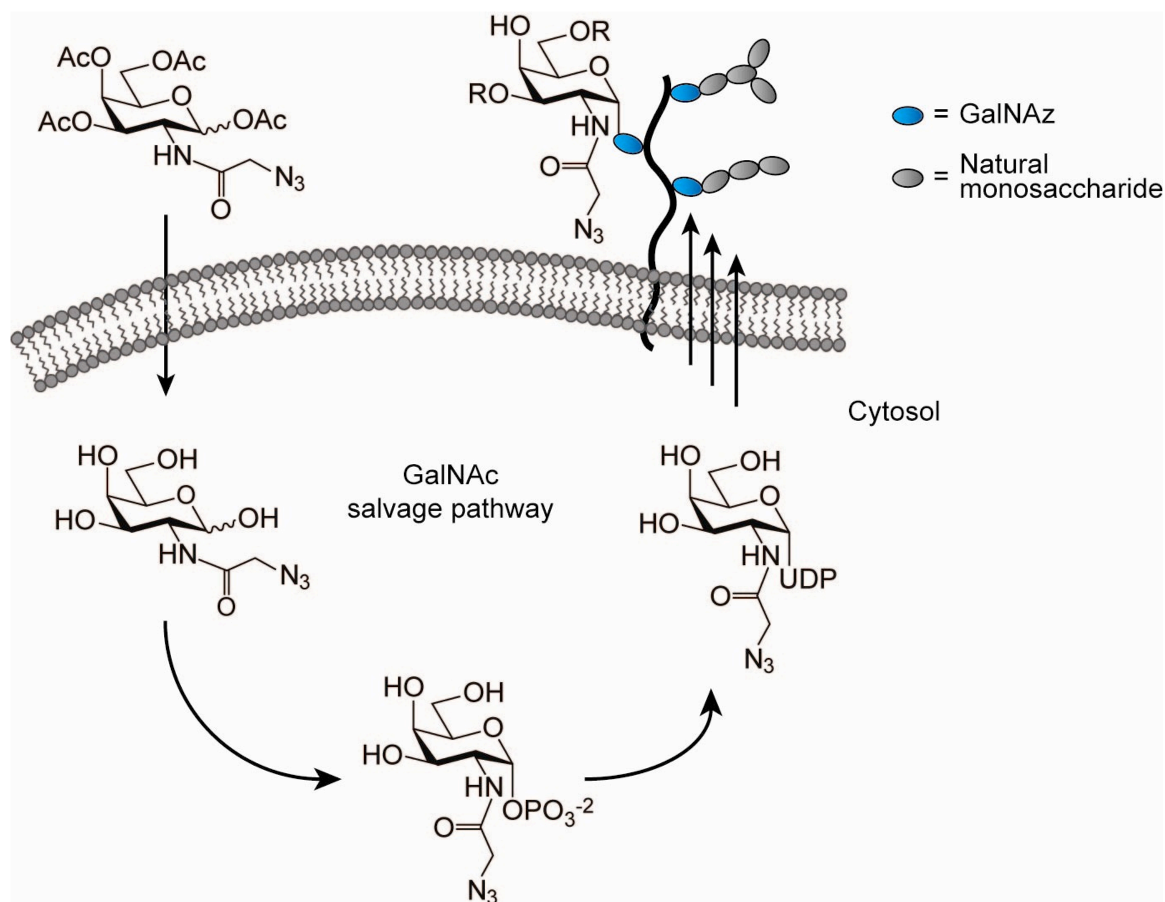

Figure S1. Strategy for the metabolic labeling of cell-surface glycans with $Ac_4GalNAz$. $Ac_4GalNAz$ passively diffuses across the plasma membrane, is deacetylated by cytosolic esterases, and enters the GalNAc salvage pathway, where it is metabolically converted to the corresponding activated nucleotidyl sugar (UDP-GalNAz). UDP-GalNAz is in turn transported into the Golgi apparatus and appended to glycoconjugates by a family of glycosyltransferases. The resulting azido glycoconjugates are then trafficked to the cell surface. Ac, acetyl; R, additional sugar residues.

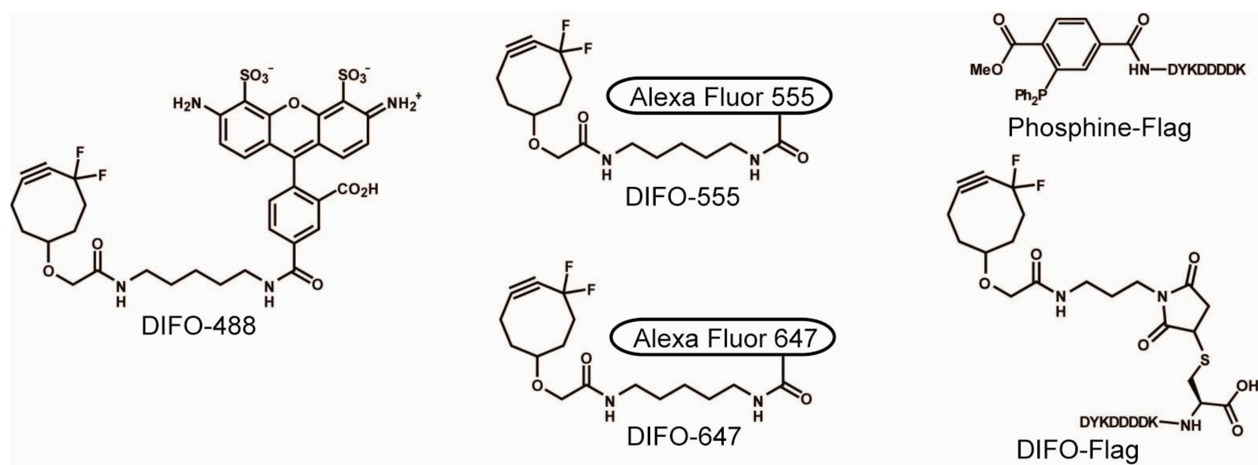

Figure S2. Panel of DIFO and phosphine probes used in this study for visualization and capture of azide-labeled glycoconjugates.

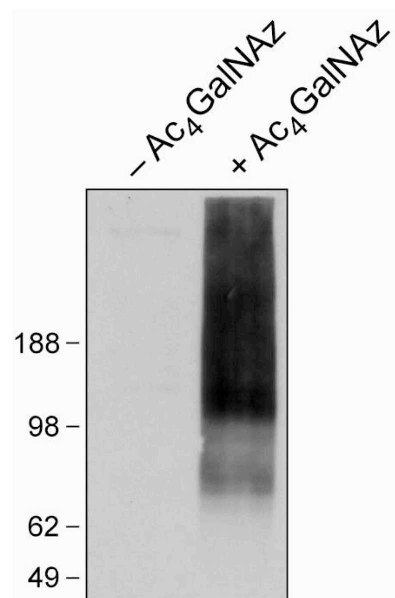

Figure S3. Immunoblot analysis of species from ZF4 cells metabolically labeled with $Ac_4GalNAz$. ZF4 cells were incubated in the absence (– $Ac_4GalNAz$) or presence (+ $Ac_4GalNAz$) of $Ac_4GalNAz$ (100 μM, 3 d), lysed, and reacted with DIFO-Flag (10 μM, 12 h). An immunoblot was performed using standard procedures, detecting with HRP-conjugated anti-Flag. Equal protein loading was confirmed using Ponceau S stain.

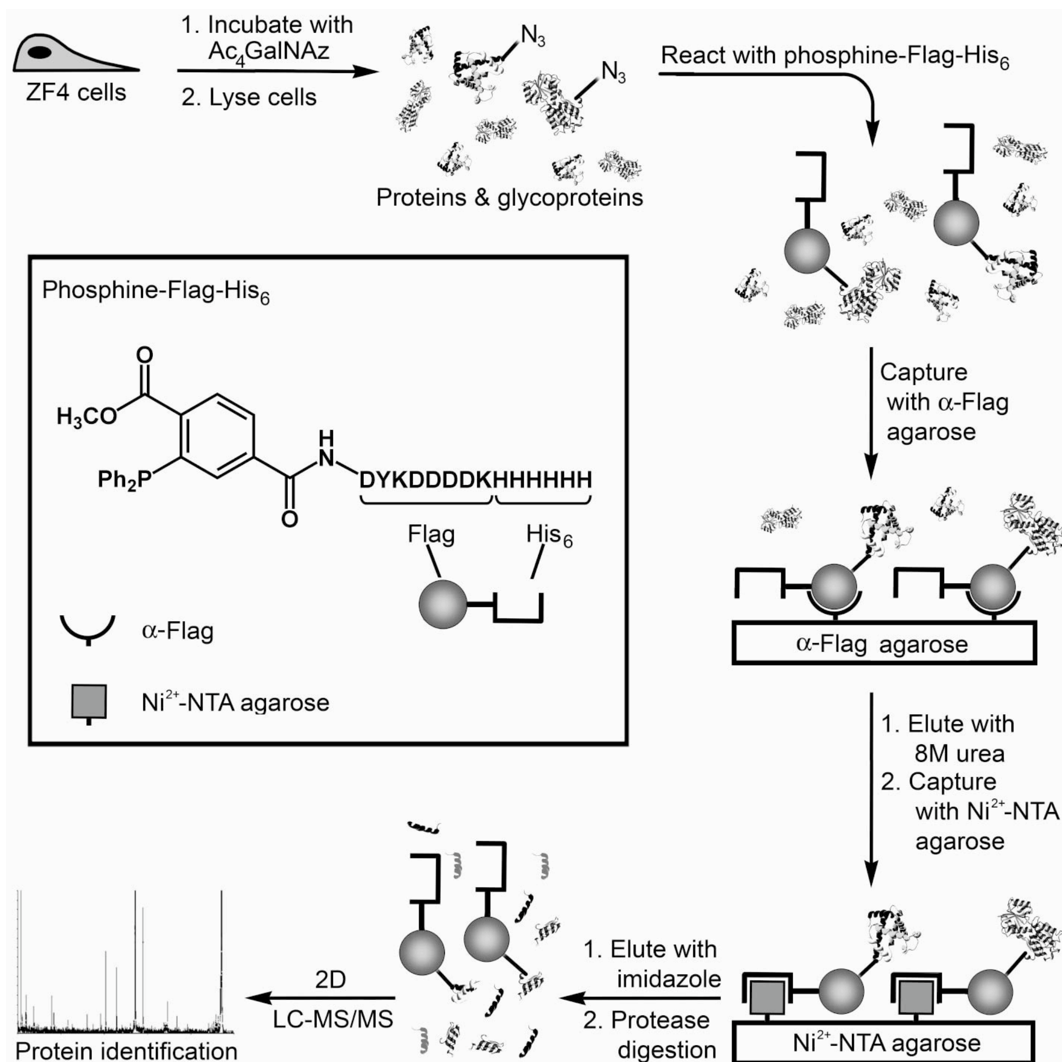

Figure S4. Strategy for glycoproteomic analysis of ZF4 cells metabolically labeled with $Ac_4GalNAz$. ZF4 cells are incubated with $Ac_4GalNAz$ (100 µM, 3 d), lysed, and reacted with phosphine-Flag-His_6 (500 µM, 24 h). Excess phosphine-Flag-His_6 is removed by two size-exclusion columns, and the glycoproteome eluate is purified sequentially by treatment with anti-Flag agarose and then Ni-NTA agarose. The eluate is then digested with trypsin, and the resultant peptide mixture is reduced with dithiothreitol, alkylated with iodoacetamide, desalted using a C18 ZipTip, and analyzed by MUDPIT.

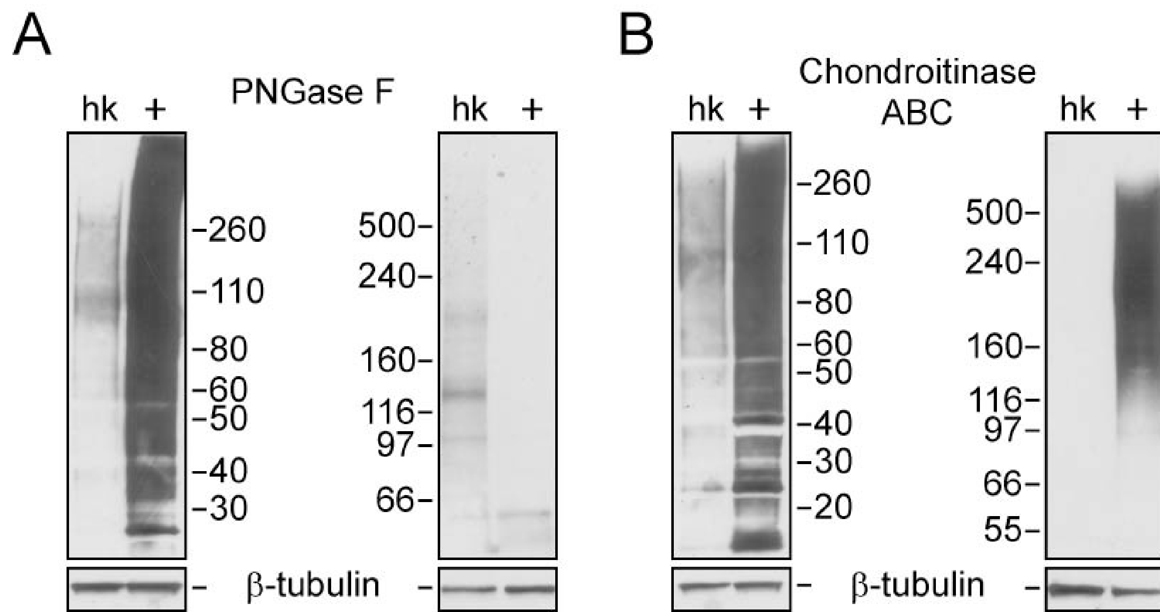

Figure S5. Biochemical analysis of glycoproteins from zebrafish metabolically labeled with $Ac_4GalNAz$. (**A**) Lysates from 120 hpf zebrafish larvae incubated with $Ac_4GalNAz$ were treated with heat killed (hk) or active (+) PNGase F, reacted with phosphine-Flag (250 μM, 12 h), and probed with either HRP-conjugated anti-Flag (top left panel), HRP-conjugated concanavalin A (top right panel), or anti-β-tubulin (bottom panels). (**B**) Lysates from 120 hpf zebrafish larvae incubated with $Ac_4GalNAz$ were treated with heat killed (hk) or active (+) chondroitinase ABC, reacted with phosphine-Flag (250 μM, 12 h), and probed with either HRP-conjugated anti-Flag (top left panel), anti-chondroitin sulfate ΔDi-Os (top right panel), or anti-β-tubulin (bottom panels). The numbers indicated denote apparent molecular weight (kDa).

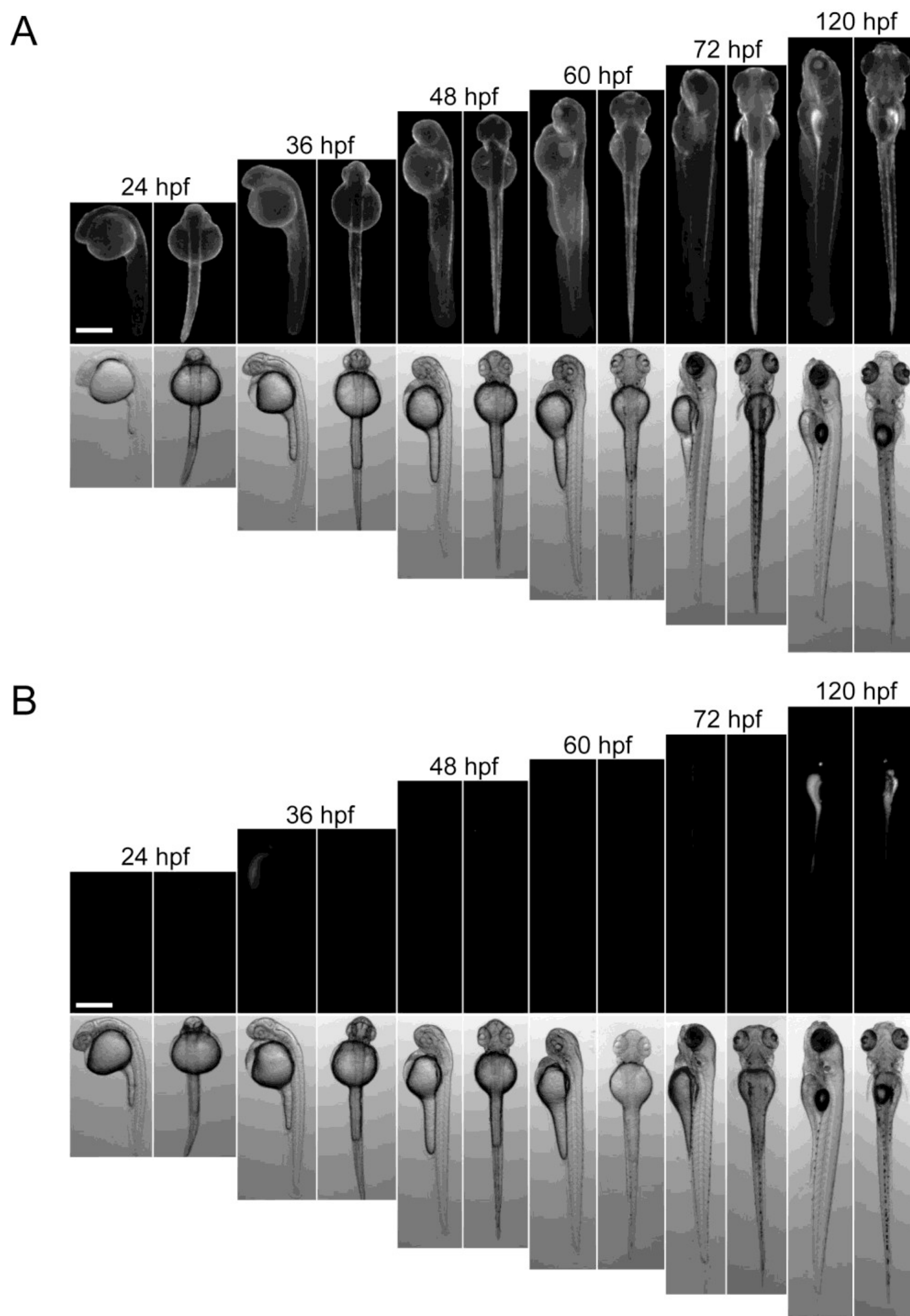

Figure S6. Timecourse of metabolic labeling of zebrafish embryos with $Ac_4GalNAz$ and $Ac_4GalNAc$ from 24–120 hpf and single-color imaging after reaction with DIFO-647 to demonstrate both the lack of developmental phenotype caused by the azido sugar and the feasibility of DIFO-647 detection of cell-surface azido glycans in vivo during embryonic and early larval development. Zebrafish embryos were treated with

$Ac_4GalNAz$ (**A**) or $Ac_4GalNAc$ (**B**) from 3 hpf until the time indicated using the general labeling procedure. The embryos were then washed and reacted with DIFO-647 (100 μM, 1 h). Following the reaction, the zebrafish were treated with tricaine, mounted for imaging in 0.6% low melting point agarose, and imaged by epifluorescence microscopy. The resulting images were deconvolved using the nearest neighbor deconvolution algorithm in Slidebook 4.2. Scale bar, 500 μm.

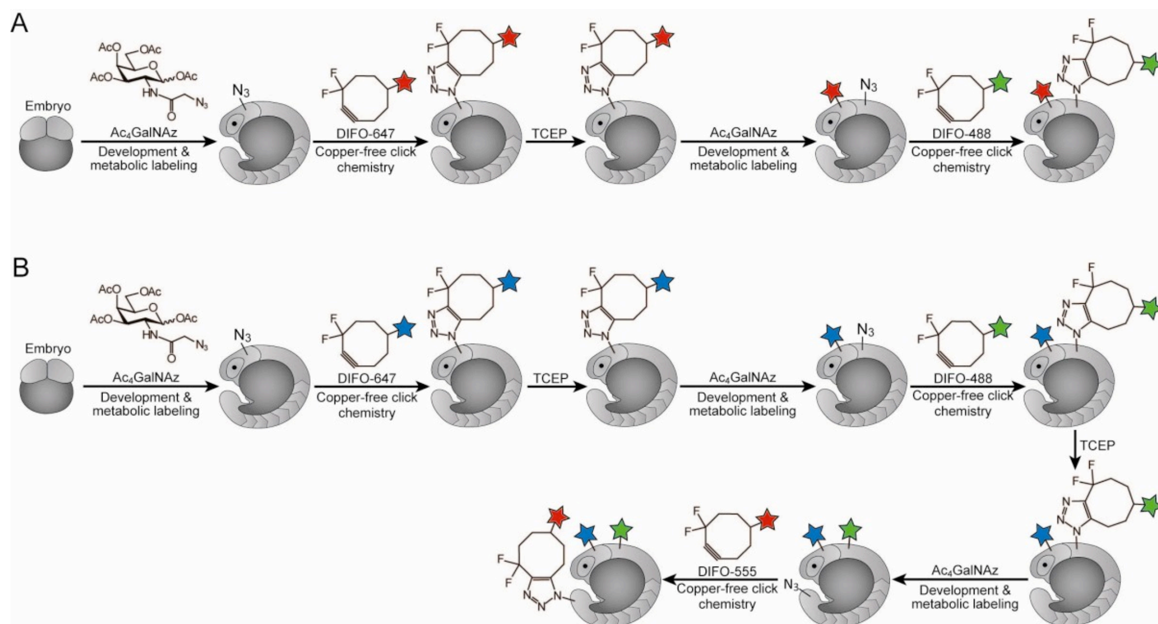

Figure S7. Strategy for two- and three-color labeling of zebrafish with DIFO-fluorophore conjugates. (**A**) Schematic for two-color labeling of zebrafish embryos with DIFO-647 and DIFO-488. Embryos are metabolically labeled with $Ac_4GalNAz$ until 60 hpf, washed, and then reacted with DIFO-647 (100 µM, 1 h, red). The embryos are washed, reacted with TCEP (50 mM, 10 min), washed again, and returned to embryo medium supplemented with $Ac_4GalNAz$ for an additional 0–1 h to allow for metabolic incorporation of azides into new cell-surface glycans. The embryos are washed and then reacted with DIFO-488 (100 µM, 1 h, green). (**B**) Schematic for three-color labeling of zebrafish embryos with DIFO-647, DIFO-488, and DIFO-555. Embryos are metabolically labeled with $Ac_4GalNAz$ until 60 hpf, washed, and then reacted with DIFO-647 (100 µM, 1 h, blue). The embryos are washed, reacted with TCEP (50 mM, 10 min), washed again, and returned to embryo medium supplemented with $Ac_4GalNAz$ for an additional 1 h to allow for metabolic incorporation of azides into new cell-surface glycans. The embryos are washed and

then reacted with DIFO-488 (100 μM, 1 h, green). The embryos are washed, reacted with TCEP (50 mM, 10 min), washed again, and returned to embryo medium supplemented with $Ac_4GalNAz$ for an additional 9 h to allow for metabolic incorporation of azides into new cell-surface glycans. The embryos are washed and then reacted with DIFO-555 (100 μM, 1 h, red).

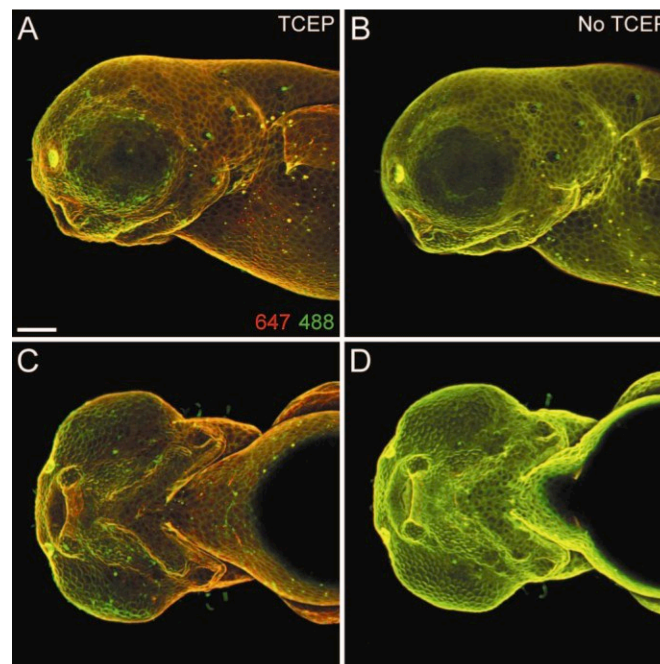

Figure S8. TCEP incubation is required for optimal temporal resolution of glycan biosynthesis. Zebrafish embryos (72 hpf) were metabolically labeled with $Ac_4GalNAz$ using the general procedure, reacted with DIFO-647 (100 μM, 1 h), washed, treated with 50 mM TCEP (**A**, **C**) or embryo medium (**B**, **D**) for 10 min, incubated with $Ac_4GalNAz$ for 1 h, washed, and then reacted with DIFO-488 (100 μM, 1 h). The embryos were then washed, transferred to tricaine, mounted for imaging in 0.6% low melting point agarose, and imaged by laser scanning confocal microscopy. Shown are lateral (**A**, **B**) or ventral (**C**, **D**) views of z-projection fluorescence images. Red, DIFO-647; green, DIFO-488. Scale bar: 100 μm.

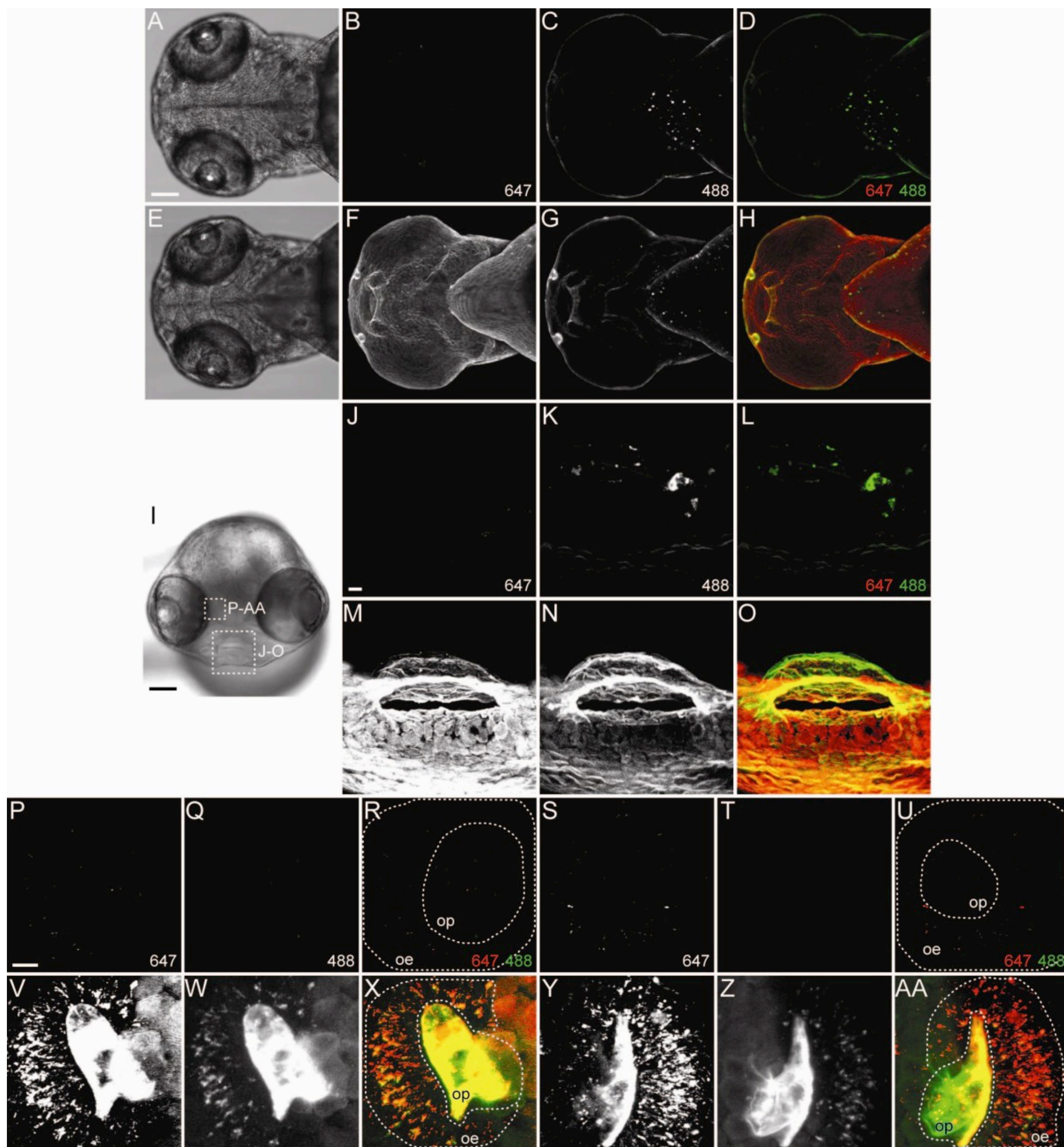

Figure S9. Two-color labeling of O-linked glycans in live zebrafish with 2-h time resolution. Zebrafish embryos (60 hpf) were metabolically labeled with $Ac_4GalNAc$ (**A**–**D**, **I**–**L**, **P**–**U**) or $Ac_4GalNAz$ (**E**–**H**, **M**–**O**, **V**–**AA**) using the general procedure, reacted with DIFO-647 (100 μM, 1 h), washed, treated with TCEP (50 mM, 10 min), washed, and then immediately reacted with DIFO-488 (100 μM, 1 h). The embryos

were then washed, transferred to tricaine, mounted for imaging in 0.6% low melting point agarose, and imaged by laser scanning confocal microscopy. (**A**–**H**) Single z-plane brightfield (**A**, **E**) or maximum intensity z-projection fluorescence images (**B**–**D**, **F**–**H**) of a ventral view of the head and jaw regions. **i**, Single z-plane brightfield image of a frontal view of the head. (**J**–**O**) Maximum intensity z-projection fluorescence images of the mouth region highlighted in (**I**). (**P**–**AA**) Maximum intensity z-projection fluorescence images of the olfactory pit (op) and olfactory bulb (ob) region highlighted in (**i**). Red: DIFO-647 (60–61 hpf); green: DIFO-488 (61–62 hpf). Scale bars: **A**–**I**, 100 µm; **J**–**AA**, 10 µm.

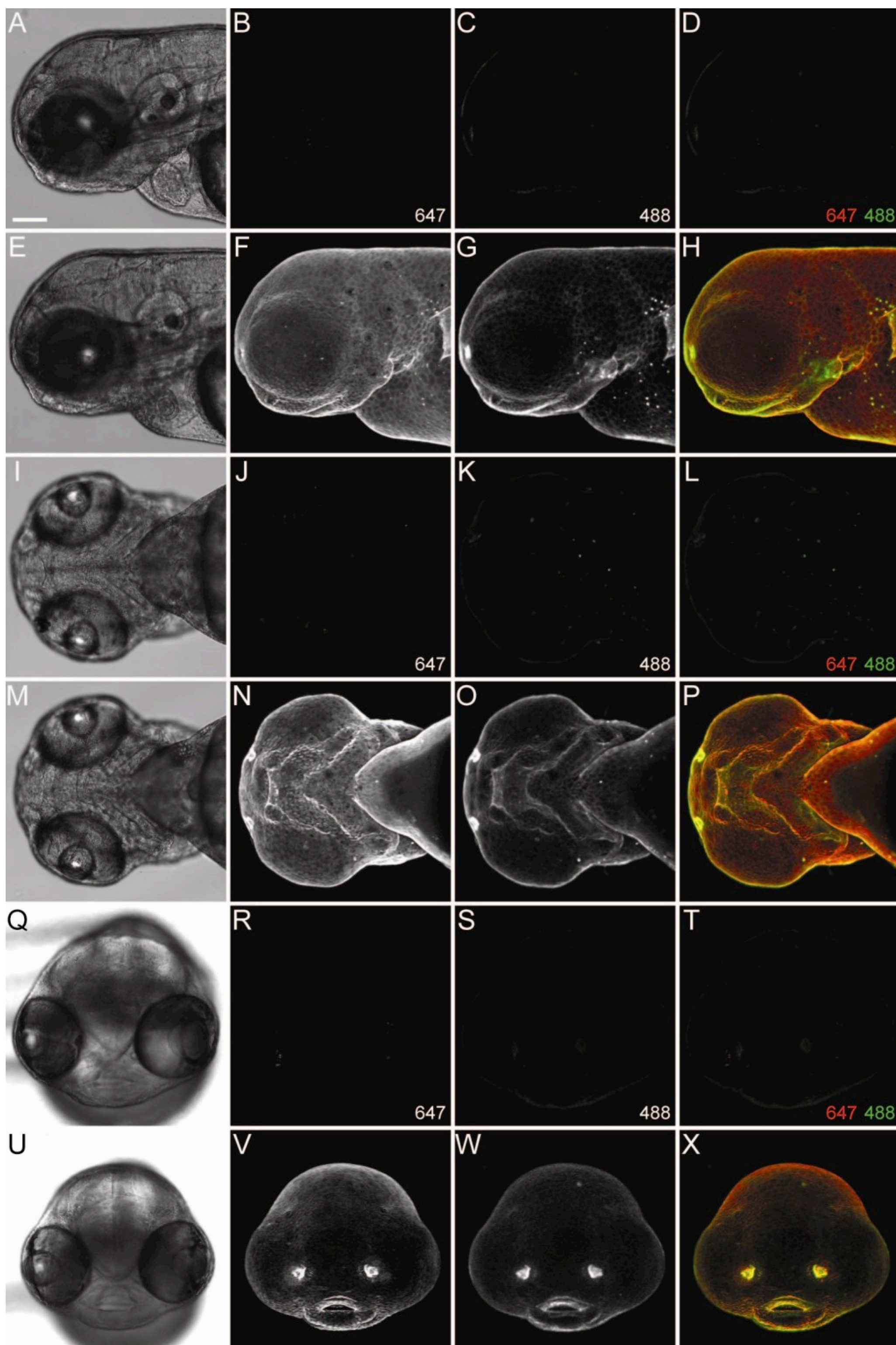

Figure S10. Two-color labeling of O-linked glycans in live zebrafish with 3-h time resolution. Zebrafish embryos (60 hpf) were metabolically labeled with Ac_4GalNAc

(**A**–**D**, **I**–**L**, **Q**–**T**) or Ac_4GalNAz (**E**–**H**, **M**–**P**, **U**–**X**) using the general procedure, reacted with DIFO-647 (100 μM, 1 h), washed, treated with TCEP (50 mM, 10 min), incubated with the appropriate sugar for 1 h, washed, and then reacted with DIFO-488 (100 μM, 1 h). The embryos were then washed, transferred to tricaine, mounted for imaging in 0.6% low melting point agarose, and imaged by laser scanning confocal microscopy. (**A**–**H**) Single z-plane brightfield (**A**, **E**) or maximum intensity z-projection fluorescence (**B**–**D**, **F**–**H**) images of a lateral view of the head and jaw regions. (**I**–**P**) Single z-plane brightfield (**I**, **M**) or maximum intensity z-projection fluorescence (**J**–**L**, **N**–**P**) images of a ventral view of the head and jaw regions. (**Q**–**X**) Single z-plane brightfield (**Q**, **U**) or maximum intensity z-projection fluorescence (**R**–**T**, **V**–**X**) images of a frontal view of the head. Red: DIFO-647 (60–61 hpf); green: DIFO-488 (62–63 hpf). Scale bar: 100 μm.

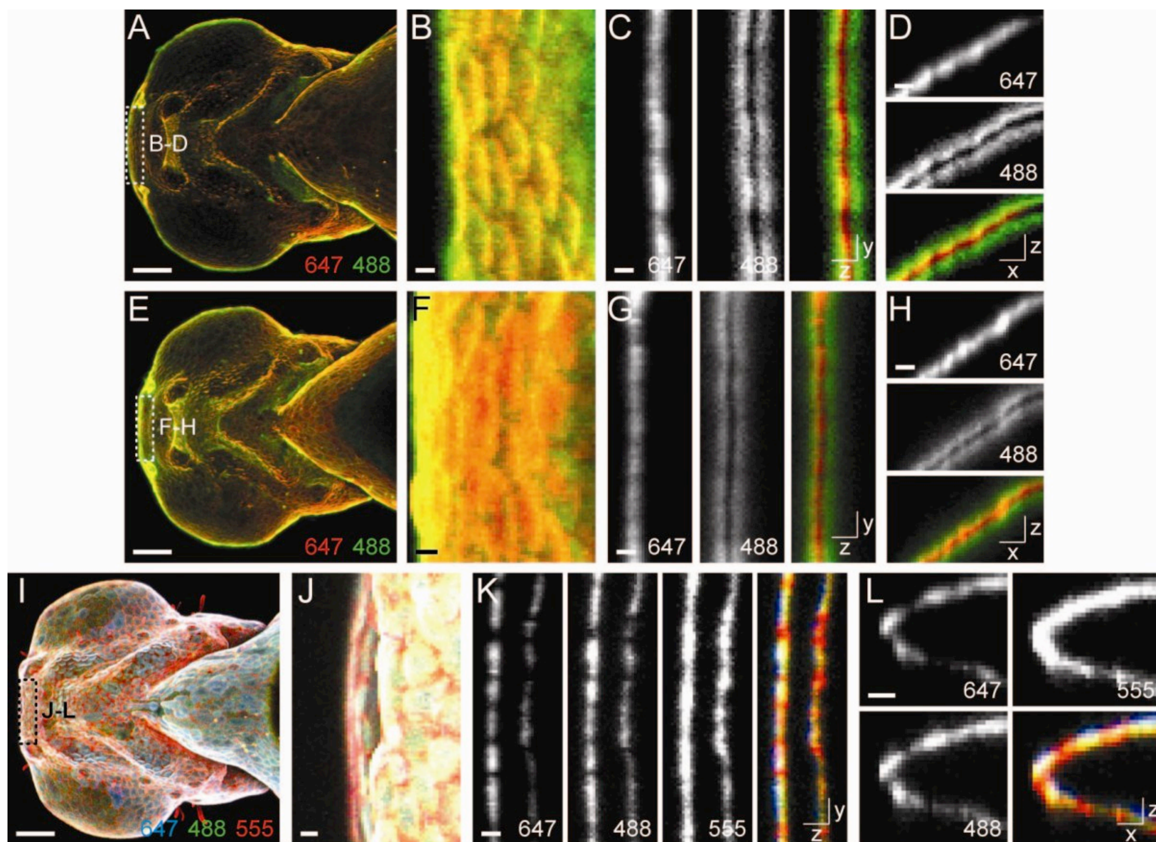

Figure S11. Dynamic imaging of layered organization of glycans in zebrafish between 60–72 hpf. Zebrafish embryos (60 hpf) were metabolically labeled with $Ac_4GalNAz$ using the general procedure, reacted with either DIFO-647 and then DIFO-488 in the 2-h (**A**–**D**) or 3-h (**E**–**H**) time resolution protocols, or a sequence of DIFO-647, DIFO-488, and DIFO-555 in the 12-h time resolution protocol (**I**–**L**). In all cases, the embryos were then washed, transferred to tricaine, mounted for imaging in 0.6% low melting point agarose, and imaged by laser scanning confocal microscopy. (**A**, **E**, **I**) Maximum intensity z-projection fluorescence images of a ventral view. (**B**, **F**, **J**) Maximum intensity z-projection fluorescence images of the regions highlighted in **A**, **E**, and **I**, respectively. (**C**, **G**, **K**) Single x-plane fluorescence images of the regions highlighted in **A**, **E**, and **I**, respectively. (**D**, **H**, **L**)

Single y-plane fluorescence images of the regions highlighted in **A**, **E**, and **I**, respectively. (**A**–**D**) Red: DIFO-647 (60–61 hpf); green: DIFO-488 (61–62 hpf). (**E**–**H**) Red: DIFO-647 (60–61 hpf); green: DIFO-488 (62–63 hpf). (**I**–**L**) Blue: DIFO-647 (60–61 hpf); green: DIFO-488 (62–63 hpf); red: DIFO-555 (71–72 hpf). Scale bars: **A**, **E**, **I**, 100 μm; **B**–**D**, **F**–**H**, **J**–**L**, 5 μm.

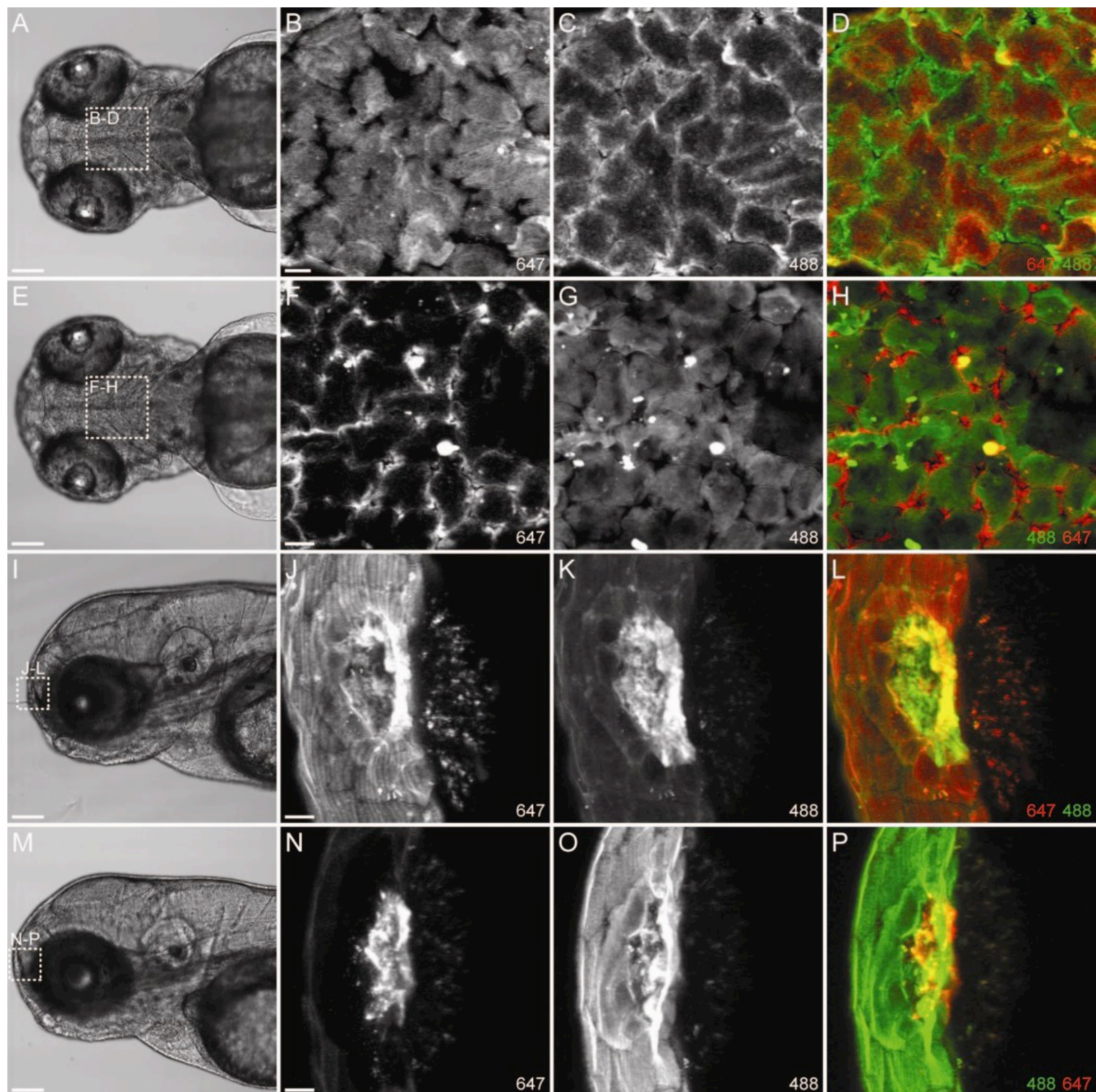

Figure S12. Permutation of the order of addition of DIFO-fluorophores does not change spatiotemporal labeling patterns. Zebrafish embryos (60 hpf) were metabolically labeled with $Ac_4GalNAz$ using the general procedure, and reacted with either DIFO-647 (**A–D**, **I–L**) or DIFO-488 (**E–H**, **M–P**) for 1 h at 100 μM. The embryos were washed, treated with TCEP (50 mM, 10 min), and incubated in $Ac_4GalNAz$ for 1 h, washed, and labeled with the complementary DIFO-fluorophore (100 μM, 1 h). The embryos were then washed, transferred to tricaine, mounted for imaging in 0.6% low melting point agarose, and imaged by laser scanning confocal

microscopy. (**A**, **E**) Brightfield image of a ventral view. (**B–D**, **F–H)** Monochrome and merge z-projection fluorescence images of the pharyngeal epidermal regions highlighted in (**A**) and (**E**) respectively. (**I**, **M**) Brightfield image of a lateral view. (**J–L**, **N–P)** Monochrome and merge z-projection fluorescence images of the olfactory regions highlighted in (**I**) and (**M**) respectively. Red: DIFO-647; green: DIFO-488. Scale bars: **A**, **E**, **I**, **M**, 100 µm; **B**–**D**, **F**–**H**, **J**–**L**, **N–P**, 10 µm.

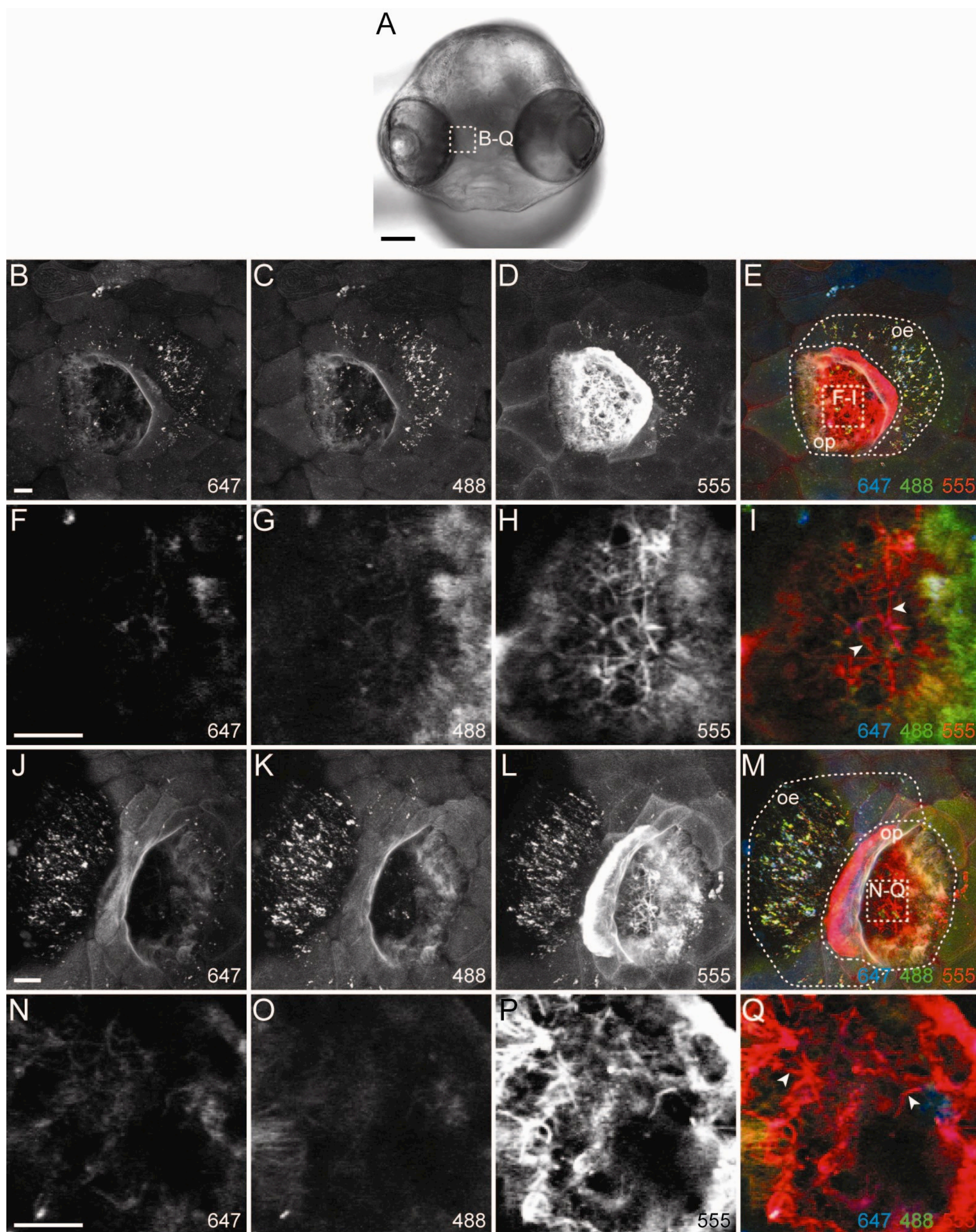

Figure S13. Three-color labeling of O-linked glycans in the olfactory organ of live zebrafish over 12 h. Zebrafish embryos (60 hpf) were metabolically labeled with $Ac_4GalNAz$ using the general procedure, reacted with DIFO-647 (100 μM, 1 h), washed, treated with TCEP (50 mM, 10 min), incubated with the appropriate sugar for

1 h, washed, and then reacted with DIFO-488 (100 µM, 1 h). The fish were then reacted with TCEP a second time (50 mM, 10 min) and returned to the appropriate sugar for 9 h, washed, and reacted with DIFO-555 (100 µM, 1 h). The embryos were then washed, transferred to tricaine, mounted for imaging in 0.6% low melting point agarose, and imaged by laser scanning confocal microscopy. (**A**) Single z-plane brightfield image of a frontal view. (**B**–**E**, **J**–**M**) Monochrome (**B**–**D**, **J**–**L**) and merged (**E**, **M**) maximum intensity z-projection fluorescence images of the region highlighted in (**A**) in two separate animals (**B**–**E** and **J**–**M**, respectively). (**F**–**I** and **N**–**Q**) Monochrome (**F**–**H**, **N**–**P**) and merged (**I**, **Q**) maximum intensity z-projection fluorescence images of the regions highlighted in (**E**) and (**M**) respectively, in two separate animals (**F**–**I** and **N**–**Q**). Solid arrowheads indicate representative examples of labeling of cilia. op, olfactory pit; ob, olfactory bulb. Blue: DIFO-647 (60–61 hpf); green: DIFO-488 (62–63 hpf); red: DIFO-555 (71-72 hpf). Scale bars: **A**, 100 µm; **B**–**Q**, 10 µm.

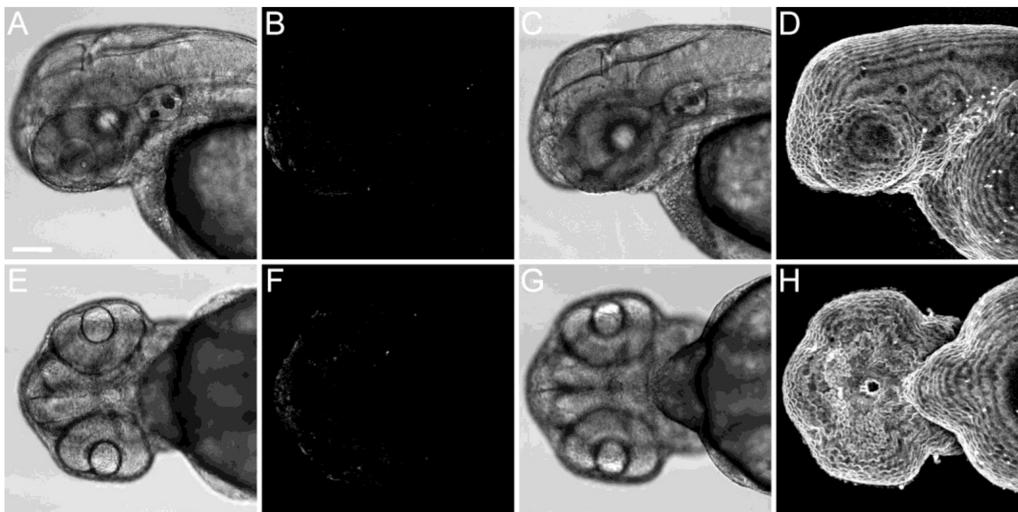

Figure S14. In vivo imaging of sialic acids in zebrafish. Embryos were treated with no sugar (**A**, **B**, **E**, and **F**) or $Ac_4ManNAz$ (**C**, **D**, **G**, and **H**) from 3–48 hpf, washed, and reacted with DIFO-488 (100 μM, 1 h). Following the reaction, the zebrafish were washed, treated with tricaine, mounted for imaging in 0.6% low melting point agarose, and imaged by laser scanning confocal microscopy. Shown are lateral (**A–D**) and ventral (**E–H**) views of brightfield (**A**, **C**, **E**, and **G**) or z-projection fluorescence (**B**, **D**, **F**, and **H**) images. Scale bar, 100 μm.

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Movie S1. Z-series of two-color labeling of the mouth region (frontal view). Zebrafish embryos were metabolically labeled with Ac_4GalNAz using the general procedure and reacted, starting at 60 hpf, with DIFO-647 and, subsequently, DIFO-488 using the 2-h time resolution protocol described in Fig. 3A–D. Panels (from left): DIFO-647 fluorescence, DIFO-488 fluorescence, brightfield, fluorescence merge (red: DIFO-647; green: DIFO-488). Scale bar: 10 μm.

Movie S2. Z-series of two-color labeling of the head and jaw region (lateral view). Zebrafish embryos were metabolically labeled with Ac_4GalNAz using the general procedure and reacted, starting at 60 hpf, with DIFO-647 and, subsequently, DIFO-488 using the 2-h time resolution protocol described in Fig. 3A–D. Panels (from left): DIFO-647 fluorescence, DIFO-488 fluorescence, brightfield, fluorescence merge (red: DIFO-647; green: DIFO-488). Scale bar: 10 μm.

Movie S3. Z-series of two-color labeling of the head and jaw region (ventral view). Zebrafish embryos were metabolically labeled with Ac_4GalNAz using the general procedure and reacted, starting at 60 hpf, with DIFO-647 and, subsequently, DIFO-488 using the 3-h time resolution protocol described in Fig. 3E–H. Panels (from left): DIFO-647 fluorescence, DIFO-488 fluorescence, brightfield, fluorescence merge (red: DIFO-647; green: DIFO-488). Scale bar: 100 μm.

Movie S4. Zoom-in of z-series of two-color labeling of the head and jaw region (ventral view). Zebrafish embryos were metabolically labeled with Ac_4GalNAz using the general procedure and reacted, starting at 60 hpf, with DIFO-647 and, subsequently, DIFO-488 using the 3-h time resolution protocol described in Fig. 3E–H. Panels (from left): DIFO-647 fluorescence, DIFO-488 fluorescence, brightfield, fluorescence merge (red: DIFO-647; green: DIFO-488). Scale bar: 25 µm.

Movie S5. Z-series of two-color labeling of the olfactory organ (frontal view). Zebrafish embryos were metabolically labeled with Ac_4GalNAz using the general procedure and reacted, starting at 60 hpf, with DIFO-647 and, subsequently, DIFO-488 using the 3-h time resolution protocol described in Fig. 3E–H. Panels (from left): DIFO-647 fluorescence, DIFO-488 fluorescence, brightfield, fluorescence merge (red: DIFO-647; green: DIFO-488). Scale bar: 10 µm.

Movie S6. Z-series of three-color labeling of the head and jaw region (lateral view). Zebrafish embryos were metabolically labeled with Ac_4GalNAz using the general procedure and reacted sequentially, starting at 60 hpf, with DIFO-647, DIFO-488, and DIFO-555 using the 12-h time resolution protocol described in Fig. 3I–N. Panels (from left): DIFO-647 fluorescence, DIFO-488 fluorescence, DIFO-555 fluorescence brightfield, fluorescence merge (blue: DIFO-647; green: DIFO-488; red: DIFO-555). Scale bar: 100 µm.

Movie S7. Z-series of three-color labeling of the head and jaw region (ventral view). Zebrafish embryos were metabolically labeled with Ac_4GalNAz using the general procedure and reacted sequentially, starting at 60 hpf, with DIFO-647, DIFO-488, and DIFO-555 using the 12-h time resolution protocol described in Fig. 3I–N. Panels

(from left): DIFO-647 fluorescence, DIFO-488 fluorescence, DIFO-555 fluorescence brightfield, fluorescence merge (blue: DIFO-647; green: DIFO-488; red: DIFO-555). Scale bar: 100 μm.

Movie S8. Z-series of three-color labeling of a hair cell (frontal view). Zebrafish embryos were metabolically labeled with $Ac_4GalNAz$ using the general procedure and reacted sequentially, starting at 60 hpf, with DIFO-647, DIFO-488, and DIFO-555 using the 12-h time resolution protocol described in Fig. 3I–N. Panels (from left): DIFO-647 fluorescence, DIFO-488 fluorescence, DIFO-555 fluorescence brightfield, fluorescence merge (blue: DIFO-647; green: DIFO-488; red: DIFO-555). Scale bar: 2.5 μm.

Movie S9. Z-series of three-color labeling of the olfactory organ (frontal view). Zebrafish embryos were metabolically labeled with $Ac_4GalNAz$ using the general procedure and reacted sequentially, starting at 60 hpf, with DIFO-647, DIFO-488, and DIFO-555 using the 12-h time resolution protocol described in Fig. 3I–N. Panels (from left): DIFO-647 fluorescence, DIFO-488 fluorescence, DIFO-555 fluorescence brightfield, fluorescence merge (blue: DIFO-647; green: DIFO-488; red: DIFO-555). Scale bar: 10 μm.