Supporting Information

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SI Materials and Methods

Generation of *Cosmc*$^{-/y}$ and *Cosmc*$^{flox/y}$ Mouse ES Cells. We engineered a targeting vector with three Lox^{P} sites in the core 1 β3galactosyltransferase specific molecular chaperone (*Cosmc*) allele, which flanked the *Cosmc* exon and an inserted *Neo* cassette. The linearized targeting vector is shown in Fig. 1*A*. The targeting vector was electroporated into HZ2.2 embryonic stem cells derived from a 129SvEv/TAC male mouse (Emory University transgenic facility). After 300 μg/mL G418 (Invitrogen) and 2 mmol/L ganciclovir (Calbiochem) selection, ES cell clones with correct homologous recombination were screened by PCR and confirmed by Southern blots. Clone 3A12 was transiently transfected with an expression vector encoding Cre recombinase to delete *Cosmc* and the *Neo* gene cassette to generate ES cells with a null *Cosmc* (*Cosmc*$^{-/y}$), or *Neo* gene cassette only to create ES cells with a floxed *Cosmc* (*Cosmc*$^{flox/y}$).

Generation of *Cosmc*$^{flox/+}$, *Cosmc* mosaic, *Cosmc*$^{+/-}$, and *Cosmc*$^{-/y}$ Mice, and *Cosmc*$^{+/-}$ and *Cosmc*$^{-/y}$ Mouse Embryos. The *Cosmc*$^{-/y}$ and *Cosmc*$^{flox/y}$ ES cells were microinjected into C57BL/6J blastocysts, and then implanted into pseudopregnant mice. Chimeras among the offspring were bred with C57BL/6J female mice to generate female mice with *Cosmc*$^{flox/+}$. Chimeras from *Cosmc*$^{-/y}$ could not produce germ line, although all chimeras from *Cosmc*$^{flox/y}$ were germ-line-transmittable. Genotypes of mice were determined by PCR of DNA from tail biopsies or from portions of embryos or yolk sacs. WT and floxed alleles were identified using PCR with specific primers (Table S3).

To generate *Cosmc* mosaic mice, the *Cosmc*$^{flox/+}$ mice were crossed with *EIIa-Cre* transgenic mice (JAX Lab #003724). The promoter of the adenovirus *EIIa* gene is active in oocytes and preimplantation embryos. The *Cosmc*$^{flox/+}$/*EIIa-Cre* and *Cosmc*$^{flox/y}$/*EIIa-Cre* offspring analyzed were mosaic *Cosmc* knockout (KO) and WT genotypes. To create heterozygous female mice (*Cosmc*$^{+/-}$), the mosaic male *Cosmc*$^{flox/y,\ -/y}$/*EIIa-Cre* mouse with apparent normal development was in segregation breeding with C57BL/6J female mice to create *Cosmc*$^{+/-}$. To generate *Cosmc*$^{-/y}$ mice, *Cosmc*$^{+/-}$ mice, and embryos, the heterozygous female with apparent normal development were bred with C57BL/6J male mice. The strategies for generating the mice or embryos with different genotyping (*Cosmc*$^{flox/+}$, *Cosmc* mosaic, *Cosmc*$^{+/-}$, *Cosmc*$^{-/y}$) are shown (Fig. 2*A*). Genotypes of mice were determined by PCR of DNA from tail snips. Mice were kept in a specific pathogen-free barrier facility. Except for *EIIa-Cre* mice, all mice were of mixed genetic background (129SvEv/TAC and C57BL/6J). Animal studies were performed according to the Institutional Animal Care and Use Committee protocol approved by Emory University.

Preparation of Embryo Extracts. E10.5 embryos were resuspended in an appropriate volume of 25 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and proteinase inhibitor mixture (Roche Molecular Biochemicals) and homogenized by sonication on an ice-bath for 5 s four times. The supernatants were obtained by centrifugation of homogenate at 700 × *g* for 10 min, and the extracts were obtained by adding 0.5% Triton X-100 to the supernatant and solubilizing on ice for 30 min.

Glycosyltransferase Assays. T-synthase and β1,4 GalT activity from murine embryos and Tn(+) spleen cells extracts were measured using the acceptor GalNAcα-phenyl and GlcNAc-β-S-pNp, respectively, as previously described (1).

Southern Blot. Genomic DNA (gDNA) (10 μg) from different ES cells and tissues of WT and mosaic mice were digested in a 200 μL reaction with 100 units of SacI or NheI and HindIII (New England BioLabs) at 37 °C overnight. Southern blot was performed as previously described (2). Probe 1 is ~700 bp DNA from the right up-stream of the start of left arm in the targeting sequence (Fig. 1*A*), used for Southern blot of gDNA from ES cells. Probe 2 is 1,090 bp of *Bgl-II* fragment downstream of the right arm targeting sequence (Fig. 1*A*), used for Southern blot of gDNA from tissues of mice. The probes were labeled with [α-^{32}P]-dCTP using the random labeling kit following the manufacturer's protocol.

Western Blot. Embryo extracts (40 μg of protein) or ES cell extracts with or without prior Neuraminidase (Sialidase) (Roche), Sialidase plus O-Glycanase, or PNGase F (New England Biolabs) treatments were resolved by SDS/PAGE under reducing conditions and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). For peanut agglutinin (PNA) and *Helix pomatia* agglutinin (HPA) binding, the membranes were blocked with 5% nonfat dry milk and incubated with 2 μg/mL HRP conjugated PNA or 1 μg/mL HRP conjugated HPA (EY Laboratories) in TBS at room temperature for 1 h. For *Canavalia ensiformis* agglutinin (ConA), *Ricinus communis* agglutinin-1 (RCA), and *Sambucus nigra* agglutinin (SNA) binding (Vector Laboratories), the membrane was blocked with 0.5% BSA, 0.5% Tween-20 and incubated with 0.1, 0.2, and 0.004 μg/mL biotinylated lectins, respectively. For T-synthase detection by Western blot, an anti-T-synthase antibody (C1GALT1) was used (Santa Cruz Biotechnology, Inc.) at a 1:100 dilution. Binding was detected with ECL chemiluminescent substrate (HighSignal West Pico; Pierce Chemical Co.).

N-Glycan Analysis of Glycoproteins from Mouse ES Cells. WT or *Cosmc*-KO ES cells were grown in ES cell media with leukemia inhibiting factor (LIF) in gelatin coated 20-cm dishes at 37 °C, 5% CO_2. Approximately 2 × 10^6 cells were solubilized in CHAPS buffer [150 mM NaCl, 0.5% CHAPS (3-[3-(cholamideopropyl) dimethylammonio-1-propanesulfonate, 20Mm Tris-HCl (pH7.5), 2.5 mM sodium pyrophosphate, 1mM ethylenediamine tetraacetic acid (EDTA)]. The lysates were centrifuged at 100,000 × *g* for 90 min at 4 °C. The pellets were resuspended in CHAPS buffer and sonicated for 10 min at 4 °C. The solubilized material was reduced by 10 mM DTT (DTT) at 56 °C for 45 min followed by alkylation with 55 mM iodoacetamide by incubation in the dark at room temperature for 60 min. The mixture was then treated with trypsin (20:1) (Sigma-Aldrich) at 37 °C overnight, follow by heat-inactivation of enzyme at 95 °C for 10 min. Beta-mercaptoethanol (Sigma-Aldrich) was added into solution to final 1% concentration and boiled at 98 °C for 10 min in a water bath. The mixture was cooled and 1 M sodium phosphate buffer, pH 7.4 (final concentration 50 mM) was added. PNGase F (5,000 units, New England Biolabs) was added into the solution and incubated at 37 °C overnight. The mixture was cooled and Milli Q water was added to a final volume of 20 mL. The sample was applied to activated Sep-pak C18 column (Waters, 2 g) and the flow-through was collected. The flow-through was loaded into activated carbograph and washed with water to remove salts. The released N-glycans were eluted from the carbograph column by 30% acetonitrile, 0.1% Trifluoroacetic acid (TFA), 1.5 mL, three times. The glycans were dried by spin-vac overnight. The dried oligosaccharides were permethylated by solid phase method (3). After permethylation, the N-glycans were dissolved in 10 μL 50% methanol solution. Samples (0.5 μL) were spotted on a MALDI plate followed by 0.5 μL matrix and dried under vacuum. The matrix used

was 10 mg/mL 2,5-Dihydroxybenzoic acid (Sigma-Aldrich) with 1 mM sodium acetate in 50% acetonitrile with 0.1% TFA. All MALDI-TOF data were collected on Applied Biosystems 4800 MALDI-TOF-TOF using positive reflection mode.

RT-PCR, PCR. The mRNA from ES cells and mouse tissues was isolated using FastTrack MAG Mrna Isolation Kit (Invitrogen), and gDNA from cells and mouse tails was prepared using DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocols. The RT-PCR for mouse *Cosmc* and *G3PDH* were performed with 5 ng of mRNA as template with a SuperScript First-stand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. The PCR were carried out with Phusion High-Fidelity PCR kit (New England Biolabs) in a 50-μL reaction containing 100 ng of gDNA as template and 100 nmol/L of each primer. For cDNA of Cosmc, PCR was performed with primers (Table S3) and the PCR products were analyzed on a 1% Tris-acetate EDTA agarose gel.

Immunohistochemistry. $Cosmc^{+/y}$ and $Cosmc^{-/y}$ ES cells were grown in six-well plates coating with 0.1% gelatin. When the cells became 60 to 70% confluent, cells were fixed with 4% paraformaldehyde for 1 h. Staining procedure was done according to the manufacturer's protocol (Zymed Laboratories). Briefly, the cells were incubated with 5 μg/mL anti-Tn monoclonal antibody (mAb, mouse IgM) at room temperature for 1 h, then incubated with peroxidase-labeled goat anti-mouse IgM (1:500) for 1 h at room temperature. The cells were washed with PBS after each incubation. The slides were developed with the chromogen AEC single solution for 3 to 5 min and counterstained with Mayer's hematoxylin. The cells were covered with crystal mount media and dried overnight. Tissues from mosaic *Cosmc* mice were fixed in 10% neutral pH formalin overnight at room temperature, sectioned at 5 μm thickness, and deparaffinized sections were blocked with a goat serum block (Zymed Laboratories) and streptavidin/biotin blocking kit (Vector Laboratories), then incubated overnight with biotinylated anti-Tn antigen mAb overnight at 4 °C. The staining was visualized using HRP-conjugated streptavidin (1:500 Vector Laboratories) for 1 h at room temperature. The slides were developed with the chromogen AEC single solution for 3 to 5 min and counterstained with Mayer's hematoxylin. The tissues were covered with mount media and visualized under a microscope (model: 1× 51S8F-3; Olympus) using SPOT software (Olympus).

Whole-Mount Staining. E10.5 embryos fixed overnight in 4% paraformaldehyde and were treated in blocking buffer (1% Triton X-100, 10% FCS in PBS) for 2 h at room temperature and subsequently incubated with Alexa488-labeled anti-Tn mAb (10 μg/mL) for 4 days with gentle rotation at 4 °C. Embryos were washed in PBS 1% Triton plus 10% FCS for 3 h. The embryos were covered with mount media and visualized under a fluorescence microscope (model: 1× 51S8F-3; Olympus) using SPOT software (Olympus). Unstained whole embryos were photographed with a Leica PLHNAPG 1.0× LEICA MZ FLIII camera, and software QCCapture was used to collect the images.

Fluorescent Lectin Staining. The formalin fixed embryo sections (E11.5) were deparaffinized, treated with neuraminidase, blocked with streptavidin/biotin blocking kit (Vector Laboratories), and incubated with biotinylated PNA or HPA lectins (10 μg/mL) at room temperature for 1 h. After three washes, the staining was visualized using Alexa-488 conjugated streptavidin (1:500, Vector Laboratories) for 1 h at room temperature. Stained embryos sections were photographed with a Leica PLHNAPG 1.0× LEICA MZ FLIII camera, and software QCCapture was used to collect the images.

Isolation of Tn(+) and Tn(−) Cells From Spleens of Mosaic *Cosmc*-Null Mice. WT and mosaic mouse spleens were separated into a single-cell suspension by a standard preparation. For depletion of mouse B cells expressing surface IgM, the spleen cells were incubated with rat anti-mouse IgM microbeads (Miltenyi Biotec). Magnetic cell sorting procedure was done according to the manufacturer's protocol. Unlabeled cell fractions were incubated with biotinylated anti-Tn antigen mAb (2 μg/mL) for 10 min at 4 °C. The cell pellet was labeled with streptavidin microbeads (Miltenyi Biotec) and separate into Tn(+) and Tn(−) cells with MACS column and separator.

Glycan Array Analysis. Glycan microarrays were prepared as described previously (4). Briefly, aminoalkyl glycosides were covalently coupled to N-succinimidyl-activated glass slides (1 × 3 in.) in a sodium phosphate buffer, pH 8.5, 0.005% Tween 20. Slides were immersed in 50 mM ethanolamine, 50 mM sodium borate, pH 9.0, for 1 h, rinsed with water, dried under a stream of microfiltered air, and stored in a desiccator until use. Biotinylated lectins were diluted in TSM Binding Buffer containing 1% BSA and 0.05% Tween 20 and added to the array for 1 h at room temperature in a dark humid chamber. The slides were washed by successive immersion in TSM containing 0.05% Tween 20 (four times), TSM (four times), and then incubated with Alexa 488-labeled streptavidin. After 1 h at room temperature in a dark humid chamber, the slide was washed by successive immersion in TSM containing 0.05% Tween 20 (four times), TSM (four times), and water (four times). The slide was dried by microcentrifugation. An image of bound fluorescence was obtained using a microarray scanner (ScanArray Express, PerkinElmer Life Sciences). The integrated spot intensities were determined using Imagene software (BioDiscovery).

1. Ju T, Cummings RD, Canfield WM (2002) Purification, characterization, and subunit structure of rat core 1 Beta1,3-galactosyltransferase. *J Biol Chem* 277(1):169–177.
2. Ju T, et al. (2008) Human tumor antigens Tn and sialyl Tn arise from mutations in Cosmc. *Cancer Res* 68:1636–1646.
3. Kang P, Mechref Y, Klouckova I, Novotny MV (2005) Solid-phase permethylation of glycans for mass spectrometric analysis. *Rapid Commun Mass Spectrom* 19:3421–3428.
4. Blixt O, et al. (2004) Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. *Proc Natl Acad Sci USA* 101:17033–17038.

Fig. S1. Southern blot of ES cell clones. (*A*) WT and *Cosmc* KO gDNA was digested with NheI and blotted with Probe 1. (*B*) gDNA from WT and *Cosmc* KO ES cells was digested with SacI and blotted with Probe 2. See Fig. 1*A* for notation of probes. Lanes 1 to 8 of *Cosmc* KO correspond to the eight selected clones.

Fig. S1

Fig. S2. Lectin blot analyses of $Cosmc^{+/y}$ and $Cosmc^{-/y}$ ES cells. Blots of cell extracts from WT ($Cosmc^{+/y}$) and *Cosmc* deficient ES cells ($Cosmc^{-/y}$) were probed with (*A*) HPA, (*B*) PNA, (*C*) ConA, (*D*) RCA, and (*E*) SNA. The extracts were incubated with or without Sialidase (S), Sialidase plus O-Glycanase (O), or PNGase F (P) before electrophoresis and blotting. Jurkat cells transfected with *Cosmc* were used as a positive control. The same volume and amount of cell extract from WT and $Cosmc^{-/y}$ were analyzed on each lectin blot.

Fig. S2

Fig. S3. MALDI-TOF-TOF analysis of N-glycan on glycoproteins from mouse ES cells. The structures of N-glycans from (*A*) WT and (*B*) Cosmc-deficient ES cells were analyzed. *Insets* for *A* and *B*: sialylated structures.

Fig. S3

Fig. S4. Transcription of the Lox^{P}-*Cosmc* in kidney and spleen between WT and transgenic animals (*A*). (*B–D*) Southern blot analyses of the deletion (Δ) of *Cosmc* from multiple organs, including heart (H), spleen (Sp), kidney (K), liver (Li), lung (L), small intestine (SMI), large intestine (LINT), stomach (S), and thymus (Th) for Group I Mosaics (*B*); Group II Mosaics (*C*); WT littermate controls (*D*) corresponding to Figs. 2 *B–D*, respectively. (*E–H*) Immunohistochemistry of tissues from a $Cosmc^{+/-}$ mouse with anti-Tn mAb. (*E*) Kidney (K), (*F*) lung (L), (*G*) small intestine (SMI), and (*H*) large intestine (LINT) tissue sections. Brown staining corresponds to areas of tissue positive for Tn expression. (Scale bar, 50 μm.)

Fig. S4

Fig. S5. Western blot of T-synthase in the extracts from WT, $Cosmc^{+/-}$, and $Cosmc^{-/y}$ embryos at E12.5. T-synthase bands are seen as doublet bands in the WT and $Cosmc^{+/-}$; no bands were seen in the $Cosmc^{-/y}$ embryonic extract. β-actin was used as a loading control.

Fig. S5

Fig. S6. Binding of biotinylated PNA to Galβ1–3GalNAc residues and biotinylated HPA to terminal α-GalNAc residues on a glycan microarray. PNA binding at 10 μg/mL (*A*) and 1 μg/mL (*B*), with the glycan structures and binding data represented as relative fluorescence units (RFUs) for the major glycans bound, listed in order of decreasing RFUs (*C* and *D*). HPA binding at 10 μg/mL (*E*) and 1 μg/mL (*F*), with the glycan structures and binding data represented as RFUs for the major glycans bound, listed in order of decreasing RFUs (*G* and *H*).

Fig. S6

Table S1. Offspring from mosaic male mice (*Cosmc*$^{-/y,flox/y}$) in Group II Mosaic mice bred with C57BL/6J female mice

	Male	Female	Total
Cosmc$^{+/y}$ *or Cosmc*$^{+/+}$	69 (61.6%)	30 (26.7%)	99 (88.3%)
Cosmc$^{+/-}$	–	13 (11.6%)	13 (11.6%)

Table S2. Offspring from heterozygous female (*Cosmc*$^{+/-}$) bred with C57BL/6J male (*Cosmc*$^{+/y}$) mice

Genotype	Male	Female	Total
Wild-type *Cosmc*$^{+/+}$ or *Cosmc*$^{+/y}$	39 (37.1%)	52 (49.5%)	91 (86.7%)
Heterozygous *Cosmc*$^{+/-}$	–	14 (13.4%)	14 (13.4%)
Hemizygous *Cosmc*$^{-/y}$	0	–	0

Table S3. Primers used for PCR and RT-PCR

Genotype/transcript	Primer sequence
WT *Cosmc*	P1 5′-GCAACACAAAGAAACCCTGGG-3′ P2 5′-CAAATCCCTTGGCACTGGTATG-3′
Floxed *Cosmc*	P1 5′-GCAACACAAAGAAACCCTGGG-3′ P3 5′-TCGTCTTTGTTAGGGGCTTGC-3′
cDNA *Cosmc*	Forward primer 5′-ATCACTATGCTAGGCCACATTAGGATTGGA-3′ Reverse primer 5′-GGAGGTAAGAAAACCAATGCATCATTGAAAA-3′