

Supplementary Information

Salmonid polysialyltransferases to generate a variety of sialic acid polymers

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Supplementary information

Supplementary Figure S1: Production in transfected HEK293 cells and biochemical characterization of recombinant polysialyltransferases *Cma* ST8Sia II-r1 and ST8Sia II-r2.

Supplementary Figure S2: SDS-PAGE and WB with the specific mAb735 to detect products formed after sialylation reaction with human and fish ST8Sia IV enzymes.

Supplementary Figure S3: ³¹P NMR spectra of chemo-enzymatically synthesized activated CMP-Sias.

Supplementary Figure S4: Polysialylation status of HEK293 cells.

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Supplementary Figure S7 Exosialylation of HEK293 cells with *Hsa*ST8Sia IV.

Supplementary Figure S8: On the top, amino acid sequence of the anti-polySia scFv of mAb735 and localization of hydrogen bound interactions.

Supplementary Figure S9: Original blots

Supplementary Table S-1: Description of sialylated glycanic structures of acceptor substrates used in MPSA.

Supplementary Table S-2: PCR oligonucleotides used for amplification of fish cDNAs

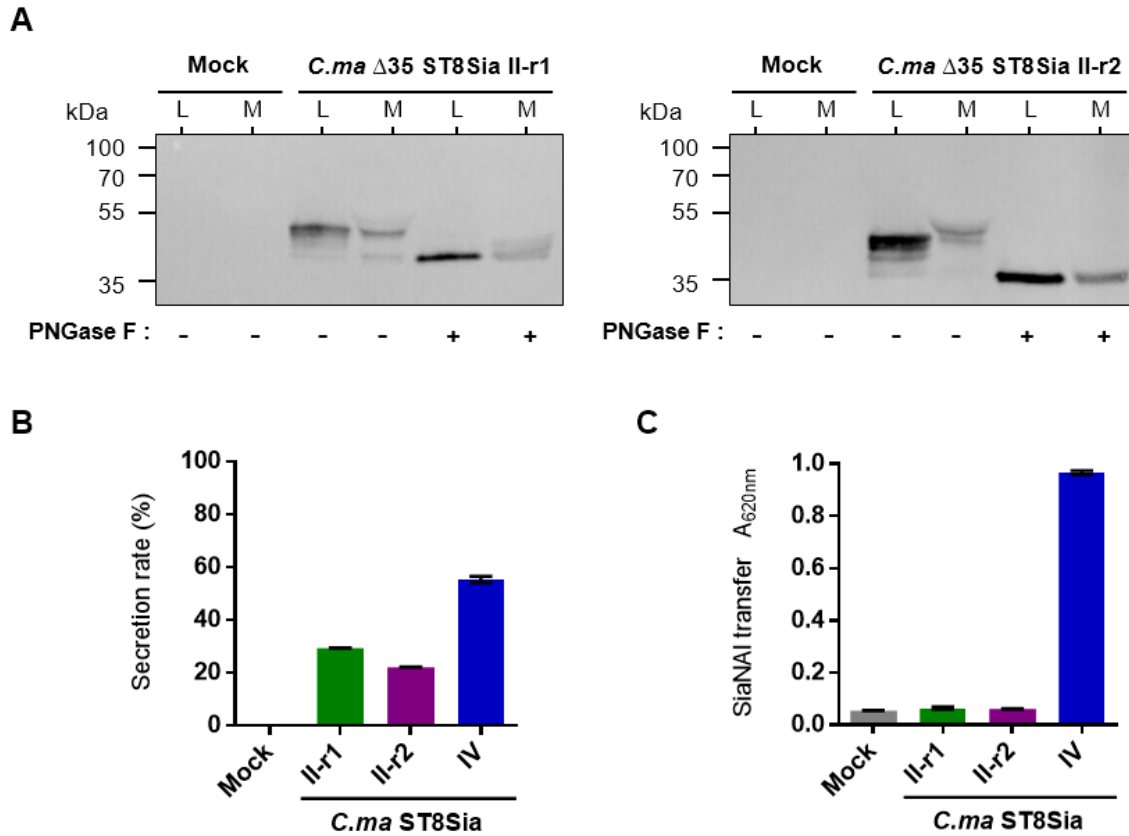


Figure S1: Production in transfected HEK293 cells and biochemical characterization on CD166/ALCAM using the MPSA of recombinant polysialyltransferases *Cma*ST8Sia II-r1 and ST8Sia II-r2. Plasmids for the expression of $\Delta 28$ ST8Sia IV (blue), $\Delta 35$ ST8Sia II-r1, (green) and $\Delta 35$ ST8Sia II-r2 (purple) were transfected in HEK293 cells. Culture media and cells were collected 72 h post-transfection. Seven μ g of cell lysates (L) and 20 μ L of culture media (M) of HEK293 transfected cells with $\Delta 35$ ST8Sia II-r1 (left panel) and $\Delta 35$ ST8Sia II-r2 (right panel) were treated overnight with 50 U of PNGase F according to the manufacturer's instructions. Reaction products were denatured in Laemmli buffer and analyzed on WB. **(A)** Representative WB with PNGase-F treated and native samples. Samples were loaded, separated on SDS-PAGE (8 % acrylamide gel) and transferred on nitrocellulose membrane. WB was carried out with the anti-3 $\times$ FLAG antibody (1 μ g/mL). Original blot is presented in supplementary figure S9. Molecular weight markers are indicated on the left side. **(B)** Secretion efficiency of each *Cma* polysialyltransferases. WB signal intensities of culture media and cell lysate were analyzed by densitometry (ImageJ). Secretion of each isoform is reported in percentage (%) relative to total enzyme quantities in the culture media and cell lysates quantified on each WB (n=2). **(C)** Biochemical characterization on CD166/ALCAM using the MPSA. Sialylation reactions were performed in the MPSA 4 h at 27 °C using 400 ng of CD166/ALCAM, 100 μ M of CMP-SiaNAI and 20 μ L of either *Cma* $\Delta 35$ ST8Sia II-r1 (green) or *Cma* $\Delta 35$ ST8Sia II-r2 (purple) or 20 μ L of *Cma* $\Delta 28$ ST8Sia IV (blue). Mock transfected cell culture medium (grey) was used as negative control. Azido-PEG3-biotin was then covalently attached to the alkyne group of each transferred SiaNAI through copper catalyzed alkyne-azide cycloaddition (CuAAC) and biotin was detected by the anti-biotin antibody coupled with HRP (32 ng/mL). TMB (3,3',5,5'-Tetramethylbenzidine) substrate was used and absorbance was measured with a microplate spectrophotometer at 620 nm. Error bars represent SEM (n=3).

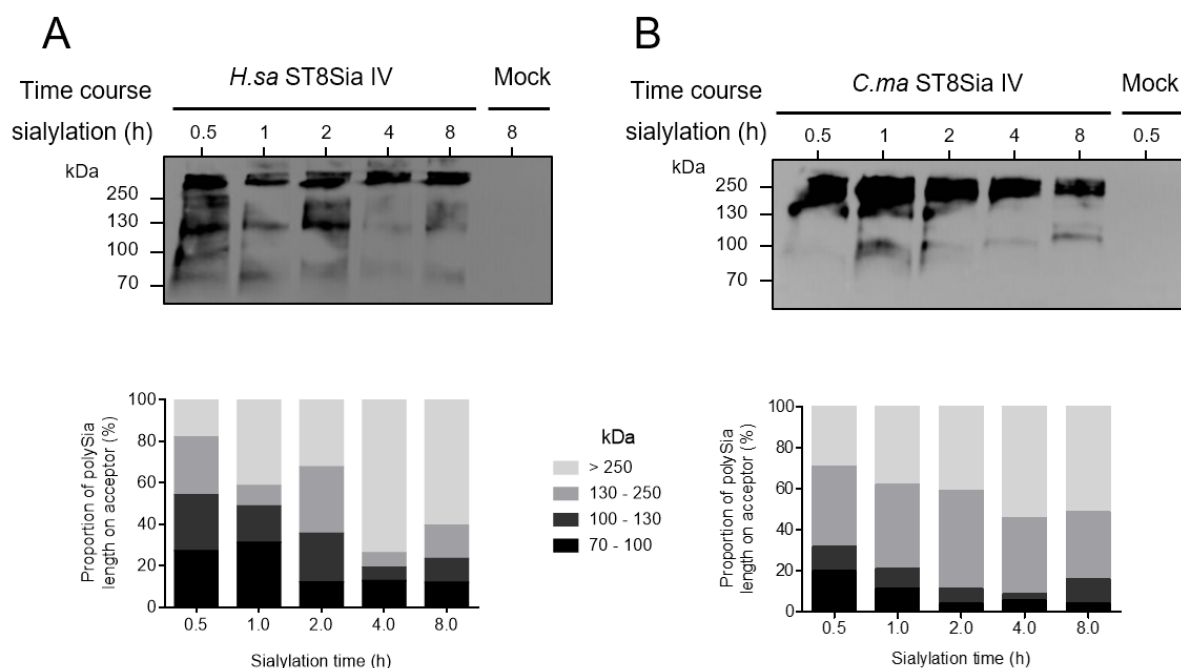


Figure S2: SDS-PAGE and Western blot with the specific mAb735 to detect products formed after sialylation reaction with human and fish ST8Sia IV enzymes. Time course sialylation reactions were performed from 0.5 to 8 h at 27 °C with 100 μ M of natural donor substrate CMP-Neu5Ac on 6 μ g of CD166/ALCAM with 150 ng of *H.sa* ST8Sia IV or 20 μ L of *C.ma* ST8Sia IV or Mock. Sialylated products were heated for 5 min at 65 °C in Laemmli buffer to preserve the polySia chains, separated on SDS-PAGE (6 % acrylamide gel) and sialylated proteins were transferred onto nitrocellulose. Western blot analyses shown on the top were carried out with the mAb735. Original blots are presented in supplementary figure S9. Molecular weight markers are indicated on the left side. Densitometry analyses were performed with ImageJ and the percentage of polySia chains length according to band sizes is reported below for **A**) the human and **B**) the coregone enzyme (n=2).

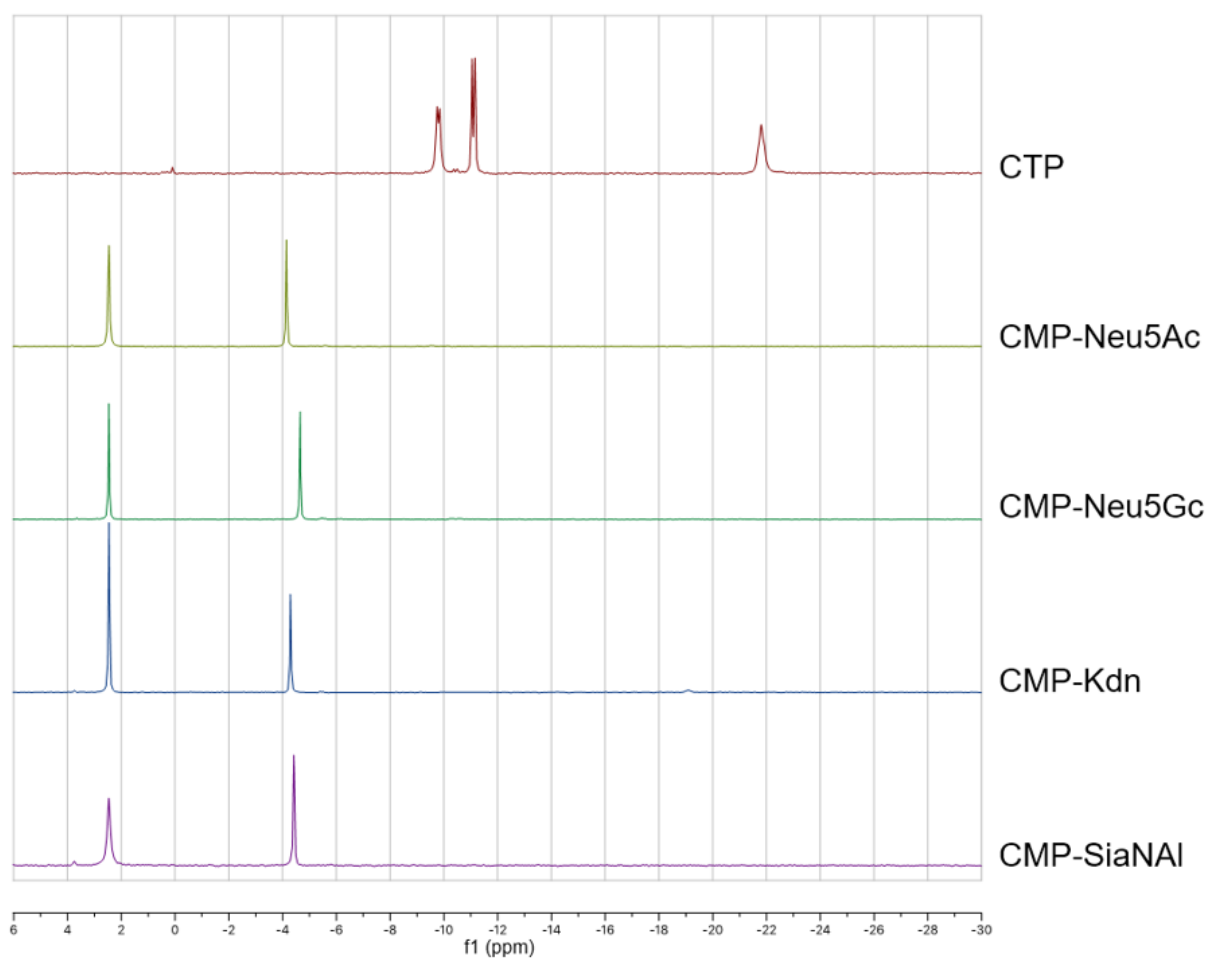


Figure S3: ^{31}P NMR spectra of chemo-enzymatically synthesized activated CMP-sialic acids. Enzymatic reactions were performed in equimolarity of CTP and each Sia (1:1) with 0.3 U CMP-Sialic acid Synthase (CSS) from *N. meningitidis* for CMP-SiaNAI, CMP-Neu5Ac and CMP-Neu5Gc and from rainbow trout for CMP-Kdn and 0.5 U PPase in Tris-HCl 100 mM, MgCl_2 20 mM buffer (pH 8.5) during 1 h at 37 °C. Products integrity was checked by 1D NMR spectrometry of ^{31}P in a Brüker Avance II 400 MHz NMR spectrometer. Peaks shift (ppm) from CTP spectrum (red) after one hour of synthesis to CMP-Neu5Ac (olive), CMP-Neu5Gc (green), CMP-Kdn (blue) and CMP-SiaNAI (purple) spectra indicates 100 % formation and purity of the four products formed.

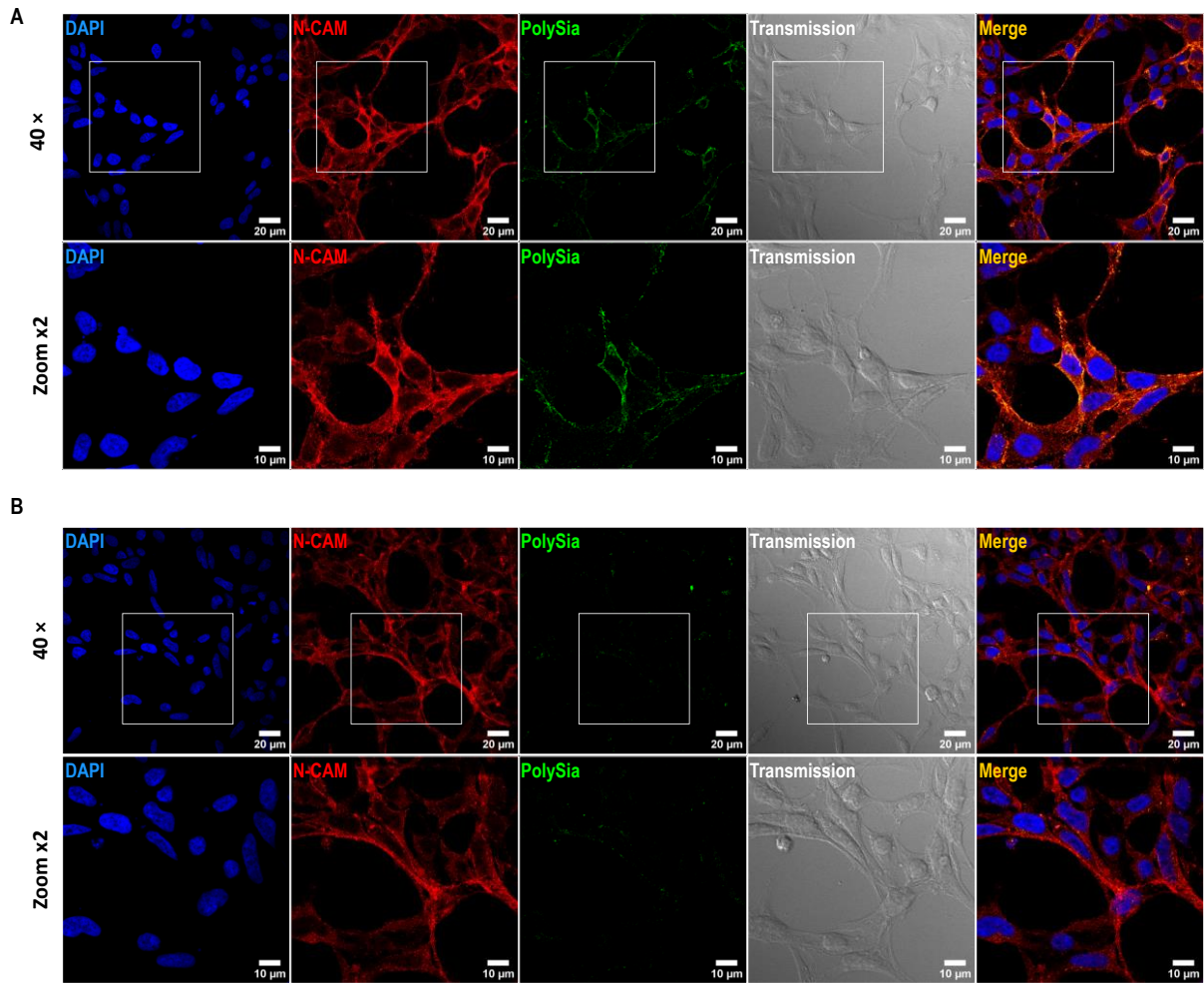


Figure S4: Polysialylation status of HEK293 cells. A) HEK293 cells without endoN treatment show polysialylation labelling on cell surface using mAb735 and anti-CD56/NCAM antibodies. B) After EndoN treatment, mAb735 detection signal (green) is abolished. Nuclei are stained with DAPI (blue). Outline of the cells is visualized by transmission in grey. DAPI, NCAM and polySia signals are superposed in Merge. Fluorescence was detected through a Carl Zeiss confocal microscope and Transmission correspond to transmitted light microscopy images. Scale bars: 20 µm for acquisitions at 40 × and 10 µm for the Zoom × 2 pictures.

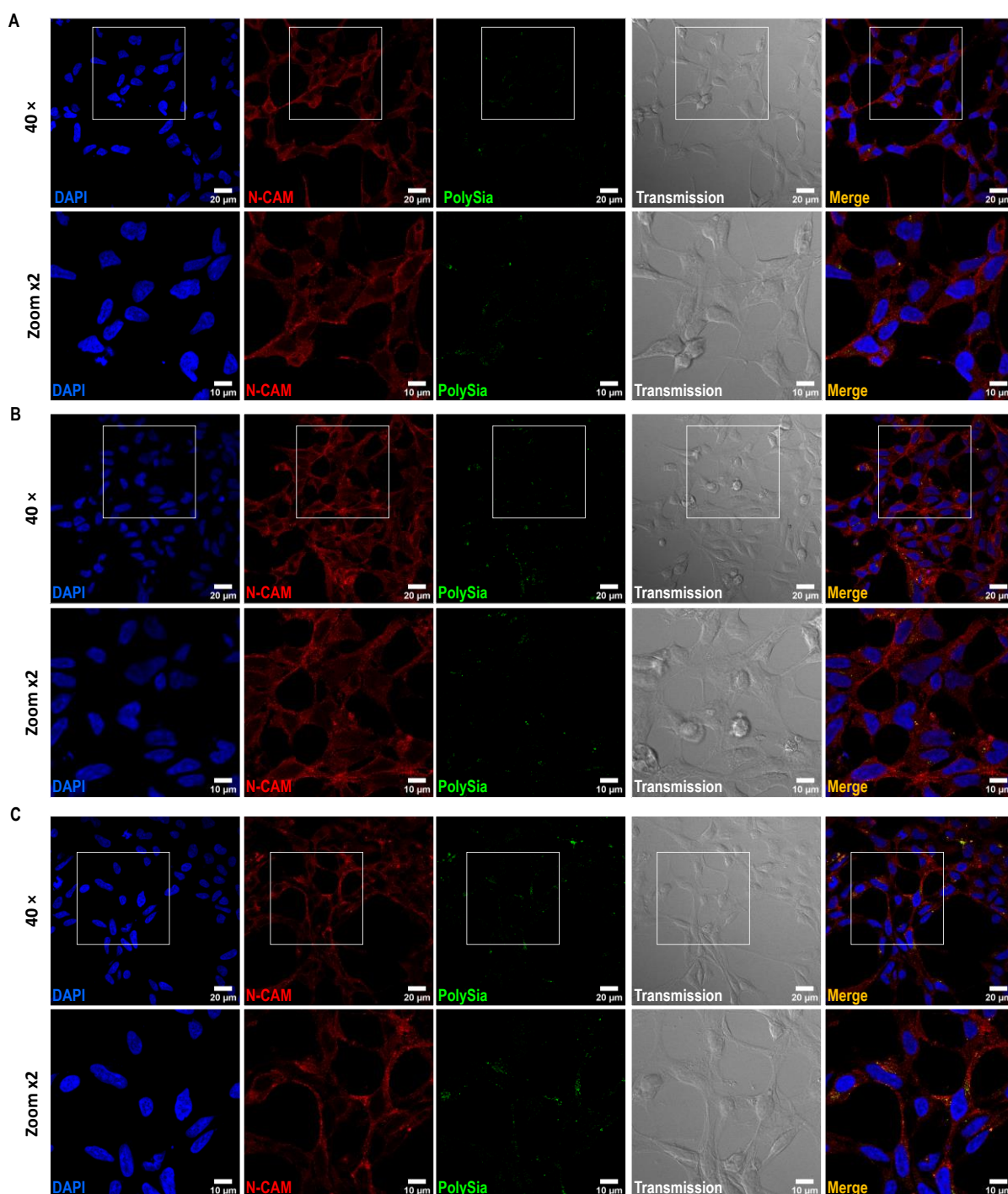


Figure S5: Exosialylation of HEK293 cells with Mock enzyme source: After EndoN treatment, sialylation reactions were carried out 4 h at 27°C on fixed cells with 40 μL of Mock medium and 500 μM of donor substrates: A) CMP-Neu5Ac, B) CMP-Neu5Gc and C) CMP-Kdn. Polysialylation on cell surface is detected using mAb735 antibody (green) and NCAM is detected using anti-CD56/NCAM (red) antibody. Nuclei are stained with DAPI (blue). Outline of the cells is visualized by transmission in grey. DAPI, NCAM and polySia signals are superposed in Merge. Fluorescence was detected through a Carl Zeiss confocal microscope and Transmission correspond to transmitted light microscopy images. Scale bars: 20 μm for acquisitions at 40 × and 10 μm for the Zoom × 2 pictures.

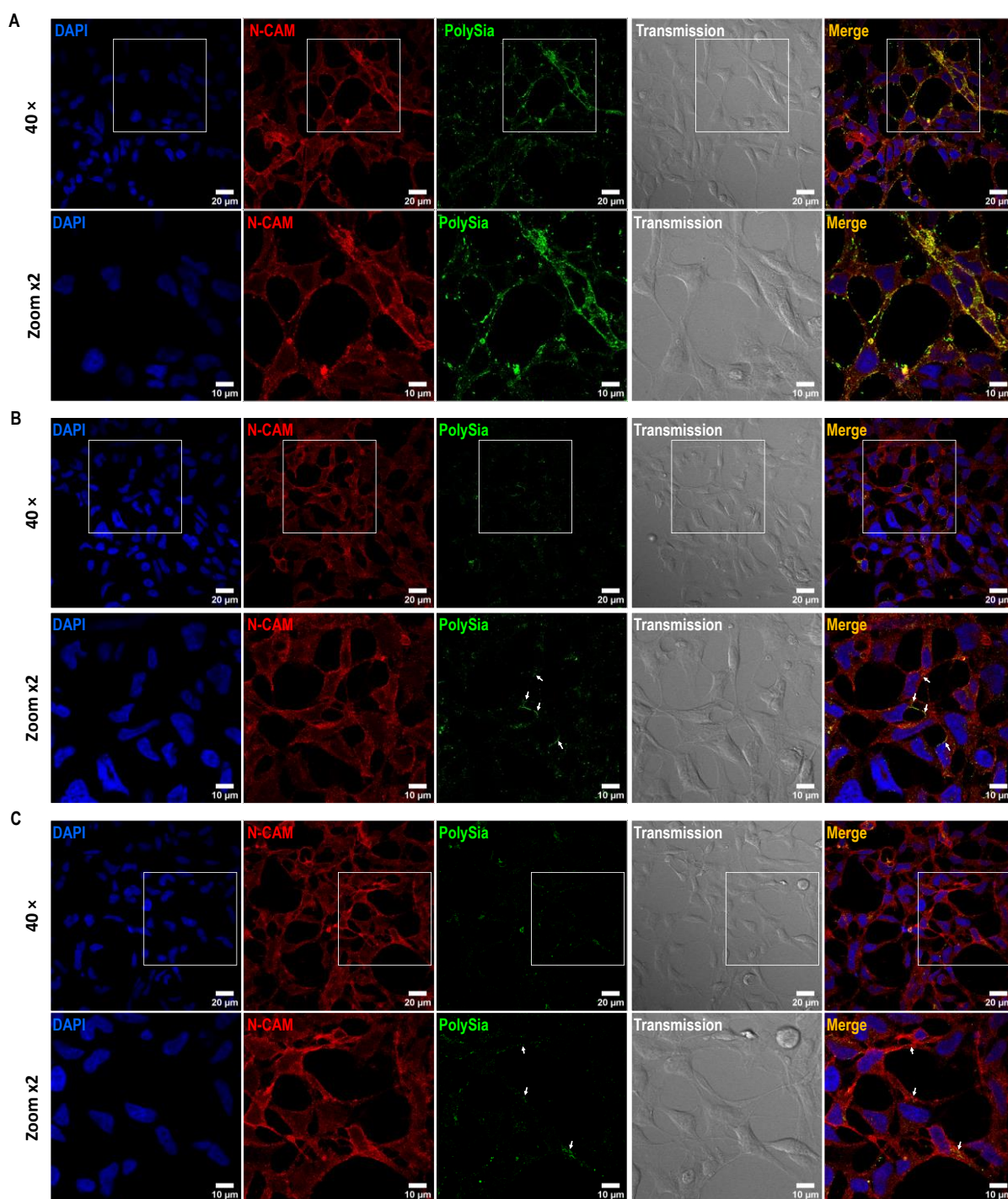


Figure S6: Exosialylation of HEK293 cells with *CmaST8Sia IV*. After endoN treatment, sialylation reactions were carried out 4 h at 27 °C on fixed cells with 40 µL of *Cma* enzyme and 500 µM of donor substrates: A) CMP-Neu5Ac, B) CMP-Neu5Gc and C) CMP-Kdn. Polysialylation on cell surface is detected using mAb735 antibody and NCAM is detected using anti-CD56 antibody. Nuclei are stained with DAPI (blue). Outline of the cells is visualized by transmission in grey. DAPI, NCAM and polySia signals are superposed in Merge. Fluorescence was detected through a Carl Zeiss confocal microscope and Transmission correspond to transmitted light microscopy images. White arrows indicate polySias. Scale bars: 20 µm for acquisitions at 40 × and 10 µm for the Zoom × 2 pictures.

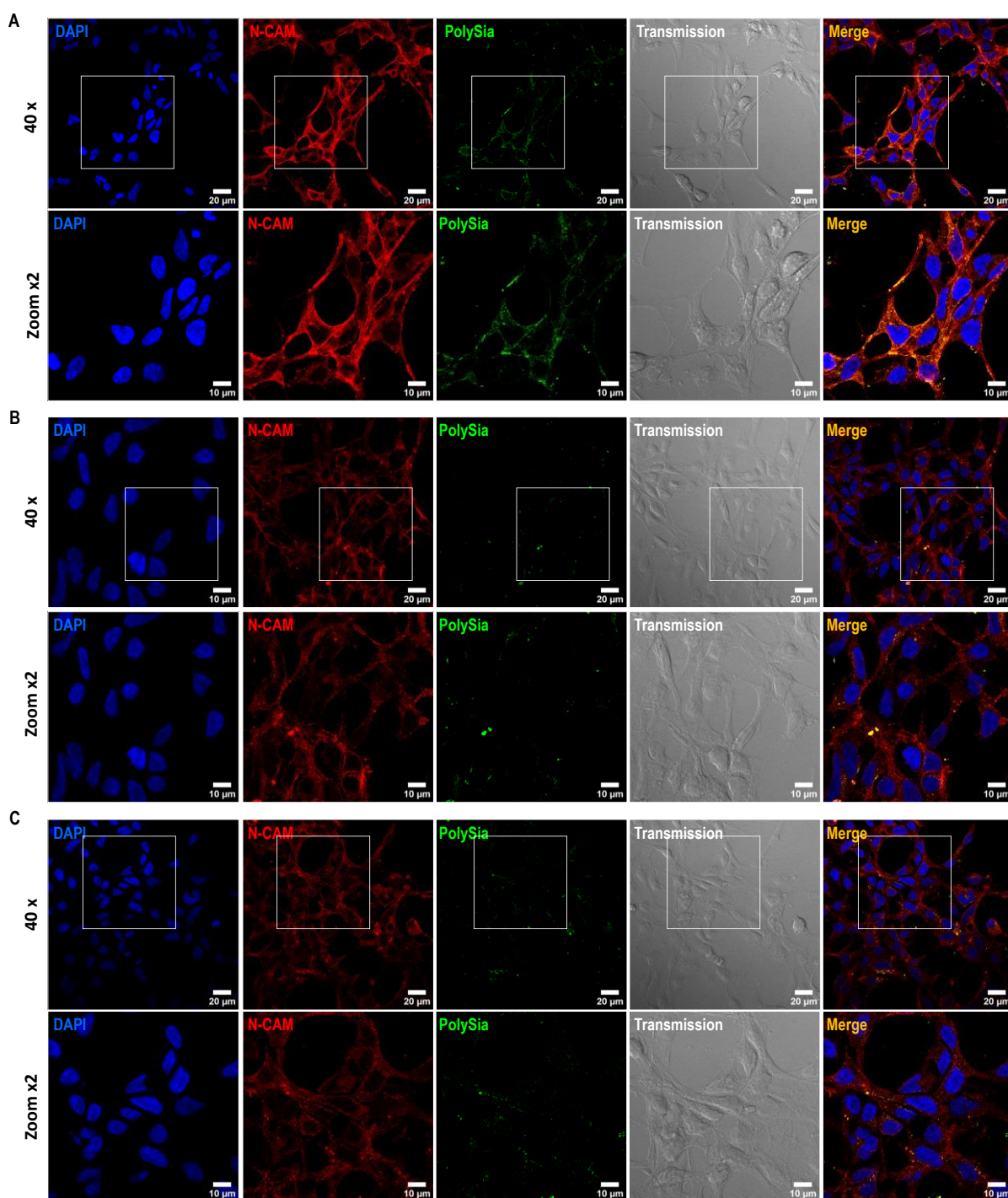
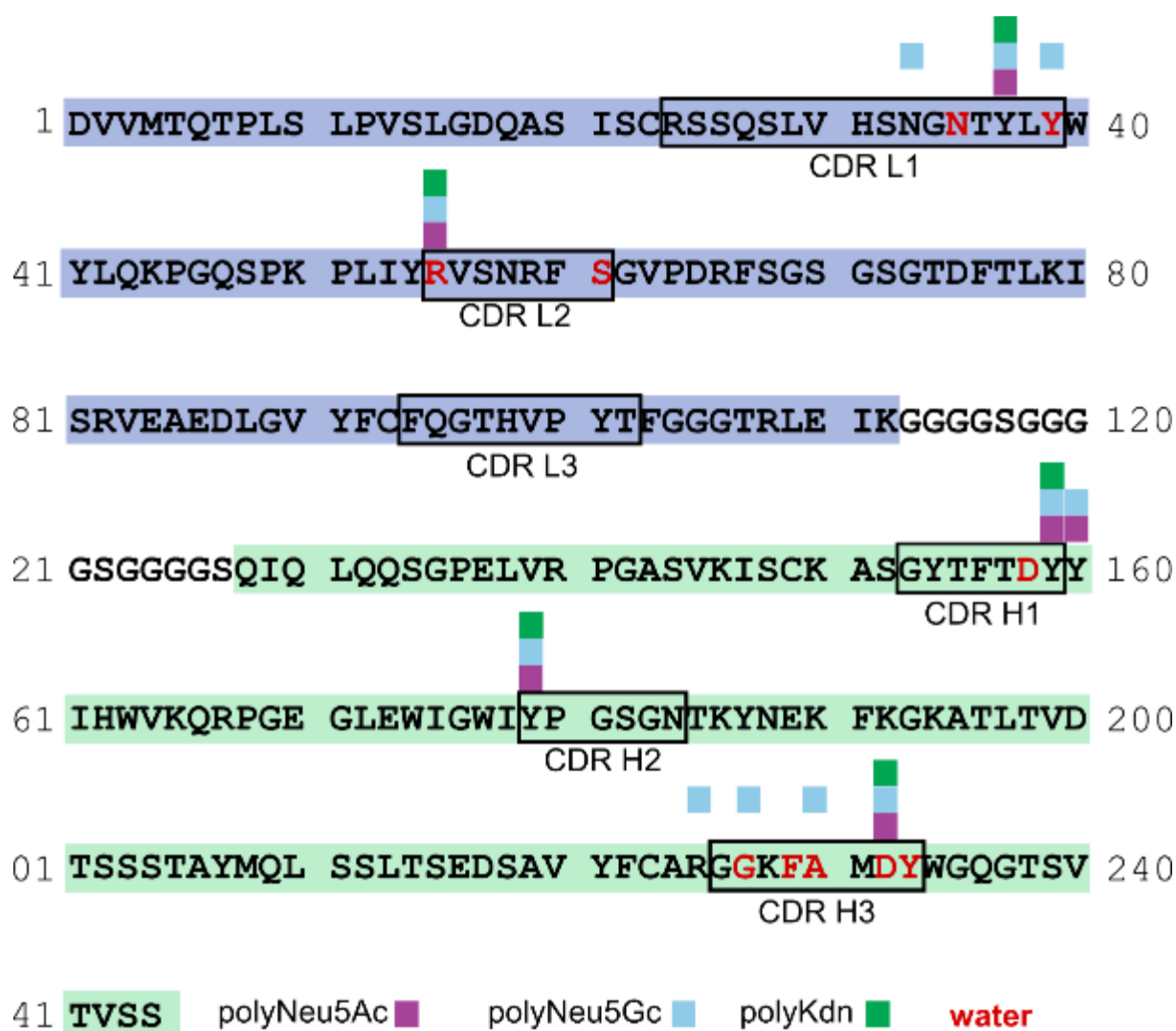


Figure S7 Exosialylation of HEK293 cells with *Hsa* ST8Sia IV. After endoN treatment, sialylation reactions were carried out 4 h at 27 °C on fixed cells with 200 ng of the human ST8Sia IV enzyme and 500 µM of donor substrates: A) CMP-Neu5Ac, B) CMP-Neu5Gc and C) CMP-Kdn. Polysialylation labelling on cell surface is detected using mAb735 and anti-CD56/NCAM antibodies. Nuclei are stained with DAPI (blue). Outline of the cells is visualized by transmission in grey. DAPI, NCAM and polySia signals are superposed in Merge. Fluorescence was detected through a Carl Zeiss confocal microscope and Transmission correspond to transmitted light microscopy images. Scale bars: 20 µm for acquisitions at 40 × and 10 µm for the Zoom ×2 pictures.



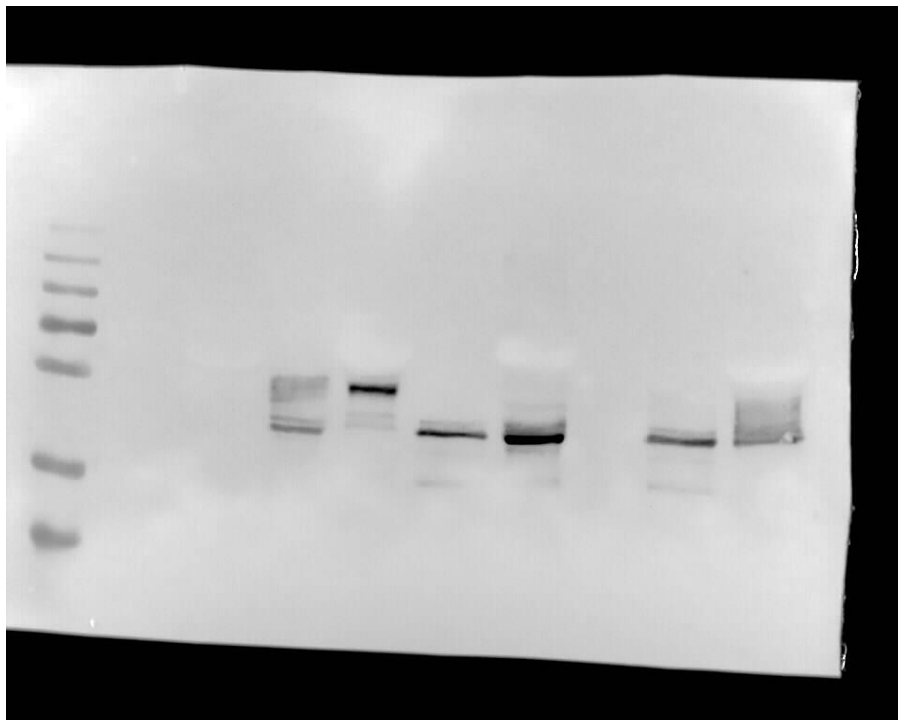
Amino Acid	CDR	Neu5Ac (equivalent)	Neu5Gc (equivalent)	Kdn (equivalent)
Asn33	L1		Sia3 (Sia7)	
Asn35	L1	Indirect	Indirect	Indirect
Tyr37	L1	Sia3 (Sia7)	Sia3 (Sia7)	Sia3 (Sia7)
Tyr39	L1	Indirect	Sia3 (Sia7)	Indirect

Arg55	L2	Sia2 (Sia6) Indirect	Sia2 (Sia6) Indirect	Sia2 (Sia6) Indirect
Ser61	L2	Indirect	Indirect	Indirect
Asp158	H1	Indirect	Indirect	Indirect
Tyr159	H1	Sia2 (Sia6)	Sia2 (Sia6)	Sia2 (Sia6)
Tyr160	H1	Sia4 (Sia8)	Sia4 (Sia8)	
Tyr179	H2	Sia4 (Sia8)	Sia4 (Sia8)	Sia4 (Sia8)
Arg225			Sia2 (Sia6)	
Gly227	H3	Indirect	Sia2 (Sia6)	Indirect
Phe229	H3	Indirect	Indirect	Indirect
Ala230	H3	Indirect	Sia2 (Sia6)	Indirect
Asp232	H3	Sia2 (Sia6) Indirect	Sia2 (Sia6) Indirect	Sia2 (Sia6) Indirect
Tyr233	H3	Indirect	Indirect	Indirect

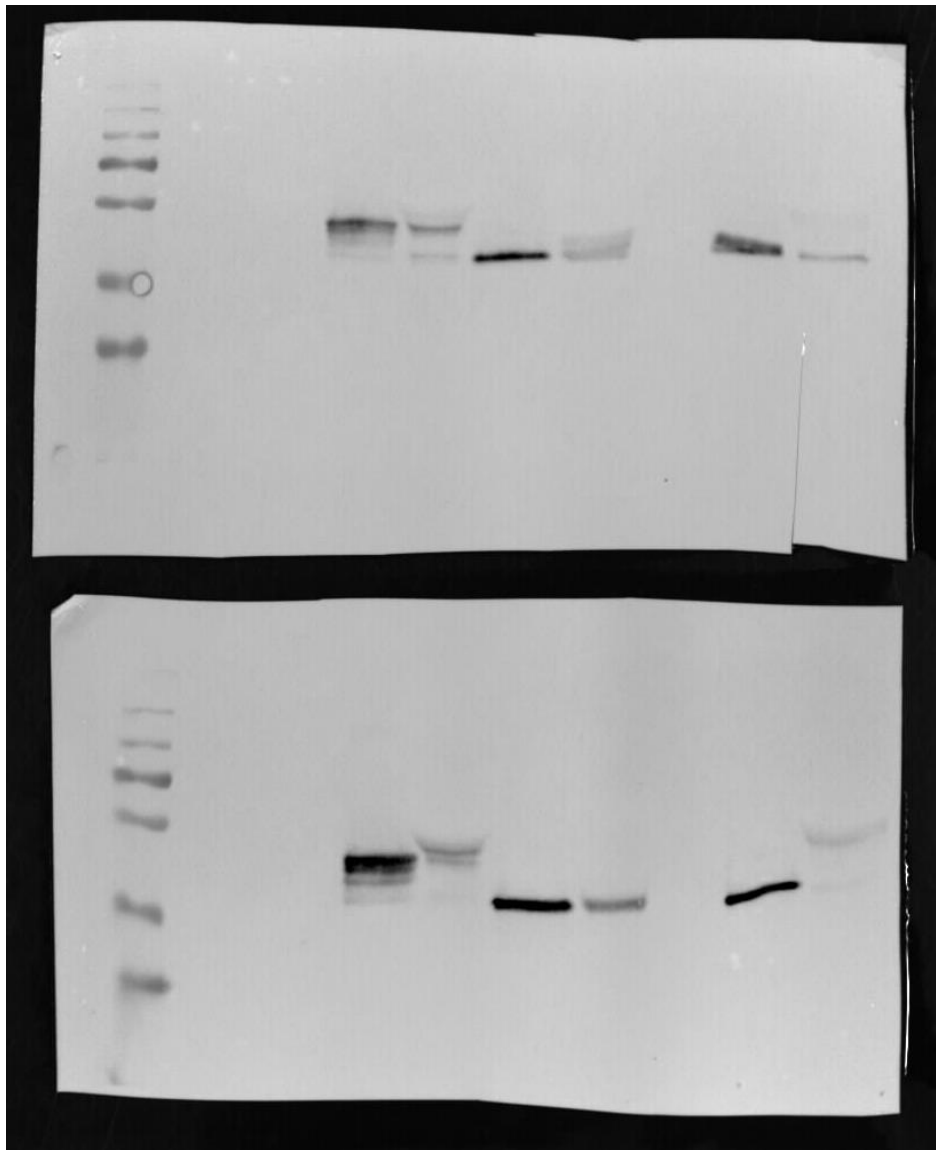
Figure S8: On the top, amino acid sequence of the anti-polySia scFv of mAb 735 and localization of hydrogen bound interactions. VL and VH domains are colored in blue and green background, respectively. The complementarity-determining regions (CDRs) L1, L2, L3, H1, H2, H3 are indicated with black boxes. Colored boxes above the sequence mark residues that make direct interaction with polyNeu5Ac (purple), polyNeu5Gc (light blue) and polyKdn (green), whereas residues involved in water-mediated indirect interactions with the antigen are shown in red. Interactions are summarized in the table below.

Figure S9: Original and unprocessed images A. of figure 2; B. of supplemental figure S1; C. of figure 4; D. of figure 5; E. of supplemental figure S2; F. of figure 7

A. Figure 2: WB anti-FLAG *C.ma*ST8Sia IV

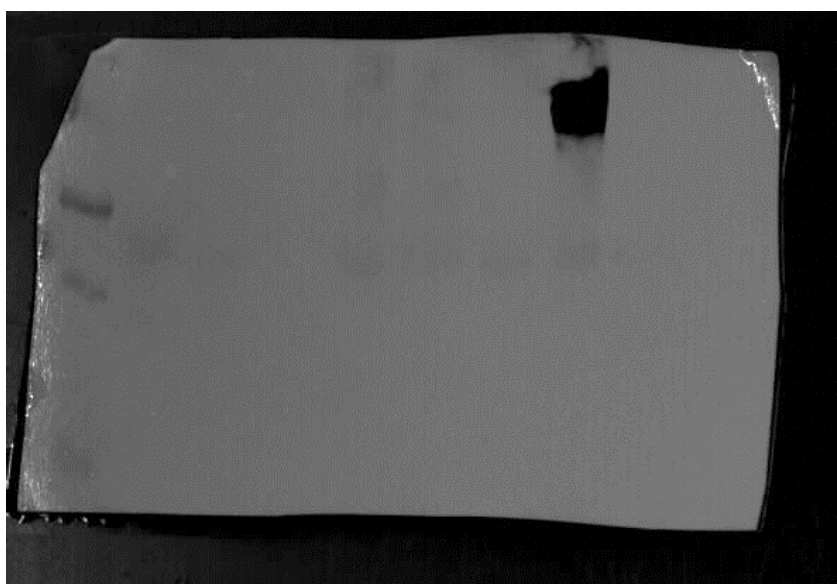
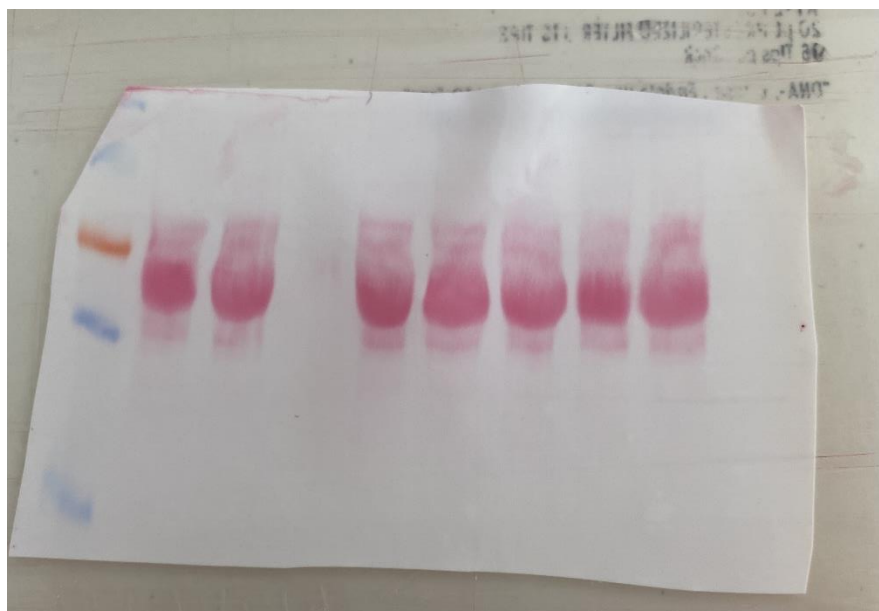


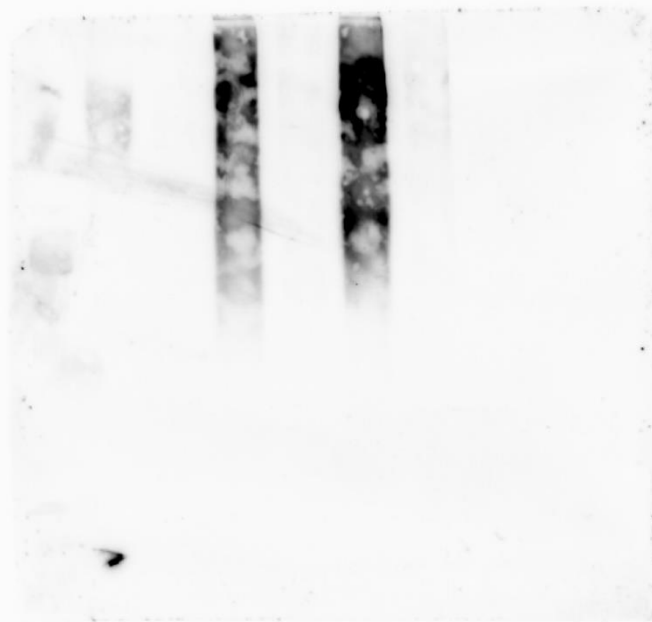
B. Supplemental figure S1: WB anti-Flag *C.ma*ST8Sia II-r1 and *C.ma*ST8Sia II-r2



C. Figure 4: Controls by colorimetric analysis and WB with anti-polySia of the polysialylation status of the various actors of polysialylation prior sialylation reaction (Figure 4A).

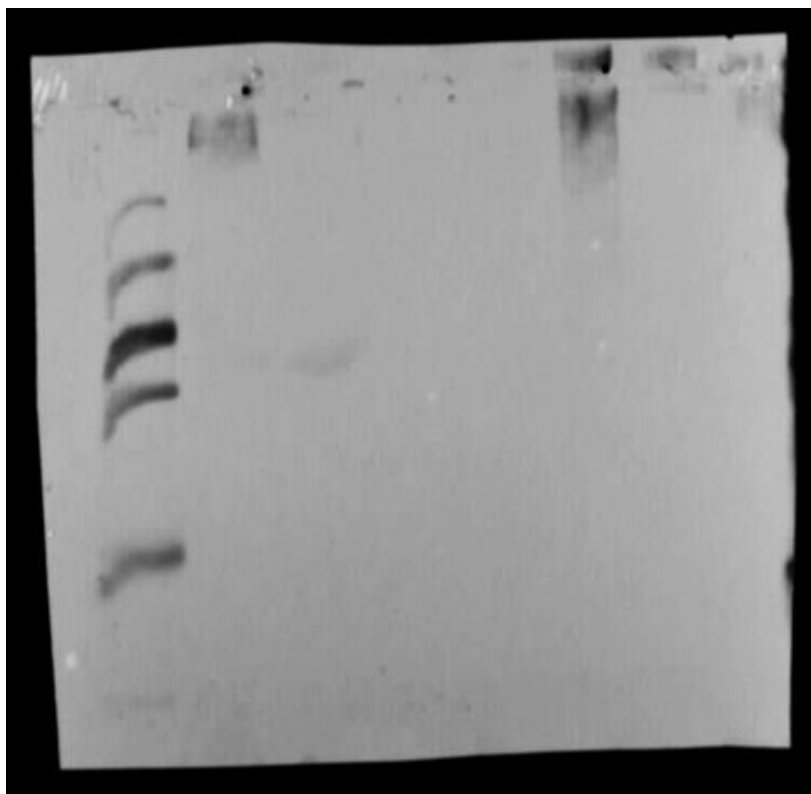
Controls by WB with anti-polySia of the ALCAM polysialylation status after polysialylation reaction (Figure 4C)



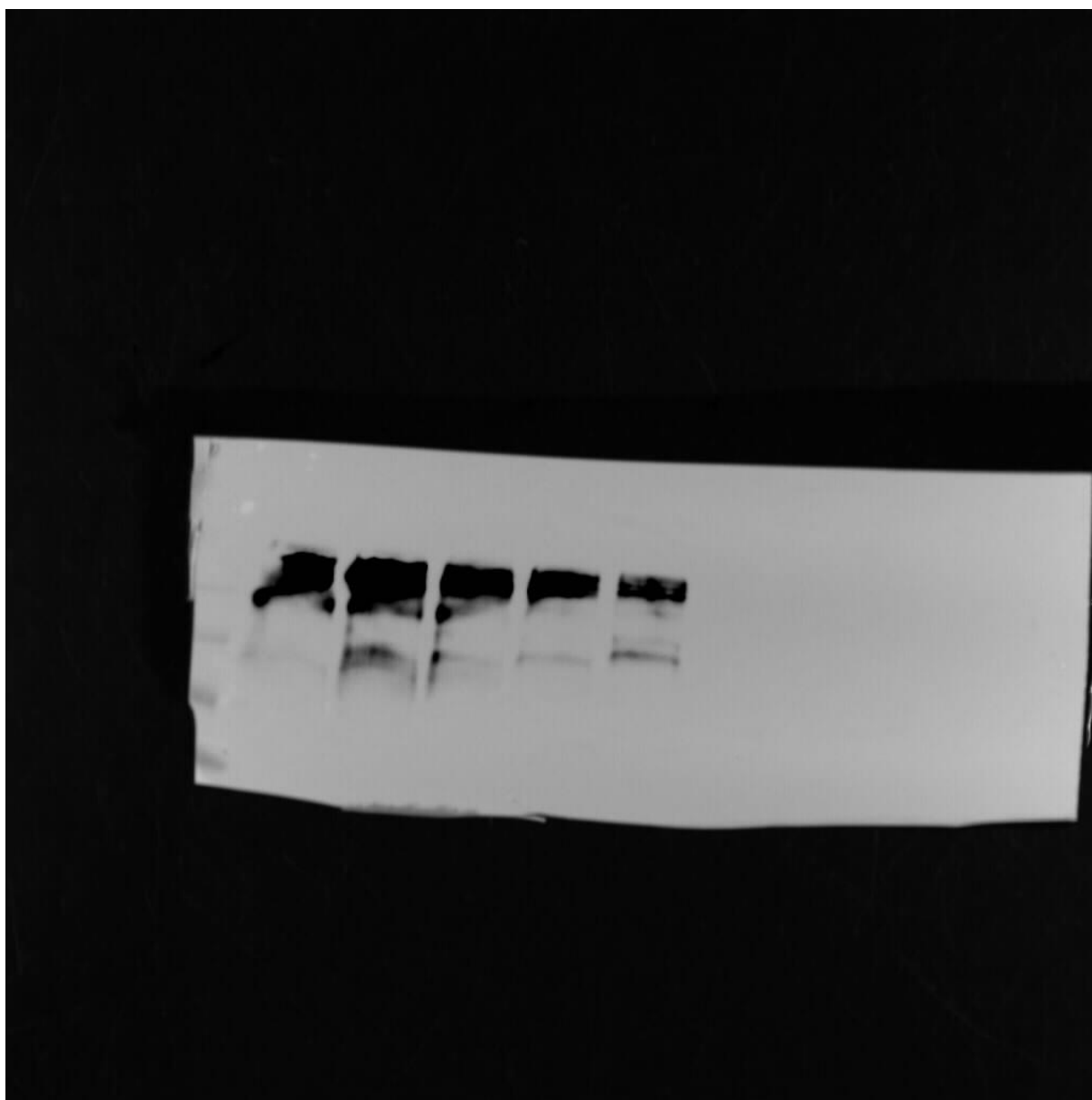
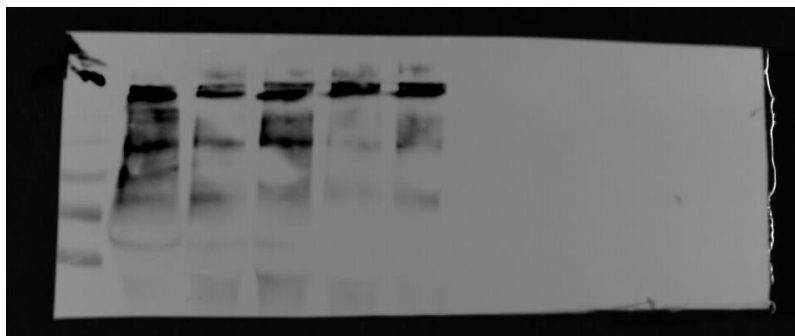


D. Figure 5 WB anti-polySia after sialylation with CMP-Neu5Ac, CMP-Neu5Gc and CMP-Kdn





E. Supplemental figure S2: WB anti-polySia and time-course of polySia formation with CMP-Neu5Ac using human ST8Sia IV and fish ST8Sia IV



F. Figure 7: Chemiluminescence and colorimetric analysis of WB with anti-polySia

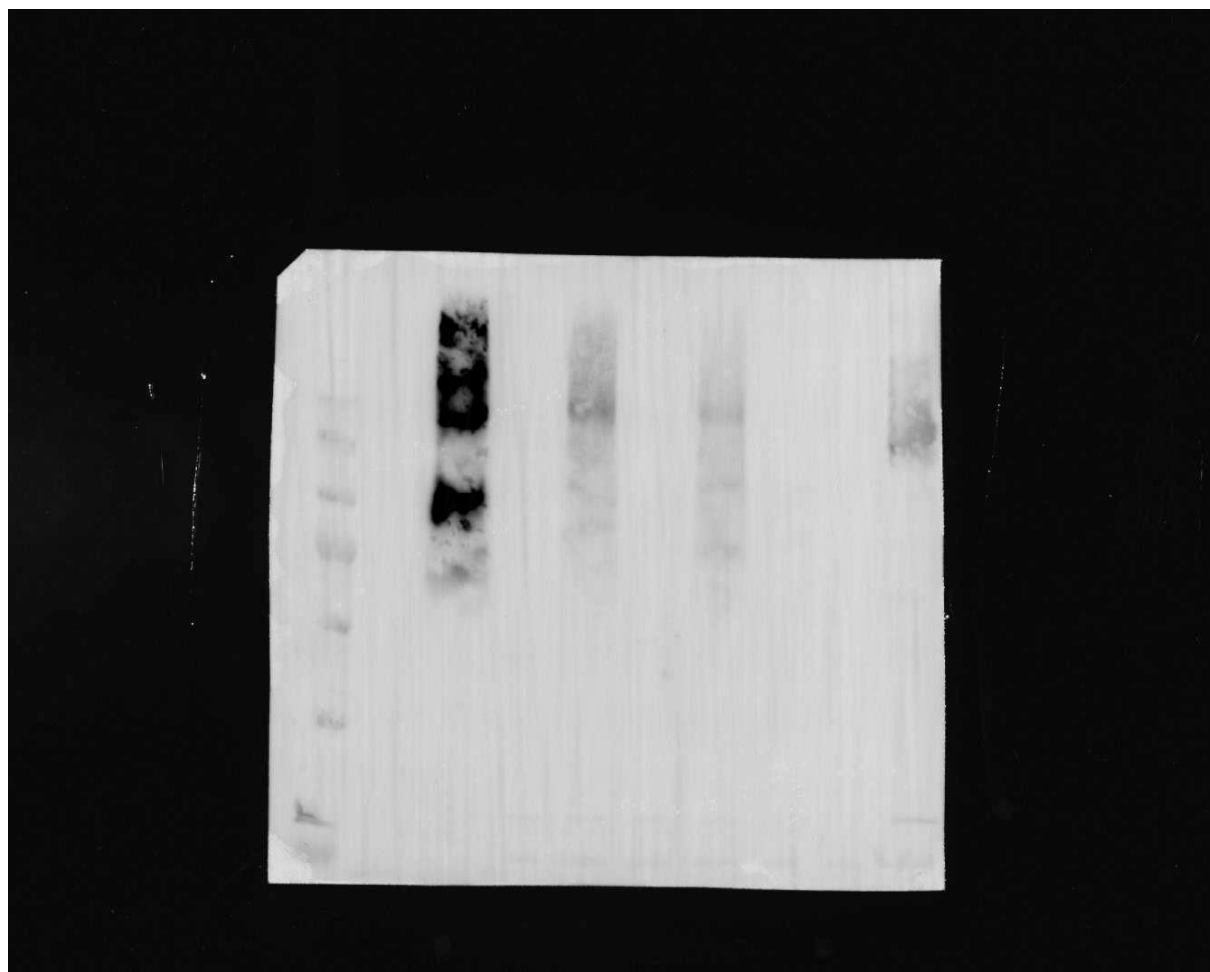
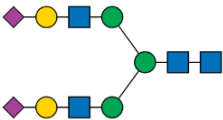
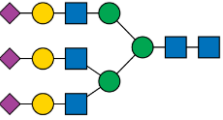
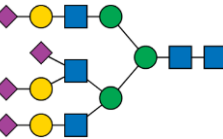


Table S-1: Description of sialylated glycanic structures of acceptor substrates used in enzymatic assay MPSA. Glycan representation was done with GlycoGlyph ¹ according to the SNFG nomenclature ².

Acceptor substrate	N-/O-glycans sialylated	Symbolic representation	Sialylated glycanic structure	References
Fetuin	N- : 3 tri-antennary sialylated N-glycans		Neu5Acα2-6(3)Galβ1-4GlcNAcβ1-2Manα1-3[Neu5Acα2-6(3)Galβ1-4GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-Asn	3
			Neu5Acα2-6(3)Galβ1-4GlcNAcβ1-2[Neu5Acα2-6(3)Galβ1-4GlcNAcβ1-4]Manα1-3[Neu5Acα2-6(3)Galβ1-4GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-Asn	
			Neu5Acα2-6(3)Galβ1-4GlcNAcβ1-2[Neu5Acα2-6(3)Galβ1-4[Neu5Acα2-6]GlcNAcβ1-4]Manα1-3[Neu5Acα2-6(3)Galβ1-4GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-Asn	
	O- : core-1 mucin-type		Neu5Acα2-6GalNAc-Ser/Thr	
			Neu5Acα2-3Galβ1-3GalNAc-Ser/Thr	



Neu5Ac α 2-3Gal β 1-3[Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-6]GalNAc-Ser/Thr



Gal β 1-3[Neu5Ac α 2-6]GalNAc-Ser/Thr

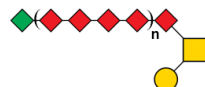


Neu5Ac α 2-3Gal β 1-3[Neu5Ac α 2-6]GalNAc-Ser/Thr

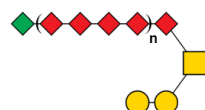
PSGP-L

PSGP-H

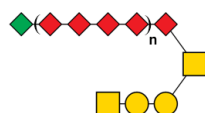
O- : core-1 and core-2 mucin-type disialylated (-L) or oligo- / polysialylated from 2 to 20 Sia (-H)



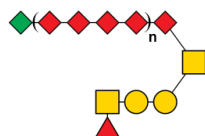
Kdn α 2-8(Neu5Acyl α 2-8) $n \approx 2-20$
Neu5Acyl α 2-6[Gal β 1-3]GalNAc-Ser/Thr



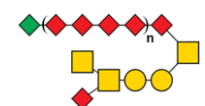
Kdn α 2-8(Neu5Acyl α 2-8) $n \approx 2-20$
Neu5Acyl α 2-6[Gal β 1-4Gal β 1-3]GalNAc-Ser/Thr



Kdn α 2-8(Neu5Acyl α 2-8) $n \approx 2-20$
Neu5Acyl α 2-6[GalNAc β 1-3Gal β 1-4Gal β 1-3]GalNAc-Ser/Thr

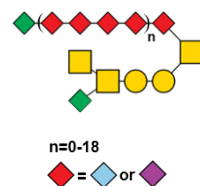


Kdn α 2-8(Neu5Acyl α 2-8) $n \approx 2-20$
Neu5Acyl α 2-6[Fuca1-3GalNAc β 1-3Gal β 1-4Gal β 1-3]GalNAc-Ser/Thr



Kdn α 2-8(Neu5Acyl α 2-8) $n \approx 2-20$
Neu5Acyl α 2-6[GalNAc β 1-4[Neu5Acyl α 2-3]GalNAc β 1-3Gal β 1-4Gal β 1-3]GalNAc-Ser/Thr

4

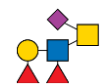


Kdn α 2-8(Neu5Acyl α 2-8)_{n $\approx$ 2-20}
 Neu5Acyl α 2-6[GalNAc β 1-4[Kdn α 2-3]GalNAc β 1-3Gal β 1-4Gal β 1-3]GalNAc-Ser/Thr

Bovine Submaxillary Mucin (BSM)	O- : Core -1, Core -2, Core -3, Core -5		Neu5Acyl α 2-6GalNAc-Ser/Thr	5
			Neu5Aca α 2-6[GlcNAc β 1-3]GalNAc-Ser/Thr	
			Neu5Aca α 2-3Gal β 1-3GalNAc-Ser/Thr	
			Neu5Gca α 2-6[GalNAc α 1-3]GalNAc-Ser/Thr	
			Neu5Gca α 2-6[Gal β 1-4GlcNAc β 1-3]GalNAc-Ser/Thr	
			Neu5Aca α 2-6[Fuca α 1-3(4)Gal β 1-3]GalNAc-Ser/Thr	
			Neu5Aca α 2-3Gal β 1-3[GlcNAc β 1-6]GalNAc-Ser/Thr	
			Neu5Acyl α 2-6[Fuca α 1-3(4)Gal β 1-4GlcNAc β 1-3]GalNAc-Ser/Thr	



Neu5Acα2-6[GalNAcβ1-4[Fuca1-3]Galβ1-4GlcNAcβ1-3]GalNAc-Ser/Thr



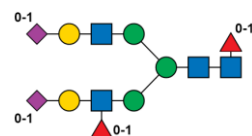
Neu5Acα2-6[Fuca1-3(4)Galβ1-4[Fuca1-3]GlcNAcβ1-3]GalNAc-Ser/Thr



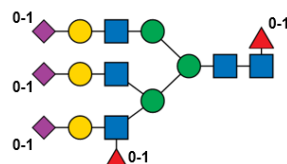
6

Orosomucoid
(α-1-acid
glycoprotein)

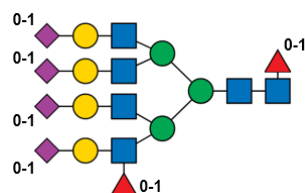
N- ; di-, tri- and
tetra-antennary
LacNAc sialylated
N-glycans



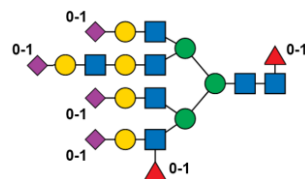
(Neu5Acα2-6(3))₀₋₁Galβ1-4[(Fuca1-3)₀₋₁]GlcNAcβ1-2Manα1-3[(Neu5Acα2-6(3))₀₋₁Galβ1-4GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAcβ1-4[(Fuca1-6)₀₋₁]GlcNAc-Asn



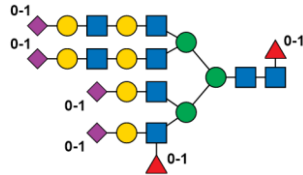
(Neu5Acα2-6(3))₀₋₁Galβ1-4[(Fuca1-3)₀₋₁]GlcNAcβ1-2[(Neu5Acα2-6(3))₀₋₁Galβ1-4GlcNAcβ1-4]Manα1-3[(Neu5Acα2-6(3))₀₋₁Galβ1-4GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAcβ1-4[(Fuca1-6)₀₋₁]GlcNAc-Asn



(Neu5Acα2-6(3))₀₋₁Galβ1-4[(Fuca1-3)₀₋₁]GlcNAcβ1-2[(Neu5Acα2-6(3))₀₋₁Galβ1-4GlcNAcβ1-4]Manα1-3[(Neu5Acα2-6(3))₀₋₁Galβ1-4GlcNAcβ1-2[(Neu5Acα2-6(3))₀₋₁Galβ1-4GlcNAcβ1-4]Manα1-6]Manβ1-4GlcNAcβ1-4[(Fuca1-6)₀₋₁]GlcNAc-Asn



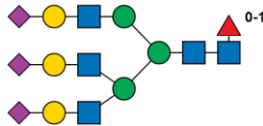
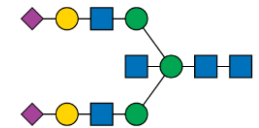
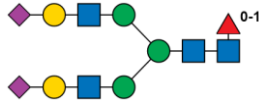
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4]Man α 1-6]Man β 1-4GlcNAc β 1-4[(Fuc α 1-6)₀₋₁]GlcNAc-Asn

(Neu5Ac α 2-6(3))₀₋₁Gal β 1-4[(Fuc α 1-3)₀₋₁]GlcNAc β 1-2[(Neu5Ac α 2-6(3))₀₋₁Gal β 1-4GlcNAc β 1-4]Man α 1-3[(Neu5Ac α 2-6(3))₀₋₁Gal β 1-4GlcNAc β 1-4Gal β 1-4GlcNAc β 1-2[(Neu5Ac α 2-6(3))₀₋₁Gal β 1-4GlcNAc β 1-4Gal β 1-4GlcNAc β 1-4]Man α 1-6]Man β 1-4GlcNAc β 1-4[(Fuc α 1-6)₀₋₁]GlcNAc-Asn

CD166/ALCAM N- : bi-, tri- and tetra-antennary sialylated N-glycans (α 2.6- and α 2.3-Sia)

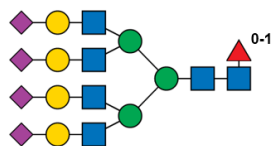


Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-2Man α 1-3[Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-2Man α 1-6]Man β 1-4GlcNAc β 1-4[(Fuc α 1-6)₀₋₁]GlcNAc-Asn

7, 8

Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-2Man α 1-3[GlcNAc β 1-4][Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-2Man α 1-6]Man β 1-4GlcNAc β 1-4GlcNAc-Asn

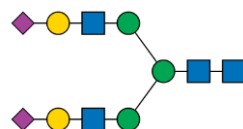
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Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-
2[Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-
4]Man α 1-3[Neu5Ac α 2-3Gal β 1-
4GlcNAc β 1-2[Neu5Ac α 2-3Gal β 1-
4GlcNAc β 1-2]Man α 1-6]Man β 1-
4GlcNAc β 1-4[(Fuc α 1-6)₀₋₁]GlcNAc-Asn

DNase I

N- : LacNAc
sialylated N-glycans



Neu5Ac α 2-6(3)Gal β 1-4GlcNAc β 1-
2Man α 1-3[Neu5Ac α 2-6(3)Gal β 1-
4GlcNAc β 1-2Man α 1-6]Man β 1-
4GlcNAc β 1-4GlcNAc-Asn

9

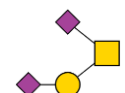
NRP-2

O- : Core-1, Core-2

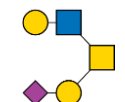


Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-6[Gal β 1-
3]GalNAc-Ser/Thr

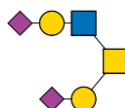
10



Neu5Ac α 2-3Gal β 1-3[Neu5Ac α 2-
6]GalNAc-Ser/Thr



Gal β 1-4GlcNAc β 1-6[Neu5Ac α 2-3Gal β 1-
3]GalNAc-Ser/Thr



Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-
6[Neu5Ac α 2-3Gal β 1-3]GalNAc-Ser/Thr

Table S-2: List of sense and antisense oligonucleotides used to generate cDNA encoding truncated form of the *C. maraena st8sia2 r-1*, *st8sia2 r-2* and *st8sia4* lacking their first 35, 35 or 28 amino acid residues, respectively. CDNAs were amplified by PCR using previous construct p3×FLAG-CMV10 as DNA templates for *st8sia4* and *st8sia2 r-1* and pcDNA3.1 for *st8sia2 r-2*, leading to $\Delta 35$ ST8Sia II-r1, $\Delta 35$ ST8Sia II-r2 or $\Delta 28$ ST8Sia IV constructs.

<i>st8sia4</i> :	Full-length	5'-CCCAAGCTTATGCTCTCACGGAAACGC-3' (sense)
		5'-CGGGGTACCTTAAGATTCGCACTTCGAAGTCG-3' (antisense)
	$\Delta 28$ ST8Sia IV	5'-GCAAAGCTTGAAGAACATCAGGAAGCTCAAGTC-3' (sense)
		5'-CGGGGTACCTTAAGATTCGCACTTCGAAGTCG-3' (antisense)
<i>st8sia2 r-1</i> :	Full-length	5'-CCCAAGCTTATGCAGTTAGAATTCCGAACGCTG-3' (sense)
		5'-CGGGGTACCTTACATTCCTGCATCACAAGAGCC-3' (antisense)
	$\Delta 35$ ST8Sia II-r1	5'-AAAAAGCTTGGAGGTTCCAGAACATTGTACTTG-3' (sense)
		5'-CTTGCCCCTTGCTCCATACCAC-3' (antisense)
<i>st8sia2 r-2</i> :	Full-length	5'-CCCAAGCTTATGCAGTTAGAATTCCGAACATTG-3' (sense)
		5'-CGGGGTACCTTACGTTCTGATCACAAGAGCC-3' (antisense)
	$\Delta 35$ ST8Sia II-r2	5'-AAAAAGCTTGGAGGTTCCAGAAAATTGTACATGCAC-3' (sense)
		5'-AAAGGTACCTTAAGTTCTGATCACAAGAGCCAGT-3' (antisense)

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