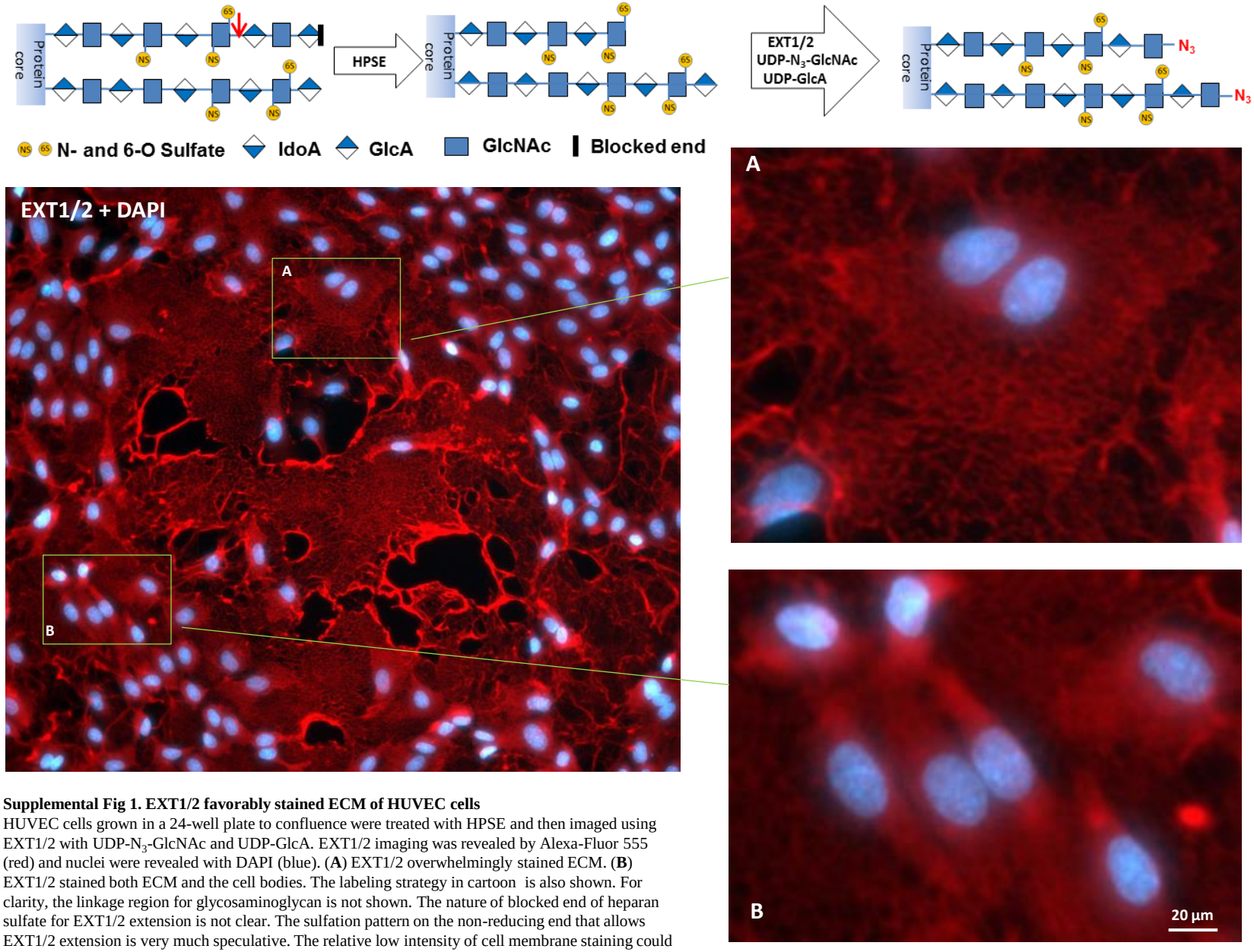
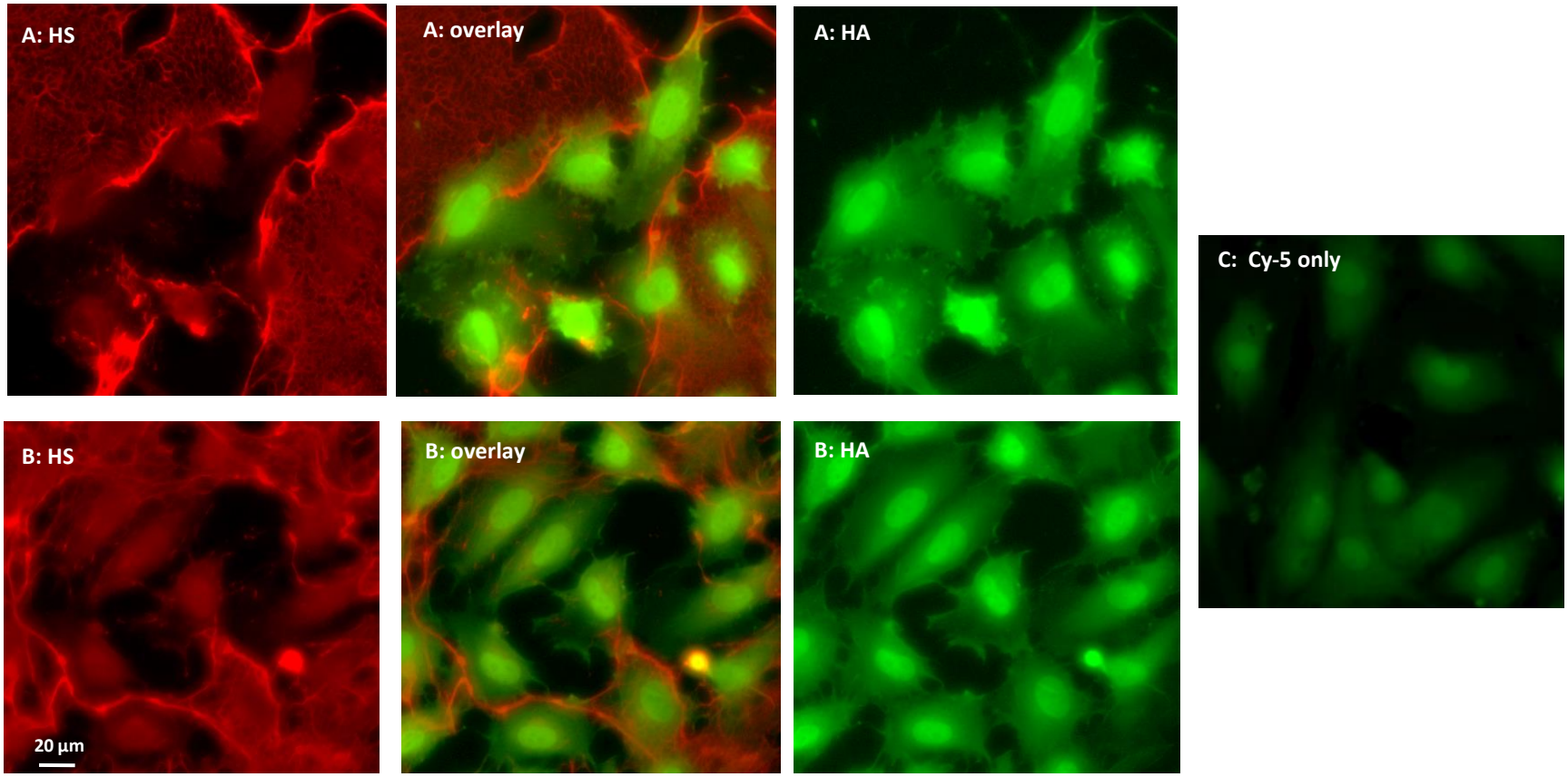
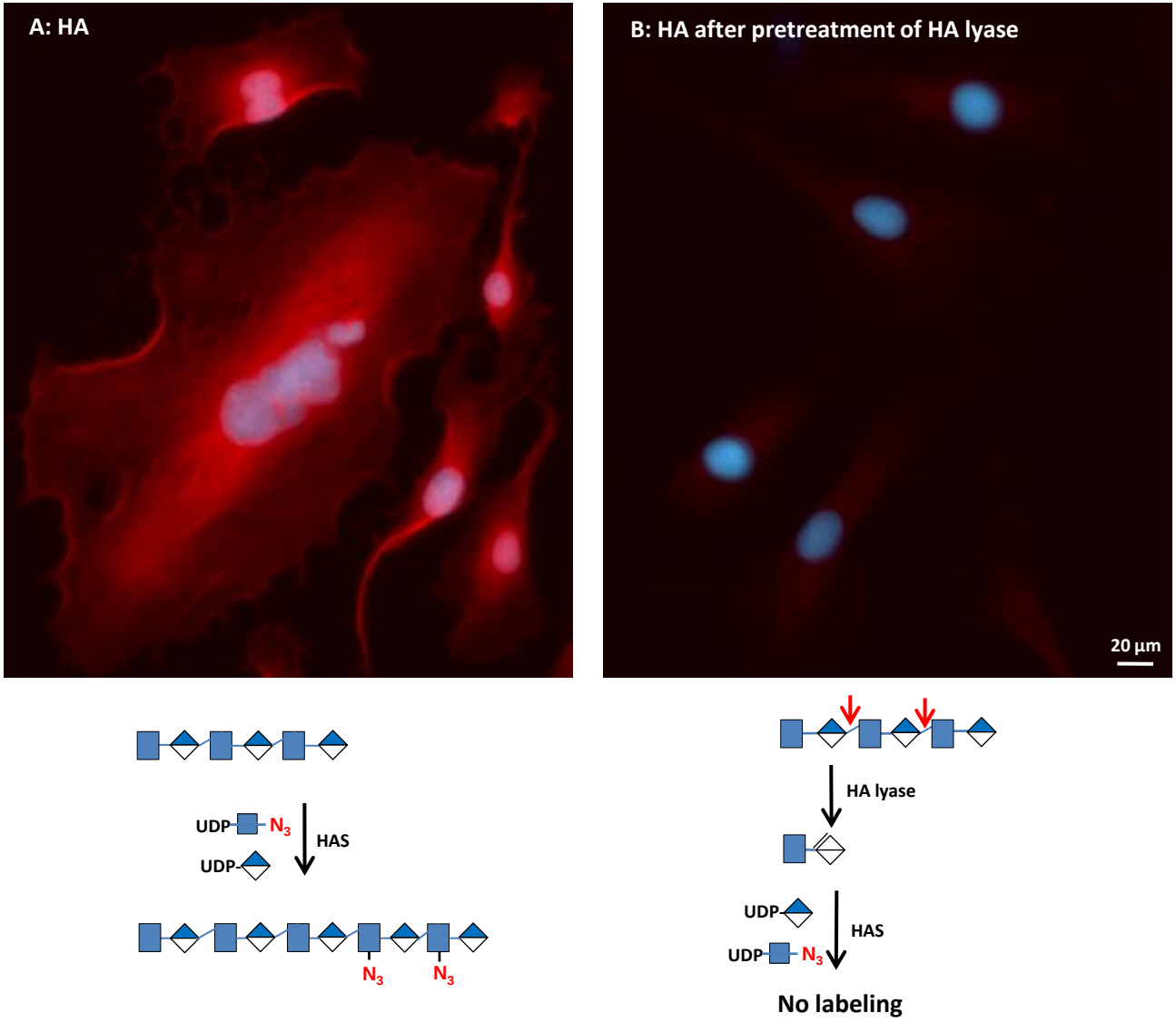


Supplemental Fig 1. EXT1/2 favorably stained ECM of HUVEC cells
HUVEC cells grown in a 24-well plate to confluence were treated with HPSE and then imaged using EXT1/2 with UDP-N_3-GlcNAc and UDP-GlcA. EXT1/2 imaging was revealed by Alexa-Fluor 555 (red) and nuclei were revealed with DAPI (blue). (**A**) EXT1/2 overwhelmingly stained ECM. (**B**) EXT1/2 stained both ECM and the cell bodies. The labeling strategy in cartoon is also shown. For clarity, the linkage region for glycosaminoglycan is not shown. The nature of blocked end of heparan sulfate for EXT1/2 extension is not clear. The sulfation pattern on the non-reducing end that allows EXT1/2 extension is very much speculative. The relative low intensity of cell membrane staining could be due to that higher percentage of cell membrane heparan sulfate chains are blocked and are resistant to HPSE digestion.



Supplemental Fig 2. HS and HA staining on HUVEC cells

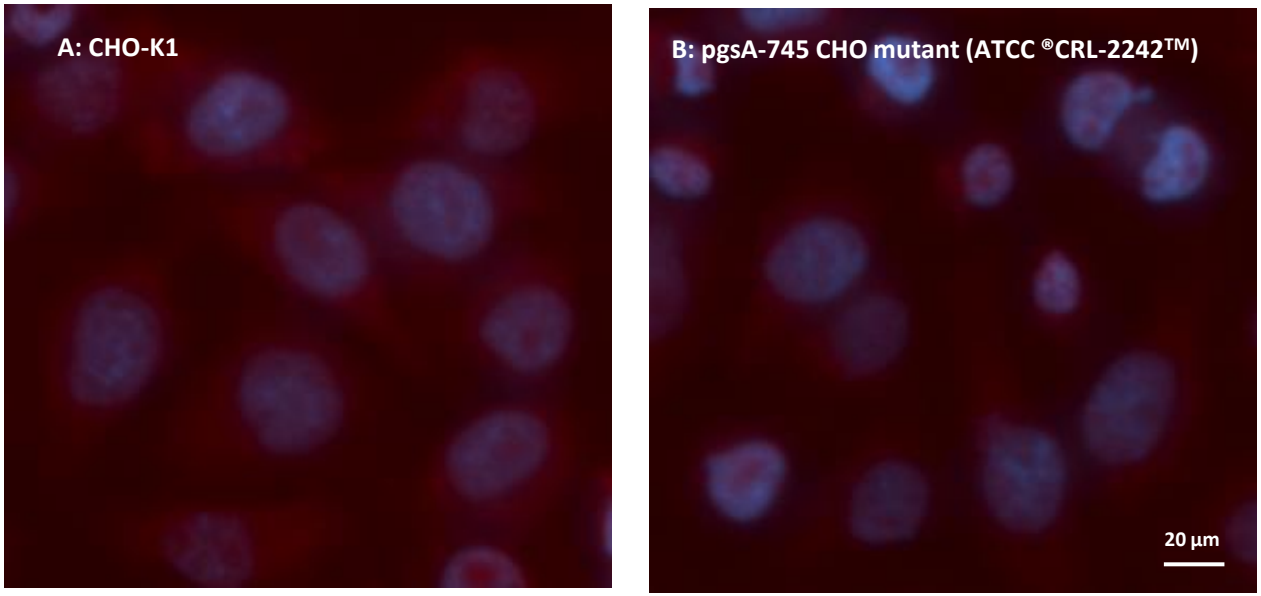
(A) And (B) HUVEC cells were grown in a 24-well plate to sub-confluence and were imaged for HS (red) and HA (green). HS was first labeled using EXT1/2 in the presence of UDP-N_3-GlcNAc and UDP-GlcA. The labeled HS was conjugated to biotin and revealed by streptavidin-Alexa Fluor 555. The cells were further labeled for HA using *Pasteurella multocida* hyaluronan synthase (HAS) in the presence of UDP-N_3-GlcNAc and UDP-GlcA. The labeled HA was directly conjugated to Cy5-Alkyne for imaging. (C) Negative control for Cy5-Alkyne staining. In the control, HUVEC cells were directly conjugated to Cy5-Alkyne. For viewing regions that were relatively weakly stained, the gamma ratting was set to 1.5.



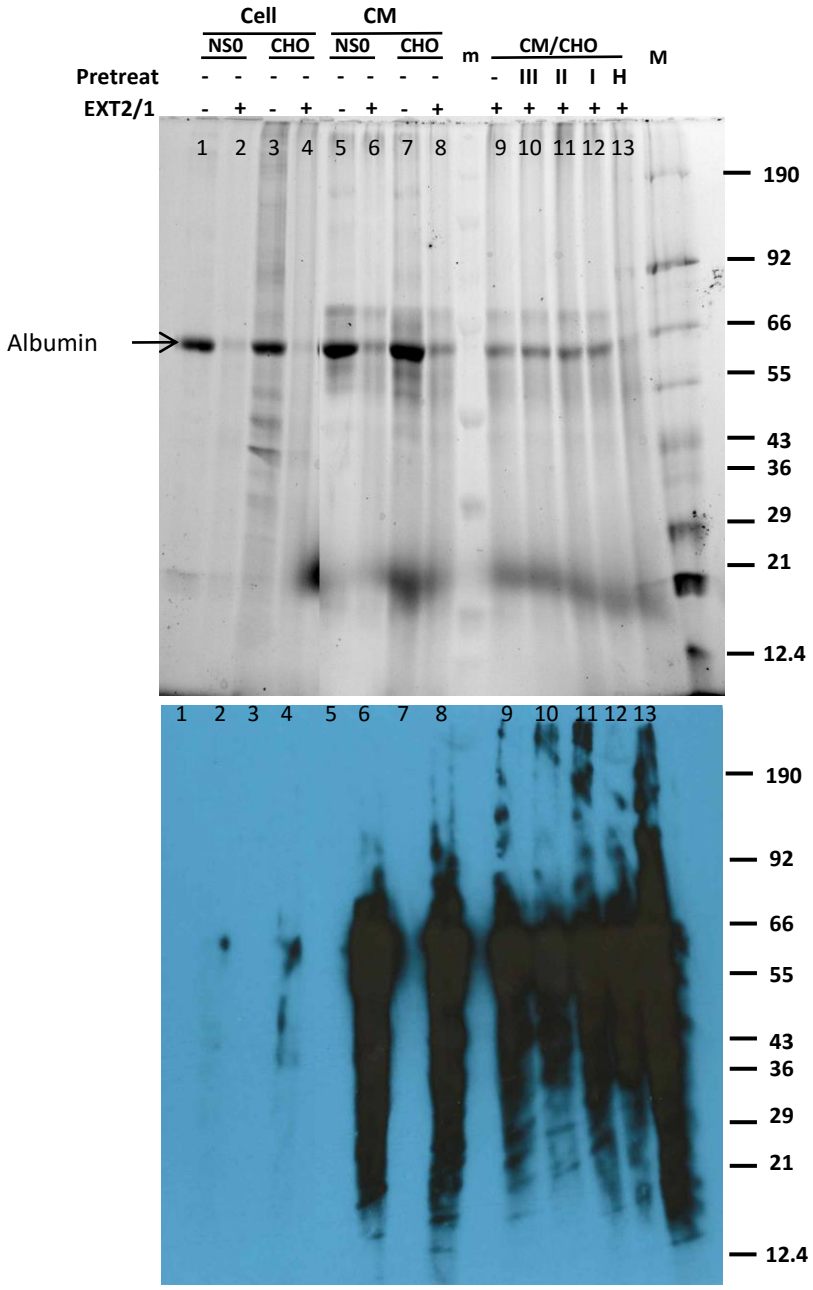
Supplemental Fig 3. HA staining on HUVEC cells was abolished by HA lyase.

HA was revealed by Alexa Fluor 555 (red) and nuclei were stained with DAPI (blue).

(**A**) HUVEC cells grown in a 24-well plate to confluence were labeled by HAS in the presence of UDP-azido-GlcNAc and UDP-GlcA. (**B**) The HUVEC cells in an adjacent well of the 24 well plate were first treated with HA lyase and then labeled by HAS in the presence of UDP-azido-GlcNAc and UDP-GlcA. HA lyase treatment resulted unsaturated $\Delta^{4,5}$-uronic acid at the non-reducing end of HA, rendering it unrecognizable by HAS.



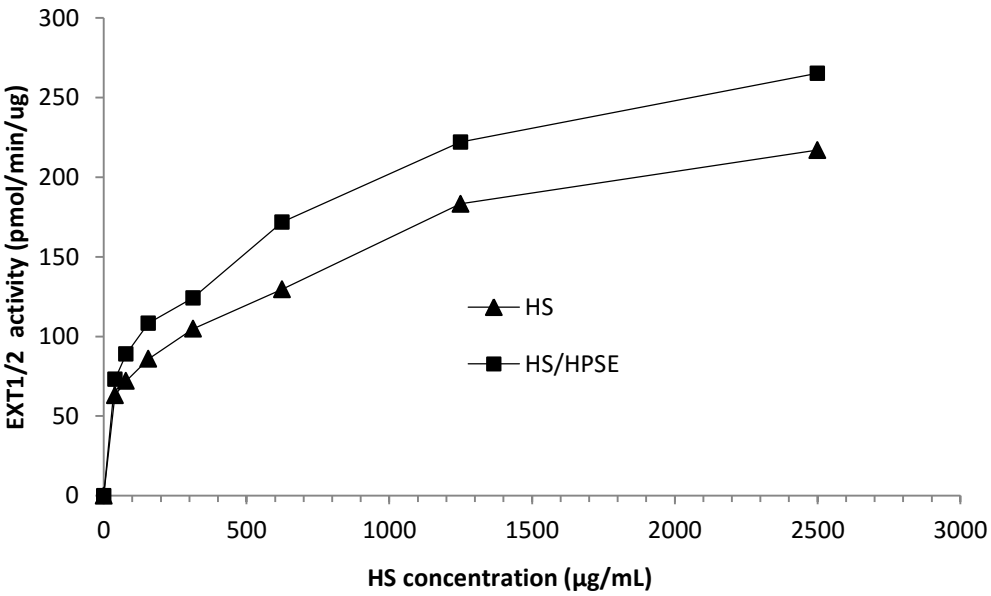
Supplemental Fig 4. HS staining on wild type (CHO-K1) (A) and CHO mutant cell (pgsA-745) (B). HS was labeled by EXT1/2 in the presence of UDP-N_3-GlcNAc and UDP-GlcA under exactly the same condition, then linked to biotin via click chemistry and revealed by Streptavidin conjugated Alexa Flour 555 (red) and nuclei were stained with DAPI (blue). For comparison, all imaging parameters including for exposure time and contrast adjustment were kept the same. CHO-K1 cells show only slightly stronger staining than pgsA-745 CHO mutant. Besides, no staining was found to be deposited on ECM in both cases.



Supplemental Fig 5. Detecting heparan sulfate in cell lysate and conditioned medium (CM) using EXT1/2

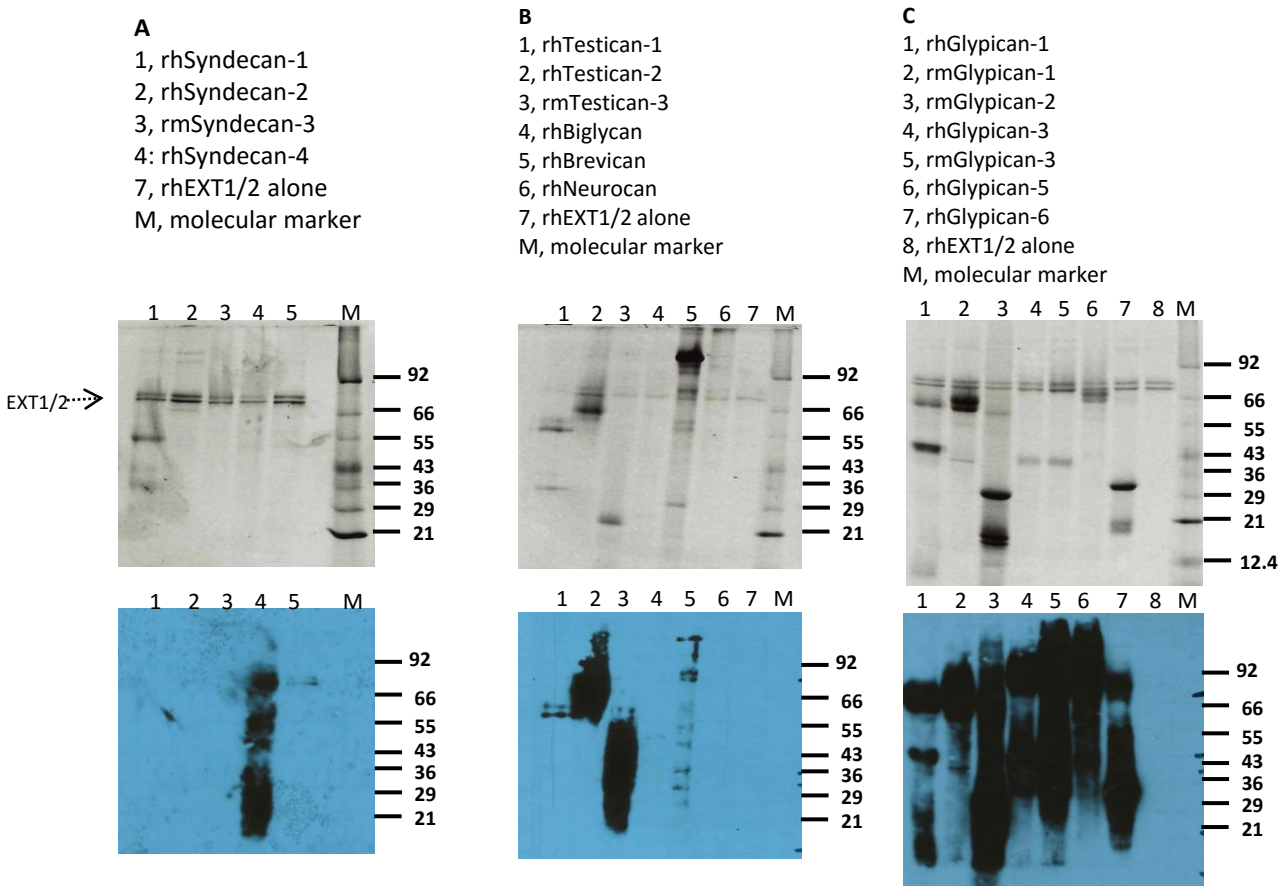
NS0 and CHO-K1 cells and their conditioned medium (CM) (corresponding to about 1 μg of protein for each labeling) without and with pretreatment of heparinase and heparanase were labeled with EXT1/2 in the presence of UDP-azido-GlcNAc (for detailed labeling procedures, refer to *Wu, ZL et al. Glycobiology* (2017) *27:518-524*). The labeled samples were then conjugated to biotin via click chemistry. All samples were separated on SDS-PAGE (upper panel), followed by blotting with Streptavidin-HRP (lower panel). While little labeling was observed on cell fraction, extensive labeling is observed in both the conditioned medium of NS0 and CHO-K1 cells. While heparinase III that is specific for heparan sulfate reduced the labeling, HPSE increased the labeling, which is consistent to Figure 4. The remaining labeling in heparinarse III pretreated sample maybe due to incomplete digestion.

I, heparinase I;
II, heparinase II;
III, heparinase III;
m, Bio-Rad pre-stained molecular marker;
M, regular molecular weight marker.



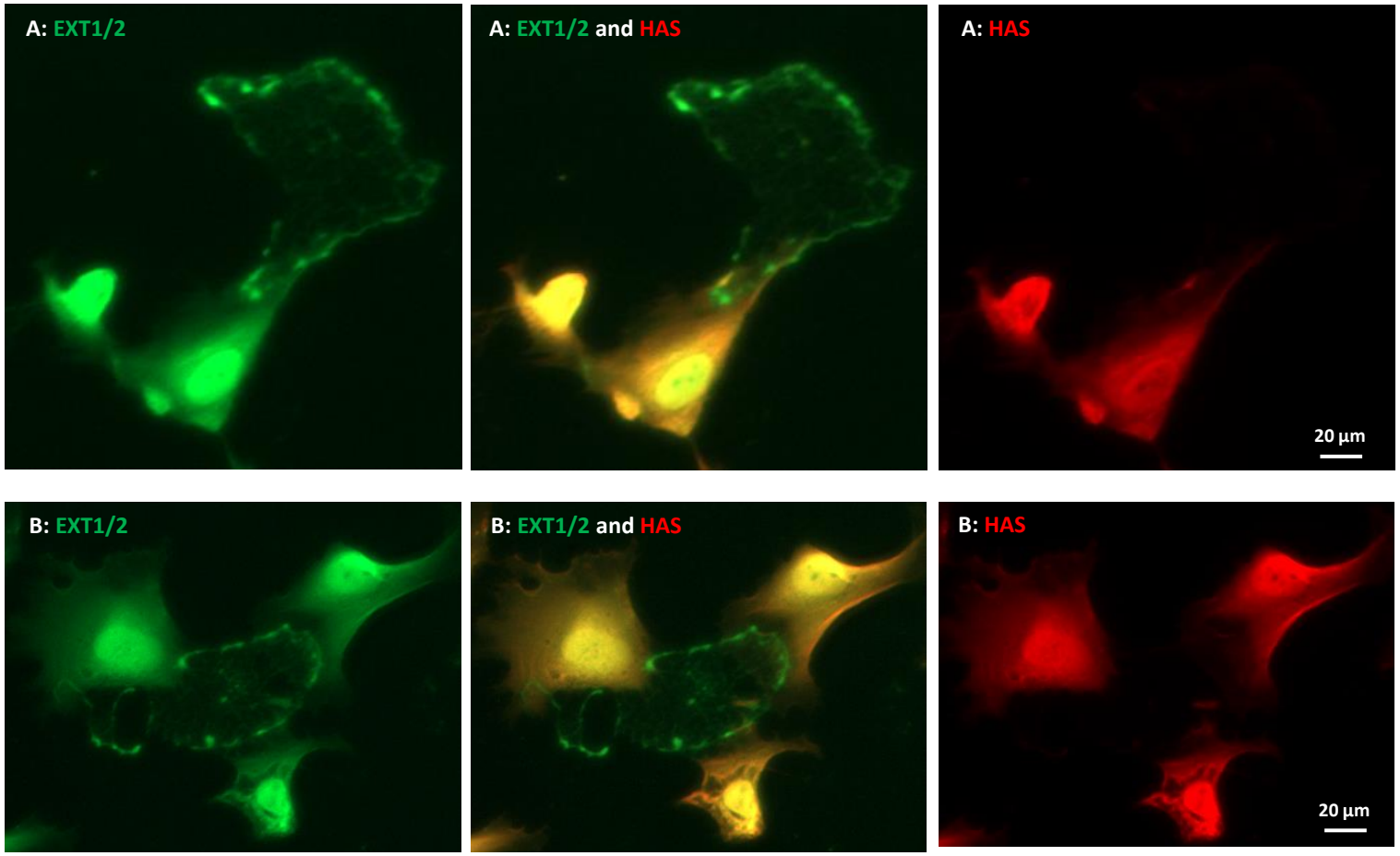
Supplemental Fig. 6. Effect of HPSE digestion of HS on EXT1/2 activity

EXT1/2 activity was compared on untreated HS (triangles) (HS was from Celsus Glycoscience, Inc.) and HPSE treated HS (squares, labeled as HS/HPSE) at different concentrations using EA001 Universal Glycosyltransferase Activity Kit (R&D Systems/Bio-Techne). For each reaction, various amount of untreated or HPSE treated HS was incubated with 0.3 µg of EXT2/1 in the presence of 0.2 mM UDP-GlcA, 0.2 mM UDP-GlcNAc, 0.1 µg Coupling Phosphatase 1 and 10 mM Mn^{2+} in 50 µL of 25 mM Tris buffer pH 7.5 at 37°C for 20 minutes. The phosphate released from the by-product UDP by Coupling Phosphatase 1 was measured using malachite reagents (Wu, Z. et al Glycobiology, 2010, 21: 727). The specific activities at different HS concentrations were then calculated and plotted against the HS inputs. HPSE treatment clearly increased the activity of EXT1/2, suggesting that the newly created non-reducing ends of HS by HPSE digestion made contributions to the increase of EXT1/2 activity. For HPSE treatment, 500 µg HS was mixed with 1 µg of HPSE in 20 µL of 50 mM MES pH 5.5 and incubated at 37°C for 20 minutes.

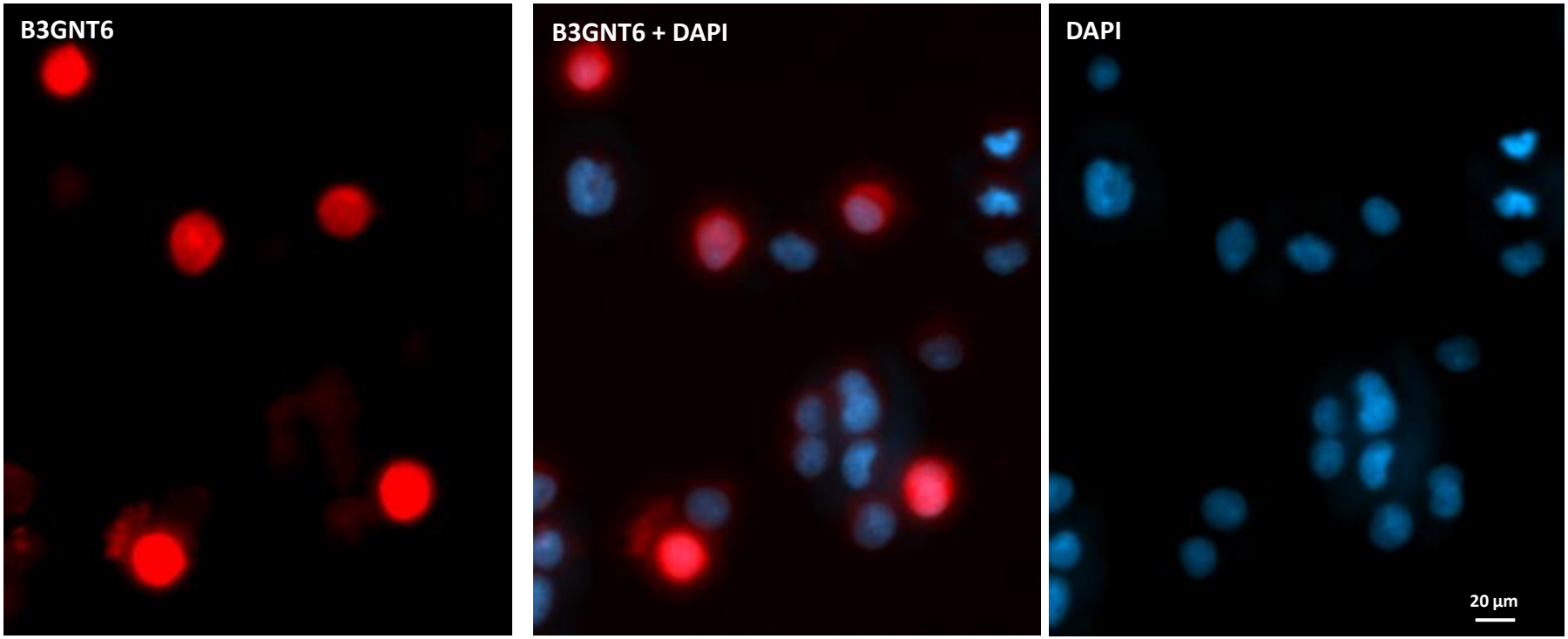


Supplemental Fig 7. Labeling of various heparan sulfate proteoglycans

To investigate whether there is any substrate preference for EXT1/2 labeling, various known heparan sulfate proteoglycans were labeled by EXT1/2 via click chemistry. The SDS gels were visualized via TCE (trichloroethanol) staining. TCE reacts with the indole ring of the amino acid tryptophan. However, many heparan sulfate proteoglycans lack tryptophan and were not revealed by TCE staining, or, were very poorly stained by TCE due to heavy glycosylation. In all three panels, the top portions are SDS-PAGE stained with TCE and the lower portions are membrane blots revealed with streptavidin-HRP (details of the methods can be found at *Wu, ZL et al. Carbohydrate Research (2015) 412:1-6*). A) Syndecans labeling (for detailed labeling procedures, refer to *Wu, ZL et al. Glycobiology* (2017) *27:518-524*). B) Testican, biglycan, brevican, and neurocan labeling. C) Glypican labeling. As results, Syndecan 4, testican 2 and 3, and all glypicans were labeled strongly. Those proteoglycans that were not labeled might not have heparan sulfate or contained heparan sulfate chains that couldn't be extended by EXT1/2. All tested proteoglycans are either commercially available from R&D Systems or can be obtained through customer requests.

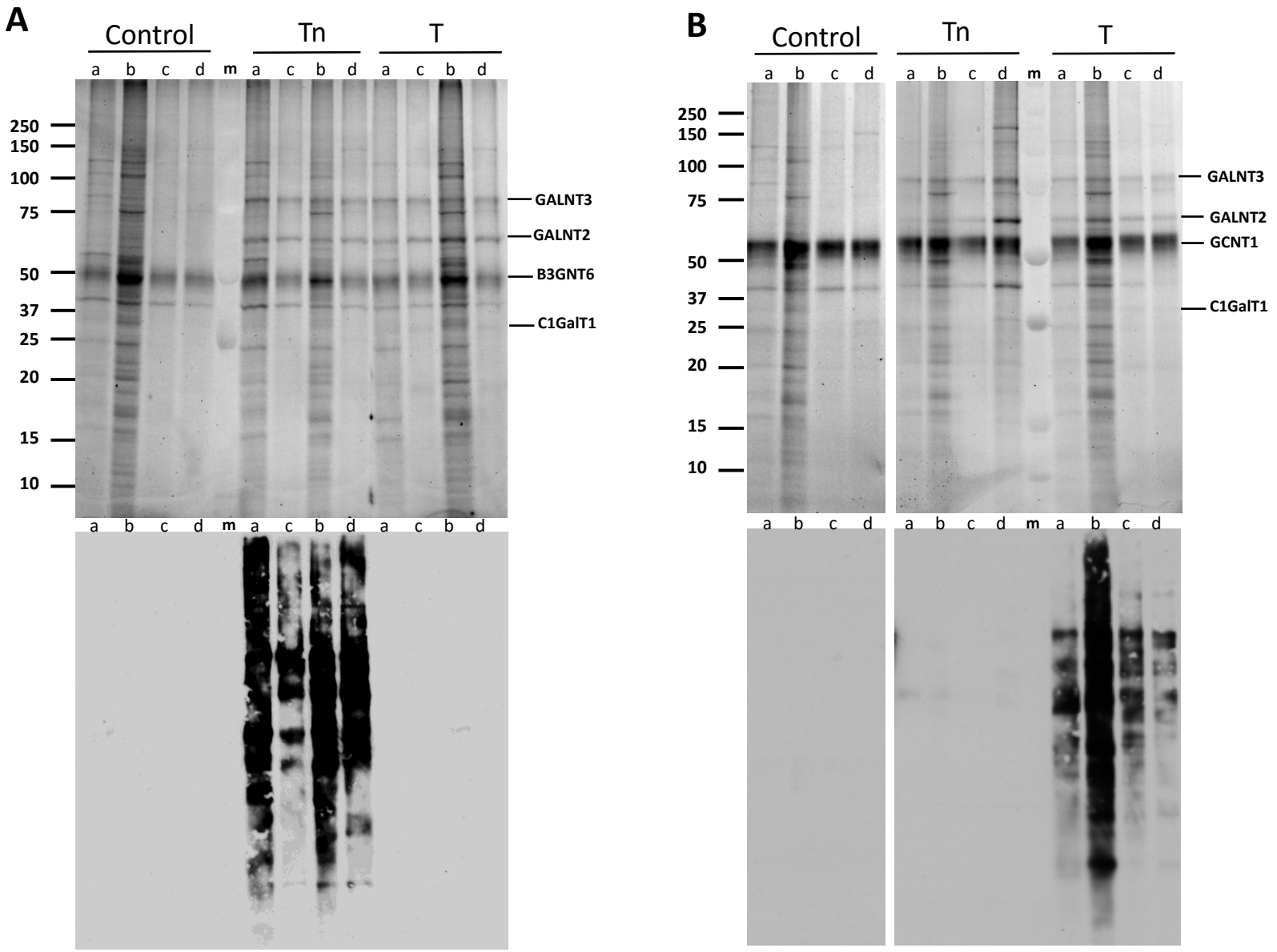


Supplemental Fig 8. Formation of extracellular matrix revealed by HS staining
HUVEC cells grown in a 24-well plate to a non-confluence stage were first treated with HPSE and then imaged for HS by EXT1/2 with UDP-azido-GlcNAc and UDP-GlcA. HS imaging was revealed by Alexa-Fluor 488 (green). Cell body was imaged by HAS with UDP-azido-GlcNAc and UDP-GlcA and revealed by Alexa-Fluor 555 (red). Two cases are shown here (**A** and **B**). In each case, the secreting HS resembles a casting net. All figures were normalized to the highest pixel value without change of gamma rating.

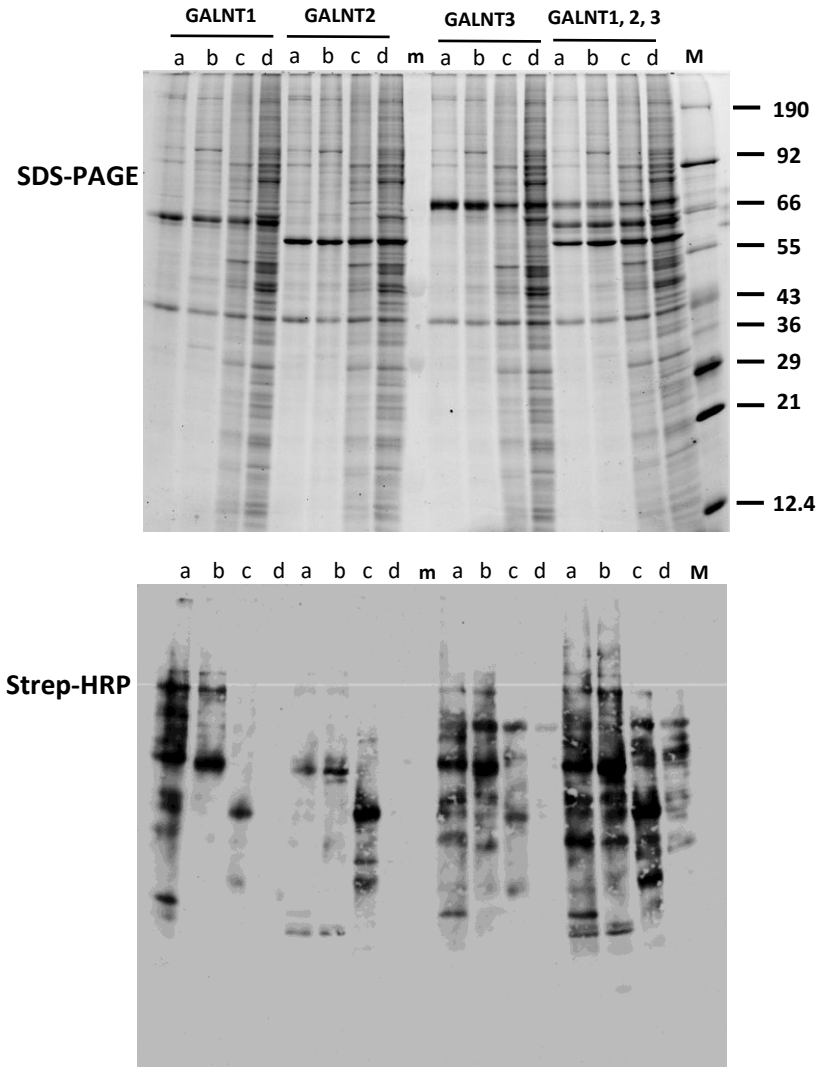


Supplemental Fig 9. Tn antigen on Jurkat cells revealed by B3GNT6

Jurkat cells grown in suspension were fixed with 4% paraformaldhyde. The cells were labeled for Tn antigen using B3GNT6 with UDP-azido-GlcNAc as the donor substrate and then conjugated to biotin via click chemistry and finally revealed by Alexa Fluor® 555 Streptavidin (ThermoFisher Scientific). Nuclei were stained with DAPI (blue). Some of the cells were strongly positive while the rest were much weakly stained.

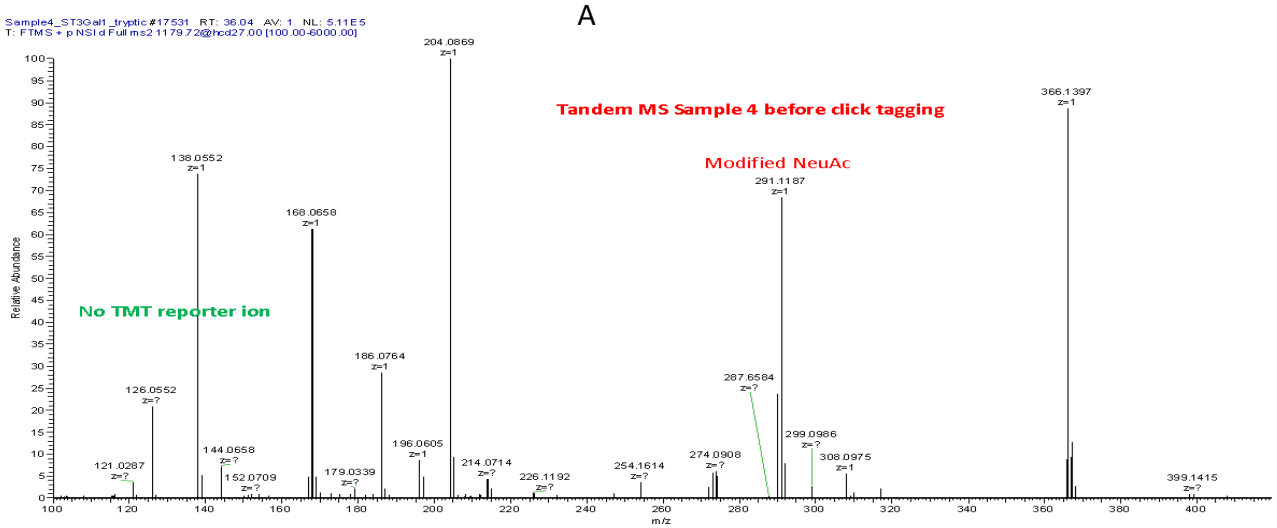


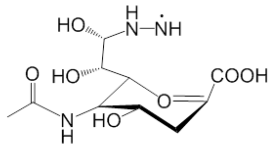
Supplemental Fig 10. Specific detection of T and Tn antigens installed on cellular extracts Tn or T antigens were installed to the nuclear and cytoplasmic extracts of HUVEC and HEK 293 cells and then detected with B3GNT6 and GCNT1, using the same strategy as Fig. 6A. (**A**) Detection of Tn antigen installed on the cell extracts by B3GNT6. (**B**) Detection of T antigen installed on cell extracts by GCNT1. Upper panels are SDS-PAGE of the samples, and lower panels are blotting with Streptavidin-HRP. a, cytoplasmic extract from HUVEC cells; b, cytoplasmic extract from HEK 293 cells; c, nuclear extract from HUVEC cells; d, nuclear extract from HEK cells; m, pre-stained molecular weight marker.



Supplemental Fig 11. Different substrate specificities and synergistic effect of GALNTs

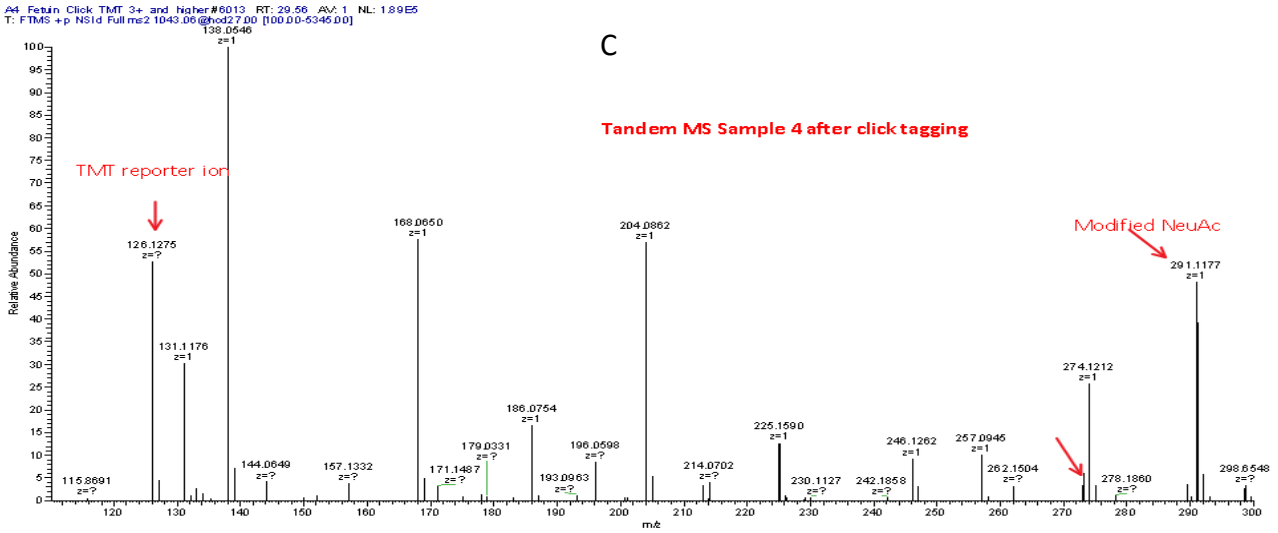
Nuclear and cytoplasmic extracts of HUVEC and HEK 293 cells were labeled with GALNT1, 2, and 3 individually or in combination in the presence of UDP-azido-GalNAc. All samples were then conjugated to biotin via click chemistry and separated on SDS-PAGE (upper panel), followed by blotting with Streptavidin-HRP (lower panel). The three enzymes showed different band patterns of labeling, which is particular evident comparing those of GALNT2 and GALNT3 labeling. More interestingly, the three enzymes showed synergistic effect on labeling (lane "d" under GALNT1,2,3). a, nuclear extract from HUVEC cells; b, nuclear extract from HEK cells; c, cytoplasmic extract from HUVEC cells; d, cytoplasmic extract from HEK 293 cells; m, Bio-Rad pre-stained molecular marker; M, regular molecular weight marker.

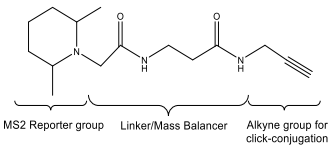




Chemical Formula: $C_{10}H_{17}N_3O_7^{\bullet}$
Exact Mass: 291.1066

Structure of 291 oxonium ion derived from NeuNAz (273 is a water loss from this ion) is a indication of the incorporation of NeuNAc





Structure of Alkyne-functionlized TMT reagent . TMT reagent can be linked to azido sugar via click chemistry. The presence of TMT in mass spectrometry of a labeled sample is another indication of the incorporation of azido sugars.

Supplemental Fig 12. Tandem MS analysis of a glycopeptide of fetal bovine fetuin labeled with NeuNAz. As a proof of a principle, asialo fetal bovine fetuin was labeled with NeuNAz (Azido functionalized NeuAc) with rhST6Gal 1 (R&D Systems) and then digested with trypsin. A)The digested sample was directly analyzed by tandem mass spectrometry. The presence of 291 oxonium ion is a indication of the incorporation of NeuNAz. B) The digested sample was first reacted to Alkyne functionalized Tandem Mass Tags (TMT zero alkyne, Thermo Scientific) via click chemistry and then analyzed by tandem mass spectrometry. Structure for the alkyne TMT zero tags and fragments generated upon collisional dissociation are again the indication of the incorporation of azido-sugar to the sample. The fragment at *m/z* 126.13 is a reporter ion used for confirming presence of TMT.

A

>NP_776409.1 alpha-2-HS-glycoprotein precursor [Bos taurus]
MKSFVLLFCLAQLWGCHSIPLDPVAGYKEPACDDPDTEQAALAAVDYINKHLPRGYKHTLNQIDSVKVWPRRPTGEVYDIEIDTLETTCHVLDPTPLANCSVRQQT
QHAVEGDCDIHVLKQDGQFSVLFTKCDSSPDSAEDVRKLCPDCPLLAPLNDSRVVHAVEVALATFNAESNGSYLQLVEISRAQFVPLPVSVSVEFAVAATDCIAKEV
VDPTKCNLLAEKQYGFCKGSVIQKALGGEDVRVTCTLFQTQPVIPQPQPDGAEAEAPSAVPDAAGPTPSAAGPPVASVVVGPSVVAVPLPLHRAHYDLRHTFSGV
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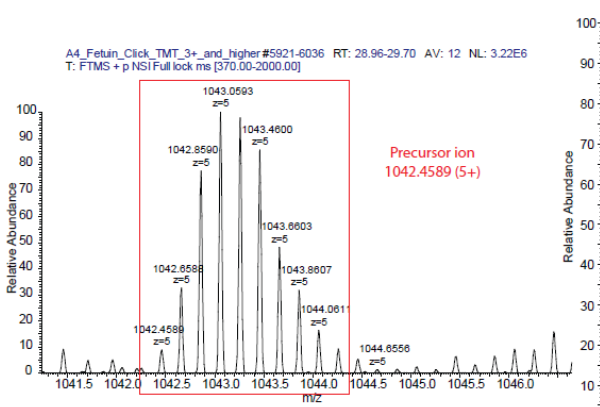
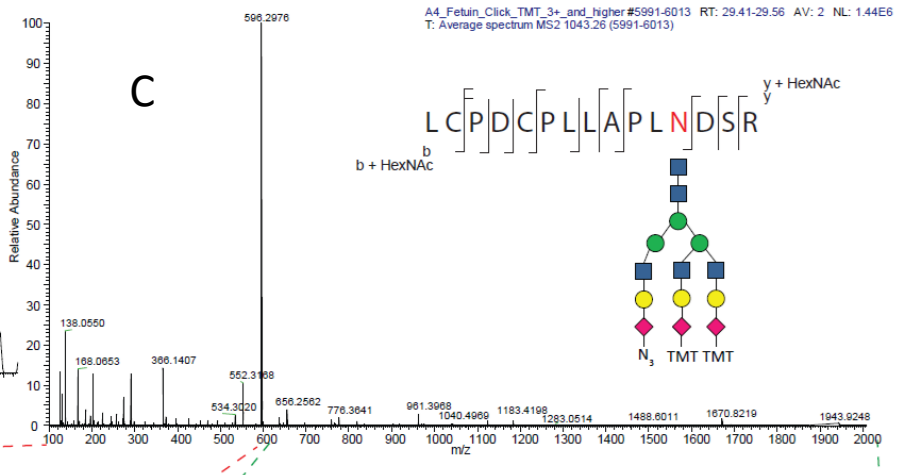
B



C



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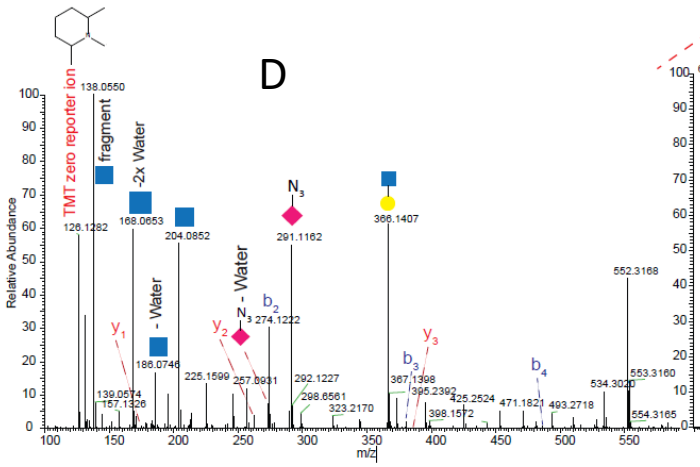
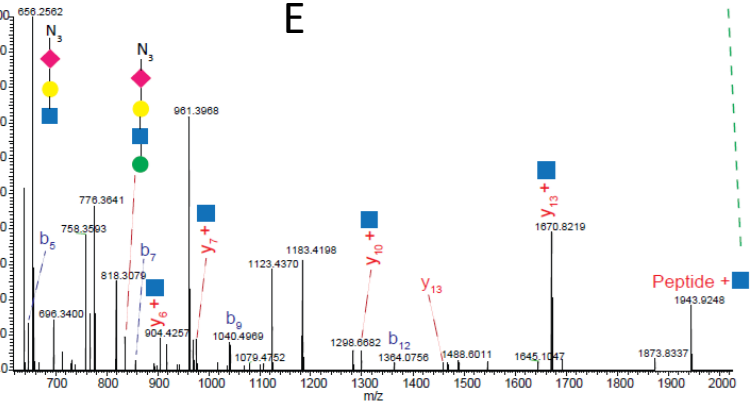


E



Supplemental Fig 13. MS and tandem MS of a fetuin glycopeptide labeled with azido sialic acids that were further clicked to alkyne functionalized TMT. A) sequence of the glycopeptide indicated by blue color. The glycosylation site is in red. B) The mass spectrum of the glycopeptide. C) MS2 of the glycopeptide. D) and E) are the enlarged regions of C).