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An enzymatic pathway in the human gut microbiome that converts A to universal O type blood

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SUPPLEMENTARY DISCUSSION

GH109 catalytic activity on RBC surface antigens

Tests of the activity of the newly discovered GH109 enzymes on red blood cells were disappointing since, apart from the *EmGH109*, which had already proven to be functional, none of the GH109 enzymes tested showed any activity on red blood cells at comparable enzyme concentration. This may be due to poor association with the RBC surface. Red blood cells have a net negative surface charge, largely due to the presence of sialic acids ¹. This would tend to repel any protein with like charge. Liu *et. al.* ² showed that the active site of *EmGH109* has a slight positive charge, allowing the enzyme to bind on the cell surface and cleave the α -N-acetyl-galactosamine. Indeed successful conversions required a low ionic strength buffer, consistent with this observation. Later, Chapanian *et al.* ³ showed that an increase of local enzyme concentration on RBCs could be achieved by using macromolecular crowding agents such as dextran 40k, allowing useful conversions by *EmGH109* in a “high” ionic strength buffer system. However, even with those additives none of the other GH109s discovered proved usefully active on RBCs, rendering them unsuitable for blood type conversion.

In vivo function of *FpGalNAcDeacetylase* and *FpGalNase*

The natural *in vivo* functions of the *FpGalNAcDeacetylase* and *FpGalNase* from *Flavonifractor plautii*, a common human gut microorganism, are still under investigation. The presence of signal peptides on the enzymes suggests that they are exported into the gut lumen, where they would be able to digest A antigen structures presented on mucins, other glycoproteins or cell surfaces ⁴. Investigations of the genes downstream of *FpGalNAcDeAc* and *FpGalNase* on the whole genome from *Flavonifractor plautii* strain YL31 revealed a major facilitator superfamily (MSF) transporter, potentially able to transport the released sugar into the bacterium along with an isomerase that could convert galactosamine-phosphate to its keto sugar, tagatose-phosphate (or bis-phosphate) and two aldolases that could catalyse the conversion of this phosphosugar to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Supplementary Fig. 20). Such enzymes have been proposed previously as being involved in the catabolism of galactosamines ⁵, thus it seems probable that the pathway in *F. plautii* is providing metabolic intermediates for cellular metabolism.

More broadly, enzymes that modify the acetylation state of sugars as part of anabolic or catabolic pathway are well known. One example is in heparan sulfate catabolism ^{6,7} where the glucosamine moiety exposed after de-N-sulfation of the polysaccharide is first acetylated by a glucosamine acetyltransferase, generating an acetamidoglycan that can then be cleaved by an α -N-acetyl-glucosaminidase. Another example is the formation of exopolysaccharide biofilms in fungi like *Aspergillus fumigatus*. An important step in the synthesis is the deacetylation of N-acetyl-galactosamine in galactosaminogalactans (GAG) to generate the final form ⁸.

Conversion of A-type blood

Not only do the *FpGalNAcDeacetylase* and *FpGalNase* exhibit very good kinetic parameters for the deacetylation and subsequent cleavage of the A antigen in solution, but also they function well, at very low enzyme concentrations, on actual red blood cells. Of yet more importance is the very high substrate specificity of *FpGalNAcDeAc* since, to this point, the only substrate cleaved

has been the α -N-acetyl-galactosamine on the A antigen. The hydrolase, *FpGalNase* has broader substrate acceptance, but as far as we are able to determine and detect, there are no other (non-acetylated) galactosamines on red blood cells. Certainly when we incubated O⁺ and B⁺ RBC samples with both enzymes no release of galactosamine could be detected (Supplementary Fig. 12). This is also consistent with the general opinion in the literature about the glycan structures on RBC's ^{9,10}.

Since the H antigen is only produced after the second reaction, performed by the *FpGalNase*, additional experiments were performed to verify the complete cleavage of the GalN antigen intermediate on the RBCs. A FACS analysis of A⁺RBC treated with either *EmGH109* or *FpGalNAcDeAc* and *FpGalNase* showed the same level of H antigen in the two cases. If GalN intermediates remained, a lower H antigen count would be observed for the two-enzyme system. Additionally, A⁺RBCs treated only with *FpGalNAcDeAc* thus presenting a GalN antigen, show strong agglutination reactions when added to autologous or allogeneic A serum samples. However, when they are treated with *FpGalNAcDeAc* in combination with *FpGalNase*, agglutination is not seen (Supplementary Fig. 21, Supplementary Table 18). We concluded from these experiments that no residual GalN antigen intermediate is present on A⁺RBCs that have been treated with *FpGalNAcDeAc* and *FpGalNase*.

Blood type conversion could be performed with equal efficiency either in blood plasma directly or in the standard buffer systems (at neutral pH, without EDTA) that are used for the preparation of RBCs in blood banks ¹¹. Thus it would be possible to add the enzyme mix directly to the blood bag shortly before starting A⁺ blood collection or at a later processing stage when the RBCs had been separated. Our conversion tests, using Anti-A antibody binding as our metric, suggested that at RT and even at 4°C most of the A antigens are converted after 30-60 min using enzyme concentrations of only 5 μ g/mL in samples with 10% hematocrit (Supplementary Fig. 13). This is a more than suitable timeframe, since most collected blood units are processed on the following day. Given the low enzyme concentrations required, their removal from the RBCs will be readily achieved in subsequent wash and separation steps.

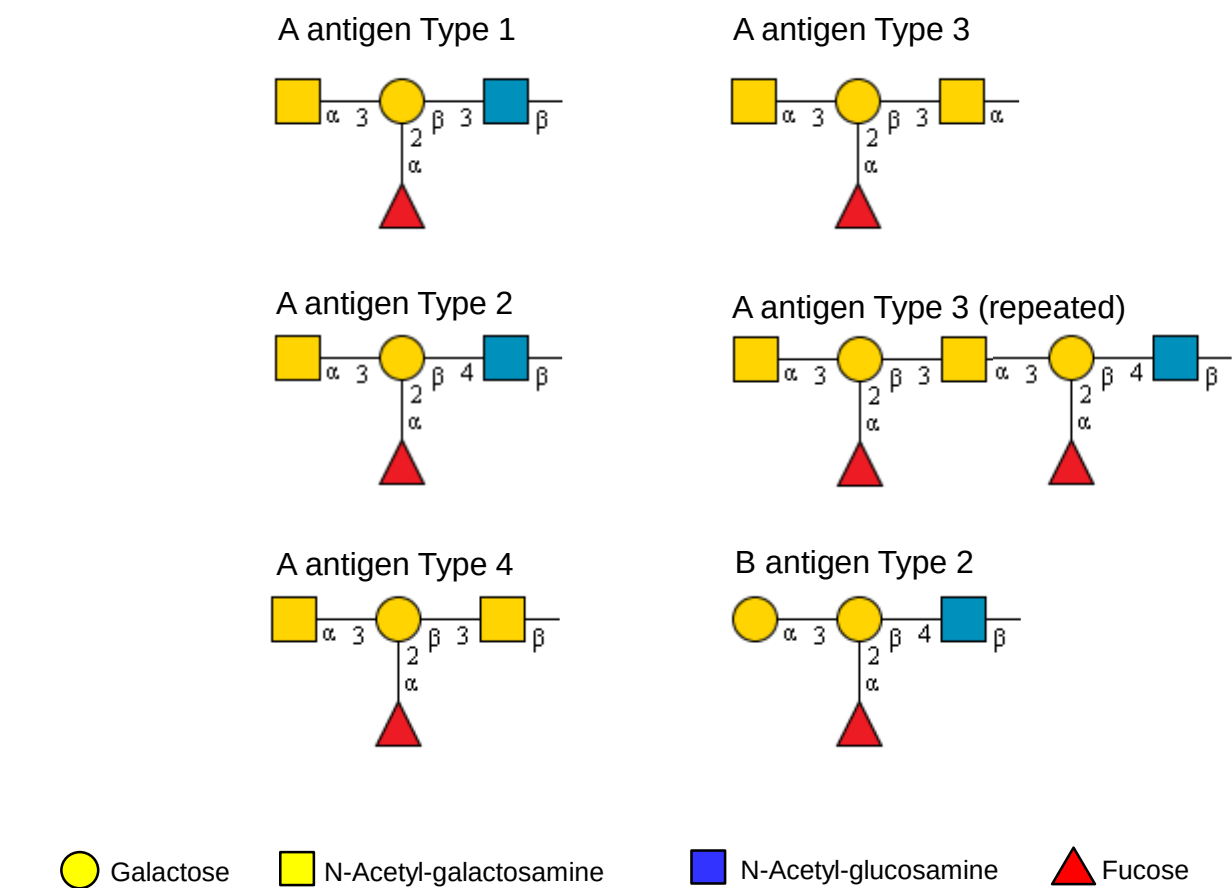
We assessed our ability to convert a whole unit of packed A⁺ RBCs (309 ml, 60% hematocrit) with our enzyme system. For this trial we elected to use a large amount of each enzyme (24 mg) to ensure complete and rapid conversion. Indeed, upon checking at 24 h at 4°C all A antigens had been converted to the H antigens (Supplementary Table 15). Based on the initial enzyme concentration tests, future whole blood bag conversion tests can be performed with much lower enzyme concentrations, (around 5 mg each or lower). None of the other published A antigen- cleaving enzymes function at such low concentrations, without use of special buffer conditions ^{2,12}.

SUPPLEMENTARY LITERATURE

- 1 Eylar, E. H., Madoff, M. A., Brody, O. V. & Oncley, J. L. The contribution of sialic acid to the surface charge of the erythrocyte. *J Biol Chem* **237**, 1992-2000 (1962).
- 2 Liu, Q. P. *et al.* Bacterial glycosidases for the production of universal red blood cells. *Nat Biotechnol.* **25**, 454-464, doi: http://www.nature.com/nbt/journal/v25/n4/supinfo/nbt1298_S1.html (2007).
- 3 Chapanian, R. *et al.* Enhancement of biological reactions on cell surfaces via macromolecular crowding. *Nat Commun* **5**, doi:Artn 4683Doi 10.1038/Ncomms5683 (2014).

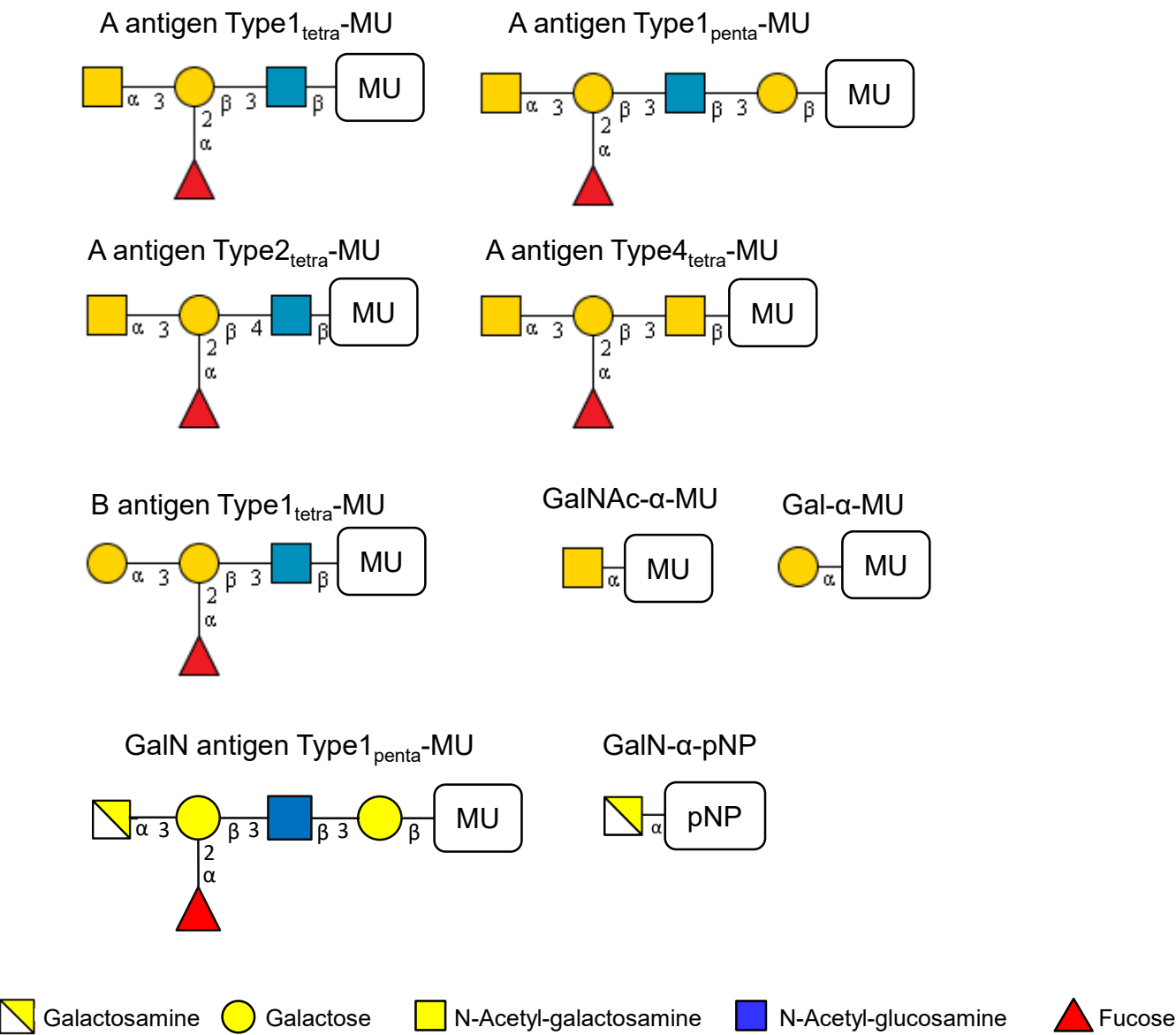
- 4 Rossez, Y. *et al.* Almost all human gastric mucin O-glycans harbor blood group A, B or H antigens and are potential binding sites for *Helicobacter pylori*. *Glycobiology* **22**, 1193-1206, doi:10.1093/glycob/cws072 (2012).
- 5 Brinkkotter, A., Kloss, H., Alpert, C. A. & Lengeler, J. W. Pathways for the utilization of N-acetyl-galactosamine and galactosamine in *Escherichia coli*. *Mol Microbiol* **37**, 125-135, doi:DOI 10.1046/j.1365-2958.2000.01969.x (2000).
- 6 Bame, K. J. & Rome, L. H. Acetyl Coenzyme a-Alpha-Glucosaminide N-Acetyltransferase - Evidence for a Transmembrane Acetylation Mechanism. *J Biol Chem* **260**, 1293-1299 (1985).
- 7 Kowalewski, B. *et al.* Arylsulfatase G inactivation causes loss of heparan sulfate 3-O-sulfatase activity and mucopolysaccharidosis in mice. *P Natl Acad Sci USA* **109**, 10310-10315, doi:10.1073/pnas.1202071109 (2012).
- 8 Lee, M. J. *et al.* Deacetylation of Fungal Exopolysaccharide Mediates Adhesion and Biofilm Formation. *mBio* **7**, e00252-00216, doi:10.1128/mBio.00252-16 (2016).
- 9 Haltiwanger, R. S., Wells, L., Freeze, H. H. & Stanley, P. In: Varki A, Cummings RD, Esko JD, *et al.*, editors. *Essent. Glycobiol.* Chapter 13, 151-160, doi: 10.1101/glycobiology.3e.013 (2015).
- 10 Aoki, T. A Comprehensive Review of Our Current Understanding of Red Blood Cell (RBC) Glycoproteins. *Membranes* **7**, doi:Artn 5610.3390/Membranes7040056 (2017).
- 11 Hess, J. R. Conventional blood banking and blood component storage regulation: opportunities for improvement. *Blood Transfus-Italy* **8**, S9-S15, doi:10.2450/2010.003S (2010).
- 12 Yu, C. Y., Xu, H., Wang, L. S., Zhang, J. G. & Zhang, Y. P. Human RBCs blood group conversion from A to O using a novel alpha-N-acetylgalactosaminidase of high specific activity. *Chinese Sci Bull* **53**, 2008-2016, doi:10.1007/s11434-008-0248-y (2008).
- 13 Mitchell, A. L. *et al.* InterPro in 2019: improving coverage, classification and access to protein sequence annotations. *Nucleic Acids Res* **47**, D351-D360, doi:10.1093/nar/gky1100 (2019).
- 14 Ren, J. *et al.* DOG 1.0: illustrator of protein domain structures. *Cell Res* **19**, 271-273, doi:10.1038/cr.2009.6 (2009).

Supplementary Figure 1 : Different linkage types of blood group antigens



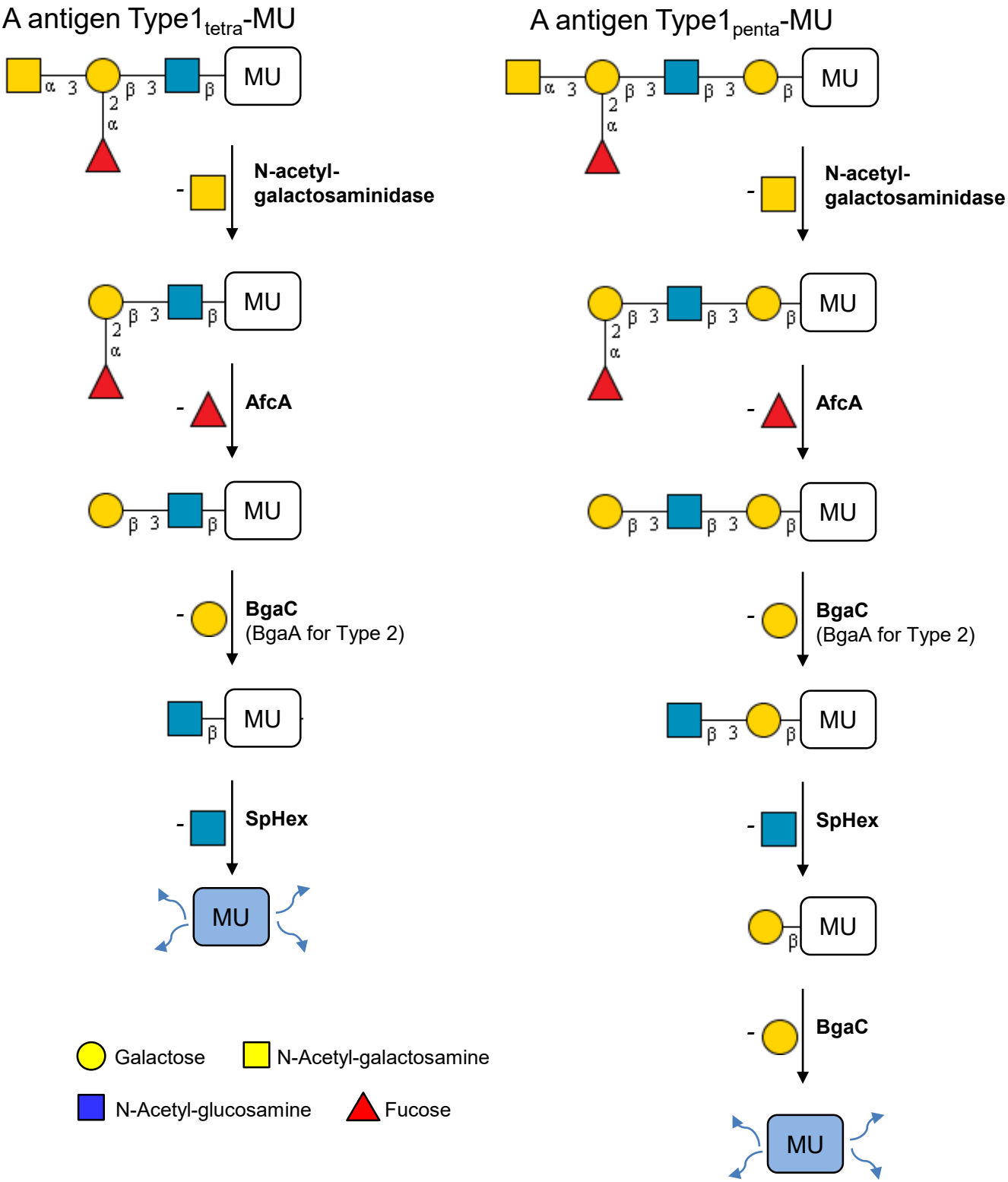
Overview of the principal structures of A antigen sub types. Their display varies based on the tissue type. The main difference lies in the bond connecting the 4th sugar from the non-reducing end; Type 1 linkage is -β1,3-GlcNAc-β-, Type 2 is -β1,4-GlcNAc-β-, Type 3 is -β1,3-GalNAc-α-, Type 3 (repeated) is a Type 2 followed by an Type 3 linkage and Type 4 is -β1,3-GalNAc-β-. B antigen are, in most cases, only linked in a Type 2 fashion. Sugars shown, using the Consortium for Functional Glycomics notation⁶⁴.

Supplementary Figure 2 : Substrates



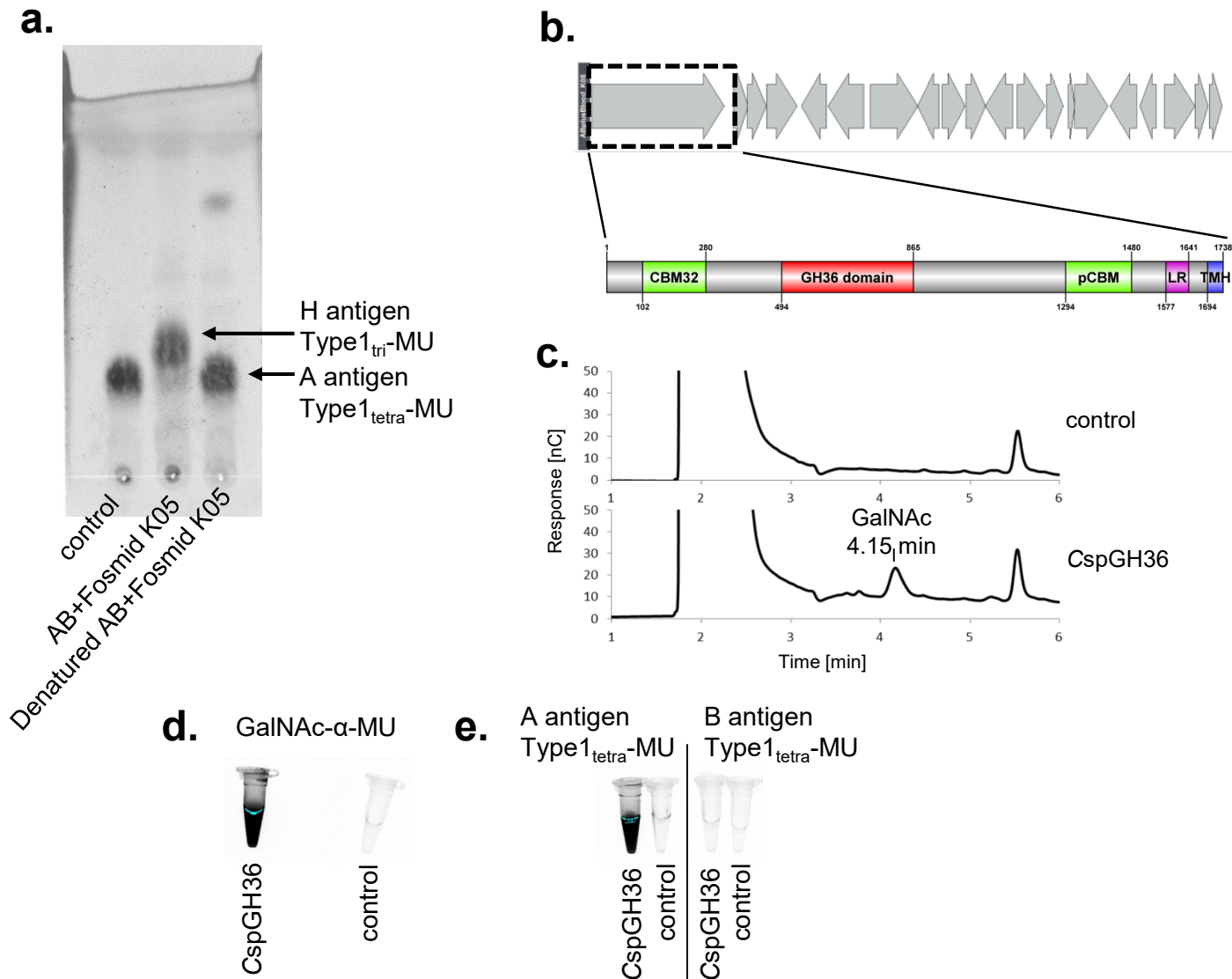
Overview of the substrates used in this study for screening or kinetic analysis. All are used as their MU (methylumbelliferyl alcohol) glycosides, except the galactosaminide (GalNH₂) which employs a pNP (*para*-nitrophenol) leaving group . Sugars are shown using the Consortium for Functional Glycomics notation⁶⁴.

Supplementary Figure 3 : Coupled enzyme assay



Detection of cleavage of the terminal sugar group (GalNAc for A and Gal for B antigen, not presented here) for the A antigen Type1_{tetra}-MU or A antigen Type1_{penta}-MU was achieved with a coupled enzyme assay. The addition of a α-fucosidase (AfcA), β-galactosidase (BgaA or BgaC) and β-hexosaminidase (SpHex) allowed the stepwise cleavage of the glycan only if the terminal GalNAc or Gal had been removed. This resulted in the release of free fluorophore 4-Methylumbelliferyl alcohol (MU). Sugars are shown using the Consortium for Functional Glycomics notation⁶⁴.

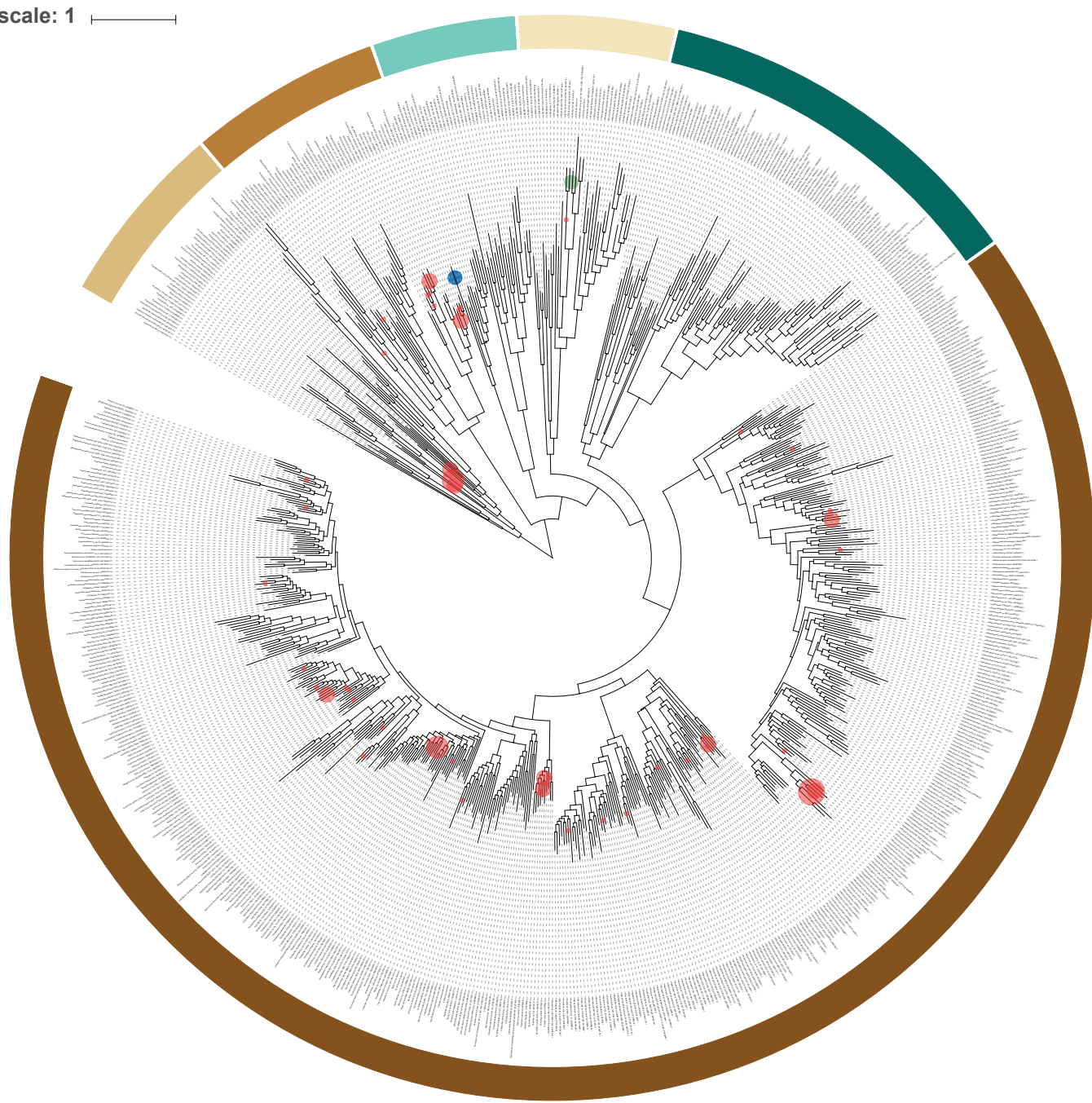
Supplementary Figure 4 : Analysis of A antigen Type1_{tetra}-MU activity on AB⁺Fosmid_K05



Reactions were carried out using the standard conditions described. As controls the expression of an empty vector was used: for Fosmids pCC1FOSTM and for purified proteins pET28a. **a.** Cell lysates from cultured AB⁺Fosmid_K05 (native and boiled) and a control were incubated with A antigen Type1_{tetra}-MU and analysed via TLC. The uncropped version of the TLC is presented in Supplementary Figure 22. **b.** The sequence identified in the sub-library of AB⁺Fosmid_K05 incubated with A antigen Type1_{tetra}-MU in a coupled screening assay is marked in the dotted box: it contained only one ORF. Protein domain structure of CspGH36. Catalytic domain is red, Carbohydrate-binding modules (CBM) are green, Listeria repeat (LR) lilac and a transmembrane helix (TMH) blue. **c.** HPAE-PAD assay with mucin from porcine stomach Type II with and without CspGH36. Sugars were identified based on standards. **d.** Activity of CspGH36 and a control on GalNAc-α-MU. The cleaved methylumbelliferyl alcohol was visualised by 365 nm excitation. **e.** Activity test of CspGH36 and a control on A antigen Type1_{tetra}-MU and B antigen Type1_{tetra}-MU. The assays were performed together with SpHex, AfcA and BgaC. Experiments were performed 2 (**a.**, **d.**, **e.**) or 3 (**c.**) times independently from each other with similar results.

Supplementary Figure 5 : GH36 phylogeny

Tree scale: 1



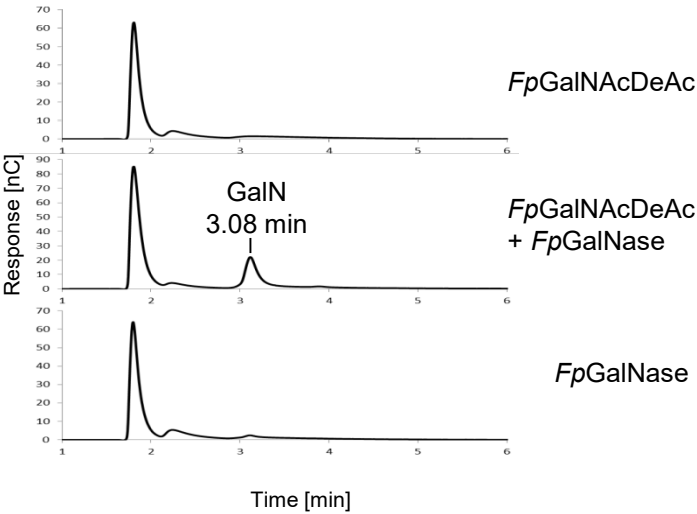
Clusters

Cluster 1	Cluster 3	Putative Cluster 5
Cluster 2	Cluster 4	Putative Cluster 6

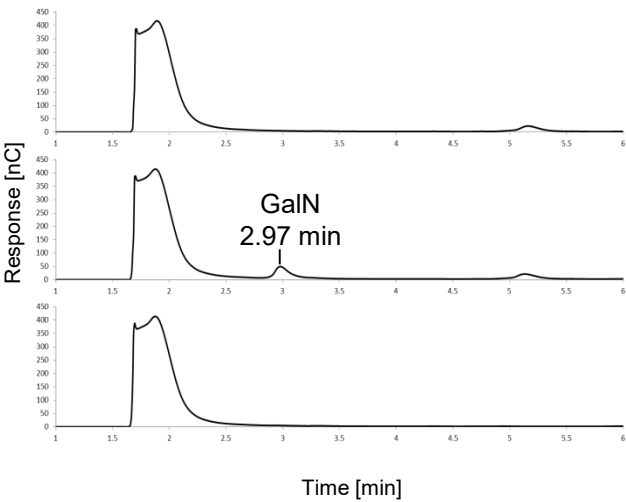
Phylogeny of 738 GH36 genes from CAZy database.. The different clusters of GH36 subfamilies are represented by the outer colored bars. Red dots mark locations of characterized GH36 enzymes used to identify/assign the clusters. The sequence marked with the blue dot is the newly discovered *FpGalactosaminidase* (*FpGalNase*), a member of the putative cluster 5 and the green dot marks the position of *CspGH36*, located in cluster 4. An additional new putative cluster was also identified and numbered here with a 6. The selection of 738 genes was chosen from a set of 3537 sequences, downloaded from CAZy database, filtered using a hidden Markov Model (HMM) and then clustered at 70% similarity

Supplementary Figure 6 : HPAE-PAD analysis

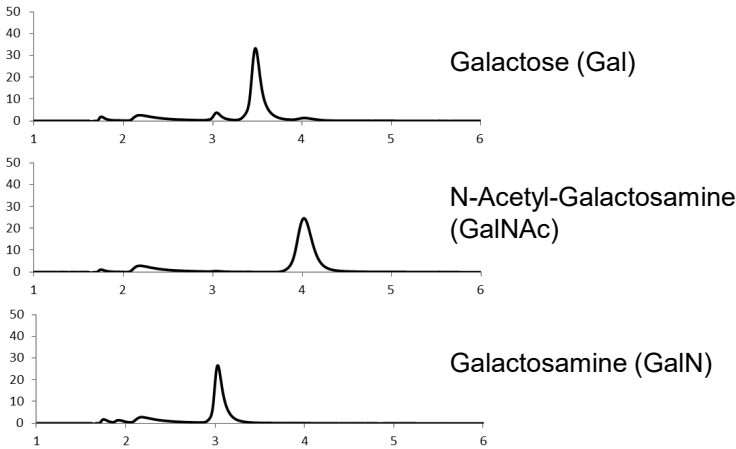
a. A antigen Type1_{tetra}-MU



b. Mucin

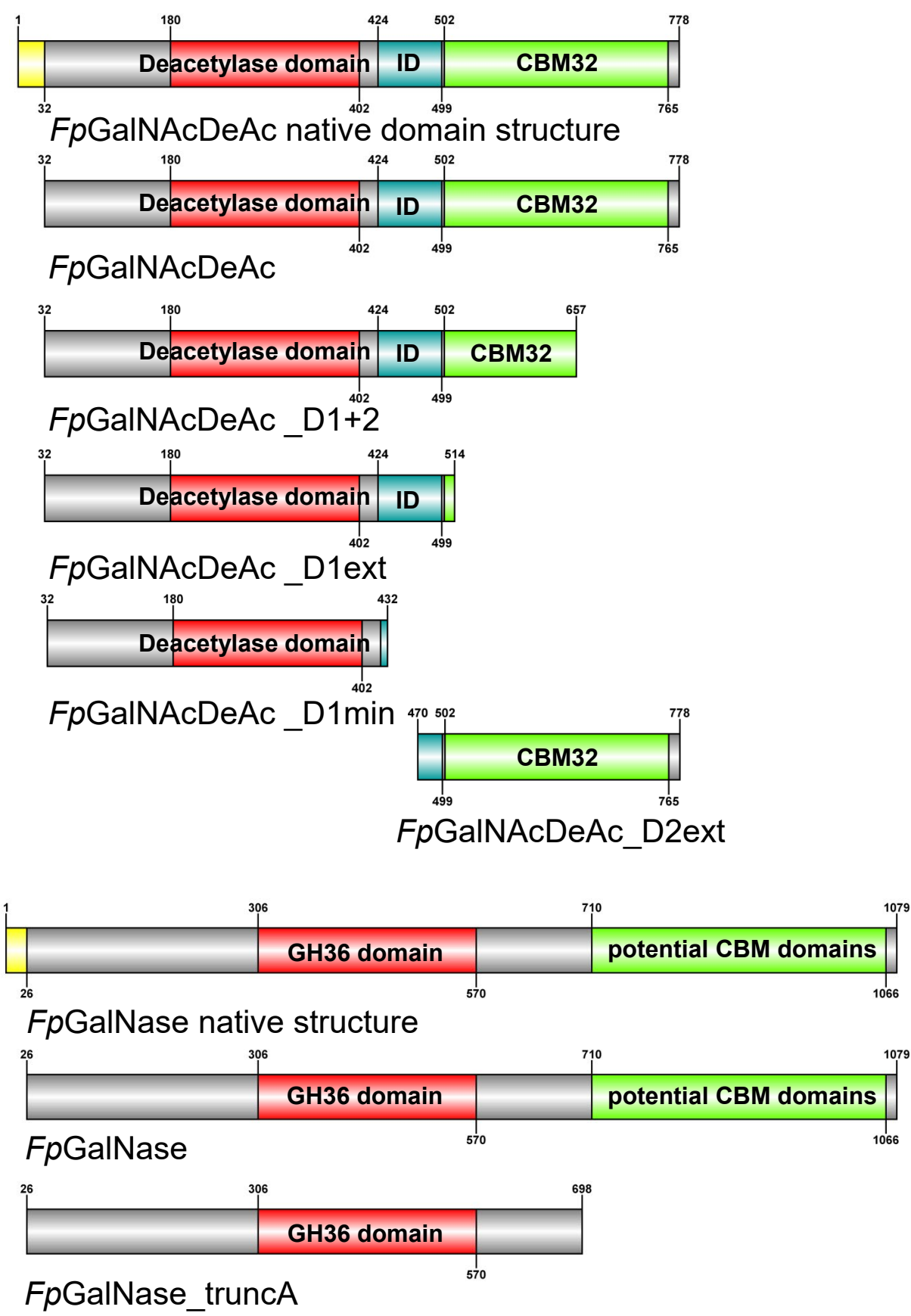


c. Standards



HPAE-PAD chromatography was used to identify monosaccharides released upon cleavage of substrate. **a**, Using A antigen Type1_{tetra}-MU as substrate, only the combination of *FpGalNAcDeAc* and *FpGalNase* catalysed cleavage, releasing galactosamine. **b**, Using mucin from porcine stomach Type II as substrate, only the combination of *FpGalNAcDeAc* and *FpGalNase* could release galactosamine. **c**, Sugars were identified based on standards. All experiments were performed 3 times independently from each other with similar results.

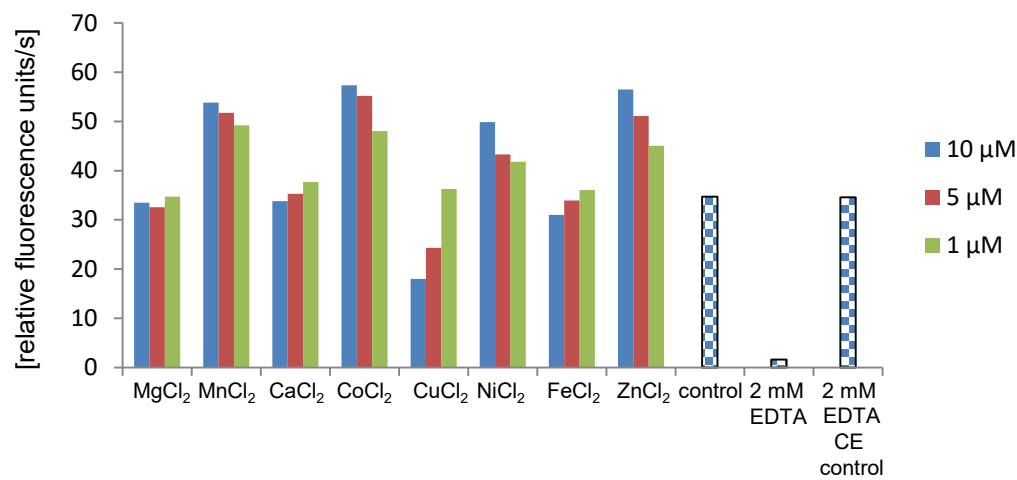
Supplementary Figure 7 : Protein domain structures



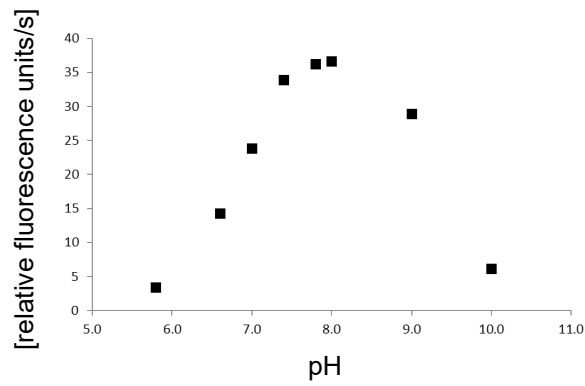
The protein domain structure is presented based on the homologous superfamilies identified by InterPro prediction¹³ via DOG2.0¹⁴. Signal peptides are shown in yellow, catalytic domains in red, carbohydrate-binding modules in green and the Invasin domain in blue. The truncations of the proteins employed are displayed below.

Supplementary Figure 8 : *Fp*GalNAc deacetylase characterization

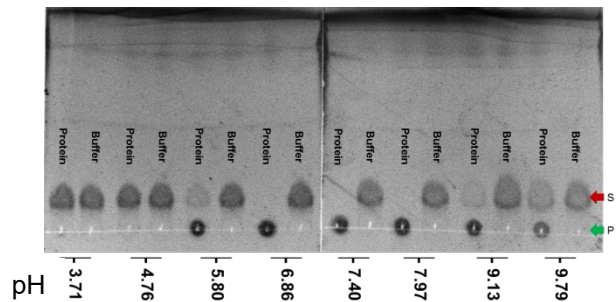
a.



b.

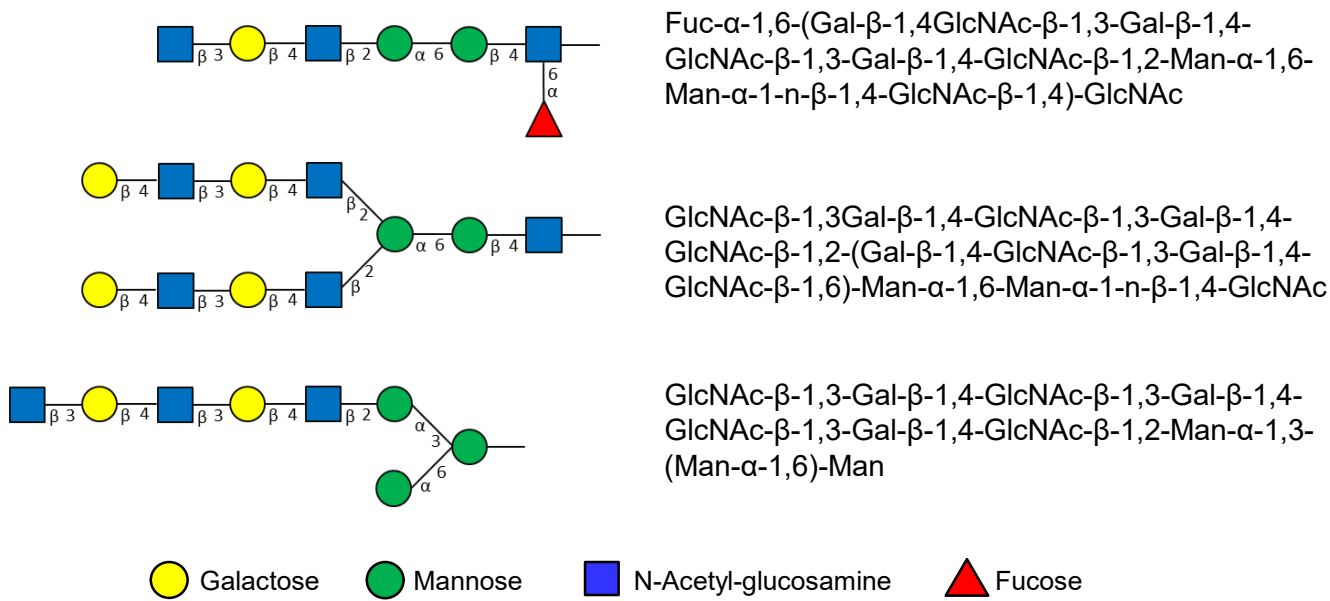


c.



Characterisation of the activity of *Fp*GalNAc deacetylase (*Fp*GalNAcDeAc) on A antigen Type1_{penta}-MU substrate. **a**, Dependence of *Fp*GalNAcDeAc on divalent metal ions. Checkered columns are those of control reactions. **b**, pH-dependence of enzyme activity (shown as relative fluorescence units per second). **c**, TLC analysis of pH-dependence. The red arrow marks the substrate and the green the product. The uncropped version of the TLC is presented in Supplementary Figure 22. Reactions were carried out using standard conditions. Experiments were performed 2 times independently from each other with similar results.

Supplementary Figure 9 : *FpGalNAcDeAc_D2ext* Glycan array data

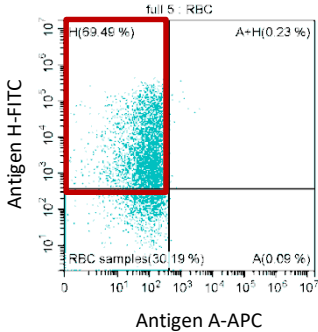


The top three glycan hits identified on the CFG array when screened against FITC-labeled *FpGalNAcDeAc_D2ext*. Analysis of binding motifs was performed with the webtool <https://glycopattern.emory.edu/>. Sugars are shown using the Consortium for Functional Glycomics notation⁶².

Supplementary Figure 10 : Influence of the putative CBM domain from *FpGalNase* on enzymatic conversion of RBCs

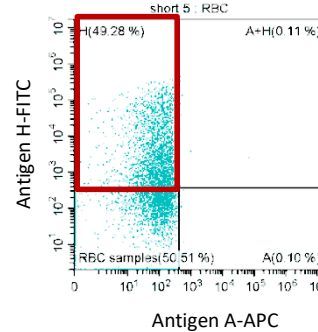
FpGalNAcDeAc and *FpGalNase*

a. 5 µg/mL

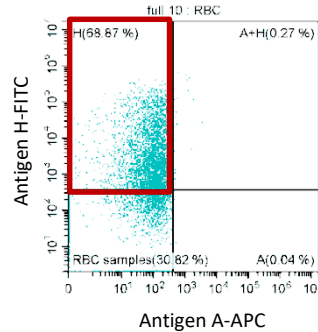


FpGalNAcDeAc and *FpGalNase truncA*

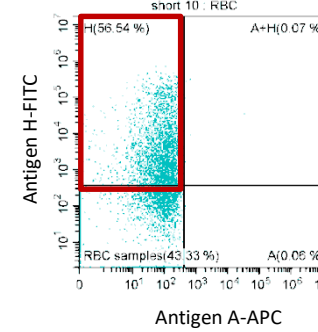
b. 5 µg/mL



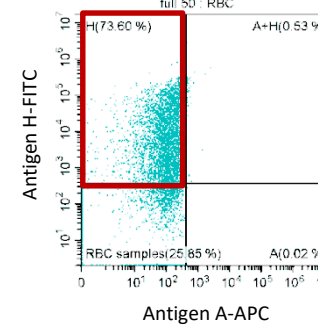
c. 10 µg/mL



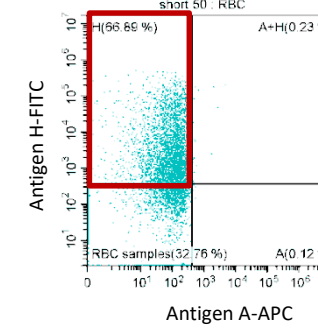
d. 10 µg/mL



e. 50 µg/mL

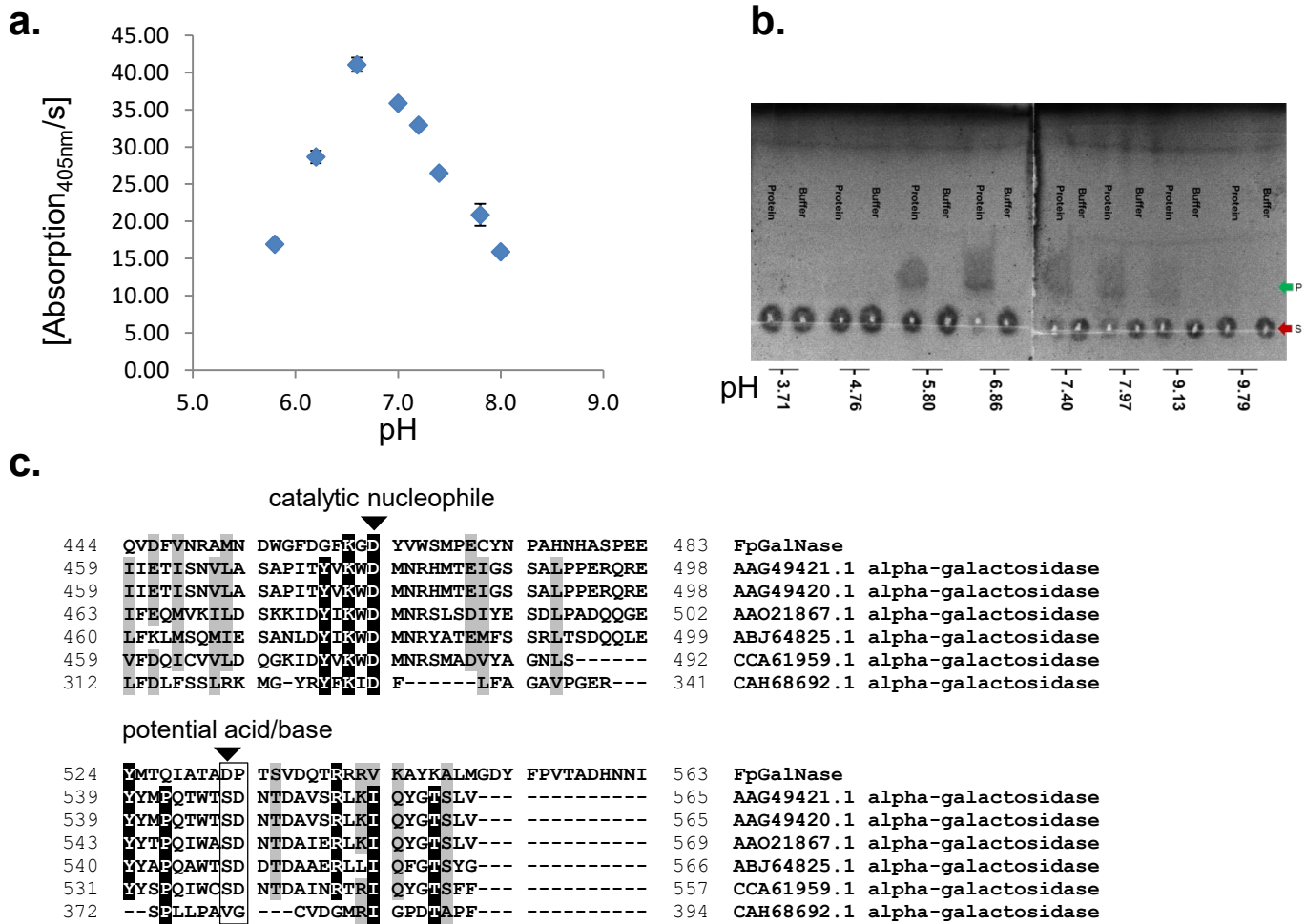


f. 50 µg/mL



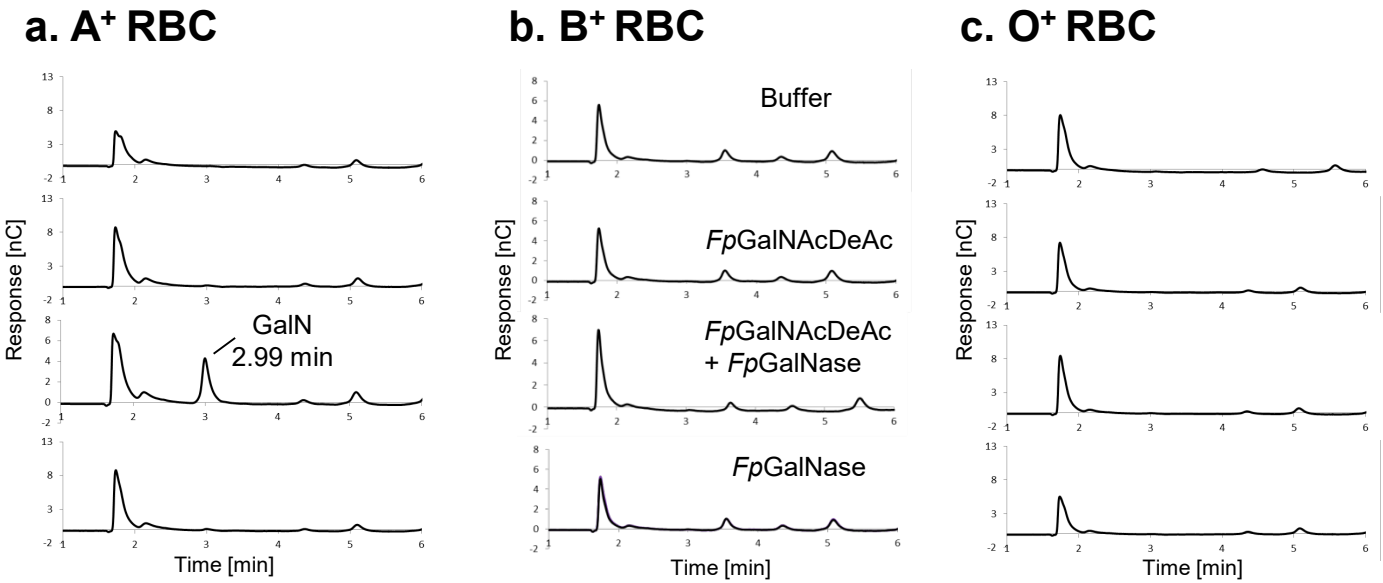
FACS screening of RBCs after treatment with *FpGalNAcDeAc* and *FpGalNase* or *FpGalNase_truncA*. APC-anti-A antibody and anti-H antibody + secondary FITC-labelled antibody were used for the labeling of A or H antigens on the treated RBC's. **a**, A⁺RBC's treated with 5 µg/mL *FpGalNAcDeAc* and *FpGalNase*. **b**, A⁺RBC's treated with 5 µg/mL *FpGalNAcDeAc* and *FpGalNase_truncA*. **c**, A⁺RBC's treated with 10 µg/mL *FpGalNAcDeAc* and *FpGalNase*. **d**, A⁺RBC's treated with 10 µg/mL *FpGalNAcDeAc* and *FpGalNase_truncA*. **e**, A⁺RBC's treated with 50 µg/mL *FpGalNAcDeAc* and *FpGalNase*. **f**, A⁺RBC's treated with 50 µg/mL *FpGalNAcDeAc* and *FpGalNase_truncA*. The red boxes mark the quadrant for H antigen quantification. Reactions were carried out using standard conditions. The experiment was repeated with 2 independent A⁺ RBC donors, showing similar results.

Supplementary Figure 11 : *FpGalactosaminidase* characterization



Characterization of the *FpGalactosaminidase* (*FpGalNase*). **a**, Relative activity (shown as $A_{405\text{ nm}}$ per second) for GalN- α -pNP at different pH values. (n=3, error bars = standard deviation). **b**, TLC analysis of pH optimum for cleavage of GalN antigen Type1_{tetra}-MU. The red arrow marks the substrate and the green arrow marks the product. The uncropped version of the TLC is presented in Supplementary Figure 22. Experiments were performed 2 times independently from each other with similar results. **c**, Sequence alignment of portions of the GH36 enzyme sequence showing the catalytic nucleophile D463 and the potential general acid/base residue D532.

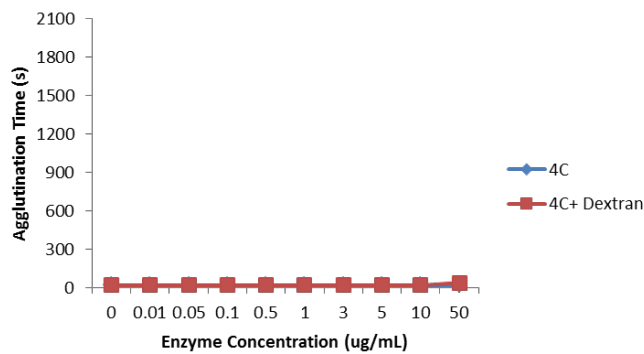
Supplementary Figure 12 : HPAE-PAD analysis of sugar release from RBCs after enzyme treatment.



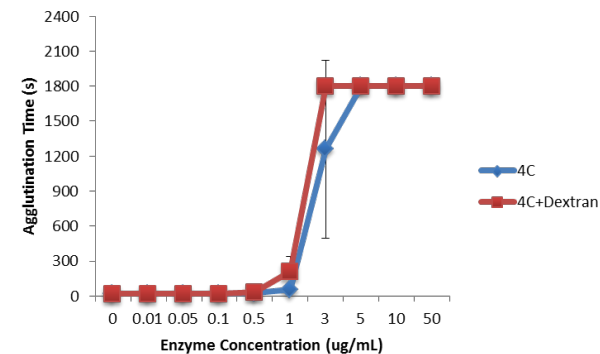
HPAE-PAD chromatography was used to identify monosaccharides released upon enzymatic treatment of RBCs: enzymes used are shown in centre panel. **a**, A⁺ red blood cells (RBC): only the combination of *FpGalNAcDeAc* and *FpGalNase*, released a sugar, namely galactosamine. **b**, B⁺ RBCs: no sugar release was seen. **c**, O⁺ RBCs: no sugar release was seen. Sugars were identified based on standards, presented in Supplementary Fig. 6. All experiments were performed 2 times independently from each other with similar results.

Supplementary Figure 13 : Anti-A antibody titration of residual A antigen after cleavage with different concentrations of enzymes

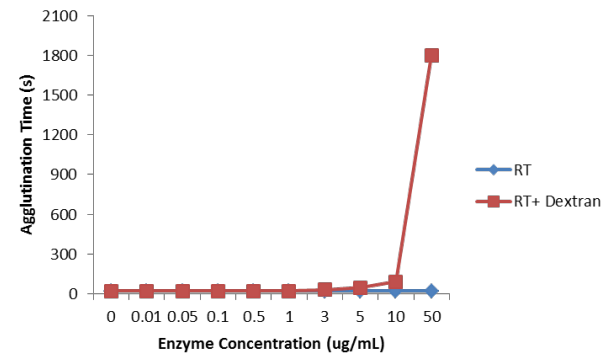
a. 4°C *EmGH109*



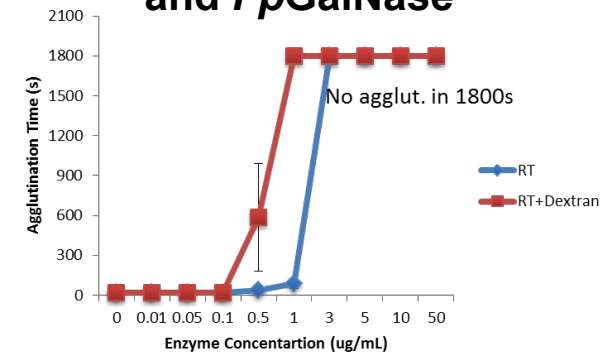
b. 4°C *FpGalNAcDeAc* and *FpGalNase*



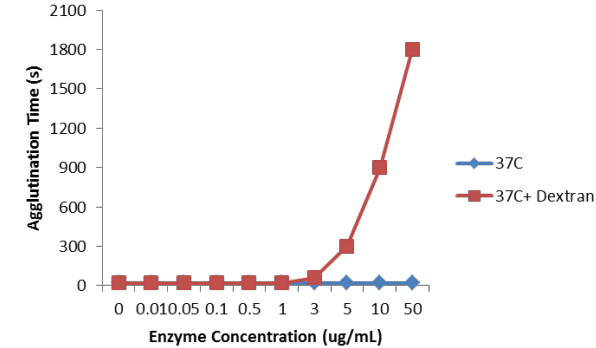
c. RT *EmGH109*



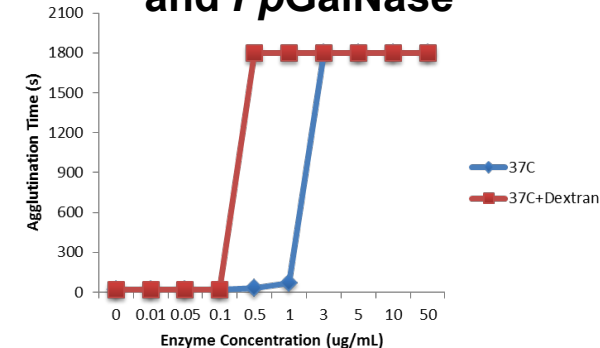
d. RT *FpGalNAcDeAc* and *FpGalNase*



e. 37°C *EmGH109*



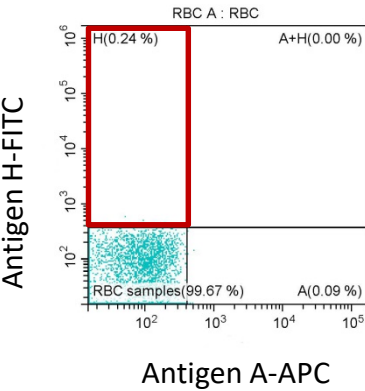
f. 37°C *FpGalNAcDeAc* and *FpGalNase*



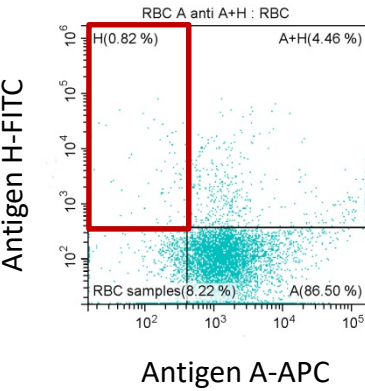
Anti-A antibody titration of the remaining A antigens on RBCs after enzyme conversion. Different concentration of enzymes (a, c, e *EmGH109*; b, d, f *FpGalNAcDeAc* and *FpGalNase*) with and without Dextran 40k were incubated with A⁺RBC's at different temperatures (a, b 4°C; c, d RT; e, f 37°C) for 1 h. Activities of the enzymes were determined as a function of the time till agglutination (0 - 1800 seconds). All experiments were performed with one A⁺ RBC and repeated 3 times independently, error bar represents standard deviation.

Supplementary Figure 14 : FACS RBC antibody controls

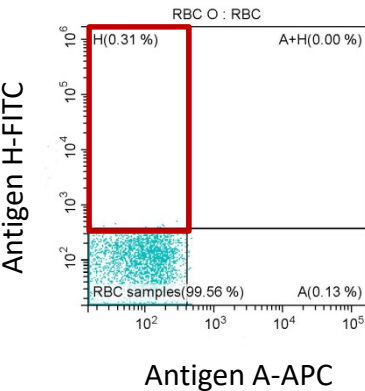
a. Control A⁺ RBC



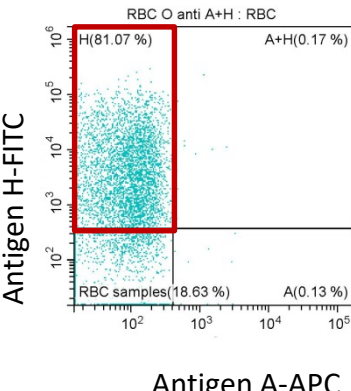
b. A⁺ RBC + Antibodies



c. Control O⁺ RBC



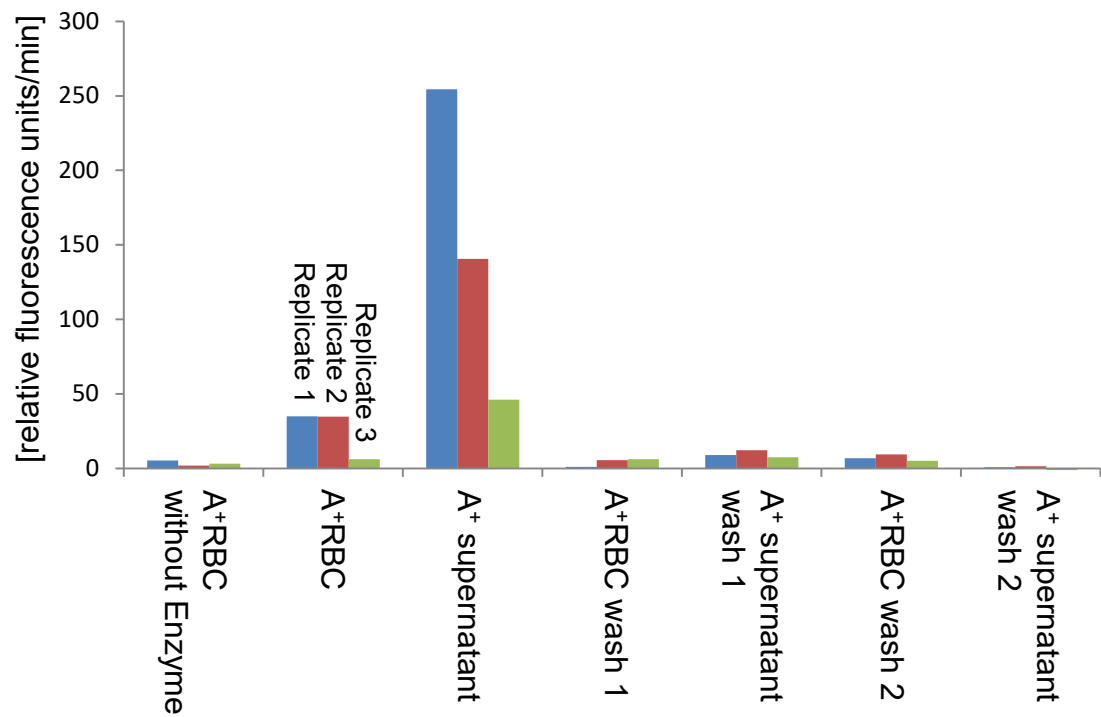
d. O⁺ RBC + Antibodies



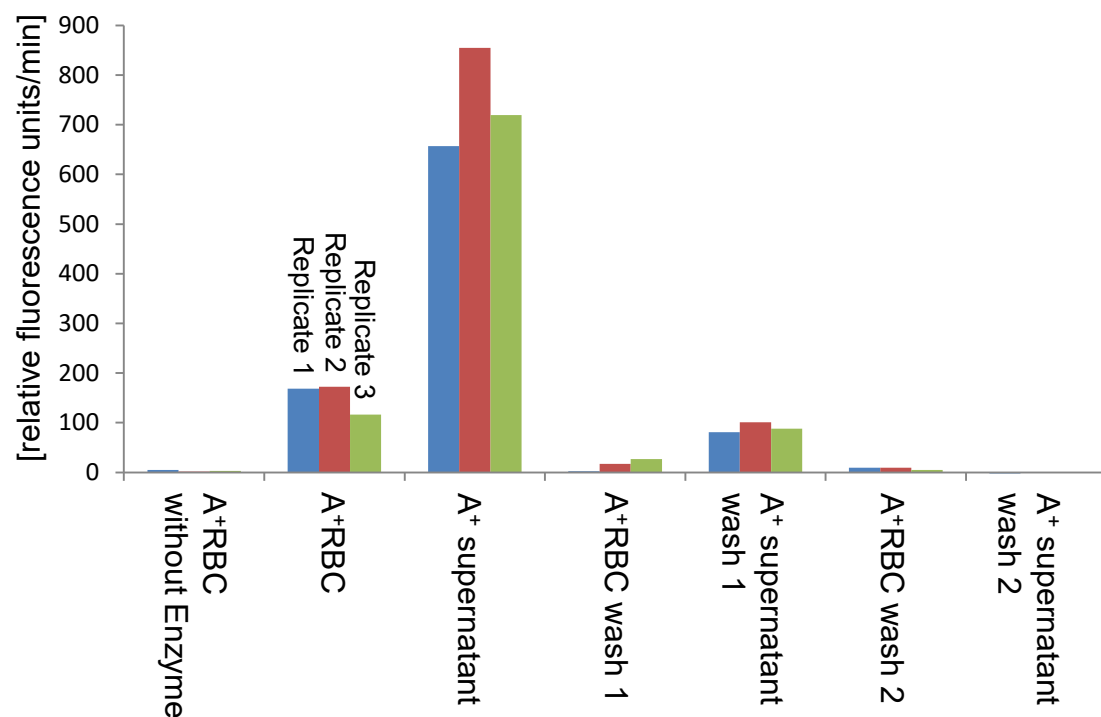
FACS screening of RBCs after incubation with different antibodies. **a**, control A+RBCs without antibodies. **b**, A⁺RBCs treated with APC-anti-A antibody and anti-H antibody + secondary FITC-labelled antibody. **c**, control O+RBC's without antibodies. **d**, O+RBC's treated with APC-anti-A antibody and anti-H antibody + secondary FITC-labelled antibody. The red boxes mark the quadrant for H antigen quantification. The figures display 1000 single cell events in the analysis gate. Reactions were carried out using standard conditions as described. The experiment was repeated with 2 independent A⁺RBC and O⁺RBC donors, showing similar results.

Supplementary Figure 15 : Removal of enzymes from RBCs

A⁺RBC with 5 ug/mL *FpGalNAcDeAc* + *FpGalNAse*

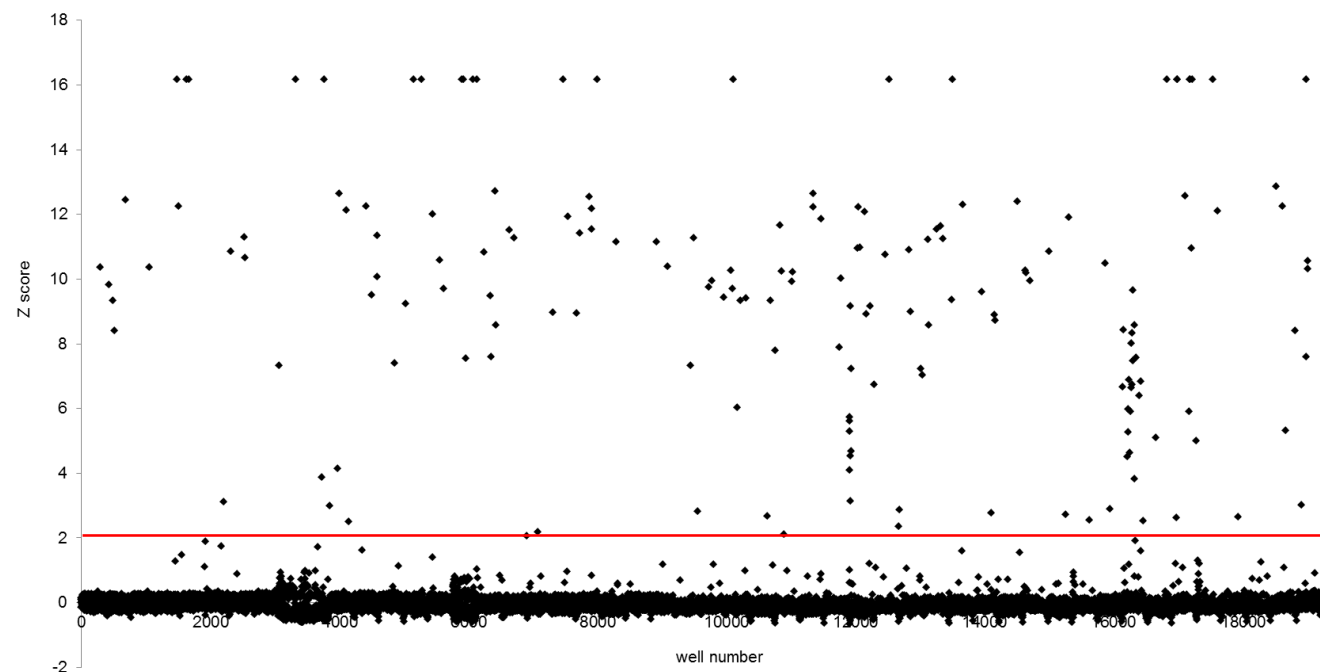


A⁺RBC with 20 ug/mL *FpGalNAcDeAc* + *FpGalNAse*



Removal of the utilized enzymes from the RBC was investigated by addition of A antigen Type2_{tetra}-MU substrate and the coupled enzyme mix to the RBC's and their supernatants after each wash. Reactions were carried out in triplicate using standard conditions.

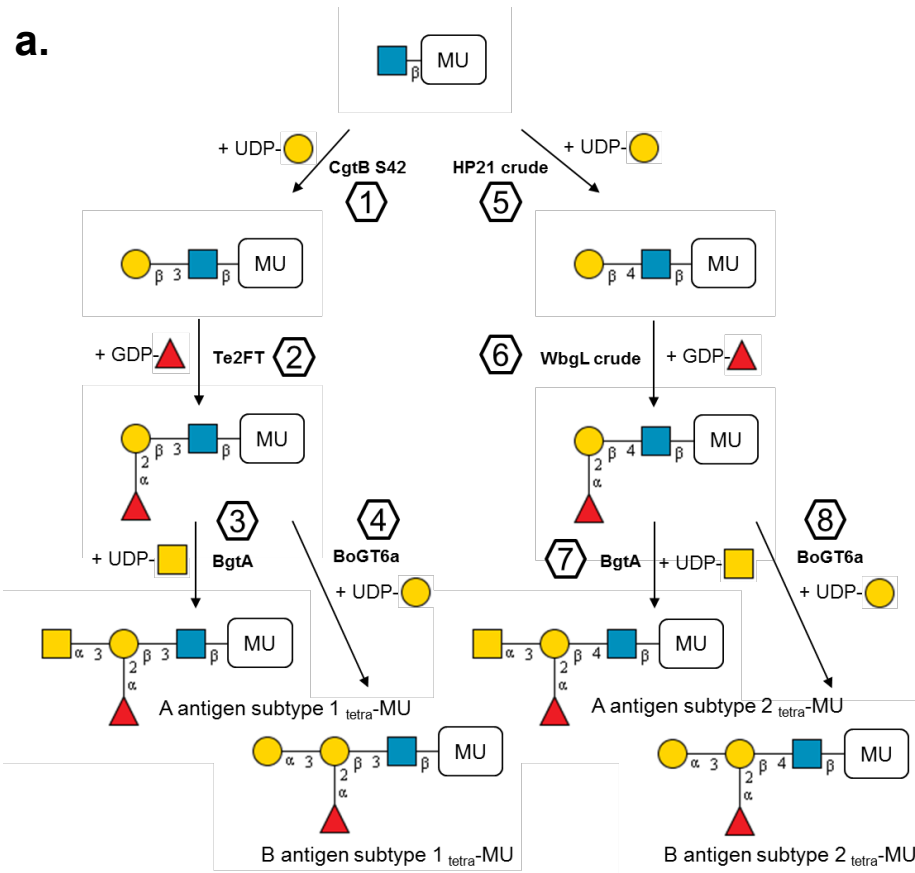
Supplementary Figure 16: Z-score for library screening



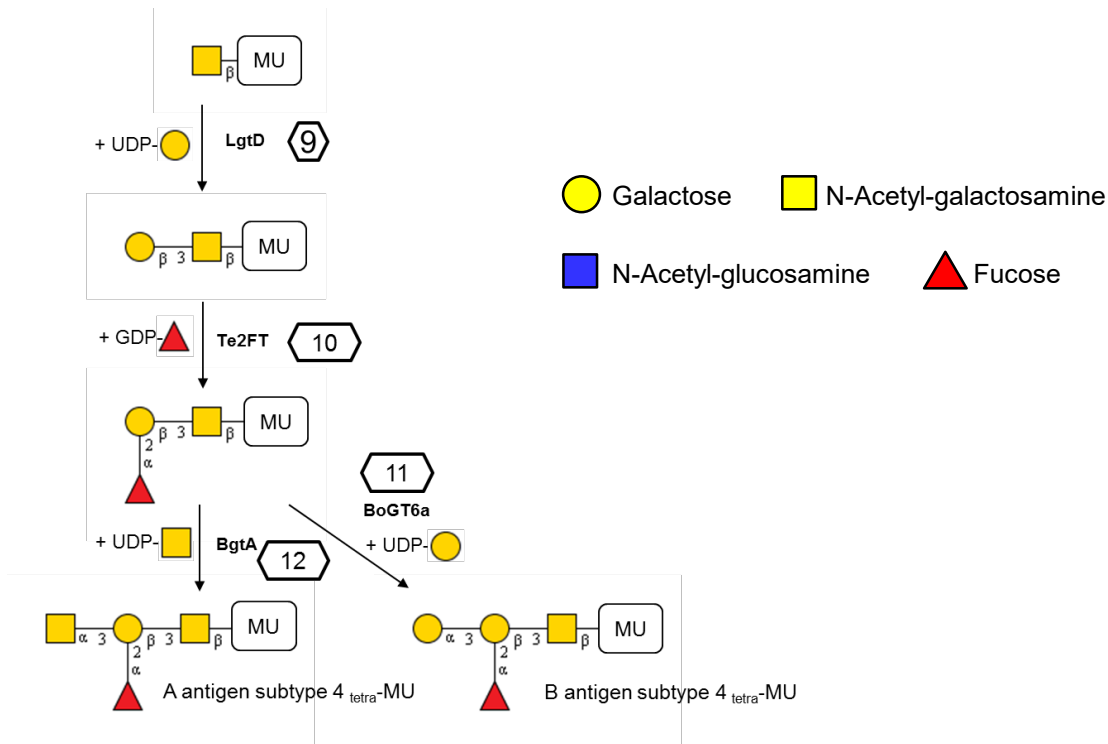
Z-score of the initial screen against 50 μM Gal- α -MU and GalNAc- α -MU substrates. The red line shows the chosen cut off at Z score > 2. Z-score = (fluorescence – mean value) / standard deviation

Supplementary Figure 17 : Overview of Antigen synthesis

a.

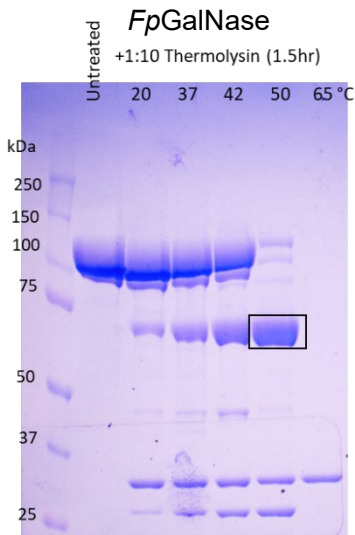


b.



Overview of the enzymatic syntheses of the A and B tetrasaccharide-MU glycosides. Reactions were carried out using standard conditions. Sugars are shown using the Consortium for Functional Glycomics notation⁶⁴. **a**, Synthesis of Type 1 and 2 antigens, starting from GlcNAc-β-MU. **b**, Synthesis of Type 4 Antigen, starting from the GalNAc-β-MU. Numbers indicate the enzymes used, as listed in Supplementary Table 17.

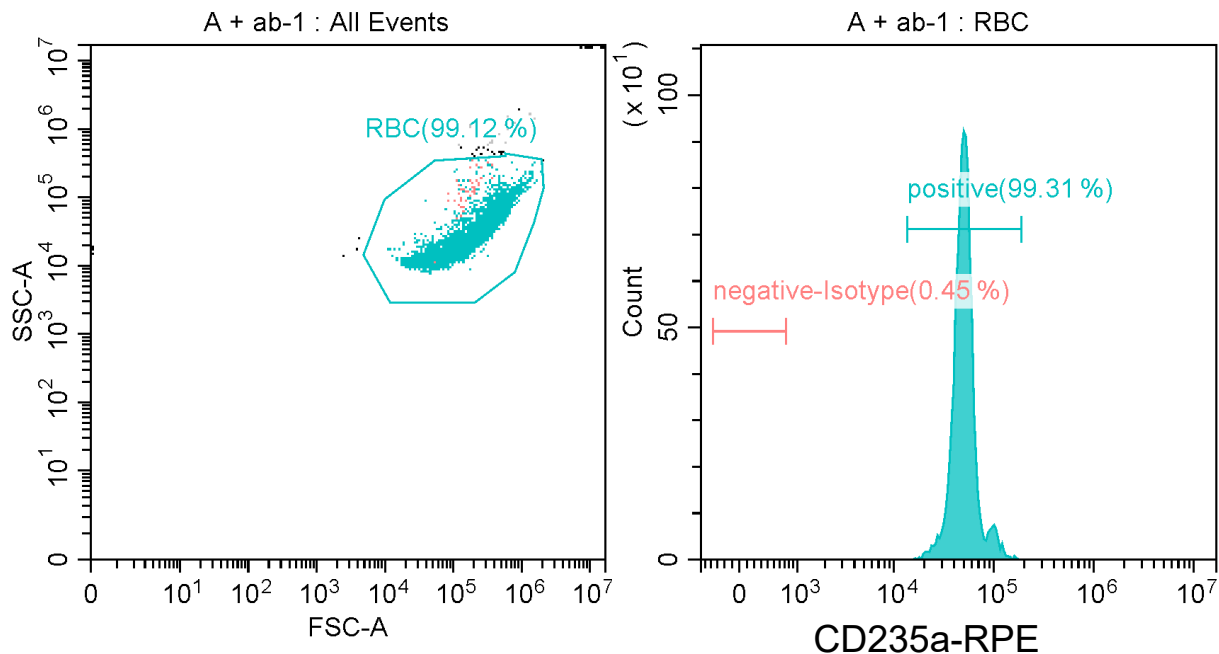
Supplementary Figure 18 : Thermolysin digestion of *FpGalNase*



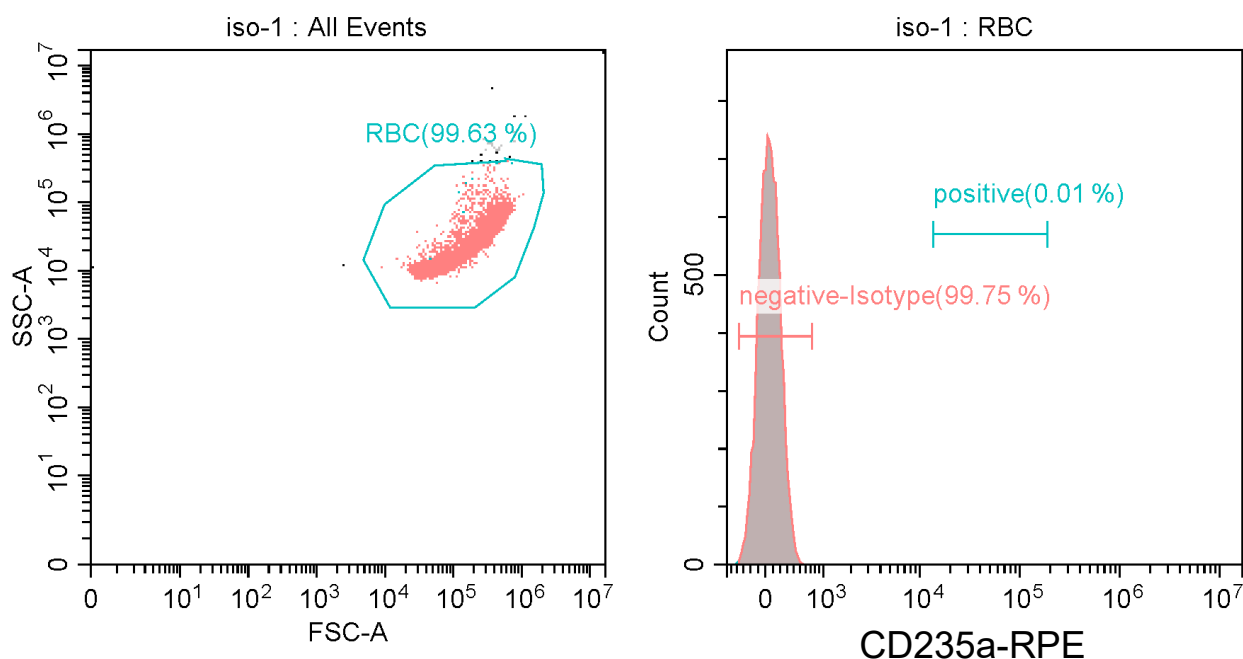
SDS-PAGE of *FpGalNase* incubated with 1:10 Thermolysin for 1.5 h at different temperatures. The boxed protein band marks the protein truncation used for MS analysis. The uncropped version of the SDS-PAGE is presented in Supplementary Figure 22. The experiment was performed one time.

Supplementary Figure 19 : Gating Strategy

a



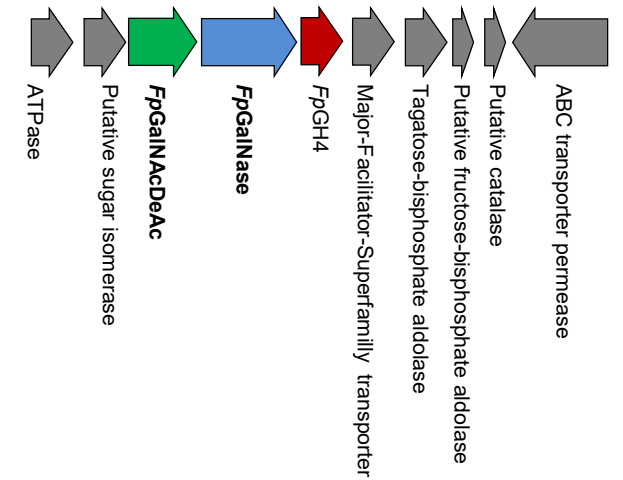
b



Flow cytometry gating using naïve RBCs treated with RPE-conjugated anti CD235a (Glycophorin A) antibody. SSC/FSC and histogram graft of **a**. RBC treated with anti CD235a antibody (RBC population confirmed by the increase in RPE signaling) and **b**. RBC treated isotype control (negative control)

Supplementary Figure 20: Gene cluster

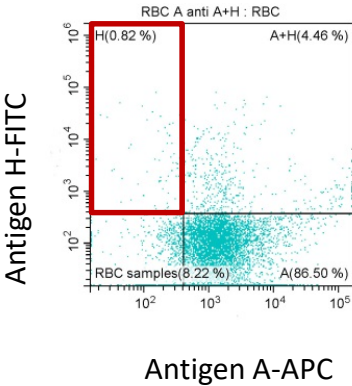
Flavonifractor plautii strain YL31, complete genome



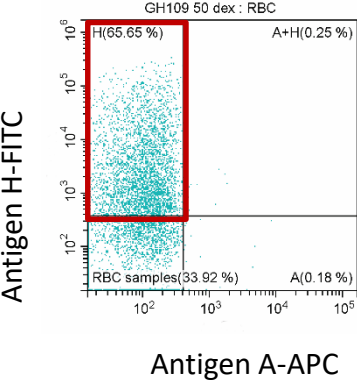
Overview of the ORF around *FpGalNAcDeAc* and *FpGalNase* on *Flavonifractor plautii* strain YL31 . The arrows show the direction of the ORF of each gene.

Supplementary Figure 21 : Residual GalN antigen

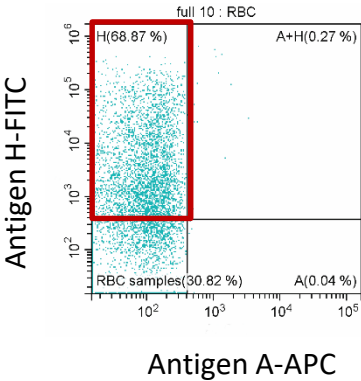
a. Control A⁺ RBC



b. A⁺ RBC + *EmGH109* (50 µg/ml + Dextran 40k)



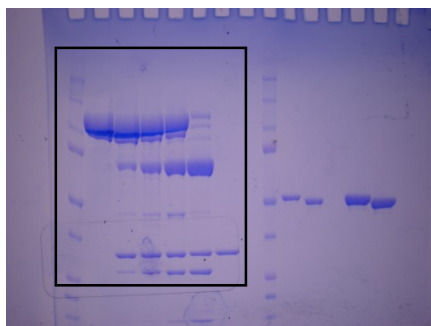
c. A⁺ RBC + *FpGalNAcDeAc* and *FpGalNase* (10 µg/ml each)



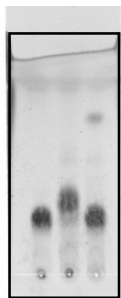
Validation of complete conversion of A to H antigens on RBCs. FACS analysis of A⁺RBCs; **a**, untreated A⁺RBCs, **b**, A⁺RBC + *EmGH109* (50 µg/ml + Dextran 40k) and **c**, A⁺RBC + *FpGalNAcDeAc* and *FpGalNase* (10 µg/ml each) incubated for 1 h at 37°C. The enzymatically modified RBC's were treated with Anti-H-antibody (plus secondary FITC-labelled antibody) and APC labelled Anti-A-antibody to determine the presence of the respective antigens. The region containing H antigens is marked with a red box. 10 000 single cell events collected in the analysis gate. The experiment was repeated with 2 independent A⁺RBC donors, showing similar results.

Supplementary Figure 22 : Full length blots

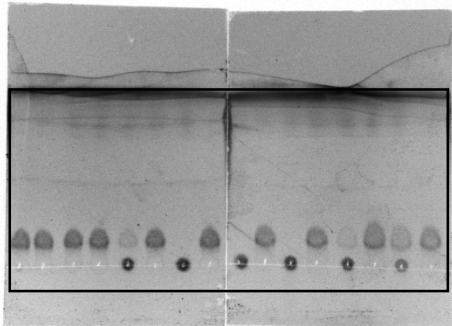
a. uncropped Supplementary Figure 18



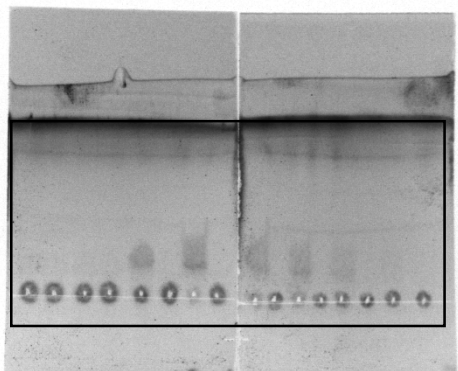
b. uncropped Supplementary Figure 4



c. uncropped Supplementary Figure 8



d. uncropped Supplementary Figure 11



Presentation of the full length SDS-PAGE gels (and TLCs) **a.** Supplementary Figure 18, **b.** Supplementary Figure 4, **c.** Supplementary Figure 8, **d.** Supplementary Figure 11). The black box represents the cropped version shown in the respective Supplementary Figures.

Supplementary Table 1 : A⁺ RBC conversion of CspGH36

Enzyme	Blood type	Dextran 40k	Enzyme conc. [ug/ml]	Anti-A MTS
non	A ⁺			4
CspGH36	A ⁺		50	4
CspGH36	A ⁺	300 mg/ml	50	3

Reactions contained 10% hematocrit.

Reactions were carried out using standard conditions as described. MTS card results for CspGH36 in 1xPBS pH 7.4, with and without Dextran 40k. Incubation was 1 h at 37°C. A score of 4 for the MTS cards means that the antigens are still present and 0, the best result, that no antigen could be detected. The experiment was repeated with 2 independent A⁺RBC donors, showing similar results.

Supplementary Table 2: Protein accession numbers

enzyme name	CAZy database	classification	accession #	fosmid	organism
FpGalNAcDeacetylase	CBM32	carbohydrate-binding protein	WP_009260926.1	AB+Fosmid_N08	Flavonifractor plautii
FpGalactosaminidase	GH36	alpha-galactosidase	WP_044942952.1	AB+Fosmid_N08	Flavonifractor plautii
FpGH4	GH4	alpha-glucosidase/alpha-galactosidas	WP_024723251.1	AB+Fosmid_N08	Clostridiales
BsGH109	GH109	oxidoreductase NAD-binding domain protein	CDA48352.1	AB+Fosmid_E11	Bacteroides stercoris
BvGH109_2	GH109	oxidoreductase NAD-binding domain protein	WP_057279443.1	AB+Fosmid_G15/G12/O11/L22/K22	Bacteroides vulgatus
BvGH109_1	GH109	gfo/ldh/MocA family oxidoreductase	WP_005848863.1	AB+Fosmid_I04/N03/J22	Bacteroides vulgatus
CspGH36	GH36		MK500922	AB+Fosmid_K05	Collinsella sp.

Supplementary Table 3 : Kinetic parameters (k_{cat}/K_m) for cleavage of A antigens of different types by GH109 enzymes

Proteins	Type1 _{tetra} -MU	Type2 _{tetra} -MU	Type4 _{tetra} -MU
<i>Em</i> GH109	1.77 ± 0.06	1.64 ± 0.06	1.09 ± 0.03
<i>Bv</i> GH109_1	1.21 ± 0.02	1.08 ± 0.05	0.59 ± 0.01
<i>Bv</i> GH109_2	1.04 ± 0.03	0.98 ± 0.04	0.52 ± 0.01
<i>Bs</i> GH109	2.39 ± 0.06	2.77 ± 0.07	0.93 ± 0.02
	k_{cat}/K_M [s ⁻¹ *mM ⁻¹]	k_{cat}/K_M [s ⁻¹ *mM ⁻¹]	k_{cat}/K_M [s ⁻¹ *mM ⁻¹]

Reactions were carried out using standard conditions as described.
Values indicate averages (n = 3 independent samples) ± standard error.

Supplementary Table 4 : MTS card data for GH109 enzymes

a

Enzyme	Blood type	Dextran 40k	Enzyme conc. [ug/ml]	Anti-A MTS
non	A ⁺			4
<i>EmGH109</i>	A ⁺		150	4
<i>EmGH109</i>	A ⁺		100	4
<i>EmGH109</i>	A ⁺	300 mg/ml	20	0
<i>EmGH109</i>	A ⁺	300 mg/ml	15	0
<i>EmGH109</i>	A ⁺	300 mg/ml	10	1
<i>EmGH109</i>	A ⁺	300 mg/ml	5	2
<i>BvGH109_1</i>	A ⁺		100	4
<i>BvGH109_1</i>	A ⁺	300 mg/ml	100	4
<i>BvGH109_2</i>	A ⁺		100	4
<i>BvGH109_2</i>	A ⁺	300 mg/ml	100	4
<i>BsGH109</i>	A ⁺		100	4
<i>BsGH109</i>	A ⁺	300 mg/ml	100	4

Reactions contained 10% hematocrit.

b

Enzyme	Blood type	Buffer system	Enzyme conc. [ug/ml]	Anti-A MTS
non	A ⁺			4
non	O ⁺			0
<i>EmGH109</i>	A ⁺	1xPBS pH 7.4	100	4
<i>EmGH109</i>	A ⁺	1xPBS pH 7.4 300mg/ml Dextran 40k	100	0
<i>EmGH109</i>	A ⁺	100% blood plasma	100	4

Reactions contained 10% hematocrit.

Reactions were carried out using standard conditions as described. **a**, MTS card results for different GH109 treatments in 1xPBS pH 7.4, with and without Dextran 40k. Incubation was 1 h at 37°C. **b**, Test of *EmGH109* in different buffer systems. A score of 4 for the MTS cards means that the antigens are still present and 0, the best result, that no antigen could be detected. The experiments were repeated with 2 independent A⁺RBC donors, showing similar results.

Supplementary Table 5 : Antigen cleavage activity of fosmid N08 candidate enzymes

	A antigen Type1 _{tetra} -MU [RFU]	B antigen Type1 _{tetra} -MU [RFU]
Empty Fosmid	15807	2305
AB ⁺ Fosmid_N08	>100000*	9184
<i>FpCBM32</i>	13799	3940
<i>FpGH36</i>	13530	>100000*
<i>FpGH4</i>	13280	3759
	<i>18h 37°C incubation</i>	<i>18h 37°C incubation</i>
<i>FpCBM32 + FpGH36</i>	>100000*	16045
<i>FpCBM32 + FpGH4</i>	16843	3484
<i>FpGH36 + FpGH4</i>	19037	29594
	<i>30 min 37°C incubation</i>	<i>8h 37°C incubation</i>

* 100000 RFU is the maximal signal detectable by the fluorimeter, nothing above was detected.
 The experiments were performed 2 times independently with similar results.
 [RFU] = relative fluorescence units.

Supplementary Table 6 : Substrate specificities

Overview of the substrate specificity for *Fp*GalNAc deacetylase (*Fp*GalNAcDeAc), *Fp*Galactosaminidase (*Fp*GalNase) and *Fp*GH4

Protein name	Substrate	Activity
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GlcNAc- β -1,3-Gal- β -MU	yes
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GlcNAc- β -MU	yes
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,3-(Fucose- α -1,2-)-Gal- β -1,4-GlcNAc- β -MU	yes
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GalNAc- β -MU	yes
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,3-(Fucose- α -1,2-)-Gal- β -b-pNP	yes
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,3-Gal- β -1,3-GlcNAc- β -MU	no
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,4-GalNAc- α -1,4-GalNAc- α -O(CH ₂) ₈ CO ₂ CH ₃	no
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,4-Gal- α -1,4-GalNAc- α -O(CH ₂) ₈ CO ₂ CH ₃	no
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,4-GalNAc- α -1,4-Gal- α -O(CH ₂) ₈ CO ₂ CH ₃	no
<i>Fp</i> GalNAcDeAc	GalNAc- α -1,4-Gal- α -1,4-Gal- α -O(CH ₂) ₈ CO ₂ CH ₃	no
<i>Fp</i> GalNAcDeAc	Gal- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GlcNAc- β -MU	no
<i>Fp</i> GalNAcDeAc	2,3- α -Sialyllactose-BODIPY	no
<i>Fp</i> GalNAcDeAc	GalNAc- α -MU	traces*
<i>Fp</i> GalNAcDeAc	GalNAc- β -MU	no
<i>Fp</i> GalNAcDeAc	GlcNAc- α -MU	no
<i>Fp</i> GalNase	GalN- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GlcNAc- β -1,3-Gal- β -MU	yes
<i>Fp</i> GalNase	GalN- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GlcNAc- β -MU	yes
<i>Fp</i> GalNase	GalN- α -1,3-(Fucose- α -1,2-)-Gal- β -1,4-GlcNAc- β -MU	yes
<i>Fp</i> GalNase	GalN- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GalNAc- β -MU	yes
<i>Fp</i> GalNase	Gal- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GlcNAc- β -MU	yes
<i>Fp</i> GalNase	Gal- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GalNAc- β -MU	yes
<i>Fp</i> GalNase	GalNAc- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GlcNAc- β -MU	no
<i>Fp</i> GalNase	GalN- α -1,3-(Fucose- α -1,2-)-Gal- β -b-MU	yes
<i>Fp</i> GalNase	GalN- α -1,4-GalN- α -1,4-GalN- α -O(CH ₂) ₈ CO ₂ CH ₃	traces*
<i>Fp</i> GalNase	GalN- α -1,4-Gal- α -1,4-GalN- α -O(CH ₂) ₈ CO ₂ CH ₃	yes
<i>Fp</i> GalNase	GalN- α -1,4-GalN- α -1,4-Gal- α -O(CH ₂) ₈ CO ₂ CH ₃	traces*
<i>Fp</i> GalNase	GalN- α -1,4-Gal- α -1,4-Gal- α -O(CH ₂) ₈ CO ₂ CH ₃	yes
<i>Fp</i> GalNase	2,3- α -Sialyllactose-BODIPY	no
<i>Fp</i> GalNase	GalNAc- α -MU	no
<i>Fp</i> GalNase	Gal- α -pNP	traces*
<i>Fp</i> GalNase	GalN- α -pNP	yes
<i>Fp</i> GalNase	GlcN- α -pNP	no
<i>Fp</i> GH4	GalNAc- α -MU	no
<i>Fp</i> GH4	GalNAc- β -MU	no
<i>Fp</i> GH4	GlcNAc- α -MU	no
<i>Fp</i> GH4	GlcNAc- β -MU	no
<i>Fp</i> GH4	Gal- α -MU	yes
<i>Fp</i> GH4	Glc- α -MU	no
<i>Fp</i> GH4	Glc- β -MU	no
<i>Fp</i> GH4	GalN- α -MU	yes
<i>Fp</i> GH4	GlcN- α -MU	yes
<i>Fp</i> GH4	GalUA- α -pNP	no
<i>Fp</i> GH4	GalNAc- α -1,3-(Fucose- α -1,2-)-Gal- β -1,3-GlcNAc- β -1,3-Gal- β -MU	no

*Activity was only present if incubated with 100x more enzyme for 48 h at 37°C

Supplementary Table 7 : Kinetic parameters for cleavage of different antigen substrates by *FpGalNAcDeAc* and *FpGalNase* constructs

Protein		Substrate	K_M [μ M]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} mM $^{-1}$]
<i>FpGalNAcDeAc</i>	<i>full length</i>	A antigen Type1 _{penta} -MU	340 ± 35	4.86	14.28
	<i>_D1ext</i>	A antigen Type1 _{penta} -MU	212 ± 37	5.37	25.23
	<i>_D1+2</i>	A antigen Type1 _{penta} -MU	265 ± 43	9.99	37.59
	<i>full length</i>	A antigen Type1 _{tetra} -MU	-	-	23.49 ± 0.63
	<i>full length</i>	A antigen Type2 _{tetra} -MU	-	-	23.37 ± 0.71
	<i>full length</i>	A antigen Type4 _{tetra} -MU	-	-	33.27 ± 2.08
<i>FpGalNase</i>	<i>full length</i>	GalN antigen Type1 _{penta} -MU	64.5 ± 7.6	2.35	36.48
	<i>_trunca</i>	GalN antigen Type1 _{penta} -MU	54.7 ± 3.1	1.86	34.08
	<i>full length</i>	GalN antigen Type1 _{tetra} -MU	-	-	45.70 ± 3.57
	<i>full length</i>	GalN antigen Type2 _{tetra} -MU	-	-	47.27 ± 7.49
	<i>full length</i>	GalN antigen Type4 _{tetra} -MU	-	-	30.05 ± 2.33
	<i>full length</i>	B antigen Type1 _{tetra} -MU	-	-	0.02
	<i>full length</i>	GalN- α -pNP	700 ± 151	0.03	0.04

Values indicate averages (n = 3 independent samples) ± standard error.

Supplementary Table 8 : Relative activities for different potential Inhibitors of *FpGalNAcDeAc*.

% rel. Activity	Additive
100	Buffer
83	2% DMSO
88	4% DMSO
93	1 µM EDTA
26	10 µM EDTA
6	100 µM EDTA
112	1 µM Marimastat
106	10 µM Marimastat
94	100 µM Marimastat
48	1 mM Marimastat
88	1x Pierce Protease Inhibitor , EDTA-free
93	2x Pierce Protease Inhibitor , EDTA-free
88	4x Pierce Protease Inhibitor , EDTA-free

Reactions were carried out using standard conditions
Values indicate averages (n = 3 independent samples).

Supplementary Table 9 : Influence of the *Fp*GalNAcDeAc CBM32 domain on enzymatic conversion of RBCs

MTS card data for *Fp*GalNAcDeAc and its truncations *Fp*GalNAcDeAc_D1ext and *Fp*GalNAcDeAc_D1+2 for blood type conversion of A⁺RBC's. A score of 4 on the MTS cards means that the antigens are still present and 0, the best result, that no antigen could be detected. The experiment was repeated with 2 independent A⁺RBC donors, showing similar results.

Enzyme	Blood type	Dextran 40k	Enzyme conc. [ug/ml]	Anti-A MTS
non	A ⁺			4
<i>Fp</i> GalNAcDeAc	A ⁺		3	0
<i>Fp</i> GalNAcDeAc	A ⁺	300 mg/ml	0.5	0
<i>Fp</i> GalNAcDeAc_D1ext	A ⁺		100	1
<i>Fp</i> GalNAcDeAc_D1ext	A ⁺		50	3
<i>Fp</i> GalNAcDeAc_D1ext	A ⁺	300 mg/ml	10	0
<i>Fp</i> GalNAcDeAc_D1+2	A ⁺		100	3
<i>Fp</i> GalNAcDeAc_D1+2	A ⁺		50	4
<i>Fp</i> GalNAcDeAc_D1+2	A ⁺	300 mg/ml	10	0

Reactions contained 10% hematocrit.

Supplementary Data Table 10: X-ray Data and Structure Refinement statistics for *FpGalNAcDeAc_D1min*

Structure	Br anomalous PDB: 6N1A	B_tri complex PDB: 6N1B
Data collection		
Space group	P 21 21 21	P 21 21 21
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	51.58, 69.19, 104.33	51.71, 69.24, 104.81
α , β , γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	46.2 – 1.60 (1.63 – 1.60) ^a	46.4 – 1.30 (1.32 – 1.30)
<i>R</i> _{merge} (%)	9.5 (145.5)	5.4 (34.0)
<i>I</i> / σI	27.4 (1.8)	16.3 (1.9)
Completeness (%)	97.7 (79.2)	97.3 (69.1)
Redundancy	22.9 (13.3)	5.5 (1.9)
CC _{1/2}	1.00 (0.63)	1.00 (0.76)
Refinement		
Resolution (Å)	1.60	1.30
No. reflections	48871	90596
<i>R</i> _{work} / <i>R</i> _{free} (%)	13.9 / 16.7	14.7 / 16.2
No. atoms		
Protein	5771	5727
B_tri ligand	---	55
Glycerol / ions	24 / 3	24 / 4
Water	365	359
<i>B</i> -factors		
Protein	18.8	12.7
Ligand	---	20.2
Glycerol / ions	35.9 / 26.8	22.0 / 9.1
Water	30.0	27.1
R.m.s. deviations		
Bond lengths (Å)	0.021	0.027
Bond angles (°)	1.94	2.23

^a Values in parentheses refer to the highest resolution bin

Supplementary Table 11: Specific activity of *FpGalNAcDeAc_D1min* and its mutants for the cleavage of A antigen Type2_{tetra}-MU

mutation site	k_{cat}/K_M [$s^{-1}mM^{-1}$]
WT	492 ± 124
E64A	N.D.
E64L	N.D.
C99A	140 ± 6
C99S	75.5 ± 2.5
D100N	0.073 ± 0.005
H252A	0.084 ± 0.012
H252F	0.030 ± 0.002
Y315F	0.168 ± 0.039

N.D. = no detectable activity
Values indicate averages (n = 3 independent samples) $\pm$ standard error.

Supplementary Table 12 : Specific activity for FpGalNase_truncA and its mutations D463A/G/S for GalN- α -MU cleavage.

Enzyme	mutation site	k_{cat}/K_M [$s^{-1} \cdot mM^{-1}$]
<i>FpGalNAase_truncA</i> (GalN- α -pNP)	WT	0.113 ± 0.002
	D463A	N.D.
	D463G	N.D.
	D463S	N.D.

Reactions were carried out using standard conditions as described.
N.D. = not detectable.
Values indicate averages (n = 3 independent samples) $\pm$ standard error.

Supplementary Table 13 : MTS card results for treatment of A⁺, B⁺ and AB⁺ RBCs with *Em*GH109, *Fp*GalNAcDeAc and *Fp*GalNase.

A score of 4 for the MTS cards means that the antigens are still present and 0, the best result, that no antigen could be detected. The experiment was repeated with two independent A⁺ RBC donors, showing similar results.

Enzyme	Blood type	Dextran 40k	Enzyme conc. [ug/ml]	Anti-A MTS	Anti-B MTS
non	A ⁺			4	0
non	B ⁺			0	4
non	AB ⁺			4	4
<i>Em</i> GH109	A ⁺	300 mg/ml	15	0	
<i>Fp</i> GalNAcDeAc	A ⁺		10	0	0
<i>Fp</i> GalNAcDeAc	A ⁺		3	0	
<i>Fp</i> GalNAcDeAc	A ⁺		2	1	
<i>Fp</i> GalNAcDeAc	A ⁺	300 mg/ml	0.5	0	
<i>Fp</i> GalNAcDeAc	A ⁺	300 mg/ml	0.4	2	
<i>Fp</i> GalNAcDeAc	AB ⁺		3	0	4
<i>Fp</i> GalNase	B ⁺		100		4
<i>Fp</i> GalNase	B ⁺	300 mg/ml	100		4
<i>Fp</i> GalNase	AB ⁺		100	4	4
<i>Fp</i> GalNase	AB ⁺	300 mg/ml	100	4	4
<i>Fp</i> GalNAcDeAc + <i>Fp</i> GalNase	A ⁺	300 mg/ml	0.5 each	0	
<i>Fp</i> GalNAcDeAc + <i>Fp</i> GalNase	A ⁺		10 each	0	0
<i>Fp</i> GalNAcDeAc + <i>Fp</i> GalNase	AB ⁺	300 mg/ml	0.5 each	0	4
<i>Fp</i> GalNAcDeAc + <i>Fp</i> GalNase	AB ⁺		2 each	0	4

Reactions contained 10% hematocrit.

Supplementary 14 : Enzymatic blood type conversion

MTS card score and agglutination data for *FpGalNAcDeAc*- and *FpGalNase*-catalysed conversion of blood types of A⁺ and A⁻ RBC from different blood donors. Anti-A MTS score reflects the de-acetylation of the A antigen and the Anti H agglutination score the subsequent cleavage of the galactosamine, producing the H antigen.

blood type	buffer system	# of donors	Anti-A MTS score					# of donors	Anti H antibody agglutination score					
			4	3	2	1	0		0	1	2	3	4	5
A ⁺ RBC	1x PBS pH 7.4 + Dextran 40k	26	26	0	0	0	0	19	8	1	5	5	0	0
A ⁺ RBC + Enzymes	1x PBS pH 7.4 + Dextran 40k	26	0	0	0	0	26	19	0	0	0	0	1	18
	1x PBS pH 7.4	13	0	2	1	0	10	6	0	0	0	0	1	5
	100 % blood plasma	17	0	0	0	0	17	10	0	0	0	0	0	10
A ⁻ RBC	1x PBS pH 7.4 + Dextran 40k	10	10	0	0	0	0	10	5	0	0	5	0	0
A ⁻ RBC + Enzymes	1x PBS pH 7.4 + Dextran 40k	10	0	0	0	0	10	10	1	0	0	0	1	8
	1x PBS pH 7.4	5	0	0	0	0	5	5	1	0	0	0	1	3
	100 % blood plasma	5	0	0	0	0	5	5	0	0	0	0	1	4

Reactions contained 10% hematocrit.

A antigen removal was determined by MTS cards (0 = best score) and H antigen presence was investigated through agglutination assays (5 = best score). Presented are untreated RBC's and RBC's enzymatically converted in different buffers.

Supplementary Table 15: Whole blood bag conversion

MTS card score and agglutination data for type conversion of whole blood pack (C0510 18 990033 A+) mixed with SAGM. Final volume was 309 ml with 60% hematocrit.

	Samples	Enzyme conc. [ug/mL]	Anti-A MTS Score	Anti-H Agglutination Score
1 st Trial	O ⁺ RBC (from donor #4)	0	0	5
	control A ⁺ RBC	0	4	0
	4°C 24h	40	0	5
	4°C 48h	40	0	5
	4°C 72h	40	0	5
2 nd Trial	O ⁺ RBC (from donor #4)	0		5
	control A ⁺ RBC	0		0
	4°C 24h	40		5
	4°C 48h	40		5
	4°C 72h	40		5

A antigen removal was determined by MTS cards (0 = best score) and H antigen presence was investigated through agglutination assays (5 = best score). Presented are untreated RBC's and RBC's enzymatically converted in different buffers. The experiment was performed once..

Supplementary Table 16: Primer list

Name	Sequence
<i>FpGalNAcDeAc_withoutSignalP_fw</i>	ATGGTCTCGCCATGCAGACTCCAGCGAGTCCG
<i>FpGalNAcDeAc_D1min_rv</i>	ATGGTCTCGATTCTTACGTCGTGTAGCCGGGGTC
<i>FpGalNAcDeAc_D1ext_rv</i>	ATGGTCTCGATTCTTAATCACTGGAGGTATATTTTCACGACC
<i>FpGalNAcDeAc_D1+2_rv</i>	ATGGTCTCGATTCTTACGCAGGCTCGATTGGACCATAC
<i>FpGalNAcDeAc_D2ext_fw</i>	ATGGTCTCGCCATGATGTGGCGACGGTGGATGAG
<i>FpGalNAcDeAc_rv</i>	ATGGTCTCGATTCTTATTCTCCACATACGAAAAATAGTCG
<i>FpGalNase_withoutSignalP_fw</i>	ATGGTCTCGCCATCGTGGTAAAAAGTTTCATATCACTCAC
<i>FpGalNase_truncA_rv</i>	ATGGTCTCGATTCTTATGCGTTAGTGGTATAAGTCAAATAGTC
<i>FpGalNase_rv</i>	ATGGTCTCGATTCTTATTCCGAAATTTCCACCGCTTTAAC
<i>FpGH4_fw</i>	ATGGTCTCGCCATGATAAAATTACATTTCTTGCGCAGG
<i>FpGH4_rv</i>	ATGGTCTCGATTCTTAATGATACTCCGGCAACCGATC
<i>pET28_goldengate_site_fw</i>	ATGGTCTCCGAATTCGAGCTCCGTCGAC
<i>pET28_goldengate_site_rv</i>	ATGGTCTCGATGGCTGCCGCGCGGCAC
<i>BvGH109_1_withoutSignalP_fw</i>	ACGGTCTCACGGCAAACCGTAAGTTCAGGAGATTC
<i>BvGH109_1_rv</i>	ACGGTCTCAATTCTTATTTCTTTTTCTTCTTAGCTTTTCG
<i>BvGH109_2_withoutSignalP_fw</i>	ACGGTCTCACGGCATGTTGCTGTCTATTAGCGGC
<i>BvGH109_2_rv</i>	ACGGTCTCAATTCTCATTACTTTTTTACTTTTCAGCTTTTTAGC
<i>BsGH109_withoutSignalP_fw</i>	ACGGTCTCACGGCTCTTCACCGACATCCACTGTG
<i>BsGH109_rv</i>	ACGGTCTCAATTCTCACATTTCTTTTCAGCCTGCGCC
<i>EmGH109_withoutSignalP_fw</i>	ATGGTCTCGCGGCCCTAAAAAGGTAAGAATAGCTTTTATC
<i>EmGH109_rv</i>	ATGGTCTCGATTCTTAGTAGTCGTCATTTATTGCAAATG
<i>pET16_goldengate_site_fw</i>	ATGGTCTCCGAATCATATGCTCGAGGATCCGGCTGC
<i>pET16_goldengate_site_rv</i>	ATGGTCTCGGCCGCTGCTGTGATGATGAT
<i>FpGalNase_mut_D463A_fw</i>	GATGGCTTCAAGGGAGCGTATGTGTGGAGTATG
<i>FpGalNase_mut_D463A_rv</i>	CATACTCCACACATACGCTCCCTTGAAGCCATC
<i>FpGalNase_mut_D463G_fw</i>	GATGGCTTCAAGGGAGGCTATGTGTGGAGTATG
<i>FpGalNase_mut_D463G_rv</i>	CATACTCCACACATAGCCTCCCTTGAAGCCATC
<i>FpGalNase_mut_D463S_fw</i>	GATGGCTTCAAGGGATCTTATGTGTGGAGTATG
<i>FpGalNase_mut_D463S_rv</i>	CATACTCCACACATAAGATCCCTTGAAGCCATC
<i>FpGalNAcDeAc_mut_C99S_fw</i>	CTACCACAACCTCTGACGCTGATATGGTC
<i>FpGalNAcDeAc_mut_C99S_rv</i>	ATCAGCGTCAGAGTTGTGGTAGCGGGTG
<i>FpGalNAcDeAc_mut_C99A_fw</i>	CTACCACAACGCGGACGCTGATATGGTC
<i>FpGalNAcDeAc_mut_C99A_rv</i>	ATCAGCGTCCGCGTTGTGGTAGCGGGTG
<i>FpGalNAcDeAc_mut_H252F_fw</i>	GCCCCCTGGTTTCAGGATGTCCAG
<i>FpGalNAcDeAc_mut_H252F_rv</i>	CATCCTGCGCCCAGGGGGCCAACTG
<i>FpGalNAcDeAc_mut_H252A_fw</i>	GCCCCCTGGGCGCAGGATGTCCAG
<i>FpGalNAcDeAc_mut_H252A_rv</i>	CATCCTGCGCCCAGGGGGCCAACTG
<i>FpGalNAcDeAc_mut_D100N_fw</i>	CGTACCACAACCTGCAACGCTGATATGG
<i>FpGalNAcDeAc_mut_D100N_rv</i>	CCATATCAGCGTTGCAGTTGTGGTAGCG
<i>FpGalNAcDeAc_mut_Y315F_fw</i>	GATGATTTCCAGATCTTTCGCTCCAGTTTTTAC
<i>FpGalNAcDeAc_mut_Y315F_rv</i>	GTA AAAA ACTGGAGCGAAAGATCTGGAATCATC
<i>FpGalNAcDeAc_mut_E64A_fw</i>	CTCCATCTACGCGAACCCGTCCATCGTTG
<i>FpGalNAcDeAc_mut_E64A_rv</i>	ATGGACGGGTTTCGCGTAGATGGAGATCC
<i>FpGalNAcDeAc_mut_E64L_fw</i>	CTCCATCTACCTGAACCCGTCCATCGTTG
<i>FpGalNAcDeAc_mut_E64L_rv</i>	ATGGACGGGTTTCAGGTAGATGGAGATCC

Supplementary Table 17: Enzymes used for A antigen synthesis

Enzymes utilized for A and B tetrasaccharide synthesis.

Enzyme	Full Name	Donor	Step	Purification	References
CgtBS42	β-1,3-galactosyltransferase	UDP-Gal	1	NiNTA	Yang <i>et al.</i> , 2010
HP0826	β-1,4-galactosyltransferase	UDP-Gal	5	crude	Namdjou <i>et al.</i> , 2008
LgtD	β-1,3-N-acetylgalactosaminyltransferase	UDP-Gal	9	NiNTA	Hood <i>et al.</i> , 2001
WbgL	α-1,2-fucosyltransferase	GDP-Fucose	6	crude	Engels <i>et al.</i> , 2014
Te2FT	α-1,2-fucosyltransferase	GDP-Fucose	2, 10	NiNTA	Zhao <i>et al.</i> , 2016
BgtA	α-1,3-N-acetylgalactosaminyl transferase	UDP-GalNAc	3, 7, 12	NiNTA	Yi <i>et al.</i> , 2008
BoGt6a	α-1,3-N-acetylgalactosaminyl/galactosyl transferase	UDP-Gal	4, 8, 11	NiNTA	Tumbale <i>et al.</i> , 2009

Step numbers refer to steps shown in Supplementary Fig. 17

Supplementary Table 18 : Residual GalN antigen

Enzyme treated A⁺RBCs were analyzed using an observational agglutination assay. RBC samples were mixed with autologous or allogeneic A serum samples in a 1:1 ratio and observed for agglutination for 30 min at RT. The data shows that no residual GalN antigen intermediate is left on those RBCs treated with *Fp*GalNase. The experiment was performed one time with one A⁺RBC donor.

Samples	Agglutination Score	
	Autologous	Allogeneic
B ⁺ RBCs	5	5
O ⁺ RBCs	0	0
A ⁺ RBCs	0	0
<i>Fp</i> GalNAcDeAc treated A ⁺ RBCs	5	5
<i>Fp</i> GalNAcDeAc plus <i>Fp</i> GalNase treated A ⁺ RBCs	0	0
<i>Em</i> GH109 treated A ⁺ RBCs	0	0

Reactions contained 10% hematocrit.

Agglutination was determined by observation 5 is the best score, showing no agglutination after 30 min and 0 is the worst score with agglutination within 2 min.