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Glyco-engineered CHO cell lines producing alpha-1-antitrypsin and C1 esterase inhibitor with fully humanized N-glycosylation profiles

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Abbreviations:

A1AT, alpha-1-antitrypsin; **AATD**, alpha-1-antitrypsin deficiency; **AUC**, area under curve; **B3gnt2**, UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2; **Cas9**, CRISPR-associated protein 9; **C1INH**, C1 esterase inhibitor; **CHO**, Chinese hamster ovary; **CRISPR**, clustered regularly interspaced short palindromic repeats; **FACS**, fluorescence-activated cell sorting; **FITC**, Fluorescein isothiocyanate; **Fut8**, alpha-(1,6)-fucosyltransferase; **Glul**, glutamate-ammonia ligase; **HAE**, hereditary angioedema; **HM**, high–mannose; **indel**, insertion or deletion; **IVC**, integral of viable cells; **KO**, knock-out; **mAb**, monoclonal antibody; **Mgat4A**, mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase isozyme A; **Mgat4B**, mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase isozyme B; **Mgat5**, mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetylglucosaminyltransferase; **MSX**, methionine sulfoximine; **sgRNA**, single guide RNA; **SNA**, sambucus nigra agglutinin; **Sppl3**, signal peptide peptidase like 3;
St3gal3, ST3 beta-galactoside alpha-2,3-sialyltransferase 3;
St3gal4, ST3 beta-galactoside alpha-2,3-sialyltransferase 4;
St3gal6, ST3 beta-galactoside alpha-2,3-sialyltransferase 6;
ST6GAL1, ST6 beta-galactoside alpha-2,6-sialyltransferase 1;
VCD, viable cell density; **WT**, wild type

Abstract

Recombinant Chinese hamster ovary (CHO) cells are able to provide biopharmaceuticals that are essentially free of human viruses and have N-glycosylation profiles similar, but not identical, to humans. Due to differences in N-glycan moieties, two members of the serpin superfamily, alpha-1-antitrypsin (A1AT) and plasma protease C1 inhibitor (C1INH), are currently derived from human plasma for treating A1AT and C1INH deficiency. Deriving therapeutic proteins from human plasma is generally a cost-intensive process and also harbors a risk of transmitting infectious particles. Recombinantly produced A1AT and C1INH (*rh*A1AT, *rh*C1INH) decorated with humanized N-glycans are therefore of clinical and commercial interest.

Here, we present engineered CHO cell lines producing *rh*A1AT or *rh*C1INH with fully humanized N-glycosylation profiles. This was achieved by combining CRISPR/Cas9-mediated disruption of 10 gene targets with overexpression of human ST6GAL1. We were able to show that the N-linked glyco-structures of *rh*A1AT and *rh*C1INH are homogeneous and similar to the structures obtained from plasma-derived A1AT and C1INH, marketed as Prolastin®-C and Cinryze®, respectively. *rh*A1AT and *rh*C1INH produced in our glyco-engineered cell line showed no detectable differences to their plasma-purified counterparts on SDS-PAGE and had similar enzymatic *in vitro* activity. The work presented here shows the potential of expanding the glyco-engineering toolbox for CHO cells to produce a wider variety of glycoproteins with fully humanized N-glycan profiles. We envision replacing plasma-derived A1AT and C1INH with recombinant versions and thereby decreasing our dependence on human donor blood, a limited and possibly unsafe protein source for patients.

1 Introduction

Chinese hamster ovary (CHO) cells serve an important role in the biotechnology industry as the primary workhorse for the production of recombinant protein therapeutics [1]. Many of these therapeutics are glycoproteins that contain one or more N-glycan and/or O-glycan chains. As N-glycans can potentially affect protein folding, immune regulation, cellular homeostasis and the biological half-life of proteins [2, 3], it is considered a critical quality attribute and much effort has been put forth to improve features of protein N-glycosylation. The production of diverse N-glycan structures is a major contributor to the heterogeneity of protein products derived from CHO cells. The inherent heterogeneity of CHO N-glycan profiles is especially a drawback when one distinct N-glycan structure is desired on the protein product. Two examples of human plasma proteins with distinct, homogeneous N-glycan structures are found within the serpin superfamily, alpha-1-

antitrypsin (A1AT) and C1 esterase inhibitor (C1INH) [4]. Patients with the genetic disorders alpha-1-antitrypsin deficiency (AATD) or hereditary angioedema (HAE-C1INH) have decreased plasma levels of functional A1AT or C1INH, respectively, and are currently treated with prophylactic augmentation therapy of plasma purified A1AT or C1INH[5, 6]. Augmentation therapy is cost intensive [5], and C1INH purified from pooled donor plasma has been associated with hepatitis C virus infections prior to the introduction of virucidal methods[7]. Despite current dedicated virus inactivation steps, cases of Hepatitis G transmission have been reported [8] and non-enveloped viruses can still be transmitted via plasma-derived products [9]. Nevertheless, approved C1INH formulas are concentrates purified from human donors (Berinert®, Cinryze) despite containing undesired protein impurities identified as α1-antichymotrypsin, ceruloplasmin and Factor C3 [6].

Both native human plasma A1AT (*pl*A1AT) and C1INH (*pl*C1INH) possess a N-glycan profile with ~60–80% diantennary, disialylated, non-fucosylated (A2G2S2) structures with human-like alpha-2,6-linked sialic acids. *pl*A1AT has three N-glycosylation sites [4], where natural A2G2S2 structures are not essential for biological activity but enhance *in vivo* half-life and *in vitro* protein stability [10, 11]. *pl*C1INH is thought to be one of the most heavily glycosylated plasma proteins and harbors ten O-linked and six N-linked glycan structures [12]. The six N-linked glycan moieties with A2G2S2 as predominant structure have been shown to be increase serum half-life and are reported to increase *in vivo* efficacy [13–15].

The ability to generate high A2G2S2 N-glycan proportions on *rh*A1AT/*rh*C1INH may be critical for improving product quality. Although efforts have been reported, generating high proportions of A2G2S2 N-glycans has not to our knowledge been published. For instance, CHO cells lack active St6 beta galactoside alpha-2,6-sialyltransferase 1 (ST6GAL1) to cap N-glycans with alpha-2,6-linked sialic acids [16]. *rh*A1AT and *rh*C1INH were produced in various platforms [17-29]. However, these approaches revealed low

productivity or the N-glycosylation was far from the profile of *pl*A1AT or *pl*C1INH and therefore cleared rapidly from the human blood making intravenous administration impractical.

Commercially available *rh*C1INH from transgenic rabbits shows activity similar to *pl*C1INH and has decreased virus transmission risk. However, it differs in N-glycosylation profiles from *pl*C1INH and therefore reveals a risk of allergy, a dissatisfactory pharmacokinetic profile and consequently is unlikely to be of use in prophylaxis [30]. Glycosylation-engineering in primary human cells aimed to mimic O-glycan profiles of *pl*C1INH. However, complete sialylation of *rh*C1INH N-glycan structures was not achieved [31].

To address this, we aimed to engineer the heterogeneous CHO-S N-glycan profile towards a predominantly non-fucosylated biantennary A2G2 structure by combining the publicly available CHO-K1 genome sequence [32], clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) for multiplexing gene editing [33], and reported CHO glycosyltransferases [34],. To this end, we made functional knockouts of the Glul-gene and nine glycosylation-gene targets (10x KO, Suppl. Table 1). We hypothesized that *rh*A1AT and *rh*C1INH produced in this genetic background with parallel co-expression of ST6GAL1 would display an N-glycan profile similar to *pl*A1AT and *pl*C1INH with predominant A2G2S2 N-glycan structures. We present CHO clones producing *rh*A1AT/*rh*C1INH similar to *pl*A1AT/*pl*C1INH when assessed by N-glycan analysis, protein activity, SDS-PAGE and isoelectric focusing. CHO derived *rh*A1AT and *rh*C1INH with fully humanized N-glycan profiles have the potential to replace the cost-intensive and possibly unsafe plasma-based augmentation therapy of AATD and HAE-C1INH patients without compromising activity and N-glycosylation.

2 Materials and methods

2.1 sgRNA, GFP_2A_Cas9 and A1AT/C1INH_ST6GAL1_Glul plasmid design

GFP_2A_Cas9 and single guide RNA (sgRNA) plasmids were constructed as previously described [33]. The sgRNA target design for Mgat4A, Mgat4B, Mgat5, St3gal3, St3gal4, St3gal6, B3gnt2, Fut8, Sppl3 and Glul was performed using "CRISPy" [35]. The target sites for the mentioned genes and the oligos for sgRNA cloning are listed in Suppl. Table S1 and Table S2, respectively.

Plasmids for co-expression of A1AT/C1INH and ST6GAL1 were constructed with uracil-specific excision reagent cloning method as previously described [36, 37] (Suppl. Fig. 1). The DNA sequences of the plasmids are listed in Suppl. Table S5.

2.2 Cell cultivation and transfection for genome editing

CHO-S suspension cells were incubated in a humidified incubator at 120 rpm, 37˚C, 5% CO_2, passaged to 2-3 x 10^5 cells/mL every 2-3 days and transfected in 6-well plates (BD Biosciences, San Jose, CA) as described previously [33]. The GFP_2A_Cas9 / sgRNA plasmid ratios for each transfection was 1:1 of which the plasmid load of sgRNA was divided equally by the amount of different sgRNAs used per transfection (Suppl. Table S4). To measure FACS sorting efficiency, pmaxGFP® vector (Lonza, Basel, Switzerland) transfection was performed as well. Cells were harvested for fluorescence-activated cell sorting (FACS) 48 h post transfection.

2.3 Single cell cloning of genome edited cells using FACS

Prior to FACS, cells were filtered through a 40 µm cell strainer into a FACS-compatible tube.

Single fluorescent-positive (GFP) cells were sorted into 384-well plates (Corning, New York, NY) containing 30 µL CD CHO medium supplemented with 8 mM L-glutamine, 1.5% HEPES buffer and 1% Antibiotic-Antimycotic (Gibco, Waltham, MA) per well as described previously [38]. For cell sorting, fluorescent-positive cell populations were gated based on non-transfected WT CHO-S cells. Two weeks after cell sorting cell colonies were moved to

96-well flat-bottom plates (BD Biosciences) and expanded for deep sequencing analysis and batch cultivation.

2.4 Deep sequencing analysis

Confluent colonies from 96-well flat-bottom replicate plates were harvested for genomic DNA extraction. DNA extraction was performed using QuickExtract DNA extraction solution (Epicentre, Illumina, Madison, WI) according to the manufacturer's instruction. The library preparation was based on Illumina 16S Metagenomic Sequencing Library Preparation and deep sequencing was carried out on a MiSeq Benchtop Sequencer (Illumina, San Diego, CA). The protocol for amplifying the targeted genomic sequences, amplicon purification, adapter-PCR and following quality analysis was based on previously published work [33]. PCR primers are presented in Suppl. Table S3.

2.5 Transfection and expression in polyclonal cell lines by applying MSX-selection

Cells were seeded in 250 mL Corning vent cap shake flasks (Sigma-Aldrich) as duplicates with cell densities ~1 x 10^6 cells/mL in 60 mL CD CHO medium supplemented with 8 mM L-glutamine (Life Technologies) and transfected with 75 µg of A1AT-Glul-ST6GAL1 plasmid or 75 µg of C1INH-Glul-ST6GAL1 plasmid (Suppl. Fig. 1) using FreeStyle™ MAX reagent together with OptiPRO SFM medium (Life Technologies) according to the manufacturer's recommendations. 1µL/mL anti-clumping agent was added 24 h after transfection. pmaxGFP® vector (Lonza) transfection was performed to measure transfection efficiencies. Two days after transfection, cells were transferred into 60 mL CD CHO medium lacking L-glutamine (Life Technologies) and supplemented with 1µL/mL anti-clumping agent and 0 µM, 10 µM, 30 µM or 50 µM MSX (EMD Millipore, Billerica, MA). Cell densities and viabilities were determined once per day using the NucleoCounter NC-250 Cell Counter (ChemoMetec). The cells were passaged in fresh selection medium every 2-3 days until viability and doubling time reached stable values. Polyclonal cell lines (pools) were seeded in duplicates at ~1 x 10^6 cells/mL with corresponding MSX

concentrations. Cell densities and viabilities were determined once per day and supernatants of the pools were harvested three days after seeding and pooled within duplicates for purification of *rh*A1AT and *rh*C1INH.

2.6 Single cell cloning of cells from polyclonal cell pools using FACS

Non-stained single cells were sorted from pools as described above. For cell sorting, all viable cells were gated for sorting into 384-well plates with L-glutamine-free medium. Two weeks after cell sorting the clones were moved to 96-well flat-bottom plates (BD Biosciences) and expanded to shake flask format in CD CHO medium supplemented with 1µL/mL anti-clumping agent, 25 µM MSX and lacking L-glutamine.

2.7 Screening cell pools and single cell clones for human-like α-2,6-sialic acid linkage formation with lectin staining

For lectin staining of cells, triplicates of 10,000 cells per sample were diluted in 200 µL of 0.22 µm pore size filtered CD CHO medium (Life Technologies) supplemented with 5 µg/mL Hoechst 33342 (Merck, Darmstadt, Germany) and 1 µg/mL Fluorescein isothiocyanate (FITC) labeled *Sambucus nigra* agglutinin (SNA) lectin (Biomol, Hamburg, Germany). After 60 min incubation in the dark at 37°C and 5% CO_2, the cells were washed with 200 µL CD CHO medium and then washed twice with 200 µL phosphate buffered saline (PBS) (300g, 5 min, RT). The samples were resuspended in 200 µl PBS and transferred to 96-well plate for final centrifugation at 300 g for one minute. The percentage of FITC SNA positive cells was determined in a 96-well optical-bottom microplate (Greiner Bio-One, Frickenhausen, Germany) using a Celigo Imaging Cell Cytometer (Nexcelom Bioscience, Lawrence, MA). Cells were identified using the blue channel (Hoechst-positive cells), and the green channel (FITC SNA-positive cells) was used to detect cells with alpha-2,6-sialic acid linkage. A Hoechst/FITC SNA-stained CHO-S WT sample was gated to distinguish between FITC-positive and FITC-negative cells.

2.8 Batch cultivation: cell growth analysis and N-glycosylation profiling

For batch cultivation and N-glycan analysis, cells were seeded at 0.4 x 10^6 cells/mL in 250 mL Corning vent cap shake flasks (Sigma-Aldrich, St. Louis, MI) as duplicates in 60 mL CD CHO medium supplemented with 1 µL/mL anti-clumping agent (Life Technologies). CHO-S WT and non-producing parental 10x KO cell lines were additionally supplemented with 8 mM L-glutamine. *rh*A1AT/*rh*C1INH producing clones were cultivated in L-glutamine-free medium at all times and passaged in medium containing 25 µM MSX until the batch cultivation was initiated. Cell densities and viabilities were determined once per day using the NucleoCounter NC-250 Cell Counter (ChemoMetec) until the viability was <70%, at which point the culture was terminated. Supernatant samples with total secreted protein (secretome) from CHO-S WT and parental, non-producing 10x KO cell lines were taken five days after seeding and pooled within biological replicates. The volume for secretome samples was calculated to harbor 20 x 10^6 cells. For all shake flasks, additional supernatant samples were taken by centrifuging 1 mL of cell suspension for 5 minutes at 1000 g and storage of supernatant at -80°C until further analysis.

2.9 *rh*A1AT and *rh*C1INH purification

*rh*A1AT and *rh*C1INH were purified using CaptureSelect affinity resins (Thermo Fisher Scientific) according to the manufacturer's instructions. *rh*A1AT was further purified by size exclusion chromatography on a Superdex 200 increase 10/300GL column (GE Healthcare) equilibrated in PBS.

2.10 Titer assessment of *rh*A1AT/*rh*C1INH producing clones

*rh*A1AT and *rh*C1INH titers were determined using biolayer interferometry on an Octet RED96 (Pall, Menlo Park, CA, USA) as described previously for A1AT [39]. After hydration in PBS, streptavidin biosensors (18-5021, Fortebio, Pall) were functionalized with CaptureSelect biotin anti-A1AT conjugate or CaptureSelect biotin anti-C1INH conjugate (Thermo Fisher Scientific) at 5 µg/mL in PBS, and blocked in PBS containing 1 µg/mL biocytin (600 and 300 s incubation steps, respectively). Standards were prepared in spent

CHO-S medium using plasma-derived A1AT (Athens Research & Technology) at 100, 50, 25, 12.5, 6.3, 3.1, and 1.6 μg/mL or C1INH (R&D systems) at 40, 20, 10, 5, 2.5, 1.25, and 0.625 μg/mL. Samples and standards were diluted two-fold and contained 0.1% BSA w/v, 0.1% tween-20 v/v, and 500 mM NaCl. When needed, samples were further diluted to fall within the range of the standard dilution series. After equilibration in spent CHO-S medium (120 s), samples and standards were measured for 300 s with a shaking speed of 1000 rpm at 30°C. Regeneration was performed with 50 mM TRIS, 2 M $MgCl_2$, pH 7.5. Assays were performed in 96-well black microplates (Greiner Bio-One, Kremsmünster, Austria). Octet System Data Analysis 7.1 software was used to calculate binding rates and absolute A1AT and C1INH concentrations.

2.11 SDS-PAGE, isoelectric focusing and PNGase treatment

SDS-PAGE was performed on Novex 4-12% Tris-Glycine mini gels and isoelectric focusing (IEF) was performed on Novex pH 3-10 IEF gels (Thermo Fisher Scientific) as per the manufacturer's instructions. Deglycosylation with PNGase F was performed according to the manufacturer's instructions (New England Biolabs, Ipswich, MA).

2.12 Activity assays

A1AT inhibitory activity was determined using the EnzChek Elastase Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. In short, A1AT (8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.13, and 0.06 μM) was incubated with purified active porcine pancreatic elastase and fluorescently labelled substrate (DQ-elastin). Measurement of fluorescence was performed after 45 min at room temperature (Excitation: 485 nm, slit width 9.0 nm; Emission: 530 nm, slit width 13.5 nm).

C1INH inhibitory activity was determined using the Technochrom C1INH Assay Kit (TechnoClone, Vienna, Austria). In short, plasma containing C1INH activity (120%, 60%, 30%) and samples (~0.25 μM) were incubated with substrate-buffer mixture for 3 min at

room temperature, after which 50% acetic acid was added. Extinction was measured at 405 nm.

2.13 N-Glycan analysis

N-glycans were derivatized with GlycoWorks RapiFluor-MS N-Glycan Kit (Waters, Milford, MA) according to the manufacturer's instruction. Briefly; 12 µg purified protein or 12 µl of 10x concentrated (Amicon Ultra-15, Merck) secretome sample was used for each sample. Labeled N-Glycans were analyzed by LC-MS as described previously [33]. Separation gradient from 30% to 43% 50 mM ammonium formate buffer and MS were run in positive mode. Amount of N-Glycan was measured by integrating the peaks with Thermo Xcalibur software (Thermo Fisher Scientific, Waltham, MA) giving the normalized, relative amount of the glycans.

2.14 Proteomics

Preparation of protein extract from CHO cells were done as previously described in Bonde et al., 2016 [40]. Liquid chromatography was performed on a Cap-LC system (Thermo scientific) coupled to an 75 µm x 15 cm 2µm C18 easy spray column (Thermo Scientific). The flow rate 1.2 ul and using a stepped gradient, going from 4% to 40% acetonitrile water over 50 minutes. The samples were sprayed into an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Scientific). MS-level scans with resolution set to 60,000; Data dependent MS2 selection with HCD collision energy set to 28%. The resulting data were analyzed using MaxQuant. Data was searched against the Chinese hamster database retrieved from Uniprot with proteome Id UP000001075.

3 Results

3.1 Growth profile and N-glycan profile of clonal 10x KO cell lines

The aim of our study was to produce *rh*A1AT and *rh*C1INH in CHO cells with N-glycan profiles similar to human *pl*A1AT and *pl*C1INH. Our approach was to engineer the heterogeneous N-glycan profile of CHO-S WT cells towards a homogeneous A2G2S2 N-glycan structure, which is the predominant N-glycan on *pl*A1AT/*pl*C1INH. To this end, we generated out-of-frame insertions or deletions (indels) in eight glycosyltransferase genes (Mgat4A, Mgat4B, Mgat5, St3gal3, St3gal4, St3gal6, B3gnt2, Fut8) as well as in the genes Sppl3 and Glul (Suppl. Table S4) over four successive rounds of multiplexed CRISPR/Cas9 gene editing. Two clones with indels in the targeted genes were subjected to growth analysis and N-glycan profiling.

Two clones (10x KO A and 10x KO B) with out-of-frame indels in all ten gene targets were obtained and both showed a pronounced increase in batch culture longevity when compared to the parental CHO-S WT cell line (Fig. 1A).

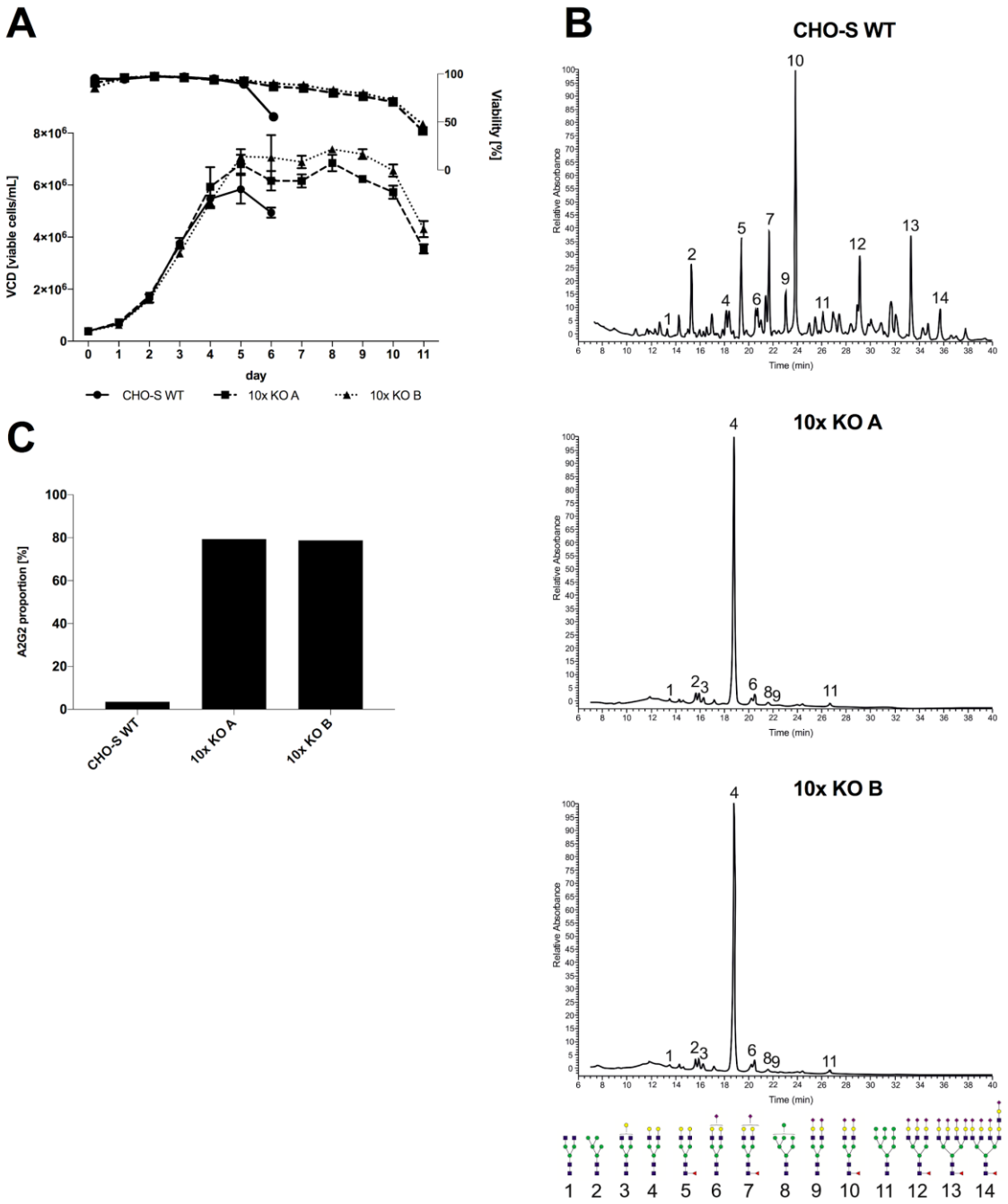

Fig. 1: Growth and N-glycan structure analysis of CHO-S WT and 10x KO cell lines. (A) Viable cell density (VCD) and viability of batch cultures of CHO-S WT and two clonal cell lines (10x KO A and 10x KO B) with indels in eight glycosyltransferases as well as Glul and Sppl3. Error bars indicate the standard deviation of triplicate parallel cultures. (B) N-glycan profiling of total secreted proteins from CHO-S WT and the 10x KO A and 10x KO B clones. In the chromatogram, elution time indicated on the x-axis and y-axis represents signal intensity normalized to highest peak. (C) Proportion of non-fucosylated, biantennary N-glycans with terminal galactose (A2G2) in total secreted proteins from CHO-S WT and the 10x KO A and 10x KO B clones.

CHO-S WT reached maximal viable cell density of ~6 x 10^6 cells/mL on day five and cell viability declined rapidly to <50% on day 6. In contrast, the 10x KO A and 10x KO B clones had cell viabilities >75% until day 10 of the batch cultivation and reached higher maximal viable cell density than CHO-S WT.

N-glycan analysis of the CHO-S WT secretome resulted in more than 25 annotated N-glycan structures (Fig. 1B) where the A2G2S2 structure, predominantly found on *pl*A1AT and *pl*C1INH, was not detected. The majority of CHO-S WT N-glycans contained core-fucosylation. The N-glycans produced by CHO-S WT cells appear diverse and comprise high-mannose structures as well as non-galactosylated, fully and partially sialylated di-, tri- and tetra-antennary structures (all with alpha-2,3-linked sialic acids). A2FG2S2 was found as the main N-glycan on total secreted proteins of CHO-S WT. In contrast, the N-glycan profiles of 10x KO A and 10x KO B are more homogeneous (Fig. 1B) with all structures lacking core-fucosylation. In addition, only relatively small amounts of CHO-specific alpha-2,3-linked sialylation were present.

After disruption of the targeted genes, the proportion of A2G2 within N-glycan structures of total secreted proteins was increased from 3.5% (CHO-S WT) to 79% in both 10x KO clones (Fig. 1C). We concluded that the 10x KO A and B clones were suitable host cell lines in our effort to generate humanized N-glycans.

3.2 Introducing human-like sialylation in 10x KO cell lines

On the basis of A2G2 secretome N-glycan structures of clone 10x KO B, we aimed to develop clonal cell lines expressing ST6GAL1 and *rh*C1INH or ST6GAL1 and *rh*A1AT. We envisioned that such cell lines are capable to produce *rh*A1AT or *rh*C1INH with predominant A2G2S2 N-glycan structures as found on *pl*A1AT and *pl*C1INH. The functional Glul-KO selection system was confirmed by MSX-dosage dependent recovery times of cell viabilities from transfected cell pools (Suppl. Fig. 3A). Passaging of the different

transfection pools was performed until viability and doubling times were stable. We then conducted FACS-based single cell cloning with the 50 μM MSX-selected cells. During the expansion of the generated clones, only clones exhibiting predominant FITC-SNA staining and detectable levels of *rh*A1AT/*rh*C1INH in supernatants on coomassie-stained SDS-PAGE gels were selected (Suppl. Fig. 2). Based on these criteria, two *rh*A1AT (A1-1 and A1-2) and two *rh*C1INH (C1-1 and C1-2) producing clones were selected for further characterization.

SNA lectins are reported to bind predominantly to sialic acids of N-glycans linked to the galactose residue in a human-like alpha-2,6-sialylation. Analyzing FITC-SNA-stained CHO-S WT, we found relatively low levels of alpha-2,6-sialylation (Fig. 2A). To determine the proportion of cells with human-like sialylation, FITC-SNA stained CHO-S WT samples were used to gate between FITC-positive and FITC-negative cells (Suppl. Fig. 2A). Within the two 50 μM MSX-selected polyclonal cell lines, <30% of the cells were found to comprise alpha-2,6-linked sialic acids on N-glycans of cell surface proteins (Fig. 2B). In comparison, 82-90% of the cells in the populations of the selected four clones (A1-1, A1-2, C1-1 and C1-2) had the desired alpha-2,6-linked sialic acids on their N-glycans.

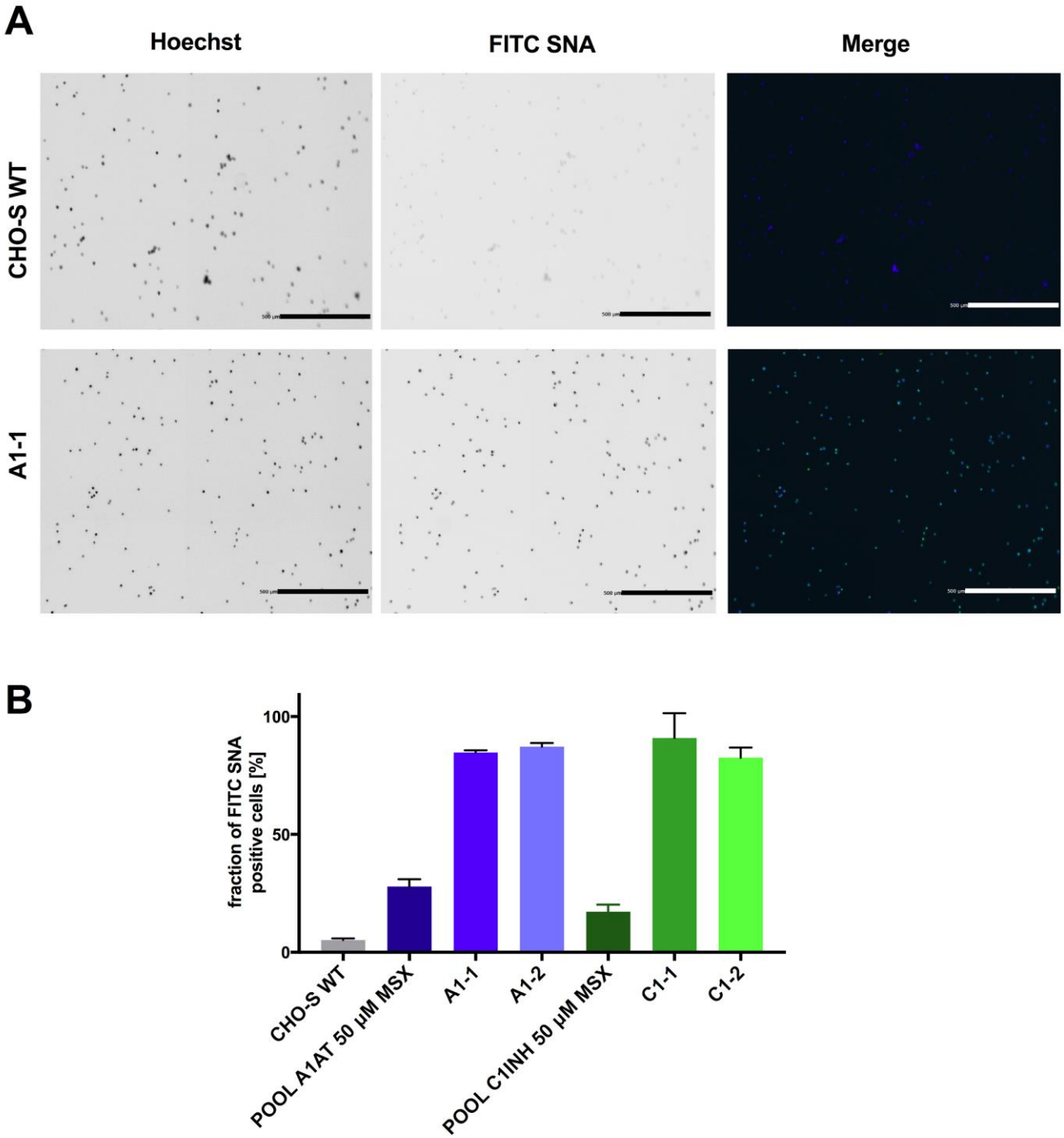

Fig. 2: FITC-SNA lectin staining of selected poly- and monoclonal cell lines. (A) Fluorescent images of CHO-S WT and A1-1 cell line. Cells were stained for alpha-2,6-sialic acid linkage with FITC-SNA (green) and for nuclei with Hoechst (blue). The bottom right corner bar displays a length of 500 µm (B) Comparison of FITC-SNA positive cells. FITC-SNA lectin staining of CHO-S WT, two 50 µM MSX polyclonal cell lines and four selected clones. Bars indicate the proportion of cells with positive FITC signal due to SNA lectin binding on alpha-2,6-linked sialic acids on the cell surface. Error bars represent standard deviation of three individual measurements per sample.

3.3 Extended culture longevity retained in *rh*A1AT and *rh*C1INH-producing clones

Both the two *rh*A1AT- and the two *rh*C1INH-producing clones showed the extended culture longevity as well as maximum VCD similar to the parental 10x KO B cell line (Fig. 3A). Viabilities of all clones were >75% until day 9 and clone C1-2 maintained cell viability >75% until day 11, similar to the non-producing parental 10x KO B.

In both *r*Ch1INH-producing clones, *rh*C1INH titers increased from day 0 until day 5, but then stagnated at ~40 µg/mL until the end of the batch cultures (Fig. 3B). In comparison, *rh*A1AT titers from clones A1-1 and A1-2 increased continuously to 123 µg/mL and 117 µg/mL , respectively. Despite increasing numbers of viable cells, the stagnation of C1INH titers at ~40 µg/mL for clones C1-1 and C1-2 in the second half of the batch cultures leads to the assumption that *rh*C1INH is unstable in the cell culture. By SDS-PAGE gel analysis of late phase supernatant samples we observed protein bands migrating just below *rh*C1INH (~70-100 kDa) which were not present in the non-producing parental clone (Suppl. Fig. 4A).

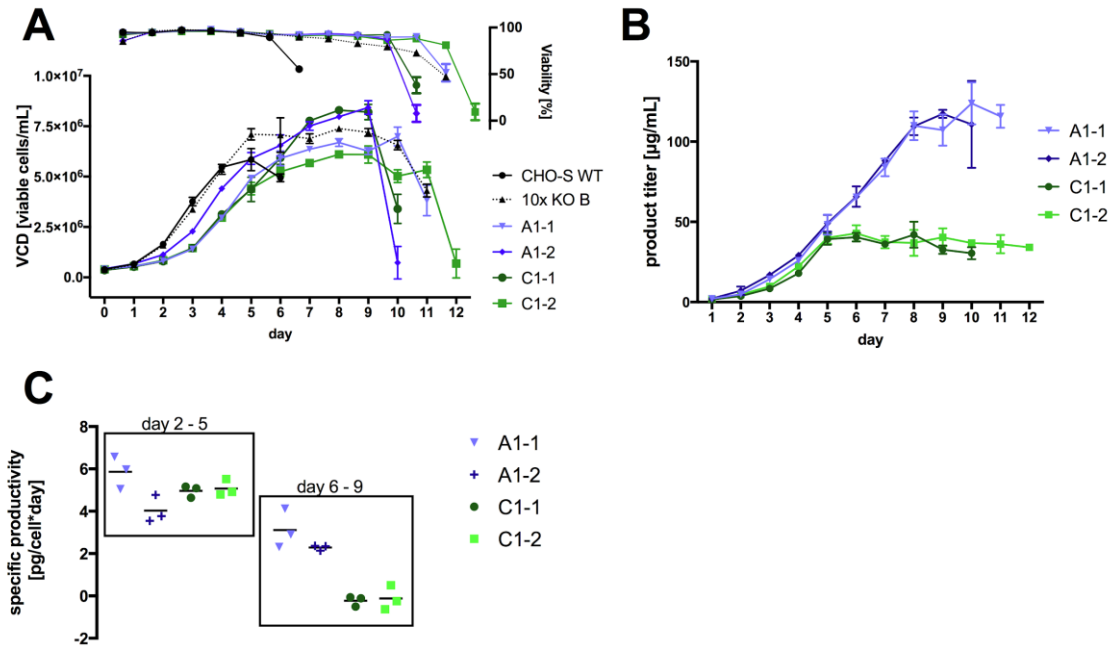

Fig. 3: Growth profiles, product titers and specific productivities of selected producing and non-producing clones. (A) Viable cell densities and cell viabilities of CHO-S WT, 10x KO B the *rh*A1AT- (A1-1 and A1-2) and *rh*C1INH- (C1-1 and C1-2) producing clonal cell lines

measured in batch cultures. Error bars indicate range of duplicate parallel cultures. (B) *rh*A1AT and *rh*C1INH titer in supernatants during the batch culture experiment. Error bars indicate standard deviation of three individual measurements from two shake flasks per clone. (C) Specific productivities of the *rh*A1AT and *rh*C1INH-producing clonal cell lines in the batch culture experiment. Average specific productivity was calculated from day 2 – 5 and from day 6 – 9. Colored symbols represent average measured specific productivity for shake flask duplicates. Black lines shows the average specific productivity based on the three measurements of shake flask duplicates.

On the basis of *rh*A1AT/*rh*C1INH concentration in the cultivation supernatant and integral of viable cells (IVC), we determined the average specific productivity of the four clones during day 2 – 5 and day 6 - 9 (Fig. 3C). In the early phase, the specific productivity of *rh*A1AT for clones A1-1 and A1-2 was 5.8 and 4.0 pg/cell*day, respectively, decreasing to 2–3 pg/cell*day in the late phase (day 6–9). On the contrary, the two *rh*C1INH-producing clones expressed *rh*C1INH at ~5 pg/cell*day in the early phase, whereas their specific productivity in the late phase decreased to ~0 pg/cell*day. Overall, cell growth of all four clones was comparable to the parental cell line whereas product titers of A1AT-producing clones were increased compared to C1INH-producers.

3.4 Activity and N-glycosylation profile of CHO-produced *rh*A1AT and *rh*C1INH are similar to plasma-derived products

We purified and characterized *rh*A1AT and *rh*C1INH to investigate the impact of our N-glycosylation engineering approach on product quality and protein activity. Therefore we compared the protein products produced in clonal cell lines derived from 10x KO B (*rh*A1AT and *rh*C1INH) to the CHO-S WT and plasma-derived counterparts (*pl*A1AT and *pl*C1INH).

SDS-PAGE gel analysis revealed that purified *rh*A1AT and *rh*C1INH produced in the four clones seem to have hydrodynamic volumes (molecular weight) similar to *pl*A1AT and *pl*C1INH without detectable impurities as seen in *pl*C1INH (Fig. 4A). *rh*A1AT and *rh*C1INH produced in CHO-S WT background did not co-migrate with *pl*A1AT and *pl*C1INH,

respectively. However, after deglycosylation with PNGaseF, all recombinantly produced proteins aligned with corresponding bands of *pl*A1AT and *pl*C1INH with the exception of *rh*C1INH produced in a CHO-S WT background displayed an additional protein band at ~65 kDa.

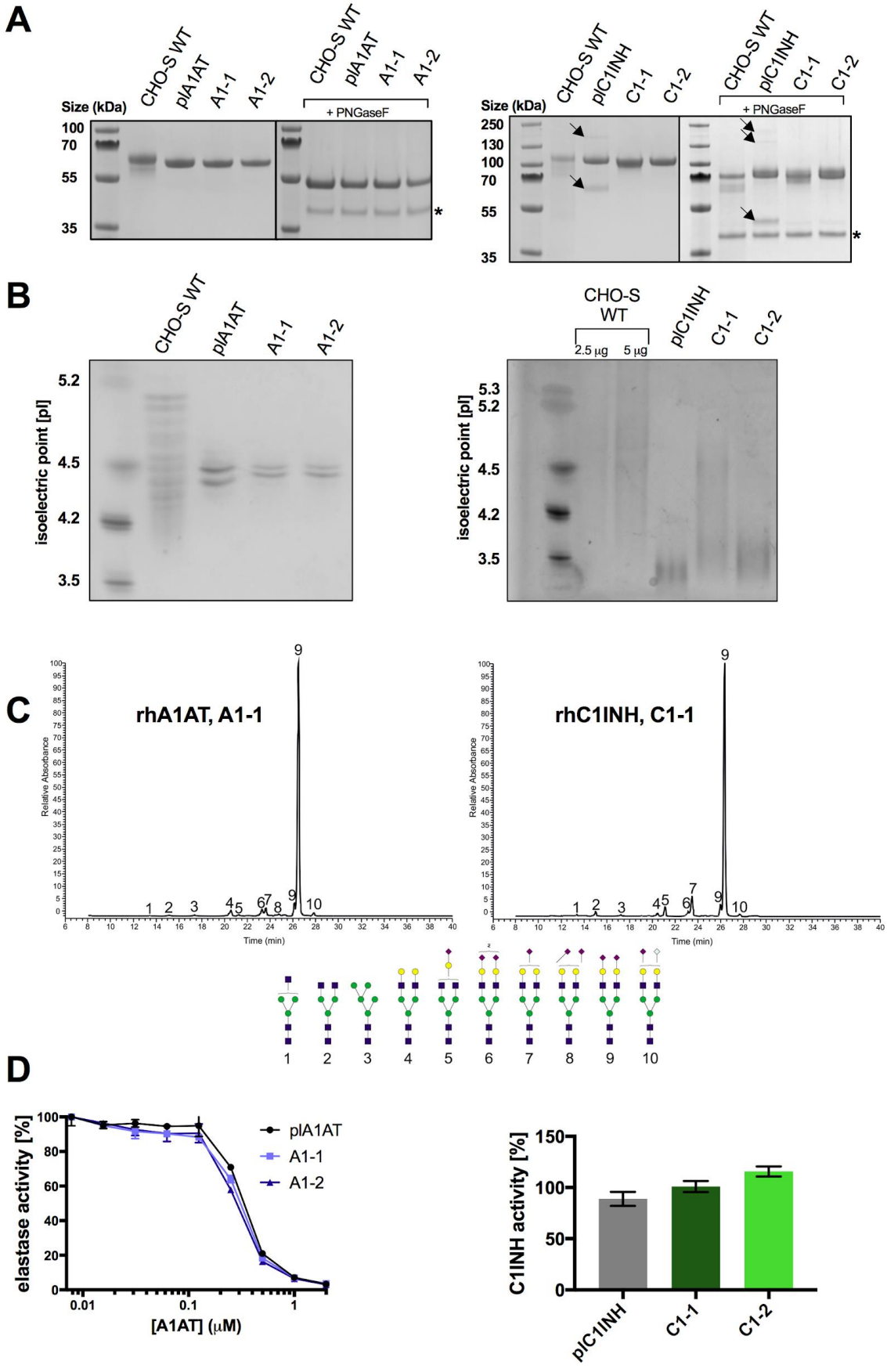
A
Size (kDa)
CHO-S WT
plA1AT
A1-1
A1-2
+ PNGaseF
100
70
55
35
plC1INH
C1-1
C1-2
250
130
B
isoelectric point [pI]
5.2
4.5
4.2
3.5
2.5 µg
5 µg
5.3
C
rhA1AT, A1-1
rhC1INH, C1-1
Relative Absorbance
Time (min)
D
elastase activity [%]
[A1AT] (µM)
C1INH activity [%]

Fig. 4: Characterization of purified *rh*A1AT and *rh*C1INH. (A) SDS-PAGE gel analysis of commercially available Cinryze (*pl*C1INH) and Prolastin-C (*pl*A1AT) as well as *rh*A1AT and *rh*C1INH purified from polyclonal CHO-S WT or from monoclonal cell lines derived from 10x KO B. Removal of N-glycans by PNGaseF was performed where indicated. PNGaseF migrating as a ~40 kDa band is indicated with an asterisk and impurities of *pl*C1INH are indicated with arrows. (B) IEF gel analysis of same proteins as described for panel A. 2.5 µg purified protein was analysed per sample if not indicated otherwise. (C) N-glycan structures annotated from *rh*A1AT and *rh*C1INH produced in clones A1-1 and C1-1. (D) Left panel: *In vitro* assay measuring the inhibition of elastase activity at different concentrations of *pl*A1AT and *rh*A1AT purified from clones A1-1 and A1-2. Error bars indicate range of duplicate measurements. Maximum proteolytic activity of porcine elastase was set to 100%. Right panel: *In vitro* activity assessment of *pl*C1INH and *rh*C1INH purified from clones C1-1 and C1-2. As described in the assay, 1 IU/ml C1INH activity was set to 100%. Error bars indicate range of duplicate measurements.

To further characterize the CHO-produced *rh*A1AT and *rh*C1INH, we performed IEF gel analysis (Fig. 4B). *rh*A1AT from clones A1-1 and A1-2 manifested in two bands with isoelectric points (pI) at ~ pH 4.5 similar to *pl*A1AT. In contrast, *rh*A1AT produced in a CHO-S WT background displayed more than nine detectable isoforms with pI between pH 4 – 5.

IEF gel analysis of *rh*C1INH produced in a CHO-S WT background resulted in isoforms with pI ranging from pH ~4 – 5.5. A high degree of heterogeneity was also found in purified *rh*C1INH produced in clone C1-1. However, *rh*C1INH produced in clone C1-2 was less heterogeneous with pI at pH ~3.5 similar to *pl*C1INH.

In N-glycan analysis of purified *rh*A1AT and *rh*C1INH from CHO-S WT cells we detected a higher degree of heterogeneity compared to N-glycan structures on *rh*A1AT and *rh*C1INH from polyclonal 10x KO cell pools (Suppl. Fig. 3B). The polyclonal cell lines revealed two predominant sugar structures on both proteins (A2G2 and A2G2S2 N-glycans), whereas we could not detect the A2G2S2 structure on products from CHO-S WT. Moreover, the amount of predominant N-glycan structures on *rh*A1AT and *rh*C1INH was decreased from two (polyclonal pools) to one (monoclonal producers), identified as A2G2S2 N-glycan (Fig. 4C).

All four 10x KO-derived monoclonal cell lines produced *rh*A1AT and *rh*C1INH with higher proportion of A2G2S2 structures than *pl*A1AT and *pl*C1INH (Suppl. Fig. 2C and Suppl. Fig. 3C). The proportion of A2G2S2 in *rh*A1AT and *rh*C1INH was approximately 88 - 92% and 84%, respectively, and 82% for *pl*A1AT and 66% for *pl*C1INH .

Finally, we investigated the activity of purified *rh*A1AT and *rh*C1INH. *rh*A1AT activity was determined by its inhibitory function of elastase activity (Fig. 4D, Suppl. Fig. 4B). Similar to *pl*A1AT and *rh*A1AT from CHO-S WT, a decrease in elastase activity was detected at A1AT concentrations >0.1 µM for *rh*A1AT from clones A1-1 and A1-2. In addition, 50% of elastase inhibition was reached at ~0.3 µM A1AT for *pl*A1AT as well as *rh*A1AT. *In vitro* activity of purified *rh*C1INH produced by clones C1-1 and C1-2 was similar or higher compared to *pl*C1INH.

4 Discussion

We aimed to produce *rh*A1AT and *rh*C1INH in CHO-S with N-glycan profiles similar to *pl*A1AT and *pl*C1INH, which to our knowledge has not yet been achieved by recombinant expression. First, the heterogeneous N-glycan profile of CHO-S WT cells was changed to more homogeneous profiles in bespoke cell lines with predominant A2G2 N-glycan structures (Fig. 1B). Disrupting nine N-glycosylation-related genes increased the A2G2 proportion on total secreted protein from 3.5% in CHO-S WT-derived cells to ~80% in 10x KO cell lines (Fig. 1C). This supports the previously suggested strategy to decrease N-glycan branching and alpha-2,3-sialylation by disrupting Mgat4A, Mgat4B, Mgat5, St3gal3, St3gal4 and St3gal6 [34]. The impact of gene disruptions on cell culture performance was assessed in batch cultures. Interestingly, the monoclonal cell lines with disruption in ten gene targets showed enhanced growth characteristics compared to CHO-S WT cells (Fig. 1A). Overexpression of the Glul gene has previously been found to decrease ammonia levels, which might explain improved CHO cell growth of the four characterized producer

clones in L-glutamine-free medium [39]. However, the cause for the boosted cell growth of Glul-lacking 10x KO cell lines in L-glutamine-supplemented medium (Fig. 1A) remains to be explored in further studies. Additionally, when CHO-S cells are subjected to a single cell sorting-based selection procedure, we suspect that the protocol generates monoclonal cell lines with an extended culture longevity phenotype compared to the parental cell line. Thus, we do not suggest that knocking out the 10 gene targets lead to the observed extended culture longevity phenotype.

Since the disruption of the ten targets did not seem to interfere with cell culture performance, we performed co-expression of ST6GAL1 and *rh*A1AT or ST6GAL1 and *rh*C1INH in the 10x KO-derived clone B. After transfection, we observed a MSX-concentration dependent recovery of the transfected cell pools and successful killing of untransfected 10x KO B after 5 days of growth in L-glutamine-free medium similar to a previous study (Suppl. Fig. 3A) [41]. However, untransfected CHO-S WT cells were also able to recover up to the highest MSX-concentration of 50 µM, which is in accordance to previous work [36]. As shown in the killing curve of the untransfected 10x KO cells, the advantage of the Glul-KO system here seems to be the elimination of untransfected cells.

Surprisingly, after selection at 50 µM MSX, the polyclonal cell lines did not show the desired predominant A2G2S2 glycosylation of purified *rh*A1AT and *rh*C1INH as we found incomplete sialylation on both proteins (Suppl. Fig. 3B). Similar lack of sialylation in the polyclonal cell lines was found after FITC-SNA lectin staining where only <30% of cells were identified to have alpha-2,6-sialylation (Fig. 2B). This might be due to incomplete vector integration into the genomic DNA or chromosome instability leading to a heterogeneous cell population with reduced stability of the integrated elements as reported earlier [42]. The cell pools most likely harbor a mixture of cells which do not all express the ST6GAL1-protein successfully. However, we were able to discard clones with

incomplete sialylation by single cell cloning and screening for FITC-SNA lectin positive clones producing *rh*A1AT or *rh*C1INH.

Although the stagnating *rh*C1INH titer at day 5, the reported *rh*C1INH titer here is higher than previously reported production platforms with maximum titers of only 6 µg/mL in insect cells [29] and 30 µg/mL in *P. pastoris* [27] albeit the media we used was without any optimization for protein production. Further investigation needs to be carried out to understand the stagnation of *rh*C1INH titers. However, it can be speculated that the rate of *rh*C1INH production is similar to the rate of *rh*C1INH degradation, preventing an increase of *rh*C1INH titer in the cultivation supernatant.

In comparison to *pl*C1INH (Cinryze), the *rh*C1INH from C1-1 and C1-2 did not show α1-antichymotrypsin, ceruloplasmin and Factor C3 impurities on SDS-PAGE gels [8] (Fig. 4A), implying that CHO-based cell platforms possibly can supply HAE-C1INH patients with higher purity than human plasma.

With titers between 300 – 400 µg/mL, human neuronal cell lines produce *rh*A1AT with higher titers than our clones A1-1 and A1-2. However, *rh*A1AT from these neuronal cells exhibits core-fucosylation, is not fully sialylated, and therefore differs largely from *pl*A1AT N-glycosylation [24]. Similarly, earlier studies expressed *rh*A1AT in CHO with titers of up to 1.15 g/L, though differing from *pl*A1AT by revealing core-fucosylation and alpha-2,3-sialylation [25, 26, 43].

In contrast to the production platforms listed earlier, *rh*A1AT and *rh*C1INH produced in our 10x KO cell lines are not only exceeding sialylation levels of *pl*A1AT and *pl*C1INH (Suppl. Fig. 2C), but also reveal human-like alpha-2,6-sialylation instead of alpha-2,3-sialylation. Previous work reported only 2.6% A2G2S2 structures on *rh*A1AT expressed in CHO-K1, however with 2,3- sialic-acid linkage [26].

Interestingly, the increased sialylation of *rh*A1AT from the two clones had no impact on *in vitro* activity (Fig. 4D). This is in accordance with previous work, which showed that A1AT

activity is not linked to its N-glycosylation and CHO WT produced *rh*A1AT has similar activity to *pl*A1AT [10, 38]. Furthermore, differences of CHO-S WT- and 10x KO-derived *rh*A1AT were made visible using IEF gel analysis, where *rh*A1AT from the two clones revealed similar patterns to *pl*A1AT (Fig. 4B).

As presented in Fig. 4B, in contrast to *rh*A1AT, *rh*C1INH differed partially in IEF gel analysis profile from *pl*C1INH. Increased *rh*C1INH sialylation (Suppl. Fig. 2C) might lead to altered charge distribution and consequently cause changes in IEF gel patterns. For *rh*C1INH from clone C1-1, we observed a double-band in SDS-PAGE analysis although N-glycans were removed by PNGaseF treatment (Fig. 4A). This might indicate that O-glycan charge variants are responsible for the heterogeneity observed in IEF gel analysis and SDS-PAGE of *rh*C1INH from C1-1 (Fig. 4A and 4B) as described previously [44].

In summary, our work describes a strategy to successfully engineer the heterogeneous N-glycosylation profile of CHO-S WT cells to the specific A2G2S2 N-glycan structure with the purpose of producing *rh*A1AT and *rh*C1INH with N-glycan profiles similar to human plasma-derived products. We used CRISPR/Cas9 to disrupt ten genes and then overexpressed *rh*A1AT or *rh*C1INH on a ST6GAL1- and Glul-encoding plasmid. After selection with MSX and single cell cloning, we identified clones expressing *rh*A1AT or *rh*C1INH with titers of up to 124 µg/mL and 42 µg/mL, respectively (Fig. 3B). Purified *rh*A1AT and *rh*C1INH were similar to the plasma-derived counterparts judged by SDS-PAGE analysis (Fig. 4A), degree and type of sialylation (Fig. 2B, Suppl. Fig. 2C, Suppl. Fig. 3C) and *in vitro* activity (Fig. 4D). Thus, the work presented shows the promise and potential of replacing cost-intensive and possibly unsafe plasma-derived augmentation therapy for AATD and C1INH-HAE patients by CHO- produced *rh*A1AT and *rh*C1INH. This strategy is in compliance with the Medical and Scientific Advisory Council (MASAC) recommendation of replacing plasma-derived products with recombinant products for treatment of diseases [45].

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Author contributions

A. H. H., S. K., H. G. H., M. R. A., H. F. K., B. V., J. A., S. N. and T. A. designed the experiments. T. A. and J. A. constructed the knock-out cell lines. S. N. designed and cloned the expression plasmids. T. A. generated and analyzed the final cell lines, performed protein expression and wrote the manuscript. S. K. performed protein purification and analysis. A. H. H. conducted N-glycosylation analysis. A. H. H., S. K., H. G. H., J. A., M. R. A., B. V., H. F. K. and G. M. L. guided the project, contributed to experimental design and commented and corrected the manuscript.

Competing interests

A patent based on this work has been filed. The authors on the patent are A. H. H., S. K., B. V., H. F. K. . The International Patent Application No. is EP17204071. The remaining authors declare no competing financial interests.

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Supplementary Material for

Glyco-engineered CHO cell lines producing alpha-1-antitrypsin and C1 esterase inhibitor with fully humanized N-glycosylation profiles

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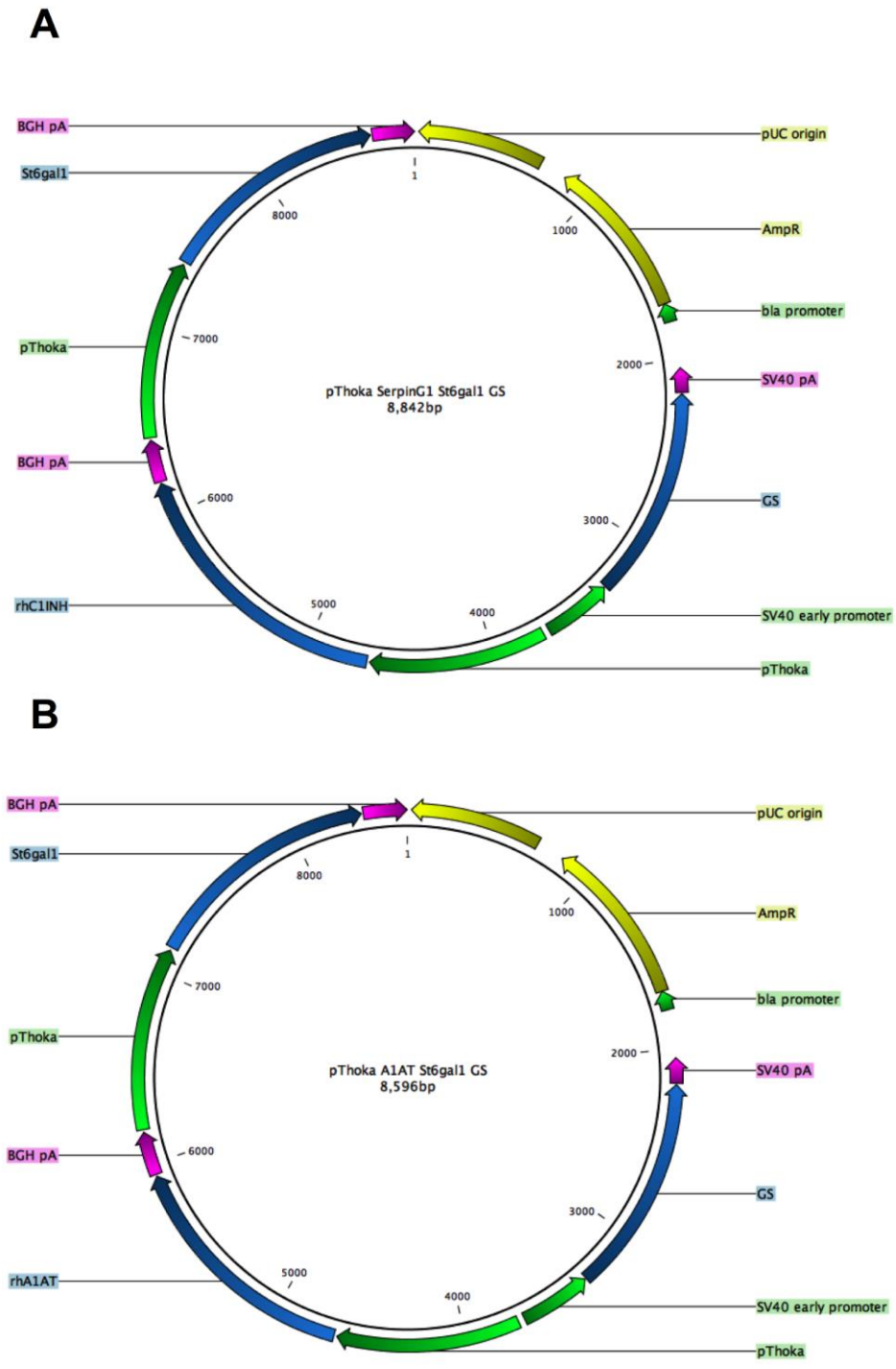
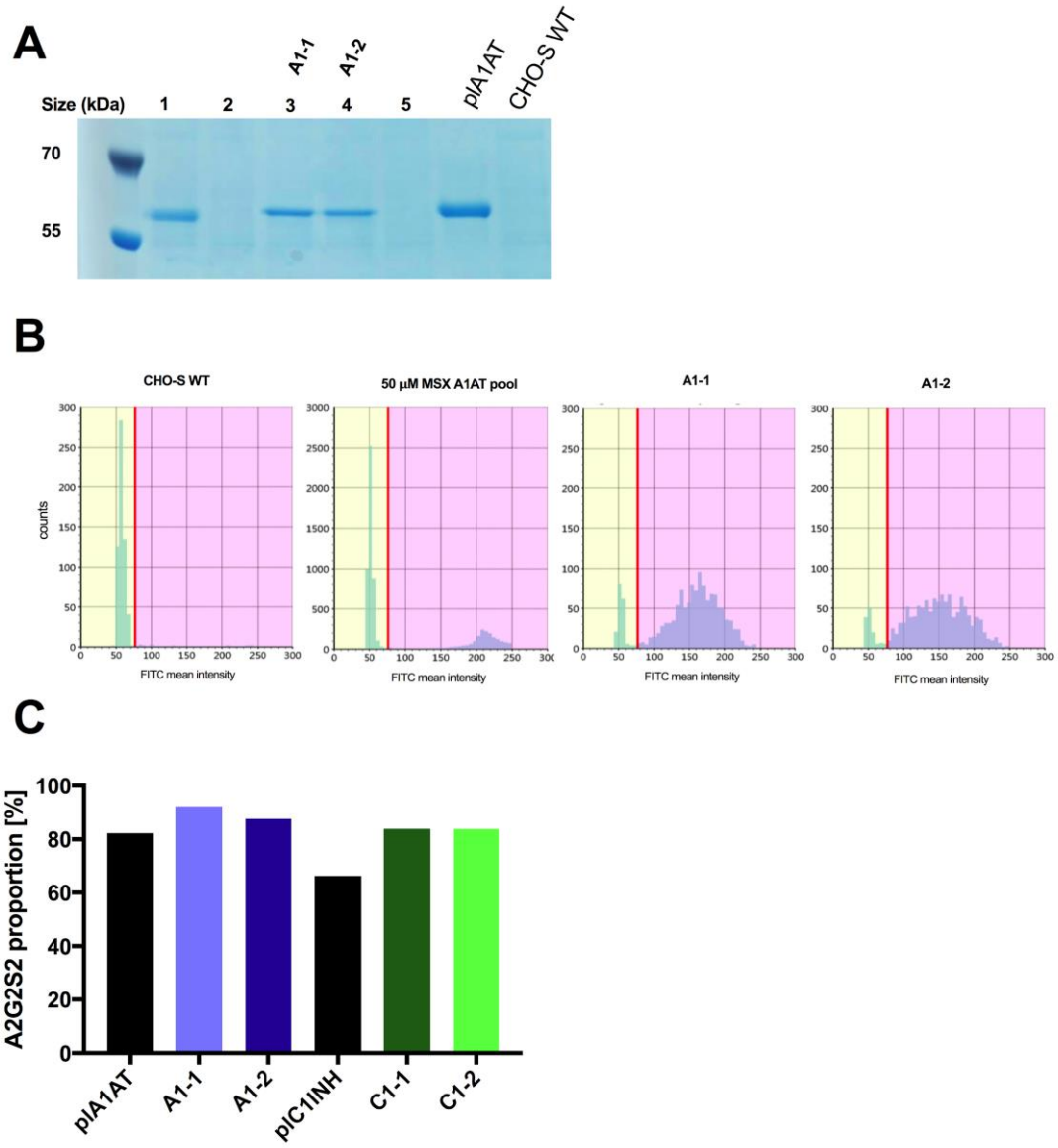
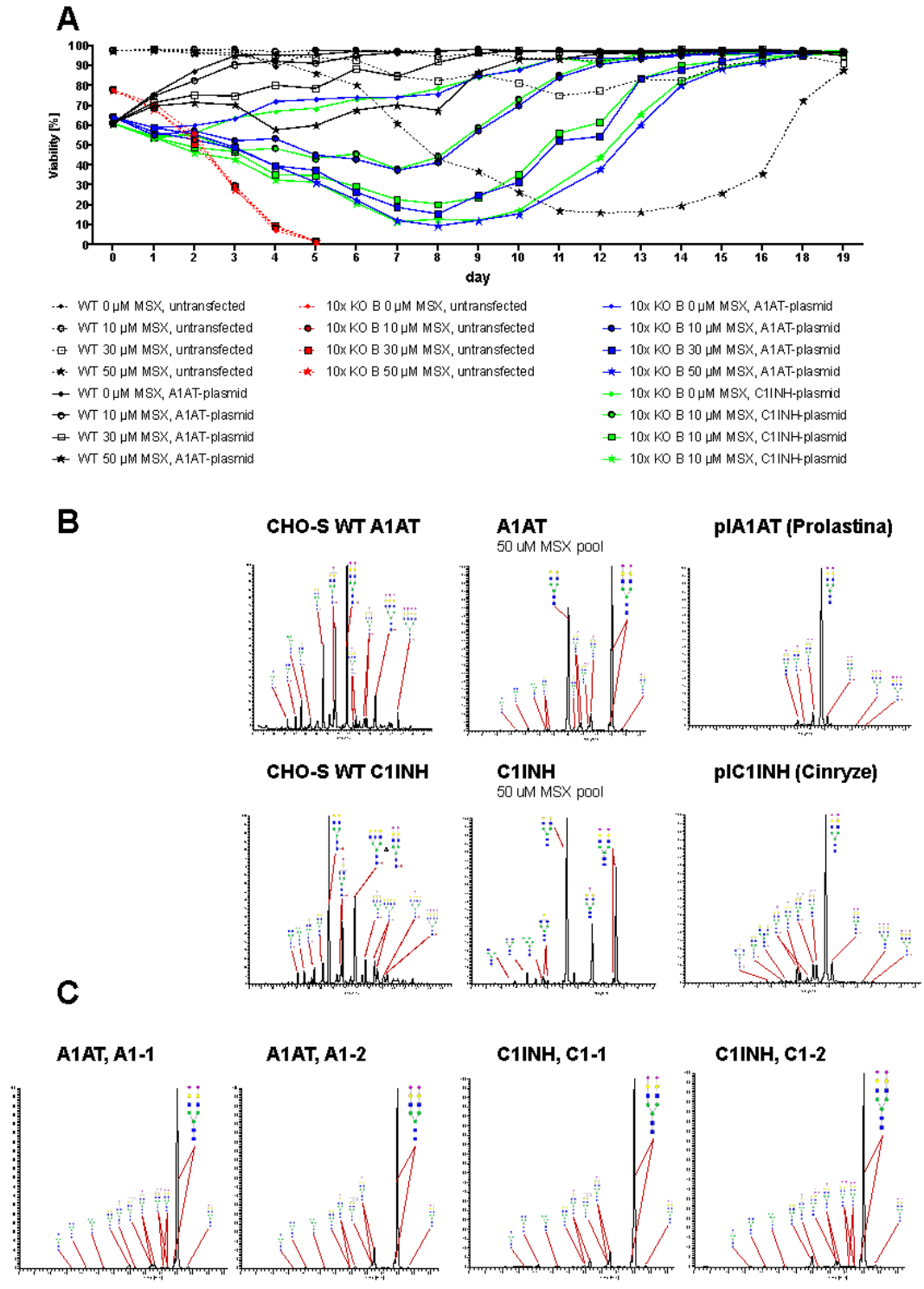


Suppl. Fig. 1: Vector design for overexpression. (A) *rh*C1INH and ST6GAL1 are under the control of mCMV-hEF-1a-5' consisting of an mCMV enhancer element, a hEF-1a promoter element, and a 5' HTLV untranslated region. The plasmid backbone consists of an ampicillin resistance cassette and SV40 early promoter in front of the Glul (GS) sequence. Poly-A sequences (purple) terminate Glul, *rh*A1AT and *rh*C1INH cassettes. (B) *rh*A1AT and ST6GAL1 are under the control of mCMV-hEF-1a-5'. The plasmid backbone consists of an ampicillin resistance cassette and

SV40 early promoter in front of the Glul (GS) sequence. Poly-A sequences (purple) terminate Glul, *rh*A1AT and *rh*C1INH cassettes.

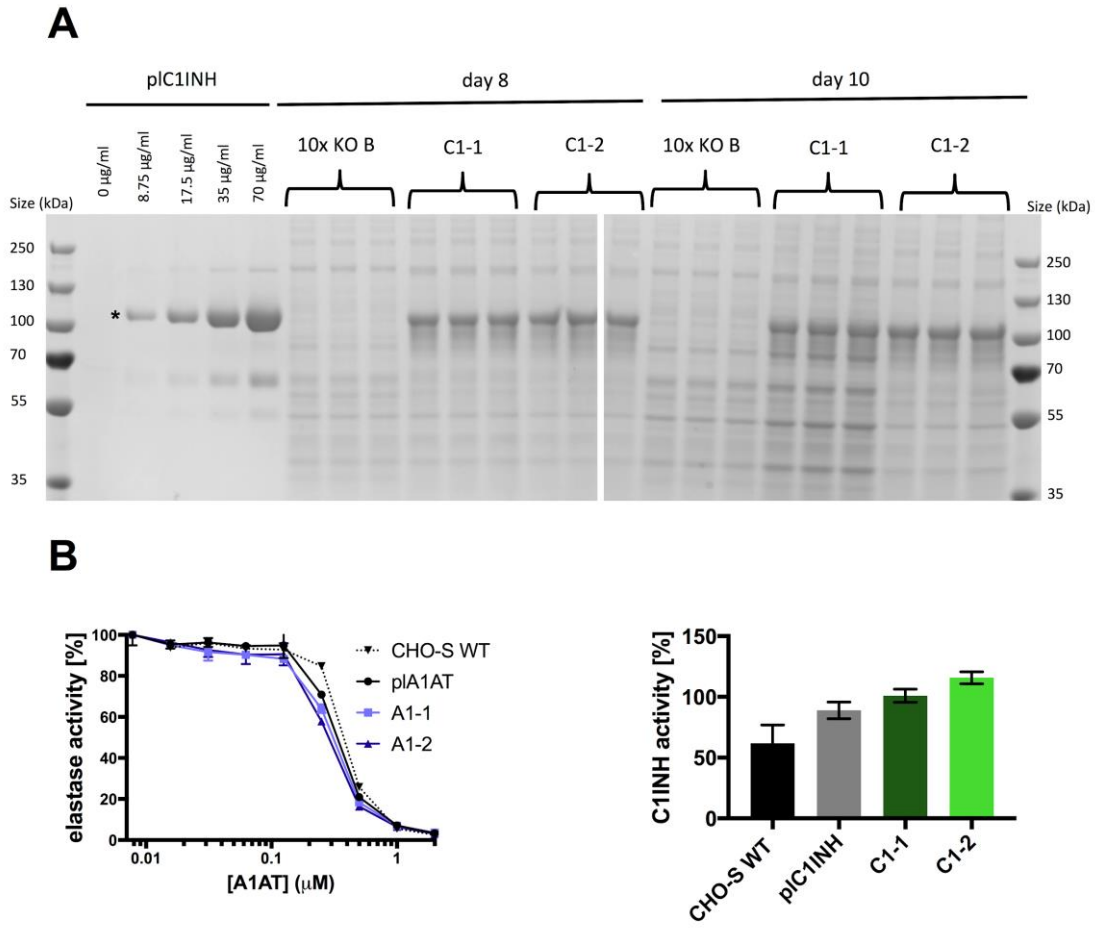


Suppl. Fig. 2: Screening for FITC SNA positive and *rh*A1AT producing clones. (A) Supernatant of monoclonal cell lines (clone 1 - 5) on SDS PAGE to screen for *rh*A1AT producing clones. Control samples consist of plA1AT and supernatant from non-producing CHO-S WT cells. (B) Cells of CHO-S WT, the 50 µM MSX A1AT polyclonal cell line and the clones A1-1 and A1-2 were stained with FITC-SNA. The histograms show the number of cells on y-axis and FITC mean intensity on the x-axis. CHO-S WT cells were used for gating between FITC-positive and -negative cells (red line). (C) N-glycan analysis of purified A1AT and C1INH versions. A2G2S2 proportions of purified *rh*A1AT and *rh*C1INH from different clones compared to *pl*A1AT and *pl*C1INH.



Suppl. Fig. 3: Viability of cells during MSX-based selection and N-glycan analysis of A1AT and C1INH derived from plasma as well as polyclonal and monoclonal cell lines. (A) Cell viability of non-transfected and transfected cell lines during selection with different concentrations of MSX. (B) N-glycan histograms of purified A1AT and C1INH from transiently transfected CHO-S WT and 50 µM MSX-selected polyclonal cell lines in comparison to Cinryze and Prolastin-C. (C) N-glycan

analysis of purified *rh*A1AT and *rh*C1INH from four selected monoclonal producing cell lines.



Suppl. Fig. 4: Analysis of cell culture supernatants and protein activity after protein purification. (A) SDS-PAGE gel analysis of supernatants from C1INH-producing clones and the 10x KO B parental cell line during batch cultivation. 10 µL of supernatant from day 8 and 10 of triplicate cultures as well as *pl*C1INH at different concentrations were analyzed by SDS-PAGE under reducing conditions. Intact C1INH is migrating slightly above 100 kDa as indicated by an asterisk. (B) A1AT activity assay of *pl*A1AT, *rh*A1AT transiently expressed in CHO-S WT and *rh*A1AT from stable clones A1-1 and A1-2 (left panel) as well as C1INH activity assay of *pl*C1INH, *rh*C1INH transiently expressed in CHO-S WT and *rh*C1INH from stable clones C1-1 and C1-2 (right panel).

Table S1: sgRNA target sequences. The bases in red mark the PAM site

Gene name of target	hypothesized KO effect	Target sequence (5'→ 3')
MGAT4A	decreased branching	GTCTACATTCGTCACTGTCGGGG
MGAT4B	decreased branching	GCTTCAGTCGCGGATCCTCTGGG
MGAT5	decreased branching	GGATGGCTACCCCCACTGCGAGG
ST3GAL3	decreased sialylation	GATCCTAGCCCACTTTCGAAAGG
ST3GAL4	decreased sialylation	GTGTCGTCGTTGTGTTGTGGTGG
ST3GAL6	decreased sialylation	GGAGTTGTGATCATTGTGAGCGG
B3GNT2	decreased elongation	GTTGGGCAAGACGCCCCCCGAGG
FUT8	no core-fucosylation	GTCAGACGCACTGACAAAGTGGG
SPPL3	hyper-glycosylation	AGAGAGACGGACGCTCCAATAGG
GLUL*	Gln-dependent growth	TCCCAAATCAGCAAACAGACTGG

*the Glul sgRNA efficiency during KO-generation of the presented sequence was very low compared to other target sgRNAs and we recommend the usage of a different design

Table S2: Oligos for sgRNA expression vector cloning.

Oligo Name	Oligo sequence (5’ → 3’)
gRNA_MGAT4A_411545_fwd	GGAAAGGACGAAACACCGTCTACATTCGTCACTGTCGGTTTTAGAGCTAGAAAT
gRNA_MGAT4A_411545_rev	CTAAAACGACAGTGACGAATGTAGACCGGTGTTTCGTCCTTTCCACAAGATAT
gRNA_MGAT4B_1280368_fwd	GGAAAGGACGAAACACCGCTTCAGTCGCGGATCCTCTGTTTTAGAGCTAGAAAT
gRNA_MGAT4B_1280368_rev	CTAAAACAGAGGATCCGCGACTGAAGCGGTGTTTCGTCCTTTCCACAAGATAT
gRNA_MGAT5_327084_fwd	GGAAAGGACGAAACACCGGATGGCTACCCCCACTGCGGTTTTAGAGCTAGAAAT
gRNA_MGAT5_327084_rev	CTAAAACCGCAGTGGGGGTAGCCATCCGGTGTTTCGTCCTTTCCACAAGATAT
gRNA_ST3GAL3_244730_fwd	GGAAAGGACGAAACACCGATCCTAGCCCACTTTCGAAGTTTTAGAGCTAGAAAT
gRNA_ST3GAL3_244730_rev	CTAAAACTTCGAAAGTGGGCTAGGATCGGTGTTTCGTCCTTTCCACAAGATAT
gRNA_ST3GAL4_964386_fwd	GGAAAGGACGAAACACCGTGTCGTCGTTGTGTTGTGGGTTTTAGAGCTAGAAAT
gRNA_ST3GAL4_964386_rev	CTAAAACCACAACACAACGACGACACCGGTGTTTCGTCCTTTCCACAAGATAT
gRNA_ST3GAL6_1812502_fwd	GGAAAGGACGAAACACCGGAGTTGTGATCATTGTGAGGTTTTAGAGCTAGAAAT
gRNA_ST3GAL6_1812502_rev	CTAAAACCTCACAATGATCACAACTCCGGTGTTTCGTCCTTTCCACAAGATAT
gRNA_B3GNT2_1273293_fwd	GGAAAGGACGAAACACCGTTGGGCAAGACGCCCCCCGGTTTTAGAGCTAGAAAT
gRNA_B3GNT2_1273293_rev	CTAAAACCGGGGGGCGTCTTGCCCAACGGTGTTTCGTCCTTTCCACAAGATAT
gRNA_FUT8_681494_fwd	GGAAAGGACGAAACACCGTCAGACGCACTGACAAAGTGTTTTAGAGCTAGAAAT
gRNA_FUT8_681494_rev	CTAAAACACTTTGTCAGTGCGTCTGACGGTGTTTCGTCCTTTCCACAAGATAT
gRNA_SPPL3_213040_fwd	GGAAAGGACGAAACACCAGAGAGACGGACGCTCCAATGTTTTAGAGCTAGAAAT
gRNA_SPPL3_213040_rev	CTAAAACATTGGAGCGTCCGTCTCTCTGGTGTTTCGTCCTTTCCACAAGATAT
gRNA_GLUL_941540_fwd	GGAAAGGACGAAACACCGGCCCAGGGAAGCCATCGGAGTTTTAGAGCTAGAAAT
gRNA_GLUL_941540_rev	CTAAAACTCCGATGGCTTCCCTGGGCCGGTGTTTCGTCCTTTCCACAAGATAT

Table S3: Primer list for deep sequencing (MiSeq). The primers contain overhang sequences compatible with Illumina Nextera XT indexing
(forward primeroverhang: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG,
reverse primer overhang: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG).

primer name	sequence (5′ → 3′)
MiSeq_MGAT4A_411545_fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGACAGACAGAAGGCAAATCTACG
MiSeq_MGAT4A_411545_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTAACAGCTACACAGGAAGAGCA
MiSeq_MGAT4B_1280368_fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGGATGGGGTGTATGGAGGT
MiSeq_MGAT4B_1280368_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTGCAGACTGCTCTCCTTGG
MiSeq_MGAT5_327084_fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATGAATCTCATGGTTTCCTTTGT
MiSeq_MGAT5_327084_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTTCAAGACTCAACTCTTTCCC
MiSeq_ST3GAL3_244730_fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGGAAACAGCATGGGCAAAC
MiSeq_ST3GAL3_244730_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTGGAATGTGGATGGTGGC
MiSeq_ST3GAL4_964386_fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACACCTGATGACCACATCGT
MiSeq_ST3GAL4_964386_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCAGGGTCCACTTCTGGATT
MiSeq_ST3GAL6_1812502_fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCACTGTCTTACTACCCACAGGA
MiSeq_ST3GAL6_1812502_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTTTCATTATATTCAAGAGCCAC
MiSeq_B3GNT2_1273293_fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTCACCCACCGGAGAAACAG
MiSeq_B3GNT2_1273293_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGAAGGCAAGCAATTCGGGA
MiSeq_FUT8_681494_fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGCCCCCATGACTAGGGATA
MiSeq_FUT8_681494_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCTGCGTTCGAGAAGCTGAAA
MiSeq_SPPL3_213040_fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGTGGAGTAACTTACCTGCTGT
MiSeq_SPPL3_213040_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAGTGGTGAGTGTGTCCTGT
MiSeq_GLUL_941540_fwd2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAACCAGCACCCCTGGTT
MiSeq_GLUL_941540_rev2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGCTGCCAGTCTGTTTGC

Table S4: Indels generated in ten targeted genes by CRISPR/Cas9 multiplexing.

Multiplexing round	1					2		3		4
Gene	MGAT4A	MGAT4B	MGAT5	ST3GAL4	ST3GAL6	ST3GAL3	B3GNT2	GLUL	SPPL3	FUT8
10x KO clone A	+2	-1	+1	-5/+1	+1	+1/+2	-1	-13/-10/-2	+1	+1
10x KO clone B	+2	-1	+1	-5/+1	+1	+1/+2	-1	-13/-10/-2	+1	-7/-1

Table S5: Nucleotide sequences for overexpression vectors.

vector	
SerpinG1-plasmid AmpR Bla promoter SV40pA GLUL SV40 promoter mCMV-hEF-1a-5' promoter C1INH/ SerpinG1	agacgtcaTGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACC AGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCG GGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCAC GAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCAC TGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTAC GGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCC GGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGA TCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAG GATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTT ACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGA TAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTAT CAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTT GCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCT CGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGG TTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATT CTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGC GACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAAC GTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTT CAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACA CGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATA 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GCTCCCTTGGAGCCTACCTAGACTCAGCCGGCTCTCCACGCTTTGCCTGACCCTGCTTGCTCAACTCTACGTCTTTGTTTCGTTT TCTGTTCTGCGCCGTTACAGATCCAAGCTGTGACCGGCGCCTACagtgcgatCGCCACCATGGCCAGCAGACTGACACTGCTGACCC TGCTGCTCCTCCTGCTGGCTGGAGACAGGGCTTCCTCCAACCCCAACGCCACCAGCAGCAGCTCCCAGGACCCTGAGTCCCTCCA GGACAGGGGAGAAGGCAAGGTCGCCACCACCGTCATCTCCAAAATGCTCTTCGTCGAGCCCATCCTCGAGGTCAGCTCCCTCCCC ACCACAAACAGCACAACCAACAGCGCCACCAAGATCACCGCCAACACCACCGACGAACCCACAACCCAGCCCACCACAGAGCCTA CAACACAGCCTACCATCCAGCCTACCCAACCCACCACCCAGCTCCCTACCGACTCCCCTACCCAGCCTACCACAGGCTCCTTTTGT CCCGGACCTGTGACCCTGTGCTCCGACCTGGAGTCCCATAGCACAGAGGCTGTCCTCGGAGATGCCCTGGTGGATTTCAGCCTCA AACTCTACCACGCCTTCAGCGCCATGAAGAAGGTCGAGACCAATATGGCCTTCTCCCCCTTTAGCATCGCCAGCCTGCTCACCCA AGTCCTGCTCGGAGCCGGCGAGAATACCAAGACCAACCTGGAGAGCATCCTGTCCTACCCTAAGGACTTCACCTGCGTCCACCA GGCCCTCAAGGGCTTTACCACCAAAGGAGTCACATCCGTCAGCCAGATCTTCCATTCCCCTGACCTCGCCATTAGGGACACATTC

BGHpA mCMV-hEF-1a-5' promoter ST6GAL1 BGHpA	GTGAACGCCTCCAGGACCCTGTACAGCAGCTCCCCTAGGGTCCTGTCCAACAACAGCGACGCCAACCTGGAGCTCATTAATACA TGGGTGGCCAAGAATACAAACAACAAGATTAGCAGGCTCCTGGATAGCCTGCCTTCCGACACCAGGCTCGTGCTCCTCAATGCC ATCTACCTCTCCGCCAAGTGGAAGACCACATTCGACCCCAAGAAAACAAGGATGGAGCCCTTTCACTTTAAAAATAGCGTGATC AAGGTGCCCATGATGAACAGCAAGAAGTACCCTGTCGCCCACTTCATCGACCAGACCCTGAAGGCTAAGGTGGGACAGCTCCAA CTGTCCCATAATCTGAGCCTGGTCATCCTCGTGCCTCAGAACCTGAAGCACAGGCTGGAGGACATGGAACAGGCCCTGTCCCCC AGCGTGTTTAAGGCCATCATGGAAAAACTCGAGATGTCCAAGTTTCAACCCACCCTCCTCACCCTGCCCAGAATTAAGGTCACC ACAAGCCAGGACATGCTCAGCATTATGGAGAAGCTCGAGTTCTTCGATTTCTCCTACGACCTCAACCTCTGCGGCCTGACAGAA GACCCTGACCTGCAGGTGAGCGCCATGCAGCACCAGACAGTGCTGGAGCTCACCGAGACAGGAGTGGAAGCTGCTGCCGCCTCC GCTATTTCCGTGGCCAGGACCCTCCTGGTGTTCGAGGTGCAACAACCCTTCCTGTTCGTCCTGTGGGACCAACAACACAAGTTCC CTGTGTTCATGGGCAGAGTCTACGACCCCAGAGCCTGAacacagtctCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTC CCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAG TAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGG ATGCGGTGGGCTCTATGGATTAAGCTCGCGTAGTCAATGGGAAAAACCCATTGGAGCCAAGTACACTGACTCAATAGGGACTT TCCATTGGGTTTTGCCCAGTACATAAGGTCAATAGGGGGTGAGTCAACAGGAAAGTCCCATTGGAGCCAAGTACATTGAGTCA ATAGGGACTTTCCAATGGGTTTTGCCCAGTACATAAGGTCAATGGGAGGTAAGCCAATGGGTTTTTCCCATTACTGACATGTAT ACTGAGTCATTAGGGACTTTCCAATGGGTTTTGCCCAGTACATAAGGTCAATAGGGGTGAATCAACAGGAAAGTCCCATTGGA GCCAAGTACACTGAGTCAATAGGGACTTTCCATTGGGTTTTGCCCAGTACAAAAGGTCAATAGGGGGTGAGTCAATGGGTTTT TCCCATTATTGGCACATACATAAGGTCAATAGGGGTGACTAGTCAGTGGGCAGAGCGCACATCGCCCACAGTCCCCGAGAAGTT GGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCC GCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAGTAGTTGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCA GAACACAGCTGAAGCTTCGAGGGGCTCGCATCTCTCCTTCACGCGCCCGCCGCCCTACCTGAGGCCGCCATCCACGCCGGTTGAG TCGCGTTCTGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCCGCCGTCTAGGTAAGTTTAAAGCTCAGGTCGAGACCGGGC CTTTGTCCGGCGCTCCCTTGGAGCCTACCTAGACTCAGCCGGCTCTCCACGCTTTGCCTGACCCTGCTTGCTCAACTCTACGTCT TTGTTTCGTTTTCTGTTCTGCGCCGTTACAGATCCAAGCTGTGACCGGCGCCTACagtgcgatCGCCACCATGATCCACACCAACCT GAAGAAGAAATTCTCCTGCTGCGTGCTGGTGTTCCTGCTGTTCGCCGTGATCTGCGTGTGGAAAGAGAAGAAGAAGGGCTCCTA CTACGACTCCTTCAAGCTGCAGACCAAAGAATTCCAGGTGCTGAAGTCCCTGGGCAAGCTGGCCATGGGCTCCGACTCTCAGTC CGTGTCCTCCAGCTCTACCCAGGACCCCCACAGAGGCAGACAGACCCTGGGCTCTCTGAGAGGCCTGGCCAAGGCTAAGCCTGA GGCCTCCTTCCAGGTGTGGAACAAGGACTCCTCCAGCAAGAACCTGATCCCCCGGCTGCAGAAGATCTGGAAGAACTACCTGTC CATGAACAAGTACAAGGTGTCCTACAAGGGCCCTGGCCCTGGCATCAAGTTCTCTGCCGAGGCCCTGAGATGCCACCTGAGGGA CCATGTGAACGTGTCCATGGTGGAAGTGACCGACTTCCCATTCAACACCTCCGAGTGGGAGGGCTACCTGCCCAAAGAGTCCAT CCGGACCAAGGCTGGCCCTTGGGGCAGATGTGCTGTGGTGTCCTCTGCCGGCTCCCTGAAGTCCTCTCAGCTGGGCAGAGAGAT CGACGACCACGACGCCGTGCTGCGGTTTAATGGCGCCCCTACCGCCAACTTCCAGCAGGACGTGGGCACCAAGACCACCATCCGG CTGATGAACTCCCAGCTCGTGACAACCGAGAAGCGGTTCCTGAAGGACTCCCTGTACAACGAGGGCATCCTGATCGTGTGGGAC CCCTCCGTGTACCACTCCGACATCCCCAAGTGGTATCAGAACCCCGACTACAACTTCTTCAACAACTACAAGACCTACCGGAAGC TGCACCCCAACCAGCCCTTCTACATCCTGAAGCCCCAGATGCCCTGGGAGCTGTGGGACATTCTGCAGGAAATCTCCCCCGAGGA AATCCAGCCCAACCCCCCTTCCTCTGGCATGCTGGGCATCATTATCATGATGACCCTGTGCGACCAGGTGGACATCTACGAGTT TCTGCCCTCCAAGAGAAAGACCGACGTGTGCTACTACTACCAGAAGTTCTTCGACTCCGCCTGCACCATGGGCGCCTACCACCCT CTGCTGTACGAGAAGAACCTCGTGAAGCACCTGAACCAGGGCACCGACGAGGATATCTACCTGCTGGGCAAGGCCACCCTGCCT GGCTTCAGAACCATCCACTGCTGAacacagtctCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCT TGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTA TTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCT ATGG
A1AT plasmid AmpR Bla	agacgtcaTGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACC AGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCG GGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCAC GAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCAC TGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTAC GGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCC GGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGA TCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAG GATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTT ACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGA TAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTAT CAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTT GCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCT CGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGG TTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATT CTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGC GACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAAC GTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTT CAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACA CGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATA TTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCGACGGATCGGG

promoter SV40pA GLUL SV40 promoter mCMV-hEF-1a-5' promoter A1AT BGHpA mCMV-hEF-1a-5' promoter	AGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTG TGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTG CTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGACAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGC AGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTCCG CGGTTAGTTTTTGTATTGGAAGGGCTGGTCGCCAGTCTCATTGAGAAGGCATGTGCGGACGATGGCTTCTGTCACTGCAAAGGG GTCACAATTGGCAGAGGGGCGGCGGTCTTCAAAGTAACCTTTCTTCTCCTGGCCGACAGTCCGGGGAATGCGGATGCTGGCACT GCGATTGGCGACACCAGCAGAAAAGTCGTTGATGTTGGACGTTTCGTGGAACCCAGTCAGACGACGGGCATTGTCCAGGCCCCC CTTGGGATCGTAGGCTCGAATGTGGTACCGGTGCCGCTTGCTTAGTTTCTCGATGGCCTCCTCGATGTGCTTCAGACCATTCTC CTCCCGCATGGCCTTGGTGCTAAAGTTGGTATGGCAGCCTGCACCATTCCAGTTCCCAGGAATGGGCTTGGGGTCAAAGGTTGC TATTACCCCAAAGTCTTCACATACTCGATGCAAGATGAAACGGGCCACCCAGAGATGATCTCCCATGCGGATTCCTTCACAGGG TCCTATTTGGAATTCCCACTGGGCAGGCATGACCTCAGCATTTGTTCCTGTAATCTTGACCCCAGCATACAAGCAGGCGCGGTA GTGAGCCTCCACGATATCCCTGCCATAGGCTTTGTCTGCGCCCACACCACAGTAATACGGACCTTGGGGCCCAGGAAAGCCATT GGAAGGCCAACCAAAAGGGTGCCCATCTGTTCCCATCAGAGTATACTCCTGTTCCATTCCAAACCAGGGGTGCTGGTTGCTCAC CATGTCCATTATCCGTTTACACGAGTGCCTTAAATTGGTCTCTGCAGGCTTCCGGTTGTACTTGAAAACTTCACAGAACACCAG CTTGTTGGGATCTCTGCGGAAGGGGTCCCGAAACATGGCAACAGGGCTGAGATACATGTCACTGTTGGAGCCCTCAGACTGAAA GGTACTAGAGCCATCAAAATTCCACTCAGGTAACTCTTCTACACACTTGGGCTCACAGTCCAGGGTGCGGGTTTTGCAGCGCAG TCCTTCTCCAGTACCATCAACCCAGATATACATGGCTTGGACTTTCTCACCCTGGGGCAGGCACAAGTACATTTGCTTGATGTT TTTGTTCAAGTGGGAACTTGCTGAGGTGGCCATATCGATCGAAAATGGATATACAAGCTCCCGGGAGCTTTTTGCAAAAGCCTA GGCCTCCAAAAAAGCCTCCTCACTACTTCTGGAATAGCTCAGAGGCAGAGGCGGCCTCGGCCTCTGCATAAATAAAAAAAATTA GTCAGCCATGGGGCGGAGAATGGGCGGAACTGGGCGGAGTTAGGGGCGGGATGGGCGGAGTTAGGGGCGGGACTATGGTTGCT GACTAATTGAGATGCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACACCTGGTTGCTGACTAATTGAGA TGCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACACCCTAACTGACACACATTCCACAGACGTCGCTCGA TGTACGGGCCAGATATACGCGTAGTCAATGGGAAAAACCCATTGGAGCCAAGTACACTGACTCAATAGGGACTTTCCATTGGGT TTTGCCCAGTACATAAGGTCAATAGGGGGTGAGTCAACAGGAAAGTCCCATTGGAGCCAAGTACATTGAGTCAATAGGGACTT TCCAATGGGTTTTGCCCAGTACATAAGGTCAATGGGAGGTAAGCCAATGGGTTTTTCCCATTACTGACATGTATACTGAGTCAT TAGGGACTTTCCAATGGGTTTTGCCCAGTACATAAGGTCAATAGGGGTGAATCAACAGGAAAGTCCCATTGGAGCCAAGTACA CTGAGTCAATAGGGACTTTCCATTGGGTTTTGCCCAGTACAAAAGGTCAATAGGGGGTGAGTCAATGGGTTTTTCCCATTATTG GCACATACATAAGGTCAATAGGGGTGACTAGTCAGTGGGCAGAGCGCACATCGCCCACAGTCCCCGAGAAGTTGGGGGGAGGG GTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCC GAGGGTGGGGGAGAACCGTATATAAGTGCAGTAGTTGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAGCTG AAGCTTCGAGGGGCTCGCATCTCTCCTTCACGCGCCCGCCGCCCTACCTGAGGCCGCCATCCACGCCGGTTGAGTCGCGTTCTGC CGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCCGCCGTCTAGGTAAGTTTAAAGCTCAGGTCGAGACCGGGCCTTTGTCCGGC GCTCCCTTGGAGCCTACCTAGACTCAGCCGGCTCTCCACGCTTTGCCTGACCCTGCTTGCTCAACTCTACGTCTTTGTTTCGTTT TCTGTTCTGCGCCGTTACAGATCCAAGCTGTGACCGGCGCCTACagtgcgatCGCCACCATGCCCAGCTCCGTGAGCTGGGGCATTC TCCTCCTCGCTGGCCTGTGCTGTCTGGTGCCTGTGAGCCTGGCCGAAGACCCCCAAGGAGACGCTGCTCAGAAGACAGACACAT CCCACCATGACCAGGACCACCCCACCTTCAATAAGATCACCCCTAACCTCGCTGAGTTTGCCTTTTCCCTCTACAGGCAACTGGC CCACCAGAGCAACTCCACCAATATCTTCTTTAGCCCTGTGAGCATCGCCACAGCCTTCGCCATGCTGAGCCTGGGCACCAAGGCT GATACACATGACGAGATCCTGGAAGGACTGAACTTCAACCTGACCGAGATCCCCGAGGCCCAGATCCACGAGGGCTTCCAGGAA CTGCTGAGGACCCTGAACCAGCCTGACAGCCAGCTCCAGCTCACCACCGGCAATGGCCTCTTCCTGAGCGAGGGCCTCAAGCTCG TGGATAAGTTCCTGGAAGACGTGAAGAAGCTGTACCACTCCGAAGCCTTCACAGTGAACTTTGGCGACACAGAGGAGGCCAAG AAGCAGATCAACGACTATGTGGAGAAGGGCACCCAGGGCAAGATCGTGGACCTCGTGAAGGAGCTGGATAGGGACACCGTGTT CGCTCTCGTGAACTATATCTTCTTCAAGGGCAAGTGGGAGAGGCCCTTCGAGGTGAAAGACACAGAGGAAGAGGACTTCCACGT CGACCAAGTGACCACAGTCAAGGTCCCCATGATGAAGAGACTGGGCATGTTCAACATCCAGCATTGCAAAAAGCTGAGCAGCTG GGTGCTGCTCATGAAGTATCTCGGCAACGCCACAGCCATCTTCTTCCTGCCCGATGAGGGCAAGCTCCAGCATCTGGAAAACGA GCTCACCCACGACATTATCACCAAGTTTCTGGAGAACGAAGACAGGAGGAGCGCTAGCCTCCACCTCCCCAAACTCAGCATCAC CGGCACATATGACCTGAAGTCCGTCCTCGGCCAGCTGGGCATCACAAAGGTCTTCTCCAACGGCGCCGACCTGAGCGGAGTCAC AGAAGAGGCTCCCCTGAAGCTGAGCAAGGCTGTGCATAAGGCCGTGCTGACAATTGACGAGAAAGGCACAGAGGCTGCCGGAG CCATGTTCCTGGAAGCTATCCCCATGAGCATCCCCCCCGAGGTGAAATTCAACAAACCCTTCGTGTTCCTGATGATCGAGCAGA ACACCAAGTCCCCCCTCTTCATGGGCAAGGTCGTGAACCCCACCCAGAAGTAAacacagtctCTGTGCCTTCTAGTTGCCAGCCATC TGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCA TCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAG CAGGCATGCTGGGGATGCGGTGGGCTCTATGGATTAAGCTCGCGTAGTCAATGGGAAAAACCCATTGGAGCCAAGTACACTGAC TCAATAGGGACTTTCCATTGGGTTTTGCCCAGTACATAAGGTCAATAGGGGGTGAGTCAACAGGAAAGTCCCATTGGAGCCAA GTACATTGAGTCAATAGGGACTTTCCAATGGGTTTTGCCCAGTACATAAGGTCAATGGGAGGTAAGCCAATGGGTTTTTCCCAT TACTGACATGTATACTGAGTCATTAGGGACTTTCCAATGGGTTTTGCCCAGTACATAAGGTCAATAGGGGTGAATCAACAGGA AAGTCCCATTGGAGCCAAGTACACTGAGTCAATAGGGACTTTCCATTGGGTTTTGCCCAGTACAAAAGGTCAATAGGGGGTGA GTCAATGGGTTTTTCCCATTATTGGCACATACATAAGGTCAATAGGGGTGACTAGTCAGTGGGCAGAGCGCACATCGCCCACAG TCCCCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTC GTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAGTAGTTGCCGTGAACGTTCTTTTTCGCAAC GGGTTTGCCGCCAGAACACAGCTGAAGCTTCGAGGGGCTCGCATCTCTCCTTCACGCGCCCGCCGCCCTACCTGAGGCCGCCATC CACGCCGGTTGAGTCGCGTTCTGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCCGCCGTCTAGGTAAGTTTAAAGCTCAG GTCGAGACCGGGCCTTTGTCCGGCGCTCCCTTGGAGCCTACCTAGACTCAGCCGGCTCTCCACGCTTTGCCTGACCCTGCTTGCT CAACTCTACGTCTTTGTTTCGTTTTCTGTTCTGCGCCGTTACAGATCCAAGCTGTGACCGGCGCCTACagtgcgatCGCCACCATGA TCCACACCAACCTGAAGAAGAAATTCTCCTGCTGCGTGCTGGTGTTCCTGCTGTTCGCCGTGATCTGCGTGTGGAAAGAGAAGA AGAAGGGCTCCTACTACGACTCCTTCAAGCTGCAGACCAAAGAATTCCAGGTGCTGAAGTCCCTGGGCAAGCTGGCCATGGGCT CCGACTCTCAGTCCGTGTCCTCCAGCTCTACCCAGGACCCCCACAGAGGCAGACAGACCCTGGGCTCTCTGAGAGGCCTGGCCAA GGCTAAGCCTGAGGCCTCCTTCCAGGTGTGGAACAAGGACTCCTCCAGCAAGAACCTGATCCCCCGGCTGCAGAAGATCTGGAA GAACTACCTGTCCATGAACAAGTACAAGGTGTCCTACAAGGGCCCTGGCCCTGGCATCAAGTTCTCTGCCGAGGCCCTGAGATG CCACCTGAGGGACCATGTGAACGTGTCCATGGTGGAAGTGACCGACTTCCCATTCAACACCTCCGAGTGGGAGGGCTACCTGCC CAAAGAGTCCATCCGGACCAAGGCTGGCCCTTGGGGCAGATGTGCTGTGGTGTCCTCTGCCGGCTCCCTGAAGTCCTCTCAGCT

ST6GAL1 BGHpA	GGGCAGAGAGATCGACGACCACGACGCCGTGCTGCGGTTTAATGGCGCCCCTACCGCCAACTTCCAGCAGGACGTGGGCACCAA GACCACCATCCGGCTGATGAACTCCCAGCTCGTGACAACCGAGAAGCGGTTCCTGAAGGACTCCCTGTACAACGAGGGCATCCT GATCGTGTGGGACCCCTCCGTGTACCACTCCGACATCCCCAAGTGGTATCAGAACCCCGACTACAACTTCTTCAACAACTACAA GACCTACCGGAAGCTGCACCCCAACCAGCCCTTCTACATCCTGAAGCCCCAGATGCCCTGGGAGCTGTGGGACATTCTGCAGGA AATCTCCCCCGAGGAAATCCAGCCCAACCCCCCTTCCTCTGGCATGCTGGGCATCATTATCATGATGACCCTGTGCGACCAGGTG GACATCTACGAGTTTCTGCCCTCCAAGAGAAAGACCGACGTGTGCTACTACTACCAGAAGTTCTTCGACTCCGCCTGCACCATG GGCGCCTACCACCCTCTGCTGTACGAGAAGAACCTCGTGAAGCACCTGAACCAGGGCACCGACGAGGATATCTACCTGCTGGGC AAGGCCACCCTGCCTGGCTTCAGAACCATCCACTGCTGAacacagtctCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCT CCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGA GTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGG GATGCGGTGGGCTCTATGG

Table S6: Proteomics-based identification of proteins in whole cell lysates from CHO KO and CHO WT cells. Of the KO's only Glul could be detected. KO of Glul is verified as no hits in KO cell line.

Protein IDs	Fasta headers	Intensity 10x KO cell line_1	Intensity 10x KO cell line_2	Intensity WT_1	Intensity WT_2
G3HLB3	tr|G3HLB3|G3HLB3_CRIGR Glutamine synthetase OS=Cricetulus griseus	0	0	34989000	34830000
G3HG36	tr|G3HG36|G3HG36_CRIGR Glutamine synthetase OS=Cricetulus griseus	0	0	28628000	26788000