ANTIBODY EXPRESSION AND ENGINEERING USING *Saccharomyces cerevisiae*

by

ANJALI SHENOY

(Under the Direction of Adam W. Barb)

ABSTRACT

Antibody Dependent Cellular Cytotoxicity is an important mechanism for immunotherapeutics. This mechanism involves target cells with surface antigens, antibodies, and immune effector cells such as macrophages and natural killer (NK) cells. The Fc domain of the antibody engages with the Fc γ Receptors (FcγRIIIa) on the cell surface of macrophages or NK cells. This interaction has a strict N-glycan requirement restricting the production of antibodies to mammalian expression platforms. In addition to this, the amino acid composition of Fc also influences its affinity towards the receptor.

This work represents two disparate approaches to enhance FcγRIIIa receptor binding. We first combined protein and N-glycan engineering to create a novel glycoengineered Yeast Surface Display (YSD) platform (EBY100-(GPD)-EndoS2) to simultaneously meet both these objectives. This platform could be used for engineering Fc through the creation of YSD libraries and for the production of Rituximab. This yeast expressed Rituximab possessed the desired engineered glycoform (>99%). In addition to this observation, the antibody also displayed properties in terms of Fab binding and receptor engagement similar to commercial antibody. We are currently in the process of validating hits identified using this platform for antibody engineering. In addition to

this, we have also utilized YSD to create library of novel aglycosylated disulphide variants to eliminate the requirement of the N-glycan completely. Our best hits displayed significant receptor-binding affinity with K_D 1-3μM.

Another part of this work describes creation of a novel yeast platform to boost protein expression for ^{15}N-labeling of Fc. Isotope labeling has been restricted to either mammalian or prokaryotic expression systems, yeast however combines advantages of both these platforms. We created yeast strain EBY100-Kar2p-T2A-PDI by utilizing a ribosome skip site to simultaneously produce both proteins, possibly lowering E.R stress, and resulting in increased target protein expression. With this platform, we recovered 2.2 mg/L of uniformly ^{15}N-labeled human immunoglobulin (Ig)G1 Fc domain with 90.6% 15N labeling. NMR spectroscopy revealed a high degree of similarity between the yeast and mammalian expressed IgG1 Fc domains. In the future, we could utilize this platform to ^{15}N and ^{13}C label Fc to further understand kinetics and map the binding interface between Fc and the FcγRIIIa.

INDEX WORDS: Antibody Dependent Cellular Cytotoxicity, N-Glycosylation, *Saccharomyces cerevisiae*, Ribosome skip site, Yeast surface display

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CHAPTER 1

INTRODUCTION

Monoclonal antibodies contribute significantly to the market revenue generated by the pharmaceutical industry, one such example includes the therapeutic Adalimumab (Humira) that generated $16 million in 2016. There are several other mAbs that are in the process of being approved or have been approved some examples include Obinutuzumab and Tafasitamab both against Hodgkin's lymphoma. Hence, any progress in improving the production or enhancing therapeutic function is invaluable in antibody development. Many mAbs utilize Antibody Dependent Cellular Cytotoxicity (ADCC) and Complement Dependent Cellular Cytotoxicity (CDC) as their mechanism of therapeutic function. Several groups, including ours, have worked towards enhancing the ADCC response of monoclonal antibodies. In this work, we have focused on increasing the affinity of the Fc domain (human IgG1) against FcγRIIIa (found on macrophages and natural killer cells). This interaction is influenced by several factors but namely the amino acid and N-glycan composition of the Fc domain. This work focuses predominantly enhancing the affinity by focusing on either one (amino acid composition) or both (amino acid and N-glycan composition) simultaneously. To achieve this goal, we have utilized yeast surface display (YSD) which is a powerful platform that can screen 10^7-10^8 variants within a library with ease.

This work includes a literature review (Chapter 2) that describes the various approaches by which yeast strains *Saccharomyces cerevisiae* and *Pichia pastoris* have been glycoengineered to create the desired N-glycans. This chapter highlights the considerable amount of effort required to achieve this goal. Understanding the previous efforts undertaken is key in designing new

glycoengineered yeast strains. We then utilize some of the ideas described in this chapter to design a novel glycoengineered yeast surface display strain EBY100-(GPD)-EndoS2 (described in Chapter 3). We used this platform to produce glycoengineered antibody and Fc yeast surface display libraries.

In Chapter 4, we proceed with strain engineering to create a strain (EBY100-Kar2p-F2A-PDI) to boost heterologous protein expression. We demonstrated that this platform can be used to boost production of Rituximab. Rituximab is a monoclonal antibody used as a therapeutic against B-cell lymphoma and other autoimmune diseases. We then proceeded to utilize this platform to ^{15}N-label the Fc domain for NMR. This work represents a significantly cheaper and easier approach for isotope labeling compared to a previous protocol developed for mammalian expression systems.

The strict N-glycan requirement on the Fc domain has restricted the production of antibodies to mammalian expression systems. These platforms are expensive and prone to contamination In Chapter 5, we utilize YSD to create novel aglycosylated Fc domain that still engage the FcγRIIIa receptor. The Fc variants described in this section could possibly be expressed in prokaryotic expression systems (incapable of N-glycosylation) and still retain their therapeutic function lowering production costs considerably.

In Chapter 6, we utilize the EBY100-(GPD)-EndoS2 strain (described in Chapter 3) to screen for glycoengineered Fc domains with enhanced affinity for the FcγRIIIa receptor. This combines both protein and N-glycan engineering to create novel Fc variants. Very little has been done in this aspect, despite the importance of both these aspects in influencing the affinity of FcγRIIIa binding.

Finally, in the concluding chapter we summarize and hypothesize potential future directions to build upon the work described in this dissertation

CHAPTER 2[1]

RECENT ADVANCES TOWARDS ENGINEERING GLYCOPROTEINS USING MODIFIED YEAST DISPLAY PLATFORMS

[1] Anjali Shenoy and Adam W. Barb, Accepted by Glycosylation 2022, Reprinted here with the permission of Springer Nature Nov 2022

Abstract

Yeast are capable recombinant protein expression hosts that provide eukaryotic post-translational modifications such as disulphide bond formation and N-glycosylation. This property has been used to create surface display libraries for protein engineering, however, Yeast Surface Display (YSD) with common laboratory strains has limitations in terms of diversifying glycoproteins due to the incorporation of a high level of mannose residues which often obscure important epitopes and are immunogenic in humans. Developing new strains for efficient and appropriate display will require combining existing technologies to permit efficient glycoprotein engineering. Foundational efforts generating knockout strains, which lack characteristic hypermannosylation reactions, exhibited morphological defects and poor growth. Later strains with 'humanized' N-glycosylation machinery surmounted these limitations by targeting a small suite of glycosylhydrolase and glycosyltransferase enzymes from other taxa to the endoplasmic reticulum and Golgi. Advanced yeast strains also provide key modifications at the glycan termini that are essential for the full function of many glycoproteins. Here we review progress in glycoprotein engineering when glycosylation is required for full function using advanced yeast expression platforms and the suitability of each for YSD of glycoproteins.

2.1 Introduction

Appropriate post-translational modifications are required for the efficacy of most biopharmaceuticals, including therapeutic monoclonal antibodies (mAbs) and erythropoietin, necessitating the use of appropriate recombinant expression hosts. The attachment of asparagine (N)-linked carbohydrate chains (glycans) and subsequent remodelling during secretion is essential for proper half-life, clearance rate and immunogenicity [1][2]. Currently, Chinese hamster ovary (CHO) cells are the predominant system used for mAb expression but require complex media and precise conditions. Furthermore, mammalian systems are prone to contamination from endotoxins, mycoplasmas, prions, and other

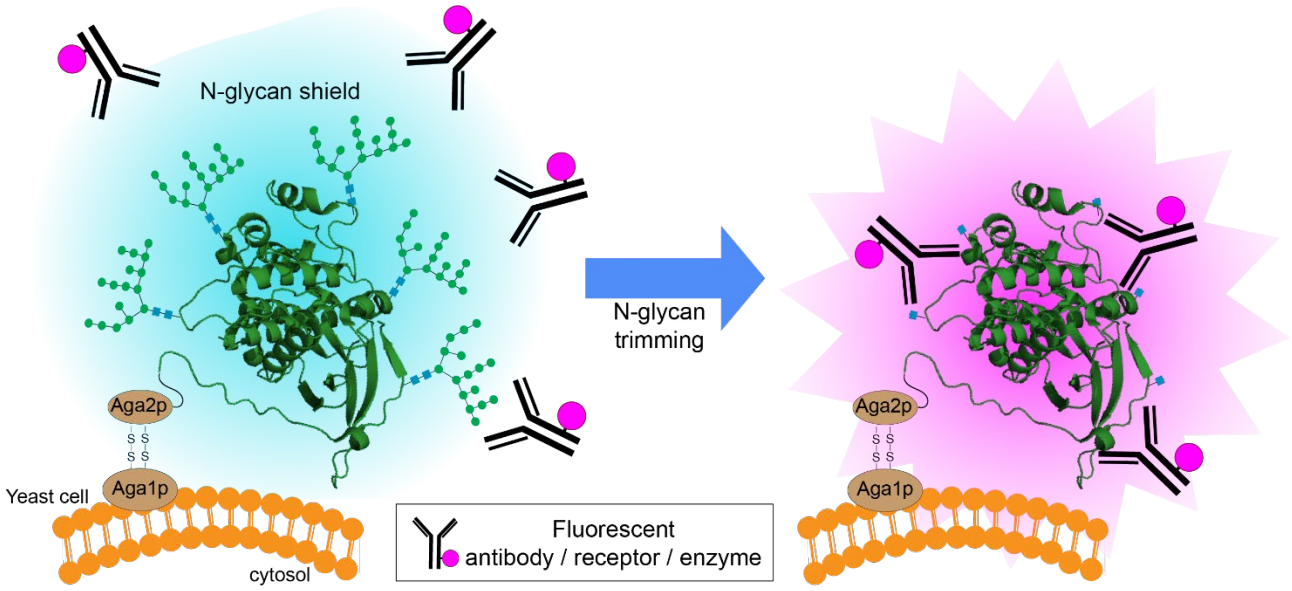

Figure 2.1 Anchoring glycoproteins on the yeast surface results in glycan shielding which prevents epitope detection. Trimming the large oligomannose glycoforms exposes the epitopes for detection.

factors. These limitations have promoted the development of other expression systems. Prokaryotic expression systems are largely incapable of N-glycosylation, and therefore have not been used for this purpose. It should be noted that chemoenzymatic remodelling following

prokaryotic expression shows promise for future commercial use, but this technology remains limited [3][4].

Yeasts provide eukaryotic expression conditions that are capable of human therapeutic expression with Endoplasmic Reticulum (ER)-localized foldases, chaperones and N-glycosylation machinery. Yeast are likewise robust and widely used for industrial applications, however, yeast produce N-glycans with a high mannose content that are powerful ligands for innate immune receptors in the human body leading to increased clearance and immunogenicity [5]. In addition, the high mannose yeast glycoforms sterically limit interactions with large ligands (Figure 2.1).These limitations motivated several groups to develop yeast strains with humanized N-glycosylation machinery to combine the multiple advantages of yeast as an expression platform and the strict N-glycan requirements of human therapeutics (thoroughly covered in many articles including [5][6][7][8][9]). Problems faced in this pursuit included ensuring correct enzyme localization, the availability of substrate nucleotide sugars, homogeneity of the secreted product and compositional differences compared to proteins expressed from mammalian cells.

In addition to their use as an industrial expression host, yeast also provide the ability to create and screen libraries for protein engineering. Yeast Surface Display (YSD) was developed to address limitations with existing phage display technologies including protein solubility [10]. YSD however, utilizes eukaryotic expression, which has a sophisticated quality control machinery in the ER and provides appropriate folding and secretion machinery for surface display [11]. In addition to this, YSD libraries have been found to be twice as diverse as comparable phage display libraries [12]. Thus, yeast is an excellent expression platform for glycoprotein engineering. YSD has been used for several applications such as developing recyclable biocatalysts, vaccines or for the display of peptides and antibodies [13]. In this review we will focus on the recent advances in

engineering N-glycosylation in yeast (*Pichia pastoris* and *Saccharomyces cerevisiae*) followed by the application of these optimized strains for protein engineering.

2.2. Producing human n-glycoforms in yeast

Mammalian and yeast expression systems exhibit many similarities but also notable differences in their N-glycosylation machineries. Both systems generate a common $Man_8GlcNAc_2$ glycoform before diverging. Mammalian expression systems produce mostly complex-type N-glycans whereas yeast expression systems produce oligomannose glycoforms (Figure 2.2). *Saccharomyces cerevisiae* elongates N-glycans with several Golgi-resident mannosyltransferases that can add up to 200 mannose residues [14]. Heterologous proteins secreted by *Pichia pastoris* catalyze similar mannosylation reactions but at much lower levels [15]. Therefore, therapeutic proteins expressed

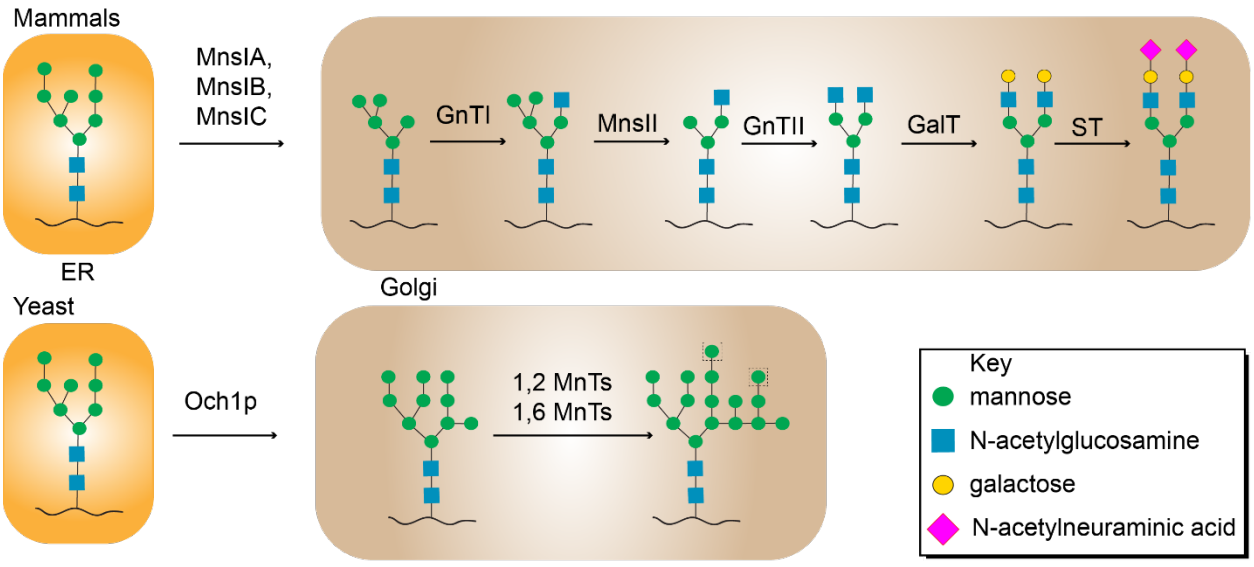

Figure 2.2 Comparison between mammalian and fungal N-glycosylation the mammalian and fungal N-glycosylation pathways are similar up to the point the $Man_8GlcNAc_2$ glycoform is produced. The two pathways then diverge in the Golgi. The mammalian N-glycosylation machinery produces a biantennary complex-type glycoform whereas the fungal system produces an oligomannose glycoform as its end product. The extent of branching is dependent on the yeast strain

in common yeast strains possess undesirable oligo-mannose glycoforms which are recognized by lectins leading to rapid clearance of therapeutics from the body [16]. The differences in N-glycosylation machineries must be resolved to express therapeutics using a yeast expression system. For this, several groups have attempted to create strains where the N-glycosylation machinery has been "humanized."

Initial efforts removed the enzymes that catalyzed non-human N-glycosylation reactions. These knockout strains can be easily created in *Saccharomyces cerevisiae* due to efficient homologous recombination [17]. Mannosyltransferases that catalyzed hypermannosylation reactions were eliminated in these strains, including Och1p, a transmembrane enzyme found in the Golgi [18][19]. One group knocked out multiple mannosyltransferases to obtain a Δoch1Δmnn1Δalg3 strain, which produced a $Man_8GlcNAc_2$ or $Man_5GlcNAc_2$ glycoform on the expressed protein [20] (Figure 2.3). However, this *Saccharomyces cerevisiae* knockout strain suffered from substantial defects including temperature sensitivity, cell division defects, morphological defects and heterogenous glycoform profiles [21][22]. One group induced a low fidelity polymerase, DNA Polymerase δ, to incorporate mutations within the genome and isolated knock-out strain variants of the Δoch1Δmnn4Δmnn1 strain that could overcome these defects though it is unclear where the mutations are located [23].

Other efforts resulted in useful *Pichia pastoris* strains [24]. Chiba and coworkers targeted an α-1,2 mannosidase from *Asperigillus niger* to the ER in *Pichia pastoris.* This strain expressed target proteins with a $GlcNAc_2Man_5$ glycoform (Figure 2.3b) [25]. Similarly, an α-1,2 mannosidase from *Trichoderma reesei* was targeted to the *Pichia pastoris* ER utilizing the HDEL retention signal to remove greater than 85% of the α-1,2 mannose residues on target proteins

(Figure 2.3) [26]. However, simply targeting the enzyme to the appropriate organelle proved insufficient to generate N-glycan homogeneity.

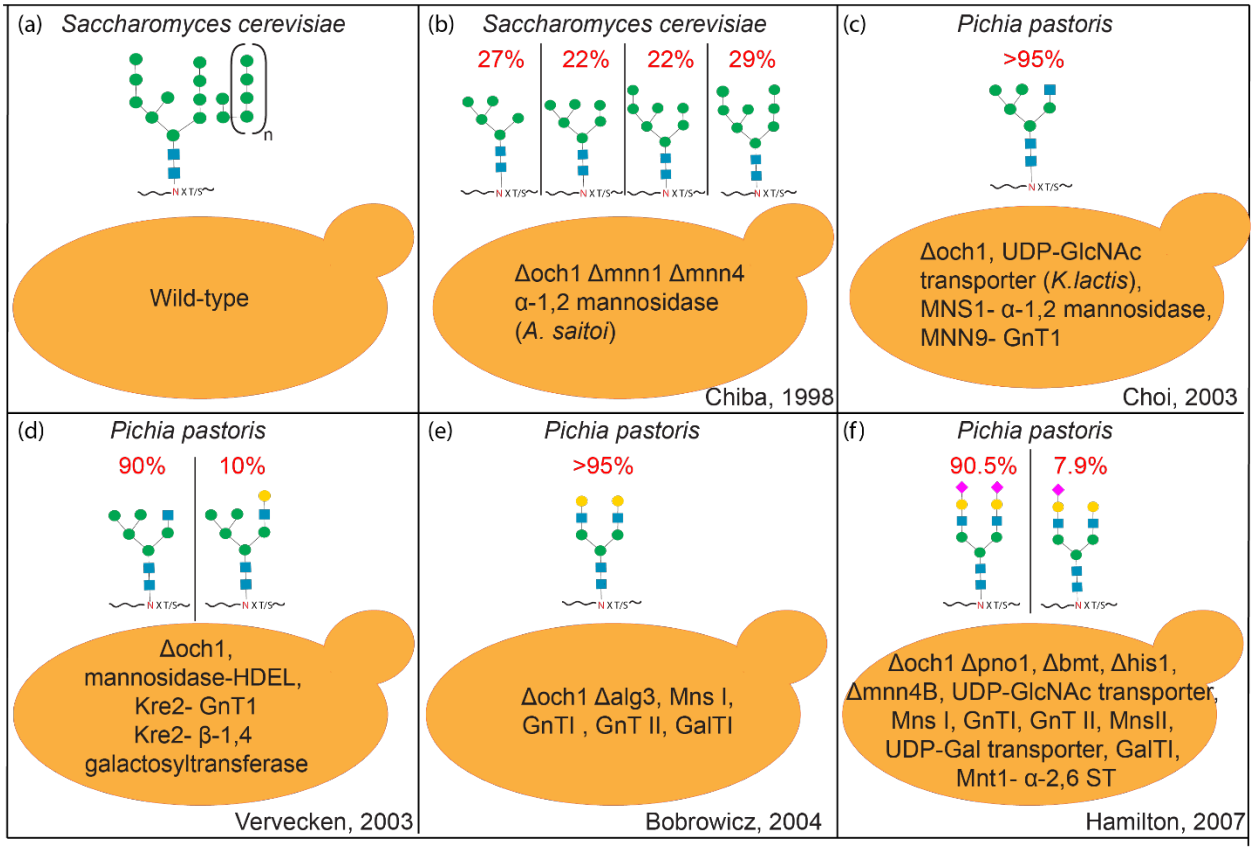

Figure 2.3 Targeting glycosyltransferases and glycosylhydrolases to the appropriate organelle (ER or Golgi) resulted in a higher abundance of the desired glycoform compared to previous methods. The figure above shows the different glycoengineered strains created in both *Pichia pastoris* and *Saccharomyces cerevisiae* to fulfil this purpose.

The suitable combination of peptide leader sequence and catalytic domain has been shown to be necessary for ensuring optimum enzymatic activity. Gerngross and co-workers created a screen using mass spectrometry to analyze the enzymatic efficiency of a variety of chimeric enzymes (differing in their N-terminal leader sequence and catalytic domain). The N-glycan attached to a target protein, Kringle 3, reported the enzymatic efficiency of the chimeric library. They found the chimeric enzyme containing the MNS1 or MNN10 leader peptides (*Saccharomyces cerevisiae*) fused to the catalytic domain from a *C. elegans* α-1,2 mannosidase

possessed the highest enzymatic efficiency of 70-80%. The second screen developed for the enzyme β-1,2 N-Acetylglucosaminyltransferase (GnT1) identified the human GnT1 enzyme with the MNN9 leader peptide (*Saccharomyces cerevisiae*) as the most efficient, converting most N-glycans exclusively to the $GlcNAcMan_5GlcNAc_2$ glycoforms [27].

The presence of a sufficient amount of nucleotide sugars as substrate for enzymatic reactions emerged as another critical factor to consider while engineering yeast strains. Yeast synthesize UDP-GlcNAc, GDP-Man, UDP-Gal and UDP-Glc. However, the nucleotide sugars UDP-Fuc and CMP-N-acetylneuraminic acid that serve as donors for mammalian glycosyltransferases are not produced and therefore, synthesis machineries for these sugar nucleotides must be engineered [28][29]. Yeast strains with engineered sugar nucleotide transporters have been created to ensure substrate availability in the appropriate organelle including the UDP-GlcNAc transporter from *K. lactis* which was integrated into the *Pichia pastoris* genome. Overexpressing Kre2-GnT1 signal peptide: enzyme chimera in the Δoch1 knock-out strain of *Pichia pastoris* was sufficient to convert a majority of the $Man_5GlcNAc_2$ glycoform to $GlcNAc\text{-}Man_5GlcNAc_2$ (>95%) (Figure 2.3c)[27].

A combination of targeting enzymes and engineering nucleotide sugar transporters within yeast strains produced hybrid and complex type N-glycans. Targeting a galactosyltransferase to the Golgi with a Kre2 signal sequence in the Δoch1 *Pichia pastoris* knockout strain converted just 10% of the $GlcNAcMan_5GlcNAc_2$ glycoform to $GalGlcNAcMan_5GlcNAc_2$ (Figure 2.3d). This may have been limited by a low amount of UDP-Gal in the Golgi though supplementing the culture with additional galactose did not increase conversion efficiency [30]. Creating a Δalg3 knockout within a previously created knockout strain (producing $GlcNAcMan_5GlcNAc_2$ glycoform) resulted in a strain that produced the $GlcNAcMan_3GlcNAc_2$ glycoform. The strain was then further

engineered to express GnTII and a β-1,4 galactosyltransferase targeted to the Golgi as well as a UDP-Gal transporter. This strain produced proteins with a nearly homogeneous $Gal_2GlcNAc_2Man_3GlcNAc_2$ glycoform (Figure 2.3e) [31].

Yeast also lacks the CMP-sialic acid synthesis machinery, CMP-sialic acid transporters and sialyltransferases. These issues were addressed through the creation of a *Pichia pastoris* strain that was able to sialylate target proteins at the glycan termini, though less than 60% of the biantennary glycans analyzed contained two sialic acid modifications. To improve conversion efficiency, a library of chimeric enzymes with variable combinations of N-terminal leader sequences and catalytic domains was screened to identify the optimal combination of the Mnt1 leader sequence (*Saccharomyces cerevisiae*) and mouse α-2,6 sialyltransferase resulting 90.5% conversion (Figure 2.3f) [29].

Pichia pastoris strains, capable producing complex type N-glycans, have been exploited to express a variety of therapeutics, relying upon the availability of several promoters for the simultaneous expression of multiple biologics including hGH (Human Growth Hormone) and IFN a-2b (Interferon a-2b), excipient HSA (Human Serum Albumin) or a cocktail of antibodies anti-PD1 (Programmed Cell Death Protein) and anti-CTLA-4 (Cytotoxic T-lymphocyte Associated Protein). This system would lower downstream processing, cost and time needed for production [32]. Similarly, the antibody Rituximab was produced in glycoengineered *Pichia pastoris* [33]. Rituximab is a monoclonal antibody used as a therapeutic against B-cell lymphoma and other autoimmune diseases.

Several groups have focused on creating a high throughput method for large scale expression of antibodies from these glycoengineered strains. An automated system for antibody expression combining overlap extension PCR and a glycoengineered *Pichia pastoris* strain

generated antibodies at half the cost as compared to a mammalian expression system [34]. Another attempt to automate antibody production from glycoengineered yeast strain involved purifying antibodies from culture medium using protein A capture in a 96-well format to allow for processing a large number of antibody samples for quality control purposes [35]. Aspects associated with the large-scale production of antibodies from glycoengineered *Pichia pastoris* have also been optimized [36].

2.2.1 Increasing N-glycosylation efficiency

Removing enzymes that synthesize N-glycan precursors, including the formation of Lipid Linked Oligosaccharide donors (LLO), results in shorter glycoforms that are not efficiently transferred to the polypeptide. This section focuses on engineering N-glycosylation by optimizing this transfer process along with flipping the LLO across the ER membrane and hence, obtaining yeast strains that produce the desired glycoforms. The N-glycan transfer reaction is catalysed by a multi-subunit enzyme known as the Oligosaccharyl Transferase (OST). This 9-subunit complex transfers a 14 residue ($Glc_3Man_9GlcNAc_2$) N-glycan from a lipid carrier (dolichol phosphate) to N-X-(S/T) sequons in a nascent polypeptide chain (X is any amino acid except proline). The OST subunits in yeast are either essential for growth, including Wbp1, Swp1, Stt3, Ost1 and Ost2 or non-essential, including Ost4, Ost5, Ost3 and Ost6. [37]. Stt3 is the catalytic subunit within the OST complex responsible for both peptide recognition and catalysing N-glycan transfer to the nascent protein. Studies related to the N-glycosylation machinery of *Campylobacter jejuni* revealed a set of Pgl genes responsible for the process. The Pgl subunit in this organism has a high sequence similarity to the Stt3 subunit of OST in yeast [38].

Stt3 deletion in *Saccharomyces cerevisiae* is deleterious but can be compensated with expression of the Stt3 subunit from *Toxoplasma gondii* [39]. However, considerations of glycan specificity are important for engineering Stt3 domains from other organisms in yeast. Yeast OST has a strong preference for the $Glc_3Man_9GlcNAc_2$ glycoform. The K_m of the reaction was 10-fold higher when DolPP-$GlcNAc_2Man_9Glc_3$ was used as the substrate compared to DolPP-$GlcNAc_2$ [40]. This glycan specificity is also determined by the entire OST complex and not by the individual catalytic subunit Stt3 alone. This was shown by replacing the Stt3 subunit of *Saccharomyces cerevisiae* with the homologous Stt3 subunit from *Trypanosoma cruzi* in the OST complex. Trypanosomes Stt3 subunits display a greater preference for smaller N-glycans ($Man_{7-9}GlcNAc_2$). The changes in N-glycosylation of a target protein, CPY, was studied using an Δalg5 knockout yeast strain. The Δalg5 produces glycoform $Man_9GlcNAc_2$-Dol PP which is a preferred substrate for *T. cruzi* Stt3 subunit (Figure 2.4) [41]. However, this subunit in complex with yeast

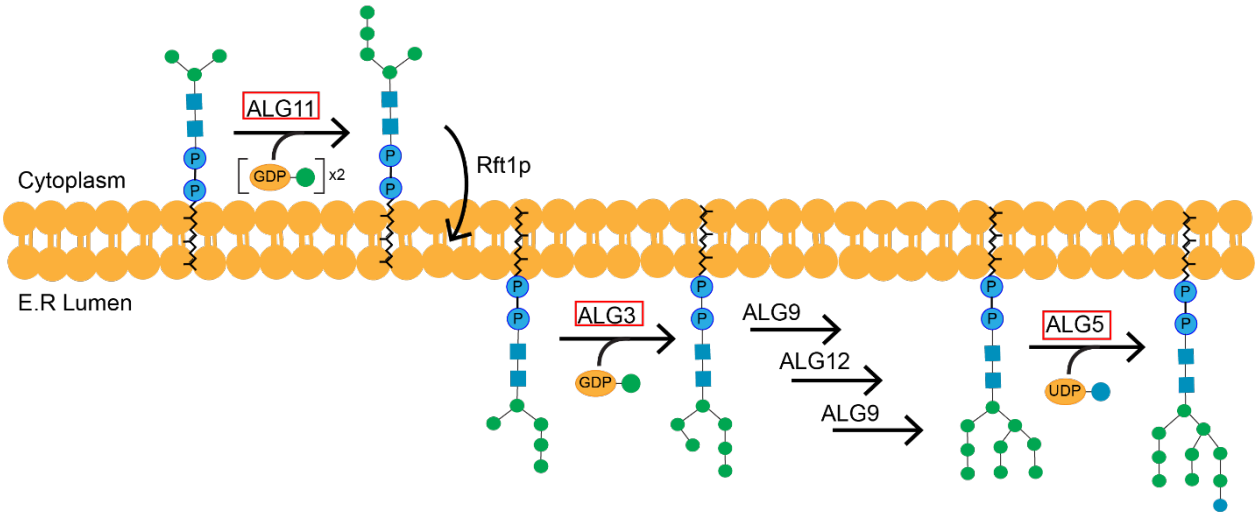

Figure 2.4 Lipid Linked Oligosaccharide (LLO) biosynthetic pathway The figure above shows a portion of the Lipid Linked Oligosaccharide (LLO) biosynthetic pathway. A few of the enzymes involved in this pathway have been knocked out (highlighted in *red*) in yeast strains to modify glycan composition.

OST was unable to efficiently transfer this N-glycan ($Man_9GlcNAc_2$) to a target protein resulting in lower glycosylation levels [42].

The Stt3 domain of *Leishmania major* (LmStt3) subunit has a greater preference for $Man_6GlcNAc_2$ and $Man_9GlcNAc_2$ glycoforms [43]. There are four paralogs of Stt3 in *Leishmania major*. Hese and coworkers determined these paralogs could compensate for defects observed in Δstt3 strains of *Saccharomyces cerevisiae*. As expected, LmStt3 was able to transfer truncated N-glycans more efficiently than their glycosylated counterparts ($Glc_3Man_9GlcNAc_2$) [44].

In one example, a series of strains with Alg gene knockouts were created to provide appropriate substrates to the engineered Stt3 domain, (Figure 2.4). Alg3 adds a mannose residue to convert $Man_5GlcNAc_2$-DolPP to $Man_6GlcNAc_2$-DolPP [45]. Alg11 catalyzes the last step of N-glycan processing on the cytoplasmic side of the ER ($Man_4GlcNAc_2$ to a $Man_5GlcNAc_2$ glycoform) prior to flipping across the ER membrane [46]. A combination of the Δalg3 Δalg11 knockout in a single strain produced an accumulation of the $Man_3GlcNAc_2$ LLO glycoform. Utilizing this strain, it was possible for one group to simultaneously engineer N-glycosylation efficiency and glycoforms in *Saccharomyces cerevisiae* [47].

Stt3 domains themselves may not necessarily be sufficient, inefficient flipping of the ideal LLO to the ER lumen sometimes proves limiting. The yeast flippase, Rft1p exhibits relaxed glycan specificity with a slight preference for $Man_5GlcNAc_2$ glycoforms [48]. An artificial flippase Flc*2p was engineered into the yeast strain to flip the $Man_3GlcNAc_2$ glycoform. Another problem in this strain is the inefficient transfer of the N-glycan from LLO to the polypeptide, this is due to the fact that the yeast OST has a preference for the glycoform $Glc_3Man_9GlcNAc_2$ over the $Man_3GlcNAc_2$ glycoform. This was addressed by introducing the Stt3 subunit from *Leishmania brasiliensis* (can transfer $Man_3GlcNAc_2$). Once the N-glycan transfer was complete, the protein

underwent later N-glycan modifications in the Golgi by the chimeric enzymes Kre2-GnT1 and Mnn2-GnT2. This represents a different approach to express proteins having a complex type N-glycan that does not require targeting α-1,2 mannosidases to the appropriate location. Mass spectrometry data revealed $GlcNAc_2Man_3GlcNAc_2$ as the most abundant glycoform, however there were some incompletely processed glycoforms along with some amount of $Man_3GlcNAc_2$ glycoforms (Figure 2.5).

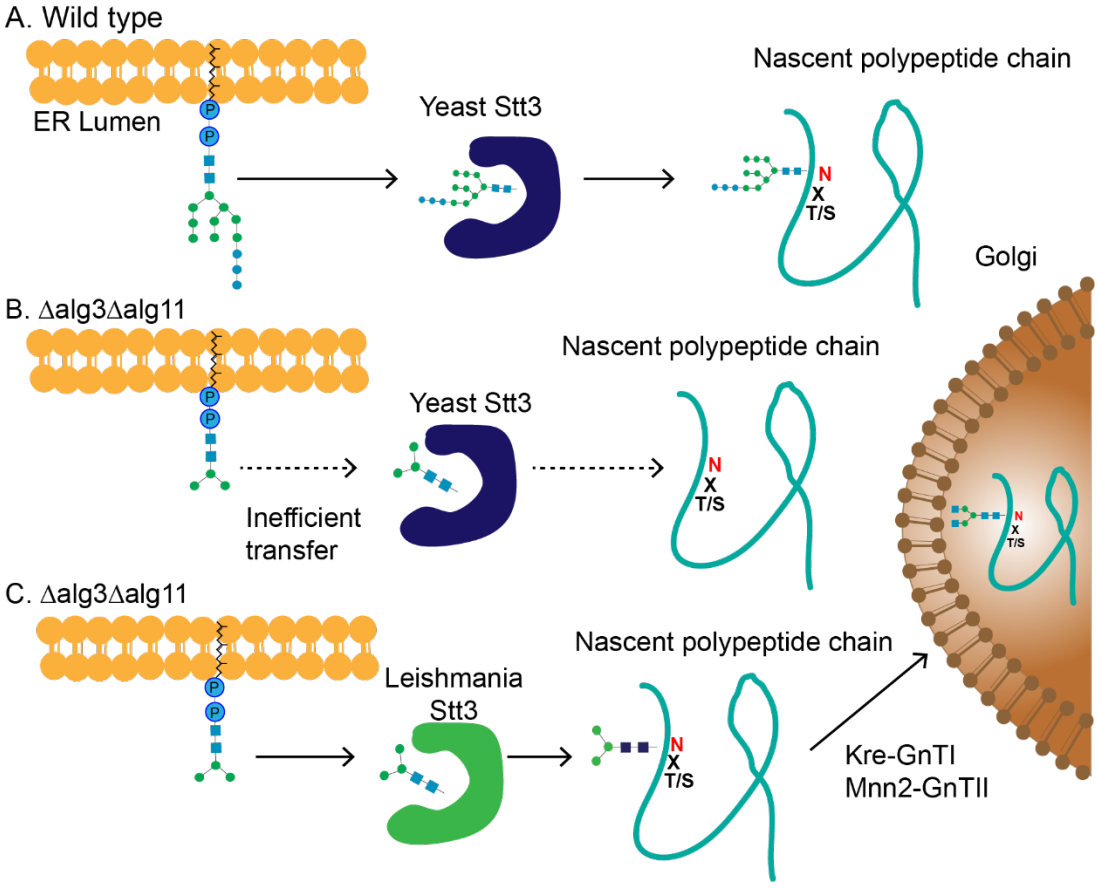

Figure 2.5 An approach to engineer N-glycosylation in yeast by targeting enzyme involved in LLO biosynthesis. (**a**) The preferred substrate for yeast Oligosaccharyl Transferase (OST) is $Glc_3Man_9GlcNAc_2$. This N-glycan is efficiently transferred from the dolichol diphosphate lipid donor to the N-X-(T/S) on a nascent polypeptide chain. (**b**) In the Δalg3Δalg11 knockout strain, the $Man_3GlcNAc_2$ glycoform is accumulated and results in this N-glycan being inefficiently transferred to the nascent polypeptide. (**c**) Replacing yeast OST complex with the *Leishmania major* Stt3 subunit, with a preference for the accumulated glycoform, overcomes this limitation. These truncated N-glycans are then available for modification with mammalian enzymes.

A common problem associated with fungal expression systems is the lower N-glycan occupancy (75-85%) compared to the mammalian expression system (>99%). The occupancy increased to >99% and was comparable to results obtained from antibodies expressed in CHO cells by expressing the LmStt3-D paralog in *Pichia pastoris*, with no significant influence on the resulting glycoforms [49].

2.2.2 Post purification methods to add mammalian glycans to yeast-expressed proteins

Previous sections covered the use of glycoengineered yeast strains to produce proteins with desired glycoforms. However, the glycoform often lacks a high degree of homogeneity. Some groups have used purified glycan modifying enzymes to engineer N-glycans. Two approaches to generate highly homogeneous protein preparations following purification of yeast-expressed glycoproteins include the use of mammalian glycosyltransferases in vitro or with the use of prokaryotic glycan hydrolases. The former subject is covered extensively in detail elsewhere [50][51][52][53][54].

Glycan hydrolase-treated proteins represent one approach to eliminate glycan heterogeneity in therapeutics when extended N-glycans are not required. Endoglycosidase (Endo) H (isolated from *Streptomyces plicatus*) trims yeast-produced oligomannose N-glycans to a single *N*-acetylglucosamine residue and efficiently trims glycans following purification (Figure 2.6) [55]. Alternatively, co-culturing two *Pichia pastoris* strains expressing either the target glycoprotein or Endo H and generated antibody comparable to antibody treated with Endo H following purification [56].

Many prokaryotic glycan hydrolases possess the unique ability to transfer N-glycans from a donor (peptide/protein with an attached N-glycan or sugar oxazoline) to an acceptor protein

through a transglycosylation reaction. Synthetic sugar oxazolines are transition state analogues that promote efficient reactions that proceed with minimal product degradation [57].

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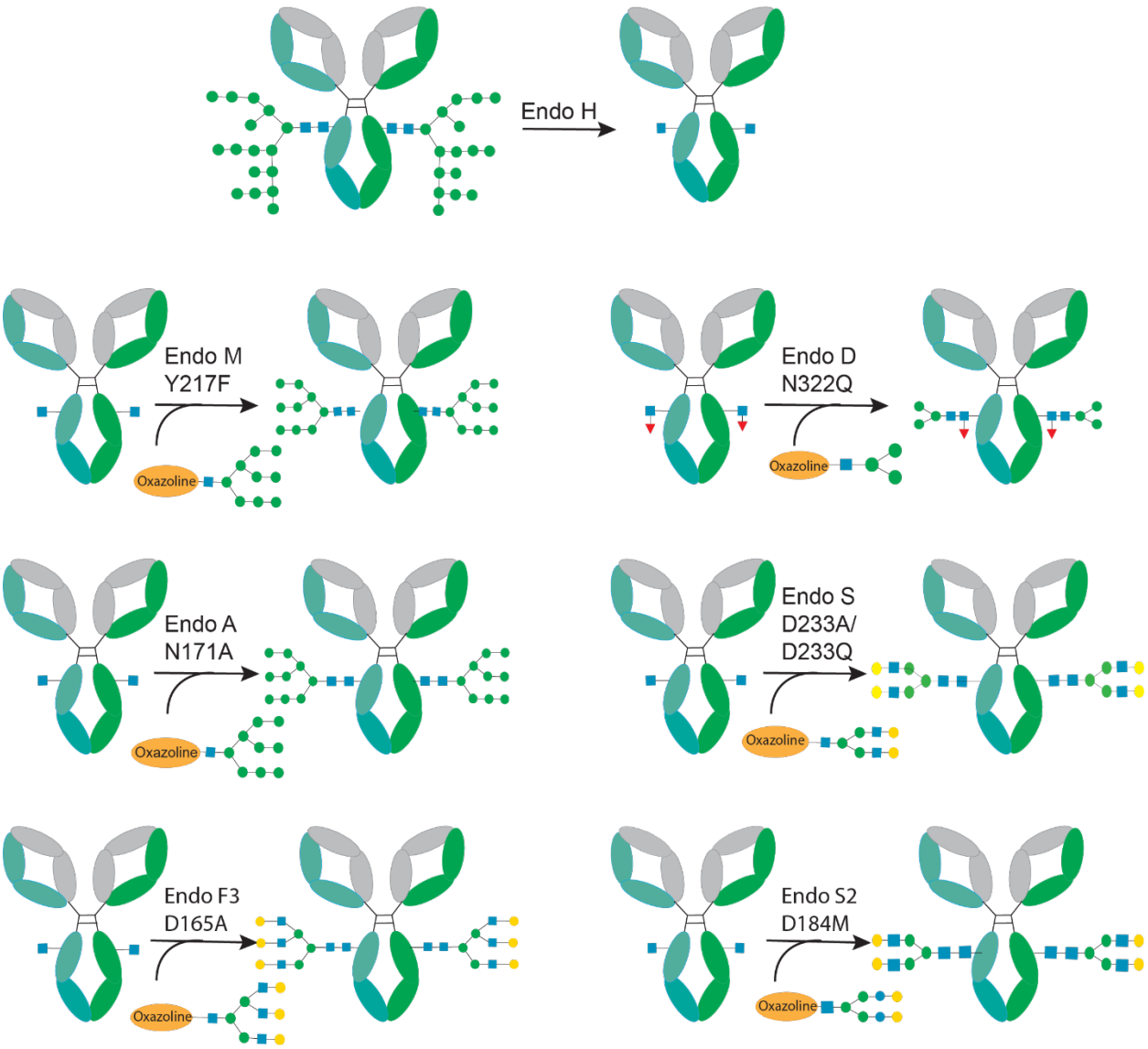

Figure 2.6 Enzymes used for modifying yeast-produced N-glycans modification of glycoproteins post-purification. Endo H trims oligomannose glycoforms to a single N-acetylglucosamine residue (GlcNAc) that serves as a substrate for transglycosylase reactions. Endo M, A, D and F3 recognize a broad range of protein substrates. EndoS and Endo S2 are largely specific for immunoglobulins with EndoS2 capable of adding both complex and hybrid-type N-glycans to IgG1.

Endo M (GH85 family) possesses little native transglycosylation ability. Umekawa and coworkers identified the Endo M Y217F variant that demonstrated improved transglycosylation activity with reduced product hydrolysis (Figure 2.6) [58]. Other enzymes capable of catalyzing a variety of transglycosylation reactions include Endo A N171A that transfers an oligomannose Nglycan [4], Endo F3 D165A that transfers triantennary N-glycans [59] and Endo D that transfers $Man_3GlcNAc_1$ to a fucosylated donor (Figure 2.6) [3].

Protein (substrate) specific glycosidases have likewise provided a scaffold for protein-specific transglycosylases. Endo S (isolated from *Streptomyces pyogenes*) specifically hydrolyzes complex type N-glycans from immunoglobulin G [60][61]. This enzyme is an endo β-1,4-N-acetylglucosamindase that cleaves the chitobiose core of a complex type N-glycan to generate IgG with a single N-linked *N*-acetylglucosamine attached to N297 and the hydrolyzed N-glycan product [60]. A crystal structure of inactive Endo S (D233A/E235L) revealed that the glycosidase domain of this enzyme forms a $(\beta/\alpha)_8$ barrel with a large central channel, the loops surrounding this domain form contacts with the N-glycan, thus determining N-glycan specificity [62]. Sequence alignment with a related enzyme, Endo F3, revealed a residue responsible for stabilizing the oxazolinium ion during catalysis (D233 in Endo S)[63]. Site directed mutagenesis to an A or Q residue at this site introduced efficient transglycosylase activity including the ability to transfer from an activated sugar-oxazoline donor with a complex-type glycan to IgG bearing a single *N*-acetylglucosamine in an hour [64]. This mutant enzyme likewise proved efficient with sialylated oxazoline donors that provided >90% conversion of an antibody.

Comparable variants of a related enzyme, EndoS2, likewise display transglycosylation activity though with a broader substrate profile. EndoS2 (also isolated from *Streptomyces pyogenes*) is also a β-1,4-N-acetyl-D-endoglycosidase that can digest biantennary sialylated

complex-type N-glycans from both IgG and the acute phase protein AGP [65]. EndoS2 and Endos share a 37% amino acid identity including active site conservation with the conserved GH18 amino acid motif (DXXDXXE) and conserved tryptophan residues [65]. However, EndoS2 exhibits a broader N-glycan specificity compared to Endo S, including the ability to digest complex-, hybrid- and oligomannose-type glycoforms [66]. This broad glycan specificity was later explained through crystal structures where it was shown that both the catalytic glycosylhydrolase (GH) and carbohydrate binding (CB) domains work in concert to accommodate a larger number of glycoforms with the active site of the enzyme [67]. Mutation of the EndoS2 D184 residue, responsible for stabilizing the oxazoline ion during catalysis, introduced transglycosylase activity. The EndoS2 D184M variant efficiently transferred a complex type N-glycan from the oxazoline substrate to IgG that was previously trimmed to display a single N-acetylglucosamine residue at a rate that was at least six times greater than the best EndoS variant. In one example, this enzyme converted >95% of the substrate antibody with sialylated N-glycans. EndoS2 D184M also transferred oligo-mannose and hybrid-type N-glycans, though with lower efficiency [68].

The discovery of a highly efficient EndoS2 variant having transglycosylation activity has allowed one group to use a combination approach that expressed antibodies with a *Pichia pastoris* expression platform lacking mannosyltransferases followed by enzymatic treatment, generating a homogeneously glycosylated product. The expressed antibody N-glycans were trimmed to a single N-acetylglucosamine residue following purification using Endo E. This product was next modified at an 80% level by EndoS2 D184M to generate antibodies with an $Gal_2GlcNAc_2Man_3GlcNAc_2$ glycoform [69].

2.3. Yeast Surface Display (YSD) using glycoengineered yeast strains

This review has focused on glycoengineering yeast to produce mammalian glycoforms, though another advantage of using a yeast expression platform is the ability to create and screen yeast display libraries. Due to its sophisticated protein quality control machinery and ability to carry out eukaryotic post-translational modifications, yeast is an excellent platform to diversify glycoproteins. This display technology involves screening through diverse protein libraries for a desired phenotype using techniques such as Fluorescence Assisted Cell Sorting (FACS) and Magnetic Assisted Cell Sorting (MACS) [70]. Through this technique it has been possible to identify several high affinity binders with a single chain variable antibody fragment (scFv) exhibiting affinity as high as 48 fM affinity [71].

Other proteins that have been diversified through this method include antibody antigen binding (Fab) domains that recognize a variety of antigens. Examples with low nM to μM affinity include Anti-HIV antibodies (10-43 nM) [72] and anti TNF-α antibodies [73], as well as the Major Histocompatibility Complex HLA-DR1 [74] and T-Cell coreceptor CD4 (5.3 - 8.8 μM) [75]. YSD has also developed an antibody with 2.1 pM affinity against H1N1 [76].

Fab domains do not contain a conserved N-glycosylation site, unlike the crystallizable fragment (Fc) of IgG1 that also requires an N-glycan for receptor binding. Though Fab display on yeast is straightforward, it is much more challenging to display function forms of Fc and other glycoproteins due to improper modifications and folding defects as well as hypermannosylation. One notable example is the Epidermal Growth Factor Receptor (EGFR) with extensive post translational modifications including 25 disulphide bonds and 12 potential sites of N-glycosylation. This protein is partially misfolded when displayed on the yeast surface as determined through binding with epitope-specific monoclonal antibodies through flow cytometry.

Therefore, it was necessary to screen for mutations within this protein to allow for better folding on the surface [77]. Similar problems were observed in the case of T cell receptors (TCR) displayed on the yeast surface. [78]. Problems associated with hypermannosylation were observed when the HIV gp120 and gp140 proteins were displayed on the yeast surface. The large N-glycans on the glycoproteins blocked antibody binding to specific epitopes which resulted in problems detecting protein expression levels [79].

The N-glycan processing in *Pichia pastoris* is less extensive as compared to *Saccharomyces cerevisiae*. Several glycoengineered *Pichia pastoris* strains have been created that are capable of producing complex-type glycoforms. As a result, Pichia Surface Display (PSD) was developed to display complex-type glycoforms on the yeast surface. PSD utilized the same agglutination system as *Saccharomyces cerevisiae* (YSD) to display protein. A GS115 (wild type) and a Man_5 glycoform producing *Pichia pastoris* strain were used to validate this technique [80]. To create display libraries using PSD, the cells are transformed with linearized plasmid through integration of the DNA into the genome, however, in the case of YSD, cells are transformed using an episomal plasmid DNA. As a result, there is higher percentage of cells that test positive for surface protein in PSD as compared to YSD [81]. Antibodies expressed in both CHO cells and glycoengineered strain of *Pichia pastoris* were comparable in terms of biochemical characteristics [82]. This implies that affinities measured through PSD would be comparable to those measured using protein isolated from mammalian systems.

2.4. Other notable applications of *Pichia* and *Saccharomyces* surface display

YSD has also been leveraged for vaccine development. In this application, the antigen of interest is anchored to the yeast surface. These cells are then injected into mice to induce an immune response. The antibodies produced in response to the antigen are isolated from the mice

and studied further. It has been shown that β-glucan from *Saccharomyces cerevisiae* can work as an adjuvant [83]. Some antigens that were used in this application include portions of the Hepatitis B antigen (HBsAg) [84], the VP7 capsid protein of Grass Carp Rheovirus (GCRV) [85], Eno1 protein of *Candida albicans* [86] and H5 antigen from Highly Pathogenic Avian Influenza (HPAI). These proteins were expressed on the surface of either wildtype or glycoengineered deletion strains [84] [85]. The rationale behind expressing antigens in deletion strains was to eliminate hypermannosylation and allow for epitopes that would otherwise be shielded by large N-glycans to be exposed and elicit a better immune response.

PSD was used to create libraries of camelid heavy chain antibodies utilizing a strain that generated Man_5 glycoforms. The library size is estimated to be 10^7 and considerably smaller compared to YSD libraries (10^8-10^9). With two rounds of sorting, antibodies having K_D values of 1.6 and 3.2 nM were isolated. These K_D values were determined through a titration experiment with antigen-GFP through flow cytometry [87]. Other aspects of this surface display technology have been modified for further improvement. One group identified anchor protein ScSed1p to anchor the protein of interest (Fab domain) onto the yeast surface. This anchor protein served as an alternative to the GPI anchor which has typically been used in yeast cells to display proteins on the surface [88]. This anchor protein was later used by another group to anchor Fc onto the surface to create PSD libraries of antibodies against antigen PCK9. This expression platform could simultaneously select for and secrete a display protein of interest [89]. Another indirect approach for display of proteins through PSD was developed by making use of *E. coli* derived proteins Im7 and CL7 (K_D 10^{-14} – 10^{-17}). The induced yeast cells (expressed scSed1p-Im7) were stained with purified CL7-GFP which resulted in more than 99% of the induced yeast cells expressing Sed1p-Im7-CL7-GFP complex on the yeast surface [90].

PSD has also been used to immobilize enzymes of the yeast surface for catalysis, and these methods likewise can be used for engineering ER, Golgi and secreted enzymes that often contain extensive processing and modifications. Immobilizing enzymes onto the yeast surfaces is considered to be a milder alternative to chemical methods [91]. Some examples include enzyme Lipase B52 [92], Phytase [93] and Trehalose synthase [94]. In some cases, it was found that the enzyme when displayed on *Pichia pastoris* was more stable than when expressed on *Saccharomyces cerevisiae*. However, it took a longer induction time to induce protein expression in *Pichia pastoris* as compared to *Saccharomyces cerevisiae* [92].

Recently, groups have focused on increasing display efficiency and developing novel approaches to display the protein on the yeast surface. One group displayed protein of interest (Nanobody library) by fusing its C-terminus to the N-terminus of Aga2p, the Aga2p was then fused to Acyl Carrier Protein (ACP). This fusion with ACP allows for orthogonal labelling (CoA-647) or immobilizing onto a chip (CoA-biotin) for affinity measurements. Such a display platform would make validating hits obtained from sorting libraries less cumbersome [95]. In order to increase surface expression levels, one group created a bicistronic system to express both heavy chain and light chain of the antibody Fab domain under a single GAL1 promoter through ribosome skipping [96]. Another group focused on increasing the amount of functional yeast surface displayed protein, by expressing both Protein Disulphide Isomerase (PDI) and ER resident chaperone Kar2p along with displaying Fab domains (against TNF-α) on the surface. This approach resulted in lower heavy chain and light chain expression on the cell surface, however greatly increased the amount of functional Fab that could bind antigen TNF-α compared to the total amount of Fab expression [97].

2.5 Discussion

The availability of several glycoengineered *Pichia pastoris* and *Saccharomyces cerevisiae* strains combined with the ability to create diverse protein display libraries provides unique opportunities to engineer glycoproteins that contain appropriate modifications. PSD platform provides proof of concept that glycoproteins can be displayed on the surface of glycoengineered yeast strains. However, this technology entails a cumbersome procedure to create protein libraries (integration of linearized plasmid) as compared to YSD (assembly of libraries in-vivo through homologous recombination). The PSD transformation protocol may result in a higher percentage of cells expressing surface displayed protein, but the resulting libraries will be smaller (10^6-10^7 vs. 10^8-10^9 for *Saccharomyces cerevisiae*).

One major advantage of utilizing PSD is the availability of several glycoengineered strains, utilizing these strains for protein engineering purposes would allow for the displayed protein to have the desired glycoform. However, in the case of *Saccharomyces cerevisiae*, it has been difficult to create strains capable of producing homogeneous glycoforms, this is perhaps due to the fact to the extensive hypermannosylation found on protein expressed in this strain that blocks epitopes from detection using large reagents like antibodies, thus interfering with surface expression detection. Furthermore, eliminating these reactions impacts strain viability through off-target effects that limit the ability to efficiently sort and screen. Another possible approach to address non-homogeneous glycan profiles in these strains would be to use transglycosylase variants of enzymes to remodel glycoproteins. This protocol entails multiple enzymatic steps and sugar oxazolines to carry out the reaction. Remodelling glycoproteins in this manner is feasible within a laboratory setting, however, it may face challenges during scale up to achieve biologic therapeutic production.

Recent developments in this field have focused on developing a more efficient method to stain cells for FACS and increasing the amount of functional yeast displayed protein on the surface. But so far there has not been a platform developed which would allow for creation of diverse libraries of glycoproteins with homogenous glycoforms. Here we defined the progress towards such a goal and note that efficient glycoprotein display and engineering is likely close at hand. The development of such a platform will allow effective screening of vast and diverse glycoproteins libraries including T cell receptors, major histocompatibility complexes or Fc domains that are currently not possible with existing display technologies.

2.6 References

1. Sinclair, A. M., & Elliott, S. (2005). Glycoengineering: The effect of glycosylation on the properties of therapeutic proteins. *Journal of Pharmaceutical Sciences*, *94*(8), 1626–1635. https://doi.org/10.1002/jps.20319

2. Egrie, J. C., Dwyer, E., Browne, J. K., Hitz, A., & Lykos, M. A. (2003). Darbepoetin alfa has a longer circulating half-life and greater in vivo potency than recombinant human erythropoietin. *Experimental Hematology*, *31*(4), 290–299. https://doi.org/10.1016/S0301-472X(03)00006-7

3. Fan, S. Q., Huang, W., & Wang, L. X. (2012). Remarkable transglycosylation activity of glycosynthase mutants of endo-D, an endo-β-N-acetylglucosaminidase from Streptococcus pneumoniae. *Journal of Biological Chemistry*, *287*(14), 11272–11281.https://doi.org/10.1074/jbc.M112.340497

4. Huang, W., Li, C., Li, B., Umekawa, M., Yamamoto, K., Zhang, X., & Wang, L. X. (2009). Glycosynthases enable a highly efficient chemoenzymatic synthesis of N-Glycoproteins carrying intact natural N-Glycans. *Journal of the American Chemical Society*, *131*(6), 2214–2223. https://doi.org/10.1021/ja8074677

5. Wildt, S., & Gerngross, T. U. (2005). *THE HUMANIZATION OF N -GLYCOSYLATION PATHWAYS IN YEAST*. https://doi.org/10.1038/nrmicro1087

6. Chen, R. (2015). The sweet branch of metabolic engineering : cherry - picking the low - hanging sugary fruits. *Microbial Cell Factories*, 1–10. https://doi.org/10.1186/s12934-015-0389-z

7. Piirainen, M. A., Ruijter, J. C. De, Koskela, E. V, & Frey, A. D. (2014). Glycoengineering of yeasts from the perspective of glycosylation efficiency. *New BIOTECHNOLOGY*, *31*(6), 532–537. https://doi.org/10.1016/j.nbt.2014.03.001

8. De Pourcq K, De Schutter K, Callewaert N. Engineering of glycosylation in yeast and other

fungi: current state and perspectives. *Appl Microbiol Biotechnol*. 2010;87(5):1617-1631. doi:10.1007/s00253-010-2721-1

9. Khan, A. H., Bayat, H., Rajabibazl, M., Sabri, S., & Rahimpour, A. (2017). Humanizing glycosylation pathways in eukaryotic expression systems. *World Journal of Microbiology and Biotechnology*, *33*(1). https://doi.org/10.1007/s11274-016-2172-7

10. Boder, E., Wittrup, K. Yeast surface display for screening combinatorial polypeptide libraries. *Nat Biotechnol* **15,** 553–557 (1997). https://doi.org/10.1038/nbt0697-553
[11]Ellgaard, L., Helenius, A. Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* **4,** 181–191 (2003). https://doi.org/10.1038/nrm1052

12. Bowley, D. R., Labrijn, A. F., Zwick, M. B., & Burton, D. R. (2007). Antigen selection from an HIV-1 immune antibody library displayed on yeast yields many novel antibodies compared to selection from the same library displayed on phage. *Protein Engineering, Design and Selection*, *20*(2), 81–90. https://doi.org/10.1093/protein/gzl057

13. Schreuder, Maarten P, Mooren, A. T. A., Toschka, H. Y., Verrips, C. T., & Klis, F. M. (1996). *Immobilizing proteins on the surface of yeast cells*. 115–120.

14. PROTEIN GL YCOSYLA TION IN YEAST M. A. Kukuruzinskai, M. L. E. Berghi,2, and B. J. Jacksoni, Ann. Rev. Biochem. 1987. 56:915-44

15. Cereghino, J. L., & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast Pichia pastoris. *FEMS Microbiology Reviews*, *24*(1), 45–66. https://doi.org/10.1016/S0168-6445(99)00029-7

16. Goetze, A. M., Liu, Y. D., Zhang, Z., Shah, B., Lee, E., Bondarenko, P. V., & Flynn, G. C. (2011). High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. *Glycobiology*, *21*(7), 949–959. https://doi.org/10.1093/glycob/cwr027

17. Baudin, A., Ozier-kalogeropoulos, O., Denouel, A., Lacroute, F., & Cullin, C. (1993). A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. *Nucleic Acids Research*, *21*(14), 3329–3330. https://doi.org/10.1093/nar/21.14.3329

18. Nakayama, K., Nagasu, T., Shimma, Y., Kuromitsu, J., & Jigami, Y. (1992). OCH1 encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides. *The EMBO Journal*, *11*(7), 2511–2519. https://doi.org/10.1002/j.1460-2075.1992.tb05316.x

19. Nakayama, K. I., Nakanishi-Shindo, Y., Tanaka, A., Haga-Toda, Y., & Jigami, Y. (1997). Substrate specificity of α-1,6-mannosyltransferase that initiates N-linked mannose outer chain elongation in Saccharomyces cerevisiae. *FEBS Letters*, *412*(3), 547–550. https://doi.org/10.1016/S0014-5793(97)00634-0

20. Nakanishi-Shindo, Y., Nakayama, K. I., Tanaka, A., Toda, Y., & Jigami, Y. (1993). Structure of the N-linked oligosaccharides that show the complete loss of α-1,6-polymannose outer chain from och1, och1 mnn1, and och1 mnn1 alg3 mutants of Saccharomyces cerevisiae. *Journal of Biological Chemistry*, *268*(35), 26338–26345

21. Nagasu, T., Shimma, Y. -I, Nakanishi, Y., Kuromitsu, J., Iwama, K., Nakayama, K. -I, Suzuki, K., & Jigami, Y. (1992). Isolation of new temperature-sensitive mutants of Saccharomyces cerevisiae deficient in mannose outer chain elongation. *Yeast*, *8*(7), 535–547. https://doi.org/10.1002/yea.320080705

22. Zhou, J., Zhang, H., Liu, X., Wang, P. G., & Qi, Q. (2007). Influence of N-glycosylation on Saccharomyces cerevisiae morphology: A Golgi glycosylation mutant shows cell division defects. *Current Microbiology*, *55*(3), 198–204. https://doi.org/10.1007/s00284-006-0585-5

23. Abe, H., Takaoka, Y., Chiba, Y., Sato, N., Ohgiya, S., Itadani, A., Hirashima, M., Shimoda,

C., Jigami, Y., & Nakayama, K. I. (2009). Development of valuable yeast strains using a novel mutagenesis technique for the effective production of therapeutic glycoproteins. *Glycobiology*, *19*(4), 428–436. https://doi.org/10.1093/glycob/cwn157

24. Martinet, W., Maras, M., Saelens, X., Jou, W. M., & Contreras, R. (1998). Modification of the protein glycosylation pathway in the methylotrophic yeast Pichia pastoris. *Biotechnology Letters*, *20*(12), 1171–1177. https://doi.org/10.1023/A:1005340806821

25. Chiba, Y., Suzuki, M., Yoshida, S., Yoshida, A., Ikenaga, H., Takeuchi, M., Jigami, Y., & Ichishima, E. (1998). Production of human compatible high mannose-type (Man5GlcNAc2) sugar chains in Saccharomyces cerevisiae. *Journal of Biological Chemistry*, *273*(41), 26298–26304. https://doi.org/10.1074/jbc.273.41.26298

26. Callewaert, N., Y, W. L., Cadirgi, H., Geysens, S., Saelens, X., Min, W., & Y, R. C. (2001). Use of HDEL-tagged Trichoderma reesei mannosyl oligosaccharide 1,2-alpha-D-mannosidase for N-glycan engineering in Pichia pastoris. *FEBS Lett*. 2001;503(2-3):173-178. doi:10.1016/s0014-5793(01)02676-x

27. Choi, B., Bobrowicz, P., Davidson, R. C., Hamilton, S. R., Kung, D. H., Li, H., Robert, G., Nett, J. H., Wildt, S., Gerngross, T. U., Choi, B., Bobrowicz, P., Davidson, R. C., Hamilton, S. R., Kung, D. H., Li, H., Miele, R. G., Nett, J. H., Wildt, S., & Gerngross, T. U. (2003).*Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast Pichia pastoris*. PNAS April 29, 2003 100 (9) 5022-5027; https://doi.org/10.1073/pnas.0931263100

28. Muraoka, M., Miki, T., Ishida, N., Hara, T., & Kawakita, M. (2007). Variety of nucleotide sugatransporters with respect to the interaction with nucleoside mono- and diphosphates. *Journal of Biological Chemistry*, *282*(34), 24615–24622. https://doi.org/10.1074/jbc.M611358200

29. Hamilton, S. R., Davidson, R. C., Sethuraman, N., Nett, J. H., Jiang, Y., Rios, S., Bobrowicz, P., Stadheim, T. a, Li, H., Choi, B., Hopkins, D., Wischnewski, H., Roser, J., Mitchell, T., Strawbridge, R. R., Hoopes, J., Wildt, S., & Gerngross, T. U. (2006). Humanization of yeast to

produce complex terminally sialylated glycoproteins. *Science*. 2006;313(5792):1441-1443. doi:10.1126/science.1130256

30. Vervecken, W., Kaigorodov, V., Callewaert, N., Geysens, S., De Vusser, K., & Contreras, R. (2004). In vivo synthesis of mammalian-like, hybrid-type N-glycans in Pichia pastoris. *Applied and Environmental Microbiology*, *70*(5), 2639–2646. https://doi.org/10.1128/AEM.70.5.2639-2646.2004

31. Bobrowicz P, Davidson RC, Li H, et al. Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast Pichia pastoris: production of complex humanized glycoproteins with terminal galactose. *Glycobiology*. 2004;14(9):757-766. doi:10.1093/glycob/cwh104

32. Cao, J., Perez-Pinera, P., Lowenhaupt, K., Wu, M. R., Purcell, O., De La Fuente-Nunez, C., & Lu, T. K. (2018). Versatile and on-demand biologics co-production in yeast. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-017-02587-w

33. Li, H., Sethuraman, N., Stadheim, T. A., Zha, D., Prinz, B., Ballew, N., Bobrowicz, P., Choi, B., Cook, W. J., Cukan, M., Houston-cummings, N. R., Davidson, R., Gong, B., Hamilton, S. R., Hoopes, J. P., Jiang, Y., Kim, N., Mansfield, R., Nett, J. H., … Gerngross, T. U. (2006). *Optimization of humanized IgGs in glycoengineered Pichia pastoris*. *Nat Biotechnol*. 2006, 24(2):210-215. doi:10.1038/nbt1178

34. Shah, K.A., Clark, J.J., Goods, B.A., Politano, T.J., Mozdzierz, N.J., Zimnisky, R.M., Leeson, R.L., Love, J. and Love, K.R. (2015), Automated pipeline for rapid production and screening of HIV-specific monoclonal antibodies using pichia pastoris. Biotechnol. Bioeng., 112: 2624-2629. doi:10.1002/bit.25663

35. Jiang, Y., Li, F., Button, M., Cukan, M., Moore, R., Sharkey, N., & Li, H. (2010). A high-throughput purification of monoclonal antibodies from glycoengineered Pichia pastoris. *Protein*

Expression and Purification, *74*(1), 9–15. https://doi.org/10.1016/j.pep.2010.04.016

36. Potgieter, T. I., Kersey, S. D., Mallem, M. R., Nylen, A. C., & D'Anjou, M. (2010). Antibody expression kinetics in glycoengineered Pichia Pastoris. *Biotechnology and Bioengineering*, *106*(6), 918–927. https://doi.org/10.1002/bit.22756

37. Knauer, R., & Lehle, L. (1999). The oligosaccharyltransferase complex from yeast. *Biochimica et Biophysica Acta - General Subjects*, *1426*(2), 259–273. https://doi.org/10.1016/S0304-4165(98)00128-7

38. Wacker M, Linton D, Hitchen PG, et al. N-linked glycosylation in Campylobacter jejuni and its functional transfer into E. coli. *Science*. 2002;298(5599):1790-1793. doi:10.1126/science.298.5599.1790

39. Shams-Eldin, H., Blaschke, T., Anhlan, D., Niehus, S., Müller, J., Azzouz, N., & Schwarz, R. T. (2005). High-level expression of the Toxoplasma gondii STT3 gene is required for suppression of the yeast STT3 gene mutation. *Molecular and Biochemical Parasitology*, *143*(1), 6–11. https://doi.org/10.1016/j.molbiopara.2005.04.008

40. *N* -Glycosylation of Yeast Proteins Characterization of the Solubilized Oligosaccharyl Transferase, Chandra B. Sharma, Ludwig Lehle, Widmar Tanner, European Journal of Biochemistry, https://doi.org/10.1111/j.1432-1033.1981.tb05306.

41. Huffaker, T. C., & Robbins, P. W. (1983). Yeast mutants deficient in protein glycosylation. *Proceedings of the National Academy of Sciences of the United States of America*, *80*(24 I), 7466–7470. https://doi.org/10.1073/pnas.80.24.7466

42. Castro, O., Movsichoff, F., & Parodi, A. J. (2006). Preferential transfer of the complete glycan is determined by the oligosaccharyltransferase complex and not by the catalytic subunit. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(40), 14756–14760. https://doi.org/10.1073/pnas.0607086103

43. All in One: Leishmania Major STT3 Proteins Substitute for the Whole Oligosaccharyltransferase Complex in Saccharomyces Cerevisiae, Farnoush Parsaie Nasab [1], Benjamin L Schulz, Francisco Gamarro, Armando J Parodi, Markus Aebi, Mol Biol Cell 2008 Sep;19(9):3758-68 doi: 10.1091/mbc.e08-05-0467. Epub 2008 Jul 2

44. Hese, K., Otto, C., & Routier, H. (2009). *The yeast oligosaccharyltransferase complex can be replaced by STT3 from Leishmania major*. *19*(2), 160–171. https://doi.org/10.1093/glycob/cwn118

45. Davidson, R. C., Nett, J. H., Renfer, E., Li, H., Stadheim, T. A., Miller, B. J., Miele, R. G., Hamilton, S. R., Choi, B. K., Mitchell, T. I., & Wildt, S. (2004). Functional analysis of the ALG3 gene encoding the Dol-P-Man: Man5GlcNAc2-PP-Dol mannosyltransferase enzyme of P. pastoris. *Glycobiology*, *14*(5), 399–407. https://doi.org/10.1093/glycob/cwh023

46. Cipollo, J. F., Trimble, R. B., Chi, J. H., Yan, Q., & Dean, N. (2001). The Yeast ALG11 Gene Specifies Addition of the Terminal α1,2-Man to the Man5GlcNAc2-PP-dolichol N-Glycosylation Intermediate Formed on the Cytosolic Side of the Endoplasmic Reticulum. *Journal of Biological Chemistry*, *276*(24), 21828–21840. https://doi.org/10.1074/jbc.M010896200

47. Nasab, F. P., Aebi, M., Bernhard, G., & Frey, A. D. (2013). A combined system for engineering glycosylation efficiency and glycan structure in Saccharomyces cerevisiae. *Applied and Environmental Microbiology*, *79*(3), 997–1007. https://doi.org/10.1128/AEM.02817-12

48. Helenius, J., Ng, D. T. W., Marolda, C. L., Walter, P., Valvano, M. A., & Aebi, M. (2002). *Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein*. *1436*(1996), 447–450.

49. Choi, B., Warburton, S., & Lin, H. (2012). *Improvement of N -glycan site occupancy of*

therapeutic glycoproteins produced in Pichia pastoris. 671–682. https://doi.org/10.1007/s00253-012-4067-3

50. Li, C., & Wang, L. X. (2018). Chemoenzymatic Methods for the Synthesis of Glycoproteins. *Chemical Reviews*, *118*(17), 8359–8413. https://doi.org/10.1021/acs.chemrev.8b00238

51. Wang, L. X., & Amin, M. N. (2014). Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chemistry and Biology*, *21*(1), 51–66. https://doi.org/10.1016/j.chembiol.2014.01.001

52. Giddens, J. P., Lomino, J. V., DiLillo, D. J., Ravetch, J. V., & Wang, L. X. (2018). Site-selective chemoenzymatic glycoengineering of Fab and Fc glycans of a therapeutic antibody. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(47), 12023–12027. https://doi.org/10.1073/pnas.1812833115

53. Li, T., DiLillo, D. J., Bournazos, S., Giddens, J. P., Ravetch, J. V., & Wang, L. X. (2017). Modulating IgG effector function by Fc glycan engineering. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(13), 3485–3490. https://doi.org/10.1073/pnas.1702173114T.

54. Castilho, A. (2015). Glyco-engineering: Methods and protocols. *Glyco-Engineering: Methods and Protocols*, *1321*, 1–439. https://doi.org/10.1007/978-1-4939-2760-9

55. Robbins, P. W., Wirth, D. F., & Hering, C. (1981). Expression of the Streptomyces enzyme endoglycosidase H in Escherichia coli. *Journal of Biological Chemistry*, *256*(20), 10640–10644

56. Wang, F., Wang, X., Yu, X., Fu, L., Liu, Y., Ma, L., & Zhai, C. (2015). High-level expression of endo-β-N-acetylglucosaminidase H from Streptomyces plicatus in Pichia pastoris and its application for the deglycosylation of glycoproteins. *PLoS ONE*, *10*(3). https://doi.org/10.1371/journal.pone.0120458

57. Fujita, M., Shoda, S. ichiro, Haneda, K., Inazu, T., Takegawa, K., & Yamamoto, K. (2001). A novel disaccharide substrate having 1,2-oxazoline moiety for detection of transglycosylating activity of endoglycosidases. *Biochimica et Biophysica Acta - General Subjects*, *1528*(1), 9–14. https://doi.org/10.1016/S0304-4165(01)00164-7

58. Umekawa, M., Huang, W., Li, B., Fujita, K., Ashida, H., Wang, L. X., & Yamamoto, K. (2008). Mutants of Mucor hiemalis endo-β-N-acetylglucosaminidase show enhanced transglycosylation and glycosynthase-like activities. *Journal of Biological Chemistry*, *283*(8), 4469–4479. https://doi.org/10.1074/jbc.M707137200

59. Giddens, J. P., Lomino, J. V., Amin, M. N., & Wang, L. X. (2016). Endo-F3 glycosynthase mutants enable chemoenzymatic synthesis of core-fucosylated triantennary complex type glycopeptides and glycoproteins. *Journal of Biological Chemistry*, *291*(17), 9356–9370. https://doi.org/10.1074/jbc.M116.721597

60. Collin, M., & Olsén, A. (2001). EndoS, a novel secreted protein from Streptococcus pyogenes with endoglycosidase activity on human IgG. *EMBO Journal*, *20*(12), 3046–3055. https://doi.org/10.1093/emboj/20.12.3046

61. Allhorn, M., Olin, A. I., Nimmerjahn, F., & Colin, M. (2008). Human IgC/FcγR interactions are modulated by streptococcal IgG glycan hydrolysis. *PLoS ONE*, *3*(1). https://doi.org/10.1371/journal.pone.0001413

62. Trastoy, B., Klontz, E., Orwenyo, J., Marina, A., Wang, L. X., Sundberg, E. J., & Guerin, M. E. (2018). Structural basis for the recognition of complex-Type N-glycans by Endoglycosidase S. *Nature Communications*, *9*(1), 1–11. https://doi.org/10.1038/s41467-018-04300-x

63. Allhorn, M., Olsén, A., & Collin, M. (2008). EndoS from Streptococcus pyogenes is hydrolyzed by the cysteine proteinase SpeB and requires glutamic acid 235 and tryptophans for

IgG glycan-hydrolyzing activity. *BMC Microbiology*, *8*, 1–10. https://doi.org/10.1186/1471-2180-8-3

64. Wei Huang, John Giddens, Shu-Quan Fan, Christian Toonstra, and Lai-Xi Wang, Chemoenzymatic Glycoengineering of Intact IgG Antibodies for Gain of Functions, *Journal of the American Chemical Society* 2012 *134* (29), 12308-12318 DOI: 10.1021/ja3051266

65. Sjögren, J., Struwe, W. B., Cosgrave, E. F. J., Rudd, P. M., Stervander, M., Allhorn, M., Hollands, A., Nizet, V., & Collin, M. (2013). EndoS2 is a unique and conserved enzyme of serotype M49 group A Streptococcus that hydrolyses N-linked glycans on IgG and α1-acid glycoprotein. *Biochemical Journal*, *455*(1), 107–118. https://doi.org/10.1042/BJ20130126

66. Sjögren, J., Cosgrave, E. F. J., Allhorn, M., Nordgren, M., Björk, S., Olsson, F., Fredriksson, S., & Collin, M. (2015). EndoS and EndoS2 hydrolyze Fc-glycans on therapeutic antibodies with different glycoform selectivity and can be used for rapid quantification of high-mannose glycans. *Glycobiology*, *25*(10), 1053–1063. https://doi.org/10.1093/glycob/cwv047

67. Klontz, E. H., Trastoy, B., Deredge, D., Fields, J. K., Li, C., Orwenyo, J., Marina, A., Beadenkopf, R., Günther, S., Flores, J., Wintrode, P. L., Wang, L. X., Guerin, M. E., & Sundberg, E. J. (2019). Molecular Basis of Broad Spectrum N-Glycan Specificity and Processing of Therapeutic IgG Monoclonal Antibodies by Endoglycosidase S2. *ACS Central Science*, *5*(3), 524–538. https://doi.org/10.1021/acscentsci.8b00917

68. Li, T., Tong, X., Yang, Q., Giddens, J. P., & Wang, L. X. (2016). Glycosynthase mutants endoglycosidase S2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling. *Journal of Biological Chemistry*, *291*(32), 16508–16518. https://doi.org/10.1074/jbc.M116.738765

69. Liu, C. P., Tsai, T. I., Cheng, T., Shivatare, V. S., Wu, C. Y., Wu, C. Y., & Wong, C. H. (2018). Glycoengineering of antibody (Herceptin) through yeast expression and in vitro

enzymatic glycosylation. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(4), 720–725. https://doi.org/10.1073/pnas.1718172115.

70. Chao, G., Lau, W. L., Hackel, B. J., Sazinsky, S. L., Lippow, S. M., & Wittrup, K. D. (2007). *Isolating and engineering human antibodies using yeast surface display*. *1*(2), 755–769. https://doi.org/10.1038/nprot.2006.94

71. Boder, E. T., Midelfort, K. S., & Wittrup, K. D. (2000). Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(20), 10701–10705. https://doi.org/10.1073/pnas.170297297

72. Walker, L. M., Bowley, D. R., & Burton, D. R. (2009). Efficient Recovery of High-Affinity Antibodies from a Single-Chain Fab Yeast Display Library. *Journal of Molecular Biology*, *389*(2), 365–375. https://doi.org/10.1016/j.jmb.2009.04.019

73. A general method for greatly improving the affinity of antibodies by using combinatorial libraries, Arvind Rajpal, Nurten Beyaz, Lauric Haber, Guido Cappuccilli, Helena Yee, Ramesh R. Bhatt, Toshihiko Takeuchi, Richard A. Lerner, Roberto Crea, Proceedings of the National Academy of Sciences Jun 2005, 102 (24) 8466-8471; DOI: 10.1073/pnas.0503543102

74. Boder, E. T., Bill, J. R., Nields, A. W., & Marrack, P. C. (2005). *Yeast Surface Display of a Noncovalent MHC Class II Heterodimer Complexed With Antigenic Peptide*. *0239099*. https://doi.org/10.1002/bit.20616

75. Wang, X. X., Li, Y., Yin, Y., Mo, M., Wang, Q., Gao, W., Wang, L., & Mariuzza, R. A. (2011). Affinity maturation of human CD4 by yeast surface display and crystal structure of a CD4-HLA-DR1 complex. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(38), 15960–15965. https://doi.org/10.1073/pnas.1109438108

76. Shembekar, N., Mallajosyula, V. V. A., Mishra, A., Yeolekar, L., Dhere, R., Kapre, S., Varadarajan, R., & Gupta, S. K. (2013). Isolation of a High Affinity Neutralizing Monoclonal Antibody against 2009 Pandemic H1N1 Virus That Binds at the "Sa" Antigenic Site. *PLoS ONE*, *8*(1), 1–10. https://doi.org/10.1371/journal.pone.0055516

77. Kim, Y., Bhandari, R., Cochran, J. R., Kuriyan, J., & Wittrup, K. D. (2006). *Directed Evolution of the Epidermal Growth Factor Receptor Extracellular Domain for Expression in Yeast*. *1035*(December 2005), 1026–1035. https://doi.org/10.1002/prot.20618

78. Michele C. Kieke, Eric V. Shusta, Eric T. Boder, Luc Teyton, K. Dane Wittrup, and David M. Kranz (1999), Selection of functional T cell receptor mutants from a yeast surface-display library, PNAS May 11, 1999 96 (10) 5651-5656; https://doi.org/10.1073/pnas.96.10.5651

79. Mathew, E., Zhu, H., Connelly, S. M., Sullivan, M. A., Brewer, M. G., Piepenbrink, M. S., Kobie, J. J., Dewhurst, S., & Id, M. E. D. (2018). *Display of the HIV envelope protein at the yeast cell surface for immunogen development*

80. Jacobs, P. P., Ryckaert, S., Geysens, S., De Vusser, K., Callewaert, N., & Contreras, R. (2008). Pichia surface display: Display of proteins on the surface of glycoengineered Pichia pastoris strains. *Biotechnology Letters*, *30*(12), 2173–2181. https://doi.org/10.1007/s10529-008-9807-1

81. Antibodies, S. D. (n.d.). *Chapter 8 Pichia Surface Display : A Tool for Screening Single Domain Antibodies*. *911*, 125–134. https://doi.org/10.1007/978-1-61779-968-6

82. Ha, S., Wang, Y., & Rustandi, R. R. (2011). Biochemical and biophysical characterization of humanized IgG1 produced in Pichia pastoris. *MAbs*, *3*(5), 453–460. https://doi.org/10.4161/mabs.3.5.16891

83. Berner VK, Sura ME, Hunter KW Jr. (2008) Conjugation of protein antigen to microparticulate beta-glucan from Saccharomyces cerevisiae: a new adjuvant for intradermal and

oral immunizations. *Appl Microbiol Biotechnol*, 80(6):1053-1061. doi:10.1007/s00253-008-1618-8

84. Schreuder, M.P, C. Deen, W.J.A Boersma, P.H Pouwels, and F.M Klis (1996). "Yeast Expressing Hepatitis B Virus Surface Antigen Determinants on Its Surface: Implications for a Possible Oral Vaccine." *Vaccine* 14.5: 383-88.

85. Luo, S., Yan, L., Zhang, X., Yuan, L., Fang, Q., Zhang, Y. A., & Dai, H. (2015). Yeast surface display of capsid protein VP7 of grass carp reovirus: Fundamental investigation for the development of vaccine against hemorrhagic disease. *Journal of Microbiology and Biotechnology*, *25*(12), 2135–2145. https://doi.org/10.4014/jmb.1505.05041

86. Shibasaki, S., Aoki, W., Nomura, T., Miyoshi, A., Tafuku, S., Sewaki, T., & Ueda, M. (2013). An oral vaccine against candidiasis generated by a yeast molecular display system. *Pathogens and Disease*, *69*(3), 262–268. https://doi.org/10.1111/2049-632X.12068

87. Ryckaert S, Pardon E, Steyaert J, Callewaert N (2010) Isolation of antigen-binding camelid heavy chain antibody fragments (nanobodies) from an immune library displayed on the surface of Pichia pastoris. *J Biotechnol*. 145(2):93-98. doi:10.1016/j.jbiotec.2009.10.010

88. Lin, S., Houston-cummings, N. R., Prinz, B., Moore, R., Bobrowicz, B., Davidson, R. C., Wildt, S., Stadheim, T. A., & Zha, D. (2012). A novel fragment of antigen binding (Fab) surface display platform using glycoengineered Pichia pastoris. *Journal of Immunological Methods*, *375*(1–2), 159–165. https://doi.org/10.1016/j.jim.2011.10.003

89. Shaheen HH, Prinz B, Chen MT, Pavoor T, Lin S, et al. (2013) A Dual-Mode Surface Display System for the Maturation and Production of Monoclonal Antibodies in Glyco-Engineered *Pichia pastoris*. PLOS ONE 8(7): e70190. https://doi.org/10.1371/journal.pone.0070190

90. Li S, Qiao J, Lin S, Liu Y, Ma L. (2019) A Highly Efficient Indirect *P. pastoris* Surface Display Method Based on the CL7/Im7 Ultra-High-Affinity System. *Molecules*, 24(8):1483. Published 2019 Apr 15. doi:10.3390/molecules24081483

91. Surface Display—An Alternative to Classic Enzyme Immobilization Mateja Lozanˇci´c, Amir Sk. Hossain, Vladimir Mrša and Renata Tepari´c , Catalysts 2019, 9, 728; doi:10.3390/catal9090728.

92. Jiang, Z., Gao, B., Ren, R., Tao, X., Ma, Y., & Wei, D. (2008). Efficient display of active lipase LipB52 with a Pichia pastoris cell surface display system and comparison with the LipB52 displayed on Saccharomyces cerevisiae cell surface. *BMC Biotechnology*, *8*, 1–7. https://doi.org/10.1186/1472-6750-8-4

93. Harnpicharnchai P, Sornlake W, Tang K, Eurwilaichitr L, Tanapongpipat S. Cell-surface phytase on Pichia pastoris cell wall offers great potential as a feed supplement. *FEMS Microbiol Lett*. 2010;302(1):8-14. doi:10.1111/j.1574-6968.2009.01811.x

94. Yang Shaojie, Lv Xin, Wang Xihui, Wang Junqing, Wang Ruiming, Wang Tengfei (2017) Cell-Surface Displayed Expression of Trehalose Synthase from Pseudomonas putida ATCC 47054 in Pichia Pastoris Using Pir1p as an Anchor Protein, Frontiers in Microbiology, 10.3389/fmicb.2017.02583

95. Uchański, T., Zögg, T., Yin, J., Yuan, D., Wohlkönig, A., Fischer, B., Rosenbaum, D. M., Kobilka, B. K., Pardon, E., & Steyaert, J. (2019). An improved yeast surface display platform for the screening of nanobody immune libraries. *Scientific Reports*, *9*(1), 1–12. https://doi.org/10.1038/s41598-018-37212-3

96. Rosowski, S., Becker, S., Toleikis, L., Valldorf, B., Grzeschik, J., Demir, D., Willenbücher, I., Gaa, R., Kolmar, H., Zielonka, S., & Krah, S. (2018). A novel one-step approach for the

construction of yeast surface display Fab antibody libraries. *Microbial Cell Factories*, *17*(1), 1–11. https://doi.org/10.1186/s12934-017-0853-z

97. Mei, M., Li, J., Wang, S., Lee, K. B., Iverson, B. L., Zhang, G., Ge, X., & Yi, L. (2019). Prompting Fab Yeast Surface Display Efficiency by ER Retention and Molecular Chaperon Co-expression. *Frontiers in Bioengineering and Biotechnology*, *7*(November), 1–11. https://doi.org/10.3389/fbioe.2019.00362

CHAPTER 3[2]

EXPRESSION AND DISPLAY OF GLYCOENGINEERED ANTIBODIES AND ANTIBODY FRAGMENTS WITH AN ENGINEERED YEAST STRAIN

[2] Anjali Shenoy, Srisaimaneesh Yalamanchili, Alexander R. Davis and Adam W. Barb, Accepted by Antibodies 2021, 10(4), 38, Reprinted here with the permission of MDPI Nov 2022

Abstract:

Interactions with cell surface receptors enhance the therapeutic properties of many important antibodies, including the low-affinity Fc γ Receptors (FcγRs). These interactions require proper processing of the immunoglobulin G Fc N-glycan, and eliminating the N-glycan abolishes binding, restricting antibody production to mammalian expression platforms. Yeasts, for example, generate extensively mannosylated N-glycans that are unsuitable for therapeutics. However, Fc with a specifically truncated N-glycan still engages receptors with considerable affinity. Here we describe the creation and application of a novel *Saccharomyces cerevisiae* strain that specifically modifies the IgG1 Fc domain with an N-glycan consisting of a single N-acetylglucosamine residue. This strain displayed glycoengineered Fc on its surface for screening yeast surface display libraries and also served as an alternative platform to produce glycoengineered Rituximab. An IgG-specific endoglycosidase (EndoS2) truncates the IgG1 Fc N-glycan. EndoS2 was targeted to the yeast ER using the signal peptide from the yeast protein disulfide isomerase (PDI) and a yeast ER retention signal (HDEL). Furthermore, >99% of the yeast expressed Rituximab displayed the truncated glycoform as determined by SDS-PAGE and ESI-MS analyses. Lastly, the yeast expressed Rituximab engaged the FcγRIIIa with the expected affinity (K_D = 2.0 ± 0.5 μM) and bound CD20 on Raji B cells.

3.1 Introduction

Monoclonal antibodies have revolutionized the treatment of many cancers, autoimmune diseases and infectious diseases with notable high-profile examples targeting Ebola virus and COVID-19 [1–4]. Due to the strict requirement of proper post-translational modification, commercial antibodies are predominantly expressed using mammalian cells including Chinese Hamster Ovary (CHO) cells. Mammalian expression platforms produce glycoproteins with appropriate processing including asparagine(N)-linked carbohydrates (glycans) at Asn 297 of the IgG1 heavy chain that is required for interactions with the Fc γ receptors (FcγRs). These interactions trigger antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) that participate in clearing diseased tissue and pathogens [5,6]. N-glycosylation is a co-translational modification that also affects complement activation by antibodies and half-life in the serum [7–10]. Despite superior processing capabilities, mammalian expression systems require more specialized equipment and more complex growth media.

Alternative microbial expression platforms have the potential to surmount limitations associated with mammalian systems. Among these, yeasts (*P. pastoris* and *S. cerevisiae*) provide a distinct advantage as eukaryotes capable of N-glycosylation as well as other eukaryotic post-translational modifications (Figure 3.1) [11]. Unfortunately, antibodies produced in yeast contain oligomannose N-glycans which are rapidly cleared in the peripheral compartment [12]. Ideal expression platforms generate antibodies with N-glycan compositions similar to serum IgG, which include highly processed forms with extensive modification of the non-reducing termini [13]. It is notable that commercial mammalian anti-body expression systems are imperfect and can produce

undesirable amounts of oligo-mannose glycoforms (5–15%) that cannot be completely prevented by adjusting growth conditions [14].

It is possible to produce highly homogeneous protein N-glycans with alternative expression systems by enzymatic remodeling. As an alternative, several groups have attempted to introduce the glycan processing machinery into engineered yeast strains, with varying levels of success [15–20] though more successful attempts are reported in *Pichia pastoris* as compared to *Saccharomyces cerevisiae* [15,20,21]. However, competing mannosyltransferases in the Golgi lead to undesirable glycoforms in glycoengineered yeast strains and lower amounts of the glycoform of interest. Alternatively, Endoglycosidase F (Endo F) and EndoH remove the majority of the yeast N-glycan, which can extend to hundreds of residues in contrast to the ~8–11 IgG N-glycan residues in human serum (Figure 3.1) [22,23]. These enzymes hydrolyze N-glycans leaving

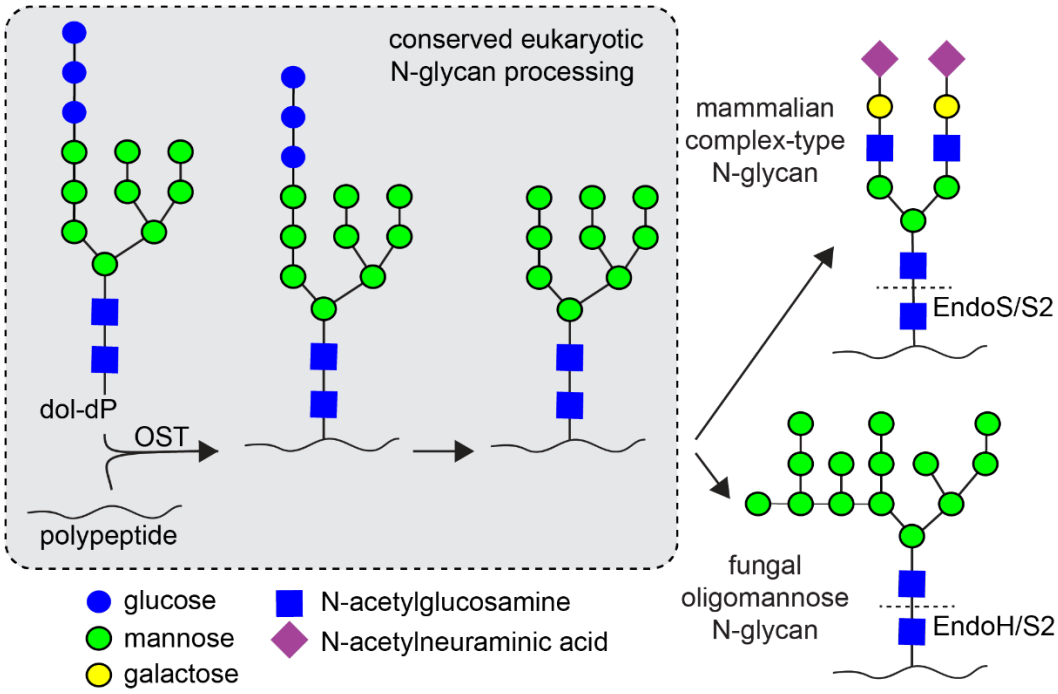

Figure 3.1. **N-glycan processing in yeast and mammals** N-glycan processing in yeasts and mammals shares a common starting point. However, the end products are substantially different with yeasts capable of extensive mannosylation. The dashed line indicates the site of endoglycosidase (endo) cleavage; EndoS and EndoS2 cleave IgG1 N-glycans. OST—oligosaccharyltransferase; dol-dP—dolichol diphosphate.

a single N-acetylglucosamine residue that is identical in N-glycans produced by both yeast and mammals. A comparable effort targeted EndoH to the ER in a plant, *Nicotiana benthamiana*, resulting in N-glycan cleavage with no notable growth defects, though plants appear less dependent on N-glycosylation than mammalian cells [24]. This aspect allowed for an approach entailing in-vivo deglycosylation of Rituximab with EndoH in *Nicotiana benthamiana* followed by the subsequent addition of a complex-type glycan using an EndoS2 transglycosylase variant [25].

Surprisingly, antibodies and antibody fragments displaying this single-residue N-acetylglucosamine N-glycan bind FcγRIIIa with only a 10-fold reduction in affinity and elicit FcγR-mediated cellular immune responses [26,27]. The immunoglobulin-specific endoglycosidases Endo S and EndoS2 provide an alternative approach to generate antibodies with homogeneous mammalian N-glycans [28,29]. EndoS2 cleaves N-glycans from IgG1 with a broader range of N-glycan substrates including complex, oligomannose and hybrid type N-glycans [30].

Here, we report the development of a yeast expression system for antibody expression. Combining the protein-expressing capabilities of *S. cerevisiae* with glycan remodeling into a single cell is expected to provide a cost benefit over post-purification protein remodeling and also provides antibody expression with inexpensive medium conditions using a host organism widely used for commercial production [31]. Another advantage of utilizing *Saccharomyces cerevisiae* over *Pichia pastoris* to create this dual application strain is the library size that can be achieved is far larger and less cumbersome to create [32]. The *Saccharomyces cerevisiae* strain described here

is additionally capable of displaying the IgG1 Fc fragment on the surface, a required component of protein engineering using a yeast surface display [33].

3.2. Material and Methods

3.2.1. Material

All materials were purchased from Millipore Sigma unless otherwise noted. GFP-FcγRIIIa was prepared as previously described [34].

3.2.2. Strain and Media

The *S. cerevisiae* strain EBY100 (GAL1-AGA1:URA3 ura3-52 trp1 leu2Δ1 his3 Δ200 pep4: HIS2 prb1 Δ1.6R can1 GAL) was purchased from the American Type Culture Collection (ATCC-MYA4941). EBY100 cells were grown in YPD rich medium. EBY100-EndoS2 yeasts were grown in minimal medium supplemented with tryptophan (Trp; YNB-Dropout + Trp = 6.7 g/L Yeast Nitrogen Base Without Amino Acids and 1.5 g/L Yeast Synthetic Drop Out Medium Supplements without Uracil, Tryptophan and Leucine along with 2 mg/mL L-Trp). EBY100-EndoS2 + pYD1-Fc yeast cells were grown in a minimal medium lacking leucine and tryptophan (YNB-Dropout = 0.67 g/L Yeast Nitrogen Base Without Amino Acids and 0.15 g/L Yeast Synthetic Drop Out Medium Supplements without Uracil, Tryptophan and Leucine). Protein expression was induced in YPGal = 10 g/L Yeast Extract + 20 g/L Peptone + 2% Galactose (w/v).

3.2.3. Cloning of Fc into the Yeast Surface Display Plasmid pYD1

pYD1 was purchased from Addgene. A codon optimized Fc insert for expression in *S. cerevisiae* was ordered from IDT. This insert was cloned into the pYD1 plasmid using restriction sites Nhe1 and EcoR1. The final plasmid pYD1-Fc was validated through DNA sequencing (Eurofins).

3.2.4. Creation and Validation of the EBY100-EndoS2 Strains

The construct encoding the app8 signal peptide with EndoS2, a Flag tag followed by the HDEL sequence was synthesized by IDT, then cloned into the Yeast Integrating Plasmid (YIP) pAG305 (Addgene) to integrate EndoS2 into the LEU2 site in the yeast genome. Once the sequence was validated through DNA Sequencing, the plasmid was linearized and used to transform EBY100. The integration was validated through yeast colony PCR. The positive cells were then transformed with the pYD1-IgG1 Fc yeast surface display plasmid. This transformation was again validated through a PCR-based colony screen using primers listed in Table S1. To determine whether the endogenous EndoS2 can cleave the N-glycan off Fc, the cells were first grown in YNB-Dropout medium [6.7 g/L Yeast Nitrogen Base without amino acids and ammonium sulfate (Sigma Y1251) and 1.5 g/L Yeast Synthetic Dropout Medium Supplement (Sigma Y1771)] supplemented with 2% glucose and then induced in YNB-Dropout supplemented with 2% galactose for 24 h. The OD_{600} of the culture was measured and a volume of culture corresponding to ~2×10^8 colony-forming units was taken to prepare protein samples for a Western blot (0.1 OD_{600}~10^6 cells).

3.2.5. Protein Sample Preparation

Protein samples for Western blots were prepared by concentrating cells with centrifugation for 2 min at 1500 g. The pellet was then washed three times with 100 µL of 1 × PBS, followed by an incubation in 100 µL of 0.1 M NaOH and incubation at RT for 5 min. The cells were then spun down at 1500 g for 2 min, then resuspended in 50 µL of 2 × SDS PAGE sample buffer. The cells were incubated at 95 °C for 5 min. The sample was then centrifuged for 10 min at 1500 g. Supernatant (10 µL) was diluted 1:1 using 2 × SDS PAGE sample buffer and was loaded onto a 12% SDS polyacrylamide gel. The protein was transferred to a polyvinylidene difluoride (PVDF) membrane using the Invitrogen Power Blotter (PB0010). The membrane was blocked in TBS +

Tween20 (TBST) with 5% dry milk for one hour at room temperature on an orbital shaker. This was followed by incubation with rabbit anti-hIgG primary antibody (Target: IgG; Rabbit IgG; AB_228410 ThermoFisher) in 5% dry milk in 1 × TBST overnight at 4 °C on an orbital shaker. The membrane was washed three times for 5 min with TBST buffer, followed by incubation with secondary HRP conjugated antirabbit antibody (Target: Rabbit IgG Clone: Polyclonal Goat IgG; Research and Diagnostic Systems, dilution 1:2000) in 5% milk in 1 × TBST buffer for 2 h at room temperature on an orbital shaker. The membrane was washed three times with 1 × TBST buffer for 5 min and then imaged using ChemiDoc XRS + Image System (Bio-Rad, Hercules, CA, USA) using the ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

3.2.6. Flow Cytometry to Determine Fc Surface Expression

EBY100-EndoS2 + pYD1-Fc cells were first grown in YNB-Dropout + 2% glucose overnight, pelleted by centrifugation and then induced in YNB-Dropout + 2% galactose for 24 h at 30 °C with shaking. Cells (~10^6) from this culture were used to prepare samples for flow cytometry. The pellet was washed with 100 µL of 1 × PBS and then washed with 100 µL of 1 × PBSA (1 × PBS + 1% Bovine Serum Albumin). The cells were incubated with rabbit anti-hIgG on ice for 45–60 min in the dark. The cells were then washed with 100 µL of 1 × PBSA. The cell pellet was then incubated with phycoerythrin-conjugated antirabbit IgG on ice for 45–60 min in the dark. The cell pellet was then washed twice with 100 µL 1 × PBS. Similarly, for GFP-FcγRIIIa staining, a cell pellet ~10^6 cells were incubated with 50 µL of ~2 mM GFP-FcγRIIIa for 1 h at 4 °C in the dark. The samples were analyzed with a FACSCanto to determine Fc surface expression and receptor FcγRIIIa binding.

3.2.7. Expression and Purification of IgG1 Fc and Rituximab

A single yeast clone verified through the colony screen was inoculated into a 5 mL YNB-Dropout medium supplemented with 2% glucose and incubated with shaking at 30 °C for 16 h. This culture was then added to 100 mL YNB-Dropout supplemented with 2% galactose, then incubated with shaking for 24 h at 30 °C. The culture was then centrifuged for 10 min at 3000 g. The supernatant was discarded. The cell pellet was then resuspended with 100 or 500 mL YPGal and was incubated with shaking for 72 h at 20 °C. This culture was then centrifuged twice at 3000 g for 10 min to remove cell debris. The supernatant was diluted 2-fold with 25 mM MOPS 100 mM NaCl, pH 7.4 (Buffer A) and then loaded onto a Protein A column (2 mL). The column was then washed with 12 mL of Buffer A. Protein was eluted in 1 mL fractions of 100 mM glycine, pH 3.0 and was collected in tubes containing 500 µL of 1 M Tris pH 8 to immediately neutralize it. The protein samples were then loaded onto a 12% SDS-PAGE gel to be analyzed. For samples analyzed with size exclusion chromatography: 8–10 mL of concentrated sample was applied to a Superdex 200 column using an ÄKTA Go FPLC (Cytiva) prewashed with Buffer A and eluted with Buffer A.

3.2.8. Purification Using Concanavalin A Column

Concanavalin A Sepharose resin (1 mL) was washed with 5 column volumes of Equilibration Buffer. Purified IgG1 Fc was diluted 1:1 with Equilibration Buffer (1× PBS pH 7.4, 0.1 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$), then loaded onto the column by gravity. The column was then washed twice using 5 mL of Equilibration Buffer. The protein was then eluted from the column as 1 mL fractions using Elution Buffer (1× PBS pH 7.4, 0.2 M d-mannose). Each fraction (10 µL) was analyzed using a 12% SDS-polyacrylamide gel that was stained with Coomassie brilliant blue.

2.2.9. Cloning the Heavy Chain and Light Chain of Rituximab into pESC-TRP1

The constructs for the app8-heavy chain and app8-light chain were synthesized by IDT. These constructs were then inserted into pESC-TRP1 (Agilent) through restriction endonuclease cloning using Sal1 and Nhe1 (heavy chain) and EcoRI and Nhe1 (light chain) and ligated with T4 DNA Ligase according to the manufacturer's directions. The completed sequence was validated through DNA sequencing (Eurofins).

3.2.10. Mass Spectrometry

Purified Rituximab (5 μg) from the EBY100-(GPD) EndoS2 strain was resuspended in 50 mM ammonium carbonate pH 8.0, 10% methanol, boiled for 5 min at 95 °C, then cooled on ice for 5 min. The sample was then treated with 5 mM dithiothreitol and incubated at 37 °C for 1 h, then 14 mM iodoacetamide was added and the sample incubated in the dark for 30 min at room temperature before adding trypsin (~1.8 μg) and incubating at 37 °C overnight. The samples were then lyophilized. The samples were then resuspended in binding buffer (15 mM ammonium acetate, 85% acetonitrile, pH 3.5). This solution was loaded onto a Cellulose/HILIC column, which was then washed three times with 10 μL of binding solution. Glycopeptides were eluted with 15 mM ammonium acetate, 10% acetonitrile, pH 3.5 to collect three elution fractions (10 μL each).

3.2.11. Surface Plasmon Resonance

Rituximab was immobilized on a CM5 chip (Cytiva) as previously described in [35] using a BiaCore T200 instrument (Cytiva). Increasing concentrations of GFP-FcγRIIIa were applied to the chip surface and each concentration was continuously applied until equilibrium was reached. Affinity constants were calculated using the T200 system software after double normalization (first to the surface-deactivated reference channel and then to a sample with zero concentration collected using the lane of interest) and a 1:1 binding model with time zero = zero response units. Data were

averaged from a 5 s window after equilibrium was reached. Reported errors indicate errors of the curve fitting procedure.

3.2.12. Flow Cytometry for Fab Functionality with Raji B-Cells

Raji B-cells (5×10^5) were washed with 1× PBS. Antibody dilutions were prepared in 1× PBS + 1% FBS. The cells were spun down at 400 g for 5 min. The cells were blocked with 90 µL 1× PBS + 1% FBS + 10 µL human serum and incubated for 5 min on ice. Antibody dilutions of the yeast-derived Rituximab and commercial Rituximab were added to the cells as separate samples. The cells were incubated on ice for 1 h. The cells were centrifuged at 400 g for 5 min and resuspended in 100 µL of 1× PBS twice. This was followed by another blocking step with human serum described above and the addition of 1 µg per sample of rabbit anti-hIgG1 antibody (Target: Human IgG; Clone: Rabbit; RRID: AB_228410). This incubation was carried out on ice for 30 min. The cells were centrifuged at 400 g for 5 min twice and resuspended in 100 µL of 1× PBS twice. This was followed by another blocking step with human serum described above followed by the addition of a secondary PE-conjugated donkey antirabbit antibody (Target: Human IgG; Clone: Donkey: RRID: AB_2563484). This incubation was carried out on ice for 30 min. The cells were centrifuged at 400 g for 5 min and resuspended in 100 µL of 1× PBS twice. The samples were then blocked with 1× PBS + 1% paraformaldehyde for 20 min at RT. The samples were then run on the CytoFLEX and analyzed.

3.3. Results

3.3.1. IgG1 Fc with a Hydrolyzed N-Glycan Expressed on Yeast Binds GFP-FcγRIIIa.

We integrated *endoH* into *S. cerevisiae* (EBY100) and fused DNA encoding a signal peptide from the yeast protein disulfide isomerase (PDI) gene and a yeast ER retention signal (HDEL) [36]. We selected the HO locus because integration at this site occurs with high efficiency and negligible

impact on cell growth [37]. EndoH expression in this strain is controlled by the GAL1 promoter to minimize deleterious effects on cell growth. Unfortunately, this strain exhibited growth defects and inefficient N-glycan cleavage. It is possible that because EndoH cleaves all N-glycosylated yeast proteins, growth was inhibited by off-target cleavage of glycans on proteins that are important for proper cell function therefore, we pursued incorporating an IgG-specific N-glycan hydrolase.

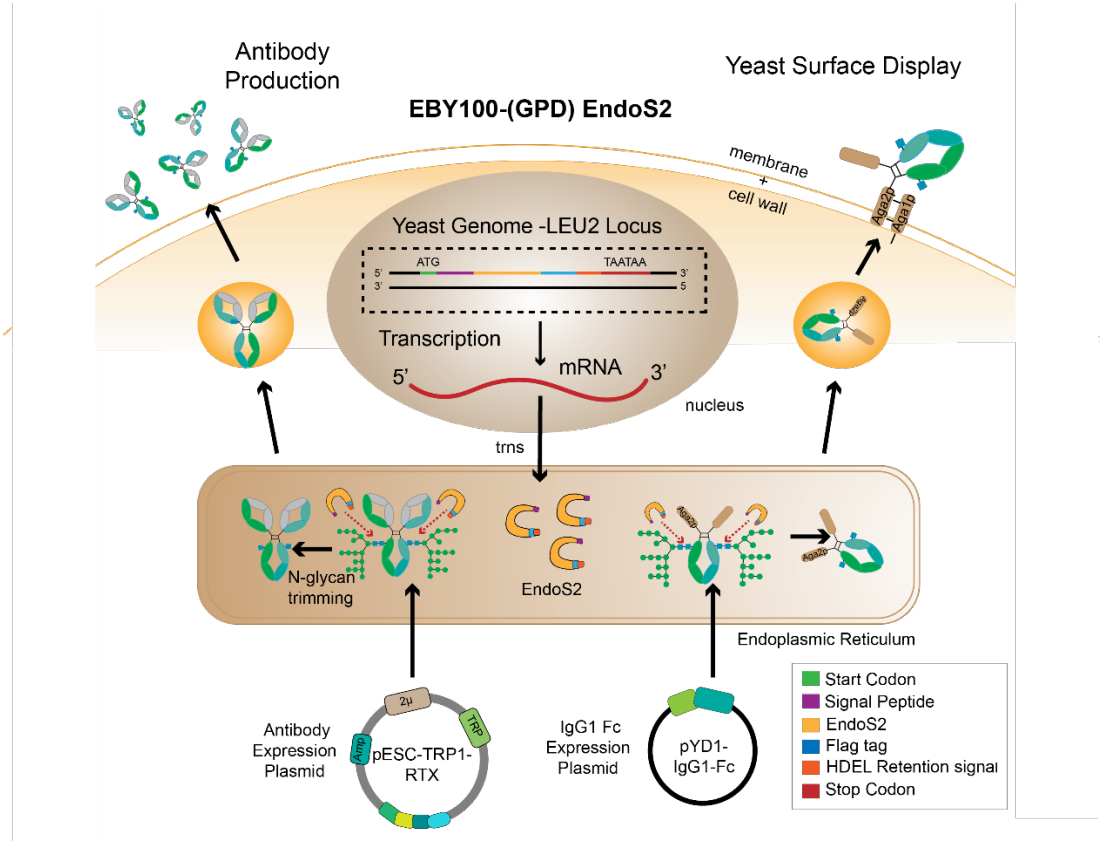

Figure 3.2. Schematic of EBY100-(GPD)-EndoS2 strain EBY100-(GPD) EndoS2 is a dual-function yeast strain that can display glycoengineered Fc on the yeast surface for protein engineering or produce glycoengineered antibodies.

We integrated *endoS2* into the yeast LEU2 locus [38]. This inserted construct likewise targeted EndoS2 to the ER and included an ER retention tag, as described for EndoH, above (Figure 3.2). We observed a shift in mobility of yeast surface displayed IgG1 Fc expressed in the EBY100-

(GAL1) EndoS2 comparable to the aglycosylated version of the same protein (Figure 3.3E). This result suggests that endogenously produced EndoS2 efficiently cleaved the Fc N-glycan. However, this strain showed low surface expression of IgG1 Fc and low GFP-FcγRIIIa binding (Figure 3.3C, H). It is possible that utilizing the GAL1 promoter for expressing both the EndoS2 and IgG1 Fc resulted in lowered expression of both proteins.

To overcome reduced expression, we next incorporated *endoS2* with a constitutive GPD promoter into the EBY100 strain and continued to express IgG1 Fc from pYD1 with the GAL1 promoter. These promoters reportedly permitted high levels of simultaneous expression [29]. The EBY100-(GPD) EndoS2 strain resulted in superior Fc surface expression and GFP-FcγRIIIa surface staining (Figure 3.3D, I). Furthermore, we observed efficient N-glycan cleavage (Figure 3.3J).

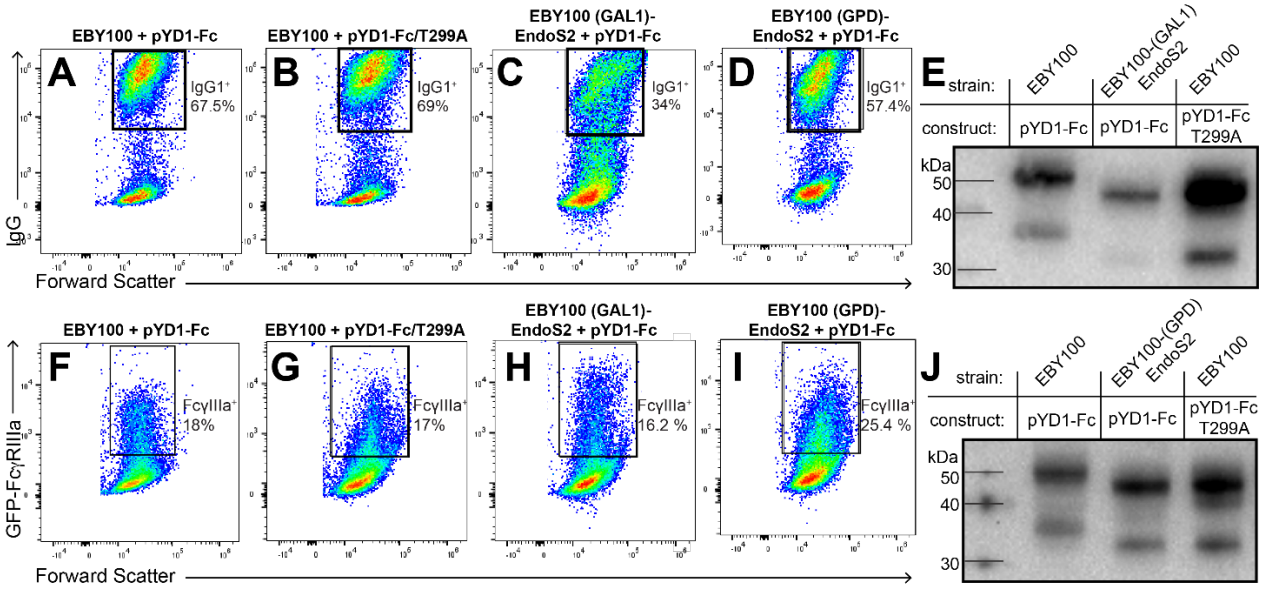

Figure 3.3. IgG1 Fc display and processing by the EndoS2 yeast strains and the respective controls (pYD1-Fc and pYD1-Fc/T299A). Flow cytometry for IgG1 Fc using an anti-IgG1 antibody (**A**–**D**) and GFP-FcγRIIIa binding (**F**–**I**). Boundaries for the highlighted boxes were drawn based on negative controls (not shown). (**E**,**J**) Western blots showing mobility of the Aga2p-IgG1 Fc fusions. T299A is an aglycosylated IgG1 Fc variant which shows reduced binding to GFP-FcγRIIIa.

3.3.2. IgG1 Fc with a Truncated N-glycan is Secreted by EBY100-(GPD) EndoS2

We attempted to extract IgG1 Fc to further validate the glycoform produced by the EBY100-(GPD) EndoS2 strain. The Aga2p-Fc expressed from the pYD1-Fc plasmid is anchored onto the yeast surface through disulfide bonds with the yeast cell wall protein Aga1p [33]. We failed to break these bonds using dithiothreitol and recover a sufficient amount of sample for analysis [39,40]. Furthermore, the Aga2p-fused IgG1 Fc appears as two bands in SDS-PAGE with the upper band likely resulting from Aga2p O-glycosylation (Figure 3.3C, F). As an alternative, we introduced a plasmid to secrete IgG1 Fc into the medium by appending the engineered N-terminal app8 secretion factor in place of the Aga2p fusion [41].

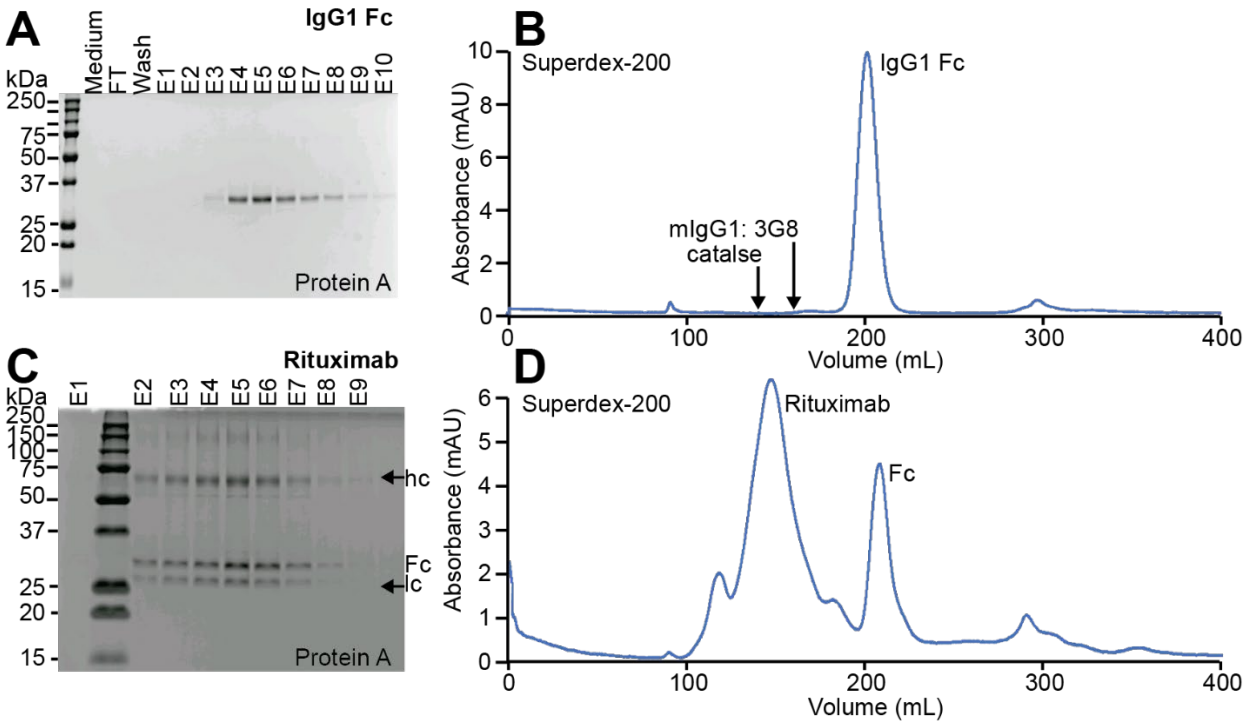

Figure 3.4. Purification of IgG1 Fc and Rituximab expressed from EBY100-(GPD) EndoS2. IgG1 Fc purified using Protein A chromatography (**A**) and size-exclusion chromatography (**B**). Rituximab purified using Protein A chromatography (**C**) and size-exclusion chromatography (**D**). The largest peak in D at 140 mL is Rituximab. Arrows indicate the Rituximab heavy and light chain. (**A**, **C**) are 12% reducing SDS-PAGE gels.

We purified IgG1 Fc secreted by both the EBY100 and EBY100-(GPD) EndoS2 strain from the spent culture medium using Protein A chromatography [42]. Protein A, a cell wall component of *Streptococcus aureus*, binds the IgG1 Fc domain [43]. The IgG1 Fc proteins isolated from both yeast strains bound Protein A and were eluted using 100 mM glycine, pH 3.0 (the single N-acetylglucosamine form is shown in Figures 2.4A)

We next probed the secreted IgG1 Fc for the presence of oligomannose-type N-glycans using

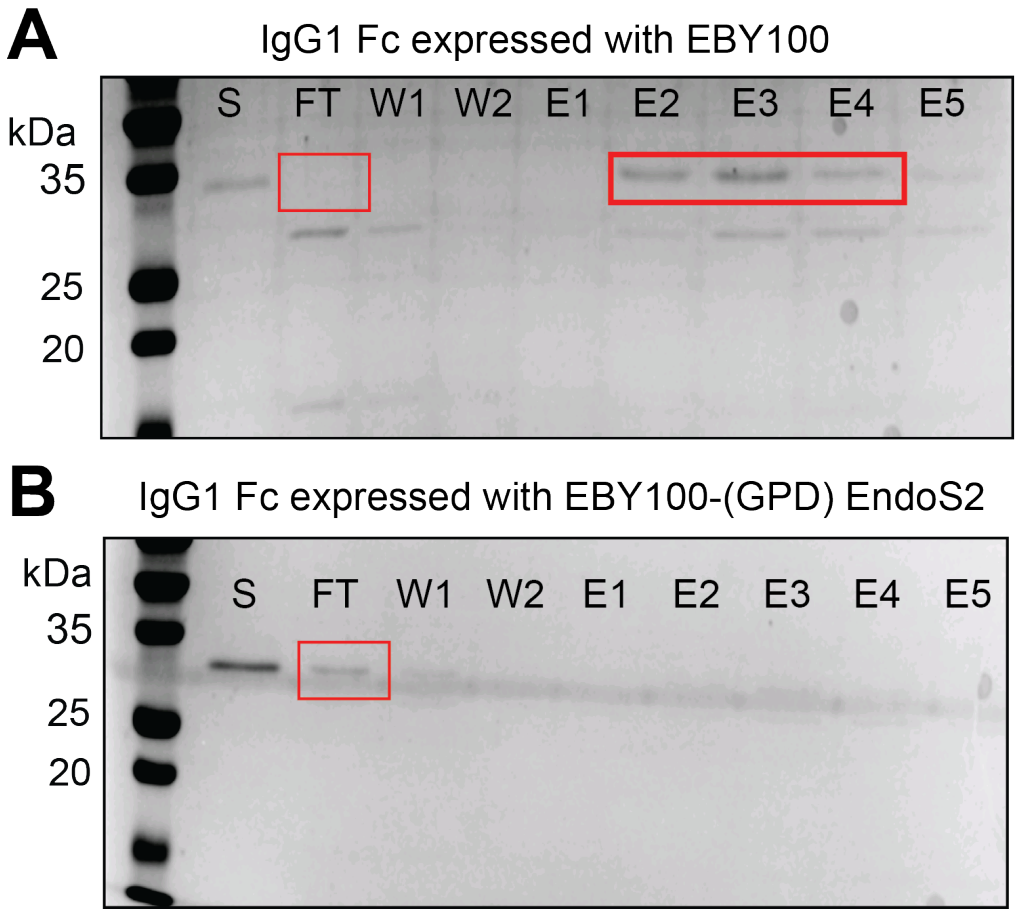

Figure 3.5. Identification of oligomannose N-glycans on secreted IgG Fc. Concanavalin A purification of Fc derived from the (**A**) EBY100 and the (**B**) EBY100-(GPD) EndoS2 strains. "S" is the starting material before loading on the column, "FT" is the flow-through unbound fraction, "W" wash and "E" elutions.

a concanavalin A-sepharose resin. IgG1 Fc isolated from wildtype EBY100 cells is expected to possess an oligomannose glycoform, and we observed binding of the majority of this protein to the concanavalin A column (Figure 3.5A). A small amount of Coomassie stained material is observed at a substantially lower molecular weight in the flow-through fraction. This may represent a contaminating protein in the preparation or aglycosylated Fc. Furthermore, we observed that the majority of the IgG1 Fc isolated from our glycoengineered EBY100-(GPD) EndoS2 strain did not bind the concanavalin A column, which is consistent with the EndoS2-catalyzed removal of the N-glycan leaving a single N-acetylglucosamine residue (Figure 3.5B).

3.3.3. Glycoengineered Rituximab Expressed with EBY100-(GPD) EndoS2

We next determined whether the EBY100-(GPD) EndoS2 strain would produce a full-length glycoengineered antibody and serve as an alternative antibody expression platform. Wong and coworkers created glycoengineered antibodies with glycoengineered *Pichia pastoris* and utilized a bidirectional promoter to produce both heavy chain and light chain from a single construct [44]. We adopted a similar approach using the pESC-TRP1 plasmid with a bidirectional GAL1-GAL10 promoter to express Rituximab (Figure 3.2). We chose to use the GAL1 promoter for expressing the heavy chain based on the reported stronger expression compared to GAL10 [45]. Similar to the IgG1 Fc expression construct, we appended the app8 secretion factor at the N-termini of both the heavy chain and light chain. The pESC-TRP1-app8RTXhc-app8RTXlc was transformed into the EBY100-(GPD) EndoS2 strain. A positive colony was then grown and induced to produce full-length Rituximab.

3.3.4. Purification of Full-Length Rituximab from the EBY100-(GPD) EndoS2 Strain

We purified full-length Rituximab from the spent medium of an induced yeast culture using a Protein A column. Rituximab isolated from the EBY100-(GPD) EndoS2 strain bound protein A, signifying that the antibody was properly folded, and eluted with 100 mM glycine, pH 3.0, as is expected for IgG1 (Figure 3.4C).

Yeast-expressed Rituximab eluted from a Superdex 200 column at 140 mL as shown in Figure 3.4D. This retention time is between the peaks for catalase (206 kDa) and 3G8 (a mouse IgG1; 145 kDa, Figure 3.4B, D) and is consistent with a heterotetrameric assembly containing two heavy chains and two light chains with an expected molecular mass of 145 kDa. Additionally, we noted the presence of IgG1 Fc in the purified sample that is likely the result of proteolysis during Rituximab expression and retained during the protein A separation step (Figure 3.4D). We also observed some minor peaks in Rituximab likely resulting from aggregation and proteolysis (Figure .4D). We recovered 0.24 mg of purified Rituximab from a 500 mL culture at a yield of 0.48 mg/L, which was more than twice the amount of Rituximab we were able to recover from our unmodified yeast strain (0.2 mg/L).

We characterized the purified yeast-expressed Rituximab by electrospray ionization tandem mass spectrometry (Figure 3.6). The most predominant glycopeptide peak belonged to the N297-containing tryptic peptide modified with a single N-acetylhexosamine residue. An MS2 spectrum of this species identified individual peaks following collision-induced degradation, including the loss of a single N-acetylhexosamine residue coincident with the loss of N297, indicating N297 as the likely site of the carbohydrate addition (Figure 3.6B). Based on comparing the relative intensity of MS1 peaks for the N297-containing species, glycopeptides with a single N-acetylglucosamine (99.36%) appeared as the most abundant glycoform with lesser amounts of Man_9 (0.04%), Man_{10}

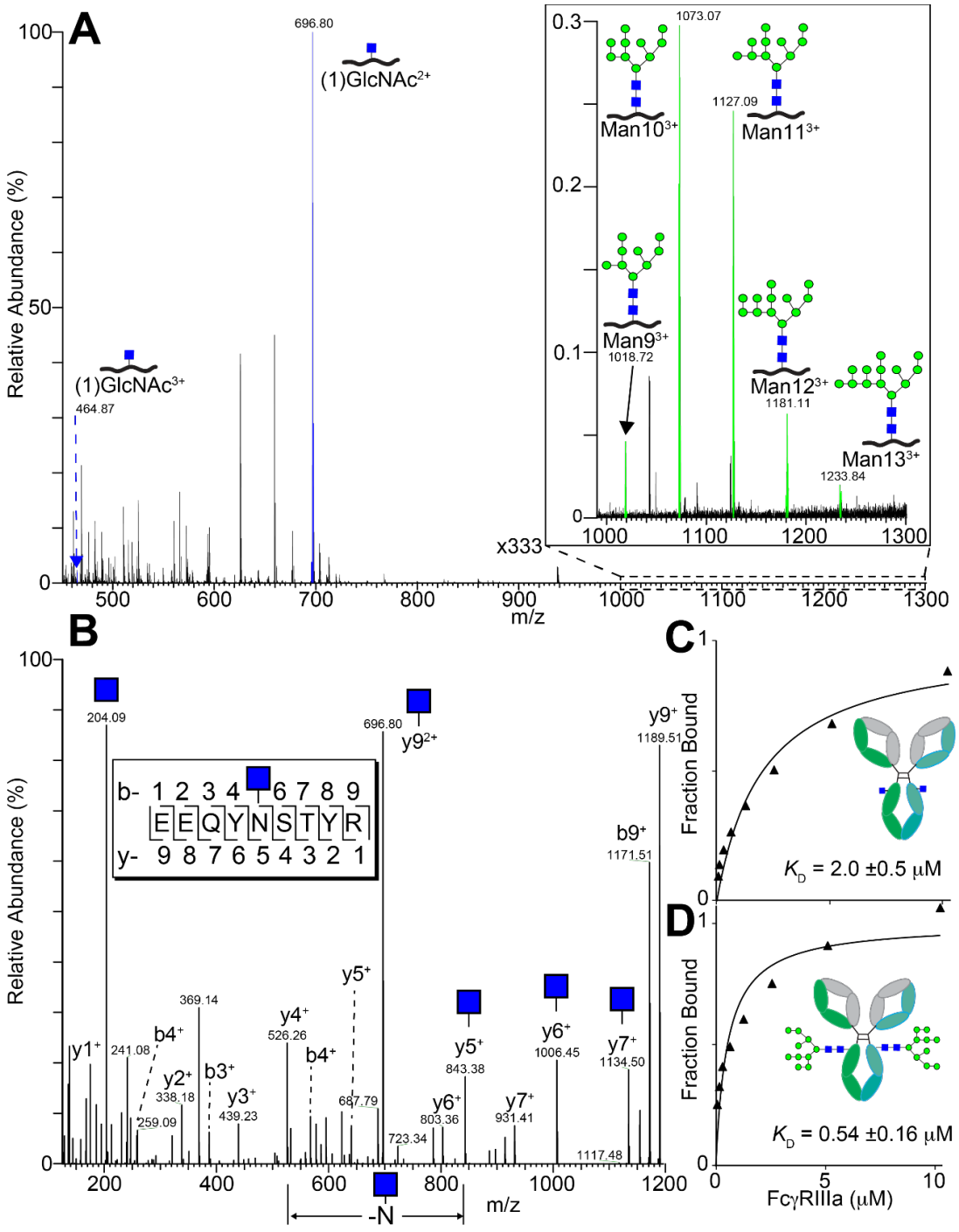

Figure 3.6. Yeast-expressed Rituximab composition and receptor binding. (**A**) Total ion current from ESI-MS spectra of trypsinized Rituximab expressed from the EBY100-(GPD) EndoS2 strain summed from 11–15 min; doubly charged ions corresponding to oligomannose N-glycoforms were not observed. Isobaric ions were not distinguished; thus, each cartoon represents multiple possible configurations. (**B**) ESI-MS/MS of the (1) GlcNAc glycopeptide from panel A showing a single GlcNAc residue at N297. Rituximab displaying either truncated (**C**) or yeast N-glycans (**D**) binding to high affinity FcγRIIIa (V158) variant as determined by surface plasmon resonance.

(0.29%), Man_{11} (0.24%), Man_{12} (0.06%) and Man_{13} (0.01%; Figure 3.6A). Furthermore, we identified peptides covering 93.9 and 100% of the heavy and light chain sequences, respectively, and found no spectra corresponding to the app8 secretion tag on the light chain and 24 app8 peptides out of 1845 total for the heavy chain, indicating a high level of app8 cleavage during antibody secretion. The high rate of EndoS2-catalyzed N-glycan cleavage determined by MS is consistent with the high level of IgG1 Fc processing observed in SDS-PAGE and concanavalin A binding (Figures 2.3 and 2.5). These results demonstrate that the endogenous EndoS2 efficiently cleaved the N-glycan from Rituximab and IgG1 Fc.

3.3.5. Functionality of Rituximab Isolated from EBY100-(GPD) EndoS2.

We next assessed the functionality of the EBY100-(GPD) EndoS2 expressed Rituximab. First, we analyzed binding to GFP-FcγRIIIa using surface plasmon resonance (SPR) by immobilizing Rituximab to a chip and flowing over various amounts of GFP-FcγRIIIa (Figure 3.6C). We determined a K_D of 2.0 ± 0.5 μM which was slightly lower than the 5.8 μM value previously reported for an identical glycoform of IgG1 Fc [27]. Rituximab with the non-truncated yeast N-glycan shows ~4-fold greater affinity (Figure 3.6D), which is identical to previous measurements of IgG1 Fc with an oligomannose N-glycan [27].

We next tested the ability of the EBY100-(GPD) EndoS2 expressed anti-CD20 Rituximab to bind CD20-expressing Raji B-cells by incubating this antibody with Raji B-cells and detecting the amount of Rituximab on the cell surface using flow cytometry. We saw a clear increase in anti-IgG staining following this treatment that was comparable to a commercial Rituximab preparation, indicating both Rituximab antibodies efficiently bound Raji B cells a ta concentration of 40 nM (Figure 3.7). Furthermore, we observed the same amount of staining for both the commercial and

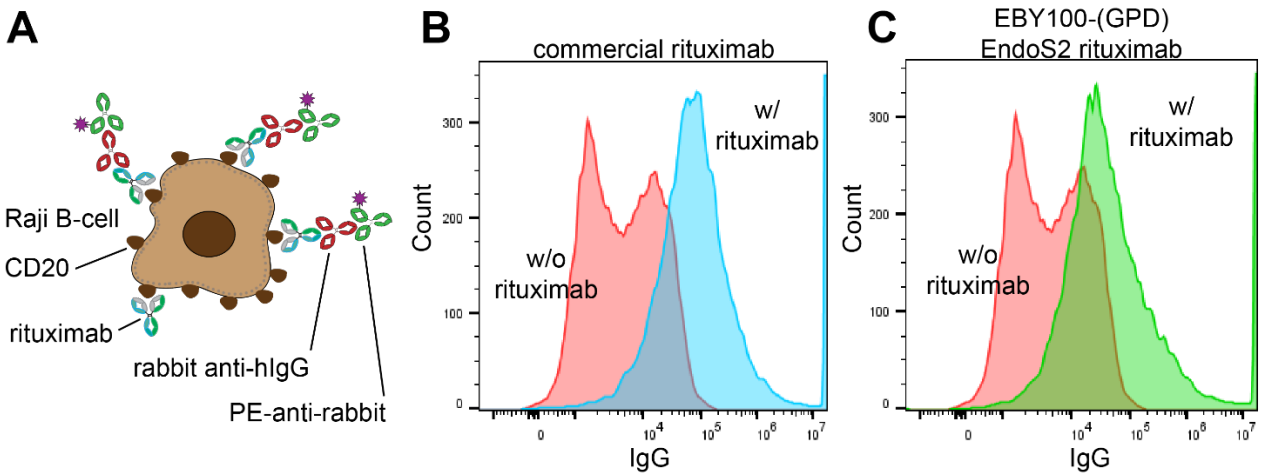

Figure 3.7. Binding of yeast-expressed Rituximab to Raji B cells. (**A**) Schematic diagram of the detection strategy. Flow cytometry analysis of Raji B cells incubated with the two detection antibodies shown in panel A, with or without commercially sourced Rituximab (**B**) or EBY100-(GPD) EndoS2 expressed Rituximab(**C**).

yeast derived Rituximab at 400 nM [46].

3.4. Discussion

The IgG1 Fc N-glycan plays a crucial role in the interaction between IgG1 and FcγRIIIa. The IgG1 Fc N-glycan does not directly engage the receptor; however, it has been shown to stabilize the C'E loop that interacts with the receptor [27]. The IgG1 Fc N-glycan also forms contacts with aromatic amino acids on the Fc domain through CH-π bonds, providing further stabilization [27]. The composition of the N-glycan has also been shown to influence FcγRIIIa binding affinity [48,49]. Removal of this N-glycan abolishes receptor binding; however, our group demonstrated that trimming the Fc N-glycan to a single N-acetylglucosamine residue still allows for receptor engagement (10-fold lower affinity compared to wildtype interaction) [27]. The efficacy of this truncated glycoform has likewise been shown through cell-based assays [50]. Therefore, we sought to create a yeast strain that could produce antibodies with

the single N-acetylglucosamine glycoform to achieve the two goals of (i) preserving FcγRIIIa binding and (ii) removing the oligomannose glycoforms that promote clearance.

One approach to creating this strain would be to knockout ER and Golgi resident mannosyltransferases to trim down the N-glycan. However, such knockout strains have shown defects in morphology and several growth defects that are expected to reduce commercial viability [51]. Therefore, we pursued a targeted approach to trim off the IgG1 Fc N-glycan without causing growth defects in yeast. We achieved this through the creation of the EBY100-(GPD) EndoS2 strain. In this strain, the use of EndoS2 specifically cleaves the N-glycan from Fc and eliminates off-target effects. We observed a high level of N-glycan processing (>90%) in Rituximab produced by this strain through mass spectrometry experiments. This result surmounts previous efforts that report substantial difficulty glycoengineering *S. cerevisiae* [21].

The EBY100-(GPD) EndoS2 strain also proved effective to express full-length functional Rituximab. Previously, full-length antibodies were produced in *S. cerevisiae* at very low levels of 50 μg/L [52]. Work performed by Wittrup, and co-workers lead to the development of the app8 secretion tag that has allowed for enhancement in antibody production [41]. Utilizing this secretion tag, we were able to recover ~500 μg/L of highly processed Rituximab with a high level of processing and complete removal of the app8 secretion tag. Though it is unlikely that this yeast platform will affect commercial therapeutic antibody manufacture that utilizes highly optimized CHO cells and boasts yields >2 g/L, there is substantial potential to improve yield in this strain with further strain engineering and promote laboratory-scale antibody development efforts [11,53].

Antibodies expressed with the EBY100-(GPD) EndoS2 strain are likewise ideal substrates for post-purification glycan remodeling. Novel transglycosylase EndoS and EndoS2 variants are available for modification of antibodies and require antibodies with an N-glycan consisting of only a single N-acetylglucosamine residue and a synthetic sugar donor [55,56]. These reactions boast a high level of efficiency [57]. One antibody N-glycan remodeling approach entailed a combination of glycoengineered yeast strains and chemoenzymatic reactions to engineer Herceptin with a complex-type N-glycoform. This method required producing Herceptin in knock-out *Pichia pastoris* strains, followed by the removal of the N-glycan using an endoglycosidase and then adding the N-glycan back using transglycosylase EndoS2 variants [44]. The EBY100-(GPD) EndoS2 strain can be used to replace separate expression and in vitro truncation steps and reduce cost considerably due to its high efficiency of N-glycan cleavage. The antibody produced by the EBY100-(GDP) EndoS2 strain can then be remodeled following purification to have the desired glycoform using any of these transglycosylase enzyme variants.

In addition to antibody expression, the EBY100-(GPD) EndoS2 strain displays glycoengineered Fc on the yeast surface, can be used to screen yeast surface display libraries, and offers an inexpensive alternative platform to produce glycoengineered antibodies. In summary, we report the development and characterization of a novel *S. cerevisiae* that efficiently expresses antibodies and antibody fragments that contain a truncated N297 glycan.

3.5 References

1.Duehr, J.; Wohlbold, T.J.; Oestereich, L.; Chromikova, V.; Amanat, F.; Rajendran, M.; Gomez-Medina, S.; Mena, I.; Tenoever, B.R.; García-Sastre, A.; et al. Novel Cross-Reactive Monoclonal Antibodies against Ebolavirus Glycoproteins Show Protection in a Murine Challenge Model vaccines and antiviral agents Crossm. *J. Virol.* 2017, *91*, 652–669.

2.Fan, P.; Chi, X.; Liu, G.; Zhang, G.; Chen, Z.; Liu, Y.; Fang, T.; Li, J.; Banadyga, L.; He, S.; et al. Potent neutralizing monoclonal antibodies against Ebola virus isolated from vaccinated donors. *mAbs* 2020, *12*, 1742457, https://doi.org/10.1080/19420862.2020.1742457.

3.Khoury, D.S.; Cromer, D.; Reynaldi, A.; Schlub, T.E.; Wheatley, A.K.; Juno, J.A.; Subbarao, K.; Kent, S.J.; Triccas, J.A.; Davenport, M.P. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nat. Med.* 2021, *27*, 1205–1211, https://doi.org/10.1038/s41591-021-01377-8.

4.Gaebler, C.; Wang, Z.; Lorenzi, J.C.C.; Muecksch, F.; Finkin, S.; Tokuyama, M.; Cho, A.; Jankovic, M.; Schaefer-Babajew, D.; Oliveira, T.Y.; et al. Evolution of antibody immunity to SARS-CoV-2. *Nature* 2021, *591*, 639–644, https://doi.org/10.1038/s41586-021-03207-w.

5.Tay, M.Z.; Wiehe, K.; Pollara, J. Antibody-Dependent Cellular Phagocytosis iAntiviral Immune Responses. *Front. Immunol.* 2019, *10*, 332, https://doi.org/10.3389/fimmu.2019.00332.

6.van Erp, E.A.; Luytjes, W.; Ferwerda, G.; van Kasteren, P.B. Fc-Mediated Antibody Effector Functions during Respiratory Syncytial Virus Infection and Disease. *Front. Immunol.* 2019, *10*, 548.

7.Yu, M.; Brown, D.; Reed, C.; Chung, S.; Lutman, J.; Stefanich, E.; Wong, A.; Stephan, J.-P.; Bayer, R. Production, characterization and pharmacokinetic properties of antibodies with N-linked Mannose-5 glycans. *mAbs* 2012, *4*, 475–487, https://doi.org/10.4161/mabs.20737.

8.Chen, X.; Liu, Y.D.; Flynn, G.C. The effect of Fc glycan forms on human IgG2 antibody clearance in humans. *Glycobiology* 2008, *19*, 240–249, https://doi.org/10.1093/glycob/cwn120.

9.Liu, L.; Stadheim, A.; Hamuro, L.; Pittman, T.; Wang, W.; Zha, D.; Hochman, J.; Prueksaritanont, T. Pharmacokinetics of IgG1 monoclonal antibodies produced in humanized Pichia pastoris with specific glycoforms: A comparative study with CHO produced materials. *Biologicals* 2011, *39*, 205–210, https://doi.org/10.1016/j.biologicals.2011.06.002.

10.Boune, S.; Hu, P.; Epstein, A.L.; Khawli, L.A. Principles of N-Linked Glycosylation Variations of IgG-Based Therapeutics: Pharmacokinetic and Functional Considerations. *Antibodies* 2020, *9*, 22, https://doi.org/10.3390/antib9020022.

11.Shukla, A.A.; Wolfe, L.S.; Mostafa, S.S.; Norman, C. Evolving trends in mAb production processes. *Bioeng. Transl. Med.* 2017, *2*, 58–69, https://doi.org/10.1002/btm2.10061.

12.Goetze, A.M.; Liu, Y.D.; Zhang, Z.; Shah, B.; Lee, E.; Bondarenko, P.V.; Flynn, G.C. High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. *Glycobiology* 2011, *21*, 949–959, https://doi.org/10.1093/glycob/cwr027.

13.Moremen, K.W.; Tiemeyer, M.; Nairn, A. Vertebrate protein glycosylation: Diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol.* 2012, *13*, 448–462, https://doi.org/10.1038/nrm3383.

13.Dalziel, M.; Crispin, M.; Scanlan, C.N.; Zitzmann, N.; Dwek, R.A. Emerging Principles for the Therapeutic Exploitation of Glycosylation. *Science* 2014, *343*, 1235681, https://doi.org/10.1126/science.1235681.

14.Vervecken, W.; Kaigorodov, V.; Callewaert, N.; Geysens, S.; De Vusser, K.; Contreras, R. In Vivo Synthesis of Mammalian-Like, Hybrid-Type N-Glycans in Pichia pastoris. *Appl. Environ. Microbiol.* 2004, *70*, 2639–2646, https://doi.org/10.1128/aem.70.5.2639-2646.2004.

15.Hamilton, S.R.; Bobrowicz, P.; Bobrowicz, B.; Davidson, R.C.; Li, H.; Mitchell, T.; Nett, J.H.; Rausch, S.; Stadheim, T.A.; Wischnewski, H.; et al. Production of Complex Human Glycoproteins in Yeast. *Science* 2003, *301*, 1244–1246, https://doi.org/10.1126/science.1088166.

16.Callewaert, N.; Y, W.L.; Cadirgi, H.; Geysens, S.; Saelens, X.; Min, W.; Y, R.C. Use of HDEL-Tagged Trichoderma Reesei Mannosyl Oligosaccharide 1, 2- K—D -Mannosidase for N -Glycan Engineering in Pichia Pastoris. *FEBS Lett.* 2001, *503*, 173–178.

17.Chiba, Y.; Suzuki, M.; Yoshida, S.; Yoshida, A.; Ikenaga, H.; Takeuchi, M.; Jigami, Y.; Ichishima, E. Production of Human Compatible High Mannose-type (Man5GlcNAc2) Sugar Chains in Saccharomyces cerevisiae. *J. Biol. Chem.* 1998, *273*, 26298–26304, https://doi.org/10.1074/jbc.273.41.26298.

18.Choi, B.-K.; Bobrowicz, P.; Davidson, R.C.; Hamilton, S.R.; Kung, D.H.; Li, H.; Miele, R.G.; Nett, J.H.; Wildt, S.; Gerngross, T.U. Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast Pichia pastoris. *Proc. Natl. Acad. Sci. USA* 2003, *100*, 5022–5027, https://doi.org/10.1073/pnas.0931263100.

19.Bobrowicz, P.; Davidson, R.C.; Li, H.; Potgieter, T.I.; Nett, J.H.; Hamilton, S.R.; Stadheim, T.A.; Miele, R.G.; Bobrowicz, B.; Mitchell, T.; et al. Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast Pichia pastoris: Production of complex humanized glycoproteins with terminal galactose. *Glycobiology* 2004, *14*, 757–766, https://doi.org/10.1093/glycob/cwh104.

20.Nasab, F.P.; Aebi, M.; Bernhard, G.; Frey, A.D. A Combined System for Engineering Glycosylation Efficiency and Glycan Structure in Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* 2013, *79*, 997–1007, https://doi.org/10.1128/aem.02817-12.

21.Wang, F.; Wang, X.; Yu, X.; Fu, L.; Liu, Y.; Ma, L.; Zhai, C. High-Level Expression of Endo-β-N-Acetylglucosaminidase H from Streptomyces plicatus in Pichia pastoris and Its Application for the Deglycosylation of Glycoproteins. *PLoS ONE* 2015, *10*, e0120458, https://doi.org/10.1371/journal.pone.0120458.

22.Wildt, S.; Gerngross, T.U. The humanization of N-glycosylation pathways in yeast. *Nat. Rev. Genet.* 2005, *3*, 119–128, https://doi.org/10.1038/nrmicro1087.

23.Mamedov, T.; Cicek, K.; Gulec, B.; Ungor, R.; Hasanova, G. In vivo production of non-glycosylated recombinant proteins in Nicotiana benthamiana plants by co-expression with Endo-β-N-acetylglucosaminidase H (Endo H) of Streptomyces plicatus. *PLoS ONE* 2017, *12*, e0183589, https://doi.org/10.1371/journal.pone.0183589.

24.Bennett, L.D.; Yang, Q.; Berquist, B.R.; Giddens, J.P.; Ren, Z.; Kommineni, V.; Murray, R.P.; White, E.L.; Holtz, B.R.; Wang, L.-X.; et al. Implementation of Glycan Remodeling to Plant-Made Therapeutic Antibodies. *Int. J. Mol. Sci.* 2018, *19*, 421, https://doi.org/10.3390/ijms19020421.

25.Kao, D.; Danzer, H.; Collin, M.; Groß, A.; Eichler, J.; Stambuk, J.; Lauc, G.; Lux, A.; Nimmerjahn, F. A Monosaccharide Residue Is Sufficient to Maintain Mouse and Human IgG Subclass Activity and Directs IgG Effector Functions to Cellular Fc Receptors. *Cell Rep.* 2015, *13*, 2376–2385, https://doi.org/10.1016/j.celrep.2015.11.027.

26.Subedi, G.P.; Barb, A.W. The Structural Role of Antibody N-Glycosylation in Receptor Interactions. *Structure* 2015, *23*, 1573–1583, https://doi.org/10.1016/j.str.2015.06.015.

27.Collin, M. EndoS, a novel secreted protein from Streptococcus pyogenes with endoglycosidase activity on human IgG. *EMBO J.* 2001, *20*, 3046–3055, https://doi.org/10.1093/emboj/20.12.3046.

28.Sjögren, J.; Struwe, W.; Cosgrave, E.; Rudd, P.M.; Stervander, M.; Allhorn, M.; Hollands, A.; Nizet, V.; Collin, M. EndoS2 is a unique and conserved enzyme of serotype M49 group A Streptococcus that hydrolyses N-linked glycans on IgG and α1-acid glycoprotein. *Biochem. J.* 2013, *455*, 107–118, https://doi.org/10.1042/bj20130126.

29.Sjogren, J.; Cosgrave, E.F.J.; Allhorn, M.; Nordgren, M.; Björk, S.; Olsson, F.; Fredriksson, S.; Collin, M. EndoS and EndoS2 hydrolyze Fc-glycans on therapeutic antibodies with different glycoform selectivity and can be used for rapid quantification of high-mannose glycans. *Glycobiology* 2015, *25*, 1053–1063, https://doi.org/10.1093/glycob/cwv047.

30.Gomes, A.M.V.; Carmo, T.S.; Carvalho, L.S.; Bahia, F.M.; Parachin, N.S. Comparison of Yeasts as Hosts for Recombinant Protein Production. *Microorganisms* 2018, *6*, 38, https://doi.org/10.3390/microorganisms6020038.

31.Lin-Cereghino, J.; Wong, W.W.; Xiong, S.; Giang, W.; Luong, L.T.; Vu, J.; Johnson, S.D.; Lin-Cereghino, G.P. Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris. *BioTechniques* 2005, *38*, 44–48, https://doi.org/10.2144/05381bm04.

32.Boder, E.T.; Wittrup, K.D. Yeast Surface Display for Screening Con1binatorial Polypeptide Libraries. *Nat. Biotechnol.* 1997, *15*, 553–557.

33.Subedi, G.P.; Johnson, R.W.; Moniz, H.A.; Moremen, K.W.; Barb, A. High Yield Expression of Recombinant Human Proteins with the Transient Transfection of HEK293 Cells in Suspension. *J. Vis. Exp.* 2015, e53568, https://doi.org/10.3791/53568.

34.Subedi, G.P.; Hanson, Q.M.; Barb, A.W. Restricted Motion of the Conserved Immunoglobulin G1 N-Glycan Is Essential for Efficient FcγRIIIa Binding. *Structure* 2014, *22*, 1478–1488, https://doi.org/10.1016/j.str.2014.08.002.

35.Mei, M.; Zhai, C.; Li, X.; Zhou, Y.; Peng, W.; Ma, L.; Wang, Q.; Iverson, B.L.; Zhang, G.; Yi, L. Characterization of aromatic residue–controlled protein retention in the endoplasmic reticulum of Saccharomyces cerevisiae. *J. Biol. Chem.* 2017, *292*, 20707–20719, https://doi.org/10.1074/jbc.m117.812107.

36.Voth, W.P.; Richards, J.D.; Shaw, J.M.; Stillman, D.J. Yeast Vectors for Integration at the HO Locus. *Nucleic Acids Res.* 2001, *29,* e59-e5

37.Gnügge, R.; Liphardt, T.; Rudolf, F. A shuttle vector series for precise genetic engineering ofSaccharomyces cerevisiae. *Yeast* 2015, *33*, 83–98, https://doi.org/10.1002/yea.3144.

38.Uchański, T.; Zögg, T.; Yin, J.; Yuan, D.; Wohlkönig, A.; Fischer, B.; Rosenbaum, D.M.; Kobilka, B.K.; Pardon, E.; Steyaert, J. An improved yeast surface display platform for the screening of nanobody immune libraries. *Sci. Rep.* 2019, *9*, 382, https://doi.org/10.1038/s41598-018-37212-3.

39.Wang, S.; Cho, Y.K. Yeast surface display of full-length human microtubule-associated protein tau. *Biotechnol. Prog.* 2019, *36*, e2920, https://doi.org/10.1002/btpr.2920.

40.Rakestraw, J.A.; Sazinsky, S.L.; Piatesi, A.; Antipov, E.; Wittrup, K.D. Directed evolution of a secretory leader for the improved expression of heterologous proteins and

full-length antibodies inSaccharomyces cerevisiae. *Biotechnol. Bioeng.* 2009, *103*, 1192–1201, https://doi.org/10.1002/bit.22338.

41.Fishman, J.B.; Berg, E.A. Protein A and Protein G Purification of Antibodies. *Cold Spring Harb. Protoc.* 2019, *2019*, https://doi.org/10.1101/pdb.prot099143.

42.Deisenhofer J. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-A resolution. Biochemistry. 1981 Apr 28;20(9):2361-70. PMID: 7236608.

43.Liu, C.-P.; Tsai, T.-I.; Cheng, T.; Shivatare, V.S.; Wu, C.-Y.; Wong, C.-H. Glycoengineering of antibody (Herceptin) through yeast expression and in vitro enzymatic glycosylation. *Proc. Natl. Acad. Sci. USA* 2018, *115*, 720–725, https://doi.org/10.1073/pnas.1718172115.

44.Li, A.; Liu, Z.; Li, Q.; Yu, L.; Wang, D.; Deng, X. Construction, and characterization of bidirectional expression vectors inSaccharomyces cerevisiae. *FEMS Yeast Res.* 2008, *8*, 6–9, https://doi.org/10.1111/j.1567-1364.2007.00335.x.

45.Rougé, L.; Chiang, N.; Steffek, M.; Kugel, C.; Croll, T.I.; Tam, C.; Estevez, A.; Arthur, C.P.; Koth, C.M.; Ciferri, C.; et al. Structural biology. *Science* 2020, *367*, 1224–1230.

46.Subedi, G.P.; Hanson, Q.M.; Barb, A.W. Restricted Motion of the Conserved Immunoglobulin G1 N-Glycan Is Essential for Efficient FcγRIIIa Binding. *Structure* 2014, *22*, 1478–1488, https://doi.org/10.1016/j.str.2014.08.002.

47.Shields, R.L.; Lai, J.; Keck, R.; O'Connell, L.Y.; Hong, K.; Meng, Y.G.; Weikert, S.H.A.; Presta, L.G. Lack of Fucose on Human IgG1 N-Linked Oligosaccharide Improves Binding to Human FcγRIII and Antibody-dependent Cellular Toxicity. *J. Biol. Chem.* 2002, *277*, 26733–26740, https://doi.org/10.1074/jbc.m202069200.

48.Subedi, G.P.; Barb, A.W. The immunoglobulin G1 N-glycan composition affects binding to each low affinity Fc γ receptor. *mAbs* 2016, *8*, 1512–1524, https://doi.org/10.1080/19420862.2016.1218586.

49.Allhorn, M.; Olin, A.I.; Nimmerjahn, F.; Collin, M. Human IgG/FcγR Interactions Are Modulated by Streptococcal IgG Glycan Hydrolysis. *PLoS ONE* 2008, *3*, e1413, https://doi.org/10.1371/journal.pone.0001413.

50.Zhou, J.; Zhang, H.; Liu, X.; Wang, P.G.; Qi, Q. Influence of N-Glycosylation on Saccharomyces cerevisiae Morphology: A Golgi Glycosylation Mutant Shows Cell Division Defects. *Curr. Microbiol.* 2007, *55*, 198–204, https://doi.org/10.1007/s00284-006-0585-5.

51.Horwitz, A.H.; Chang, C.P.; Better, M.; Hellstromt, K.E.; Robinson, R.R. Secretion of functional antibody and Fab fragment from yeast cells. *Proc. Natl. Acad. Sci. USA* 1988, *85*, 8678–8682.

52.Kelley, B. Industrialization of mAb production technology: The bioprocessing industry at a crossroads. *mAbs* 2009, *1*, 443–452, https://doi.org/10.4161/mabs.1.5.9448.

53.Shukla, A.A.; Wolfe, L.S.; Mostafa, S.S.; Norman, C. Evolving trends in mAb production processes. *Bioeng. Transl. Med.* 2017, *2*, 58–69, https://doi.org/10.1002/btm2.10061.

54.Li, T.; Tong, X.; Yang, Q.; Giddens, J.; Wang, L.-X. Glycosynthase Mutants of Endoglycosidase S2 Show Potent Transglycosylation Activity and Remarkably Relaxed Substrate Specificity for Antibody Glycosylation Remodeling. *J. Biol. Chem.* 2016, *291*, 16508–16518, https://doi.org/10.1074/jbc.m116.738765.

55.Huang, W.; Giddens, J.; Fan, S.-Q.; Toonstra, C.; Wang, L.-X. Chemoenzymatic Glycoengineering of Intact IgG Antibodies for Gain of Functions. *J. Am. Chem. Soc.* 2012, *134*, 12308–12318, https://doi.org/10.1021/ja3051266.

56.Parsons, T.B.; Struwe, W.B.; Gault, J.; Yamamoto, K.; Taylor, T.A.; Raj, R.; Wals, K.; Mohammed, S.; Robinson, C.V.; Benesch, J.L.P.; et al. Optimal Synthetic Glycosylation of a Therapeutic Antibody. *Angew. Chem.* 2016, *128*, 2407–2413, https://doi.org/10.1002/ange.201508723.

CHAPTER 4[3]

METABOLIC ^{15}N LABELING OF THE N-GLYCOSYLATED IMMUNOGLOBULIN G1 FC WITH AN ENGINEERED *SACCHAROMYCES CEREVISIAE* STRAIN

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Abstract:

The predominant protein expression host for NMR spectroscopy is *Escherichia coli*, however, it does not synthesize appropriate post-translation modifications required for mammalian protein function and is not ideal for expressing naturally secreted proteins that occupy an oxidative environment. Mammalian expression platforms can address these limitations; however, these are not amenable to cost-effective uniform ^{15}N labeling resulting from highly complex growth media requirements. Yeast expression platforms combine the simplicity of bacterial expression with the capabilities of mammalian platforms, however yeasts require optimization prior to isotope labeling. Yeast expression will benefit from methods to boost protein expression levels and developing labeling conditions to facilitate growth and high isotope incorporation within the target protein. In this work, we describe a novel platform based on the yeast *Saccharomyces cerevisiae* that simultaneously expresses the Kar2p chaperone and protein disulfide isomerase in the ER to facilitate the expression of secreted proteins. Furthermore, we developed a growth medium for uniform ^{15}N labeling. We recovered 2.2 mg/L of uniformly ^{15}N-labeled human immunoglobulin (Ig)G1 Fc domain with 90.6% ^{15}N labeling. NMR spectroscopy revealed a high degree of similarity between the yeast and mammalian expressed IgG1 Fc domains. Furthermore, we were able to map the binding interaction between IgG1 Fc and the Z domain (three helix bundle from Protein A derived from *Streptomyces aureus*) through chemical shift perturbations. This platform represents a novel cost-effective strategy for ^{15}N-labeled immunoglobulin fragments.

4.1 Introduction

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique to characterize protein function, structure and motion that provides atomic-level resolution [1–3]. High efficiency incorporation of stable isotopes is essential for assigning individual atoms to peaks in spectra. Protein expression for NMR experiments has mostly been limited to prokaryotic or mammalian expression systems [4]. Prokaryotic systems offer several advantages including rapid growth, low cost, and isotope incorporation using metabolic precursors added to the growth medium. However, prokaryotes are not suitable for expressing many eukaryotic proteins, leading to misfolding and formation of insoluble aggregates (inclusion bodies). Furthermore, standard prokaryotic expression systems are not capable of adding mammalian post-translational modifications which confer many functional properties to the target protein. In contrast, mammalian expression platforms synthesize appropriate modifications, however they have substantial limitations including additional equipment requirements, complex medium formulations that preclude metabolic incorporation of protein labeling precursors from a single nitrogen or carbon source, and a BSL2 requirement [5–8]. Unicellular yeasts are microbes with the advantages of both prokaryotic and mammalian expression platforms including simple growth media, rapid growth, an oxidizing ER lumen for disulfide bond formation, machinery for eukaryotic post-translational modifications (N-glycosylation) and chaperones [5]

The majority of yeast labeling protocols, however, have been developed in the methylotrophic yeast *Pichia pastoris*, which has widely been used to express heterologous proteins in contrast to other commercially relevant species including *Kluyveromyces lactis* and *Saccharomyces cerevisiae*[5,9–13]. A number of techniques for expressing proteins using *P. pastoris* are available. In most cases, expression of heterologous proteins in *P. pastoris* was driven

by the strong AOX1 promotor which is induced by methanol [14]. The amount of methanol in the medium must be optimized to allow for sufficient protein expression but prevent cell lysis [9]. Genetic manipulation in *P. pastoris* is typically achieved through integration of a plasmid into the genome through homologous recombination [14], therefore limiting DNA copy number that would be expected to increase protein expression levels [5,9]. Several groups also addressed medium optimization through supplementation during labeling to boost cell growth and protein expression without negatively affecting isotope-incorporation efficiency [5,9–11]. Despite the availability of several deletion strains, proteolysis, improper folding, and protein degradation limit labeling and purifying protein from *P. pastoris*. This appears to be a greater problem when using minimal medium to supply a single carbon and nitrogen source for cost-effective isotope labeling [12]. Optimizing the construct design to allow for proper secretion from *P. pastoris* and addition of sorbitol in the labeling medium were observed to reduce the amount of proteolytic degradation without depleting gene expression [5,12]. Multiple studies have successfully purified several milligrams of isotope labeled target proteins from *Pichia pastoris* by using fermenters and other specialized equipment which boosted the yield above the 3-4 mg/L found using shake flasks [15,16].

As an alternative, *S. cerevisiae* is heavily utilized for bread and beer making, as a model organism in the laboratory [17,18] and has been used for the expression of several therapeutics [19]. However, despite the wide application of *Saccharomyces cerevisiae*, there have been few reports of isotope labeling. Hong and coworkers genetically engineered *S. cerevisiae* to produce cholesterol with selective ^{13}C labeling to study interactions with an exogenous viral protein [13]. Similarly, adding [^{13}C]-glucose to the growth medium labeled N-glycans. In this example, a

knockout strain (Golgi-resident mannosyltransferases) accumulated glycoproteins in the E.R., and N-glycans were hydrolyzed

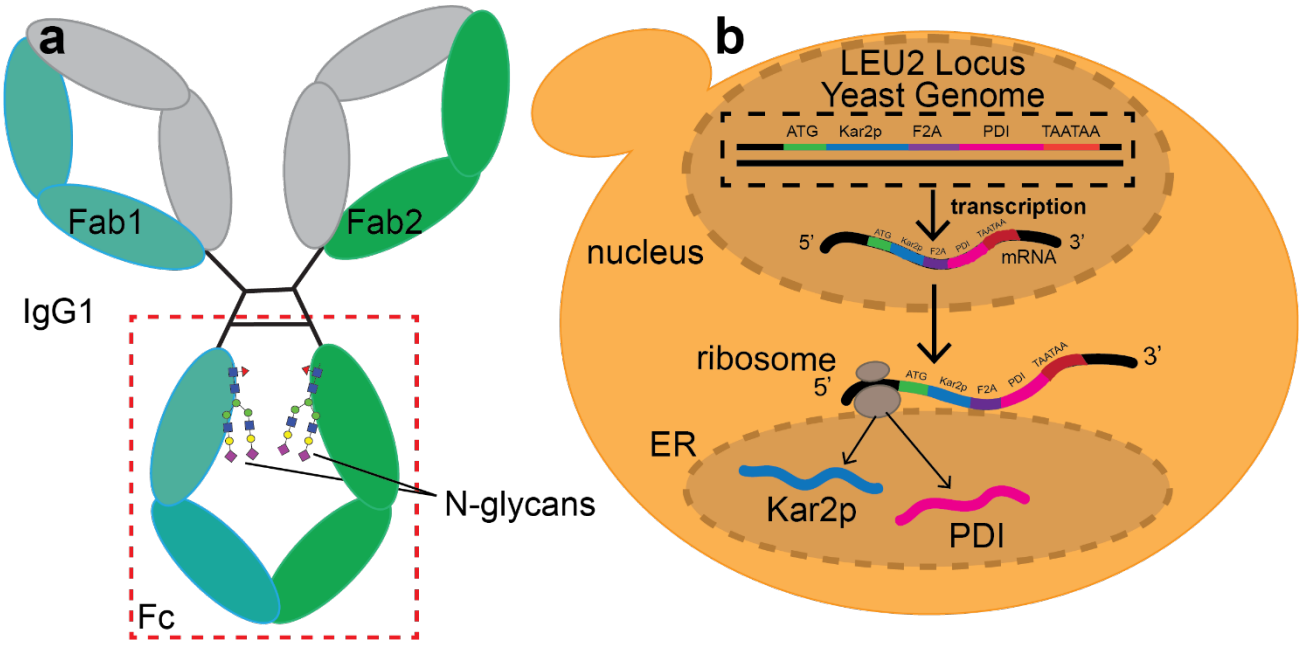

Figure 4.1 Immunoglobulin G1 and EBY100-Kar2p-F2A-PDI yeast strain a. Immunoglobulin G1 **b**. The EBY100-Kar2p-F2A-PDI yeast strain developed herein allows for the simultaneous expression of both yeast chaperone Kar2p and yeast Protein Disulphide Isomerase (PDI) from a single transcript. These proteins aid protein folding within the endoplasmic reticulum.

following protein purification for further study [20,21]. Recently, Wall and Hough reported ^{15}N labeling of a cytosolic *S. cerevisiae* protein fragment Nsp1 using a mixture of yeast nitrogen base, supplemented with [^{15}N]-ammonium chloride to study hydrophobic interactions using in-cell NMR [22]. A *S. cerevisiae* growth medium formulation for ^{15}N-labeling secreted glycoproteins using a simple metabolic precursor is not available to our knowledge. In this work, we describe an approach utilizing a novel engineered *S. cerevisiae* strain to express [^{15}N]-labeled proteins.

We developed an expression and labeling strategy to produce proteins that are naturally secreted by the native host cell. Many proteins are targeted to the lumen of the E.R. during translation,

whereupon E.R.-resident modification machinery and chaperones fold and modify proteins in an oxidative environment. Of particular interest are antibodies secreted into the blood that are a critical product of the adaptive immune system. Fully active antibodies, particularly of the immunoglobulin G (IgG) class, require disulfide bond formation and N-glycosylation for activity (Figure1a) [23,24]. Here we apply novel expression and labeling technologies to IgG1 and the IgG1 Fc domain from humans.

^{15}N-labeling of the IgG Fc domain was previously reported by Kato and co-workers using mammalian cells. IgG1 Fc is a 50 kDa homodimer formed by two C-terminal halves of the IgG1 two heavy chains, is glycosylated at Asn297, and mediates interactions between the antibody and cell surface receptors [25]. This truly heroic expression and labeling effort was labor intensive and required supplementing the growth medium with multiple isotope-labeled compounds including ^{15}N-labeled algal lysate and seven amino acids [6,7,26,27]. A very recent contribution reports a modified mammalian cell line and a somewhat simplified medium for this same purpose [28]. Here we describe a novel approach to achieve [^{15}N]-labeling from a single metabolic precursor using an engineered *S. cerevisiae* strain.

4.2. Materials and Methods

All materials were purchased from Millipore Sigma unless otherwise noted.

4.2.1 Strain

The *S. cerevisiae* strain EBY100 (GAL1-AGA1:URA3 ura3-52 trp1 leu2Δ1 his3 Δ200 pep4: HIS2 prb1 Δ1.6R can1 GAL) was purchased from the American Type Culture Collection (ATCC-MYA4941). DNA cloning experiments used *Escherichia coli* DH5α cells.

4.2.2 Construct Design

Gene fragments for app8 and human IgG1 Fc were codon optimized for expression in *Saccharomyces cerevisiae* and synthesized by IDT [29]. These two fragments were amplified by PCR. The PCR fragments were gel extracted and used for a fusion PCR reaction to append the app8 fragment at the 5' end of the Fc-encoding fragment. DNA encoding app8-Fc was then cloned into the Sal1 and Nhe1 site of the pESC-TRP1 vector (Agilent). The app8-Fc expression was driven by the GAL1 promotor. The pESC-RTX plasmid was described previously (Shenoy et al. 2021a).

Codon-optimized gene fragments encoding Kar2p, PDI and Kar2p-F2A-PDI were synthesized by IDT. These fragments were then cloned into the XbaI and HindIII restriction sites of pAG305-GPD vector (Addgene) to produce vectors: pAG305-PDI, pAG305-Kar2p and pAG305-Kar2p-F2A-PDI. DNA sequence was verified by Sanger sequencing (Eurofins).

Creating the EBY100-Kar2p, EBY100-PDI and EBY100-F2A-PDI yeast strains

Each vector was linearized with a BstEII digestion (Promega). The linearized plasmid was purified on a 1% agarose gel and the DNA fragment was gel extracted using a Qiagen Gel Extraction kit. Roughly 200 ng of the linearized DNA was used to transform EBY100 yeast cells using the Lithium Acetate (LiAc) method to create EBY100-PDI; EBY100-Kar2p and EBY100-Kar2p-F2A-PDI strains, respectively[31]. Transformants were selected on YNB-Dropout + Trp plates. The integrations were verified with PCR. We then transformed each of these strains with the pESC-TRP1-RTX plasmid or the pESC-TRP1-app8-Fc plasmid, selected transformants with YNB-Dropout medium + agar and identified for positive transformants using yeast colony PCR.

Table 4.1. Media conditions for rituximab production

Yeast Strain	Growth Media (Prior to transformation)	Media conditions (Post transformation with pESC-TRP1)	Induction Media for RTX Production
EBY100-(PDI / Kar2p / Kar2p-F2A-PDI)	YNB-Dropout + Trp+ 2% Glucose	YNB + Dropout + 2% Glucose	0-24h: YNB-Dropout + 2% Gal 24-96h: YPGal
EBY100	YPD	YNB-CAA + 2% Glucose	0-24h: YNB-CAA + 2% Gal 24-96h: YPGal

YNB-Dropout = 6.7 g/L Yeast Nitrogen Base (YNB; without Amino Acids and Ammonium Sulphate) and 1.5 g/L Yeast Synthetic Drop Out Medium Supplements (without uracil, tryptophan, and leucine); YNB-CAA: 6.7 g/L Yeast Nitrogen Base (YNB; without Amino Acids) and 5 g/L Casamino acids; YPD/YPGal: 10 g/L yeast extract + 20 g/L peptone + 2 % (glucose or galactose; w/v); Trp: 2 mg/mL

4.2.3 Rituximab Expression from the EBY100-PDI/Kar2p/Kar2p-F2A-PDI strains

Media conditions used for growth and induction of different yeast strains described in Table I. Overnight cultures (5 mL) were used to start a 50 mL induction culture at an OD_{600} = 0.5, followed by incubation with shaking for 24 h at 30 ºC. Cells were pelleted at 3000 g for 10 min. The supernatant was discarded and cells were used to start another induction culture in 50 mL in YPGal. We incubated this culture for an additional 72 h at 20 ºC. The culture was centrifuged twice at 3000 g for 10 min. The supernatant was collected, and its pH was adjusted to 6.5 to allow for binding with Protein A. The entire supernatant was applied to a Protein A column. The column was then washed with 5 mL Buffer A (20 mM MOPS and 100 mM sodium chloride, pH 7.4). The column was eluted with 100 mM Glycine pH 3 as 1 mL fractions into tubes containing 500 µl 1 M TRIS pH 8.0. All elution fractions were pooled, frozen, then lyophilized. The protein was then resuspended in 200 µl 1M Tris pH 8. 10 µl of each sample was then resuspended in 10 µl 2x SDS Sample buffer, boiled at 95 ºC for 5 min and centrifuged in a microcentrifuge at 3000 rpm for 1

min. This sample was then loaded onto a 12% SDS polyacrylamide gel. The protein was transferred onto a polyvinylidene difluoride (PVDF) membrane using an Invitrogen Power Blotter (PB0010). After transfer, the membrane was blocked in TBS+Tween20 (TBST) with 5% powdered milk (w/v) for 1 h at RT on an orbital shaker. This was followed by incubation with rabbit anti-hIgG primary antibody (RRID: AB_228410, ThermoFisher) in 5% dry milk in TBST overnight at 4 ºC on an orbital shaker. The membrane was washed three times for 10 min with TBST buffer. Followed by incubation with secondary Goat Anti-Rabbit IgG (H+L) Alexa Fluor Plus 680 (RRID: AB_2633283, Fisher Scientific, dilution: 1:2000) in 5% powdered milk in 1x TBST buffer for 2 h at RT on an orbital shaker. The membrane was washed three times with 1x TBST buffer for 10 min and then imaged using LiCOR (LiCor Odyssey CLx). Western blot intensities were quantified using ImageJ.

4.2.3 ^{15}N labeling and purifying app8-Fc

The *S. cerevisiae* EBY100-Kar2p-F2A-PDI strain was transformed with the pESC-TRP1-app8-Fc plasmid and grown overnight in a 5 ml culture in YNB-Dropout medium at 30 ºC. This culture was used to inoculate a 100 mL culture, in medium containing 1.7 g/L YNB (without amino acids and ammonium sulphate), 91.8 mM $^{15}NH_4Cl$, 38 mM Na_2HPO_4, 71.7 mM NaH_2PO_4 and 2% glucose. The medium was sterilized by autoclaving prior to use. This culture was incubated at 30 ºC for 24 h with shaking. A portion of these cells were centrifuged to remove spent medium and used to inoculate a 1 l culture at a final OD_{600} = 0.5 in NMR induction medium (with 2% YPGal) containing 1.7 g/L YNB (w/o amino acids and ammonium sulphate), 37.2 mM $^{15}NH_4Cl$, 38 mM Na_2HPO_4, 71.7 mM NaH_2PO_4, 2% galactose, 0.2 g/L Yeast Extract, 0.4 g/L Peptone, 1 g/L of a powdered multivitamin (Green Wise Men's Multivitamin, Publix Grocery). Similarly, to induce cells in an NMR induction media with 5% YPGal (same as the previous medium except with 0.5

g/L Yeast Extract, 1 g/L Peptone). Media were mixed under gentle warming to dissolve soluble components, then centrifuged at 3000 g for 10 min to remove insoluble debris and sterilized by passage through a 0.2 mm filter prior to use. The induction cultures were incubated at 30 ºC for 48 h with orbital shaking. Culture supernatant was clarified by centrifugation (2x 3000 g for 10 min), then adjusted to pH 6.5 with dropwise addition of 10 M sodium hydroxide.

The pH-adjusted culture supernatant was diluted 1:1 with 25 mM sodium phosphate, 150mM sodium chloride pH 7.2, then applied to a 5 mL Protein A column (Cytiva) preequilibrated with 15 mL of the same buffer. The flow rate was 4.5 mL/min. The protein was eluted with Elution buffer (100 mM glycine, pH 3). Elution fractions (2 mL) were collected in tubes containing 1 mL of 1M Tris pH 8.0. The column was then washed once again with Buffer A.

4.2.4 Endo F1 digestion

EndoF1 was expressed and purified as described [32]. EndoF1 was then coupled to AminoLink Coupling Resin according to the manufacturer protocol (Thermo Scientific) at a final concentration at 6 mg/mL. The resin was then exchanged into 25 mM MOPS, 100 mM sodium chloride, pH 7.4. The resin was then used to digest the IgG Fc N-glycans. For this digestion, we used approximately 150 µl of the 50% EndoF1-resin slurry (v/v) with approximately 2 mg of Fc. The EndoF1 protein resin and Fc were incubated overnight at RT on an inverting rotator. The resin was removed with a MicroBio spin column (BioRad).

4.2.5 Mass spectrometry of ^{15}N-labeled app8-Fc

Lyophilized protein samples were reconstituted in HPLC-grade water at 1 mg/mL. Each sample (1 µL) was diluted to 500 µL in 50:50:0.1 methanol: water: formic acid. To observe the IgG1 Fc monomers, 5 µL of each solution was treated with 380 µM TCEP at 37 °C for 30 min. Then, the reduced Fc solutions (1 µL) were diluted to 500 µL in 50:50:0.1 methanol: water: formic acid.

Mass spectra were collected using a SolariX XR 12 T Fourier transform ion cyclotron resonance mass spectrometer (Bruker). Samples were introduced by direct infusion into an electrospray ionization source at a rate of 120 μL/h using a capillary inlet voltage of 4500 V. Mass spectra were collected in positive mode in the range of m/z 250-3000. The transient length was 1.3 s and 2 M points were collected. For every spectrum, 100 scans were averaged. In some cases, the large number of ions present in the cell made it difficult to obtain a signal transient of sufficient quality, and so a 300-400 m/z quadrupole isolation window was set to only allow 5-8 charge states of the protein into the analyzer cell. Mass spectra were processed using Data Analysis (Bruker Scientific), and protein monoisotopic masses were determined using the SNAP^{TM} algorithm.

The SNAP^{TM} algorithm is designed to determine the monoisotopic masses for natural abundance proteins, and therefore was unable to determine the monoisotopic molecular weight of the ^{15}N labeled Fc. Instead, the average mass of the ^{15}N labeled was used to determine the percent ^{15}N incorporation. A smoothing function was applied to the high-resolution mass spectrum for the labeled and unlabeled protein. The average mass for both proteins was determined using the charge deconvolution software in Data Analysis. The difference in average mass between the unlabeled and labeled proteins provided the degree of ^{15}N enrichment.

4.2.6 Solution NMR Spectroscopy

The Z domain was expressed in LB medium as previously described [33]. Protein concentrations were determined by absorbency at 280 nm using a NanoDrop spectrophotometer. EndoF1-digested IgG1 Fc was exchanged into a buffer containing 20 mM sodium phosphate, 100 mM potassium chloride, 50 mM trimethylsilylpropanesulfonate (DSS), pH 7.2 using a 10 kDa MWCO centrifugal filter. NMR spectra were collected at a 50 ºC sample temperature on a 21.1 T spectrometer equipped with a Bruker NEO console and 5 mm TXO cryoprobe. The Bruker trosyetf3gpsi pulse

sequence was used with 512 and 128 complex points in the direct and indirect dimensions, respectively, and 8 - 64 scans. Spectra were processed with a sine-squared line broadening function applied in the direct dimension and a sine function with linear prediction to 128 points in the indirect dimension. Spectra were processed in NMR Pipe [34] and analyzed in NMRViewJ [35].

4.3 Results

4.3.1 Engineering the EBY100 strain with ER-targeted chaperones

Though targeted for secretion, we observed a high abundance of protein retained in cells following expression of the antibody rituximab (not shown). To improve secretion, we explored expressing constitutive ER-localized proteins that promote folding. Co-expression of the intrinsic Kar2p chaperone and protein disulphide isomerase (PDI) was previously shown to exhibit variable effects on the level of heterologous protein expression; in some cases, there is a significant boost in expression [36], but in some cases there was only a minor increase [37]. There have also been reports of increased ER stress in yeast cells expressing multiple heterologous proteins simultaneously [38,39]. We engineered *S. cerevisiae* to simultaneously co-express the Kar2p and PDI in the ER lumen. To reduce ER stress, we incorporated a F2A ribosome skip site and combined the Kar2P and PDI open-reading-frames into a single transcript [40]. F2A was previously used to successfully produce multiple proteins simultaneously in mammalian expression systems [40–42]. The cleavage efficiencies of these 2A peptides have been estimated to be around 60-70% in mammalian cells but can be increased by placing two of these peptides in tandem [43]. The functionality of these peptides can also be modulated through changes in amino acid sequence [42]. Thus, protein expression with the 2A peptide provides options for further fine-tuning, if needed. Lastly, we integrated the Kar2p-F2A-PDI construct into the yeast genome to allow for

better retention of the construct during culture as compared to plasmids which require continual selection (Figure 3.1(b)) [39].

4.3.2 Yeast engineering increased antibody production

We next determined the effect of yeast strain engineering on heterologous antibody production. Antibodies, including IgG1, are challenging targets for heterologous expression in yeast due to the heterotetrametic assembly (two heavy chains, two light chains), formation of ten disulfide bonds and N-glycosylation of each heavy chain. We transformed each yeast strain with the pESC-TRP-

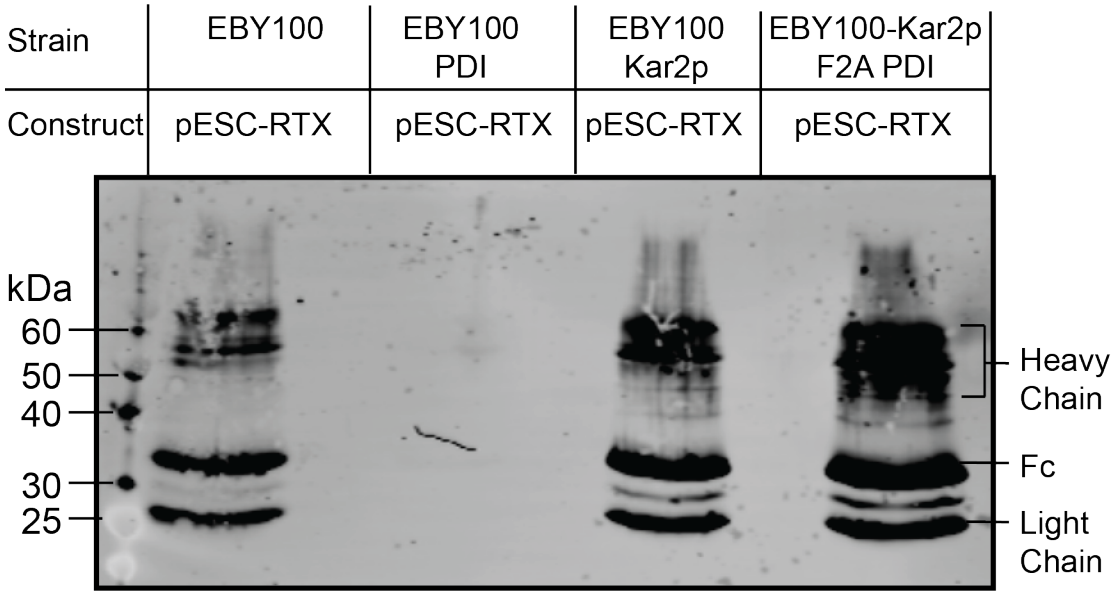

Figure 4.2. Comparison between antibody production with the engineered *S. cerevisiae* strains. Each of the lanes in the Western blot represent the production of Rituximab (RTX). Rituximab is a heterotetramer composed of two (N-glycosylated) heavy chains >50 kDa and two light chains (~25 kDa). Cleavage of the heavy chain is evident by the appearance of the Fc domain and is expected for an antibody secreted by yeast.

RTX plasmid that encodes the heavy and light chains for rituximab expression (Shenoy et al. 2021a). We observed increased rituximab protein expression in the yeast strain containing both Kar2p and PDI, compared to the original strain, EBY100, and the strain with only Kar2P (Figure 3.2, 100%, 72.6%, 72.6% relative intensity, respectively). Curiously, the EBY100-PDI strain

failed to produce protein in multiple repeated experiments despite showing comparable culture growth.

The pattern of IgG1 bands observed in the anti-IgG1 western blot are similar in all cases, indicating that the secreted antibody, purified from the culture supernatant, is comparable in all samples. However, the amount of protein differed between these cultures. In all cases, we observed a smearing in the heavy chain likely due to varying levels of N-glycan remodeling. We also observed IgG1 Fc, likely resulting from heavy chain proteolysis in the hinge region following secretion. We were encouraged by the EBY100-Kar2p-F2A-PDI strain to express and label app8-Fc for NMR.

4.3.3 ^{15}N-labeling of Fc using an engineered yeast strain

We applied the engineered yeast strain to express the IgG1 crystallizable fragment (Fc). In contrast to prior reports utilizing mammalian expression and highly complex media formulations, we explored expressing uniform [^{15}N]-labeled IgG1 Fc in yeast using a minimal growth medium.

Our goal was to identify a minimal medium to facilitate cell growth and protein expression that supported a high level of isotope incorporation. Minimal media is designed to supply simple carbon and nitrogen sources in contrast to complex undefined growth medium based on peptone or yeast extracts. Unfortunately, minimal media often reduces yeast growth and protein production. Our base medium was yeast nitrogen base (YNB), which despite the name contains mostly vitamins and metals, and is combined with ammonium sulfate and a carbon source to make a complete growth medium. We explored using YNB supplementation with [^{15}N]-ammonium

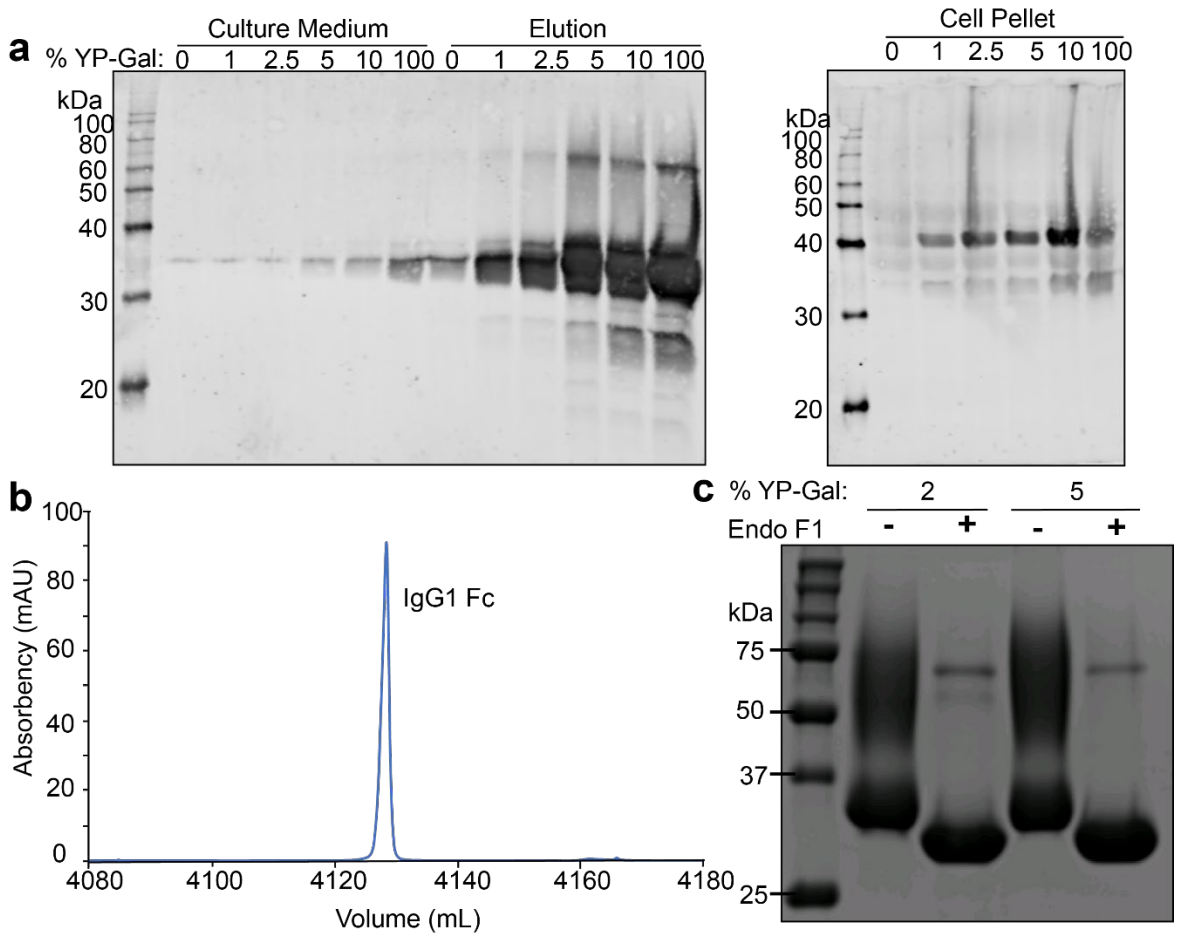

Figure 4.3 Expression and purification of IgG1 Fc from the EBY100-Kar2p-F2A-PDI strain. **a**. Optimization of culture medium conditions by adding rich medium (YP-Gal). IgG1 Fc detected in an anti-IgG western blot of the spent medium, cell pellet and as eluted from a Protein A column. **b**. IgG1 Fc domain purified using a Protein A column. **c**. Endo F1 digestion of purified Fc, using samples purified from two different media condition and stained with Coomassie

chloride, additional biotin, an over-the-counter multivitamin, and low percentages of rich medium (YPGal). We determined that YNB with [^{15}N]-ammonium chloride, supplemented with a multivitamin and 2-5% rich medium provided suitable yield with predicted low levels of ^{14}N contamination from the (unlabeled) rich medium (Figure 3a). Interestingly, expression yields

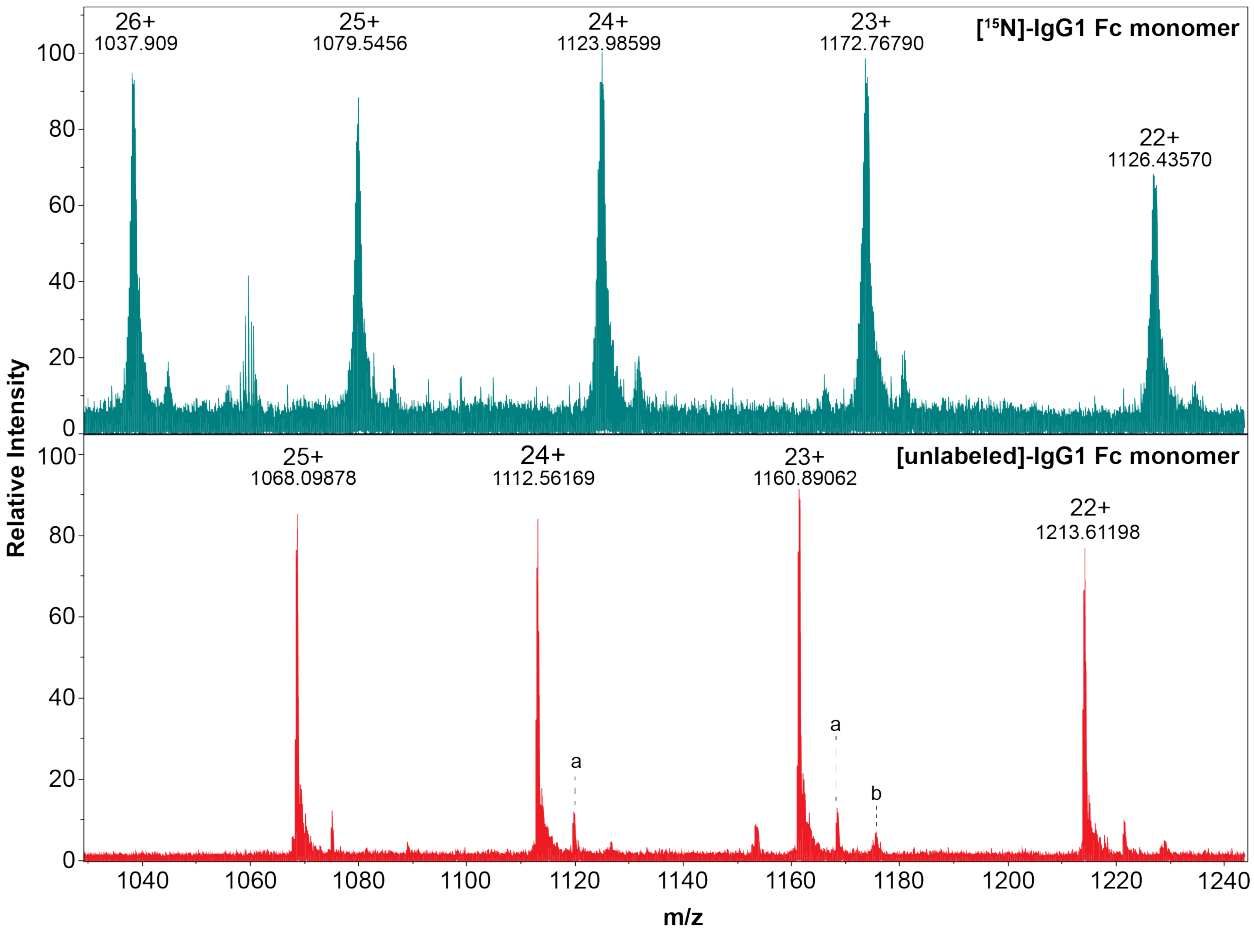

Figure 4.4. FT-ICR-MS spectrum of intact IgG1 Fc. Charge states and m/z values are shown. Average molecular masses are reported in Table 2. "a" corresponds to an increased molecular mass of 163.057 Da, and "b" is an additional 162.164 Da

improved dramatically from 0% to 5% rich medium, indicating that the minimal medium still lacks at least one key component that is in abundance at levels greater than 5%. Also, the cell pellet showed clear retention of the app8-Fc fusion protein, with the secreted protein detected in the spent culture medium and the elution fractions appeared highly processed to remove the app8 secretion tag.

Growth in the minimal medium reduced the pH of the culture supernatant to 2.7-3.0, introducing the additional challenge to protein stability during expression as well as an acidic pH which prevented purification. Adding sodium phosphate to the medium alleviated acidification,

resulting in final cultures ranging from pH 5.5-6. These optimization steps improved expression yields and purification. Interestingly, it is important to note that expression was highly repeatable in Erlenmeyer flasks, but never observed in baffled Fernbach flasks.

We recovered 4.5 mg of [^{15}N]-IgG1 Fc from a 2.0 l culture using the engineered EBY100-Kar2p-F2A-PDI *S. cerevisiae* strain and [^{15}N]-minimal medium supplemented with 5 % rich medium (Fig 3b). A comparable 2.7 l culture supplemented with 2 % rich medium yielded 1.9 mg. One limitation of yeast is the extensive mannosylation of N-glycans that can extend to hundreds of residues [44]. This extensive modification is visible as a dark, high molecular weight smear in a reducing SDS-polyacrylamide gel with the purified protein (Figure 4.3c). We can achieve N-glycan homogeneity by treating the sample with endoglycosidase (Endo)F1 to trim the N-glycan back to a single N-acetylglucosamine residue. This glycoform retains binding affinity for a critical Fc receptor, Fc g receptor IIIa [24,45]. EndoF1 treatment eliminated the smearing, producing a single band. This result also indicated the expressed material is N-glycosylated to a high percentage, as no digested or smaller bands appeared in the untreated samples. Importantly, we also did not observe smaller protein degradation products in this Fc sample indicating negligible proteolysis of the purified protein.

Analysis of [^{15}N]-IgG1 Fc expressed with 5% rich medium using intact protein MS revealed an increased molecular mass relative to IgG1 Fc expressed in unlabeled medium (Figure 4.4). The observed mass for the unlabeled protein differed slightly from the molecular mass calculated using the protein sequence with a mass difference of 0.13-0.20 Da (Table 2). Comparing the labeled and unlabeled proteins revealed that ^{15}N is present at 90.55% of the sites. Furthermore, we observed

no evidence of proteolysis in either preparation, consistent with the SDS-PAGE analysis (Figure 4.3c).

Table 4.2. Average IgG1 Fc masses from FT-ICR-MS

IgG1 Fc	Calc. mass (Da)	Obs. mass (Da)	error (ppm)
[unlabeled] monomer	26695.91000	26695.70355	7.73
[^{15}N] monomer	27008.97899	26980.04891	n/a
[unlabeled] dimer	53379.71360	53379.84824	2.52
[^{15}N] dimer	54005.85158	53952.17020	n/a

4.3.4 NMR of the [^{15}N]-IgG1 Fc expressed in S. cerevisiae

An ^{15}N-^{1}H HSQC-TROSY spectrum of the [^{15}N]-IgG1 Fc showed well dispersed peaks consistent with this mainly beta-sheet and loop protein (Figure 4.5). These peaks superimposed very well with a spectrum assigned by Kato and co-workers using mammalian expressed IgG1 Fc [7](Figure 4.5b). We quantified the difference in peak positions by calculating the average of the absolute differences in peak position, resulting in an average difference of 0.02 ppm and 0.15 ppm (^{1}H and ^{15}N, respectively). The similarity between these NMR spectra, combined with the high N-glycosylation of the yeast-expressed IgG1 and the ability to purify through a protein- protein affinity interaction (Protein A) supports the conclusion that yeast expressed properly folded [^{15}N]-IgG1 Fc.

We next analyzed Fc binding to the IgG-binding three-helix Z domain [46]. The location of the binding interaction is known; however, it is not known if binding only affects IgG residues proximal to the interaction site or throughout the protein [47,48]. Adding increasing

amounts of the Z domain to [^{15}N]-IgG1 Fc showed substantial chemical shift perturbations throughout the 2d NMR spectra (Figure 4.6). Most peaks exhibited intermediate to slow chemical exchange kinetics, though a few with small chemical shift perturbations showed intermediate to

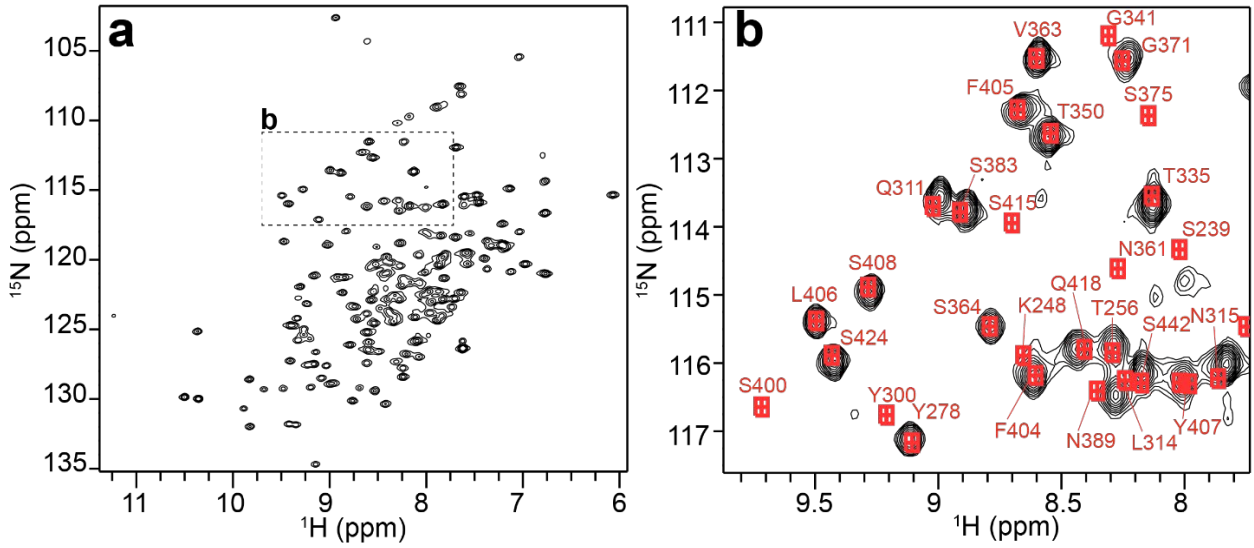

Figure 4.5. Solution NMR of the [^{15}N]-labeled IgG1 Fc expressed from the engineered EBY100-Kar2p-F2A-PDI yeast strain (5% YP-Gal). **a**. ^{1}H-^{15}N HSQC-TROSY of Fc at 21.1 T and 50 ºC. **b**. The enlarged section shows similarity between the observed peaks and those assigned by Kato and coworkers (BMRB – 27453). Labels indicate amino acid residue position and type.

to rapid exchange (Figure 4.6b/c), consistent with an expected affinity of 10-50 nM [49]. Mapping the shifted residues to a structural model of the IgG1 Fc domain revealed the sites most impacted by binding, including those in slow exchange, are found predominantly near the Z domain binding site (Figure 4.6d). Interestingly, the A strand was also affected by titration; this strand normally interacts with distal portions of the IgG1 Fc N-glycan [50,51]. Unaffected peaks are primarily located far from the binding site and at the dimer interface formed by the Cg3 domains. These spectra demonstrate clear binding of the Z domain and chemical shift perturbations throughout the IgG1 Fc domain.

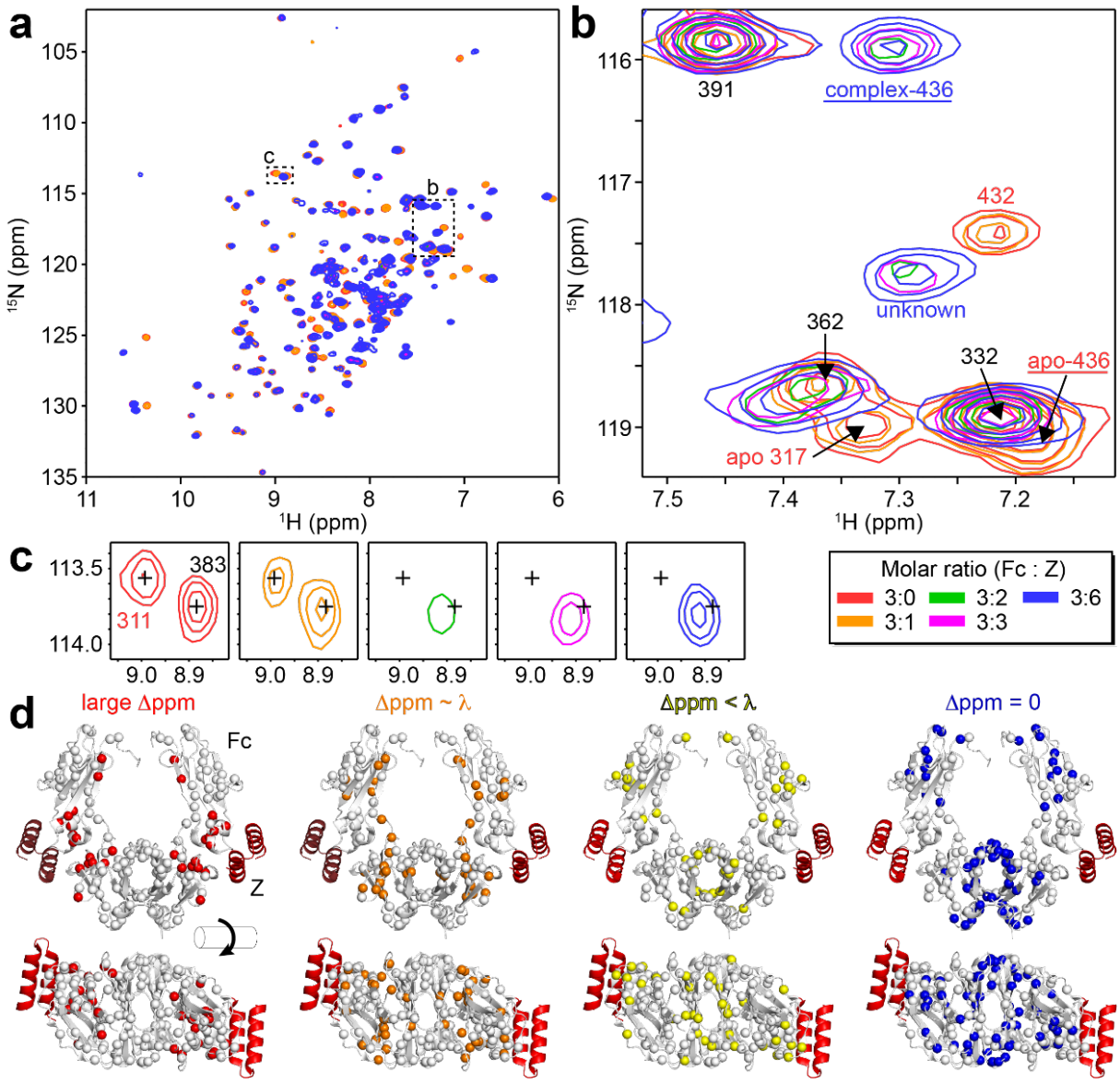

Figure 4.6 Interaction of [^{15}N]-labeled IgG1 Fc (2% YP-Gal) with the Z domain. **a**. ^{1}H-^{15}N HSQC-TROSY spectra collected during an Fc-Z titration; molar ratios are indicated below panel (**b**). **b**. Section enlarged from panel (**a**) to show large displacement of the Y436 peak. The assignment for the 436 peak in the complexed spectrum was made based on Barb and Subedi (2016) JBNMR 64(1):75-85. **c**. Section enlarged to show fast exchange for the 383 peak, but slow exchange for the 311 peak. **d**. Peaks with similar chemical shift perturbation regimes mapped onto the IgG1 Fc structure (white ribbon, from pdb 5vu0) showing the location of Z domain binding, (red ribbon, two of three helices shown from pdb 1l6x).

4.4. Discussion

Here we describe two advances in protein production for NMR spectroscopy. First, we developed a *S. cerevisiae* strain with increased capacity to secrete a complex glycosylated and disulfide-bonded heterooligomeric antibody, rituximab. Although antibodies have been purified in *P. pastoris*, no attempt has focused on isotope labeling antibodies using this expression platform thus far [52]. It is important to note that overexpression of PDI and Kar2p may not always boost expression levels, and the expression of each protein will likely require some optimization to produce maximal yields.

Second, we also report the application of this strain to express IgG1 Fc using a minimal medium formulation that incorporated ^{15}N from a single metabolic precursor. It is possible that this approach will be applicable to the expression and labeling of other proteins, particularly naturally secreted proteins. It is also notable that the medium formulation does provide the capability to incorporate ^{13}C labels using [^{13}C]-galactose. Unfortunately, [^{13}C]-galactose is prohibitively expensive for large cultures and other strategies will be required for inexpensive metabolic ^{13}C labeling in this system.

Heterologous protein expression in yeast usually utilizes *P. pastoris* that can produce large quantities of protein, though limitations as noted above are well described. Accordingly, several groups developed labeling conditions for NMR spectroscopy. In most cases, protein expression levels were significantly boosted by the use of fermenters and other specialized equipment in comparison to the shake flask method which yield amounts comparable to the amount obtained in this study (~3-4 mg/L)(Kjaergaard et al. 2007a; Ali et al. 2019a). In comparison, there are fewer labeling protocols and examples using *S. cerevisiae*, despite the wide adoption of this yeast for

biological research and relative ease of genetic manipulation. Furthermore, S. cerevisiae is easily transformed and readily expresses proteins from plasmids into growth medium with neutral pH,

unlike *P. pastoris*. These data suggest *S. cerevisiae* has the potential to be an important expression host for NMR spectroscopy with the capability to perform essential post-translational modifications and an ability to produce properly folded mammalian proteins. This work represents an advance in an underdeveloped application of *S. cerevisiae*.

In recent years, state legislatures in the United States have considered bills that would block the use of fetal tissue for any purpose, including HEK293 cells that are of fetal origin. HEK293 cells are a vital resource for glycoprotein expression, and though limited, for isotope labeled proteins. The development of novel scientific resources, including yeast platforms to express and label glycoproteins, may provide an important alternative should one of these bills be made into law.

4.5 References

1. Kleckner, I.R.; Foster, M.P. An Introduction to NMR-Based Approaches for Measuring Protein Dynamics. *Biochim Biophys Acta Proteins Proteom* 2011, *1814*, 942–968.

2. Neudecker, P.; Lundström, P.; Kay, L.E. Relaxation Dispersion NMR Spectroscopy as a Tool for Detailed Studies of Protein Folding. *Biophys J* 2009, *96*, 2045–2054.

3. Boehr, D.D.; Dyson, H.J.; Wright, P.E. An NMR Perspective on Enzyme Dynamics. *Chem Rev* **2006**, *106*, 3055–3079, doi:10.1021/cr050312q.

4. Yanaka, S.; Yagi, H.; Yogo, R.; Yagi-Utsumi, M.; Kato, K. Stable Isotope Labeling Approaches for NMR Characterization of Glycoproteins Using Eukaryotic Expression Systems. *J Biomol NMR* **2018**, *71*, 193–202, doi:10.1007/s10858-018-0169-2.

5. Yao, Y.; Wang, J.; Viroonchatapan, N.; Samson, A.; Chill, J.; Rothe, E.; Anglister, J.; Wang, Z.Z. Yeast Expression and NMR Analysis of the Extracellular Domain of Muscle Nicotinic Acetylcholine Receptor α Subunit. *Journal of Biological Chemistry* **2002**, *277*, 12613–12621, doi:10.1074/jbc.M108845200.

6. Yamaguchi, Y.; Nishimura, M.; Nagano, M.; Yagi, H.; Sasakawa, H.; Uchida, K.; Shitara, K.; Kato, K. Glycoform-Dependent Conformational Alteration of the Fc Region of Human Immunoglobulin G1 as Revealed by NMR Spectroscopy. *Biochim Biophys Acta Gen Subj* **2006**, *1760*, 693–700, doi:10.1016/j.bbagen.2005.10.002.

7. Yagi, H.; Zhang, Y.; Yagi-Utsumi, M.; Yamaguchi, T.; Iida, S.; Yamaguchi, Y.; Kato, K. Backbone 1H, 13C, and 15N Resonance Assignments of the Fc Fragment of Human Immunoglobulin G Glycoprotein. *Biomol NMR Assign* **2015**, *9*, 257–260, doi:10.1007/s12104-014-9586-7.

8. Yanaka, S.; Yamazaki, T.; Yogo, R.; Noda, M.; Uchiyama, S.; Yagi, H.; Kato, K. NMR Detection of Semi-Specific Antibody Interactions in Serum Environments. *Molecules* **2017**, *22*, doi:10.3390/molecules22101619.

9. Fan, Y.; Emami, S.; Munro, R.; Ladizhansky, V.; Brown, L.S. Isotope Labeling of Eukaryotic Membrane Proteins in Yeast for Solid-State NMR. In *Methods in Enzymology*; Academic Press Inc., 2015; Vol. 565, pp. 193–212.

10. Rodriguez, E.; Rama Krishna, N. *An Economical Method for 15 N/ 13 C Isotopic Labeling of Proteins Expressed in Pichia Pastoris 1*; 2001; Vol. 130;.

11. Sugiki, T.; Ichikawa, O.; Miyazawa-Onami, M.; Shimada, I.; Takahashi, H. Isotopic Labeling of Heterologous Proteins in the Yeast Pichia Pastoris and Kluyveromyces Lactis. *Methods in Molecular Biology* **2012**, *831*, 19–36, doi:10.1007/978-1-61779-480-3_2.

12. Munro, R.; de Vlugt, J.; Ladizhansky, V.; Brown, L.S. Improved Protocol for the Production of the Low-Expression Eukaryotic Membrane Protein Human Aquaporin 2 in Pichia Pastoris for Solid-State NMR. *Biomolecules* **2020**, *10*, doi:10.3390/biom10030434.

13. Elkins, M.R.; Sergeyev, I. v.; Hong, M. Determining Cholesterol Binding to Membrane Proteins by Cholesterol 13C Labeling in Yeast and Dynamic Nuclear Polarization NMR. *J Am Chem Soc* **2018**, *140*, 15437–15449, doi:10.1021/jacs.8b09658.

14. Cereghino, J. L. & Cregg, J. M. *Heterologous protein expression in the methylotrophic yeast Pichia pastoris*. *FEMS Microbiology Reviews*, Volume 24, Issue 1, January 2000, Pages 45–66, https://doi.org/10.1111/j.1574-6976.2000.tb00532.x

15. Kjaergaard, M.; Gårdsvoll, H.; Hirschberg, D.; Nielbo, S.; Mayasundari, A.; Peterson, C.B.; Jansson, A.; Jørgensen, T.J.D.; Poulsen, F.M.; Ploug, M. Solution Structure of

Recombinant Somatomedin B Domain from Vitronectin Produced in Pichia Pastoris . *Protein Science* **2007**, *16*, 1934–1945, doi:10.1110/ps.072949607.

16. Ali, R.; Clark, L.D.; Zahm, J.A.; Lemoff, A.; Ramesh, K.; Rosenbaum, D.M.; Rosen, M.K. Improved Strategy for Isoleucine 1H/13C Methyl Labeling in Pichia Pastoris. *J Biomol NMR* **2019**, *73*, 687–697, doi:10.1007/s10858-019-00281-1.

17. Parapouli, M.; Vasileiadis, A.; Afendra, A.S.; Hatziloukas, E. Saccharomyces Cerevisiae and Its Industrial Applications. *AIMS Microbiol* 2020, *6*, 1–31.

18. Xie, Y.; Han, X.; Miao, Y. An Effective Recombinant Protein Expression and Purification System in Saccharomyces Cerevisiae. *Curr Protoc Mol Biol* **2018**, *123*, doi:10.1002/cpmb.62.

19. Kulagina, N.; Besseau, S.; Godon, C.; Goldman, G.H.; Papon, N.; Courdavault, V. Yeasts as Biopharmaceutical Production Platforms. *Frontiers in Fungal Biology* **2021**, *2*, doi:10.3389/ffunb.2021.733492.

20. Chiba, Y.; Suzuki, M.; Yoshida, S.; Yoshida, A.; Ikenaga, H. Production of Human Compatible High Mannose-Type (Man 5 GlcNAc 2) Sugar Chains in Saccharomyces Cerevisiae *. **1998**, *273*, 26298–26304.

21. Kamiya, Y.; Yamamoto, S.; Chiba, Y.; Jigami, Y.; Kato, K. Overexpression of a Homogeneous Oligosaccharide with 13C Labeling by Genetically Engineered Yeast Strain. *J Biomol NMR* **2011**, *50*, 397–401, doi:10.1007/s10858-011-9525-1.

22. Wall, K.P.; Hough, L.E. In-Cell NMR within Budding Yeast Reveals Cytoplasmic Masking of Hydrophobic Residues of FG Repeats. *Biophys J* **2018**, *115*, 1690–1695, doi:10.1016/j.bpj.2018.08.049.

23. Wigzell, H. *Biological Significance of Carbohydrate Chains on Monoclonal Antibodies (Tunicamycin/Fc Receptor/Complement/Protein A/Immunocomplex)*; 1983; Vol. 80;.

24. Subedi, G.P.; Barb, A.W. The Structural Role of Antibody N-Glycosylation in Receptor Interactions. *Structure* **2015**, *23*, 1573–1583, doi:10.1016/j.str.2015.06.015.

25. Huber, R., Deisenhofer, J., Colman, P. *et al.* Crystallographic structure studies of an IgG molecule and an Fc fragment. *Nature* **264,** 415–420 (1976). https://doi.org/10.1038/264415a0

26. Yamaguchi Y, Kim H, Kato K, Masuda K, Shimada I, Arata Y. Proteolytic fragmentation with high specificity of mouse immunoglobulin G. Mapping of proteolytic cleavage sites in the hinge region. J Immunol Methods. 1995 Apr 26;181(2):259-67. doi: 10.1016/0022-1759(95)00010-8. PMID: 7745255.

27. Sawada J, Terao T, Itoh S, Maeda M, Tsuji A, Hosoda H, Nambara T. Production and characterization of monoclonal antibodies to 17 alpha-hydroxyprogesterone. J Steroid Biochem. 1987 Oct;28(4):405-10. doi: 10.1016/0022-4731(87)91058-2. PMID: 3312820

28. Yanaka, S.; Yagi, H.; Yogo, R.; Onitsuka, M.; Kato, K. Glutamine-Free Mammalian Expression of Recombinant Glycoproteins with Uniform Isotope Labeling: An Application for NMR Analysis of Pharmaceutically Relevant Fc Glycoforms of Human Immunoglobulin G1. *J Biomol NMR* **2022**, doi:10.1007/s10858-021-00387-5.

29. Rakestraw, J.A.; Sazinsky, S.L.; Piatesi, A.; Antipov, E.; Wittrup, K.D. Directed Evolution of a Secretory Leader for the Improved Expression of Heterologous Proteins and Full-Length Antibodies in Saccharomyces Cerevisiae. *Biotechnol Bioeng* **2009**, *103*, 1192–1201, doi:10.1002/bit.22338.

30. Shenoy, A.; Yalamanchili, S.; Davis, A.R.; Barb, A.W. Expression and Display of Glycoengineered Antibodies and Antibody Fragments with an Engineered Yeast Strain. *Antibodies* **2021**, *10*, doi:10.3390/antib10040038.

31. Gietz, R.D.; Woods, R.A. Transformation of Yeast by Lithium Acetate/Single-Stranded Carrier DNA/Polyethylene Glycol Method. *Methods Enzymol* **2002**, *350*, 87–96, doi:10.1016/S0076-6879(02)50957-5.

32. Meng, L.; Forouhar, F.; Thieker, D.; Gao, Z.; Ramiah, A.; Moniz, H.; Xiang, Y.; Seetharaman, J.; Milaninia, S.; Su, M.; et al. Enzymatic Basis for N-Glycan Sialylation: Structure of Rat A2,6-Sialyltransferase (ST6GAL1) Reveals Conserved and Unique Features for Glycan Sialylation. *Journal of Biological Chemistry* **2013**, *288*, 34680–34698, doi:10.1074/jbc.M113.519041.

33. Barb, A.W.; Subedi, G.P. An Encodable Lanthanide Binding Tag with Reduced Size and Flexibility for Measuring Residual Dipolar Couplings and Pseudocontact Shifts in Large Proteins. *J Biomol NMR* **2016**, *64*, 75–85, doi:10.1007/s10858-015-0009-6.

34. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J Biomol NMR. 1995 Nov;6(3):277-93. doi: 10.1007/BF00197809. PMID: 8520220.

35. Johnson BA. Using NMRView to visualize and analyze the NMR spectra of macromolecules. Methods Mol Biol. 2004;278:313-52. doi: 10.1385/1-59259-809-9:313. PMID: 15318002.

36. Shusta EV, Raines RT, Plückthun A, Wittrup KD. Increasing the secretory capacity of Saccharomyces cerevisiae for production of single-chain antibody fragments. Nat Biotechnol. 1998 Aug;16(8):773-7. doi: 10.1038/nbt0898-773. PMID: 9702778.

37. Hou, J.; Tyo, K.E.J.; Liu, Z.; Petranovic, D.; Nielsen, J. Metabolic Engineering of Recombinant Protein Secretion by Saccharomyces Cerevisiae. *FEMS Yeast Res* **2012**, *12*, 491–510, doi:10.1111/j.1567-1364.2012.00810.x.

38. Rakestraw, A.; Wittrup, K.D. Contrasting Secretory Processing of Simultaneously Expressed Heterologous Proteins in Saccharomyces Cerevisiae. *Biotechnol Bioeng* **2006**, *93*, 896–905, doi:10.1002/bit.20780.

39. Mei, M.; Li, J.; Wang, S.; Lee, K.B.; Iverson, B.L.; Zhang, G.; Ge, X.; Yi, L. Prompting Fab Yeast Surface Display Efficiency by ER Retention and Molecular Chaperon Co-Expression. *Front Bioeng Biotechnol* **2019**, *7*, 1–11, doi:10.3389/fbioe.2019.00362.

40. de Felipe, P.; Hughes, L.E.; Ryan, M.D.; Brown, J.D. Co-Translational, Intraribosomal Cleavage of Polypeptides by the Foot-and-Mouth Disease Virus 2A Peptide. *Journal of Biological Chemistry* **2003**, *278*, 11441–11448, doi:10.1074/jbc.M211644200.

41. Cruz-Teran, C.A.; Tiruthani, K.; Mischler, A.; Rao, B.M. Inefficient Ribosomal Skipping Enables Simultaneous Secretion and Display of Proteins in Saccharomyces Cerevisiae. *ACS Synth Biol* **2017**, *6*, 2096–2107, doi:10.1021/acssynbio.7b00144.

42. Yu KK, Aguilar K, Tsai J, Galimidi R, Gnanapragasam P. Use of Mutated Self-Cleaving 2A Peptides as a Molecular Rheostat to Direct Simultaneous Formation of Membrane and Secreted Anti-HIV Immunoglobulins. *PLoS ONE* **7**, (2012). e50438. https://doi.org/10.1371/journal.pone.0050438

43. Liu, Z.; Chen, O.; Wall, J.B.J.; Zheng, M.; Zhou, Y.; Wang, L.; Ruth Vaseghi, H.; Qian, L.; Liu, J. Systematic Comparison of 2A Peptides for Cloning Multi-Genes in a Polycistronic Vector. *Sci Rep* **2017**, *7*, doi:10.1038/s41598-017-02460-2.

44. Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi BK, Hopkins D, Wischnewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S, Gerngross TU. Humanization of yeast to produce complex terminally sialylated glycoproteins. Science. 2006 Sep 8;313(5792):1441-3. doi: 10.1126/science.1130256. PMID: 16960007.

45. Kao, D.; Danzer, H.; Collin, M.; Groß, A.; Eichler, J.; Stambuk, J.; Lauc, G.; Lux, A.; Nimmerjahn, F. A Monosaccharide Residue Is Sufficient to Maintain Mouse and Human IgG Subclass Activity and Directs IgG Effector Functions to Cellular Fc Receptors. *Cell Rep* **2015**, *13*, 2376–2385, doi:10.1016/j.celrep.2015.11.027.

46. Nilsson B, Moks T, Jansson B, Abrahmsén L, Elmblad A, Holmgren E, Henrichson C, Jones TA, Uhlén M. A synthetic IgG-binding domain based on staphylococcal protein A. Protein Eng. 1987 Feb-Mar;1(2):107-13. doi: 10.1093/protein/1.2.107.

47. Tashiro M, Tejero R, Zimmerman DE, Celda B, Nilsson B, Montelione GT. High-resolution solution NMR structure of the Z domain of staphylococcal protein A. J Mol Biol. 1997 Oct 3;272(4):573-90. doi: 10.1006/jmbi.1997.1265.

48. Idusogie, E.E.; Presta, L.G.; Gazzano-Santoro, H.; Totpal, K.; Wong, P.Y.; Ultsch, M.; Meng, Y.G.; Mulkerrin, M.G. Mapping of the C1q Binding Site on Rituxan, a Chimeric Antibody with a Human IgG1 Fc. *The Journal of Immunology* **2000**, *164*, 4178–4184, doi:10.4049/jimmunol.164.8.4178.

49. Cedergren L, Andersson R, Jansson B, Uhlén M, Nilsson B. Mutational analysis of the interaction between staphylococcal protein A and human IgG1. *Protein Eng* (1993). Jun;6(4):441-8. doi: 10.1093/protein/6.4.441

50. Barb, A.W.; Prestegard, J.H. NMR Analysis Demonstrates Immunoglobulin G N-Glycans Are Accessible and Dynamic. *Nat Chem Biol* **2011**, *7*, 147–153, doi:10.1038/nchembio.511.

51. Subedi, G.P.; Hanson, Q.M.; Barb, A.W. Restricted Motion of the Conserved Immunoglobulin G1 N-Glycan Is Essential for Efficient FcγRIIIa Binding. *Structure* **2014**, *22*, 1478–1488, doi:10.1016/j.str.2014.08.002.

52. Schaefer, J. v.; Plückthun, A. Engineering Aggregation Resistance in IgG by Two Independent Mechanisms: Lessons from Comparison of Pichia Pastoris and Mammalian Cell Expression. *J Mol Biol* **2012**, *417*, 309–335, doi:10.1016/j.jmb.2012.01.027.

CHAPTER 5

ENGINEERING AGLYCOSYLATED Fc VARIANTS WITH DISULPHIDE LINKAGES TO ENGAGE FC γ RECEPTOR IIIA

Abstract:

Antibody Dependent Cellular Cytotoxicity (ADCC) is an important mechanism utilized by monoclonal antibodies to achieve therapeutic activity. This mechanism involves Immunoglobulin IgG1 and FcγRIIIa found on the surface of macrophages and monocytes. This interaction has a strict N-glycan requirement. The Fc N-glycan stabilizes the C'E loop which forms a contact surface with FcγRIIIa. Removal of the antibody N-glycan destabilizes this loop and disrupts receptor binding. Thus, the N-glycan required and has restricted expression of immunoglobulins to mammalian expression platforms. In addition to this, work from our group has shown that the FG and C'E loop (loops in the Fc Cγ2 domain) form stabilizing intramolecular non-covalent interactions in mouse Fc. Due to the high degree of similarity between mouse and human Fc, it is possible that this relationship is conserved in human Fc. In this work, we aim to create aglycosylated Fc variants by stabilizing the C'E through disulphide linkages with different loops (BC, FG) in the Cγ2 domain of Fc. We diversified these variants through yeast surface display (YSD) to improve affinity. Through this approach we recovered aglycosylated Fc clones with ~2-4 μM affinity to the FcγRIIIa. These affinities are comparable to previously discovered aglycosylated Fc variants. We also believe that the engineered disulfides within the Cγ2 domain will confer a high degree of thermostability to these variants. Future analysis will include

expressing these Fc clones as full-length antibodies and characterizing their function through an ADCC assay.

5.1 Introduction

Antibody Dependent Cellular Cytotoxicity (ADCC) is an important mechanism for immunotherapy. ADCC, which plays an important role in tumor clearance requires interactions between target-coated antibodies and cell surface antibody-binding receptors (Lee et al. 2017). Once the target cells are completely encapsulated by antibodies in the serum (opsonization), the immune effector cells are activated and destroy the target cells by releasing granzyme and perforin. Several groups are working towards understanding and increasing the affinity of the interaction between the Fc domain of the antibody and the receptor FcγRIIIa [2]– [7].

Analyzing the crystal structure between Fc and FcγRIIIa, making targeted mutations at the binding interface was one of the approaches employed to improve binding affinities with receptor by mutating key residue required for this interaction, however this approach does not probe the residues outside the binding interface that can influence binding affinities (Sondermann et al. 2000; Shields et al. 2001a). Some examples include IgG1 Fc residues P329 and D265 that form contacts with FcγRIIIa and the Fc N-glycan respectively, mutations to which abolish receptor binding (Shields et al. 2001a). Another group employed computational modeling to design variants with enhanced FcγRIIIa binding. The variants obtained were then tested for various antibody-elicited immune responses including ADCC, CDC and Antibody Dependent Cellular Phagocytosis (ADCP) (Lazar et al. 2006).In addition to potential limitations, designing Fc variants with optimized Activating/Inhibiting ratios (A/I) is challenging considering the high degree of homology between the various FcγR receptors (93%) (Richards et al. 2008). The activating aspect of this ratio is determined by affinities that Fc has towards activating receptors such as FcγRIIIa

(activating immune response) and the inhibiting response is determined by affinities towards inhibiting receptors such as FcγIIIB.

Some groups have reported success by combining beneficial mutations to create novel Fc variants with enhanced receptor binding. Some notable examples are the GASDALIE (3-fold increase in affinity) and SDALIE Fc variants with enhanced receptor binding created by Bjorkman and coworkers (Ahmed et al. 2016). Another approach involved breaking the symmetry of the homodimeric Fc by incorporating distinct alpha and beta polypeptides with the rationale that the IgG1 Fc domain engages asymmetrically with the FcγIIIa receptor (Mimoto et al. 2013; Liu et al. 2014). Most approaches described thus far are not high-throughput and would only allow for the screening of a limited number of variants at a time. Yeast Surface Display provides a powerful alternative where 10^7-10^8 variants can be screened (Boder and Wittrup 1997a). Stavenhagen and co-workers utilized this platform to discover a glycosylated Fc variant with enhanced receptor binding Fc variant 18 (F243L/R292P/Y300L/V305I/P396L) (Nordstrom et al. 2011a). However, all Fc variants described are N-glycosylated. The N-glycan plays an important role in stabilizing the C'E loop that forms a contact with the FcγRIIIa receptor (Subedi and Barb 2015b). Elimination of this N-glycan leads to the collapse of this loop abrogating FcγIIIa binding. This has largely restricted the expression of antibodies to mammalian expression systems (Examples: Human Embryonic Kidney (HEK) cells and Chinese Hamster Ovarian (CHO) cells) which require specialized culture equipment and are prone to contamination.

Salzinsky and co-workers discovered aglycosylated Fc variants that could engage receptor and showed that the Fc N-glycan is not strictly required for FcγIIIa receptor binding (Sazinsky et al. 2008a)(Chen et al. 2017a). Jung and co-workers used a combination of mutations found in both glycosylated and aglycosylated contexts to design aglycosylated Fc with increased FcγRIIIa

binding (Yoon et al. 2019a). When Fc is glycosylated, mutations are mostly localized in the hinge and the upper Cγ2 domain (Cγ2 and Cγ3 domains make up the Fc), whereas when Fc is aglycosylated, mutations are typically distributed throughout (Yoon et al. 2019b).

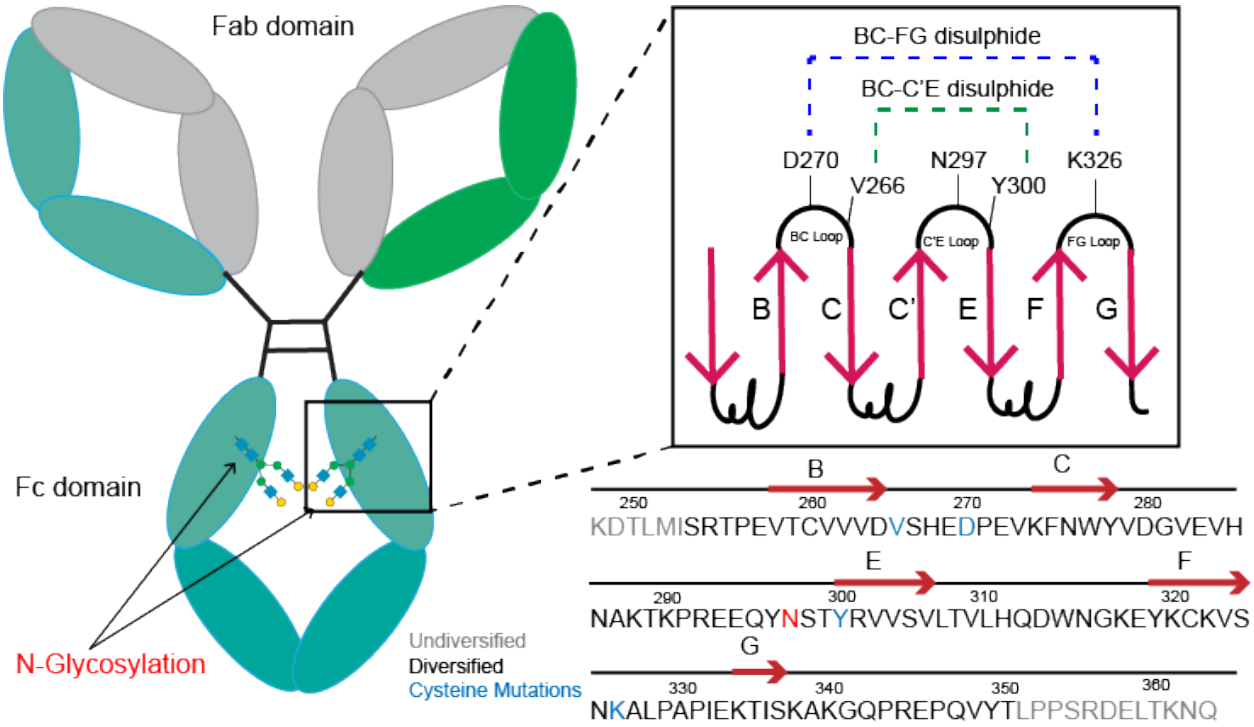

Figure 5.1. Structure and engineering of IgG1 Fc to possess disulphide linkages. IgG1 antibody interacts with FcγIIIa receptor through its Fc domain. The Fc domain is split into two domains – Cγ2 and Cγ3 domains. The Cγ2 domain contains the N297 residue which is a conserved site of N-glycosylation. This figure highlights the location of the three loops in the Cγ2 domain – BC, C'E and FG along with the location of the cysteine mutations within these loops.

Work from our group has shown that mutations in the flanking loops (BC and FG loops) in mouse Fc influence the chemical environment (observed through NMR) and hence affect receptor binding. This result indicates that these loops are possibly interconnected (Falconer and Barb 2018a). Mouse Fc shares a high degree of similarity to human Fc with the highest similarity reported in the cγ2 domain. Therefore, human and mice Fc domains could share similarities in behavior. In this work, we describe a novel approach to create aglycosylated Fc domains by linking

the BC, C'E and FG loop (in the cγ2 Fc domain) through disulfide bonds. Most groups have identified enhanced variants by chance, in this approach we aim to directly address the destabilization of the C'E loop by engineering disulfide bonds between the loops (Figure 5.1). In addition, we believe the addition of these disulfide bonds in the Fc would enhance thermostability of this domain (Wozniak-Knopp et al. 2010, 2012).

5.2. Materials and Methods

5.2.1 Strain and Media

The yeast surface display strain, EBY100 [ATCC-MYA4941] (GAL1-AGA1:URA3 ura3-52 trp1 leu2Δ1 his3 Δ200 pep4: HIS2 prb1 Δ1.6R can1 GAL), was used to create the Fc disulphide mutants and create libraries and purchased from ATCC. EBY100 cells were grown in rich medium YPD (Sigma Y1375) prior to transformation. Cells transformed with the yeast surface display plasmid pYD1 were plated on Minimal Dextrose plates (0.67g/L Yeast Nitrogen Base without Amino Acids (Sigma Y0626), 0.1 mg/L Leucine and 2% Glucose). Yeast Surface Display Libraries were grown/induced in YNB-CAA [6.7g/L Yeast Nitrogen Base Without Amino Acids (Sigma Y0626) and 0.5 g/L Casamino acids (Millipore Sigma CAS 65072-00-6)] with 2% glucose/galactose).

5.2.2 *Creation and Surface Expression Determination of Disulphide variants*

The Fc disulphide mutants - Fc T299A/D270C/K326C, Fc T299A/V266C/Y300C and Fc T299A/D270C/K326C/V266C/Y300C were created using Site Directed Mutagenesis (SDM) through fusion PCR(Hoa et al. 1989). The fusion PCR product was cloned into the pYD1 plasmid backbone using the Nhe1 and EcoR1restriction sites. The mutations within the Fc insert were confirmed through DNA Sequencing. These plasmids were transformed into EBY100 cells through standard yeast transformation protocol (Gietz and Schiestl 2007) A yeast colony screen

was used to determine positive transformants. A positive clone was grown in overnight at 30℃ in YNB-CAA + 2 % glucose and then induced at an OD_{600}= 0.5 in YNB-CAA +2% galactose. To determine Fc surface expression, around 10^6 induced cells were used to prepare flow cytometry samples. The cells were spun down at 1500 g for 1 min to remove the supernatant. The pellet was washed with 100 µl 1xPBS followed by a wash with 100 µl 1x PBSA (1x PBS +1% BSA). The cells were then incubated with Rabbit Anti hIgG (Primary Antibody) for an hour in the dark at 4℃. The cells were then washed with 100 µl of 1x PBSA and then incubated with PE Anti-Rabbit (Secondary Antibody) for another hour at 4 ℃ in the dark. These samples were run on the FACSCanto to determine surface expression of the three individual Fc disulphide variants.

5.2.3 *Creation of Yeast Surface Display (YSD) libraries*

To minimize the amount of unmutated colonies within the library that result from acarryover intact plasmid template, we amplified separate fragments of the pYD1 plasmid to contain the selection marker (TRP1), the CEN/ARS element and BC-FG (Fc) fragment respectively. The plasmid fragments (TRP1 and CEN/ARS) were amplified using Pfu Turbo. The BC-FG fragment was diversified using error-prone PCR (epPCR). This was achieved using Taq Polymerase with low concentrations of template (100 pg) with 5mM $MgCl_2$ and 0.5 mM $MnCl_2$. The epPCR products were gel extracted using a Qiagen Gel Extraction kit. The epPCR gel extracted products and the backbone fragments were used to transform EBY100 through standard yeast transformation protocols (Gietz and Woods 2002).

5.2.4 *Sorting and diversifying YSD Libraries*

The library was expanded in YNB-CAA +2% glucose overnight and then induced in YNB-CAA +2% galactose for 24 h. For sorting, approximately 10^7 cells were stained with 363 nM Protein A-AF647 and 2 µM GFP-CD16a. AF647-Protein A reagent was used to detect Fc surface expression.

In the first round of sorting, 2% of the top double positive cells AF647(+) GFP (+) were selected. The sorted cells were then grown up in ~1ml of YNB-CAA +2% glucose. These cells were then miniprepped using the Zymoprep kit to isolate plasmid. This plasmid was then used as a template for the next round of mutagenesis. In subsequent rounds of sorting, 0.1% of the top double positive cells were selected. This process was repeated until we no longer saw enrichment of the double positive cell population. The enriched library was diluted and plated onto minimal dextrose plates to isolate individual colonies. The colonies were then inoculated into a 96-well plate containing ~200µl YNB-CAA +2% glucose and was sent to Genewiz for DNA Sequencing.

5.2.5 Expressing selected clones as full-length Antibodies

DNA constructs for the Fc regions of Yeast Surface Display Clones – Clone 8, 11 and 16 (Supplementary Material) and the beginning portion of Rituximab Heavy chain RTX HC were synthesized by IDT. The starting HC fragment and each of the Fc were amplified individually using Pfu Polymerase (Agilent) and then combined through fusion PCR using 2x GoTaq (Promega). These full-length HC DNA fragments were then amplified to include restriction sites for EcoR1 and BstX1 to clone into pEF1. The ligation products pEF1-HC (Clone 8/11/16) were transformed into DH5α cells, colony screened by colony PCR, and their sequences were verified through Sanger Sequencing. Qiagen Mega Prep Kit was used to recover sufficient amount of DNA to transfect HEK293F cells. Similarly, the Rituximab light chain (RTX LC) was synthesized by IDT and was amplified to include restriction sites Not1 and HindIII to clone into vector pGENT2. The ligation product pGENT2-RTX-LC was transformed into transformed into DH5α cells, screened by colony PCR, and their sequences were verified through Sanger Sequencing. Qiagen Mega Prep Kit was used to recover sufficient amount of DNA to transfect HEK293F cells.

5.2.6 Surface Plasmon Resonance for the selected clones

Rituximab was immobilized on a CM5 chip (Cytiva) as previously described in (insert reference) using a BiaCore T200 instrument (Cytiva). Increasing concentrations of GFP-FcγRIIIa were applied to the chip surface and each concentration was continuously applied until equilibrium was reached. Affinity constants were calculated using the T200 system software after double normalization (first to the surface-deactivated reference channel and then to a sample with zero concentration collected using the lane of interest) and a 1:1 binding model with time zero = zero response units. Data were averaged from a 5 s window after equilibrium was reached. Reported errors indicate errors of the curve fitting procedure.

5.2.7 Antibody Dependent Cellular Cytotoxicity for the selected clones

Raji B-cells (5 x10^6) were resuspended in 5 mL of BATDA Loading buffer (1x PBS, 10% FBS, 20 mM HEPES) with 5 µl of BATDA reagent at 37 ℃, 5% CO2 for 30 minutes. The cells were spun down at 100 g for 5 min and washed with 10 mL of Wash Buffer (1x PBS, 20 mM HEPES). After the wash steps, cells were resuspended in the appropriate volume of Assay Media to get a final cell count of 200,000 cells/mL. Approximately 10,000 cells were used per well of the assay and a small volume of the supernatant was taken out to use for the background controls. A range of 2-8x10^6 YTS-CD16a cells were used to set up this assay. These cells were resuspended in the appropriate volume of assay medium to get a final cell concentration of 4x10^6 cells/mL. Approximately ~20,000 YTS CD16a cells were used to get a Target: Effector cell ratio of 1:20. Antibody was added to wells containing both Raji B-cells and YTS CD16a cells for a final concentration of 20 µg/mL. This plate included controls for background, spontaneous and maximum lysis. The 96-well V-bottomed plate was spun down at 500 rpm for 5 min. The plate was then incubated at 37℃ for 2 hours. Supernatant (20 µl) from each well was transferred to a flat-bottomed plate for detection. The supernatant was mixed with 200 µl europium solution to

each well. The plate reader (BioTek Synergy Neo2) was used to take readings of the plate. The specific lysis was calculated using formulae described in (Miller et al. 2012).

5.3. Results

5.3.1 Creation of Disulphide mutants to create novel Fc variants

The elimination of the N-glycan from the C'E loop of Fc causes loop destabilization and abrogates FcγIIIa binding (Subedi and Barb 2015a). In our tailored approach we aim address this C'E loop destabilization through

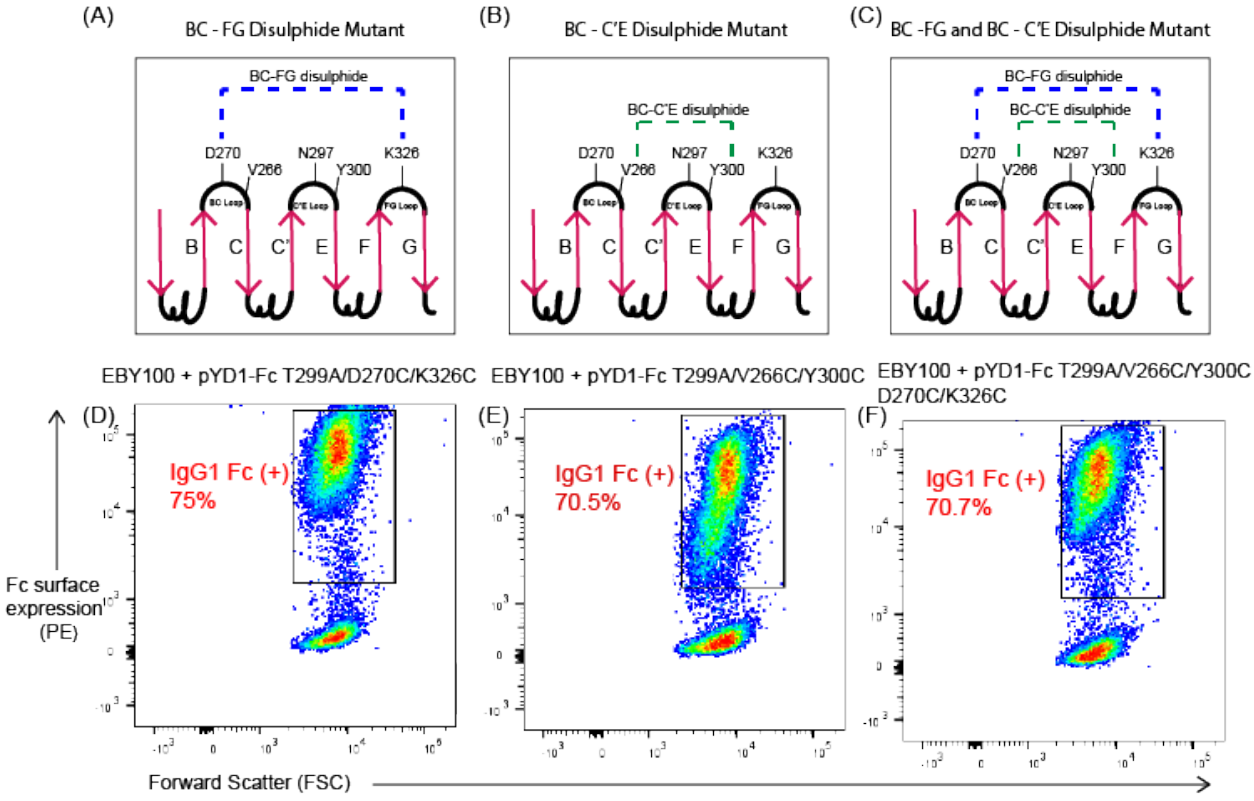

Figure 5.2. Expression of Fc disulphide variants on yeast cell surface Top (A-C) shows the location of the three Fc aglycosylated disulphide variants (a) Fc T299A/D270C/K326C (BC & FG disulphide variant) (B) Fc T299A/V266C/Y300C (BC & C'E disulphide variant) (C) Fc T299A/D270C/K326C/V266C/Y300C (BC & FG and BC & C'E disulphide variant) Bottom (d-f) Shows Fc yeast surface expression of these clones (D) EBY100 + pYD1-Fc T299A/D270C/K326C (E) EBY100 + pYD1-Fc T299A/V266C/Y300C (F) EBY100 + pYD1-Fc T299A/D270C/K326C/V266C/Y300C. The PE fluorescence was used as a readout for Fc surface expression. The Fc expression levels for all three disulphide variants was comparable to Fc wildtype yeast surface expression (data not shown).

the creation of disulphide bonds between loops in the cγ2 domain of Fc. In order to determine residues that could be used for this purpose we used Disulphide by Design (Dombkowski 2003). This software returns residues at the proper orientation to form disulphide bonds. The possible combinations provided were D270C K326C (BC-FG disulphide) and V266C Y300C (BC-C'E disulphide). We characterized these variants following expression using the HEK cell line and purified using a Protein A column. We determined the K_D of these variants for receptor FcγRIIIa through Surface Plasmon Resonance (SPR). Through SPR it was determined that only the D270C K326C (BC-FG disulphide) bound the FcγRIIIa receptor, whereas the other two clones did not (data not shown).

In addition to this, we expressed these three disulphide variants (aglycosylated) on the yeast surface by expressing the respective surface display plasmids in EBY100. Surface expression of these disulphide variants were determined through flow cytometry. We used a PE conjugated Anti-hIgG antibody to detect Fc surface expression as shown in Figure 5.2. All three disulphide variants expressed at levels comparable to the Fc WT on the yeast surface and showed poor FcγRIIIa receptor binding on the yeast surface (data not shown).

5.3.2 Enriching and diversifying library using disulphide mutant as a starting point for the library

We decided to use the double disulphide variant connecting the BC-FG and BC-C'E loops [D270C K326C and V266C Y300C] as a starting point for Yeast Surface Display. Our hypothesis was if the engineered disulphide bond resulted in an improvement in FcγIIIa binding it would be maintained after subsequent rounds of diversification and sorting. To diversify our libraries, we used error-prone PCR to amplify the region from the BC to FG loop of the cγ2 domain in Fc. We

then assembled yeast surface display libraries by combining backbone fragments along with the epPCR product. For sorting we stained our induced library with Protein A-AF647 and 2 µM GFP-FcγRIIIa. We utilized Protein A for staining as it can only bind to properly folded Fc. In the first round we drew a broader gate to select for the 2% $AF647^{+}$ GFP^{+} binders. We used our undiversified double disulphide Fc variants as our reference to draw sorting gates. In subsequent rounds, 0.1 % $AF647^{+}$ GFP^{+} were selected. After four rounds of sorting, we observed a 40-fold enrichment of $AF647^{+}$ GFP^{+} cells compared to our undiversified control shown in Figure 5.3. Further rounds did not lead to an increase in enrichment.

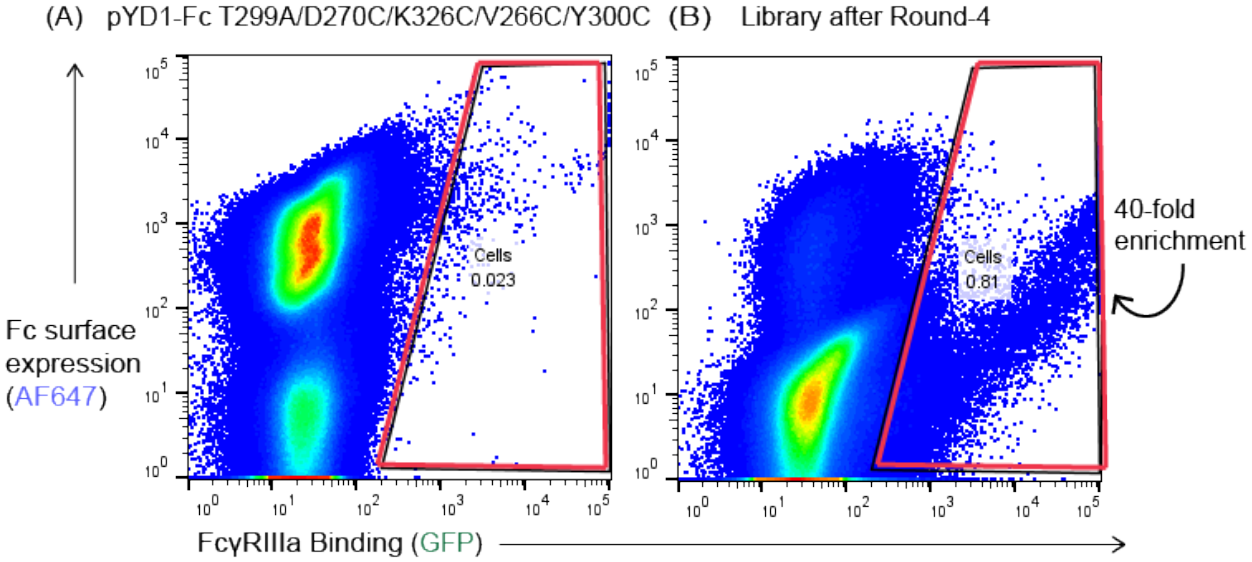

Figure 5.3. Screening of Fc yeast surface display library. Panel (A) shows the starting material of the library – EBY100 + pYD1-Fc T299A/D270C/K326C/V266C/Y300C stained with 2µM FcγIIIa-GFP and Protein A-AF647. (B) Represents the library derived from (a) after four rounds of mutagenesis. The library showed a 40-fold enrichment compared to the wildtype control

5.3.4 Panning to enrich for the best clones within the library

In addition, we enriched for the best binders to help narrow our focus to the best clones within our library. We repeatedly sorted yeast surface display clones for GFP-FcγIIIa binding followed by a sort based on Fc surface expression. A similar approach was used by Georgiou's group, where

they utilized a panning approach to distinguish between low, medium, and high affinity clones within their library(Wang et al. 2018).

5.3.5 DNA sequence analysis of the clones derived from sorting and panning

We analyzed clones using Sanger Sequencing. Through our screening approach, we recovered several diverse full-length Fc clones in which 94% were aglycosylated. Of these aglycosylated clones, 49% of these clones possessed disulphide bonds. The majority of the clones retained the cysteine residues to form a disulphide bond between the BC & C'E loop. A few clones possessed a disulphide bond between the BC & FG loop. We did not recover any clones with all four engineered cysteines remaining.

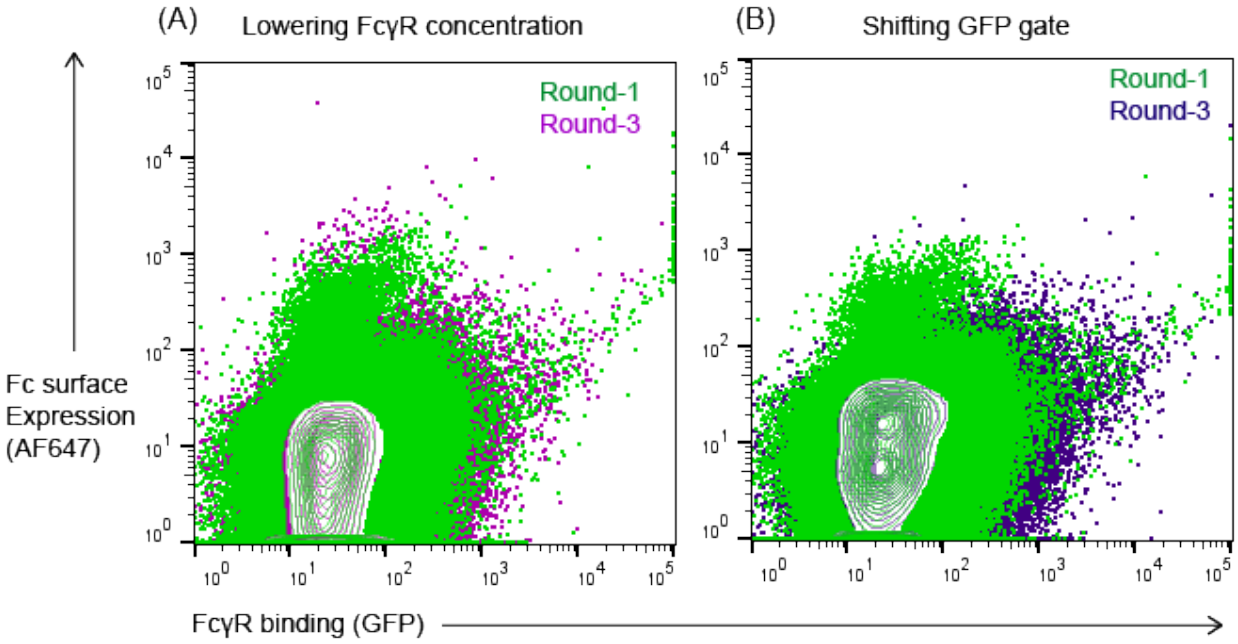

Figure 5.4. Panning Approaches to enrich library.To prioritize the best clones within the library – we utilized a panning approach there were two strategies we used to achieve this. (A) Represents a panning approach where we lowered the concentration of receptor FcγIIIa (Round 1- 1µM; Round-2 – 500 nM; Round 3- 250 nM) (B) Represents a panning approach where during each round of sorting we shifted the GFP axis to select for the best clones within the library

Further sequence analysis of our clones revealed that most of the mutations were distributed throughout the Fc domain with several of them localized in the C'E loop and surrounding regions

(Figure 5). This observation is consistent with reports from several groups who have previously created aglycosylated clones (see Table 4.1 and Figure 5.5). However, we observed a number of mutations localized in the BC loop. This result is interesting considering mutations have mostly been found localized in the C'E and FG loops and our clones represent an inversion of that trend.

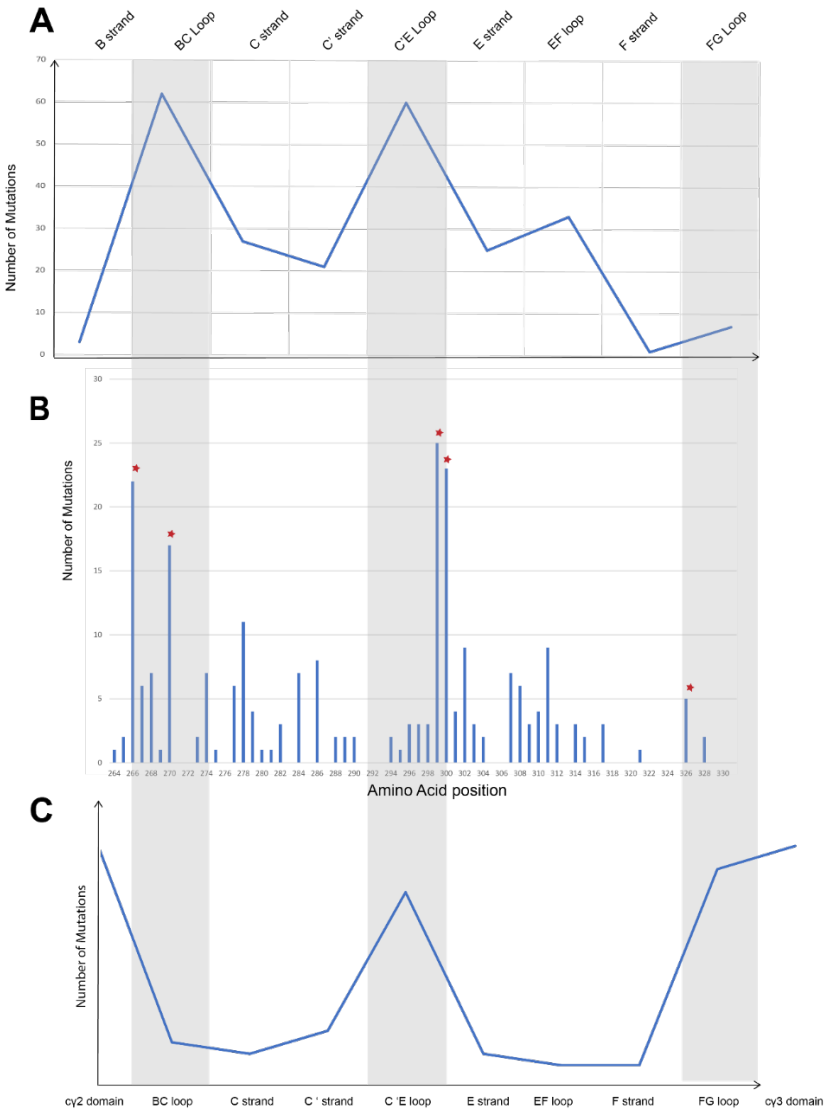

Figure 5.5. Distribution of mutations in different portions of the Fc domain (A) Represents the distribution of mutations in different portions of the Fc domain. Through this plot, we can observe that we had a higher number of mutations in the BC and C'E loop and fewer in the FG loop. (B) Represents the location of the individual mutations at specific amino acid positions in the Fc domain (C) Represents distribution of mutations in different positions of the Fc domain in literature

5.3.5 *Affinity Determination of Yeast Surface Display Clones with receptor FcγIIIa using SPR*

We next selected individual clones for further analysis. We selected clones that maintained a disulphide linkage between any of the three loops -BC, C'E and FG (Clone-38,55 and 58). In our panning approach, we observed a clustering of three main groups of Fc clones. We selected one clone to represent each of these groups (Clones 36,54 and 57). We then narrowed down which YSD clones from both sorting and panning to characterize further with SPR. Fc domains corresponding to these clones were purified from HEK293F cells and coupled to a CM5 SPR chip for K_D determination. The K_D values obtained from each of the selected clones along with their source of origin is shown in Figure 5.6. The values obtained for K_D of these clones are comparable to what was previously observed by previous groups (Ott et al. 2008; Chen et al. 2017b; Yoon et al. 2019c).

Table 5.1 Fc mutations resulting in enhanced receptor CD16a binding

Fc mutations resulting in increased FcγIIIa binding	**K_D (nM) of the clones**	**N-glycan status**	**References**
T256A K290A S298A E333A K334A A339T	1.32 (Ratio wrt WT) 1.31 1.34 1.27 1.39 1.34	Glycosylated	(Shields et al. 2001a)
G236A; S239D; A330L; I332E S239D; A330l; I332E	124 nM (FcγIIIa V158) 100 nM (FcγIIIa F158) 201nM (FcγIIIa F158)	Glycosylated	(Ahmed et al. 2016)

I332E S239D/I332E	30 nM (FcγIIIa V158) 2nM (FcγIIIa V158)	Glycosylated	(Lazar et al. 2006)
N297D N297H T299A/K326E	0.4 (Ratio wrt WT) 0.4 ~1	Aglycosylated	(Sazinsky et al. 2008a)
F243L/ R292P/Y300L/V305I/P393L	40 nM FcγIIIa V158 99 nM FcγIIIa F158	Glycosylated	(Stavenhagen et al. 2007a)
G236A/S239D/I332E S239D/I332E	23 nM FcγIIIa V158 95 nM FcγIIIa V158	Glycosylated	(Richards et al. 2008)
S239D/S298A/E356K/D399K (Chain A) S239D/A300L/I332E/K392D/ K409D (Chain B)	19 nM FcγIIIa (V158) 22 nM FcγIIIa (F158)	Glycosylated	(Liu et al. 2014)
S239D/A330L/I332E (Chain A) L234Y/G236W/S298A(Chain B) L234Y/L235Q/G236W/S239M/H268M/D270E (Chain A) D270E/K326E/A330M/K334E (Chain B)	42 nM FcγIIIa (V158) 1054-fold enhancement	Glycosylated Glycosylated	(Mimoto et al. 2013)
N297D/S298T/K326I/A327Y/ L328G N297H/S298A/K326I/A327Y/ L328G T299A/K326I/A327Y/L328G	1-3 μM FcγIIIa 1-2 μM FcγIIIa Wildtype affinity	Aglycosylated	(Chen et al. 2017a)

L235V/F243L/R292P/Y300L/ P396L	89 nM – 415 nM FcγIIIa (V158) 161 nM – 1059 nM FcγIIIa (F158)	Glycosylated	(Nordstrom et al. 2011a)
V264E/S298G/T299A/K326I/ A327Y/L328G/T350A/E282V/ N390D/T384A/M428L	1.537μM FcγIIIa (V158)	Aglycosylated	(Yoon et al. 2019a)

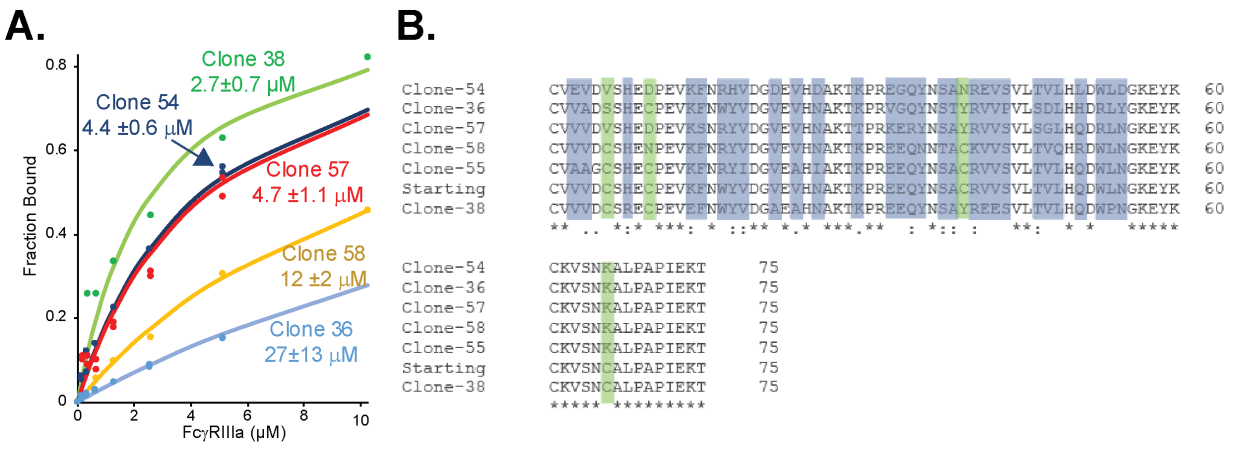

Figure 5.6. Characterization of selected YSD Fc clones (**A**) K_D values of selected aglycosylated Fc yeast surface display clones with the fit (**B**) Sequence Alignment of the Yeast Surface Display clones with respect to the starting material used to create libraries

5.4. Discussion

Thus far, we have developed a novel approach to engineer aglycosylated Fc domain with enhanced FcγRIIIa binding. Work done by our group with these disulphide variants isolated from HEK293F cells showed an increase melting temperature compared to wildtype. Engineering disulphide bonds within the Fc domain could possibly result in a more thermally stable Fc. Previously discovered aglycosylated Fc variants were found by chance. In this approach, we specifically targeted the destabilization caused by the lack of the N-glycan on the C'E loop. Through our screening approach we were able to achieve a 40-fold enrichment compared to our wildtype control. We then

selected novel clones determined by sequencing to characterize further. We then determined the affinities of these selected clones (K_D's) through SPR. We found that the affinities of these clones (K_D 1-3 μM) are comparable to the affinities of previously aglycosylated clones. However, we believe the engineered disulphide bonds can provide additional structural stabilization to the Fc clones as there is a possibility that aglycosylated clones could be structurally unstable due to the lack of the N-glycan. Currently, we are working towards assembling and purifying two of the selected Fc clones (Clone-38 and 54) as full-length anti-CD20 antibodies (rituximab). Antibodies containing these Fc clones will allow us to examine the ADCC activity. We originally assembled and purified these antibodies from engineered yeast strain -EBY100-Kar2p-F2A-PDI (described in chapter 3) but did not recover enough full-length antibody due to proteolysis. We are currently attempting to purify these clones from HEK293F cells.

Identifying aglycosylated Fc variants would allow for the expression of antibody from prokaryotic expression platforms rather than mammalian expression platforms, significantly lowering production cost. In addition to this, it would completely eliminate the problem of N-glycan heterogeneity originating from mammalian expression. However, an issue that can arise is increased clearance of aglycosylated antibodies from serum compared to their glycosylated counterparts. However, work done by Salzinsky and co-workers have shown that the clearance rate although slightly reduced, is not adversely affected by the lack of a N-glycan [6].

5.5. References

1. Lee, C.H.; Romain, G.; Yan, W.; Watanabe, M.; Charab, W.; Todorova, B.; Lee, J.; Triplett, K.; Donkor, M.; Lungu, O.I.; et al. IgG Fc Domains That Bind C1q but Not Effector Fc3 Receptors Delineate the Importance of Complement-Mediated Effector Functions. *Nat Immunol* **2017**, *18*, 889–898, doi:10.1038/ni.3770.

2. Park, H.I.; Yoon, H.W.; Jung, S.T. The Highly Evolvable Antibody Fc Domain. *Trends Biotechnol* 2016, *34*, 895–908. doi: 10.1016/j.tibtech.2016.04.005. Epub 2016 May 10. PMID: 27173171.

3. Ju, M.S.; Jung, S.T. Aglycosylated Full-Length IgG Antibodies: Steps toward next-Generation Immunotherapeutics. *Curr Opin Biotechnol* 2014, *30*, 128–139. doi: 10.1016/j.copbio.2014.06.013. Epub 2014 Jul 16. PMID: 25035939.

4. Traxlmayr, M.W.; Faissner, M.; Stadlmayr, G.; Hasenhindl, C. Directed Evolution of Stabilized IgG1-Fc Scaffolds by Application of Strong Heat Shock to Libraries Displayed on Yeast Biochimica et Biophysica Acta Directed Evolution of Stabilized IgG1-Fc Scaffolds by Application of Strong Heat Shock to Libraries Displa. *BBA - Proteins and Proteomics* **2012**, *1824*, 542–549, doi:10.1016/j.bbapap.2012.01.006.

5. Sazinsky SL, Ott RG, Silver NW, Tidor B, Ravetch JV, Wittrup KD. Aglycosylated immunoglobulin G1 variants productively engage activating Fc receptors. Proc Natl Acad Sci U S A. 2008 Dec 23;105(51):20167-72. doi: 10.1073/pnas.0809257105.

6. Chen, T.F.; Sazinsky, S.L.; Houde, D.; DiLillo, D.J.; Bird, J.; Li, K.K.; Cheng, G.T.; Qiu, H.; Engen, J.R.; Ravetch, J. v.; et al. Engineering Aglycosylated IgG Variants with Wild-Type or

Improved Binding Affinity to Human Fc Gamma RIIA and Fc Gamma RIIIAs. *J Mol Biol* **2017**, *429*, 2528–2541, doi:10.1016/j.jmb.2017.07.001.

7. Yoon, H.W.; Jo, M.; Ko, S.; Kwon, H.S.; Lim, C.S.; Ko, B.J.; Lee, J.C.; Jung, S.T. Optimal Combination of Beneficial Mutations for Improved ADCC Effector Function of Aglycosylated Antibodies. *Mol Immunol* **2019**, *114*, 62–71, doi:10.1016/j.molimm.2019.07.007.

8. Sondermann P, Huber R, Oosthuizen V, Jacob U. The 3.2-A crystal structure of the human IgG1 Fc fragment-Fc gammaRIII complex. Nature. 2000 Jul 20;406(6793):267-73. doi: 10.1038/35018508.

9. Shields, R.L.; Namenuk, A.K.; Hong, K.; Meng, Y.G.; Rae, J.; Briggs, J.; Xie, D.; Lai, J.; Stadlen, A.; Li, B.; et al. High Resolution Mapping of the Binding Site on Human IgG1 for FcγRI, FcγRII, FcγRIII, and FcRn and Design of IgG1 Variants with Improved Binding to the FcγR. *Journal of Biological Chemistry* **2001**, *276*, 6591–6604, doi:10.1074/jbc.M009483200.

10. Lazar GA, Dang W, Karki S, Vafa O, Peng JS, Hyun L, Chan C, Chung HS, Eivazi A, Yoder SC, Vielmetter J, Carmichael DF, Hayes RJ, Dahiyat BI. Engineered antibody Fc variants with enhanced effector function. Proc Natl Acad Sci U S A. 2006 Mar 14;103(11):4005-10. doi: 10.1073/pnas.0508123103.

11. Richards, J.O.; Karki, S.; Lazar, G.A.; Chen, H.; Dang, W.; Desjarlais, J.R. Optimization of Antibody Binding to FcγRIIa Enhances Macrophage Phagocytosis of Tumor Cells. *Mol Cancer Ther* **2008**, *7*, 2517–2527, doi:10.1158/1535-7163.MCT-08-0201.

12. Ahmed, A.A.; Keremane, S.R.; Vielmetter, J.; Bjorkman, P.J. Structural Characterization of GASDALIE Fc Bound to the Activating Fc Receptor FcγRIIIa. *J Struct Biol* **2016**, *194*, 78–89, doi:10.1016/j.jsb.2016.02.001.

13. Liu, Z.; Gunasekaran, K.; Wang, W.; Razinkov, V.; Sekirov, L.; Leng, E.; Sweet, H.; Foltz, I.; Howard, M.; Rousseau, A.M.; et al. Asymmetrical Fc Engineering Greatly Enhances Antibodydependent Cellular Cytotoxicity (ADCC) Effector Function and Stability of the Modified Antibodies. *Journal of Biological Chemistry* **2014**, *289*, 3571–3590, doi:10.1074/jbc.M113.513366.

14. Mimoto, F.; Igawa, T.; Kuramochi, T.; Katada, H.; Kadono, S.; Kamikawa, T.; Shida-Kawazoe, M.; Hattori, K. Novel Asymmetrically Engineered Antibody Fc Variant with Superior FcγR Binding Affinity and Specificity Compared with Afucosylated Fc Variant. *MAbs* **2013**, *5*, 229–236, doi:10.4161/mabs.23452.

15. Boder, E.T.; Wittrup, K.D. Yeast Surface Display for Screening Combinatorial Polypeptide Libraries;Nat. Biotechnol. 1997; 15:553–557. doi: 10.1038/nbt0697-553

16. Nordstrom, J.L.; Gorlatov, S.; Zhang, W.; Yang, Y.; Huang, L.; Burke, S.; Li, H.; Ciccarone, V.; Zhang, T.; Stavenhagen, J.; et al. Anti-Tumor Activity and Toxicokinetics Analysis of MGAH22, an Anti-HER2 Monoclonal Antibody with Enhanced Fcγ Receptor Binding Properties. *Breast Cancer Research* **2011**, *13*, doi:10.1186/bcr3069.

17. Subedi, G.P.; Barb, A.W. The Structural Role of Antibody N-Glycosylation in Receptor Interactions. *Structure* **2015**, *23*, 1573–1583, doi:10.1016/j.str.2015.06.015.

18. Sazinsky SL, Ott RG, Silver NW, Tidor B, Ravetch JV, Wittrup KD. Aglycosylated immunoglobulin G1 variants productively engage activating Fc receptors. Proc Natl Acad Sci U S A. 2008 Dec 23;105(51):20167-72. doi: 10.1073/pnas.0809257105.

19. Chen, T.F.; Sazinsky, S.L.; Houde, D.; DiLillo, D.J.; Bird, J.; Li, K.K.; Cheng, G.T.; Qiu, H.; Engen, J.R.; Ravetch, J. v.; et al. Engineering Aglycosylated IgG Variants with Wild-Type or

Improved Binding Affinity to Human Fc Gamma RIIA and Fc Gamma RIIIAs. *J Mol Biol* **2017**, *429*, 2528–2541, doi:10.1016/j.jmb.2017.07.001.

20. Yoon, H.W.; Jo, M.; Ko, S.; Kwon, H.S.; Lim, C.S.; Ko, B.J.; Lee, J.C.; Jung, S.T. Optimal Combination of Beneficial Mutations for Improved ADCC Effector Function of Aglycosylated Antibodies. *Mol Immunol* **2019**, *114*, 62–71, doi:10.1016/j.molimm.2019.07.007.

21. Yoon, H.W.; Jo, M.; Ko, S.; Kwon, H.S.; Lim, C.S.; Ko, B.J.; Lee, J.C.; Jung, S.T. Optimal Combination of Beneficial Mutations for Improved ADCC Effector Function of Aglycosylated Antibodies. *Mol Immunol* **2019**, *114*, 62–71, doi:10.1016/j.molimm.2019.07.007.

22. Falconer, D.J.; Barb, A.W. Mouse IgG2c Fc Loop Residues Promote Greater Receptor-Binding Affinity than Mouse IgG2b or Human IgG1. *PLoS One* **2018**, *13*, doi:10.1371/journal.pone.0192123.

23. Wozniak-Knopp, G.; Stadlmann, J.; Rüker, F. Stabilisation of the FC Fragment of Human IgG1 by Engineered Intradomain Disulfide Bonds. *PLoS One* **2012**, *7*, doi:10.1371/journal.pone.0030083.

24. Wozniak-Knopp, G.; Bartl, S.; Bauer, A.; Mostageer, M.; Woisetschläger, M.; Antes, B.; Ettl, K.; Kainer, M.; Weberhofer, G.; Wiederkum, S.; et al. Introducing Antigen-Binding Sites in Structural Loops of Immunoglobulin Constant Domains: Fc Fragments with Engineered HER2/Neu-Binding Sites and Antibody Properties. *Protein Engineering, Design and Selection* **2010**, *23*, 289–297, doi:10.1093/protein/gzq005.

25. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene. 1989 Apr 15;77(1):51-9. doi: 10.1016/0378-1119(89)90358-2. PMID: 2744487.

26. Gietz, R.D.; Schiestl, R.H. High-Efficiency Yeast Transformation Using the LiAc/SS Carrier DNA/PEG Method. *Nat Protoc* **2007**, *2*, 31–34, doi:10.1038/nprot.2007.13.

27. Gietz, R.D.; Woods, R.A. Transformation of Yeast by Lithium Acetate/Single-Stranded Carrier DNA/Polyethylene Glycol Method. *Methods Enzymol* **2002**, *350*, 87–96, doi:10.1016/S0076-6879(02)50957-5.

28. Miller, A.S.; Tejada, M.L.; Gazzano-Santoro, H. Development of an ELISA Based Bridging Assay as a Surrogate Measure of ADCC. *J Immunol Methods* **2012**, *385*, 45–50, doi:10.1016/j.jim.2012.08.004.

29. Subedi, G.P.; Barb, A.W. The Structural Role of Antibody N-Glycosylation in Receptor Interactions. *Structure* **2015**, *23*, 1573–1583, doi:10.1016/j.str.2015.06.015.

30. Dombkowski, A.A. Disulfide by Design™: A Computational Method for the Rational Design of Disulfide Bonds in Proteins. *Bioinformatics* **2003**, *19*, 1852–1853, doi:10.1093/bioinformatics/btg231.

31. Wang, B.; Dekosky, B.J.; Timm, M.R.; Lee, J.; Normandin, E.; Misasi, J.; Kong, R.; McDaniel, J.R.; Delidakis, G.; Leigh, K.E.; et al. Functional Interrogation and Mining of Natively Paired Human v H:V L Antibody Repertoires. *Nat Biotechnol* **2018**, *36*, 152–155, doi:10.1038/nbt.4052.

32. Stavenhagen, J.B.; Gorlatov, S.; Tuaillon, N.; Rankin, C.T.; Li, H.; Burke, S.; Huang, L.; Johnson, S.; Bonvini, E.; Koenig, S. Fc Optimization of Therapeutic Antibodies Enhances Their Ability to Kill Tumor Cells in Vitro and Controls Tumor Expansion in Vivo via Low-Affinity Activating Fcγ Receptors. *Cancer Res* **2007**, *67*, 8882–8890, doi:10.1158/0008-5472.CAN-07-0696

CHAPTER 6

GLYCOENGINEERED Fc VARIANTS SELECTED USING A NOVEL YEAST STRAIN

Abstract:

The IgG1 Fc N-glycan and the Fc amino acid sequence play important roles in influencing receptor binding affinities. Very little work has been done combining these aspects to engineer Fc with optimal binding with the Fc γ Receptor IIIa to enhance Antibody Dependent Cellular Cytotoxcity (ADCC). In this work, we utilize novel glycoengineered yeast strain to create Fc yeast surface display (YSD) libraries. This strain was created by targeting immunoglobulin specific enzyme EndoS2 to the yeast Endoplasmic Reticulum (ER) to achieve N-glycan cleavage by utilizing the target peptide from yeast disulphide isomerase (PDI) and E.R retention signal HDEL. This results in the display of the Fc domain on the yeast surface with a single N-Acetylglucoasmine residue (GlcNAc). We were able to achieve 20-fold enhancement in binders compared to our wildtype control. In addition to this, we also report a novel approach to selectively enrich for glycosylated YSD Fc clones from our library by selectively labeling the GlcNAc moiety on the yeast surface displayed Fc through copper-free click chemistry. We are currently in the process of producing our selected YSD Fc clones in the form of a full-length antibody Rituximab. We will then utilize techniques such as Surface Plasmon Resonance (SPR) and ADCC to characterize these clones to better understand their functionality.

6.1 Introduction

Antibody dependent cell-mediated cytotoxicity (ADCC) is an important mechanism in immunotherapy that requires three components – antibodies, target cells with the appropriate surface antigen, and effector cells. The antibodies opsonize antigens and recruit immune effector cells (example Macrophages or Natural Killer cells) to destroy the target cell. Immunoglobulin G1 antibodies (IgG1) possess a conserved N-glycosylation site (N297) which is required for receptor FcγRIIIa engagement. This N-glycan does not directly interact with the receptor but helps stabilize the C'E loop that forms a binding interface with the receptor (Subedi and Barb 2015c). The monosaccharide units of the N-glycan form CH-π bonds with aromatic amino acids in Fc stabilizing the N-glycan and influencing FcγRIIIa binding (Subedi et al. 2014b). In addition to this, our group along with others have shown that N-glycan composition influences FcγRIIIa binding affinities (Peipp et al. 2008; Subedi and Barb 2016). This extensive body of work establishes the role of the Fc N-glycan in FcγRIIIa binding. In addition to this, several groups have identified specific mutations within the Fc binding interface to enhance FcγRIIIa binding (Shields et al. 2001b; Stavenhagen et al. 2007b; Sazinsky et al. 2008b; Nordstrom et al. 2011b; Chen et al. 2017b; Yoon et al. 2019c). However, despite the equally important roles played by the N-glycan and amino acid composition in influencing binding, very little work has been done in combining protein and N-glycan approaches simultaneously to screen for Fc with enhanced receptor binding. Yeast Surface Display would be an attractive platform to combine both protein and N-glycan engineering given the ability of yeasts to N-glycosylate heterologous protein and create diverse libraries with relative ease (Boder and Wittrup 1997b). However, fungal N-glycosylation pathways result in an oligomannose glycoform on heterologous expressed protein resulting in rapid

clearance from serum(Goetze et al. 2011). Most mammalian proteins are expected to display predominantly complex-type N-glycans. This limitation has led to several groups to engineer yeast strains to express proteins with hybrid or complex type N-glycans

(Chiba et al. 1998b; Choi et al. 2003; Vervecken et al. 2004; de Pourcq et al. 2010; Shenoy Anjali and Barb 2022)

. Shaheen and co-workers utilized glycoengineeredstrains of *Pichia patoris* to screen for Fab domains (displayed as full-length Antibody) for antigen binding (Shaheen et al. 2013). A majority of these glycoengineered yeast strains were created with *Pichia pastoris* rather than *Saccharomyces cerevisiae*, this is due to the high degree of hypermannosylation that occurs in the Golgi of *Saccharomyces cerevisiae*. However, *S. cerevisiae* is potentially an easier platform to create display libraries given its proficiency in homologous recombination.

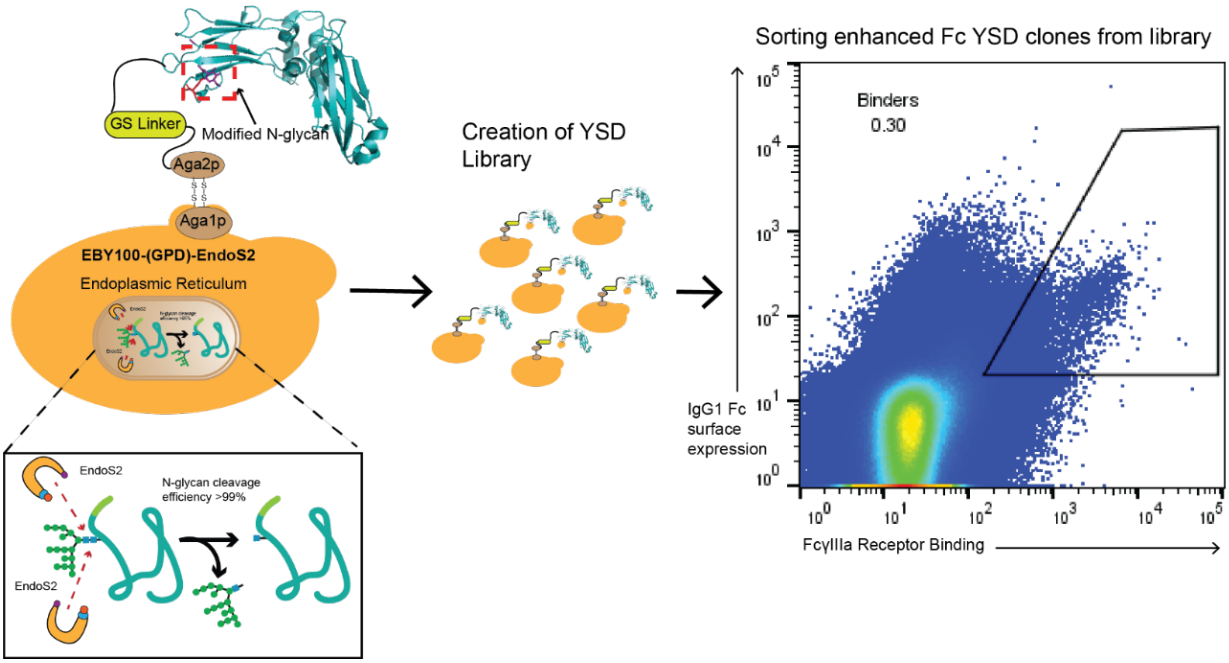

Figure 6.1. Schematic of glycoengineered Saccharomyces cerevisiae strain EBY100-(GPD)-EndoS2 for library screening. Glycoengineered *Saccharomyces cerevisiae* strain EBY100-(GPD)-EndoS2 was used to create YSD libraries and was sorted through FACS to selected for Fc variants with enhanced FcγIIIa receptor binding

We have previously described the creation of the *S. cerevisiae* yeast strain, EBY100-(GPD)-EndoS2 with a high degree of N-glycan processing (>95%) (Shenoy et al. 2021b). This strain cleaves the endogenous oligomannose glycoform on Fc to a single N-acetylglucoasmine residue. This strain was created by targeting the immunoglobulin-specific glycosylhydrolase EndoS2 to the yeast Endoplasmic Reticulum (E.R) (Sjögren et al. 2013, 2015). The N-glycan cleavage achieved in this strain also removes the highly immunogenic oligomannose portion of the N-glycan that leads to rapid clearance from serum (Goetze et al. 2011)

hIgG1 Fc with a single N-acetylglucosamine residue (GlcNAc) has been shown to engage receptor FcγRIIIa (10-fold lower than Fc with a complete complex-type N-glycan)(Subedi and Barb 2015c; Kao et al. 2015b). In this work, we utilize this novel glycoengineered yeast strain to create yeast surface display libraries to screen for Fc variants with enhanced FcγRIIIa binding (Figure 6.1). We also report a novel approach to screen YSD Fc clones for the desired glycoform (single GlcNAc) by labeling through Click Chemistry.

6.2. Materials and Methods

6.2.1 Strain and Media

The generation of the yeast surface display strain, EBY100 [ATCC-MYA4941] (GAL1-AGA1:URA3 ura3-52 trp1 leu2Δ1 his3 Δ200 pep4: HIS2 prb1 Δ1.6R can1 GAL) was used to create the EBY100-(GPD)-EndoS2 was described [19]. The EBY100-(GPD)-EndoS2 strain (prior to transformation) was grown in YNB-Dropout (Sigma Aldrich Y151) + 2% glucose + Tryptophan [6.7 g/L YNB without ammonium sulphate and amino acids + 1.5g/L Dropout mix (Leu-, Ura-, Trp-)] (Sigma Aldrich Y1771). Yeast Surface Display Libraries were plated on YNB-Dropout Agar plates and liquid cultures were grown/induced in YNB-Dropout containing 2% glucose/galactose.

6.2.2 Creation of Yeast Surface Display Libraries

Yeast surface display libraries were assembled in the glycoengineered yeast strain EBY100-GPD-EndoS2 strain. We used error-prone PCR to diversify a fragment of the Fc sequence containing the BC to the FG loops, with a low fidelity Taq polymerase in the presence of 5mM MgCl2, 0.5 mM MnCl2 and low template DNA concentrations (100 pg). The product was purified using a Qiagen Gel Extraction kit. The error-prone PCR product and the two backbone fragments (described in Chapter 4) were used to transform EBY100-GPD-EndoS2 using standard yeast transformation protocols [26].

6.2.3 Induction and Sorting Yeast Surface Display Libraries

The library was expanded in YNB-Dropout +2% glucose medium overnight. This culture was used to start a 100 mL Induction culture at an OD600 of 0.5. The culture was induced with galactose 24 hours at 30°C. Approximately 10^7 induced cells were used to prepare samples for sorting. The cells were spun down and washed with 100 µl of 1x PBS, followed by a wash with 100 µl 1x PBSA. The cells were labeled with 363 nM Protein A-AF647 and 2µM CD16a-GFP at 4°C in the dark. For the initial rounds of sorting a broad gate was drawn to select for ~2% AF647+GFP+ cells. These sorted cells were grown up in 1mL YNB-Dropout +2% glucose. The plasmid was isolated from these cells using a Zymoprep kit (Zymo Research 50444107). Further rounds of diversification were carried out selecting for the top 0.1% of AF647+GFP+ cells. The cells from the enriched library were diluted and plated on YNB-Dropout plates to get individual colonies. These colonies were grown up in a 96-well plate and sent for Sanger Sequencing (Genewiz).

5.2.4 Library enrichment using Click Chemistry

This was an approached used to selected for full-length Fc with a single N-Acetylglucosamine residue (GlcNAc) within the enriched library. Approximately 10^7 induced yeast cells were washed

with a 100 µl of 1xPBS. The supernatant was discarded. The cells were incubated with 5 mM UDP-Az-Gal (Carbosynth MU11565), 5U Gal T (Sigma SAE0093) in 1xPBSA (1x PBS +1% BSA) for 1 hour at 37 ℃ with mixing. The cells were spun down and washed with 100 µl of 1x PBSA. Then labeled with 100 µg/mL of Az Dye AF488-DBCO (Click Chemistry Tools) and 0.75 µg of AF647 Anti-Flag Antibody (Thermo Fisher, AB_2610655) in the dark at 4 ℃ for 1 hour. The cells were washed twice with 100 µl 1x PBS. AF647+AF488+ cells were sorted for using the MoFlo Asterios.

6.2.5 Cloning and Isolation of EndoS2 YSD clones as full-length Rituximab Antibodies

DNA constructs for the Fc regions of Yeast Surface Display Clones – Clone 8, 11 and 16 (Supplementary Material) and the beginning portion of Rituximab Heavy chain RTX HC were synthesized by IDT. The starting HC fragment and each of the Fc were amplified individually using Pfu Polymerase (Agilent) and then combined through fusion PCR using 2x GoTaq (Promega). These full-length HC DNA fragments were then amplified to include restriction sites for EcoR1 and BstX1 to clone into pEF1. The ligation products pEF1-HC (Clone 8/11/16) were transformed into DH5α cells, colony screened by colony PCR, and their sequences were verified through Sanger Sequencing. Qiagen Mega Prep Kit was used to recover sufficient amount of DNA to transfect HEK293F cells. Similarly, the Rituximab light chain (RTX LC) was synthesized by IDT and was amplified to include restriction sites Not1 and HindIII to clone into vector pGENT2. The ligation product pGENT2-RTX-LC was transformed into transformed into DH5α cells, screened by colony PCR, and their sequences were verified through Sanger Sequencing. Qiagen Mega Prep Kit was used to recover sufficient amount of DNA to transfect HEK293F cells.

Table 6.1. List of Primers used for RTX Heavy Chain Fusion PCR and Cloning

Primer Name	DNA Sequence	Function
pGRTXLCF	5' CAG CGG CCG CAT GAG ACT GC '3	To clone RTX LC into the pGENT2 vector
pGRTXLCR	5' CGT AAG CTT TTA GCA CTC GCC TCT GTT G '3	To clone RTX LC into the pGENT2 vector
EndoS2_F	5' CTG CCC CGC ACC TGA ACT CCT G '3	RTX Heavy Chain Fusion Reaction
EndoS2_R	5' CAGGAGTTCAGGTGCGGGGCAG '3	RTX Heavy Chain Fusion Reaction
pEFRTX_Fnew	5' CAC CCA GAA TTC GCG GCC GCC A '3	Cloning into pEF1a vector
pEFRTX_R	5' GGT CCA GGA AGT TGT TAT CAT TTA CCC GG '3	Cloning into pEF1a vector

6.3. Results

6.3.1 Creation of Yeast Surface Display libraries using the glycoengineered EBY100-(GPD)-EndoS2 yeast strain

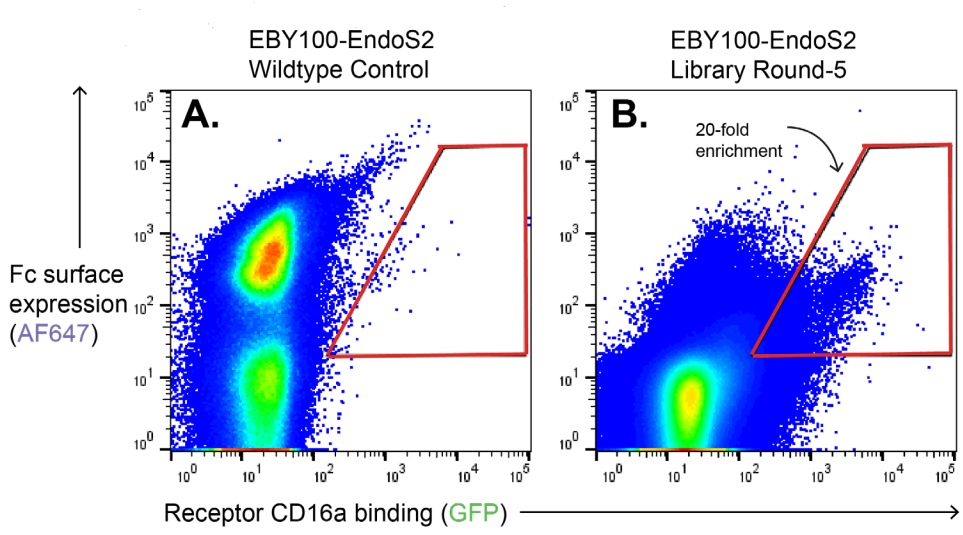

Figure 6.2 Sorting of Yeast Surface Display Library using EBY100-(GPD)-EndoS2 strain (A) Flow cytometry-based analysis of the EBY100-(GPD)-EndoS2 strain expressing wildtype Fc on its cell surface. The Y-axis indicates Fc surface expression measured by Protein A-AF647 binding. The X-axis shows receptor GFP-CD16a binding. The red boxes in A and B serve for comparison between the two samples

We utilized our glycoengineered strain EBY100-(GPD)-EndoS2 strain (Shenoy et al. 2021b) to screen for minimally glycosylated Fc variants with enhanced FcγRIIIa binding. Induced yeast cells were stained with AF647- Protein A and FcγRIIIa-GFP to select for both Fc surface expression and receptor binding. Labeling with Protein A would allow for the enrichment of properly folded Fc on the yeast surface as this protein is sensitive to the correct folding of the Fc (Deisenhofer et al. 1975). In addition to this, Protein A and receptor FcγIIIa do not interact with each other and therefore would not interfere in each other's binding to yeast surface displayed Fc (Gouda et al. 1992). In the initial rounds of sorting, a more generous gate was drawn to select for the top 2% of GFP^+ $AF647^+$ cells. In subsequent rounds, a more stringent gate (0.1-0.2%) was drawn to enrich our clones for enhance Fc surface expression and receptor binding. In addition to this, we also drew a diagonal gate shown in Figure 6.2, to be able to select for clones that express low levels of surface Fc but bind receptor FcγRIIIa with high affinities. After five rounds of sorting, we achieved a maximal 20-fold enrichment in GFP^+ $AF647^+$ positive cells and proceeded with DNA sequencing our hits.

6.3.2 DNA Sequence Analysis of Yeast Surface Display Clones

We then sequenced ~96 of our enriched clones. We identified several full-length glycosylated clones. We mapped out each of these mutations to their specific location within the loops of the cγ2 domain (BC, C'E and FG) as shown in Figure 6.3. We observed a number of mutations localized within the C'E loop and surrounding regions, which is not surprising considering the important role of the N-glycan in stabilizing this specific loop (Subedi and Barb 2015c). This localization has also been observed

in various Fc variants with enhanced FcγRIIIa binding, both variants with and without the N-glycan (Stavenhagen et al. 2007b; Sazinsky et al. 2008b; Nordstrom et al. 2011b; Chen et al. 2017b; Yoon et al. 2019c). We also observed very few mutations (~5 mutations per clone) in some

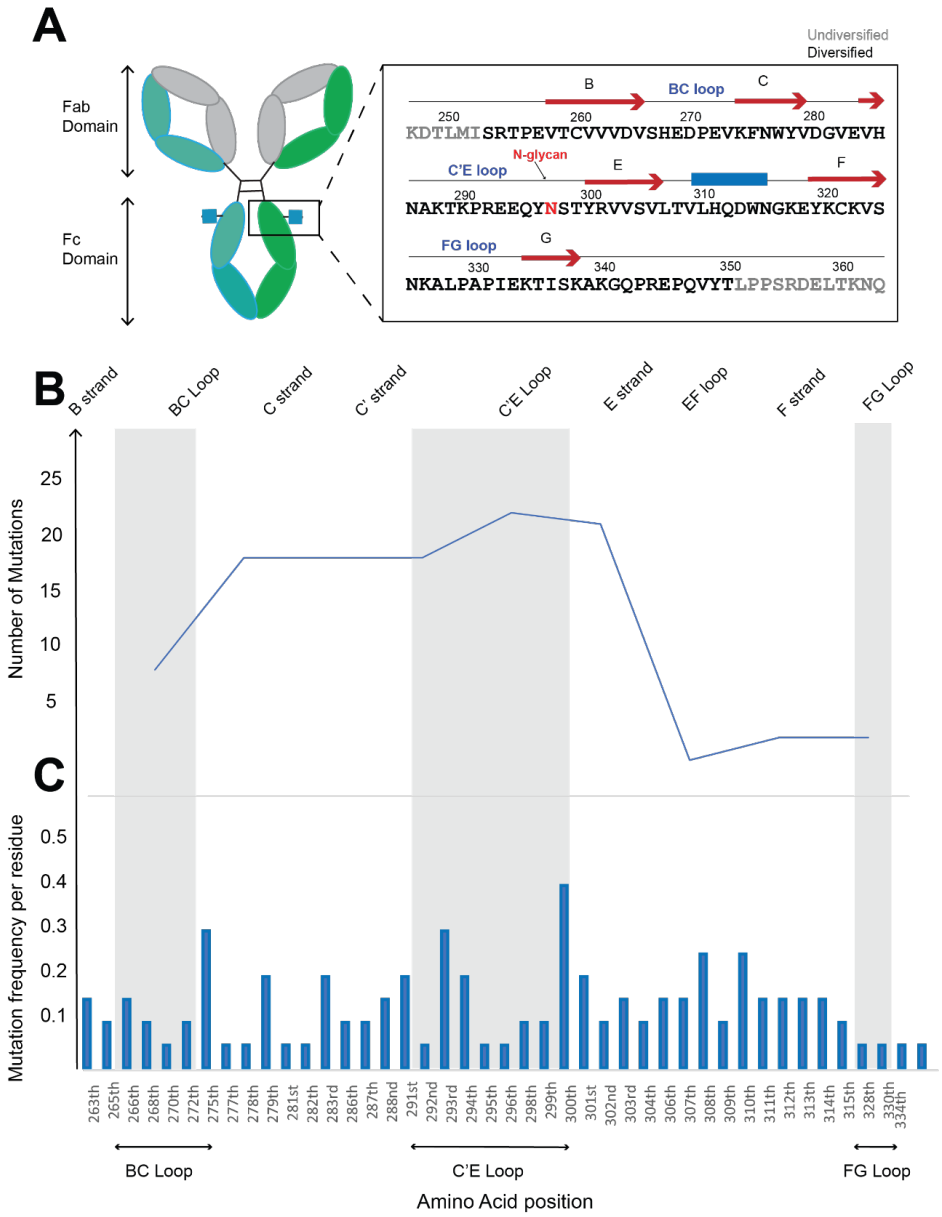

Figure 6.3 Distribution of mutations in different portions of the Fc domain (A) Black text highlights the diversified region in the context of full-length human IgG1 (B) Distribution of mutations within the Fc domain of the antibody (C) Specific amino acid positions where mutations are located

of the clones, relative to the aglycosylated clones (~8.4 mutations per clone) described in Chapter 4 of this work, this could be due to the partial stabilization provided by the single GlcNAc resulting in fewer mutations needed to achieve the desired phenotype of enhanced receptor FcγRIIIa binding. In addition to this we also observed some clones with mutations disrupting the N(X)S/T sequon in the C'E loop resulting in an aglycosylated Fc (We observed roughly 45-50% of our YSD clones were aglycosylated).

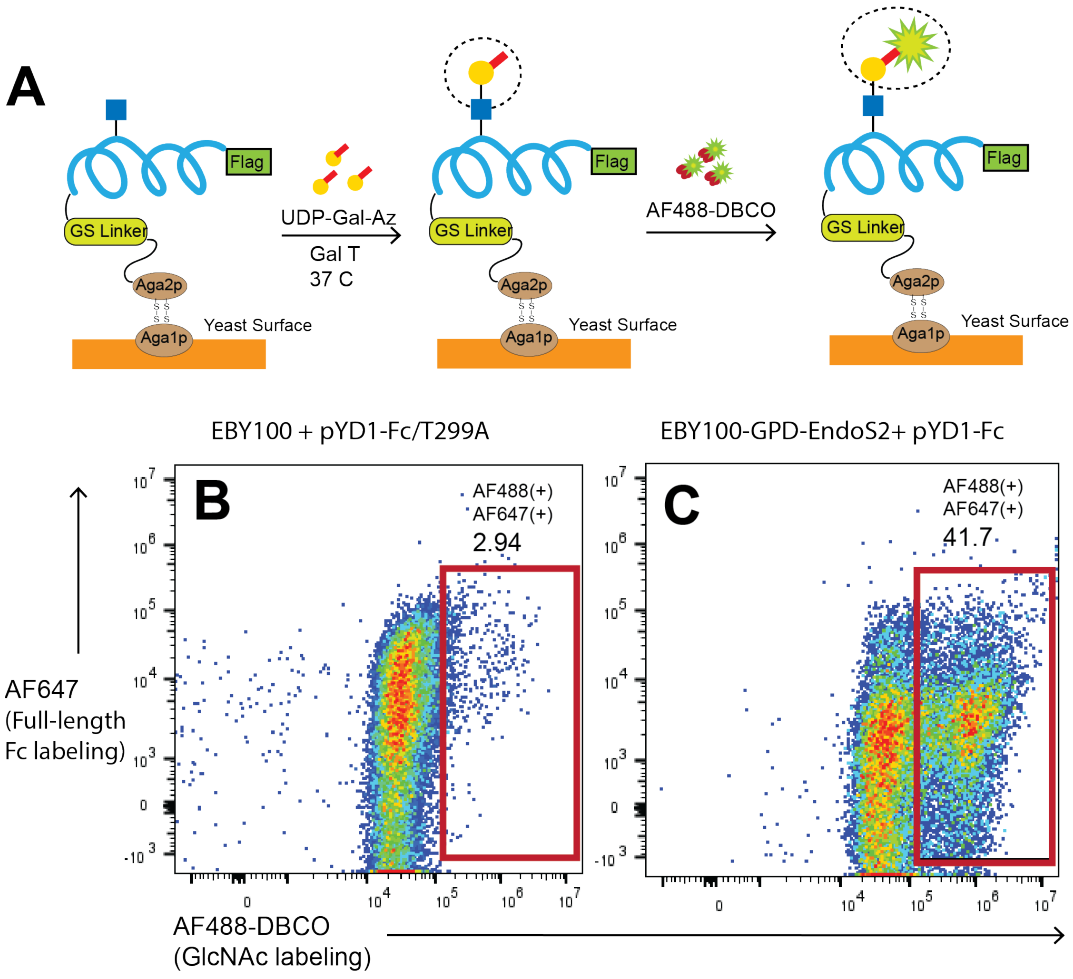

Figure 6.4. "GlcNAc" labeling using Click Chemistry (A) Schematic representing Click Chemistry mechanism labeling GlcNAc on the yeast surface (B) Click Chemistry GlcNAc labeling (AF488-DBCO) on aglycosylated control EBY100 + pYD1-Fc T299A cell surface (C) Click Chemistry GlcNAc labeling (AF488-DBCO) on EBY100-(GPD)-EndoS2 + pYD1-Fc cell surface

6.3.3 Click Chemistry to specifically label the GlcNAc residue on yeast surface displayed Fc

In order to enrich for glycosylated clone within our library, we created a click chemistry labeling technique shown in Figure 6.4A. Bertozzi and co-workers have shown that GlcNAc residues in N-glycans in *Saccharomyces cerevisiae* can be specifically labeled through click chemistry by engineering the metabolic pathway in yeast [25]. In this approach we used a combination of enzyme β-1,4 galactosyltransferase along with the UDP-Gal-Az substrate analog to specifically label the GlcNAc residue on the yeast surface displayed Fc.

We first incubated our induced yeast surface display library with UDP-Gal Az and a β-1,4 galactosyltransferase. This enzyme specifically adds Gal to a GlcNAc substrate and is unlikely to add Gal residues to any other glycans on the yeast cell surface [26]. In order to maximize labeling on the yeast cell surface, excessive amounts of enzyme and the UDP-Gal-Az were added to ~10^7 induced yeast cells. Following this the cells were labeled with DBCO-AF488 which reacts with the Gal-AZ-GlcNAc moiety on the yeast surface displayed Fc.

Conditions for effective labeling were optimized. Incubation at 37℃ resulted in a greater extent of labeling as compared to incubation at 30 ℃. This result is expected considering the maximum enzyme activity of β-1,4 galactosyl transferase is at 37 ℃. Surprisingly, incubation at this temperature did not affect cell viability. We used an aglycosylated control (EBY100 + pYD1-Fc T299A) to validate the approach. This control possesses Fc with disrupts the NX(S/T) sequon resulting in the display of aglycosylated Fc on the yeast surface. Since we observed no staining in this sample ($AF488^+$, $AF647^+$) it indicates that the click chemistry reagents do not non-specifically label N-glycans on the yeast surface and just on Fc. We used the same labeling conditions to label these cells along with EBY100-(GPD)-EndoS2 + pYD1-Fc cells. These cells were also labeling with an AF647 Anti-Flag antibody to test for Fc surface expression (Fc-flag). We observed a

distinctive AF488^{+} AF647^{+} population in our EBY100-(GPD)-EndoS2 cells (Figure 6.4C) which was not observed in our aglycosylated control (Figure 6.4B) depicting the high specificity of this approach in targeting and effectively labeling the single Gal-Az-GlcNAc moiety on surface displayed Fc.

6.3.4 Click Chemistry GlcNAc labeling to screen for glycosylated hits within library

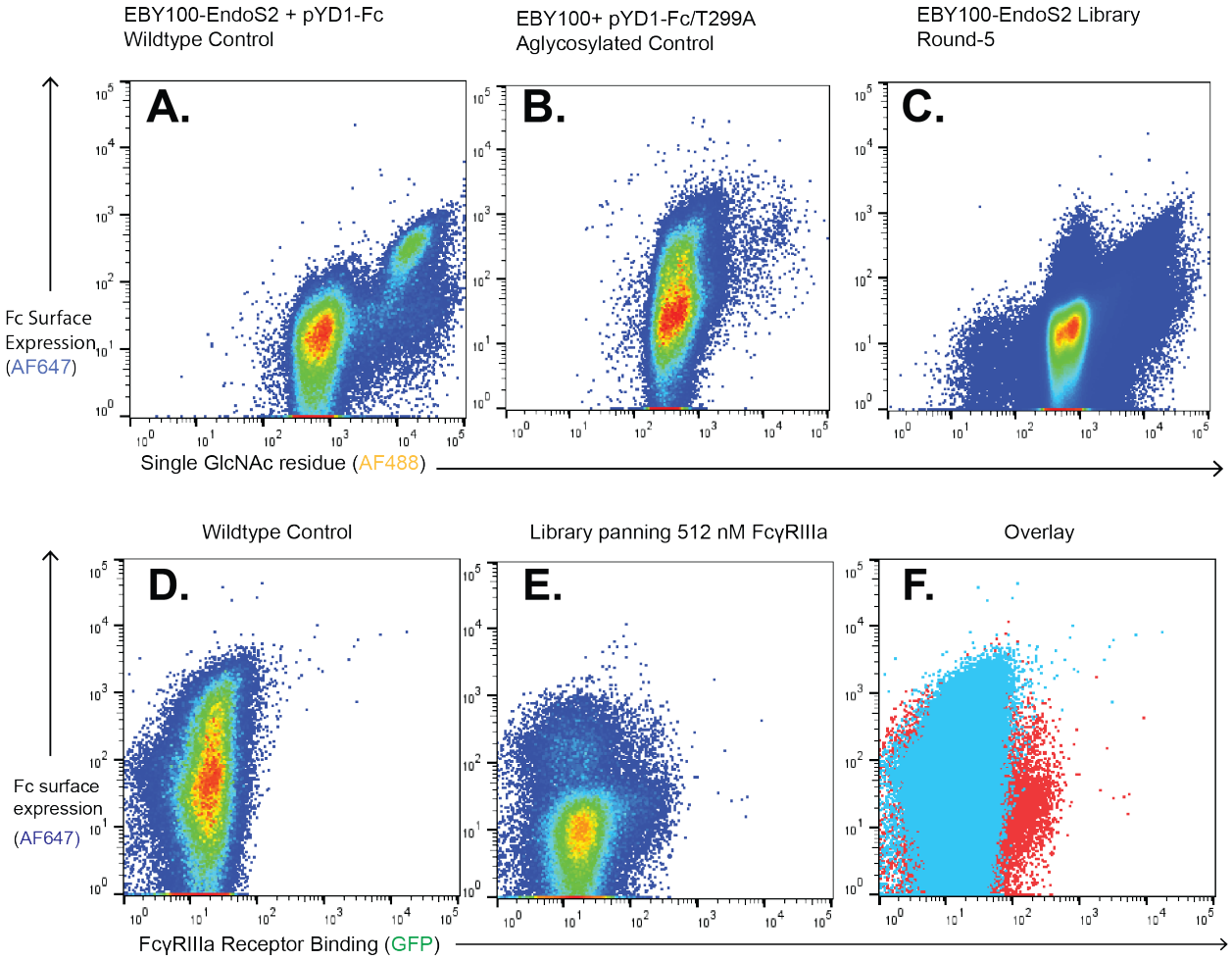

Figure 6.5 Sorting YSD library using "GlcNAc" labeling (A) EBY100-EndoS2 expressing wildtype Fc on its cell surface. (B) EBY100 yeast strain expressing an aglycosylated Fc on its cell surface (C) EBY100-EndoS2 Library. Fc surface expression was measured by AF647 fluorescence along the Y-axis. The presence of the single GlcNAc was measured by AF488 fluorescence measured along the X-axis. (D) Wildtype control EBY100-(GPD)-EndoS2 + pYD1-Fc stained with 512 FcγRIIIa and Anti-Flag AF647 antibody (E) EBY100-(GPD)-EndoS2 Fc library stained with the same reagents as the wildtype sample (F) overlay of the two samples to show enrichment of FcγRIIIa binding

We then applied the Click Chemistry approach to detect GlcNAc on the displayed Fc of our induced library. Following the labeling approach described previously, we labeled our induced yeast cells with the AF647 Anti-Flag antibody and DBCO-AF488. The Flag tag was fused to the C-terminus of the Fc and selected for using an AF647 Anti-Flag antibody to promote selection of full length clones. The cells were sorted for both Fc surface expression (Fc-Flag) and the desired glycoform (Single GlcNAc) shown in Figure 6.5C. These cells were again sorted for Fc surface expression (Fc Flag) and FcγRIIIa binding shown in Figure 6.5E. There was a significant enrichment

```
Clone-11      PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVEVDVSHEDPEVKSNWYVGGVEVHN      60
Clone-16      PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKSNWYVDGVEVHN      60
WT            PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHN      60
Clone-8       PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSLEDPEVKFNWYVDGVEVHN      60
              ************************************ **** ****** ****.******

Clone-11      ASTKSREKQYNSTYRVASVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP      120
Clone-16      ASTKSREKQYNSTYRVASVLTVLHQDWLNGKEYKCKVSNRALPAPIEKTISKAKGQPREP      120
WT            AKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP      120
Clone-8       TKTKPREEQYNSTSRVVSVLTVLHQNWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP      120
              :.** **:***** **.********:************:**********************

Clone-11      QVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL      180
Clone-16      QVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL      180
WT            QVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL      180
Clone-8       QVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL      180
              ************************************************************

Clone-11      YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKAWI      224
Clone-16      YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKAWI      224
WT            YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKAWI      224
Clone-8       YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKAWI      224
              ******************************************
```

Figure 6.6. Sequence Alignment of selected YSD clones Sequence Alignment between Wildtype, Clone-8, Clone-11, and Clone-16

in these yeast surface clones in terms of FcγRIIIa binding compared to the wildtype control as seen in the overlay between the two sorting samples shown in Figure 6.5F. These enriched cells were

then grown up and sequenced through Sanger Sequencing to obtain DNA sequence information from the enriched clones. Based on DNA Sequencing of clones from Round-1 and Round-2, there was a significant jump in the percentage of glycosylated clones from 37.5% to 71%.

Interestingly, we noticed mutations in Clone-11 and Clone-16 that could result in more structural stability in the C'E loop. The C'E loop of hIgG1 contains residues (P291, E293 and E294) that are not well-suited to a solvent exposed environment and are possibly contributing to structural instability in this area. Some mutations found in these clones include A287T, P291S, E293K and Y300S. Moreover, mutations to other well-suited residues could possibly improve hIgG1 Fc affinity to the FcγRIIIa receptor. hIgG1 and mIgG2c Cγ2 domain share a high degree of homology. The hIgG1 Cγ2 domain possesses a disordered C'E loop compared to the mIgG2c domain has a more well-defined secondary structure (DE loop) in the same region. This structural attribute could be a reason for the higher affinity of the mIgG2c antibody to its respective receptor FcγIVa compared to hIgG1 to FcγIIIa [27,28]. The mutations found in some of these Yeast surface display clones could possibly provide some structural stability to this disordered loop.

6.4. Discussion

In this work, we utilized the novel glycoengineered yeast strain, EBY100-(GPD)-EndoS2 strain to create yeast surface display libraries to screen for Fc variants with enhanced FcγRIIIa receptor binding. Both N-glycan and amino acid composition of Fc influences the affinity of receptor binding. This work combines both these aspects to engineer Fc. We were able to enrich our yeast surface display libraries 20-fold compared to our wildtype control. We also developed a novel approach to screen Fc based on its N-glycan through a click chemistry approach labeling the GlcNAc residue on yeast surface displayed Fc. We have now selected three novel clones to express

in HEK293F, determine affinity to receptor using SPR followed by ADCC to determine functional activity.

The single GlcNAc residue on the C'E loop provides some stabilization to this loop providing a "more stabilized" starting point compared to aglycosylated Fc to create YSD libraries. However, with Fc with a single N-Acetylglucosamine residue is lacking several advantageous CH-π interaction the aromatic amino acids on Fc forms with the monosaccharide units on the N-glycan [2]However, it has been cumbersome to engineer the yeast strain *Saccharomyces cerevisiae* to produce heterologous protein with a complex type glycoform. There have been more successful attempts reported using yeast strain *Pichia pastoris*. However, library generation in this strain entails a cumbersome process of integrating the YSD library within the genome.

In addition to this, hits picked up from this YSD library can be engineered to have a complex type glycoform by using novel transglycosylase variants of EndoS2 (EndoS2 D184M or D184C). These enzymes can add back a complex type glycoform with high efficiency resulting in >95% conversion with very little hydrolysis of the final product [29]. In the future, we could possibly utilize these unique enzymes to engineer *Saccharomyces cerevisiae* to display Fc with a complex type N-glycan.

6.5. References

1. Subedi, G.P.; Barb, A.W. The Structural Role of Antibody N-Glycosylation in Receptor Interactions. *Structure* **2015**, *23*, 1573–1583, doi:10.1016/j.str.2015.06.015.

2. Subedi, G.P.; Hanson, Q.M.; Barb, A.W. Restricted Motion of the Conserved Immunoglobulin G1 N-Glycan Is Essential for Efficient FcγRIIIa Binding. *Structure* **2014**, *22*, 1478–1488, doi:10.1016/j.str.2014.08.002.

3. Subedi, G.P.; Barb, A.W. The Immunoglobulin G1 N-Glycan Composition Affects Binding to Each Low Affinity Fc γ Receptor. *MAbs* **2016**, *8*, 1512–1524, doi:10.1080/19420862.2016.1218586.

4. Peipp, M.; van Bueren, J.J.L.; Schneider-Merck, T.; Bleeker, W.W.K.; Dechant, M.; Beyer, T.; Repp, R.; van Berkel, P.H.C.; Vink, T.; van de Winkel, J.G.J.; et al. Antibody Fucosylation Differentially Impacts Cytotoxicity Mediated by NK and PMN Effector Cells. *Blood* **2008**, *112*, 2390–2399, doi:10.1182/blood-2008-03-144600.

5. Stavenhagen, J.B.; Gorlatov, S.; Tuaillon, N.; Rankin, C.T.; Li, H.; Burke, S.; Huang, L.; Johnson, S.; Bonvini, E.; Koenig, S. Fc Optimization of Therapeutic Antibodies Enhances Their Ability to Kill Tumor Cells in Vitro and Controls Tumor Expansion in Vivo via Low-Affinity Activating Fcγ Receptors. *Cancer Research* **2007**, *67*, 8882–8890, doi:10.1158/0008-5472.CAN-07-0696.

6. Yoon, H.W.; Jo, M.; Ko, S.; Kwon, H.S.; Lim, C.S.; Ko, B.J.; Lee, J.C.; Jung, S.T. Optimal Combination of Beneficial Mutations for Improved ADCC Effector Function of Aglycosylated Antibodies. *Molecular Immunology* **2019**, *114*, 62–71, doi:10.1016/j.molimm.2019.07.007.

7. Chen, T.F.; Sazinsky, S.L.; Houde, D.; DiLillo, D.J.; Bird, J.; Li, K.K.; Cheng, G.T.; Qiu, H.; Engen, J.R.; Ravetch, J. v.; et al. Engineering Aglycosylated IgG Variants with Wild-Type or

Improved Binding Affinity to Human Fc Gamma RIIA and Fc Gamma RIIIAs. *Journal of Molecular Biology* **2017**, *429*, 2528–2541, doi:10.1016/j.jmb.2017.07.001.

8. Shields, R.L.; Namenuk, A.K.; Hong, K.; Meng, Y.G.; Rae, J.; Briggs, J.; Xie, D.; Lai, J.; Stadlen, A.; Li, B.; et al. High Resolution Mapping of the Binding Site on Human IgG1 for FcγRI, FcγRII, FcγRIII, and FcRn and Design of IgG1 Variants with Improved Binding to the FcγR. *Journal of Biological Chemistry* **2001**, *276*, 6591–6604, doi:10.1074/jbc.M009483200.

9. Nordstrom, J.L.; Gorlatov, S.; Zhang, W.; Yang, Y.; Huang, L.; Burke, S.; Li, H.; Ciccarone, V.; Zhang, T.; Stavenhagen, J.; et al. Anti-Tumor Activity and Toxicokinetics Analysis of MGAH22, an Anti-HER2 Monoclonal Antibody with Enhanced Fcγ Receptor Binding Properties. *Breast Cancer Research* **2011**, *13*, doi:10.1186/bcr3069.

10. Sazinsky SL, Ott RG, Silver NW, Tidor B, Ravetch JV, Wittrup KD. Aglycosylated immunoglobulin G1 variants productively engage activating Fc receptors. Proc Natl Acad Sci U S A. 2008 Dec 23;105(51):20167-72. doi: 10.1073/pnas.0809257105.

11. Boder, E.T.; Wittrup, K.D. Yeast Surface Display for Screening Combinatorial Polypeptide Libraries;Nat. Biotechnol. 1997; 15:553–557. doi: 10.1038/nbt0697-553

12. Goetze, A.M.; Liu, Y.D.; Zhang, Z.; Shah, B.; Lee, E.; Bondarenko, P. v.; Flynn, G.C. High-Mannose Glycans on the Fc Region of Therapeutic IgG Antibodies Increase Serum Clearance in Humans. *Glycobiology* **2011**, *21*, 949–959, doi:10.1093/glycob/cwr027.

13. De Pourcq K, De Schutter K, Callewaert N. Engineering of glycosylation in yeast and other fungi: current state and perspectives. Appl Microbiol Biotechnol. 2010 Aug;87(5):1617-31. doi: 10.1007/s00253-010-2721-1.

14. Choi, B.K.; Bobrowicz, P.; Davidson, R.C.; Hamilton, S.R.; Kung, D.H.; Li, H.; Miele, R.G.; Nett, J.H.; Wildt, S.; Gerngross, T.U. Use of Combinatorial Genetic Libraries to Humanize N-

Linked Glycosylation in the Yeast Pichia Pastoris. *Proc Natl Acad Sci U S A* **2003**, *100*, 5022–5027, doi:10.1073/pnas.0931263100.

15. Vervecken, W.; Kaigorodov, V.; Callewaert, N.; Geysens, S.; de Vusser, K.; Contreras, R. In Vivo Synthesis of Mammalian-like, Hybrid-Type N-Glycans in Pichia Pastoris. *Applied and Environmental Microbiology* **2004**, *70*, 2639–2646, doi:10.1128/AEM.70.5.2639-2646.2004.

16. Shenoy Anjali and Barb, A.W. Recent Advances Toward Engineering Glycoproteins Using Modified Yeast Display Platforms. In *Glycosylation: Methods and Protocols* ; Davey, G.P., Ed.; Springer US: New York, NY, 2022; pp. 185–205 ISBN 978-1-0716-1685-7.

17. Chiba Y, Suzuki M, Yoshida S, Yoshida A, Ikenaga H, Takeuchi M, Jigami Y, Ichishima E. Production of human compatible high mannose-type (Man5GlcNAc2) sugar chains in Saccharomyces cerevisiae. J Biol Chem. 1998 Oct 9;273(41):26298-304. doi: 10.1074/jbc.273.41.26298.

18. Shaheen, H.H.; Prinz, B.; Chen, M.; Pavoor, T.; Lin, S.; Houston-, N.R.; Moore, R.; Stadheim, T.A.; Zha, D. A Dual-Mode Surface Display System for the Maturation and Production of Monoclonal Antibodies in Glyco- Engineered Pichia Pastoris. PLOS ONE, 2013, *8*, 1–10, doi:10.1371/journal.pone.0070190.

19. Shenoy, A.; Yalamanchili, S.; Davis, A.R.; Barb, A.W. Expression and Display of Glycoengineered Antibodies and Antibody Fragments with an Engineered Yeast Strain. *Antibodies* **2021**, *10*, doi:10.3390/antib10040038.

20. Sjögren, J.; Cosgrave, E.F.J.; Allhorn, M.; Nordgren, M.; Björk, S.; Olsson, F.; Fredriksson, S.; Collin, M. EndoS and EndoS2 Hydrolyze Fc-Glycans on Therapeutic Antibodies with Different Glycoform Selectivity and Can Be Used for Rapid Quantification of High-Mannose Glycans. *Glycobiology* **2015**, *25*, 1053–1063, doi:10.1093/glycob/cwv047.

21. Sjögren, J.; Struwe, W.B.; Cosgrave, E.F.J.; Rudd, P.M.; Stervander, M.; Allhorn, M.; Hollands, A.; Nizet, V.; Collin, M. EndoS2 Is a Unique and Conserved Enzyme of Serotype M49 Group A Streptococcus That Hydrolyses N-Linked Glycans on IgG and A1-Acid Glycoprotein. *Biochemical Journal* **2013**, *455*, 107–118, doi:10.1042/BJ20130126.

22. Kao, D.; Danzer, H.; Collin, M.; Groß, A.; Eichler, J.; Stambuk, J.; Lauc, G.; Lux, A.; Nimmerjahn, F. A Monosaccharide Residue Is Sufficient to Maintain Mouse and Human IgG Subclass Activity and Directs IgG Effector Functions to Cellular Fc Receptors. *Cell Reports* **2015**, *13*, 2376–2385, doi:10.1016/j.celrep.2015.11.027.

23. Deisenhofer J. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-A resolution. Biochemistry. 1981 Apr 28;20(9):2361-70. PMID: 7236608.

24. Hiroaki Gouda, Miki Shiraishi, Hideo Takahashi, Koichi Kato, Hidetaka Torigoe, Yoji Arata, and Ichio Shimada. NMR Study of the Interaction between the B Domain of Staphylococcal Protein A and the Fc Portion of Immunoglobulin G, *Biochemistry* **1998** *37* (1), 129-136, DOI: 10.1021/bi970923f

25. Breidenbach, M.A.; Gallagher, J.E.G.; King, D.S.; Smart, B.P.; Wu, P.; Bertozzi, C.R. Targeted Metabolic Labeling of Yeast N-Glycans with Unnatural Sugars. *Proc Natl Acad Sci U S A* **2010**, *107*, 3988–3993, doi:10.1073/pnas.0911247107.

26. Qasba PK, Ramakrishnan B, Boeggeman E. Structure, and function of beta -1,4-galactosyltransferase. Curr Drug Targets. 2008 Apr;9(4):292-309. doi: 10.2174/138945008783954943. PMID: 18393823; PMCID: PMC2365515.

27. Dekkers, G.; Bentlage, A.E.H.; Plomp, R.; Visser, R.; Koeleman, C.A.M.; Beentjes, A.; Mok, J.Y.; van Esch, W.J.E.; Wuhrer, M.; Rispens, T.; et al. Conserved FcγR- Glycan

Discriminates between Fucosylated and Afucosylated IgG in Humans and Mice. *Molecular Immunology* **2018**, *94*, 54–60, doi:10.1016/j.molimm.2017.12.006.

28. Falconer, D.J.; Barb, A.W. Mouse IgG2c Fc Loop Residues Promote Greater Receptor-Binding Affinity than Mouse IgG2b or Human IgG1. *PLoS ONE* **2018**, *13*, doi:10.1371/journal.pone.0192123.

29. Huang, W.; Giddens, J.; Fan, S.Q.; Toonstra, C.; Wang, L.X. Chemoenzymatic Glycoengineering of Intact IgG Antibodies for Gain of Functions. *J Am Chem Soc* **2012**, *134*, 12308–12318, doi:10.1021/ja3051266.

CHAPTER 7

CONCLUSION

7.1. *Simultaneous N-glycan and protein engineering using yeast surface display (YSD)*

Despite the importance of both amino acid sequence and N-glycan composition of hIgG1 Fc for FcγRIIIa binding, very little has been done in combining these aspects to engineer Fc. Through the years, yeast surface display platforms have been developed using *Saccharomyces cerevisiae* strain EBY100 [1–6]This strain is highly efficient at homologous recombination however cumbersome to glycoengineer with high efficiencies resulting in deleterious strains or high N-glycan heterogeneity [7,8]. This can be attributed to the high levels of N-glycan processing that occurs in the Golgi due to an extensive network of mannosyltransferases present. Yeast strain *Pichia pastoris* has been relatively easier to glycoengineer however, cumbersome to create yeast surface display libraries [8–12]. Surface Display Libraries with this strain require the integration of the linearized library into the genome resulting in a smaller display library. In this case, a tradeoff has to be made between ease of library generation and efficiency of N-glycan engineering.

Work done by our group has shown that Fc with a single N-Acetylglucoasmine residue can still engage the FcγRIIIa receptor although with a 10-fold lower affinity compared to the corresponding wildtype interaction [13]. Truncating the N-glycan results in a loss of advantageous CH-π bonds between aromatic amino acids on the Fc and the monosaccharide units on the N-glycan, this results in a lowered affinity towards the receptor [14]. However, it

would also get rid of the highly immunogenic oligomannose portion of the yeast N-glycan [15]We decided to engineer yeast strain EBY100 to produce EBY100-(GPD)-EndoS2 a novel yeast display platform that is capable of displaying Fc with a single N-Acetylglucosamine (GlcNAc) [16]. This platform was created by targeting the immunoglobulin specific EndoS2 to the Endoplasmic Reticulum achieving high N-glycan processing efficiency (>95%) [17]. It is possible to screen for Fc hits that show enhanced binding with receptor FcγRIIIa and then utilize EndoS2 transglycosylase variants to add back the desired glycoform [18]. These reactions proceed with very high efficiencies (>95% as observed by LC-MS) and can add a broad range of N-glycans onto the antibody [18,19]. This class of enzyme have been used to successfully engineer the N-glycan on antibody Herceptin (produced in glycoengineered *Pichia pastoris*)[20].

We could also further engineer the EBY100-(GPD)-EndoS2 platform to display Fc with a complex type glycoform using these transglycosylase EndoS2 variants described previously. This enzyme EndoS2 D184M could be targeted to the yeast Golgi to further process the Fc (with a single GlcNAc) to have a complex type glycoform. Bertozzi and co-workers have shown that Saccharomyces cerevisiae strains can be engineered with a transporter to take up UDP-GlcNAc analogues and incorporate into their N-glycans [21]. We could engineer this strain (EBY100-(GPD)-EndoS2) to have a transporter for the uptake of oxazoline substrate (for complex type N-glycan). The fungal glycoform could then be processed in the E.R (cleavage of oligomannose glycoform by EndoS2) and then in the Golgi (addition of complex type N-glycan by EndoS2 D184M) to then display Fc with a complex type glycoform on the yeast surface.

7.2 *Isotope Labeling using novel yeast strain EBY100-Kar2p-T2A-PDI*

Yeast is an attractive model for isotope labeling of proteins as it combines advantages of prokaryotic expression platforms due to its ease of usage and cost, and its ability to express heterologous proteins with post-translational modifications. There have been several successful labeling attempts in *Pichia pastoris* [22,23]. However, very little has been done with respect to yeast strain *Saccharomyces cerevisiae* despite its wide usage. *Pichia pastoris* is often the yeast strain of choice when it comes to protein expression given its ability to produce large quantities of protein [24]. *Saccharomyces cerevisiae* has been trickier in this regard to produce heterologous protein [25].

Several groups in the past have resorted to using folding aids Kar2p (Hsp70) and PDI to boost heterologous protein expression [26–29]However, the effect the expression of these proteins would have on heterologous protein expression is hard to determine. Expressing several proteins simultaneously can result in E.R stress and result in lowered protein expression. In order to both boost expression of Fc as well as lower E. R stress in the cell, we simultaneously expressed Kar2p (Hsp70) and PDI by utilizing a ribosome skip site [EBY100-Kar2p-T2A-PDI] [30] .We also made use of the app8 secretion tag discovered by Wittrup and co-workers to facilitate better secretion of the protein into the media [31]. This platform worked very well to ^{15}N-label Fc with a ~91% ^{15}N isotope incorporation. The ^{15}N-HSQC spectra of this protein superimposed very well with a spectrum from a mammalian derived ^{15}N-labeled Fc [32]. However, the conditions used for ^{15}N-labeling cannot be used for ^{13}C labeling. Therefore, labeling conditions with this strain has to optimized to meet this goal.

Dual ^{15}N and ^{13}C labeling of Fc can possibly allow for understanding protein interactions between IgG1 Fc domain and receptor FcγRIIIa would be particularly useful in engineering related therapeutics. This has been challenging due to comparatively low affinity binding between Fc domain and FcγRIIIa receptor due to a broadening of peaks in the observed spectra resulting in difficulty discerning bound vs unbound state. Using a technique such as NMR would allow for a better understanding of the dynamics and interfaces involved in this interaction.

7.3 References

1. Kieke MC, Shusta EV, Boder ET, Teyton L, Wittrup KD, Kranz DM. Selection of functional T cell receptor mutants from a yeast surface-display library. Proc Natl Acad Sci U S A. 1999 May 11;96(10):5651-6. doi: 10.1073/pnas.96.10.5651.

2. Gai, S.A.; Wittrup, K.D. Yeast Surface Display for Protein Engineering and Characterization. *Current Opinion in Structural Biology* **2007**, *17*, 467–473, doi:10.1016/j.sbi.2007.08.012.

3. Kondo, A.; Ueda, M. Yeast Cell-Surface Display - Applications of Molecular Display. *Applied Microbiology and Biotechnology* **2004**, *64*, 28–40, doi:10.1007/s00253-003-1492-3.

4. Cherf, G.M.; Cochran, J.R. Chapter 8 Applications of Yeast Surface Display for Protein Engineering. Methods in Molecular Biology, **2015**, *1319*, doi:10.1007/978-1-4939-2748-7.

5. Pepper LR, Cho YK, Boder ET, Shusta EV. A decade of yeast surface display technology: where are we now? Comb Chem High Throughput Screen. 2008 Feb;11(2):127-34. doi: 10.2174/138620708783744516.

6. Boder, E.T.; Wittrup, K.D. Yeast Surface Display for Screening Combinatorial Polypeptide Libraries;Nat. Biotechnol. 1997; 15:553–557. doi: 10.1038/nbt0697-553

7. Zhou, J.; Zhang, H.; Liu, X.; Wang, P.G.; Qi, Q. Influence of N-Glycosylation on Saccharomyces Cerevisiae Morphology: A Golgi Glycosylation Mutant Shows Cell Division Defects. *Current Microbiology* **2007**, *55*, 198–204, doi:10.1007/s00284-006-0585-5.

8. Chiba Y, Suzuki M, Yoshida S, Yoshida A, Ikenaga H, Takeuchi M, Jigami Y, Ichishima E. Production of human compatible high mannose-type (Man5GlcNAc2) sugar chains in Saccharomyces cerevisiae. J Biol Chem. 1998 Oct 9;273(41):26298-304. doi: 10.1074/jbc.273.41.26298.

9. Vervecken, W.; Kaigorodov, V.; Callewaert, N.; Geysens, S.; de Vusser, K.; Contreras, R. In Vivo Synthesis of Mammalian-like, Hybrid-Type N-Glycans in Pichia Pastoris. *Applied and Environmental Microbiology* **2004**, *70*, 2639–2646, doi:10.1128/AEM.70.5.2639-2646.2004.

10. Ha, S.; Wang, Y.; Rustandi, R.R.; Choi, B.K.; Bobrowicz, P.; Davidson, R.C.; Hamilton, S.R.; Kung, D.H.; Li, H.; Miele, R.G.; et al. In Vivo Synthesis of Mammalian-like, Hybrid-Type N-Glycans in Pichia Pastoris. *Proc Natl Acad Sci U S A* **2013**, *3*, 2639–2646, doi:10.1128/AEM.70.5.2639-2646.2004.

11. Parsaie Nasab F, Aebi M, Bernhard G, Frey AD. A combined system for engineering glycosylation efficiency and glycan structure in Saccharomyces cerevisiae. Appl Environ Microbiol. 2013 Feb;79(3):997-1007. doi: 10.1128/AEM.02817-12.

12. Shaheen, H.H.; Prinz, B.; Chen, M.; Pavoor, T.; Lin, S.; Houston-, N.R.; Moore, R.; Stadheim, T.A.; Zha, D. A Dual-Mode Surface Display System for the Maturation and Production of Monoclonal Antibodies in Glyco- Engineered Pichia Pastoris. PLOS ONE, **2013**, *8*, 1–10, doi:10.1371/journal.pone.0070190.

13. Subedi, G.P.; Barb, A.W. The Structural Role of Antibody N-Glycosylation in Receptor Interactions. *Structure* **2015**, *23*, 1573–1583, doi:10.1016/j.str.2015.06.015.

14. Subedi, G.P.; Hanson, Q.M.; Barb, A.W. Restricted Motion of the Conserved Immunoglobulin G1 N-Glycan Is Essential for Efficient FcγRIIIa Binding. *Structure* **2014**, *22*, 1478–1488, doi:10.1016/j.str.2014.08.002.

15. Goetze, A.M.; Liu, Y.D.; Zhang, Z.; Shah, B.; Lee, E.; Bondarenko, P. v.; Flynn, G.C. High-Mannose Glycans on the Fc Region of Therapeutic IgG Antibodies Increase Serum Clearance in Humans. *Glycobiology* **2011**, *21*, 949–959, doi:10.1093/glycob/cwr027.

16. Shenoy, A.; Yalamanchili, S.; Davis, A.R.; Barb, A.W. Expression and Display of Glycoengineered Antibodies and Antibody Fragments with an Engineered Yeast Strain. *Antibodies* **2021**, *10*, doi:10.3390/antib10040038.

17. Collin, M.; Olsén, A. EndoS, a Novel Secreted Protein from Streptococcus Pyogenes with Endoglycosidase Activity on Human IgG. *EMBO Journal* **2001**, *20*, 3046–3055, doi:10.1093/emboj/20.12.3046.

18. Li, T.; Tong, X.; Yang, Q.; Giddens, J.P.; Wang, L.X. Glycosynthase Mutants of Endoglycosidase S2 Show Potent Transglycosylation Activity and Remarkably Relaxed Substrate Specificity for Antibody Glycosylation Remodeling. *Journal of Biological Chemistry* **2016**, *291*, 16508–16518, doi:10.1074/jbc.M116.738765.

19. Huang, W.; Giddens, J.; Fan, S.Q.; Toonstra, C.; Wang, L.X. Chemoenzymatic Glycoengineering of Intact IgG Antibodies for Gain of Functions. *J Am Chem Soc* **2012**, *134*, 12308–12318, doi:10.1021/ja3051266.

20. Liu, C.P.; Tsai, T.I.; Cheng, T.; Shivatare, V.S.; Wu, C.Y.; Wu, C.Y.; Wong, C.H. Glycoengineering of Antibody (Herceptin) through Yeast Expression and in Vitro Enzymatic Glycosylation. *Proc Natl Acad Sci U S A* **2018**, *115*, 720–725, doi:10.1073/pnas.1718172115.

21. Breidenbach, M.A.; Gallagher, J.E.G.; King, D.S.; Smart, B.P.; Wu, P.; Bertozzi, C.R. Targeted Metabolic Labeling of Yeast N-Glycans with Unnatural Sugars. *Proc Natl Acad Sci U S A* **2010**, *107*, 3988–3993, doi:10.1073/pnas.0911247107.

22. Ali, R.; Clark, L.D.; Zahm, J.A.; Lemoff, A.; Ramesh, K.; Rosenbaum, D.M.; Rosen, M.K. Improved Strategy for Isoleucine 1H/13C Methyl Labeling in Pichia Pastoris. *Journal of Biomolecular NMR* **2019**, *73*, 687–697, doi:10.1007/s10858-019-00281-1.

23. Kjaergaard, M.; Gårdsvoll, H.; Hirschberg, D.; Nielbo, S.; Mayasundari, A.; Peterson, C.B.; Jansson, A.; Jørgensen, T.J.D.; Poulsen, F.M.; Ploug, M. Solution Structure of Recombinant Somatomedin B Domain from Vitronectin Produced in Pichia Pastoris . *Protein Science* **2007**, *16*, 1934–1945, doi:10.1110/ps.072949607.

24. Karbalaei, M.; Rezaee, S.A.; Farsiani, H. Pichia Pastoris: A Highly Successful Expression System for Optimal Synthesis of Heterologous Proteins. *Journal of Cellular Physiology* 2020, *235*, 5867–5881.

25. Horwitz, Arnold H., C. Paul Chang, Marc Better, Karl Erik Hellstrom, and Randy R. Robinson. Secretion of Functional Antibody and Fab Fragment from Yeast Cells. *Proceedings of the National Academy of Sciences of the United States of America* 85, no. 22 (1988): 8678–82.

26. Zhang, W.; Zhao, H.L.; Xue, C.; Xiong, X.H.; Yao, X.Q.; Li, X.Y.; Chen, H.P.; Liu, Z.M. Enhanced Secretion of Heterologous Proteins in Pichia Pastoris Following Overexpression of Saccharomyces Cerevisiae Chaperone Proteins. *Biotechnology Progress* **2006**, *22*, 1090–1095, doi:10.1021/bp060019r.

27. Ryabova LA, Desplancq D, Spirin AS, Plückthun A. Functional antibody production using cell-free translation: effects of protein disulfide isomerase and chaperones. Nat Biotechnol. 1997 Jan;15(1):79-84. doi: 10.1038/nbt0197-79. PMID: 9035111.

28. Rakestraw, A.; Wittrup, K.D. Contrasting Secretory Processing of Simultaneously Expressed Heterologous Proteins in Saccharomyces Cerevisiae. *Biotechnology and Bioengineering* **2006**, *93*, 896–905, doi:10.1002/bit.20780.

29. de Ruijter, J.C.; Koskela, E. v.; Frey, A.D. Enhancing Antibody Folding and Secretion by Tailoring the Saccharomyces Cerevisiae Endoplasmic Reticulum. *Microbial Cell Factories* **2016**, *15*, doi:10.1186/s12934-016-0488-5.

30. Shenoy, A.; Davis, A.R.; Roberts, E.T.; Amster, I.J.; Barb, A.W. Metabolic15N Labeling of the N-Glycosylated Immunoglobulin G1 Fc with an Engineered Saccharomyces Cerevisiae Strain. *Journal of Biomolecular NMR* **2022**, doi:10.1007/s10858-022-00397-x.

31. Rakestraw, J.A.; Sazinsky, S.L.; Piatesi, A.; Antipov, E.; Wittrup, K.D. Directed Evolution of a Secretory Leader for the Improved Expression of Heterologous Proteins and Full-Length Antibodies in Saccharomyces Cerevisiae. *Biotechnology and Bioengineering* **2009**, *103*, 1192–1201, doi:10.1002/bit.22338.

32. Yamaguchi, Y.; Nishimura, M.; Nagano, M.; Yagi, H.; Sasakawa, H.; Uchida, K.; Shitara, K.; Kato, K. Glycoform-Dependent Conformational Alteration of the Fc Region of Human Immunoglobulin G1 as Revealed by NMR Spectroscopy. *Biochimica et Biophysica Acta - General Subjects* **2006**, *1760*, 693–700, doi:10.1016/j.bbagen.2005.10.002.