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Abigail Saltzman 4/10/23

Deep Mutational Scanning to Identify IgG1 Fc Mutations Affecting its Affinity to EndoS2

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Abstract
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Endoglycosidases are an important group of enzymes that hydrolyze oligosaccharides from glycoproteins. Because IgG antibodies use N-linked glycan on their fragment crystallizable (Fc) region to promote immune responses via effector functions, they can be inactivated by endoglycosidases. *Streptococcus pyogenes,* a gram-positive bacterial species, produces two novel endoglycosidases, EndoS and EndoS2 to evade host immune responses. EndoS and EndoS2 recognize both the IgG glycan and the Fc of the IgG. Specificity for the Fc glycoprotein backbone, rather than only the glycan, makes EndoS and EndoS2 unique among carbohydrate active enzymes and attractive targets to the field of immunotherapy. To take advantage of these enzymes or to counteract their natural pathogenic activity, their mechanism of substrate recognition must be understood. Consequently, describing the binding modes of EndoS2 and EndoS to the IgG Fc is an area of active research. The binding modes of the two enzymes to the Fc region are known to be quite similar. However, EndoS2 is of particular interest due to its ability to hydrolyze a wider range of glycans than EndoS. In this paper, we tested the effect of every single point mutation in the Fc and hinge sequence on Endo-S2 binding affinity using deep mutational scanning (DMS) and a mammalian surface display platform. We employed flow cytometry and cell sorting to collect the Fc mutants with the highest and lowest affinity for EndoS2 (top and bottom 15 %). Our data can help confirm which portions of the Fc are likely not involved with EndoS2 contact, thus narrowing down which residues may be most central to the complex formation. The scan also provides preliminary insight into which specific mutations at the relevant residues would affect EndoS2 binding. Identifying these mutations would be beneficial to the development of monoclonal antibodies with a different EndoS2 affinity, which could be used clinically.

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Introduction

Immunoglobulins (IgGs), the most abundant antibody in human serum, contribute to the complex and highly specific ways that the body responds to pathogens. The upper portion of an IgG contains two identical fragment antigen-binding (Fab) regions, which are responsible for recognizing pathogen antigens. The lower portion, attached via a flexible hinge region, is the fragment crystallizable (Fc) region, a homodimer stabilized with disulfide bonds. The Fc region interacts with other immune system agents and enlists them to effectively fight infections. The Fc homodimer contains two protomers, FcP1 and FcP2, that are divided into two domains: CH2 and CH3.[1] A vital feature of the IgG Fc region is a conserved glycosylation site at the Asn-297 residue in the CH2 domain (figure 1). There are over 33 different oligosaccharides that are found N-linked to the Fc regions of IgGs. These glycans all have a $Man_3GlcNAc$ penta-saccharide core and are post translationally turned into either complex type (CT), high-mannose (HM) or hybrid type (Hy) glycans.[2] In humans these glycans are naturally biantennary complex type glycans made up of over 20 different unique combinations of monosaccharides. This diversity allows for countless precise and efficient interactions with other immune system receptors, leading to an array of effector functions. The receptors that interact with IgGs include Fc-gamma receptors (FcγRs) on the surface of leukocytes and complement components (C1qs). Stimulation of these receptors leads to downstream effector functions such as antibody-dependent–cellular-cytotoxicity (ADCC) and complement-dependent–cellular-cytotoxicity (CDCC).[3]

These important immune responses, among others, are severely inhibited when the IgG Fc is missing the Asn-297 glycan. Consequently, certain pathogens have developed immune system evasion techniques that target this glycosylation site and therefore promote their own survival. One such pathogen is *Streptococcus pyogenes* (group A Streptococcus; GAS), a gram-

positive bacterial species that is responsible for Group-A Streptococcal infections, which can range from mild to severe. GAS has been found to express a group of enzymes called endo-β-N-acetylglucosaminidases (endoglycosidases). These enzymes can hydrolyze the β-1,4-N-acetyl-D-glucose amine linkages of glycoproteins like IgGs.

EndoS and EndoS2 are the two of the most prominent endoglycosidases found natively expressed in GAS and can both remove the Asn-297 Fc glycan. EndoS is a 108 kDa protein that was discovered in the M1 serotype of GAS and EndoS2, a 95 kDa protein, was found in the M49 serotype.[4-5] EndoS can hydrolyze the CT glycans on IgG while EndoS2 can hydrolyze all three subsets of IgG glycans: CT, HM, and H_y.[6] However, unlike most enzymes that act on carbohydrates, EndoS and EndoS2 do not only display specificity for various glycoforms, but for the glycoprotein backbone, the IgG Fc. In fact, EndoS and EndoS2 are rendered inactive when the IgG antibody is denatured, despite the glycan's presence.[5] This suggests a catalytically vital interaction between the structure of these enzymes and the Fc region of the IgG that the glycan is attached to, in addition to an interaction with the glycan itself. This rare glycoprotein selectivity makes EndoS and EndoS2 potentially useful in treating a variety of illnesses. Since these enzymes deactivate IgGs, they may be able to counteract excessive immune responses in patients with autoimmune disorders. In addition, EndoS and EndoS2 synthases, mutants that reattach glycans rather than hydrolyze them, are promising tools to the field of immunotherapy. These variants could help synthesize IgGs that elicit stronger immune responses to cancers and infectious diseases.[7]

To manipulate the glycoprotein specificity of these enzymes and develop these therapies, the structures of EndoS and EndoS2, as well as the mechanism of interaction between them and the IgG Fc region, must be understood. While the amino acids sequence identity of the two

enzymes is only 37%, they are structurally similar, both being multi-modular and adopting a distinctive V shape. The domains of EndoS, in order of N-terminus to C-terminus, are the glycoside hydrolase (GH) domain, the leucine-rich repeat (LRR) domain, the hybrid IgG domain, a ß-sandwich domain, and three-helix bundle (3H) domain (Figure 2a).[8] EndoS2 can be divided into the same domains, but the 3H domain is absent (figure 2b).[6] A single particle cryo-EM structure of EndoS docking to the Fc region of IgG1 monoclonal antibody revealed valuable details of the enzyme's substrate recognition mechanism. While currently there is no cryo-EM structure for EndoS2 in complex with the Fc, comparisons of cryo-EM structures of EndoS and EndoS2 alone suggest that they interact with the IgG1 Fc region in a very similar manner. The crystal structure of EndoS and Fc demonstrated that the GH and β- sandwich domains are responsible for gripping the Fc region. Consequently, the Fc region sits within the opening of the enzyme's 'V,' while orienting the Asn-297 glycan towards the GH domain, where the active site is. In addition to the contact with the Asn-297 glycan, the GH domains of EndoS and EndoS2 have substantial peptide-peptide interactions (PPIs) with the CH2 domain of the FcP1 protomer. Some FcP1 residues identified as participants in these PPIs are P271, E272, R292, Y296, and S298. These interactions include hydrogen bonding, hydrophobic interactions, and salt bridges.[9]

The β-sandwich domain is another prominent contributor to the enzymes' contact with the Fc region and their consequent specificity for IgGs. This domain was previously thought to be a carbohydrate binding module (CBM) due to its structural similarities to the CBMs within the CAZy classification system.[8] However, the cryo-EM structure revealed that the glycan is not interacting with the β-sandwich at all. In reality, this domain participates in PPIs with residues on the Fc, specifically at the CH3 and CH2 joint of the FcP1. One essential residue of EndoS, W803, is known to have a π-π interaction with the H435 residue found in the Fc CH3 domain,

and a hydrophobic interaction with the I253 residue found in the CH2 domain. Another important aromatic residue, Y909, interacts with the side chains of Q311, L314 and N315 of the CH3 domain. EndoS2 is believed to participate in similar interactions through its W712 and Y820 residues, which are at positions analogous to those of W803 and Y909 on EndoS, respectively.[9]

Alanine scans for both the Fc and EndoS proteins explored if mutating the residues that were identified in the cryo-EM structure would affect the Asn-297 glycan's rate of removal. This is vital information to have if these enzymes are to be used for therapeutic glycan remodeling of IgGs. It was discovered that mutating the W803 residue to alanine made EndoS completely dysfunctional, leading to zero glycan hydrolysis. Altering many of the residues throughout the CH2 and CH3 domains of the Fc protein also significantly affected the hydrolytic activity of EndoS. However, the region of mutations that led to the most notable decrease in hydrolysis was clearly the CH2-CH3 joint (figure 3).[9]

The relevant PPIs between the Fc region and EndoS/EndoS2 can potentially be manipulated to produce antibodies with a different affinity for these enzymes. This can be done by inserting amino acids substitutions at the relevant Fc residues. The structural analysis and alanine scans that have been previously conducted may be helpful in pinpointing where the most effective substitutions would be. However, these experiments do not provide much insight into which, if any, specific amino acid alteration would increase or decrease the strength of a PPI at a relevant residue.

In this experiment, an initial exploration into potentially useful Fc mutations is conducted using a method called deep mutational scanning (DMS).[10] This technique utilizes a library of gene mutants that include all the possible single point mutations in the Fc-hinge sequence (amino

acids 216-447). The Fc mutants in this library, once transfected and displayed on the surface of mammalian cells, can be assessed for their affinity to EndoS and EndoS2. This is done using Fluorescent-activated cell sorting (FACS). This method uses fluorescent tags to evaluate the frequency of an enzyme binding to a substrate expressed on a cell's surface. A BD FACS Aria II instrument was used to isolate the cells expressing Fc mutants with particularly high or low affinity specifically for EndoS2. This paper presents the preliminary findings from the deep mutational scan. This may provide insight into the molecular mechanism of Fc and EndoS2 binding. In addition, Fc mutants with a different affinity for EndoS2 could have clinical applications. For example, an Fc with a higher EndoS2 affinity may be a more efficient tool in the chemoenzymatic synthesis of monoclonal IgGs. Higher affinity IgGs could also act as competitive inhibitors, protecting an infected host's native IgGs from hydrolysis. Alternatively, lower affinity Fc mutants could be useful in creating monoclonal antibodies that evade attack by endoglycosidases and simultaneously promote valuable immune responses.[11]

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Materials and Methods

Expression and purification of two inactive EndoS2 mutants

Plasmids for two EndoS2 mutants, pET32-Avi-EndoS2-E186L (TC129) and pET32-EndoS2-E186L-Avi (TC130) fused to a CPD domain and a polyhistidine tag at the C-terminus, were obtained from the Sundberg lab collection. Proteins were expressed in *Escherichia coli* BL21(DE3) cells. Following a standard bacterial transformation protocol using ampicillin resistance as a selection marker, a single BL21 colony was cultured at 37°C in one liter of LB medium with 100 µg/ml of ampicillin. Once the bacterial density reached an OD_{600} value of 0.6-0.8, expression was induced by adding isopropyl b-D-1-thio-galactopyranoside (IPTG) to the bacterial culture at a final concentration of 0.5 mM. The bacterial culture was left for expression overnight at 22°C. The cells were then harvested through centrifugation and resuspended using phosphate buffer saline (PBS) with 10% glycerol. The cells were lysed using sonication and centrifuged again to remove cell debris. Ni^{2+}-immobilized metal-affinity chromatography was used for purification of proteins. The lysate was passed through the nickel resin columns binding the proteins via their CPD domains. Phytic acid was used to cleave the proteins from their CPD domains and release them from the nickel columns. The presence of the EndoS2 proteins in the purified samples were verified on an SDS-Page gel. The samples were dialyzed using PBS to remove the left over phytic acid and protein concentration was measured using an Eppendorf Bio Spectrometer.

Biotinylation of EndoS2 mutants

The Avi-tags attached to the two EndoS2 mutants theoretically provide an anchor for a biotin molecule to be attached. Both mutants were put through a biotinylation process using the Avidity BirA500 kit and protocol. After an hour of the initial incubation at 30° C, another 13 µL of 500uM d-biotin and 2 µl of 1 mg/ml Bir A ligase was added and the reactions were incubated for

another hour at 30°C. After dialysis in PBS buffer, a streptavidin assay was run. Sample buffer and PBS were added to 5-10 µg of both protein mutants to a total volume of 30µl. The samples were boiled at 90°C for 3 minutes, spun down and cooled at RT. The samples were split into two aliquots of 15 µl. In total there were 4, 15 µl aliquots, 2 for TC129 and 2 for TC130. To one of each protein's samples, 5µl of 2mg/ml streptavidin was added. The proteins were incubated at room temperature for 5 minutes and run on an SDS-PAGE gel to be analyzed.

Mammalian cells transfection

The pLVX-IRES-ZsGreen1 expression plasmid, containing a Myc-tag, GFP sequence, a transmembrane region, and an ampicillin resistance gene, was obtained from Fillip Frank. The Sundberg lab then cloned the wildtype sequence of the IgG1 Fc and hinge regions (amino acids 216-447) into the pLVX plasmid using Gibson Assembly. Meanwhile, a stable cell line of HEK293T cells was generated (Sundberg Lab). These cells were maintained in a 37°C shaker and diluted by a factor of 10 every other day. The day before transfection these cells were counted and diluted to a concentration of 0.5 million cells/ml with FreeStyle™ F17 Expression Medium. The cells were put back into the shaker overnight. The next day the cells were counted and diluted to a concentration of 1 million cells/ml. 8smls of Opti-MEM™ reduced serum medium was added to a 15 ml tube. 112 µg of pLVX WT plasmids was added to the medium and the tube was inverted 10x. Next, 350 µl of polyethylenimine (PEI) was added and the tube was inverted 20x. The transfection mixture was incubated at room temperature for 20 minutes then added dropwise to the HEK293T cells. The cells were placed back into the shaker and were incubated for 36 hours.

EndoS2 binding to Fc

1.2 million HEK293T cells transfected with the pLVX-Fc wildtype plasmid were collected, washed with PBS-BSA 1% and aliquoted evenly to 12 wells of a Nunc™ 96 well U-bottom plate in volumes of 20 µl. EndoS2 TC129, the mutant selected based on the results of the streptavidin binding assay, was then added to the first 11 wells through a 2-fold serial dilution. No EndoS2 was added to the HEK293T cells in the final well. The starting EndoS2 concentration ranged from 20 µM. Once the enzyme was added to the cells, they were incubated for one hour at room temperature, with agitation. Cells were washed 3 times, then stained with anti-Myc-(71D10)-PE antibodies to detect Fc and PerCPeFluor-710 streptavidin (Invitrogen) to detect EndoS2 on the cells' surface. This was done in a volume of 150 µl with a dilution factor of 1:300, fluorophores to PBS-BSA 1%. The cells were agitated for 1 hour on a shaker at room temperature. Next, the cells were washed three times and viewed on the flow cytometer. Gating was employed to isolate cells that were GFP+ and anti-Myc+. Median PerCP values were used to assess EndoS2 binding to Fc on the cell surface and to calculate the enzyme's K_d.

Deep mutational surface-display Fc library generation

A 'library' of Fc mutants was ordered from Twist Bioscience. The company created a library of constructs, each with a single amino acid substitution at positions 216-447 of the hinge and Fc regions of IgG1. The company attempted to switch each wildtype residue for all 19 alternative amino acids. Each successfully mutated construct was encoded with a 15-nucleotide long bar code directly following the Fc sequence. The Sundberg lab then cloned this library into the pLVX-IRES-ZsGreen1 lentiviral expression plasmid using Gibson assembly. Pacific Biosciences was enlisted to sequence the cloning products and assign the 15 nucleotide bar codes to their

respective mutants. Stable cell lines expressing this library were generated using LV-MAX lentiviral production system (ThermoFisher).

Fluorescence-activated cell sorting of Fc library to select mutants with different affinity to EndoS2

30 million HEK293T cells expressing the Fc library on their surface were collected, washed, and resuspended in PBS-BSA 1%. EndoS2 was then added to the cells with a final enzyme concentration of 3.9 µM. The final volume of the mixture was 500 µl. After one hour of incubation with EndoS2, cells were washed and incubated in 150 µl PBS-BSA 1% with PerCP-710 and Myc-Tag (71D10) at 1:300 dilution of fluorophore to PBS. The cells were incubated for another hour, washed, and sorted on the BD FACS Aria II instrument (BD) in the Emory Flow Cytometry Core. The cells population was plotted on graphs with the Anti-Myc signal on the y axis and the PerCP signal on the x axis. Anti-Myc signal was used to account for differences in expression. Cells expressing Fc mutants with higher affinity for EndoS2 had stronger PerCP signals. Diagonal gates were used to select cells with the highest, the top 15 %, and lowest, bottom 15 %, PerCP-710 signal. $3x10^5$ and $1.0 x10^6$ cells were collected in LV-MAX production medium and processed for identification of mutations.

High-throughput sequencing of sorted cell populations

Cells with the high and low PerCP-710 signal were washed with PBS and the RNA was extracted. The extraction was performed with the GeneJet RNA purification kit (ThermoFisher). A High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) was used to produce cDNA of the Fc mutants that exhibited higher and lower binding to EndoS2. Primers that would only bind to the barcode region of the mutants were used to PCR-amplify the cDNA. The amplification was performed using the Q5 PCR protocol (New England Biolabs). The PCR

reaction was run for 30 cycles and the product was verified on a 1% agarose DNA gel. The cDNA was then put through the same PCR protocol again, but for only 8 PCR cycles to decrease the chance of mutagenesis. PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) and submitted to Emory Genomic core for sequencing. Specifically, the purified amplicons were appended with dual-indexed bar codes using the library amplification protocol of the Illumina NexteraXT DNA Library Preparation kit (the tagmentation protocol was skipped). Libraries were validated by capillary electrophoresis on an Agilent 4200 TapeStation, pooled, and sequenced on an Illumina NovaSeq 6000 at PE100 or PE26x91 to achieve a depth of approximately 10 million reads per sorted sample and 50 million reads for each reference sample.

Site directed mutagenesis of Fc wildtype to introduce mutations with different affinity for EndoS2

Using the Emory Genomics Core data, 5 of the mutations that most significantly increased EndoS2 affinity, and another 5 that most significantly decreased affinity, were chosen to be introduced into the Fc wildtype via site-directed mutagenesis PCR. Once the mutants were selected, a set of forward and reverse primers for each mutant was ordered from ITD (Integrated DNA Technologies). The 10 sets of primers were designed with the base pair deviations from Fc wildtype necessary for introducing the selected amino acid mutation. The 125 ng of each primer was added to 100 ng of the pLVX- Fc wildtype plasmid. The other reagents, dNTP, reaction buffer and PfuUltra high-fidelity DNA polymerase, were sourced from the Agilent quick change II site directed mutagenesis kit. 5µl of 10x reaction buffer, 1µl of dNTP and the appropriate amount of sterilized H_2O necessary to reach a final volume of 50 µl was added to each PCR

reaction. Finally, 1µl of Pfu (2.5 U/ µl) was added and mixed. The thermocycler settings found in the Agilent quick change II protocol were used with the following alterations: denaturing was done for 2 minutes, elongation for 2.5 min/kb and the PCR was run for 34 cycles, rather than 16-18.[10] After the PCR protocol was complete, 1µl of Dpn1 restriction enzyme (10 U/µl), also found in the Agilent kit, was added to each reaction to eliminate residual template DNA.

Plasmid amplification and retrieval of Fc mutants

To amplify the site-directed mutagenesis PCR product 4µl from each PCR product was mixed into 45µl aliquots of 10 xl Gold high competency cells. The cells were put on ice for 30 minutes and then heat shocked at 42° C for 45 seconds. Next, 900µl of LB were mixed with the cells and they were placed back on ice for 2 minutes. The cells were then placed in a 37° C shaker for 1 hour and then spun down for 1 minute at 13000 rpm. The supernatant was discarded, and all the cells were plated on ampicillin plates. The plates were left at 37° C overnight. The following day single colonies were picked up from the plate and placed in 10 mls of Nzy+ broth, with 100 µg/µl ampicillin. The precultures were kept in the 37°C shaker for 12-16 hours. The next day, the precultures were spun down and the bacterial DNA was purified using the QIAGEN miniprep kit and reagents. Once purified, 100 ng of each DNA sample was sent to GENEWIZ for sequencing to confirm the presence of the mutations.

Results

Expression, purification and biotinylation of EndoS2 mutants

Two constructs of catalytically inactive EndoS2, TC129 and TC130 were expressed and purified (figure 4). These mutants differ only in the position of their Avi-tag. In TC129, the Avi-tag is at the N-terminus before the GH domain, while in TC130 it is positioned at the C-terminus after the ß-sandwich domain. An Avi-tag is a recognition site for the BirA ligase that will attach biotin to the lysine residue inside the tag. Biotinylated EndoS2 will subsequently be labelled with a streptavidin-linked fluorophore, allowing for its detection by flow cytometry. Both mutant proteins were expressed in E. coli, purified using His-tag and Ni^{2+}-immobilized metal-affinity chromatography, and biotinylated. To assess the biotinylation efficiency we performed a gel shift assay using streptavidin. A fully biotinylated protein will completely shift on SDS-PAGE upon pre-incubation with streptavidin. As a control we incubated a sample of non-biotinylated protein, a sample of protein without streptavidin added and streptavidin alone. All samples were run in parallel (Figure 4). The SDS-PAGE gel showed that there was a weight shift of ~66 kDa after TC129 was incubated with streptavidin, confirming that TC129 was successfully biotinylated. However, TC130 did not show observable biotinylation of the TC130 mutant, as there was no weight difference between the protein with and without streptavidin added (figure 5). Later repetitions yielded the same result, demonstrating that EndoS2 is likely unable to be biotinylated at the C terminus. Therefore, TC129 was the only EndoS2 mutant used for the rest of the project.

A mammalian surface display platform for IgG1 Fc and hinge domains

To display the Fc and hinge regions on the surface of mammalian cells the proteins' sequence (amino acids 216-447) was cloned into the lentiviral expression plasmid (pLVX-IRES-ZsGreen). This vector contains an N-terminal Myc-tag followed by a signal peptide (SP) (derived from

IgG4) and a C-terminal transmembrane region (TM) (derived from PDGFR). Following the TM region is a 3' internal ribosomal entry site (IRES) and GFP, which was used as a selection marker for expression. Post cloning, the plasmid included the Fc and hinge regions positioned between the Myc-tag and the transmembrane region. These neighboring domains allow the Fc and hinge regions to be displayed on a mammalian cell's surface. Because the Myc-tag should be expressed outside the cell, an anti-Myc fluorophore can be used to confirm surface display (figure 6). To validate the surface-display system, a stable cell line expressing the IgG1-Fc WT was generated (Sundberg Lab) using the lentiviral system. This cell line could be used to assess the ability of EndoS2 to bind to an IgG1 Fc when the region is expressed on the cell surface. The HEK293 cells expressing Fc were incubated with decreasing concentrations of EndoS2 via a 2-fold dilution. The cells were then stained with SA-PerCp-eFluor 710, which binds to the biotin molecule on EndoS2 and anti-Myc-PE antibodies which attaches to the Myc tag at the N-terminal of Fc (Figure 7). We then used flow cytometry to measure Fc binding across 11 EndoS2 concentrations, enabling the calculation of a dissociation constant for the binding of Fc to EndoS2. The Kd was calculated using normalized Median Fluorescence Intensity (MFI) of the PerCP-710 signal (figure 8). Previously, quantitative line shape analysis of methyl-TROSY spectra showed that EndoS2D186L binds Rituximab-Fc with a dissociation constant of 3.1μM (Sundberg Lab Jono paper) and similar Kd of ~9 μM obtained by surface plasmon resonance for the binding of EndoS2D186L to IgG1-CT.[6] A Kd of 3.9 μM measured by our technique is consistent with that data obtained using recombinant proteins.

A deep mutational scanning library of IgG1 Fc and hinge regions

A site-saturation library containing almost all possible amino acid substitutions of the Fc and hinge regions (amino acids 216-447) was synthesized by Twist Bioscience. Theoretically, this library could produce 4408 different plasmids, as there are 19 possible amino acid substitutions for 232 amino acids. However, Pacific Biosciences' long-read sequencing of cloned plasmids' barcodes revealed that 2 Fc region residues, L242 and A431, failed to mutate. Ultimately, PacBio assigned unique barcodes to 4376 single amino acid mutants (figure 9).

Identification of Fc mutants with different affinity for EndoS2

The HEK293T cells transfected with the Fc mutant library were sorted on the BD Aria II instrument using diagonal gates. The cells expressing Fc mutants that had the highest (15%) and lowest (15%) EndoS2 affinity were collected (figure 10). After RNA extraction, reverse transcription, and PCR-amplification, the DNA barcodes for the Fc mutants with higher and lower affinity for EndoS2 were sent to Emory genomics core for sequencing. The unsorted library of Fc mutants was used to assess which mutants disproportionately appeared in either the top 15% or bottom 15% of binders. This was done by calculating enrichment scores for each mutant in both sorted populations. Enrichment scores represent the ratio of how frequently a mutant appears in the selected population to its frequency in the reference population, the unsorted mutation library. These scores were normalized and the mutants that had the highest and lowest affinities were assigned an enrichment score of 1. The enrichment scores provided initial insight into which mutations may positively and negatively affect Fc binding to EndoS2. In general, the data alternates between residues with many higher affinity mutants and residues with many lower affinity mutants, with large sections of amino acids where mutations had negligible effect on binding affinity. Most mutations that affected EndoS2 and Fc binding were found in the CH2 domain. As expected, no hinge region mutations (amino acids 216-227)

showed up in either binder population and, outside of a few residues around the H435 hydrophobic site, mutants in the CH3 domain residues did not have a significant effect either. Residues within the CH2 domain that had many high affinity mutants included 241, 262-266, 271, 295 and 300-303 and residues with low affinity mutants were 257, 292, 296 and 309-312. In The CH3 domain residues 429, 430, 434, and 435 each had multiple low binder mutations.

Additionally, at the site of glycosylation itself, the Asn-297 residue, almost every mutation was abundant in the bottom binder population. At residue 299, while most mutations decreased binding, a switch from threonine to serine, mutation T299S, increased EndoS2 binding with an enrichment score of 1. At residue I253, a switch to threonine had a top binder enrichment score of 1, but a switch to leucine left the mutant with an enrichment score of 1 in the low binder population. Residue S298 had mutations with enrichment scores of ~0.8 in both high and low binder populations. No mutations of E272, which is thought to participate in hydrophobic interactions with W314 of EndoS, had a significant presence in either binder population (ES (Enrichment Scores) <0.58). Only a switch to Tyrosine in Fc residue L314, which is thought to have hydrophobic contact with Y909 of EndoS, earned a high enrichment score in either binder population (ES=0.97). No mutations of N315 were particularly strong or weak binders; this residue was also predicted to interact with Y909 of EndoS (figures 11-12)

Validation of DMS experiments

To validate results from DMS experiments we introduced 3 individual mutations into the pLVX-Fc WT plasmid. This was done using site directed mutagenesis. 2 of these mutants, V323Y and F241R, had enrichment scores of 1 in the high binder DMS populations and the third mutation, H435W, had an enrichment score of 1 in the low binder DMS population. Binding to point mutants will be evaluated using EndoS2 titrations on surface-displayed Fc.

Comparing EndoS2 binding affinity and EndoS hydrolysis rates of Fc mutants

Hydrolysis assays were performed on 12 different single amino acid substitution Fc mutants. 2 of these substitutions were to Phenylalanine, 1 was to Glycine, 1 was to Tryptophan and the rest were to Alanine. The rate of EndoS glycan hydrolysis of these Fc mutants was measured using intact mass spectrometry. The relative rates of activity for removal of the first glycan of the Fc mutants were normalized to that of the Fc wildtype.[9] Because all mutants were present in the site saturated library used for DMS in this study, the same mutants tested for hydrolytic activity with EndoS have now been tested for their binding affinity to EndoS2. To assess whether there is any correlation between an Fc mutant's affinity for EndoS2 and its rate of hydrolysis by EndoS, a graph plotting the enrichment scores of these mutants (calculated from the DMS data) against their relative rates of activity (from the MS data) was created. A best fit line for the graph had an R^2 value of 0.14, demonstrating no apparent correlation between the two data sets (figure). From this data there were 2 distinctive outlier mutants, I253A and E293A, whose binding affinity diverged greatly from the hydrolytic rate. Once these outliers were removed from the dataset, the correlation between hydrolytic rate and binding affinity increased significantly. The new best fit line had an R^2 value of 0.77 (figure 13).

Discussion

The Asn-297 glycan on IgGs is an important aspect of their function. The glycans interact with other immune receptors whose downstream effects help fight pathogens. Hydrolyzing this glycan is a potent strategy that some pathogens have employed to evade the immune system. The gram-positive bacterial species, *Streptococcus pyogenes (GAS),* produces two unique endoglycosidases, EndoS and EndoS2 that can hydrolyze an IgG glycan and therefore damage an IgGs utility. EndoS2 can hydrolyze a wider range of glycans than EndoS, making EndoS2 a particularly promising tool for the chemoenzymatic synthesis of antibodies. While Endos and EndoS2 recognize the Asn-297 glycan, they also require the IgG Fc region to successfully form the enzyme-substrate complex. This glycoprotein specificity indicates the contribution of peptide-peptide interactions (PPIs) between the Fc region and the Endoglycosidases. However, the nature and relevance of these PPIs, and the exact residues involved, are still not fully understood.

In this study, we elucidate more about the protein contact between EndoS2 and the Fc region using a deep mutational scan and mammalian surface display. This method allowed us to test the EndoS2 binding affinity of all possible IgG1 Fc and hinge region (amino acids 216-447) point mutations. While the data is preliminary, it can provide insight into which Fc amino acids are most pertinent to EndoS2 binding.

Interestingly, the findings show that mutations in many residues led to a sweeping decrease or increase in binding, indiscriminate of which amino acid the residue was mutated to. This may seem intuitive in the case of an overall decrease in affinity, as perhaps the specific orientation and charge of the wildtype residue is simply the most ideal for binding. However, one may expect amino acids with qualities most divergent from those of the wildtype residue to cause the greatest decrease, but this was often not the case in the data. For example, at a polar

residue, Q311, every mutation led to a decrease in binding, but the substitutions that caused the least aggressive damage were hydrophobic residues, alanine and tryptophan.

This general trend is also present within the high binder Fc mutant population. For instance, every mutation at residue V264, outside of isoleucine, led to an increase in binding. This may seem sensible when one considers the somewhat chemically inert nature of valine; it is conceivable that an impactful PPI at this residue is possible, but not currently being taken advantage of. This would suggest that the Fc's contact with EndoS2 has the potential to be more extensive. As with Q311, the affinity effect of mutations at V266 did not follow any obvious pattern that was in line with the mutant residues' characteristics. At this position, a switch to aromatic phenylalanine and a switch to charged aspartic acid both earned top binder enrichment scores greater than 0.9. This could mean that multiple types of PPIs are possible at this residue, promoting a dynamic binding model for Fc and EndoS2.

The aspect of the DMS data that is perhaps more conclusive are the positions where no mutations influenced EndoS2 binding affinity. There were large stretches of Fc residues that had no mutants appear in either the top or bottom binder population. As expected, this was the case for the hinge region (residues 216-227), this confirms that the hinge does not play an essential role in EndoS2 binding. Other non-significant mutations were at CH2 domain residues 274-288 and throughout most of the CH3 domain (amino acids 340-447), though there was notable presence of mutants at CH3 residues 429-435 in the low binder population.

Prior to our deep mutational scan, information on the Fc and EndoS binding mechanism had been gathered from cryo-EM structural analysis, alanine mutational scans and hydrolytic activity measurements. Because the DMS was performed with EndoS2, and the previous experiments used EndoS, care must be taken when comparing the datasets. Nonetheless,

comparative structural analysis of EndoS and EndoS2 suggests that they bind the Fc in very similar ways and considering the past and present data together is still valuable.

A recent cryo-EM structure of the Fc bound to EndoS helped identify some of the existing PPIs in the enzyme-Fc complex. However, the knowledge that certain PPIs are present does not delineate that these PPIs are imperative for successful complex formation. Still, cross-reference of previously identified PPIs with the new DMS dataset could help confirm that certain Fc residues are important to EndoS2 binding. The EndoS-Fc cryo-EM structure revealed that Fc residues: R292, E294, P271, E272, Y296, S298, I253, H435, Q311, L314 and N315, are all involved in PPIs with the GH and ß-sandwich domains of EndoS.[9] Our DMS data showed that the EndoS2 affinity of Fc was particularly sensitive to mutational changes in many of these same residues. Most of the residues mentioned had corresponding mutants with enrichment scores of over 0.7 in either the top binder, low binder, or both populations. Of this residue group, only E272, L314 and N315 showed little change in EndoS2 affinity for all mutations.

However, mutations at these residues with the highest enrichment scores did not usually correspond to the amino acids that would strengthen the PPI predicted by the cryo-EM structure. For example, when Fc residue I253, seen to have hydrophobic contact with W803 of EndoS, was mutated to isoleucine, another hydrophobic residue, the resulting construct had an enrichment score of 1 in the low binder population. Furthermore, some of the top binders at this residue included I253S and I253T, which have polar side chains.

The importance of these residues was also previously examined by mutating them to alanine and measuring the new hydrolytic activity of EndoS. However, the association between these mutants' hydrolytic activity and binding affinity is still unclear. When the enrichment scores from our DMS EndoS2 experiment were graphed against the earlier study's hydrolytic

activity measurements for EndoS (performed on the same Fc alanine mutants), the correlation was quite low (R^2 of 0.14). However, when two particularly uncorrelated mutants, I253A and E293A, were removed, the R^2 value became 0.77. There are many theories that could explain these outliers. For example, perhaps I253A binds too tightly, slowing hydrolysis. On the other hand, this residue may simply operate differently in EndoS and EndoS2. Performing a DMS of Fc with EndoS or measuring the hydrolytic rate of EndoS2 on Fc alanine mutants would reveal more.

While more research is needed to say anything definitively, the data from our deep mutational scan can help narrow down which Fc residues are central to EndoS2 binding. It seems reasonable to conclude that the Fc residues, for which no mutants show stronger or weaker EndoS2 binding, are probably not useful targets for mutagenesis. As for mutants displaying an altered affinity for EndoS2, the fact that many correspond with residues already predicted to participate in PPIs from the Fc-EndoS cryo-EM structure, validates the DMS data as a whole and confirms the similar nature of EndoS and EndoS2. However, the incongruences between the two datasets and the lack of pattern in accordance with side chain qualities make interpreting our deep mutational scan difficult.

In addition, it is important to note that just because a mutation was prevalent in the top or low binder population does not guarantee it has a different affinity for EndoS2. This could be a consequence of a construct's ability to be stably expressed on the mammalian cell surface to a greater or lesser extent than the wildtype. Therefore, to confirm that mutants of interest truly have altered EndoS2 binding affinity, an experiment to determine the K_d of these mutants should be performed. Still, we have demonstrated that the deep mutational scan and mammalian surface display platform are useful methods for efficiently testing the effects of over 4300 mutations in

the IgG1 Fc. The data presented can provide more basis for further research into the mechanism of EndoS2 and ultimately contribute to the development of monoclonal antibodies with a different affinity for the novel enzyme.

Figures

1

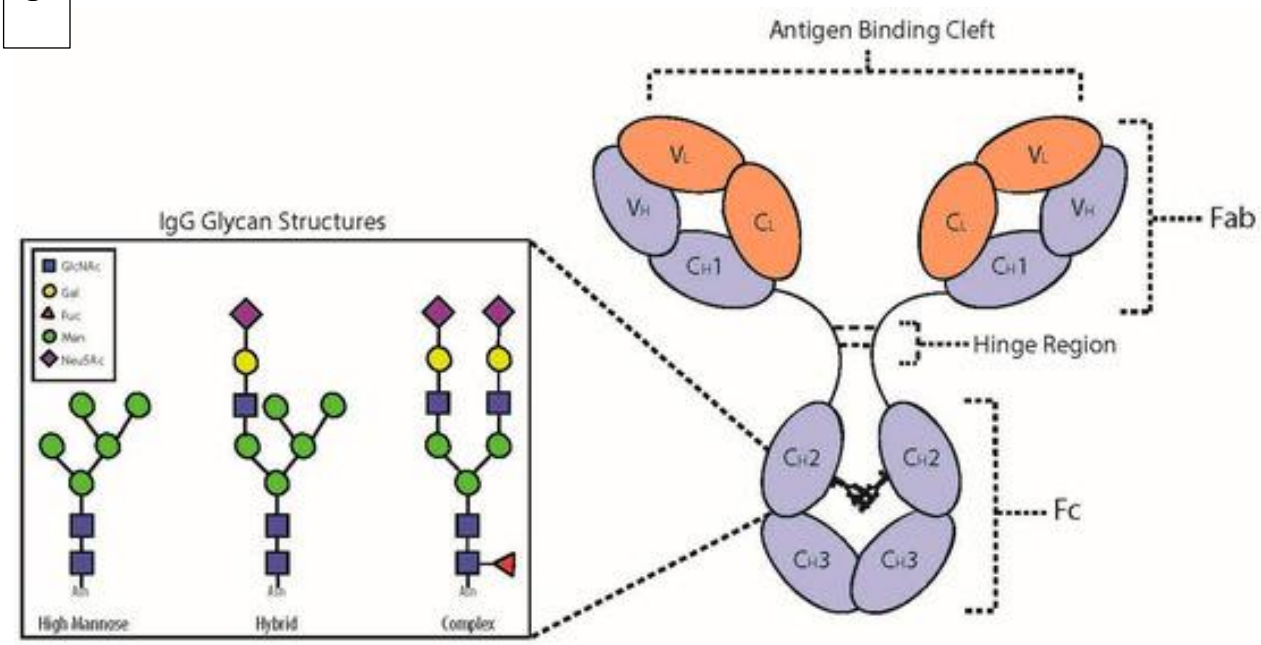


Figure 1: **A depiction of wildtype IgG and 3 glycan types**. This cartoon shows the Fab, Fc and hinge regions of IgG. The CH2 and CH3 domains of the Fc region are also labeled. The dotted lines show where the conserved glycosylation site, Asn-297, and the 3 glycan subtypes that can be N-linked at this residue.[14]

2

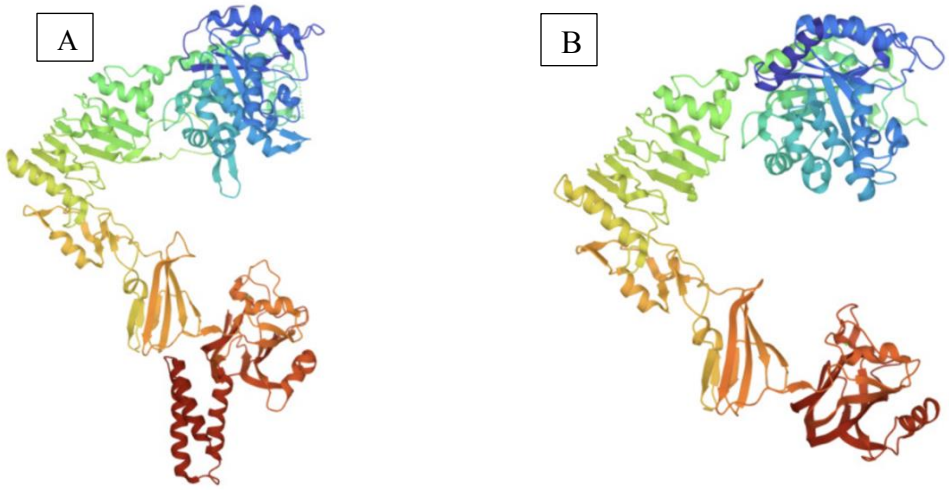


Figure 2: **RCSB structures for EndoS and EndoS2** A) The structure of EndoS includes 5 domains: the glycoside hydrolase (GH) domain (blue), the leucine-rich repeat (LRR) domain (green), the hybrid IgG domain (yellow), a ß-sandwich domain (orange), and three-helix bundle (3H) domain (red). B) The structure of EndoS2, the three-helix bundle (3H) domain (red) present in EndoS is absent in EndoS2.

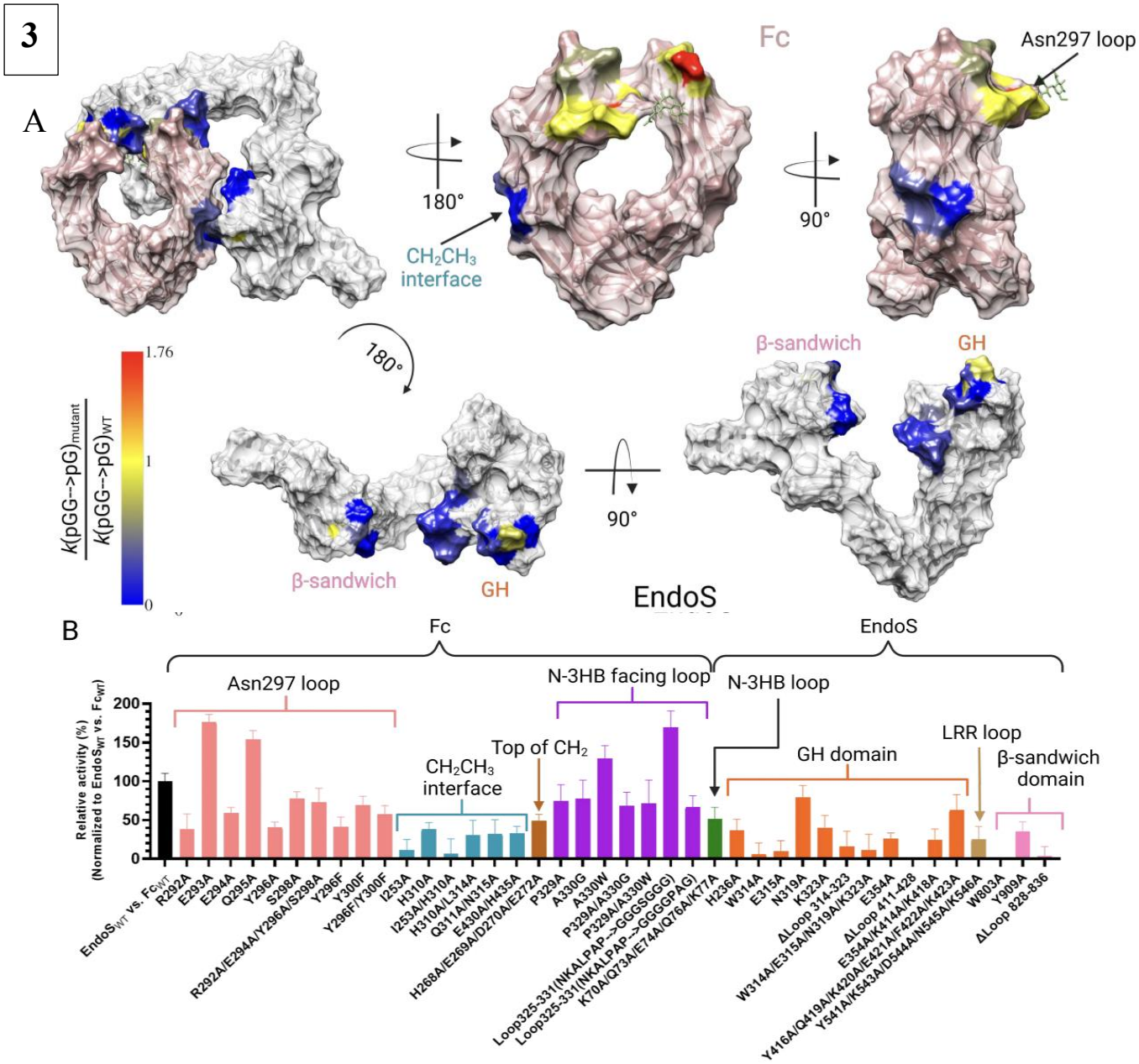


Figure 3: **Alanine scans and hydrolytic measurement experiments previously performed on EndoS.** A) A visual representation of EndoS in complex with the Fc region of IgG1. The colors represent the effect of alanine mutations at certain residues on EndoS and the Fc. The color scale ranges from red, indicating the highest increase in hydrolytic activity due to an alanine mutation, to blue, indicating the greatest decrease in hydrolytic activity due to an alanine mutation. These rates were measured using LC-MS kinetic analyses. B) Relative hydrolytic rate of EndoS and Fc alanine mutants, normalized with the hydrolytic rate of EndoS and Fc wildtype. All rates represent removal of first glycan from the Fc, all rates were statistically significant (multiple comparison test, Tukey method, $p < 0.0001$).[9]

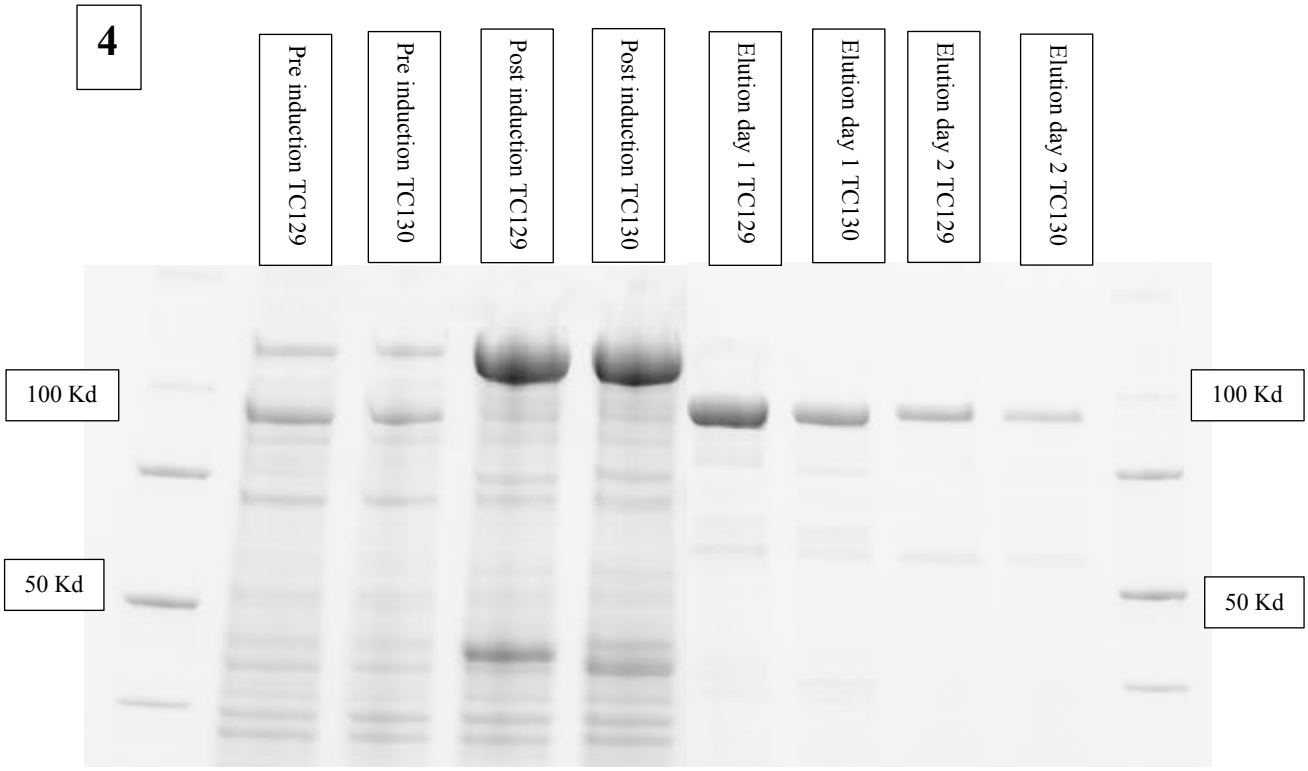


Figure 4: **Expression and purification of two inactive EndoS2 mutants.** Rows 1-2 show the boiled protein samples of Bl21 cells before induction with IPTG. Rows 3-4 show the post induction expression of pET32-Avi-EndoS2-E186L (TC129) and pET32-EndoS2-E186L-Avi (as TC130). Both were expressed at 22℃ overnight, with 0.5mM IPTG, which induces the lac-operon. Rows 4-8 show elutions from nickel resin of both mutants, elutions are smaller in size due to the absence of the CPD domain from the C terminus, which was cleaved during purification with phytic acid.

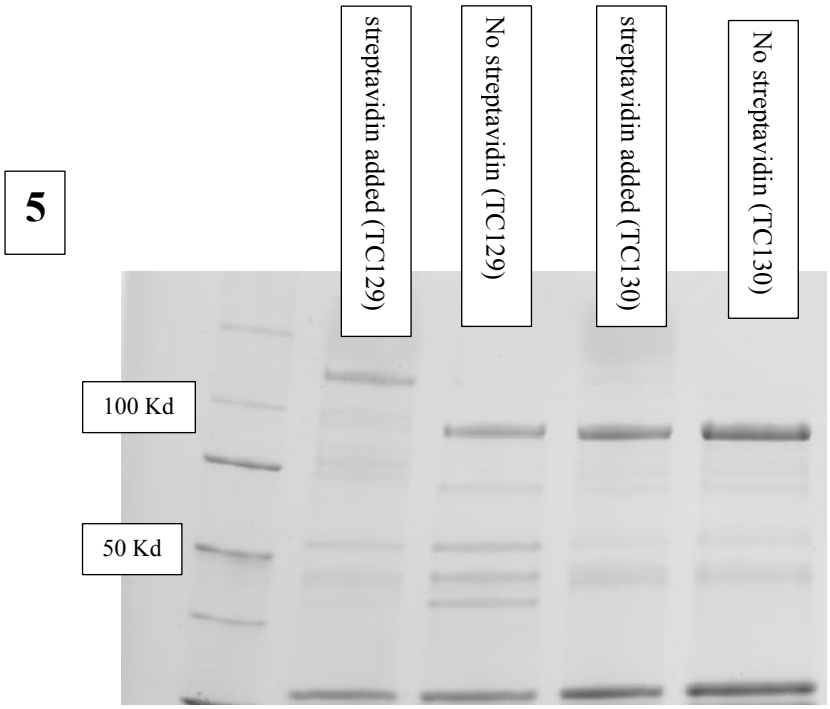


Figure 5: **Streptavidin assay to assess success of biotinylation reaction**. Due to Biotin's small size, Streptavidin binding must be done first so that biotinylation can be confirmed. If Streptavidin can bind, a shift in protein size should be visible. This shift is clear in the first column and proves the presence of Biotin and streptavidin on Endo-S2 with the Avi tag on the N terminus (TC129). No shift in third column demonstrates that streptavidin did not bind to Endo-s2 with the Avi tag on the C terminus (TC130).

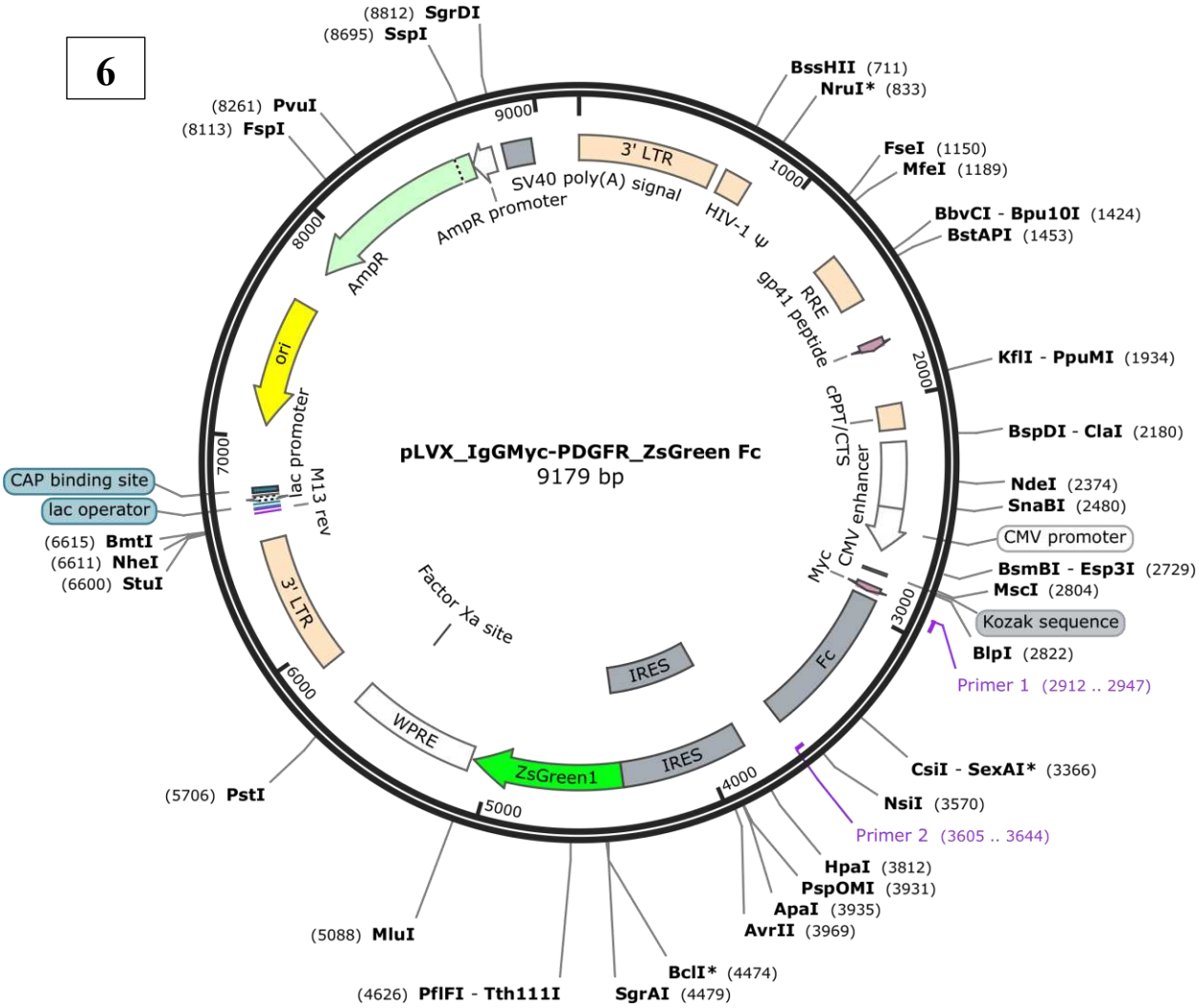


Figure 6: **Plasmid map for the Fc-hinge regions (amino acids 216-447), cloned into the lentiviral expression plasmid (pLVX-IRES-ZsGreen).** This vector contains an N-terminal Myc-tag followed by a signal peptide (SP) (derived from IgG4) and a C-terminal transmembrane region (TM) (derived from PDGFR). Also included is a 3' internal ribosomal entry site (IRES) and GFP, which was used as a selection marker for expression. An ampicillin marker is also present, used to select for the plasmid during bacterial transformation.

7

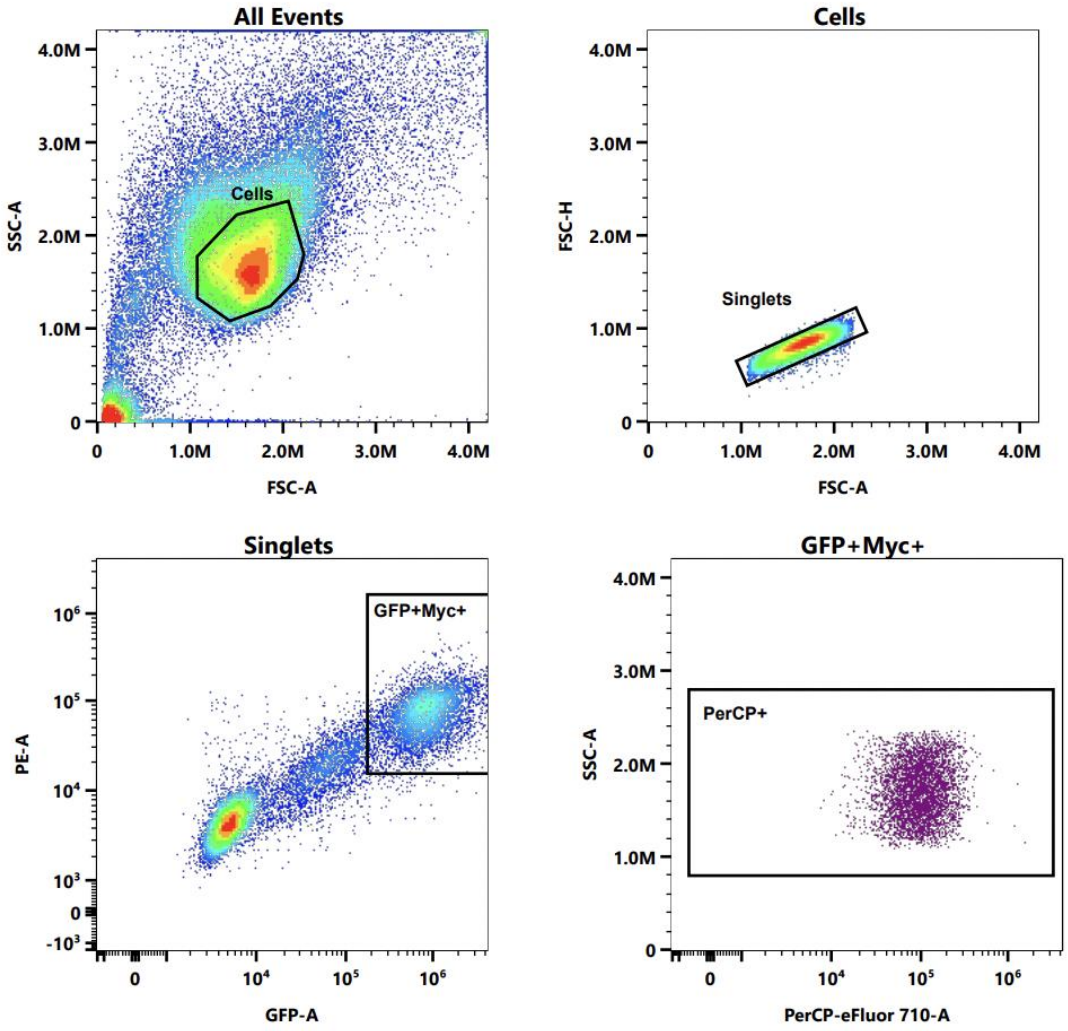


Figure 7: **Validation of EndoS2 binding to Fc wildtype on the mammalian cell surface**. Gating scheme for EndoS2 titrations using HEK293T cells stably expressing surface-displayed Fc protein. First singlet and healthy cells are selected for using gates. Another gate is used to select GFP+ and Myc+ cells. Finally, PerCP+ signal, used to detect EndoS2 on the cell surface, is measured in the remaining cell populations.

8

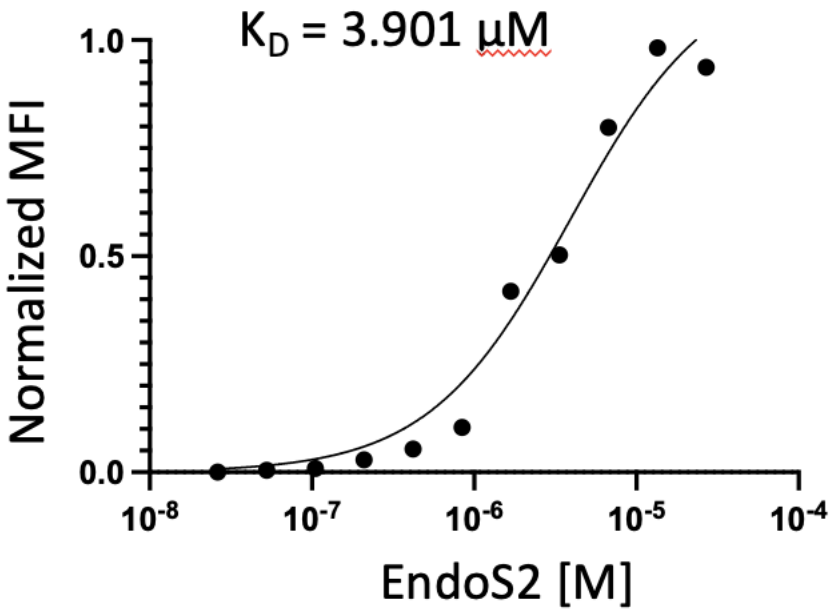
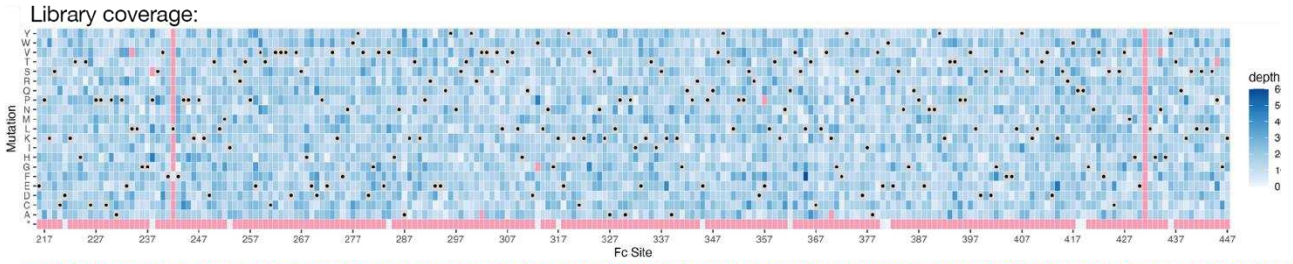


Figure 8: **Titration curve of EndoS2 binding to cell surface displayed IgG1 Fc.** The normalized Median Fluorescence Intensity (MFI) of the PerCP-710 signals emitted on the flow cytometer was collected for all 11 EndoS2 concentrations were collected. These values were plotted along the y axis, along the x axis are the 11 corresponding EndoS2 [M] concentrations that were added to the cells via a 2-fold serial dilution. The K_d was calculated using Prizm software. The resulting K_d is 3.9 µM.

9

A



B

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL
PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS
LSPGK

216-227 HINGE
228-340 CH2
341-447 CH3

Figure 9: A) **A visualization of the entire Fc mutational space.** The wildtype amino acid is shown for each position with a black dot. Failed mutations are shown in pink. Amino acids correspond to amino acids 216-447 of the Fc and hinge regions. 4376 mutations were successful out of 4408 possible mutants. B) The wildtype amino acid sequence for the Fc and hinge region of the IgG1 antibody. The hinge region is in red, the CH2 domain in blue, and the CH3 domain in purple.

10

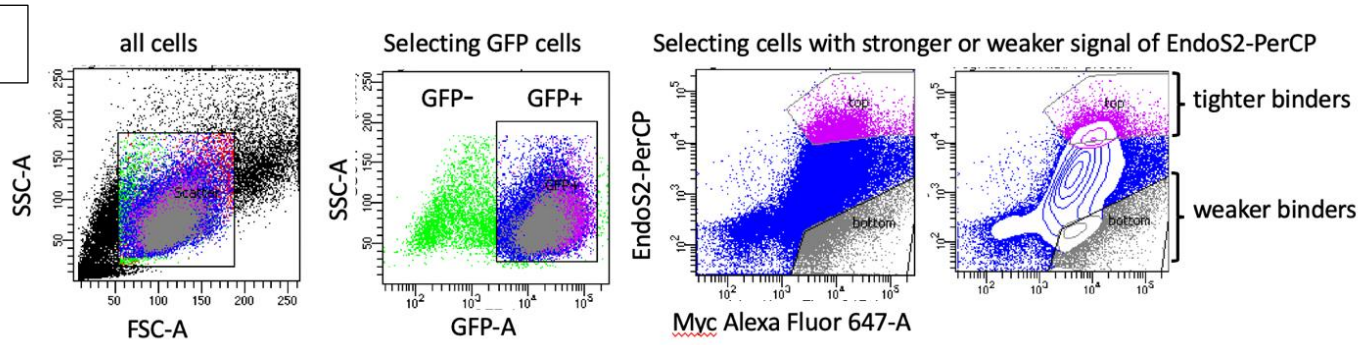


Figure 10) **Sorting of HEK293T cells expressing the Fc and hinge mutant library**. Fc expression and surface display (Anti-Myc Alexa Fluor 647-A) and EndoS2 binding (PerCP) were measured by flow cytometry. Gating was used to select GFP+ and Myc+ cells. Diagonal gates are then used to isolate 15% of cells with tighter (top) and weaker (bottom) binding to EndoS2 (PerCP). These cells were sorted on a BD FACS Aria II instrument.

11

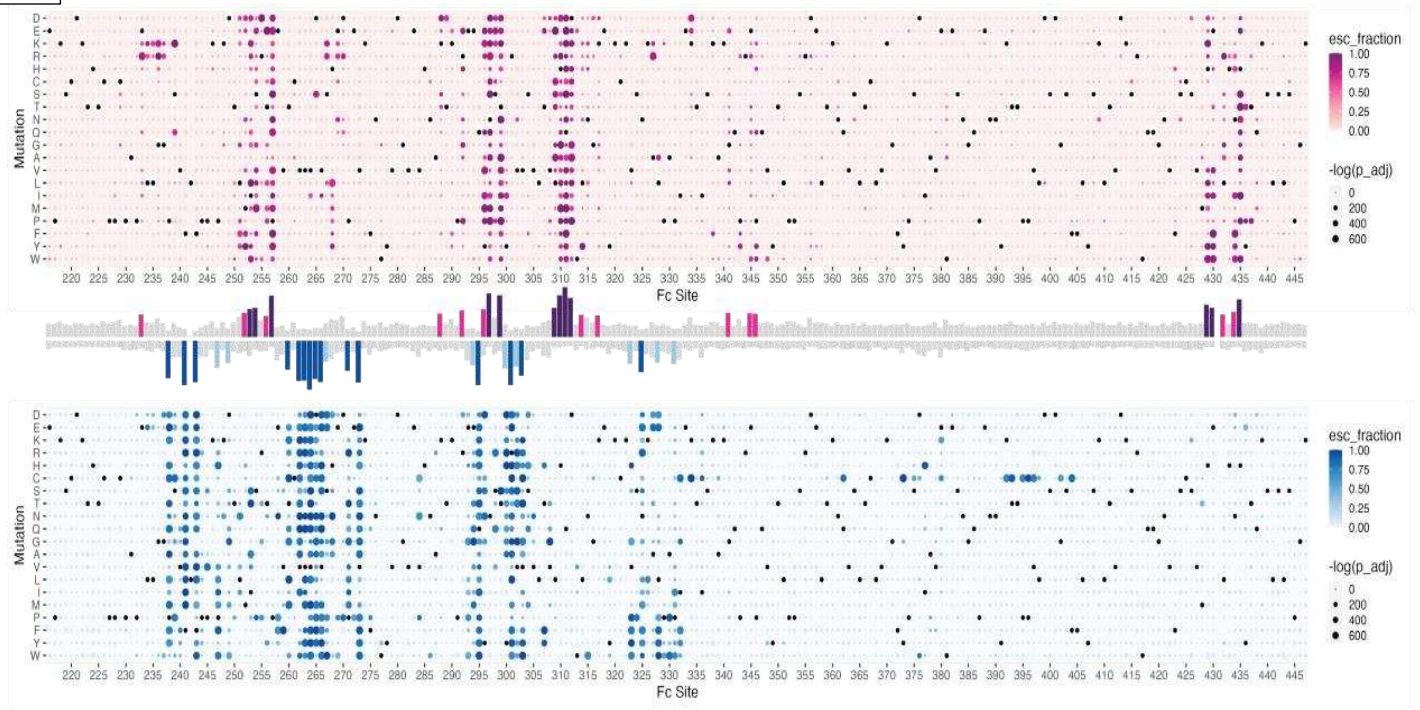


Figure 11: **Deep mutational scan data to evaluate the impact of mutations in IgG1 Fc on its interaction with EndoS2**. These heatmaps display each mutation in the Fc mutant library as a color-coded bubble. The darker shade the bubble is the higher the enrichment score of a mutant and the more prevalent that mutant is in the corresponding dataset. The pink dataset comes from the weaker binding mutants of Fc and the blue dataset comes from the from the tighter binding mutants of Fc. The size of the bubbles represents the adjusted p value (calculated using fisher's exact test) of the enrichment scores.

12

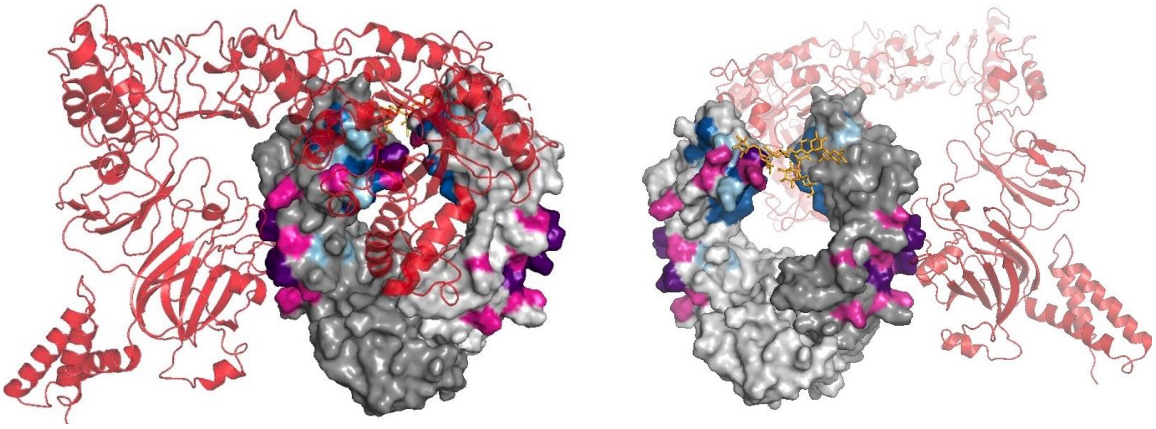
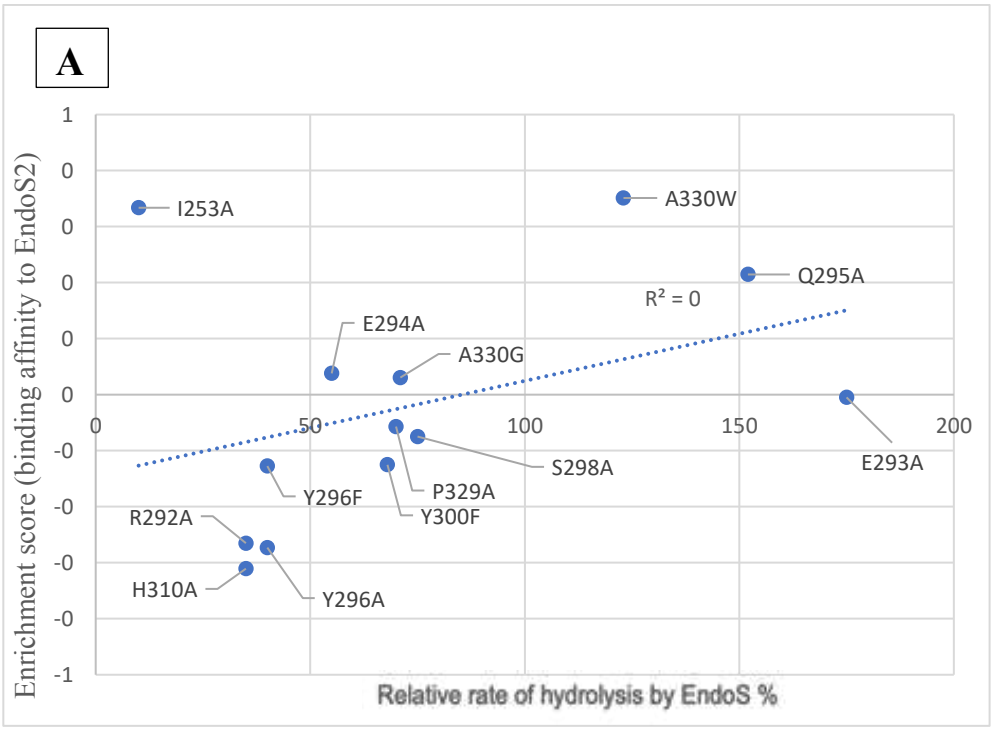


Figure 12: **EndoS in complex with the Fc region color-coded using DMS data.** PyMol structure of Fc with color coding based on the enrichment scores from the DMS data. The blue portions represent residues where mutations lead to an increase in EndoS2 affinity. The residues that decreased EndoS2 binding are color coded in pink. As with the heatmaps in figure 11 the darkest spots represent the mutations with the highest enrichment scores. EndoS is shown docking rather than EndoS2 because the crystal structure for Fc in complex with EndoS2 has not been made.

13


A
Enrichment score (binding affinity to EndoS2)
Relative rate of hydrolysis by EndoS %
I253A
A330W
Q295A
R² = 0
E294A
A330G
S298A
E293A
Y296F
P329A
Y300F
R292A
H310A
Y296A

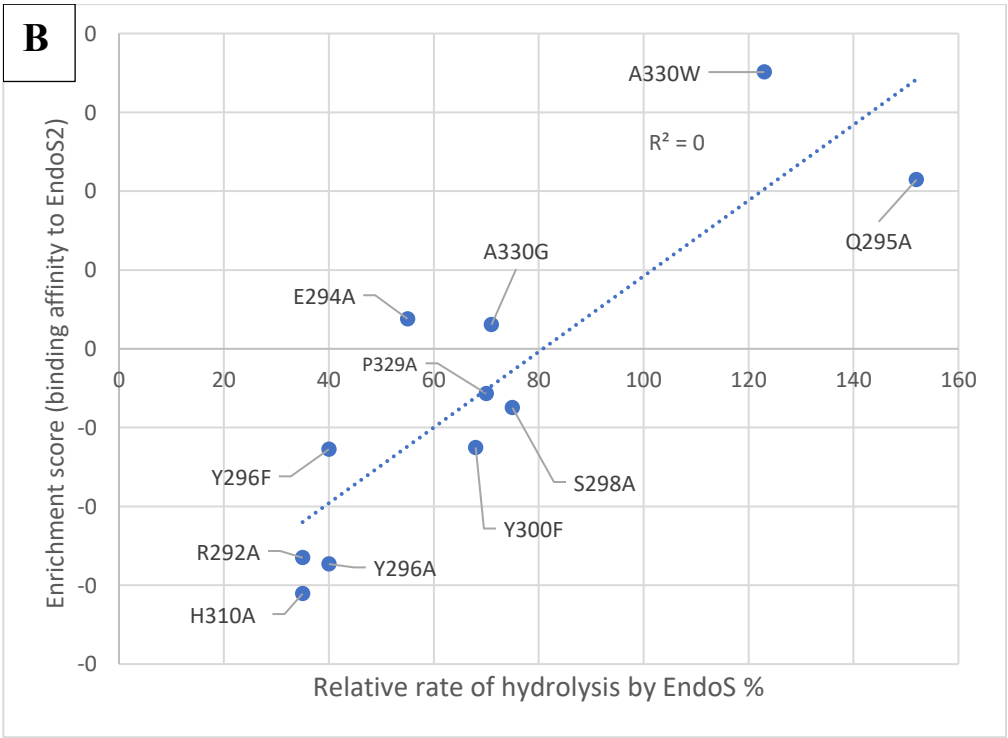

B
Enrichment score (binding affinity to EndoS2)
Relative rate of hydrolysis by EndoS %
A330W
R² = 0
Q295A
A330G
E294A
P329A
S298A
Y296F
Y300F
R292A
Y296A
H310A

Figure 13: **Enrichment scores (EndoS2 binding affinity) vs Relative rate of Hydrolysis by EndoS**. The normalized deglycosylation rates of mutant IgG1 Fc regions with point alanine substitutions by active EndoS wildtype are plotted on the x axis. These were calculated using LC-MS kinetic analyses.[9] The DMS normalized enrichments scores for the same alanine mutants, representing their affinity for EndoS2 when they are expressed on the mammalian cell surface are plotted on the y axis. A) Data for all relevant mutants was graphed and a linear regression was applied to assess the correlation of the two variables. The resulting R^2 is 0.1442. B) Two distinctive outliers I253A and E293A were removed from the graph and a new linear regression was applied. The R^2 for the best fit line was then 0.77.

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