Antibody Therapeutics, 2024, Vol. 7, No. 1 ***88–95***
https://doi.org/10.1093/abt/tbae002
Advance Access Publication on 18 January 2024

Brief Report

Effects of Fc glycosylation on the activity of WNT mimetic agonistic antibodies

Hui Chen[1], Sung-Jin Lee[2], Brian Ouyang[1], Nicholas Suen[1], Jay Ye[1], Chenggang Lu[2] and Yang Li[3,*]

[1]Protein Sciences, Surrozen Inc., South San Francisco, CA 94080, USA, [2]Discovery Biology, Surrozen Inc., South San Francisco, CA 94080, USA, and [3]Research, Surrozen Inc., South San Francisco, CA 94080, USA

Received: September 12, 2023; Revised: January 12, 2024; Accepted: January 16, 2024

ABSTRACT

Monoclonal antibodies have been explored in a broad range of applications including receptor agonism. Given the importance of receptor conformation in signaling, the agonistic activity of antibodies that engage these receptors are influenced by many parameters. Tetravalent bispecific antibodies that target the frizzled and lipoprotein receptor-related protein receptors and subsequently activate WNT ("Wingless-related integration site" or "Wingless and Int-1" or "Wingless-Int") signaling have been constructed. Because WNT activation stimulates stem cell proliferation and tissue regeneration, immune effector functions should be eliminated from therapeutic antibodies targeting this pathway. Here, we report an unexpected effect of Fc glycosylation on the agonistic activity of WNT mimetic antibodies. Our findings underscore the importance of antibody format, geometry and epitope in agonistic antibody design, and highlight the need to establish appropriate early discovery screening strategies to identify hits for further optimization.

Statement of Significance: Agonistic antibodies are sensitive to formats, geometries and epitopes. Tetravalent bispecific WNT mimetic antibodies have been described. Chen et al. report an unexpected effect of Fc glycosylation on the agonistic activity of WNT mimetics and highlight the importance of establishing appropriate discovery screening strategies to identify early hits for optimization.

KEYWORDS: glycosylation; bispecific antibody; agonist; WNT; FZD; LRP; surrogate; WNT mimetic

INTRODUCTION

As a class, therapeutic antibodies have advantages of stability, extended serum half-life and a mature manufacturing process; the number of therapeutic antibodies has been rapidly increasing, and >100 antibody drugs have been approved to date [1]. The most abundant circulating immunoglobulins (Igs) are the IgG isotype, which includes four subclasses: IgG1, IgG2, IgG3 and IgG4. Most approved monoclonal antibody therapies are of the IgG1 subclass. Antibody molecules are bifunctional: the N-terminal fragment antigen-binding region (Fab) specifically binds target antigens, whereas the fragment crystallizable region (Fc) on the C-terminal half of the molecule engages in immune effector functions through binding to Fc-γ receptors (FcγRs) and complement component 1q (C1q). The engagement of FcγRs induces cellular immune responses that lead to antibody-dependent cell-mediated cytotoxicity and antibody-dependent cellular phagocytosis, as well as to inflammation through the induction of cytokine secretion. C1q binding results in complement-dependent cell-mediated cytotoxicity and complement-dependent cell-mediated phagocytosis [2]. Whereas the immune effector functions are critical in certain therapeutic contexts, such as tumor killing, with the expansion of drug targets, the cytotoxic effector functions of an antibody may be undesirable and could potentially lead to safety risks in other settings. The Fc region also interacts with the neonatal Fc receptor, which is located primarily on vascular endothelial cells, thus leading to IgG recycling and an increased plasma half-life [3]. Extensive structural and mutagenesis studies over many years have identified key residues that either directly contribute to or allosterically modulate interactions between antibodies and various Fc receptors and complement factors [4]. Protein engineering

*To whom correspondence should be addressed. Yang Li, 171 Oyster Point Blvd, Suite 400, South San Francisco, CA 94080, USA. Tel: (650)475-2804; Email: yang@surrozen.com

of these sites has enabled the generation of antibodies with enhanced or diminished effector function and extended or shortened plasma half-life. For example, IgGs contain a conserved glycosylation site at amino acid Asn297 (N297) in the heavy chain constant domain 2 (CH2). Mutation of N297 to Ala (N297A), Gly (N297G), or Gln (N297Q) eliminates IgG glycosylation, and the aglycosylated IgG consequently undergoes a conformational change in the CH2 domain that significantly decreases binding to FcγRs and C1q, and largely eliminates effector function [5]. Mutations in the direct binding surface to FcγRs and C1q, such as the combination of Leu234Ala/Leu235Ala/Pro329Gly (LALAPG) mutations, also effectively inhibit effector functions [6].

WNT ("Wingless-related integration site" or "Wingless and Int-1" or "Wingless-Int") signaling is highly conserved in the animal kingdom and plays critical roles in embryonic development, and in adult tissue homeostasis and injury repair. WNT ligands bind frizzled (FZD) family receptors and the co-receptor low-density lipoprotein receptor-related protein (LRP), thereby inducing WNT/β-catenin signaling, which is essential in regulating stem/progenitor cell function [7]. There are 10 FZDs (FZD_{1-10}) and 2 LRPs (LRP5 and LRP6). Bispecific molecules that induce complex formation between FZD and LRP have been found to mimic WNT ligand function and induce WNT/β-catenin signaling [8–10]. WNT mimetics based on various antibody modalities have been constructed and identified tetravalent bispecific format design being optimal for signaling [9–11]. Given the important roles of WNTs in stem cell biology, these agonistic antibodies may have therapeutic utility in promoting endogenous repair mechanisms and inducing tissue regeneration through stimulating tissue stem cell proliferation and differentiation. Therefore, for such regenerative medicine applications, effector functions must be eliminated from these WNT mimetic bispecific agonistic antibodies. Here, we studied the effects of various effectorless Fc mutations on bispecific WNT mimetic antibodies, in particular on a set of tetravalent bispecific molecules utilizing variable heavy domain of heavy chain (VHH) and IgG fusions in the symmetrical VHH-IgG format [11]. The VHH domains bind LRP and the IgG portion binds FZD with different specificities, the VHH domain is attached to IgG on various locations including N-terminus of heavy or light chains or C-terminus of heavy or light chains. Our results revealed an unexpected effect of Fc glycosylation on agonistic activity.

MATERIALS AND METHODS

Molecular cloning

All constructs except immunoglobulin G-degrading enzyme of *Streptococcus pyogenes* (IdeS) were cloned into the pcDNA3.1(+) mammalian expression vector (Thermo Fisher Scientific Cat# V79020). FA-L6 wild type (WT) was constructed by fusing the VHH domain to the N-terminus of the light chain (LC) of FA IgG1 with a 5-mer linker (GSGSG); FA-L6 N297 mutants were obtained by mutating N297 in the Fc domain to G, A and Q; FA-L6 LALAPG was obtained by introducing LALAPG mutations into the Fc domain [6]. FA-L6, FA-L5 and FA-36 were constructed by fusing the VHH domain to the N-terminus of the light chain of FA IgG1 LALAPG with a GSGSG linker. FB-L6, FB-L5 and FB-36 were generated by fusing the VHH domain to the N-terminus of the light-chain of FB IgG1 LALAPG with a GSGSG linker. FA-L6_NH, FA-L6_CH, FA-L6_CL were constructed by fusing L6 to either the N-terminus or C-terminus of heavy chain (HC) or C-terminus of light chain of FA IgG1 LALAPG. IdeS (D30-N341) was cloned into pET-28b(+) with a G4S linker (GGGGS) followed by a 6 × His tag at the C-terminus. L6 complementarity-determining region 3 (CDR3) was mapped and sequences determined using Distributed Bio Abgenesis software. Alanine scanning mutagenesis was performed using QuikChange II Site-directed mutagenesis kit (Agilent Cat# 200523) to individually mutate each CDR3 residue to alanine.

Protein production

All recombinant proteins except IdeS protein were produced in Expi293F cells (Thermo Fisher Scientific Cat# A14527) through transient transfection. The proteins were first purified with CaptivA Protein A affinity resin (Repligen Cat# CA-PRI-0005) then further purified through Superdex 200 Increase 10/300 GL (Cytiva Life Sciences Cat# GE28–9909-44) size-exclusion chromatography with 1× HBS buffer (20 mM HEPES, pH 7.4 and 150 mM NaCl). The proteins were subsequently examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and estimated to have >90% purity. IdeS protein was produced in the transformed BL21-DE3 (New England Biolabs Cat# C2527H) bacterial strain [12]. After pre-charged nickel resin (Sigma-Aldrich Cat# 5893801001) purification, the purified IdeS protein was dialyzed in 1× HBS buffer and purity was examined by SDS-PAGE to have > 90% purity.

$F(ab')_2$ generation

FA-L6 WT $F(ab')_2$ was generated by incubation of FA-L6 WT with IdeS protease in a 5:1 (w/w) ratio in 1× HBS buffer at room temperature for 4 h; FA-L6 N297G $F(ab')_2$ was generated in the same way, except that the ratio of FA-L6 N297G to IdeS protease was 5:2 (w/w), because the efficiency of IdeS digestion of FA-L6 N297G was lower than that of FA-L6 WT. The digested proteins were then purified with CaptureSelect™ LC-Lambda[Hu] Affinity Matrix (Thermo Fisher Cat# 084905) by binding at room temperature for 2 h, washing with 1× HBS and elution with 0.1 M glycine, pH 3.3, and immediate neutralization with a 1/10 volume of 1 M Tris–HCl, pH 8.0 and 1.5 M NaCl. The proteins were subsequently examined by SDS-PAGE and estimated to have >90% purity.

Peptide N-glycosidase F (PNGase F) treatment to remove glycan on the Fc domain

PNGase F treatment was performed by incubation of 20 μg purified IgG1 proteins with 1 μL PNGase F (Promega Cat# V483A) in 1× HBS buffer at 37°C for 2 h. The

proteins were subsequently examined by SDS-PAGE to confirm glycan removal, as indicated by faster migration after PNGase F treatment.

Super top-flash assays

WNT signaling activity was measured in HEK293 cells containing a luciferase gene under the control of a WNT-responsive promoter, as previously reported [13]. In brief, cells were seeded at a density of 10 000 per well in 96-well plates 24 h before treatment, in the presence of 3 μM inhibitor of WNT production-2 (IWP2) (Thermo Fisher Scientific Cat# 35–331-0) to inhibit the production of endogenous WNTs and the presence of 20 nM Fc-R-Spondin 2. Cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega Cat# E1531), and luciferase activity was measured with a Luciferase Assay System (Promega Cat# E1500) according to the vendor's suggested procedures. Data are representative of three independent experiments performed in triplicates and are shown as mean ± standard deviation (SD).

Mouse small intestinal organoid growth assay

Activity of Fc mutated WNT mimetics was assessed in a mouse small intestinal organoid growth assay as previously described [10]. Slight modifications made to the published protocol are specified below. Mouse small intestinal organoids (STEMCELL technologies Cat# 70931) were maintained in mouse IntestiCult Organoid Growth Medium (STEMCELL technologies Cat# 06005) and split at a 1 to 8 ratio after fully grown for 7 days to set up the growth assay in 48-well tissue culture plates. Each treatment had 8 replicate wells. Protein treatments were diluted in Basal Media (DMEM: F12K (Gibco Cat# 12634–010) at 1:1 ratio with 10 mM HEPES (Thermo Fisher Scientific Cat# 15630), 1× Penicillin/Streptomycin (Lonza Cat# 17-603E), 1× GlutaMAX (Thermo Fisher Scientific Cat# 35050061), 1× N2 supplement (Thermo Fisher Scientific Cat# 17502048), 1× B27 supplement (Thermo Fisher Scientific Cat# 17504044), 1.25 mM N-acetylcysteine (Sigma-Aldrich Cat# A0737), 50 ng/mL recombinant human epidermal growth factor (EGF) (Peprotech Cat# AF-100-15), 50 ng/mL recombinant human Noggin (Peprotech Cat# 120-10C) and 500 ng/mL recombinant human R-Spondin 1 (R&D Systems Cat# 4645-RS-025)) with 1 μM IWP2. Media and treatments were changed once on Day 5 after plating. Images were acquired on day 6, one each from five different wells per condition, on a Nikon Eclipse Ts2 microscope with a Nikon Digital Sight 1000 camera. One representative image was selected for each treatment and shown in the figure.

Dynamic light scattering

Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter (Dh) and polydispersity of various molecules on an UNcle (UNchained Laboratories) instrument. Sample was applied to an 8.8 μL quartz capillary cassette and measured at 0.25 mg/mL in 1× HBS, pH 7.4 buffer at 25°C with four acquisitions of 5 s each. Data was analyzed by the software Uncle Analysis V6.01 and plotted using Prism (Graphpad) based on three independent experiments performed in duplicate.

Thermal stability measurement

Melting temperatures were determined using an UNcle instrument based on the barycentric mean of intrinsic fluorescence emission spectra in the thermal ramp 15°C to 95°C with the ramp rate of 1°C per min. The proteins were 0.25 mg/mL in 1× HBS, pH 7.4. Data were analyzed by the software Uncle Analysis V6.01. Melting temperatures were defined as the maximum point of the first derivative of the melting curve.

Affinity measurement

The binding kinetics of FA-L6 WT, FA-L6 N297A and FA-L6 N297G to human FZD_8 cysteine-rich domain (FZD_8 CRD) and to human LRP6E3E4 was determined through bio-layer interferometry (BLI) with an Octet Red 96 (PALL ForteBio) instrument at 30°C and 1000 rpm with Anti-Human Fc Capture (AHC) (Sartorius Cat# 18–5060) or Streptavidin (SA) biosensors (Sartorius Cat# 18–5019). For monovalent binding analyses, FA-L6 WT, FA-L6 N297A and FA-L6 N297G proteins were diluted to 50 nM in running buffer (PBS, 0.05% Tween-20, 0.5% BSA, pH 7.2), captured by the AHC biosensor and dipped into wells containing FZD_8 CRD, or LRP6E3E4 at different concentrations in running buffer, or into a well containing only running buffer, as a reference. For bivalent binding analyses, biotinylated FZD_8 CRD or LRP6E3E4 proteins were diluted to 50 nM in running buffer (PBS, 0.05% Tween-20, 0.5% BSA, pH 7.2), captured by the SA biosensor, and dipped into wells containing FA-L6 WT, FA-L6 N297A, FA-L6 N297G, or WNT mimetic CDR3 mutant proteins at different concentrations in running buffer, or into a well containing only running buffer, as a reference. The dissociation of the interaction in running buffer was followed. The K_D for each binder was calculated in Octet System software.

RESULTS

N297 mutations decrease WNT mimetic activity

We have previously reported the construction of WNT mimetics based on various antibody modalities [10, 11]. Our results have indicated that tetravalent bispecific engagement of FZDs and LRPs is optimal for signaling. To eliminate any potential cytotoxicity toward stem or progenitor target cells, we first introduced the N297G mutation into the Fc region of the tetravalent bispecific WNT mimetic FA-L6 [11] in the VHH-IgG format (Fig. 1A). FA is the IgG portion of the molecule that binds $FZD_{1,2,5,7,8}$, and L6 is the VHH portion that binds LRP6 [11]. Unexpectedly, whereas FA-L6 with a WT IgG1 Fc molecule (denoted FA-L6 WT or FA-L6 Fc WT henceforth) was fully active in WNT responsive HEK293 Super Top-Flash (STF) cell assays, FA-L6 N297G showed a significant loss of activity (Fig. 1B). To understand

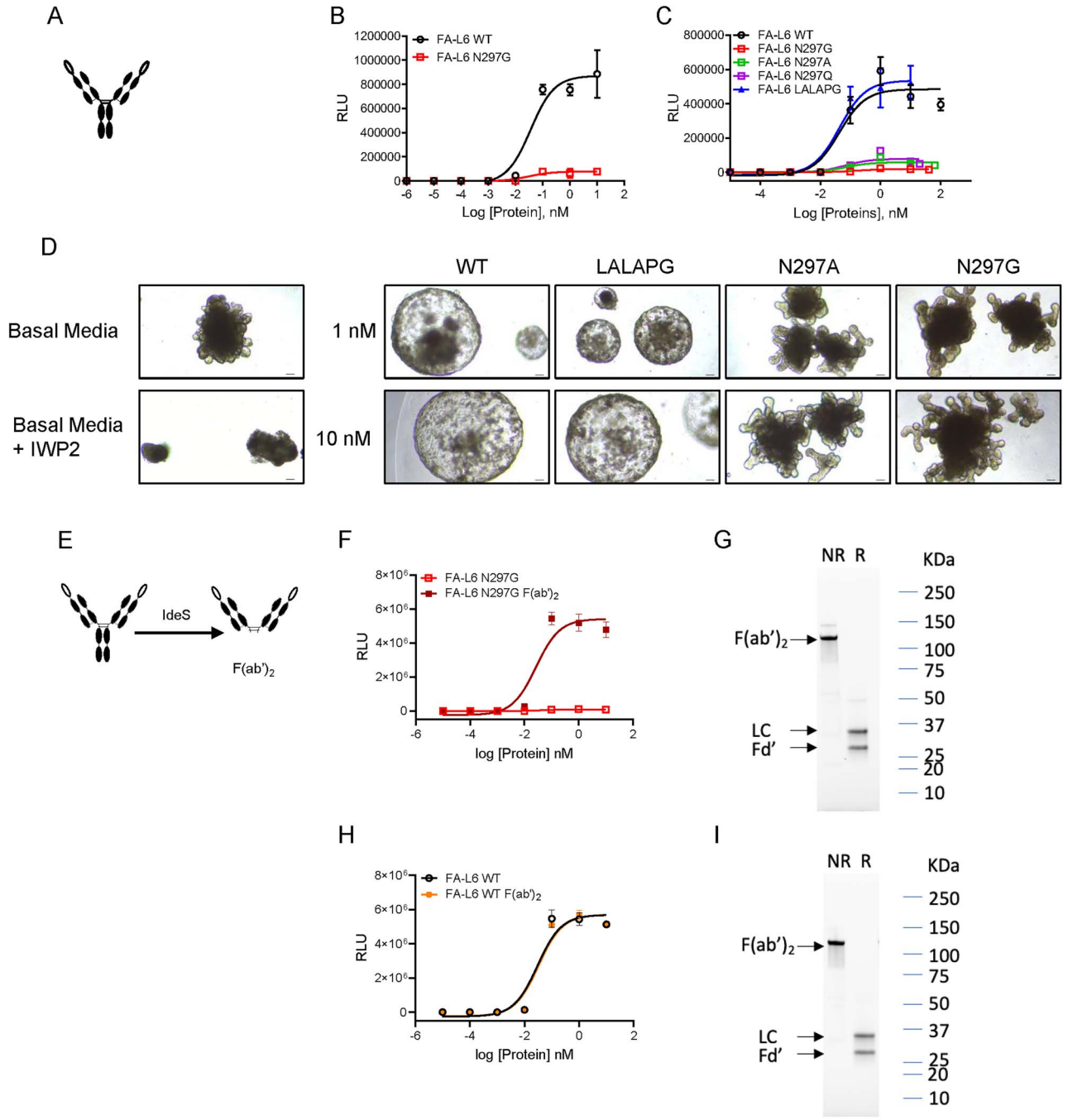


Figure 1. N297 mutations disrupt WNT mimetic activity. (A) Diagram of the WNT mimetic molecule FA-L6. (B) Mutation of N297 to G significantly disrupts the activity of FA-L6 WNT mimetic. (C) Mutations of N297 significantly disrupt the activity of FA-L6 WNT mimetic, whereas the LALAPG mutation does not. (D) Activity of FA-L6 Fc mutations tested in a mouse small intestinal organoid growth assay. Representative images of mouse small intestinal organoids treated with Basal media, Basal media +1 μM IWP2 or with Basal media +1 μM IWP2 plus 1 nM FA-L6 WT, 10 nM FA-L6 WT, 1 nM FA-L6 LALAPG, 10 nM FA-L6 LALAPG, 1 nM FA-L6 N297A, 10 nM FA-L6 N297A, 1 nM FA-L6 N297G and 10 nM FA-L6 N297G. Bars: 100 μm. (E–G) The removal of the N297G Fc domain by IdeS (E) rescues the molecule's STF activity (F). (G) SDS-PAGE of IdeS digested FA-L6 N297G. (H and I) Removal of the Fc domain from FA-L6 WT does not affect the molecule's activity. (H) STF activity of untreated and IdeS treated FA-L6 WT. (I) SDS-PAGE of IdeS digested FA-L6 WT.

whether this finding was unique to the N297G mutant, and whether interaction with FcγRs and C1q might have been required for the WNT mimetic activity observed with the Fc WT molecule, we generated the additional Fc variants FA-L6 N297A, N297Q and LALAPG. Whereas all N297 mutants had similarly low activity, FA-L6 LALAPG showed activity similar to that of the Fc WT molecule (Fig. 1C). This finding suggested that FcγRs and C1q did not contribute to the WNT mimetic activity observed with the Fc WT molecule, and that Fc glycosylation

is important for this WNT mimetic molecule. Mouse small intestinal (mSI) organoid culture represents another WNT dependent *in vitro* functional assay system. In WNT-free basal medium, stem cells located at the tip of finger-like protrusions (structures resembling intestinal crypts) receive WNT signal from neighboring Paneth cells and continuously produce differentiated epithelial cells which migrate toward and shed into the lumen of a mSI organoid after death (Fig. 1D top left panel). When endogenous WNT production is inhibited by Porcupine inhibitor and exogenous WNT source is introduced to a mSI organoid culture, high WNT activity promotes robust proliferation of stem cells and progenitor cells but prevents cell differentiation so organoids continuously grow larger as semi-transparent spheres [10]. In the presence of lower WNT activity, progenitor cells proliferate but unlike high WNT conditions where the organoids grow as large semi-transparent spheres, the lower WNT activity conditions produce mSI organoids with protruding "fingers" similar but longer as compared to culturing in WNT-free basal media. These morphological differences of mSI organoids in WNT-high, -low and -free media make it an ideal system for visualizing activities of WNT mimetics. We treated mSI organoids with FA-L6 WT, FA-L6 N297A, FA-L6 N297G and FA-L6 LALAPG at 1 nM or 10 nM in the presence of a Porcupine inhibitor to block secretion of endogenous WNT. When secretion of endogenous WNT was blocked and in the absence of exogenous WNT (basal media + IWP2), mSI organoids gradually degenerated as stem cells depleted over the course of one-week treatment (Fig. 1D lower left panel). With either 1 nM or 10 nM FA-L6 WT or FA-L6 LALAPG treatment, mSI organoids stayed as spheres in shape and continuously grew bigger, indicating high WNT activities from such treatments (Fig. 1D). In contrast, treatment with 1 nM or 10 nM FA-L6 N297A or FA-L6 N297G produced organoids with long protruding fingers, indicating low WNT activity from these Fc glycosylation mutations (Fig. 1D). These additional functional data from mSI organoids is consistent with the STF data on the impact of Fc glycosylation on FA-L6 WNT mimetic activity observed in Fig. 1C STF reporter assay.

To further eliminate the potential contribution of FcγR to agonistic activity, we performed IdeS protease [14] digestion of the FA-L6 N297A molecule, thus generating an FA-L6 $F(ab')_2$ fragment with tetravalent bispecific FZD/LRP binding arms intact but without the Fc region (Fig. 1E). The removal of the Fc region from FA-L6 N297A fully restored the agonistic activity to the level of the FA-L6 Fc WT molecule (Fig. 1F and G). IdeS digestion of FA-L6 with WT Fc had no effect on its activity (Fig. 1H and I). These results further supported that FcγRs are not required for FA-L6 WNT mimetic activity.

To further confirm that glycosylation on N297 is important for the activity of FA-L6 WNT mimetic, we performed PNGase F treatment on FA-L6 to remove glycans [15]. Glycan removal was confirmed by SDS-PAGE wherein the treated FA-L6 migrated faster than the untreated molecule (Fig. S1A); moreover, similarly to the N297 mutants, its STF activity was diminished (Fig. 2A). These results suggest that IgG1 Fc glycosylation is critical for the STF activity of FA-L6 WNT mimetic.

The glycan requirement is unique to the L6 binding arm

To understand whether the requirement for glycosylation is a general feature of WNT mimetic antibodies or was unique to the particular FZD and LRP binders used in FA-L6, we tested another FZD binder (FB, with $FZD_{1,2,7}$ specificity), two other LRP binders (LRP5 binder L5 and LRP5/6 binder #36, WO2019/126401) in combination with FA and L6 [11]. The PNGase F treated FA-L5 and FA-36 showed faster migration than the untreated molecule, thereby demonstrating the treatment effects (Fig. S1A). In contrast to that of FA-L6, the STF activity of FA-L5 and FA-36 were not affected by PNGase F treatment (Fig. 2B and C). Similarly, PNGase F treated FB-L5 and FB-36 retained full activity, whereas FB-L6 significantly lost STF activity (Fig. 2D–F). These results suggested that the LRP binder L6 containing WNT mimetics, was uniquely sensitive to Fc glycosylation. Next, we tested whether this glycosylation dependent STF might be sensitive to the location of L6 on FA. Because the initially tested FA-L6 had L6 appended to the N-terminus of LC, we generated three additional geometries in which the L6 arm was appended to the N-terminus (FA-L6_NH) or C-terminus of HC (FA-L6_CH), or the C-terminus of LC of FA (FA-L6_CL). All tested molecules with L6 appended to different locations showed faster migration in SDS-PAGE (Fig. S1B), and a significant decrease in STF activity after PNGase F treatment (Fig. 2G–I).

The hydrodynamic diameter of FA-L6 N297 mutants are smaller than FA-L6 WT

We compared the hydrodynamic diameter of the molecules FA-L6 WT and N297 mutants (FA-L6 N297G and FA-L6 N297A) on an UNcle instrument by DLS. Interestingly, both N297 mutants showed smaller hydrodynamic diameter than the FA-L6 WT, which implied that the mutations caused a conformational change in the protein (Fig. 3A left panel). In contrast, FA-L5 WT and N297 mutants (FA-L5 N297G and FA-L5 N297A) showed only a trend of hydrodynamic diameter difference (Fig. 3A right panel). Both sets of molecules with the N297 mutations showed similarly decreased thermal stability compared to their WT molecules (Fig. 3B).

N297 mutants modestly decrease the receptor affinity of L6

To understand whether the N297 residue mutation affects the binding of L6 to LRP6, we conducted bio-layer interferometry (BLI) assays. We directly measured the binding of FA-L6 WT and its N297 mutants (FA-L6 N297A and FA-L6 N297G) to recombinant LRP6 protein. FA-L6 WT, FA-L6 N297A, or FA-L6 N297G proteins were immobilized on the BLI surface, and binding was tested with different doses of recombinant LRP6 or FZD_8 proteins. Both mutations at N297 modestly decreased the binding affinity of FA-L6 WNT mimetic to LRP6 (Fig. 3C and E), whereas binding of FZD_8 was unaffected (Fig. 3D and F).

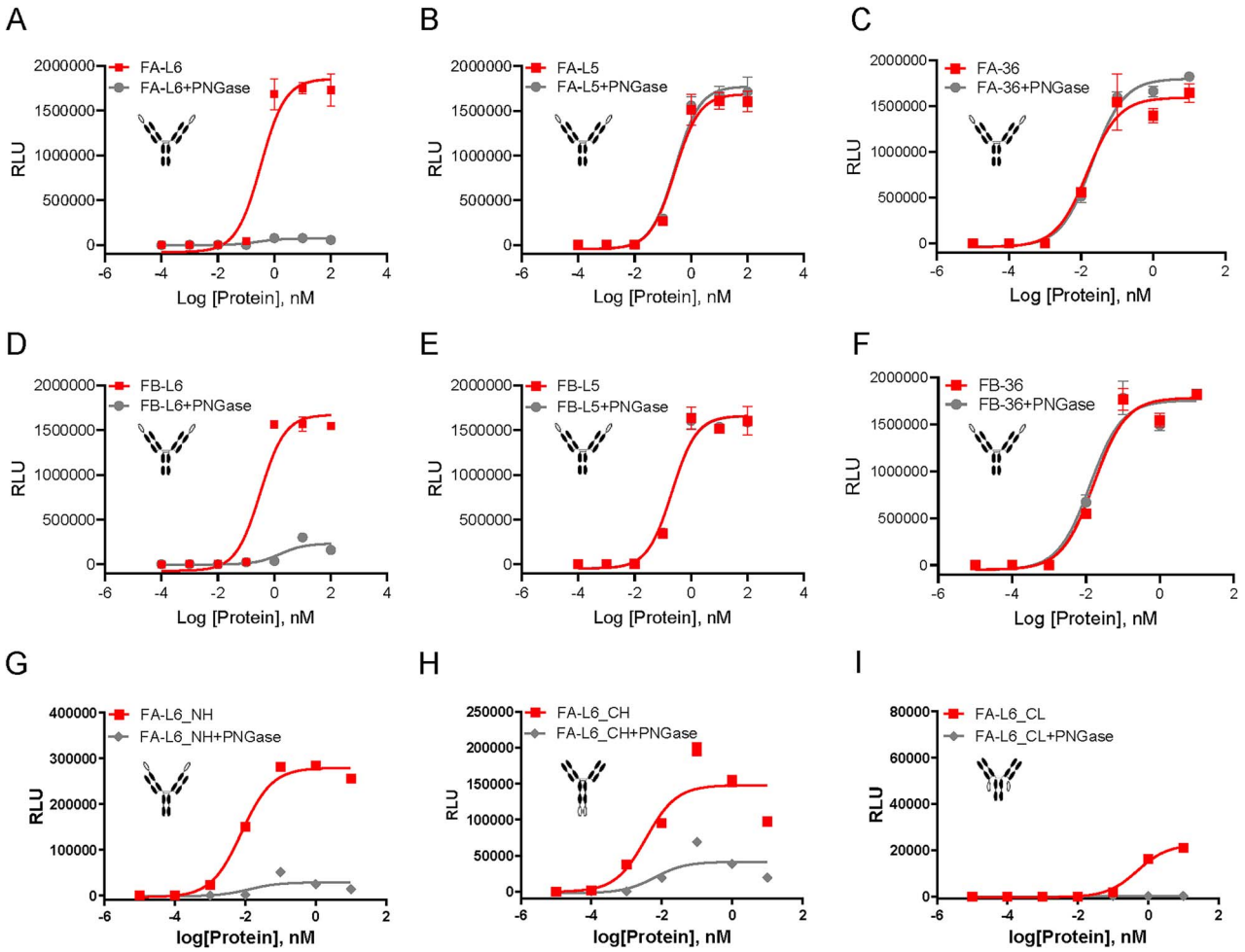


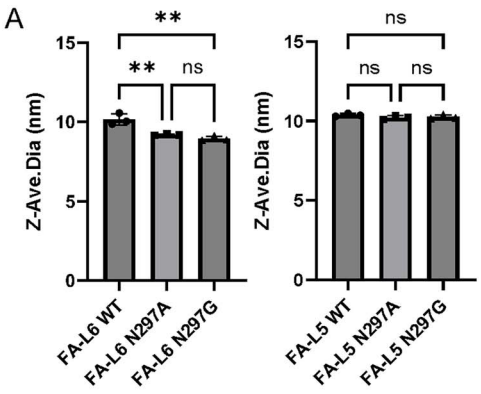
Figure 2. Glycan removal at N297 through PNGase F treatment disrupts the activity of L6 appended WNT mimetics. PNGase F treatment significantly decreases the activity of FA-L6 (A) but not FA-L5 (B) or FA-36 (C). PNGase F treatment significantly decreases the activity of FB-L6 (D) but not FB-L5 (E) or FB-36 (F). (G–I) Molecules with L6 appended to different locations on FA show a significant loss of STF activity after PNGase F treatment.

Since the effects of N297 mutations on binding affinity of FA-L6 were modest, we interrogated whether L6 CDR3 alanine-scanning mutations with similarly modest reduction of binding affinity could cause activity decreases like the N297 mutations. All amino acid residues of the L6 CDR3 were singly mutated to alanine and bivalent binding affinities were measured for each alanine mutated FA-L6. Indeed, Ala mutations S6A and P3A, which caused small affinity decreases (K_D value increase of ~2–3 times) both strongly impacted activities of the corresponding mutated FA-L6 WNT mimetics, reducing Emax by 5-fold or more in STF (Fig. S2). These data suggest that the binding impairments due to de-glycosylation on N297 could have contributed to the diminished activity observed for FA-L6 N297 mutants.

DISCUSSION

Monoclonal antibodies have become a modality of choice for developing new therapeutics. With advances in the understanding of antibody structure and function, and the multitude of available engineering options, antibodies are no longer antagonists that merely bind targets of interest and block their functions. Many ingenious approaches, such as multispecific antibodies, have enabled new uses and activities not normally achieved with conventional antibodies. Agonistic antibodies that mimic natural ligand functions are one such new application of this modality. Given the importance of receptor conformations in signaling, agonistic antibodies are sensitive to antibody formats, geometries, epitopes and other factors [16].

We and others have reported the construction of agonistic WNT mimetic platforms by using various antibody formats [8–11]. These molecules are all bispecific and engage both the FZD and LRP receptors on stem/progenitor cells, thus increasing cell survival and inducing proliferation and tissue regeneration. In molecules containing an Fc domain, various mutations have been introduced to eliminate effector function [9–11]. Here, we demonstrated that glycosylation mutations had unexpected negative effects on the agonistic activity of some WNT mimetics. The glycosylation on N297 in the CH2 domain of Fc is critical to maintain an "open" conformation that allows binding to FcγRs,



B

	Tm (°C)		Tm (°C)
FA-L6 WT	66.5 ± 1.6	FA-L5 WT	66.3 ± 0.6
FA-L6 N297A	60.8 ± 1.0	FA-L5 N297A	61.2 ± 0.8
FA-L6 N297G	62.5 ± 0.7	FA-L5 N297G	62.6 ± 1.1

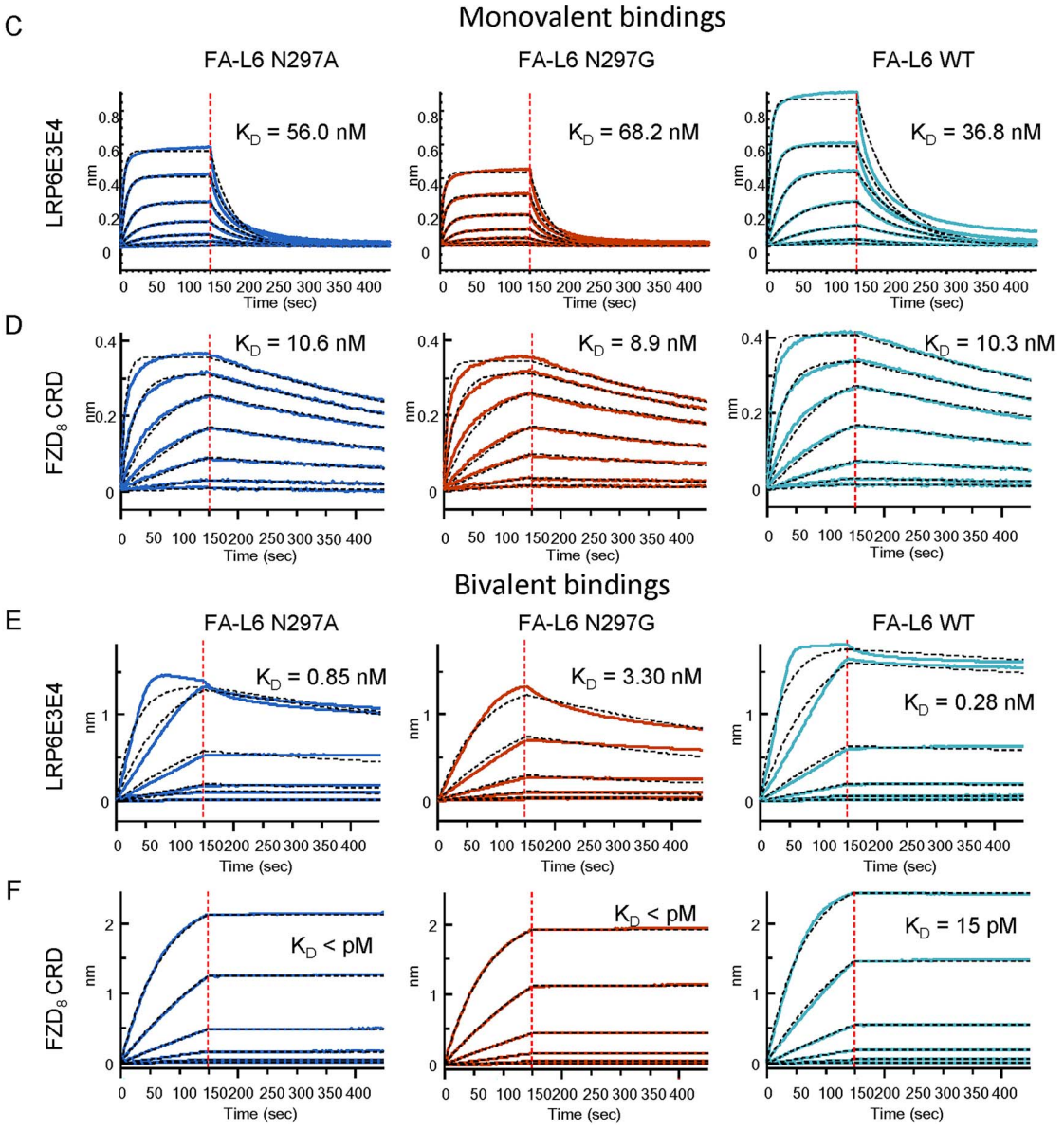


Figure 3. The biophysical property comparison of FA-L6 N297 mutants to FA-L6 WT molecule. (A) The hydrodynamic diameter of FA-L6 N297 mutants (FA-L6 N297G and FA-L6 N297A) are smaller than FA-L6 WT. FA-L5 WT and N297 mutants (FA-L5 N297G and FA-L5 N297A) showed no significant hydrodynamic diameter difference. $^{**}P < 0.01$. (one-way ANOVA). (B) Both sets of N297 mutants showed decreased thermal stability (Tm) compared to their WT molecules. (C–F) Binding of FA-L6 and its N297 mutants to LRP6E3E4 or FZD_8 CRD. The monovalent (C, D) and bivalent (E, F) binding affinities of FA-L6 WT, FA-L6 N297A and FA-L6 N297G to LRP6E3E4 or FZD_8 CRD, measured with an Octet Red 96 system. Dotted lines indicate simulated fits.

whereas absence of glycosylation may result in a more dynamic CH2 domain [17]. This change in the "openness" of the CH2 domain may alter the conformation of the WNT mimetic FA-L6 as demonstrated by the changes in the hydrodynamic diameter which may then affect the binding of the VHH L6 arms to the LRP6 receptor (as evidenced by the modest decrease in affinity in BLI experiments (Fig. 3)), and the signaling competency of the assembled receptor complex. Interestingly, changes in the hinge region and the presentation of the Fab arms have been reported to affect the activity of other agonistic antibodies [18, 19]. The effects observed herein appear to be specific to one LRP6 binder, L6, thus further supporting the previously described sensitivity of agonistic antibodies to epitopes and geometries [16, 20].

In conclusion, we described the effects of CH2 glycosylation on the agonistic activity of WNT mimetics. Our results highlight the importance of designing appropriate initial screening scaffolds to avoid false negatives for hit identification. Our findings suggest that starting with WT Fc and introducing effectorless mutations later in the discovery process may be desirable.

SUPPLEMENTARY DATA

Supplementary Data are available at ABT Online.

ACKNOWLEDGMENTS

The authors thank Teppei Yamaguchi, Haili Zhang, Menaka Bolaki Biala, Yan Pomogov and Yongfeng Fan for technical support and discussions; and Craig Parker for critical reading of the manuscript.

AUTHOR CONTRIBUTIONS

HC, SJL and CL Conceptualization, Formal analysis, Supervision, Investigation, Visualization, Methodology, Writing—original draft; Writing—review and editing; BO, NS, JY, Resources, Investigation, Visualization, Methodology, Writing—review and editing; YL, Conceptualization, Formal analysis, Resources, Supervision, Funding acquisition, Visualization, Methodology, Writing—original draft; Writing—review and editing.

CONFLICT OF INTERESTS STATEMENT

All authors are current or former full-time employees and shareholders of Surrozen, Inc. YL is Executive Vice President of Research at Surrozen, Inc. Patent applications are pending for the work described herein.

DATA AVAILABILITY STATEMENT

Data reported in this paper are available upon request.

FUNDING

This work was funded by Surrozen, Inc.

ETHICS AND CONSENT STATEMENT

Not applicable.

ANIMAL RESEARCH STATEMENT

Not applicable.

REFERENCES

1. Mullard, A. FDA approves 100th monoclonal antibody product. *Nat Rev Drug Discov* 2021; **20**: 491–5.
2. Delidakis, G, Kim, JE, George, K *et al.* Improving antibody therapeutics by manipulating the fc domain: immunological and structural considerations. *Annu Rev Biomed Eng* 2022; **24**: 249–74.
3. Wang, X, Mathieu, M, Brezski, RJ. IgG fc engineering to modulate antibody effector functions. *Protein Cell* 2018; **9**: 63–73.
4. Liu, R, Oldham, RJ, Teal, E *et al.* Fc-engineering for modulated effector functions—improving antibodies for cancer treatment. *Antibodies (Basel)* 2020; **9**: 9.
5. Leabman, MK, Meng, YG, Kelley, RF *et al.* Effects of altered FcgammaR binding on antibody pharmacokinetics in cynomolgus monkeys. *MAbs* 2013; **5**: 896–903.
6. Lo, M, Kim, HS, Tong, RK *et al.* Effector-attenuating substitutions that maintain antibody stability and reduce toxicity in mice. *J Biol Chem* 2017; **292**: 3900–8.
7. Nusse, R, Clevers, H. Wnt/beta-catenin Signaling, disease, and emerging therapeutic modalities. *Cell* 2017; **169**: 985–99.
8. Janda, CY, Dang, LT, You, C *et al.* Surrogate Wnt agonists that phenocopy canonical Wnt and beta-catenin signalling. *Nature* 2017; **545**: 234–7.
9. Tao, Y, Mis, M, Blazer, L *et al.* Tailored tetravalent antibodies potently and specifically activate Wnt/frizzled pathways in cells, organoids and mice. *Elife* 2019; **8**: e46134.
10. Chen, H, Lu, C, Ouyang, B *et al.* Development of potent, selective surrogate WNT molecules and their application in defining frizzled requirements. *Cell Chem Biol* 2020; **27**: 598–609.e4.
11. Fowler, TW, Mitchell, TL, Janda, CY *et al.* Development of selective bispecific Wnt mimetics for bone loss and repair. *Nat Commun* 2021; **12**: 3247.
12. Wenig, K, Chatwell, L, von Pawel-Rammingen, U *et al.* Structure of the streptococcal endopeptidase IdeS, a cysteine proteinase with strict specificity for IgG. *Proc Natl Acad Sci USA* 2004; **101**: 17371–6.
13. Zhang, Z, Broderick, C, Nishimoto, M *et al.* Tissue-targeted R-spondin mimetics for liver regeneration. *Sci Rep* 2020; **10**: 13951.
14. von Pawel-Rammingen, U, Johansson, BP, Bjorck, L. IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J* 2002; **21**: 1607–15.
15. Pucic, M, Knezevic, A, Vidic, J *et al.* High throughput isolation and glycosylation analysis of IgG-variability and heritability of the IgG glycome in three isolated human populations. *Mol Cell Proteomics* 2011; **10**: M111 010090.
16. Dickopf, S, Georges, GJ, Brinkmann, U. Format and geometries matter: structure-based design defines the functionality of bispecific antibodies. *Comput Struct Biotechnol J* 2020; **18**: 1221–7.
17. Borrok, MJ, Jung, ST, Kang, TH *et al.* Revisiting the role of glycosylation in the structure of human IgG fc. *ACS Chem Biol* 2012; **7**: 1596–602.
18. White, AL, Chan, HT, French, RR *et al.* Conformation of the human immunoglobulin G2 hinge imparts superagonistic properties to immunostimulatory anticancer antibodies. *Cancer Cell* 2015; **27**: 138–48.
19. Grujic, O, Stevens, J, Chou, RY *et al.* Impact of antibody subclass and disulfide isoform differences on the biological activity of CD200R and betaklotho agonist antibodies. *Biochem Biophys Res Commun* 2017; **486**: 985–91.
20. Shi, SY, Lu, YW, Liu, Z *et al.* A biparatopic agonistic antibody that mimics fibroblast growth factor 21 ligand activity. *J Biol Chem* 2018; **293**: 5909–19.