Targeted protein degradation systems to enhance Wnt signaling

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Abstract

Molecules that facilitate targeted protein degradation (TPD) offer great promise as novel therapeutics. Human hepatic lectin, asialoglycoprotein receptor (ASGR) is selectively expressed on hepatocytes. We have previously engineered an anti-ASGR1 antibody-mutant RSPO2 (RSPO2RA) fusion protein (called SWEETS^{TM}) to drive tissue-specific degradation of ZNRF3/RNF43 E3-ubiquitin ligases, leading to hepatocyte specific enhanced Wnt signaling, proliferation, and restored liver function in mouse models. Such an antibody-RSPO2RA fusion molecule is currently in human clinical trials. In the current study, we identified two new ASGR1 and ASGR1/2 specific antibodies, 8M24 and 8G8. High-resolution crystal structures of ASGR1:8M24 and ASGR2:8G8 complexes revealed that these antibodies bind to distinct epitopes on the opposite sides of ASGR, away from the substrate binding site. Both antibodies enhanced Wnt-activity when assembled as SWEETS molecules with RSPO2RA through specific effects sequestering E3 ligases. In addition, 8M24-RSPO2RA and 8G8-RSPO2RA efficiently downregulated ASGR1 through TPD mechanisms. These results demonstrate the possibility of combining different therapeutic effects and different degradation mechanisms in a single molecule.

Keywords: SWEETS, TPD, LYTAC, TransTAC, PROTAC, AbTAC, PROTAB, REULR, DUBTAC, protein stabilizer, ASGR, ASGR1, ASGR2, ASGPR, WNT, R-spondin, R-spondin mimetic

Introduction

Wnt ("Wingless-related integration site" or "Wingless and Int-1" or "Wingless-Int") signaling pathway is essential for proper biogenesis, homeostasis, and regeneration of many organs and tissues (Steinhart and Angers, 2018). In WNT/β-catenin signaling system, a WNT ligand simultaneously engages frizzled (FZD) family of receptors (FZD_{1-10}) and co-receptors LRP5 or LRP6 (low-density lipoprotein receptor-related protein 5/6) resulting in nuclear stabilization of β-catenin leading to expression of specific target genes responsible for cellular proliferation and regeneration. Targeted delivery of molecules that could modulate or stimulate Wnt-signal in a tissue or cell specific manner is critical for the treatment of various diseases where tissue regeneration could confer therapeutic benefits (Fowler et al., 2021; Liu et al., 2022; Nguyen et al., 2022; Nusse and Clevers, 2017; Xie et al., 2022).

Wnt signaling is finetuned by a variety of biochemical mechanisms (Rim et al., 2022). One negative feedback regulatory mechanism involves membrane E3 ubiquitin-protein ligases (E3 ligases), ZNRF3 (zinc and ring finger 3) and RNF43 (ring finger protein 43) proteins, which mark FZD and LRP receptors for proteasomal degradation *via* ubiquitination and endocytosis (Koo et al., 2012, Hao et al., 2012). In contrast, RSPO (R-spondins 1 to 4) proteins enhance Wnt-signal by facilitating clearance of the negative regulators, ZNRF3/RNF43, through LGR4-6 (leucine-rich repeat-containing G-protein coupled receptors 4, 5, 6) from the cell membrane *via* endocytosis of the RSPO:ZNRF3/RNF43:LGR ternary complex (Carmon et al., 2011; Chen et al., 2013). RSPO proteins bind to ZNRF3/RNF43 and LGR *via* their Furin Fu1 and Fu2 domains, respectively. Removal of ZNRF3/RNF43 E3 ligases from the cell membrane stabilizes FZD and LRP receptors and amplifies Wnt signaling. Therefore, disease conditions where Wnt ligands are present but signaling is limited could potentially have therapeutic benefits by targeted delivery of RSPOs.

ASGR (asialoglycoprotein receptor also known as ASGPR) mediates clearance of desialylated, galactose- or N-acetylgalactosamine-terminating plasma glycoproteins *via* receptor mediated endocytosis for lysosomal degradation (Schwartz et al., 1986; Weigel and Yik, 2002). ASGR is thought to exist as a heterotrimer of two ASGR1 and one ASGR2 polypeptides referred to as H1 and H2, respectively (Henis et al., 1990; Saxena et al., 2002). Both ASGR1 (H1) and ASGR2 (H2) polypeptides are type-II membrane proteins with a short N-terminal cytosolic domain followed by a single-transmembrane helix, and extracellular region

comprising a helical stalk-region that mediate oligomerization *via* a coiled-coil structure and a carbohydrate recognition domain (CRD) at their C-terminus (Meier et al., 2000). Human ASGR1 and ASGR2 share 54% sequence identity between them. ASGR is a calcium dependent C-type lectin highly expressed in mammalian hepatocytes and has been explored for targeted delivery of drugs to liver (Huang et al., 2017; Sanhueza et al., 2017).

Taking advantage of the liver-specific ASGR expression and its ability to induce endocytosis and lysosomal degradation of bound proteins, we have previously described a tissue targeted RSPO mimetic system (termed SWEETS for **S**urrozen **W**nt signal **E**nhancer **E**ngineered for **T**issue **S**pecificity), where a mutant-RSPO2 domain (RSPO2RA) fused to an anti-ASGR1 antibody, that specifically upregulated WNT target genes in hepatocytes, stimulated hepatocytes proliferation, and restored liver function in a diseased mouse model (Zhang et al., 2020). Since RSPO2RA retained the ability to bind the two E3 ligases but lost the ability to bind LGRs, SWEETS-induced proximity of E3 ligases to ASGR1 resulted in hepatocyte-specific endocytosis and degradation of E3 ligases, leading to stabilization of FZD and enhancement of Wnt signal. To further these efforts, here we describe the discovery and characterization of two new ASGR antibodies, namely 8M24 and 8G8. High-resolution crystal structures of ASGR1CRD:8M24 and ASGR2CRD:8G8 complexes revealed that the binders have distinct epitopes on the opposite surface of ASGR and provided insights into their specificities. SWEETS molecules assembled by fusion of RSPO2RA to 8M24 (8M24-RSPO2RA) and to 8G8 (8G8-RSPO2RA) showed robust Wnt-signal activation in cell-based assays. Further analysis also showed that such bispecific molecules not only induced internalization and lysosomal degradation of E3 ligases, but they also induced ASGR degradation through proteasomal and lysosomal pathways. These ASGR-targeted SWEETS molecules represent a unique targeted protein degradation (TPD) platform, that functions *via* multiple mechanisms, and expands the opportunities to treat liver diseases using regenerative therapeutics.

Results:

8M24 is a human ASGR1 specific binder and 8G8 binds to both ASGR1 and ASGR2

Mice immunizations were performed using the recombinantly expressed and purified extracellular domains of human ASGR1 (hASGR1, residues 62-291) and human ASGR2 (hASGR2, residues 66-292). Hybridoma lines were screened by ELISA against immunogens and ASGR CRDs, hASGR1CRD (residues 154-291) and hASGR2CRD (residues 177-311). Selected hybridoma lines, 8M24 and 8G8, were sequenced and recombinant Fabs or IgGs were cloned, expressed, and purified for further characterization. Analytical size exclusion chromatography (SEC) was used to investigate complex formation with ASGR1CRD and ASGR2CRD from both human and mouse. Compared to the antigens or 8M24-Fab, an early eluting peak (9.10 min), corresponding to antigen-Fab complex was observed between 8M24-Fab and hASGR1CRD but not between 8M24-Fab and mASGR1, hASGR2, or mASGR2 CRDs (Supplementary Fig. S1A). In contrast, early eluting peaks at 9.03, 9.50, 9.35, and 9.50 minute were observed for 8G8-Fab with all four antigens (Supplementary Fig. S1B). These observations suggest that 8M24 binds specifically to hASGR1, while 8G8 is dual specific and binds both ASGR1 and ASGR2.

Further, biolayer interferometry (BLI) was performed to corroborate SEC results. For BLI, 8M24 and 8G8 IgG1 antibodies were captured on an anti-Human IgG Fc capture (AHC) sensor and CRD of the antigens were used as analyte to determine monovalent affinity. As shown in Fig. 1A, 8M24 antibody shows tight binding and specific interaction to hASGR1 with K_D in the sub-picomolar range (Table 1) and did not bind to mASGR1, hASGR2, and mASGR2 (Fig. 1B). In contrast, 8G8 antibody bound to all four antigens (Fig. 1C-1E) with the strongest interaction for hASGR1, followed by hASGR2 and mASGR1 and weakest with mASGR2 (Table 1). Overall BLI results agree with those of SEC and establish that 8M24 antibody is specific to human ASGR1 and 8G8 binds to both human ASGR1 and ASGR2 and cross reactive to their mouse counterparts. Subsequently, we pursued crystal structure determination of hASGR1:8M24 and hASGR2:8G8 complexes to gain molecular insights into their specificities.

Structure of hASGR1CRD:8M24 complex

hASGR1CRD:8M24 complex crystallized in the $P2_12_12_1$ space group with one complex molecule per asymmetric unit. The refined structure of hASGR1CRD:8M24 complex, determined at a resolution of 1.7 Å, shows good stereochemistry with R_{cryst} and R_{free} factors of 17.3% and 20.5%, respectively (Table 2). Overall structure of hASGR1CRD:8M24 is shown in Fig. 2A. Structure of ASGR1CRD from its 8M24 complex can be superimposed onto apo-hASGR1CRD (PDB code: 1DV8;Meier *et al.*, 2000) with r.m.s.d. (root mean square deviation) of 0.44 Å over 128 $C^{\propto}$ atoms revealing that the binding of 8M24 to hASGR1 does not induce any significant conformational changes of the antigen. The binding site of 8M24 on hASGR1 is away from the substrate binding site marked by glycerol, used as a cryoprotectant while freezing crystals, bound to the Ca^{2+} ion (Fig. 2A & 2B). Upon complex formation both hASGR1 and 8M24 buries an average surface area of 792 $Å^2$ and epitope residues of 8M24 on hASGR1 that are within 4.5 Å from the antibody form a close-knit cluster (Fig. 2B).

The strong interaction between hASGR1 and 8M24, with sub-picomolar affinity, is predominantly contributed by residues from the helix ∝1 and those flanking ∝1 (Fig. 2C) with residues from the ß6 strand determining specificity of 8M24 for hASGR1 over ASGR2 (Fig. 3A). Lys173 of hASGR1, near the N-terminus of the helix ∝1, forms a hydrogen bond with the main-chain carbonyl oxygen of Thr105 from the HCDR3 loop of 8M24 (Fig. 2D). Lys173 also interacts strongly with Asp177 within the helix ∝1 of hASGR1, positioning later for hydrogen bonding interaction with Asn32 from the LCDR1 of 8M24. Further, this Asn32 (LCDR1) also interacts with sidechain of Ser106 from HCDR3. hASGR1 Lys173 is conserved in hASGR2 and substituted with Arg and Leu in mASGR1 and mASGR2, respectively (Fig. 3A). Introduction of longer-polar residue Arg or hydrophobic Leu would likely disrupt the structural conformation of closely juxtaposed interacting residues near the N-terminus of the ∝1 helix (Fig. 2D) and thus might explain lack of interaction between 8M24 and mASGR1 and mASGR2. Towards the C-terminus of hASGR1 ∝1 helix, conserved or similar residues Leu184, Glu185, and Asp186 are involved in strong hydrogen bonding interaction with 8M24 (Fig. 2E & 3A). Conserved Glu185 forms ionic and hydrogen bonds with Arg101 and His107 from the HCDR3 of 8M24 and hASGR1 Asp186 forms similar interactions with Arg54 and Asn57 of HCDR2 (Fig. 2E). Detailed structural analyses reveal that, in addition to the above mentioned Lys173, Asn180 may also contribute to the specificity of 8M24 for human ASGR1 (Fig. 3B). The sidechain amide-nitrogen Asn180 of hASGR1 forms a strong hydrogen with the

mainchain peptide carbonyl-oxygen of Phe91 from the LCDR3 of 8M24. Asn180 also form a C-H=O hydrogen bond with the C∝ atom of Trp92 from the LCDR3 of 8M24. Further, the sidechain of Asn180 is snugly fit into a cavity formed by Phe91-Trp92-Gly93 (LCDR3 loop) and Asn32 (LCDR1 loop) of 8M24 as can be seen from the tight overlap of atomic surfaces formed by these residues (Fig. 3B). hASGR1 Asn180 is replaced by residues with longer sidechains such as lysine in mASGR1, hASGR2, and glutamine in mASGR2. It is likely that residues with longer sidechains are not well accommodated at this position given the tight juxtaposition of Asn180 against the Phe91-Trp92-Gly93 (LCDR3 loop) and Asn32 (LCDR1 loop) of 8M24. Other critical sequence differences in the ASGR1 and ASGR2 are noted near the C-terminus of their CRDs (Fig. 3A). The Thr279 and Leu281 residues, conserved in ASGR1, are replaced by residues with longer, positively charged sidechains such as arginine and lysine in ASGR2. The sidechain hydroxyl of hASGR1 Thr279 forms a hydrogen bond with the Arg54 from the HCDR2 of 8M24 (Fig. 2E). It is likely that the positively charged residues of hASGR2 and mASGR2 near the C-terminus of their CRD are detrimental for their interaction with the 8M24 and thus contribute towards the specificity of 8M24 for the human ASGR1.

Structure of hASGR2CRD:8G8 complex

hASGR2CRD:8G8 complex crystallized in the *H*32 space group with one complex molecule per asymmetric unit. The structure of hASGR2CBD:8G8 complex was determined, by molecular replacement method, at a resolution of 1.9 Å, and refined to R_{cryst} and R_{free} factors of 16.6% and 20.4%, respectively, with good stereochemistry (Table 2). Overall structure of hASGR2CRD:8G8 is shown in Fig. 4A. Structures of hASGR2CRD (determined here) and apo hASGR1CRD (PDB code: 1DV8;Meier *et al.*, 2000) could be superimposed on each other with r.m.s.d. of 0.74 Å over 125 C∝ atoms with sequence identify of 65%. Thus, both the overall fold of ASGR2CRD made up of six ß-strands flanked by two ∝-helices on either side and three Ca^{2+} ions important for structural stability are conserved between the structures apo hASGR1 and hASGR2 CRDs. This suggests that binding of 8G8 to ASGR2 does not induce any significant conformational changes in the antigen. Further, the overall structures of hASGR1 and hASGR2 CRDs from the respective 8M24 and 8G8 complexes can be superimposed on each other with r.m.s.d. of 0.85 Å over 126 C∝ atoms. Such a superposition reveals that both the 8G8 and 8M24 binds away from the ASGR2 and ASGR1 substrate binding sites with non-

overlapping interaction surfaces, on the opposite sides of the antigens, around the helices ∝2 and ∝1, respectively (Fig. 3C).

8G8 binds to hASGR2CRD between the ß1, ß2, and ß6 strands and the helix ∝2, away from the substrate binding site marked by glycerol bound to the Ca^{2+} ion (Fig. 4A & 4B). Upon complex formation, both hASGR2 and 8G8 buries an average surface area of 663 $Å^2$ and epitope residues of 8G8 on hASGR2 that are within 4.5 Å from the antibody form a close-knit cluster (Fig. 4B). Among the seventeen epitope residues, 5 out of 6 from the ß1 strand, 2 out of 3 from the ß2 strand, 3 out of 7 from the ∝2 helix, and the Arg297 from the ß6 strand are conserved or similar between human, mouse, and rat ASGR1 and ASGR2 (Fig. 3A). hASGR2CRD epitope residues Glu183, Lys222, Gln226, His227, Asn229, and Arg297 are involved in hydrogen bonding/ionic interactions with paratope residues from the LCDR3, HCDR2, and HCDR3 of 8G8 (Fig. 4C). Among these, Glu183, His227, and Arg297 are conserved in human and mouse ASGR1 and ASGR2 (Fig. 3A) and therefore are unlikely to contribute towards wide-ranging affinities with the 8G8 antibody (Table 1). Asn229, which is a glycine in h/mASGR1 and serine in mASGR2, interacts with the heavy-chain Ser57 only through its main-chain peptide carbonyl oxygen (Fig. 4E & 3A). The side-chain nitrogen of Gln226 is involved in hydrogen bonding interactions with Gln50 (HCDR2) and Thr94 (LCDR3; Fig. 4E). Gln226 of ASGR2 is diversely substituted by His, Arg, and Lys respectively in hASGR1, mASGR1, and mASGR2 (Fig. 3A). Low affinity (Table 1) binding to mASGR2 can be explained by interaction with Lys222 of hASGR2 which is conserved in hASRG1 (residue Lys200) but substituted by Asn and Asp residues in mASGR1 and mASGR2, respectively (Fig. 3A). In addition, Lys222 forms strong ionic and hydrogen bonds with Asp93 of LCDR3 (Fig. 4D) and therefore, an Asp substitution will reduce affinity of mASGR2:8G8 interaction significantly.

Thus, high-resolution crystal structures have provided molecular insights into specificities of 8M24 and 8G8 antibodies. Structures also revealed that their epitopes on ASGR are both non-overlapping and away from the glycoprotein binding site on the receptor.

8M24 and 8G8 RSPO2RA fusions lead to enhanced Wnt signal

RSPO proteins bind to ZNRF3/RNF43 and LGRs *via* their Furin Fu1 and Fu2 domains, respectively. We have previously engineered a F105R/F109A mutant of human RSPO2

(RSPO2RA), which showed null binding to LGRs (Zhang *et al.*, 2020). Fusion of RSPO2RA to anti-ASGR1 antibody, 4F3 (4F3-RSPO2RA, also termed 4F3-SWEETS), which binds to the stalk region of ASGR1, leads to hepatocyte-specific RSPO mimetic activity (Zhang *et al.*, 2020). Since the binding sites of 8M24 and 8G8 located on the CRD of ASGR are different from that of 4F3, RSPO2RA fusions of 8M24 and 8G8 were constructed to examine the effectiveness of these ASGR epitopes on RSPO mimetic activity. RSPO2RA is fused to the N-terminus of 8M24 and 8G8 heavy chains using a 15-mer (GGGGS)x3 linker resulting in 8M24-RSPO2RA and 8G8-RSPO2RA (Fig. 5A). These two new 8M24- and 8G8-SWEETS molecules also showed robust Wnt signaling activity in the presence of a synthetic Wnt source, comparable to that of 4F3-RSPO2RA SWEETS in human hepatocyte cell line HuH-7 cells (Fig. 5B). To demonstrate the RSPO mimetic activity depends on ASGR expression, 8M24-RSPO2RA and 8G8-RSPO2RA were then tested in HEK293 cells, which do not express ASGR. As shown in Fig. 5C, parental HEK293 cells transfected with a pcDNA empty expression vector barely respond to the treatment of 4F3-RSPO2RA, 8M24-RSPO2RA, or 8G8-RSPO2RA, and no Wnt signaling enhancement was observed above the negative control protein, αGFP-RSPO2RA. In contrast, HEK293 cells transfected with ASGR1 expression vector responded to the treatment of all three SWEETS molecules and enhanced Wnt signaling (Fig. 5D). These results revealed that the two new epitopes on the CRD of the ASGR receptor, bound by 8M24 and 8G8, also serve as effective epitopes to facilitate clearance of E3 ligase from the cell surface leading to enhanced Wnt signaling.

4F3-RSPO2RA, 8M24-RSPO2RA, and 8G8-RSPO2RA also induce degradation of ASGR1

After demonstrating that SWEETS molecules enhance Wnt signaling, which likely occurs via ASGR-mediated elimination of E3 ligases, we investigated whether these bispecific SWEETS molecules affect ASGR1 protein levels. We reasoned that juxtaposing E3 ligases to ASGR by SWEETS binding may induce ubiquitination and subsequent degradation of ASGR proteins. Whole cell extracts from HuH-7 and another hepatocyte cell line, HepG2 cells, treated with various concentrations of the three SWEETS molecules were subjected to Western blot analysis with a commercially available anti-ASGR1 antibody (Fig. 6A and Fig. S2A). The total amount of ASGR1 was reduced in a dose-dependent manner after treatment with SWEETS molecules, with 4F3-RSPO2RA and 8M24-RSPO2RA being more potent than 8G8-RSPO2RA.

ASGR1 degradation induced by SWEETS molecules were very effective with only ~20% Western blot signal remaining at higher concentrations (>1 nM) compared to the untreated samples (Fig. 6A and Fig. S2A). Next, we evaluated the kinetics of the SWEETS-mediated ASGR1 degradation. As shown in Fig. 6B and Fig. S2B, the onset of the degradation was fairly rapid, ~40% reduction was already observed at 4 hours post treatment (for 4F3- and 8M24-SWEETS), and maximal reduction was achieved by 8 hours. Treatments with the anti-ASGR1 antibodies lacking the RSPO2RA domain did not result in the same level of ASGR1 reduction, suggesting that the SWEETS induced proximity of E3 ligase to ASGR1 facilitated ASGR1 degradation (Fig. 6C and Fig. S2C). To determine the route of ASGR1 degradation, we tested the effect of the autophagy-lysosomal pathway inhibitor bafilomycin A1 and the proteasomal pathway inhibitor MG132 on ASGR1 degradation promoted by SWEETS. As shown in Fig. 6D and Fig. S2D, we observed that either bafilomycin A1 or MG132 treatment significantly impaired ASGR1 degradation, suggesting that SWEETS can promote ASGR1 degradation through both lysosomal and proteasomal pathways. Since these two main degradation pathways have been shown to be linked because blocking one activates the other (Ding et al., 2007; Korolchuk et al., 2009), in addition to assessing the ASGR1 degradation, we examined whole protein ubiquitination and LC3B lipidation to monitor whether each pathway was compromised when inhibitors were treated. Only MG132 treated group resulted in a significant increase in whole protein ubiquitination, whereas only bafilomycin A1 treated group showed accumulation of autophagosome marker LC3B-II, demonstrating that the inhibitors had little effect on the other degradation pathway (Fig. 6D and Fig. S2D). Moreover, treatment of ubiquitin-activating enzyme inhibitor (E1 ligase inhibitor), TAK-243, showed a reduction of ASGR1 degradation, implicating that the catalytic function of E3 ligases is involved in SWEETS-mediated ASGR1 degradation (Fig. 6D and Fig. S2D). Collectively, our data demonstrate that all three SWEETS molecules can act as a TPD platform by degrading ASGR through both lysosomal and proteasomal pathways.

Discussions

Targeted protein degradation technologies have emerged as a promising therapeutic approach over the past two decades (Alabi and Crews, 2021; Luh et al., 2020; Zhao et al., 2022). Protein homeostasis involves a complex network of myriad biochemical processes that mediate protein synthesis, folding, post-translational modification, transport, and removal of damaged proteins. Central to the removal of damaged protein are proteasomes and lysosomes. Proteasomes degrade transiently-lived and misfolded proteins earmarked by the ubiquitin system (Damgaard, 2021). Lysosomes facilitate degradation of long-lived proteins, protein aggregates, entire organelles and intracellular parasites *via* endocytosis, phagocytosis and autophagy. Targeted degradation of intracellular, extracellular, and cell-surface proteins with unwanted function is an exciting alternative to finding inhibitors, especially in the case of "undruggable protein targets". Since the first proof concept by PROTAC (PROteolysis TArgeting Chimeras; Sakamoto et al., 2001), a variety of TPD molecular formats, which mainly target intracellular proteins, have emerged including molecular glue, double mechanism degraders, dTAGs, Trim-away, TF-PROTAC, dual-PROTAC, AUTAC, ATTEC, and AUTOTAC (Zhao *et al.*, 2022). Targeted protein degraders that focus on cell surface and extracellular protein have also emerged in recent years, these include LYTAC, TransTAC, AbTAC, PROTAB, REULR, bispecific aptamer chimera, GlueTAC, and KineTAC (Zhao *et al.*, 2022). The TPD systems involving either ASGR or ZNRF3/RNF43 E3-ligases include, LYTAC containing an ASGR specific ligand fused to an antibody against a POI (protein of interest, Ahn et al., 2021); and AbTAC (Cotton et al., 2021), PROTAB (Marei et al., 2022), or REULR (Siepe et al., 2023), which are bispecific antibodies that bring E3 ligases to the proximity of a POI.

Our previously described tissue targeted RSPO mimetic system (SWEETS) is a TPD technology that is applied to Wnt signaling modulation (WO2018/140821, WO2020/014271, Zhang *et al.*, 2020). The action of RSPOs is not cell-specific due to the broad distribution of their co-receptors, LGRs. This property hinders their application in regenerative medicine. By redirecting the E3 binding domain (RSPO2RA) to ASGR1 through a bispecific SWEETS molecule, RNF43 and ZNRF3 were eliminated specifically from hepatocyte cell surface via ASGR1-mediated endocytosis and lysosomal degradation. While the SWEETS molecules are tissue targeted RSPO mimetics, this approach of degrading cell surface E3 ligases is similar to the TPD system that is described as LYTAC (WO2020/132100, Ahn *et al.*, 2021).

The previously described ASGR-targeted SWEETS molecule contains an anti-ASGR1 antibody whose epitope is situated on the stalk domain of the receptor (Zhang *et al.*, 2020). Here we present structural characterization of 8M24 and 8G8, two antibodies bound to the CRD of ASGR1 and ASGR2, respectively, distinct from 4F3. These new ASGR epitopes, in the context of 8M24-RSPO2RA and 8G8-RSPO2RA fusion molecules also confer RSPO mimetic activities similar to stalk targeted 4F3 antibody. Biophysical experiments revealed that while 8M24 antibody is specific to human ASGR1, 8G8 antibody binds to both human ASGR1 and ASGR2, and also cross-reactive with mouse ASGRs (Fig. S1, Fig. 1, and Table 1). High resolution crystal structures of hASGR1:8M24 and hASGR2:8G8 Fab complexes revealed (i) structural basis of their specificities toward ASGRs (Fig. 2, Fig. 3, and Fig. 4A, B); (ii) that the epitope residues for these two antibodies are situated on the opposite surfaces centered around helix ∝1 and helix ∝2, respectively (Fig. 3C); (iii) neither directly overlaps with natural ligand pocket (Fig. 2, 3, 4). Further, structures of hASGR1 and hASGR2 described here also reveals binding sites for glycerol molecules (PDB codes: 8TS0 and 8URF) that can potentially be exploited towards designing novel small-molecule binders of ASGR that can be used as ASGR inhibitors, hepatocyte-targeting, and/or ASGR-engaging TPDs (Huang *et al.*, 2017; Sanhueza *et al.*, 2017).

In addition to the LYTAC function mediated degradation of E3 ligases, we also detected enhanced degradation of ASGR1 by SWEETS, as a result of bringing E3 ligases (RNF43 and ZNRF3) in the proximity of ASGR1 and subsequent degradation of ASGR1 through proteosome and lysosome (Fig. 6). Therefore, from the perspective of enhanced ASGR1 degradation, the SWEETS molecules also have what are now described as AbTAC (Cotton *et al.*, 2021), PROTAB (Marei *et al.*, 2022), and REULR (Siepe *et al.*, 2023) TPD activities. This E3-mediated degradation is an efficient and rapid process. Despite being one of the highest expressed receptors, with an estimate of ~1 million copies in a human hepatocyte (Miki et al., 2001), up to ~80% of ASGR1 was degraded within 8 hours of incubation with SWEETS (Fig. 6). A rare loss of function ASGR1 variant has been associated with a reduced risk of coronary artery disease (CAD) (Nioi et al., 2016). Achieving sustained inhibition of ASGR1 *via* conventional anti-ASGR1 antibody approach as a potential therapy for CAD may be challenging due to the chronic nature of the disease and high ASGR1 expression levels on hepatocytes. The ability of SWEETS to achieve efficient and sustained degradation of ASGR1

offers a new approach to eliminate this receptor potentially in the setting of CAD. These results suggest that rapid and efficient degradation of other receptors when linked to E3 ligases could also occur via this strategy. Given its dual effects on E3 and ASGR1, SWEETS may also combine a liver regenerative therapy with treatment for CAD in patients where multiple comorbidities may be present. This dual effect on E3 and its intended target in other TPD systems has also been observed. For example, treatment of an IGF1R targeting PROTAB (ZNRF3-IGF1R) resulted in both the reduction of IGF1R and the stabilization of WNT receptors, FZD and LRP, similar to SWEETS (Marei *et al.*, 2022). While in certain settings, for example, concomitant ASGR1 degradation offers protection against CAD, in other cases, a more specific SWEETS/PROTAB/AbTAC/REULR TPD system might be necessary. Additional design parameters, such as linker length, orientation, epitope, E3 chosen, stoichiometry of target vs. E3, etc., may need to be optimized to achieve specificity or monoTAC activity.

And finally, beyond LYTAC and AbTAC/PROTAB/REULR functions, SWEETS are ultimately a protein stabilization platform on FZD/LRP receptors leading to enhanced cellular sensitivity to WNTs. While different from DUBTAC (Henning et al., 2022)-induced target stabilization, elimination of E3 ligases specific for target of interest represent an additional approach for protein stabilization. Therefore, it is interesting to note that ASGR-coupled SWEETS molecules embody multiple “TAC” activities.

In conclusion, here we describe a TPD system leading to cell-specific enhancement of Wnt signaling. Multiple mechanisms of protein degradation and stabilization were observed with this system. Such an approach could enable combination of different pathways where beneficial, but also highlight the need to optimize design rules where monoTAC specificity is required. The tools described here could be applied broadly to other applications, for example, anti-ASGR-anti-growth factor receptor or checkpoint inhibitor combinations or anti-E3-anti-growth factor receptor or checkpoint inhibitor combination could be derived for various cancer treatments. These and other possibilities could be the focus of future studies.

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Data Availability

Atomic coordinates and structure factor of the hASGR1CRD:8M24 and hASGR2CRD:8G8 complex structures are available in the Protein Data Bank with accession codes 8TS0 and 8URF, respectively.

Author contributions: PS and HJ Conceptualization, Formal analysis, Supervision, Resources, Investigation, Visualization, Methodology, Writing—original draft, Writing—review and editing; ZZ Conceptualization, Formal analysis, Supervision, Investigation, Visualization, Methodology, Writing—review and editing; HC Formal analysis, Supervision, Investigation, Visualization, Methodology, Writing—review and editing; NS, YY, ZH, TL, RB, JY, and SL Resources, Investigation, Visualization, Methodology, Writing—review and editing; WCY, Conceptualization, Resources, Supervision, Funding acquisition, Writing—review and editing; YL, Conceptualization, Formal analysis, Resources, Supervision, Funding acquisition, Visualization, Methodology, Writing—original draft; Writing—review and editing.

Declaration of interests: All authors are current or former full-time employees and shareholders of Surrozen, Inc. YL is Executive Vice President, Research at Surrozen, Inc. A patent application is pending for the work described in this manuscript.

Table 1: Kinetics parameters of binding of ASGR1 and ASGR2 to 8M24 and 8G8 antibodies.

Load Sample	Analyte	KD (nM)	KD (M)	kon(1/Ms)	kdis(1/s)
8M24-IgG	hASGR1CRD	<1.0E-3	<1.0E-12	7.39E+05	<1.0E-07
8G8-IgG	hASGR1CRD	1.69	1.69E-09	5.48E+05	9.25E-04
8G8-IgG	mASGR1CRD	340	3.40E-07	5.34E+05	1.81E-01
8G8-IgG	hASGR2CRD	331	3.31E-07	2.59E+05	8.56E-02
8G8-IgG	mASGR2CRD	51,500	5.01E-05	6.91E+03	3.46E-01

Note: 8M24-IgG does not bind to mASGR1, hASGR2, and mASGR2 (Fig. 1B).

Table 2: Crystallography structure determination statistics.

Data collection	hASGR1CRD:8M24	hASGR2CRD:8G8
PDB code	8TS0	8URF
Beamline	ALS BCSB 5.0.2	ALS BCSB 5.0.2
Wavelength (Å)	0.9999	0.9999
Space group	$P2_12_12_1$	*H32*
Unit-cell dimensions (Å)	a=38.9, b=90.3, c=167.8	a=b=102.41, c=358.98
Matthew's coefficient ($Å^3$/Da)	2.17	2.71
Solvent content (%)	43.20	54.56
Resolution (Å)	38.91 - 1.70 (1.73 - 1.70)	38.90 – 1.85 (1.94 – 1.90)
Number of unique reflections	66288 (3439)	57655 (3681)
Completeness (%)	100 (100)	100 (100)
$CC_{1/2}$ (%)	99.9 (40.7)	99.9 (44.0)
$\langle I/\sigma(I)\rangle$	14.7 (0.9)	16.3 (1.2)
R_{meas}	0.107 (2.923)	0.109 (3.273)
R_{pim}	0.030 (0.789)	0.025 (0.718)
Multiplicity	13.0 (13.6)	19.8 (20.7)
Refinement		
Resolution (Å)	38.05 – 1.70 (1.73 – 1.70)	33.54 – 1.90 (1.97 – 1.90)
Number of unique reflections	66188 (2660)	57636 (5730)
Number of reflections for R_{free}	3233 (152)	
R_{cryst} (%)	17.3	16.6
R_{free} (%)	20.5	20.35
r.m.s.d.'s from ideal values:		
Bond length (Å)	0.007	0.008
Bond angles (°)	0.870	0.940
Average B-factors ($Å^2$):		
Protein	32.8	48.6
Water molecules	41.0	51.6
Ligands	47.7	57.3
Ramachandran plot:		
MolProbity residues in		
Favored region (%)	97.63	98.15
Allowed region (%)	2.37	0.41
Outliers (%)	0.00	0.00

Values in parentheses correspond to the highest-resolution shell.

Figure Legends:

Fig. 1. Biolayer interferometry (BLI) profiles of antigen CRDs to antibodies. (A) Binding of hASGR1 to 8M24-IgG1; (B) Non-binding of mASGR1, hASGR2, and mASGR2 to 8M24-IgG1; (C) Binding of hASGR1 to 8G8-IgG1; (D) Binding of hASGR2 to 8G8-IgG1; (E) Binding of mASGR1 to 8G8-IgG1; and (F) Binding of mASGR2 to 8G8-IgG1. Binding profiles for hASGR1, hASGR2, mASGR1, and mASGR2 are shown in brown, magenta, orange, and red traces, respectively.

Fig. 2. Structure of hASGR1:8M24 complex. (A) A cartoon representation of overall structure of hASGR1CRD:8M24-Fab complex. The heavy- and light- chains of 8M24 are colored orange and olive, respectively, with their CDR loops marked as H1, H2, H3, L1, L2, and L3. hASGR1CRD traced in blue to red from the N- to C-terminus. Three calcium ions are represented as magenta spheres. A glycerol molecule bound to the calcium ion at the ASGR substrate binding site is highlighted in the stick representation. (B) Epitope of 8M24 highlighted on the surface of hASGR1. Antigen residues that are within 4.5 Å from 8M24 heavy- and light-chains are shown in orange and olive colors, respectively. (C) Residues of hASGR1 involved in polar interactions with the 8M24 antibody of shown as sticks on the secondary structure elements (cyan traces) with most of the residues situated on the helix ∝1 of hASGR1. (D). A close-up view of polar interactions between 8M24 and hASGR1 near the N-terminus of helix ∝1. (E) A close-up view of polar interactions between 8M24 and hASGR1 near the C-terminus of helix ∝1.

Fig. 3. Comparison of hASGR1:8M24 and hASGR2:8G8 complex structures. (A). An alignment, made with MultAlin (Corpet, 1988) and ESPript (Gouet et al., 2003) of human, mouse, and rat ASGR1 and ASGR2 sequences with the secondary structure elements of hASGR1CRD (PDB Code: 1DV8, Meier *et al.*, 2000) depicted at the top and labeled according to the color scheme in Fig. 1A. Paratope residues from the heavy- and light- chains of 8M24 are marked with orange and olive circles, respectively. Paratope residues from the heavy- and light- chains of 8G8 are marked with purple and teal circles, respectively. Important residues are highlighted with a magenta downward arrow. hASGR2 residue Ser172 is marked for

reference to the sequence numbering. (B). A dot representation showing snug-fit of hASGR1 Asn180 of the cavity formed by Phe91-Trp92-Gly93 (LCDR3 loop) and Asn32 (LCDR1 loop) of 8M24. hASGR1 Asn180 is replaced by either Lys or Gln h/m/rASGR2 and m/rASGR1, respectively. (C). Superimposed structures of hASGR1:8M24 and hASGR2:8G8 complexes illustrating that the interaction surfaces of antibodies are situated on the opposite surfaces of the antigens are non-overlapping. The overall folds of hASGR1 and hASGR2 are shown in cyan and green, respectively, with their N- and C- terminus highlighted as blue and red spheres. Three calcium ions are represented as magenta spheres. A glycerol molecule bound to the calcium ion at the ASGR substrate binding site is highlighted in the stick representation.

Fig. 4. Structure of hASGR2:8G8 complex. (A) A cartoon representation of overall structure of hASGR2CRD:8G8-Fab complex. The heavy- and light- chains of 8G8 are colored in purple and teal, respectively, with their CDR loops marked as H1, H2, H3, L1, L2, and L3. hASGR2CRD traced in blue to red from the N- to C-terminus. Three calcium ions are represented as magenta spheres. A glycerol molecule bound to the calcium ion at the ASGR substrate binding site is highlighted in the stick representation. (B) Epitope of 8G8 highlighted on the surface of hASGR2. Antigen residues that are within 4.5 Å from 8G8 heavy- and light-chains are shown in purple and teal colors, respectively. (C) Residues of hASGR2 involved in polar interactions with the 8G8 antibody of shown as sticks on the secondary structure elements (green traces) with most of the residues situated on the helix ∝2 of hASGR2. (D). A close-up view of polar interactions between 8M24 and hASGR2 near the N-terminus of helix ∝2. (E) A close-up view of polar interactions between 8M24 and hASGR2 near the C-terminus of helix ∝2.

Fig. 5. Both 8M24 and 8G8 RSPO2RA SWEETS molecules enhance Wnt signal: (A) Diagrams of the SWEETS molecules. RSPO2RA is fused at the N-terminus of the heavy chain of IgG. (B) Both 8M24 and 8G8 RSPO2RA SWEETS molecules enhance WNT signaling in HuH-7 STF cells which has the WNT response reporter. (C, D) Both 8M24 and 8G8 RSPO2RA SWEETS molecules also enhance WNT signaling in ASGR1 overexpressed HEK293 STF cells which has the WNT response reporter, but not in parental HEK293 cells without ASGR1

overexpression. Data are representative of three independent experiments performed in triplicates and are shown as mean ± standard deviation (SD).

Fig. 6. SWEETS induce degradation of ASGR1 in HuH-7 cells. (A) Dose-dependent ASGR1 degradation promoted by different concentrations of 4F3-, 8M24-, and 8G8-RSPO2RA SWEETS. (B) Time course of ASGR1 degradation treated with 10nM of 4F3-, 8M24-, and 8G8-RSPO2RA for 24h. (C) Western blot analysis demonstrating the efficacy of ASGR1 degradation in cells treated with 4F3-, 8M24-, and 8G8-RSPO2RA SWEETS compared to ASGR1 antibodies lacking the RSPO2RA domain. (D) Western blot data showing total protein levels of ASGR1, Ubiquitin, and LC3B in HuH-7 cells pre-treated with DMSO, lysosomal pathway inhibitor bafilomycin A1 (Baf.A1), proteasome inhibitor MG132, and E1 ubiquitin ligase inhibitor TAK-243 to test which degradation pathways govern ASGR1 degradation by SWEETS. Data in (A-C) are representatives of three independent experiments, while data in (D) are representatives of two independent experiments. For (A-B), total ASGR1 levels were normalized to generate graphs representing the mean of those three experiments. In (C), data are represented as mean ± s.e.m. of normalized total ASGR1 levels and one-way ANOVA with Tukey's post hoc test was used for statistical analysis. *$p<0.05$, **$p<0.01$.

Fig. S1: 8M24 is specific to human ASGR1 and 8G8 binds to both ASGR1 and ASGR2 with mouse cross-reactivity: (A) Interaction of 8M24-Fab with human and mouse ASGR1 and ASGR2 CRDs. Left-shifted peak, corresponding to complex formation, is observed only with hASGR1-CRD, revealing complex formation. (B) Interaction of 8G8-Fab with human and mouse ASGR1 and ASGR2 CRDs. Left-shifted peak, corresponding to complex formation, is observed for four proteins human and mouse ASGR1 and ASGR2 CBDs. Elution time is shown in the x-axis and the absorption unit in the y-axis are not to the actual scale. Samples were analyzed on an Agilent AdvanceBio SEC 300Å, 2.7 µm, 7.8 x 300 mm column, equilibrated with 20 mM HEPES pH 7.5, 150 mM sodium chloride, mounted on a Vanquish (Thermoscientific, USA) UHPLC system.

Fig. S2. ASGR1 degradation promoted by SWEETS was determined in an additional hepatocyte cell line, HepG2 cells. (A) ASGR1 degradation induced by different

concentrations of 4F3-, 8M24-, and 8G8-RSPO2RA showing dose-response curve of SWEETS. (B) Time course of ASGR1 degradation treated with 10nM of 4F3-, 8M24-, and 8G8-RSPO2RA for 24h. (C) Western blot analysis showing the comparisons of ASGR1 degradation induced by SWEETS and ASGR1 antibody. (D) Total protein levels of ASGR1, Ubiquitin, and LC3B in HepG2 cells pre-incubated with DMSO, lysosomal pathway inhibitor bafilomycin A1 (Baf.A1), proteasome inhibitor MG132, and E1 ubiquitin ligase inhibitor TAK-243 to determine the degradation pathway for SWEETS-mediated ASGR1 degradation. The Western blot data in (A-C) are representatives of three independent experiments, whereas data in (D) are representatives of two independent experiments. For (A-B), total ASGR1 levels were normalized by total loading control protein levels (Vinculin) to generate graphs representing the mean of those three experiments. For (C), data are represented as mean ± s.e.m. of normalized total ASGR1 levels and one-way ANOVA with Tukey's post hoc test was used for statistical analysis. **$p<0.01$, ***$p<0.001$.

Methods

Protein Expression and Purification

All proteins were cloned into pcDNA3.1(+) vector with the human kappa light-chain secretion signal sequence at the N-terminus and transiently expressed in Expi293 cells using the FectoPro transfection agent (Polyplus Transfection NY USA). In 4F3-RSPO2RA, 8M24-RSPO2RA, and 8G8-RSPO2RA SWEETS molecules, F105R/F109A mutant of human RSPO2 furin domain was fused at the N-terminus of heavy chain of respectively antibodies via a 15-mer (G4S)x3 linker. Antigens hASGR1, mASGR1, hASGR2, mASGR2, 8M24-Fab, and 8G8-Fab were expressed with His-tag and purified using the cOmplete His-Tag purification resin (Roche, USA) following standard protocols. 8M24 and 8G8 antibodies, 4F3-RSPO2RA, 8M24-RSPO2RA, and 8G8-RSPO2RA SWEETS were purified using either CaptivA resin (Repligen, USA) under gravity-flow or using a prepacked MabSelect SuRe (Cytiva, USA) column attached to an AKTA FPLC. All proteins were further polished on a Superdex 200 size-exclusion chromatography column equilibrated with 2xHBS (40 mM HEPES pH 7.5, 300 mM sodium chloride) buffer.

Determination Binding Affinity and Specificity Using BLI

For the BLI (biolayer interferometry) assays, the 8M24 and 8G8 antibodies were prepared at 50 nM in 1x phosphate buffer saline + Tween (0.05%) (PBST) + 0.5 mg/mL bovine serum albumin (BSA) (assay buffer) (Teknova P1192, Fisher BP1600) and captured on Anti-hIgG Fc Capture (AHC) Biosensors (Sartorius 18-5060). Capture was recorded for 40 seconds. Association was measured by transferring the IgG loaded sensors to wells containing CRDs of hASGR1, mASGR1, hASGR2, and mASGR2 at three-fold dilution series from 1 uM to 4 nM for 120 seconds. Dissociation was measured by placing sensors in wells containing only assay buffer for 150 seconds. Assays were performed in duplicates. Kinetics parameters were determined using instrument analysis software to fit a global 1:1 kinetic model with Rmax linked across all concentrations.

hASGR1CRD:8M24 and hASGR2CRD:8G8 crystallization and structure determination:

Purified hASGR1CRD and hASGR2CRD were mixed with the 8M24 and 8G8 Fabs, respectively, at 1.1:1 molar ratio and incubated with carboxy-peptidase A and B at a w/w ratio

of 100:1 for over-night at 4°C. Complex formation was confirmed by observation of a single-major peak on SuperdexS200 Increase (10/300 GL) column pre-equilibrated in 20mM HEPES pH 7.5 and 150 mM sodium chloride and by SDS-PAGE. Initial crystallization screen (hASGR1CRD:8M24 at 20 mg/mL and hASGR2CRD:8G8 at 28 mg/mL), using commercially available reagents with were performed using Mosquito (TTP LabTech) liquid handler and equilibrated at 18°C inside an EchoTherm incubator (Torrey Pines Scientific USA). Crystallization conditions were further optimized by grid-screens or microseed matrix screen (D'Arcy et al., 2014). Diffraction quality crystals of hASGR1CRD:8M24 complex grew in buffer containing 100 mM SPG (succinic acid, sodium phosphate monobasic monohydrate, Glycine buffer) pH 9.0 and 25 % w/v PEG 1500. Crystal was cryo-protected using 20% glycerol in the well-solution. Diffraction quality crystals of hASGR2CRD:8G8 complex grew in buffer containing 100 mM sodium HEPES pH 7.5, 100 mM calcium chloride, and 30 % (w/v) PEG 400. Crystal was cryo-protected using 16% glycerol in the well-solution. X-ray diffraction datasets were collected at the Berkeley Center for Structural Biology at the Advanced Light Source (ALS), Berkeley CA, and processed with XDS (Kabsch, 2010) and xdsme (Legrand, 2017) programs. Structures were determined by molecular replacement method using Phaser (McCoy et al., 2007) with a poly-alanine model of ASGR1CRD (PDB code: 5JPV; Sanhueza *et al.*, 2017) and variable and constant domains Fab, followed by refinement using Phenix (Liebschner et al., 2019) and validation by MolProbity (Williams et al., 2018). Crystallography models were manually inspected and built using COOT. Analyses of refined crystal structures, and image creations were performed using MOE (CCG, https://www.chemcomp.com/) and PyMol (https://pymol.org/).

SuperTop Flash (STF) Assay

Wnt signaling activity was measured using cell lines containing a luciferase gene controlled by a Wnt-responsive promoter (STF reporter) as previously reported (Janda et al., 2017). In brief, cells were seeded at a density of 10,000 per well in 96-well plates 24 h prior to treatment, then treated with various proteins overnight in the presence of 100 pM WNT mimetic FA-L6 (Fowler *et al.*, 2021). Cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI) and activity was measured with Luciferase Assay System (Promega, Madison, WI) using vendor suggested procedures. Data were plotted as average −/+ standard deviation of

triplicates and fitted by non-linear regression using Prism (GraphPad Software, San Diego, CA). For overexpression of exogenous receptors, cells were transiently transfected with plasmids containing receptors of interest under eukaryotic expression promoters (ASGR1 was clone OHU03658D from GenScript), then split into 96-well plates (20,000 cells per well) for STF assay 24 h post transfection.

Western blot analysis

For ASGR1 degradation assay, HuH-7 and HepG2 cells at a density of $1x10^5$ cells per well (24-well plate) were plated a day before the experiment. 10nM of 4F3-RSPO2RA, 8M24-RSPO2RA, 8G8-RSPO2RA, 4F3-IgG, 8M24-IgG, 8G8-IgG, or the control (αGFP-RSPO2RA) were treated for 24h. For the dose-response experiment, indicated concentrations of SWEETS or controls were treated for 24h. For the time course experiment, 10nM of each SWEETS molecule was incubated for the indicated duration. When lysosomal pathway inhibitor bafilomycin A1 (10nM, Millipore Sigma 1661), proteasome inhibitor MG132 (1µM, Selleck Chemicals S2619), E1 ubiquitin ligase inhibitor TAK-243 (50nM, Selleck Chemicals S8341) or DMSO (as the control vehicle) was treated, inhibitors were pre-treated for 18h, 10nM of SWEETS or control (αGFP-RSPO2RA) was then added for 6h in the presence of inhibitors or DMSO. Following incubation, cells were washed in ice-cold TBST and lysed in RIPA buffer (Thermo Fisher, 89900) supplemented with 1% Halt protease inhibitor (Thermo Fisher, 87786), 1% Halt phosphatase inhibitor (Thermo Fisher, 78420), and 0.1% Benzonase (Millipore Sigma, E1014) on ice for 20min. Whole-cell lysates were prepared by collecting supernatants after centrifugation at 14,000g for 20min at 4C°. Protein concentration was determined by BCA assay (Thermo Fisher, A53225/A53226). Samples were prepared by mixing with 4x Laemmli Sample Buffer (Bio-Rad, 1610747) supplemented with BME (2-Mercaptoethanol) and incubated for 5min at 95C°. Prepared samples with equal amounts of proteins were run on 10% Mini-PROTEAN TGX Precast Gels (Bio-rad, 4561033) (for ASGR1 blot) or 4-20% Mini-PROTEAN TGX Precast Gels (Bio-Rad, 4568093) (for Ubiquitin and LC3B blot) using Tris/Glycine/SDS running buffer (Bio-Rad, 1610732). Gels were transferred to nitrocellulose membranes (Bio-Rad, 1704158EDU) using Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were blocked with SuperBlock T20 (TBS) Blocking Buffer (Thermo Fisher, 37536) for 1h at room temperature (RT). Following blocking, the membranes were incubated with

primary antibodies overnight at 4C°. Blots were washed 3 times for 5min each with TBST and subsequently incubated with HRP secondary antibodies for 1hr at RT. Blots were washed 3 times for 5min each with TBST, developed using enhanced chemiluminescent (ECL) HRP substrates (Pierce, 34580), and visualized using ChemiDoc Imaging System (Bio-Rad). Image Lab (Bio-rad) was used to quantify the signal intensity of bands.

Antibodies

Rabbit polyclonal anti-Human ASGR1 antibody (Thermo Fisher, PA5-80356), 1:1,000; Rabbit polyclonal anti-LC3B antibody (Novus, NB100-2220), 1:1,000; Mouse monoclonal anti-Ubiquitin (eBioP4D1) antibody (Invitrogen, 14-6078-82), 1:500; Mouse monoclonal anti-Human Vinculin (V284) antibody (Bio-Rad, MCA465GA), 1:1,000; Goat pAb to rabbit IgG H&L (HRP) (Abcam, ab205718), 1:20,000; Goat pAb to mouse IgG H&L (HRP) (Abcam, ab205719), 1:20,000.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA). Detailed information about statistical analyses for each data are stated in the figure legends.

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Figure 1

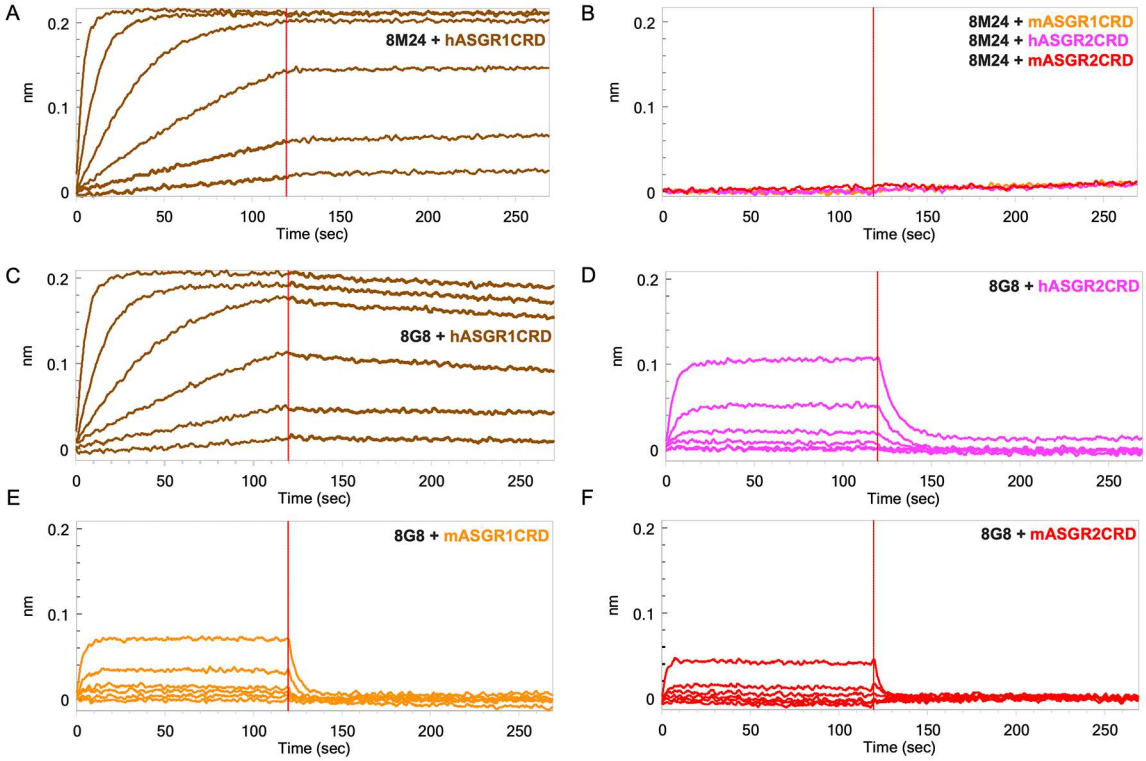

A
8M24 + hASGR1CRD
B
8M24 + mASGR1CRD
8M24 + hASGR2CRD
8M24 + mASGR2CRD
C
8G8 + hASGR1CRD
D
8G8 + hASGR2CRD
E
8G8 + mASGR1CRD
F
8G8 + mASGR2CRD
nm
0
0.1
0.2
Time (sec)
0
50
100
150
200
250

Figure 2

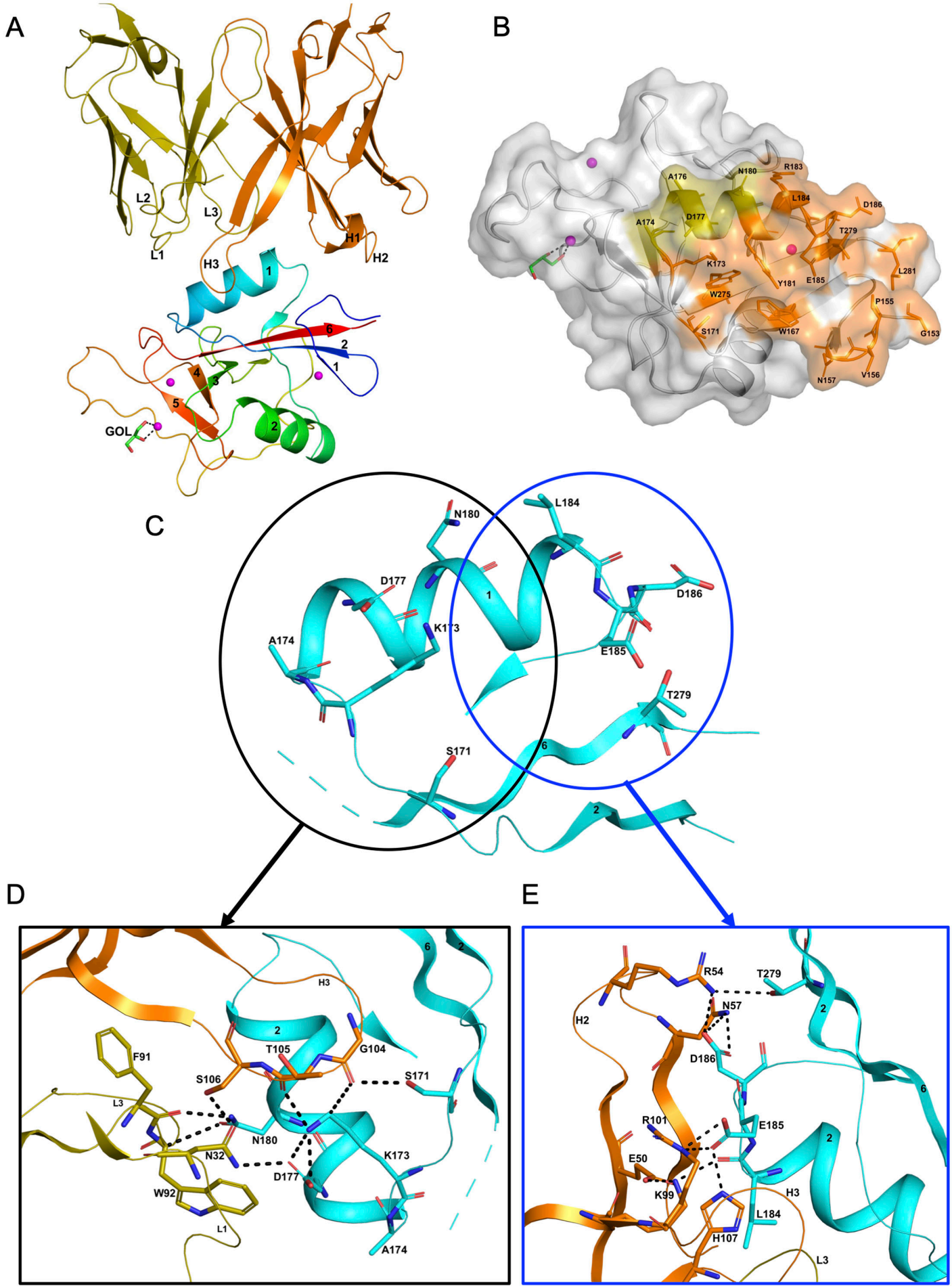
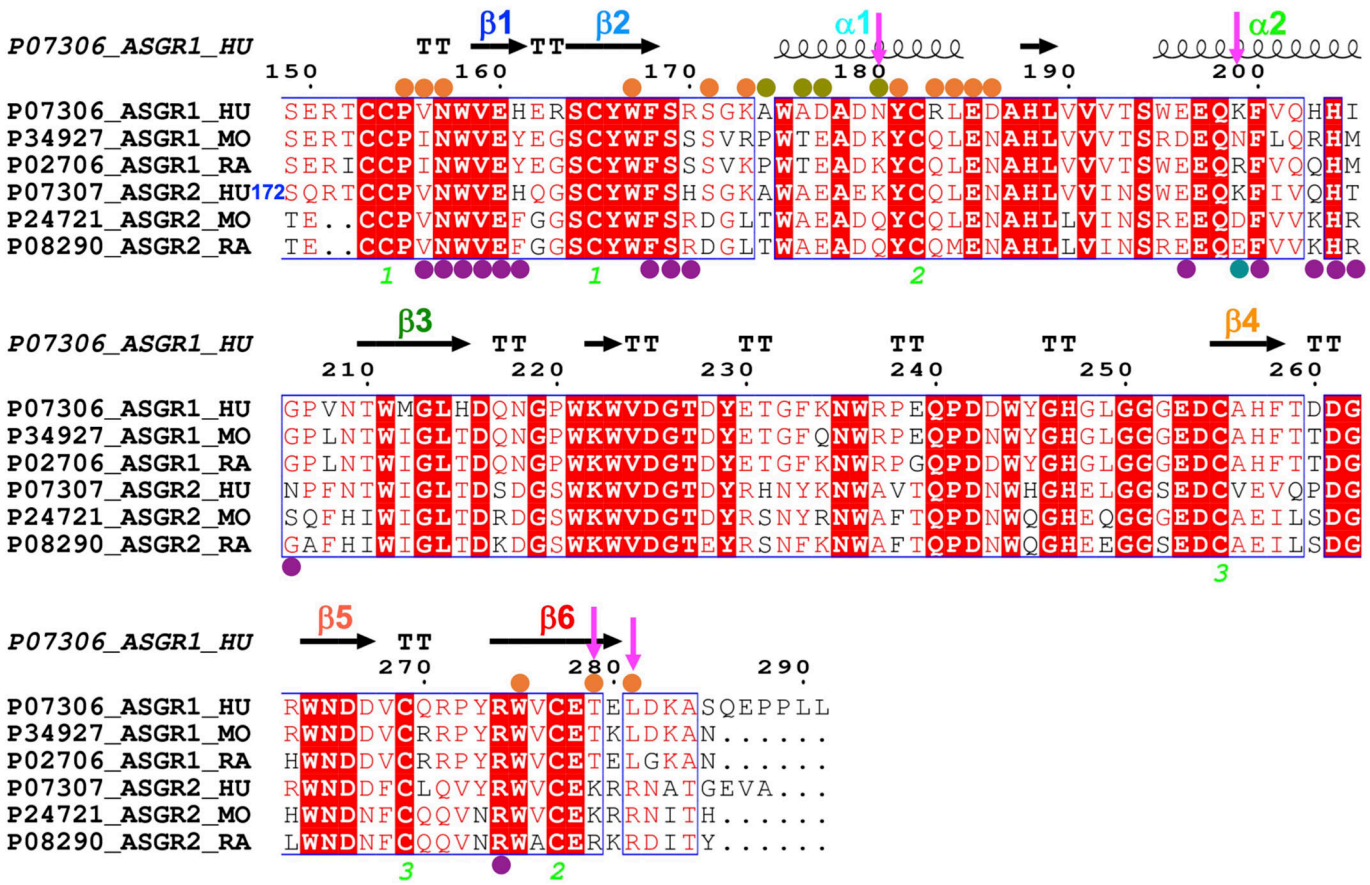

A
L2
L3
L1
H3
H1
H2
GOL
B
A176
N180
R183
L184
D186
A174
D177
T279
K173
Y181
E185
L281
W275
P155
S171
W167
G153
N157
V156
C
N180
L184
D177
D186
A174
K173
E185
T279
S171
D
F91
L3
S106
T105
G104
S171
N180
N32
K173
D177
W92
L1
A174
H3
E
R54
T279
N57
H2
D186
R101
E185
E50
K99
H3
L184
H107
L3

Figure 3

A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β1
β2
α1
α2
150 160 170 180 190 200
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P34927_ASGR1_MO SERTCCPINWVEYEGSCYWFSSSVRPWTEADKYCQLENAHLVVVTSRDEQNFLQRHM
P02706_ASGR1_RA SERICCPINWVEYEGSCYWFSSSVKPWTEADKYCQLENAHLVVVTSWEEQRFVQQHM
P07307_ASGR2_HU 172 SQRTCCPVNWVEHQGSCYWFSHSGKAWAEAEKYCQLENAHLVVINSWEEQKFIVQHT
P24721_ASGR2_MO TE..CCPVNWVEFGGSCYWFSRDGLTWAEADQYCQLENAHLLVINSREEQDFVVKHR
P08290_ASGR2_RA TE..CCPVNWVEFGGSCYWFSRDGLTWAEADQYCQMENAHLLVINSREEQEFVVKHR
P07306_ASGR1_HU
β3
β4
210 220 230 240 250 260
P07306_ASGR1_HU GPVNTWMGLHDQNGPWKWVDGTDYETGFKNWRPEQPDDWYGHGLGGGEDCAHFTDDG
P34927_ASGR1_MO GPLNTWIGLTDQNGPWKWVDGTDYETGFQNWRPEQPDNWYGHGLGGGEDCAHFTTDG
P02706_ASGR1_RA GPLNTWIGLTDQNGPWKWVDGTDYETGFKNWRPGQPDDWYGHGLGGGEDCAHFTTDG
P07307_ASGR2_HU NPFNTWIGLTDSDGSWKWVDGTDYRHNYKNWAVTQPDNWHGHELGGSEDCVEVQPDG
P24721_ASGR2_MO SQFHIWIGLTDRDGSWKWVDGTDYRSNYRNWAFTQPDNWQGHEQGGGEDCAEILSDG
P08290_ASGR2_RA GAFHIWIGLTDKDGSWKWVDGTEYRSNFKNWAFTQPDNWQGHEEGGSEDCAEILSDG
P07306_ASGR1_HU
β5
β6
270 280 290
P07306_ASGR1_HU RWNDDVCQRPYRWVCETELDKASQEPPLL
P34927_ASGR1_MO RWNDDVCRRPYRWVCETKLDKAN.....
P02706_ASGR1_RA HWNDDVCRRPYRWVCETELGKAN.....
P07307_ASGR2_HU RWNDDFCLQVYRWVCEKRRNATGEVA...
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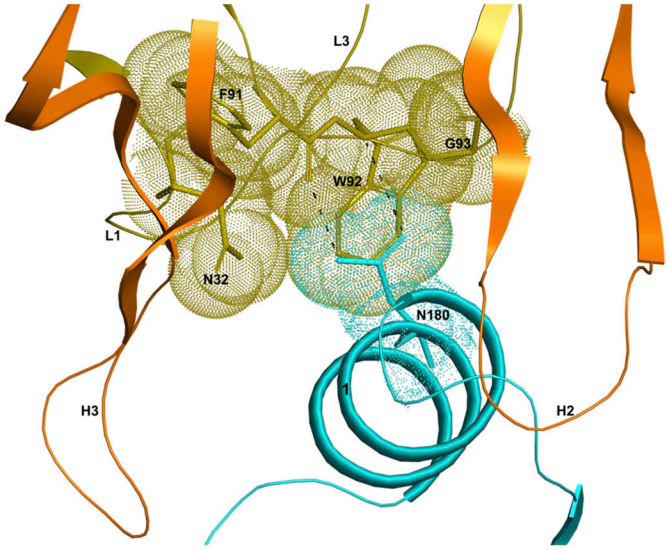
B


L3
F91
G93
W92
L1
N32
N180
H3
H2

C

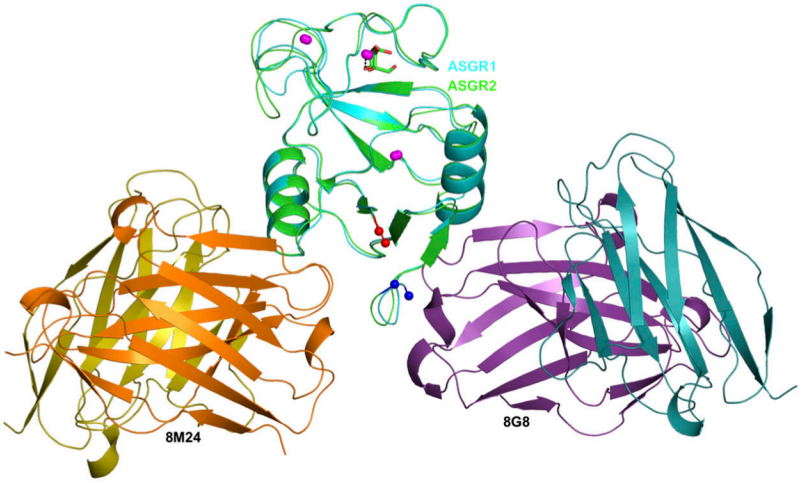

ASGR1
ASGR2
8M24
8G8

Figure 4

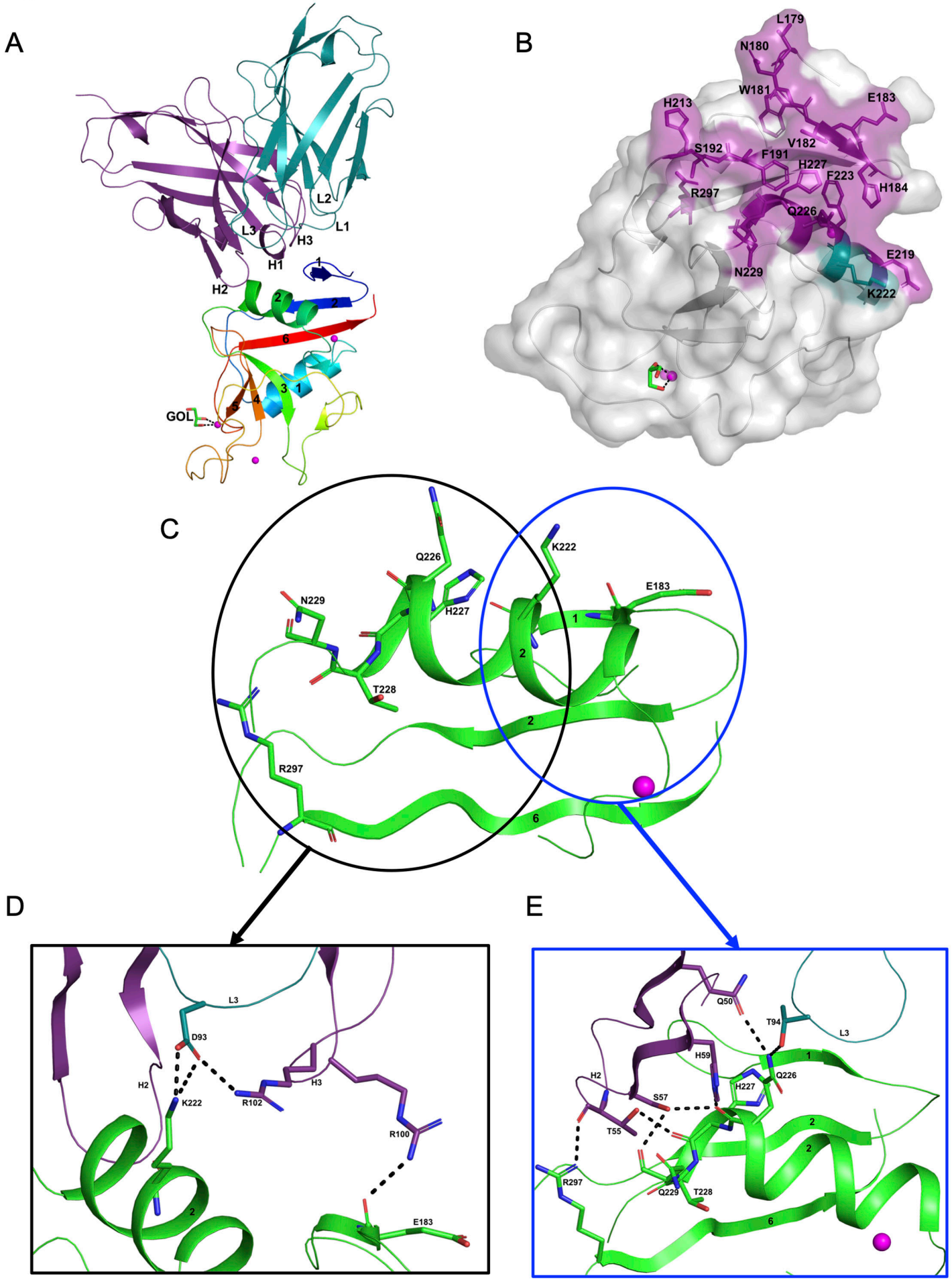
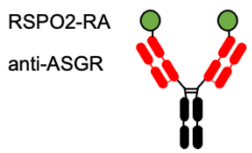
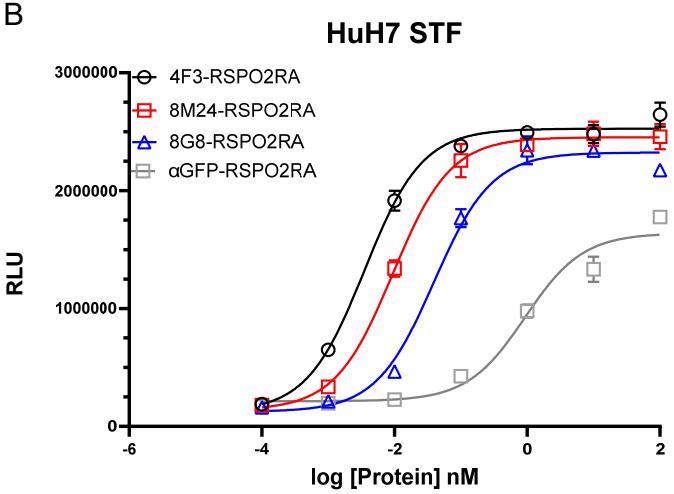

A
L2
L1
L3
H3
H1
H2
GOL
B
L179
N180
W181
E183
H213
V182
S192
F191
H227
F223
H184
R297
Q226
E219
N229
K222
C
Q226
K222
E183
N229
H227
T228
R297
D
L3
D93
H2
K222
R102
H3
R100
E183
E
Q50
T94
L3
H59
H2
Q226
H227
S57
T55
R297
Q229
T228

Figure 5

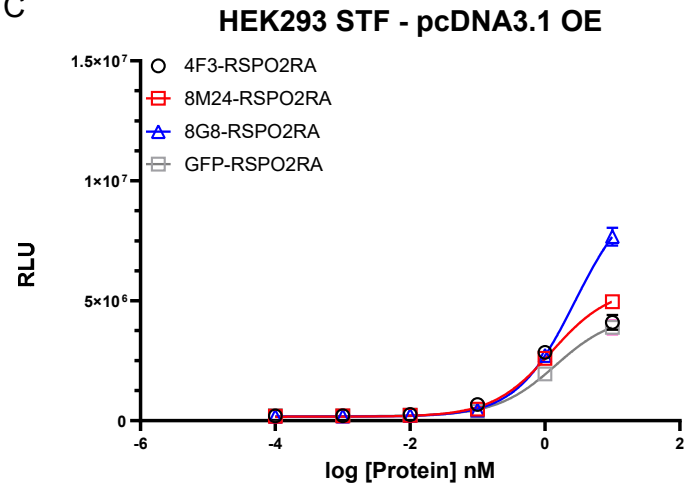
A



B



C



D

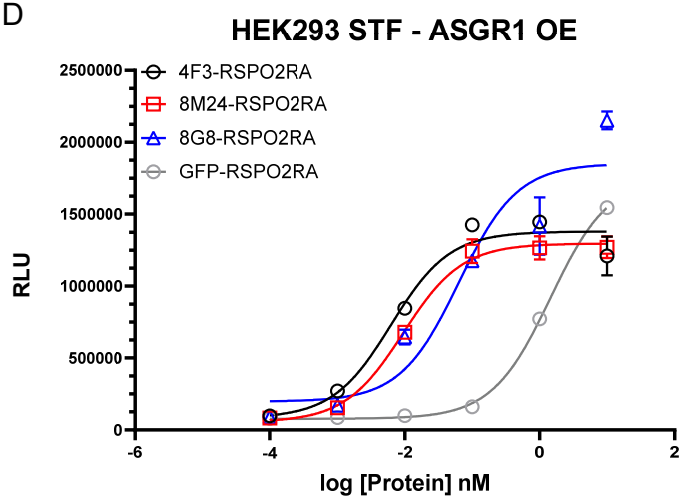
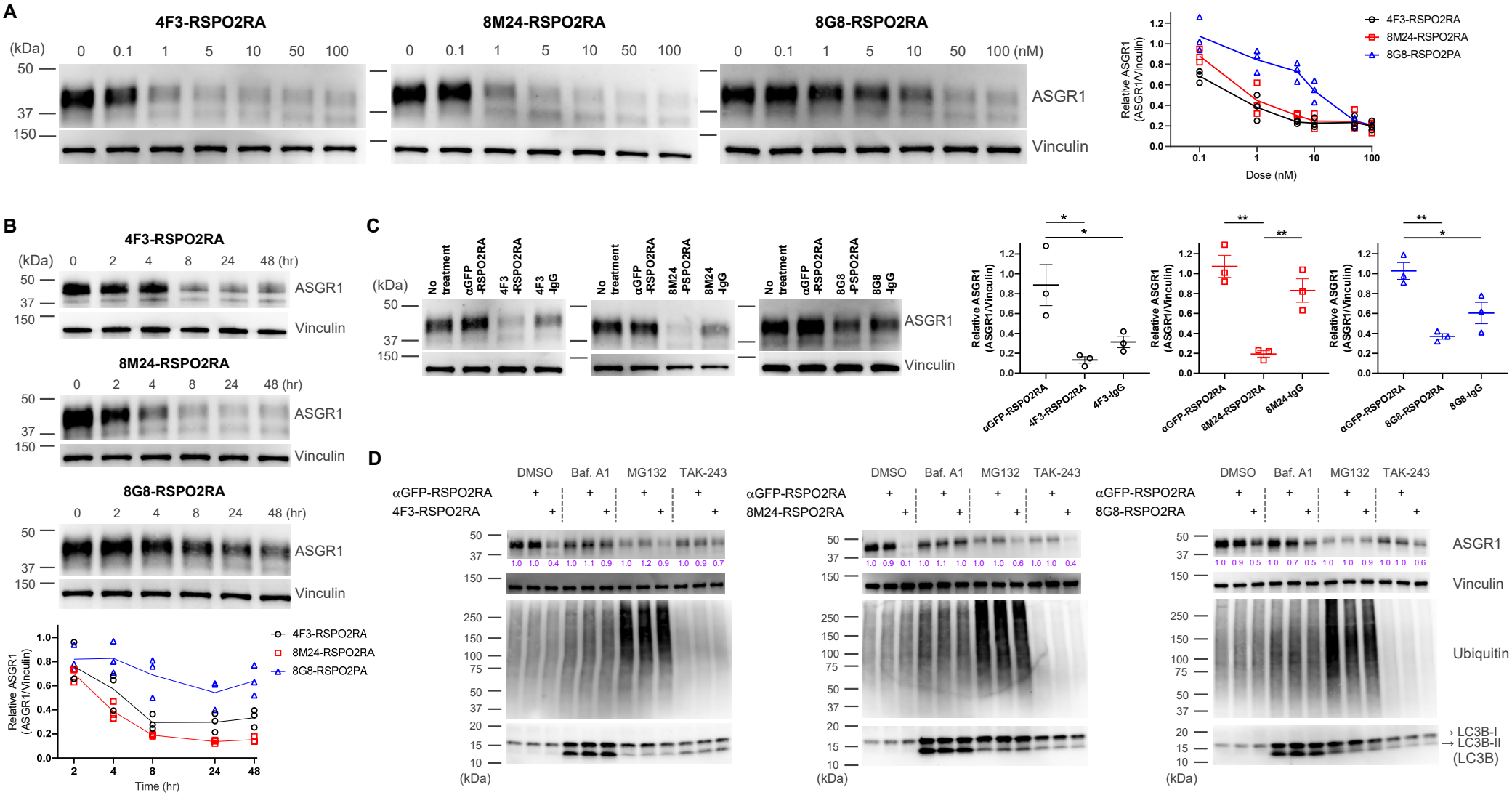
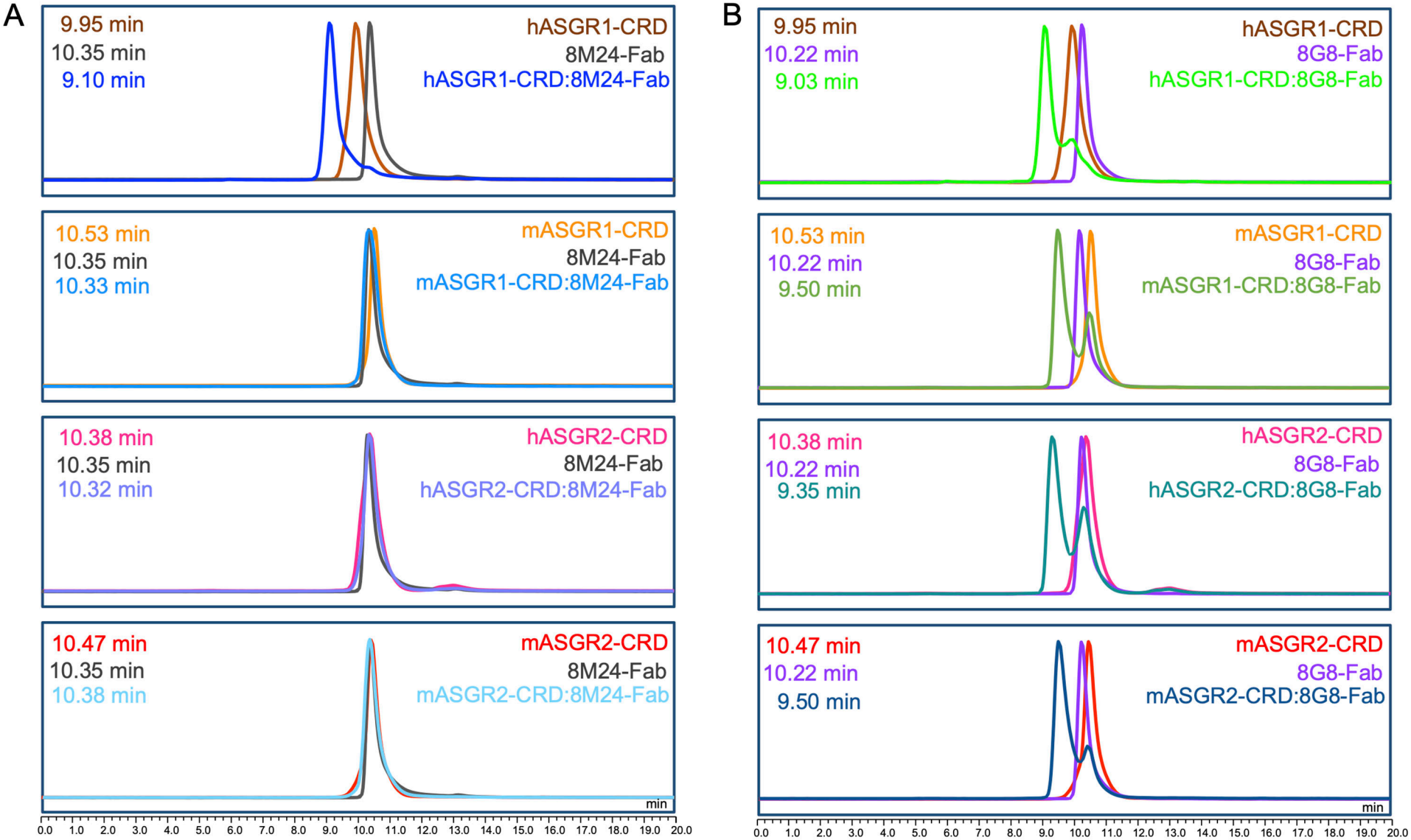
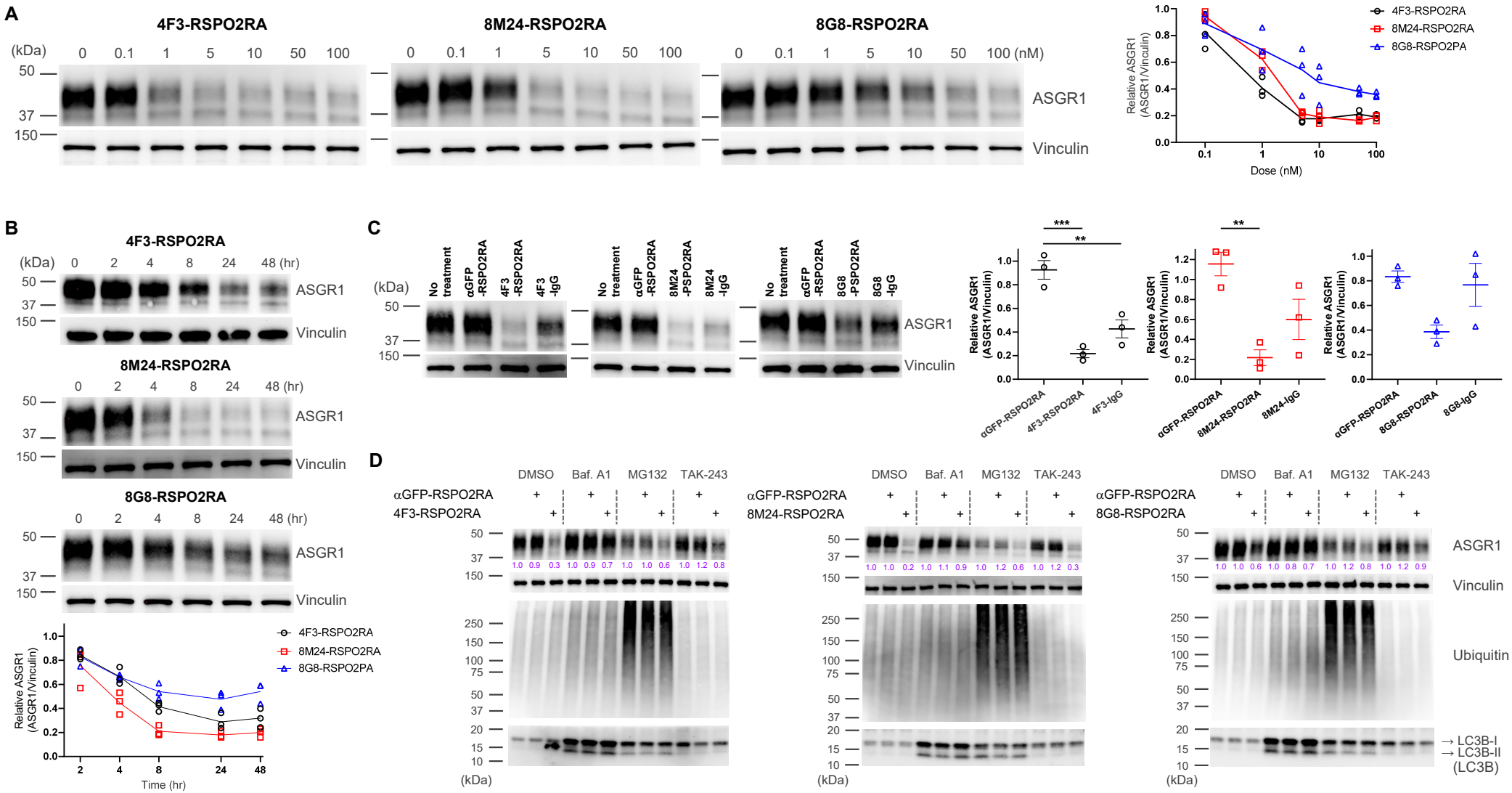


Figure 6


A
4F3-RSPO2RA
8M24-RSPO2RA
8G8-RSPO2RA
(kDa)
0 0.1 1 5 10 50 100
0 0.1 1 5 10 50 100
0 0.1 1 5 10 50 100 (nM)
50
37
150
ASGR1
Vinculin
Relative ASGR1 (ASGR1/Vinculin)
Dose (nM)
4F3-RSPO2RA
8M24-RSPO2RA
8G8-RSPO2PA
B
4F3-RSPO2RA
8M24-RSPO2RA
8G8-RSPO2RA
0 2 4 8 24 48 (hr)
Time (hr)
C
No treatment
αGFP -RSPO2RA
4F3 -RSPO2RA
4F3 -IgG
8M24 -PSPO2RA
8M24 -IgG
8G8 -PSPO2RA
8G8 -IgG
αGFP-RSPO2RA
4F3-IgG
8M24-IgG
8G8-IgG
D
DMSO
Baf. A1
MG132
TAK-243
αGFP-RSPO2RA
4F3-RSPO2RA
8M24-RSPO2RA
8G8-RSPO2RA
Ubiquitin
LC3B-I
LC3B-II
(LC3B)

Supplementary Figure 1



Supplementary Figure 2