An intact C-terminal end of albumin is required for its long half-life in humans

Jeannette Nilsen1,2, Esben Trabjerg3, Algirdas Grevys1,2, Claudia Azevedo4, Stephen O. Brennan5, Maria Stensland1,6, John Wilson7, Kine Marita Knudsen Sand1,8, Malin Bern1,2, Bjørn Dalhus9, Derry C. Roopenian7, Inger Sandlie1,8, Kasper Dyrberg Rand10 & Jan Terje Andersen1,2

Albumin has an average plasma half-life of three weeks and is thus an attractive carrier to improve the pharmacokinetics of fused therapeutics. The half-life is regulated by FcRn, a cellular receptor that protects against intracellular degradation. To tailor-design the therapeutic use of albumin, it is crucial to understand how structural alterations in albumin affect FcRn binding and transport properties. In the blood, the last C-terminal residue (L585) of albumin may be enzymatically cleaved. Here we demonstrate that removal of the L585 residue causes structural stabilization in regions of the principal FcRn binding domain and reduces receptor binding. In line with this, a short half-life of only 3.5 days was measured for cleaved albumin lacking L585 in a patient with acute pancreatitis. Thus, we reveal the structural requirement of an intact C-terminal end of albumin for a long plasma half-life, which has implications for design of albumin-based therapeutics.
Albumin acts as a versatile carrier of a wide range of substances, such as fatty acids, steroids, thyroid hormones, and metals, and plays an important role in stabilizing the extracellular fluid volume. It is expressed by hepatocytes in the liver as a single polypeptide of 585 amino acids, and consists of 67% α-helices, which fold into three homologous domains denoted DI, DII, and DIII. In the blood, it is the most abundant protein (40 g/L), with a long plasma half-life of ~3 weeks in humans.

Albumin is of interest for the pharmaceutical industry because of its ability to bind a wide range of small chemical drugs that consequently affect their pharmacokinetics. In addition, it is increasingly utilized to improve the pharmacokinetics of short-lived bioactive therapeutic peptides and proteins by genetic fusion of such molecules to the N- or C-terminal end. Two human serum albumin (HSA) fused drugs have been approved for clinical use; Tanzeum® and Idelvion®, which contain glucagon-like peptide 1 and recombinant coagulation factor IX, respectively. Both drugs are genetically fused to the N-terminal of HSA, which prolongs the half-lives of both proteins considerably shortened in the cellular studies. As a consequence, this mechanism is based on strict pH-dependent binding between albumin and FcRn, which is not easily explained by available co-crystal structures, such as fatty acids, steroids, thyroid hormones, and metals, and plays an important role in stabilizing the extracellular fluid volume. It is expressed by hepatocytes in the liver as a single polypeptide of 585 amino acids, and consists of 67% α-helices, which fold into three homologous domains denoted DI, DII, and DIII. In the blood, it is the most abundant protein (40 g/L), with a long plasma half-life of ~3 weeks in humans.

The biological mechanism responsible for its persistence in blood was revealed when Chaudhury et al. discovered that albumin binds a cellular receptor, namely the neonatal Fc receptor (FcRn). At the time, FcRn was already known to bind antibodies of the IgG class and play a key role in controlling their homeostasis. The discovery that FcRn is a key controller of albumin homeostasis opens new possibilities for rational design of albumin-based therapeutics, such as to tailor the half-life of drugs by engineering the interaction. One of the first examples of such engineering is an HSA variant with a single-point mutation in DIII (K573P), which improves binding to hFcRn by more than tenfold and extends the plasma half-life from 5.4 to 8.8 days in cynomolgus monkeys. The variant is one of a panel of engineered HSA variants with altered pharmacokinetics that together constitute the so-called Veltis® technology.

To further optimize the therapeutic use of albumin, it is crucial to fully understand how structural alterations and fusion to albumin affects its ability to bind and to be transported by FcRn. In this study, we demonstrate that truncations and amino acid substitutions in the last C-terminal α-helix of albumin strongly reduce binding to the receptor, where even removal of the last residue (L585) has a negative effect on FcRn binding. HDX-MS analysis shows that deletion of L585 causes structural stabilization in regions of the C-terminal DIII that otherwise require flexibility to allow optimal binding. Furthermore, lack of L585 resulted in reduced rescue from intracellular degradation and a shorter serum half-life in hFcRn transgenic mice. In agreement, we found that the truncated variant was quickly eliminated from the blood of a patient diagnosed with acute gallstone-induced pancreatitis and measured a half-life of 3.5 days only. Moreover, exposure to carboxypeptidase A, an enzyme found at elevated levels in plasma of individuals with pancreatitis, resulted in cleavage of L585 from the C-terminal end of HSA, also in the approved drug Tanzeum®, and consequently reduced hFcRn binding. Thus, we provide evidence for the requirement of a structurally intact C-terminal end of HSA to maintain optimal binding to hFcRn and a long plasma half-life, which should guide design of the next generation of albumin-based therapeutics.

**Results**

C-terminal modified HSA variants show reduced FcRn binding. The three homologous domains (DI, DII, and DIII) of albumin are connected by flexible loops and form a heart-shaped structure, where DIII harbors the principal binding site for FcRn. Interestingly, we have observed that introducing mutations in the last C-terminal helix of DIII affects binding to hFcRn, which is not easily explained by available co-crystal structural models. We became aware of four naturally occurring HSA variants with alterations in this C-terminal region, which are named Bazzano, Catania, Rugby Park, and Venezia. Bazzano and Catania are products of a single-nucleotide deletion and a subsequent frameshift, resulting in truncated variants lacking three amino acids and with replacement of the last 19 or 6 C-terminal amino acid residues, respectively. Rugby Park and Venezia are truncated by seven amino acids, and have their C-terminal ends altered from position 572, due to a splice error in intron 13 (Rugby park) or a 30-bp deletion and a 5-bp insertion resulting in skipping of exon 14 (Venezia).

We produced recombinant versions of the variants and observed only minor differences in expression levels in comparison with WT HSA (Fig. 1b, c). To measure receptor binding, surface plasmon resonance (SPR) measurements were conducted by immobilizing the HSA variants followed by injecting titrated amounts of monomeric hFcRn at pH 5.5. Dissociation constants (Kd) were calculated by fitting the sensorgrams to a simple 1:1 Langmuir binding model or a steady-state affinity-binding model. All four variants bound the receptor less well than the WT, where Catania and Venezia showed a 6.6- and 7.6-fold
decrease in affinity, respectively, while Bazzano and Rugby Park bound with 9–10-fold weaker affinity (Fig. 1d–h, Table 1).

As an attempt to explain the effects of the naturally occurring C-terminal alterations on receptor binding, we scrutinized a co-crystal structural model of WT HSA in complex with hFcRn (Fig. 1i). None of the residues of the last C-terminal α-helix of HSA contact the receptor, but several of them form intramolecular interactions with the second last α-helix (Fig. 1j). Both α-helices are part of the hydrophobic pocket that accommodates W53 of FcRn, where the second last α-helix contains residues (e.g., F551) that form hydrophobic interactions with the tryptophan. Thus, truncating and substituting amino acid residues of the last C-terminal α-helix, as in the mutated variants, probably disrupt some of the helix–helix interactions and consequently the binding pocket, resulting in the negative effect on hFcRn binding.

**L585 is required for optimal FcRn binding.** Survey of albumin sequences from 20 species indicates that all lack the C-terminal L585 found in albumin from orangutan, chimpanzee, and human (Fig. 2a). Moreover, clinical data demonstrate that 4–15% of circulating albumin in humans also lack this residue. To address the effect of loss of L585, we expressed a truncated recombinant version of HSA (L585X) (Fig. 2b). Circular dichroism (CD) analysis at 25 °C and pH 5.5 showed very similar ellipticity spectra as that of the WT (Fig. 2c). Binding to hFcRn at pH 5.5 and pH 7.4 was first compared by ELISA, where the receptor was captured onto a mutated hIgG1 variant that binds strongly to FcRn at both pH conditions. The HSA variants were

---

**Table 1 SPR-derived kinetics for binding of HSA variants to hFcRn.**

| HSA variant | $k_a$ (10$^4$ M$^{-1}$ s$^{-1}$) | $k_d$ (10$^{-2}$ s$^{-1}$) | $K_{Dc}$ (μM) | $K_{Dc}$ (μM) |
|-------------|-----------------|-----------------|-----------------|-----------------|
| WT          | 4.8             | 3.3             | 0.7             | 1.1             |
| L585X       | 1.5             | 5.6             | 3.7             | 5.3             |
| Bazzano     | NA              | NA              | NA              | 10.0            |
| Catania     | NA              | NA              | NA              | 7.3             |
| Rugby Park  | NA              | NA              | NA              | 11.3            |
| Venezia     | NA              | NA              | NA              | 8.4             |

*The HSA variants were immobilized on CM5 chips and serial dilutions of hFcRn were injected at pH 5.5.

The kinetic rate constants were obtained using a simple first-order (1f) Langmuir bimolecular interaction model.

The steady state affinity constant was obtained using an equilibrium (Res) binding model.

NA, not acquired due to fast kinetics.

---

**Fig. 1 Genetic HSA variants with modified C-terminal ends show reduced FcRn binding.** a An overview of the C-terminal sequences of genetic HSA variants. b A 12% SDS-PAGE gel stained with Coomassie Blue showing 2 μg of purified recombinant WT HSA and genetic variants. The gel was cropped and the full-length gel is shown in Supplementary Fig. 1. c Relative expression levels of WT HSA and genetic variants by transiently transfected HEK293E cells. Protein secreted into the growth medium was quantified by ELISA, and the values represent the mean ± s.d. of four replicates. d SPR sensograms showing binding of titrated amounts (0, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 μM) of monomeric hFcRn injected over immobilized (~200 RU) WT HSA at pH 5.5. e–h SPR sensograms showing binding of titrated amounts (0, 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 μM) of monomeric hFcRn injected over immobilized (~500 RU) WT HSA and genetic variants. The gel was cropped by ELISA, where the values represent the mean ± s.d. of four replicates.

---

**ARTICLE**
The rate at which backbone amides exchange their protons with deuterium from solvent provides information about the conformational dynamics of proteins, as the rate of exchange of backbone amides is primarily determined by the stability of local formational dynamics of proteins, as the rate of exchange of backbone amides is primarily determined by the stability of local backbones amides. Furthermore, backbone amides are heavily structured region, such as in stable α-helices and β-strands, to a backbone amide in less structured or disordered regions. The exchange rate can vary up to seven orders of magnitude by lowering the temperature and pH. The quenched samples were digested by pepsi, before the mass of the resulting peptides were determined by liquid chromatography–mass spectrometry (LC–MS). Deuterium incorporation for 77 peptides was followed for both the WT and the truncated variant, covering 91% of the sequence (Supplementary Figs. 3 and 4). Most peptide segments of HSA did not display a difference in HDX between WT and L585X (Fig. 3e; Supplementary Fig. 3). Also, no difference was observed for the peptides corresponding to the last part of the C-terminal α-helix, when correcting for the difference in the “experimental maximally” labeled samples (Fig. 3a). However, the HDX differed in three other regions in DIII; residues 409–420 (Fig. 3b), 496–506 (Fig. 3c), and 530–550 (Fig. 3d), which are spatially in close proximity to the C-terminal end in crystal structures of HSA alone as well as when in complex with hFcRn (Fig. 3f, g). The significance of these reductions in HDX was further supported in the data from several overlapping peptides comprising these regions of DIII (Supplementary Fig. 3). Interestingly, for all three identified regions, the rate of HDX was slower for L585X, which suggests that these regions are less dynamic in the truncated variant. Thus, deletion of L585 causes local structural stabilization in distinct regions of DIII.

**Deletion of L585 causes structural stabilization in DIII.** As the HSA-hFcRn co-crystal structure does not directly explain the effect of removing L585, we studied the structural dynamics of HSA with and without the C-terminal leucine using hydrogen/deuterium exchange mass spectrometry (HDX-MS) at pH 5.5. The rate at which backbone amides exchange their protons with deuterium from solvent provides information about the conformational dynamics of proteins, as the rate of exchange of backbone amides is primarily determined by the stability of local hydrogen bonding. The exchange rate can vary up to seven orders of magnitude when comparing a backbone amide in a heavily structured region, such as in stable α-helices and β-strands, to a backbone amide in less structured or disordered regions (non-hydrogen bonded). Briefly, HDX was initiated by diluting WT HSA or L585X into deuterated buffer. The exchange reaction was stopped at various time points from 15 s up to 2960 min (i.e., sampled across a time range of approximately four orders of magnitude) by lowering the temperature and pH. The quenched samples were digested by pepsi, before the mass of the resulting peptides were determined by liquid chromatography–mass spectrometry (LC–MS). Deuterium incorporation for 77 peptides was followed for both the WT and the truncated variant, covering 91% of the sequence (Supplementary Figs. 3 and 4). Most peptide segments of HSA did not display a difference in HDX between WT and L585X (Fig. 3e; Supplementary Fig. 3). Also, no difference was observed for the peptides corresponding to the last part of the C-terminal α-helix, when correcting for the difference in the “experimental maximally” labeled samples (Fig. 3a). However, the HDX differed in three other regions in DIII; residues 409–420 (Fig. 3b), 496–506 (Fig. 3c), and 530–550 (Fig. 3d), which are spatially in close proximity to the C-terminal end in crystal structures of HSA alone as well as when in complex with hFcRn (Fig. 3f, g). The significance of these reductions in HDX was further supported in the data from several overlapping peptides comprising these regions of DIII (Supplementary Fig. 3). Interestingly, for all three identified regions, the rate of HDX was slower for L585X, which suggests that these regions are less dynamic in the truncated variant. Thus, deletion of L585 causes local structural stabilization in distinct regions of DIII.

**HSA lacking L585 is more rapidly degraded.** To study the consequence of lack of L585 on FcRn-mediated recycling, we used the newly developed human endothelial cell-based recycling assay (HERA). Briefly, human microvascular endothelial cells (HMEC1) that overexpress hFcRn were grown in wells until 95–100% confluency. The cells were starved for 1 h, before equal amounts of WT HSA or L585X were added and incubated for 4 h to allow uptake. The cells were washed, and fresh pH 7.4 buffer was added. After another 4 h incubation, the amount of HSA released from the cells was quantified using ELISA, which revealed that only half the amount of the truncated variant was recycled compared with full-length HSA (Fig. 4a).
**Fig. 3 HDX-MS analysis shows structural stabilization in three regions of HSA lacking L585.** a–e Deuterium uptake plots for peptide segments of WT HSA (black) and L585X (red) of (a) the C-terminal peptides (i.e., with or without L585), b–d the peptide regions that showed a statistically significant difference in deuterium incorporation and (e) a peptide that showed no difference. The values were normalized to the deuterium uptake of the corresponding peptide in the “maximally labeled” control sample. The data points represent the mean ± s.d. of triplicates, except for 2960 min, where n = 1. *Marks a statistically significant change in HDX as defined in the “Methods” section. f, g The C-terminal peptide is highlighted in green, whereas the structural regions stabilized by removal of L585 are highlighted in orange (409–420), red (496–506), and dark blue (530–550) on the crystal structures of HSA (DI; pink, DII; wheat, DIII; light cyan) (f) alone and (g) in complex with hFcRn (HC; light green, β2m; gray). The regions that were not covered in the HDX data are colored in black. The figures were made using PyMOL with the crystallographic data of HSA (PDB ID 1AO6) and the WT HSA-hFcRn co-complex (PDB ID 4N0F)26.

---

**Fig. 4 HSA lacking L585 shows decreased cellular recycling and serum half-life in mice.** a HERA showing relative cellular recycling of WT HSA and L585X. Equal amounts of each variant (1000 nM) were incubated with HMEC1 cells for 4 h, followed by extensive washing and another 4 h incubation step before sample collection. The amounts of recycled WT HSA and L585X were quantified by ELISA, and the obtained data are shown as mean ± s.d. of three independent experiments where n = 3. ****p < 0.0001, by unpaired Student’s t test. b, c Elimination curves of WT HSA (open circle) and L585X (filled circle) in hFcRn transgenic mice. Tg32-Alb−/− mice received 4 mg/kg HSA via intraperitoneal injection (IP) on day 0 (n = 4) (b). Tg32 mice received 2 mg/kg HSA via intravenous injection (IV) on day 0 (n = 5) (c). The serum levels of HSA are presented as percentage remaining in the circulation compared to day 1. The curves represent the mean ± s.d of four or five mice. Mean β-phase half-lives (t1/2) ± s.e.m are shown in days. *p = 0.0245, **p = 0.0023, by unpaired Student’s t test.
Next, we measured the serum half-life of HSA with and without L585 in two humanized mouse models, the so-called Tg32 and Tg32-Alb−/− mouse strains. While both strains express hFcRn instead of the mouse receptor, the latter also lacks expression of mouse albumin. In the albumin knockout mice (Tg32-Alb−/−), a serum half-life of about 14 days was measured for L585X as compared with 18 days for the WT (Fig. 4b). In the presence of high levels of endogenous albumin (Tg32), the truncated variant was eliminated even more rapidly relative to full-length HSA, showing 1.7-fold shorter serum half-life (decreased from 2.7 to 1.6 days) (Fig. 4c). Notably, both variants showed monophasic log-linear decay curves, supporting the lack of immunogenicity and of an antibody response, even in the presence of large amounts of competing endogenous albumin. The transgenic mice used have low levels of immunogenicity and of an antibody response, even in the presence of large amounts of competing endogenous albumin. Thus, C-terminal truncation leads to less efficient cellular recycling and reduced serum half-life as a result of altered FcRn-binding kinetics. HSA lacking L585, which binds hFcRn with fivefold weaker affinity due to a slower on-rate and a faster off-rate, is thus less likely to bind the receptor in the endosomes, especially in the presence of large amounts of competing endogenous albumin, and instead follow the default pathway to the lysosomes.

Truncated HSA lacking L585 has a short half-life in humans. Patients with pancreatitis have elevated pancreatic protease activity in the circulation and as a result, the level of HSA lacking L585 may increase dramatically. We collected six blood samples from a patient with acute gallstone induced pancreatitis over a time period of 104 h, starting at the time the patient was admitted to the hospital. To monitor the condition, we measured the plasma activity of amylase and lipase (Fig. 5a), which are commonly used diagnostic markers of pancreatitis. Plasma samples taken the first day showed markedly elevated activity of both amylase (974–1023 U/I) and lipase (7880–7926 U/I), which had decreased substantially by the 30 h blood sample (291 U/I and 714 U/I, respectively), supporting that the pancreas was recovering. Over the 104 h time period, the total serum albumin concentration decreased slightly from 40.9 g/L to 35.9 g/L (Supplementary Fig. 5a). The mass and heterogeneity of purified HSA were assessed using LC–ESI–MS, which showed that a constrained molecular weight was reduced from 66 kDa to 60 kDa. The truncated variant was isolated from a healthy individual in which L585 accounted for ~3% of the total serum albumin (Supplementary Fig. 5c). As measured for the recombinant version, plasma-derived L585X showed reduced hFcRn binding compared with full-length HSA (Fig. 5c, d).

Carboxypeptidase A (CPA), also referred to as CPA1, is a pancreatic protease that

Fig. 5 Plasma-derived L585X shows reduced binding to hFcRn and a short serum half-life in a patient with acute pancreatitis. a, b Plasma activity of amylase (upward triangle) and lipase (downward triangle) (a) and the percentage of L585X (b) over time in blood of a patient with acute pancreatitis. The percentage of L585X was calculated from the mass spectra on the total HSA (Supplementary Fig. 5). c ELISA showing binding of equal amounts (225 nM) of WT HSA and plasma-derived L585X to hFcRn at pH 5.5. d SPR sensorgrams showing binding of equal amounts (2000 RU) of plasma-derived WT HSA and L585X injected over immobilized (~2000 RU) hFcRn at pH 5.5. Injections were performed at 25 °C, and the flow rate was 50 μl/min.

Fig. 6 HSA exposed to CPA shows reduced hFcRn binding. a Relative hFcRn binding at pH 5.5 of HSA variants exposed (striped bars) or nonexposed (white bars) to CPA. Calculated from ELISA results in (b) and in Supplementary Fig. 6, where nonexposed was set to 1. The data are shown as the mean ± s. d. of three independent experiments where n = 2. b ELISA showing binding to hFcRn at pH 5.5 of WT HSA (circle) and K573P (triangle) that had been exposed (filled black) or not exposed (filled white) to CPA.
cleaves aromatic and aliphatic amino acid residues from the C-terminal of proteins. It is the enzyme predicted to cleave the C-terminal L585 of HSA. This is supported by increased serum concentrations of CPA observed in parallel with elevated serum levels of L585X in patients with pancreatitis. We incubated WT HSA with and without CPA purified from human pancreas at 37 °C for 5 h followed by MS analysis, which confirmed that exposure to the enzyme generated a product without L585 (Supplementary Table 1). The protocol was repeated with the commercially available N-terminal HSA-drug fusion, Tanzeum. After exposure to CPA, more than 99% of the C-terminal peptides detected were lacking L585, whereas the leucine was present in the nonexposed sample (Supplementary Table 1). In addition, we included the engineered variant, HSA K573P, which has improved pH-dependent binding affinity for hFcRn and extended half-life in nonhuman primates. Incubation with CPA resulted in complete removal of L585 from the C-terminal end of the engineered variant (Supplementary Table 1). The effect of losing L585 on hFcRn binding was then evaluated by ELISA, which demonstrated weaker binding of the enzyme-exposed HSA and fusion construct as compared with the nonexposed variants (Fig. 6a; Supplementary Fig. 6). The same was observed for HSA K573P, however, the cleaved version still bound more strongly compared with non–cleaved WT HSA (Fig. 6a, b). Thus, CPA cleaves the C-terminal L585 of HSA and causes reduced FcRn binding.

**Discussion**

Albumin is the most abundant protein in blood and plays a key role as carrier of primarily small hydrophobic and insoluble molecules throughout the body. To take advantage of its long plasma half-life and improve in vivo efficacy of drugs, one can either target drugs to HSA via albumin-binding molecules, use site-specific conjugation to C34 of HSA or genetically fuse proteins of interest to the N- or C-terminal end of HSA. In this context, knowledge about how FcRn binds and controls the plasma half-life and transport of albumin, as well as how structural alterations and fusion to albumin affect these properties, will improve design of novel albumin-based therapeutics with tailored pharmacokinetics.

We have previously reported that the C-terminal domain (DIII) of HSA contains the main binding site for hFcRn, and that DI modulates the interaction. More than 70 genetic variants of HSA have been identified. Here, we studied the Bazzano, Catania, Rugby Park, and Venezia variants, which have amino acid substitutions and truncations in the C-terminal end. The variants were first identified during routine clinical electrophoresis run on serum from heterozygous carriers of one normal and one mutated albumin gene, in which the variants are present at lower levels than the normal protein. Thus, they are secreted into the circulation, but it is not known whether the genetic modifications affect the rate of expression and secretion by liver cells. Furthermore, CD analysis has demonstrated that the modifications in the C-terminal end increase or decrease the overall α-helical content and thermal stability. In this study, we found that all four variants have reduced binding affinity toward hFcRn. The weakest binding was measured for Rugby Park, which is present at only 8% of the total serum albumin, followed by Bazzano, Venezia, and Catania representing 18%, 30%, and 30%, respectively. Thus, the FcRn-binding affinity of the variants correlate with their serum level in heterozygous carriers.

Furthermore, we demonstrate that a fully intact C-terminal end of HSA is required for optimal binding to hFcRn, as removal of the very last L585 residue resulted in more than fivefold weaker affinity due to slower association and faster dissociation. HDX-MS data support that differences in structural dynamics contribute to reduced binding of the truncated variant, as deletion of the leucine led to structural stabilization of three regions in DIII, that have been reported to undergo large conformational changes upon receptor binding (up to 19 Å in Ca–Ca distances). The regions are located in or in close proximity to the interaction interface in the co-crystal structure, and it has previously been shown that targeted mutagenesis within these segments affect receptor binding. The data support that the presence of L585 causes a functionally important local destabilization and structural flexibility within DIII that is in favor of hFcRn binding.

From an evolutionary perspective, it is interesting that the C-terminal leucine residue is not conserved, as it is only expressed by human, chimpanzee, and orangutan. These are also the only species that express albumin with a lysine instead of a proline in position 573, which also has great influence on FcRn binding. Thus, in regard to binding affinity for FcRn, the L585 residue compensates for the lack of P573 in HSA. Since transporting cargo is a main function, we speculate that different requirements to albumin in this regard may have been the driving force resulting in these species differences. Notably, the leucine influences the structural dynamics of DIII of HSA, it may well be that its presence also affects the ligand binding properties of the domain.

Interestingly, HSA that lacks the last C-terminal L585 residue is present in the blood of healthy individuals, where it has been measured to constitute 4–15% of the circulating albumin pool. In line with this, we found that ~3% of the total albumin in plasma from a healthy control lacked the leucine. Like the recombinant version, reduced binding to hFcRn was measured for L585X isolated from human plasma. As such, reduced receptor binding provides an explanation for its short in vivo half-life of 3.5 days. This was measured in a patient with acute gallstone-induced pancreatitis, which is a condition where the pancreatic duct that connects the pancreas to the small intestine is blocked, leading to premature activation of pancreatic enzymes that attack the pancreas. At most, we found that 60% of the total albumin was lacking L585, which is in line with previous reports showing levels ranging from 17 to 93% in patients suffering from pancreatic disease. The truncated form is most likely a result of exposure to CPA, which is synthesized by the pancreas and normally released as a zymogen into the gut, but found at elevated levels in plasma of individuals with pancreatitis. Similarly, a tenfold increase in the plasma concentration of CPA, and a high percentage of circulating L585X, has been measured in a severely traumatized patient. We speculate whether cleavage of L585 may be a way to promote the clearance of albumin that carries metabolic waste products that accumulate during acute disease. Interestingly, as the leucine is not conserved, this would not necessarily apply to all species. Future studies should address whether various ligands whose binding induce conformational changes in HSA, could make the protein more or less susceptible to enzymatic cleavage. Moreover, as the basal level of the activated form of CPA in plasma is very low, this raises questions as to where and when HSA is cleaved under normal conditions.

Our findings also have implications for HSA-based therapeutics with a free C-terminal end, as exemplified here with a N-terminal drug fusion approved for clinical use. Incubation of the drug with CPA resulted in reduced binding to hFcRn due to loss of L585. Thus, exposure to the enzyme in vivo will affect the pharmacokinetics. Instead, genetic fusion of a drug of interest to the C-terminal of HSA may be favorable to prevent enzymatic cleavage, as long as the drug does not represent a substrate for
CPA itself or compromise hFcRn binding. We previously demonstrated that genetic fusion of a small peptide or a single-chain-variable fragment to both the N- or C-terminal end of HSA slightly decreased binding to hFcRn. Thus, it is crucial to address whether or not a particular fusion of interest interferes with binding to hFcRn, as it may vary depending on the nature of the fused protein. Importantly, to secure long serum half-life, engineered HSA variants with improved binding to hFcRn, such as the K573P variant, may be used to compensate for diminished receptor binding caused by C-terminal truncation or design.

Methods

**Construction and production of HSA variants.** A pcDNA3.1 vector containing cDNA-constructs encodes for full-length HSA has been described previously and cDNA fragments encoding modified DIII sequence (K573P, L585X, Bazzano; (567)) of the hFcRn HC genetically fused to the C-terminal end of the fused protein. Importantly, to secure long serum half-life, engineered HSA variants with improved binding to hFcRn, such as the K573P variant, may be used to compensate for diminished receptor binding caused by C-terminal truncation or design.

**Production of recombinant hFcRn.** A vector containing cDNA that encodes for the three ectodomains (α1–α3) of the hFcRn HC genetically fused to the Schistosoma japonicum glutathione S-transferase (GST) and the human β-subunit was used. Soluble GST-tagged hFcRn was expressed by transient transfection HEK293E cells as described above, and purified using a GSTrap FF column (GE Healthcare). Briefly, the column was pre-equilibrated with 1× PBS/0.05% sodium azide, before the supernatant was applied at a flow rate of 1-2 ml/min. The column was washed with 100 ml 1× PBS/0.05% sodium azide, before the bound protein was eluted with 50 ml 0.1 M Glycine-HCl (pH 3.0) and neutralized with 1 M Tris-HCl (pH 8.0). The collected protein was up-concentrated and buffer-exchanged to 1× PBS using Amicon Ultra-15 10 K columns (Millipore). Size-exclusion chromatography was performed using a Superdex 200 increase 10/300 GL column (GE Healthcare). The monomeric fraction was collected and up-concentrated using Amicon Ultra-0.5 30 K columns (Millipore). Protein concentrations were determined using a DS-11 spectrofluorometer (Novex) and distilled H2O. The samples were loaded onto a 12% Bis-Tris NuPAGE SDS-PAGE gel in parallel with SpectraTM Multicolor Broad Range Protein Ladder (Thermo Fisher), and separated at 200 V for 22 min in MES buffer, followed by staining with Coomassie Brillant Blue (Bio-Rad).

**HDX-MS.** All reagents were purchased from Sigma-Aldrich in analytical grade, except for the immobilized pepsin beads (Thermo Scientific). The exchange reaction was initiated by diluting HSA variants (20 pmol) 1:9 with 99% D2O (111.2 mM Na2HPO4, 44 mM citric acid, pH 5.5) over immobilized HSA with a flow rate of 10 or 40 μl/min at 25 °C. Relative binding of serum-derived HSA (WT HSA and L585X) was measured by injecting equal amounts (1 μM) over immobilized hFcRn at a flow rate of 50 μl/min and temperature of 25 °C. Phosphate buffer (67 mM phosphate buffer, 0.16 mM HEPES, 0.15 M NaCl, 0.05% surfactant P20) at pH 7.4 was used as running and dilution buffer, while HEPES buffer (0.01 M HEPES, 0.15 M NaCl, 0.005% surfactant P20) at pH 7.4 was used for regeneration of the flow cells. All binding curves were zero adjusted, and the reference cell value was subtracted. Binding kinetics and affinity values were estimated using the Langmuir 1:1 ligand binding model or an equilibrium (Req) binding model provided by the BIACore Evaluation 4.1 software or Biacore T200 Evaluation Software, version 3.0.

**HDX-MS.** All reagents were purchased from Sigma-Aldrich in analytical grade, except for the immobilized pepsin beads (Thermo Scientific). The exchange reaction was initiated by diluting HSA variants (20 pmol) 1:9 with 99% D2O (111.2 mM Na2HPO4, 44 mM citric acid, pH 5.5) over immobilized HSA with a flow rate of 10 or 40 μl/min at 25 °C. Relative binding of serum-derived HSA (WT HSA and L585X) was measured by injecting equal amounts (1 μM) over immobilized hFcRn at a flow rate of 50 μl/min and temperature of 25 °C. Phosphate buffer (67 mM phosphate buffer, 0.16 mM HEPES, 0.15 M NaCl, 0.05% surfactant P20) at pH 7.4 was used as running and dilution buffer, while HEPES buffer (0.01 M HEPES, 0.15 M NaCl, 0.005% surfactant P20) at pH 7.4 was used for regeneration of the flow cells. All binding curves were zero adjusted, and the reference cell value was subtracted. Binding kinetics and affinity values were estimated using the Langmuir 1:1 ligand binding model or an equilibrium (Req) binding model provided by the BIACore Evaluation 4.1 software or Biacore T200 Evaluation Software, version 3.0.

**CD spectroscopy.** CD spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco International). Measurements were performed on WT HSA and L585X (150 μg/ml) in 10 mM PBS (pH 5.5) without NaCl at 20 °C using a quartz cuvette (Starna) with a path length of 0.1 cm. Each sample was scanned five times at 20 nm/minute (bandwidth of 1 nm, response time of 1 s) with wavelength range set to 190–260 nm. The data were averaged, and the spectrum of a sample-free control was subtracted.
reduced samples by tandem MS using both data-dependent and data-independent acquisitions methods.

Locally during radiolabelling with Glu−fibrinopeptide B was applied to all recorded spectra. To perform peptide identifications, the data were analyzed in PLGS 3.0, which correlate precursor- and fragment ions to a local database containing the sequence of WT HSA, pepsin, and randomized sequences of these two proteins as well. To determine the deuterium uptake for each peptide, the HDX-MS data were analyzed using the software Dynamic 3.0 (Waters Inc.). To allow access to the HDX data of this study, the HDX Data Summary Table (Supplementary Table 2) and the HDX Data Table (Supplementary Data 1) are included in the Supporting Information according to the community-based recommendations.

HERA. In total, 7.5 × 10^5 HMEC1 cells stably expressing HA-hFcRn-RFP69 were seeded into 24-well plates per well (Costar) and cultured for 1 day in growth medium. After washing twice with PBS, cells were incubated for 1 h in Hank’s balanced salt solution (HBSS) (Life Technologies). WT HSA or L585X (100 nM) diluted in 250 μl HBSS (pH 7.4) were added per well and incubated for 4 h. The medium was removed, and the cells were washed five times with ice-cold HBSS (pH 7.4). Growth medium supplemented with MÉM nonessential amino acids (Thermo Fisher) and penicillin for 24 h after 4 h. To quantify the amount of HSA in the samples, a two-way anti-HSA ELISA was used as described below.

Mouse studies. Two hFcRn transgenic mouse strains were used in the study: Hemizygous Tg32 mice (B6.Cg-Fcgrtm1Def/J Tg(CFGR7)32Dcr Dcr/J; The Jackson Laboratory, Bar Harbor, ME) and homozygous Tg32-Alb−/− albumin deficient mice (B6.Cg-Alb−/− Fcgrtm1Def/J Tg(CFGR7)32DcrDcr/J; The Jackson Laboratory, Bar Harbor, ME). Mice (male, 11–16 weeks, 20–25 g, five mice/group) received WT HSA or L585X (4 mg/kg) in 20 ml/kg 1× PBS by intraperitoneal injection, and blood (25 μl) was drawn from the retro-orbital sinus at 1, 8, 14, 20, 30, 45, and 58 days post injection. Tg32 mice (male, 7–8 weeks, 20–25 g, five mice/group) received WT HSA or L585X (2 mg/kg) in 10 ml/kg 1× PBS by intravenous injection, and blood (100 μl) was drawn from the retro-orbital sinus at 1, 3, 5, 7, and 10 days post injection. The blood samples were mixed with 1% K3-EDTA and maintained on ice until centrifugation at 17,000×g for 5 min at 4 °C. Plasma was isolated and diluted 1:10 in 50% glycerol/PBS solution and stored at −20 °C.

The animals were carried out at The Jackson Laboratory (Tg32 study by JAX Service, Bar Harbor, ME), and were performed in accordance with guidelines and regulations approved by the Animal Care and Use Committee at The Jackson Laboratory. To quantify the amount of HSA in plasma (diluted 1:200-1:400 in PBSTM), a two-way anti-HSA ELISA was used as described below. The plasma concentration was presented as percentage remaining in the circulation at different time points post injection compared to the concentration on day 1 (100%). The β−half-life was calculated using the formula: \[ t_{1/2} = \frac{\log 0.5}{\log A_0/A_t} \times t, \] where \( t_{1/2} \) is the half-life of the HSA variant evaluated, \( A_0 \) is the amount of HSA remaining, \( A_t \) is the amount of HSA on day 1, and \( t \) is the elapsed time.

Two-way anti-HSA ELISA. Polyclonal goat anti-HSA (1.0 μg/ml) (Sigma-Aldrich) or monoclonal mouse anti-HSA (1.0 μg/ml) (Abcam) diluted in PBSTM were added to 96-well ELISA plates (Costar) and incubated overnight at 4 °C. The wells were blocked with PBSM overnight at 4 °C, and then washed three times with PBSTM. Serial dilutions of HSA (500.0–0.2 ng/ml) in PBSTM were applied in parallel with collected growth medium, HERA samples or plasma, followed by 1 h incubation at RT. The wells were washed three times with PBSTM, before bound HSA was detected using anti-goat IgG antibody conjugated to horseradish-peroxidase (1:10,000) (Bethyl Laboratories, Inc.). The wells were washed three times with PBSTM and developed with p-nitrophenyl-phosphate substrate (10 μg/ml) (Sigma-Aldrich) diluted in diethanolamine buffer. The absorbance was measured at 405 nm using the Sunrise spectrophotometer (TECAN).

ESI-MS and isolation of L585X from human plasma. The patient included in the study experienced a single acute event of gallstone induced pancreatitis. Blood samples were taken upon hospital admission and then analyzed. The samples were analyzed using an ESI-MS coupled to Q Exactive Quadrupole-Orbitrap mass spectrometer (Thermo Fisher) with the EASY Spray PepMap RSLC column (C18, 2 μl, 100 Å, 75 μm × 25 cm) and column temperature at 60 °C. A 60 min gradient and injections of 3 μl were used. Raw files were submitted to MaxQuant software (version 1.6.1.0) for protein identification and rough quantification. Searches were done against the sequences of WT HSA and L585X. Parameters were set as follow: carbamidomethylation as fixed modification, and protein N-acetylation and methionine oxidation as variable modifications. First search error window of 20 ppm and main search error of 4.5 ppm with two allowed miscreations was used. Quantification was done on the peptide level by comparing the intensity of the C-terminal peptide lacking L585 against the intensity of the corresponding WT peptide within the same sample, without correlating for possible differences in ionization efficiency of the single peptides.

Sequence and structural analysis. The albumin sequences of human (AA89977), orangutan (NP_001127106.2), chimpanzee (XP_517233.3), macaque (NP_001182578.1), panda (XM_002928492.3), elephant (AAT90502.1), horse (XP_001075972.1), donkey (AAV28861), cattle (AAAS1411), goat (ACF01391), sheep (NP_001099376), pig (AAAP39881), dog (CAAB46867.1), cat (CAAC59279.1), rabbit (NP_001075813), opossum (XM_001364821.2), hamster (ABRE86005.1), guinea pig (AAQ20888.1), rat (AAH53539.1), and mouse (AAH49971) were downloaded from the National Center for Biotechnology Information. The alignments were made using Clustal software.

Statistics and reproducibility. Statistical analysis of the data obtained by HDX-MS was performed using Excel software (Microsoft). HDX-MS was performed with triplicates of each albumin variant for all time points, except for the 2960 min sample where \( n = 1 \). For data points performed in triplicate, the comparative analyses were performed with either a homoscedastic or a heteroscedastic Student’s t-test, depending on the equality of the variances of the compared data points. The equality of the variances was determined by an F-test with the significance level set to 0.05. A peptide was only considered to have a significant difference in HDX if one of its data points met the following two criteria: (1) a significant difference in deuterium uptake (\( p < 0.05 \)), and (2) the absolute difference in deuterium uptake should be larger than two times the pooled standard deviation (SD)\( \times 2 \times \sqrt{\text{SD}_{\text{pool}}^2 + \text{SD}_{\text{MS/MS}}^2} = 0.13D \)) (Supplementary Fig. 7). For the last (2960 min) time point, for which replicate data were not obtained, statistical significance was determined if the change exceeded a 99% confidence limit (±0.26 D) following the method described by Houde et al. and Arora et al.17,23. This 99% confidence limit was estimated from 385 single measurements of standard deviations from each protein state.

Statistical analysis of the data obtained by HERA and half-life measurements in mice was performed using GraphPad Prism 8 (GraphPad Software Inc.). The HERA experiment was performed three times, where three wells for each albumin variant was included each time. Half-life measurements were performed once in Tg32-Alb−/− mice and once in Tg32 mice, where four and five mice per albumin variant were included, respectively. Statistical significance was assessed by an unpaired Student’s t-test (with 95% confidence level and a two-sided \( p < 0.05 \) defined as a significant difference).
Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Data that support the findings of this study are stored in excel file format and are available from the corresponding author upon reasonable request. Source data underlying graphs presented in the main figures are available in Supplementary Data 1 and 2.

Received: 30 September 2019; Accepted: 17 March 2020;
Published online: 20 April 2020

References
1. Peters, T. Jr., All About Albumin: Biochemistry, Genetics, and Medical Applications (Academic Press, 1995).
2. Curry, S., Mandelkow, H., Brick, P. & Franks, N. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. Nat. Struct. Biol. 5, 827–835 (1998).
3. Sugio, S., Kashima, A., Mochizuki, S., Noda, M. & Kobayashi, K. Crystal structure of human serum albumin at 2.5 Å resolution. Protein Eng. 12, 439–446 (1999).
4. Curry, S. Lessons from the crystallographic analysis of small molecule binding to human serum albumin. Drug Metab. Pharmacokinet. 24, 342–357 (2009).
5. Bern, M., Sand, K. M., Nilsen, J., Sandlie, I. & Andersen, J. T. The role of albumin receptors in regulation of albumin homeostasis: Implications for drug delivery. J. Control Release 211, 144–162 (2015).
6. Rendell, M. S. Albilugitude: a unique GLP-1 receptor agonist. Expert Opin. Biol. Ther. 16, 1557–1569 (2016).
7. Santagostino, E. Transforming the treatment for hemophilia B patients: update on the clinical development of recombinant fusion protein linking recombinant coagulation factor IX with recombinant albumin (rIX-FP). Thromb. Res. 141(Suppl 3), S5–S8 (2016).
8. Chaudhury, C. et al. The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan. J. Exp. Med. 197, 313–322 (2003).
9. Ghetie, V. et al. Abnormally short serum half-lives of IgG in beta 2-microglobulin-deﬁcient mice. Eur. J. Immunol. 26, 690–696 (1996).
10. Israel, E. J., Wilsker, D. F., Hayes, K. C., Schoenfeld, D. & Simister, N. E. Increased clearance of IgG in mice that lack beta(2)-microglobulin: Possible protective role of FcRn. J. Immunol. 89, 573–578 (1996).
11. Junghans, R. P. & Anderson, C. L. The protection receptor for IgG catabolism is the beta(2)-microglobulin-containing neonatal intestinal transport receptor. Proc. Natl Acad. Sci. USA 93, 5512–5516 (1996).
12. Ward, E. S. & Ober, R. J. Chapter 4: multiskipping by exploitation of intracellular transport functions the many faces of FcRn. Adv. Immunol. 103, 77–115 (2009).
13. Ghetie, V. et al. Abnormally short serum half-lives of IgG in beta 2-microglobulin-deﬁcient mice. Eur. J. Immunol. 26, 690–696 (1996).
14. Israel, E. J., Wilsker, D. F., Hayes, K. C., Schoenfeld, D. & Simister, N. E. Increased clearance of IgG in mice that lack beta(2)-microglobulin: Possible protective role of FcRn. J. Immunol. 89, 573–578 (1996).
15. Junghans, R. P. & Anderson, C. L. The protection receptor for IgG catabolism is the beta(2)-microglobulin-containing neonatal intestinal transport receptor. Proc. Natl Acad. Sci. USA 93, 5512–5516 (1996).
16. Ward, E. S. & Ober, R. J. Chapter 4: multiskipping by exploitation of intracellular transport functions the many faces of FcRn. Adv. Immunol. 103, 77–115 (2009).
17. Prabhak, P. et al. Elucidation of intracellular recycling pathways leading to exocytosis of the Fc receptor, FcRn, by using multispectral plane microscopy. Proc. Natl Acad. Sci. USA 104, 5889–5894 (2007).
18. Ward, E. S. et al. From sorting endosomes to exocytosis: association of Rab4 and Rab11 GTPases with the Fc receptor, FcRn, during recycling. Mol. Biol. Cell 16, 2028–2038 (2005).
19. Ober, R. J., Martinez, C., Vaccaro, C., Zhou, J. & Ward, E. S. Visualizing the site and dynamics of IgG salvage by the MHC class I-related receptor, FcRn. J. Immunol. 172, 2021–2029 (2004).
20. Ober, R. J., Martinez, C., Lai, X., Zhou, J. & Ward, E. S. Exocytosis of IgG as mediated by the receptor, FcRn: an analysis at the single-molecule level. Proc. Natl Acad. Sci. USA 101, 11076–11081 (2004).
21. Ward, E. S., Zhou, J., Ghetie, V. & Ober, R. J. Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans. Int. Immunol. 15, 187–195 (2003).
22. Pyzik, M. et al. Hepatic FcRn regulates albumin homeostasis and susceptibility to liver injury. Proc. Natl Acad. Sci. USA 114, E2862–E2871 (2017).
23. Schmidt, E. G. W. et al. Direct demonstration of a neonatal Fc receptor (FcRn)-driven endosomal sorting pathway for cellular recycling of albumin. J. Biol. Chem. 292, 13312–13322 (2017).
24. Greys, A. et al. A human endothelial cell-based recycling assay for screening of FcRn targeted molecules. Nat. Commun. 9, 1–14 (2018).
25. Chia, J. et al. Half-life-extended recombinant coagulation factor IX-human albumin fusion protein is recycled via the FcRn-mediated pathway. J. Biol. Chem. 293, 6363–6373 (2018).
26. Burmeister, W. P., Gastinel, L. N., Simister, N. E., Blum, M. L. & Bjorkman, P. J. Crystal structure at 2.2 Å resolution of the MHC-related neonatal Fc receptor. Nature 372, 336–343 (1994).
show dramatic differences in immunoglobulin G and albumin binding. J. Biol. Chem. 285, 4826–4836 (2010).

51. Lankisch, P. G., Apte, M. & Banks, P. A. Acute pancreatitis. Lancet 386, 85–96 (2015).

52. Auld, D. S. in Handbook of Proteolytic Enzymes, 3rd edn (eds Neil D. R. & Guy S.) 1289–1301 (Academic Press, 2013).

53. Brennan, S. O., Fellowes, A. P. & George, P. M. Albumin banks peninsula: a new view of a classical protein from mass spectrometry. Biochim. Biophys. Acta 1433, 321–326 (1999).

54. Roth, M. & Rohn, R. A Determination of pancreatic carboxypeptidase A in human blood serum. Clin. Chim. Acta 135, 65–71 (1983).

55. Kemik, O. et al. Serum procarboxypeptidase A and carboxypeptidase A levels in pancreatic disease. Hum. Exp. Toxicol. 31, 447–451 (2012).

56. Peters, T. Jr., Kragh-Hansen, U. & Minchiotti, L. Altered chain-length and glycosylation modify the human and mouse albumin bind their respective neonatal Fc receptors differently. Sci. Rep. 8, 14648 (2018).

57. Firan, M. et al. The MHC I-related receptor, FcRn, plays an essential role in the maternofetal transfer of gamma-globulin in humans. Clin. Chim. Acta 292, 113–124 (2000).

58. Iwao, Y. et al. Immunohistochemical localization of the neonatal Fc receptor affects Fc effector functions. J. Immunol. Methods 146, 1–10 (1991).

59. Andersen, J. T. et al. Single-chain variable fragment albumin fusions bind the neonatal Fc receptor (FcRn) in a species-dependent manner: implications for in vivo half-life evaluation of albumin fusion therapeutics. J. Biol. Chem. 288, 24277–24285 (2013).

60. Stewart, J. D. & Gilvarg, C. Determination of the activity of carboxypeptidase A in the blood of healthy human adults. Clin. Chim. Acta 281, 19–28 (1999).

61. Rousseaux, J. et al. [Bisublimainemia in pancreatitis: structural modifications of human serum albumin by proteolytic enzymes of the pancreas (author’s transl)]. Clin. Chim. Acta 71, 35–46 (1976).

62. Bar-Or, D., Rael, L. T., Bar-Or, R., Slone, D. S. & Craun, M. L. The formation and rapid clearance of a truncated albumin species in a critically ill patient. Clin. Chim. Acta 365, 346–349 (2006).

63. Andersen, J. T. et al. Single-chain variable fragment albumin fusions bind the neonatal Fc receptor (FcRn) in a species-dependent manner: implications for in vivo half-life evaluation of albumin fusion therapeutics. J. Biol. Chem. 288, 24277–24285 (2013).

64. Bernstein, G. et al. Prolonged and increased expression of soluble Fc receptors, IgG and a TCR-Ig fusion protein by transiently transfected adherent 293E cells. J. Immunol. Methods 298, 93–104 (2005).

65. Firan, M. et al. The MHC class I-related receptor, FcRn, plays an essential role in the maternofetal transfer of gamma-globulin in humans. Int. Immunol. 13, 993–1002 (2001).

66. Greys, A. et al. Fc engineering of human IgG1 for altered binding to the neonatal Fc receptor affects Fc effector functions. J. Immunol. 194, 5497–5508 (2015).

67. Trabjerg, E., Kartberg, F., Christensen, S. & Rand, K. D. Conformational characterization of nerve growth factor-beta reveals that its regulatory prodomain domain stabilizes three loop regions in its mature part. J. Biol. Chem. 292, 16665–16676 (2017).

68. Masson, G. R. et al. Recommendations for performing, interpreting and reporting hydrogen deuterium exchange mass spectrometry (HDX-MS) experiments. Nat. Methods 16, 595–602 (2019).

69. Wellen, A. W. et al. Multivalent immune complexes divert FcRn to lysosomes by exclusion from recycling sorting tubules. Mol. Biol. Cell 24, 2398–2405 (2013).

70. Bennett, M. J., Barakat, K., Huzil, J. T., Tuszyński, J. & Schiemer, D. C. Discovery and characterization of the laulimalide-microtubule binding mode by mass shift perturbation mapping. Chem. Biol. 17, 725–734 (2010).

71. Houde, D., Berkowitz, S. A. & Engen, J. R. The utility of hydrogen/deuterium exchange mass spectrometry in biopharmaceutical comparability studies. J. Pharm. Sci. 100, 2071–2086 (2011).

72. Arora, J. et al. Charge-mediated Fab-Fc interactions in an IgG1 antibody induce reversible self-association, cluster formation, and elevated viscosity. ACS Comb. Sci. 8, 1561–1574 (2016).

Acknowledgements

We are grateful to Satheesha Sivaganesan for excellent technical assistance. We thank Dr. Wayne L. Lencer for (Boston Children’s Hospital, Harvard Medical School and Harvard Digestive Diseases center) for the HMECI cell line stably expressing HA-hFcRn-EGFP. This work was supported in part by the Research Council of Norway through its Center of Excellence funding scheme (project no. 179573). J.T.A. and J.N. were supported by the Research Council of Norway (Grant no. 230526) and South-Eastern Norway Regional Health Authority (Grant no. 40018). E.T. and K.D.R. were supported by the Benzon Foundation and the Independent Research Foundation Denmark (Sapere Aude Grant-DFF-4184-00537A). A.G. and K.M.K.S. were supported by the University of Oslo. C.A. was supported by Fundação para a Ciência e a Tecnologia (FCT Portugal) (SFRH/BD/117598/2016). M.B. was supported by the Research Council of Norway through its program for Global Health and Vaccination Research (GLOBVAC) (Grant no. 143822) and program for biotechnology and innovation (BIOTEK2021) (Grant no. 267606). B.D. was supported by South-Eastern Norway Regional Health Authority (Grant no. 2015095) (Regional Core Facility for Structural Biology).

Author contributions

J.N., E.T., A.G., S.O.B., M.S., B.D., D.C.R., I.S., K.D.R., and J.T.A. designed research; J.N., E.T., A.G., S.O.B., M.S., J.W., K.M.K.S., M.B., and J.T.A. performed research; J.N., E.T., A.G., S.O.B., M.S., D.C.R., I.S., K.D.R., and J.T.A. analyzed the data; J.N., E.T., I.S., and J.T.A. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s42003-020-0903-7.

Correspondence and requests for materials should be addressed to J.T.A.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020