Structure-based mutagenesis reveals the albumin-binding site of the neonatal Fc receptor

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Albumin is the most abundant protein in blood where it has a pivotal role as a transporter of fatty acids and drugs. Like IgG, albumin has long serum half-life, protected from degradation by pH-dependent recycling mediated by interaction with the neonatal Fc receptor, FcRn. Although the FcRn interaction with IgG is well characterized at the atomic level, its interaction with albumin is not. Here we present structure-based modelling of the FcRn–albumin complex, supported by binding analysis of site-specific mutants, providing mechanistic evidence for the presence of pH-sensitive ionic networks at the interaction interface. These networks involve conserved histidines in both FcRn and albumin domain III. Histidines also contribute to intramolecular interactions that stabilize the otherwise flexible loops at both the interacting surfaces. Molecular details of the FcRn–albumin complex may guide the development of novel albumin variants with altered serum half-life as carriers of drugs.

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**Results**

**HSA DIIIb is crucial for pH-dependent binding to hFcRn.** Albinum consists of three homologous domains (DI, DII and DIII), each comprising \( \alpha \)-helices stabilized by a complex network of twelve cysteine residues forming six disulfide bridges\(^2\). The three domains are linked by loops and form a heart-shaped structure (Fig. 1b). To investigate how each individual domain contributes to hFcRn binding, several domain variants (DI – DIII, DII – DIII, DI – DII, DIII) as well as wild-type (WT) HSA (Fig. 1c) were expressed in yeast (Fig. 1d). The binding of each variant to immobilized recombinant hFcRn was measured by surface plasmon resonance (SPR). Equal amounts of the HSA variants were injected at pH 6.0 and pH 7.4. The HSA variant consisting solely of DIII bound hFcRn, whereas the variant lacking DIII did not bind to FcRn at all (Fig. 1e). Furthermore, the DI – DII variant bound slightly stronger to hFcRn than DIII alone (Table 1). DII, on the other hand, did not seem to contribute to binding.

Each of the three HSA domains has two subdomains, a and b. To address the importance of the C-terminal subdomain DIIIb, we engineered an HSA variant where this domain was deleted (HSA – DIIIb). Lack of DIIIb completely abolished hFcRn binding (Fig. 1f). For comparison, we included a recombinant version of HSA – Bartin, which lacks DIII except from the first 29 amino acids\(^3\). These results demonstrate that an intact DIII is crucial for receptor binding.

**A point mutation alters hFcRn binding in HSA Casebrook.** HSA is normally non-glycosylated, but a few exceptions exist because of rare mutations\(^4\). One such variant (Casebrook) has a single nucleotide substitution that changes the coding from Asp to Asn at amino-acid residue 494 (ref. 19), localized in the stretch of amino acids (residue 490 – 510) that form a long loop connecting subdomains DIIa and DIIib (Figs 2a and 3a). This point mutation introduces a glycosylation motif (\(^{494}\)Asn-Glu-Thr\(^{496}\)) and attachment of an N-linked oligosaccharide. We studied migration in SDS – PAGE and hFcRn binding of a number of recombinant HSA variants that allowed us to dissect the role of the oligosaccharide and individual amino acids at \(^{494}\)Asn-Glu-Thr\(^{496}\). The recombinant version of Casebrook (D494N) migrated more slowly than WT HSA in SDS – PAGE, which reflects attachment of oligosaccharides (Fig. 2b). Moreover, in six variants, D494A, D494Q, E495Q, E495A, T496A and D494N/T496A, the glycosylation motif was disrupted, and consequently, all of these mutants migrated like their WT counterpart (Fig. 2b).

All variants were tested for binding to immobilized hFcRn by SPR, and distinct binding differences were detected at acidic pH.
with a hierarchy from strongest to weakest binding as follows; WT > T496A > D494N/T496A > D494N/E495Q > E495A > D494A > D494Q (Fig. 2c–f; Table 1). The same trend was obtained by ELISA (Supplementary Fig. S1). The binding kinetics revealed major differences in dissociation rates for most mutants, while recombinant Casebrook (D494N) showed a twofold reduced affinity resulting from both altered association and dissociation constants. Mutation of Asp-494 and Glu-495 to a neutral alanine or a glutamine had a large effect on receptor binding, whereas mutation of the flanking Thr-496 had only a small effect. The HSA Casebrook variant isolated from a heterozygous individual bound hFcRn similar to its recombinant counterpart (Fig. 2f; Supplementary Fig. S2; Table 1).

The Casebrook variant is present at a two to three-fold lower level than normal HSA in serum of heterozygous individuals. To investigate whether HSA shows twofold reduced binding affinity for hFcRn (Fig. 2h; Table 1). The carboxylic side chain of Asp-494 forms a charged-stabilized salt-bridge with Arg-472 as well as hydrogen bonds with both Gln-417 and Thr-496. N-linked glycosylation of Asn-494 will reduce the hydrogen-bonding capacity and eliminate the negative component of the salt-bridge, which may be important for stabilizing the loop. In support of this is the finding that a Q417A mutation also destabilizes the N-terminal end of the loop encompassing residues 490–495, and thus affect its conformation and ability to interact with hFcRn.

Besides Asp-494, Glu-495 and Thr-496 at the N-terminal end of the loop, we targeted two charged residues (Lys-500 and Glu-501) in addition to Pro-499 in the middle of the loop (Fig. 3a) by mutagenesis and investigated the effect on hFcRn binding. We found moderate effects of P499A and E501A, while mutation of the positively charged Lys-500 dramatically reduced binding to the receptor by more than 30-fold (Fig. 3b; Table 1).

Crucial roles of conserved histidines in HSA DIII. Guided by the fact that histidine residues are key players in the strictly pH-dependent IgG–FcRn interaction, we assessed the role of the four histidines found within HSA DIII. Of these, three are highly conserved across species (His-446, His-510 and His-535) and one is not (His-440) (Supplementary Table S2). Whereas His-440 and His-464 are found within DIIIa, His-510 is localized to the end of the loop.

Structural implications of HSA Casebrook. To investigate whether the HSA mutants (T496A, D494N/T496A, D494N, E495Q, E495A, D494A and D494Q) had any major impact on the global structure, their secondary structural elements were determined by circular dichroism (CD). No major difference from that of WT HSA was observed for any of the mutants at either pH 7.4 or pH 6.0 (Supplementary Fig. S3; Supplementary Table S1).

Next, we inspected a crystal structure of HSA20, and found Asp-494 to be involved in an intramolecular network of polar interactions involving amino acids in both DIIIa and DIIIb (Fig. 2a). The hisidines found within HSA DIII. Of these, three are highly conserved across species (His-446, His-510 and His-535) and one is not (His-440) (Supplementary Table S2). Whereas His-440 and His-464 are found within DIIIa, His-510 is localized to the end of the loop.

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To obtain a molecular explanation, we inspected a crystal structure of the Fc region of IgG (His-310 and His-435) for hFcRn binding, which parallels the requirement for histidines in the non-conserved His-440 did not (Fig. 3c; Table 1). Thus, we pinpoint to hFcRn at pH 6.0. Mutation of each of the three conserved histidines within DIII as fundamental for pH-dependent hFcRn binding, which parallels the requirement for histidines in the Fc region of IgG (His-310 and His-435).

Connecting DIIa and DIIb, and His-535 is found in one of the α-helices of DIIIb (Fig. 3a). All four histidines were mutated individually to glutamine, and the corresponding mutants were expressed in yeast (Supplementary Fig. S4) before they were tested for binding to hFcRn at pH 6.0. Mutation of each of the three conserved histidines almost completely abolished binding, whereas mutation of the non-conserved His-440 did not (Fig. 3c; Table 1). Thus, we pinpoint these three histidines within DIII as fundamental for pH-dependent hFcRn binding, which parallels the requirement for histidines in the Fc region of IgG (His-310 and His-435).

Furthermore, alanine substitutions of two positively charged lysines (Lys-536 and Lys-538) and Pro-537 in the vicinity of His-535 were also shown to moderately attenuate binding to hFcRn (Fig 3d; Table 1). Taken together, the binding data define a core structural area on DIII important for pH-dependent hFcRn binding.

Mapping the HSA binding site on hFcRn. We have previously identified a highly conserved histidine residue localized to the α2-domain of both mouse and human FcRn HC to be crucial for albumin binding (His-168 and His-166, respectively)21,22. To obtain a molecular explanation, we inspected a crystal structure of hFcRn that was recently solved under acidic conditions (pH 4.2) (Fig. 1a)23. We found that His-166 is engaged in a network of intramolecular interactions that involves charge-stabilized hydrogen bonds with Glu-54 and Tyr-60 found on a surface-exposed loop within the α1-domain (residue 51–60) (Fig. 4a). At low pH, His-166 will carry a positive charge, and we propose that uncharged His-166 of immobilized hFcRn to 1 μM of WT HSA and recombinantly produced Casebrook (D494N/T496A) (c) Representative SPR sensorgrams showing binding of immobilized hFcRn to 1 μM of WT HSA and Casebrook isolated from a heterozygote patient. (g) Competitive binding of WT HSA and Casebrook to hFcRn at pH 6.0. The receptor (100 nM) was injected in the presence of titrated amounts of WT or Casebrook HSA (0.015–1.000 nM) over immobilized HSA. (h) SPR sensorgrams showing binding of immobilized hFcRn to 1 μM of WT HSA and Q417A at pH 6.0.

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

| Albumin variant | $k_a$ (10^3 per Ms) | $k_d$ (10^-3 per s) | $k_d$ (µM)^a | $k_d$ (µM)^b |
|----------------|---------------------|---------------------|--------------|--------------|
| WT             | 5.9 ± 0.1           | 7.0 ± 0.2           | 1.1          | 2.4          |
| DIII           | 2.6 ± 0.0           | 7.2 ± 0.0           | 27.6         | 17.4         |
| DI-DII         | 3.2 ± 0.1           | 4.5 ± 0.1           | 14.1         | 15.0         |
| Q417A          | 5.0 ± 0.0           | 1.1 ± 0.1           | 2.2          | 3.3          |
| H440Q          | 5.1 ± 0.0           | 7.0 ± 0.1           | 1.3          | 2.5          |
| H464A          | NA^d               | NA                  | NA           | NA           |
| D494N          | 3.8 ± 0.0           | 8.5 ± 0.0           | 2.2          | 4.6          |
| (Casebrook)    | D494A              | 5.9 ± 0.1           | 2.0 ± 0.0    | 3.6          | 4.2          |
| D494Q          | 5.4 ± 0.2           | 25.5 ± 0.1          | 4.7          | 5.3          |
| E495Q          | 4.2 ± 0.0           | 13.1 ± 0.0          | 3.1          | 3.2          |
| E495A          | 3.8 ± 0.1           | 13.0 ± 0.0          | 3.4          | 3.1          |
| T496A          | 5.4 ± 0.0           | 7.6 ± 0.2           | 1.4          | 2.5          |
| D494N/T496A    | 5.4 ± 0.1           | 8.5 ± 0.2           | 1.5          | 2.5          |
| Casebrook      | 3.6 ± 0.1           | 9.7 ± 0.1           | 2.7          | 3.8          |
| P499A          | 2.6 ± 0.0           | 12.1 ± 0.0          | 4.6          | 3.9          |
| K500A          | 14.3 ± 0.2          | 47.8 ± 0.0          | 33.4         | 25.0         |
| E501A          | 5.1 ± 0.0           | 9.8 ± 0.0           | 1.9          | 2.6          |
| H510Q          | NA                  | NA                  | NA           | 12.1         |
| HS10Q          | NA                  | NA                  | NA           | 16.2         |
| HS53Q          | 4.4 ± 0.2           | 9.3 ± 0.1           | 2.1          | 3.7          |
| KS536A         | 3.7 ± 0.1           | 14.3 ± 0.2          | 3.9          | 5.5          |
| K538A          | 3.9 ± 0.0           | 7.1 ± 0.0           | 1.8          | 2.9          |
| HSA 568Stop    | NA                  | NA                  | NA           | 17.0         |
| HSA Dilla      | ND                  | ND                  | ND           | ND           |

^a The kinetic rate constants were obtained using a simple first-order (1:1) Langmuir bimolecular interaction model, which assumes that one HSA molecule binds one FcRn. The kinetic values represent the average of triplicates.

^b The steady-state affinity constant was obtained using an equilibrium (Reg) binding model supplied by the BiAevaluation 4.1 software. The affinities derived from equilibrium binding data represent the average of triplicates.

^c ND, not determined because of no or very weak binding.

^d NA, not acquired because of fast binding kinetics.
His-166 in hFcRn and the residues His-464, His-510 and His-535
2.6 Å resolution structure solved at pH 4.2 (refs 8, 23). The program of hFcRn; the 2.7 Å resolution structure solved at pH 8.2 and the
A proposed mode of binding

Discussion

FcRn has evolved to protect IgG and albumin from catabolism5,18,22. Although FcRn binding to IgG has been studied in great detail for
decades, its recently discovered interaction with albumin is poorly understood at the molecular level. In the present study, we provide
mechanistic evidence for the importance of several interaction interfaces on both molecules, revealing how FcRn and HSA interact
in a pH-sensitive fashion that facilitates cellular recycling.

Our finding that DIII alone, unlike DI-DII, could bind to the receptor, modulated by pH, conclusively shows that DIII harbours
a tenfold reduced binding affinity was found when His-161 acidic pH (Fig. 5b). This is in agreement with our previous finding
where a tenfold reduced binding affinity was found when His-161 was mutated22. The complex could be further reinforced by a salt-
bridge formed between Glu-168 of hFcRn and Lys-524 of DIII, a

Another cleft on the surface of HSA is formed between the DIIIa–
DIIb connecting loop and one of the other α-helices of DIII (resi-
dues 520–535). Here His-161 of FcRn may interact with Glu-531 at acidic pH (Fig. 5b). This is in agreement with our previous finding
where a tenfold reduced binding affinity was found when His-161 was mutated22. The complex could be further reinforced by a salt-
bridge formed between Glu-168 of hFcRn and Lys-524 of DIII, a

prediction that is supported by the fact that mutation of Glu-168 moderately reduces binding to HSA (Supplementary Fig. 6).

Moreover, His-535 may interact favourably with Phe-157 while His-464 is localized close to a β-hairpin within hFcRn encompass-
ning residues 99–102 that is wedged in-between DI and DIIAs in HSA (Fig. 5c). Here FcRn Asp-101 has several possible partners
in DI such as Arg-197 and Lys-190, however, they must necessar-
ily undergo some conformational changes in order to get close to
Asp-101. Interestingly, this β-hairpin has two different conforma-
tions in the low and high pH-forms of hFcRn,8,23, suggesting that
Asp-101 is indeed located in a flexible element of hFcRn.

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mechanistic evidence for the importance of several interaction interfaces on both molecules, revealing how FcRn and HSA interact
in a pH-sensitive fashion that facilitates cellular recycling.

Our finding that DIII alone, unlike DI-DII, could bind to the receptor, modulated by pH, conclusively shows that DIII harbours the
principal core-binding site for FcRn, as in agreement with previous reports9,17. However, we also show that binding to DIII alone is
considerably weaker than that for full-length HSA, a finding that suggests that there may be a moderate contribution to receptor

in HSA in the protein–protein interface20. The software returned one model for the docking of DIII against hFcRn with an ordered
loop at pH 4.2 and eight models for hFcRn at pH 8.2, lacking loop residues 52–59. Of these eight models, five evidently showed incompa-
tible poses as judged by the position of HSA DI and DII, and were rejected. The three remaining models were closely related and had
the same general structural pose. Superimposing of the low pH form of hFcRn on these then showed that the structured loop made
no severe conflicts with the docked HSA.

The final selected model reveals interaction areas that fit very well with the obtained binding data (Fig. 5a). Particularly, the long
loop between subdomains DIIIa and DIIb (490–510) as well as the last C-terminal α-helix of HSA form a crevice on the surface of
HSA into which the pH-dependent and flexible loop in hFcRn (residues 51–60) may bind (Fig. 5b). The structure of hFcRn shows that
His-166 stabilizes this loop through an intramolecular interaction with Glu-54 (Fig. 4a); however, the model suggests that His-
166 may additionally be engaged in binding to Glu-505 of HSA (Fig. 5b). Glu-505 may also interact with Arg-162 of the receptor. A
key role of His-510 is supported by the fact that it is predicted to interact with Glu-54 within the pH-dependent α1-domain loop (Fig. 5b). Mutation of His-510 (H510Q) reduced binding by 14-fold (Fig. 3c; Table 1). Thus, His-166 in hFcRn and His-510 in HSA seem to be involved in regulating an ionic network in the core of the hFcRn–HSA interaction interface.

Furthermore, the predicted involvement of the C-terminal α-helix of HSA is supported by the finding that deletion of the last 17 amino acids of DIIIb (568Stop) reduced binding affinity to hFcRn by more than 17-fold (Supplementary Fig. 5; Table 1). The model also predicts salt-bridges between Lys-150 and Glu-151 in hFcRn on one hand and Glu-501 and Lys-500 in HSA (Fig. 5b). This is in line with the binding data that show reduced binding capacity of HSA variants mutated at these positions (Fig. 3b).

Figure 3 | Conserved histidines are fundamental for binding to hFcRn. (a) Structural location of selected residues in DIII of HSA. Residues in the
loop connecting the subdomains DIIa and DIIb selected for mutagenesis (Asp-494, Glu-495, Lys-500 and Glu-501) as well as extra residues close
to the connecting loop, such as the conserved histidines (His-464, His-510 and His-536), Lys-536 and Pro-537 are displayed as ball-and-stick (maroon). The non-conserved His-440 is distally localized. The last C-terminal α-helix is highlighted in yellow. Representative SPR
sensorgrams showing binding of immobilized hFcRn to 1μM of WT HSA and (b) P499A, K500A and E501A, and (c) H440Q, H464Q, H510Q and H535Q as well as (d) K526A, P537A and K538A at acidic pH (6.0).
Figure 4 | His-166 of hFcRn stabilizes a flexible loop in a pH-dependent manner. Close-up view of the FcRn HC loop area at different pH conditions. (a) At low pH (4.2), the positively charged His-166 forms charge-stabilized hydrogen-bond interactions with Glu-54 and Tyr-60 within the surface-exposed loop in hFcRn. (b) At high pH (8.2), the uncharged His-166 looses the interactions with Glu-54 and Tyr-60, and the loop between residues Trp-51 and Tyr-60 becomes flexible and structurally disordered (represented by the dashed line). (c) Binding of 0.5 μg ml⁻¹ of hFcRn WT and mutants (E54Q, Q56A and H166A) to titrated amount of HSA (0.3–200 μg ml⁻¹) coated in ELISA wells at pH 6.0. n = 4. All data are presented as mean ± s.d.

binding from DI or DII, either directly or indirectly. Furthermore, a DI–DIII construct-bound hFcRn slightly stronger than DIII. One may speculate that this is due to structural stabilization of DIII or that DI interacts with hFcRn when fused to DIII, although DI in the DI–DIII fusion has a different location than DI in WT HSA. Interestingly, the model suggests that there may be a minor interaction between DI and hFcRn.

Several naturally occurring HSA variants, with mutations in DIII, have altered blood levels. One such variant, Casebrook, with a point mutation that introduces an N-linked oligosaccharide attachment site, constitutes about 35% of total HSA in heterozygous individuals. Introduction of the mutation in rabbit albumin resulted in a 50% reduction in half-life when injected into rabbits. On the basis of these observations, we engineered an HSA variant mimicking Casebrook and found that it had a twofold reduced affinity for hFcRn. This was indeed also the case for Casebrook isolated from a heterozygous individual.

When inspecting the crystal structure of hFcRn solved at acidic pH, we found the partially exposed and protonated His-166 to be engaged in stabilizing a loop that was disordered at basic pH, through binding to an acidic (Glu-54) and a polar (Tyr-60) amino acid. The disorder of the loop is likely explained by loss of protonation of His-166 at basic pH, which then regulates the flexibility and conformation of the loop in a pH-dependent manner.

Four histidine residues in HSA DIII were individually mutated to glutamines, and three highly conserved histidines (His-464, His-510 and His-535; Supplementary Table S2) were found to be important for binding at acidic pH. This fact as well as the prerequisite for hFcRn His-166 for binding to HSA, were used to guide the hFcRn–HSA models output by the docking algorithm. The best model reveals that DIII forms the major interaction interface with the α1–α2-platform of the receptor, with predicted key residues being His-510 and Glu-505 in HSA, as well as Glu-54 in hFcRn.

Of the remaining two conserved histidines on DIII, His-535 may reinforce the HSA–hFcRn complex by aromatic stacking or stabilization of this important loop. His-464, on the other hand, may interact, directly or indirectly, with a flexible β-hairpin element in hFcRn. Interestingly, this β-hairpin loop is the most flexible part of hFcRn, except for the pH-dependent loop stabilized by His-166, as judged by a comparison of low and high pH crystal structures. The flexible β-hairpin is in contact with both the α-helix in HSA that contains His-464 as well as a long loop in DI, suggesting an indirect conformational ‘tuning’ of the hFcRn–HSA interface involving DI and DIIIs. Our data support a study showing that mutation of the conserved histidines to alanine resulted in increased clearance of HSA DIII fused to antibody fragments when injected into mice.

The principal function of albumin is to transport fatty acids that are bound asymmetrically to hydrophobic pockets within or between the three domains. DI harbours two high affinity binding sites, and the fatty acids bind close to the loop between DIIIs and DIIb, which also includes several residues found to affect hFcRn binding. Comparison of the fatty acid bound and free states of HSA shows no substantial rearrangements within DIII, but a considerable shift in orientation of DI relative to DIII (Supplementary Fig. S8). In effect, superimposing DIII in the fatty acid binding HSA onto the corresponding domain in our structural model reveals that DI may move away from hFcRn when binding to fatty acids.

The half-life regulatory function of FcRn may be utilized for therapy, as discussed elsewhere. Obviously, drugs may fail to show convincing effects in vivo if their half-lives are short as a consequence of their size being below the renal clearance threshold. This limits transition from lead candidate to drugs on the market. A solution may be genetic fusion of the therapeutic to albumin or the IgG Fc, which have shown to improve biodistribution and pharmacokinetics.

The serum half-life of IgG may also be altered, as demonstrated for engineered IgGs with mutations in their IgG1 Fc that result in improved pH-dependent FcRn binding, and consequently extended half-life in vivo. No examples have so far been presented for albumin, except for the observation that mouse albumin binds more strongly to hFcRn than HSA. The structural hFcRn–HSA model presented in this study may guide the development of HSA variants with altered half-life, which could be attractive for delivery of both chemical and biological drugs.
Tumours and inflamed tissues show increased accumulation of albumin as a result of leaky capillaries and defective lymphatic drainage. Consequently, albumin-based therapeutics or diagnostics accumulate at the site of tumour or inflammation. Because of tissue toxicity of the fused molecules, fine-tuning of albumin half-life may be an attractive approach to improve tumour targeting and imaging, as previously shown for IgGs with attenuated affinity for FcRn. The HSA variants described in this paper, with substantially reduced or intermediate FcRn binding affinities, may serve as attractive candidates.

**Methods**

**Production of hFcRn variants.** Production of hFcRn has previously been described. Gene-segments encoding mutant hFcRn variants (E54Q, Q56A and E168A) were ordered from Genscript, subcloned and produced as described.

**Production of HSA variants.** *Escherichia coli* DH5α was used for manipulation, propagation and preparation of HSA DNA. Expression cassettes (NotI cassettes) contain the *Saccharomyces cerevisiae* PRB1 promoter, a leader sequence (MKWVS-FISLLFLSSAYSRSLDXR (FL) fused in-frame with the HSA gene) and the *S. cerevisiae* ADH1 terminator sequence. Plasmids were generated using standard cloning techniques (Supplementary Table S3) or a mixture of this and gap-repair (Supplementary Table S4). DNAs containing mutations within the

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**Figure 5 | A proposed hFcRn-HSA docking model.** (a) An overview of the docked molecules in two orientations showing the FcRn HC (green), β2m (grey) and the three HSA α-helical domains DI (pink), DII (orange) and DIII (cyan/blue). The HSA DIII is split into DIIla (cyan) and DIIlb (blue). (b) Close-up view of the interaction interface between hFcRn (green cartoon) and HSA (blue surface) in the docking model. The C-terminal end of HSA (dark blue) and the loop corresponding to residues 490–510 between subdomains DIIla and DIIlb form a crevice on the HSA surface into which the pH-dependent and flexible loop in hFcRn (residues 51–59) might bind. His-166 of hFcRn may form strong, charge-stabilized interactions with HSA residues Glu-54 and Glu-505. HSA Glu-505 could further interact with hFcRn Arg-162. Possible salt-bridges are formed between Lys-150 and Glu-151 of hFcRn with Glu-501 and Lys-500 of HSA. A cleft on the HSA surface is formed between the loop connecting DIIla and DIIlb and the α-helix encompassing residues 520–535. His-161 of hFcRn may interact with Glu-531 of HSA at low pH, and the complex could be further reinforced by the salt-bridge between hFcRn Glu-168 and HSA Lys-524. (c) Interaction interface between hFcRn (green surface) and HSA (pink, blue and cyan cartoon) in the docking model. A β-hairpin loop in hFcRn is wedged in-between domains DI (pink) and DIIla (cyan) in HSA. The hFcRn Asp-110 could be a partner to either Lys-190 or Arg-197 of HSA following some structural rearrangements in this interface. The conserved His-464 is located in the DIIla α-helix contacting the β-hairpin loop.
ALB gene (GeneArt GmbH or DNA2.0) were subcloned into NotI/Sacl-digested pDB2243 (a derivative of pAYE316 (ref. 38) to create plasmids pDB3876–pDB3882 (Supplementary Table S3). Expression cassettes (NotI fragments) were obtained from pDB3876–pDB3886 and ligated into NotI-digested pSAC35 (ref. 39) to generate pDB3885–pDB3887 (Supplementary Table S3).

Plasmids used for gap-repair were generated by digesting pDB2244 (containing NotI cassette from pDB2243 in NotI-digested pSAC35) with SwaI and the 9.54 kb fragment (containing the S. cerevisiae LEU2 gene, and the HSA NcoI cassette) was ligated into pDB3927. Expression cassettes were generated using DNA fragments (NotI/BglII or SacI/SphI) containing mutations, subcloned into AvrII/SphI or SacI/SphI-digested pDB3927 (Supplementary Table S4), and then generated in S. cerevisiae by gap repair.

A truncated HSA variant (residues 1–186) was generated using PCR and gap-repair with the oligonucleotides xAP314 and xAP307 (5′-CTTCAGAGAATCTCAGTGCT-3′ and 5′-GAATTCGATCTTAAGAGAAGATCGTC-3′, respectively) was equilibrated using the Qiagen PCR Purification kit. 100 ng of each DNA encoding glutathione S-transferase (GST)-tagged HSA WT-GST, HSA–DII–GST and HSA–Bartin was measured using hFcRn (2 μg ml⁻¹) coated in wells following by washing and detection as described above.

To Biocart (Catanayama Chemicals). Measurements were performed with HSA (2 mg ml⁻¹) in 10 mM PBS (pH 6.0) without NcoI added, at 23 °C using a quartz cuvette (Starna) with a path length of 0.1 cm. Each sample was scanned 7 times at 20 nm min⁻¹ (bandwidth of 1 nm, response time of 1 s) with wavelength range set to 190–290 nm. The data were averaged and the spectrum of a sample-free control was subtracted. Secondary structural elements were calculated using the neural network program CDNN version 2.1 and the supplied neural network based on the 33-member basis set 44.

Docking procedure. Docking models of HSA and hFcRn were generated using the ZDOCK Fast Fourier Transform docking program 45. The coordinates for HSA DIII (a.a. 382–582) were retrieved from the crystal structure of HSA at 2.5 Å (PDB code 1b10) 46. Two different models of hFcRn were used: the 2.7 Å resolution structure of hFcRn at pH 8.2 (PDB code 1exu) and the 2.6 Å resolution structure at pH 4.2 (PDB code 3m17) 47. The β2m domain was included in the docking. The ZDOCK program was run with preferences for docking poses with the two histidines, His-161 and His-166, in hFcRn, and residues His-404, His-510 and His-353 in hFcRn that are part of the protein–ligand interface. All crystal structure figures were designed using PyMOL (DeLano Scientific).

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J.T.A., B.D., J.C., A.P. and I.S. designed research; J.T.A., B.D., J.C., M.B.D, A.P. and S.O.B. contributed new reagents/analytic tools; J.T.A., B.D., J.C., M.B.D, A.P. and S.O.B. performed research; J.T.A., B.D., J.C., M.B.D, A.P. and S.O.B. wrote the paper.

Additional information
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