Transferrin receptor targeting by de novo sheet extension


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The de novo design of polar protein–protein interactions is challenging because of the thermodynamic cost of stripping water away from the polar groups. Here, we describe a general approach for designing proteins which complement exposed polar backbone groups at the edge of beta sheets with geometrically matched beta strands. We used this approach to computationally design small proteins that bind to an exposed beta sheet on the human transferrin receptor (hTfR), which shuttles interacting proteins across the blood–brain barrier (BBB), opening up avenues for drug delivery into the brain. We describe a design which binds hTfR with a 20 nM Kd, is hyperstable, and crosses an in vitro microfluidic organ-on-a-chip model of the human BBB. Our design approach provides a general strategy for creating binders to protein targets with exposed surface beta edge strands.

computational protein design | drug delivery | neurological disease | transferrin receptor | blood–brain barrier

While most protein–protein interfaces are composed primarily of sidechain–sidechain interactions, backbone hydrogen bonding can also play a role. For example, beta sheet hydrogen bonds across protein–protein interfaces are present in complexes of PDZ domains with their peptide targets, SUMO–SIM interactions, and Serpin-protease complexes among others (1, 2); in each case, the result is an extended beta sheet that spans both partners. Such backbone interactions can contribute to interaction specificity even though backbone hydrogen bonding groups are present on all residues: Formation of hydrogen bonds with the correct geometry requires precise alignment of often twisted or curved beta strands—the structure of the interacting edge strand in a binder must be matched to the structure of the edge strand it interacts with in the target. Pathological processes such as the formation of amyloid fibrils also involve beta sheet extension (3), and inhibitors that hydrogen bond to the beta sheet have been developed to block such extension (4, 5). Design approaches have been used to create homodimeric structures with extended beta sheets (6, 7) that rely on symmetrical/self-docking of scaffolds to form homodimers. But to date, methods for designing heterodimeric complexes through beta sheet extension in which one component (the target protein) is fixed have not been described, even though targets with an exposed edge strand constitute a substantial class of therapeutically interesting molecules (1, 2).

A challenge in designing binding proteins is how to form an extensive binding interface while avoiding the energetically unfavorable burial of non-hydrogen-bonded polar groups on the target. Indeed, previous de novo binder design efforts have focused primarily on sidechain–sidechain interactions with hydrophobic patches on target protein surfaces with few polar groups (8, 9). With this approach, polar regions of a target protein surface are difficult to design binders against as it is very challenging to make sidechain-mediated hydrogen bonds to all the exposed polar groups simultaneously; in particular, the many exposed C=O and N–H groups at the edges of beta sheets are difficult to fully engage with sidechain hydrogen bonds. We reasoned that designed binding proteins with edge beta strands complementary in shape to an exposed beta strand in the target protein could overcome this challenge, as the multiple strand–strand hydrogen bonds could compensate for the loss of interactions with water.

Results

We developed a computational design approach for designing binding proteins with beta sheets geometrically poised to pair with exposed beta strands in target proteins of interest. We first align short two-stranded beta sheet motifs to the target protein edge strands and then use gradient-based minimization of the backbone coordinates to optimize the hydrogen bonding interactions across the interface with the target (Fig. 14). These optimized beta strands are then grafted (9) onto small de novo designed protein scaffolds with geometrically matching beta sheets, yielding a docked

Significance

The de novo design of proteins that bind natural target proteins is useful for a variety of biomedical and biotechnological applications. We describe a design strategy to target protein containing an exposed beta edge strand. We use the approach to design binders to the human transferrin receptor which shuttles back and forth across the blood–brain barrier. Such binders could be useful for the delivery of therapeutics into the brain.

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After filtering docking based on hydrogen bond geometry and buried surface area across the interface, Rosetta flexible backbone combinatorial sequence design is used to design additional specific sidechain–sidechain interactions across the interface and to stabilize the designed scaffold.

We sought to use our protocol to design a human transferrin receptor (hTfR) binding protein. hTfR transports transferrin (the major carrier of iron in the body) across the blood–brain barrier (BBB) via receptor mediated transcytosis, and this process has been exploited to deliver therapeutic payloads into the brain parenchyma that would otherwise be blocked by the BBB (10). For example, antibodies and nanoparticles linked to larger complicated molecules such as transferrin or anti-TfR antibodies have been shown to cross the BBB into the brain parenchyma in a hTfR dependent manner (11–13). Thus, hTfR is an attractive target candidate for the development of BBB traversing vehicles (10).

We targeted the region surrounding an edge strand located in the hTfR apical domain (Fig. 1B). This domain is distant from the transferrin binding site (which is important to avoid competitive binding) and is exposed and therefore suitable for beta sheet extension. We first experimented with de novo–designed ferredoxins as a base scaffold for the grafting step of the protocol after strand docking, as these scaffolds contain beta sheets into which the docked strand can be grafted and helices for additional contacts to hTfR. We found that while such scaffolds could make good edge strand interactions after the grafting/matching step, the number of additional contacts between the ferredoxin helices and hTfR were limited (Fig. 1C). To increase the buried surface area in the interface we followed two strategies. In strategy 1, we reasoned that truncating the ferredoxin C-terminal strand would shift the helix closer to hTfR, allowing for even larger burial of surface area in the interface than in strategy 1.

In strategy 2, Expansion of the truncated scaffolds with additional helices A and B allows for an even larger burial of surface area in the interface than in strategy 1.

Fig. 1. Design of edge strand–mediated complexes. (A) Design pipeline. After detection of exposed edge strands, a library of two-stranded beta sheet motifs was used to generate a docked strand: one of the two strands in each motif are aligned to the edge strands such that the second strand in the motif forms nonclashing beta sheet hydrogen bonds with the target that was subsequently minimized to optimize the backbone hydrogen bonds. Scaffolds from an in silico library are then superimposed or grafted onto this strand and scaffold residues are optimized to make favorable interactions with the target through the interface strand and flanking helices. (B) The homodimeric human transferrin receptor ectodomain [PDB: 3kas (32)] contains an exposed edge strand in the apical domain (red box). (C) Full-length designed ferredoxins can be docked to hTfR via strand E6, but there is little packing between helix H5 and hTfR (red X). (D) Strategy 1: Removal of strand E6 and instead docking via strand E1 allows for better packing interactions between helix H5 and hTfR. (E) Strategy 2: Expansion of the truncated scaffolds with additional helices A and B allows for an even larger burial of surface area in the interface than in strategy 1.
(14, 15), we expanded the truncated scaffold by adding a polyvaline helix at the N terminus to form a second interface with the target. Thousands of backbone structures were generated, in some of which the secondary interface helix was stabilized with another buttressing helix (Fig. 1E and SI Appendix, Fig. S1). A library of 649 selected designs were ordered for strategy 1 on an oligonucleotide array, and 50 synthetic genes were ordered of the larger strategy 2 designs and tested for binding using yeast surface display (Fig. 2A and B). Of the 649 designs from strategy 1, none bound to hTIR, despite having high in silico folding propensity and high interface shape complementarity (SI Appendix, Figs. S1B and S2). However, for strategy 2, one design (designated 2DS25) clearly bound fluorescently labeled hTIR (Fig. 2A and B). The flaw in the strategy 1 designs was likely the still low interface buried surface area, despite truncation of the C-terminal strand of the ferredoxin domain, suggesting 2DS25 binds the targeted area on hTIR (SI Appendix, Fig. S3).

We next expressed and purified 2DS25 from *Escherichia coli* using immobilized metal affinity chromatography (IMAC). The protein eluted as a monodisperse peak from size-exclusion chromatography (SEC) and was confirmed to be a monomer in solution by native mass spectrometry (SI Appendix, Fig. S3). Circular dichromism (CD) spectroscopy showed that 2DS25 is highly stable: The melting temperature is above 95 °C, and the guanidine–HCl concentration required for 50% denaturation was 5.7 M (Fig. 2D and SI Appendix, Fig. S3 E and F). Purified 2DS25 bound the hTIR ectodomain in biolayer interferometry experiments (Fig. 2E). Mutation of key residues in the designed binding site abolished binding, suggesting that complex formation is through the designed interface (Fig. 2E). In a yeast surface competition experiment, 2DS25 competed for binding with Machupo virus (MACV) GP1, a viral glycoprotein which binds the TIR apical domain, suggesting 2DS25 binds the targeted area on TIR (SI Appendix, Fig. S3G).

To probe the sequence determinants of folding and binding, and to facilitate determination of the structure of the 2DS25–hTIR complex, we created a site saturation mutagenesis (SSM) library in which each position on 2DS25 was substituted with all other 20 amino acids one at a time and screened for hTIR binding using fluorescence-activated cell sorting (SI Appendix, Fig. S4). Deep sequencing revealed that the designed core residues of 2DS25 were conserved, suggesting 2DS25 folds as designed. The key interface residues were also conserved, while affinity-increasing substitutions were identified around the interface. Combination of these enriched substitutions yielded higher affinity variants (see Materials and Methods).

The crystal structures of two increased affinity variants (2DS25.1 and 2DS25.5, SI Appendix, Table S2) in complex with hTIR were determined to be resolutions of 3.1 and 2.8 Å, respectively. The structures are virtually identical with an rmsd of 0.26 Å (SI Appendix, Fig. S5). In both cases, the design binds the Apical domain of hTIR using beta sheet extension and overall closely resembles the computational design model (Fig. 3A and SI Appendix, Table S1). The structure of 2DS25 superimposes closely on the computational model with an rmsd of 1.2 Å (Fig. 3B); this
interface residues are the same (Fig. S6 and S7), likely due to differences in the structure of the TIR apical region compared to that in the structure (Protein Data Bank [PDB]: 3kas) used in the design calculations (SI Appendix, Fig. S6B). The structural differences in the receptor are at both the backbone and side chain level, in particular at Tyr211; a superposition of all hTfR apo and holo structures in the PDB shows this region is flexible and can exist in multiple conformations (Fig. 3D).

As the conformation of hTfR observed in our crystal structure differs from those in other structures of the receptor, we investigated what our design protocol produced when targeted against this new conformation. In this new design round, we also loosened constraints of χ torsion angles of aromatic residues normally imposed during design calculations to allow for more strained but overall favorable π–π interactions present in the crystal structure (SI Appendix, Figs. S7 and S8). We selected 48 designs and expressed them in E. coli. Of the 48 designs ordered, 24 were soluble after SEC, and 7 designs showed a binding signal in biolayer interferometry (SI Appendix, Fig. S9A), a substantial improvement in success rate compared to the previous design round. We proceeded with three designs for further biophysical characterization and found that they bound with affinities ranging from 400 to 700 nM (Fig. 3E and SI Appendix, Figs. S9B, S13 and Table S2). The 2.5 Å resolution crystal structure of design 3DS18 in complex with hTfR very closely matches the computational model (Fig. 3F and SI Appendix, Fig. S10 A and B). The docked complexes of 3DS18 and 2DS25 are similar on the backbone level, but apart from the side chains in the docked-strand edge strand, none of the interface residues are the same (SI Appendix, Fig. S10 C and D).

### 2DS25 Crosses the BBB In Vitro.

Binding affinity is a key factor determining transcytosis efficiency of compounds targeting hTfR. For instance, monovalent or low-affinity antibodies were found to transcytose more efficiently than higher affinity antibodies, which instead were targeted for lysosomal degradation (11, 12, 18), suggesting an optimal $K_D$ exists for transcytosis. We hence took advantage of the SSM data to create a range of designs with different $K_D$’s (see Materials and Methods). The majority of the mutants that improved binding map to the interface between hTfR and 2DS25 and likely optimize packing interactions and electrostatic contacts (Fig. 4A–C and SI Appendix, Fig. S11A). Two mutants (A44G and I66L) that improved binding occurred in the core of 2DS25 distal to the interface; these may produce subtle conformational alterations that stabilize the interface (SI Appendix, Fig. S11B). Biolayer interferometry of five variants revealed $K_D$’s ranging from 20 to 400 nM (Fig. 4D and SI Appendix, Figs. S12 and S13 and Table S2).

We explored the potential of our designs for BBB traversal using a recently developed human in vitro BBB model that was created using microfluidic organ chip technology (19). These BBB chips contain two parallel microfluidic channels separated by a porous extracellular matrix-coated membrane; the membrane is lined by human-induced pluripotent stem cell-derived brain microvascular endothelial cells (iPS-BMVECs) on one side, creating a “vascular” channel and primary human brain pericytes and astrocytes on the other side of the membrane, creating the central nervous system (CNS) channel. These chips recapitulate key features of the human BBB including a permeability barrier similar to that observed in vivo, the expression of physiologically relevant multiple drug transporters and hTIRs that can recapitulate shuttling of anti-TIR antibodies across the BBB in vitro (19) and undergo in vivo-like transcytosis of brain-seeking extracellular vesicles in vitro (20). Following injection of three Alexa488-labeled designs spanning two orders of magnitude in $K_D$ (2DS25 2 μM, 2DS25.3 200 nM, and 2DS25.5 20 nM) into the vascular channel, we observed transport of 2DS25.3 and 2DS25.5 into the cerebral channel (Fig. 4E). Transport across the BBB was slightly higher for 2DS25.5 ($K_D$ 20 nM) compared to 2DS25.3 ($K_D$ 200 nM).

![Fig. 3.](Image)

**Fig. 3.** Structural analyses of 2DS25 complexes. (A) Overview of the designed model and crystal structure (gray: hTfR, yellow: 2DS25). (B) Superposition of the 2DS25.5 (yellow) and the designed model (light gray). (C) Superposition of the 2DS25.5-hTfR crystal structure (dark gray and yellow) and designed model (light gray) at the interface. (D) Overlay of hTfR ectodomain structures in the PDB (1cx8, 1de4, 1suv, 3kas, 6d04, 3s9l, 6h5i, 6wrv, and 6wrv). Edge strand backbones are colored in blue. Tyr211 is shown in thick yellow sticks. (E) Equilibrium binding curves 2DS25 and the designs 3DS2, 3DS10, and 3DS18. (F) Overlay of the crystal structure 3DS18 in complex with hTfR (green and dark gray) and the designed model (light gray).
An interface knockout (KO) variant of 2DS25 showed lower penetration into the CNS channel than 2DS25.3 and 2DS25.5, indicating the transport was specific. Transport of the base design 2DS25 was comparable to the interface KO, suggesting that the low $K_d$ (2 μM) hampers transport into the CNS channel. In all performed assays, barrier integrity was maintained throughout the experiment (Fig. 4F).

**Discussion**

Our method for computationally designing small proteins that bind to exposed beta strands and neighboring regions on protein targets considerably expands the possibilities for protein inhibitor design. “One-sided” interface design in which a protein is de novo designed to bind to a fixed target protein with high specificity and affinity has been largely limited until now to targets with surface hydrophobic patches, which can be complemented by appropriately shaped hydrophobic clusters on the designed protein. Our method now makes available the much more polar and less concave regions surrounding edge beta strands and hence increases the number of proteins of interest which can be targeted.

The relative orientation of the edge strand and flanking helices on our designed scaffolds were important for success. Truncation of the base ferredoxin scaffold and addition of helices yielded scaffolds with improved binding metrics when docked. Inspection of other edge strand–containing targets suggests that the overall structural context of the target edge strand is often similar to that in hTfR, with a central edge strand flanked by helices albeit with relative orientations that are different from in hTfR. Exhaustive sampling of the positions of helices relative to the edge strand on a designed scaffold should give rise to a family of scaffolds that can engage a large number of edge strand–containing target proteins.

The advantage of computational design over antibody and other selection methods in being able to choose the region of the target being bound is clear in the hTfR case; we selected a site far away from the transferrin binding site to avoid competition. The observation of strand shifting in the target structure highlights the importance of targeting regions which are relatively immobile; in the case of hTfR, a superposition of the available structures indicates high mobility at the edge strand, which we failed to take into account in the first design rounds. Moving forward, design against multiple target crystal structures or conformations produced by molecular dynamics simulations and other methods may be useful, particularly in cases in which multiple crystal structures are not available.

Our results on the in vitro BBB traversal of 2DS25.3 and 2DS25.5 are encouraging, but it must be emphasized that for more detailed characterization of the BBB traversal properties of our designs, in vivo experiments beyond the scope of this study will be required. Assessment of the kinetics of BBB traversal both in vitro and in vivo as well as comparison against current state of the art BBB shuttling systems (21, 22) will be important to evaluate the full potential of our designs for BBB traversal. Once further characterized, our small, stable designed hTfR binder, and similar designs against other targets at the BBB may provide new possibilities for transporting therapeutics and other molecular cargo into the brain. The small size (10 kDa) and different binding mode to hTfR may offer improved access to the brain compared to antibodies and the cognate ligand transferrin (which is 76 kDa). Given the high stability and modularity, and likely robustness to genetic fusion and chemical coupling, our

![Fig. 4. In vitro BBB traversal of 2DS25 variants. (A) Positions on 2DS25 that improve binding (C-alpha atoms as orange spheres). (B) The A27V substitution was highly enriched in site saturation mutagenesis and creates a snug packing interaction with a hydrophobic patch on hTfR. (C) On the opposite side of the interface, E74H, R77S, and K78T may improve electrostatic complementarity. (D) Bilayer interferometry equilibrium binding curves of 2DS25 and optimized variants. (E) Transcytosis of 2DS25 variants in the in vitro human BBB chip model. Measurement is taken after 3 h. *P value 0.033, **P < 0.0001, Dunnett’s test. (F) Barrier integrity of the BBB chips at 3 h. Bar plots show mean values with SD as error bars.](https://doi.org/10.1073/pnas.2021569118)
designs could have a distinct advantage over larger more complex molecules for fusion/coupling to therapeutic cargos.

Materials and Methods

Protein Design.

Identification of target edge strands. The transferrin receptor target protein (PDB: 3kas) was relaxed in the Rosetta energy function using coordinate constraints after removing hetero atom records. All target protein edge strands were identified visually by inspection in a molecular graphics viewer or programmatically by calculating the atomic solvent accessible surface area (aSASA) of all backbone H and O atoms present in residues that were in beta conformation. Strands with a length of at least three residues and an average aSASA value above 2 were considered solvent exposed and hence edge strands suitable for strand docking.

Geometric matching beta motifs to edge strands. The C-alpha atoms of short parallel and antiparallel two-stranded beta sheets derived from the PDB were aligned onto the target edge strand. The aligned segments of the motifs were next deleted. The docked strands were then either trimmed down further or extended at either the N or C terminus, creating a range of strands with different lengths. These docks were relaxed using gradient descent-based minimization in the presence of the target using Rosetta FastRelax to optimize backbone hydrogen bond interactions with the target edge strand. Docks failing a specified threshold value (typically −4) for the backbone hydrogen bond score term in Rosetta (hbond_lr_bb) were discarded. Matching, the resulting protein–protein complexes were repacked at the interface using the PackRotamersMover followed by carboxyl–amino sidechain hydrogen bond score term in Rosetta (hbond_lr_bb) were discarded.

Protein Purification and Expression.

De novo designed ferredoxin-like peptides and their variants were purchased from Integrated DNA Technologies or Genscript (see the spreadsheet in SI Appendix for detailed construct information) using the Daedalus expression system (30). After clearing the N-terminal expression tag with TEV, the protein was further purified by SEC. Peak fractions were biotinylated using an in vitro biotinylation kit (Avidity). Biotinylated TFR was further purified by Superdex 200 Increase 10/300GL in SEC buffer. Peak fractions were concentrated to ~1.5 mg/mL, flash-frozen, and stored at −80 °C.

For structural studies, a soluble fragment of the hTFR 1 ectodomain (residues 121 to 760) was cloned into the pHSec expression vector (31). We produced TFR in human embryonic kidney 293S GnT1−/− cells (ATCC CRL-3022) in suspension culture and maintained in serum-free Freestyle 200 (Fisons) containing 10 mM Hepes pH 7.5, 50 mM NaCl. Peak fractions were verified by SDS-polyacrylamide gel electrophoresis, a second PCR was performed to add pETCON adaptor sequences to both DNA ends to facilitate cloning into the yeast surface display vector pETCON. This gene pool was again purified by gel extraction.

Library Generation.

The gene library for the first generation hTFR binders was ordered from Agilent Technologies with flanking adaptor sequences to allow amplification of the genes. qPCR using Kapa HiFi Hotstart Ready Mix (Kapa Biosystems) was performed to amplify the library in order to prevent over-amplification of the genes. qPCR using Kapa HiFi Hotstart Ready Mix (Kapa Biosystems) was performed to amplify the library in order to prevent over-amplification of the genes. qPCR using Kapa HiFi Hotstart Ready Mix (Kapa Biosystems) was performed to amplify the library in order to prevent over-amplification of the genes. qPCR using Kapa HiFi Hotstart Ready Mix (Kapa Biosystems) was performed to amplify the library in order to prevent over-amplification of the genes. qPCR using Kapa HiFi Hotstart Ready Mix (Kapa Biosystems) was performed to amplify the library in order to prevent over-amplification of the genes.

Yeast Surface Display and Deep Sequencing. Myc-tagged designs were displayed on the yeast surface as Aga2p fusion proteins. The diversity of the libraries was below 10% in all cases. Yeast cells were grown at 30 °C in C-trp-ura-2% glucose

$$\Delta G_m = \frac{\Delta G_0}{m}$$

where $m$ is the observed signal, $S_0$ is the signal of the folded baseline, and $S_D$ is the signal of the denatured baseline. $S_D$ was obtained by

$$FD = 1 / (1 + e^{-\frac{S - S_0}{S_D - S_0}/G_0})$$

and $G_0$ is the observed signal,
media for 16 to 24 h before expression was induced by transferring cells to synthetic galactose medium supplemented with casamino acids media (SGC-AA) for 16 to 24 h at 30 °C. Cells were harvested by centrifugation and washed twice with PBSF (PBS supplemented with 1% bovine serum albumin). Cells were subsequently incubated with biotinylated hTFR for 0.5 to 2 h at room temperature before being washed twice with PBSF. These cells were next labeled with streptavidin–phycoerythrin (PE) and an FITC conjugated anti-Myc antibody (Immunochemistry Consultants Laboratory) for 20 min before being washed again. For initial screening for binding signals, biotinylated hTFR was preincubated with streptavidin–PE (in nitrogen) for 10 min before the complex was added to cells enabling the identification of weak binders by using avid binding conditions. Samples were sorted or measured in a Sony SH800 cell sorter or Accuri Flow cytometer (BD Biosciences) using the FITC and PE signals. Sorted cells were collected and grown in C-trp-ura+2% glucose media for 24 to 48 h before being frozen at −80 °C for later analyses. Serum albumin was added against 100 nM, 20 nM, and 7 nM hTFR, whereas the combination libraries were selected against 250 nM, 10 nM, 1 nM, 0.5 nM, 0.250 nM, and 0.125 nM hTFR.

DNA preparation for deep sequencing was performed as described before (35). DNA was sequenced using MiSeq sequencer with a 600-cycle reagent kit (Illumina). Reads were aligned with paired-end read merger software (36). Sequences were finally analyzed using custom scripts based on the Enrich software (37).

Combination Variants Generation. After deep sequencing analyses of the SSML library, we identified 13 positions in which individual mutations improved binding. Two approaches were followed to further optimize the binding affinity. First, a subset of selected mutants was manually combined and ordered as synthetic genes for testing in binding assays. This approach yielded 2DS25.3.

In the second approach we generated a combination library. We ordered two overlapping Ultramer oligonucleotides (Integrated DNA Technologies) containing 13 specified positions. For the 13 specified positions, sequences were finally analyzed using custom scripts based on the Enrich software (37).

Binding Interferometry. Binding assays were performed on an OctetRED96 BLI system (ForteBio) using streptavidin-coated biosensors. Biosensors were equilibrated for at least 10 min in Octet buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20) supplemented with 1 mg/mL bovine serum albumin (SigmaAldrich). For each experiment, the biotinylated hTFR ectodomain was immobilized onto the biosensors by dipping the biosensors into a solution with 10 to 50 nM hTFR for 200 to 500 s, followed by dipping in fresh octet buffer to establish a baseline for 200 s. Titrations were executed at 25 °C while rotating at 1,000 rpm. Association of designs to TFR on the biosensor was allowed by dipping biosensors in solutions containing designed proteins diluted in octet buffer for 90 s. After reaching equilibrium, the biosensors were dipped into fresh buffer solution in order to monitor the dissociation kinetics for 900 to 1,500 s. In single concentration assays, 1 μM design was used diluted in octet buffer. For equilibrium binding titrations, kinetic data were collected and processed using a 1:1 binding model to obtain the equilibrium binding response Req using the data analysis software 9.1 of the manufacturer. Multiple binding experiments with different proteins and concentrations under different hTFR immobilization densities were performed to ensure reproducibility. Representative binding curves are presented in the main text. Both global kinetic fitting using lower concentration data and steady-state saturation fits using data from all concentrations were performed for Kp calculations. For steady-state fits, in each design, seven Req values were fitted with a custom Python script to a saturation binding curve to obtain Bmax and the equilibrium dissociation constant KD.

In Vitro Human BBB Chip Traaversal Assays. Single cysteine variants (E3C) of 2DS25, 2DS25_KO, 2DS25.3, and 2DS25.5 used in the human BBB chip studies were expressed and purified as described above in the presence of reducing agent TCEP. Proteins were labeled with Alexa Fluor 488 CS maleimide (Thermo Fisher) and purified according to the manufacturer’s protocol. In vitro human BBB chips were generated as previously described (19). The human-induced pluripotent stem (IPS) cell line iMR90-C4 (WiCell Research Institute) was differentiated into human iPS-BMVECs using a hypoxia-induced differentiation protocol for mimicking the embryological developmental conditions for obtaining high expression of functional tight and adherens junctions, as well as efflux proteins and hTFR.

Two-channel microfluidic devices (obtained from Emulate Inc.) were activated with Sulfo-SANPAH (Thermo Fisher) treatment prior to coating the channels with collagen IV (400 μg/mL; Sigma Aldrich) and fibronectin (100 μg/mL; Sigma Aldrich) overnight. Both channels of the chip were rinsed with PBS and then with astrocyte medium before seeding cells. Primary human astrocytes (SciCell) and pericytes (SciCell) were cocultured in the brain channel of the BBB chip by mixing together 0.7 × 106 cells/mL astrocytes and 0.3 × 106 cells/mL pericytes in the astrocyte medium, seeding them in the apical channel of the chip, and then incubating them under static conditions at 37 °C. After 1 h, unattached astrocyte and pericytes were removed by washing both channels of the chip with endothelial cell (EC) medium containing fibroblast growth factor (20 ng/mL; R&D Systems) and retinoic acid (RA) (10 μM; Sigma Aldrich) (EC+RA), and then 2 μL iPS-BMVECs (2.3 × 106 cells/mL) were seeded in the basal channel, and the device was immediately placed upside down to allow the HMVECs to adhere to the porous membrane. After overnight incubation under static conditions at 37 °C, the chip was placed right side up and both channels of the BBB chip replaced with EC medium without growth factors were cultured under static conditions for one additional day. The next day (2 d after seeding) the BBB chips were attached to specialized chip holders with medium reservoirs (Pods; Emulate Inc.) to provide a source of EC medium to flow through both channels (60 μL/h) using the automated control features of the Zoë Culture Instrument (Emulate Inc.).

On the third day after seeding, Alexa Fluor 488 labeled 2DS25 variants at 400 nM in EC medium were flowed through the vascular channel of the BBB chip at 60 μL/h. All samples included 50 μg/mL cascad blue molecule (Thermo Fisher) to simultaneously monitor the barrier integrity of the chips during the experiments. Effluent samples were collected from both vascular and apical channels at 3 h intervals. The fluorescent intensity of the samples was normalized by the background intensity of the samples that were prepared with PBS and were flash frozen in well solution containing 20% (volume/volume) glycerol and 8% (volume/volume) polyethylene glycol monomethyl ether 550 and were flash frozen in well solution containing 20% (weight/volume) glycerol.

The 2DS25.5:ShTFR complex grew in 0.1 M BICINE pH 8.5 and 8% (weight/volume) polyethylene glycol monomethyl ether 550 and were flash frozen in well solution containing 20% (volume/volume) glycerol. Crystals of the 2DS25.5:ShTFR complex grew in 0.1 M BICINE pH 8.5 and 8% (weight/volume) polyethylene glycol monomethyl ether 550 and were flash frozen in well solution containing 20% (weight/volume) glycerol. Crystals of the 2DS25.1:ShTFR complex grew in 1.1 M Sodium Malonate pH 7.0, 0.1 M Heps pH 7.0, and 0.5% Jefflamine ED-2001 pH 7.0 and were flash frozen in mother liquor. Diffraction data for the complexes were collected at a wavelength of 0.979 and temperature of 100 K on Northeastern Collaborative Access Team Advanced Photon Source (APS) beamlines 24-ID-C and 38-ID-D.

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24-DE (APS, Argonne National Laboratory). Structures were determined by molecular replacement using PHASER with coordinates for TFR1 (PDB: 3kas) and coordinates for models of the respective designs as search models.

Data Availability. Crystal structures have been deposited in the RCSB PDB with the accession nos. 6WRX, 6WRV, and 6WRR. Additional supporting data has been deposited in the online Zenodo repository (https://zenodo.org/record/4594115) (47). All other study data are included in the article and/or supporting information.

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