Structure of the trypanosome transferrin receptor reveals mechanisms of ligand recognition and immune evasion

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To maintain prolonged infection of mammals, African trypanosomes have evolved remarkable surface coats and a system of antigenic variation. Within these coats are receptors for macromolecular nutrients such as transferrin. These must be accessible to their ligands but must not confer susceptibility to immunoglobulin-mediated attack. Trypanosomes have a wide host range and their receptors must also bind ligands from diverse species. To understand how these requirements are achieved, in the context of transferrin uptake, we determined the structure of a Trypanosoma brucei transferrin receptor in complex with human transferrin, showing how this heterodimeric receptor presents a large asymmetric ligand-binding platform. The trypanosome genome contains a family of around 14 transferrin receptors, which has been proposed to allow binding to transferrin from different mammalian hosts. However, we find that a single receptor can bind transferrin from a broad range of mammals, indicating that receptor variation is unlikely to be necessary for promiscuity of host infection. In contrast, polymorphic sites and N-linked glycans are preferentially found in exposed positions on the receptor surface, not contacting transferrin, suggesting that transferrin receptor diversification is driven by a need for antigenic variation in the receptor to prolong survival in a host.

Iron is essential for numerous cellular processes, including oxygen and electron transport. In mammals, iron is transported within serum and tissue fluids while bound to a transport protein, transferrin. Transferrin facilitates iron distribution while preventing toxic effects of free iron ions. Transferrin has two lobes, each binding a single Fe ion. These lobes adopt ‘open’ iron-free conformations in apo-transferrin, or ‘closed’ iron-bound conformations in holo-transferrin. Changes in pH and transferrin receptor binding each induce changes in transferrin conformation, modulating iron binding and release.

Acquisition of iron by mammalian cells requires a transferrin receptor. The receptor–transferrin complex is endocytosed and trafficked to an endosomal compartment. Here, acidification and receptor binding combine to induce a change in transferrin conformation from holo- to apo-, releasing iron. The receptor–transferrin complex is then recycled to the plasma membrane, where increased pH causes release of apo-transferrin, freeing the receptor for further uptake cycles.

Many pathogens scavenge iron from their mammalian hosts through uptake of transferrin. Bacterial pathogens have the challenge of inducing iron release from transferrin without involvement of an acidic endocytic compartment. In Neisseria, this is mediated by transferrin receptors on the outer membrane that bind holo-transferrin and induce release of iron at neutral pH. In contrast, African trypanosomes, such as T. brucei, are eukaryotic pathogens with an active endocytic system. They express transferrin receptors which are endocytosed to carry iron-loaded transferrin into endocytic compartments. How the receptor binds transferrin and the effect of pH changes in mediating iron and transferrin release are uncertain.

The trypanosome transferrin receptors show no sequence similarity to mammalian transferrin receptors. Instead, they are heterodimers of related proteins ESAG6 and ESAG7, attached to cell membranes by a single glycosylphosphatidylinositol (GPI) anchor on ESAG6. Both subunits are predicted to share a similar fold with variant surface glycoproteins (VSGs), which coat trypanosome surfaces. Transferrin receptors must recognize their ligand in the context of this densely packed VSG coat and be endocytosed in vesicles that bud from the flagellar pocket. Immunoglobulins access both trypanosome cell surface and flagellar pocket, raising the question of how transferrin receptors avoid detection.

The T. brucei genome contains a family of transferrin receptors, with 14 identified in the Lister 427 isolate, although only one is expressed at a time. The receptors vary in sequence, and growth in culture containing sera from different species has been shown to select for expression of different receptors. This suggested a model in which variation in receptor sequence was proposed to allow uptake of transferrin from different mammals, thereby enabling host promiscuity. Here, we used structural and biophysical analyses to investigate how trypanosome transferrin receptors bind transferrin, to determine their role in facilitating iron release and to investigate how polymorphisms affect species specificity and immune recognition.

Phylogenetic analysis of ESAG6 and ESAG7 sequences separates the receptors into groups, with BESS1 and BESS17 distant in the phylogenetic trees (Extended Data Fig. 1a). Crystals of BESS1 transferrin receptor bound to human transferrin formed and diffracted to 3.42Å resolution. Further crystals were obtained using a variant of BESS17 in which the eight N-linked glycosylation sites were mutated. These diffracted to 2.75Å resolution, allowing structure determination by molecular replacement (Fig. 1a and Extended Data Figs. 1b and 2).

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The structure reveals an elongated heterodimer of ESAG6 and ESAG7, each containing three long α-helices (Fig. 1). The N-terminal two helices map closely onto the corresponding long helices of the VSGs, while a third helix strengthens the fold (Extended Data Fig. 3). At the membrane-proximal side, each subunit contains a short α-helix that forms a wedge between the two subunits of the heterodimer. Thirty residues at the C-terminal end of ESAG6 were not resolved and most probably form a flexible polypeptide linking the receptor to the C-terminal GPI anchor. The membrane-distal surface of each subunit lacks secondary structure and is formed of a complex array of intertwined loops. As ESAG6 and ESAG7 have 80% sequence identity, it is unsurprising that they share similar folds (Fig. 1b). However, the membrane-distal loops adopt different conformations, allowing both subunits to contribute to an asymmetric binding site for a single transferrin molecule.

The extensive dimerization interface between the two subunits is stabilized by a network of hydrogen bonds, and subtle differences in loop conformations towards the membrane-distal end of each subunit are likely to favour formation of productive heterodimers rather than homodimers.

The receptor fits into a cleft in transferrin, making contacts primarily with the N-terminal domain of the N- and C-lobes, with the most extensive interface involving the C-lobe. (Fig. 1 and Extended Data Figs. 4 and 5). A structural comparison reveals that human12,21 and trypanosome receptors use entirely different structural features to bind a similar surface of transferrin (Fig. 2a). This may reduce the likelihood of transferrin escape mutants that prevent uptake into trypanosomes. When bound to the trypanosome receptor, the N-lobe of transferrin is in the open apo conformation while the C-lobe is in the closed holo-conformation, and electron

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**Fig. 1** | The structure of the trypanosome transferrin receptor. **a**, The structure of the trypanosome transferrin receptor heterodimer (ESAG6 in dark blue and ESAG7 in light blue) bound to human transferrin (red). The iron ion is shown as an orange sphere. **b**, An alignment of ESAG and ESAG7 showing the divergence of the membrane-distal loops to create an asymmetric binding site for transferrin.
In particular, we aimed to determine whether the receptor responds to pH changes in a similar way. For this purpose, we investigated whether the complex responds to pH changes in a similar way. In particular, we aimed to determine whether the receptor stimulates iron release, as our structure, determined from crystals grown at pH 6.5, showed an empty N-lobe and an iron-bound C-lobe (Fig. 2b). This led us to ask whether this state is due to a low pH or due to the presence of the receptor. We first determined apo- and holo-transferrin affinities at four different pH values (Fig. 2c). Binding of apo-transferrin was unaltered by pH change. In contrast, holo-transferrin bound most tightly at pH 7.5, while at pH 6.5, or lower, it had an affinity close to that of apo-transferrin. Compared with human transferrin receptor, bind-
ing of trypanosome transferrin receptor to transferrin varies as they experience pH changes during progression through the endo-

Fig. 2 | The role of the trypanosome transferrin receptor in pH-dependent transferrin binding and iron release. a, Structures of complexes of the trypanosome and human transferrin receptors bound to transferrin were aligned on the transferrin component. Transferrin is red, the trypanosome transferrin receptor heterodimer is blue (ESAG6 in dark blue and ESAG7 in light blue) and the human transferrin receptor is purple. b, Alignment of transferrin (red) bound to the trypanosome transferrin receptor (blue) with apo-transferrin (yellow; PDB: 2HAV) and holo-transferrin (green; PDB: 3V83). This shows that, when bound to the trypanosome transferrin receptor, the N-lobe of transferrin is in the apo conformation while the C-lobe is in the holo, iron-bound conformation. c, Analysis by surface plasmon resonance of binding to human transferrin by the trypanosome transferrin receptor at different pH values. Each concentration series was performed once. d, Analysis by pPIXE of the amount of iron bound to transferrin or transferrin in complex with the trypanosome transferrin receptor at different pH values. Data points represent technical replicates (n = 3) while bars represent the mean. RU, response units; Tf, transferrin.

density likely to be Fe^{3+} was found only in the C-lobe (Fig. 2b). This is in contrast to serum transferrin, in which both lobes are partially occupied by iron^{20}.22.

Binding of human transferrin receptor to transferrin varies as they experience pH changes during progression through the endo-

We next used microbeam particle-induced X-ray emission (μPIXE) to assess the amount of iron that remains bound to trans-
ferrin at four different pH values, either alone or bound to the receptor (Fig. 2d and Extended Data Fig. 7). Determination of the number of iron atoms relative to the number of sulfur atoms allowed calculation of the amount of iron per transferrin. At pH 7.5, either in the presence or absence of receptor, there were ~0.6 iron

The role of the trypanosome transferrin receptor in pH-dependent transferrin binding and iron release.

acidification, the trypanosome transferrin receptor reduces release. Coupled with the retention of apo-transferrin binding at neutral pH, this makes it likely that, far from recycling transferrin, the trypano-
some receptor maintains binding. Any remaining iron may then be released as a result of transferrin degradation in the lysosome.

African trypanosomes contain multiple transferrin receptors, with around 14 in the Lister 427 genome. This contrasts with other receptors, such as the single-copy haptoglobin-haemoglobin receptor^{17}. It has been proposed that diversity in transferrin recep-
tors accommodates differences in transferrin sequences, allowing trypanosomes to infect a wide range of mammals^{18}. To test this, we studied binding of BES1 and BES17 receptors (Extended Data Fig. 8) to transferrin from eight different mammalian species, representing a spectrum of the mammalian evolutionary tree (Extended Data Fig. 4).

The binding of transferrin variants was measured by surface plasmon resonance (Fig. 3c and Extended Data Fig. 6). BES17 bound all eight transferrin variants tested, with affinities ranging from 2.8 to 500 nM. As these are all significantly below the transferrin concentrations of ~30 μM in serum and ~15 μM in inter-
stitial fluid^{20}, BES17 would be nearly saturated with transferrin in all of these mammals. This will ensure efficient transferrin uptake in physiological conditions. Additionally, receptor saturation will ensure that the most exposed surface of the receptor is covered by transferrin and therefore not available for recognition by immuno-
globulins, reducing risk of immune detection and clearance.

The second receptor, BES1, bound more weakly to all eight transferrin variants, with submicromolar affinity for transferrin from just three species (Extended Data Fig. 6), and little or no binding detected to four. In no case was the affinity of any transferrin for BES1 greater than that for BES17. To assess which molecular features of BES1 reduce transferrin binding, we first made chimeric receptors containing ESAG6 from BES1 and ESAG7 from BES17 or vice versa. We assessed binding of these to human transferrin. BES17 binds with an affinity of 13.1 nM while BES1 shows negligible...
However, both chimeras showed an intermediate affinity (243 nM and 353 nM), revealing that both ESAG6 and ESAG7 contribute to binding (Extended Data Figs. 6 and 9). We next selected four residues close to the transferrin binding site that are polymorphic (ESAG6 residue 139, and ESAG7 residues 246, 229 and 233) (Extended Data Fig. 8). Mutation of each of these four sites in BES1 increased its affinity for both rat and human transferrin, with four mutations leading to the greatest increase in affinity (BES1mut) (Extended Data Figs. 6 and 9). This mutant bound all eight transferrin variants with a higher affinity than BES1 receptor (Fig. 3 and Extended Data Fig. 6).

We next tested whether growth of BES1 receptor-expressing T. brucei occurs in sera containing transferrin molecules for which this receptor has low affinity. We compared growth of trypanosomes in fetal calf serum (dissociation constant (K_d) = 80.1 nM) with that in rabbit (K_d = 3.5 μM), pig (weak, K_d not determinable) or horse (weak, K_d not determinable) sera (Fig. 3d). In all four cases, cells were grown for 72 h in fetal calf serum (cow transferrin), before exchange into media containing 10% of exclusively cow, rabbit, pig or horse transferrin.

While growth rates over the next 309 h were not identical, trypanosomes grew in all four sera. Indeed, transferrins with very different affinities for BES1 receptor supported similar growth rates, with doubling times in cow transferrin of ~5.9 h, similar to those in horse transferrin of ~6.3 h. We next assessed, by sequencing reverse-transcribed messenger RNA, whether trypanosomes had switched transferrin receptor expression (Extended Data Fig. 10). Trypanosomes grown for 72 h in fetal calf serum expressed BES1. After a further 309 h in different sera, the predominant receptor remained BES1 in all four cases. However, a small fraction of other receptors were present, showing that the experiment had been conducted for long enough to detect switching. Nevertheless, the fraction of other receptors expressed was no greater when trypanosomes were grown in low-affinity horse or pig transferrin than in high-affinity cow transferrin or moderate-affinity rabbit transferrin. Therefore, while these different receptors may have greater affinity
for transferrin than the original BES1, they are no more likely to be expressed when cells are grown in transferrin with low BES1 affinity, suggesting a lack of selection to switch away from BES1 based on transferrin affinity.

These findings suggest that BES1 has not evolved to bind more tightly to transferrin from a particular species, as it has reduced affinity for all transferrin variants, most probably due to evolutionary drift in the absence of selection for high-affinity transferrin binding. This, together with the discovery that a single transferrin receptor can bind to transferrin from all tested mammals with sub-micromolar affinity, makes it likely that multiple transferrin receptors are not required for iron acquisition in multiple mammals and that selection for multiplication and divergence of this receptor family is due to an evolutionary pressure other than the need for transferrin uptake in different mammals.

As receptor variation appears unlikely to be required to facilitate a broad host species range, it might instead aid long-term avoidance of the host adaptive immune response through serial expression of antigenic variants. Shannon sequence entropies were calculated for each residue of the transferrin receptor to assess their degree of polymorphism and the location of polymorphic residues. If variation is driven by a selection pressure to alter transferrin binding, we would expect residues that directly contact transferrin to be most divergent. However, if diversification is driven by evasion of immune...
A more likely hypothesis is that multiple receptors are present to avoid inhibition of transferrin uptake by antibodies raised against the transferrin binding site. Switching receptor to a novel antigenic variant could reset the system to prevent such competition. Indeed, our studies support a model in which evolution of a panel of transferrin receptors most probably allows antigenic variation, as we find that polymorphism is concentrated not predominantly in residues that contact transferrin, but across the most exposed, membrane-distal third of the receptor.

Such a panel will allow antigenic switching to prevent antibodies from competing for transferrin binding and will aid immune evasion. Perhaps the broader and most exposed platform of the transferrin receptor is more likely to be detected by antibodies than the narrower haptoglobin-haemoglobin receptor, increasing the likeliness of detection. A panel of transferrin receptors has therefore evolved to allow a population of trypanosomes to survive, under immune onslaught, for longer in their mammalian host.

Methods

Transferrin receptor expression and cloning. To produce glycosylated transferrin receptors, the open reading frames of BES17 ESAG6 (BES17e6) and BES1 ESAG6 (BES1e6) were modified to remove the native signal peptide and predicted GPI anchor addition site. These genes (residues 18–375 for BES17 and 20–377 for BES1) were optimized, synthesized (GeneArt) and subcloned into the pDest12 vector for mammalian expression from the Cytomegalovirus promoter. The CD33 signal peptide was used for recombinant protein secretion and C-terminal AviTag and decahistidine tags preceded by a (GS)3 linker were included. The open reading frames of BES17 ESAG7 (BES17e7) and BES1 ESAG7 (BES1e7) were modified to remove the native signal peptide: Genes (residues 18–337 for BES17 and 20–338 for BES1) were codon optimized, synthesized and subcloned into the pDest12 vector. The CD33 signal peptide was used for secretion and a C-terminal (GS)3 linker and Strep-II tag were present. Glycosylated transferrin receptor was used for all studies except for structure determination.

To produce non-glycosylated, tagless transferrin receptor for structural studies, the open reading frame of BES17e6 was modified to remove the signal peptide and predicted GPI anchor addition site. A TEV cleavable N-terminal polyhistidine-tag was added and predicted N-linked glycosylation sites were mutated, by changing asparagine to aspartic acid (N26Q, N110Q, N235Q, N250Q, N360Q). The C-terminal linker and tags were deleted. BES17e7 was modified by removal of the signal peptide, removal of glycosylation sites by mutation of asparagine to aspartic acid (N26Q, N110Q, N234Q) and deletion of the C-terminal linker and Strep-II tag. These open reading frames were cloned into the pDest12 vector for mammalian cell expression from Cytomegalovirus promoter with a CD33 signal peptide.

For protein production, G22 CHO cells were grown in 500 ml serum-free CCMe8 medium (SACF). Cells were cotransfected with recombinant BES17e6 and BES17e6 plasmids in equal mass ratios and were fed on day 1, day 3 and day 6 by the addition of 3.3% F9 and 0.2% F10 cell feed (MedImmune). Cells were collected on day 8 and the aqueous phase containing secreted recombinant protein was recovered after centrifugation.

Transferrin receptor complexes were purified by nickel affinity chromatography using a HisTrap EXCEL 5 ml column (GE Healthcare). To prepare complexes for crystallization, human holo-transferrin (Sigma Aldrich, T4132) was mixed with non-glycosylated transferrin receptor in a 1:1 molar excess with antibodies raised against the transferrin receptor. Kifunensine (Sigma Aldrich, K1140) was used to block proteolysis. The purified complexes were then subjected to size exclusion with a Superdex 200 16/60 chromatography column (GE Healthcare) with a running buffer of 10 mM Tris pH 8, 150 mM NaCl. For structural studies of glycosylated receptor, BES17e6 and BES17e7 plasmids were cotransfected into G22 CHO cells in the presence of 5 µM Kifunensine (Sigma Aldrich, K1140) to generate homogeneous mannose-rich glycans and were isolated by nickel affinity chromatography. Carboxybisepoxide B (Roche, 1010323001) was also added before size exclusion chromatography.

For surface plasmon resonance, G22 CHO cells were cotransfected with BES17e6 and BES17e7 (BES17); BES16 and BES17e7 (BES1); BES16 G139R mutant and BES17e7 Y246S, I229V, C232R (BES1mut); BES16e7 and BES17e7 (BES1e7); BES16 and BES17e7 chimera (BES16e7 BES17e7); BES16 G139R mutant and BES17e7 (BES1 ESAG6 G139R); BES16 and BES17e7 Y246S and I229V mutant (BES1 ESAG7 S246Y I229V); BES16 G139R mutant or BES17e7 S246Y I229V mutant (BES1 ESAG6 G139R ESAG7 S246Y I229V). All receptors were isolated by nickel affinity chromatography as described in the previous paragraph.

Crystallization and structure determination. Concentrated protein (13.7 mg ml⁻¹) was subjected to sitting drop vapour diffusion crystallization trials in SwisSci
96-well plates by mixing 100 nL protein with 100 nL reservoir solution. Crystals of complexes containing either glycosylated or non-glycosylated BSE17 transferrin receptor and human transferrin were obtained in 12% (w/v) polyethylene glycol 5,000 MME, 12% 2-methyl-2,4-pentanediol, 0.1 M MES, pH 6.5 at 18°C. Crystals were transferred into 12% (w/v) polyethylene glycol 5,000 MME, 25% 2-methyl-2,4-pentanediol and 0.1 M MES, pH 6.5, and were then cryo-cooled in liquid nitrogen for storage and data collection. Data were collected on beamline 103 at the Diamond Light Source at 100 K with a wavelength of 1 A and were indexed and scaled using the HKL package. Crystals of the glycan-lacking mutant diffracted to 2.7 Å resolution and were used for subsequent structure determination. Phaser was used to determine a molecular replacement solution. First, a search was conducted to locate human transferrin with the two lobes separated into separate search models (residues 3–330 and 339–674 from Protein Data Bank (PDB) code 1KF0)25. The solution from this search fixed in place, a second step used a highly refined search (Model containing just the helical core of a dimeric VSG (residues 7–112 and 239–251 from PDB code 2VSG))29. This identified one copy of the helical core of a dimeric VSG and scaled using XDS26. Crystals of the glycan-lacking mutant diffracted to pH 4.8, 150 mM NaBr; (2) 50 mM sodium citrate pH 5.5, 150 mM NaBr; (3) 50 mM Bis-Tris pH 6.5, 150 mM NaBr; and (4) 50 mM Bis-Tris pH 7.5, 150 mM NaBr. In each case, buffers had been titrated with citric acid to the desired pH. As a control, human transferrin, in the absence of receptor, was purified through the same protocol. Samples were concentrated to 1 mM for analysis to the retained iron. The measurements were carried out at the Ion Beam Centre, University of Surrey, UK. A 2.5-MeV proton beam of diameter 2.0 μm was used to induce characteristic X-ray emission from dried protein droplets (volume per droplet ~0.1 μl) under vacuum. The X-rays were detected in a solid-state lithium-drifted silicon detector with high-energy resolution. By scanning the proton beam in x and y over the dried sample, spatial maps were obtained of all elements heavier than magnesium present in the sample. Quantitative information, using sulfur as an internal standard, was obtained by collecting three or four point spectra from each droplet. These spectra were analysed with GUPIX27 within DAN32 (ref. 27) to extract the relative amount of each element, particularly iron, in the sample. Comparison of the quantities of sulfur and iron allowed determination of the number of iron ions per protein complex.

**Surface plasmon resonance analysis.** Native transferrin variants for surface plasmon resonance analysis were purified from serum using transferrin receptor affinity chromatography. Cow serum (Sigma Aldrich, B9433), horse serum (Sigma Aldrich, H1270), pig serum (Sigma Aldrich, P9783), mouse serum (Sigma Aldrich, M5905), rabbit serum (ThermoFisher, 16120099), rat serum (ThermoFisher, 10710 C), and human serum (ThermoFisher) were used. Due to limited volumes of donor serum, human holo-transferrin (Sigma Aldrich, T4132) was also used after confirming by surface plasmon resonance that there were no significant differences in binding kinetics. Human apo-transferrin (Sigma Aldrich, T1147) was dialysed in the presence of 10 mM EDTA into 10 mM HEPES pH 7.4, 150 mM NaCl for 15 h to remove any residual traces of free iron. BSE17 receptor was immobilized via amine coupling to a 1-ml HiTrap NHS-activated HP column (GE Healthcare) following the manufacturer's protocol. Serum was passed over the column and washed with PBS. Transferrin was eluted with 100 mM citrate pH 3.5 and dialysed into PBS. It was loaded with iron by incubating with a fourfold molar excess of iron (III) sulfate for 1 h in the presence of 5 mM sodium bicarbonate32. Free iron was removed by dialysis into 10 mM HEPES pH 7.4, 150 mM NaCl. Avi-tagged transferrin receptors for surface plasmon resonance were biotinylated by incubation of 30 μM receptor with 0.4 μM BirA, 0.3 mM biotin and 5 mM ATP at 25 °C for 16 h.

Surface plasmon resonance was performed using the Biotin CAPture kit (GE Healthcare) on a Biacore T200 instrument (GE Healthcare). Biotinylated receptor was immobilized onto flow path 2 of a Biotin CAPture chip (GE Healthcare) to a total of ~500 response unit, while flow path 1 was left without receptor as a blank. Most experiments were run at 25°C in 10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% Tween-20. However, for the pH assay, a phosphate-citrate buffer system was used to mimic the endocytic pH range with 50 mM citric acid, 150 mM NaCl, 0.05% Tween-20 and 50 mM NaH2PO4, 150 mM NaCl, 0.05% Tween-20 mixed to achieve pH 6.5, 5.5 and 4.8. Twofold serial dilutions of purified native transferrin were injected over the receptor-coupled chip for 240 s, followed by a 500 s dissociation time. The chip was regenerated using CAPture regeneration solution (GE Healthcare) after each cycle. Inhibition of the receptor-bound complexes in the blank reference flow cell was used to eliminate non-specific signals. Blank-subtracted sensograms were fitted to a 1:1 interaction model and kinetic rates were determined using T200 evaluation software.

**Data availability** Crystallographic data, including molecular models and reflection data, are available in the Protein Data Bank with accession codes 6SOY and 6SOZ. All other data and constructs are available on request from the corresponding authors.

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Author contributions
C.E.T. performed protein production, crystallization and surface plasmon resonance analysis. C.E.T. and M.K.H. determined the crystal structure. C.E.T., O.J.S.M. and M.C. conducted growth and expression analyses. P.G.W. and E.F.G. performed and analysed μPIXE experiments. A.L.G., S.R., T.J.V. and R.M. participated in design and experimental coordination. C.E.T., M.C. and M.K.H. devised the study and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41564-019-0589-0. Supplementary information is available for this paper at https://doi.org/10.1038/s41564-019-0589-0. Correspondence and requests for materials should be addressed to M.C. or M.K.H. Reprints and permissions information is available at www.nature.com/reprints. Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. © The Author(s), under exclusive licence to Springer Nature Limited 2019
Extended Data Fig. 1 | Phylogenic analysis of transferrin receptors. a. Evolutionary trees showing the relatedness of different transferrin receptor variants within the blood stream expression sites of the Lister 427 strain. The BES1 and BES17 receptors are highlighted in red. b. Comparison of the BES17 ESAG6 and ESAG7 proteins. The black box indicates the signal sequence and the dashed black box indicates the GPI-anchor addition site. Blue cylinders represent the helical regions of the structure. Red stars mark residues which make direct contacts with human transferrin. Orange highlighting of the text indicates residues which differ between ESAG6 and ESAG7.
### Data collection

|                      | Non-glycosylated | Glycosylated |
|----------------------|-------------------|--------------|
| Space group          | C121              | C121         |
| Cell dimensions      |                   |              |
| $a$, $b$, $c$ (Å)    | 163.49, 108.11, 115.00 | 128.18, 117.87, 134.55 |
| $\alpha$, $\beta$, $\gamma$ (°) | 90.0, 128.74, 90.0 | 90.0, 111.5, 90.0 |
| Resolution (Å)       | 46.3 – 2.75 (2.80 - 2.75) | 39.6 – 3.42 (3.48 – 3.42) |
| $R_{merge}$          | 9.3 (99.9)        | 4.9 (116.3)  |
| $I / \sigma I$       | 9.1 (1.2)         | 11.6 (1.1)   |
| Completeness (%)     | 98.9 (99.8)       | 99.0 (99.5)  |
| Redundancy           | 59.95             | 152.06       |

### Refinement

|                      | Non-glycosylated | Glycosylated |
|----------------------|-------------------|--------------|
| Resolution (Å)       | 2.75Å             | 3.42Å        |
| No. reflections      | 38216             | 25066        |
| $R_{work} / R_{free}$| 18.3/23.3         | 21.1/24.1    |
| No. atoms            |                   |              |
| Protein              | 10005             | 10048        |
| Ligand/ion           | 15                | 163          |
| Water                | 38                | 0            |
| $B$-factors          |                   |              |
| Protein              | 79.0              | 216.9        |
| Ligand/ion           | 111.3             | 265.0        |
| Water                | 66.6              | 0            |
| R.m.s. deviations    |                   |              |
| Bond lengths (Å)     | 0.01              | 0.01         |
| Bond angles (°)      | 1.21              | 1.22         |

*One crystal was used to obtain each data set.
*Values in parentheses are for highest-resolution shell.

**Extended Data Fig. 2** | Data collection and refinement statistics. Table of data collection and refinement statistics for crystal structures.
Extended Data Fig. 3 | Comparison of the transferrin receptor with VSGs and the haptoglobin-haemoglobin receptor. a. Structural alignment of the transferrin receptor with known VSG structures, 1VSG (orange), 2VSG (yellow) and 5LY9 (pink) and the Trypanosoma brucei haptoglobin–haemoglobin receptor (TbHpHbR, red). b. Sequence alignment of the two subunits of the transferrin receptor with the three VSGs. Orange highlighting indicates divergent residues.
Extended Data Fig. 4 | Sequence alignment of mammalian transferrin variants analysed in this study. a, Phylogenetic tree of placental mammals adapted from ref. 36. b, Sequence alignment of selected transferrin variants. Variable residues are highlighted in orange and residues which directly contact the BES17 transferrin receptor are indicated by red stars. Green circles identify residues which contact the human transferrin receptor.
### Extended Data Fig. 5 | Table of interactions

Table of interactions between the trypanosome transferrin receptor and transferrin.

| Chain | Residue | Group   | Chain | Lobe | Residue | Group   | Type of interaction |
|-------|---------|---------|-------|------|---------|---------|--------------------|
| A     | R139    | Side chain | C     | N    | A54     | Backbone CO | Hydrogen bond      |
| A     | D221    | Side chain | C     | C    | H349    | Side chain  | Hydrogen bond      |
| A     | T222    | Side chain | C     | C    | H349    | Side chain  | Hydrogen bond      |
| A     | T222    | Side chain | C     | C    | E372    | Side chain  | Hydrogen bond      |
| A     | G228    | Backbone CO | C     | C    | R352    | Side chain  | Hydrogen bond      |
| A     | G228    | Backbone CO | C     | C    | S370    | Backbone NH | Hydrogen bond      |
| A     | Y248    | Side chain | C     | C    | H349    | Side chain  | Aromatic stacking  |
| A     | Y266    | Side chain | C     | C    | H349    | Side chain  | Hydrogen bond      |
| B     | Y18     | Side chain | C     | N    | T330    | Side chain  | Hydrogen bond      |
| B     | Y18     | Side chain | C     | N    | R324    | Side chain  | Cation-π            |
| B     | Y18     | Side chain | C     | N    | N325    | Side Chain  | Hydrogen bond      |
| B     | E19     | Side chain | C     | N    | Y71     | Side chain  | Hydrogen bond      |
| B     | E19     | Side chain | C     | N    | R324    | Side chain  | Hydrogen bond      |
| B     | E19     | Side chain | C     | N    | K312    | Side chain  | Hydrogen bond      |
| B     | N20     | Side chain | C     | C    | N383    | Side chain  | Hydrogen bond      |
| B     | G138    | Backbone CO | C     | C    | R352    | Side chain  | Hydrogen bond      |
| B     | S140    | Backbone CO | C     | C    | R352    | Side chain  | Hydrogen bond      |
| B     | S140    | Side chain | C     | C    | S359    | Side chain  | Hydrogen bond      |
| B     | S141    | Side chain | C     | C    | C368    | Backbone NH | Hydrogen bond      |
| B     | Q142    | Side chain | C     | C    | R352    | Side chain  | Hydrogen bond      |
| B     | Q142    | Side chain | C     | C    | C368    | Backbone CO | Hydrogen bond      |
| B     | N150    | Side chain | C     | C    | S359    | Side chain  | Hydrogen bond      |
| B     | N150    | Side chain | C     | C    | E367    | Side chain  | Hydrogen bond      |
| B     | R213    | Side chain | C     | C    | E367    | Side chain  | Hydrogen bond      |
| B     | D222    | Side chain | C     | N    | N76     | Side chain  | Hydrogen bond      |
| B     | H265    | Side chain | C     | N    | P74     | Side chain  | Stacking           |
Extended Data Fig. 6 | Surface plasmon resonance analysis. Measurement of kinetic binding parameters for transferrin receptor variants to transferrins. BES1mut has a G139R mutation in ESAG6 and S246Y I229V and C223R in ESAG7. ESAG6 is e6 and ESAG7 is e7 in the table.

| Receptor | pH | Transferrin | $K_0$ (nM) | $k_{on}$ (M$^{-1}$s$^{-1}$) | $k_{off}$ (s$^{-1}$) |
|----------|----|-------------|-------------|-----------------------------|-------------------|
| BES17    | 7.4| Human       | 13.1        | 1.6$\times$10$^5$           | 2.0$\times$10$^3$ |
| BES17    | 7.4| Rabbit      | 15          | 1.8$\times$10$^5$           | 2.7$\times$10$^3$ |
| BES17    | 7.4| Mouse       | 75.3        | 3.6$\times$10$^5$           | 2.8$\times$10$^2$ |
| BES17    | 7.4| Rat         | 500         | 1.3$\times$10$^5$           | 6.3$\times$10$^2$ |
| BES17    | 7.4| Cow         | 2.8         | 2.7$\times$10$^5$           | 7.5$\times$10$^4$ |
| BES17    | 7.4| Goat        | 1.4         | 2.5$\times$10$^5$           | 3.5$\times$10$^4$ |
| BES17    | 7.4| Horse       | 3.7         | 2.4$\times$10$^5$           | 8.9$\times$10$^4$ |
| BES17    | 7.4| Pig         | 6.2         | 1.5$\times$10$^5$           | 9.1$\times$10$^4$ |
| BES1     | 7.4| Human       | ND          | ND                          | ND                |
| BES1     | 7.4| Rabbit      | 3550        | 5.3$\times$10$^4$           | 1.9$\times$10$^1$ |
| BES1     | 7.4| Mouse       | 351         | 2.6$\times$10$^5$           | 9.0$\times$10$^2$ |
| BES1     | 7.4| Rat         | 2868        | 1.2$\times$10$^5$           | 3.6$\times$10$^1$ |
| BES1     | 7.4| Cow         | 80.1        | 4.5$\times$10$^5$           | 3.6$\times$10$^2$ |
| BES1     | 7.4| Goat        | 238         | 2.8$\times$10$^5$           | 6.7$\times$10$^2$ |
| BES1     | 7.4| Horse       | ND          | ND                          | ND                |
| BES1     | 7.4| Pig         | ND          | ND                          | ND                |
| BES1mut  | 7.4| Human       | 303         | 6.6$\times$10$^4$           | 2.0$\times$10$^2$ |
| BES1mut  | 7.4| Rabbit      | 98.8        | 2.1$\times$10$^5$           | 3.9$\times$10$^3$ |
| BES1mut  | 7.4| Mouse       | 146         | 2.9$\times$10$^5$           | 4.2$\times$10$^2$ |
| BES1mut  | 7.4| Rat         | 1107        | 1.3$\times$10$^5$           | 1.4$\times$10$^3$ |
| BES1mut  | 7.4| Cow         | 3.2         | 6.5$\times$10$^5$           | 2.1$\times$10$^3$ |
| BES1mut  | 7.4| Goat        | 7.6         | 4.1$\times$10$^5$           | 3.1$\times$10$^3$ |
| BES1mut  | 7.4| Horse       | 254         | 3.1$\times$10$^5$           | 8.0$\times$10$^2$ |
| BES1mut  | 7.4| Pig         | 1270        | 9.6$\times$10$^4$           | 1.2$\times$10$^1$ |
| BES1e6   | 7.4| Human       | 353         | 8.9$\times$10$^4$           | 3.1$\times$10$^2$ |
| BES1e6   | 7.4| Human       | 243         | 1.2$\times$10$^5$           | 2.8$\times$10$^2$ |
| BES17e6  | 7.4| Human       | 2607        | 7.2$\times$10$^4$           | 1.9$\times$10$^1$ |
| BES17e6  | 7.4| Rat         | 2791        | 1.1$\times$10$^5$           | 3.1$\times$10$^1$ |
| BES17    | 7.4| Human       | 1150        | 7.3$\times$10$^4$           | 8.3$\times$10$^2$ |
| BES17    | 7.4| Rat         | 2068        | 1.3$\times$10$^5$           | 2.7$\times$10$^1$ |
| BES17    | 7.4| Human       | 411         | 7.2$\times$10$^4$           | 2.9$\times$10$^2$ |
| BES17    | 7.4| Rat         | 1070        | 1.4$\times$10$^5$           | 1.6$\times$10$^1$ |

**Receptors**: BES1, BES1mut, BES17, BES1mut

**Transferrins**: Human, Rabbit, Mouse, Rat, Cow, Goat, Horse, Pig

**Kinetic Parameters**: $K_0$, $k_{on}$, $k_{off}$
Extended Data Fig. 7 | Elemental analysis by mPXIE to determine the number of Fe ions per transferrin or transferrin:TfR complex at different pH values. Plots of counts per channel for emission induced by different photon energies, annotated with the element responsible for emission. Comparison of the quantities of Fe and S, together with knowledge of the protein sequences, and the numbers of S atoms, allowed determination of the number of Fe ions per transferrin or transferrin:TfR complex. Data shown is representative of three technical replicates.
Extended Data Fig. 8 | Comparison of the sequences of BES17 and BES1 transferrin receptors. Sequence alignments of ESAG6 subunits and ESAG7 subunits. Numbering is according to the BES17 sequence, which matches that of the crystal structure. Residues highlighted in orange vary between the BES1 and BES17 variants. Residues indicated by a red star directly contact human transferrin in the BES17 receptor. Residues indicated by a blue triangle are those mutated in the BES1mut mutant. Residues boxed with a continuous line represent the putative signal peptides while those boxed with a discontinuous line represent the GPI-anchor addition sequence.
Extended Data Fig. 9 | SPR analysis of transferrin receptor variants and mutants. **a.** Analysis of the binding of chimeric receptors formed between the ESAG6 and ESA7 subunits of the BES1 and BES17 transferrin receptor variants to human transferrin by surface plasmon resonance. **b.** Analysis of the binding to human and rat transferrin of mutants of the BES1 transferrin receptor. Each concentration series was performed once.
Extended Data Fig. 10 | Analysis of transferrin receptor expression during growth in sera from different mammals. a Sequences of ESAG7 from different transferrin receptor variants within the blood stream expression sites of the Lister 427 strain, showing nucleotide sequences equivalent to 778 to 837 in the BES1 sequence. Below this are shown the predominant sequences found in Trypanosoma brucei cells grown in serum exclusively containing cow, horse, rabbit and pig transferrin for 309 hours. The red lettering and red star indicate a sequence difference at position 798 in which a T is unique to the BES1 receptor. b Sequencing chromatograms of the expressed transferrin receptor variants in cells, showing nucleotides equivalent to 791 to 805 in the BES1 sequence. The star marks residue 798, which is T in BES1 and C in the other receptors in the Lister 427 strain. The top chromatogram shows that Trypanosoma brucei grown in fetal calf serum (cow) for 72 hours predominantly express BES1. These cells were transferred into media containing serum from cow, pig, horse or rabbit and were grown for a further 119 hours (the 191h time point) and 309 hours (the 381h time point) and the chromatograms show the transferrin receptor sequences expressed at these times remains predominantly BES1, with lower levels of other receptors in all four sera. A similar outcome was seen for ESAG6 sequences (not shown).
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Software and code

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Data collection
Crystallographic data was collected using software at Diamond Light Source beamline I03. SPR data was collected using standard Biacore T200 Control Software v 2.0. The software used to collected microPIXE data is bespoke. It is described in the experimental procedures and is standard for this technique.

Data analysis
Crystallographic data was analysed using XDS, Phaser and Buster; all standard and freely available software packages. Surface plasmon resonance data was analysed using the standard Biacore Evaluation Software v 2.0. MicroPIXE data was analysed using published software as described.

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Life sciences study design

Data from a single crystal was used to determine each of the two crystal structures shown. Surface plasmon resonance data, as presented, was conducted once with multiple independent measurements, at different concentrations, recorded for each experimental pair of binding partners. The microPIXE measurements were recorded as three technical replicates, with the average and the individual data points shown. Growth curves were representative of two independent replicates and sequencing was performed twice.

Data exclusions

No data was excluded

Replication

Crystallographic data for the glycosylated and non-glycosylated forms of the receptor show consistency of structure. Surface plasmon resonance data sets were not repeated but involve multiple independent measurements at different concentrations within each curve series, which are consistent. For microPIXE, individual measurements were taken in triplicate and variation is shown as individual data points. For growth curves, two independent experiments gave indistinguishable outcomes.

Randomization

We did not consider randomization necessary as the measurements taken did not involve subjective selection of which data images to show. The structural biology, surface plasmon resonance analysis, microPIXE analysis, growth curves and sequencing data are shown in full without selection.

Blinding

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