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**ONLINE METHODS**

**General.** Biotin (Sigma) was dissolved in DMSO at 100 mM. Avidin (Sigma) was dissolved in PBS at 23 μM. SDS-PAGE was performed at 200 V with the gel box (X Cell SureLock, Invitrogen) surrounded by ice to prevent dissociation of the streptavidin subunits during electrophoresis. Structures of streptavidin without biotin (1swa) and with biotin (1mk5) were displayed and aligned using PyMOL (DeLano Scientific). *Fig. 3b* was constructed with PyMOL based on crystal structures 1swe, 2iuu and 2ve9. Note that FtsK-γ is shown in the conformation bound to KOPS and is likely to change when translocating12.

**Plasmid construction.** Streptavidin in this paper refers to core streptavidin with His6 at the C-terminus in pET21a(+). Traptavidin (GenBank GU952124) was generated by introducing the S52G R53D mutation into streptavidin by QuikChange™ (Stratagene) using the primer S52G R53D F (Supplementary Table 1) and its reverse complement. The mutations were confirmed by DNA sequencing. AP-IGF1R was constructed from pcDNA3 containing human IGF1R (a kind gift from Val Macaulay, University of Oxford) by PCR of two fragments, the first fragment with primers IGFA and IGFB and the second fragment with IGFC and IGFD (Supplementary Table 1). The fragments were joined by overlap extension PCR, digested with NheI and NotI and ligated into pcDNA3.1. The acceptor peptide17 (GLNDIFEAQKIEWHE) was thus inserted after the IGF1R signal sequence along with a 6 amino acid spacer before the start of the N-terminus of mature IGF1R. BirA-ER (*E. coli* biotin ligase targeted to the endoplasmic reticulum) and pECFP-H2B (human histone H2B fused to enhanced cyan fluorescent protein) have been previously described18.

**Streptavidin and traptavidin expression and purification.** An overnight culture of streptavidin or traptavidin, picked from a freshly grown colony of *E. coli* BL21 (DE3) RIPL (Stratagene), was diluted 100-fold into LB ampicillin, grown to OD600 0.9 at 37 °C, induced with 0.5 mM IPTG, and incubated for a further 4 h at 37 °C. Inclusion bodies were isolated from the cell pellet of a 750 mL culture by rocking with 10 mL 300 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.8 mg/mL lysozyme, 1% Triton X-100 pH 7.8 for 30 min at 25 °C followed by 9 min pulsed sonication on ice at 40% amplitude on a Sonics Vibra-Cell sonicator. Following centrifugation at 27,000 g for 15 min, the inclusion body pellet was washed three times in 10 mL 100 mM NaCl, 50 mM Tris, 0.5% Triton X-100 pH 7.8 and then dissolved in 6 M guanidinium hydrochloride pH 1.5 (GuHCl). Protein in GuHCl was refolded by rapid dilution into PBS at 4 °C and stirring overnight19. Ni-NTA resin (Qiagen), equilibrated in 300 mM NaCl, 50 mM Tris, 10 mM imidazole pH 7.8, was added and rotated overnight at 4
°C. The next day, the resin was isolated by centrifugation, washed once with 5 mL 300 mM NaCl, 50 mM Tris, 30 mM imidazole pH 7.8 and then added to a poly-prep column (Bio-Rad) for elution with 5 mL 300 mM NaCl, 50 mM Tris, 200 mM imidazole pH 7.8. The eluate was dialyzed three times against PBS. Protein concentration was determined after dialysis from OD_{280} using ε_{280} of 34,000 M⁻¹cm⁻¹. Typical yields were 8 mg per liter of culture for streptavidin and 5 mg per liter of culture for traptavidin. Streptavidin and traptavidin were labeled with Alexa Fluor 555 by adding 1/10 volume of 1 M NaHCO₃ pH 8.3 and then a 10-fold molar excess of Alexa Fluor 555 succinimidyl ester (Invitrogen) (stock dissolved at 1mg/mL in dry dimethylformamide) and incubating for 4 h at 25 °C. Free dye was separated using 1 mL packed volume Sephadex G-25 (Sigma) in a poly-prep column. Fractions containing labeled protein were pooled and free dye was further removed by three rounds of dialysis in PBS.

**Biotin-4-fluorescein off-rate assay.** The off-rate of biotin-4-fluorescein from avidin, streptavidin or traptavidin was measured using a PHERAswer plate-reader with 480 nm excitation and 520 nm emission (BMG LABTECH). In this assay the binding of biotin-4-fluorescein to an excess of binding protein results in quenching of fluorescein emission. As the biotin-4-fluorescein dissociates, the fluorescence recovers. The assay was performed using excess biotin, so that sites left open by biotin-4-fluorescein dissociation are re-filled by biotin immediately. 1 µM protein in 10 µL PBS was added to 12 nM biotin-4-fluorescein (Invitrogen) with 0.12 mg/mL BSA in 170 µL PBS and incubated for 1 h at 37 °C. 20 µL PBS or 20 µL 1 mM biotin in PBS was then added and fluorescence measurements immediately started at 37 °C. Percentage dissociation was calculated as (signal with biotin – signal without biotin)/(signal without quenching – signal without biotin) x100. For the signal without quenching, no biotin-binding protein was added to the biotin-4-fluorescein.

For the low pH off-rate assay, 100 nM streptavidin or traptavidin was incubated with 12 nM B4F in 100 mM NaCl, 30 mM sodium citrate pH 5.0 for 3 h at 25 °C, before incubating at 37 °C and adding 100 µM biotin and incubating for various times. Since B4F fluorescence is decreased at low pH, samples were then placed on ice to block further dissociation, adjusted to pH 7.2 with 1 M HEPES pH 8.3, and fluorescence intensity was immediately measured as above. P-values were calculated using two-tailed Student t-tests from the triplicate data at the 6 h timepoint.

**³H-biotin off-rate assay.** The dissociation kinetics of biotin from streptavidin and traptavidin were determined using a method modified from that described previously. 10 nM 8,9-[³H]-biotin (PerkinElmer LAS UK) was incubated for 1 h at 25 °C with 250 nM streptavidin or traptavidin in PBS. To initiate dissociation, non-radioactive biotin was added to a final concentration of 50 µM and incubated at 37 °C. At each time point, the protein-biotin complex was pulled down by incubation with a 50% slurry of Ni-NTA resin, equilibrated in 300 mM NaCl, 50 mM Tris, 10 mM imidazole pH 7.8, for 1 h at 25 °C, followed by centrifugation. 25 µL supernatant, containing unbound radioactive biotin, was added to the scintillation cocktail and counted in a liquid scintillation counter (LS-5000TD, BeckmanCoulter). The average radioactivity of the supernatant at each time point (x) and the radioactivity of the protein-biotin complex before addition of cold biotin (a) enabled the first-order dissociation rate constant to be determined from the plot of ln(fraction bound) [ln(a-x/a)] against time. Excel was used for linear regression and to calculate error bars, using the “LINEST” linear least squares curve-fitting routine.
**Biotin-4-fluorescein on-rate assay.** The on-rate of B4F for streptavidin or traptavidin was measured in PBS on a PHERAstar plate-reader. 20 μL 10 nM streptavidin or traptavidin was added to 180 μL 56 pM B4F and the fluorescein emission was measured every 6 s at 25 °C. The on-rate was too fast to measure using this approach at 37 °C. The concentration of free B4F was calculated as (signal with B4F – signal without B4F)/(signal without protein – signal without B4F) × 50 pM starting B4F. Linear regression using GraphPad Prism (GraphPad Software) was applied to the plot of ln [free biotin-4-fluorescein] against time, with the gradient equal to $k_{on} \times$ [streptavidin or traptavidin]. Error bars for the on-rate were calculated using the “LINEST” linear least squares curve-fitting routine in Excel.

**$^3$H-biotin on-rate assay.** The on-rate of biotin was determined by incubating 250 pM streptavidin or traptavidin with 1 nM $^3$H-biotin in PBS at 37 °C. Binding was stopped by addition of 50 μM non-radioactive biotin. Protein was pulled down by incubation with 50% slurry of Ni-NTA resin, equilibrated in 300 mM NaCl, 50 mM Tris, 10 mM imidazole pH 7.8, for 1 h at 25 °C (in this time at 25 °C dissociation of $^3$H-biotin is negligible$^{20}$), followed by centrifugation. 25 μL supernatant, containing unbound $^3$H-biotin, was added to scintillation cocktail and counted in a liquid scintillation counter (Wallac 1409, PerkinElmer). We measured the radioactivity of the supernatant in cpm at each time point and the total radioactivity present before addition of protein, to calculate [free $^3$H-biotin]. The second-order association rate constant was then determined from the plot of 1/[free $^3$H-biotin] against time. Excel was used for linear regression and to calculate error bars, using the “LINEST” linear least squares curve-fitting routine.

**Equilibrium dissociation constant.** $K_d$ was calculated from $k_{off}/k_{on}$. The standard deviation of this quotient was calculated with the formula for $(A \pm a)/(B \pm b) = (C \pm c)$, where $c = C \times \sqrt{[(a/A)^2 + (b/B)^2]}$. Our $k_{on}$ and $k_{off}$ for streptavidin are comparable with previous values$^{23, 24}$. The literature $K_d$ value for streptavidin-biotin$^{25}$ (4×10$^{-14}$ M) was determined at 25 °C, but it is known that the off-rate of streptavidin dramatically increases with temperature$^{23, 26}$.

**Thermostability assay.** To determine tetramer stability, 3 μM streptavidin or traptavidin in PBS was heated at the indicated temperature for 3 min in a Bio-Rad DNA Engine® Peltier Thermal Cycler and then immediately placed on ice$^{27-29}$. Samples were mixed with 6x SDS-PAGE loading buffer and loaded onto an 18% polyacrylamide gel. The 100% monomer positive control was mixed with SDS loading buffer prior to heating at 95 °C for 3 min. Band intensities were quantified using a ChemiDoc XRS imager and QuantityOne 4.6 software (Bio-Rad). Note that the tetramer band does not run according to molecular weight as its position is influenced by protein charge.

To determine thermostability of biotin conjugate binding, 5.0 μM streptavidin or traptavidin in PBS was incubated with 21 nM monobiotinylated DNA in a volume of 4 μL for 30 min at 25 °C. Samples were made up to 10 μL with a final concentration of 100 μM biotin, 20 mM Tris acetate, 1 mM DTT, 2 mM magnesium acetate and 20 mM potassium glutamate pH 7.5 and incubated for 5 min at 25 °C, before heating at the indicated temperature for 3 min in a Thermal Cycler and cooling to 10 °C. A 1.5% agarose gel was run at 6.0 V/cm in TAE for 45 min at 25 °C and ethidium bromide-stained DNA was visualized on a ChemiDoc XRS imager using
QuantityOne 4.6 software. Percentages were defined as $100 \times$ the intensity of the band for free DNA divided by the summed intensities of the bands for free and bound DNA. The 439 bp monobiotinylated DNA was prepared by PCR using Taq DNA polymerase with the primers Fts1 and the internally biotinylated primer bioFts2 (Eurofins) from plasmid pJEG41-N1, a derivative of pUC18 containing a lambda phage insert.

**Cell culture, biotinylation and imaging.** COS7 cells were grown in DMEM with 10% Fetal Calf Serum, 50 U/mL penicillin and 50 µg/mL streptomycin. Cells were transfected using Lipofectamine 2000 (Invitrogen) following manufacturer’s instructions with 0.25 µg AP-IGF1R, 0.2 µg BirA-ER and 0.05 µg H2B-ECFP per well of a 48 well plate. Cells were incubated with 10 µM biotin overnight for optimum biotinylation by BirA-ER. The next day, cells were washed 3 times in PBS with 5 mM MgCl$_2$ (PBS/Mg) and kept thereafter at 4 ºC. Cells were incubated for 15 min in PBS/Mg with 1% dialyzed BSA and 0.4 µM Alexa Fluor 555-conjugated traptavidin or streptavidin. For pre-blocking, 50 µM biotin was added to the fluorescent traptavidin 5 min before adding to cells. Cells were washed with PBS/Mg 3 times before imaging live. Cells were imaged using a wide-field DeltaVision Core fluorescent microscope (AppliedPrecision) with a 40 × oil-immersion lens. ECFP (436DF20 excitation, 480DF40 emission, Chroma 86002v1 dichroic) and Alexa Fluor 555 (540DF420 excitation, 600DF50 emission, Chroma 84100bs polychroic) images were collected and analyzed using softWoRx 3.6.2 software. Typical exposure times were 0.1 - 0.5 s and fluorescence images were background-corrected. Different samples in the same experiment were prepared, imaged and analyzed under identical conditions.

**AFM analysis.** AFM is described in a Supplementary Note.

**FtsK displacement assay.** For Fig. 3, a 439 bp monobiotinylated DNA fragment, was generated by PCR with Taq DNA polymerase using primers Fts1 and the internally biotinylated primer bioFts2 (Eurofins) from plasmid pJEG41-P1. This fragment contained two lots of tandem KOPS loading sites (GGGCAGGGGGGCGAGG) positioned such that upon addition of ATP, it would take FtsK loaded at KOPS ~0.5 s to translocate to streptavidin or traptavidin$^{12}$. *Pseudomonas aeruginosa* FtsK PAK4, a soluble fragment containing the C-terminal 578 residues including the α, β and γ domains and a C-terminal His$_6$-tag, was a kind gift of James Graham (Oxford University). The protein was overexpressed in *E. coli* and purified by ammonium sulfate precipitation, nickel affinity chromatography and heparin affinity chromatography. The FtsK displacement assay was performed at 25 ºC in 20 mM Tris acetate, 2 mM magnesium acetate, 20 mM potassium glutamate, 1 mM dithiothreitol (DTT) pH 7.5. 16 nM DNA fragment was incubated with 2 µM streptavidin or traptavidin for 10 min, followed by 100 µM biotin to block free biotin binding sites. PAK4 FtsK was added at 0.5 µM in Fig. 3c and 3d and at the indicated concentrations in Fig. 3e and allowed to bind to the DNA for 5 min. 2 mM ATP was added to start the reaction and samples were incubated for 3 min in Fig. 3c and 3e and for the indicated times in Fig. 3d. Reactions were stopped by adding 1 µL 0.1% SDS with 200 mM EDTA pH 8.0. Samples were incubated for a further 15 min to allow FtsK to dissociate from the DNA, mixed with 6 × gel loading buffer (1.2 M sucrose, 0.75 mM bromophenol blue; 0.93 mM xylene cyanol FF) and loaded on a 1.5% agarose gel in TAE at 6.0 V/cm for 45 min at 25 ºC. Ethidium bromide-stained DNA was visualized.
on a ChemiDoc XRS imager. % free = 100 × intensity of band for free DNA / (intensity of band for free DNA + intensity of band for bound DNA). % displaced = 100 × (% free – % free for negative control) / (100 – % free for negative control). Negative values of % displaced were rounded up to zero. P-values were calculated using a two-tailed Student t-test (n = 2).

For Supplementary Fig. 4, a 598 bp monobiotinylated DNA fragment, containing two 8 bp KOPS loading sites 226 bp from the biotinylated end, was generated by PCR with Taq DNA polymerase using primer Fts1 and the terminally biotinylated primer bioFts3 (Sigma-Genosys) from plasmid pJEG41. *E. coli* FtsK<sub>50C</sub>, a soluble fragment containing the α, β and γ domains of FtsK, was purified as described<sup>30</sup>. The FtsK displacement assay was performed at 25 °C in 25 mM Tris pH 7.5, 10 mM MgCl<sub>2</sub>. 5.9 nM DNA fragment was incubated with 0.5 µM streptavidin or traptavidin for 15 min, followed by 100 µM biotin to block free biotin binding sites. 1 µM FtsK<sub>50C</sub> was added and allowed to bind to the DNA for 5 min. 2.5 mM ATP was added to start the reaction, which was stopped after 2 min with a final concentration of 0.1% SDS and 20 mM EDTA. Samples were incubated for 20 min and then mixed with 10 × gel loading buffer (250 mM Tris pH 7.5, 20 mM EDTA, 50% glycerol, 2.5% bromophenol blue) before loading on a 1.5% agarose gel in 1 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 10 V/cm for 2 h at 25 °C. Gels were stained with SYBR Green (Invitrogen) for 2 h, washed in ddH<sub>2</sub>O for 30 min, imaged using a Fuji FLA3000 scanner, and quantified using Image Gauge software (Fuji).

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Traptavidin, a streptavidin mutant with a ~10-fold lower off-rate for biotin than streptavidin itself, has increased mechanical strength and thermostability. It should find use in a diversity of applications where the dissociation of streptavidin can be a limitation.