Electronic Supplementary Material (ESI) for Dalton Transactions.

Supporting Information

Routes of iron entry into, and exit from, the catalytic ferroxidase sites of the prokaryotic ferritin *Syn*Ftn

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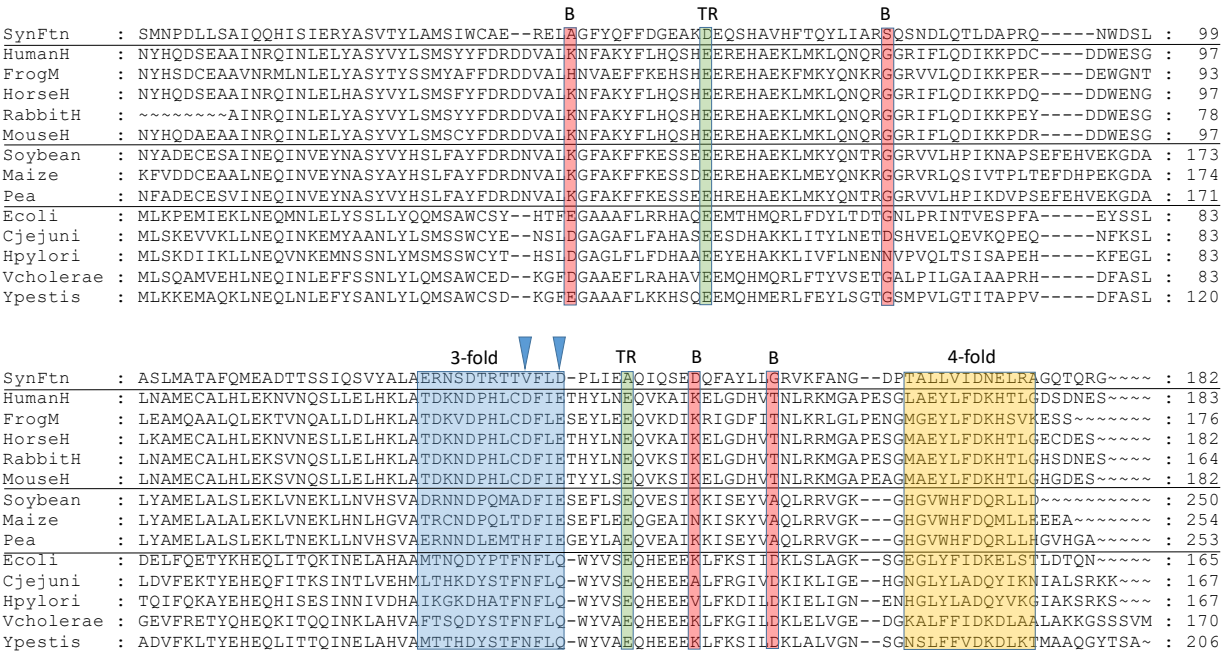

Figure S1. Comparison of the sequence of *Syn*Ftn with those of selected other ferritins. The area shaded in blue denotes those residues that comprise the 3-fold channel with the positions of the two conserved carboxylates of the animal proteins marked by blue triangles. The area shaded orange denotes the residues that comprise the 4-fold channel. Red shading indicates residues that line the B-channel of prokaryotic ferritins and green shading the positions of the 'transfer carboxylates' identified in Frog M and Human H ferritin. The proteins shown are ferritin heavy chain from Homo sapiens (HumanH), ferritin middle subunit from *Rana catesbeiana* (FrogM), ferritin heavy chain from *Equus caballus* (HorseH), ferritin heavy chain from *Oryctolagus cuniculus* (RabbitH), ferritin heavy chain from *Mus musculus* (MouseH), ferritin 1 from *Glycine max* (Soybean), ferritin 1 from *Zea mays* (Maize), ferritin 1 from *Pisum sativum* (Pea), non-heme ferritin FtnA from *Escherichia coli* (Ecoli), non-heme ferritin from *Campylobacter jejuni* (Cjejuni), non-heme ferritin from *Helicobacter pylori* (Hpylori), ferritin from *Vibrio cholera* (Vcholerae) and ferritin from *Yersinia pestis* (Ypestis).

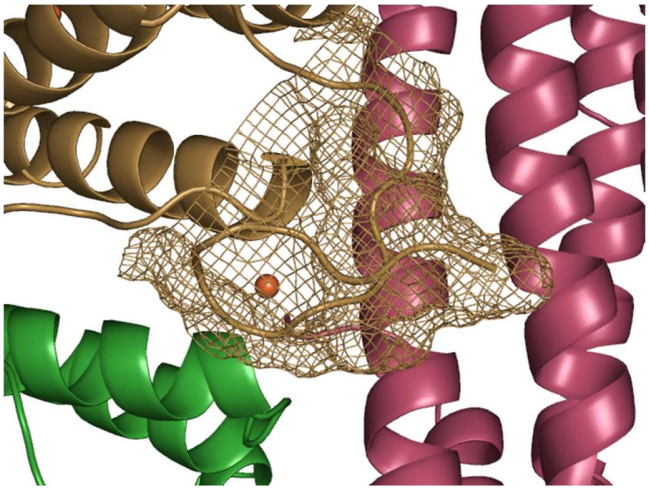

Figure S2. The B-channel of *Syn*Ftn is capped by an N terminal extension of the peptide chain. The image shows the B-channel of *Syn*Ftn formed at the intersection of three subunit monomers (PDB entry 6GKA) overlaid with an orange sphere at the position at which an iron ion was observed in the structure of E44Q *Pm*Ftn soaked overnight in a Fe^{2+} containing solution (PDB entry 4ZKH). The brown mesh represents the surface of the N terminal of *Syn*Ftn, which is an extension relative to *E. coli* FtnA, the most extensively characterized of the prokaryotic Ftn proteins.

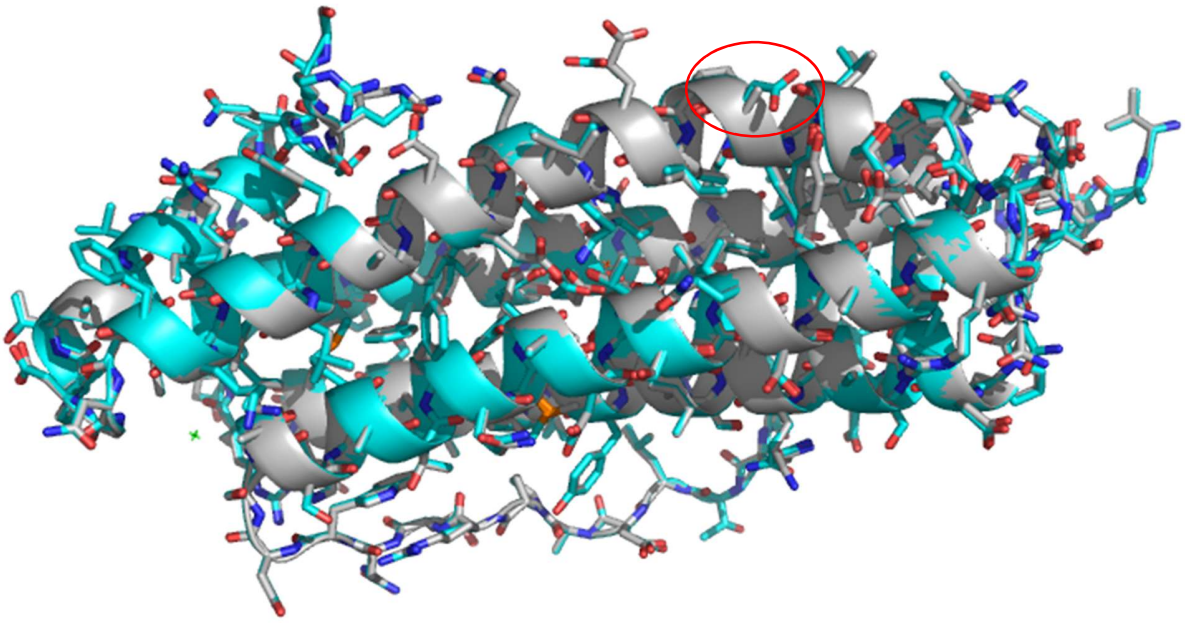

Figure S3. Comparison of the crystal structure of wild type and variant D137A *Syn*Ftn. Oxygen atoms coloured red, nitrogen blue, sulphur orange and carbon cyan (wild type) or white (D137A). The position of the mutated residue is marked by the red elipse.

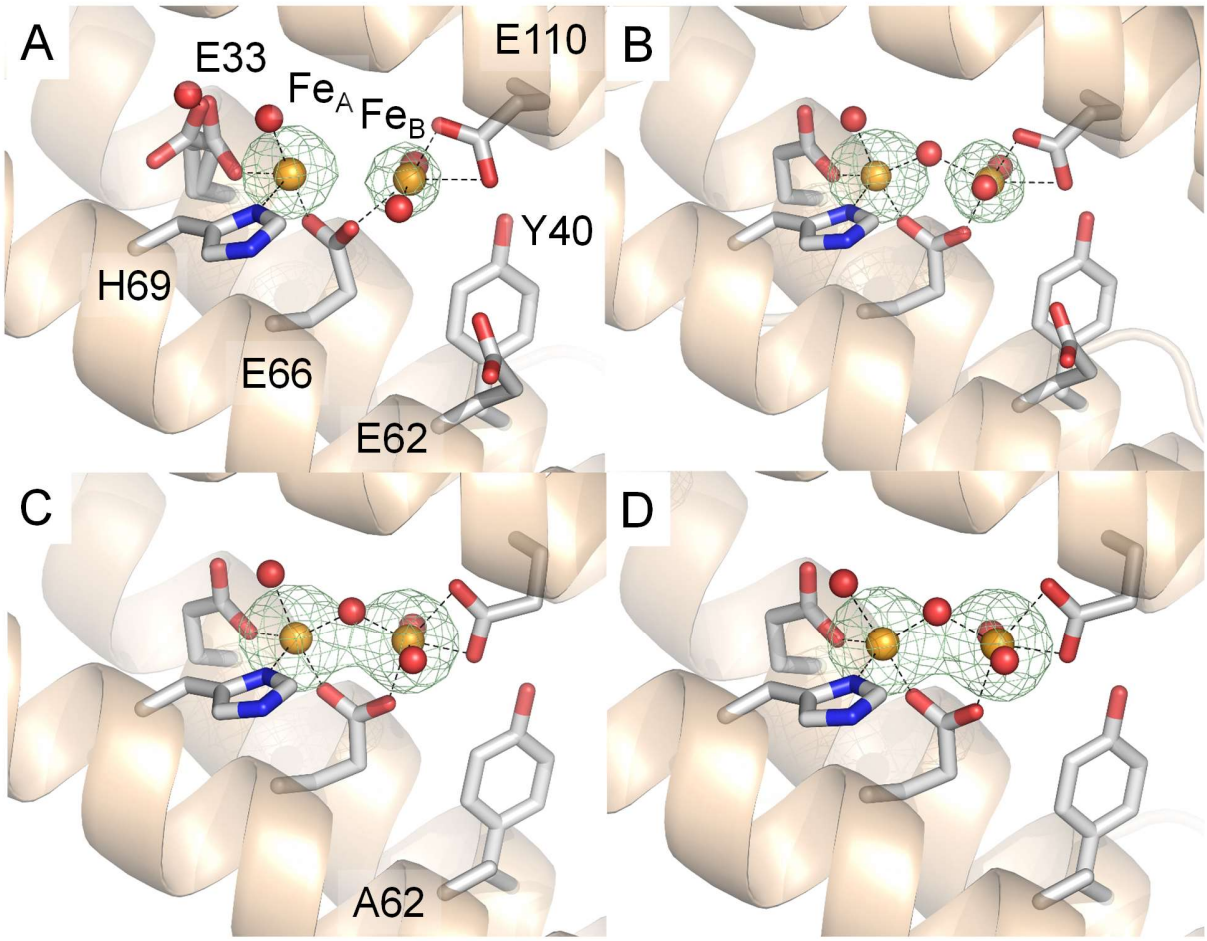

Figure S4. The ferroxidase center of Fe^{2+}-soaked *Syn*Ftn variants. The di-iron site of D137A *Syn*Ftn following the soaking of crystals in a 5 mM Fe^{2+} solution for either 2 min (A) or 20 min (B). (C-D) as (A-B) but for crystals of E62A *Syn*Ftn. Carbon is shown in white, nitrogen in blue, oxygen in red and iron as bright orange spheres with associated water molecules or (hydr)oxide ions as red spheres. The light green mesh in each of panels A to D shows the anomalous difference Fourier map calculated from data collected at the iron K-edge and contoured at 8 σ.

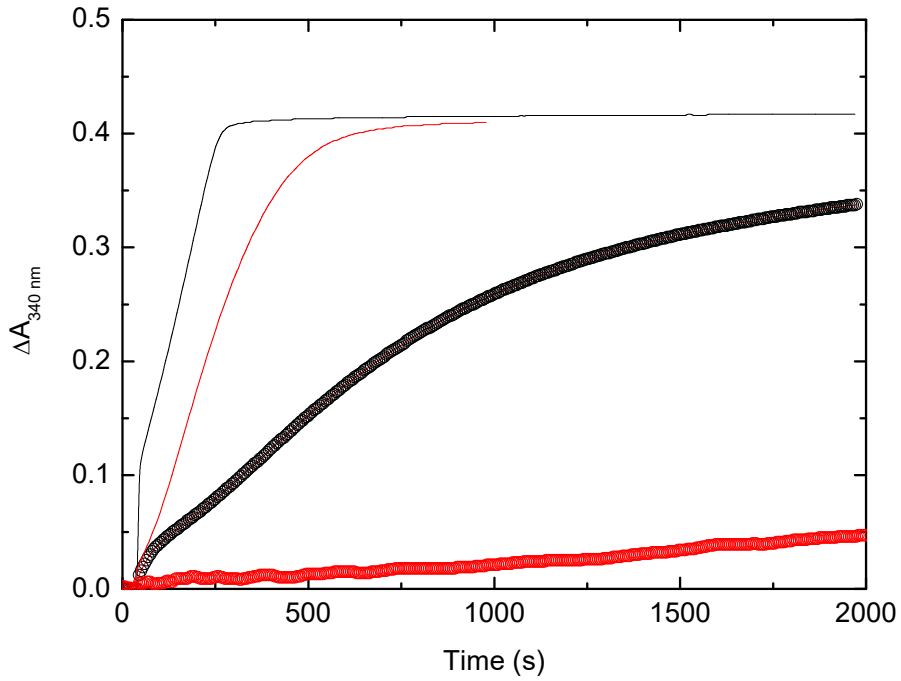

Figure S5. Inhibition of *Syn*Ftn by Zn^{2+}. The increase in absorbance at 340 nm as a function of time following the addition of 400 equivalents of Fe^{2+} to 0.5 μM protein (24mer). The response of wild type protein is shown in black and variant D137A in red, solid lines represent data for addition of Fe^{2+} to apo proteins and open circles for addition of Fe^{2+} to protein pre-incubated with 96 equivalents of Zn^{2+} (4 Zn^{2+}/monomer). All measurements were carried out in MES pH 6.5 at 25 ˚C.

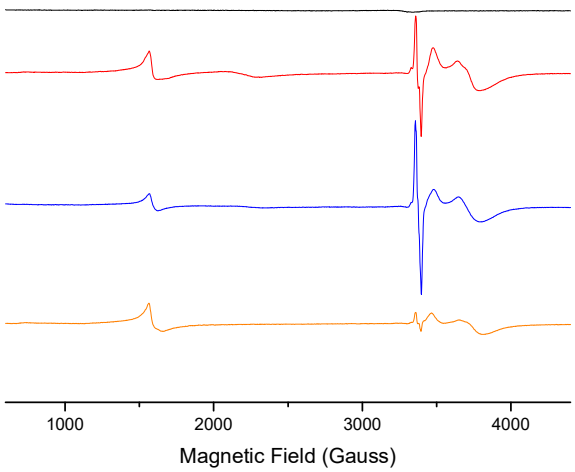

Figure S6. EPR properties of *Syn*Ftn and variants . Full sweep EPR spectra of: wild type *Syn*Ftn prior to the addition of Fe^{2+} (black); wild type *Syn*Ftn frozen 9 s after the addition of 72 equivalents of Fe^{2+} (red); variant E62A *Syn*Ftn frozen 12 s after the addition of 72 equivalents of Fe^{2+} (blue); and, variant D137A *Syn*Ftn frozen 20 s after the addition of 72 equivalents of Fe^{2+} (orange). Note that the intensity of the radical signal centred on 3386 G is variable between preparations and does not correlate with the E62A and D137A substitutions.

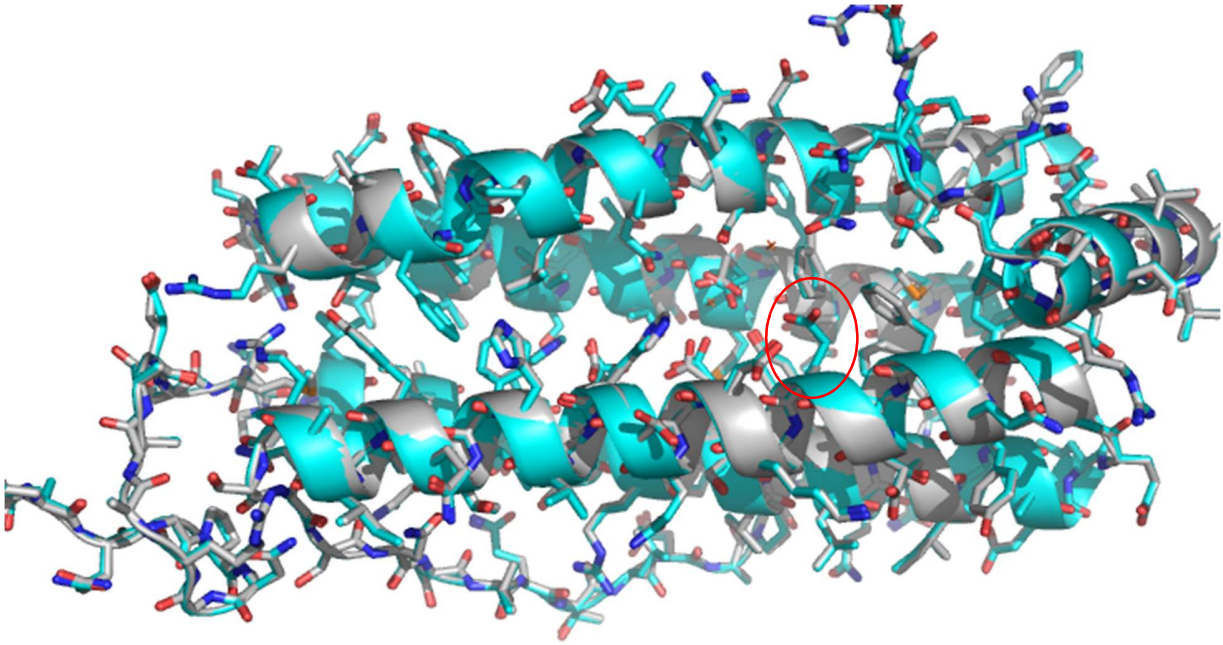

Figure S7. Comparison of the crystal structure of wild type and variant E62A *Syn*Ftn. Oxygen atoms coloured red, nitrogen blue, sulphur orange and carbon cyan (wild type) or white (E62A). The position of the mutated residue is marked by the red elipse.

Table S1. *Syn*Ftn D137A mutant. Data collection and refinement statistics.

PDB code	**6SOM**	**6SON**	**6SOO**
Fe^{2+} Soak (min)	**0**	**2**	**20**
Wavelength	0.9763	0.9795	0.9795
Resolution range	40.55 - 2.15 (2.23 - 2.15)	51.07 - 1.60 (1.66 - 1.60)	44.12 - 1.57 (1.63 - 1.57)
Space group	F 4 3 2	F 4 3 2	F 4 3 2
Unit cell	176.8 176.8 176.8 90 90 90	176.9 176.9 176.9 90 90 90	176.5 176.5 176.5 90 90 90
Total reflections	223008 (22708)	609753 (61303)	852797 (83644)
Unique reflections	13408 (1302)	31808 (3112)	33428 (3301)
Multiplicity	16.6 (17.4)	19.2 (19.7)	25.5 (25.3)
Completeness (%)	99.81 (100.00)	99.93 (100.00)	99.96 (99.97)
Mean I/sigma(I)	10.25 (1.47)	26.10 (1.58)	27.06 (1.27)
Wilson B-factor	37.51	30.84	29.22
R-merge	0.1991 (1.465)	0.05239 (1.774)	0.06179 (2.264)
R-meas	0.2055 (1.509)	0.05386 (1.821)	0.06308 (2.31)
R-pim	0.05005 (0.3592)	0.01231 (0.4085)	0.01252 (0.4565)
CC1/2	0.997 (0.693)	1 (0.644)	1 (0.605)
CC*	0.999 (0.905)	1 (0.885)	1 (0.868)
Reflections used in refinement	13406 (1302)	31807 (3112)	33427 (3300)
Reflections used for R-free	677 (65)	1539 (153)	1671 (165)
R-work	0.1855 (0.2561)	0.1582 (0.2303)	0.1652 (0.2448)
R-free	0.2397 (0.3310)	0.1890 (0.2805)	0.1957 (0.2966)

CC(work)	0.957 (0.859)	0.968 (0.868)	0.959 (0.853)
CC(free)	0.946 (0.752)	0.948 (0.810)	0.956 (0.768)
Number of non-hydrogen atoms	1514	1630	1583
Macromolecules	1393	1416	1399
Ligands	1	3	2
Solvent	120	211	182
Protein residues	178	178	178
RMS(bonds)	0.006	0.005	0.005
RMS(angles)	0.71	0.69	0.67
Ramachandran favored (%)	98.86	100.00	100.00
Ramachandran allowed (%)	1.14	0.00	0.00
Ramachandran outliers (%)	0.00	0.00	0.00
Rotamer outliers (%)	0.00	0.66	0.00
Clashscore	1.83	1.79	0.73
Average B-factor	37.46	34.81	32.62
Macromolecules	36.95	33.02	31.18
Ligands	43.73	51.64	43.96
Solvent	43.35	46.58	43.56

Statistics for the highest-resolution shell are shown in parentheses.

Table S2. *Syn*Ftn E62A mutant. Data collection and refinement statistics.

PDB code	**6SOP**	**6SOQ**	**6SOR**
Fe^{2+} Soak (min)	**0**	**2**	**20**
Wavelength	0.9795	0.9795	0.9795
Resolution range	40.58 - 1.93 (2.00 - 1.93)	36.13 - 1.67 (1.73 - 1.67)	39.46 - 1.74 (1.80 - 1.74)
Space group	F 4 3 2	F 4 3 2	F 4 3 2
Unit cell	176.9 176.9 176.9 90 90 90	177 177 177 90 90 90	176.5 176.5 176.5 90 90 90
Total reflections	128714 (12505)	211108 (13333)	194099 (16168)
Unique reflections	18381 (1789)	27809 (2527)	24709 (2426)
Multiplicity	7.0 (7.0)	7.6 (5.3)	7.9 (6.7)
Completeness (%)	99.82 (99.89)	98.90 (91.85)	99.87 (100.00)
Mean I/sigma(I)	11.11 (1.19)	22.75 (1.44)	15.09 (1.52)
Wilson B-factor	35.55	28.17	30.79
R-merge	0.1016 (1.327)	0.04735 (0.9856)	0.06909 (0.9414)
R-meas	0.1098 (1.435)	0.05083 (1.093)	0.07412 (1.021)
R-pim	0.04087 (0.5372)	0.01811 (0.4579)	0.02631 (0.3896)
CC1/2	0.998 (0.49)	0.999 (0.496)	0.998 (0.581)
CC*	0.999 (0.811)	1 (0.814)	1 (0.857)
Reflections used in refinement	18379 (1789)	27808 (2526)	24708 (2426)
Reflections used for R-free	931 (90)	1398 (120)	1248 (119)
R-work	0.1550 (0.2735)	0.1511 (0.2555)	0.1575 (0.2304)
R-free	0.1998 (0.3269)	0.1830 (0.3370)	0.1873 (0.2812)

CC(work)	0.967 (0.802)	0.969 (0.810)	0.968 (0.844)
CC(free)	0.964 (0.755)	0.945 (0.645)	0.966 (0.836)
Number of non-hydrogen atoms	1583	1637	1600
Macromolecules	1395	1398	1395
Ligands	1	4	4
Solvent	187	235	201
Protein residues	178	178	178
RMS(bonds)	0.007	0.007	0.007
RMS(angles)	1.01	1.09	1.02
Ramachandran favored (%)	99.43	99.43	100.00
Ramachandran allowed (%)	0.57	0.57	0.00
Ramachandran outliers (%)	0.00	0.00	0.00
Rotamer outliers (%)	0.00	0.00	0.00
Clashscore	1.46	2.18	1.46
Average B-factor	37.11	30.13	33.13
Macromolecules	35.79	28.09	31.68
Ligands	39.32	30.23	32.88
Solvent	46.95	42.27	43.21

Statistics for the highest-resolution shell are shown in parentheses.

Table S3. Refined fractional occupancies of metal binding sites in *Syn*Ftn mutants. Soak solutions comprised the well solution with 5 mM Fe^{2+} and the pH adjusted to 6.5. Crystals were soaked for either 2 or 20 min prior to freezing.

*Syn*Ftn variant	Soak/ min	Fractional occupancy[a]		
		Site A	Site B	Site 3FC
D137A	2	0.50	0.32	0
	20	0.56	0.54	0
E62A	2	0.74	0.51	0.26
	20	0.92	0.88	0.33

[a] Sites A and B constitute the ferroxidase centre. Site 3FC represents the iron binding site which lies on the symmetry axis in the 3-fold channel of the iron-soaked wild type ferritin structure (PDB entry 3OUY), and has a maximal occupancy of 0.33