Engineering intracellular biomineralization and biosensing by a magnetic protein

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Remote measurement and manipulation of biological systems can be achieved using magnetic techniques, but a missing link is the availability of highly magnetic handles on cellular or molecular function. Here we address this need by using high-throughput genetic screening in yeast to select variants of the iron storage ferritin (Ft) that display enhanced iron accumulation under physiological conditions. Expression of Ft mutants selected from a library of $10^7$ variants induces threefold greater cellular iron loading than mammalian heavy chain Ft, over fivefold higher contrast in magnetic resonance imaging, and robust retention on magnetic separation columns. Mechanistic studies of mutant Ft proteins indicate that improved magnetism arises in part from increased iron oxide nucleation efficiency. Molecular-level iron loading in engineered Ft enables detection of individual particles inside cells and facilitates creation of Ft-based intracellular magnetic devices. We demonstrate construction of a magnetic sensor actuated by gene expression in yeast.
magnetic approaches to biological experimentation are particularly attractive because they interact minimally with biological processes, rarely incur damage and have already led to powerful manipulation and imaging techniques. Existing magnetic biotechnologies are of limited value for studying molecular and cellular level phenomena, however. The best known magnetic measurement techniques, nuclear magnetic resonance and magnetic resonance imaging (MRI), are ill-suited for analysis of specific molecular phenomena in cells and tissue. In vivo nuclear magnetic resonance spectroscopy is too insensitive to permit robust measurements of most biomolecules. Molecular MRI measurements can be made using contrast agents that combine magnetic properties with other functionalities, but these agents need to be delivered exogenously. Techniques for magnetic modulation of biological systems have been demonstrated at cellular level, but also tend to depend on exogenous nanoparticles that are difficult to apply to biological systems. Although manipulation of cellular magnetism and magnetic image signals has also been demonstrated using genetic techniques, the effects tend to be weakened or less specific than approaches based on synthetic magnetic nanoparticles, in part because the molecular organization of magnetic material in cells is less controlled.

A strongly magnetic protein could provide a basis for robust modulation or detection of well-defined molecular-level phenomena. A promising starting point for generation of such a molecule is ferritin (Ft), an iron storage protein found in most animal, plant and bacterial cells. Ft proteins consist of a spherical shell of 24 identical or closely homologous polypeptide chains, in which a reservoir of hydrated iron oxide accumulates and can be rapidly mobilized according to physiological needs. Ft variants have been used as magnetic gene reporters, expressible cellular imaging agents and components of magnetically responsive genetic devices, but Ft is much less potent than synthetic nanoparticles of similar volume and often contains far fewer iron atoms than its core structure could in principle accommodate. In vitro manipulation of Ft mineralization has enabled the generation of highly magnetic species, but the resulting protein complexes cannot be applied in conjunction with genetic techniques and suffer similar limitations to those of synthetic nanoparticles.

To address these limitations, we designed a strategy for enhancing the magnetic properties of intracellularly expressed Ft in a systematic and high-throughput fashion. In this paper, we present our approach and its success in isolating mutant Ft variants that biomineralize iron more effectively than their natural counterparts. We characterize the selected mutants and show that their enhanced iron loading capability may arise from improvements to iron oxide nucleation. Finally, we show that the new ‘hypermagnetic’ Ft variants act as genetically encodable tools for multiscale cellular imaging, magnetic manipulation of cells and construction of intracellular magnetic devices capable of sensing molecular-level phenomena.

Results

Screening for Ft variants with enhanced biomineralization.

Our biomaterial engineering approach was based on the hypothesis that mutant Ft molecules that sequester iron compounds most effectively would also form complexes with optimal magnetic properties—a view motivated by the fact that both greater Ft iron content and denser, unhydrated iron oxide mineralization can result in higher per-particle magnetic moments. Iron accumulation by Ft variants is expected to reduce cytosolic iron concentration by mass action principles, so we established a reporting system in yeast whereby expression of Ft mutants could be evaluated for induction of a low cytosolic iron phenotype. In Saccharomyces cerevisiae, intracellular iron level is regulated by the iron-responsive transcriptional activator Aft1, which under low-iron conditions translocates into the nucleus and regulates genes involved in iron uptake. One of the genes upregulated by Aft1 encodes the cell surface high-affinity iron transporter, FTR1; by monitoring expression of an FTR1–green fluorescent protein (GFP) fusion reporter, we could therefore identify individual cells that display low cytosolic iron concentrations (Fig. 1a). This system was intended as a tool for selecting mutant Ft variants that robustly sequester cellular iron, and that would therefore induce greater FTR1–GFP expression and fluorescence than Ft variants with less potent iron binding capacity.

As a template for random mutagenesis and screening, we choose to work with a Ft from the thermophilic bacterium Pyrococcus furiosus (PFt). PFt has the advantage that it is highly thermostable and, therefore, likely to be more tolerant to mutations introduced to alter biomineralization than human heavy chain Ft (HFe, Tm = ~77°C), which has been used for the majority of biotechnological applications of Ft in the past. In addition, PFt forms homooligomeric protein shells that require only a single polypeptide, in contrast to conventional mammalian Fts that incorporate two chains, making PFt structure and chemistry simpler and more predictable. To facilitate isolation and analysis of PFt variants, we fused an affinity tag (Strep-tag II) to the N terminus of PFt to form a construct abbreviated SPFt (Supplementary Fig. 1a). The tag had...
minimal effect on protein folding and iron loading functions of
the protein (Supplementary Figs 1b–e). SPFt was expressed in
yeast cells bearing the FTR1–GFP reporter and induced
elevated fluorescence, compared with control cells bearing no
SPFt or harbouring a compromised SPFt with E94G and K142R
substitutions that eliminate ferroxidase activity of the protein
(Fig. 1b). Results of fluorescence microscopy were further
validated by fluorescence-activated cell sorting (FACS) analysis
(Fig. 1c). Fluorescence histograms from cells transfected with a
SPFt expression plasmid displayed a peak of notably higher
fluorescence, indicating upregulation of the fluorescent reporter;
a second peak with fluorescence comparable to vector control
transfected cells was observed under saturating growth conditions
and probably arises from SPFt expression plasmid loss in some
cells. These results were consistent with the explanation that SPFt
expression sequesters cytosolic iron and boosts FTR1–GFP
reporter expression.

To isolate mutants that preferentially biomineralize more iron
in vivo, we subjected the entire Pft coding sequence in SPFt to
PCR-based random mutagenesis. After transfection, this resulted
in a library of 10 million yeast clones expressing randomly
mutated SPFt variants with an average mutation rate of one
nucleotide change per gene (Supplementary Fig. 2). This relatively
low mutation rate was chosen to avoid accumulation of
deleterious mutations, which could obscure beneficial but rare
mutations. The yeast library was incubated in a minimum media
and sorted by FACS to obtain cells exhibiting highest levels of
FTR1–GFP fluorescence. Cells in the top 5% were propagated for
a subsequent round of sorting (Fig. 2a), and the procedure was
repeated. After four rounds (Fig. 2b), we sequenced the sorted
population and identified mutations that were enriched among
the selected yeast cells (Supplementary Table 1). Following
resting of the individual-enriched mutations, three were chosen
for further analysis: L55P, F57S and F123S.

Characterization of selected SPFT mutants. To confirm the Ft
dependence of iron reporter expression in the selected clones,
plasmids for SPFT L55P, F57S and F123S were isolated and
retransformed for reanalysis by FACS; fluorescence histograms
were consistent with the screening results (Fig. 2c). As an
additional test of the iron accumulation phenotype, we incubated
the three selected clones in iron-supplemented media and
measured the total cellular iron content (Fig. 2d) and iron content
of purified SPFT proteins (Fig. 2e,f). The most effective of the SPFT
mutants, L55P, induced 1.6 ± 0.2 (mean ± s.e., n = 3) times
greater cellular iron accumulation than wild-type SPFT and
2.6 ± 0.3 times greater accumulation than HFT. Compared with
SPFT, the L55P mutant also exhibited almost double the number
of iron atoms per Ft 24-mer, indicating that the cellular
biomineralization phenotype originates largely from an increase
in iron sequestration by Ft at the molecular level. For both L55P
and F57S mutants, significant enhancement of cellular iron
accumulation (Student’s t-test, P = 0.002, n = 6 for L55P and
P = 0.003, n = 6 for F57S) and molecular-level Ft iron loading
(P = 0.00003, n = 6 for L55P and P = 0.02, n = 4 for F57S) were
observed. Further gains could not readily be obtained by
combining these mutations or by performing additional random
mutagenesis on the isolated clones. The results nevertheless
prove for the first time that intracellular Ft biomineralization

![Figure 2](https://example.com/figure2.png)

**Figure 2 | Selection of SPFT mutants by high-throughput genetic screening.** (a) Summary of the fluorescence-activated cell sorting (FACS)-based yeast
genetic screening procedure. Control yeast cells lacking the FTR1–GFP reporter (neg) or positive cells harbouring the reporter and a SPFT gene library (Lib)
were grown in minimum media. The yeast populations were presorted to remove debris and aggregated cells, and then used to establish a criterion (green
outline) designed to reject cells lacking a functional reporter construct. From among Lib cells that passed this criterion, roughly 5% of cells which displayed
the highest GFP fluorescence intensities (black label) were selected during each FACS run. Multiple rounds of selection and regrowth were performed
(arrow) to enrich library mutants which induced the highest levels of fluorescent reporter expression. (b) A histogram showing the distribution of GFP
fluorescence intensity in the yeast cell population transformed with the initial library (Lib, red), and following one to four successive rounds of enrichment
(S1–S4). (c) Flow cytometry distributions of GFP fluorescence intensity of yeast cells transformed with SPFt (red) and three mutants identified through
the screen, L55P (green), F57S (cyan) and F123S (magenta) incubated in minimal media overnight. Cytosolic iron content of intact yeast (d) and
molecular-level iron loading by purified SPFT variants (e) was measured for each of the selected mutants using a bathophenanthroline disulfonate-binding assay
following 16 h incubation of the corresponding cells in iron-rich medium. Error bars denote s.e.m. of three or more independent measurements. (f) Native
gel analysis of purified SPFT and mutant nanoparticles stained with Coomassie blue for protein content (top) and Prussian blue for iron content (bottom),
showing substantially increased iron content of the selected SPFT mutants.
Mechanistic analysis of biominer alization by SPFt variants. In an attempt to understand the mechanism by which primary sequence mutations in SPFt lead to enhanced iron accumulation in the selected Ft holomers, we performed a series of characterization experiments. By inspecting the crystal structure of Pf1, we saw that all three mutant residues point towards the inside of the iron storage cavity and lie on the B and D helices close to a site thought to be involved in oxidation of Fe2+ ions that enter the Pf1 core (Fig. 3a). We speculated that the mutations might therefore affect either the enzymatic functionality of Pf1 or the structure of the iron oxide core itself. To test these ideas, we began by measuring the iron assimilation and release kinetics of the SPFt variants. No significant differences in iron oxidation (the initial step in core formation) or iron release rates were found (Supplementary Table 2).

To examine potential structural effects of the mutations, we characterized the purified protein nanoparticles by high-resolution cryo-electron microscopy (cryo-EM), a powerful technique that allows imaging of proteins in the near-native environment. Micrographs confirmed that SPFt and the variants all form 12 nm cage-like structures as expected (Fig. 3b). Image autocorrelation analysis indicated the presence of electron dense centres of 4–8 nm diameter for each variant (Supplementary Fig. 5). The L55P variant exhibited a marginally wider autocorrelation profile than other variants, possibly indicating a larger mean core size, while the F123S displayed the narrowest profile. There was more striking variation in the frequency of electron dense cores discernible among the four SPFt variants, however. Only 68.3 ± 1.3% (mean ± s.e.m.) of wild-type SPFt nanoparticles contained dark core structures, whereas 96.1 ± 0.1%, 87.0 ± 0.3% and 78.3 ± 1.5% of the L55P, F57S and F123S mutants, respectively, appeared electron dense (Fig. 3c). Increased core formation in each mutant was significant with respect to SPFt (t-test; P = 0.03 for L55P, P = 0.04 for F57S, P = 0.04 for F123S; n = 2 samples with 400 particles per sample), suggesting that an increased ability of the mutant proteins to nucleate mineral core formation might largely account for their ability to accumulate a larger number of iron atoms per protein molecule. This explanation might also be compatible with the finding that the selected SPFt mutations could not be combined to further improve iron loading, given the possibility that enhanced mineral
nucleation and growth at one site might not be compatible with nucleation directed at another site, and that competing nucleation and growth at multiple sites decreases homogeneity of mineral crystal formation.

**Magnetic sorting and imaging using hypermagnetic SPFt.** Our strategy for engineering hypermagnetic SPFt variants was predicated on the notion that iron sequestration by SPFt mutants would accompany enhanced magnetic properties. To demonstrate this, we explored the utility of hypermagnetic SPFt variants in imaging and high-gradient magnetic cell separation (HGMS) applications. For MRI experiments, the same yeast samples used for the iron assays in Fig. 2d were pelleted and imaged in a 7 T magnet using a spin-echo acquisition sequence. The transverse relaxation rate (1/T2) of cells transformed with the most iron-rich Ft mutant, L55P, was significantly higher than that of cells expressing wild-type SPFt (58.2 ± 3.7 s⁻¹ vs 30.0 ± 2.5 s⁻¹, t-test P = 0.001, n = 4) or human Hft (21.9 ± 0.9 s⁻¹, P = 0.001, n = 3), indicating that the hypermagnetic mutant L55P indeed shows higher sensitivity as an intracellularly expressed MRI contrast agent (Fig. 4a). The ability of SPFt L55P to enhance magnetic capture in HGMS was assessed by comparing the mutant protein to wild-type SPFt and Ft-free control cells. Yeast cells expressing SPFt L55P were retained with four times greater efficacy than cells transformed with SPFt (Fig. 4b), demonstrating that the increased cellular magnetization due to expression of hypermagnetic mutant protein nanoparticles significantly improved the sensitivity of magnetic cell sorting process (t-test; P = 0.007, n = 3). HGMS and imaging results could not be explained by differences in the protein expression levels of wild-type SPFt versus the hypermagnetic mutant L55P; these variants expressed at similar levels of 0.45 ± 0.03% and 0.53 ± 0.07% of total soluble protein per cell, respectively. Moreover, the normalized transverse relaxation rates (1/T2 per mM Fe) of cells transformed with wild-type SPFt and the hypermagnetic mutants L55P, F57S and F123S were all very similar: 17.4 ± 2.0, 21.5 ± 1.9, 19.2 ± 1.3 and 19.2 ± 2.5 mM⁻¹ s⁻¹ for wild type, L55P, F57S and F123S, respectively. This indicates that the observed differences in magnetic behaviour are primarily due to the variation in the number of iron atoms accumulated in these cells, rather than to differences in per-iron relaxivity or magnetic moment.

Because enhanced mineral accumulation and magnetism is explicitly associated with SPFt nanoparticles, as opposed to cellular mineral content more generically⁹,¹⁰,¹³, we hypothesized that the mutants identified here could provide means for engineering molecular-scale imaging markers and devices. At an ultrastructural level, SPFt mutants could for instance constitute effective genetically encoded labels for electron microscopic investigations of cells¹⁸. To address this possibility, we examined transmission electron microscopy (TEM) images of yeast transfected with SPFt L55P or with an empty control vector. Yeast expressing the SPFt variant showed distinct puncta of elevated electron density, each close in size to that expected for a Ft mineral core and visible on close examination of arbitrary cytosolic fields of view (Fig. 5a). Comparable images from control did not reveal similar puncta. As a quantitative indication of this difference, we used an automated template-matching procedure to identify approximately Gaussian electron-dense spots of 7 nm full width at half height in multiple TEM images of both SPFt-expressing and control yeast cells. Puncta that closely matched the template (correlation coefficient = 0.9) were counted in cytosolic regions only (Fig. 5b). This analysis indicated a concentration of 220 ± 40 puncta µm⁻³ from SPFt cell images (n = 10) but only 70 ± 8 puncta µm⁻³ from control images (n = 4), a significant difference (t-test P = 0.05) supporting the identification of these spots with SPFt nanoparticles. This suggests that SPFt variants could indeed function as TEM-detectable genetically encoded labels in engineered yeast and perhaps other cells.

**Construction of an intracellular sensor using SPFt L55P.** In addition to potential utility for magnetic cell sorting, cellular MRI and electron microscopic investigations, the hypermagnetic SPFt variants are potentially useful building blocks for incorporation into magnetic molecular devices. As demonstration of this idea, we constructed a SPFt-based magnetic biosensor for galactose-induced gene expression in yeast (Fig. 6a). To design the biosensor, we made use of the dependence of magnetic

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**Figure 4 | Engineered SPFt mutants are effective hypermagnetic probes in yeast.** (a) Yeast cells transformed with empty vector (Vec), human heavy chain Ft (Hft), SPFt, L55P, F57S and F123S were pelleted and imaged in a 7 T MRI scanner. Relaxation rates (1/T2 per mM Fe) of cells transformed with wild-type SPFt and the hypermagnetic mutants L55P, F57S and F123S were all very similar: 17.4 ± 2.0, 21.5 ± 1.9, 19.2 ± 1.3 and 19.2 ± 2.5 mM⁻¹ s⁻¹ for wild type, L55P, F57S and F123S, respectively. This indicates that the observed differences in magnetic behaviour are primarily due to the variation in

**Figure 5 | Detection of intracellular SPFt particles in ultrastructural cell images.** (a) Representative TEM image of a yeast cell following transfection with SPFt L55P and growth in overnight in medium containing 1 mM ferric citrate before sample preparation (left, scale bar, 500 nm). A closeup of the region identified by the dashed box at left region shows electron dense puncta such as those indicated by arrowheads (top right, scale bar, 100 nm). Similar puncta are not apparent in a comparable region from control cells (bottom right). (b) Automated analysis of TEM images from SPFt-expressing (n = 10) or control cells (n = 4) enables quantification of puncta that correlate with coefficient ≥0.9 to a Gaussian spot with full width at half height of 7 nm, comparable to the expected SPFt mineral core size. The difference in the density of puncta in SPFt L55P-expressing versus control cells is significant with t-test P = 0.05.
relaxation properties on the aggregation state of iron-loaded Ft molecules\textsuperscript{33,34}. Aggregation of purified SPFt L55P variant in buffer could be induced by mixing purified protein with streptavidin (SA) tetramers, which provide a multivalent binding moiety for SA that could be expressed in cells, where they are likely to exhibit faster and more versatile responses than could be obtained by altering Ft expression itself\textsuperscript{13}. Yeast bearing SPFt-based magnetic devices might also be applicable as whole-cell-based sensors in opaque media or living organisms.

**Discussion**

In this report, we have shown that a high-throughput protein selection strategy can be applied to enhance intracellular molecular-level biomineralization within Ft variants, resulting in proteins with the ability to induce magnetic phenotypes, influence imaging signals at multiple scales and serve as building blocks for intracellular magnetic devices. Mechanistic analysis of the SPFt mutants identified here indicated that single amino acid substitutions significantly enhanced the uniformity of mineral formation within SPFt expressed in yeast. This result could not have been predicted from the Pft structure alone, validating the random library construction approach we took, and also shows that screening for iron sequestration phenotypes can complement traditional site-directed mutagenesis studies\textsuperscript{36–38} to expand knowledge about the mechanisms of iron mineralization by Ft.
The specific mutagenesis and screening approach taken here is one of a universe of approaches that could have been taken to obtain desirable Ft biominalization mutants. Selecting for iron accumulation as opposed to magnetic properties biased the screen towards variants with increased mineral core formation (Fig. 3 and Supplementary Fig. 5), potentially enhancing the performance of selected variants as TEM labels (Fig. 5). For magnetic applications such as imaging and magnetic sorting (Fig. 4), as well as magnetic biosensing (Fig. 6), screening directly for magnetic properties might have been more direct, but magnetic assays tend to be both less sensitive and cruder. For instance, our own initial experience with magnetic column-based selection approaches (cf. Fig. 4b) revealed several sources of artifacts, ranging from nonspecific column adhesion to cell clumping, which would compromise the efficiency of a screen. Although further improvement in magnetic screening techniques is certainly possible in the future, indirect screening using optical approaches proved useful here, as in our earlier work with magnetically active MRI sensors.39 With either type of approach, careful molecular analysis of selected clones must be performed to avoid mutations unlinked to the desired phenotype (such as changes in growth rate), but nevertheless artificially favored by the screening technique.

Although all of our experiments were performed in yeast, the protein engineering principles applied here and potentially the specific clones identified could be applied for biotechnological applications in other eukaryotic or prokaryotic cellular environments. Further optimization could be necessary to enhance Ft variant expression in the desired host, or to adapt the magnetic proteins to iron homeostasis and protein folding conditions in other systems. In any context, manipulating mineral nucleation could prove to be a general and versatile route for tuning intracellular biominalization, particularly if unnatural mineral species are desired.40 Protein engineering approaches like those introduced here could also be used to engineer additional metalloproteins, and could further alter other parameters of genetically expressed magnetic biomaterials and biosensors.

**Methods**

**Yeast strain and handling methods.** We used the haploid yeast (S. cerevisiae) strain BY4742/FTRI-GFP (MATα × FTRI-GFP: HIS3 harboured leu2Δ lys2Δ ura3Δ) (gift from Dr Christopher Burd) as a host for expression of all Ft variants. We grew yeast cells in a dropout medium without histidine (SD-HIS) made with a dry culture medium (Fykova, Hollister, CA) or in a YPAD medium: 10 g l⁻¹ yeast extract (BD Biosciences, San Jose, CA), 20 g l⁻¹ of Bacto Peptone (BD Biosciences), 20 mg l⁻¹ of adenine hemisulfate and 20 g l⁻¹ glucose. We transformed yeast cells with expression plasmids using the Frozen-EZ Yeast Transformation II kit (Zymo Research, Irvine, CA).

**Construction of Strept-tag II/ferritin fusion proteins.** We used Escherichia coli NEB10β cells (New England Biolabs, Ipswich, MA) for plasmid construction. To create an expression plasmid with a dominant selectable marker, we used the PCR to amplify a geneticin resistant cassette, KanMX4 from a plasmid pFA6-kanMX4, kindly provided by Dr Peter Filippenko. We subcloned the PCR product containing KanMX4 fragment into the pHvX2 yeast expression plasmid generously supplied by Dr Hennie Van Vuuren.41 We then made a point deletion to destroy a superfluous EcoRI site by the QuickChange Lightning Kit (Agilent Technologies, Santa Clara, CA) to yield the host plasmid, pHVX2G, used for subsequent expression of Ft constructs in our experiments. We amplified ferritin gene of Pt from the genomic DNA of the bacteria (ATCC, Manassas, VA). A Strept-tag II sequence (W5HPQQEK), spacer (GTSS) and restriction sites were genetically fused at the 5' end of the Pst gene and the PCR product was subcloned into pHVX2G to yield plasmid pHVX2G-SPFt (Supplementary Table 3).

**SPFt expression and affinity purification.** For expression of SPFt, we inoculated yeast cells with expression plasmids in 1 ml of YPAD media with 200 µg ml⁻¹ Geneticin and incubated overnight at 30 °C. Then diluted the cultures into fresh media at OD600 = 0.04 and incubated them for 16 h at 30 °C before harvesting. We washed the freshly harvested yeast with 30 ml of PBS + 10 mM EDTA twice and finally resuspended in PBS. We lysed yeast cell pellet with P-PER Plus (Thermo Scientific, Waltham, MA), benzamide nuclease (EMD Millipore, Billerica, MA) and protease inhibitors according to the manufacturer’s instructions. We then centrifuged the lysate at 5,000 g for 20 min at 4 °C. SPFt protein was purified by applying the cleared lysate into the Strep-Tactin sepharose column (IBA, Göttingen, Germany) according to the manufacturer’s instructions. We then eluted the streptavidin from the column with 100 mM DTT (Electron Microscopy Sciences, Hatfield, PA), removed the excess solution with a filter paper and let it dry for 30 s. We then applied 15 µl of 1% phosphotungstic acid (pH 7.0) over the sample for about 10 s and removed the excess stain with a filter paper. The grid was blotted at room temperature for at least 1 h before imaging with a JEOL 2010 HRTEM instrument (JEOL, Tokyo, Japan)

For cryo-EM, we applied 5 µl of the protein and buffer solution on a lacey copper grid coated with a continuous carbon film and removed excess sample without damaging the carbon layer using a Gatan Cryo Plunge III (Gatan, Pleasanton, CA). We mounted the grid on a Gatan 626 cryo-holder equipped in the TEM column and kept it under liquid nitrogen throughout the transfer into the microscope and the subsequent imaging session. We imaged the SPFt samples on a JEOL 2100 FEG microscope (JEOL) using a minimum-dose method that was essential to avoid sample damage under the electron beam. We imaged at 200 kV with a magnification setting that kept the square pixel size for assessing particle size about the images on a Gatan 2k × 2k UltraScan CCD camera (Gatan, Pleasanton, CA).

To calculate the percentage of filled cores, we counted 400 particles per sample and divided the number of filled particles by 400. For each SPFt variant, we obtained cryo-EM images of the protein samples from two different batches to calculate the mean, s.e.m., and statistical parameters. We estimated core sizes by autocorrelation functions were computed from the same TEM images (three per variant) in Matlab, generating autocorrelation plots and radial profiles presented in Supplementary Fig. 5.

**Library construction.** We carried out library construction using an error-prone PCR approach.42 The entire SPFt gene except for the Strept-tag II sequence was subjected to mutagenesis over 30 error-prone amplification cycles, which yielded on average one amino acid mutation per SPFt gene. The linearized vector was prepared by digesting pHVX2G with Apal and Xhol followed by gel purification. We transformed yeast with the SPFt library according to the method developed by Benatuly et al. with a few modifications. We mixed 1.5 µg of digested plasmid and 0.5 µg of error-prone PCR product with 100 µl of electrocompetent cells (≅ 1.6 × 10⁸ cells per ml) in a disposable electroporation cuvette with 0.2 cm gap (Bio-Rad, Hercules, CA) on ice for 5 min. We electroporated the cells at 3 kV using MicroPulser electroporator (Bio-Rad), resulting in time constants ranging from 4.8 to 5.3 ms. After electroporation, we immediately transferred the cells to 1:1 mix of 1 M sorbitol:YPAD medium and incubated in 30 °C for 3 h. Then we harvested cells by centrifugation and resuspended in SD-HIS with 200 µg ml⁻¹ of Geneticin and incubated for 2 days before freezing them for long-term storage at – 80 °C. Typical transformation efficiency was 0.5–1.0 × 10⁷ transforms per µg of plasmid DNA. The library diversity was tested by sequencing randomly picked 24 colonies.

**Measurements of iron content in cells and purified protein.** We used a colormetric assay based on the protocol of Tamarit et al.43 to quantify the iron content of yeast cells and the purified protein. This method relies on the Fe²⁺ dependent optical absorbance of bathophenanthrolinedisulfonic acid (BPS) at 535 nm at pH 5.4. As standards, we dissolved known amounts of ferrous ammonium sulfate in 3% nitric acid.

For measuring the iron content of yeast cells, we digested 4.2 × 10⁶ cells by boiling in 200 µl of 3% nitric acid for 2 h, and centrifuged at 10,000 g for 5 min. To measure the concentration of iron in SPFt, a 1:1 ratio of purified protein and 3% nitric acid solution were mixed and boiled for 15 min followed by centrifugation at 10,000 g for 5 min. In both cases, the iron quantification assay was applied to the supernatant of the resulting samples. Iron loading stoichiometries of the protein samples were computed by dividing the iron concentrations by the protein concentrations, as measured by the 660 nm Protein Assay (Thermo Scientific).

**High-throughput screening.** We inoculated 1 × 10⁶ cells in a 20 ml SD-HIS medium containing 200 µg ml⁻¹ of Geneticin at 30 °C overnight (about 16–20 h). We harvested the cells in a culture tube and resuspended in a sterile PBS such that the cell density was about 5 × 10⁶ cells per ml. We filtered the cells with a sterile membrane with 40 µm pores immediately before sorting. Similarly, we prepared negative control samples using the BY4742 background strain without the FTRI–GFP reporter. We set up a flow cytometry protocol using the control yeast samples. First, the yeast population was gated with forward and side scattering channels to remove debris and aggregated cells. We then collected cells displaying green fluorescence at top ~ 5%, indicating high FTRI–GFP expression. We propagated these cells overnight in 4 ml of SD-HIS medium supplemented with 200 µg ml⁻¹ of Geneticin.
Measurement of iron oxidation and release kinetics. We monitored the kinetics of iron oxidation by SPFt variants by an optical assay. We prepared SPFt samples with 16,274-mW/cm² light in 100 mM MOPS, pH 7.0. We added ferrous ammonium sulfate solution (1 mM), made in degassed distilled water to the protein solution (final concentration of 0.1 μM) at a 500-fold molar excess of iron(II). Following a mixing dead time (~5 s), we recorded the optical absorbance of the mixture at 315 nm every 2 s for 5 min. We used a disposable cuvette with a 1 cm path length and recorded the spectra with SpectraMax M2 Microplate reader (Molecular Devices, Sunnyvale, CA). We calculated the specific activity, defined as the micromoles of iron(III) formed per minute per milligram of 24-mer SPFt sample, by dividing the change in absorbance of the reaction mixture over the first 30 s by the extinction coefficients of SPFt variants and the absorbition of protein in the reaction. Extinction coefficients for wild type SPFt, L55P, F57S and F123S were 2.6 ± 0.1, 2.7 ± 0.1 and 2.8 ± 0.1 mm cm⁻¹, respectively.

We measured the kinetics of iron release from preloaded SPFt variants by monitoring time dependent formation of the BPS complex with Fe³⁺ released from iron-loaded Ft variants. We used purified SPFt samples that were loaded aerobically with 1,000 Fe atoms per molecule. These samples were diluted to a final concentration of 0.1 μM SPFt oligomers in an iron mobilization assay buffer that included MOPS (0.1 M, pH 7.0), sodium acetate (20 mM) and BPS (1 mM). We measured the absorbance values at 535 nm every 30 s for 3 h using SpectraMax M2 Microplate reader. We took the first 3.5 min of the data and computed the initial rate of iron release using the standard curve constructed using freshly made ferrous ammonium sulfate solutions.

Measurement of magnetization curves. 100 μl of each SPFt variant dispersed in Tris buffer (0.1 mM g⁻¹ Fe) was sealed in a propylene straw using a hot press. Zero field cooled curves at 5 K were measured using a superconducting quantum interference device (MPMSXL, Quantum Design, San Diego, CA). Diamagnetic background signal was subtracted by measuring a buffer only sample sealed in the same manner.

Measurement of denaturation profiles. SYPRO dye (Life Technologies) was diluted 25-fold from the manufacturer’s 5,000 × 1 stock into 0.1 M Tris, 0.15 M NaCl, pH 8.5. 1.5 μl of each SPFt variant (0.39 mg ml⁻¹) was mixed with 1.5 μl SYPRO solution and 27 μl GdmHCl to a final concentration of 0–8 M GdmHCl. After 10 min incubation at room temperature, fluorescence intensity was measured using a plate reader with excitation at 567 nm and emission at 580 nm.

Yeast cell pellet MRI. We prepared the yeast samples as described in SPFt expression and purification section. After we washed the cells twice with PBS supplemented with 10 mM EDTA, the supernatant was decanted and 100 μl of the cell suspension was loaded into the wells of a microtiter plate. Unused wells were filled with PBS. We centrifuged the plate at 1,500g for 10 min and placed it in a 12 cm outer diameter biorad transfor transfor transfor in a 20 cm bore Bruker 7 T Avance III MRI scanner. We imaged a 2 mm slice through the cell pellet samples with the field of view of 5 × 5 cm and the data matrices were 256 × 256 points. We used T2-weighted spin echo pulse sequence with multiecho acquisition; repetition time was 2 s, and echo time ranged from 5 to 150 ms in 5 ms intervals. We used custom routines written in Matlab (Mathworks, Natick, MA) to reconstruct the images and computed relaxation time constants by fitting image intensity data to exponential decay curves.

Magnetic cell sorting. High gradient magnetic separations of yeast cells were performed using magnetic columns (Miltenyi Biotec, Bergisch Gladbach, Germany) inserted into a Franzant CNizer, Model L-1CN (S. G. Frantz Company, Inc., Tulltow, PA). Briefly, we suspended yeast cells at the density of 10⁸ cells/ml in a sorting buffer consisting of PBS supplemented with 2 mM EDTA and 0.5% BSA. After equilibrating the column with the sorting buffer, we applied the yeast cells in the column in the presence of an externally applied magnetic field of 0.6 T followed by a wash with the sorting buffer. We then switched off the magnetic field and eluted the cells from the column with the sorting buffer. We collected the flow through, the wash and the elution fractions from the yeast cell pellet. We carried out optical density measurements at 600 nm to estimate the cell densities of each fraction and computed the percentages of cells retained on the columns.

Electron microscopy analysis of SPFt particles in cells. For electron microscopy, yeast cells were grown in YPD medium supplemented with 1 mM ferric citrate overnight. Cells were then harvested, washed in PBS and spheroplasted before transferred to a fix buffer (3% glutaraldehyde, 0.1 sodium cacodylate, 5 mM CaCl₂, 5 mM MgCl₂, 2.5% sucrose). Cells were embedded in 2% low melting temperature agarose and cut into small pieces. Sample blocks were post-fixed in 1% osmium/0.1% potassium ferrocyanide in 0.1 M cacodylate and 5 mM CaCl₂ for 30 min at room temperature. Sample blocks were washed thoroughly and transferred to 1% thiocarboxydrate at room temperature for 5 min followed by another wash. The sample blocks were then dehydrated in increasingly concentrated ethanol solutions and embedded in Spurr resin. Blocks were sectioned on a Leica Ultracut UC7 (Leica Microsystems Inc., Buffalo Grove, IL), stained with 2% uranyl acetate and imaged using FEI Tecnai Spirit transmission electron microscope at 80 kV (FEI, Hillsboro, OR).

To quantitate putative Ft particles, images were first manually segmented to define cytosolic compartments, in particular by excluding membrane, extracellular space and vacuoles. A template for matching to the images was defined by specifying a dark Gaussian spot of 7 nm full width at half height on a white background of 20 × 20 nm. This template was then compared with the images using Fourier-based correlation, to identify image locations that displayed correlation coefficients of 0.9 or greater when matched to the template. Groups of one or more contiguous pixels were counted as a single particle. A total of 10 images of SPFt L55P-transfected cells and 4 images of control cells were analysed in this way and results were scaled to denote the concentration of qualifying puncta per cubic micron. This analysis was performed using custom code implemented in Matlab.

Construction of T7-tagged mutant streptavidin plasmids. We used the PCR with High-Fidelity Phusion master mix (New England Biolabs) to construct the gene of an SA variant optimized for efficient folding and selective binding of Streptag-II (Supplementary Table 1) with High-Fidelity Phusion master mix (New England Biolabs) to construct the gene of an SA variant optimized for efficient folding and selective binding of Streptag-II (Supplementary Table 1).

Bacterial expression and purification of SA variants. To express SA variants, we transformed E. coli with the plasmid, pT7-7 SA Tm and grown in M9 minimum medium supplemented with 100 μg ml⁻¹ ampicillin at 37°C. Once the culture reached OD₆₀₀ = 0.8, we induced the recombinant protein expression with 0.4 μM isopropl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 30°C. We harvested and lysed cells with BugBuster reagent (EMD Millipore) supplemented with protease inhibitor cocktail III (EMD Millipore) and Lysonasone Bioprocessing Reagent (EMD Millipore) for 50 min at room temperature. Insoluble fractions were removed by centrifugation at 10,000 g for 40 min. The soluble fraction of lysate was used for the affinity purification of optimized SA using T7-Tag Affinity Purification Kit (EMD Millipore) according to the manufacturer’s instructions. We then buffer exchanged the purified protein and concentrated into the assay buffer. Wild-type SA was purified from Sigma-Aldrich (St Louis, MO). Protein concentrations were determined using the 660 nm Protein Assay (Thermo Scientific) with BSA as a standard.

DLS measurements. We performed DLS measurements on a DynaPro DLS system (Wyatt Technology, Santa Barbara, CA), at 30°C with averaging over 72 acquisitions each and a 2 s integration time. The laser power was set to 25%. We mixed 16 μl of 0.2 μM 24-mer SPFt sample with various concentrations of SA tetramers, briefly vortexed, and incubated for 5 min before making the DLS measurements in triplicates.

Coexpression of SPFt variants and SA in yeast. To test the SPFt-based biosensing system in cells, we transformed yeast cells with two expression plasmids, pHVX2G-SPFT-L55P and pSAZtStm encoding SPFt L55P or optimized mutant SA, respectively. Control experiments were performed using plasmids encoding PfT L55P, SPFt E94G/K142R, or PfT E94G/K142R in place of SPFt L55P. We first incubated the yeast cells in such medium with 2% glucose and 10 mM FeCl₃ for 5 min before loading the cell suspension with 0.1% raffinose plus 0.1% glucose medium with 2% glucose and 10 mM FeCl₃ and centrifuged them at 10,000 g for 5 min before loading onto a 12% Mini-Protein TGX Precast gel (Bio-Rad). We ran the protein gels at 160 V for 30 min and transferred

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References


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Author contributions

Y.M. and A.J. wrote the paper. R.C. and P.A. respectively performed and supervised the magnetometry procedures. Y.M. was supported by grants DP2-OD002114, R01-NS076462, and R01-MH103160 to A.J. Y.M. was supported by a Siebel Scholar Fellowship and a Friends of the McGovern Institute Fellowship. The magnetometry experiments were conducted at the Shared Experimental Facilities supported in part by the MBRC Program of the National Science Foundation under award number DMR-1419807.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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