Supporting Online Materials

A cytosolic iron chaperone that delivers iron to ferritin

Haifeng Shi, Krisztina Z. Bencze, Timothy L. Stemmler, and Caroline C. Philpott

Materials and Methods

Yeast strains, plasmids, media, and cell lines- Yeast strains were constructed in YPH499 (*MATα ura3-52 lys2-801(amber) ade2-101(ochre) trp1-63 his3-200 leu2-1*). For the *FeRE/HIS3* fusion construct, 977 nucleotides upstream of the *FRE1* open reading frame and the entire *HIS3* coding region were amplified and cloned into pRS405, which was integrated into the *leu2-1* locus of YPH499. The coding sequence of human H-ferritin was amplified from pHL2284 and cloned into the SalI and BamHI sites of YIpDCE1. The resulting plasmid YIpDCE1-H ferritin was linearized in the *ADE2* gene with StuI and integrated into the *ade2-101* locus. The coding sequence of human L-ferritin was amplified from pHL2285 and cloned into the KpnI and XhoI sites of YEp352PGK91-2. The DraI-NheI fragment of YEp352PGK91-2-L ferritin containing PGK1 promoter- L ferritin-PGK1 terminator was inserted into the NaeI and SpeI sites of pRS404. The plasmid pRS404-PGK1-L-ferritin was linearized in *TRP1* with EcoRV and integrated into the *trp1-63* locus. The yeast strain containing *FeRE/HIS3* and human H- and L-ferritin and termed the Ferritin FeRE/HIS3 strain. A congenic strain was constructed without H- and L-ferritin and termed the No Ferritin FeRE/HIS3 strain. Rich media (YPD), yeast complete synthetic media (SC) and defined-iron media were prepared as described (*S1, S2*). A human hepatoma cell line Huh7 was maintained at 37ºC with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). A stable HEK293 cell line expressing IRP2 using the tet-on system (*S3*) was maintained in DMEM α modification with 10 % tet system approved FBS. IRP2 expression was induced by 2 μg/ml of doxycycline for 2 days.

Library construction, screening conditions, plate assay, and ferric reductase assay - The human liver cDNA library was constructed by Open Biosystems (Huntsville, AL) in the yeast expression plasmid YEp352-PGK91-1 using human liver total RNA (BD Biosciences). The Ferritin-FeRE/HIS3 strain was transformed with the human liver cDNA library and transformants were selected on defined-iron medium without uracil and histidine, supplemented with 1 mM ferrozine, 10 μM copper sulfate, and 200 μM ferrous ammonium sulfate (FAS). Transformants demonstrating enhanced growth were selected, and the plasmids were rescued and sequenced. Multiple copies of plasmids containing the full coding sequence of PCBP1 were obtained. The parent vector, YEp352-PGK91-1, and pPCBP1 were re-transformed into the Ferritin-FeRE/HIS3 strain and the No Ferritin-FeRE/HIS3 strain. The transformed strains were spotted in serial 10-fold dilutions onto the selection medium described above, with and without histidine supplementation, and incubated at 30ºC for 3 days. Ferric reductase assays were performed as described (*S4*), and reported as the mean ± SEM. Human holoferritin (Sigma) and human ferritin expressed in yeast in polyacrylamide gels were stained with Coomassie blue for protein detection and $K_4Fe(CN)_6$ for iron detection.

Protein depletion by siRNA- PCBP1 was depleted by Stealth Select RNAi (Invitrogen) with the sequences 5'-GCU CCU CUG GUA GGC AGG UUA CUA U-3' and 5'-AUA GUA ACC UGC CUA CCA GAG GAG C-3'. A non-targeting, scrambled sequence siRNA pool was used as a control. Huh7 or HEK293 cells were transfected with 50 pMol of siRNA using Lipofectamine RNAiMAX (Invitrogen). Cells were harvested at 1, 2, 3

and 4 days after transfection to determine the time course of PCBP1 depletion. Rescue of PCBP1 depletion was performed using a mutated human PCBP1 carrying 3 silent mutations. Ultimate ORF clone IOH2101, containing human PCBP1 (Invitrogen), was mutated at three positions, G978A, T981C, and T984C using two-step PCR with two pairs of primer 5'-GTA GGC AAG TCA CCA TCA CTG GCT CTG CTGC-3', 5'- GTG ATG GTG ACT TGC CTA CCA GAG GAG CCT TC-3', M13 forward primer and M13 reverse primer. The PCR product of the mutated PCBP1 gene was recombined into pcDNA-DEST47 (Invitrogen) using LR Clonase (Invitrogen). The mutated plasmid pmPCBP1 was co-transfected with PCBP1 siRNA using Lipofectamine 2000 (Invitrogen).

Real-time PCR- Quantitative real-time PCR was performed using the double-stranded DNA dye SYBR Green (Applied Biosystems) on an ABI 7500 system according to the manufacturer's protocols. The primers for detection of PCBP1 mRNA were 5'-TCA TGA CCA TTC CGT ACC AGC-3' and 5'- ATC GAG CGG AGA AAT GGT GTG-3'. β-Actin was selected as internal control reference gene. The primers for β-actin were 5'-CCT GGC ACC CAG CAC AAT G-3' and 5'- CGC CGA TCC ACA CGG AGT AC-3'. The PCBP1 values were normalized to β-actin according to Pfaffl's mathematical model for relative quantification in real time PCR (*S5*).

Ferritin iron loading- Yeast cells were grown to late log-phase in SC supplemented with 2 μCi/ml of $^{55}Fe^{3+}$ (PerkinElmer Life and Analytical Sciences) at a concentration of 10 μM. Yeast cellular proteins were extracted using the glass bead lysis method in 50 mM

Tris-HCl (pH 7.4), 150 mM NaCl, and a protease inhibitor cocktail (Roche). Huh7 cells were grown in DMEM with 10% FBS supplement with 4 μCi/ml of $^{55}Fe^{3+}$ at a concentration of 10 μM for 6, 12 and 24 hours. Cellular proteins were extracted with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton, and a protease inhibitors. Protein samples were separated on 3-8% Tris-acetate polyacrylamide gels (Invitrogen) under native conditions. Gels were dried and ^{55}Fe was detected by phosphorimaging (Molecular Dynamic). Images were obtained on Typhoon Trio (Amersham Biosciences) and analyzed using ImageQuant (Amersham Biosciences). Ferritin protein levels were determined by enzyme-linked immunosorbent assay (ELISA) (Bio-Quant, inc, San Diego, CA), according to the manufacturer's instruction. For *in vitro* ferritin iron loading, horse spleen apoferritin (Sigma) (0.1 μM) was incubated in buffer B (1 μM $^{55}Fe^{2+}$, 50 mM Tris-HCl, 100 mM NaCl, 0.1-1 mM DTT, pH 7.0) with increasing concentrations of recombinant PCBP1 (0-3 μM) or BSA (1 μM) at 25°C for 6 hrs. Iron incorporation was measured by autoradiography of ferritin separated on 3-8% polyacrylamide gels as described above.

Immunoblotting- Cellular proteins from yeast and Huh7 cells were extracted as described above. Lysates of Huh7 cells were separated on 3-8% gels under native conditions (for native ferritin detection) or denaturing SDS-PAGE and transferred to Hybond-LFP (Amersham Biosciences). Ferritins were detected using monoclonal antibody against native ferritin (USBiological) at 1:1000 dilution, with Cy3-labeled goat anti-mouse IgG at 1:1000 as secondary antibody. Images were obtained on Typhoon Trio (Amersham Biosciences) analyzed by ImageQuant (Amersham Biosciences). Yeast protein samples

were separated on 4-12% gels and transferred to Hybond-LFP. Ferritins were detected using a polyclonal anti-ferritin antibody (USBiological) at 1:5000 dilution. Anti-Pgk1p (Molecular Probes) was used to confirm equivalent loading. Tet-on IRP2 cells were extracted in 25 mM Tris (pH 8.3), 10 mM KCl, 1mM DTT, 1mM EDTA, 1 mM PMSF, 0.1 mM desferrioxamine and a protease inhibitor cocktail. Rabbit polyclonal antiserum directed against IRP2 (gift from T. Rouault) was used at 1:5000 to detect IRP2. Anti-actin (Abcam) at 1:5000 was used to confirm equivalent loading.

Immunoprecipitation- Yeast cells were lysed using glass beads in 50 mM Tris (pH 7.4), 40 mM KCl, 1 mM PMSF, 5 mM DTT, and protease inhibitor cocktail. Ferritin was immunoprecipitated using rabbit polyclonal anti-ferritin antibody (USBiological) at 1:400 dilution and protein G agarose (GE Healthcare) in lysis buffer without FAS, with 1 mM FAS, and with 1 mM FAS and 5 mM bathophenanthroline disulfonate (BPS). Samples were eluted from beads using 2x SDS PAGE sample buffer. Rabbit polyclonal anti-PCBP1 antibody (Santa Cruz Biotechnology) at 1:400 was used to detect PCBP1.

Measurement of labile iron pool- The labile iron pools (LIP) were measured using calcein-AM (Molecular Probes) as described with the following modifications (*S6, S7*). Huh7 cells cultured in 6 well plates were loaded with 0.25 μM calcein-AM in HEPES-buffered saline for 30 minutes and then washed twice. Fluorescence (F1) was recorded using Typhoon Trio (Excitation, 488 nm; Emission, 526 nm). The cells were then treated with 100 μM salicylaldehyde isonicotinoyl hydrazone (*S8*) for 15 min. to chelate the labile iron pool. The fluorescence (F2) of calcein was measured again. The cells were

harvested and lysed quantitatively. Protein concentration was measured by BCA and used to normalize F2-F1 (ΔF). The relative LIP of PCBP1-depleted cells (ΔF) was normalized to the ΔF obtained from control siRNA-treated cells.

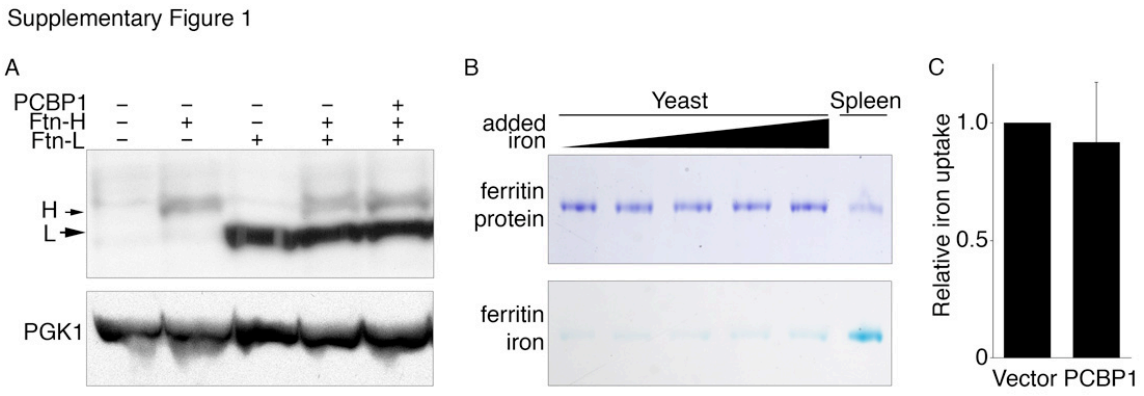
Purification of recombinant PCBP1- Recombinant His_6-PCBP1 was expressed from plasmid pET28a-αCP1 (gift from S. A. Liebhaber) and purified as described (*S9*). PCBP1 without additional vector-derived amino acids was produced using the IMPACT system (New England BioLabs, Inc.). The coding region of human PCBP1 was amplified by PCR and cloned into SapI-PstI sites of vector pTYB11. The recombinant fusion protein was purified on chitin columns and self-cleavage of the intein fusion tag was induced by 50 mM DTT according to the manufacturer's instruction. Purified His_6-PCBP1 was dialyzed against buffer A (50 mM Tris-HCl, pH 7.6, 150 mM NaCl) and untagged PCBP1 was dialyzed against 20 mM Tris-HCl, pH 8.5, 500 mM NaCl, 200 μM DTT. All buffers were sparged with argon to displace oxygen prior to use.

Iron uptake assays- Human apotransferrin (Calbiochem) was iron loaded using $^{55}Fe^{3+}$ as described (*S10*). Cells were incubated with 0.5 μM ^{55}Fe-transferrin in serum free medium for 1 hour at 37°C or on ice. The cells were washed with wash buffer (PBS supplemented with 1mM EDTA, 1 mM BPS, 1 mM KF) and lysed in 20 mM Tris-HCl (pH 6.8), 1mM EDTA, 150 mM NaCl, 1% NP-40. Twenty microliter samples were mixed with scintillation cocktail CytoScint (MP) and counted in a scintillation counter (Beckman). Protein concentrations measured by BCA were used to normalize count per minute (CPM). Iron uptake was the difference in CPM between cells incubated at 37°C and

incubated on ice. In a similar procedure, non-transferrin iron uptake was measured by incubation of cells in the presence of 4 μCi/ml of ^{55}Fe-NTA (10 μM Fe^{3+}, 40 μM NTA) in DMEM, 1% BSA, 150 μM ascorbate for 1 hour.

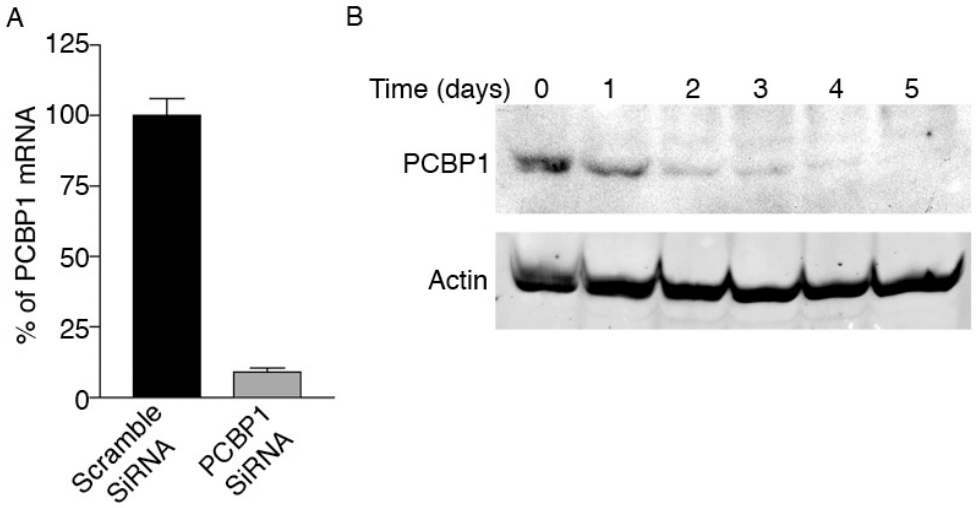
Circular Dichroism Spectroscopy (CD) and Isothermal titration calorimetry (ITC)- CD measurements were performed on multiple independent His_6-PCBP1 and untagged PCBP1 samples at 25ºC, using an Olis DSM 10 CD spectrometer (Olis Inc., Bogart, GA) equipped with a Peltier electronic temperature controller (Quantum Northwest Inc., Spokane, WA). PCBP1 samples were prepared at a concentration of 15 μM in 1 mM $NaPO_4$, pH 7.6. Following a buffer baseline subtraction spectrum, protein CD samples were loaded into a 0.1 cm quartz CD cell (Hellma Cells Inc., USA). CD spectra were collected in the far UV region (between 190-260 nm) using a 1 nm increment size; the smoothed spectrum displayed in Figure 1 represents the average of 10 individual spectra. Secondary structure elements were assessed using the CDSSTR fitting method (*S11*) implemented within the Olis Global Works software package. CD thermal denaturation studies were performed over a temperature range of 10 to 95°C. Full averaged spectra were collected in 5 ° increments over the wavelength range of 190-260 nm. ITC measurements were performed at 25°C on a MicroCal VP-ITC instrument (MicroCal Inc., Northampton, MA). Multiple independent PCBP1 samples were prepared anaerobically at protein concentrations of 30 μM in Buffer A. Titrates of 1 mM anaerobic ferrous ammonium sulfate solution ($Fe(NH_4)_2(SO_4)_2$•$6H_2O$, Fisher Scientific Co.) were injected in 10 μL volumes into a 1.4 mL protein volume at 15 min. intervals. Injector stirring speeds were held at ~550 rpm to ensure complete and expedient mixing of all solution

components. Control experiments of buffer dilution into protein were also performed. The binding isotherm was simulated using single and multiple binding site models. Data and fitting analysis were performed using the Origin 5.0 software package supplied by MicroCal. Origin 5.0 uses a nonlinear, least squares curve-fitting algorithm to determine metal association constants, stoichiometric ratios, and thermodynamic parameters for the complex.



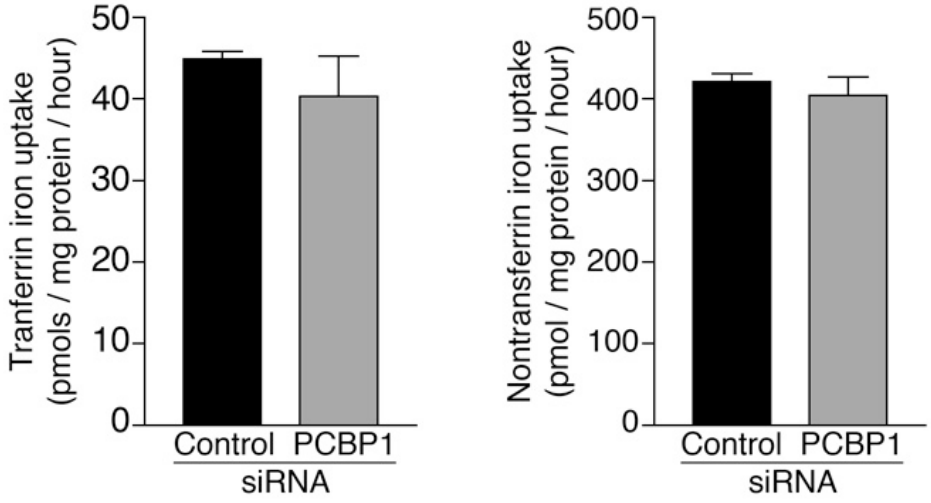
Supplementary Figure 1. Saccharomyces cerevisiae expressing human ferritin H- and L-chains assemble ferritin into a heteropolymer that migrates similarly to native human ferritin, but contains little iron. **(A)** Plasmids containing the human H- and L-ferritin coding sequences under the control of the yeast PGK1 promoter were integrated into the yeast genome. Yeast expressing H- and L-ferritin were also transformed with pPCBP1. Expression of H- and L-ferritin was detected in immunoblots of yeast lysates. Note that the relative amounts of H- and L-ferritin are unchanged by co-expression of PCBP1. **(B)** The yeast Ferritin strain was grown in YPD medium containing 0-1 mM added iron and heat-treated lysates were separated on non-denaturing gels. Purified human spleen ferritin was added as a standard. Duplicate gels were subjected to Coomassie staining for protein (upper panel) or Perl's staining for iron (lower panel). Note that although greater amounts of yeast ferritin than spleen ferritin are present, the amounts of iron incorporated into the yeast ferritin are lower. **(C)** Expression of PCBP1 did not affect total cellular iron uptake. Yeast expressing H- and L-ferritin were transformed with pPCBP1 or the parent vector and grown SC medium in the presence of 10 μM $^{55}FeCl_3$. Cells were washed and the ^{55}Fe content of equivalent numbers of cells was measured by scintillation counting. ^{55}Fe content was normalized to the vector transformed strain (n=5).

Supplementary Figure 2



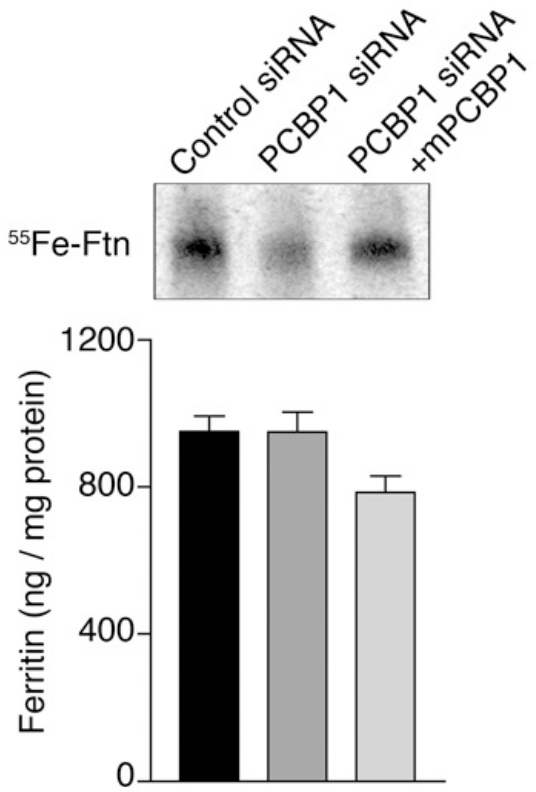
Supplementary Figure 2. PCBP1 can be depleted by siRNA in Huh7 cells. Huh7 cells were transfected with siRNA directed against PCBP1 or a control pool of siRNA in which the nucleic acid sequence was scrambled. Cells were harvested at 2 days post transfection for quantitative real time-PCR analysis of PCBP1 mRNA levels (A) or at days 0-5 post transfection for analysis of PCBP1 protein levels (B). **(A)** Actin mRNA signals were used to normalize the qRT-PCR, and the results were expressed relative to control levels of RNA (n=5). **(B)** After detection of PCBP1, membranes were stripped and re-probed with anti-actin antibody as a control for protein loading.

Supplementary Figure 3



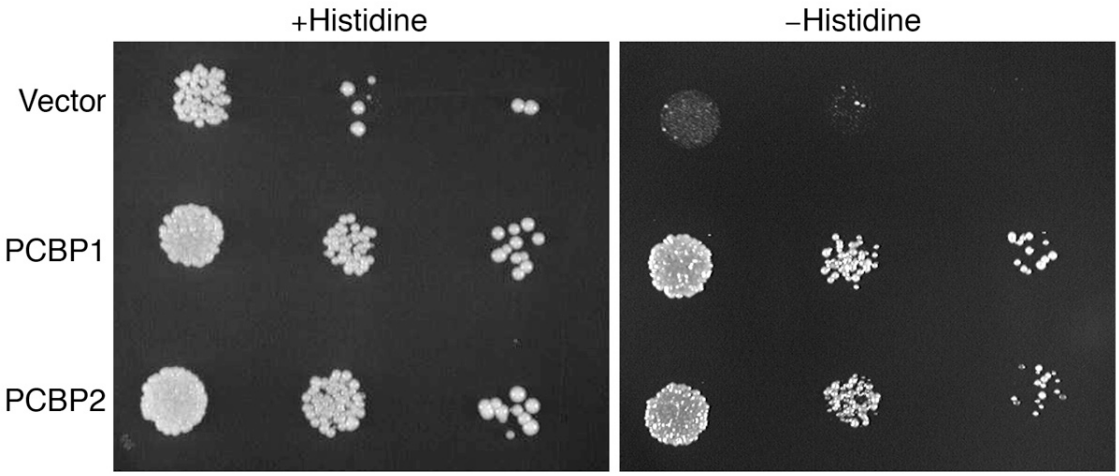
Supplementary Figure 3. Depletion of PCBP1 by siRNA did not affect uptake of ^{55}Fe-transferrin or ^{55}Fe-NTA. Huh7 cells were transfected with siRNA for PCBP1 or control sequences. On post-transfection day 4, cells were incubated with ^{55}Fe-transferrin or ^{55}Fe-NTA at 37°C or on ice in serum-free medium for 1 hr. After washing, cells were subjected to scintillation counting and protein quantitation. Cell-associated iron was normalized by protein concentration and uptake reported is difference between cells incubated at 37°C and on ice. Assays were replicated 3 and 2 times, respectively, and the data pooled for analysis. Error bars indicate SEM.

Supplementary Figure 4



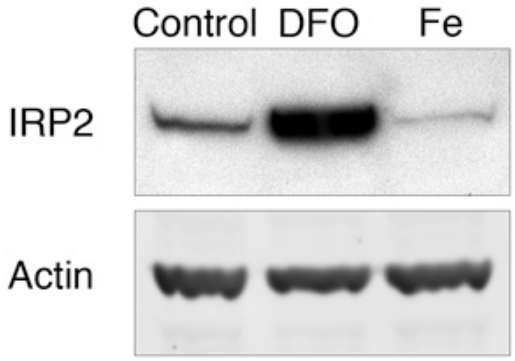
Supplementary Figure 4. Off-target effects do not account for the loss of ferritin iron loading with PCBP1 depletion. Huh7 cells were transfected with control siRNA, PCBP1 siRNA, or co-transfected with PCBP1 siRNA and an expression plasmid carrying PCBP1 with silent mutations in the coding region that prevent targeting by the siRNA. Cells were incubated with ^{55}Fe, and the iron incorporated into ferritin was measured by autoradiography of native gels (upper panel), the total ferritin was measured by ELISA (lower panel) n=2, error bars represent SEM.

Supplementary Figure 5



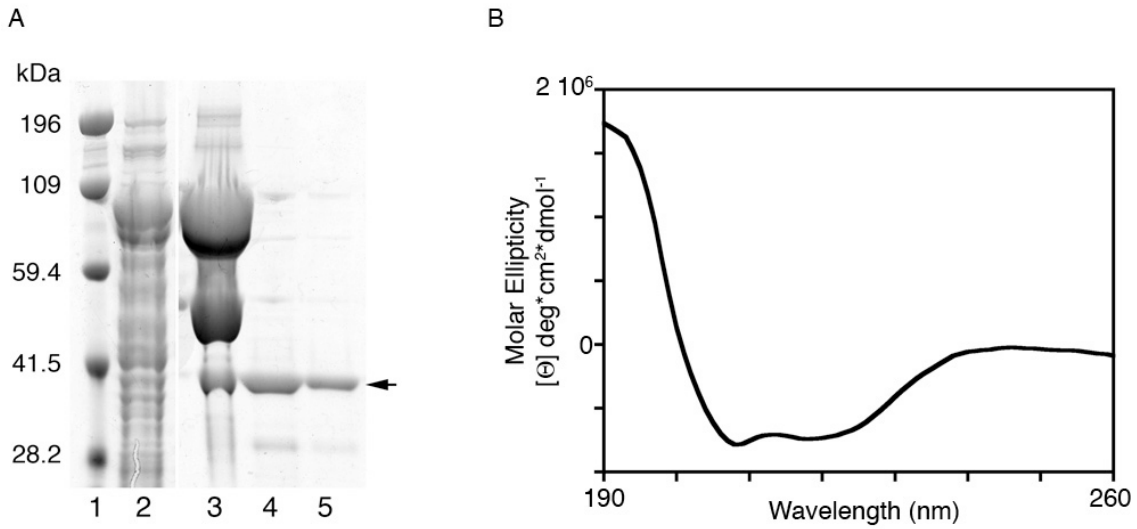
Supplementary Figure 5. PCBP2, a paralogue of PCBP1, also activates the *FeRE/HIS3* reporter when expressed in yeast containing human ferritin H- and L-chains. The Ferritin *FeRE/HIS3* strain was transformed with pPCBP1, pPCBP2, and the empty vector and plated in serial dilutions on media with and without histidine. This assay was repeated three times with identical results.

Supplementary Figure 6



Supplementary Figure 6. HEK293 cells overexpressing IRP2 from a tetracycline-inducible promoter exhibit iron dependent degradation of IRP2. HEK293 cells stably transfected with IRP2 under the control of the Tet-on system were incubated in doxycycline for 2 days followed by treatment with desferrioxamine B (DFO) at 100 μM or $FeNH_4(SO_4)_2$ (Fe) at 200 μM. for 16 hrs. Cell lysates were normalized for protein concentration. IRP2 was detected with a polyclonal rabbit anti-IRP2 antibody. Actin was detected as a loading control.

Supplementary Figure 7



Supplementary Figure 7. Purification of PCBP1 and circular dichroism spectroscopy (CD). **(A)** Intein-tagged PCBP1 was expressed in E. coli, purified on a chitin column, and induced to undergo self-cleavage to produce untagged, recombinant PCBP1. Lane 1-protein molecular weight markers, lane 2-crude lysate after induction, lane 3-residual intein-PCBP1 fusion protein retained on chitin column matrix after elution, along with retained intein tag and residual cleaved PCBP1, lanes 4- and 5- fractions eluted from column after DTT-induced cleavage of intein tag. Arrowhead indicates untagged PCBP1. **(B)** CD spectrum of purified PCBP1 at 25°C in 1 mM sodium phosphate, pH 7.6. Displayed spectrum represents an average of 10 consecutive scans. The signal minima (negative Cotton effect) observed at 208 and 222 nm, together with the signal maximum (positive Cotton effect) observed at 195 nm, suggest a large helical content in the secondary structure of PCBP1. An estimation of the helical content performed through spectral simulation indicates an average secondary structural content of 58% α-helix, 18% β-strand and 24% turns/random coils (standard deviations of 0.07).

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