Supplementary Information

A PCBP1–BolA2 chaperone complex delivers iron for cytosolic [2Fe-2S] cluster assembly

Sarju J. Patel[1], Avery G. Frey[1], Daniel J. Palenchar[1], Sooraj Achar[1], Kimberly Z. Bullough[1], Ajay Vashisht[2], James A. Wohlschlegel[2], and Caroline C. Philpott[1]*.

[1]Genetics and Metabolism Section, NIDDK, NIH, Bethesda, MD and [2]Department of Biological Chemistry, UCLA, Los Angeles, CA.

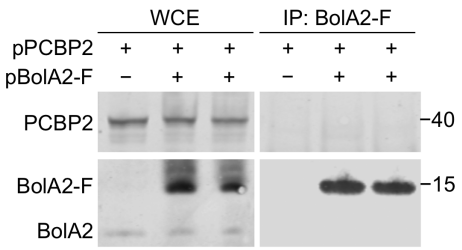
*Corresponding author: Caroline C. Philpott, M.D., Carolinep@mail.nih.gov
Chief, Genetics and Metabolism Section
Liver Diseases Branch, NIDDK, NIH
Bldg. 10 Rm. 9B-16
10 Center Drive, MSC 1800
Bethesda, MD 20892-1800
Phone: 301-435-4018; Fax: 301-402-0491

Flag-PCBP1						
	Fe	Fe	Fe*	Dfo	Dfo	Dfo*
PCBP1	1993/42	2800/54	3061/43	2561/52	2784/52	2377/33
PCBP2	248/11	436/17	904/15	331/16	520/15	682/15
BolA2	3/2 (29%)	3/3 (41%)	0	2/2 (29%)	0	0
Glrx3	0	0	0	0	0	0

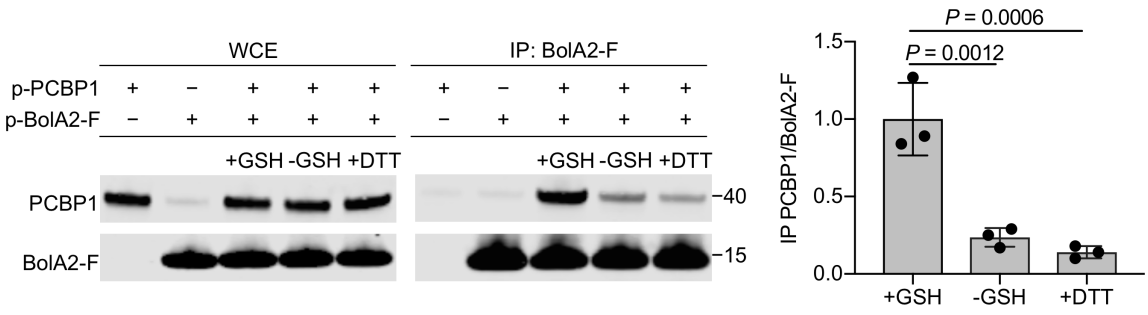
Supplementary Table 1: BolA2 detected in immunoprecipitates of Flag-PCBP1 by mass spectrometry. First number denotes total number of spectra, second number denotes unique peptides, parentheses indicate % coverage; * indicates washes at 500 mM NaCl. Data are representative of *n*=2 independent experiments.

Gene (Supplier)	Targeting sequence
Glrx3 (Stealth siRNA-Thermo Fisher)	5’-CCUACCUAUCCUCAGCUCUAUGUUU-3’
BolA2 (Stealth siRNA-Thermo Fisher)	5’-UGGUGUCGGCUAAGUUCGAGGGAAA-3’
PCBP1 (Stealth siRNA-Thermo Fisher)	5’-GCUCCUCUGGUAGGCAGGUUACUAU-3’ 5’-AUAGUAACCUGCCUACCAGAGGAGC-3’
PCBP2 (Stealth siRNA-Thermo Fisher)	5’-CCGGUGUGAUUGAAGGUGGAUUAAA-3’
Nfs1 (Dharmacon ON-Targetplus pool)	5’-GGAGCUGAUCCUCGUGAGA-3’ 5’-UCUCUAUAUGGAUGUGCAA-3’ 5’-UCCCUUACCUAAUCAACUA-3’ 5’-CUUAUGGCUGGGAGAGUGA-3’

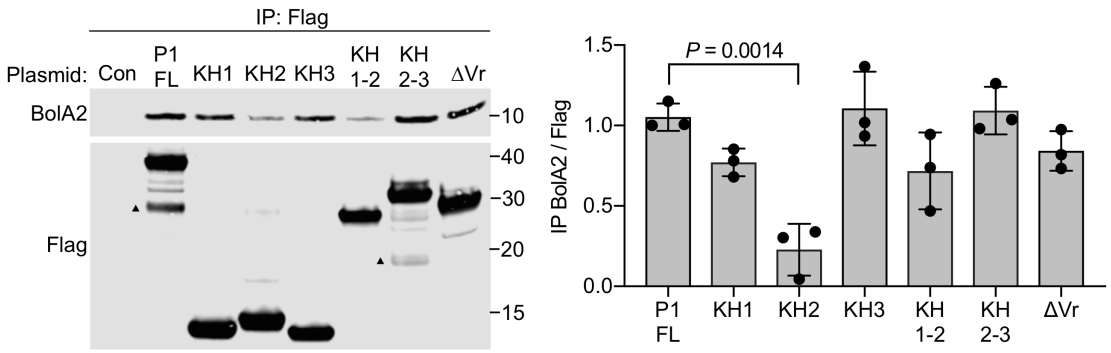
Supplementary Table 2: siRNA used in this study



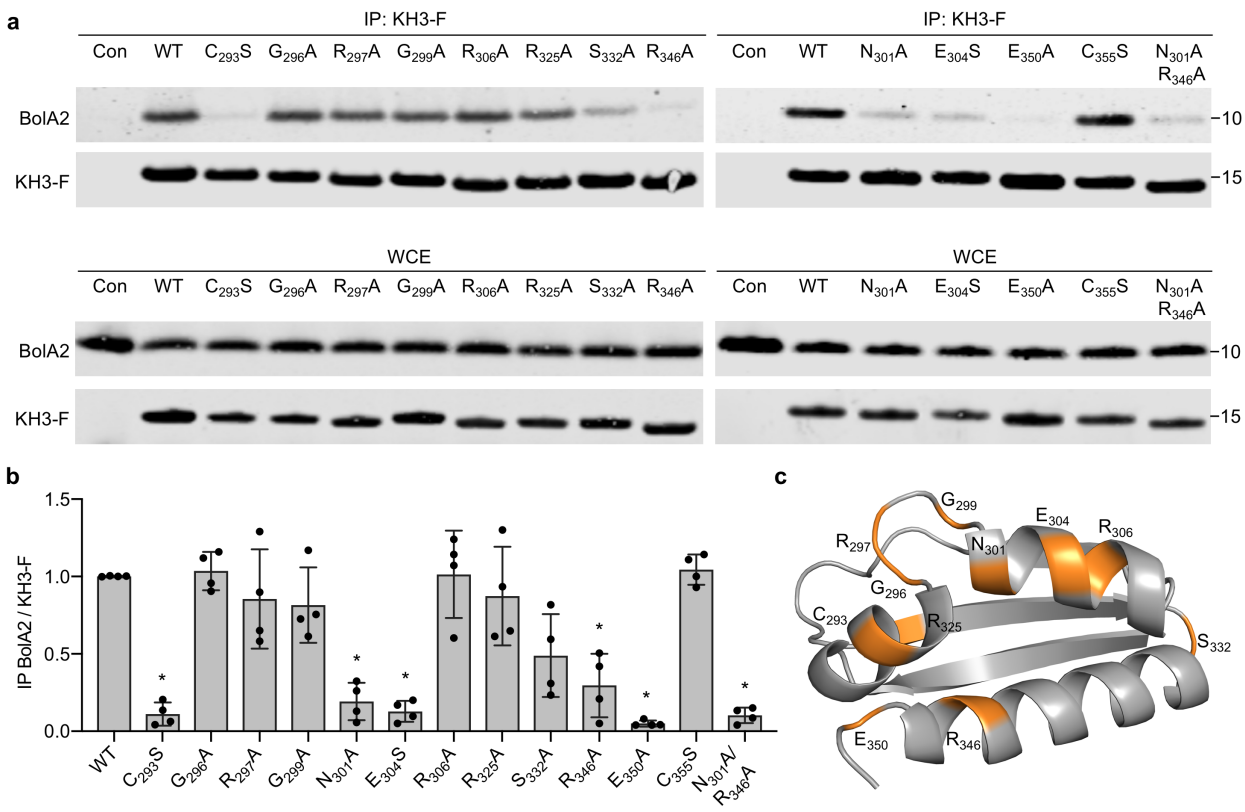
Supplementary Figure 1. PCBP2 does not bind with BolA2 in cells. HEK293 cells transfected with plasmid (p) negative control (-), PCBP2, or BolA2-Flag (BolA2-F) were lysed in buffer IP and anti-Flag immunoprecipitation (IP) performed. Whole cell extracts (WCE) and immune complexes were analyzed by immunoblot against PCBP2 and BolA2. Blots are representative of *n*=3 independent experiments with similar results. Uncropped western blots are presented in supplementary Figure 19.



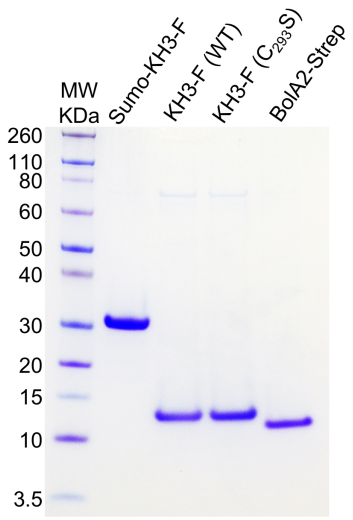
Supplementary Figure 2. Dithiothreitol does not restore PCBP1-BolA2 complex in absence of glutathione. HEK293 cells were transfected with plasmid (p) PCBP1 or BolA2-Flag (BolA2-F). Cells were harvested and lysed in buffer IP containing 5 mM glutathione (GSH) or 1 mM dithiothreitol (DTT) and anti-Flag immunoprecipitation (IP) was performed. Whole cell extracts (WCE) and immune complexes (IP: Flag) were analyzed by immunoblotting using antibodies against PCBP1 and BolA2 (*left*) with quantitation (*right*). Data are means ± SD of n=3 independent experiments. Significant P values ($P < 0.05$), as determined by two-tailed unpaired t-test relative to +GSH control, are shown. Uncropped western blots are presented in supplementary Figure 20.



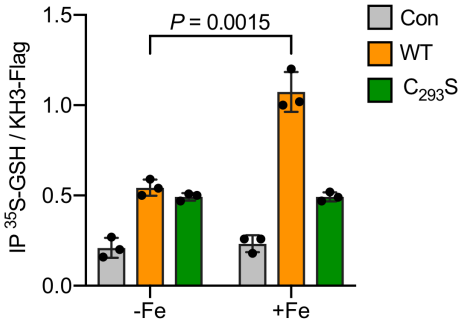
Supplementary Figure 3. Different K-homology domains of PCBP1 forms complex with BolA2. HEK293 cells were transfected with plasmid negative control (Con) or Flag tagged full length PCBP1 (P1FL) or indicated KH domains (KH1, KH2, KH3, KH1-2, KH2-3, and ΔVr). Cells were harvested, lysed and anti-Flag IP was performed. Immune complexes (IP: Flag) were analyzed by immunoblotting using antibodies against BolA2 and Flag (*left*) with quantitation (*right*). ▲ protein degradation of PCBP1. Data are means ± SD of n=3 independent experiments. Significant P values ($P < 0.05$), as determined by two-tailed unpaired t-test relative to P1FL control, are shown. Uncropped western blots are presented in supplementary Figure 21.



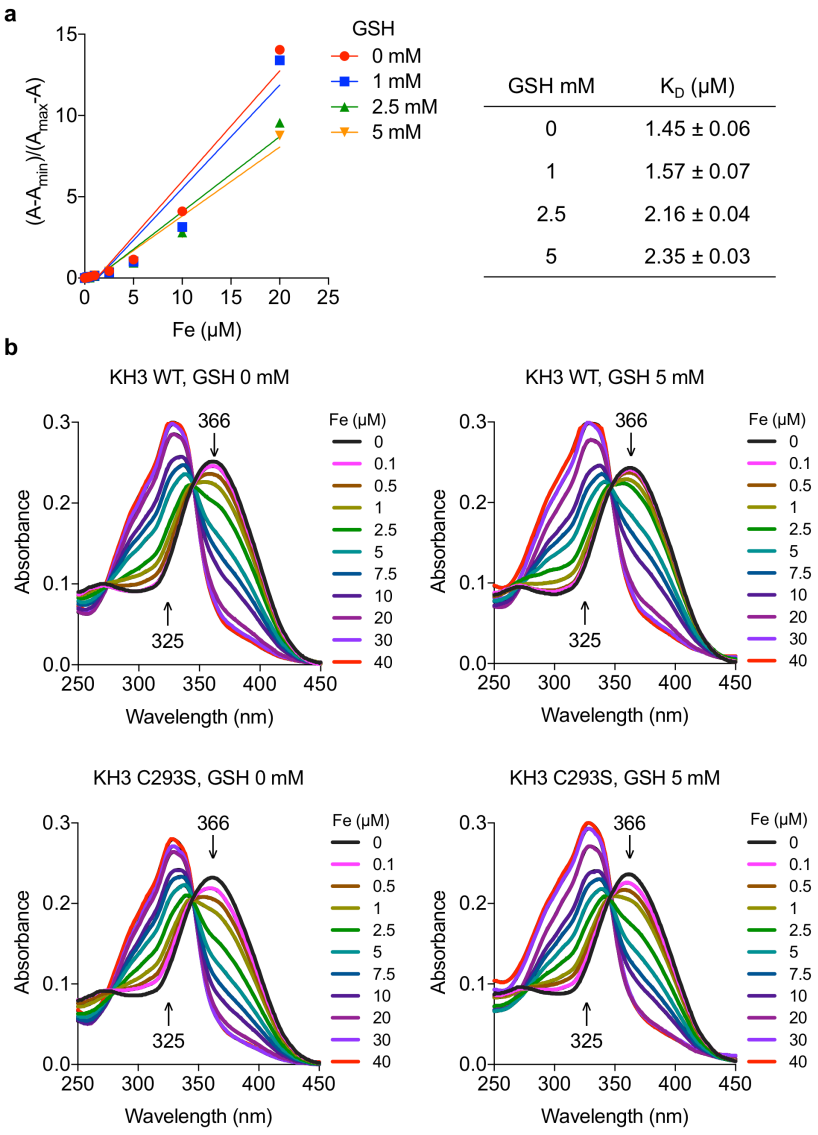
Supplementary Figure 4. Identification of residues on KH3 domain required for BolA2 interaction. a, BolA2 and different KH3-Flag variants, as indicated, were expressed in HEK293 cells and anti-Flag IP performed. WCE and immune complexes were analyzed by western blot using antibodies against BolA2 and Flag-tag. Blots are representative of *n*=4 independent experiments with similar results. **b,** Ratio of co-precipitated BolA2 to each KH3-F variant is normalized to WT. Data are means ± SD of *n*=4 independent experiments. Statistical significance level was calculated by one-way ANOVA analysis ($F = 43.91$, d.f. = 20, $P < 0.0001$) with a Dunnett's multiple comparison test, $^*P \leq 0.0001$ compared to WT. **c,** Ribbon structure of KH3 domain (PDB: 1WVN) illustrating amino acid residues tested for BolA2 interaction in orange. Uncropped western blots are presented in supplementary Figure 22.



Supplementary Figure 5. **Purification of Sumo-KH3-Flag, KH3-Flag variants (WT and C293S) and BolA2-Strep proteins.** 5 µg of purified Sumo-KH3-Flag, KH3-Flag (WT and C293S) and BolA2-Strep were resolved in SDS-PAGE and stained with coomassie brilliant blue. Data are representative of *n*=3 independent experiments with similar results.

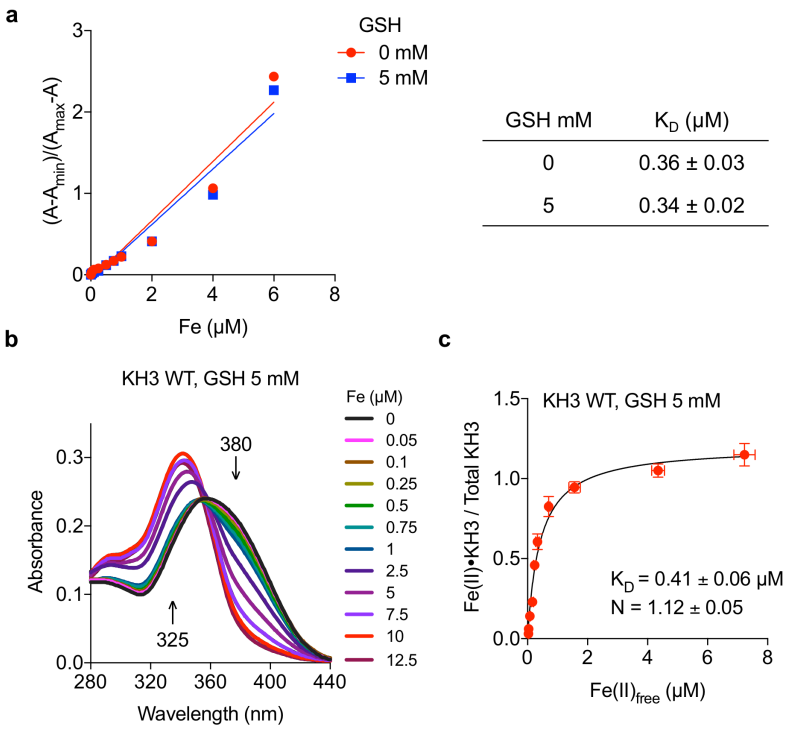


Supplementary Figure 6. Iron-dependent increase in GSH binding to WT, but not C293S KH3 domain. Purified WT or C293S KH3-F were captured on anti-Flag resin. Immobilized KH3-F or resin alone (Con) were incubated with 100 μM ^{35}S-GSH, +/- 100 μM $FeSO_4$ and analyzed for ^{35}S-GSH retention by scintillation counting. Data are means ± SD of *n*=3 independent experiments. Significant *P* values ($P < 0.05$), as determined by two-tailed unpaired *t*-test relative to WT (-Fe), are shown.

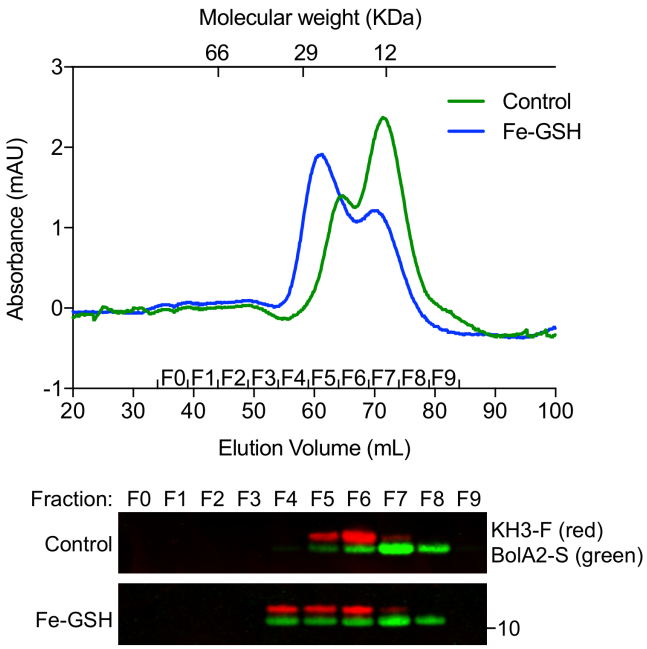


GSH mM	K_D (μM)
0	1.45 ± 0.06
1	1.57 ± 0.07
2.5	2.16 ± 0.04
5	2.35 ± 0.03

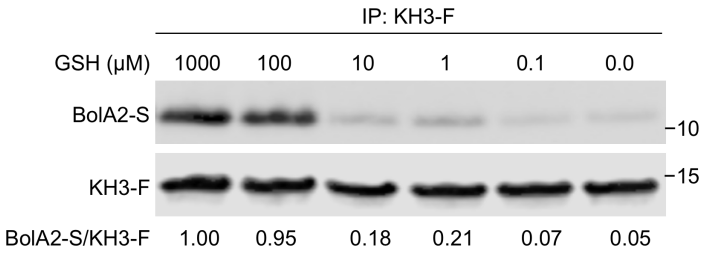
Supplementary Figure 7. **Glutathione does not interfere with iron binding to Mag-fura-2 but contribute to iron binding on KH3 domain of PCBP1. a,** Double reciprocal plot show the dependence of the emission ratio on Fe(II) concentration for Mag-fura-2 at different concentrations of glutathione (*left*). Comparison of dissociation constants (K_D, with Standard Deviations) for Mag-fura-2 with Fe(II) as determined at different concentrations of glutathione (*right*). Data are representative of two independent experiments with similar results. **b,** Absorption spectra of 10 μM Mag-fura-2 in competition with 10 μM KH3 proteins (WT or C293S) with increasing concentrations of $FeSO_4$, in absence of GSH (*left panel*) or in presence of 5 mM GSH (*right panel*). The absorbance maximum of apo-MF2 at 366 nm shifts toward 325 nm concurrent with metal binding. The change in the Mag-fura-2 absorbance at 366 nm was monitored and used to calculate the fractional saturation and apparent K_D of the protein with Fe(II) (see Figure 6b). Data are representative of *n*=4 independent experiments with similar results.



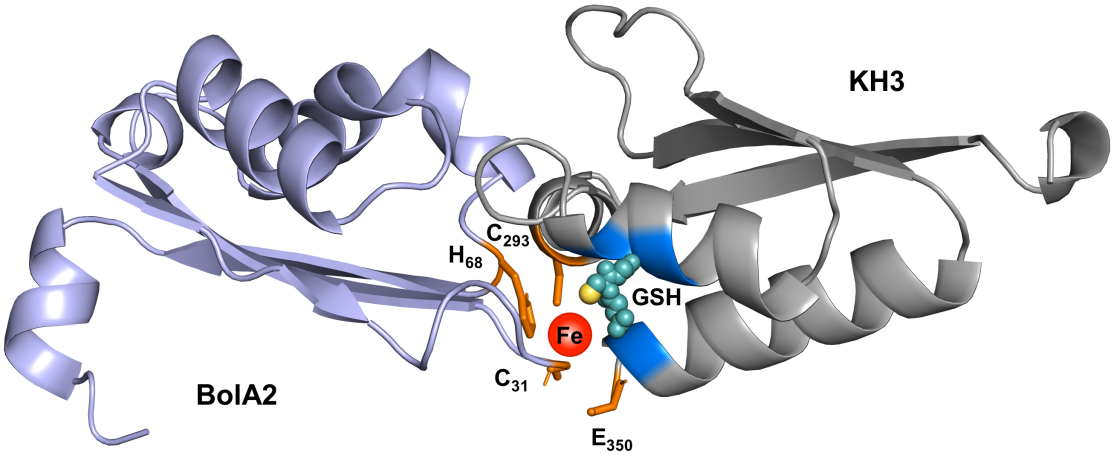
Supplementary Figure 8. **Quantitative determination of iron binding on KH3 domain of PCBP1 using Fura-4f. a,** Double reciprocal plot show the dependence of the emission ratio on Fe(II) concentration for Fura-4f at different concentrations of glutathione (*left*). Comparison of dissociation constants (K_D, with Standard Deviations) for Fura-4f with Fe(II) as determined at different concentrations of glutathione (*right*). Data are representative of two independent experiments with similar results. **b,** Absorption spectra of 10 μM Fura-4f in competition with 10 μM wild type KH3 protein with increasing concentrations of $FeSO_4$, in presence of 5 mM GSH. The absorbance maximum of apo-Fura-4f at 380 nm shifts toward 325 nm concurrent with metal binding. Data are representative of *n*=4 independent experiments with similar results. **c,** The absorbance decrement at 380 nm was monitored and used to calculate the fractional saturation of the protein with Fe(II). The data were fitted by Michaelis-Menten analysis to obtain the dissociation constant (K_D) and stoichiometry (N). Data are means ± SD of *n*=4 independent experiments.



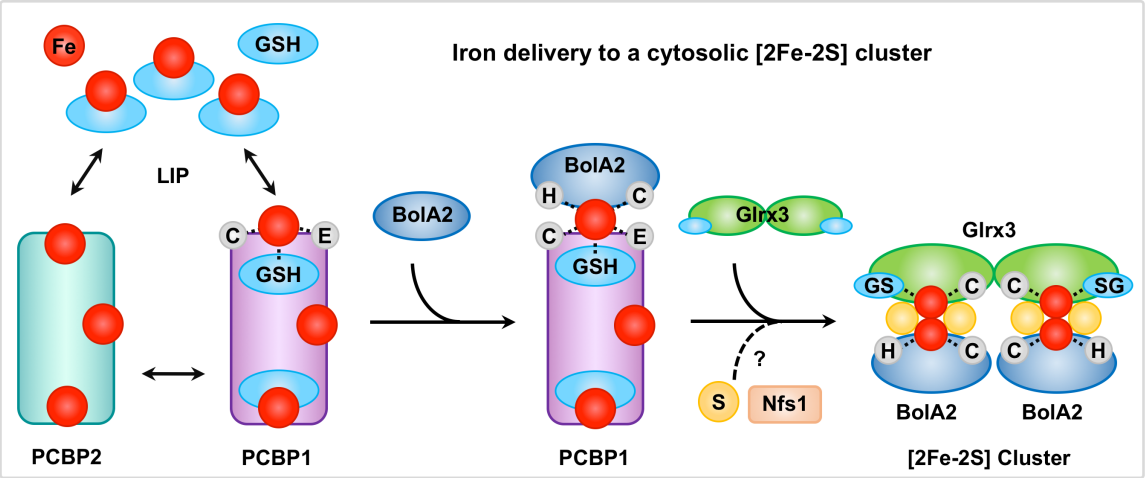
Supplementary Figure 9. **Isolation of KH3-BolA2 heterodimer complex using size exclusion chromatography.** Representative chromatograph (*upper panel*) of size-exclusion analysis of purified KH3-Flag and BolA2-Strep proteins in absence (control) or presence of 100 µM $FeSO_4$ and 1 mM GSH (Fe-GSH). Collected fractions were analyzed by Western blot using antibodies against BolA2 and Flag-tag (*lower panel*). Data are representative of two independent experiments with similar results. Uncropped western blots are presented in supplementary Figure 23.



Supplementary Figure 10. Glutathione is required for PCBP1-BolA2 complex formation *in vitro*. Purified BolA2-Strep and wild type KH3-Flag were mixed in the buffer IP containing 100 μM $FeSO_4$ with different concentration of GSH as indicated and anti-Flag IP performed. Immune complexes (IP: Flag) were analyzed by western blot and ratio of BolA2 to IP KH3-F was calculated. Blots are representative of *n*=3 independent experiments with similar results. Uncropped western blots are presented in supplementary Figure 24.



Supplementary Figure 11. A model for electrostatic interaction between BolA2 and KH3 domain. Ribbon structures of BolA2 (*light blue*) and KH3 domain (*grey*), illustrating the iron binding ligands (*orange, stick*) and GSH binding ligands (*blue*), coordinating the iron (*red, sphere*) and GSH (*teal, sphere*). Model was generated using ClusPro 2.0 protein docking platform and PyMOL version 2.2.



Supplementary Figure 12. A model for the PCBP1 mediated iron delivery to cytosolic Fe-S cluster assembly via BolA2. PCBP1 and PCBP2 are iron chaperones that play integral role in intracellular iron trafficking. Structurally PCBPs contain three tandem K-homology domains (KH1-3). Iron (Fe(II)) may be coordinated by reduced glutathione (GSH) in the cytosolic labile iron pool (LIP). Individual domain of PCBP1 can bind GSH coordinated Fe(II) with high affinity in 1:1 ratio. Cytosolic BolA-like protein (BolA2) can form a complex with Fe(II)-GSH containing PCBP1. Ferrous iron delivered by PCBP1-BolA2 complex can be combine with sulfur compound (possible exported from mitochondria), with the help of other cytosolic Fe-S cluster assembly proteins, to form a primary [2Fe-2S] cluster. Cytosolic monothiol glutaredoxin, Glrx3 and BolA2 can form heterotrimeric complex that coordinate a pair of bridging [2Fe-2S] clusters.

Figure 1a – BolA2 and PCBP1-F

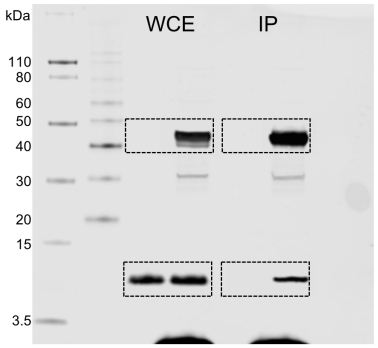


Figure 1c – PCBP1 and BolA2-F

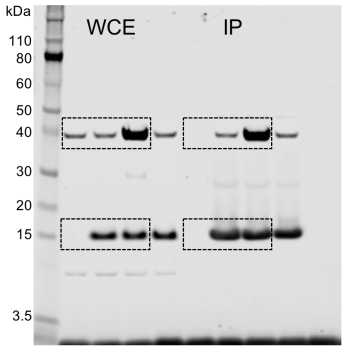


Figure 1b – BolA2-F

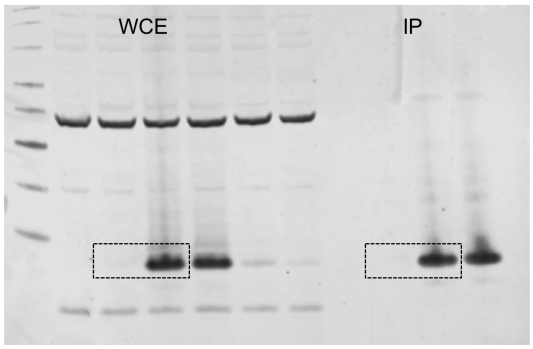


Figure 1b – PCBP1

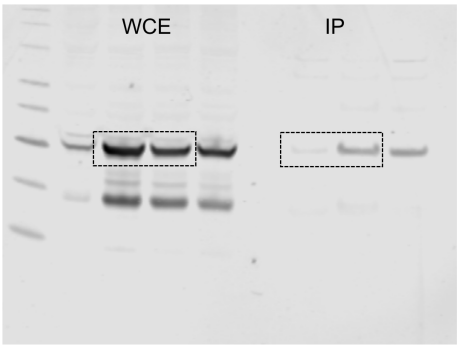


Figure 1d – PCBP1

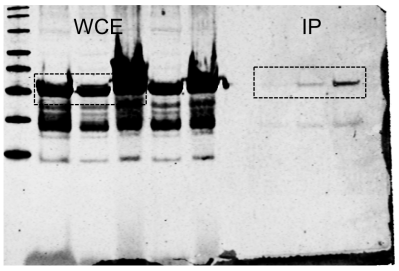


Figure 1d – Glrx3

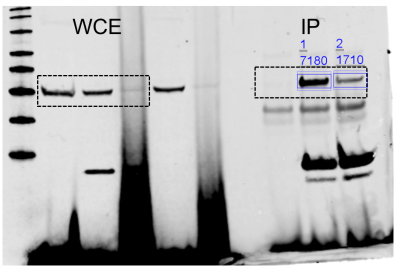


Figure 1d – BolA2-F

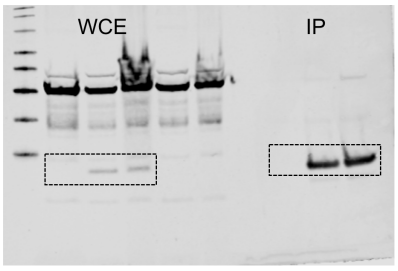


Figure 1e – PCBP1

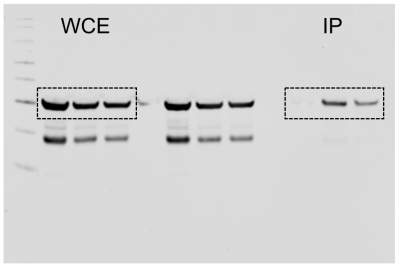


Figure 1e – Glrx3

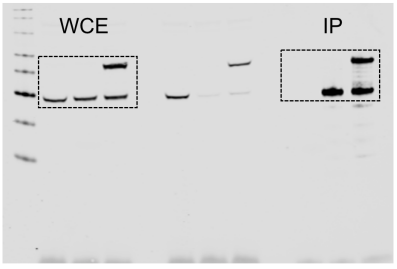
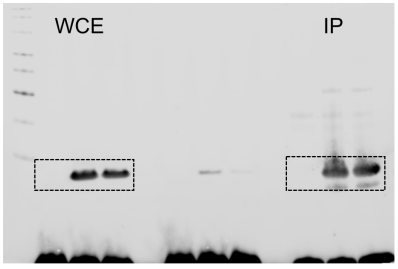


Figure 1e – BolA2-F



Supplementary Figure 13. Uncropped Western blots related to Figure 1.

Figure 2a – BolA2

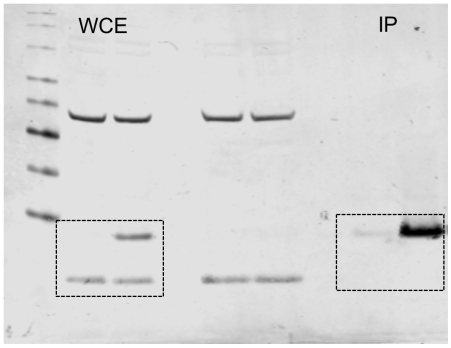


Figure 2b – Glrx3

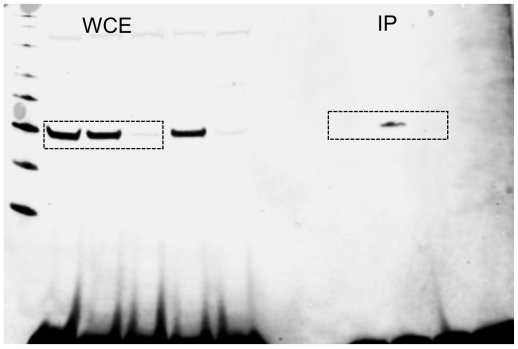


Figure 2b – BolA2

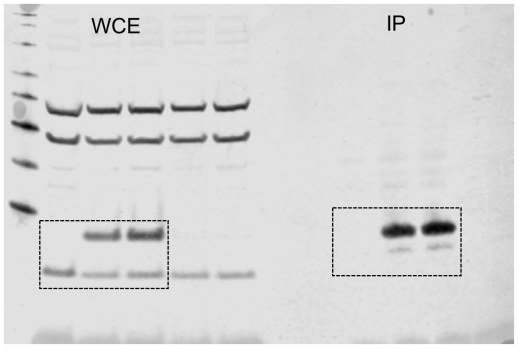


Figure 2c – PCBP1 and BolA2-F

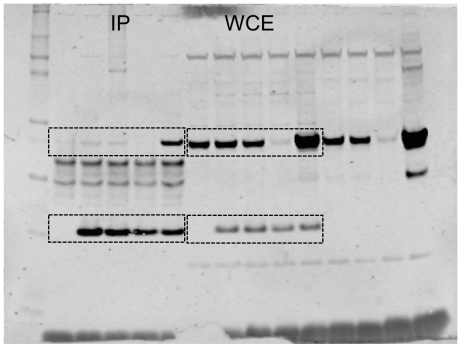
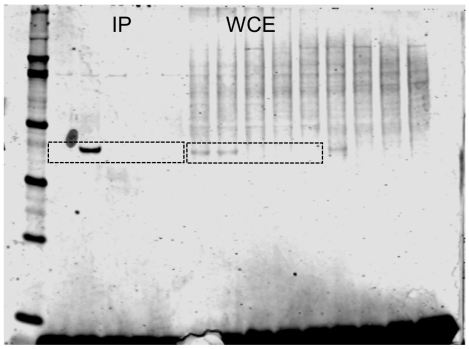


Figure 2c – Glrx3



Supplementary Figure 14. Uncropped Western blots related to Figure 2.

Figure 3a – PCBP1 and BolA2-F

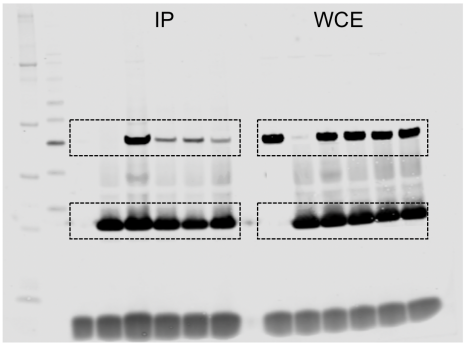


Figure 3b – Nfs1

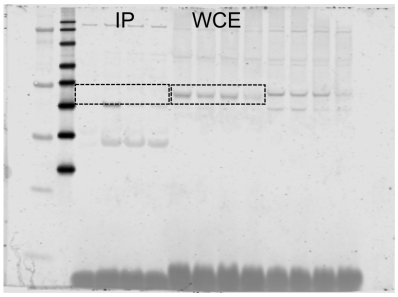


Figure 3b – Glrx3

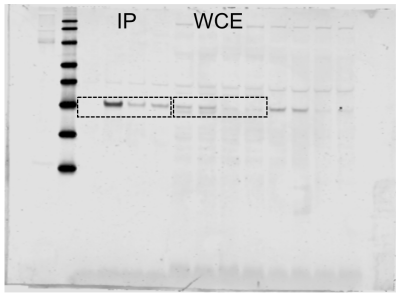


Figure 3b – PCBP1 and BolA2-F

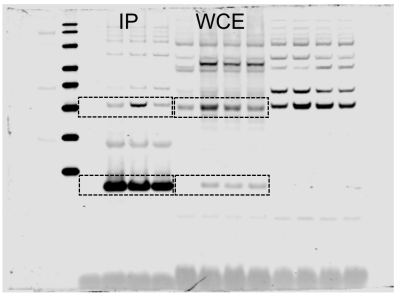


Figure 3c – PCBP1

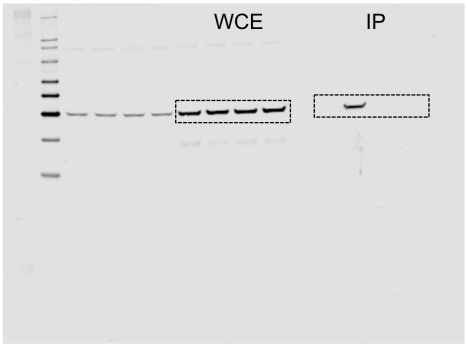
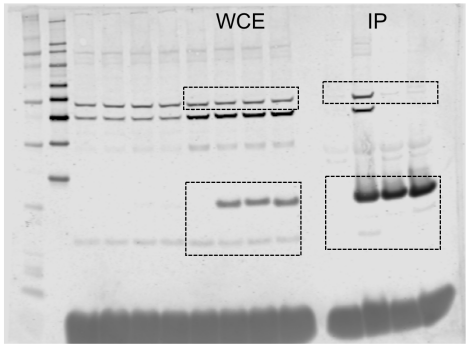


Figure 3c – Glrx3 and BolA2-F



Supplementary Figure 15. Uncropped Western blots related to Figure 3.

Figure 4a – BolA2 and PCBP1-F

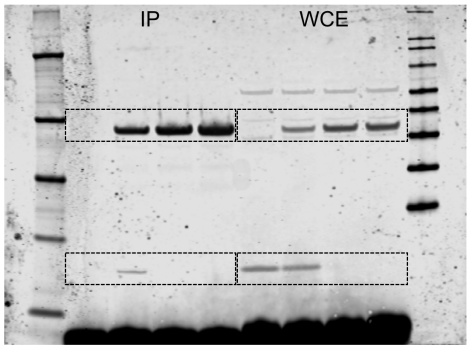


Figure 4b – PCBP1 (WCE)

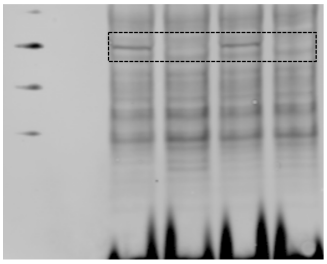


Figure 4b – PCBP2 (WCE)

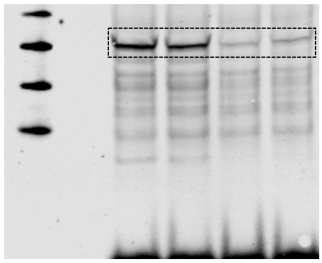


Figure 4b – Glrx3 and BolA2

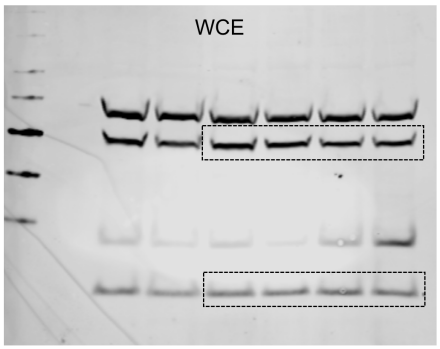


Figure 4b – Glrx3 and BolA2

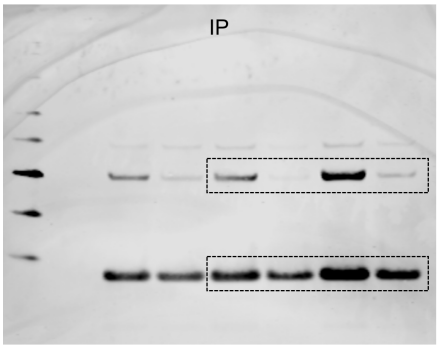


Figure 4c – Glrx3 and BolA2

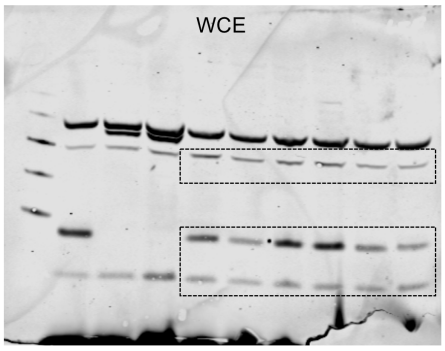
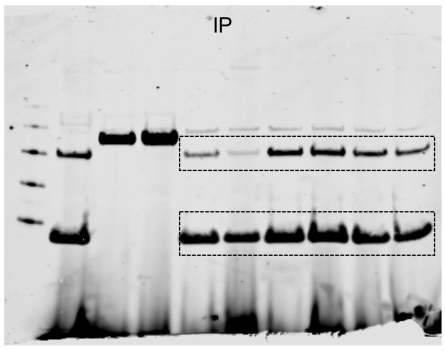


Figure 4c – Glrx3 and BolA2



Supplementary Figure 16. Uncropped Western blots related to Figure 4.

Figure 5a – BolA2 and Flag

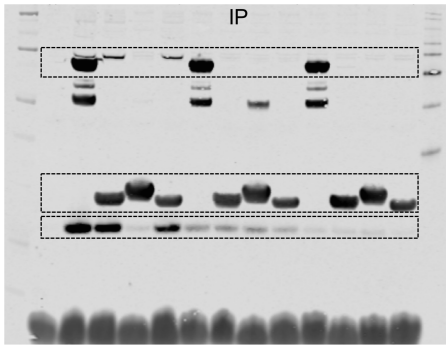


Figure 5b – Glrx3 and BolA2-F

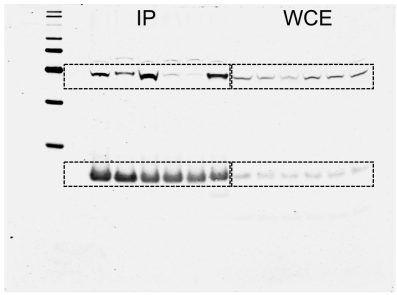


Figure 5b – PCBP1

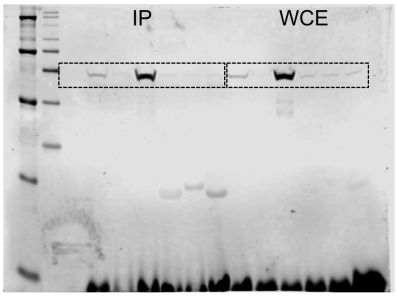


Figure 5b – Flag

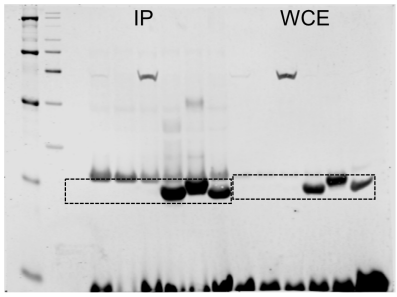


Figure 5e – Glrx3

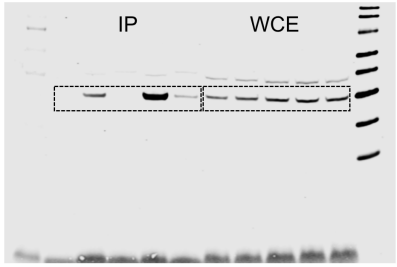


Figure 5e – BolA2-F

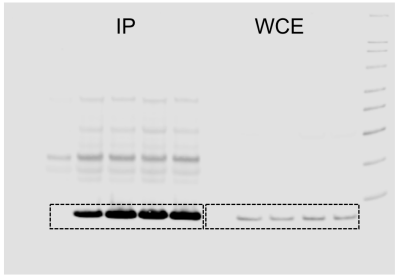
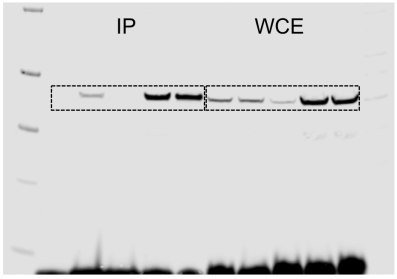
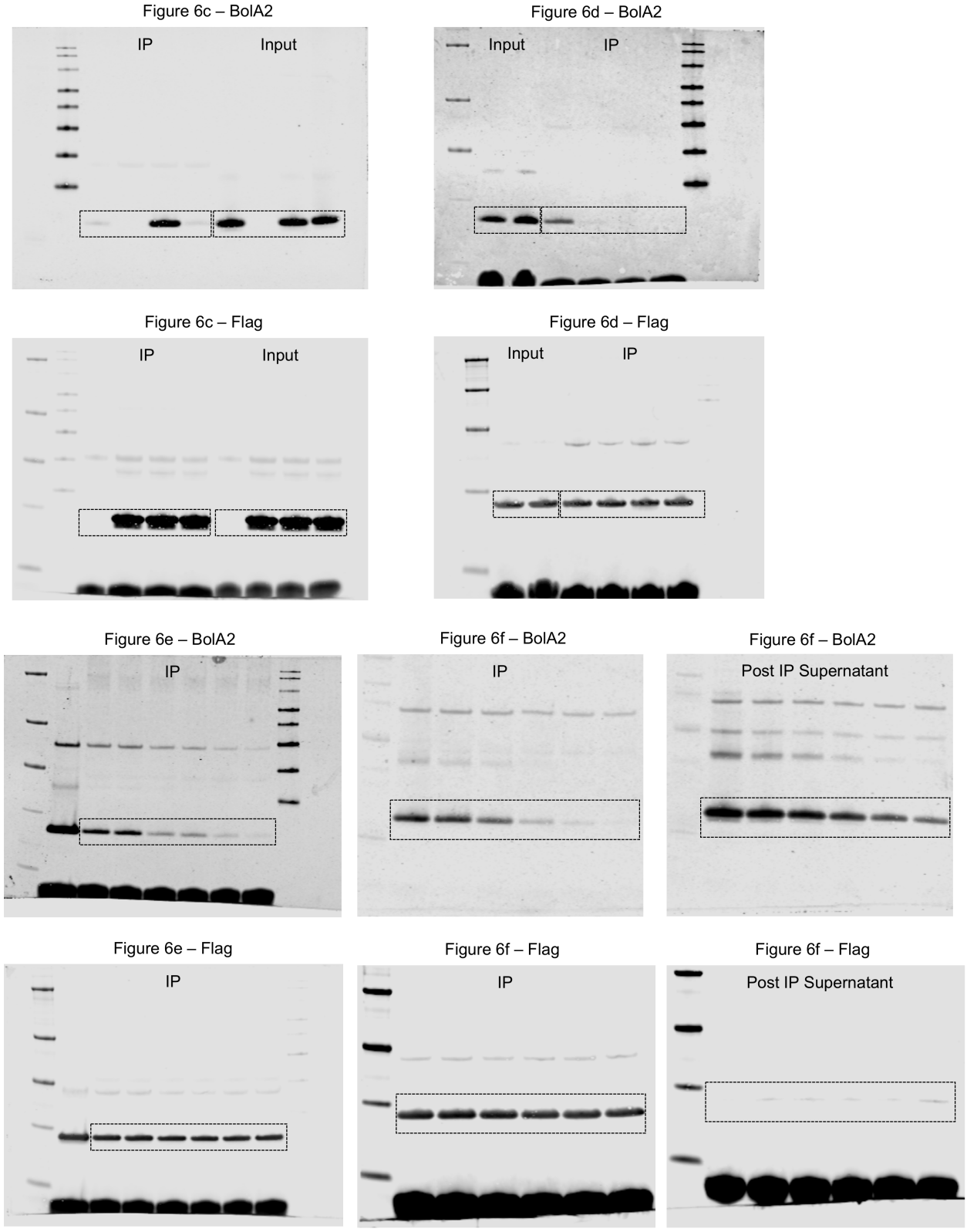


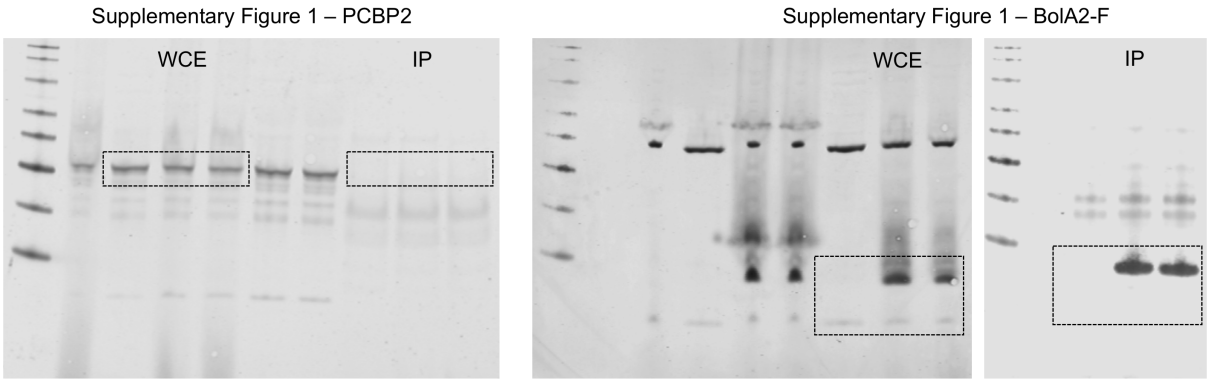
Figure 5e – PCBP1



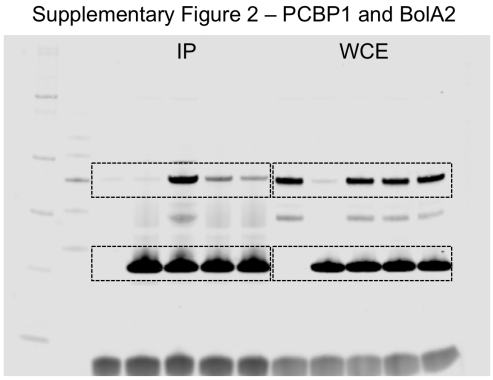
Supplementary Figure 17. Uncropped Western blots related to Figure 5.



Supplementary Figure 18. Uncropped Western blots related to Figure 6.

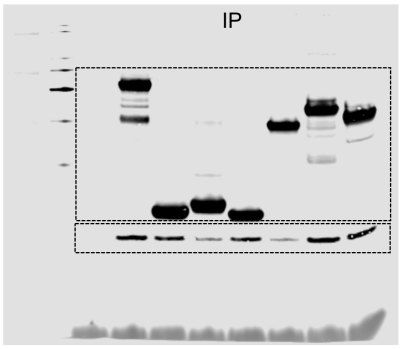


Supplementary Figure 19. Uncropped Western blots related to Supplementary Figure 1.

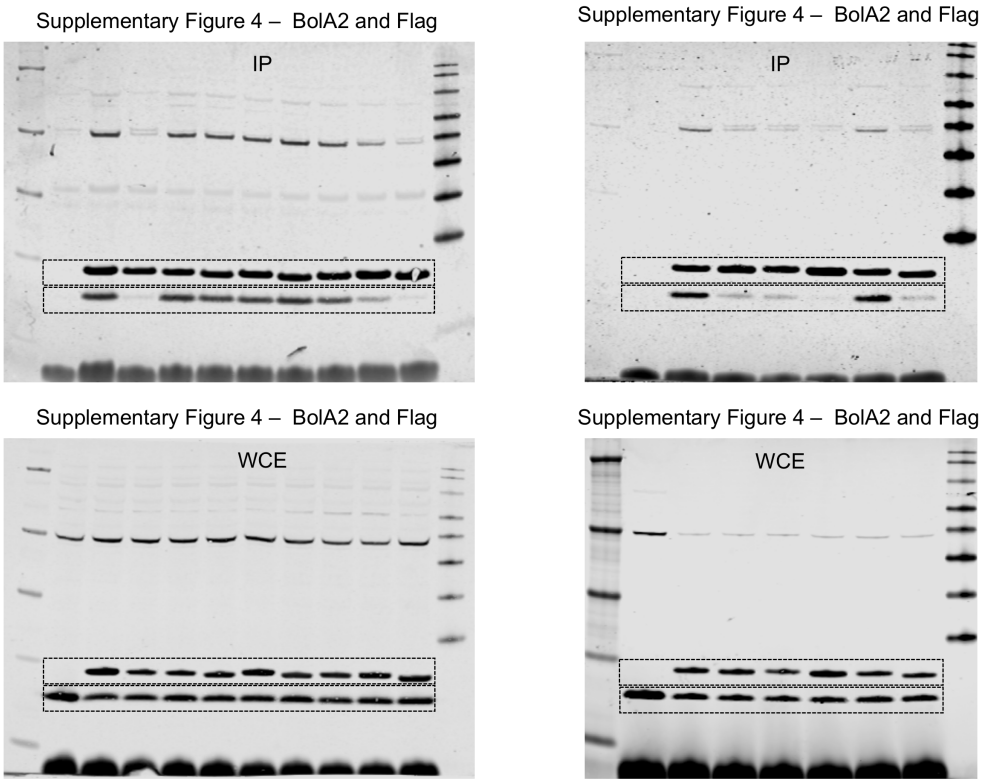


Supplementary Figure 20. Uncropped Western blots related to Supplementary Figure 2.

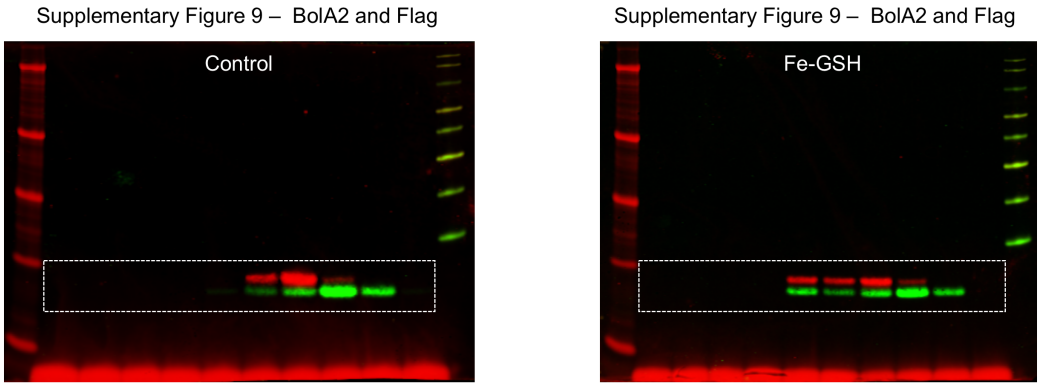
Supplementary Figure 3 – Flag and BolA2



Supplementary Figure 21. Uncropped Western blots related to Supplementary Figure 3.

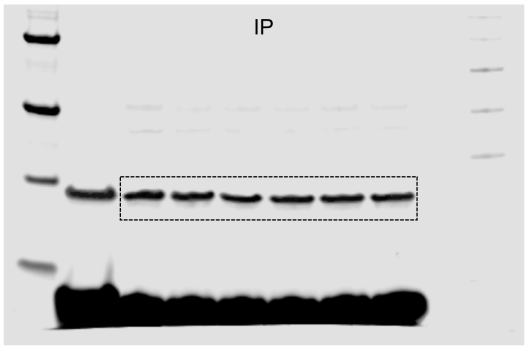


Supplementary Figure 22. Uncropped Western blots related to Supplementary Figure 4.

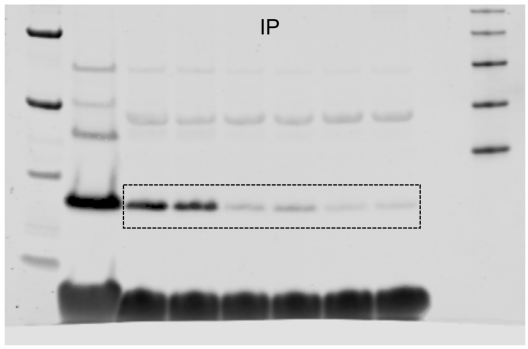


Supplementary Figure 23. Uncropped Western blots related to Supplementary Figure 9.

Supplementary Figure 10 – Flag



Supplementary Figure 10 – BolA2



Supplementary Figure 24. Uncropped Western blots related to Supplementary Figure 10.