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Supporting Online Material for

Control of Iron Homeostasis by an Iron-Regulated Ubiquitin Ligase

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Correction: On page 4, a unit has been corrected from nmol to pmol.

Control of Iron Homeostasis by an Iron-regulated Ubiquitin Ligase

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SUPPORTING ONLINE MATERIAL

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Materials and Methods

Plasmids

The full length FBXL5 cDNA (NIH_MGC_97) was obtained from Open Biosystems. The cDNA was amplified using the Advantage-HF2 polymerase (Clontech) and introduced into the pCR8/GW/TOPO vector (Invitrogen). The Quikchange system (Stratagene) was used to generate the FBXL5- Δ Fbox mutant lacking amino acids 216-240 using pCR8-FBXL5 as a template. FBXL5 and FBXL5- Δ Fbox were subcloned into pcDNA3-6xMyc and pcDNA5-FRT/TO-3xHA-3xFLAG plasmids using pCR8-FBXL5 and DEST plasmids via the Gateway cloning system (Invitrogen). FBXL5-N199 and FBXL5-C492 fragments were generated by PCR using primers containing flanking AttB1 and AttB2 sites and cloned into pDONR221 (Invitrogen). FBXL5-N199-H15A and FBXL5-N199-H57A were generated using the Quikchange system (Stratagene). These fragments were then subcloned into pcDNA5-FRT/TO-3xHA-3xFLAG or pET53-DEST (Novagen) for expression in HEK293 and *E. coli*, respectively. Plasmids expressing 2xFLAG-IRP1, 2xFLAG-IRP1-C3S, 2xFLAG-IRP2, 2xFLAG-IRP2 Δ 73, HA-Ub, Myc-CUL1, Myc-SKP1, and His₆-Smt3p were previously described (S1,S2).

Antibodies

Antibodies against human FBXL5 were generated by Swedish Human Proteome Resource as described (S3). Other antibodies used for immunoblotting were as follows: IRP2-7H6 (Santa Cruz Biotechnology), IRP2 (S4), IRP1 (S5), FLAG-M2 (Sigma), Myc-9E10 (Covance), HA-HA.11 (Covance), ferritin (Sigma), actin (Calbiochem), β -tubulin (Sigma), and GST (Cell Signaling). Horseradish peroxidase conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratories. For quantification of protein half-lives, an immunofluorescent anti-mouse secondary antibody (Rockland) was used. Immunoprecipitation reactions were performed using affinity matrices for anti-FLAG M2, anti-c-Myc, and anti-HA available from Sigma.

Cell Lines

The HEK293 and IMR90 cell lines were obtained from the American Type Culture Collection (ATCC) while Flp-InTM T-RExTM-293 was obtained from Invitrogen. Flp-InTM TRExTM-293 cells stably expressing 2xFLAG-IRP2, 2xFLAG-IRP1, and 2xFLAG-IRP1-C3S were previously described (S2). Flp-InTM T-RExTM-293 cells stably expressing 3xHA-3xFLAG-FBXL5, 3xHA-3xFLAG-FBXL5- Δ Fbox, 3xHA-3xFLAG-FBXL5-N199, and 3xHA-3xFLAG-FBXL5-C492 were generated using the Flp-In system (Invitrogen) according to the manufacturer's directions.

Cell Culture, Plasmid Transfections, and Treatments

Cell culture reagents were obtained from Invitrogen. All cell lines were cultured in complete Dulbecco's Modified Eagles medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS), 100 units/mL penicillin and streptomycin, and 2 mM

glutamine at 37°C in ambient air with 5% CO₂. Hypoxia experiments were performed at 37°C using a Modular Incubator Chamber (Billups-Rothenberg, Inc.) flushed with 1% O₂, 5% CO₂, and balanced N₂ gas and incubated at 37°C. Transient transfections were performed using either BioT (Bioland, Long Beach, CA) according to the manufacturer's protocol or linear PEI as described (S6). siRNA transfections were performed according to the manufacturer's protocol (Thermo Fisher) using Dharmafect I and siGENOME SMARTpool reagents for FBXL5 (Dharmacon #M-012424-01) or a non-targeting siGENOME control siRNA (Dharmacon #D-001210-03-05). Expression of 3xHA-3xFLAG-FBXL5 and 2xFLAG-IRP2 in stable cell lines was induced by treating cells with doxycycline for 24 hours or the times indicated at a final concentration of 100 ng/mL for protein half-life determination experiments and 500 ng/mL for all other experiments. Cells were treated with 100 µg/ml ferric ammonium citrate (FAC) (Thermo Fisher), 100 µM desferrioxamine mesylate (DFO) (Sigma), and/or or 25 µM MG132 (Z-Leu-Leu-Leu-CHO) (BIOMOL) for the times indicated.

Affinity purification of FBXL5-ΔFbox and IRP2-containing Protein Complexes

Twenty-five 15 cm tissue cultures plates each of Flp-InTM TReXTM-293 cells stably expressing His₆-3xFLAG-FBXL5-ΔFbox or 2xFLAG-IRP2 were grown, harvested, and lysed in IP buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, 1 µM pepstatin, 1 µM leupeptin and 2 µg/mL aprotinin). 200-300 mg of clarified protein lysate was then incubated at 4°C with 100 µL of equilibrated anti-FLAG M2 agarose (Sigma) for 2 hours. Beads were then washed four times using 1 mL of IP Buffer per wash before eluting with 500 µL of FLAG Elution Buffer (IP buffer lacking NP-40 and supplemented with 250 µg/mL of 3xFLAG peptide (Sigma)). Elutions were precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 10% followed by incubation on ice for 30 minutes and centrifugation at 16,000g for 10 minutes to collect the precipitate.

Proteomic Characterization of FBXL5 and IRP2 purifications

TCA precipitates from affinity-purified His₆-3xFLAG-FBXL5-ΔFbox and 2x-FLAG-IRP2 samples were digested and prepared for proteomic analysis as described (S7). The digested samples were analyzed by MudPIT (S8,S9). A 5-step multidimensional chromatographic separation was performed online and fractionated peptides were eluted directly into a LTQ-Orbitrap mass spectrometer (Thermo Fisher) in which tandem mass spectra were collected. Peptide mass spectra were analyzed using the SEQUEST and DTASelect algorithms (S10, S11). A decoy database approach was used to estimate peptide and protein level false positive rates which were less than 5% per analysis (S12). Proteins were considered candidate FBXL5 and IRP2 interacting proteins if they were identified in the relevant affinity purification but not in MudPIT analyses of other control purifications. A detailed description of the multidimensional peptide fractionation protocol, mass spectrometer settings, and bioinformatic workflow is described elsewhere (S13).

Immunoprecipitation and Immunoblotting

Cell lysates were prepared using IP buffer. Immunoprecipitations were performed using the appropriate affinity matrix equilibrated with IP Buffer and incubated with equal amounts of cell lysates at 4°C for 2 hours. Beads were washed three times with lysis buffer and resuspended in 2x SDS loading buffer. For immunoblotting, whole cell lysates and immunoprecipitates were boiled in SDS-loading buffer, separated using SDS-PAGE, transferred to Immobilon-P PVDF membranes (Millipore), and probed with the appropriate primary and secondary antibodies. Proteins were visualized using Pierce ECL western blotting substrate (Thermo Fisher).

Ubiquitination assay

HEK293 cells were transfected with plasmids expressing HA-Ubiquitin, a 2xFLAG IRP construct (2xFLAG-IRP2, 2x-FLAG-IRP1, or 2x-FLAG-IRP1-C3S), and either 6xMyc-FBXL5, 6xMyc-FBXL5-ΔFbox, or a vector control. Twenty-four hours after transfection, the medium was changed and cells were treated with 100 μg/ml FAC and 25 μM MG132 for 4 hours. Cells were harvested and lysed under denaturing conditions as described previously (S14). Ubiquitin conjugates were purified using anti-HA beads and the presence of IRP1 or IRP2 in the purified ubiquitin conjugates was detected by immunoblotting with FLAG M2 antibody.

Pulse – Chase Experiments

For endogenous IRP2 degradation analysis, Flp-InTM T-RExTM-293 cells stably expressing 3xHA-3xFLAG-FBXL5 or 3xHA-3xFLAG-FBXL5-ΔFbox were treated overnight with doxycycline in complete medium. Cells were then incubated in DMEM lacking methionine and cysteine for 30 minutes prior to a one hour pulse with 100 μCi/ml (3.7 MBq/ml) TRAN³⁵S-LABELTM (ICN Pharmaceuticals). Cells were washed three times and chased in complete medium supplemented with or without 100 μg/ml FAC for the indicated times. Cells were harvested in Triton Buffer (1% Triton, 150 mM NaCl, 50 mM Tris pH 8.0) and lysates cleared. 200-300 μg of cell extract was immunoprecipitated using IRP2 antibody and Protein A agarose (Invitrogen). Beads were washed three times with Triton Buffer and twice with PBS prior to protein elution by boiling samples in LDS sample buffer (Invitrogen). Proteins were separated on a 4-12% Bis-Tris gel (Invitrogen) using MOPS buffer (Invitrogen) and transferred to nitrocellulose prior to PhosphorImager exposure (Molecular Dynamics, Inc.). Band quantification was performed using a local average background correction with ImageQuaNT 5.0 software (Molecular Dynamics, Inc.).

For 2xFLAG-IRP2 degradation analysis with FBXL5 siRNA, Flp-InTM T-RExTM-293 cells stably expressing 2xFLAG-IRP2 (3.5×10^5) were plated on a 35 mm dish one day prior to transfection. Cells were transfected with 200 pmol FBXL5 siRNA and 4 μl DharmaFECT1 for 36 hours and then treated with doxycycline overnight. The medium was removed and the plates were washed once with PBS, once with complete medium, and then chased in complete medium supplemented with FAC. For FBXL5 degradation

analysis, Flp-InTM T-RExTM-293 cells stably expressing 3xHA-3xFLAG-FBXL5 were treated overnight with doxycycline. The medium was removed and the cells were washed as described above, and then chased in complete medium supplemented with or without FAC or DFO in normoxia or hypoxia. Cells were harvested in Triton Buffer, lysates cleared, and proteins separated on a 4-12% Bis-Tris gel using MOPS Buffer. After transferring proteins to nitrocellulose, immunoblotting was performed using FLAG-M2 and immunofluorescent secondary antibodies. Band quantification was performed using a local average background correction on an Odyssey (LI-COR) infrared imaging system.

Purification of Recombinant His₆-FBXL5-N199

One liter each of BL21(DE3) cells carrying either pET53-His₆-FBXL5-N199 or pET21-SMT3 was grown at 30°C to OD₆₀₀ ~ 0.5, treated with 1 mM IPTG, and incubated at 30°C for three additional hours. Cells were harvested, resuspended in Nickel Buffer A (50 mM Tris-HCl, pH 8.0, 200 mM KCl, 10% glycerol, 10 mM imidazole), lysed by sonication, and centrifuged at 27,000g for 20 minutes to clarify the lysate. Each lysate was incubated in batch with 0.5 mL of Ni-NTA agarose beads at 4°C for one hour, washed extensively with Nicked Buffer A, and then eluted with Nickel Buffer B (Nickel Buffer A supplemented with 500 mM imidazole). The eluates were dialyzed exhaustively against Nickel Buffer A lacking imidazole, snap-frozen in liquid N₂, and stored at -80°C.

Inductively-coupled Plasma Mass Spectrometry (ICP-MS)

Aliquots of buffer only, His₆-Smt3p, and His₆-FBXL5-N199 were mixed with an equal volume of concentrated OPTIMA grade nitric acid (Thermo Fisher) followed by digestion at 95 °C for two hours in open vessels in a dust-free environment. The dried contents of the tubes were then extracted with 500 μL of 2% OPTIMA grade nitric acid. The individual extracts were diluted to a total volume of 2 mL with 2% nitric acid. 20 μL of a 5 ppm aqueous scandium ion solution (ICP-MS grade) was then added to each tube as an internal standard and the contents vortexed vigorously. ICP measurements were then conducted on an Agilent 7500 Series ICP-MS in both He and H₂ collision gas modes. Reported measurements of Fe in each sample represent the average of 3 measurements. The individual measurements are typically obtained with a relative standard deviation of approximately 3% or less per sample.

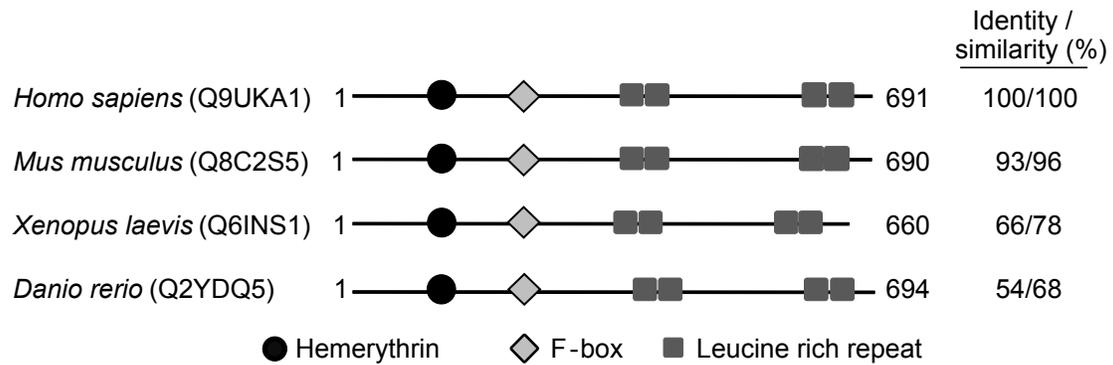


Fig. S1. Domain Organization of FBXL5. The primary sequence and domain organization of FBXL5 is strongly conserved among higher eukaryotes and consists of a N-terminal hemerythrin-like domain, a F-box domain, and four C-terminal leucine-rich repeats.

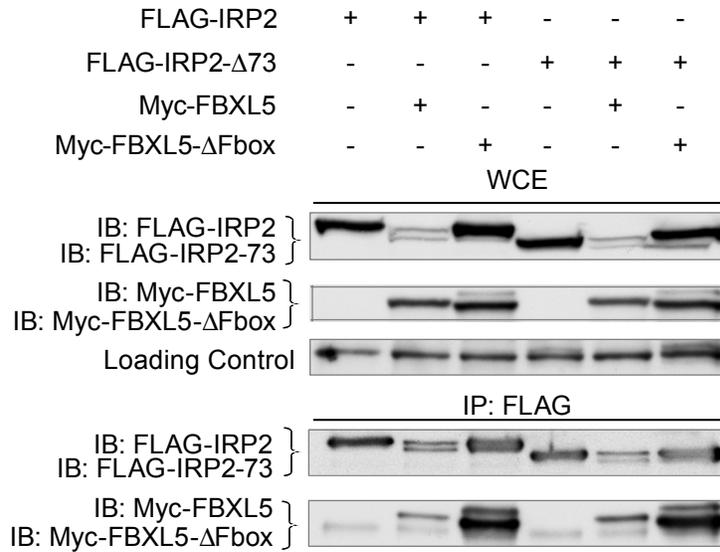


Fig. S2. The unique 73-amino acid region of IRP2 is not required for interaction with FBXL5. HEK293 cells were co-transfected with plasmids expressing FLAG-IRP2 or FLAG-IRP2-Δ73 along with Myc-FBXL5, Myc-FBXL5-ΔFbox, or control plasmids. Immunoprecipitations were performed with FLAG antibody. Whole cell extracts (WCE) and immunoprecipitates (IP) were immunoblotted with FLAG and c-Myc antibodies.

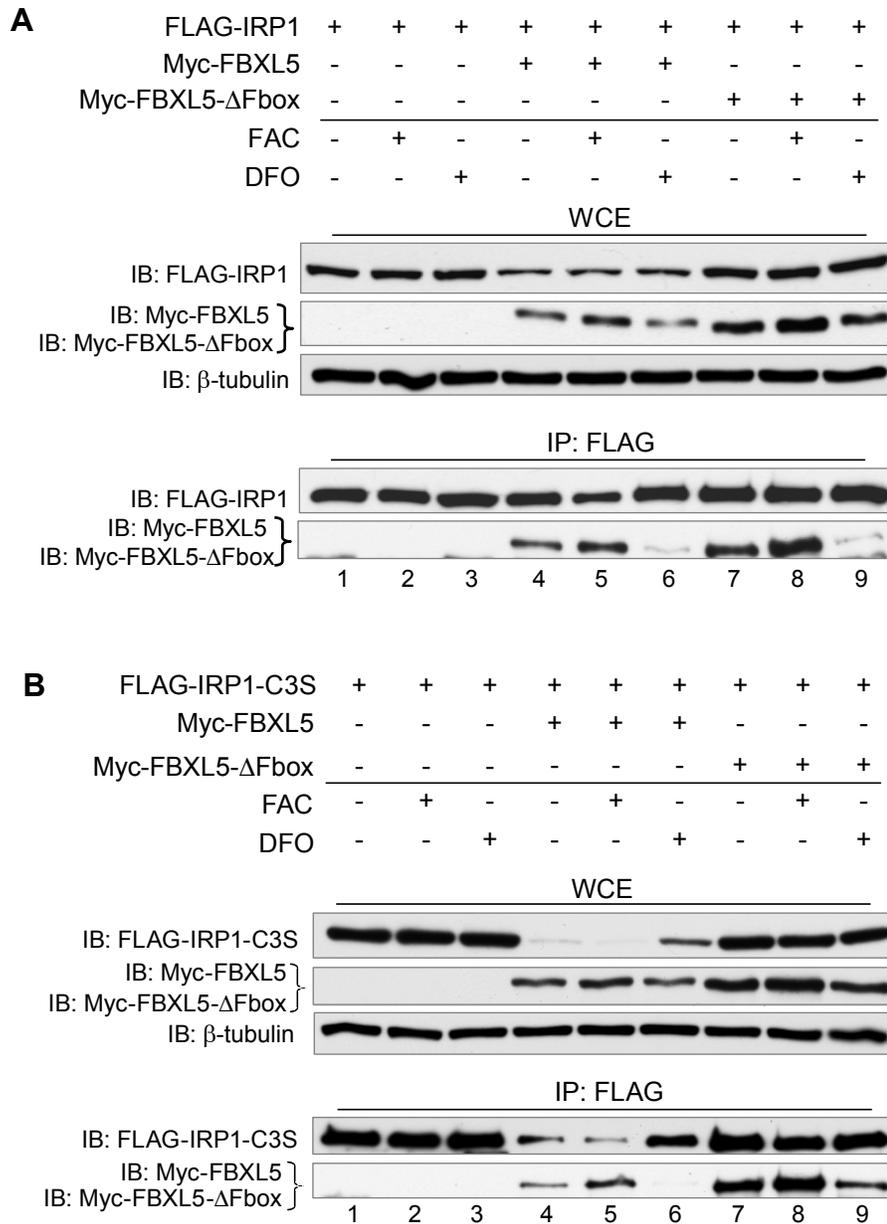


Fig. S3. The interaction of IRP1 and IRP1-C3S with FBXL5 is iron-regulated. HEK293 cells were co-transfected with FLAG-IRP1 (A) or FLAG-IRP1-C3S (B) and either Myc-FBXL5, Myc-FBXL5-ΔFbox, or a control plasmid. Transfected cells were treated with 100 μg/mL FAC or 100 μM DFO for 8 hours before harvesting. FLAG antibodies were used to immunoprecipitate FLAG-IRP1 or FLAG-IRP1-C3S. Whole cell extracts (WCE) and immunoprecipitates (IP) were immunoblotted with FLAG, c-Myc, and β-tubulin antibodies.

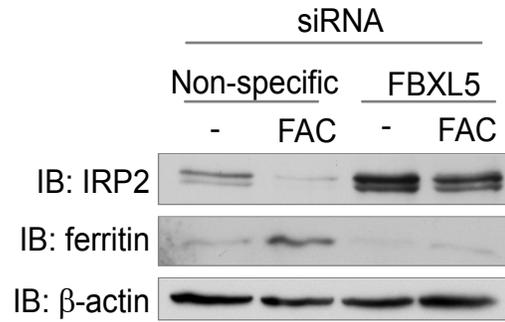
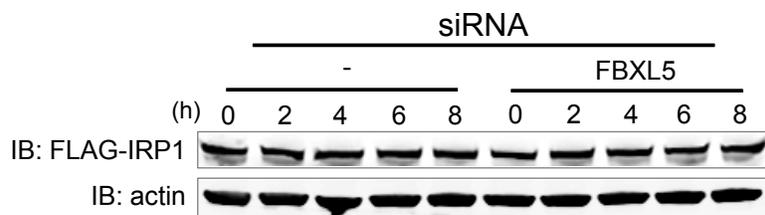


Fig. S4. FBLX5 depletion in IMR90 human diploid fibroblasts leads to IRP2 stabilization. IMR90 human diploid fibroblasts were transfected with non-specific or FBXL5 siRNAs and then treated with or without FAC for 8 hours. Whole cell extracts were immunoblotted with IRP2, ferritin, and β -actin antibodies.

A



B

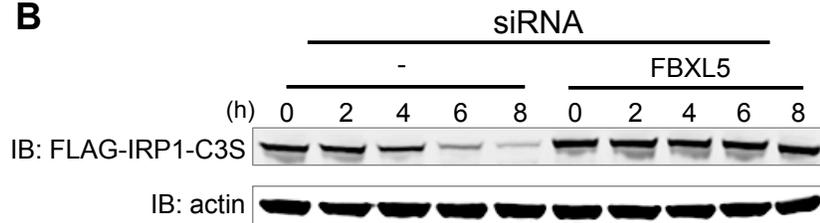


Fig. S5. FBXL5 depletion stabilizes an IRP1-3CS mutant. Flp-In TREx-293 cells stably expressing FLAG-IRP1 (A) or FLAG-IRP1-C3S (B) were treated without (-) or with FBXL5 siRNA and then doxycycline overnight. Cells were washed and chased in medium supplemented with 100 μ g/mL FAC for the indicated times. Whole cell extracts were immunoblotted with FLAG and actin antibodies.

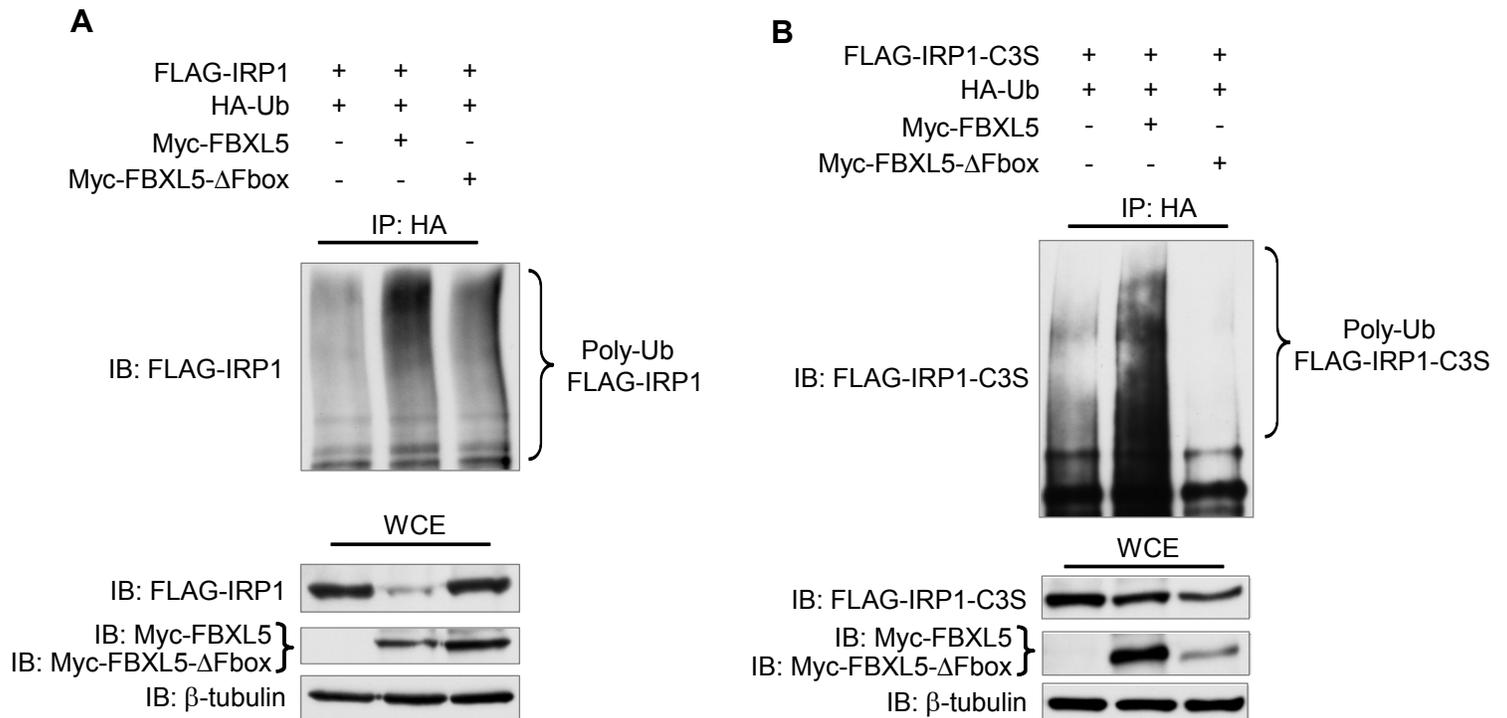


Fig. S6. FBXL5 but not FBXL5-ΔFbox overexpression stimulates the ubiquitination of IRP1 and IRP1-C3S. FLAG-IRP1 (A) or FLAG-IRP1-C3S (B) were co-transfected into HEK293 cells with HA-ubiquitin and either Myc-FBXL5, Myc-FBXL5-ΔFbox, or a control plasmid. Transfections were harvested 24 hours later with 100 μg/mL FAC and 25 μM MG132 being added to the media for the final 4 hours. Denaturing immunoprecipitations using HA antibodies were used to purify ubiquitin conjugates that were then probed with FLAG antibodies to detect ubiquitinated forms of FLAG-IRP1 and FLAG-IRP1-C3S. Whole cell extracts (WCE) were immunoblotted with FLAG, c-Myc, and β-tubulin antibodies.

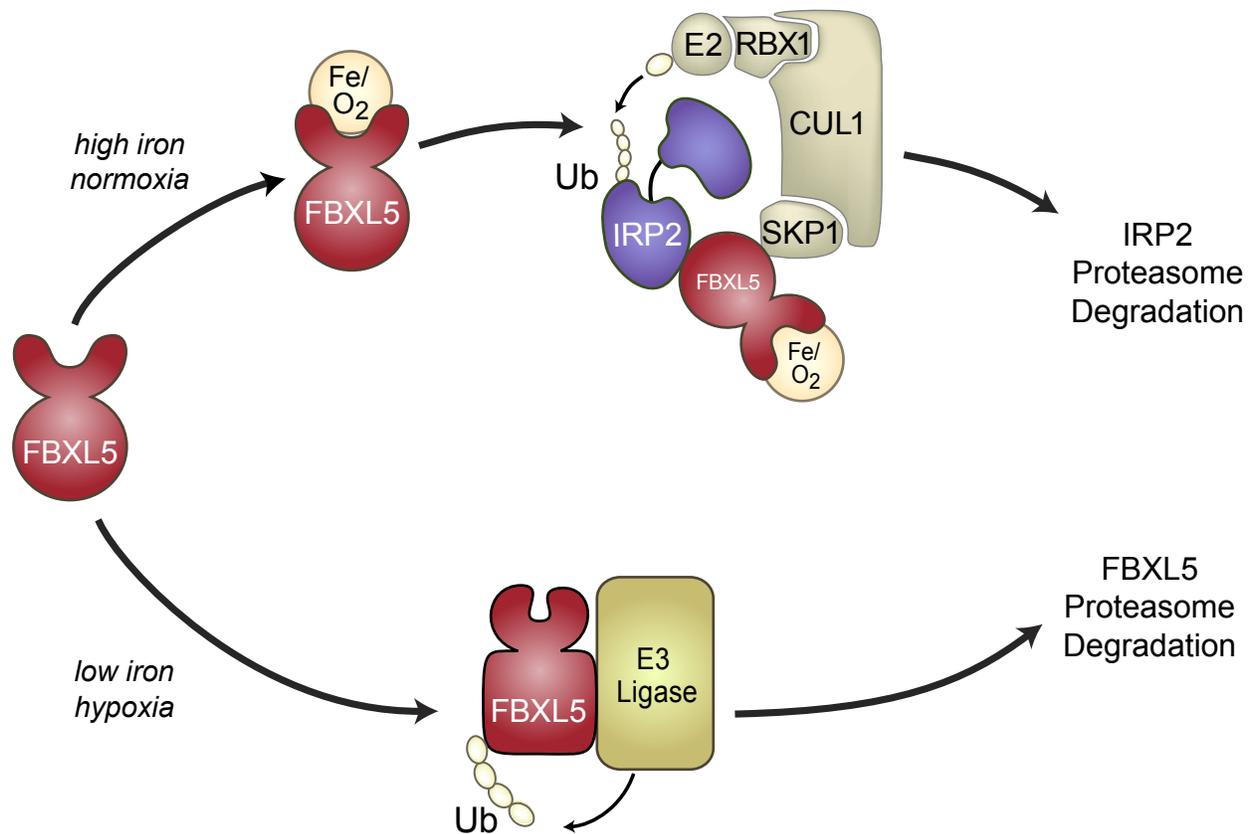


Fig. S8. Model for the regulation of FBXL5 and IRP2 by iron and oxygen. When cellular iron and oxygen levels are high, the hemerythrin-like domain of FBXL5 binds iron and oxygen resulting in increased FBXL5 stability. Stabilized FBXL5 associates with SKP1 and CUL1 and catalyzes the ubiquitination and subsequent proteasomal degradation of IRP2. In low iron or hypoxic conditions, FBXL5 is destabilized and targeted for ubiquitin-dependent degradation by proteolytic pathways.

Table S1. Proteomic Identification of FBXL5 and IRP2-associated Factors

Protein (Acc. Number)	MW (Da)	# Peptides	# Spectra	% Coverage
His-FLAG-FBXL5-ΔFbox				
FBXL5 (Q9UKA1)	78,555	12	384	16.4
IRP1 (P21399)	98,399	5	39	6.1
IRP2 (P48200)	105,012	16	156	11.7
FLAG-IRP2				
IRP2 (P48200)	105,012	135	4691	75.6
FBXL5 (Q9UKA1)	78,555	15	29	23.3
SKP1 (P63208)	18,658	5	39	6.1
CUL1 (Q13616)	89,679	10	11	16.1

The number of unique peptides, number of spectra (reflecting the fact that some peptides are identified more than once in an analysis), and % sequence coverage for each protein are indicated.

Supplementary References

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