Supporting Online Material for

An E3 Ligase Possessing an Iron Responsive Hemerythrin Domain is a Regulator of Iron Homeostasis

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MATERIALS AND METHODS

Cell Culture and Reagents

Non-tumorigenic immortalized human bronchial epithelial HBEC-30 cells ((*S1*); kindly provided by M. White), were grown in keratinocyte serum free medium supplemented with recombinant epidermal growth factor and bovine pituitary extract (Gibco). All other cell lines were grown in Dulbecco's Modified High Glucose Eagle Medium (HyClone). Tetracycline inducible cells were supplemented with 10% tetracycline-free fetal bovine serum (Clontech) while all other cells were supplemented with 10% fetal bovine serum (Atlanta Biologicals). Low (1%) O_2 incubations were carried out in a hypoxic chamber (Coy Laboratory Products). Transient transfections of plasmid DNA were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Typically, $5x10^4$ cells were plated per well of a 24-well plate and incubated for 16 hr followed by transfection with 500 ng of DNA. Neomycin-resistant cells stably expressing epitope-tagged IRP2 or FBXL5 proteins were isolated over two rounds of clonal selection in the presence of 400 μg/mL G418 (Research Products Inc). IRP1 and $IRP1^{3C>3S}$ Flp-In T-Rex HEK 293 cells were kindly provided by R. Eisenstein.

FBXL5 shRNA knockdown cells were generated by stable transfection of HEK 293 TRex cells (Invitrogen) with pSuperior vector (Oligoengine) encoding the FBXL5 shRNA: 5′-gaucccGCACAACACUGCUCUCAGAuucaagagaUCUGAGAGCAGUGUUGUGCuuuuua-3′. Individual cell lines were isolated over two rounds of clonal selection in the presence of 1 μg/mL puromycin (Sigma). FBXL5 shRNA expression was induced upon addition of 1 μg/mL tetracycline hydrochloride (Sigma) to the culture medium for 72 hr.

RNAi

Transient transfections of siRNAs (Table S4; Dharmacon) were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Typically, $2x10^4$ cells were reverse transfected in 24-well plates with 20 pmoles siRNA duplex/well. Following 48 hr incubation, cells were treated with either 100 μM ferric ammonium citrate (FAC) or 100 μM deferoxamine mesylate (DFO) and incubated an additional 16 hr.

Plasmids

Human FBXL5 cDNA (NM_012161) was amplified from a human placenta cDNA library (kindly provided by C. Mendelson) using the oligonucleotides 5′-GGAGTCTAGAACCTTCGCCAGAGCGGCAGCAGG and 5′-GGAGGGATCCACCATGGCGCCCTTTCCTGAAGAAGTGGAC and cloned into the pcDNA3.1-V5 vector (Invitrogen). FBXL5 cDNA was subcloned into the pCI-FLAG vector (kindly provided by X. Wang) using the 5′-GGAGGTCGACTCATTCGCCAGAGCGGCAGC and 5′-GGAGGGATCCACCATGGCGCCCTTTCCTGAAGAAGTGGAC oligonucleotides. Human IRP2 was amplified from a human cDNA library using the oligonucleotides 5′-AATTGGATCCACCATGGACGCCCCAAAAGCAGGATAC and 5′-CCTCCTCTAGATGAGAATTTTCGTGCCACAAAG and cloned in frame with a C-terminal 3xFLAG epitope tag into the pcDNA3.1 vector. The oligonucleotide 5′-TCGGATCCATGGCCGCCTACCCCTACGACGTGCCCGACTACGCCGGTGACGCCCCAAAAGCAGG was used to incorporate an N-terminal HA tag. Where appropriate, the overlap extension PCR method was utilized to construct deletion and point mutants using oligonucleotides listed in Table S5. Full length human IRP2 was cloned into the pPICZb vector

(Invitrogen) in frame with C-terminal 1xMyc and 6xHIS epitope tags, and then cloned into the yeast centromeric shuttle vector pRS316 (kindly provided by B. Tu) downstream of the S. Cerevisiae GAL1 promoter. The vector was transformed into a protease deficient *S. cerevisiae* W303 strain using a standard Lithium Acetate protocol (*S2*). Transformants were isolated and maintained on SD-URA medium. All constructs were confirmed by sequencing.

Immunoblot Analysis

Samples were resuspended in SDS sample buffer and proteins were resolved by SDS-PAGE prior to immunoblot analysis. Rabbit anti-IRP1 antibody was kindly provided by R. Eisenstein. The Myc 9E10 antibody was purified (*S3*) on protein G agarose (Roche) from 9E10 hybridoma (ATCC) grown in Hybridoma SFM media (Gibco). α-FBXL5 antibody was generated from rabbits immunized with 6xHis-tagged FBXL5 (1-691) expressed in *E. coli* and further affinity-purified from serum. Additional antibodies are listed in Table S6. Immune complexes were detected by enhanced chemiluminescence using peroxidase-conjugated secondary antibodies. Immunoblots using antibodies specific for tubulin were used as loading controls. Quantitation of immunoblots was performed using a Canon Model 30 LED Indirect Exposure Scanner and analyzed using Scion Image software (version 4.0.3.2).

AlphaScreen Assay

To identify candidate E3 ubiquitin ligase(s) responsible for iron-dependent IRP2 ubiquitination, a library of 800 siRNA pools (Qiagen; 4 siRNA sequences/pool/gene), each designed to suppress expression of an individual human gene, was assembled. Included in the library were siRNAs targeting genes encoding proteins known to play a role in ubiquitination as well as proteins containing domains found within previously identified E3 ligase complexes (*S4-7*). HEK 293 cells stably transfected with the HA-IRP2-FLAG expression construct were reverse-transfected with siRNA using Lipofectamine 2000 (Invitrogen) in 96-well plates. Following 48 hr incubation, cells were treated with either 25 μM FAC or 100 μM DFO and incubated for 16 hr. Media was removed and 50 μL assay mixture/well (1X PBS, 0.5% TritonX-100, 5 ng/μL streptavidin donor beads (PerkinElmer), 5 ng/μL M2 FLAG acceptor beads (PerkinElmer), 2 pg/μL biotinylated α-HA antibody (Genscript), 250 μM phenylmethylsulfonyl fluoride (PMSF; Sigma), and 1X protease inhibitor cocktail (Sigma)) was added. Plates were incubated in the dark with gentle rocking for 4 hr and data collected with an EnVision microplate reader (PerkinElmer). Assays were performed in triplicate with bars indicating standard error. For siRNA pools capable of stabilizing HA-IRP2-FLAG under iron replete conditions to levels >50% of the NT control in iron depleted cells, further validation was performed using individual siRNAs (Dharmacon) to assess effects on stabilization of endogenous IRP2 in HEK 293 cells. The four components of the SCF^{FBXL5} complex were among the top five validated siRNA targets identified in the screen.

Electrophoretic Mobility Shift Assay (EMSA)

HA-IRP2-FLAG HEK 293 cells were transfected with siRNAs and treated with either 100 μM FAC or 100 μM DFO for 16 hr. Cells were washed with 1X PBS, incubated in lysis buffer (20 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 0.2% Nonidet P-40, 1 mM DTT, 250 μM PMSF, 1X protease inhibitor cocktail (Sigma)), and centrifuged at 17,000g for 15 min. Lysate containing 10 μg total protein was incubated in the presence of 100 kcpm ^{32}P-labeled in vitro transcribed RNA encompassing the rat L-ferritin IRE in a reaction mixture containing

1 mM DTT, 0.06 U/μL RNase inhibitor (New England Biolabs), 5% glycerol, 20 mM HEPES (pH 7.4), 40 mM KCl, and 3 mM $MgCl_2$ for 1.5 hr at 4°C. For supershift reactions, 1 μL of α-IRP1 antibody or α-FLAG antibody was added to the mixture. Heparin was added to 2 mg/mL and the samples incubated another 10 min at 4°C. Protein-bound DNA complexes were resolved by electrophoresis in a 5% nondenaturing polyacrylamide gel at 4° C and visualized by phosphorimager analysis.

Quantitative Real-time Reverse Transcript (qRT)-PCR

Total RNAs were prepared using the Rneasy Mini Kit (Qiagen), treated with DNAse I (Roche) and converted to cDNA using Superscript II Reverse Transcriptase (Invitrogen) and random primers $p(dN)_6$ (Roche). qRT-PCR was performed using the Power SYBR Green PCR Master Mix (ABI) on the 7900HT Fast Real-Time PCR System (ABI) using primer sets for human TfR1 (5′-GGTGACCCTTACACACCTGGATT and 5′-TGATGACCGAGATGGTGGAA), FBXL5 (5′-CTTACCCAGACTGACATTTCAGATTC and 5′-GAAGACTCTGGCAGCAACCAA), and the 18S rRNA (5′-GATATGCTCATGTGGTGTTG and 5′-AATCTTCTTCAGTCGCTCCA) control. The thermal cycle conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melting curves were performed at 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. All tests were performed in triplicate and all experiments were repeated three times. The mathematical transformations for primary data analysis were done by SDS2.0 (ABI). The amplification data were analyzed based on the equation: R (ratio) = $2^{-(\Delta Ct\ sample-\ \Delta Ct\ control)}$. The results were expressed as fold changes of the treatment groups compared to the controls. p-values were determined by using Student's unpaired t-test.

Co-Immunoprecipitations

HA-IRP2-FLAG HEK 293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were treated with 10 μM MG132 (Boston Biochem) to block IRP2 and FBXL5 degradation for 1 hr followed by incubation with 100 μM FAC for an additional 6 hr. Cells were then washed with PBS, and incubated in lysis buffer containing 50 mM Tris/Cl (pH 8.0), 150 mM NaCl, 1.0% NP40, 250 μM PMSF, and 1X protease inhibitor cocktail (Sigma), and centrifuged at 17,000g for 15 min. Lysate (~500 μg) was incubated with 5 μL α-FLAG M2 affinity resin (Sigma) overnight, washed three times with lysis buffer, and the precipitates were subjected to immunoblot analysis. For co-immunoprecipitation of endogenous IRP2, reactions were carried out as described with two exceptions. Following incubation with MG132, FBXL5-FLAG expressing HEK 293T cells were treated with either 100 μM FAC or 100 μM DFO for 6 hrs and immunoprecipitates were eluted from the α-FLAG M2 affinity resin using a competitor FLAG-peptide prior to immunoblotting.

Recombinant Protein Expression and Purification

Bacteria, yeast, and insect cells were cultured under standard conditions unless otherwise specified. Recombinant E1 enzyme and ubiquitin were purchased from Boston Biochem. K_0-GST-Ubiquitin was expressed in *E. coli* and purified by glutathione agarose (GE Healthcare). UbcH5a E2 was expressed and purified under similar conditions, except the GST tag was released by Thrombin protease (GE Healthcare) and removed using glutathione agarose. For recombinant IRP2 expression, yeast were grown to mid-log phase in YPGL medium and induced with galactose (2% w/v) for 16 hours (*S8*). IRP2 was purified in anaerobic conditions (<1ppm

O_2) in a Coy glovebox by Ni-NTA chromatography and gel filtration on a Superdex 200 column equilibrated in deoxygenated buffer.

SCF^{FBXL5} was expressed in Sf9 cells grown in Sf900IIM media using the Bac to Bac system (Invitrogen). Briefly, SKP1, CUL1, and RBX1 were cloned into pFastBac HT vectors and full length FBXL5, or ΔF-box FBXL5 were fused to a C-terminal 3xFLAG epitope tag in the pFastBac 1 vector. Recombinant baculovirus was generated according to the manufacturer's protocol, and SCF^{FBXL5} was expressed by infecting Sf9 cells in log phase growth. Optimal expression was observed at 55-60 hr with a ratio of 4:1:6:6 of FBXL5:SKP1:CUL1:RBX1 viruses. Soluble SCF^{FBXL5} was purified by binding to FLAG M2 Resin and eluted with FLAG peptide. Eluted fractions were dialyzed in a Slide-A-Lyzer (MWCO 10kDa; Pierce) or subjected to size exclusion chromatography in a Superdex 200 column in buffer containing 10 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, and 100 mM NaCl. The subset of CUL1 protein that copurifies with FBXL5 migrates slower than the majority of overexpressed CUL1 in the input lysate, though the difference between these populations has not been determined.

Wildtype and E61A FBXL5 Hr domains were amplified by PCR using the oligonucleotides 5′-GGAGGGATCCGATGGCGCCCTTTCCTGAAGAAGTG and 5′-GGATCTAGATCACTGAGAGCAGTGTTGTGCAATCAC and cloned into the pGST-parallel vector (*S9*). Soluble GST-fusion proteins were expressed in *E. coli* grown in media supplemented with 100 μM FAC and purified using glutathione agarose. The GST fusion tag was cleaved upon incubation with TEV protease for 6 hr at 24°C and the liberated Hr domains were purified by anion exchange chromatography (HiTrap Q, GE Healthcare).

Ubiquitination Assay

Typical reactions are composed of 10 μg ubiquitin, 1 μg IRP2, 40 ng E1, 200 ng UbCH5a, and 1-3 μg of SCF^{FBXL5} in 50 μL reaction buffer containing 10 mM HEPES (pH 7.5), 10 mM KCl, 100 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, and 50 μM ferrous sulfate. Reactions were incubated at room temperature for 2 hr and resolved by electrophoresis in a 6% SDS-polyacrylamide gel.

Iron Content Measurement

UV-Visible spectra were measured with a Shimadzu UV-Visible Spectrophotometer (Model UV-1601). Iron content of the recombinant FBXL5 Hr domain was determined by the method of Beinert (*S10*) with the following modifications: 15.7 μM of protein in 50 mM Tris/Cl (pH 8.0) was denatured with 1.5% SDS, and 600 μM Ferene (3-(2-Pyridyl)-5,6-di(2-furyl)-1,2,3-triazine-5',5"-disulfonic acid disodium salt; Sigma) was employed as the iron detecting reagent. Absorbance was measured at 593nm and iron content was quantified against a standard curve generated from Claritas PPT Iron Solution Standard (CLFE2-2Y). The molar ratio of iron to FBXL5 in these preparations was calculated to be 1.1:1.0, suggesting approximately half of the protein contained a diiron center. In addition, UV-VIS absorption spectra for ferene bound to iron liberated from the hemerythrin domain showed a uniform peak with a maximum at 593 nm that was insensitive to the addition of the copper-specific chelator thiourea (data not shown), diagnostic of iron bound to ferene (*S11*).

Circular Dichroism

Circular dichroism (CD) measurements were performed in an AVIV 62 DS instrument with a 1mm path length quartz cuvette. Protein concentrations were kept at 0.11 mg/mL (6.2 μM) to prevent protein precipitation in buffer containing 10 mM Tris/Cl (pH 7.5) and 100 mM NaCl. Measurements were taken with an averaging time of 10 sec with 5 repeats. Thermal denaturation experiments were performed in a 20 mM HEPES buffer (pH 7.5) with 100mM NaCl, and data were recorded by measuring ellipticity at 222nm from 5-95°C in 1°C increments over equilibration times of 90 sec. Sodium dithionite (Fluka) and *o*-phenanthroline (Sigma) were added at 10-fold molar excess under N_2 atmosphere. Melting curves measured in reverse from 95-5°C did not overlap demonstrating that denaturation is not reversible (data not shown). Data are reported in terms of mean molar residual ellipticity.

Luciferase Gene Reporter Assay

Following 24 hr of transfection, HEK 293T cells were treated with either 100 μM FAC or 100 μM DFO and incubated an additional 24 hr under atmospheric (~20% O_2) or low (1%) O_2 conditions. Cells were lysed in buffer (30 mM Tricine (pH 7.8), 8 mM MgAc, 0.2 mM EDTA, 1% Triton) on ice, transferred to a 96-well plate, and ATP, CoA, β-mercaptoethanol, and D-luciferin (Molecular Probes) added to final concentrations of 375 μM, 375 μM, 50 mM, and 125 μM respectively. Luminescence was measured in a plate reader (Bio-Tek, Synergy HT). Assays were performed in triplicate with bars indicating standard error.

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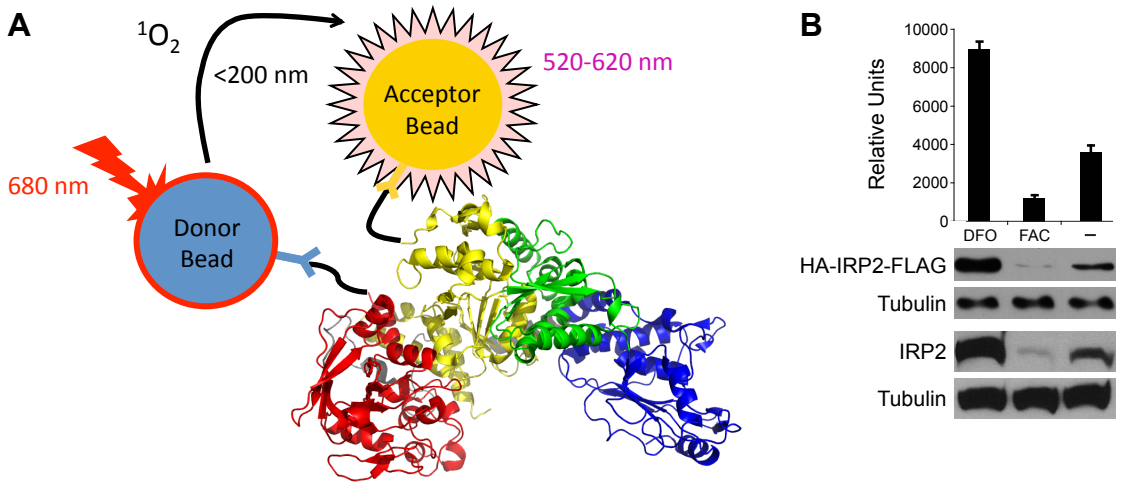

Fig. S1. A high throughput luminescent proximity assay (AlphaScreen) to measure HA-IRP2-FLAG protein levels in cell lysates. **(A)** Cartoon schematic of the AlphaScreen assay. Cellular lysates were incubated in the presence of Donor beads coated with α-HA antibodies and Acceptor beads coated with α-FLAG antibodies. Excitation of the Donor beads with long wavelength light (680 nm) results in the generation of singlet oxygen molecules with a limited lifetime, restricting their radius of diffusion in solution. If the Acceptor bead is within ~200 nm of the Donor bead, a thioxene derivative embedded within the Acceptor bead will react with singlet oxygen. The ensuing chemiluminescent reaction transfers energy to a fluorophore within the Acceptor bead that emits light at a shorter (520-620 nm) wavelength (*S12*). The IRP structure was obtained from the Protein Data Bank (2IPY). (**B**) Assessment of IRP2 protein accumulation under iron deplete (DFO) or iron replete (FAC) conditions using the AlphaScreen assay (top) or by immunoblot analysis of the HA-IRP2-FLAG reporter protein (α-FLAG antibody; middle) or endogenous IRP2 in the parental HEK 293 cell line (bottom). When HA-IRP2-FLAG is present in whole cell lysates the α-HA Donor beads and α-FLAG Acceptor beads are brought into close proximity as reflected by an amplified luminescent signal. Conversely, incubation of cells in the presence of excess iron prior to lysis results in low accumulation of HA-IRP2-FLAG and a corresponding decrease in signal. Assays were performed in triplicate with data represented as the mean +/- SE.

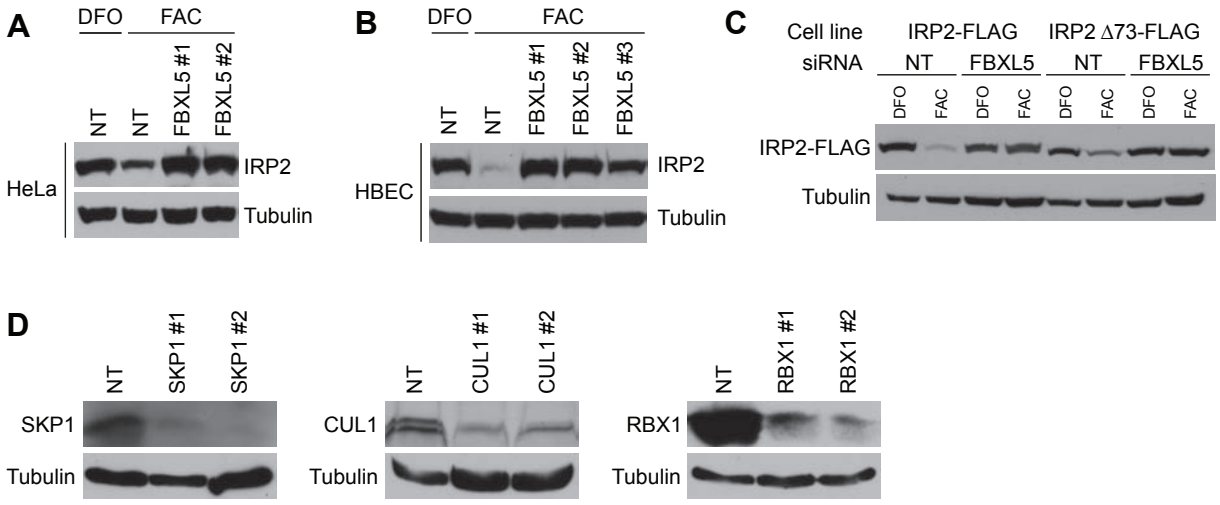

Fig. S2. RNAi-mediated knockdown of FBXL5 stabilizes IRP2. IRP2 stabilization under iron replete conditions following siRNA-mediated suppression of FBXL5 was measured by immunoblot analysis of endogenous IRP2 in (**A**) HeLa cells, (**B**) HBEC-30 cells, or (**C**) HEK 293 cell lines stably transfected with either a FLAG-tagged wildtype or IRP2-Δ73 expression construct. (**D**) Efficiency of siRNA-mediated knockdown of SKP1, CUL1, and RBX1 accumulation in HEK 293 cells as assessed by immunoblot analysis. NT, non-targeting control siRNA.

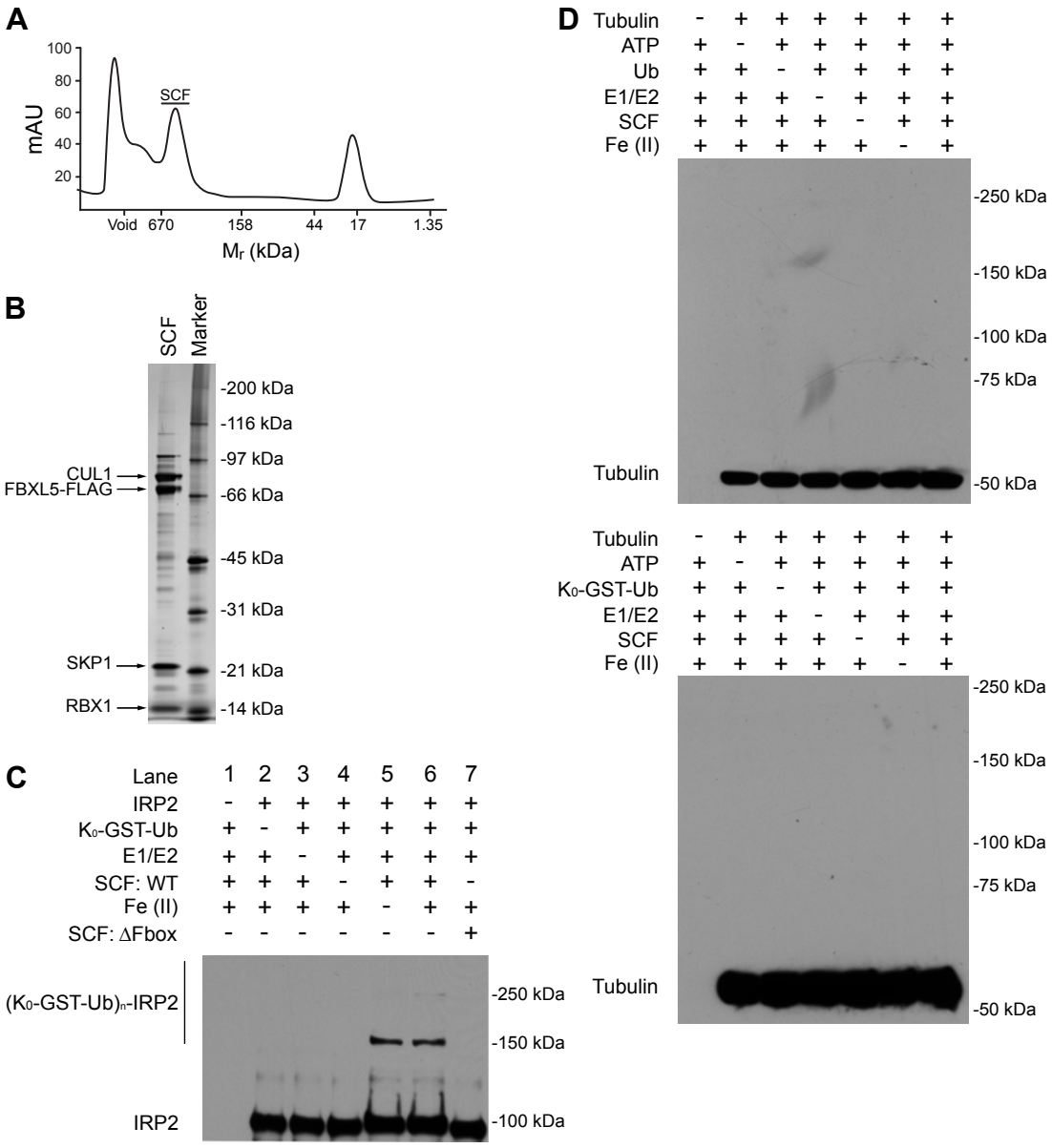

Fig. S3. Recombinant FBXL5 Assembles a SCF Complex that Ubiquitinates IRP2 in vitro. (**A**) UV-chromatogram (A280) and (**B**) silver stain of the peak fraction (SCF) from a Superdex-200 gel-filtration column loaded with FLAG-purified SCF^{FBXL5}. (**C**) FLAG-affinity purified SCF^{FBXL5}, but not ΔF-box FBXL5, is able to ubiquitinate recombinant IRP2 in vitro. (**D**) Purified recombinant SCF^{FBXL5} that ubiquitinates IRP2 in vitro (Fig. 2D) does not ubiquinate purified recombinant tubulin protein (kindly provided by L. Rice), suggesting that SCF^{FBXL5} is not simply promiscuous in vitro.

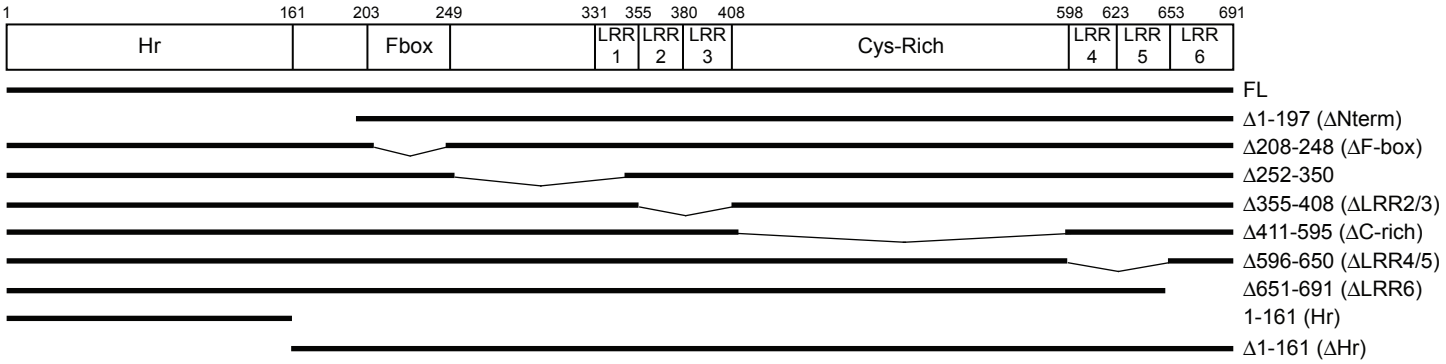

Fig. S4. Schematic of FBXL5 deletion constructs. Human FBXL5 is 691 amino acids in length and contains multiple protein domains in addition to the defining F-box, including two series of leucine rich repeat (LRR) regions, comprised of at least three putative LRRs elements each. Between the two series of LRRs is a region we termed the Cys-rich region due to the high number of conserved cysteine residues found among the vertebrate homologs. FBXL5 also contains a highly conserved N-terminus (Hr).

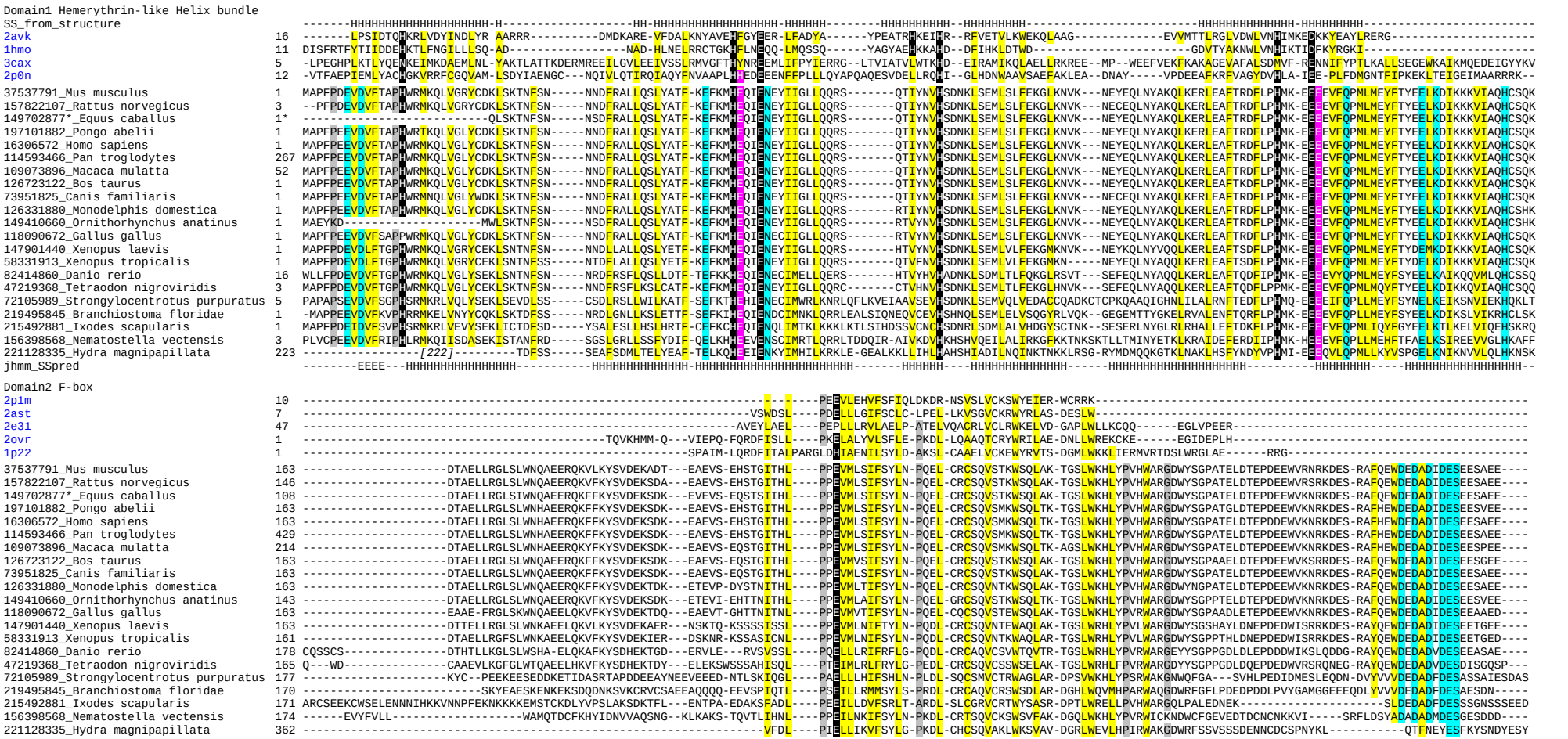

Fig. S5. Alignment of FBXL5 Sequences. FBXL5 family sequences were identified using BLAST (*S13*) against the NCBI nr sequence database. A multiple sequence alignment between the FBXL5 sequences, PSI-BLAST identified structures (2p0n and 3cax), and the next closest hemerythrin-like structures (2avk and 1hmo) was generated with PROMALS-3D (*S14*) and manually adjusted based on alignments generated with two sensitive sequence-based structure recognition methods, COMPASS (*S15*) and HHPRED (*S16*). Typically, seven residues form a diiron site buried within the helix bundle, with three histidine residues coordinating one Fe, two histidine residues coordinating a second Fe, and two carboxylates bridging the irons. As compared to known hemerythrins (*S17*, *18*), the FBXL5 family retains six out of seven invariant Fe-binding ligands (highlighted in black). Structural modeling suggests that one of two invariant FBXL5 carboxylate residues (highlighted in magenta) acts as the third ligand coordinating one of the irons. Additional invariant residues are highlighted in cyan. Positions of mainly hydrophobic residues are highlighted in yellow and positions of mainly small residues are highlighted in gray. Cys residues from the Cys-Rich region are highlighted in green, with unalignable sequence from the same region in italics. Predicted secondary structural elements are indicated below the alignment and secondary structural elements conserved in all structures are indicated above the alignment for the hemerythrin domain.

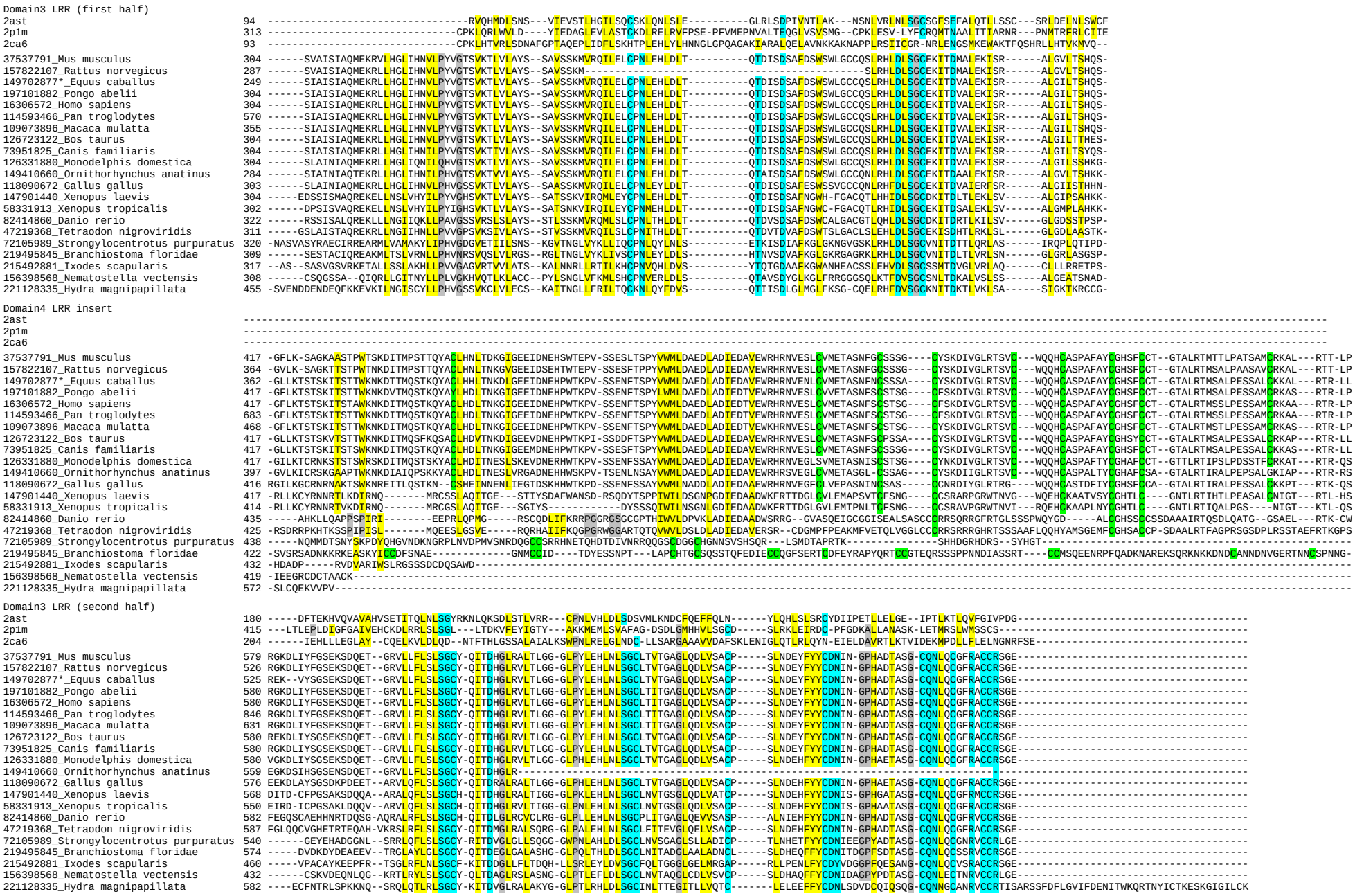

Fig. S5. Continued

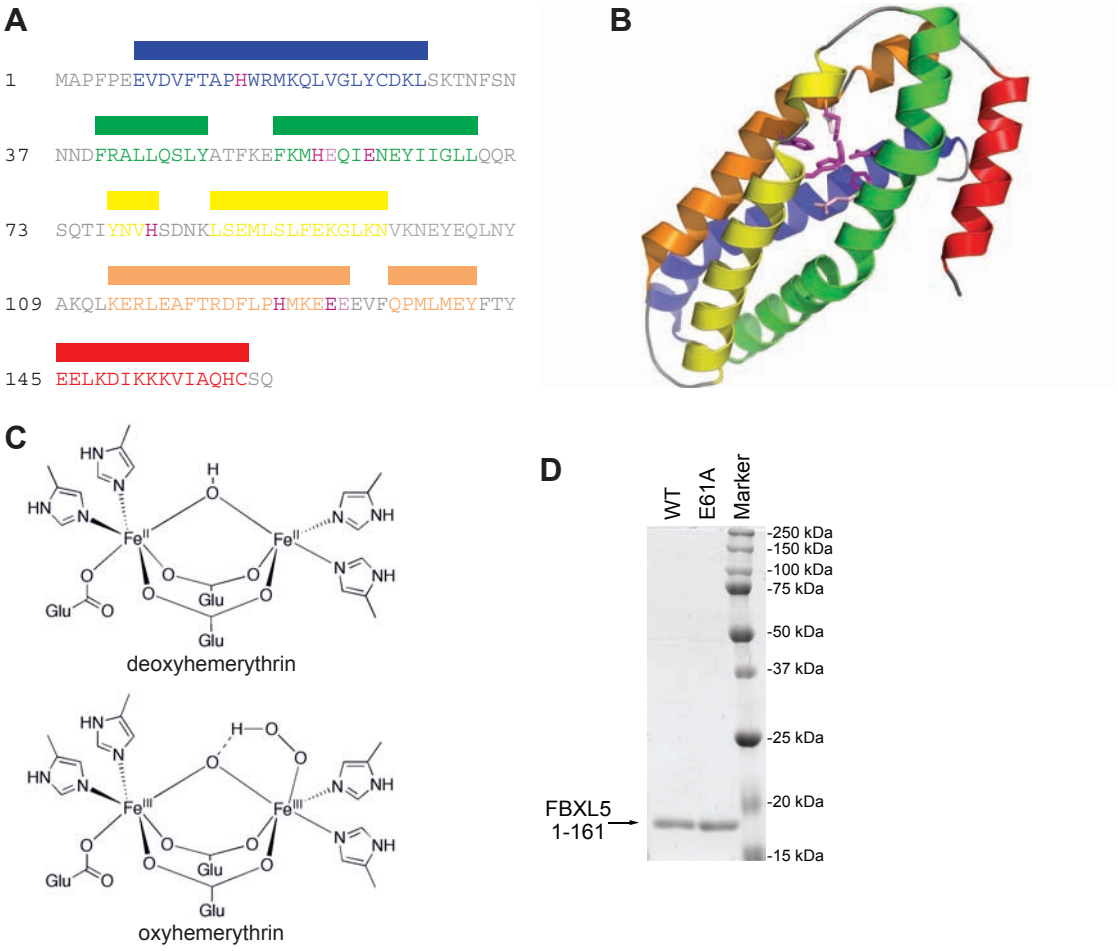

Fig. S6. FBXL5's N-terminus encodes an iron-binding hemerythrin domain. (**A**) Schematic of the predicted alpha helices (bars), conserved iron-binding ligands (magenta), and possible iron-binding ligands (pink) in the human FBXL5 hemerythrin domain. Helical bounds for FBXL5 were determined by combining information from helices observed in other structures with predictions from sequence alignments. (**B**) Model of the FBXL5 hemerythrin domain mapped upon a solved hemerythrin structure (2p0n). Six conserved Fe binding residues from hemerythrin are highlighted in magenta and residues that could replace the missing histidine are highlighted in pink. (**C**) Schematic of the oxy- and deoxyhemerythrin diiron center. (**D**) Coomassie blue staining of recombinantly expressed and purified wildtype (WT) or variant (E61A) FBXL5 hemerythrin domains.

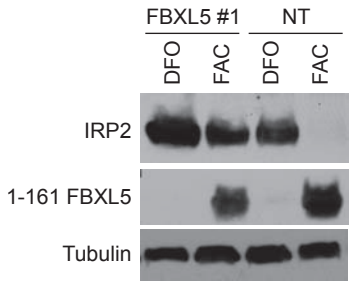

Fig. S7. Iron-dependent regulation of the FBXL5 hemerythrin domain is not affected by SCF^{FBXL5} knockdown. HEK 293T cells were cotransfected with a FLAG-tagged Hr expression construct (FBXL5 1-161) and a FBXL5 siRNA (FBXL5 #1) targeting the 3′ end of the endogenous FBXL5 transcript prior to FAC or DFO addition and immunoblotting. Efficiency of FBXL5 knockdown is indicated by IRP2 stabilization in FAC-treated cells.

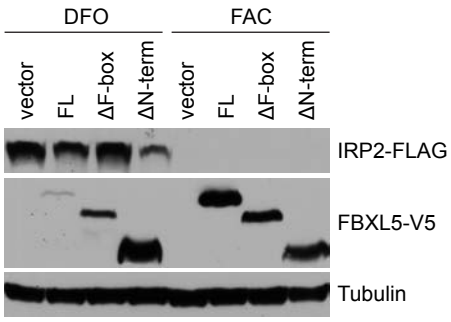

Fig. S8. Overexpression of FBXL5 lacking the hemerythrin domain reduces IRP2 accumulation in DFO-treated cells. HEK 293T cells were cotransfected with plasmids expressing IRP2-FLAG and indicated FBXL5-V5 proteins. At 8 hr post-transfection, cells were treated with FAC or DFO and incubated an additional 16 hrs.

Table S1. Quantitation of Fig. 3B Immunoblot

	FAC	DFO			DFO	FAC			
Time (hr)	0	1	2	4	6	0	1	2	4	6
IRP2	1.0	5.5	19.0	32.9	71.2	1.0	1.6	1.3	0.4	0.03
FLAG-FBXL5	1.0	0.8	0.6	0.3	0.1	1.0	1.1	22.0	62.6	70.9

Values are normalized to tubulin and expressed as fold changes from time zero

Table S2. Quantitation of Fig. 3E Immunoblot

	FL	ΔN-term	ΔF-box	Δ252-350	ΔLRR2/3	ΔC-rich	ΔLRR4/5	ΔLRR6
FAC:DFO	5.0	0.9	1.8	37.5	2.3	69.7	9.0	3.2

Values are normalized to tubulin and expressed as the ratio of FAC:DFO samples

Table S3. Quantitation of Fig. 4E Immunoblot

	Hr-Luc				Luc-Hr				Luc				E.V.
	DFO		FAC		DFO		FAC		DFO		FAC		FAC
O_2	20%	1%	20%	1%	20%	1%	20%	1%	20%	1%	20%	1%	20%
FLAG	ND	3.9	13.5	3.9	8.0	16.1	25.7	12.2	22.2	20.5	19.0	12.6	ND

Values are normalized to tubulin, ND = not determined due to insufficient signal above background

Table S4. siRNA Sequences

siRNA	Catalog #	Targeting Sequence
FBXL5 #1	D-012424-04	CCUUAGAGGUCUUAGCCUA
FBXL5 #2	D-012424-17	CCUCAAGAGUUAUGUCGAU
FBXL5 #3	D-012424-01	GCACAACACUGCUCUCAGA
SKP1 #1	D-003323-09	CGCAAGACCUUCAAUAUCA
SKP1 #2	D-003323-10	CCAAUAUGAUCAAGGGGAA
CUL1 #1	D-004086-03	GGUUAUAUCAGUUGUCUAA
CUL1 #2	D-004086-05	CAACGAAGAGUUCAGGUUU
RBX1 #1	D-004087-01	GAAGCGCUUUGAAGUGAAA
RBX1 #2	D-004087-03	GCAUAGAAUGUCAAGCUAA
NT	D-001210-01	

Table S5. PCR Primers

Construct	FBXL5 Primers
Δ1-197	5'-GGAGGATCCACCATGGTGTCAGAACACTCCACAGG
Δ208-248	5'-GGTATAACCCATTACCCTGTTCATTGGGCCAGAG 5'-ATGAACAGGGTAATGGGTTATACCTGTGGAGTGTTCTGA
Δ252-350	5'-CTTTACCCTGTTATTTTAGAGCTTTGTCCTAACC 5'-GCTCTAAAATAACAGGGTAAAGATGTTTCC
Δ355-408	5'-ATTTTAGAGCTTGGAATTCTGACATCTCATC 5'-GTCAGAATTCCAAGCTCTAAAATCTGCCTAAC
Δ411-595	5'-GCTCTTGGAATTACTGGACGTGTACTTCTGTTTC 5'-CACGTCCAGTAATTCCAAGAGCTCTGGAAATC
Δ596-650	5'-CTGATCAAGAGTGTCCTTCTCTGAATGATG 5'-CAGAGAAGGACACTCTTGATCAGATTTTTCAC
Δ651-691	5'-GGTGTCTAGAACCTGCTGAAACCAAATCCTGCAGGCCTG
Δ1-161	5'-GGAGGATCCACCATGAAGGATACTGCAGAACTCCTTAGAGG
1-161	5'-GGATCTAGATCACTGAGAGCAGTGTTGTGCAATCAC
H57A	5'-GTTCAAAATGGCTGAGCAGATTGAAAATGAATAC 5'-CAATCTGCTCAGCCATTTTGAACTCCTTGAAAG
E61A	5'-GAGCAGATTGCAAATGAATACATTATTGGTTTG 5'-GTATTCATTTGCAATCTGCTCATGCATTTTG
Construct	**IRP2 Primers**
Δ73	5'- CTTCAGTAAAGAACCTGAAACAGTGTTAAA 5'-TTTCAGGTTCTTTACTGAAGTCAATTTGTA

Table S6. Antibodies

Antibody	Vendor	Catalog #
Rabbit anti-CUL1	Santa Cruz	sc-11384
Goat anti-FBXL5	Santa Cruz	sc-54364
Mouse anti-FLAGM2	Sigma	F3165
Donkey anti-Goat HRP	Santa Cruz	sc-2056
Mouse anti-IRP2	Santa Cruz	sc-33682
Goat anti-Mouse HRP	Santa Cruz	sc-2055
Goat anti-Rabbit HRP	Santa Cruz	sc-2054
Rabbit anti-RBX1	AbCam	ab2977
Rabbit anti-SKP1	AbCam	ab10946
Mouse anti-Tubulin	Sigma	T6199
Mouse anti-V5	Invitrogen	R960-25