Iron metabolism regulates p53 signaling through direct heme-p53 interaction and modulating localization, stability and function of p53

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This PDF file includes:

Legends for Figures S1 to S7

Figure S1. Homeostatic levels of endogenous p53 protein and iron or heme are inversely correlated under the condition of iron excess. Related to Figure 1.

(A) The levels of endogenous p53 proteins in primary hepatocytes isolated from *Hfe*$^{-/-}$ mice were lower than that of wild-type. (B) Hemin destabilized endogenous p53 protein in mouse primary *Hfe*$^{+/+}$ hepatocytes in a hemin-dose dependent manner. The cells were treated with hemin at indicated concentrations for 8 hrs, before harvesting for immunoblott analysis. (C) HepG2 cells were treated with hemin (10 μM, 6 hrs) or ferric ammonium citrate (FAC; 100 μg/ml, 6 hrs). Endogenous p53 proteins were detected with immunoblotting using anti-p53. Actin was probed as an internal control.

Figure S2. Human tumor suppressor p53 protein directly interacts with heme. Related to Figure 3.

Mass spectra analysis indicated that heme (average m/z, 616.2, indicated by asterisk *) was associated with bacterially expressed and freshly purified wild-type p53 (A), but not the similarly prepared p53$_{C275, 277A}$ mutant protein (B). Spectra shown here are in monoisotopic modes. All mass spectra analyses were carried out on an Axima-QIT™ MALDI-Quadrupole Ion Trap-TOF (Shimadzu, Japan). (C) Hemin-agarose chromatography recovered endogenous p53 protein from mammalian cells. HepG2 and HCT116 cells (p53$^{+/+}$ or p53$^{-/-}$) were lysed and incubated with 20 μl hemin-agarose or agarose at 4 ℃ for 2 hrs. After extensive washing, the beads were heated in 1 X SDS-PAGE sample buffer and immunoblotted with anti-p53. (D) Surface plasmon resonance analyses of p53-heme interactions. The kinetic studies of interactions between hemin and p53 protein were performed on a Biacore 3000 (GE Healthcare). The human p53 protein was covalently coupled by its amine groups to a CM5 sensor chip (GE Healthcare). Surface plasmon resonance analyses indicated that the K_d for p53-heme interaction was at the range of ~1.0 - 2.0 μM. (E) Comparison of the sensitivities of p53 and other heme binding proteins to dialysis. BSA and ATE1

were used as positive controls of heme binding and lysozyme as the negative control. The p53, ATE1, and BSA (each at 10 μM) were incubated with hemin (10 μM) in Slide-A-Lyzer Dialysis Cassettes (7K MWCO, Thermo Scientific). Dialysis of a 0.5 ml sample against 500 ml of heme binding buffer, followed by TMB assay (Pierce) to detect the remaining hemin concentrations in dialysis cassettes at indicated time points (n=3 each). (F) Gas-sensing properties of the p53-heme complex. Ultraviolet-visible (UV-Vis) spectra analyses were carried out with p53-heme complex before (the outer blue line at the tip of the back arrow) or after nitric oxide (NO) or oxygen (O_2) infusion. The red arrow indicated spectral shifts after the infusion of increasing amounts of NO. The green arrow indicated little or no shift in the spectra after O_2 infusion.

Figure S3. The $p53_{C275, 277A}$ mutant protein has low transcriptional activity at the p21 promoter. Related to Figure 4.

HCT116 $p53^{-/-}$ cells were plated in 24-well plates and transfected with the indicated plasmids. The activity of firefly luciferase was measured with the dual luciferase assay kit (Promega) and results were obtained from at least three independent experiments, each run in triplicate. Data are presented as mean ± SEM, n = 3, ** $P < 0.01$.

Figure S4. Hemin destablilizes p53 protein through a proteasome -dependent pathway. Related to Figure 5.

HepG2 cells were treated with hemin (15 μM, 8 hrs) with or without MG132 (10 μM, 8 hrs) or an autophagy inhibitor bafilomycin A1 (BAF; 100 nM, 8 hrs).

Figure S5. Hemin induces p53 nuclear export by promoting p53-CRM1 interaction. Related to Figure 5.

(A) Leptomycin B (LMB) blocks hemin-triggered nuclear export of GFP-tagged p53. HepG2 cells transfected with GFP-tagged p53 were treated with either of LMB (5 ng/ml, 12 hrs), hemin (10 μM, 6 hrs), or both. Treated cells were fixed and stained

with DAPI. GFP-tagged p53 (Green) and DAPI stained nuclei (Blue) were visualized by fluorescence microscopy. The scale bars represent 10 μm. (B) Heme binding promotes the interaction between p53 and CRM1. Recombinant His_6-tagged human p53 protein or the pre-formed p53-heme complex (~ 8 μM) was incubated with GST only or GST-tagged CRM1 before addition of glutathione beads to the mixture. Protein-bound glutathione beads were then pelleted and washed, resolved by SDS-PAGE, and immunoblotted with anti-His or anti-GST antibodies (see supplemental methods for details). (C) Heme binds to wildtype p53 or $p53_{L348,350A}$ with comparable affinity. HepG2 cells transiently expressing HA-tagged wildtype p53 or $p53_{L348,350A}$ were lysed and incubated with agarose only or heme-agarose beads. The pelleted beads were then washed, boiled in 1 X SDS-PAGE loading buffer, resolved by SDS-PAGE, and subjected to immunoblotting analysis with anti-HA antibodies.

Figure S6, Related to Figure 6. Deferoxamine-induced tumor-suppression is dependent on endogenous p53.

Nude mice bearing tumors formed by HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells were treated with or without deferoxamine (DFO, 500 mg/kg/d over 6 consecutive days).

Figure S7, Related to Discussion. Hemin interacts with and destabilizes p63 and p73, the p53 family proteins that also bear the C-terminal CXCP motifs.

(A) The heme-binding CXCP motif is conserved in p53 family proteins. (B) Hemin-agarose batch chromatography recovered p63 and p73 when each protein was individually over-expressed in HepG2 cells. (C) HepG2 cells expressing HA-tagged p53, p63 or p73 were treated with cycloheximide (CHX, 25 μg/ml) or hemin (10 μM) as indicated for 6 hrs. Cellular extracts were subjected to immunoblotting with anti-HA. Actin was probed as an internal control.

Figure S1

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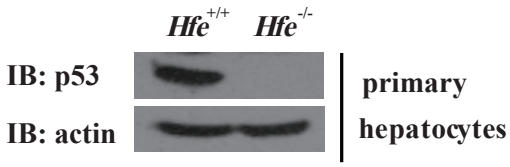

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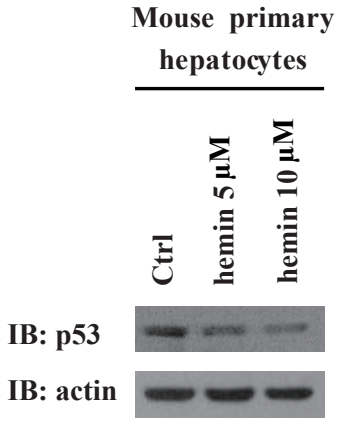

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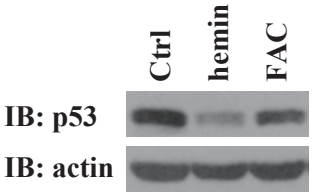
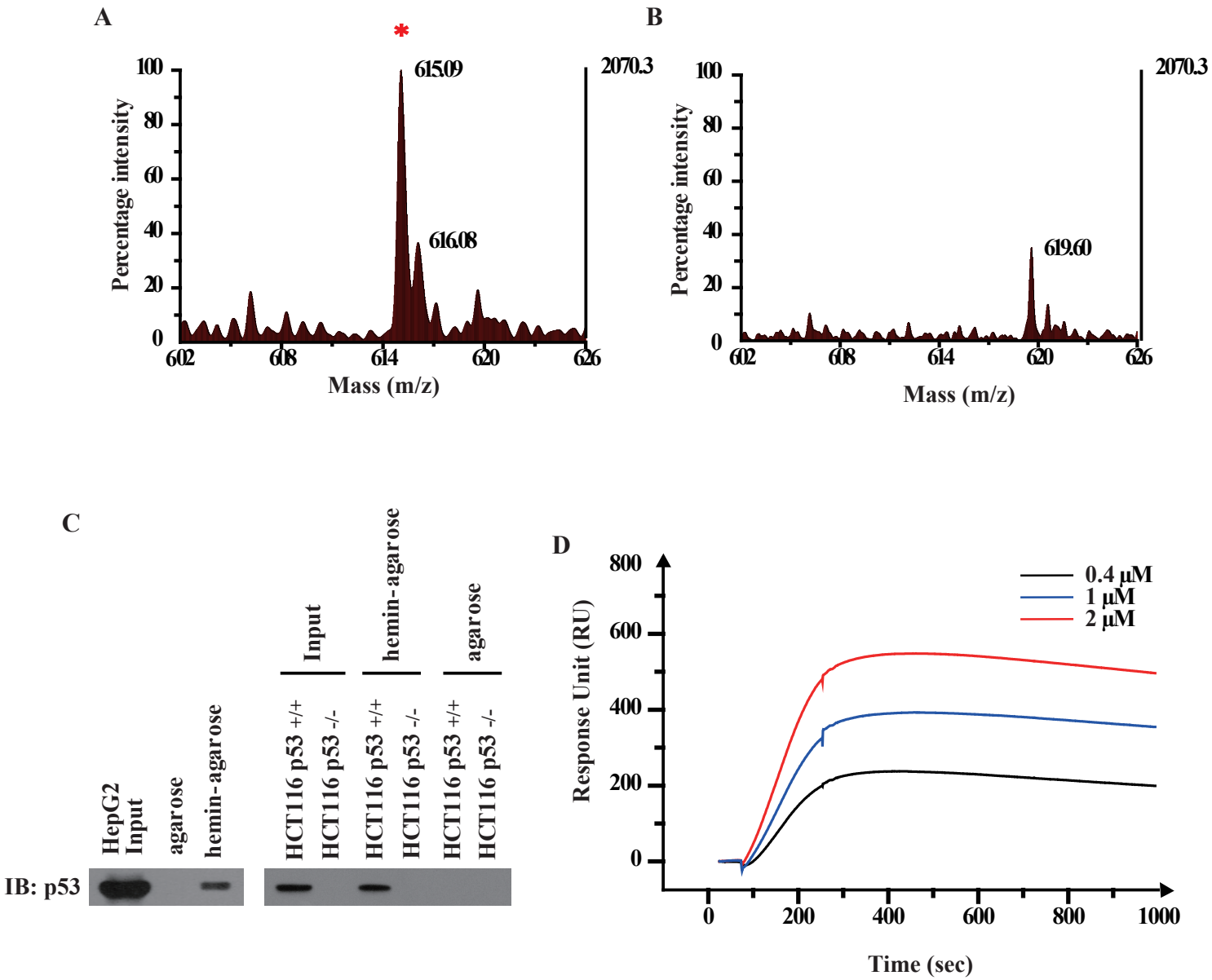


Figure S2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B
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616.08
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Percentage intensity
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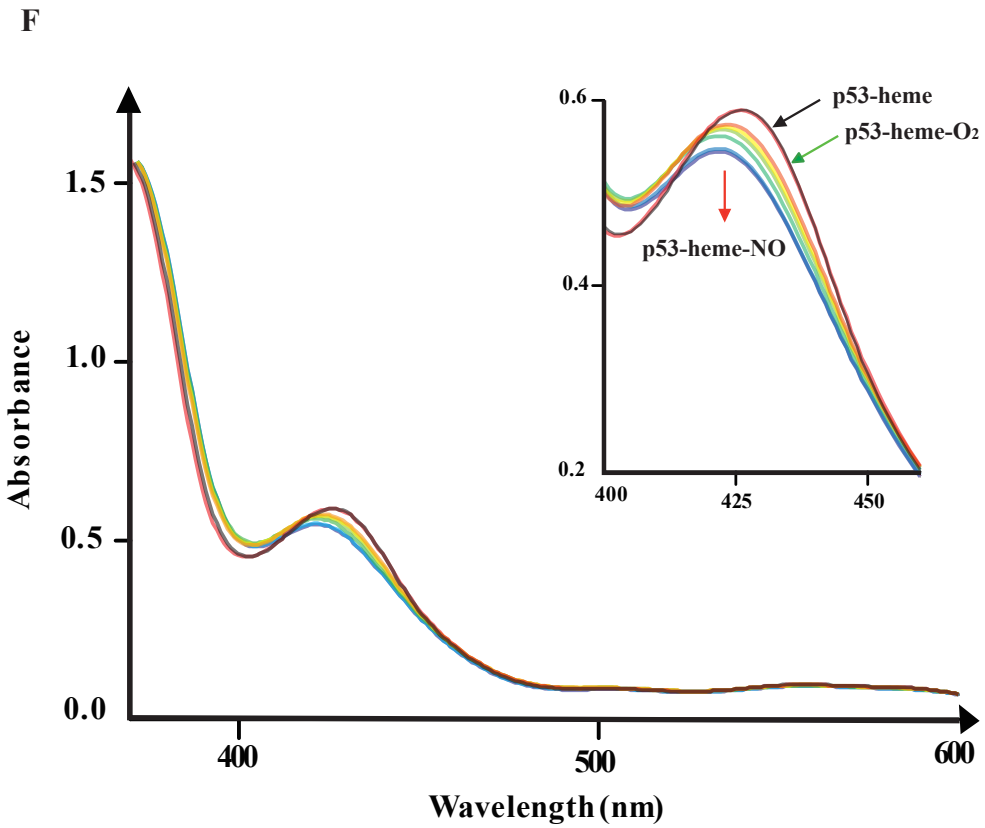
agarose
hemin-agarose
HCT116 p53 +/+
HCT116 p53 -/-
IB: p53
D
Response Unit (RU)
Time (sec)
0.4 μM
1 μM
2 μM
E
Relative % of protein remaining
lysozyme
BSA
p53
ATE1
0 hr
6 hr
12 hr
18 hr

Figure S2



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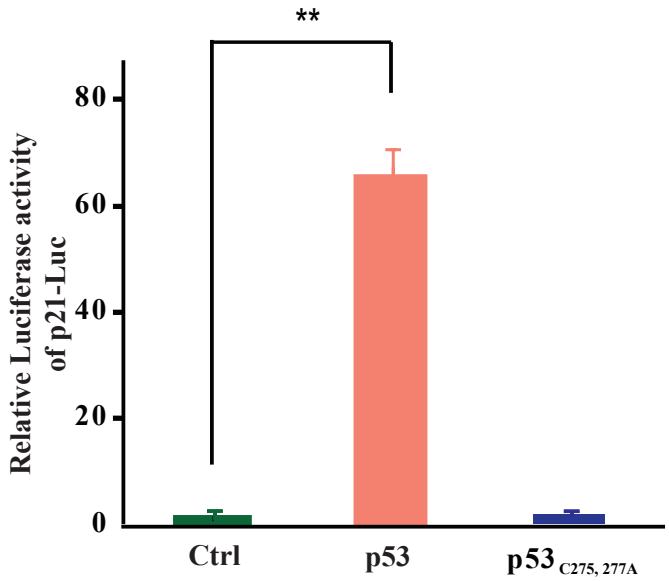

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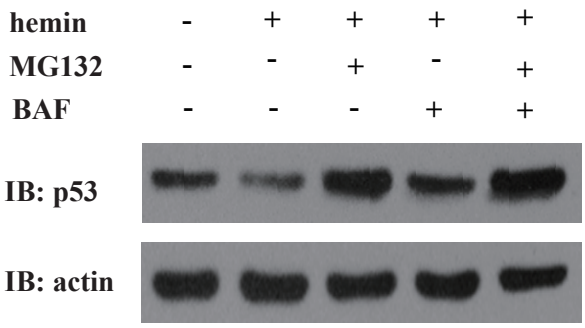

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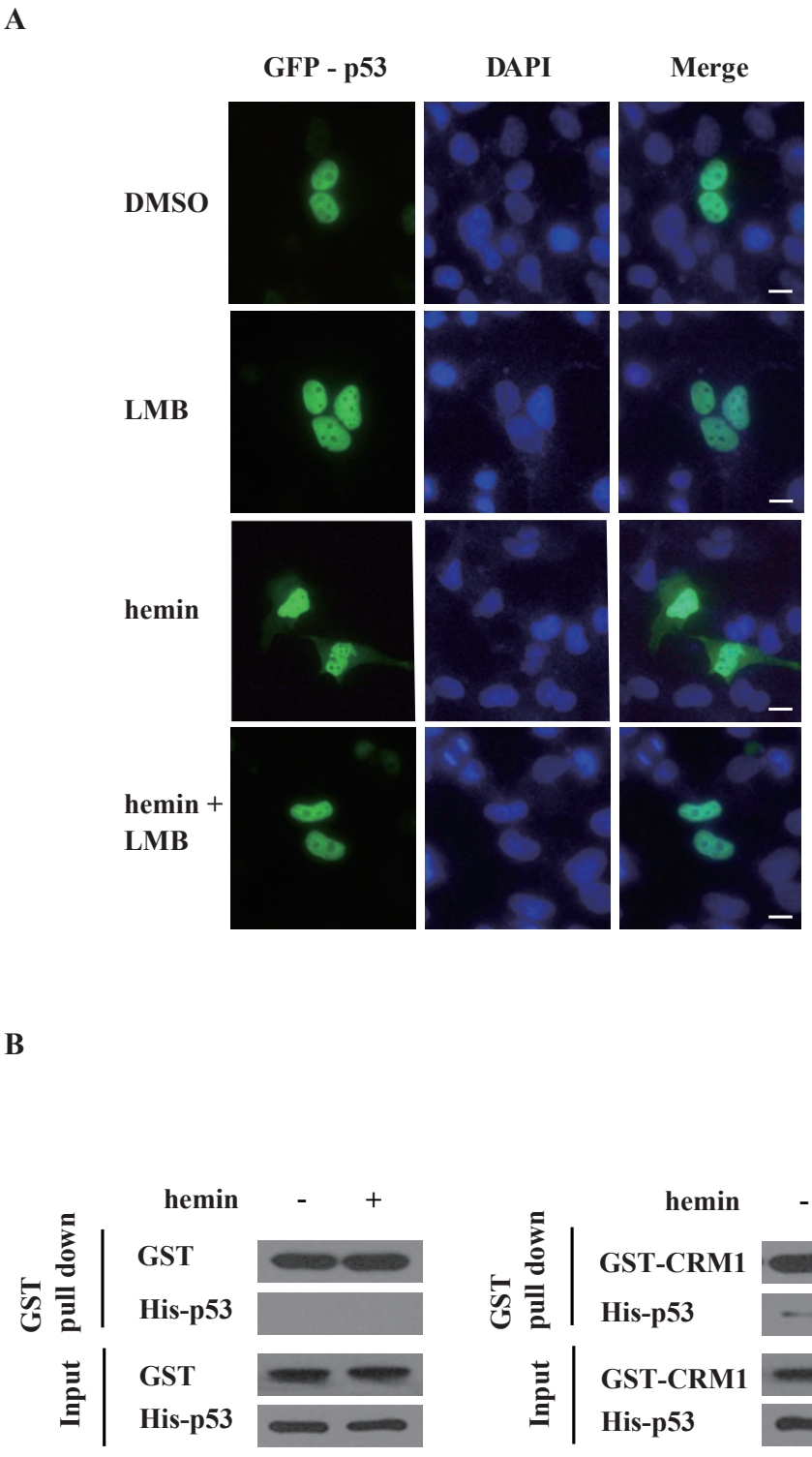
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DMSO
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B
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Input
GST
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Figure S6

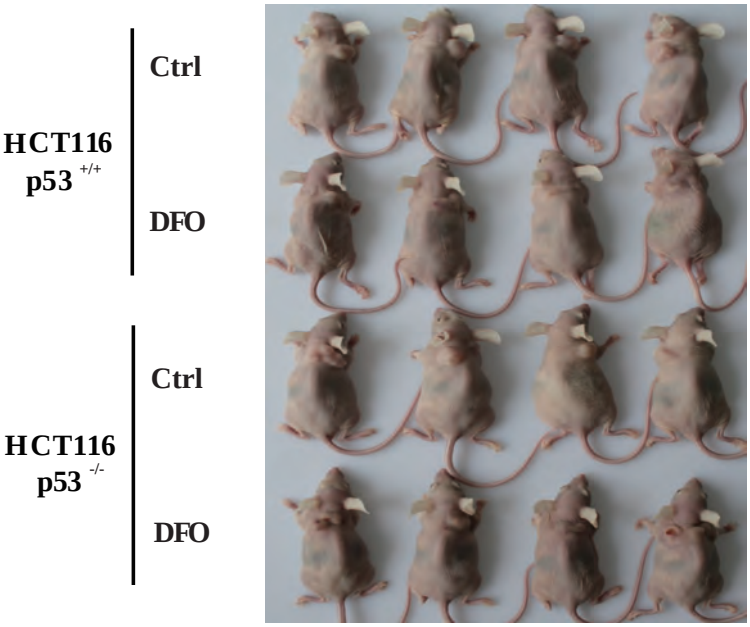
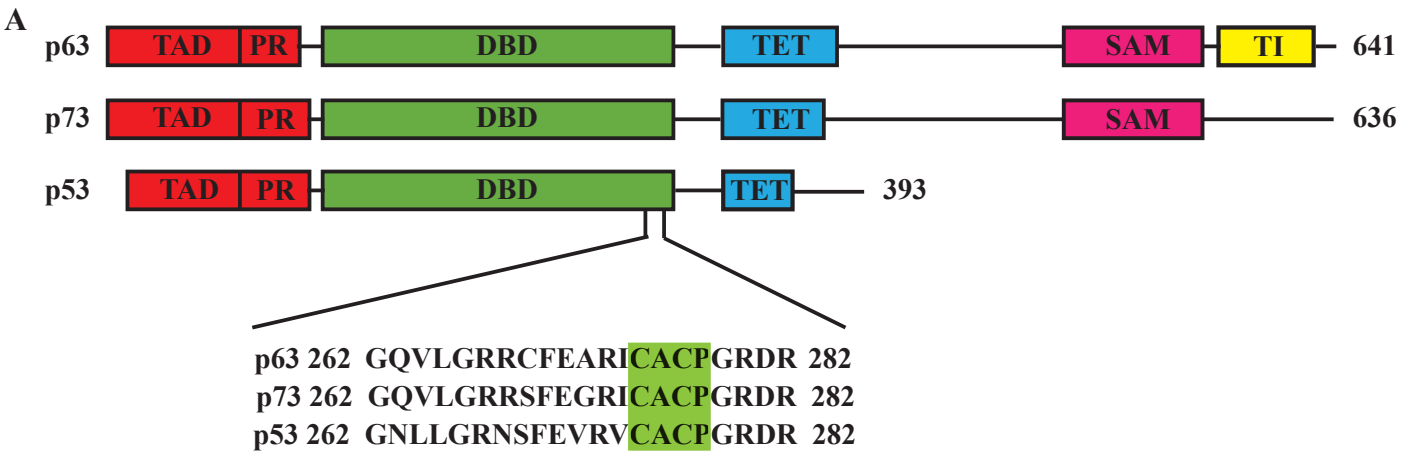


Figure S7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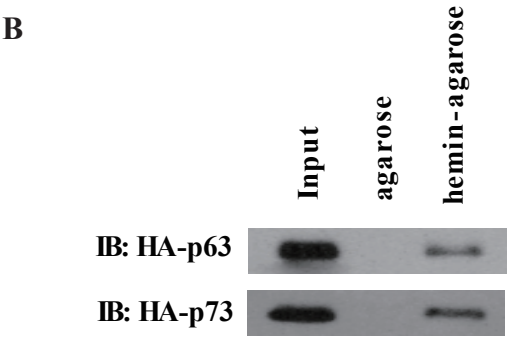
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hemin-agarose
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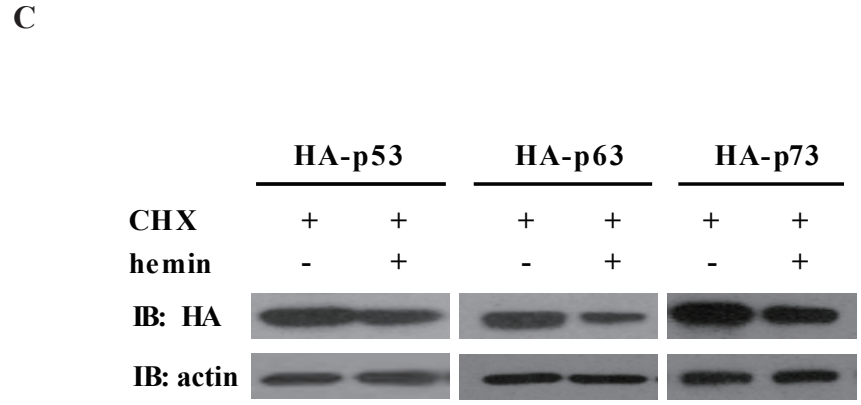
C
HA-p53
HA-p63
HA-p73
CHX
+ + + + + +
hemin
- + - + - +
IB: HA
IB: actin

Supplemental Materials

Reagents

Dulbecco's Modified Eagle Medium (DMEM), virus production-serum free medium (VP-SFM), fetal bovine serum (FBS), and Lipofectamine 2000 were obtained from Invitrogen. MG132, cycloheximide (CHX), succinylacetone (SA), Leptomycin B (LMB), Ferric ammonium citrate (FAC), deferoxamine (DFO), human ubiquitin, chicken egg white lysozyme, Bovine Serum Albumin (BSA), hemin, hemin-agarose, the compressed pure gases carbon monoxide (CO), oxygen (O_2) and nitric oxide (NO), and other chemical reagents, if not indicated otherwise, were purchased from Sigma. 1-Step Turbo TMB (3,3 ′,5,5 ′-tetramethylbenzidine) substrate for the heme assay was obtained from Pierce. The Dual-Luciferase Reporter Assay system was from Promega. The protease inhibitor cocktail and X-tremeGENE HP DNA Transfection Reagent were products from Roche. Slide-A-Lyzer Dialysis Cassettes (7K MWCO) were obtained from Thermo Scientific. The following antibodies were used: mouse anti-p53 antibody (Santa Cruz), mouse anti-HA antibody (Sigma), rabbit anti-Lamin B antibody (Santa Cruz), mouse anti-Actin antibody (Sigma), mouse anti-p21 antibody (Santa Cruz), mouse anti-Bax antibody (Santa Cruz).

Supplemental Experimental Procedures

Expression and purification of wild type and mutant p53

To express human p53 (10 μM) and its mutants bearing Cys to Ala and Pro to Ala substitutions at one or multiple Cys-Pro motifs, cDNAs encoding wild-type human p53 and the mutants were cloned into the pHUE vector to obtain the proteins with Histidine x 6 (his_6)-ubiquitin (Ub) at the N-terminus and FLAG tag at the C-terminus. The resultant plasmids were transformed into BL21 competent *E. coli* cells. Cells were then grown to an absorbance (OD_{600}) of 0.8 - 1.0 before addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration of 0.3 mM). Cells were

cultured at 16 ℃ for another 10 hours to induce expression of the recombinant p53 proteins. Proteins expressed in *E. coli* were purified with Nico-NTA QIA-Express protocol (Qiagen). Proteins purified at this stage were desalted on NAP-5 (GE Healthcare) to remove imidazole. Post-NAP5 protein fractions were then subjected to TMB assay or mass spectra analysis (see below). The N-terminal His_6-Ub tag was removed through Usp2CC cleavage and depleted with Ni-NTA beads as previously described (Hu et al., 2005). The supernatants were then mixed with pre-equilibrated anti-FLAG M2 beads (Sigma), followed by FLAG peptide elution according to the manufacturer's instruction to recover FLAG-tagged p53 protein. Proteins were dialyzed and frozen at −80 ℃ in the stock solution (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 20% glycerol, 150 mM NaCl, 1 mM Dithiothreitol (DTT)).

Gel filtration, spectroscopy analysis of hemin-protein complexes, and TMB assay

Purified FLAG-tagged human p53 (10 μM), its mutants that had Cys to Ala and Pro to Ala substitutions in one or multiple Cys-Pro motifs (10 μM), chicken egg white lysozyme (10 μM), or Bovine Serum Albumin (BSA) were incubated with hemin (50 μM) in binding buffer (20 mM HEPES, pH 7.4, 10% glycerol, 150 mM NaCl) for 10 mins on ice, then passed through pre-equilibrated NAP-5 columns (GE Healthcare) to remove the unbound hemin as previously described (Hu et al., 2008). Ultraviolet-visible (UV-Vis) spectra analyses of heme only or the heme-protein complexes were carried out on Varian or Hitachi U-3010 spectrophotometers. Hemin content was determined (in triplicate) using the TMB (3,3',5,5'-tetramethyl-benzidine)-based assay (Pierce), according to the manufacturer's instructions.

Mouse models and analysis of iron and heme contents in liver lysates or isolated primary hepatocytes

Hfe knockout mice (*Hfe*$^{-/-}$) was a kind gift from Fudi Wang, INS, CAS (with a C57/Bl/6-129/Ola genetic background, originally donated by Nancy C. Andrews, Departments of Medicine and Pediatrics, Harvard Medical School, Boston, MA). Hemin content was measured in *Hfe* $^{+/+}$ versus *Hfe* $^{-/-}$ mouse littermates.

Experimental iron overload was achieved by feeding 4-week-old male B6 wild-type mice a regular diet supplemented with 8.3 g/kg carbonyl iron (Sigma-Aldrich) for 3 weeks. Liver samples were weighed and iron content measured using an unsaturated iron-binding capacity assay as previously described (Ba et al., 2011). Liver samples containing equal amounts of total protein (30 μg) were subjected to the TMB assay (Pierce) to assess hemin content. Isolation of primary hepatocytes from *Hfe*$^{+/+}$ and *Hfe* $^{-/-}$ mice was carried out as described before (Wang et al., 2009). Hemin contents were measured (in triplicate) using the TMB (3,3',5,5'-tetramethyl-benzidine)-based assay (Pierce) for hepatocytes from *Hfe* $^{+/+}$ versus *Hfe*$^{-/-}$ mouse littermates.

Preparation of hemin solution and hemin treatment of mammalian cells

Mammalian cells (including HepG2 cells, HCT116 cells and MEF cells) were adapted to serum-free growth conditions by culture in VP-SFM (serum-free medium) (Invitrogen) as described before (Hu et al., 2008). For cell-based experiments, hemin, freshly prepared in DMSO, was added at the indicated concentrations to 80-90% confluent cells in VP-SFM. For other experiments, stock hemin (Fe^{3+} heme) solution was freshly prepared in 0.1 N NaOH for each experiment. The concentration of hemin was determined using the extinction coefficient ε_{385} = 5.84 x 10^4 $cm^{-1}M^{-1}$.

Gas-sensing assay

The p53 proteins in stock solution were exchanged into the binding buffer (20 mM HEPES, 10% glycerol, 150 mM NaCl, pH 7.4) and preserved at reduced status by adding Immobilized TCEP (Tris-2'-carboxyethyl-phosphine) Disulfide Reducing Gel (Thermo Scientific). Immediately before use, the TCEP gels were pelleted to give clear p53 protein solutions. P53 proteins (10 μM) were then incubated with 50 μM

hemin in binding buffer on ice for 15 mins and desalted using NAP-5 columns to remove free hemin. The solutions (0.5 ml) containing the recovered p53-hemin complexes were kept in sealed, air-tight cuvettes (VWR) on ice, followed by vacuuming and purging with argon for over 30 mins to remove residual oxygen from the solution. Dithionate (at a final concentration of 1.0 mM in binding buffer) was injected to reduce iron in hemin from a ferric to ferrous status. Balloons inflated with carbon monoxide (CO, ≥ 99.0%, from Sigma), nitric oxide (NO 98.5%, Sigma, passed through 1N KOH) or oxygen (O_2, ≥ 99.6%, Sigma), were attached to the cuvettes with long needles inserted into the solutions. UV-visible spectra were recorded at 1.5 mins intervals until no more shifts were observed, indicating the maximal formation of p53-heme-gas complexes.

Surface plasmon resonanc measurements

Kinetic studies of the interactions between hemin and p53 protein were performed by measuring surface plasmon resonance (SPR) using a Biacore 3000 instrument (GE Healthcare). The p53 proteins were covalently coupled to a CM5 sensor chip (GE Healthcare) using an amine coupling kit (GE Healthcare). Stock hemin solution was freshly prepared in 0.1 N NaOH, then diluted to indicated concentrations with running buffer (20 mM HEPES, 150 mM NaCl, pH 7.8) and used as the analyte. P53-heme association was monitored for a 180 s period, while the dissociation was subsequently monitored for a 600 s period.

Mass spectrometry analysis

All mass spectra analyses were carried out on an Axima-QIT™ MALDI-Quadrupole Ion Trap-TOF (Shimadzu, Japan) to assess the presence of hemin naturally bound to recombinant human p53 or $p53_{C275,\ 277A}$ proteins. P53 or $p53_{C275,\ 277A}$ eluents from NTA-Ni columns were applied to NAP-5 column to remove imidazole. The resultant solutions (1 μl) were mixed with a matrix (1 μl) containing 4-hydroxy-α-cyanocinnamic acid (α-CHC, 10 mg/ml in 50% methanol) in 0.1% TFA (trifluoroacetic acid) (v/v). All mass spectra in this work were acquired in positive ion

reflection mode after calibration with external standards. Each mass spectrum was typically collected with 150 laser spots. Mass spectra peaks at 616 Da +/- 2 Da (M/Z) indicated the presence of hemin.

Hemin-agarose chromatography

HepG2 cells or HCT116 cells ($p53^{+/+}$ or $p53^{-/-}$) were grown in 100 mm dishes with DMEM containing 10% FBS. Cells were lysed in 600 µl lysis buffer (50 mM Tris, pH7.5, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 10 µM NaF, 10 µM Na_3VO_4, 1 mM PMSF, and 1% aprotinin), then incubated with 20 µl hemin-agarose at 4 ℃ for 2 hrs. The beads were washed 4 times with the lysis buffer boiled in 1 X SDS-PAGE sample buffer (100 ℃, 10 mins), and subjected to immunoblotting with indicated antibodies.

Luciferase reporter assay

HCT116 $p53^{-/-}$ cells expressing indicated genes were seeded in 24-well plates and transfected with the luciferase reporter plasmids that contain the p53-responsive elements from the promoters of the human p21 or Bax genes. Luciferase reporter assays were carried out using a dual luciferase assay kit (Promega). Results were independently replicated in at least three experiments (each in triplicate).

Electrophoresis mobility shift assay

The electrophoresis mobility shift assay (EMSA) was performed as previously described (Jayaraman and Prives, 1995), with slight modifications. Specifically, bovine serum albumin (BSA) was replaced with 1.5 µg/ 20 µl bovine ubiquitin, which does not bind to hemin, in the DNA-protein interaction buffer (25 mM HEPES, pH7.6, 50 mM KCl, 20% glycerol, 5 mM DTT, 5 mM $MgCl_2$, 0.2 µg/20 µl poly (dI-dC). The double stranded DNA probes used for this EMSA contained the consensus p53

responsive element sequence (5'- AGG CAT GTC TAG GCA TGT CT -3', 5'- AGA CAT GCC TAG ACA TGC CT -3'). The probes were labeled with ^{32}P at the 3'-end with T4 ligase (NEB). Recombinant human p53 protein (4 μM) was then incubated with hemin at increasing concentrations (0 μM, 5 μM, 10 μM, 20 μM) on ice for 30 mins to allow for protein-hemin complex formation. The radio-labeled probe was then incubated with increasing amounts of p53 protein (0 μM, 2 μM, 4 μM) only or the above solutions containing pre-formed p53-hemin complexes. After another 30 min incubation on ice, the reaction mixtures were subjected to 6% polyacrylamide gel analysis, resolving the DNA-protein complexes from the free probes. Gels were then dried and exposed to a phosphor-imager (Kodak) before visualization on a FLA 9000 Fuji scanner.

Tryptophan fluorescence quenching assay

Fluorescence measurements were performed at room temperature (～23 ℃) using a Cary Eclipse spectrofluorometer (Varian, Ltd.) as described before (Hu et al., 2008), with slight modifications. Specifically, a 280 nm excitation, three emission scans were performed (from 290 nm to 500 nm) to determine the maximal Trp-specific fluorescent emission (340 nm) of wild type p53, or $p53_{C275,\ 277A}$ (each at 1 μM). Hemin was added to the solutions of wild type p53 protein, or $p53_{C275,\ 277A}$ to final concentrations from 0 to 18 μM. After 3 min incubations, the fluorescence intensities at 340 nm were recorded and the data were processed as previously described (Hu et al., 2008). These analyses indicated the presence of a single hemin-binding site in wild-type p53 with K_d of ~1.21 μM. The K_d for $p53_{C275,\ 277A}$ was ~15.76 μM.

GST pull down assay

GST or GST-CRM1 proteins were purified from bacteria cells (BL21 DE3) transformed with pGEX4T-1 or pGEX4T-1-CRM1(Wu et al., 2013), using standard glutathione-agarose beads according to the manufacturer's instructions (GE).

His_6-tagged p53 was cloned into the pET-28a vector for expression in BL21 expression strain. Purified His_6-tagged human p53 (20 μM) was incubated with hemin (30 μM) in GST binding buffer (20 mM HEPES, pH 7.4, 10% glycerol, 150 mM NaCl) for 10 mins on ice, then passed through pre-equilibrated NAP-5 columns (GE Healthcare) to remove unbound hemin as described before. P53 protein or the p53-hemin complex was added to the GST binding buffer (500 μl) to a final concentration of ~8 μM. GST or GST-tagged CRM1 was added to the same concentration and mixed at 4 °C for 2 hrs before glutathione beads were added and incubated for another 2 hrs. The protein-bound beads were then pelleted and washed 3 times with the GST binding buffer and subjected to immunoblotting analysis using anti-His or anti-GST antibodies.

Hemin-protein dialysis assay

BSA and ATE1 were used as positive controls of heme binding and lysozyme as the negative control. Purified p53 protein (10 μM), ATE1 protein (10 μM), lysozyme (10 μM), BSA (10 μM) were incubated with hemin (10 μM) in dialysis buffer (20 mM HEPES, pH 7.4, 10% glycerol, 150 mM NaCl) in Slide-A-Lyzer Dialysis Cassettes (7K MWCO, Thermo Scientific). Dialysis of a 0.5 ml sample against 500 ml of dialysis buffer was followed by a TMB assay (Pierce) to detect the remaining hemin concentrations in dialysis cassettes at indicated time points (n = 3 each).

Flow cytometry

For cell cycle analysis, HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells were treated with DFO (100 μM) for 24 hrs and later supplemented with FAC (100 μg/ml, 8 hrs) and hemin (10 μM, 8 hrs) as indicated. Cells were collected and fixed in 70% ethanol overnight followed by procedures described before (Pack et al., 2008). Fixed cells were stained with 50 μg/ml propidium iodide (PI) in the presence of RNase A (200 μg/ml) and subjected to cell cycle analysis using a FACS Calibur (BD Biosciences) flow cytomer.

Tumorigenicity assay and DFO treatment

Female nude mice (Bi-kai Biotech) aged 5-week-old were used. 1×10^6 HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells were suspended in Matrigel (BD Biosciences) and injected s.c. into the mice according to standard procedures. Mice bearing evident tumors were randomly divided into control and DFO treatment groups (four mice per group). DFO was dissolved in water and injected into tumors at a dose of 500 mg/kg/d over 6 consecutive days. After animals were euthanized with carbon dioxide, tumor masses were isolated and tumor weight measured.

Ubiquitylation assays

HepG2 cells transfected with His_6-tagged ubiquitin plasmids were treated with hemin (10 μM), MG132 (10 μM), or both for 6 hrs. Cells were lysed in modified RIPA buffer [50 mM Tris-Cl, pH7.4, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 0.5% sodium pyrophosphate, 0.1% SDS, and protease inhibitor cocktail (Roche)]. IP experiments were also carried out in modified RIPA buffer using anti-p53 and followed by immunoblotting with anti-His antibodies (Lee et al., 2008).

Fluorescence and immunofluorescence microscopy

Cells were seeded on gelatin-coated coverslips in 6-well plates, transfected with the indicated plasmids, and received any indicated treatments. Afterwards, cells were washed in phosphate-buffered saline (PBS) before fixation in 2% formaldehyde. Cells were subject to 4'-6-Diamidino-2-phenylindole (DAPI) staining to visualize cell nuclui and the green fluorescent protein (GFP) was directly visualized. Immunofluorescence microscopy analyses with indicated antibodies were carried out as described before (Shen et al., 2011). Immunofluorescence was visualized and

recorded on a Olympus BX51 microscope and Leica TCS SP2 laser confocal microscope. Images were processed using Leica LAS-AF software and Adobe Photoshop CS3.

Multiple sequence alignments and PDB coordinates

Proteins sequence alignments were performed with Clustal X v2.0 according to the provider's instructions (Larkin et al., 2007). PDB coordinates of the p53-DNA complex were extracted from the Protein Data Bank (pdb ID 1TSR) (Cho et al., 1994) and visualized on PyMOL (DeLano Scientific LLC, San Carlos, CA, USA).

Statistical analysis

All values are expressed as means ± SEM. The two tailed unpaired Student's *t*-test was used to assess the significance of differences between two sets of data. Differences were considered to be statistically significant when $P < 0.05$.

Supplemental References

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