Supporting Information

Lakhal-Littleton et al. 10.1073/pnas.1422373112

SI Materials and Methods

Isolation of Adult Cardiomyocytes. Hearts were cannulated and mounted on a Langendorff apparatus and then were perfused using a Liberase-containing solution (Roche) to digest the tissue for 10 min. Then the reaction was terminated using 1% BSA (Sigma Aldrich). After filtration through a 70-um gauze, cells were cultured in Medium 199 with Earl's salts, supplemented with L-carnitine (2 mM), taurine (2 mM), and L-creatine (5 mM) (medium and supplements all from Sigma Aldrich). Cells then were treated within 2 h of culture.

Cine MRI. Mice were anesthetized with 2% (vol/vol) isofluorane in O_2 and were positioned supine in a purpose-built cradle. ECG electrodes were inserted into the forepaws, a respiration loop was taped across the chest and heart, and respiration signals were monitored using a custom-built physiological motion-gating device (1). The cradle was lowered into a vertical-bore, 11.7-T MR system with a 40-mm birdcage coil (Rapid Biomedical) and visualized using a Bruker console running Paravision 2.1.1. A stack of contiguous 1-mm-thick true short-axis ECG- and respiration-gated cine-FLASH images was acquired. The entire in vivo imaging protocol was performed in ∼60 min.

Image analysis was performed using ImageJ (National Institutes of Health). LV volumes and ejection fractions were calculated from the stack of cine images as described (1).

T_2^*. Mice were anesthetized with 2% (vol/vol) isofluorane in O_2 and positioned supine in a purpose-built cradle. All experiments were performed on a 7-T preclinical MRI system (Agilent Technologies) with a multinuclear parallel imaging Direct Drive console and a small-animal gradient set (17.5 G/cm maximum strength; 87.5 $G{\cdot}cm^{-1}{\cdot}ms^{-1}$ slew rate). T^*_2 maps were determined by acquiring cardiac-gated spoiled gradient multiecho images within a single midventricular axial slice at a high spatial resolution (128 × 128 matrix; 32.0 × 32.0 mm field-of-view; 1.6-mm-thick slice with a sinc excitation; flip angle 15°; eight averages). An exponential array of eight echo times (Te) was used (Te = 1.81, 2.37, 3.10, 4.07, 5.33, 6.98, 9.15, or 12.00 ms) to ensure sensitivity to multiple decays of T^*_2. Data then were regridded appropriately in Matlab (Mathworks), zero-filled by a factor of two, and multiplied by a 2D Hamming window to reduce noise and discretization effects. After a 2D Fourier transformation, the resulting multiecho data were subjected to a threshold-based segmentation algorithm before fitting. Data then were fit (in parallel) to a single-exponential model over the echo times, using a linear least squares trust-region algorithm with bounded parameters. Cardiac T^*_2 statistics were calculated from a manually placed region of interest in the interventricular septum.

Iron Quantitation by ICP-MS. All samples were measured using the Thermo Finnigan Element 2 Sector-Field ICP-MS. Calibration was achieved using the process of standard additions; in the case of iron, spikes of 0, 0.5, 1, 10, 20, and 100 ng/g iron were added to replicates of a selected sample. An external iron standard (ICP-MS-68-A solution; High Purity Standards) was diluted and measured to confirm the validity of the calibration. Rhodium also was spiked onto each blank, standard, and sample as an internal standard at a concentration of 1 ng/g. Concentrations from ICP-MS were normalized to starting tissue weight.

1. Tyler DJ, et al. (2006) CINE-MR imaging of the normal and infarcted rat heart using an 11.7 T vertical bore MR system. *J Cardiovasc Magn Reson* 8(2):327–333.

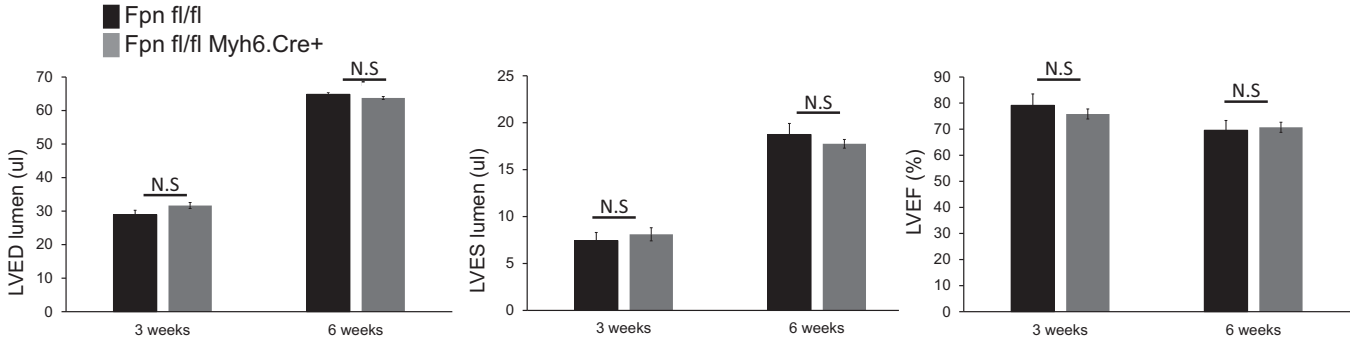
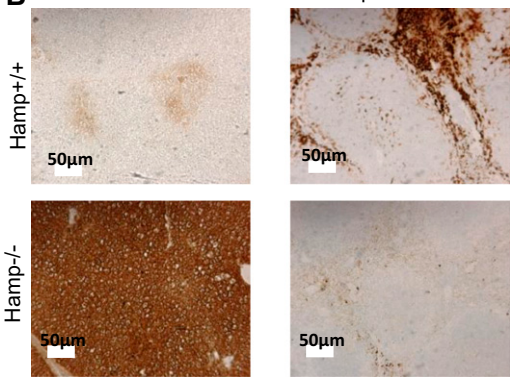


Fig. S1. Cardiac function in *Fpn fl/fl Myh6.Cre*$^+$ mice and *Fpn fl/fl* controls at age 3 and 6 wk. Values are shown as mean ± SEM. $n = 3$ per group.

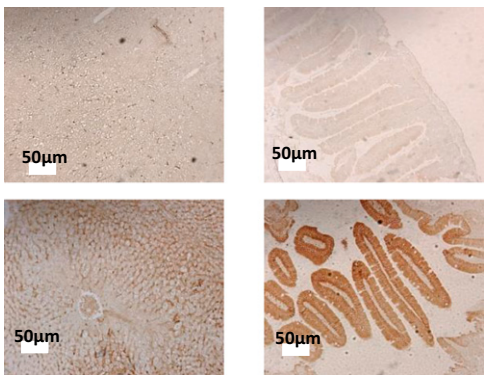
A

	3 months		6 months	
	Hamp +/+	Hamp-/-	Hamp +/+	Hamp-/-
Liver ferritin (ug/mg total protein)	1.37 ± 0.44	4.96 ± 1.85*	1.52 ± 0.65	8.79 ± 1.95*
Liver total elemental iron (ng/mg tissue)	13.1 ± 3.0	28.9 ± 5.6*	15.3 ± 2.1	36.4 ± 5.9*
Hb (g/L)	125 ± 11	149 ± 14	118 ± 18	153 ± 18*
Serum ferritin (mg/L)	0.39 ± 012	1.40 ± 0.33*	0.47 ± 0.11	1.65 ± 0.70*
Serum iron(µmol/L)	24.3 ± 12.1	49.7 ± 8.9*	26.9 ± 9.5	52.1 ± 17.3*
Serum Tf (g/L)	0.59 ± 0.17	0.64 ± 0.12	0.63 ± 0.08	0.72 ± 0.11

B



C



D

	3 months		6 months	
	Hamp +/ +	Hamp -/-	Hamp +/ +	Hamp -/-
Average Mass (mg)	56.1 ± 2.0	63.1 ± 3.6	75.5 ± 6.5	70.8 ± 4.1
LVED lumen (µl)	32.3 ± 3.2	32.7 ± 1.8	43.3 ± 3.9	45.3 ± 2.6
LVES lumen (µl)	13.3 ± 1.5	12.8 ± 2.5	15.4 ± 1.5	14.2 ± 0.5
LVEF %	57.7 ± 5.3	59.4 ± 6.5	59.4 ± 6.4	64.8 ± 4.0
RVED lumen (µl)	25.5 ± 1.8	26.9 ± 1.8	32.8 ± 1.4	39.8 ± 2.9*
RVES (µl)	8.34 ± 1.47	8.60 ± 1.02	8.41 ± 0.65	10.19 ± 0.31*
RVEF %	73.6 ± 4.8	71.6 ± 4.9	77.2 ± 3.1	73.9 ± 2.6
Heart Rate bpm	415.4 ± 13.3	418.7 ± 17.4	487.7 ± 24.4	462.9 ± 21.9
Stroke volume (µl)	18.7 ± 2.4	19.1 ± 2.3	25.3 ± 2.4	29.5 ± 2.4
Cardiac output (ml/min)	7.81 ± 1.12	7.91 ± 0.91	12.35 ± 1.20	13.35 ± 0.96
Heart/body weight ratio x1000	2.43 ± 0.14	2.60 ± 0.22	2.61 ± 0.18	2.56 ± 0.17

Fig. S2. Characterization of iron status in *Hamp*$^{-/-}$ mice and *Hamp*$^{+/+}$ littermate controls. (*A*) Summary of systemic indices of iron status in *Hamp*$^{-/-}$ mice and *Hamp*$^{+/+}$ littermate controls at age 3 and 6 mo ($n = 12$ per group). Values are shown as mean ± SEM. *$P < 0.05$. (*B*) Representative images of DAB-enhanced Perls iron staining in liver and spleen of a 3-mo-old *Hamp*$^{-/-}$ mouse and a *Hamp*$^{+/+}$ littermate control. (*C*) Representative images of FPN immunostaining in liver and duodenum of a 3-mo-old *Hamp*$^{-/-}$ mouse and a *Hamp*$^{+/+}$ littermate control. (*D*) Summary of cardiac function indices as measured by cine MRI in *Hamp*$^{-/-}$ mice and *Hamp*$^{+/+}$ littermate controls at age 3 and 6 mo ($n = 12$ per group). *$P < 0.05$.

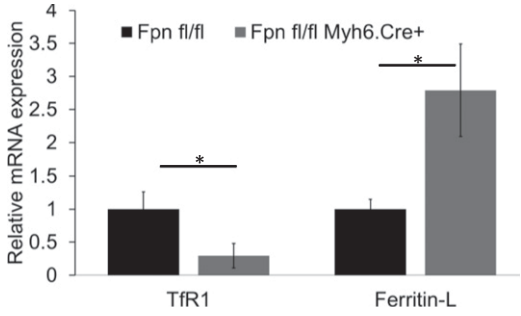


Fig. S3. Quantitative PCR expression of TfR1 and ferritin-L in hearts of 6-mo-old *Fpn fl/fl Myh6.Cre*$^{+}$ mice relative to *Fpn fl/fl* controls. $n = 6$ per group. Values are plotted as mean ± SEM. *$P < 0.05$.

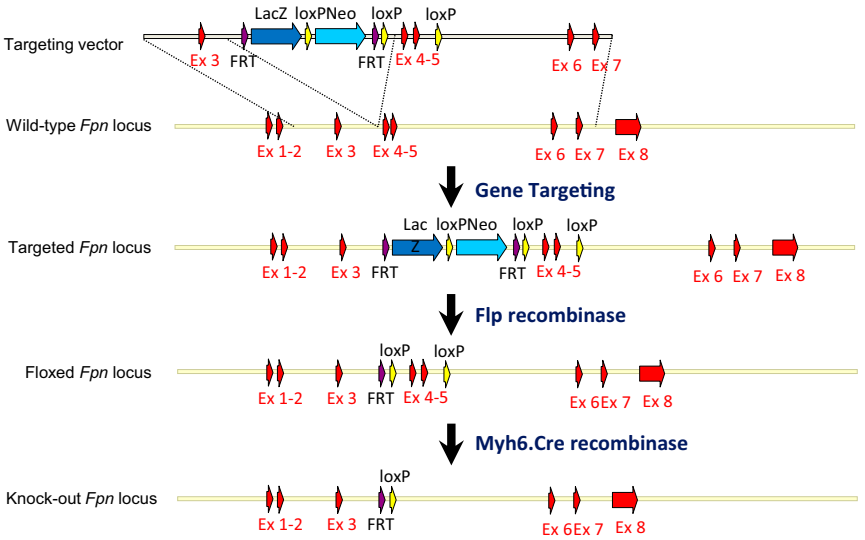


Fig. S4. Strategy for generation of *Fpn fl/fl Myh6.Cre*$^{+}$ mice. C57BL/6N mice harboring a knockout first mutation at the *Fpn* (*Slc40a1*) locus are crossed with a C57BL/6 Flp recombinase deleter mouse to remove the selection cassette and generate an *Fpn* allele with floxed exons 4 and 5, which encode the transmembrane domain. Cardiac *Fpn* knockouts then were generated by crossing homozygous *Fpn fl/fl* animals with mice transgenic for Myh6-Cre recombinase, which is under the control of the cardiomyocyte-specific myosin alpha heavy-chain 6 promoter.

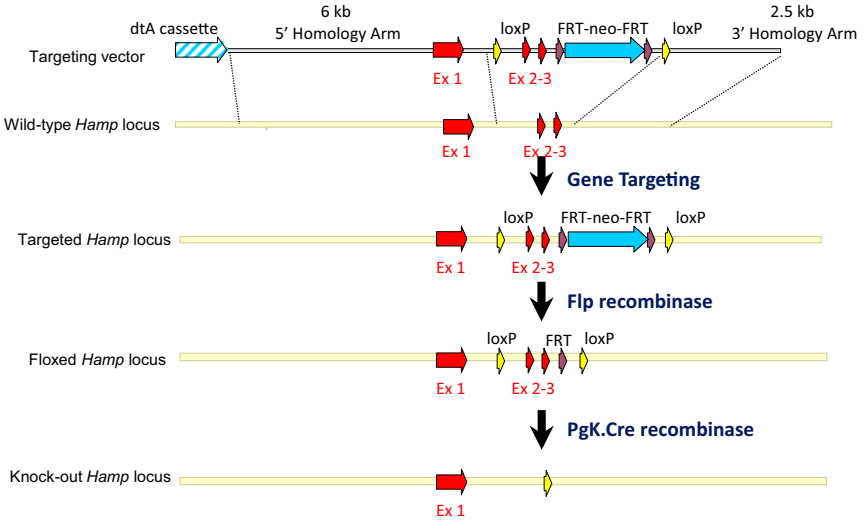


Fig. S5. Strategy for generation of *Hamp*$^{-/-}$ mice. A targeting vector was designed to introduce a floxed *Hamp* allele into mouse ES cells, with exons 2 and 3, which encode the majority of the peptide, flanked by LoxP sites. Further breeding with a C57BL/6 Flp recombinase deleter mouse allowed removal of the Neo cassette. Heterozygous ubiquitous *Hamp* knockouts (*Hamp*$^{+/-}$) were generated by crossing floxed Hamp animals with Pgk1-Cre transgenic mice.

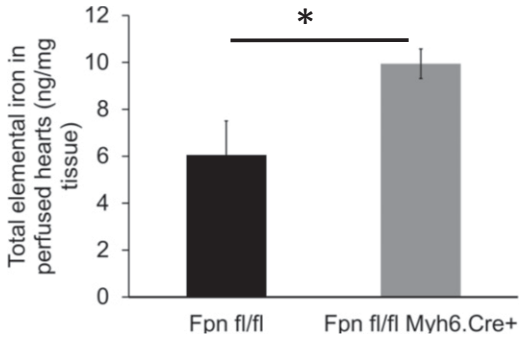


Fig. S6. Quantitation of total elemental iron in 6-mo-old *Fpn fl/fl.Myh6.Cre*$^{+}$ mice and *Fpn fl/fl* controls using perfused hearts. ($n = 3$ per group). *$P < 0.05$.

Table S1. Unchanged parameters of cardiac function in *Fpn fl/fl Myh6.Cre*$^{+}$ mice and *Fpn fl/fl* controls at age 3 and 6 mo

	3 mo		6 mo	
Parameter	*Fpn fl/fl*	*Fpn fl/fl Myh6.Cre*$^{+}$	*Fpn fl/fl*	*Fpn fl/fl Myh6.Cre*$^{+}$
Average mass, mg	65.4 ± 5.4	65.5 ± 5.2	68.1 ± 5.0	72.2 ± 4.4
RVED lumen, μL	29.2 ± 2.2	31.1 ± 2.6	29.3 ± 3.5	32.2 ± 2.1
RVES lumen, μL	8.72 ± 1.12	9.78 ± 0.98	7.76 ± 1.07	8.84 ± 1.04
RVEF, %	69.5 ± 2.4	68.6 ± 2.2	79.3 ± 1.9	76.8 ± 2.8
Stroke volume, μL	20.2 ± 1.3	21.2 ± 1.8	23.1 ± 2.4	24.6 ± 6.5
Cardiac output, mL/min	9.86 ± 0.69	10.62 ± 0.91	11.60 ± 1.35	12.73 ± 1.10
Heart rate, beats/min	486.1 ± 12.5	500.1 ± 15.3	502.1 ± 19.6	498.4 ± 69.3
Heart/body weight ratio × 1,000	2.85 ± 0.14	2.78 ± 0.17	2.69 ± 0.18	2.98 ± 0.14

Values are shown as mean ± SEM. $n = 12$ at age 3 mo and $n = 6$ at age 6 mo.

Table S2. Parameters of cardiac function in *Myh6.Cre*$^{+}$ mice and Myh6.Cre^{-} littermate controls at age 3 and 6 mo

	3 mo		6 mo	
Parameter	Myh6.Cre^{-}	*Myh6.Cre*$^{+}$	Myh6.Cre^{-}	*Myh6.Cre*$^{+}$
Average mass, mg	70.79 ± 8.36	66.43 ± 6.48	73.24 ± 7.38	72.89 ± 8.19
LVED, μL	31.75 ± 4.26	34.11 ± 5.27	38.63 ± 3.92	39.92 ± 3.75
LVES, μL	13.99 ± 2.78	13.59 ± 2.45	15.38 ± 2.56	15.92 ± 3.58
LVEF, %	67.15 ± 3.54	64.66 ± 1.91	65.5 ± 8.72	64.62 ± 7.59
RVED, μL	31.63 ± 5.90	31.62 ± 0.29	34.92 ± 4.78	35.29 ± 5.07
RVES, (μl)	8.19 ± 1.52	10.53 ± 0.11	9.16 ± 2.10	9.81 ± 3.15
RVEF, %	63.44 ± 1.59	70.52 ± 2.25	71.50 ± 6.39	72.98 ± 2.78
Stroke volume, μL	20.20 ± 3.17	22.10 ± 3.20	24.91 ± 3.89	25.60 ± 3.80
Cardiac output, mL/min	9.31 ± 1.53	10.35 ± 1.16	11.48 ± 1.67	12.80 ± 3.16
Heart rate, beats/min	484.86 ± 23.72	464.17 ± 29.30	460.70 ± 18.30	495.2 ± 32.21
Heart/body weight ratio × 1,000	2.68 ± 0.28	2.48 ± 0.27	2.75 ± 0.17	2.45 ± 0.26

Values are shown as mean ± SEM. $n = 6$ per group.