SUPPLEMENTAL DATA

Supplemental Table 1. Maternal Hematologic Parameters												
	Iron-deficient				**Iron-replete**				**Iron-loaded**			
	Non-Preg (n=6)	E12.5 (n=3)	E15.5 (n=3)	E18.5 (n=7)	Non-Preg (n=10)	E12.5 (n=6)	E15.5 (n=3)	E18.5 (n=3)	Non-Preg (n=5)	E12.5 (n=2)	E15.5 (n=3)	E18.5 (n=4)
RBC (10^6/µL)	8.1±0.3	6.9±0.2	7.3±0.2	6.6±0.6	8.6±0.5	7.4±0.5	7.8±0.3	7.4±0.3	7.5±0.5	7.2±0.1	6.4±0.3	6.3±0.1
Hb (g/dL)	12.8±0.6	9.9±0.5	9.7±0.4	8.5±0.7	12.6±0.6	10.9±0.9	11.2±0.6	10.5±0.7	12.0±0.9	10.9±0.1	10.7±0.2	10.2±0.3
HCT (%)	35.3±1.7	28.1±1.2	29.1±0.9	26.6±1.7	45.0±6.8	36.3±4.6	40.5±5.7	38.6±3.6	34.9±3.6	31.2±0.0	29.5±1.1	31.8±3.1
MCV (fL)	43.5±1.1	40.6±1.0	40.0±0.3	40.2±1.0	47.6±5.5	49.2±4.3	52.1±7.4	52.0±3.7	45.4±2.6	43.6±0.7	46.3±0.9	50.2±4.5
MCH (pg)	15.5±0.8	14.3±0.6	13.3±0.2	12.8±0.1	14.7± 0.7	14.9±1.1	14.4±0.7	14.3±1.3	16.0±0.5	15.2±0.1	16.9±0.5	16.2±0.6
Plt (10^3/µL)	924±279	1077±96	1433±17	1115±329	853±223	699±120	916±134	1013±209	942±83	1010±79	973±70	893±137
WBC	4.7±2.4	3.8±1.6	2.5±0.6	3.1±0.5	3.5±1.1	4.1±2.1	3.0±0.6	2.5±0.9	2.8±0.8	2.8±0.8	3.2±1.1	1.6±1.0

Supplemental Table 1: Maternal Hematological Parameters. Hematological parameters of dams with different iron and pregnancy states. Data are presented as mean ± SD. RBC, red blood cell count; Hb, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; Plt, platelet count; WBC, white blood cell count.

Supplemental Table 2. Embryo Hematological Parameters			
	Iron-deficient	**Iron-replete**	**Iron-overload**
	E18.5 (n=15)	E18.5 (n=7)	E18.5 (n=8)
RBC (10^6/µL)	1.4±0.2***	2.8±0.2	2.8±0.2
Hb (g/dL)	2.9±0.5***	10.4±0.5	9.7±0.9
HCT (%)	10.6±2.1***	34.6±2.7	32.6±3.3
MCV (fL)	72.9±8.2#	133.7±27.1	116.4±5.6
MCH (pg)	20.2±1.1***	36.7±2.3	34.5±1.4*
Plt (10^3/µL)	427±81	501±44	469±34
WBC	5.7±2.0	5.1±0.3	3.9±1.2

Supplemental Table 2: Embryo Hematological Parameters. Hematological parameters of E18.5 embryos from dams with different iron states. Data are presented as mean ± SD. RBC, red blood cell count; Hb, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; Plt, platelet count; WBC, white blood cell count. Statistical differences between groups were determined by one-way ANOVA for normally distributed values followed by Holm-Sidak method for multiple comparisons versus iron-replete control group (***P<0.001) or one-way ANOVA on ranks followed by Dunn's method for multiple comparisons versus iron-replete control group (#P<0.05).

Supplemental Table 3. Maternal Hematologic Parameters						
	Iron-deficient (short-term)			**Iron-loaded (10K ppm diet)**		
	Non-Preg (n=2)	E18.5 (n=5)	*P*-value	Non-Preg (n=4)	E18.5 (n=4)	*P*-value
RBC (10^6/μL)	9.0±0.7	7.3±0.2	0.250*	9.0±0.2	7.3±0.6	**0.029***
Hb (g/dL)	12.9±0.9	8.7±0.3	0.655	13.1±0.4	11.1±0.5	0.0585
HCT (%)	42.9±4.2	33.0±1.7	**0.005**	42.6±3.3	35.3±4.2	0.289
MCV (fL)	47.6±1.1	45.5±2.0	**0.009**	47.5±2.7	48.3±3.3	0.587
MCH (pg)	13.6±0.1	11.9±0.2	**0.002**	14.6±0.4	15.2±0.7	0.125
Plt (10^3/μL)	616±113	1500±379	0.275	841±118	869±250	0.889
WBC	1.9±0.5	3.0±1.2	0.894	3.4±1.1	3.1±0.5	0.369

Supplemental Table 3: Maternal hematological parameters in alternative mouse models of iron depletion and iron loading. Hematological parameters of short-term iron-depleted dams and 10 Kppm iron-loaded dams. Data are presented as mean ± SD. RBC, red blood cell count; Hb, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; Plt, platelet count; WBC, white blood cell count. Statistical analysis compares E18.5 pregnancy values for iron-deficient short-term versus long-term or iron-loading diet versus iron dextran using two-tailed Student's t-test for normally distributed values or Mann-Whitney rank sum test for non-normally distributed values (indicated by an asterisk following *P*-value).

Supplemental Table 4. Embryo Hematologic Parameters				
	Iron-deficient (short-term)	*P*-value	Iron-loaded (10Kppm diet)	*P*-value
	E18.5 (n=8)		E18.5 (n=14)	
RBC (10^6/μL)	1.6±0.4	0.118	2.7±0.2	0.594
Hb (g/dL)	3.1±0.8	0.397	9.3±0.6	0.272
HCT (%)	12.8±3.1	0.071	35.2±3.6	0.114
MCV (fL)	78.3±5.2	0.100*	133.7±27.1	**0.019**
MCH (pg)	19.0±1.4	0.050	33.9±2.3	0.535
Plt (10^3/μL)	324±108	**0.022**	411±42	**0.005**
WBC	7.4±1.5	0.142	13.8±10.5	**0.003***

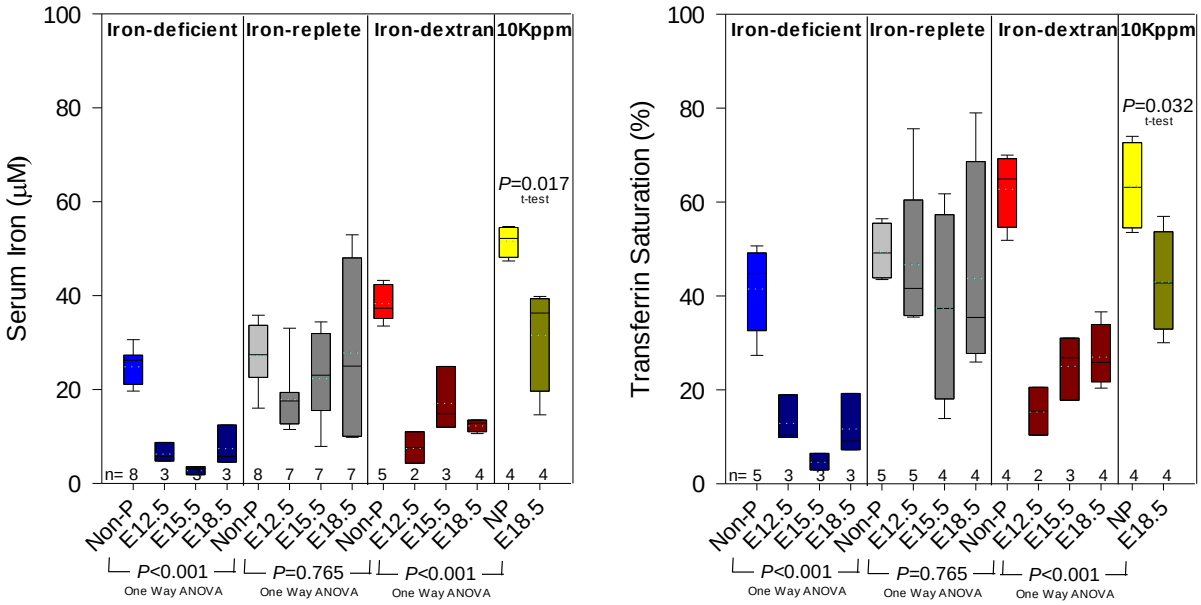
Supplemental Table 4: Embryo hematological parameters in alternative models of iron depletion or iron loading. Hematological parameters of E18.5 embryos from short-term iron-depleted dams and 10 Kppm iron-loaded dams. Data are presented as mean ± SD. RBC, red blood cell count; Hb, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; Plt, platelet count; WBC, white blood cell count. Statistical analysis compares values for iron-deficient short-term versus long-term or iron-loading diet versus iron dextran using two-tailed Student's t-test for normally distributed values or Mann-Whitney rank sum test for non-normally distributed values (indicated by an asterisk following *P*-value).

Supplemental Table 5. qPCR Primers		
Species	Gene	Sequence
Mouse	*Hprt*	Fwd: 5’-CTG GTT AAG CAG TAC AGC CCC AA-3’
		Rev: 5’-CAG GAG GTC CTT TTC ACC AGC-3’
	Hamp	Fwd: 5’-TTG CGA TAC CAA TGC AGA AGA- 3’
		Rev: 5’-GAT GTG GCT CTA GGC TAT GTT-3’
	Tfr1	Fwd: 5’-TCA TGA GGG AAA TCA ATG ATC-3’
		Rev: 5’-GCC CCA GAA GAT ATG TCG GAA-3’
	Fpn1	Fwd: 5’-ATG GGA ACT GTG GCC TTC AC-3’
		Rev: 5’-TCC AGG CAT GAA TAC GGA GA-3’
	Fpn1A	Fwd: 5’-AAA GAA GAC CCC GTG ACA GC-3’
		Rev: 5’-TCC CCG TGT TTG TTC TGA TG-5’
	Fpn1B	Fwd: 5’-GCC GGT TGG AGT TTC AAT GT-3’
		Rev: 5’-TCC CCG TGT TTG TTC TGA TG-3’
	Zc3h12A/Regnase-1	Fwd: 5’-CGA GAG GCA GGA GTG GAA AC-3’
		Rev: 5’-CTT ACG AAG GAA GTT GTC CAG GCT AG-3’
	Dmt1	Fwd: 5’-CGC TCG GTA AGC ATC TCG AA-3’
		Rev: 5’-TGT TGC CAC CGC TGG TAT CT-3’
	Hfe	Fwd: 5’-CCA CCG CGT TCA CAT TCT CT-3’
		Rev: 5’-CTG GTC ATC CAC ATA GCC CC-3’
	Hephl1	Fwd: 5’-GCA TCG GAA GTG AAG TGG AC-3’
		Rev: 5’-GGT TTG AAA TGT CCC AGG AA-3’
	Flvcr1/Mfsd7b	Fwd: 5’-TTT CCT TTG TGC CTG GAT GT-3’
		Rev: 5’-GCC CGG TGT TTA TAT TGT GC-3’
	Il6	Fwd: 5’-CTC TGC AAG AGA CTT CCA TCC AGT-3’
		Rev: 5’-CGT GGT TGT CAC CAG CAT CA-3’
	Saa1	Fwd: 5’-AGT CTG GGC TGC TGA GAA AA-3’
		Rev: 5’-ATG TCT GTT GGC TTC CTG GT-3’
Human	*FPN1 variant I*	Fwd: 5’-TTT TGC CCA AGG CTG TTG TG-3’
		Rev: 5’-TCA TGA CAC TAG GCG ACC C-3’
	FPN1 variant IIB	Fwd: 5’-ATG TAG GAT CCA CTA CCA GGG-3’
		Rev: 5’-TCA TGA CAC TAG GCG ACC C-3’
	ZC3H12A/REG1	Fwd: 5’ – CGA CAC ATA CCG TGA CCT CC – 3’
		Fwd: 5’ – TCA GGG GGC ATA AAC TTG TCA – 3’

Supplemental Table 5: qPCR Primers

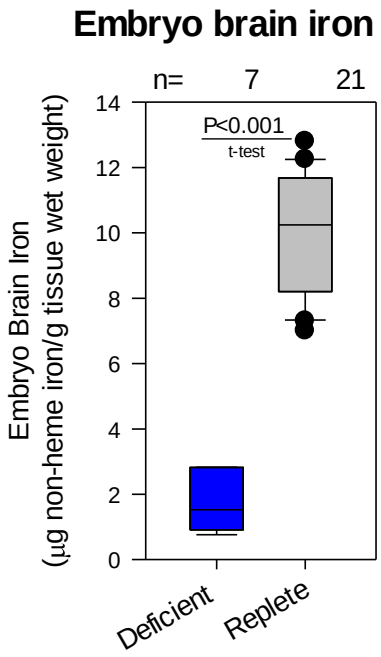
Supplemental Table 6. Antibodies	
Target protein	**Primary antibody**
Mouse FPN	Rat monoclonal antibody IC7 (western and immunofluorescence), Amgen
Human FPN	Human monoclonal antibodies 38C8 (western), 38G6 (immunofluorescence), Amgen
Hepcidin ELISA	Mouse monoclonal antibodies Ab2B10 and Ab2H4-HRP, Amgen
Mouse and human TFR1	Monoclonal antibody H68.4, ThermoFisher Scientific
Mouse and human ferritin heavy chain	Rabbit monoclonal antibody D1D4, Cell Signaling
Mouse ferritin light chain	Goat polyclonal antibody NBP1-06986, Novus
Mouse and human β-actin	Monoclonal antibody-peroxidase AC-15, SIGMA
Mouse GAPDH	Rabbit monoclonal antibody 14C10, Cell Signaling
Mouse and human electron transport chain	Total OXPHOS Rodent WB Antibody Cocktail ab110413, Abcam
Secondary antibodies:	
Anti-mouse IgG HRP (Cell Signaling #7076)	
Anti-rabbit IgG HRP (Cell Signaling #7074)	
Anti-rat IgG HRP (AbCam #ab102213)	
Pierce high sensitivity neutravidin-HRP (ThermoFisher #31030)	
Goat-anti-mouse-AF488 (ThermoFisher #A32723)	
Goat-anti-mouse-AF594 (ThermoFisher #A11032),	
Streptavidin-AF555 (ThermoFisher #S21381)	
Donkey-anti-rat-AF488 (ThermoFisher #A21208)	

Supplemental Table 6: Antibodies. 38G6 and 38C8 were biotinylated using EZ-Link Sulfo-NHS-LC-LC Biotin (Thermo 21338). FPN antibody validation is provided in Supplemental Figure 10.



Supplemental Figure 1: Maternal transferrin saturation. Females were fed standard chow (185 ppm iron) or low iron diet (4ppm iron) or high iron diet (10,000 ppm iron) 2 weeks prior to and throughout the pregnancy, or were injected with 20 mg iron dextran at time of mating. Pregnant females were analyzed at E12.5, 15.5 and 18.5. Non-pregnant (Non-P) females were subjected to an equivalent iron treatment. Serum iron of the same samples is shown for comparison.

A.



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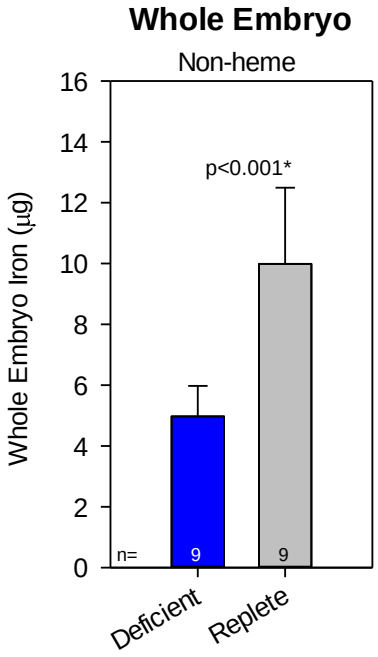
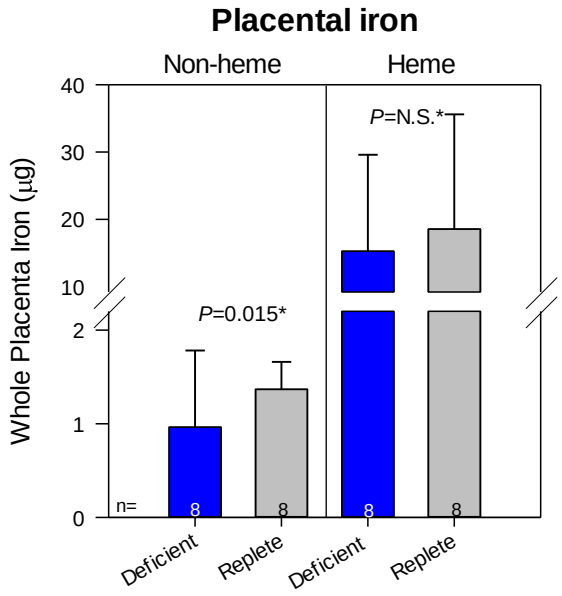
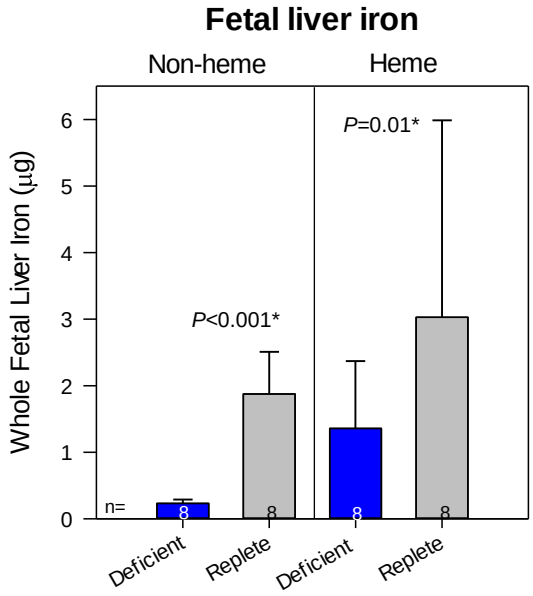


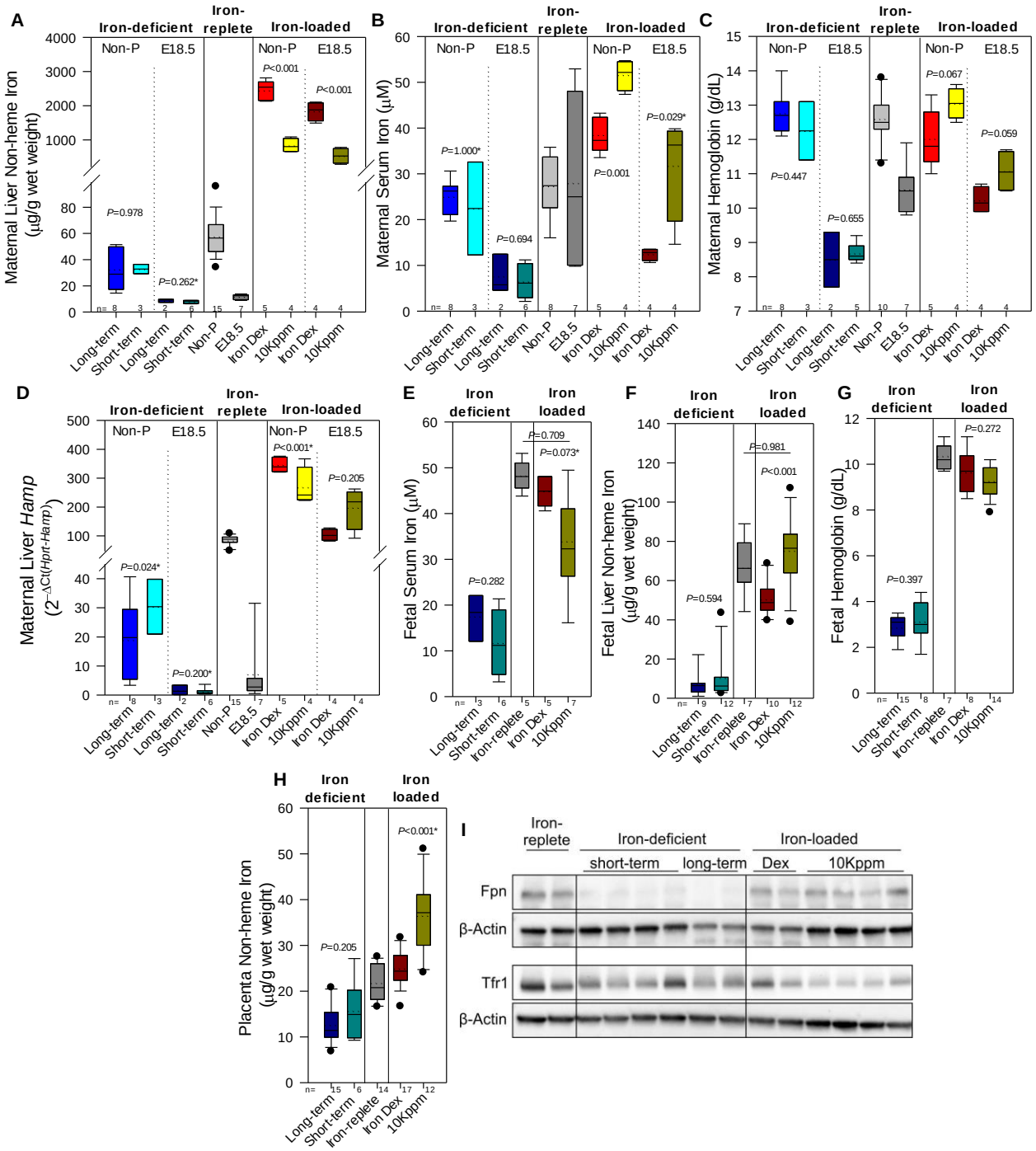
C.



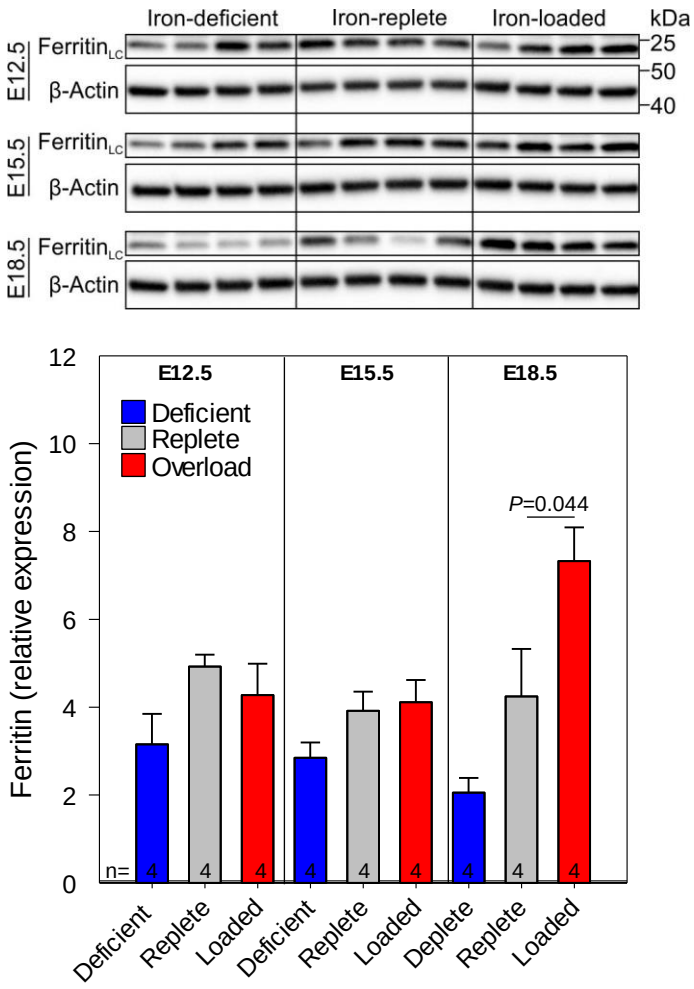
D.



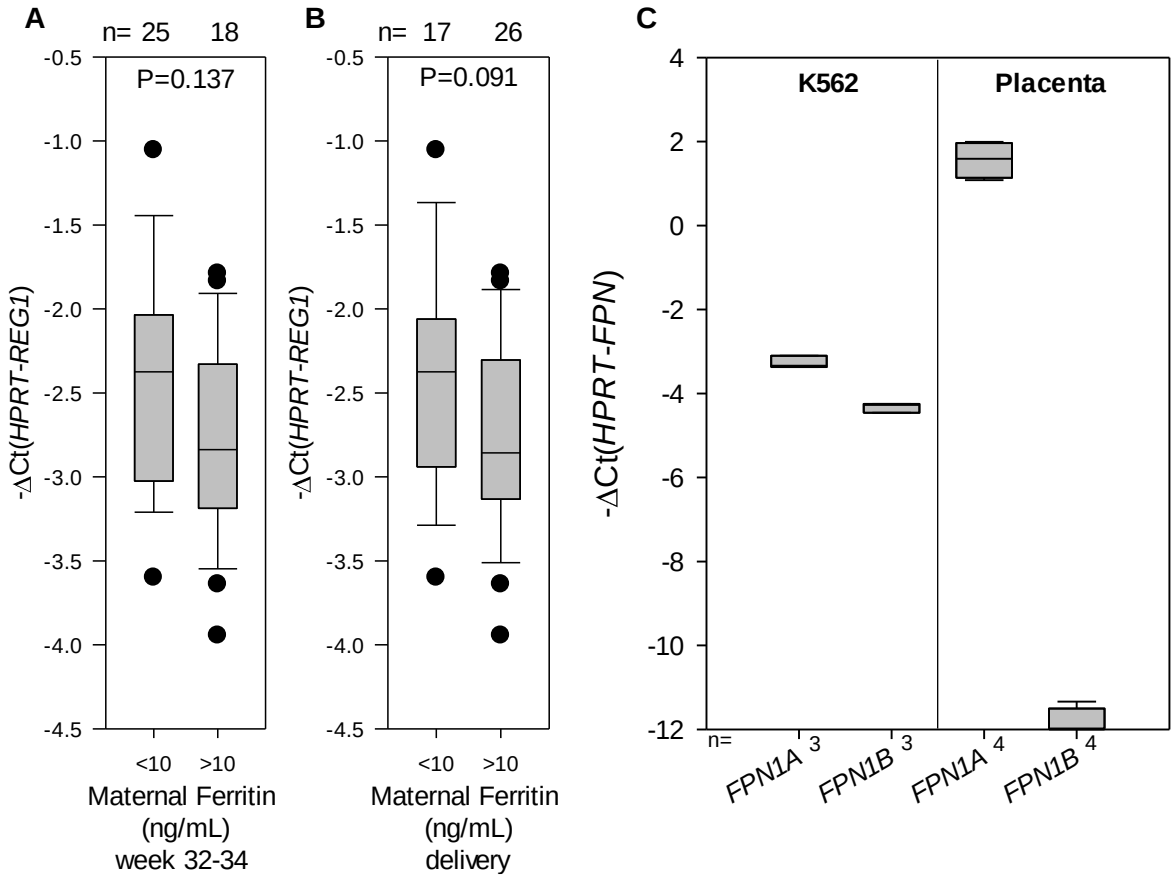
Supplemental Figure 2: The effect of maternal iron deficiency on placental and fetal iron at E18.5 compared to iron-replete pregnancy. (A) Embryo brain non-heme iron concentration. **(B)** Whole embryo non-heme iron. **(C)** Non-heme and heme iron content of whole placentas. **(D)** Non-heme and heme iron content of whole fetal livers. Statistical analyses were performed using two-tailed Student's t-test for normally distributed values or Mann-Whitney rank sum test (*) for non-normally distributed values.


A
Iron-deficient
Iron-replete
Iron-loaded
Non-P
E18.5
Maternal Liver Non-heme Iron (µg/g wet weight)
P=0.978
P=0.262*
P<0.001
P<0.001
B
Maternal Serum Iron (µM)
P=1.000*
P=0.694
P=0.001
P=0.029*
C
Maternal Hemoglobin (g/dL)
P=0.447
P=0.655
P=0.067
P=0.059
Long-term
Short-term
Non-P
E18.5
Iron Dex
10Kppm
D
Maternal Liver Hamp (2^-ΔCt(Hprt-Hamp))
P=0.024*
P=0.200*
P<0.001*
P=0.205
E
Iron deficient
Iron loaded
Fetal Serum Iron (µM)
P=0.282
P=0.709
P=0.073*
F
Fetal Liver Non-heme Iron (µg/g wet weight)
P=0.594
P=0.981
P<0.001
G
Fetal Hemoglobin (g/dL)
P=0.397
P=0.272
Iron-replete
H
Placenta Non-heme Iron (µg/g wet weight)
P=0.205
P<0.001*
I
Iron-replete
Iron-deficient
Iron-loaded
short-term
long-term
Dex
10Kppm
Fpn
β-Actin
Tfr1
β-Actin

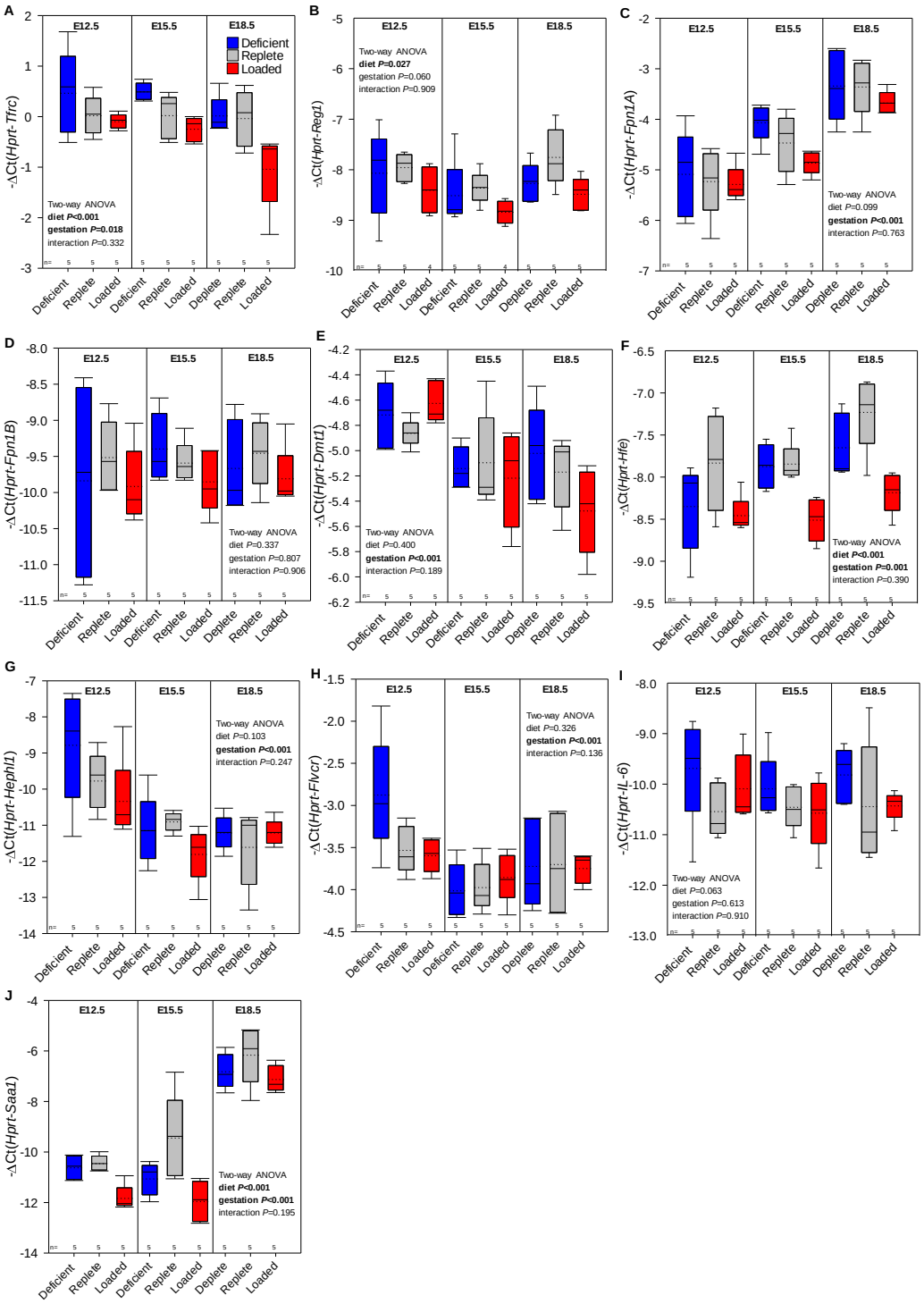
Supplemental Figure 3: Comparison of different models of iron deficiency and iron loading in pregnant mice. *Iron depletion*: long-term versus short-term iron depletion was achieved by starting females on low iron diet 2 weeks prior to pregnancy versus at mating. The diet was continued through the pregnancy. *Iron loading*: females received a single intraperitoneal injection of 20mg iron dextran at mating, or 10,000ppm iron diet 2 weeks prior to mating and throughout gestation (10Kppm). Non-pregnant animals were subjected to the same regimens and analyzed at the same time point. (**A-D**) Maternal parameters: liver iron (**A**), serum iron (**B**), hemoglobin (**C**), and hepcidin mRNA (**D**). (**E-G**) Fetal parameters: serum iron (**E**), liver iron (**F**) and hemoglobin (**G**). (**H-I**) Placental parameters: tissue non-heme iron (**H**), and Fpn and TfR1 protein levels (**I**). Statistical analyses were performed using two-tailed Student's t-test for normally distributed values or Mann-Whitney rank sum test for non-normally distributed values (indicated by an asterisk). Data for iron-deficient long-term, iron-replete and iron dextran are replicated from Figure 1 but are provided for ease of understanding.



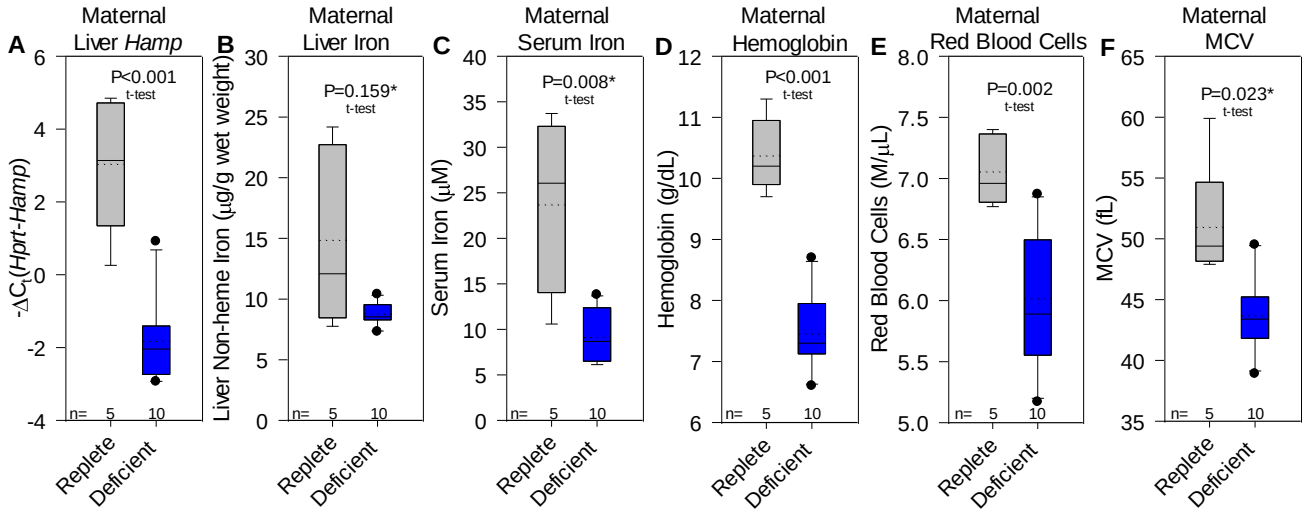
Supplemental Figure 4: Placental ferritin increases with elevated maternal iron status. Mouse placentas from Figure 1 were analyzed by western blotting to determine protein concentration of ferritin. β-Actin was used for normalization (the image is the same as in Figure 2C, but included here for convenience). Bar graph shows means +/- SE. Statistical differences between groups were determined by one-way ANOVA for normally distributed values followed by Holm-Sidak method for multiple comparisons versus iron-replete control group.



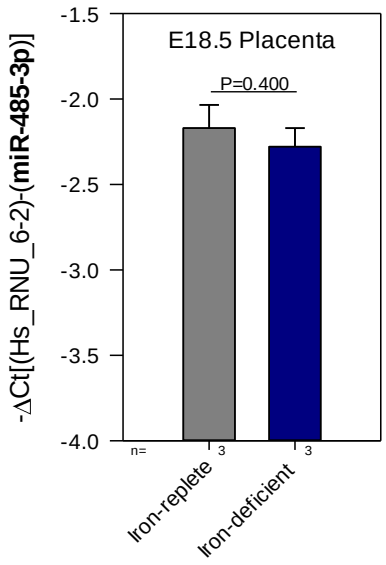
Supplemental Figure 5: *REG1* mRNA expression and *FPN* mRNA isoforms in human placenta. (**A-B**) Placentas from uncomplicated human pregnancies were analyzed by qPCR for *REG1* mRNA expression according to maternal ferritin at week 32-34 or at delivery. (**C**) FPN splicing variant "*FPN1A"* (also known as *FPN variant I* (1)) contains 5'IRE, and "*FPN1B"* (also known as FPN *variant IIB* (1, 2)) does not contain 5'IRE. The isoforms were measured by qPCR in human placental samples. *HPRT* was used as a housekeeping gene. K562 cells were used as a control sample in which a comparable amount of *FPN1A* and *1B* was reported (1).


A
-ΔCt(Hprt-Tfrc)
E12.5
E15.5
E18.5
Deficient
Replete
Loaded
Two-way ANOVA
diet P<0.001
gestation P=0.018
interaction P=0.332
B
-ΔCt(Hprt-Reg1)
Two-way ANOVA
diet P=0.027
gestation P=0.060
interaction P=0.909
C
-ΔCt(Hprt-Fpn1A)
Two-way ANOVA
diet P=0.099
gestation P<0.001
interaction P=0.763
D
-ΔCt(Hprt-Fpn1B)
Two-way ANOVA
diet P=0.337
gestation P=0.807
interaction P=0.906
E
-ΔCt(Hprt-Dmt1)
Two-way ANOVA
diet P=0.400
gestation P<0.001
interaction P=0.189
F
-ΔCt(Hprt-Hfe)
Two-way ANOVA
diet P<0.001
gestation P=0.001
interaction P=0.390
G
-ΔCt(Hprt-Hephl1)
Two-way ANOVA
diet P=0.103
gestation P<0.001
interaction P=0.247
H
-ΔCt(Hprt-Flvcr)
Two-way ANOVA
diet P=0.326
gestation P<0.001
interaction P=0.136
I
-ΔCt(Hprt-IL-6)
Two-way ANOVA
diet P=0.063
gestation P=0.613
interaction P=0.910
J
-ΔCt(Hprt-Saa1)
Two-way ANOVA
diet P<0.001
gestation P<0.001
interaction P=0.195
Deficient
Replete
Loaded
Deplete

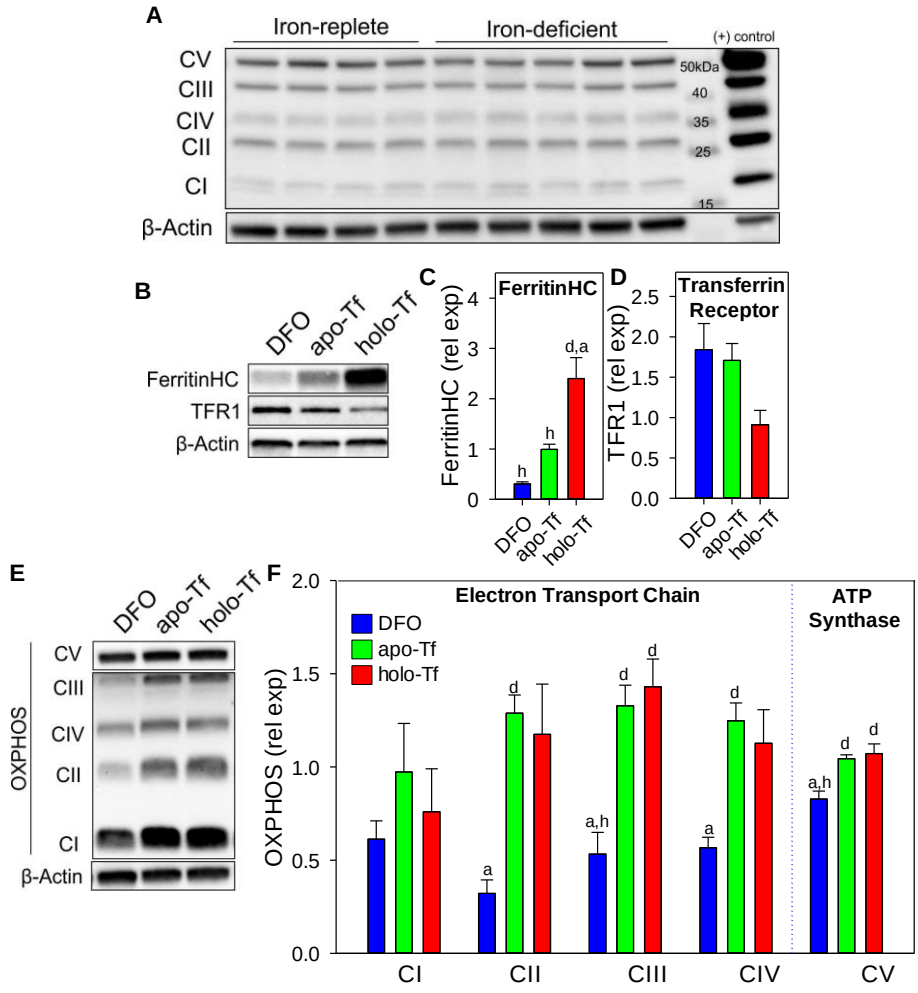
Supplemental Figure 6: Placental mRNA expression of *Tfrc* and *Fpn* in mouse pregnancies with varying maternal iron status. E12.5, 15.5 and 18.5 placentas from iron-deficient, iron-replete and iron-loaded mouse pregnancies were analyzed by qPCR to determine mRNA expression of *Tfrc* (**A**), its regulator regnase-1 (*Reg-1*) (**B**), *Fpn1* splicing variant *Fpn1A* which contains 5'IRE (**C**) and *Fpn1B* which does not contain 5'IRE (**D**), *Dmt1* (**E**), *Hfe* (**F**), *Hephl1* (**G**), *Flvcr1* (**H**), *Il-6* (**I**), *Saa1* (**J**). *Hprt* was used as a housekeeping gene. Statistical differences between groups was determined by two-way ANOVA, P values for diet, gestation and interactions are listed in the graphs along with n number. For Tfrc, diet affects expression levels ($P<0.001$) with significant differences at E18.5 between iron-deficient and iron-loaded ($P=0.005$) and between iron-replete and iron-loaded ($P=0.005$); however, gestational age does not ($P=0.332$) affect Tfrc expression. For Fpn1A, gestational age affects mRNA expression ($P<0.001$) whereas diet has no effect ($P=0.099$).



Supplemental Figure 7: Iron and hematological parameters for E18.5 *Hamp*$^{+/-}$ dams. *Hamp*$^{+/-}$ females were mated with *Hamp*$^{+/-}$ males. Females were placed on iron-replete or iron-deficient diet 1 week prior to mating. To confirm sufficient iron depletion, maternal liver hepcidin mRNA (**A**), liver iron (**B**), serum iron (**C**), hemoglobin (**D**), RBC count (**E**), and MCV (**F**) were measured. Statistical differences between groups were determined by two-tailed Student's t-test for normally distributed values or Mann-Whitney rank sum test for non-normally distributed values (indicated by an * following *P*-value).

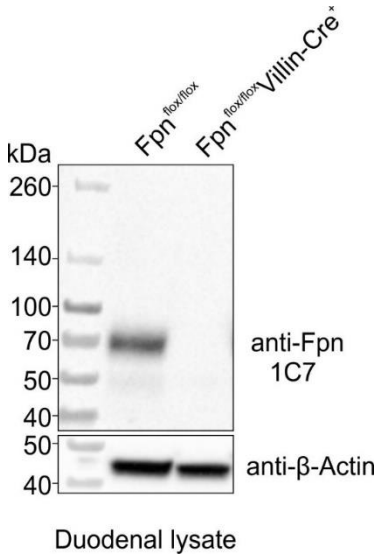


Supplemental Figure 8: miR-485-3p is not responsible for changes in placenta FPN during maternal iron deficiency. Expression levels of miR-485-3p in E18.5 placentas from iron-replete and iron-deficient pregnancies. Data are presented as mean±SE. Statistical differences were determine by Mann-Whitney rank sum test.

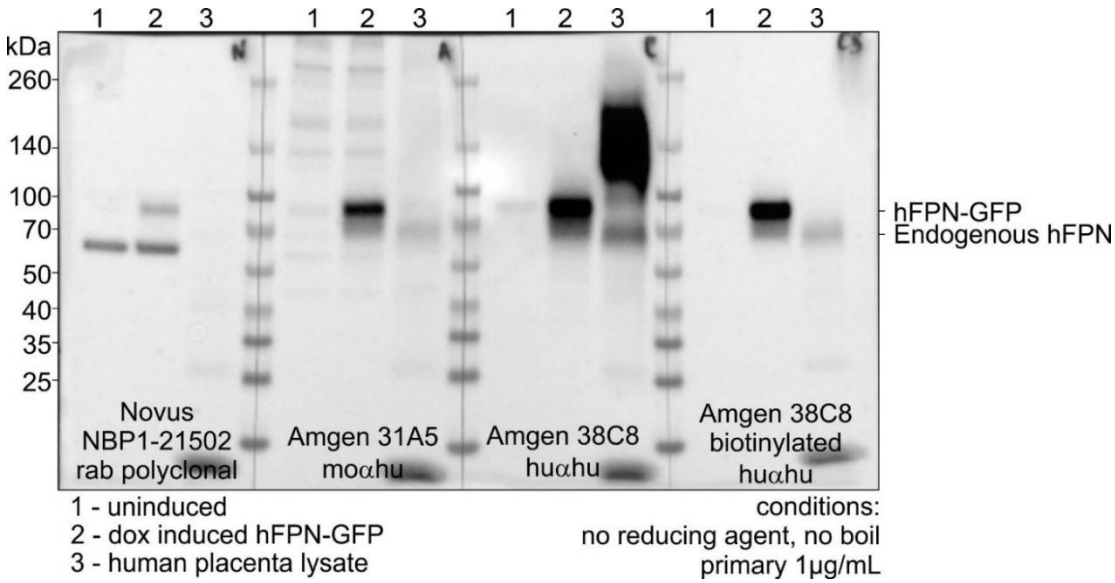


Supplemental Figure 9: Integrity of ETC complexes I-V in E18.5 mouse placentas and BeWo cells under varying iron conditions. (**A**) Placenta lysates from E18.5 iron-replete and iron-deficient pregnancies in Figure 1 were analyzed by western blot for expression of proteins involved in oxidative phosphorylation (OXPHOS): electron transport chain (ETC) complexes I (NDUFB8), II (SDHB), III (UQCRC2), IV (MTCO1) and V (ATP5A). (**B-F**) BeWo cells were treated with DFO, apo-Tf or holo-Tf (100μM each) for 24hr. (**B**) Western blot of FerritinHC and TFR1. β-Actin was used as a loading control. (**C, D**) Quantitation of ferritinHC and TFR1, relative to an untreated control within each experiment (n=3). (**E**) Western blot of ETC complexes I-V. (**F**) Quantification of ETC complexes normalized to β-Actin, and presented relative to the untreated control for each experiment (n=5). Data are presented as mean±SE. For statistical analysis, the two-tailed 1-sample Student t-test (normally distributed data) was used with 1 as the comparison. Statistical significance, $P<0.05$.

A.



B.



Supplemental Figure 10: FPN antibody validation. (A) Anti-mouse FPN antibody was validated using duodenal epithelium from intestinal-specific FPN knockout mice and their flox littermates. **(B)** Anti-human FPN antibody used in our study is Amgen 38C8 (far right). It was validated using HEK293 cells induced or not to express human FPN-GFP (lanes 1 and 2), as well as a human placental lysate expressing endogenous FPN (lane 3). Comparison to additional anti-FPN antibodies is provided.

Supplemental References

1. Cianetti L, Segnalini P, Calzolari A, Morsilli O, Felicetti F, Ramoni C, Gabbianelli M, Testa U, and Sposi NM. Expression of alternative transcripts of ferroportin-1 during human erythroid differentiation. *Haematologica.* 2005;90(12):1595-606.
2. Zhang DL, Hughes RM, Ollivierre-Wilson H, Ghosh MC, and Rouault TA. A ferroportin transcript that lacks an iron-responsive element enables duodenal and erythroid precursor cells to evade translational repression. *Cell metabolism.* 2009;9(5):461-73.