

Title

Comparison Of Non-Heme Liver Iron And Iron Metabolism Protein Levels In Superoxide Dismutase 1 Knock-Out Mice Versus Superoxide Dismutase 1 Wild-Type Mice

Abstract

Iron is an important micronutrient that is necessary for multiple cellular functions. However, iron levels must be tightly regulated in order to prevent iron-deficiency and iron-toxicity. There are many proteins involved in iron metabolism. This study focuses on iron-regulatory proteins 1 and 2 (IRP1/2), ferritin, and transferrin receptor protein (TfR). IRPs are key iron sensors that bind to iron response elements (IREs) located on mRNA when the IRP Fe-S clusters are removed, regulating translation or stability of mRNA. Ferritin is the storage form of iron. Ferritin levels must increase in iron-sufficient or overloaded conditions to store the excess iron in a safe form. TfR takes up iron and moves it into a free iron pool for utilization by the body. TfR levels must increase in iron-deficient conditions to mobilize iron to necessary tissues. When IRP binds to a ferritin IRE, ferritin mRNA translation is inhibited causing ferritin protein levels to decrease. However, when IRP binds to TfR IRE, TfR mRNA is stabilized causing TfR protein levels to increase. Reactive oxygen species (ROS) such as the superoxide anion have been known to destabilize the Fe-S cluster in IRP, possibly leading to IRP degradation in high concentrations of ROS. Superoxide dismutase 1 (SOD1) reacts with the superoxide anion to yield safer complexes (hydrogen peroxide and water). I hypothesized that SOD1 knock-out (KO) mice would have increased non-heme liver iron and ferritin levels as well as decreased TfR and IRP1/2 levels due to the increased levels of superoxide anions removing the Fe-S clusters, decreasing IRP/IRE TfR and ferritin mRNA binding. My data supported my hypothesis. I used a non-heme liver assay, hematocrit measurements, and western blots to measure the levels of non-heme liver iron and protein levels in KO and WT male mice (C57BL/6J background) at ages 8 weeks, 2.5-3 months, 4 months, 6 months, and 12 months.

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COVER SHEET

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Comparison Of Non-Heme Liver Iron And Iron Metabolism
Protein Levels In Superoxide Dismutase 1 Knock-Out Mice Versus
Superoxide Dismutase 1 Wild-Type Mice

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ABSTRACT

Iron is an important micronutrient that is necessary for multiple cellular functions. However, iron levels must be tightly regulated in order to prevent iron-deficiency and iron-toxicity. There are many proteins involved in iron metabolism. This study focuses on iron-regulatory proteins 1 and 2 (IRP1/2), ferritin, and transferrin receptor protein (TfR). IRPs are key iron sensors that bind to iron response elements (IREs) located on mRNA when the IRP Fe-S clusters are removed, regulating translation or stability of mRNA. Ferritin is the storage form of iron. Ferritin levels must increase in iron-sufficient or overloaded conditions to store the excess iron in a safe form. TfR takes up iron and moves it into a free iron pool for utilization by the body. TfR levels must increase in iron-deficient conditions to mobilize iron to necessary tissues. When IRP binds to a ferritin IRE, ferritin mRNA translation is inhibited causing ferritin protein levels to decrease. However, when IRP binds to TfR IRE, TfR mRNA is stabilized causing TfR protein levels to increase. Reactive oxygen species (ROS) such as the superoxide anion have been known to destabilize the Fe-S cluster in IRP, possibly leading to IRP degradation in high concentrations of ROS. Superoxide dismutase 1 (SOD1) reacts with the superoxide anion to yield safer complexes (hydrogen peroxide and water). I hypothesized that SOD1 knock-out (KO) mice would have increased non-heme liver iron and ferritin levels as well as decreased TfR and IRP1/2 levels due to the increased levels of superoxide anions removing the Fe-S clusters, decreasing IRP/IRE TfR and ferritin mRNA binding. My data supported my hypothesis. I used a non-heme liver assay, hematocrit measurements, and western blots to measure the levels of non-heme liver iron and protein levels in KO and WT male mice (C57BL/6J background) at ages 8 weeks, 2.5-3 months, 4 months, 6 months, and 12 months.

INTRODUCTION

Iron is a required micronutrient obtained through the diet. It is a necessary part of proteins that perform redox or non-redox functions in important cellular systems such as respiration and cell division. To achieve optimal health, iron concentrations and distribution must be tightly managed to ensure cellular requirements are met while levels do not become toxic.¹⁰

When cells become iron-deficient, multiple symptoms may arise such as anemia; impaired muscle, immune, and cognitive function; low birth weight babies; and preterm deliveries.¹⁰ Tissue-specific responses include a sudden decrease in liver iron stores, a

depletion of the functional pool of iron, and an increase in transferrin receptor (TfR) expression in tissues in order to increase iron availability within the body.⁵

On the other hand, when cells become iron-overloaded, neurodegenerative diseases may occur. Iron overload may lead to iron toxicity, the damaging of cellular structures caused by iron-induced formation of reactive oxygen species (ROS) such as the superoxide anion.¹⁰

Specific processes that control iron homeostasis are important to understand in hopes of identifying iron-related diseases and advancing clinical treatments for iron metabolism disorders as well as understanding how cells and tissues react to deficiency or overload.¹⁰

Iron metabolism involves numerous proteins. This experiment focused on four specific iron metabolism proteins: iron-regulatory proteins 1 and 2 (IRP1/2), ferritin, and TfR.

IRPs are key iron sensors of the sensory and regulatory systems necessary for iron homeostasis maintenance. They recognize and bind to iron-responsive elements (IREs) in mRNA, regulating the translation or stability of the mRNA.¹⁰

IRP1 is found within the body in two forms: with [4Fe-4S] Fe-S cluster and without Fe-s cluster.² When IRP1 contains the Fe-S cluster, it cannot bind to mRNA and vice versa (Figure 1). The presence of Fe-S cluster perturbants (i.e. superoxide anion) promotes the loss of the Fe-S cluster from IRP1, thus possibly increasing RNA binding activity between IRP1 and IREs on two types of mRNA: ferritin and TfR.⁵ When IRP1 binds to the ferritin IRE, IRP1 blocks the ability of the initiation factor complex to recruit the necessary subunit with associated factors to the mRNA, inhibiting ferritin protein

synthesis. However, when IRP1 binds to the TfR IRE, IRP1 protects the mRNA from degradation by increasing the mRNA's stability, enhancing TfR protein synthesis.¹⁰

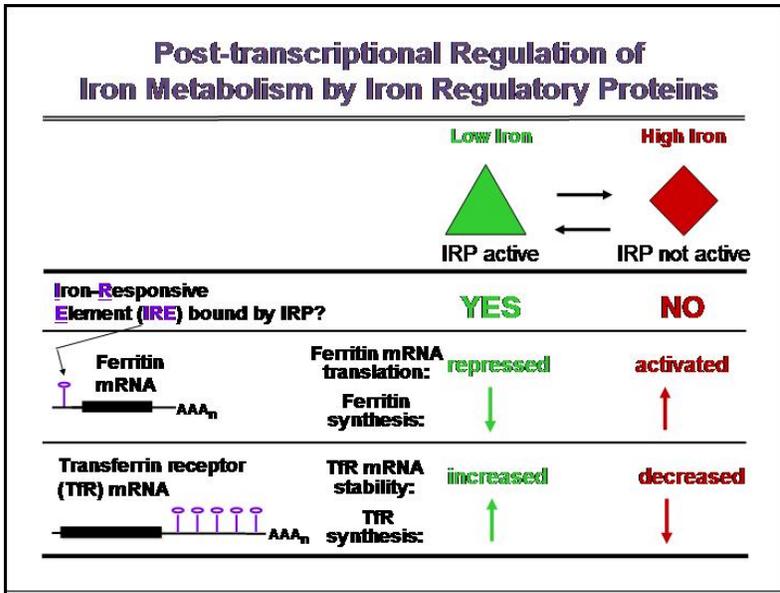


Figure 1: Diagram of post-transcriptional regulation of iron metabolism by iron-regulatory proteins. When Fe-S cluster is attached to IRP, IRP cannot bind to IRE resulting in ferritin mRNA translation to be activated and ferritin synthesis to increase. When Fe-S cluster is not attached to IRP, IRP can bind to IRE resulting in TfR mRNA stability to increase and TfR synthesis to increase.⁴

Consequently, when cells are iron-deficient, IRP1 binds to IREs with high affinity causing an increase in TfR protein; when cells are iron-sufficient, IRP1 loses its high affinity to bind IREs causing an increase in ferritin protein.¹⁰ However, it has been observed that high levels of ROS can inactivate IRP1 possibly through oxidative damage or degradation to prevent over-accumulation of RNA binding activity, resulting in decreased IRP1 levels.⁵

IRP2 does not contain the Fe-S cluster but also binds ferritin or TfR mRNA, similarly affecting the translation and stability of the mRNA as IRP1.⁹

Ferritin is an iron-binding protein that stores iron in the liver when iron is in excess. If the body becomes iron-depleted, iron is released from ferritin and mobilized throughout the body.¹⁰

TfR is a receptor found on the outside of a cell that takes up iron from extracellular space and moves it to a cytosolic free iron pool where it can be utilized by

the body when iron is needed. The iron that is not utilized is stored as ferritin or exported by another iron metabolism protein, ferroportin. Transferrin-mediated iron uptake is the primary way most cells take up non-heme iron.¹⁰

Superoxide dismutase enzyme 1 (SOD1, CuZnSOD) reacts with ROS like superoxide anion to produce peroxide and water. SOD1 deficiency promotes oxidative damage and increased sensitivity to oxidative stress. SOD1 transgenic and mutant mice have been commonly used to study the role of ROS in a variety of experiments.⁶

I hypothesized that increased levels of superoxide anions in SOD1 knock-out (KO) mice due to the lack of the SOD1 gene would increase the removal of the Fe-S cluster in IRP1, increasing IRP1 instability/degradation and decreasing IRP1/IRE mRNA binding. I further hypothesized that this change in binding would cause an increase in non-heme liver iron and ferritin levels due to an increased need to store the excess iron, a decrease in TfR levels due to decreased TfR mRNA stability and IRP1 levels due to high levels of IRP1 without Fe-S cluster increasing IRP1 degradation, and no change in IRP2 levels. I studied SOD1 KO and wild-type (WT) male mice (C57BL/6J background) at ages 8 weeks, 2.5-3 months, 4 months, 6 months, and 12 months to observe the effects that increased levels of superoxide anions have on non-heme liver iron content, blood composition, and iron metabolism protein levels as measured by a non-heme liver assay, hematocrit measurements, and western blots, respectively.

METHODS

Mouse Colonies

I used two different colonies of SOD1 KO and WT mice: a University of California- San Francisco (UCSF) colony⁶ and a St. Louis colony⁷. Both colonies were

initially compared for genotype ratios, but only the St. Louis colony was used for all further experimentation and analysis.

DNA Digestion Protocol^{6,8}

To digest the mouse ear sample for DNA extraction, a DNA digestion protocol was followed. The DNA extraction buffer was composed of 50 mM Tris pH 8.0, 20 mM NaCl, 1 mM EDTA, and 1% SDS. The Proteinase K stock solution had a concentration of 20mg/mL in DepC H₂O. To make the digestion solution, 20 parts DNA extraction buffer was added to 1 part Proteinase K stock solution. 20 µL of the digestion solution was added to each labeled microcentrifuge tube containing each mouse ear sample. Each tube was incubated in a H₂O bath for 15 minutes at 55-60°C then vortexed briefly; this step was repeated once more. Next, 180 µL of MQ H₂O was added to each tube and vortexed briefly. The tubes were finally boil in a H₂O bath for 5 minutes then cooled for 1-2 minutes and vortexed briefly.

Polymerase Chain Reaction Protocol⁶

To increase the amount of DNA per mouse ear sample, a polymerase chain reaction (PCR) protocol was followed. Solution 1 was composed of 4.4 µL MQ H₂O, 2 µL Buffer, 2.5 µL MgCl₂, and 2 µL each primer (328, 329, 330) per ear sample. Solution 2 was composed of 1.6 µL dNTP, 0.2 µL Taq (Promega), and 3.2 µL MQ H₂O per ear sample. 14.9 µL solution 1 and 0.5 µL digested DNA mouse ear sample was added to a 0.5 mL PCR tube. One drop of mineral oil was added to each tube as well as to each well in the thermocycler. The thermocycler was set to the appropriate program of heating and cooling and the tubes were placed into the wells before starting the thermocycler. Once the program was started, 5 µL solution 2 was added to each tube

during the first 5 minutes. Once the program was finished, the samples were stored at a constant 4°C.

Gel Electrophoresis Protocol^{1,6}

To determine the genotypes of each mouse ear sample after the PCR, a gel electrophoresis protocol was followed. The 20x SB buffer was composed of 8 g. NaOH/800 mL H₂O at a pH of 8.0 by adding solid boric acid. The 1x SB buffer was composed of 50 mL 20x SB buffer/1000 mL final volume. The 1x SB agarose gel was composed of 0.72 g. agarose/60 mL 1x SB buffer. The 1x SB agarose gel mixture was microwaved for 1.5-2 minutes until clear and let cool to the touch. 2 µL EtBr stock solution was added and mixed thoroughly. The gel mixture was then poured into a casting tray and the well comb was inserted. The comb was removed after the gel solidified; the gel was placed into the electrophoresis apparatus and covered with 1x SB. 15 µL thermocycled DNA sample was combined with 1.5 µL 6x sample buffer then vortexed. 16.5 µL DNA sol'n was loaded to each well using loading tips; 5 µL molecular weight marker (2 kb log ladder) was loaded into own well. The gel was run at a constant 200-225 V until the dye reached the middle of the gel. The gel was visualized under a UV box; pictures of the gel results were taken via computer.

Surgery Protocol

In order to obtain mouse liver samples, a surgery protocol was followed. Each mouse weight was measured and recorded. Each mouse was anesthetized using isofluorene. After the liver was removed and blood was drawn for hematocrit measurements, the diaphragm was perforated to ensure mouse death. Each liver was

weighed. A liver sample of ~0.1 g. was obtained and both the sample and remaining liver were immediately stored in liquid Nitrogen.

Non-Heme Liver Assay Protocol³

To measure the non-heme liver content of each mouse, a non-heme liver assay protocol was followed. The Chromagen stock was composed of 50 mg bathophenanthrolinedisulfuric acid, 0.7145 mL 70% thiglycolic acid, and MQ H₂O (final volume of 50 mL). The acid solution was composed of 12.4 mL 12.1 M HCl, 5 mL 100% TCA, and MQ H₂O (final volume of 50 mL). The 1:1 saturated NaAc: H₂O solution was composed of 18.8 g. NaAc and MQ H₂O (final volume of 50 mL). 0.5 mL acid sol'n was added to each liver sample then vortexed and incubated in H₂O bath at 65°C for 24-72 hours. The samples were cooled and brought up to 0.75 mL with acid solution then spun for 5 minutes. 50 µL sample supernatant was added to designated epindorff tube for spectrophotometry measurements. Iron standards were composed of the following:

- 1- 50 µL MQ H₂O
- 2- 50 µL acid sol'n
- 3- 50 µL 50 µg/dL Fe standard + 50 µL acid sol'n
- 4- 50 µL 200 µg/dL Fe standard + 50 µL acid sol'n
- 5- 50 µL 500 µg/dL Fe standard + 50 µL acid sol'n
- 6- 50 µL 500 µg/dL Fe standard + 50 µL acid sol'n
- 7- 50 µL 500 µg/dL Fe standard + 50 µL acid sol'n
- 8- 50 µL 1000 µg/dL Fe standard + 50 µL acid sol'n

A working chromagen reagent (WCR) was composed of 1 part chromagen stock + 10 parts 1:1 saturated NaAc:MQ H₂O solution. 1 mL WCR was added to each epindorff tube then vortexed briefly and let sit for 10 minutes. Absorbances were measured at 535

nm (visible light); the blank was the H₂O standard. Liver tissue iron (µg/g wet weight tissue) was calculated using the following equation:

$$\frac{[(At-Ab) \times Fes \times 0.75 \times (1+Ve)]}{[(As-Ab) \times W \times Ve \times 1.1]}$$

At= absorbance of test sample

Ab= absorbance of blank

As= absorbance of standard 500 (average of 5-7)

Fes= µg iron present in standard (0.2492)

W= weight of tissue dissolved (g)

Ve= volume of acid tissue extract used for calorimetric analysis in mL (0.05)

Homogenization Protocol²

To prepare the mouse liver samples for western blot analysis, a homogenization protocol was followed. The homogenization buffer was composed of 40 µL 1M citrate, 20 µL DTT, 20 µL PMSF, 80 µL each protease (SBT1, Pep), 10 mL 2x buffer, and MQ H₂O (final volume of 20mL). The liver samples were minced with a scissors.

Homogenization buffer was added to each sample tube (1:3 sample:buffer) then vortexed well. Homogenate + buffer mixture was homogenized with mortar and pestle and put into separate tubes for storage. The leftover homogenate + buffer mixture was centrifuged in Optima (TLA 55) at 45,000 rpm for 30 minutes at 4°C. The cytosol was removed after centrifugation and stored at 4°C.

BCA Protocol (Pierce BCA Protein Assay Kit #23225)

To determine the protein concentration in the homogenate and cytosol, a BCA protocol was followed. The 1x lysis buffer was composed of 2x HG buffer diluted with MQ H₂O. The BCA reagent was composed of 49 parts solution A and 1 part solution B. 10 µL of the cytosol sample was added to 40 µL 1x lysis buffer and vortexed; 5 µL of the homogenate sample was added to 45 µL 1x lysis buffer and vortexed. 5 µL of either cytosol or homogenate dilution was then added to 45 µL H₂O. 1 mL BCA reagent was

added to each sample. Samples were incubated for 30 minutes in H₂O bath at 37°C.

Spectrophotometry absorbances were measured at 562 nm (blank was H₂O) and used to determine amount of each sample necessary for final concentration of 2mg/mL.

Western Blot Protocol²

To determine the IRP1/2, ferritin, and TfR protein levels for each mouse liver sample, a western blot protocol was followed. The 12% resolving gel was composed of MQ H₂O, 30% acrylamide mix, 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate, and TEMED, while the 5% stacking gel was composed of MQ H₂O, 30% acrylamide mix, 1.0 M Tris (pH 6.8), 10% SDS, 10% ammonium persulfate, and TEMED. H₂O saturated butanol was added to 12% resolving gel before set and poured out before adding 5% stacking gel. After adding 5% stacking gel, wells were set. Gels were rinsed and placed into cassette, covered with SDS page buffer. Samples and MW was loaded and run at constant current of 15 mA until reached bottom of gel. Proteins were transferred to nitrocellulose overnight. Blots were blocked for 1 hour in non-fat dry milk, Tween 20, and PBS or Tris glycine block buffer (depending on homogenate or cytosol). Primary Abs (IRP1, IRP2, ferritin, TfR, or tubulin) were added to blots for 1 hour and washed with Tween 20 and PBS or Tris glycine solution. Secondary Abs (goat α mouse or goat α rabbit) were added to blots for 30 minutes and washed again. Blots were developed in dark room for 15-30 minutes. Bands were quantified using Optiquant computer program.

RESULTS

To genotype the 189 St. Louis and 125 UCSF mice, the polymerase chain reaction and gel electrophoresis protocols were followed (Figure 2). The KO (-/-) band was

observed at 800kb, while the WT (+/+) band was observed at 1000kb. If both bands were present in the same sample, the genotype was determined to be HET (+/-). The results of the genotyping are depicted in Figure 3. The distribution of the St. Louis and UCSF mouse genotypes (WT, KO, and Heterozygous (HET)) did not follow the expected distribution, as determined by Mendelian genetics, of WT: 25%, KO: 25%, and HET: 50%. Instead, the St. Louis mice had a distribution of WT: 34.39%, KO: 23.28%, and HET: 42.33%. The UCSF mice had a distribution of WT: 36.00%, KO: 21.60%, and HET: 42.40%. For both mouse types, the KOs and HETs observed percentages were lower than expected, while the WT percentages were higher.

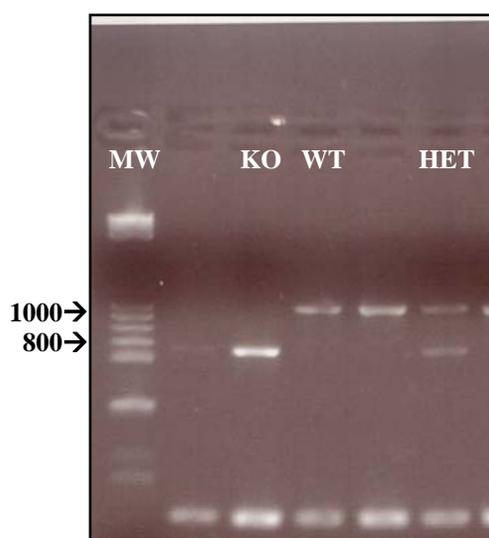


Figure 2: Photograph of PCR agarose gel visualized by gel electrophoresis. MW is 2kb log ladder. Only lower band visible at 800kb represents SOD1 KO (-/-) genotype; only upper band visible at 1000kb represents SOD1 WT (+/+) genotype; both bands visible represent heterozygous (+/-) genotype for SOD1 gene.⁶

MOUSE TYPE	GENOTYPE	NUMBER OF MICE	PERCENT (%)	MENDELIAN EXPECTED PERCENT
St. Louis	WT	65	34.39	25
	KO	44	23.28	25
	HET	80	42.33	50
UCSF	WT	45	36.00	25
	KO	27	21.60	25
	HET	53	42.40	50

Figure 3: Table displaying the breakdown of genotypes (in %) per mouse type (St. Louis or UCSF) as determined via PCR and gel electrophoresis, as well as the Mendelian expected percent of each genotype. The experimental percentages do not follow the expected percentage distribution; observed percentages for both KO and HET of both mouse types were lower than expected, while the WT percentages were higher.⁶

The C57BL/6J male mice hematocrits, ages 8 weeks to 12 months, are depicted in Figure 4. The KO mice had lower average hematocrit percentages compared to the WT mice; however, no age group had a statistically significant difference when comparing the two genotypes as determined by an unpaired, two-tailed t-test ($p < 0.05$). The greatest difference between the two genotypes was observed in the 6 months age group (14.25%), while the smallest difference was observed in the 8 weeks age group (2.45%). The 4 months age group had the smallest p-value (0.0749), while the 12 months age group had the largest p-value (0.5547).

AGE	GENOTYPE	AVERAGE(%)	P-VALUE
8 WKS	WT (n=6)	41.85	0.0837
	KO (n=6)	39.4	
2.5-3 MOS	WT (n=3)	47.58	0.1272
	KO (n=3)	41.80	
4 MOS	WT (n=3)	50.19	0.0749
	KO (n=3)	42.20	
6 MOS	WT (n=2)	45.58	0.0895
	KO (n=2)	31.33	
12 MOS	WT (n=3)	37.10	0.5547
	KO (n=3)	34.47	

Figure 4: Table displaying the C57BL/6J male mice hematocrit values (in % of whole blood composed of RBC's) for each age and genotype group. Although a trend is observed that SOD1 KO mice have lower hematocrit values than SOD1 WT mice of same age, no hematocrit values were found to be statistically different determined by unpaired, two-tailed t-test ($p < 0.05$).

The C57BL/6J male mice liver iron content (μg liver iron/g wet weight liver tissue) was determined via the non-heme liver iron assay and spectrophotometry (Figure 5). Standard error was calculated for each age and genotype by using the equation: standard deviation/ $\sqrt{\text{sample size}}$. Two age groups' average liver iron content differences were found to be statistically significant when comparing the two genotypes as determined by an unpaired, two-tailed t-test ($p < 0.05$). For both age groups, the KOs had more liver iron than the WTs. The age groups were 2.5-3 and 4 months ($p = 0.0002605$

and 0.0004298, respectively). The 6 month age group average liver iron content difference illustrated the same trend as the 2.5-3 and 4 month old mice; however, the p-value was greater than 0.05 ($p=0.07511$). The 8 week and 12 month age group average liver iron content differences were not determined to be statistically significant when comparing genotypes ($p=0.3165$ and 0.4759 , respectively). The average liver iron contents for each age and genotype were as follows: 91.87, 105.05 (8 week WT, KO); 73.29, 136.36 (2.5-3 month WT, KO); 46.00, 96.64 (4 month WT, KO); 66.10, 113.32 (6 month WT, KO); 75.00, 95.17 (12 month WT, KO).

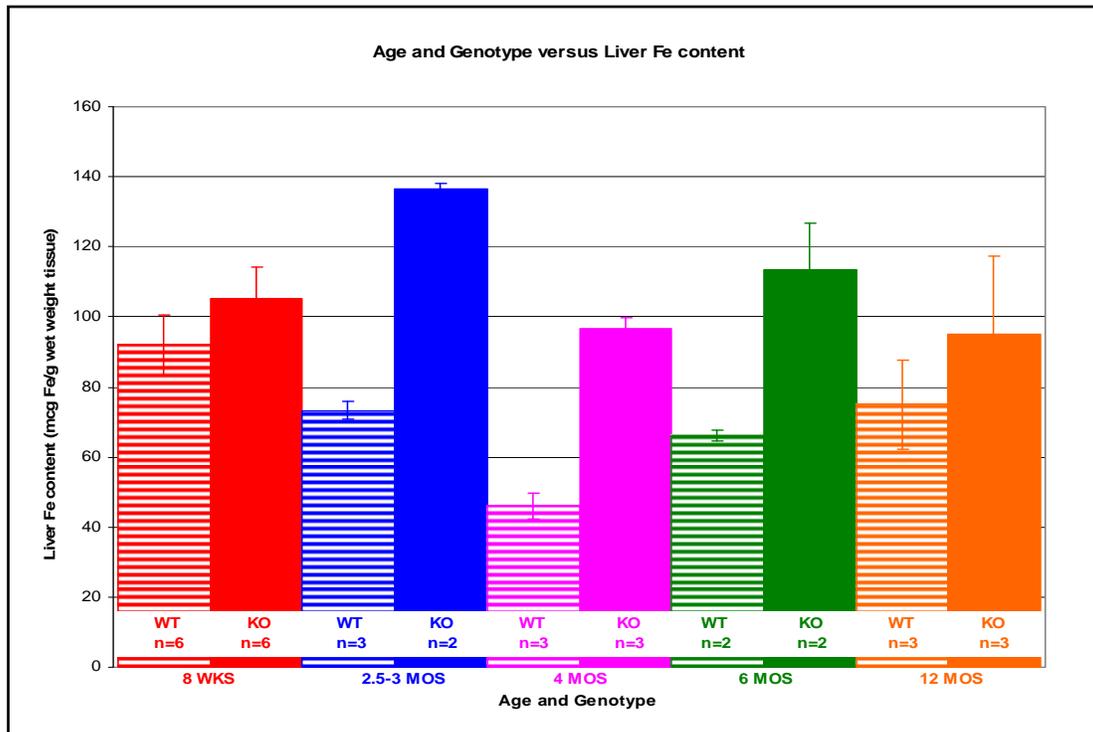


Figure 5: Histogram of C57BL/6J male mice grouped by age and genotype versus liver iron content (μg liver iron/g wet weight liver tissue) determined by the non-heme liver iron assay and spectrophotometry. Standard error bars calculated by using the equation: standard deviation/ $\sqrt{\text{sample size}}$. Statistical significance between the two genotypes per age group was determined by unpaired, two-tailed t-test ($p < 0.05$). WT versus KO average liver iron content differences were statistically significant for 2.5-3 and 4 month age groups ($p=0.0002605$ and 0.0004298 , respectively); KOs had more average liver iron than WTs. The 6 month age group average liver iron content difference illustrated the same trend; however, the p-value was greater than 0.05 ($p=0.07511$). The 8 week and 12 month age group average liver iron content differences were not determined to be statistically significant when comparing genotypes ($p=0.3165$ and 0.4759 , respectively).

The iron metabolism protein levels were determined and quantified by using the western blot protocol and OptiQuant computer program. The proteins under investigation were IRP1/2, ferritin and TfR. The protein levels were divided by the tubulin protein levels to calculate a protein:tubulin ratio for each iron metabolism protein. Each age group's protein:tubulin ratio results are illustrated in a corresponding histogram. To determine statistical significance of the difference in protein:tubulin ratio results between genotypes of a specific age group, an unpaired, two-tailed t-test was performed ($p < 0.05$). The 8 week and 12 month age groups did not have any statistically significant differences in any quantified protein:tubulin ratio levels when comparing WTs to KOs (Figures 6 and 10, respectively) as all calculated p-values were greater than 0.05. The 2.5-3 month age group did have a statistically significant difference in IRP1:tubulin ratio levels between the WTs and KOs ($p = 0.0317$) implying the KOs had a lower amount of IRP1. However, neither the IRP2 or ferritin:tubulin ratio levels were found to be significantly different ($p = 0.1432$ and 0.1525 , respectively) (Figure 7). The 4 month age group had statistically significant differences in IRP1, IRP2, and ferritin:tubulin ratio levels. IRP1 and IRP2 were found to be higher in WTs, while ferritin was found to be lower in WTs ($p = 0.0023$, 0.0004 , and 0.0012 , respectively) (Figure 8). The 6 month age group had statistically significant differences in IRP1 and ferritin:tubulin ratio levels ($p = 0.0429$ and 0.0444 , respectively). The IRP1 and ferritin amounts were found to be lower in WTs. A difference in transferrin:tubulin ratio levels in the 6 month age group was not found to be statistically significant ($p = 0.1815$) (Figure 9).

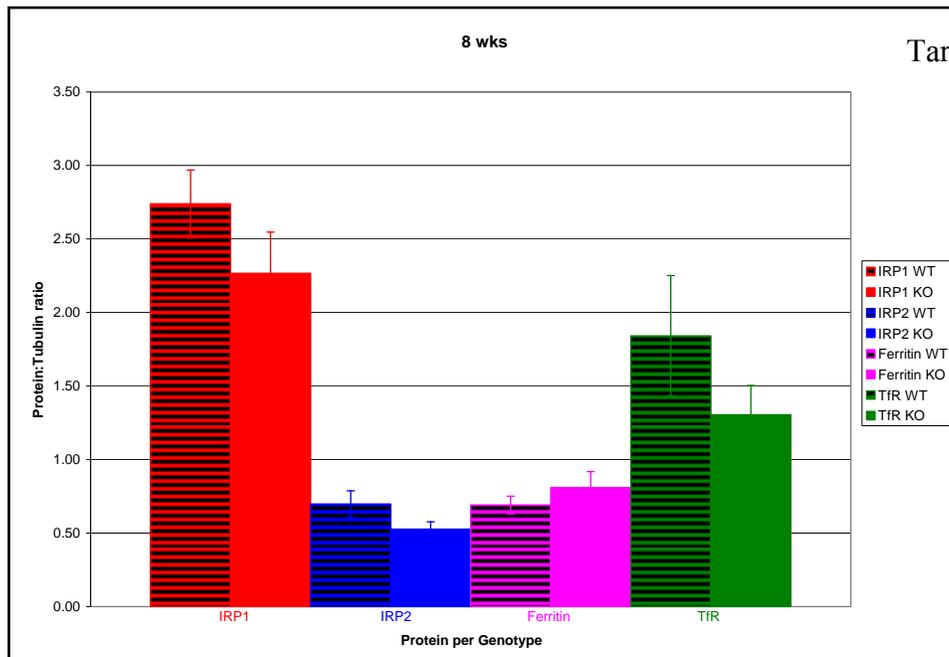


Figure 6: Histogram of 8 week old mice protein:tubulin ratio levels per genotype (WT vs. KO). Protein levels measured were IRP1, IRP2, ferritin, and TfR as measured by western blot and OptiQuant quantification. Standard error bars calculated by using the equation: standard deviation/ $\sqrt{\text{sample size}}$. Statistical significance between the two genotypes per age group was determined by unpaired, two-tailed t-test ($p < 0.05$). No statistically significant differences in any quantified protein:tubulin ratio levels when comparing WT to KOs; IRP1 ($p = 0.2209$), IRP2 ($p = 0.1300$), ferritin ($p = 0.3749$), TfR ($p = 0.2694$). WT $n = 6$; KO $n = 6$.

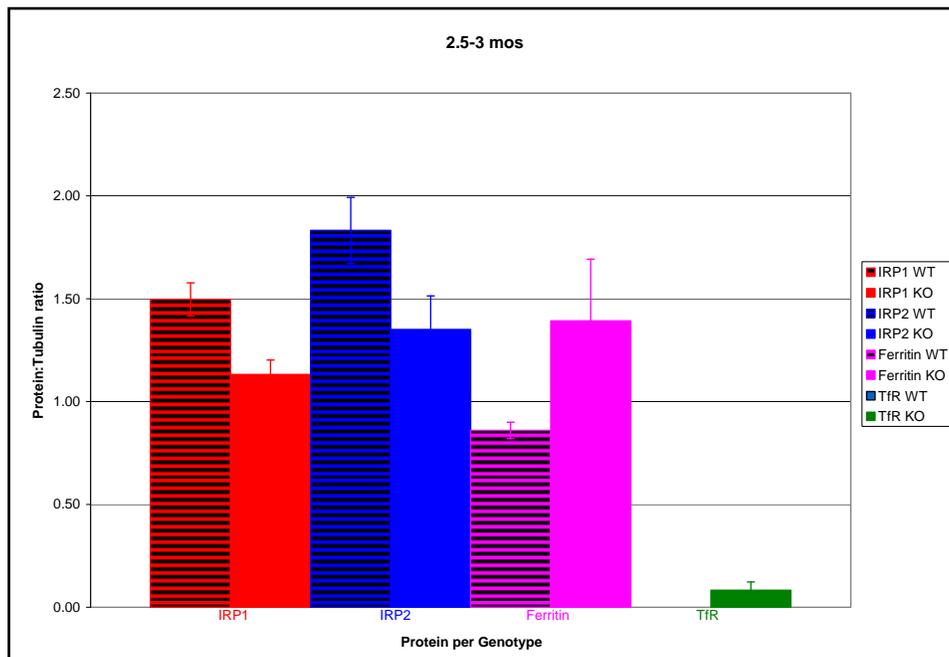


Figure 7: Histogram of 2.5-3 month old mice protein:tubulin ratio levels per genotype (WT vs. KO). Protein levels measured were IRP1, IRP2, ferritin, and TfR as measured by western blot and OptiQuant quantification. Standard error bars calculated by using the equation: standard deviation/ $\sqrt{\text{sample size}}$. Statistical significance between the two genotypes per age group was determined by unpaired, two-tailed t-test ($p < 0.05$). No statistically significant differences in quantified protein:tubulin ratio levels for proteins IRP2 or ferritin; however, statistically significant difference in quantified IRP1:tubulin ratio levels when comparing WT to KOs implying KOs had lower amount of IRP1. TfR:tubulin ratio levels were invalid due to lack of usable sample levels. IRP1 ($p = 0.0317$), IRP2 ($p = 0.1432$), ferritin ($p = 0.1525$), TfR (n/a). WT $n = 3$; KO $n = 3$.

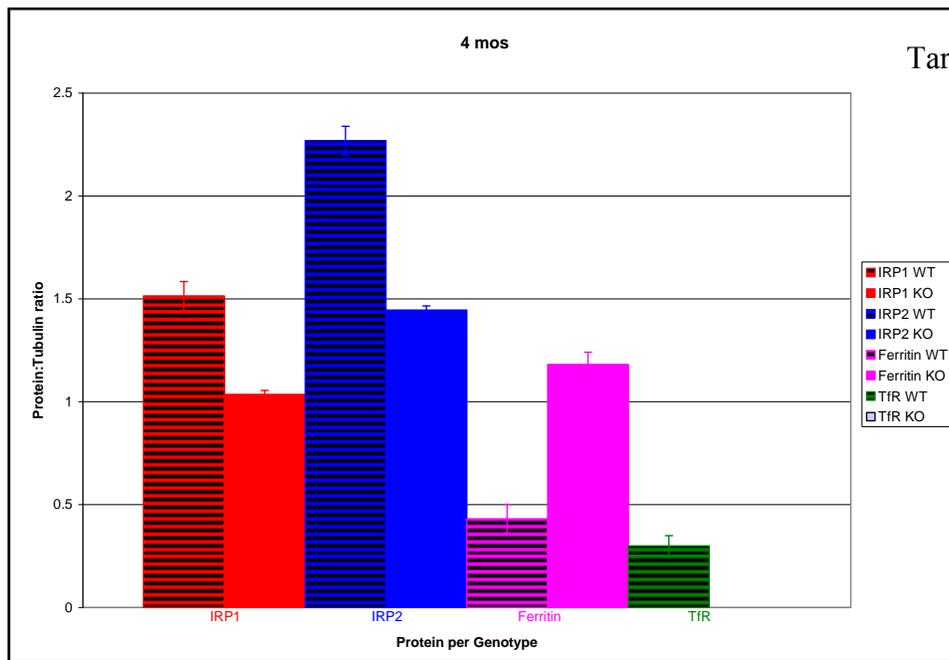


Figure 8: Histogram of 4 month old mice protein:tubulin ratio levels per genotype (WT vs. KO). Protein levels measured were IRP1, IRP2, ferritin, and TfR as measured by western blot and OptiQuant quantification. Standard error bars calculated by using the equation: standard deviation/ $\sqrt{\text{sample size}}$. Statistical significance between the two genotypes per age group was determined by unpaired, two-tailed t-test ($p < 0.05$). Statistically significant differences in all quantified protein:tubulin ratio levels except TfR when comparing WT to KO. The observed results imply WT has higher IRP1 and IRP2 levels and lower ferritin levels. TfR:tubulin ratio levels were invalid due to lack of usable sample levels. IRP1 ($p = 0.0023$), IRP2 ($p = 0.0004$), ferritin ($p = 0.0012$), TfR ($p = n/a$). WT $n = 3$; KO $n = 3$.

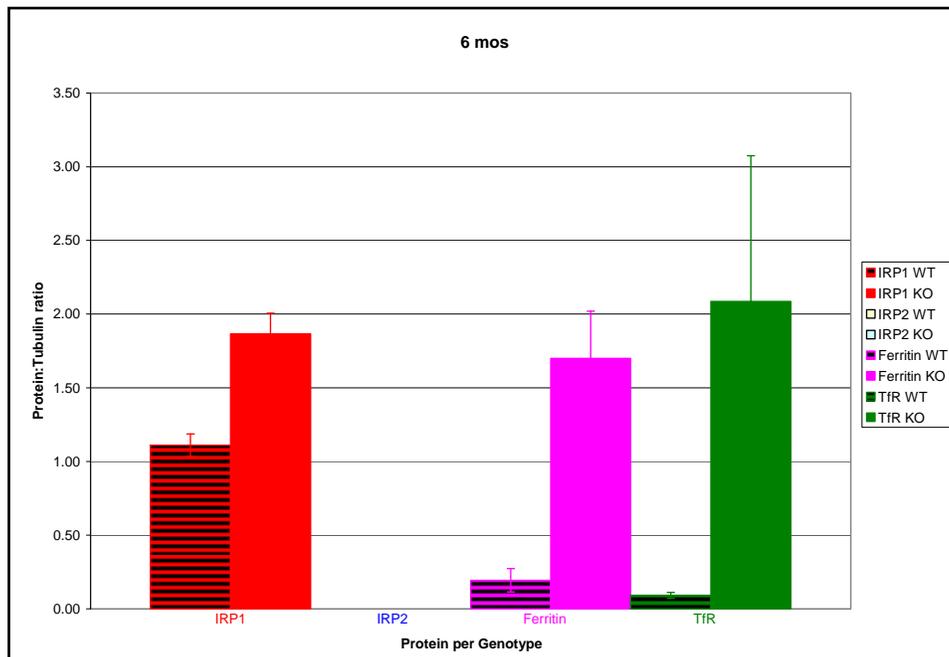


Figure 9: Histogram of 6 month old mice protein:tubulin ratio levels per genotype (WT vs. KO). Protein levels measured were IRP1, IRP2, ferritin, and TfR as measured by western blot and OptiQuant quantification. Standard error bars calculated by using the equation: standard deviation/ $\sqrt{\text{sample size}}$. Statistical significance between the two genotypes per age group was determined by unpaired, two-tailed t-test ($p < 0.05$). No statistically significant differences in any quantified protein:tubulin ratio levels except ferritin when comparing WT to KO. The observed results imply WT has lower levels of IRP1. IRP2:tubulin ratio levels were invalid due to lack of usable sample levels. IRP1 ($p = 0.0429$), IRP2 ($p = n/a$), ferritin ($p = 0.0444$), TfR ($p = 0.1815$). WT $n = 2$; KO $n = 2$.

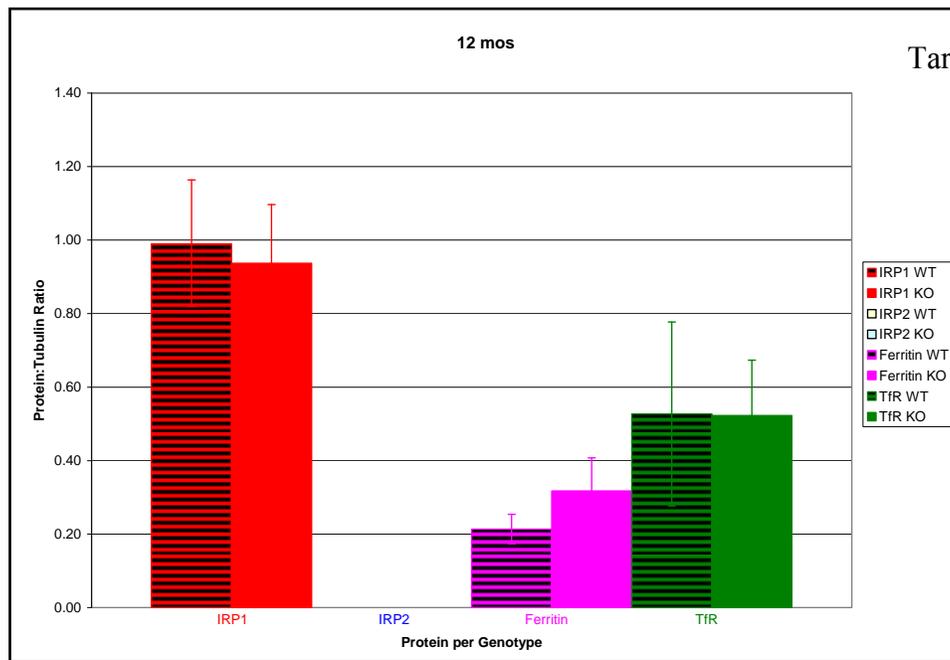


Figure 10: Histogram of 12 month old mice protein:tubulin ratio levels per genotype (WT vs. KO). Protein levels measured were IRP1, IRP2, ferritin, and TfR as measured by western blot and OptiQuant quantification. Standard error bars calculated by using the equation: standard deviation/ $\sqrt{\text{sample size}}$. Statistical significance between the two genotypes per age group was determined by unpaired, two-tailed t-test ($p < 0.05$). No statistically significant differences in any quantified protein:tubulin ratio levels when comparing WT to KO. IRP2:tubulin ratio levels were invalid due to lack of usable sample levels. IRP1 ($p = 0.8358$), IRP2 ($p = n/a$), ferritin ($p = 0.3388$), TfR ($p = 0.9891$). WT $n = 3$; KO $n = 3$.

DISCUSSION

Iron is used in many different important reactions found in the body. However, iron concentrations and distribution must be tightly managed to ensure cellular requirements are met while avoiding iron overload or iron deficiency.¹⁰ Although there are many iron metabolism proteins, this experiment focused on IRP1, IRP2, ferritin, and TfR to determine how the lack of the SOD1 gene in mice affects the protein levels compared to SOD1 WT mice of the same background, sex, and age. IRP1 unbound to Fe-S cluster binds to ferritin or TfR mRNA, either inhibiting or stabilizing translation of the mRNA, respectively.¹⁰ It has been found that superoxide anions can cause the removal of the Fe-S cluster in IRP1. This increased occurrence of Fe-S cluster removal may then cause a decrease in unbound IRP1 concentration possibly due to the increased oxidative damage or degradation of unbound IRP1 to prevent a large increase in mRNA

transcription.⁵ This decrease in IRP1 levels would result in less ferritin mRNA inhibition as well as less TfR mRNA stabilization. Since SOD1 KO mice have higher levels of superoxide anions due to lack of superoxide dismutase presence, I hypothesized SOD1 KO mice would have an increase in non-heme liver iron and ferritin levels due to an increased need to store the excess iron, a decrease in TfR levels due to decreased TfR mRNA stability and IRP1 levels due to high levels of IRP1 without Fe-S cluster increasing IRP1 degradation, and no change in IRP2 levels. After performing the non-heme liver assay, hematocrit measurements, and western blots, my hypothesis was found to be supported by my experimental data.

The non-heme liver iron contents in SOD1 KO mice at ages 2.5-3 months and 4 months were statistically higher ($p < 0.05$) when compared to the SOD1 WT mice of the same ages (Figure 5). This trend was also observed in the 6 month old mice, but possibly due to the low sample numbers, the difference was not statistically significant. The 8 week and 12 month old mice were not statistically different. It is possible the difference between the two genotypes at 8 weeks was not seen because the mice were too young to show iron metabolism differences. At 12 months, it had been previously observed that SOD1 KO mice develop liver tumors which may have impacted the resulting non-heme liver iron content.

There was no statistically significant difference ($p < 0.05$) between the SOD1 KO and SOD1 WT mice hematocrits within the same age group (Figure 4). However, a trend was observed that the KO mice have lower hematocrit levels than the WT mice. The lower percentages of red blood cells per whole blood seen in the KO mice may indicate

iron anemia possibly caused by iron sequestering of the liver as seen by increased levels of non-heme liver iron.

The IRP1 protein levels were statistically lower in SOD1 KO mice at ages 2.5-3 and 4 months when compared to SOD1 WT mice of the same age (Figures 7, 8). There was no statistically significant difference found in the 12 month or 8 week old mice; however, the trend that KO mice have lower IRP1 levels was observed (Figures 6, 9). Lower levels of IRP1 in SOD1 KO mice were hypothesized due to possible increased degradation of IRP1 caused by increased levels of ROS removing Fe-S cluster from IRP1.

The IRP2 protein level differences were not statistically significant in any age group other than the 4 months. IRP2's relationship with iron metabolism as affected in SOD1 KO mice is not fully understood. Due to the observed decrease in IRP2 levels in 4 month SOD1 KOs compared to WTs, further experimentation should be conducted to fully explain IRP2's involvement and influence.

The ferritin protein levels were statistically higher in SOD1 KO mice at age 4 months (Figure 8). There was no statistically significant difference in ferritin protein levels observed in the other age groups; however, the trend that KO mice have higher ferritin levels was observed (Figures 6, 7, 9). Higher levels of ferritin in SOD1 KO mice coincide with the lower levels of IRP1 causing less ferritin mRNA translation inhibition as well as an increased need to store iron due to the increased non-heme liver iron in KOs.

The TfR protein level differences were not statistically significant in any age group; however, a trend was observed in each age group that SOD1 KO mice have lower

levels of liver TfR (Figures 6-9). Although the differences were not significant, the trend that SOD1 KO mice have lower levels of TfR also coincides with the lower levels of IRP1 causing less TfR mRNA stability, decreasing translation and TfR protein levels as well as a lesser need for mobilization of iron due to an iron-sufficient status.

Consequently, the observed decreased levels of IRP1 and TfR support the theory that a high amount of ROS like superoxide anion causes increased degradation of IRP1, most likely due to superoxide and Fe-S cluster interaction, and resultantly decreased TfR mRNA translation stability and protein levels. Also, the observed increased levels of ferritin and non-heme liver iron content support the theory that when ROS are present, IRP1 binding to IRE decreases, no longer inhibiting ferritin mRNA translation. This increases the cells' ability to store iron in such places as the liver, reflected in the increased non-heme liver iron content in SOD1 KO mice as compared to SOD1 WT mice.

The 6 month old data was not used because the sample number was too small, and the western blot data was unreliable due to samples measured on different blots, greatly decreasing the validity of the data.

Further experimentation is necessary with a larger number of samples to increase the statistical difference between the two genotypes and the different age groups, particularly 6 months. Also, experiments to determine if any other non-heme tissue iron levels are affected by the absence of SOD1 should be explored, as well as how IRP2 and TfR are affected by increased levels of superoxide anions over time.

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REFERENCES

1. Brody, J. R. & Kern, S. K. (2004). Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. *BioTechniques*, 36, 214-216.
2. Clarke, S. L., Vasanthakumar, A., Anderson, S. A., Ponderre, C., Koh, C. M., Deck, K. M., Pitula, J. S., Epstein, C. J., Fleming, M. D., & Eisenstein, R. S. (2006). Iron-responsive degradation of iron-regulatory protein 1 does not require the Fe-S cluster. *The EMBO Journal*, 25, 544-553.
3. Cook, J. D. (1980). *Methods in Hematology, Iron*, 105-108.
4. Eisenstein, R. S. (2000). Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annual Review of Nutrition*, 20, 627-662.
5. Eisenstein, R. S. & Ross, K. L. (2003). Novel Roles for Iron Regulatory Proteins in the Adaptive Response to Iron Deficiency. *Journal of Nutrition*, 133, 1510S-1516S.
6. Elchuri, S., Oberley, T. D., Qi, W., Eisenstein, R. S., Roberts, L. J., Van Remmen, H., Epstein, C. J., & Huang, T. (2005). CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene*, 24, 367-380.
7. Ho, Y. S., Gargano, M., Cao, J., Bronson, R. T., Heimler, I., & Hutz, R. J. (1998). Reduced fertility in female mice lacking copper-zinc superoxide dismutase. *The Journal of Biological Chemistry*, 273:13, 7765-7769.
8. Sambrook, Fritsch & Maniatis. (1989). Extraction and Purification of RNA. *Molecular Cloning: A Laboratory Manual*, 7.3-7.5.
9. Starzynski, R. R., Lipinski, P., Drapier, J., Diet, A., Smuda, E., Bartłomiejczyk, T., Gralak, M. A., & Kruszewski, M. (2004). Down-regulation of iron regulatory protein 1 activities and expression in superoxide dismutase 1 knock-out mice is not associated with alterations in iron metabolism. *The Journal of Biological Chemistry*, 280:6, 4207-4212.
10. Wallander, M. L., Leibold, E. A., & Eisenstein, R. S. (2006). Molecular control of vertebrate iron homeostasis by iron regulatory proteins. *Biochimica et Biophysica Acta*, 1763, 668-689.