## Mutagenetix Phenotypic Mutation 'iron10'

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## Mutagenetix Phenotypic Mutation 'iron10'

| Mode of Inheritance | Autosomal Recessive |
| Local Stock | Sperm |
| Repository |  |
| Science Writers | Nora G. Smart |
| Authors | Xin Du, Lara Krieg |
| Last Updated | 05/13/2016 3:09 PM by Anne Murray |
| Record Created | 11/10/2010 12:08 PM by Xin Du |
| Record Posted | 06/03/2011 |
Phenotypic Description

The recessive iron10 phenotype was identified in N-ethyl-N-nitrosourea (ENU)-mutagenized G3 mice while screening for altered levels of serum iron (Figure 1). The iron10 mouse displays reduced levels of serum iron, but elevated liver iron stores (Figure 2).

Figure 1. Reduced serum iron in the iron10 mouse.

Figure 2. Elevated liver iron stores in the iron10 mouse.
Nature of Mutation

The iron10 mutation was mapped on the basis of anemia by bulk segregation analysis (BSA) of F2 intercross offspring using C57BL/10J as the mapping strain. The mutation showed strongest linkage with Chromosome 3, and causes a T to a C transition at nucleotide 3328 of the Cp transcript using Genbank record NM_001042611.1 in exon 18 of 21 total exons. Multiple transcripts of the Cp gene are displayed on Ensembl.

3312  CCTGGAACCTGGTTACTCCACTGCCACGTGACT
1028  -P--G--T--W--L--L--H--C--H--V--T--

The mutated nucleotide is indicated in red lettering and causes a leucine to proline substitution at amino acid 1033 of the encoded protein.

Protein Prediction
The mouse Cp gene encodes a 1061 or 1086 amino acid serum glycoprotein that is a member of the multicopper oxidase family of enzymes (Figure 4). Murine ceruloplasmin has 85% identity to the human protein with complete conservation of the amino acid ligands essential for binding the six copper molecules. Five of the seven N-linked glycosylation sites in human ceruloplasmin are also conserved in the murine sequence (1;2). Ceruloplasmin contains 6 copper ions per molecule that is necessary for its ferroxidase activity oxidizing ferrous iron (Fe$^{2+}$) to the ferric state (Fe$^{3+}$) as shown in the following reaction:

$$4 \text{Fe}^{2+} + 4 \text{H}^+ + \text{O}_2 = 4 \text{Fe}^{3+} + 2 \text{H}_2\text{O}.$$ 

The protein is involved in iron transport across the cell membrane (3).

The multicopper oxidase family of enzymes are characterized by the presence of three types of spectroscopically distinct copper sites (4). Ceruloplasmin contains three type I copper-binding cysteines, and charge transfer between the cysteine sulfurs and the copper at these sites results in strong absorption at 600 nanometers (nm), giving the protein an intense blue color. A single type II copper is coordinated by four imidazole nitrogens and is in close proximity to two antiferromagnetically coupled type III copper ions that absorb at 330 nm. The type II and type III coppers form a trinuclear copper cluster that is the site of oxygen binding during the catalytic cycle (5). Multicopper oxidases utilize the facile electron chemistry of bound copper ions to

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couple substrate oxidation with the four-electron reduction of dioxygen. Electrons pass from the substrate to the type I copper and then to the trinuclear copper cluster and subsequently to the oxygen molecule bound at this site (6).

Resolution of the structure of human ceruloplasmin by X-ray crystallography (1KCW; 2J5W) (Figure 5) has confirmed the presence of the trinuclear cluster as well as the identity of each of the amino acid copper ligands (7-9). Ceruloplasmin features an internal triplication with each unit consisting of two domains (10). Domains 1, 3 and 5 contain 190 amino acids, while domains 2, 4, and 6 contain roughly 150 amino acids. There are six integral copper atoms per molecule (the mononuclear sites occur in domains 2, 4 and 6 and the trinuclear site occurs between domains 1 and 6) and two labile sites with roughly 50% occupancy (8). Each of the
mononuclear copper ions is coordinated to a cysteine and two histidine residues and those in domains 4 and 6 also coordinate weakly to a methionine residue. In domain 2, the methionine is replaced by a leucine residue (7), and mutation of the copper-binding site in domain 2 failed to modify either the spectroscopic or catalytic properties of the mutated protein (11). In the mouse, the type I copper-binding sites occur at amino acids 294, 337, 342, 651, 694, 699, 704, 989, 1040 and 1045. The type II copper is coordinated with residues 120 and 992, and the type II copper-binding amino acids are 122, 179, 181, 994, 1034 and 1036 (Uniprot record Q61147).

Ceruloplasmin binds to iron using domains 4 and 6. These sites involve a histidine residue and negatively charged aspartate and glutamate residues. The access to these sites is limited to relatively small substrates by three large protuberances at the top of the molecule formed by loops from pairs of odd and even domains (8). Each of these are stabilized by the presence of sodium ions (Na\(^{2+}\)) bound near the base (9). Fe\(^{2+}\) likely releases an electron to the nearest mononuclear copper and then translocates to a holding site near the outside of the protein with Glu597 (Glu611 in the mouse; human numbering does not include the signal peptide) and Glu935 (Glu949) in domains 4 and 6 playing key roles in the translocation process. At the holding site the ferric iron would be available for collection by the iron-binding glycoprotein transferrin. Domain 1 also has a calcium (Ca\(^{2+}\))-binding site composed of Asp127 and Asp128, Lys109 and Gln124 (Asp146, Asp147, Lys128, and Gln143 in the mouse). The interaction of ceruloplasmin with red blood cells has been shown to be Ca\(^{2+}\)-dependent (12).

Along with Fe\(^{2+}\), ceruloplasmin has the ability to oxidize some organic compounds such as brain catecholamines (13-17), and can function as a nitric oxide (NO) oxidase to synthesize nitrite (18). Two distinct sites for these amine substrates have been identified (8). Synthetic amines appear to bind near the bottom of domain 4, close to an arrangement of tryptophan, methionine and histidine residues and away from the mononuclear copper-binding site. These residues are not conserved in the mouse protein. Biogenic amines, such as nor-epinephrine, epinephrine, serotonin and dopamine, bind to domain 6 near the copper-binding sites at amino acids Glu935, His940, Asp1025 (Glu949, His954 and Asp1039 in the mouse). Under physiological conditions, anions such as Cl- increase the activity of ceruloplasmin with non-iron substrates, whereas Fe\(^{2+}\) oxidation remains unaffected (19). The azide inhibitor binds to one of the type III copper atoms, while the brain-altering drug (+)-lysergic acid diethylamine (LSD) binds near Asn119 in domain 1 (Asp138 in the mouse) (8).

In mammals, alternative splicing of the Cp gene results in a glycosylphosphatidylinositol

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(GPI–anchored protein (20-24). Natural variants of the protein have been documented in humans and some of these can result in proteins with altered activity. For instance, an isoleucine to threonine change at amino acid 63 (Uniprot P00450) results in a protein that is retained in the endoplasmic reticulum due to defective glycosylation, while an aspartic acid to glutamic acid change at residue 544 results in reduced ferroxidase activity (25). N-linked glycosylation of the protein can also vary between individuals (26), and may affect protein turnover or ferroxidase activity (27). The failure to incorporate copper into ceruloplasmin results in the secretion of unstable apoceruloplasmin that lacks ferroxidase activity (28-30).

Ceruloplasmin is known to interact with several other proteins particularly those involved in iron-binding and the inflammation process. These include an interaction with the iron storage protein ferritin involving a negatively charged stretch of amino acids near the C-terminus in domain 6 (31). Ceruloplasmin also binds other proteins like myeloperoxidase (MPO), the pro-oxidant enzyme of azurophilic granules of neutrophils (32), the antimicrobial breast milk protein lactoferrin (33), and protein C (34), the inactive form of a serine protease that regulates blood clotting, inflammation and cell death. These proteins contain positively charged residues that appear to interact with ceruloplasmin (35), and the binding sites in ceruloplasmin for lactoferrin and MPO are composed of stretches of amino acids in domains 1 and 6 that include the ligands for all four copper ions of the catalytic center (36;37). Both lactoferrin and MPO have been shown to change the oxidase activity of ceruloplasmin towards various substrates and inhibit its proteolytic degradation (36-38), while ceruloplasmin inhibits MPO activity (32;38). Other leukocytic proteins that interact with ceruloplasmin include eosinophilic cationic protein, cathepsin G, azurocidin, neutrophilic elastase, proteinase 3 and 5-lipoxygenase (5-LO), the key enzyme of leukotriene synthesis (36;39;40). Such ceruloplasmin complexes also include the matrix metalloproteinases (MMP-2 and MMP-12) (41). GPI-anchored ceruloplasmin colocalizes on astrocytes with the iron exporter ferroportin (42).

The leucine to proline substitution in ceruloplasmin caused by the iron10 mutation occurs at amino acid 1033 in domain 6. This residue is adjacent to one of the type III copper-binding ligands.

Expression/Localization

In mammals, ceruloplasmin is primarily synthesized and secreted by hepatocytes in the liver (2;
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However, extrahepatic ceruloplasmin gene expression has been demonstrated in many tissues including spleen, lung, testis, brain and kidney (2;43-45). In mice, in situ hybridization found the gene to be expressed in hepatocytes, the splenic reticuloendothelial system and the bronchiolar epithelium of the lung. In the central nervous system, abundant ceruloplasmin gene expression was detected in specific populations of astrocytes within the retina and the brain as well as the epithelium of the choroid plexus. In situ hybridization utilizing ceruloplasmin cDNA clones revealed abundant expression in specific populations of glial cells within the brain microvasculature, surrounding dopaminergic melanized neurons in the substantia nigra, and within the inner nuclear layer of the retina (2). Ceruloplasmin is also localized to ependymal cells, the choroid plexus, substantia nigra, Purkinje cells and the granule cell layer of the cerebellum (42). The GPI–anchored isoform of ceruloplasmin is expressed by mammalian astrocytes and Sertoli cells (20-24), and functions in the oxidation and mobilization of iron at the blood-brain and blood-testis barriers (42).

Ceruloplasmin expression is regulated by many factors. Serum ceruloplasmin increases significantly under conditions of anemia including inflammation, infection, trauma, pregnancy and iron deficiency. The increase in ceruloplasmin levels is transcriptionally regulated by inflammatory cytokines such as interleukin (IL)-6, IL-1?, interferon (IFN)-?, and tumor necrosis factor (TNF)-? following infection and trauma (46), and hypoxia-inducible factor 1 (HIF-1) following iron deficiency (47). IL-1? is able to increase iron efflux from glia cells by coordinately inducing expression of the iron transporter ferroportin-1 (Fpn) and ceruloplasmin, a process that requires the activation of the mitogen activating protein (MAP) kinase pathway (48). The transcription factor FoxO1 also upregulates ceruloplasmin, an activity that is prevented by the presence of insulin (49). Insulin also inhibits Cp induction following cytokine stimulation (50), although this hormone may also stimulate ceruloplasmin expression under certain conditions (50). The peptide hormone hepcidin, the principal regulator of systemic iron homeostasis, decreases both ceruloplasmin and Fpn levels, although it increases the level of another iron transporter, divalent metal transporter 1 (DMT1) (51). Ceruloplasmin expression is also decreased in response to extracellular H2O2 and intracellular oxidative stress by an mRNA decay mechanism involving its 3’-untranslated region (UTR) (52).

Patients with the copper metabolic Wilson disease (WD; OMIM #277900) or Menkes disease (MD; OMIM #309400) display a marked decrease in serum ceruloplasmin (53;54). These disorders are caused by mutations in the copper-transporting P-type ATPases ATP7A (see the record for Tigrou-like) and ATP7B, resulting in copper deficiency (Menkes disease) or copper
overload (Wilson disease), respectively (54). ATP7B is localized to the trans-Golgi network (TGN) of hepatocytes, and is necessary for the incorporation of copper into ceruloplasmin in the liver. Conversely, ceruloplasmin levels are significantly increased in tumors, cancer cell lines and certain malignancies (21), as well as in patients with Alzheimer disease (AD; OMIM #104399) (55;56) and possibly schizophrenia (OMIM #181500) (57). Ceruloplasmin levels also increase in the retinas of mice and humans with glaucoma (58), and may be a protective reaction to retinal cell death.

**Background**

Ceruloplasmin is a serum ferroxidase that contains greater than 95% of the copper found in plasma, and was first isolated in 1944 (59). As mentioned above, it is a member of the multicopper oxidase family of enzymes, an evolutionarily conserved group of proteins that utilize copper to couple substrate oxidation with the four-electron reduction of oxygen to water. Unique members of this family of enzymes are present in bacteria, fungi, yeast, plants, worms, parasites, and mammals, and their substrates include manganese, iron, nitrate, bilirubin, phenols, and ascorbate (3). In addition to ceruloplasmin, several multicopper oxidases have been identified as playing a critical role in iron homeostasis. Fet3 is a ferroxidase essential for iron uptake in yeast, and hephaestin is a ceruloplasmin homologue that is required for efficient iron efflux from the placenta and enterocytes in mammals (60;61). In addition, nucleotide and amino acid sequence comparisons suggest that serum ceruloplasmin and the serum clotting factors V and VIII constitute a family of structurally related proteins (62). Despite the need for copper in ceruloplasmin function, this protein plays no essential role in the transport or metabolism of this metal (63).
Figure 6. Systemic iron homeostasis and regulation of hepcidin expression. (A) Iron is absorbed by duodenal enterocytes using the divalent metal transporter-1 (DMT-1), where it is oxidized by hephaestin to ferric iron and exported into the plasma by ferroportin. In the plasma, iron binds to transferrin (Tf). Binding of holotransferrin to the transferrin receptor (TfR1) leads to iron uptake in multiple tissues including the bone marrow, where it is
used for erythropoiesis. Senescent red blood cells are phagocytosed by spleen macrophages leading to recycling of iron back into the plasma. If iron is released into the plasma at a level that exceeds the binding capacity of transferrin, the excess iron is deposited in the liver. Hepcidin, a peptide hormone secreted by the liver, regulates iron absorption, recycling, and mobilization by binding to ferroportin and triggering its internalization and lysosomal degradation in both enterocytes and macrophages. (B) The *Hamp* gene, which encodes hepcidin, is activated in response to elevated iron levels as well as inflammatory cytokines such as IL-6. Increased plasma iron and holotransferrin levels leads to the stabilization of the transferrin receptor 2 (TfR2) resulting in the dissociation of the hemachromatosis protein (HFE) from TfR1 to TfR2. This complex has been shown to associate with hemojuvelin (HJV), a coreceptor for bone morphogenetic proteins (BMP). Binding of BMPs to the BMP receptor complex activates the BMP signaling pathway resulting in Hamp upregulation. Certain BMPs can regulate hepcidin expression independently of HJV, TfR2 and HFE. Low plasma iron leads to HJV cleavage and the production of soluble hemojuvelin (sHJV), which can bind to BMP and block the activation of BMP receptors. Two proteases, furin and TMPRSS6, have been shown to be capable of HJV cleavage. Inflammatory cytokines, such as IL-6, activate signal transducer and activator of transcription-3 (STAT3). STAT3 then binds to the *Hamp* gene promoter and induces hepcidin expression.

Although iron is necessary for a variety of metabolic processes, excessive iron levels produces oxidative stress and toxicity. Therefore the levels of iron in the body need to be tightly regulated and maintained (Figure 6). Because iron cannot be excreted, iron homeostasis is maintained by regulating the level of enteric iron absorption and by recycling iron used for erythropoiesis from the reticuloendothelial cell compartment after the catabolism of senescent erythrocytes (64). In addition, separate “iron cycles” are present to maintain iron homeostasis in the retina and brain (CNS cycle), the placenta, and the testes. Behind every blood barrier, distinct iron proteins are
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synthesized including transferrin, DMT1 (also known as SLC11A2; solute carrier family 11a member 2), Fpn, ceruloplasmin and hephaestin (3;65). DMT1 is an iron transporter located on the apical and ventral surfaces of testes Sertoli and germ cells, as well as the apical membrane of duodenal enterocytes, and allows the transport of ferric iron into the cytosol. The movement of iron from enterocytes into the bloodstream is then mediated by the iron export channel Fpn (66; 67) with the aid of hephaestin (68). Similarly, ceruloplasmin promotes cellular iron efflux in hepatocytes, reticuloendothelial, duodenal, neuronal and Sertoli cells (42;69;70), and the ferroxidase activity and presence of ceruloplasmin is required for Fpn stability (71). In addition to being important for normal release of cellular iron, ceruloplasmin can increase iron uptake by cells in situations of iron depletion (72). Both ceruloplasmin and hephaestin oxidize iron in the plasma prior to the binding of this metal to transferrin, which then mediates its transfer into cells via transferrin receptors. Within cells, iron is bound and stored in a nontoxic form by ferritin. The interaction of ceruloplasmin with ferritin ensures iron incorporation into the latter protein, while enhancing the ferroxidase reaction at the same time (31).

The liver serves as one of the main depots for iron storage in the body, and plays a central role in regulating intestinal iron absorption by secreting hepcidin, which is also known as the hepatocyte antimicrobial peptide HAMP (65;73). Hepcidin regulates iron absorption by binding to Fpn, resulting in internalization and proteolysis of the channel and preventing release of iron from intestinal cells into the plasma (74). The expression of hepcidin is regulated by plasma iron levels, erythropoietic activity, hypoxia and inflammation (75), and involves the action of several proteins including the hemachromatosis protein HFE (76), transferrin receptor 2 (TfR2; see the record for iron-man) (77;78), and hemojuvelin (HJV) (79). The membrane-bound form of HJV associates with HFE and TfR2 and binds to a cell surface receptor for bone morphogenetic proteins (BMPs), which have been shown to upregulate Hamp (80-83). Soluble HJV competitively inhibits this interaction (84;85). Hamp expression is also positively regulated by the transcription factor SMAD4, through epigenetic modification of histone H3 to a transcriptionally active form (86). Conversely, the transmembrane serine protease TMPRSS6 is a non-redundant component in a pathway that senses iron deficiency and negatively regulates Hamp expression to promote iron uptake (87;88) (see the records for mask, zorro, and masquerade). The presence of ceruloplasmin prevents hepcidin-mediated Fpn internalization (89).

An essential role for ceruloplasmin in iron metabolism was established in 1995 with the identification of patients with aceruloplasminemia (OMIM #604290) (90;91). The absence of
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Serum ceruloplasmin in patients with aceruloplasminemia leads to a slow accumulation of iron in compartments where this metal is normally mobilized for recycling. In addition to the slow accumulation of iron within the reticuloendothelial system, the absence of serum ceruloplasmin also results in increased ferrous iron in the plasma, which is rapidly removed from the circulation by the liver, pancreas, and other tissues. Iron also accumulates in the retina and brain, and patients are identified by the presence of varying signs of neurodegeneration like dementia, dysarthria, and dystonia by the fourth or fifth decade of life. The brain regions most affected display the most iron accumulation and include the substantia nigra, caudate nucleus, globus pallidus, putamen and red and dentate nucleus. Humans lacking ceruloplasmin also display insulin-dependent diabetes as a result of selective ß-islet cell loss secondary to iron deposition, and are anemic with serum iron levels decreased by about half. In addition, serum hepcidin and liver Fpn are decreased, while serum ferritin levels are increased. Iron-mediated oxidative stress is thought to contribute to tissue injury and neuronal cell death. The oxidization of brain catecholamines and NO by ceruloplasmin are also likely to be important, particularly as nitrite has been shown to confer cytoprotective effects under conditions of hypoxia [reviewed by (3;92; 93)]. A ceruloplasminemia does not result in impaired intestinal iron uptake and transport, as the oxidation of iron necessary for this process is accomplished by the homologous multicopper oxidase hephaestin (61). Individuals heterozygous for disease mutations have a partial ceruloplasmin deficiency and usually display normal iron metabolism and no clinical symptoms. A few cases of affected heterozygous patients have been reported (94-97).

Similar to patients with aceruloplasminemia, targeted knockout of the Cp gene in mice results in animals with a progressive accumulation of stored iron in the liver, spleen, cerebellum, and brainstem, mild iron deficiency anemia, and impaired motor coordination associated with loss of brainstem dopaminergic neurons (69;98). However, extensive neural and retinal degeneration does not occur unless mice are also lacking hephaestin (93;99). Hephaestin expression is upregulated in the cerebral cortex and caudate putamen, but not the substantia nigra or cerebellum of ceruloplasmin-deficient animals (100). Hephaestin-deficient mice are affected with anemia secondary to impaired iron export from the intestine and placenta (61).

The clinical characteristics of aceruloplasminemia resemble those of patients lacking serum transferrin or primary hemochromatosis (OMIM #235200), a progressive iron overload disorder in which transferrin binding capacity is exceeded by excessive iron levels (101). Hemochromatosis in both humans and mice is caused by mutations in the genes encoding many of the iron homeostasis proteins described above [reviewed by (102)]. These include HFE,
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TfR2, HJV, hepcidin and Fpn. Most of these mutations are autosomal recessive, but mutations in the gene encoding Fpn results in an autosomal dominant disorder in humans (103;104). Mutations affecting many of the other iron homeostasis proteins, including DMT1, transferrin receptor 1 (TfR1) and ferritin, also affect iron levels in the body (102), and chronic elevation of hepcidin levels causes systemic iron deficiency (105).

The increase of ceruloplasmin, ferritin, lactoferrin and other proteins involved in iron-binding and metabolism following infection is necessary to counter the infective agent's attempt to bind iron from host tissue. Similarly, the increase in ceruloplasmin described in certain neurodegenerative disorders like AD and schizophrenia may result as a protective effort to prevent further neuronal cell injury. Many neurodegenerative diseases, including AD, Parkinson’s disease (PD), Huntington’s disease (HD; OMIM #143100), and amyotrophic lateral sclerosis (ALS; OMIM #105400) display increased brain iron accumulation that may be a disruption in the complex process of cellular iron regulation and likely contributes to neuronal cell death. Natural human variants of ceruloplasmin resulting in reduced protein function have been suggested to present a vulnerability factor for iron-induced oxidative stress in PD (25).
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**Putative Mechanism**

In humans, the characterization of disease-associated *CP* mutations suggest that the mutant proteins invariably lack ferroxidase activity. Truncated proteins caused by nonsense or splice mutations are retained in the endoplasmic reticulum (ER) and cause ER-associated stress and activation of the unfolded protein stress response (106;107). Missense mutants such as I9F and P177R (numbering does not include the signal peptide) display a folding defect that leads to total or partial retention in the ER, while G631R and G969S are secreted as inactive apoproteins lacking copper (21;106;108). Amino acid substitutions that affect copper-binding residues or residues close to copper-binding sites are expected to perturb copper incorporation, and Gly631 and Gly969 are close to type 1 copper sites in domains 4 and 6 (21). The effect of other known mutations on the structure and activity of ceruloplasmin are less predictable. For instance, R701W causes severe neurological symptoms in the heterozygous state (97) and is located in a long surface-exposed loop on the flat basal region of the protein, an area not expected to contribute to copper-binding. However, this mutation results in a protein that is unable to incorporate copper likely because of defective interaction with ATP7B. Experimentally, the substitution of basic residues in the long surface loops connecting domains 2 and 3, 4 and 5 and 6 leads to defective incorporation of copper in mammalian cells. These areas may be involved in the interaction of ceruloplasmin with ATP7B. Disruption of the loop containing Arg701 in particular appears to result in dominant-negative ceruloplasmin proteins (109).

The amino acid change that occurs in ceruloplasmin of *iron10* mice alters a residue adjacent to one of the type III copper-binding ligands. It is likely that this change alters the binding of the type III copper, thus acutely affecting the ability of ceruloplasmin to oxidize various substrates including iron. The lack of ferroxidase activity would alter iron homeostasis resulting in the serum iron deficiency observed in these mice. Furthermore, it is possible that the amino acid change may result in a copper-deficient protein that lacks stability or the protein may display a folding defect leading to retention in the ER. Both of these possibilities could result in mice with aceruloplasminemia.

**Genotyping**

*Iron10* genotyping is performed by amplifying the region containing the mutation using PCR, followed by sequencing of the amplified region to detect the single nucleotide transition.

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Primers
Iron10(F): 5’- ACAGAGGCAAGCCCAAACCTGTG -3’
Iron10(R): 5’- GAAGCATACTCAACCTGTAACCTCCTCC -3’

PCR program
1) 95°C 2:00
2) 95°C 0:30
3) 56°C 0:30
4) 72°C 1:00
5) repeat steps (2-4) 29X
6) 72°C 7:00
7) 4°C 8

Primers for sequencing
Iron10_seq(F): 5’- ACTGTGGAATCAGTACCCG -3’
Iron10_seq(R): 5’- ATCATGAATTTCCTATGCTCTG -3’

The following sequence of 761 nucleotides (NCBI Mouse Genome Build 37.1, Chromosome 3, bases 19,888,821 to 19,889,581) is amplified:

```
acagaggcaag gcccaaacagt tgcgcaatcaac taggttgtgtg cctgactaat gaatgccttc
tttctcatttt tctctgtgtct cctagagttta gccatagac ccacaaatctga tagtttttag
tgataaaag gaatgctgtct aaacttctca tttgttggtg tttctaaataa acacccacaggg
agtataacgt tctgtgtct ctgacctttt cccctggaacctt tccaaaaacc tagaaatgttt
tcccacacaag cctggaacctt ctggtacttcca ctgccccagttg actgaccatgtg tccatgcttg
agatggcaact acctacactgt tttttacagt aacacaggtt aatacaagat gaatatccag
gtattgttct cttagatataa cttgaccttcg tttgtcttttg aaaattatcttg cttttgatctc
tgcatataagg atcagacacc gcataacagt gcacctgttg gcaataacac agagcatagg
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Primer binding sites are underlined; sequencing primer binding sites are highlighted in gray; the mutated T is indicated in red.
References


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104. Montosi, G., Donovan, A., Totaro, A., Garuti, C., Pignatti, E., Cassanelli, S., Trenor, C. C.,


