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## **NOVEL TMPRSS6 MUTATIONS ASSOCIATED WITH IRON-REFRACTORY IRON DEFICIENCY ANEMIA (IRIDA)**

Luigia de Falco, Francesca Totaro, Antonella Nai, Alessia Pagani, Domenico Girelli, Laura Silvestri, Carmelo Piscopo, Natascia Campostrini, Carlo Dufour, Fahd Al Manjomi, et al.

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## NOVEL TMPRSS6 MUTATIONS ASSOCIATED WITH IRON-REFRACTORY IRON DEFICIENCY ANEMIA (IRIDA)

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Key Words:	IRIDA, TMPRSS6, iron metabolism, microcytic anemia

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For Peer Review

## MUTATION IN BRIEF

## HUMAN MUTATION

NOVEL Tmprss6 MUTATIONS ASSOCIATED  
WITH IRON-REFRACTORY IRON DEFICIENCY  
ANEMIA (IRIDA)



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2 <First Author et al.>

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7 Arosio for the gift of the anti-HJV antibody.  
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10 Short Title: IRON-REFRACTORY IRON DEFICIENCY ANEMIA (IRIDA)  
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18 **ABSTRACT: Mutations leading to abrogation of matriptase-2 proteolytic activity in humans are associated**  
19 **with an iron-refractory iron deficiency anemia (IRIDA) due to elevated hepcidin levels. In this paper we**  
20 **describe 12 IRIDA patients belonging to 7 unrelated families and identify 10 (9 novel) TMPRSS6**  
21 **mutations spread along the gene sequence: 5 missense, 1 non sense and 4 frameshift. The frameshift and non**  
22 **sense mutations are predict to result in truncated protein lacking the catalytic domain. The causal role of**  
23 **missense mutations (Y141C, I212T, R271Q, S304L and C510S) is demonstrated by in silico analysis, their**  
24 **absence in 100 control chromosomes and the high conservation of the involved residues. The C510S**  
25 **mutation in the LDLRA domain in silico model causes an intra-molecular structural imbalance that impairs**  
26 **matriptase-2 activation. We also assessed the in vitro effect on hepcidin promoter and the proteolytic**  
27 **activity of I212T and R271Q variants demonstrating a reduced inhibitory effect for the former mutation,**  
28 **but surprisingly a normal function for R271Q which appears a silent mutation in vitro. Based on mRNA**  
29 **expression studies I212T could also decrease the total amount of protein produced, likely interfering with**  
30 **mRNA stability. Collectively, our results extend the pattern of TMPRSS6 mutations associated with IRIDA**  
31 **and propose a model of causality for some of the novel missense mutations.**  
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45 **KEY WORDS: IRIDA, TMPRSS6, iron metabolism, microcytic anemia**  
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## INTRODUCTION

Iron deficiency anemia is the most common form of anemia worldwide, usually secondary to inadequate dietary intake, chronic blood loss or malabsorption. Recently a genetic recessive form of iron-refractory iron-deficiency anemia (IRIDA), due to constitutively high hepcidin levels was identified (Finberg, et al., 2008).

Hepcidin, a circulating peptide synthesized mainly by the hepatocytes, is a master regulator of systemic iron homeostasis in mammals (Wrighting and Andrews, 2008). Hepcidin negatively controls the plasma iron pool, by binding and internalizing the iron exporter ferroportin on cells that release iron to the circulation, as duodenal enterocytes, macrophages and hepatocytes (Donovan, et al., 2005; Nemeth, et al., 2004). Consistently, hepcidin-deficient mice (Nicolas, et al., 2001; Viatte, et al., 2005) and humans with hepcidin mutations (Roetto, et al., 2003) develop severe iron overload. Conversely, mice with increased transgenic expression of hepcidin in the liver manifest severe iron deficiency anemia (Nicolas, et al., 2002).

Hepcidin transcription is upregulated by iron overload and inflammation and downregulated by hypoxia, iron deficiency and erythropoiesis expansion (Nemeth, 2008). Several hepcidin inhibitors have been proposed *in vitro* and in animal models. The most important inhibitor *in vivo* is the serine protease matriptase-2, encoded by *TMPRSS6* (MIM\*609862). *Tmprss6* mutant (*Mask* and *KO*) mice show iron deficiency anemia and loss of trunk hair, because of failure to suppress hepcidin expression (Du, et al., 2008; Folgueras, et al., 2008). Matriptase-2 (MT2) is highly expressed in the liver (Velasco, et al., 2002) and represses hepcidin expression by cleaving membrane-bound hemojuvelin (m-HJV) (Silvestri, et al., 2008b), the bone morphogenetic proteins (BMPs) coreceptor, which participates in the signalling pathway of SMAD proteins (Babitt, et al., 2006). Cleaving m-HJV MT2 inhibits hepcidin expression by reducing BMP signalling.

*TMPRSS6* mutations in patients with IRIDA were firstly described by Finberg et al (Finberg, et al., 2008). Until now, nineteen cases have been characterized and reported with different geographic and ethnic distribution (Edison, et al., 2009; Finberg, et al., 2008; Guillem, et al., 2008; Melis, et al., 2008; Silvestri, et al., 2009; Tchou, et al., 2009).

In this paper we describe 12 IRIDA patients belonging to 7 unrelated families and identify 10 (9 novel) *TMPRSS6* mutations, including several in the 5' end of the gene. Our results extend the pattern of *TMPRSS6* mutations associated with IRIDA, confirm the greater severity of the disease in infancy and propose a model of causality for the novel *missense* mutations.

## MATERIALS AND METHODS

### Patients

Seven families with one or more subjects with iron deficiency anemia unresponsive to oral iron and partially responsive to parenteral iron administration were collected. The pedigrees of the families are in Supporting Fig. S1, their ethnic origin, clinical and laboratory data are in Table 1 and 2. In all families, recessive transmission was suggested by parents normal hematological phenotype, the presence of affected sibling pairs and of consanguinity in two Arabian kindreds. The probands were referred because of anemia, first diagnosed in infancy. During follow up most of them required iron treatment, were unresponsive to oral iron and showed only a partial recovery after parenteral iron administration (Supporting Table S1). As shown in Table 1 the degree of anemia was variable, microcytosis [low mean corpuscular volume (MCV)] and hypochromia [low mean corpuscular haemoglobin (MCH)] were severe, serum iron and transferrin saturation were decreased and normal to low serum ferritin levels.

Hemoglobin electrophoresis was normal and genetic tests for the common alpha and beta thalassemia mutations were negative in all patients. Acquired iron deficiency, as celiac disease and bleeding disorders, and inherited causes of microcytic anemia, as the rare mutations of genes involved in intestinal iron absorption and/or erythroid iron utilization, as transferrin (Aslan, et al., 2007), *SLC40A1* encoding ferroportin (Pietrangelo, 2004) and *SLC11A2* encoding DMT1 (Iolascon, et al., 2008) were excluded. Furthermore, since haploinsufficiency of transferrin receptor 1 (*TFRC*) in *Tfrc +/-* mice causes iron deficiency (Levy, et al., 1999), mutations were excluded also in *TFRC*.

After informed consent, provided according to the Declaration of Helsinki, blood was obtained for biochemical tests and genetic analysis from probands and available family members. Blood obtained after informed consent from healthy subjects was processed within 24 hours. These studies were approved by the institutional review board of Federico II University Medical School in Naples.

## 4 &lt;First Author et al.&gt;

**Hepcidin assay**

Serum and urinary hepcidin were measured by means of recently validated a mass spectrometry-based approach, i.e. SELDI-TOF-MS using a PBSCIIc mass spectrometer, copperloaded immobilized metal-affinity capture ProteinChip arrays (IMAC30-Cu<sup>2+</sup>), and a synthetic hepcidin analogue (hepcidin-24, Peptides International, Louisville, KY) as an internal standard, as described in detail elsewhere (Swinkels, et al., 2008; Valenti, et al., 2009).

**DNA sequence analysis**

Anticoagulated (EDTA-treated) blood samples were obtained and stored at -20°C. Genomic DNA was isolated by the QIAmp DNA Blood Mini Kit (Promega Corporation, Madison, WI), according to the manufacturer's instructions.

To analyze *TMPRSS6* gene all coding exons and splice junctions were amplified by PCR and amplified fragments were directly sequenced. The *TMPRSS6* genomic sequence from GenBank accession numbers NC\_000022.9 was used as reference sequence. Detailed protocols and primer sequences are available on request. The amplified products were isolated by electrophoresis on 1% agarose gel and purified using the QIAamp purification kit (Qiagen, Valencia, CA). Direct sequencing was performed using a fluorescence-tagged dideoxy chain terminator method in an ABI 3100 automated sequencer (Applied Biosystem, Foster City, CA), according to the manufacturer's instructions.

**TMRSS6 expression analysis**

Total RNA was prepared from PBMCs using the RNA extraction kit (PreAnalytix; Qiagen) and DNaseI (Invitrogen, Carlsbad, CA) to eliminate contaminating genomic DNA. Total RNA (2µg) was reverse transcribed in a 20µL reaction using Superscript III reverse transcriptase (Superscript® VILO™ cDNA synthesis kit, Invitrogen). The *TMRSS6* cDNA from GenBank accession number NM\_153609.2 was used as a reference sequence, where the A of the ATG translation initiation site represents nucleotide +1.

Quantitative real-time-PCR (qRT-PCR) was performed by EXPRESS 2X qPCR SuperMix (Invitrogen) by using Applied Biosystems Model 7900HT Sequence Detection System. Real-time PCR primers for each gene were designed using Primer Express software version 2.0 (Applied Biosystems). The primer sequences are available upon request. PCR reactions were performed in triplicate. *TMRSS6* gene expression was calculated by using the 2-ΔΔCt method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches the detection threshold (Livak and Schmittgen, 2001). The ΔCt was computed by calculating the difference of the average Ct between the *TMRSS6* gene and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The 2-ΔΔCt is the amount of the patients' RNA relative to 10 healthy controls. The data are presented as mean ± the standard error (SE). The results were obtained on RNA samples prepared from 2 distinct PBMC samples.

**Bioinformatics analysis of TMRSS6 mutations**

By using PROGRAMM blastn-SNP ([http://www-btls.jst.go.jp/cgi-bin/Homology\\_Blast-SNP/submission\\_v3.cgi?PROGRAM\\_blastn-SNP](http://www-btls.jst.go.jp/cgi-bin/Homology_Blast-SNP/submission_v3.cgi?PROGRAM_blastn-SNP)), we investigated if these nucleotide changes corresponds to a previously identified SNP. In addition the identified *TMRSS6* mutations were ruled out as common polymorphic changes by sequencing the corresponding exons in 50 healthy individuals (100 chromosomes) with normal haematological indices.

In addition, we compared the region containing mutations from five different species at the Blocks website (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>), where human matriptase-2 reference sequence is NP\_705837.1.

The involvement of the identified missense mutations on RNA processing was assessed by using ESEfinder at <http://rulai.eshl.edu/tools/ESE/> and RESCUE-ESE at <http://genes.mit.edu/burgelab/rescue-ese> (Cartegni, et al., 2003; Fairbrother, et al., 2002). The possible impact of the amino acid substitution on the structure or function

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3 protein was predicted *in silico* by using the two web server tools Poliphen (<http://www.bork.embl-heidelberg.de/PolyPhen/>) and SIFT (<http://sift.jcvi.org/>) (Ferrer-Costa, et al., 2005; Ng and Henikoff, 2003).

4  
5 PolyPhen (=Polymorphism Phenotyping) (<http://genetics.bwh.harvard.edu/pph/>) is an automatic tool for  
6 prediction of possible impact of an amino acid substitution on the structure and function of a human protein. This  
7 prediction is based on straightforward empirical rules which are applied to the sequence, phylogenetic and  
8 structural information characterizing the substitution.

9  
10 Automatic mode of Swiss model workspace (<http://swissmodel.expasy.org/>) (Arnold, et al., 2006) was used to  
11 predict tridimensional structure of matriptase-2 wild type and mutants.

## 12 13 Functional studies

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15 The full-length human *TMPRSS6* cDNA in pcDNA3.1 was a kind gift of Prof. Carlos Lopez-Otin - Universidad  
16 de Oviedo, Spain. *TMPRSS6*<sup>I212T</sup> and *TMPRSS6*<sup>Q271R</sup> variants were obtained by mutagenesis of wild type cDNA  
17 using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's  
18 protocol. Expressing vectors encoding HJV was as previously described (Silvestri, et al., 2007).

19 HeLa and Hep3B cells were cultured as described (Silvestri, et al., 2007). Western blot analysis and the rabbit  
20 anti-HJV polyclonal antibody were as described (Silvestri, et al., 2009). Anti-FLAG antibody was from Santa Cruz  
21 Biotechnology (Santa Cruz, CA). Matriptase 2 cleavage activity was determined by analyzing soluble HJV in  
22 culture media after transfecting HeLa cells with HJV, in the presence of *TMPRSS6* wt or mutant cDNA. Cells were  
23 incubated in serum-free media, the supernatants were collected after 24 hours and concentrated using Amicon  
24 Ultra 3 kDa cut off (Millipore, Billerica, MA), cells were lysed in Ripa Buffer and 50 µg of total protein were  
25 analyzed by western blot.

26 Hep3B cells transiently transfected with 0.25 µg pGL2-basic reporter vector (Promega, Madison, WI, USA)  
27 containing the 2.9 Kb fragment of the human hepcidin promoter (Hep-Luc) in combination with pRL-TK Renilla  
28 luciferase vector (as a control of transfection efficiency, Promega) and 0.01 µg/ml of cDNA encoding wild type or  
29 mutant *TMPRSS6* with and without 0.05 µg/ml of HJV construct as described (Pagani, et al., 2008; Silvestri, et al.,  
30 2008a). Relative luciferase activity was calculated as the ratio of firefly (reporter) to renilla luciferase activity and  
31 expressed as a multiple of the activity of cells transfected with the reporter alone. Experiments were performed in  
32 triplicate.

## 33 34 RESULTS

### 35 36 Patient phenotype

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38 The hematologic data indicating iron deficiency anemia unresponsive to oral iron, with partial recovery after  
39 parenteral iron administration, were suggestive of IRIDA.

40 To further characterize the phenotype we measured serum hepcidin in almost all (Table 1). Most patients (A  
41 II1; C II1, II2, II3; E II1, II2; F II2, II3) have hepcidin levels above the normal range (4.3-7.06 nM), whereas the  
42 remaining patients (B II1; D II1; F II1) have inappropriately normal hepcidin values, considering that in iron  
43 deficiency due to causes other than IRIDA hepcidin levels are consistently reduced or undetectable ((Ganz, et al.,  
44 2008) and Table 1).

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Table 1: Clinical data of IRIDA patients.

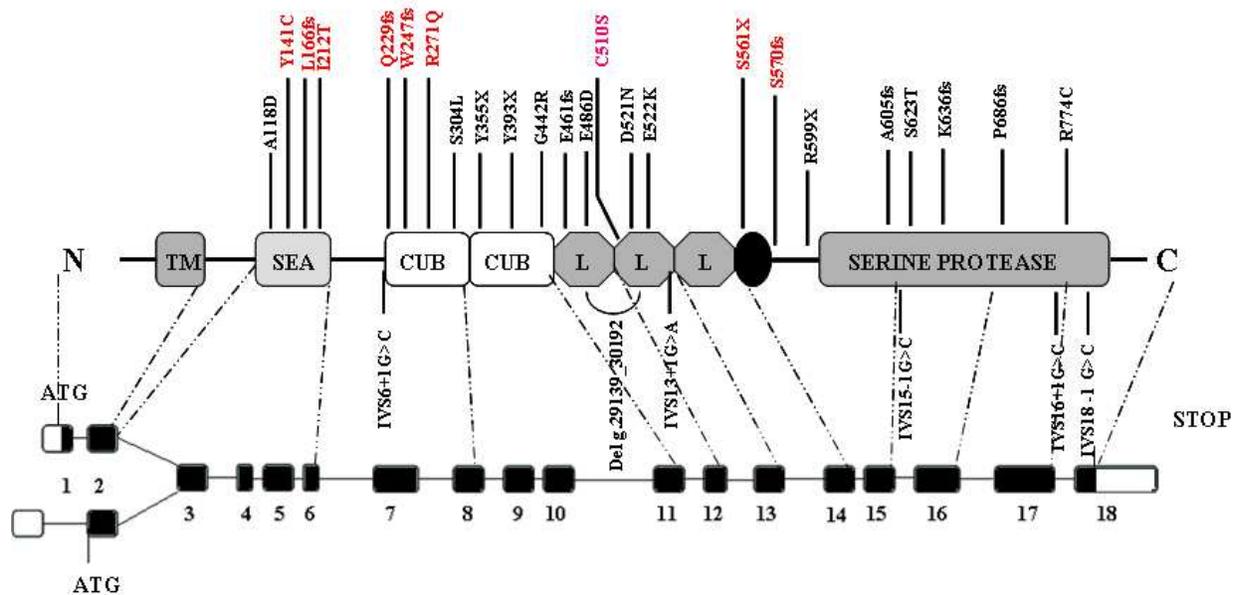
	Normal values (range)		A II-1	B II-1	C II-1	C II-2	C II-3	D II-1	E II-1	E II-2	F VI-1	F VI-2	F VI-3	G II-1
	m	f.												
Age, years/sex			8/M	5/F	7/F	5/F	3/M	3/F	8/F	11/F	6/M	8/F	2/F	9/M
Hb, g/dL	12.0-17.5	12.0-16.0	9.1	9.5	10.6	10.4	9.8	6.6	6.8	8.9	8.01	8.83	7.93	10.4
RBC, x 10 <sup>6</sup> /μL	4.2-5.6	4.0-5.4	5.3	4.70		5.0	5.49	5.28	4.66					5.3
MCV, fL	80-97		60	62.8	62.8	68	65	47	58.8	59.8	46.3	53.3	49.3	63.5
MCH, pg	25-34		17	20.2	17.8	18	16.7	12	14.5	18.5	14.3	15.9	14.9	19.6
MCHC, g/dL	32-37		29	32.2		30.6	27	26	24.7	31	30.9	29.8	30.2	
Reticulocyte count, x 10 <sup>3</sup> /μL	20-120		50	60		68	64	40	120					42.4
PLT, x 10 <sup>3</sup> /μL	130-400			420		383	410	740	647	406	778	526	592	
Serum Ferritin, μg/L	18-370	9-120	26	25	112	32	50	10	8	19	86	101	37.7	228
Serum iron, μg/dL	16-124		14	14	21	48	22	8	13	9	20	20	40	17
Transferrin saturation, %	15-35		3.7	3.7	5	9.4	6.2	2.3	4.2		3.1	3.3		5
Serum Epcidin <sup>a,*</sup> , nM	3-7		9.78	5.57	17.77	8.92	7.55	5.78	12.99	10.41	5.63	7.16	17.6	

a Reference range: n=57 normal individuals (median 4.7); b Reference range: normal individuals;

\* Values in iron deficiency anemia are 0,04-0,12 nM.

#### Identification and characterization of novel *TMPRSS6* mutations in IRIDA patients

Screening for mutations of *TMPRSS6* coding sequence and exon-intron junctions of DNA from IRIDA patients identified nine novel and one known mutations, spread along the gene sequence: five mutations were missense (Y141C, I212T, R271Q, S304L, C510S), one non sense (S561X) and four frameshift (L166fs, Q229fs, W247 (Fig.1 and Table 2).



**Figure 1:** Schematic representation of the TMPRSS6 gene and protein. Novel mutations are reported in red, known mutations are in black. N: .amino-terminus, C: carboxy-terminus. TM: transmembrane domain. SEA: sea urchin sperm protein, enteropeptidase agrin. CUB: complement protein subcomponents C1r/C1s, urchin embryonic growth factor and bone morphogenic protein 1 domain. L: low density lipoprotein receptor class A domain (LDLR). Serine Protease: serine protease domain. Black oval: cleavage activation site.

Table 2: TMPRSS6 genotype and haematological data at presentation.

Family ID	Patient number	Mutation 1 Nucleotide/amino acid change	Mutation 2 Nucleotide/amino acid change	Ethnicity	Consanguinity Yes/No	Age at diagnosis (Years)	RBC*10 <sup>12</sup> /L	Hbat diagnosis	MCV at diagnosis	Transferrin saturation (%)
A	II-1	c.536A>G(p.Y141C)	c.536A>G(p.Y141C)	Indian	n.a.	16mo	5,3	9,1	60	4
B	II-1	c.749T>C(p.I212T)	c.926G>A (p.R271Q)	Italian	N	3	4,88	8	58	5
C	II-1	c.1025C>T (p.S304L)	c.1025C>T (p.S304L)	Arabian	Y	6	n.a.	8	55	4
	II-2	c.1025C>T (p.S304L)	c.1025C>T (p.S304L)	Arabian	Y	4	5,1	8,5	60	4
	II-3	c.1025C>T (p.S304L)	c.1025C>T (p.S304L)	Arabian	Y	2	5,7	8	54	4
D	II-1	c.611delC (p.L166fs)	c.790delG(p.Q229fs)	Austrian	N	3	5,47	7,1	51	2,2
E	II-1	c.855delG(p.W247fs)	c.855delG(p.W247fs)	Greek	Y	2,5	n.a.	5,8	51	1,2
	II-2	c.855delG(p.W247fs)	c.855delG(p.W247fs)	Greek	Y	2	4,66	5,4	50	2
F	II-1	c.1796C>A (p.S561X)	c.1796C>A (p.S561X)	Arabian	Y	7	n.a.	8,83	53,3	3,3
	II-2	c.1796C>A (p.S561X)	c.1796C>A (p.S561X)	Arabian	Y	5	n.a.	8,01	46,3	3,1
	II-3	c.1796C>A (p.S561X)	c.1796C>A (p.S561X)	Arabian	Y	2	n.a.	7,93	49,3	n.a.
G	II-1	c.1642C>A (p.C510S)	c.1822_1823 insCC (p.S570fs)	Algerian	N	1,8	5,3	10,4	63,5	5

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All mutations segregated correctly within the families (Supporting Fig. S1).

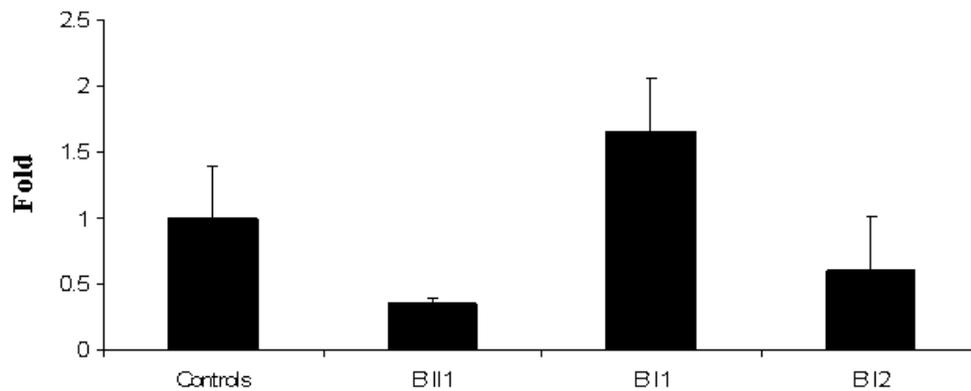
By blastn-SNP analysis we excluded that these changes correspond to previously identified SNPs. None of the disease-associated variant was present in 100 control chromosomes analyzed by direct sequencing (data not shown). In addition protein sequence analysis of MT2 (protein ID Q8IU80) from five species showed that the missense mutations (Y141C, I212T, R271Q, S304L and C510S) affect residues highly conserved across species (Supporting Fig. S2), indicating an important role of the replaced amino acids for MT2 function.

### *In silico and in vitro studies*

Non sense/frameshift mutations (L166fs, Q229fs, W247fs, S561X, L570fs) are predicted to produce either null proteins or variants that, in analogy with the *Mask* allele, lack the serine protease domain (Supporting Fig. S3).

Missense mutations might in theory interfere with the correct RNA splicing, mRNA stability or protein expression/activity or tridimensional structure.

Since we found that *TMPRSS6* gene is weakly expressed in peripheral blood mononuclear cells (PBMC), we studied its mRNA expression in PBMC of patients in comparison with normal subjects to validate the bioinformatic data. *TMPRSS6* mRNA levels were normal in patients carrying missense mutations (not shown), except in patient BII1, who showed remarkably lower levels than controls (Fig. 2). Quantitative RT-PCR of *TMPRSS6* expression showed significantly decreased levels (mean  $0.36 \pm 0.03$ ) in BII1 compared with controls (mean  $1.00 \pm 0.4$ ;  $P < .01$  by 2-tailed  $t$  test; Fig. 2). Family analysis of *TMPRSS6* mRNA showed a trend towards a decreased expression in BI-2 carrier of I212T, suggesting that this mutation is responsible of the impaired RNA expression.

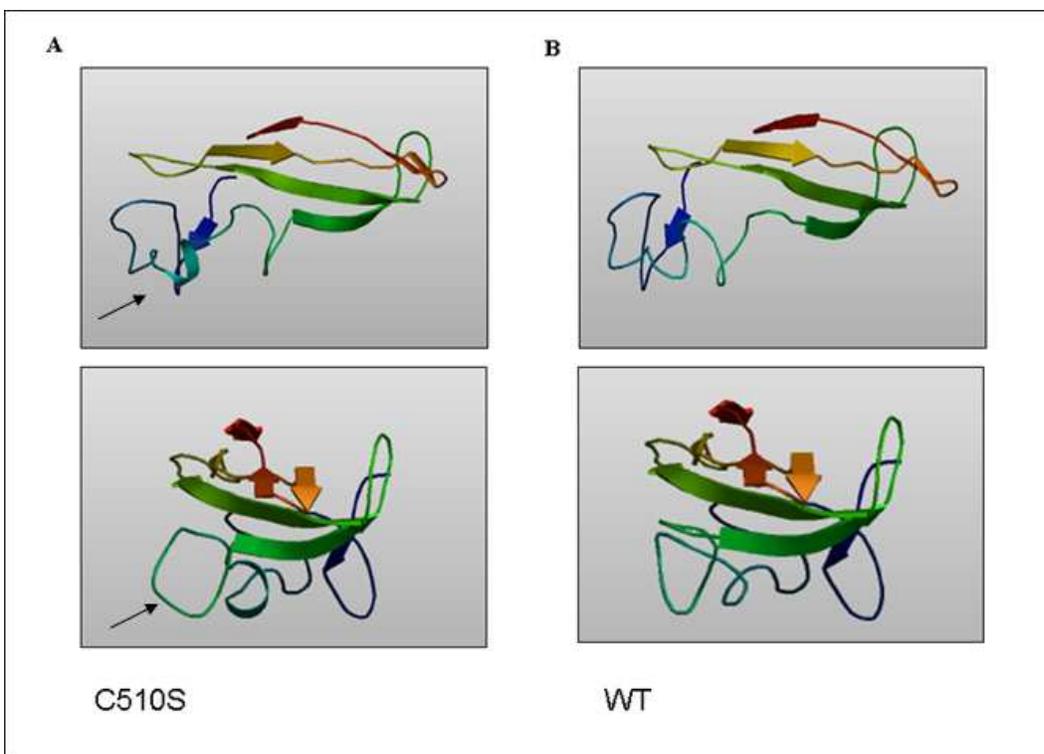


**Figure 2:** Relative gene expression of *TMPRSS6* by qRT-PCR showing significant decrease in proband B III compared to controls. Error bars represent the standard deviations (mean  $0.36 \pm 0.03$  compared with healthy subjects, mean  $1.00 \pm 0.4$ ;  $P < .01$  by 2-tailed  $t$  test).

To elucidate the possible implications of the missense mutations, that affect highly conserved amino acids, we first evaluated their impact on matriptase-2 function by in silico tools. The substitution of I212T, R271Q and

C510S were predicted not to be tolerated on protein by the SIFT program. Furthermore, all substitutions, except I212T, were predicted to be “damaging” by the multiple criteria software Polyphen.

C510S was also predicted to alter the protein tridimensional structure by Swiss model program (Fig. 3). C510S in LDLRA domain might disrupt the conserved disulphide bond linking the pro- and catalytic domains that likely maintains matriptase-2 membrane-bound. For this reason we performed a modelling of both wild type (Fig. 3B) and mutant (Fig. 3A) MT2 LDLRA domains. Interestingly, the wild type minimized models show a significant spatial shift at the auto-activation loop of this domain (Fig. 3B) that was not found in the mutant form. The results suggest that C510S may affect LDLRA domain folding leading to structural destabilization.

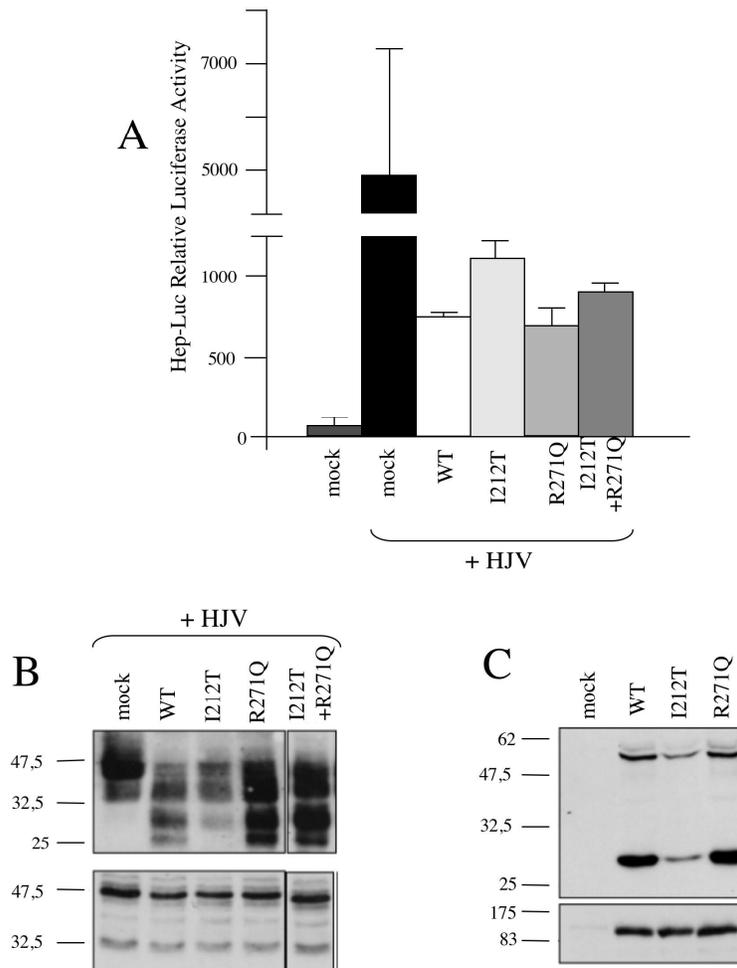


**Figure 3:** Predicted structural consequences of the LDLRA domain mutation. Wild type and mutant protein structures have been modelled by Automated mode of Swiss-Model. The modelling pipeline automatically selects suitable templates based on a Blast E-value limit, which can be adjusted upon submission ([Altschul et al.](#)). The automated template selection will favour high-resolution template structures with reasonable stereochemical properties as assessed by ANOLEA mean force potential ([Melo et al.](#)) and Gromos96 force field energy ([van Gunsteren et al.](#)).

Y141C and I212T affect SEA, R271Q and S304L CUB1 domain (Fig. 1). We performed *in vitro* functional studies testing the ability of mutants to inhibit the hepcidin promoter activation and to cleave hemojuvelin from plasma membrane in I212T and R271Q, as representative variants of SEA and CUB domain respectively.

10 &lt;First Author et al.&gt;

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4 In the hepcidin promoter luciferase assay I212T mutant showed reduced inhibitory activity, whereas R271Q  
5 inactivates hepcidin promoter as the wild type protein (Fig. 4A). Consistently I212T was less efficient in cleaving  
6 m-HJV (Fig. 4B) and in releasing specific MT2 cleavage fragments in the culture media (Fig. 4C), whereas  
7 R271Q behaves as the wild type protein in both assays.  
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**Figure 4:** In vitro studies of SEA and CUB mutations. A: Hepcidin promoter response by HJV, in the presence of *TMPRSS6* WT and mutants. A firefly luciferase reporter driven by 2.9 kb of the proximal hepcidin promoter was cotransfected into Hep3B cells with Renilla luciferase vector pRL-TK, either alone or with HJV and/or *TMPRSS6* expressing vectors. Relative luciferase activity is calculated as reported in material and method and expressed as a multiple of the activity of cells transfected with the reporter alone. Experiment was made in triplicate.

B and C: HeLa cells were transfected with HJV in the presence of the empty vector (mock), *TMPRSS6*<sup>WT</sup> (WT), *I212T* (I212T), *R271Q* (R271Q) and both mutants (I212T+R271Q). Concentrated media (upper panels) and whole cell extracts (lower panels) were loaded onto a 10% SDS-PAGE and processed for western blot analysis. Anti-HJV (B) and anti-FLAG (C) were used to detect HJV and *TMPRSS6* respectively. Scales refer to relative molecular mass in kilodaltons.

## DISCUSSION

The role of the proteolytic enzyme matriptase-2 in iron metabolism was first demonstrated in murine models. *Mask* homozygotes (Du, et al., 2008) and *Tmprss6* null mice (Folgueras, et al., 2008) are slightly smaller than their normal littermates and show microcytic anemia with low plasma iron levels and depleted iron stores. The inhibitory effect of *TMPRSS6* on the hepcidin promoter is ascribed to the cleavage of HJV from plasma membrane (Silvestri, et al., 2008b). Very recently, this model was supported by crossing *Mask* mice, lacking *Tmprss6* protease domain, with *Hjv*-deficient mice (Truksa, et al., 2009). Consistent with a genetic interaction between *Hjv* and *Tmprss6* the phenotype of *Mask* is partially corrected in *Mask, Hjv*+/+ and fully corrected in *Mask, Hjv* -/-. This finding supports *Hjv* as a major substrate for MT2 cleavage activity and suggests that IRIDA is due to the persistence of the BMP coreceptor hemojuvelin on cell surface.

The patients we have studied have all the features of IRIDA, especially the inability to respond to oral iron and the partial response to parenteral iron administration, which are due to inappropriately high hepcidin levels. The age of our patients strengthens that the degree of anemia is more evident in infancy than during adult life, as preliminary observed (Melis, et al., 2008).

Mutations previously identified in IRIDA patients (in black in Fig.1) are heterogeneous and include frame-shift, splicing, missense and nonsense (Edison, et al., 2009; Finberg, et al., 2008; Guillem, et al., 2008; Melis, et al., 2008; Ramsay, et al., 2009; Silvestri, et al., 2009; Tchou, et al., 2009). We here describe 10 further different causal mutations that add further heterogeneity to the molecular genetics of IRIDA. All these mutations appear the results of sporadic and independent events, suggesting the absence of founder effect in this disorder.

The frameshift and non sense mutations result in loss of the protein or of its catalytic domain. The causative role of the *missense* changes is inferred by the *in silico* analysis, their absence in 100 control chromosomes, by the highly evolutionary conservation of the replaced residues and by *in vitro* functional studies.

The functional consequences of mutations in serine protease, LDLRA and CUB domains were recently characterized (Silvestri, et al., 2009; Silvestri, et al., 2008b). Two mutations (D521N e E522K) in the second LDLRA were shown to be defective in their ability to activate hepcidin, to cleave m-HJV and to release soluble MT-2 (Silvestri, et al., 2009), as a serine protease mutation was (Silvestri, et al., 2008b). In agreement homology models of C510S, which occurs in LDLRA2 close to the mutations at position 521 and 522, displayed a structural imbalance as compared to wild type (Fig. 3). This mutation disrupts a disulfide bond that likely maintain matriptase-2 membrane bound and, as a consequence, impairs enzyme activation. A single CUB2 mutant studied, G442R, was partially defective in hepcidin activation and cleavage activity (Silvestri, et al., 2009) and A118D, the single SEA studied mutation, was found to cause an intra-molecular structural imbalance that correlates with the *in vitro* disruption of enzyme activation and release of matriptase-2 (Ramsay, et al., 2009). Since the effect of SEA mutation on HJV was not explored and the 442 CUB mutation had a mild effect we assessed the *in vitro* effect on hepcidin promoter and the cleavage activity of I212T and R271Q variants that were present in the same patient (proband B). Mutant I212T shows a reduced inhibitory function in the promoter assay and a partial activity on HJV cleavage. Surprisingly R271Q appears a silent mutation, both in the luciferase and in HJV cleavage assay. However, coexpressing the two mutants we observed an intermediate effect on hepcidin inhibition (Fig. 4A), but not in hemojuvelin cleavage assessed by western blot (Fig. 4B), likely due to a lower sensitivity of the latter technique. Based on mRNA expression studies I212T could also decrease the total amount of protein produced, likely interfering with mRNA stability. Either the functional tests cannot detect a functional defect resulting from the aminoacid substitution or the protein is expressed in lower amounts in the liver of the patients for whatever reasons (splicing abnormality, unstability of the protein...). We hypothesize that R271Q mutation in association with I212T *in vivo* is responsible of the IRIDA phenotype. In agreement with these findings the uncommon MT2 R446W polymorphism was proposed to probably contribute to iron deficiency anemia when carried in trans with a severe *TMPRSS6* mutation (Beutler, et al., 2009).

*TMPRSS6* is emerging as a gene extremely relevant in iron metabolism, since its polymorphic variations are associated in genome wide association studies with hemoglobin level and erythrocytes parameters in different populations (Benyamin, et al., 2009; Chambers, et al., 2009; Ganesh, et al., 2009; Soranzo, et al., 2009; Tanaka, et al., 2009). These findings suggest that variations of *TMPRSS6* even at the heterozygous state, might differentially

12 <First Author et al.>

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3 modulate hepcidin production, that might explain the great variability on iron absorption in physiologic and  
4 pathologic conditions.  
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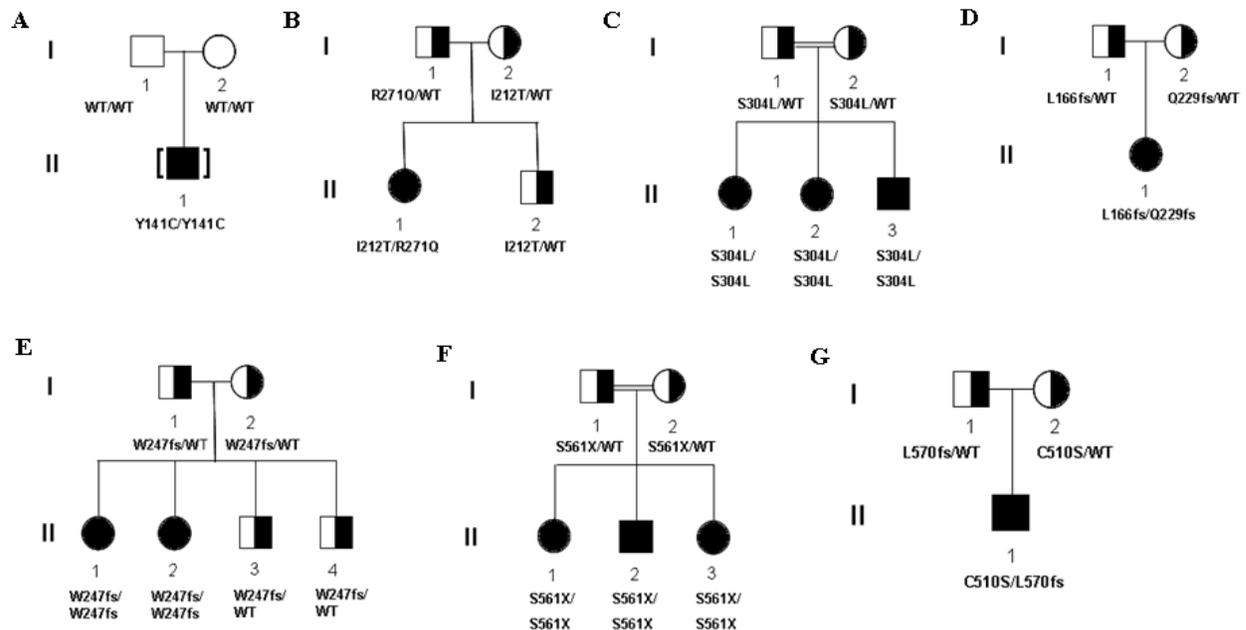
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21 SUPPLEMENTAL MATERIAL

Figure 1S: Family pedigree of the twelve subjects affected by IRIDA. *TMPRSS6* mutations identified by automated sequencing are displayed under the pedigree: open symbols, not affected; closed symbols, affected.

SEA domain (Y141C)

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Human	R	L	G	T	Y	Y	N	S	S	S	V	<b>Y</b>	S	F	G	E	G	P	L	T	C	F	F	152
Macaque	R	L	G	T	Y	Y	N	S	S	S	V	<b>Y</b>	S	F	G	E	G	P	L	T	C	F	F	150
Dog	R	L	G	T	Y	Y	N	S	S	S	V	<b>Y</b>	S	F	G	E	G	P	L	T	C	F	F	141
Cow	P	L	G	T	Y	Y	N	S	S	A	V	<b>Y</b>	S	F	G	E	G	P	F	T	C	F	F	260
Mouse	R	L	G	T	Y	Y	N	S	S	S	V	<b>Y</b>	S	F	G	E	G	P	L	T	C	F	F	154
Rat	R	L	G	T	Y	Y	N	S	S	S	I	<b>Y</b>	A	F	G	E	G	P	L	I	C	F	F	154

SEA domain (I212T)

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Human	G	L	V	I	L	E	A	S	V	K	D	<b>I</b>	A	A	L	N	S	T	L	G	C	Y	R	223
Macaque	G	L	V	I	L	E	A	S	V	K	D	<b>I</b>	A	A	L	N	S	T	L	G	C	Y	R	212
Dog	G	L	V	I	L	E	A	S	V	K	D	<b>I</b>	V	A	L	N	S	T	L	G	C	Y	R	212
Cow	G	L	V	I	L	E	A	S	V	K	D	<b>I</b>	V	A	L	N	S	T	L	G	C	Y	R	331
Mouse	G	L	V	I	L	E	A	S	V	N	D	<b>I</b>	V	V	L	N	S	T	L	G	C	Y	R	223
Rat	G	L	V	I	L	E	A	S	V	N	D	<b>I</b>	V	V	L	N	S	T	L	G	C	Y	R	223

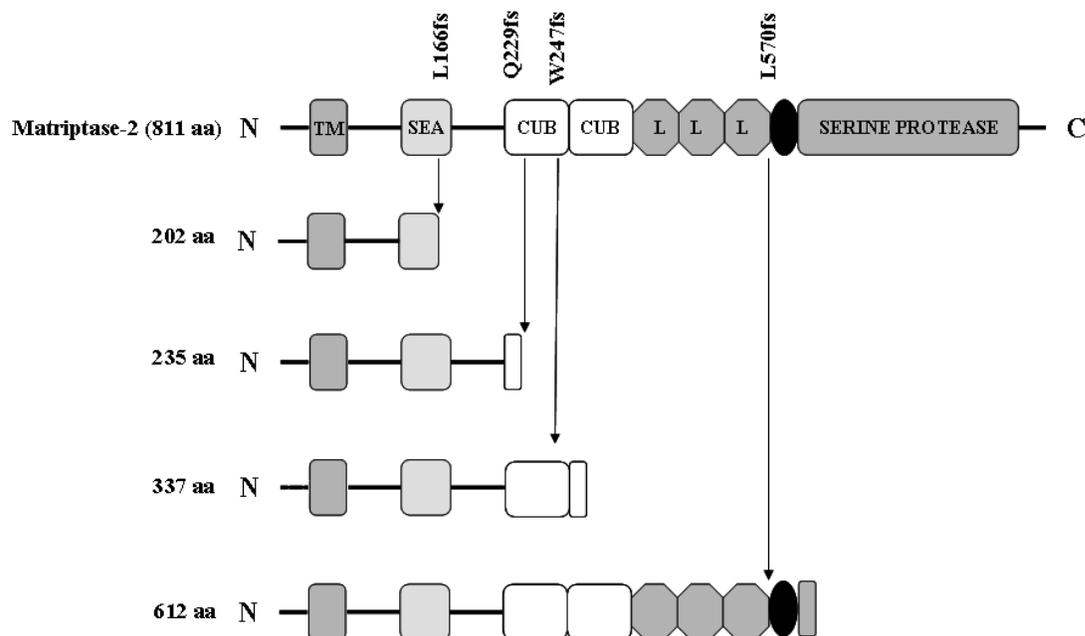
CUB1 domain (R271Q)

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Human	R	L	E	W	T	L	A	E	C	R	D	<b>R</b>	L	A	M	Y	D	V	A	G	P	L	E	282
Macaque	R	L	E	W	T	L	A	E	C	R	D	<b>R</b>	L	A	M	Y	D	V	A	G	P	L	E	271
Dog	R	L	E	W	T	L	A	E	C	R	D	<b>R</b>	L	A	M	Y	D	V	A	G	P	L	E	271
Cow	R	L	E	W	K	L	A	D	C	R	D	<b>R</b>	L	A	M	Y	D	V	A	G	P	L	E	390
Mouse	R	L	E	W	T	R	V	D	C	R	D	<b>R</b>	L	A	M	Y	D	A	A	G	P	L	E	282
Rat	Q	L	E	W	T	R	V	D	C	R	D	<b>R</b>	L	A	M	Y	D	A	A	G	P	L	E	282

**Figure 2S:** Alignment analysis of the amino acid sequences of *TMPRSS6* (Matriptase-2) from five different species, showing complete conservation of the identified mutated residue (boxed). Sequences were obtained from the following GenBank entries: Human NP\_705837; Macaque XP\_001085319; Dog XP\_531743; Cow XP\_871580; Mouse NP\_082178; Rat XP\_235768.

16 &lt;First Author et al.&gt;



**Figure 3S:** Schematic representation of the TMPRSS6 protein showing predicted protein truncation caused by frameshift mutations. The frameshift mutations result in loss of the protein or of its catalytic domain.

**Table S1:** Red cell indices and other laboratory data in three patients before and after oral treatment with iron sulfate and before and after intravenous treatment with iron gluconate.

	Patient DII-1 (age: 3 years)		Patient CII-3 (age: 3 years)		Patient CII-2 (age: 5 years)		Patient AII-1 (age: 3 years)	
	Intravenous Iron		Intravenous Iron		Intravenous Iron		Intravenous Iron	
	Before Treatment	After Treatment						
Hb, g/dL	8.3	9.5	9.8	11	10.4	11.6	9.1	10.7
MCV, fL	52	58	65	66.4	68	71.8	60	60
MCH, pg	15	16	16.7	18	18	19	17	18
Serum Ferritin, µg/L	15	74	50	113	32	133	26	25
Serum iron, µg/dL	12	14	22	34.2	48	48	14	18
Transferrin saturation, %	3	3.7	6.2	10.2	9.4	15.8	3.7	4.5