The SLC40A1 R178Q mutation is a recurrent cause of hemochromatosis and is associated with a novel pathogenic mechanism

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Methods

Plasmid constructs

The wild-type FPN1-V5 plasmid construct was generated by cloning full-length human *SLC40A1* cDNA (Genbank accession number NM_0414585.5) into the pcDNA3.1-V5-His-TOPO vector (ThemoFisher Scientific). The same strategy was adopted to generate the HLA(A)-V5 plasmid construct. All ferroportin mutations were introduced in the pcDNA3.1-FPN/V5 vector by using the QuickChange Site-Directed mutagenesis kit, according to the manufacturer's instructions (Agilent Technologies). Sequencing analyses were performed to check the integrity of all the plasmid constructs (full length *SLC40A1* cDNA sequenced after each site-directed mutagenesis).

CULTURE AND TRANSFECTION OF HUMAN EPITHELIAL KIDNEY (HEK)293T CELLS

HEK293T cells, from the American Type Culture Collection, were incubated at 37°C in a 5% CO_2 humidified atmosphere and propagated in Dulbecco's modified Eagle's medium (DMEM; Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum and 5 µg/mL plasmocin (InvivoGen). Cells were transiently transfected using JetPEI (Polyplus), according to the manufacturer's instructions, and a 2:1 transfection reagent (µL)/plasmid DNA ratio (µg).

ISOLATION OF CELL SURFACE PROTEINS AND WESTERN BLOT ANALYSIS

Membrane proteins were biotinylated and purified using the Pierce Cell Surface Protein Isolation Kit, according to the manufacturer's instructions (ThemoFisher Scientific). Western blot analysis was performed using mouse horseradish peroxidase-conjugated monoclonal antibody against V5 (ThemoFisher Scientific). The membranes were incubated with the Luminata Forte substrate, according to the manufacturer's instructions (Merck-Millipore), and digitized for pattern analysis using the GeneGnome system (Syngene).

DENSITOMETRY STUDY

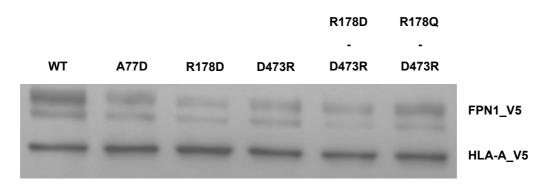
To quantitate ferroportin expression levels on the cell surface, the optic density of SLC40A1/V5 and HLA-A/V5 protein bands was measured on digital images by the GeneTools software (version 4.4, Syngene). Two normalizations were performed: ferroportin expression was first normalized with cotransfected HLA(A); then, wild-type ferroportin

expression was set at 100% and the expression of ferroportin variants was normalized to the expression of wild-type ferroportin.

55-FE RELEASE MEASUREMENTS

⁵⁵Fe loading of human apotransferrin was performed as previously described ¹⁸. For iron release experiments, a modification of the protocol described by Schimanski *et al.* ¹⁹ was used. Briefly, HEK293T cells were seeded at 1 x 10⁵cells per well in 12-well plates, grown for 8h in supplemented DMEM, and preloaded with 20 μg/ml ⁵⁵Fe-transferrin for 24h before transfection with wild-type or mutated FPN1-V5 plasmid constructs for 15h. Cells were washed once with PBS and cultured in Pro293a-CDM serum-free medium (BioWhittaker) for up to 36h. ⁵⁵Fe exported into the supernatant was collected at various time points, mixed with liquid scintillation fluid (Ultima Gold MV, Packard Bioscience) and counted for 10min in a TRI-CARB 1600 CA scintillation counter (Packard). Percentage ⁵⁵Fe export was calculated using the following formula: (⁵⁵Fe in the supernatant at each time point, divided by cellular ⁵⁵Fe at time zero) x 100.

Supplementary Figure 1.



Cell surface expression of ferroportin mutants (%)

