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

Chandran Ka,[1,2,3]* Julie Guellec,[1,3,4]* Xavier Pepermans,[5] Caroline Kannengiesser,[3,6,7] Cécile Ged,[7,8] Wim Wuyts,[9] David Cassiman,[10] Victor de Ledinghen,[11] Bruno Varet,[12] Caroline de Kerguenec,[13] Claire Oudin,[6] Isabelle Gourlaouen,[1,3] Thibaud Lefebvre,[6] Claude Férec,[1,2,7] Isabelle Callebaut[14] and Gérald Le Gac[1,2,3,7]

[1]UMR1078, INSERM, Université Bretagne Loire – Université de Bretagne Occidentale, Etablissement Français du Sang – Bretagne, Institut Brestois Santé-Agro-Matière, Brest, France; [2]Laboratoire de Génétique Moléculaire et Histocompatibilité, CHRU de Brest, Hôpital Morvan, France; [3]Laboratory of Excellence GR-Ex, Paris, France; [4]Association Gaetan Saleun, Brest, France; [5]Center for Human Genetics, University Hospital of St-Luc, Brussels, Belgium; [6]UMR1149, INSERM, Centre de Recherche sur l'Inflammation, Université Paris Diderot, AP-HP, Hôpital Bichat, Département de Génétique, France; [7]On behalf of the French National Network for the Molecular Diagnosis of Inherited Iron Overload Disorders (J. Rochette, E. Cadet, C. Kannengiesser, H. Puy, C. Ged, H. de Verneuil, G. Le Gac, C. Férec, S. Pissard, V. Gérolami), Brest, France; [8]INSERM U1035, BMGIC, CHU de Bordeaux, Laboratoire de Biochimie et Biologie Moléculaire, France; [9]Department of Medical Genetics, University and University Hospital of Antwerp, Edegem, Belgium; [10]Department of Gastroenterology-Hepatology and Metabolic Center, University Hospital of Leuven, Belgium; [11]Department of Gastroenterology and Digestive Oncology, University Hospital of Bordeaux, France; [12]Université Paris Descartes et AP-HP, Hôpital Necker, Service d'Hématologie, France; [13]AP-HP, Hôpital Beaujon, Département d'Hépatologie, Clichy, France and [14]UMR7590, CNRS, Sorbonne Universités, Université Pierre et Marie Curie-Paris, France

**CK and JG contributed equally to this work*

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Correspondence: gerald.legac@univ-brest.fr

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

^{55}Fe 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 10^5cells per well in 12-well plates, grown for 8h in supplemented DMEM, and preloaded with 20 µg/ml ^{55}Fe-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. ^{55}Fe 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 ^{55}Fe export was calculated using the following formula: (^{55}Fe in the supernatant at each time point, divided by cellular ^{55}Fe at time zero) x 100.

Supplementary Figure 1.