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RED CELLS, IRON, AND ERYTHROPOIESIS

Constitutional PIGA mutations cause a novel subtype of hemochromatosis in patients with neurologic dysfunction

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Glycosylphosphatidylinositol (GPI) anchors correctly localize 1% to 2% of human proteins to the cell membrane. Although biosynthesis of GPI anchors is commonly inactivated by somatic *Phosphatidylinositol glycan anchor biosynthesis class A (PIGA)* mutations in hematopoietic precursors in paroxysmal nocturnal hemoglobinuria (PNH), constitutional *PIGA* mutations are rare and have been implicated in severe impairment of neurologic development. Here we uncover that hypomorphic constitutional *PIGA* mutations cause a novel subtype of hereditary hemochromatosis (HH) by severely limiting GPI anchorage of hemojuvelin and ceruloplasmin.

HH is a genetic iron overload disease caused by dysfunction of the hepcidin/ferroportin regulatory axis due to mutations in several, mainly liver-expressed genes (*high Fe [HFE]*, *transferrin receptor 2 [TFR2]*, *hemojuvelin [HJV]*, *hepcidin [HAMP]*, or *ferroportin [FPN]*). The most common HH subtype, with high prevalence in the White population, presents with an adult onset and chronic phenotype and is caused by the HFE p.C282Y mutation. The non-HFE HH subtypes are relatively rare and typically manifest during juvenile age. Hepcidin, the key regulator of systemic iron homeostasis, binds to the iron exporter ferroportin,

triggering its degradation to inhibit iron export from duodenal enterocytes, hepatocytes, and macrophages. Inappropriately low hepcidin levels hallmark HH and explain increased intestinal iron absorption, progressive iron accumulation, and damage of parenchymal organs. A severe, juvenile HH subtype is caused by mutations in *HJV*, a GPI-anchored protein that enhances the bone morphogenetic protein /small mothers against decapentaplegic (SMAD) signaling pathway by functioning as a bone morphogenetic protein coreceptor.¹ Some HH-like phenotypes still lack a molecular basis.

In this study, we present the discovery of a novel mutation causing juvenile HH. We investigated a pediatric male patient (patient 1), who, in addition to early-onset epilepsy, severe developmental delay, and intellectual disability, demonstrated early systemic iron overload, meeting the diagnostic criteria for juvenile non-HFE HH.² Of note are the very high transferrin saturation (TSAT), diminished transferrin, and high serum iron and ferritin levels in the upper normal range. Together with low plasma hepcidin levels, these findings were suggestive of HH.

Table 1. Genetic, clinical, and biochemical features in 3 patients with constitutional PIGA deficiency

	Patient 1	Patient 2	Patient 3
Constitutional <i>PIGA</i> mutation	c.230G>A (p.R77Q)	c.1031T>C (p.L344P)	c.380 C>T (p.S127L)
Inheritance	Maternal	Maternal	Maternal
Sex	Male	Male	Male
Age at time of data collection	13 y	7 y	2 y
Degree of developmental delay	Moderate	Severe	Severe
Hypotonia	No	Yes	Yes
Age at seizure onset	11 mo	9 mo	6 mo
Seizure type and severity	Focal and generalized tonic-clonic seizures partially controlled by valproate, stiripentol, and clobazam	Focal and generalized tonic-clonic seizures partially controlled by topiramate, zonisamide and levetiracetam	Focal and atonic seizures, seizure free under the administration of topiramate and lacosamide
Brain anomalies	No	Multifocal cortical dysplasia of the right hemisphere (possibly explained by a hemizygous <i>pcdh19</i> variant)	No
Other congenital anomalies	Hypospadias glandis, primary enuresis nocturna	Cryptorchidism, cortical visual impairment, ichthyosis, hyper-/hypopigmentation on the neck, widely spaced teeth	Somatomegaly
Biochemical			
Ferritin (µg/L)	188 (RR: 7-140)	96 (RR: 7-140)	79 (RR: 2-63)
Transferrin saturation (%) (RR: 16-45)	96	87	70
Transferrin (g/L) (RR: 2.0-3.6)	1.87	1.97	2.23
Serum iron (µmol/L) (RR: 14-32)	45.1	43.2	39.0
Hepcidin (ng/mL) ¹⁷	2.9 (R:16.58-74.57)	4.82 (R:6.78-118.86)	3.1 (R:10.32-115.73)
FerriScan (MRI) (mg/g dry tissue) (RR: 0.17-1.8)	4.0	1.8	NA*
Serum transaminases (U/L)	Normal	Normal	Elevated (GOT 57 (RR: < 56); GPT 69 (RR: < 39))
Serum alkaline phosphatase (U/L)	243 (RR: 118-518)	223 (RR: 86-315)	411 (RR: 75-316)
Deficiency of GPI-anchored proteins and GPI anchors	↓ CD48 on a subpopulation of B- and T-lymphocytes (nonsignificant, 0.22%)	↓ CD157 on a subpopulation of monocytes (nonsignificant; 0.13%)	↓ CD58 and CD59 on a subpopulation of reticulocytes (not significant due to a very low number of reticulocytes)

c.230G>A (p.R77Q): ClinVar¹⁸ VCV000810512, likely pathogenic; PolyPhen-2¹⁹ prediction, probably damaging; c.1031T>C (p.L344P): ClinVar VCV000444793, uncertain significance; PolyPhen-2 prediction: probably damaging; c.380 C>T (p.S127L): ClinVar, not annotated; PolyPhen-2 prediction, likely damaging.

NA, not available; R, range; RR, reference range.

*Not indicated at the age of 2 y because sedation would be necessary.

In this patient, exome sequencing failed to detect known HH-associated mutations and instead identified a constitutional hemizygous missense mutation in the X-linked *PIGA*. Using next-generation panel sequencing, we could confirm this finding in 2 additional patients (patients 2 and 3), who were initially brought to medical attention because of their neurologic symptoms. Further assessment revealed signs of iron overload (Table 1), which then led to their referral to pediatric hematology. The clinical information of the 3 patients is summarized in Table 1. Supplemental Figure 1 (available on the *Blood* Web site) displaying the pedigrees of

all 3 patients shows a pattern consistent with X-chromosomal inheritance.

PIGA catalyzes the first step of GPI anchor biosynthesis, a process that is important for the dynamics and cell membrane attachment of approximately 150 human proteins.³ Somatic *PIGA* mutations have been described in patients with PNH (Online Mendelian Inheritance in Man [OMIM] 300818), a clonal hematopoietic stem cell disorder hallmarked by severe deficiency of GPI anchors that manifests with hemolytic anemia, thrombosis, and bone marrow failure.⁴ Constitutional *PIGA*

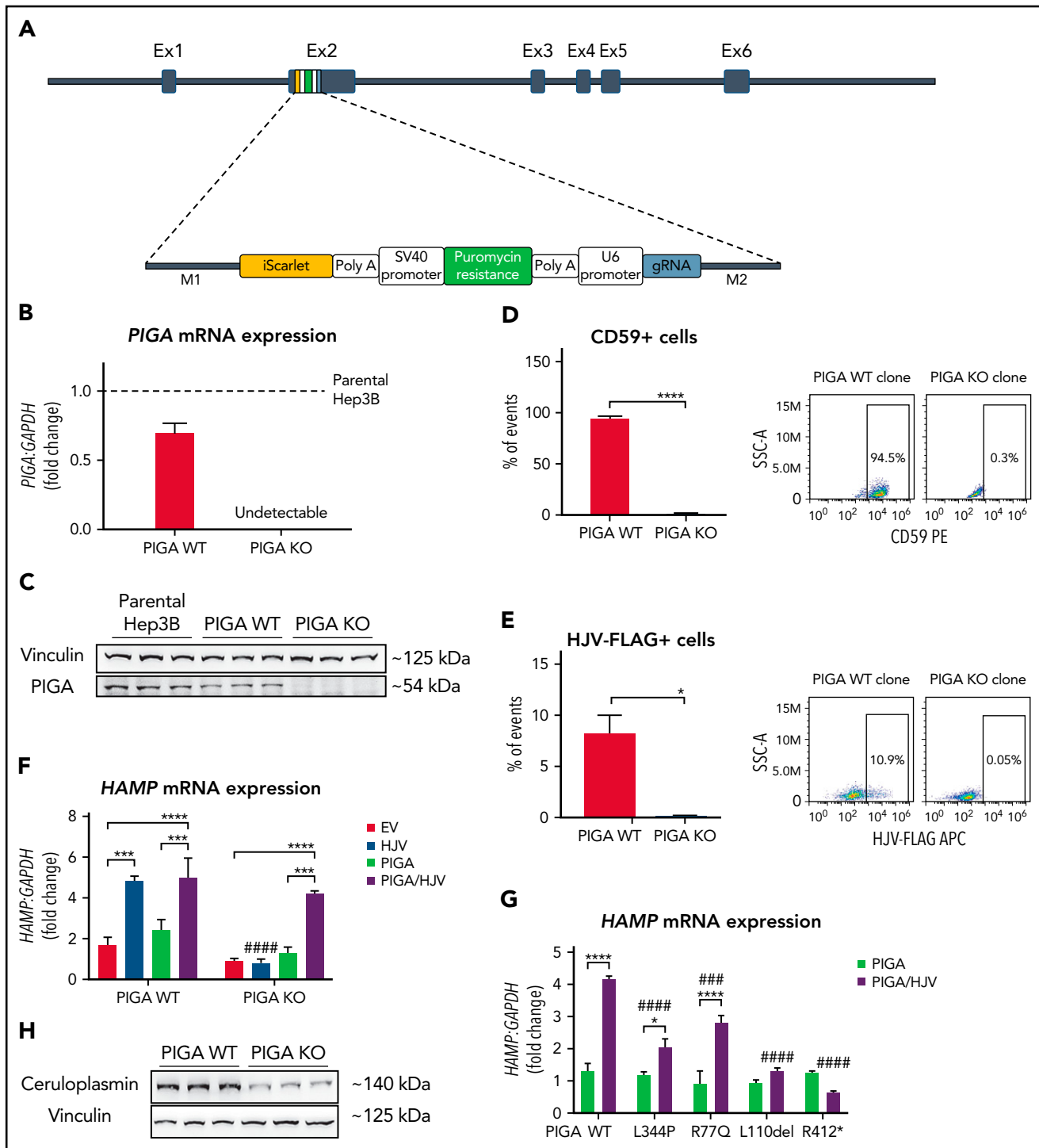


Figure 1. PIGA deficiency impairs hepcidin (HAMP) upregulation upon HJV overexpression. (A) Schematic representation of the PCR cassette integration in exon (Ex) 2 of PIGA. (B) mRNA levels of PIGA in PIGA WT and PIGA KO cells, normalized to parental Hep3B cells (dashed line). PIGA mRNA expression was normalized to the housekeeping gene GAPDH. (C) Western blot analysis of PIGA in parental Hep3B, PIGA WT, and PIGA KO cells. Vinculin was used as a loading control. (D) Fluorescence-activated cell sorter (FACS) analysis representing the percentage of CD59 hoerythrin-positive cells in PIGA WT and PIGA KO clones. (E) FACS analysis of the percentage of HJV-Flag allophycocyanin-positive cells in PIGA WT and PIGA KO cells upon HJV-Flag overexpression. In panels D and E, cells were previously gated for the exclusive inclusion of singlets (FSC-A vs FSC-H) and exclusion of dead cells (7-AAD positive). (F) Hepcidin mRNA levels in PIGA WT and PIGA KO cells after overexpression of HJV, PIGA, or HJV and PIGA vectors simultaneously. EV, empty vector. (G) mRNA levels of Hepcidin in PIGA KO cells after overexpression of PIGA^{WT}, PIGA^{L344P}, PIGA^{R77Q}, PIGA^{L110del}, and PIGA^{R412*} mutants together with HJV. Hepcidin mRNA expression was normalized to the housekeeping gene GAPDH. (H) Western blot analysis of ceruloplasmin (CP) in PIGA WT and PIGA KO cells. Vinculin was used as a loading control. Student t test/2-way analysis of variance: *[#]P < .05, **/[#]P < .01, ***/[#]P < .001, ****/[#]P < .0001; *comparisons inside each group; # relative to comparisons to the corresponding WT counterpart.

mutations causing *PIGA* deficiency are very rare and show a wide phenotypic spectrum including epileptic seizures, profound developmental delay, intellectual disability, and multiple congenital malformations.⁵ In line with previously described children with constitutional *PIGA* mutations, our patients do not show clinical or biochemical signs of PNH (details in supplemental Table 1) and do not meet the flow cytometric criteria for this condition. This is likely explained by residual activity of the hypomorphic mutations identified here, which result in a less severe reduced function than the acquired somatic null mutations causing PNH. This interpretation is consistent with the observation that not all blood cell lineages show a reduction of GPI-anchored proteins (Table 1).

PIGA deficiency has, thus far, not been known to cause HH hallmarked by hepcidin deficiency. However, HJV is a GPI-anchored protein,⁶ and we and others⁷ hypothesized that iron overload could be caused by a failure to attach GPI anchors to HJV and a subsequent inability to appropriately induce hepcidin expression by hepatocytes.

To test this hypothesis, we performed Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas12a-mediated knockout (KO)⁸ of *PIGA* in Hep3B liver cells and analyzed their capacity to control *hepcidin* expression. The *PIGA* gene is localized on the X chromosome, and therefore, we selected Hep3B cells that are derived from a male patient with hepatocellular carcinoma and consequently only contain 1 X chromosome.⁹ We inserted a polymerase chain reaction cassette (Figure 1A) containing 2 selection markers, puromycin and iScarlet, into exon 2 of the gene, disrupting the *PIGA* open reading frame in *PIGA* KO clones. Reference cells were either clones that underwent the same CRISPR/Cas12a-mediated procedure, but in which *PIGA* was not ablated (from now on referred to as *PIGA* wild-type [WT] clones) or parental Hep3B cells (Figure 1B-C). To demonstrate efficient and specific inhibition of GPI anchoring of proteins in *PIGA* KO cells, we performed flow cytometry analysis for cell surface expression of the GPI-anchored protein CD59, a diagnostic marker analyzed in patients with somatic *PIGA* mutations and PNH.⁴ As shown in Figure 1D, CD59 is only detectable in *PIGA* WT but not in *PIGA* KO cells. We next investigated the functionality of HJV in *PIGA* KO cells. Because specific, high-affinity antibodies against HJV are not available, we overexpressed a Flag-tagged version of the protein and analyzed HJV surface expression by flow cytometry analysis using an anti-Flag antibody. Our data show an almost complete absence of cells with HJV surface expression in *PIGA* KO clones (Figure 1E), whereas HJV is readily detectable on the surface of *PIGA* WT cells. Consistently, only WT cells show the expected upregulation of *hepcidin* in response to HJV overexpression,¹⁰ whereas *PIGA* KO cells fail to do so. Importantly, simultaneous transfection of HJV with a *PIGA* expression construct rescues *hepcidin* expression in *PIGA* KO cells (Figure 1F) and raises other markers of SMAD signaling, such as *SMAD6* and *SMAD7* expression (supplemental Figure 2). We next coexpressed the *PIGA* mutations identified in our patients (Table 1) or those described in patients reported previously with severe neurologic phenotypes.^{7,11} Importantly, *PIGA* KO cells show significantly lower *hepcidin* mRNA levels on HJV overexpression with all *PIGA* mutants (L344P, R77Q, L110del, and R412*) in comparison with the *PIGA* WT construct (Figure 1G). These data directly demonstrate the functional deficiency of the patients' *PIGA* alleles and

offer a molecular explanation for the increased TSAT and other iron overload parameters observed in our patients with the L344P and R77Q mutations. Of note, *hepcidin* mRNA levels are particularly low upon overexpression of the *PIGA* L110del and R412* mutations, conceivably accounting for the iron overload phenotype previously observed in the patients described by Swoboda et al⁷ with the *PIGA* L110del mutation. In comparison with patients with constitutional *PIGA* mutations displaying a more severe phenotype (exemplified by the R412* stop-gain mutation¹²), the data of the rescue experiments (Figure 1G) and the less severe clinical phenotype of the affected patients indicate that the missense mutations of our patients retain a higher residual function, thus likely explaining the milder neurologic phenotype allowing for sufficiently long survival to develop the iron overload phenotype. Moreover, our findings suggest that germline hypomorphic mutations in additional critical genes for the biosynthesis of GPI anchors may also cause iron overload over time, as has been recently demonstrated by Tremblay-Laganière et al.¹³ Interestingly, *PIGA* KO clones not only lack HJV but also show reduced levels of ceruloplasmin (CP), a ferroxidase required for efficient cellular iron export. Although the failure to express appropriate levels of HJV explains low hepcidin levels and iron accumulation, iron overload may be further aggravated by reduced CP protein expression (Figure 1H). Importantly, a GPI-anchored form of this enzyme is also expressed by astrocytes in the mammalian central nervous system.¹⁴ The neurologic phenotype in patients with inactivating *PIGA* mutations is generally attributed to the impaired attachment of GPI-anchored proteins involved in brain development to the cell membrane. These proteins include the HJV homologs repulsive guidance molecule a (RGMa) and RGMb/DRG11-responsive axonal guidance and outgrowth of neurite (DRAGON), which mediate axon guidance and growth and the formation of neuronal networks and are expressed in the central nervous system and other tissues.¹⁵ Decreased CP levels and cellular iron overload may further aggravate the neurologic symptoms of patients with constitutional *PIGA* mutations (eg, hypotonia and movement disorders), reminiscent of observations in patients with aceruloplasminemia.¹⁶ In addition, these patients are expected to have high levels of non-transferrin-bound iron (NTBI), the free toxic form of iron, because of an excessively high TSAT, which will further damage various cell types including neuronal cells.

Taken together our results connect neurologic deficits and iron overload in a novel way and uncover a new form of HH in patients with neurologic deficits and its likely molecular basis, showing that the functionality of 2 GPI-anchored proteins involved in maintaining iron homeostasis, HJV and CP, is impaired by *PIGA* mutations. These findings call for clinical assessment and treatment of potential iron overload in patients with constitutional *PIGA* mutations and long-term survival.

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Authorship

Contribution: L.M., O.M., S.C., P.F., S.A., B.H., H.S., M.L., N.G., A.E.K., and M.U.M. designed research; L.M., O.M., S.C., P.F., S.A., B.H., H.S., M.L., and N.H.-M. performed experiments; L.M., O.M., S.C., J.K., T.B., B.H., H.S., P.R.-P., T.R., N.H.-M., M.W.H., A.E.K., and M.U.M. collected and/or analyzed data; L.M., O.M., N.G., M.W.H., A.E.K., and M.U.M. wrote the manuscript; and all authors revised the manuscript.

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Footnotes

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