Investigating the key membrane protein changes during *in vitro* erythropoiesis of protein 4.2 (-) cells (mutations Chartres 1 and 2)

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The Online Supplementary Appendix

The patient was born in 1968, France. His non-consanguineous parents and two children were devoid of hematologic symptoms. The patient had no medical history before 2005 until he was hospitalized for sclerosis of bilateral varicose veins. A week following the operation, high fever, sweating and shivering developed.

A normochromic, normocytic anemia was discovered (Hb: 7.5g/dL), with 3.4×10^9/L white blood cells, including 36% lymphocytes, 6% atypical mononucleate hyperbasophilic cells and high reticulocyte count (600×10^9/L).

A parvovirus infection was established serologically. Most parameters gradually returned to normal, but a persistent increased bilirubinemia, decreased haptoglobin and elevated reticulocyte counts (immunological abnormalities were absent) were found. Sonography showed a homogenous splenomegaly (149 mm x 61 mm). Hemoglobinopathy, enzymopathy and PNH clone were ruled out and the erythrocyte membrane was examined. *Online Supplementary Table S1* shows there was a compensated anemia associated with elevated reticulocyte counts and an increased percentage of hyperdense cells.

Blood films show the presence of spherocytes and echinocytes (*Online Supplementary Figure S1A*). A number of cells look like bulky elliptocytes. Ektacytometry revealed moderate ektacytometric alterations suggestive of spherocytosis (*Online Supplementary Table S1*; *Online Supplementary Figure S1B*).

Online Supplementary Table S1. Erythrocyte indices and ektacytometric parameters reveal a compensated anemia. Erythrocyte indices were determined using the Advia 120 automat (Bayer Diagnostic France, Puteaux, France). MCH indicates mean corpuscular hemoglobin; MCHC, MCH concentration; and MCVr, mean cell volume of reticulocytes. Omin, DImax and O' are ektacytometric parameters. Normal values are shown.

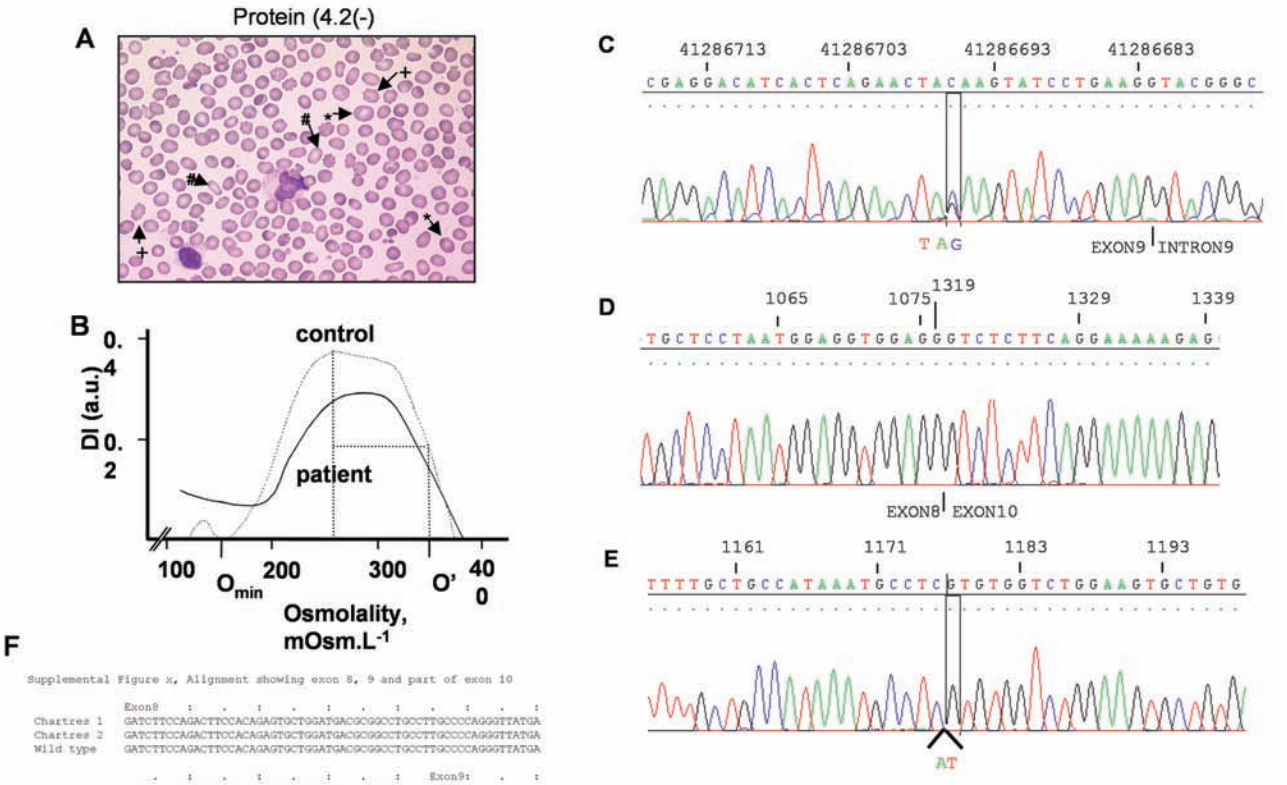
parameters	proband	normal
RBC (10^{12}/L)	4.43	4.30-5.70*
Hb (g/dL)	14.0	13.0-16.8*
Ht (%)	39	39-50*
MCV (fL)	88.0	82-98*
MCH (pg)	31.6	26.7-33.0*
MCHC (g/dL)	36.1	31.4-35.0*
Hyperdense cells[a] (%)	8.3	<4*
Reticulocytes (10^9/L;%)	252.5;5.7	<86*; <1.0*
MCVr (fL)	100.5	109.8
O_{min}	175	143-163*
DI_{max}	0.3	0.41-0 43*
O'	345	335-375*

[a]Hyperdense cells (hemoglobin concentration>41.0 g/dL)

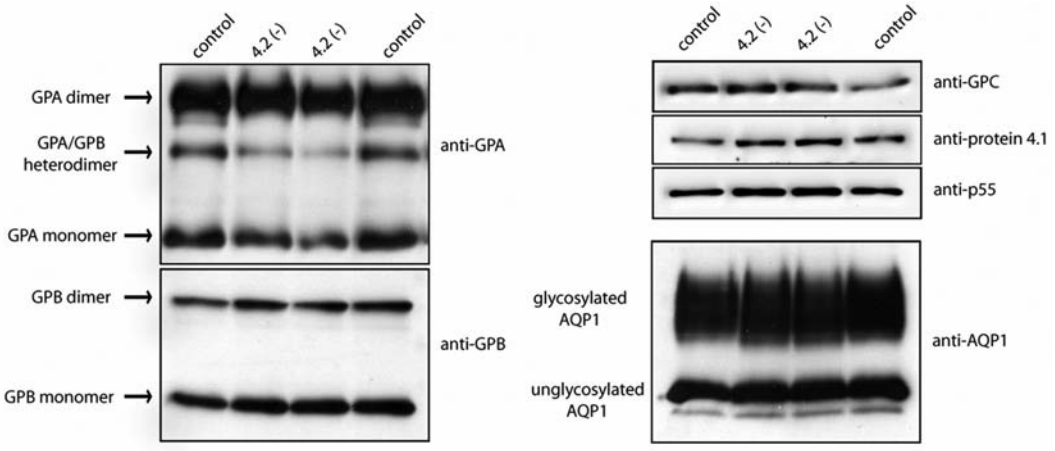
Online Supplementary Table S2. Flow cytometry data erythrocytes. Protein cell surface expression on erythrocytes. Erythrocytes were fixed in 0.01% gluteraldehyde and the cell surface expression of the indicated proteins was assessed by flow cytometry with antibodies as shown. Mean intensity values of four independent experiments are shown with their respective standard deviation. ANOVA was used to calculate statistical significance.

protein	antibody	control erythrocytes	4.2(-) erythrocytes	
control	unstained	0.367± 0.058	0.400± 0.000	
control	IgG control	0.375± 0.050	0.450± 0.058	
band 3	BRIC6	4.150± 0.768	3.375± 0.562	
GPC	BRIC4	11.300± 1.042	15.825± 2.711	
GPC	BRIC10	1.700± 0.356	2.425± 0.411	
band 3/GPA	BRIC14	19.625± 3.334	15.875± 1.443	
Rh	BRIC69	9.000± 3.102	10.775± 1.868	
RhAG	LA1818	5.750± 1.570	8.450± 2.352	
GPA	BRIC256	15.625± 3.617	21.175± 5.244	
GPA/GPB	R1.3	43.275± 4.656	44.950± 9.978	
CD47	BRIC125	7.900± 2.565	1.875± 0.189	***
CD47	BRIC126	4.375± 0.936	1.350± 0.238	***
CD44	BRIC222	0.725± 0.150	3.000± 0.927	***
CD44	BRIC235	0.925± 0.222	4.000± 0.804	***

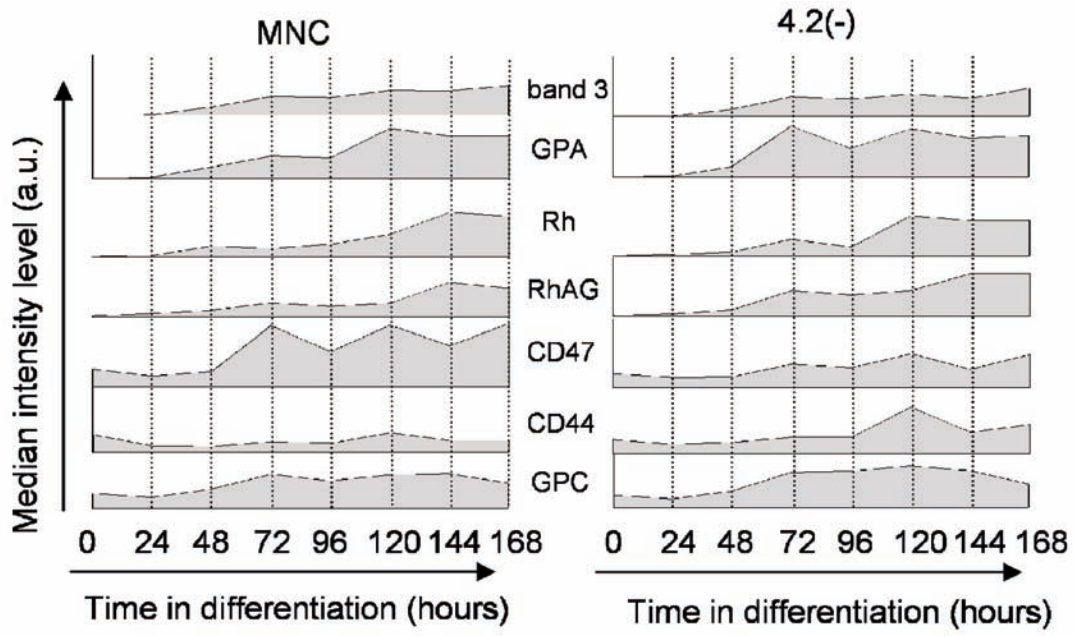
***=<0.01



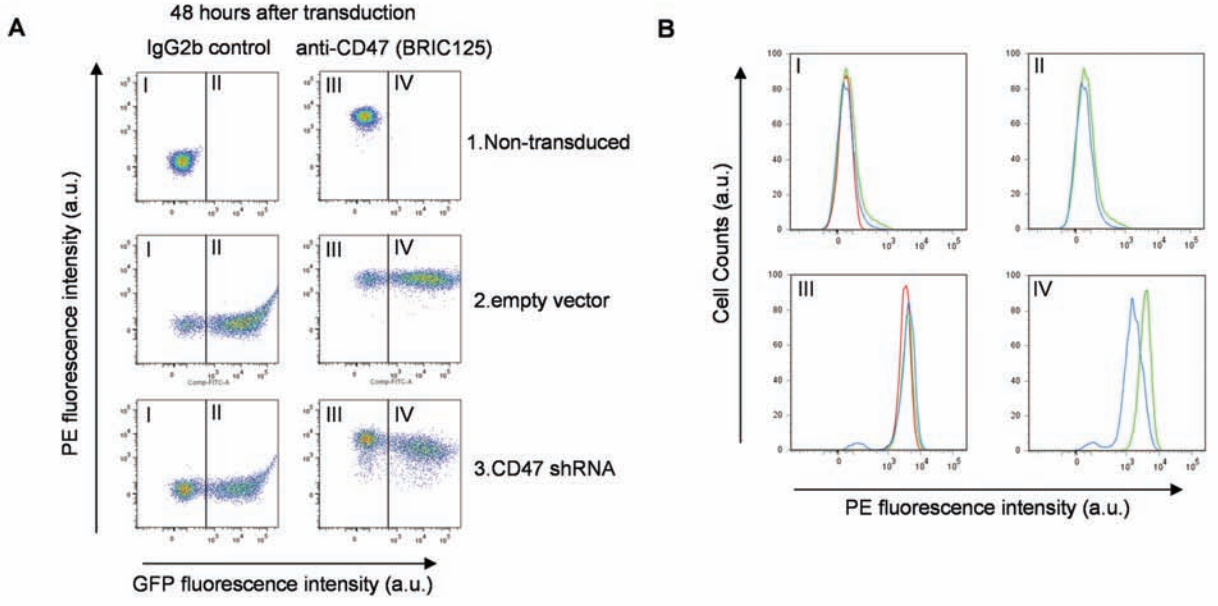
Online Supplementary Figure S1. Blood smears (A), osmotic gradient ektacytometry (B) and alignments of wt and mutant protein 4.2. (A) Many spherocytes (*) and elliptocytes (#) can be observed [echinocytes (+) are observed but are likely to be an artifact]. There was a pronounced anisocytosis. (B) Ektacytometry showed an increase in Omin, a reduction of maximum deformability, and a mild decrease of O' (see *Online Supplementary Table S1*). (C-E) mRNA isolation was carried out as described.[1] First strand cDNA was prepared using the RETROscript kit (Ambion, Texas). Protein 4.2 cDNA was amplified by PCR and sequenced (both directions) in an ABI PRISM 310 Automatic Sequencer (Applied Biosystems, Warrington, UK), as described previously.[2] Genomic DNA was isolated from buffy coats. Exons 9-11 of protein 4.2 were sequenced using exon specific primers. (C) Genomic DNA sequence of EPB42 showing the end of exon 9 and beginning of intron 9 on Chromosome 15. The heterozygous mutation C1305>G (cDNA numbering) coding for a Stop codon (TAG) is marked. (D) The cDNA sequence (protein 4.2 transcript number 2) from one allele is shown between nucleotides 1056 and 1338. The deletion of exon 9 is marked by the exon 8/exon 10 junction. (E) The cDNA sequence of the other allele is shown between nucleotides 1155 and 1198. The position of the "AT" deletion is marked. (F) Sequence analysis of exon 8, 9 and 10 of EPB42 (accession number NM_001114134) showing the disruptions in protein 4.2 Chartres 1 and 2.



Online Supplementary Figure S2. Analysis of ghosts from control and protein 4.2 erythrocytes. The patient's erythrocytes were serotyped as DCe/DCe and so a suitable DCe/DCe control was used to allow appropriate comparison throughout the experiments. 5μg ghosts were subjected to SDS-PAGE and the following proteins were detected by immunoblotting using polyclonal antibodies against protein 4.1, GPA, p55, GPC and aquaporin and monoclonal antibodies against GPB (R1.3) and α-spectrin (BRIC172).



Online Supplementary Figure S3. Cell surface marker progression during erythroblast differentiation. Comparison between erythroblasts expanded from control and protein 4.2(-) cells. Erythroblasts were induced to differentiate to reticulocytes and the expression of the indicated proteins was assessed daily by flow cytometry using specific antibodies (see *Design and Methods section*). Median intensity values were normalized against isotypic controls and plotted against time in hours (left, PBMC derived control cells; right, PBMC derived protein 4.2(-) cells).



Online Supplementary Figure S4. Lentiviral transduction of erythroblasts with shRNA against CD47. (A) Erythroblasts (day 11 in expansion) were transduced with lentiviruses pTRIP shRNACD47 (CD47 shRNA), pTRIPempty (empty vector) or not transduced (non-transduced; shRNA under control of an H1 promoter and GFP under the control of a CMV promoter). Knockdown and transduction efficiency was scrutinized after 48 h of transduction by measuring the cell surface expression of CD47 (BRIC 125) and the expression of GFP by flow cytometry. (B) The respective histograms displaying PE positiveness for the populations indicated in (A). Red lines: non-transduced cells; green lines: empty pTRIP; blue lines: pTRIPCD47shRNA. The roman numbers represent the following: I=anti-IgG2b, GFP negative population; II=anti-IgG2b, GFP positive population; III=anti-CD47 (BRIC125), GFP negative population, IV=anti-CD47 (BRIC125), GFP positive population.

References

1. Appleford NE, Wilson K, Houston F, Bruce LJ, Morrison A, Bishop M, et al. alpha-Hemoglobin stabilizing protein is not a suitable marker for a screening test for variant Creutzfeldt-Jakob disease. Transfusion. 2008;48(8):1616-26.
2. Bruce LJ, Ghosh S, King MJ, Layton DM, Mawby WJ, Stewart GW, et al. Absence of CD47 in protein 4.2-deficient hereditary spherocytosis in man: an interaction between the Rh complex and the band 3 complex. Blood. 2002;100(5):1878-85.