# **Recurring mutations in** *RPL***15 are linked to hydrops fetalis and treatment independence in Diamond-Blackfan anemia**

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#### SUPPLEMENTAL METHODS

**Pre-rRNA processing analysis.** Lymphoblastoid cell lines and HeLa cells were cultured in RPMI and DMEM, respectively (Gibco). These media were supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate (Sigma). Different 19-mer siRNAs (Eurogentec, Seraing, Belgium), whose efficiency was verified by qPCR, were used to knock down expression of *RPL15* mRNA coding for eL15 protein in HeLa cells: eL15, siRNA eL15-1 (5'-UGGUGUUAACCAGCUAAGdTdT-3') and siRNA eL15-2 (5'- UCCAGGAGCUAUGGAGAAAdTdT-3'). Each siRNA solution was added at a final concentration of 500 nM to 200 μl of cell suspension (50 x 10<sup>6</sup> cells/ml diluted in sodium phosphate buffer, pH 7.25, containing 250 mM sucrose and 1 mM MgCl<sub>2</sub>). Electro-transformation was performed at 240 V with a Gene Pulser (Bio-Rad, Hercules, CA).(1) Control HeLa cells were electro-transformed with a scramble siRNA (siRNA-negative control duplex; Eurogentec). After 10 min incubation at ambient temperature, cells were plated and grown at 37°C for 48 hours.

**RNA extraction and analysis by northern blot.** Total RNAs were extracted with Trizol from cell pellets containing 20-30 x  $10^6$  cells. The aqueous phase was further extracted with phenol-chloroform-isoamylic alcohol (25:24:1; Sigma), then with chloroform. Total RNAs were recovered after precipitation with 2-propanol. For Northern blot analyses, RNAs were dissolved in formamide, denatured for 10 min at 70°C and separated on a 1.2% agarose gel containing 1.2% formaldehyde and 1X Tri/Tri buffer (30 mM triethanolamine, 30 mM tricine, pH 7.9) (3 µg RNAs/lane). RNAs were transferred to a Hybond N<sup>+</sup> nylon membrane (GE Healthcare, Orsay, France) by passive transfer. Pre-hybridization was performed for 1 hour at 45°C in 6X SSC, 5X Denhardt's solution, 0.5% SDS, 0.9 g/ml tRNA. The 5'-radiolabeled oligonucleotide probe was incubated overnight. The sequences of the probes were:

5'-ITS1 (5'-CCTCGCCCTCCGGGCTCCGTTAATGATC-3'), ITS1-5.8S (5'-CTAAGAGTCGTACGAGGTCG-3'), ITS2 (ITS2b: 5'-CTGCGAGGGGAACCCCCAGCCGCGCA-3' and ITS2d/e: 5'-GCGCGACGGCGGACGACACCGCGGCGTC-3'), 18S (5'-TTTACTTCCTCTAGATAGTCAAGTTCGACC-3'), 28S (5'-CCCGTTCCCTTGGCTGTGGTTTCGCTAGATA-3').

Membranes were washed twice for 10 min in 2X SSC, 0.1% SDS and once in 1X SSC, 0.1% SDS, and then exposed. Signals were acquired with a Typhoon Trio Phospholmager (GE Healthcare) and quantified using the MultiGauge software.

**Polysome profiling analysis.** 400µg of total protein from freshly lysed LCLs or 1mg of total protein from freshly lysed HeLa cells were loaded onto 17-50% or 10-50% sucrose gradients, respectfully, as previously described.(2) The tubes were centrifuged at 4°C and at 40,000 rpm for 2 hours in a SW41 rotor (Optima L100XP ultracentrifuge; Beckman Coulter). The gradient fractions were collected at OD<sub>254</sub> with a Foxy Jr. gradient collector (Teledyne Isco).

**Growth curve.** 50,000 LCLs were plated in triplicate in a 12-well dish with DMEM +10%FCS and counted daily with a CASY® Cell Counter for five days. Each well was counted in triplicate each day. Statistics were performed with a Student's t-test.

*De novo* protein synthesis measurement. 100,000 LCLs were plated in 150µl methionine-free medium (DMEM containing 4.5g/L D-glucose, without L-glutamine, sodium pyruvate, L-methionine, and L-cysteine, Invitrogen

#21013). Assays performed using the Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay (Thermo Scientific) per the manufacturer's instructions using 2% paraformaldehyde for fixation and 1:1000 dilution of AHA. Fluorescence was measured by flow cytometry using an LSR-II apparatus and analyzed with FACSDiva software (BD Biosciences).

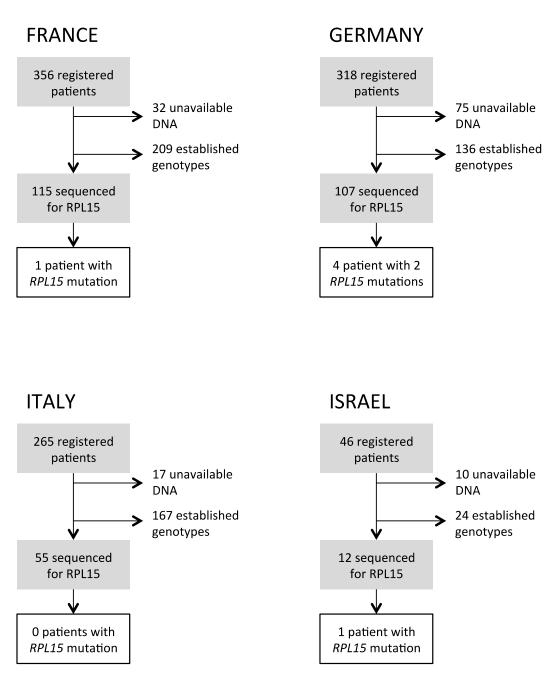
#### TABLE S1

Patient	Gen	otype	Conservation / PhysChem.	Effect (SIFT/ MT/ PP2 / PSNP)	CADD	ExAC N°/ population
1-3	c.242dupA	p.Tyr81*	Ø	Stop gain	25*	none
4	c.85C>T	p.Gln29*	Ø	Stop gain	37	none
5	c.29T>C	p.Leu10Pro	High / Moderate	D/D/B/D	27	none
6	c.458A>C	p.Lys153Thr	High / Moderate	D/D/B/N	14	none

#### Table S1. Evaluation of mutations in *RPL15* gene.

Abbreviations: Conservation, evolutionary conservation scores using Phylop and PhastCons; PhysChem., physicochemical difference between amino acids. *In silico* prediction: SIFT: D-deleterious; MT, Mutation Taster: D-disease causing; PP2, PolyPhen2: B- benign; PSNP consensus classifier: D-deleterious, N-neutral (% accuracy). CADD: Combined Annotation Dependent Depletion – score (method description: http://cadd.gs.washington.edu/info). A CADD score of 10 refers to the top 10% of deleterious variants detected in the human genome. CADD score above 20 represents the top 1% of deleterious variants. ExAC N°, number of heterozygous carriers; population, number of European individuals studied, as reported by Exome Aggregation Consortium (exac.broadinstitute.org/). Gene annotation: *RPL15* (NM\_001253379).

\* Because the duplication of A nucleotide (c242dupA) in the Tyrosine-coding triplet (TAC  $\rightarrow$  TAA A) did not yield a CADD score, we constructed the CADD score from nucleotide exchange TAC $\rightarrow$ TAA resulting in the same stop codon at position 81.



#### Figure S1. Description of EuroDBA patient registries tested for RPL15 mutations.

Four of the established registries within the EuroDBA consortium were included in this study. Numbers reflect calculations made in November 2017. In all cases, the cohorts tested for RPL15 mutations by Sanger sequencing consisted of registered patients with available DNA who had previously tested negative for mutations in all known DBA-linked genes.

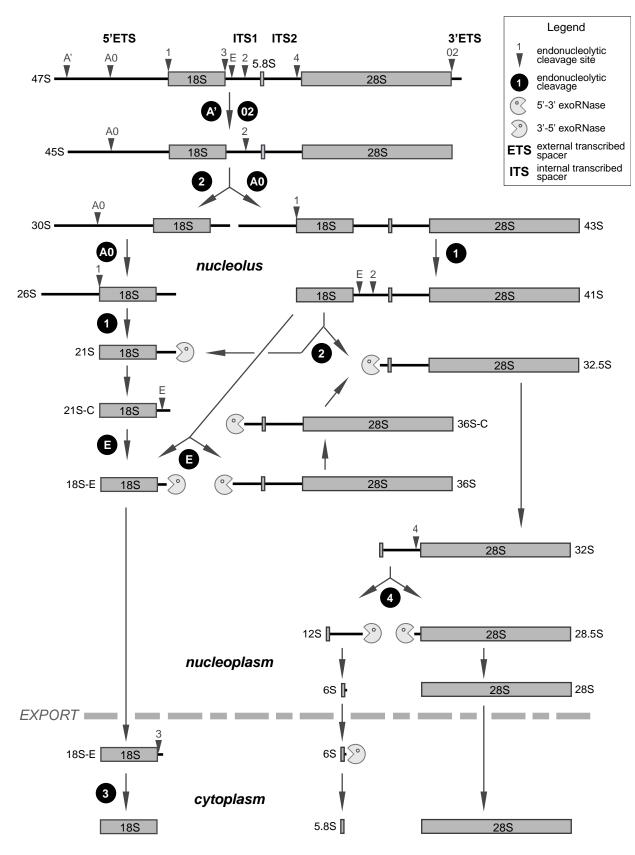
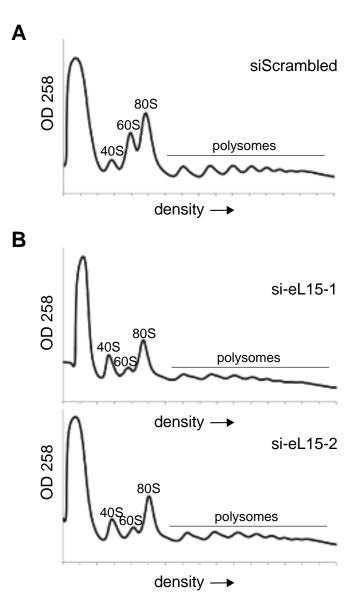
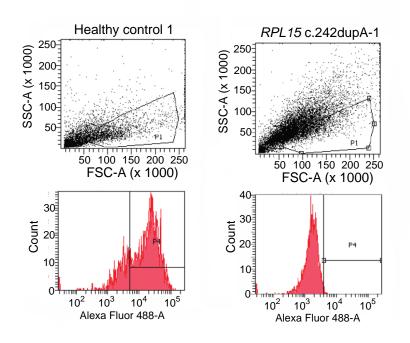


Figure S2 (Related to Figure 3). A schematic of the pre-rRNA processing pathway in mammalian cells.



# Figure S3 (Related to Figure 3). Polysome profiles of siRNA-treated HeLa cells.

**A)** Profiles of HeLa cells treated with a scrambled siRNA. **B)** Profiles of HeLa cells treated with two siRNAs against eL15 (*RPL15*). 40S subunits, 60S subunits, 80S monosomes, and polysomes are labeled.



# Figure S4 (Related to Figure 4). FACS plots of AHA labeling of LCLs for de novo protein synthesis measurements.

**A)** Plots of LCLs from a healthy control. **B)** Plots of LCLs from an individual with a *RPL15* mutation.

	Healthy cont		· · · · · · · · · · · · · · · · · · ·	trol Day 9
	Tube: KPS j7		Tube: KPS J9	
	Population	#Events %Parent %Total	Population	#Events %Parent %Total 10.000 #### 100.0
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	7AAD Neg (VIVANTES)	7,949 97.9 79.5	7AAD Neg (VIVANTES)	8,475 97.2 84.8
	IL3-R Neg	6,325 79.6 63.2	IL3-R Neg	7,828 92.4 78.3
	□ Q1 □ Q2 □ Q3 □ Q4	6,183 97.8 61.8	⊠ 01 ⊠ 02 ⊠ 03 ⊠ 04	7,531 96.2 75.3
		30 0.5 0.3	× 02	3 0.0 0.0 291 3.7 2.9
		101 1.6 1.0 11 0.2 0.1		3 0.0 0.0
	CD36 Pos	7,543 94.9 75.4	CD36 Pos	8,248 97.3 82.5
	IL3-R Pos	1,510 19.0 15.1	IL3-R Pos	573 6.8 5.7
	Anexine V Pos	116 1.5 1.2	Anexine V Pos	139 1.6 1.4 67 0.8 0.7
	CD34 Pos	344 4.3 3.4		67 0.8 0.7
	<i>RPL15</i> c.242 d	upA-1 Day 7	<i>RPL15</i> c.242 c	lupA-1 Day 9
	Tube: CD J7 Population	#Evente & Perent & Total	Tube: CD J9	#Evente @ Berent @ Tetel
	All Events	#Events %Parent %Total 10,000 #### 100.0	Population All Events	#Events %Parent %Total 10,000 #### 100.0
	POP D'INTERET	4,918 49.2 49.2		4,464 44.6 44.6
	7AAD Neg (VIVANTES)	4,049 82.3 40.5	7AAD Neg (VIVANTES)	3,883 87.0 38.8
	IL3-R Neg	2,431 60.0 24.3	IL 3-R Neg	3,024 77.9 30.2
		2,303 94.7 23.0		2,759 91.2 27.6
	Q2	18 0.7 0.2 74 3.0 0.7		10 0.3 0.1 235 7.8 2.4
	□ ⊠ 01 □ ⊠ 02 □ ⊠ 03 □ ⊠ 04	74 3.0 0.7 36 1.5 0.4		235 7.8 2.4 20 0.7 0.2
	CD36 Pos	3,600 88.9 36.0	CD36 Pos	3,441 88.6 34.4
	IL3-R Pos	1,538 38.0 15.4	IL3-R Pos	804 20.7 8.0
	Anexine V Pos	319 7.9 3.2 431 10.6 4.3	Anexine V Pos CD34 Pos	409 10.5 4.1 308 7.9 3.1
	L		2,000,000	
	<i>RPL15</i> c.242 d	upA-2 Day 7	<i>RPL15</i> c.242 c	lupA-2 Day 9
	Tube: MD J7		Tube: MD J9	
	Population	#Events %Parent %Total	Population	#Events %Parent %Total
	All Events	6,144 #### 100.0	All Events	10,000 #### 100.0
	POP D'INTERET	3,288 53.5 53.5	POP D'INTERET	6,364 63.6 63.6
	IL3-R Neg	2,735 83.2 44.5 1,305 47.7 21.2	7AAD Neg (VIVANTES)	5,413 85.1 54.1 4,286 79.2 42.9
		1,120 85.8 18.2	IL3-R Neg	4,286 79.2 42.9 3,898 90.9 39.0
	□ ⊠ 01 □ ⊠ 02 □ ⊠ 03 □ ⊠ 04	24 1.8 0.4	⊠ 01 ⊠ 02 ⊠ 03 ⊠ 04	16 0.4 0.2
	— 🖂 Q3	107 8.2 1.7		321 7.5 3.2
	Q4	54 4.1 0.9	- X Q4	51 1.2 0.5
	CD36 Pos	2,279 83.3 37.1	CD36 Pos	4,750 87.8 47.5
	IL3-R Pos	1,405 51.4 22.9	IL3-R Pos	1,079 19.9 10.8
	Anexine V Pos	130 4.8 2.1 461 16.9 7.5	Anexine V Pos	530 9.8 5.3 355 6.6 3.6
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Tube: K Populatic All Eve	PS J7 on ents OP DINTERET TAAD Neg (Vivantes) OP APos Alfa4 Pos Alfa4 Neg	#Events %Parent %Total     10,000     #### 100.0     7,539 75.4 75.4     5,143 66.2 51.4     4 0.1 0.0     1,247 24.2 12.5     4,944 96.1 49.4     183 3.6 1.8   upA-1 Day 7      #Events %Parent %Total     10,000     #### 100.0     7,539 75.4 75.4     4 0.1     0.0     1,247 24.2 12.5	Tube: KPS J9 Population All Events POP DINTERET POP DINTERET POP AP Os Band3 Pos Alfa4 Pos Population Alf Por Pop Dinteret Pop Dinteret Pop Dinteret Pop Dinteret Pop Dinteret Pop Dinteret Pop Dinteret Pop Dinteret Pop Dinteret Pop Dinteret Pos Pos	#Events %Parent %T     10,000     ####     10,200     76.7     3,269     41.6     3,016     92.3     3,009     92.0     3,140     96.1     114     3.5  UupA-1 Day 9      #Events     %Parent %     10,000     ####     1     4,552     45.5     2,487     54.6     2,082     83.7     2,088     83.7
Populatiti All Eve Populatiti All Eve Populatiti All Eve Populatiti	PS J7 on ents P DINTERET 7AAD Neg (Vivantes) OPA Pos Alfa4 Neg	#Events         %Parent         %Total           10,000         ###         100.           7,539         75.4         75.4           5,143         68.2         51.4           4         0.1         0.0           1,247         24.2         12.5           4,944         96.1         49.4           183         3.6         1.8	Tube: KPS J9 Population All Events POP DINTERET POP POS Band 3 Pos Alfa4 Pos Alfa4 Neg Population MI Events Population MI Events Pop DINTERET Population MI Events Pop DINTERET Population Alfa4 Pos Alfa4 Pos	#Events         %Parent         %T           10,000         ####         11           7,866         78.7         3,269         41.6           3,016         92.3         3,009         92.0         3,140         96.1         3,114         96.1         3,114         3,5           lupA-1 Day 9         #Events         %Parent         %         1         4,552         2,487         54.6         2,082         3.7         2,068         33.2         3         9         1.6         3,23         3         9         1.6         3,016         3,140         96.1         3         114         3.5         3.5         3         114         3.5         3         <
Populatiti All Events PC Populative: K All Events All Events PC Populative: N	PS J7 on ents P DINTERET 7 AAD Neg (Vivantes) OPA Pos Alfa4 Pos Alfa4 Neg	#Events         %Parent         %Total           10,000         ####         100.0           7,539         75.4         75.4           4         0.1         0.0           1,247         24.2         12.5           4,944         96.1         49.4           183         3.6         1.8           upA-1 Day 7         #remt         %Total           10,000         ####         100.0           7,539         75.4         75.4           5,143         68.2         51.4           4         0.1         0.0           7,539         75.4         75.4           5,143         68.2         51.4           4         0.1         0.0           7,539         75.4         75.4           5,143         68.2         51.4           4         0.1         0.0           1,247         24.2         12.5           4,944         96.1         49.4           183         3.6         1.8      upA-2         Day 7         7	Tube: KPS J9 Population POP DINTERET OPA Pos Band3 Pos Alfa4 Pos Alfa4 Pos Alfa4 Pos Population Alfevents POP DINTERET POP DINTERET POP DINTERET Alfa4 Pos Band3 Pos Alfa4 Pos Alfa4 Pos Band3 Pos Band3 Pos Alfa4 Pos Band3 Pos Band3 Pos Band3 Pos Alfa4 Pos Band3 P	#Events         %Parent         %T           10,000         ####         11           7,866         78.7         11           3,209         41.6         3,016         92.3           3,016         92.3         3,009         92.0         3,140         96.1           114         3.5         114         3.5         114         3.5           lupA-1 Day 9         #Events         %Parent         %         10,000         ####         1         4,552         45.5         2,487         54.6         2,082         83.7         2,068         83.2         39         16         2,100         84.4           lupA-2 Day 9         I         44.4         I         10,000         ####         11         10,000         ####         11         10,000         #####         11         10,000         #####         11         4,552         45.6         2,082         83.7         2,068         83.2         39         16         2,100         84.4         10,000         #####         11         10,000         #####         11         10,000         #####         11         10,000         #####         11         10,000         10,000         10,000
ube: K All Event	PS J7 on ents OP D'INTERET OPA Neg (Vivantes) OPA Pos Band3 Pos Afra4 Pos Afra4 Neg	#Events         %Parent         %Total           10,000         ###         1000           7,539         75.4         75.4           5,143         68.2         51.4           4         0.1         0.0           1,247         24.2         12.5           4,944         96.1         49.4           183         3.6         1.8	Tube: KPS J9 Population All Events POP DINTERET POP DINTERET Population Alfa4 Pos Alfa4 Pos	#Events         %Parent         %T           10,000         ####         11           7,866         76.7         1           3,269         41.6         1           3,016         92.3         1           3,009         92.0         1           3,140         96.1         114           114         3.5
opulatiti All Eventson PC Copulatiti All Eventson PC Cube: M	PS J7 on ents OP DINTERET FAD Neg (Vivantes) OP APos Alfa4 Neg	#Events         %Parent         %Total           10,000         ####         100.0           7,539         75.4         75.4           4         0.1         0.0           1,247         24.2         12.5           4,981         49.1         10.0           1,247         24.2         12.5           4,984         96.1         49.4           183         3.6         1.8	Tube: KPS J9 Population All Events POP DINTERET POP DINTERET POP AP Dos Band 3 Pos Alfa4 Pos	#Events         %Parent         %T           10,000         ####         11           7,866         78.7         12           3,016         92.3         3,016         92.3           3,009         92.0         3,140         96.1           114         3.5         114         3.5           IupA-1 Day 9           #Events         %Parent %           2,487         54.6         2,082         83.7           2,088         83.2         39         1.6         3.16           2,100         64.4         10000         ####         1           4,000         ####         1         1.0000         ####         1
opulatiti All Eve PC PC Cube: K All Eve Cube: K Cube: N Cube: N All Eve All Eve	PS J7 on ents OP DINTERET OP DINTERET OP DINTERET OP AND Neg (Vivantes) Afra4 Pos Afra4 Pos Afra4 Pos Afra4 Neg	#Events         %Parent         %Total           10,000         ####         100.00           7,539         75.4         75.4           5,143         68.2         51.4           4         0.1         0.0           1,247         24.2         12.5           4,944         98.1         49.4           183         3.6         1.8	Tube: KPS J9 Population All Events POP DINTERET POP DINTERET POP DINTERET POP DINTERET Alfa4 Pos Alfa4 Pos Alfa4 Pos Population All Events POP DINTERET POP DINTERET POP DINTERET POP DINTERET POP DINTERET POP DINTERET POP DINTERET POP DINTERET POP DINTERET Alfa4 Neg	#Events         %Parent         %T           10,000         ####         11           7,866         76.7           3,269         41.6           3,016         92.3           3,009         92.0           3,140         96.1           114         3.5           lupA-1         Day 9           #Events         %Parent           10,000         ####           14,552         45.5           2,487         54.6           2,068         83.2           39         1.6           2,100         84.4           lupA-2         Day 9           #Events         %Parent %T           10,000         ####
ropulation	PS J7 on ents OP DINTERET FAD Neg (Vivantes) OP APos Alfa4 Neg	#Events %Parent %Total     10,000     7,539     75.4     75.4     75.4     75.4     75.4     75.4     75.4     75.4     75.4     10.0     1,247     24.2     12.5     4,944     96.1     49.4     183     3.6     18  upA-1 Day 7      #Events %Parent %Total     10,000     ####     100.0     7,539     75.4     75.4     10.0     7,539     75.4     75.4     10.0     7,539     75.4     10.0     7,539     75.4     10.0     7,539     10.0     7,539     10.0     7,529     ####     100.0     3,236     43.0     43.0     43.4     40.1     10.0     7,529     ####     100.0     3,236     43.0     43.	Tube: KPS J9 Population All Events POP DINTERET POP DINTERET	#Events         %Parent         %T           10,000         ####         11           7,866         78.7         12           3,016         92.3         3,016         92.3           3,009         92.0         3,140         96.1           114         3.5         114         3.5           IupA-1 Day 9           #Events         %Parent %           2,487         54.6         2,082         83.7           2,088         83.2         39         1.6         3.16           2,100         64.4         10000         ####         1           4,000         ####         1         1.0000         ####         1
vopulatiti All Eve PC vopulatiti All Eve PC vopulatiti PC vopulatiti All Eve PC vopulatiti All Eve PC vopulatiti All Eve PC vopulatiti All Eve PC vopulatiti All Eve vopulatiti All Eve vopulatititi vopulatiti vopulatiti vopulatiti vopulatiti v	PS J7 on ents OP DINTERET PAD Neg (Vivantes) OP APos Alfa4 Pos Alfa4 Neg	#Events %Parent %Total     10,000     7,539     75.4     75.4     75.4     75.4     75.4     75.4     75.4     10.0     1,247     24.2     12.5     4,944     96.1     49.4     183     3.6     1.8  upA-1 Day 7      #Events %Parent %Total     10,000     ####     100.0     7,539     75.4     75.4     10.0     1,247     4.2     12.5     4,944     96.1     49.4     183     3.6     1.8  upA-2 Day 7      #Events %Parent %Total     7,529     ####     100.0     3,226     43.0     43.0     43.0     43.0     43.0     43.0	Tube: KPS J9 Population All Events POP DINTERET POP DINTERET POP DAP Ros Band3 Pos Alfa4 Pos Alfa4 Pos Alfa4 Pos Alfa4 Pos All Events POP DINTERET POP DINTERET All Events Alfa4 Pos Alfa4 Pos All Events Alfa4 Pos Alfa4 Pos Population Alf Events POP DINTERET POP DINTERET P	#Events         %Parent         %T           10,000         ####         11           7,866         76.7         1.259           3,016         92.3         3.009         92.0           3,009         92.0         3.140         96.1           114         3.5         1.14         3.5           lupA-1 Day 9         %Parent         %           10,000         ####         1           4,552         45.5         2.487         54.6           2,082         83.7         2.068         83.2           39         1.6         2.100         84.4           lupA-2 Day 9         ####         11           4,118         41.2         2.183         53.0           1,650         75.6         1.629         74.6
ropulation	PS J7 on ents OP DINTERET PAD Neg (Vivantes) OP Arfa4 Pos Affa4 Neg RPL15 c.242 dt MD J7 ion PTAD Neg (Vivantes) POP DINTERET PTAD Neg (Vivantes) PTAD PTAD PTAD PTAD PTAD PTAD PTAD PTAD	#Events %Parent %Total     10,000     7,539     75.4     75.4     5,143     68.2     51.4     4     0.1     0.0     1,247     24.2     12.5     10.0     1,247     3.6     1.8  upA-1 Day 7      #Events %Parent %Total     10,000     7,539     75.4     75.4     75.4     10.0     1,247     24.2     12.5     4,944     96.1     49.4     183     3.6     1.8  upA-2 Day 7      #Events %Parent %Total     7,529     ####     100.0     3,236     43.0     43.0     3,236     43.0     43.0     43.0     2,140     66.1     28.4     0	Tube: KPS J9 Population All Events POP DINTERET POP DINTERET	#Events         %Parent         %T           10,000         ####         11           7,866         78.7         1.8           3,016         92.3         3.016         92.3           3,009         92.0         3.140         96.1           114         3.5         114         3.5           lupA-1 Day 9         #Events         %Parent         %           10,000         ####         1         4,552         45.5           2,487         54.6         2,082         83.7         2,068         83.2         39         1.6         2,100         84.4           lupA-2 Day 9         #Events         %Parent         %T         10,000         ####         11         4,118         4,12         4,118         11.4         1.5         11.6         1.6         2,100         84.4         11.4         1.5         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.5         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.6         1.

**Figure S5 (Related to Figure 5). FACS results of red cell culture assays. A)** Results from blood sample from a healthy control on days 7 and 9, Annexin V, CD34<sup>+</sup>, and CD36<sup>+</sup> staining. **B)** DBA patient carrying the mutation *RPL15* c.242 dupA-1 on days 7 and 9, Annexin V, CD34<sup>+</sup>, and CD36<sup>+</sup> staining. **C)** DBA patient carrying the mutation *RPL15* c.242 dupA-2 on days 7 and 9, Annexin V, CD34<sup>+</sup>, and CD36<sup>+</sup> staining. **D)** Results from blood sample from a healthy control on days 7 and 9, Band-3+ and Alpha-4+ staining. **E)** DBA patient carrying the mutation *RPL15* c.242 dupA-1 on days 7 and 9, Band-3+ and Alpha-4+ staining. **F)** DBA patient carrying the mutation *RPL15* c.242 dupA-2 on days 7 and 9, Band-3+ and Alpha-4+ staining. **F)** DBA patient carrying the mutation *RPL15* c.242 dupA-2 on days 7 and 9, Band-3+ and Alpha-4+ staining.

# References

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