Variable readthrough responsiveness of nonsense mutations in hemophilia A

Lluis Martorell,[1,2] Vicente Cortina,[3] Rafael Parra,[4] Jordi Barquinero[1,*] and Francisco Vidal[2,5,6*]

[1]Gene and Cell Therapy Laboratory, Vall d'Hebron Institut de Recerca (VHIR), Universitat Autònoma de Barcelona (UAB); [2]Congenital Coagulopathies Laboratory, Banc de Sang i Teixits (BST); [3]Vall d'Hebron Core Laboratory (Section of Thrombosis and Haemostasis), Hospital Vall d'Hebron; [4]Banc de Sang i Teixits-Hospital Vall d'Hebron; [5]Molecular Diagnosis and Therapy Unit, VHIR-UAB and [6]CIBER de Enfermedades Cardiovasculares (CIBERCV), Barcelona, Spain

**JB and FV contributed equally to this work as co-senior authors*

 doi:10.3324/haematol.2018.212118

Received: November 23, 2018.

Accepted: June 11, 2019.

Pre-published: June 13, 2019.

Correspondence: *JORDI BARQUINERO* - jordi.barquinero@vhir.org

FRANCISCO VIDAL PEREZ - fvidal@bst.cat

Supplementary Appendix

Supplementary Methods

Patients and isolation of skin fibroblasts (continuation)

The three patients and the healthy control had previously been recruited for a study (HEMO-iPSC project, e-Rare-2) aimed at generating cellular models of HA based on patient-specific induced pluripotent stem cells (iPSCs) that can be differentiated *in vitro* to either hepatocyte-like or endothelial-like cells. The patients were selected on the basis of the PTC type, conservation of the amino acid originally encoded at the mutated site and their willingness to participate in the study.

For isolation of skin fibroblasts, a 3-mm full-thickness skin punch biopsy was obtained from the volar surface of the forearm. Primary fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) (all from Biowest, Nuaillé, France) at 37°C in 5% CO_2.

Generation of F8 variants harboring PTC mutations (continuation)

The constructs purchased from GeneArt (Thermo Fisher Scientific Inc. Waltham, MA, USA) included the plasmid with the WT sequence, that was generated from oligo assembly, and the twelve *F8*BDD variants harboring PTC mutations distributed along the *F8* cDNA, and including representatives of the two most common types of stop codons (the N-TGA-N and N-TAG-N, which represent the 83.5% of the nonsense mutations described in the HA database, while only 16.5% cases are of the N-TAA-N type). Furthermore, the nonsense mutations described in our study, distributed throughout all the functional FVIII domains and a variety of genetic contexts (p.Q462X, p.Q1705X, p.Q1764X, p.W274X, p.W1726X, p.W2015X, p.W2131X, p.R1715X, p.R1822X, p.R1960X, p.R2071X and p.R2228X). All the PTC-containing variants were generated by site-directed mutagenesis and were finally cloned into the NotI/XhoI site of the pcDNA3.1 Zeo (-) vector. Zeocin resistance was selected to avoid the use of potential RTAs such as geneticin, gentamicin or other aminoglycosides for the selection of resistant clones. For CHO cell

transfection the *F8BDD* mutant constructs were recovered using the endotoxin-free Qiagen maxi kit (Qiagen. Hilden, Germany) according to the manufacturer's recommendations.

RTA treatment (continuation)

After 48 h of RTA exposure for the indicated concentrations, the supernatants and the cells were collected and stored at -80ºC until needed. For protein extraction cells were washed three times with PBS and cell homogenates were recovered in RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0) (all from Sigma-Aldrich). For RNA isolation cells were washed once with PBS and recovered in RLT buffer using the RNeasy® mini kit (Qiagen).

F8 mRNA analysis (continuation)

The cDNAs generated were quantified by either real-time quantitative PCR (qRT-PCR) in patient's fibroblast or by a semi-quantitative PCR (qPCR) in CHO cells. Briefly, qRT-PCR was carried out using TaqMan Universal master mix II with Uracil-N-glycosylase (Thermo Fisher Scientific) following the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase gene (4333764F) served as the endogenous control and *F8* mRNA was detected using the TaqMan assay (Hs00252034_m1). The qRT-PCR reactions were performed in a LightCycler®480 II (Roche Life Science). Relative mRNA levels were assessed according to the ΔCt method. [1] On the other hand, the semi-quantitative PCR (qPCR) was carried out using platinum Taq DNA polymerase (Thermo Fisher Scientific) and the *F8*RNA3.1 and *F8*RNA5.2 as *F8*BDD-specific primers, as previously described. [2] Cycloheximide (CHX) (Sigma-Aldrich) was used overnight at 1 μg/ml.

FVIII Ag levels of F8BDD variants (continuation)

For determination of FVIII Ag levels, supernatants of either control (untreated) or RTA-treated cells were collected after 48 h and diluted 1:25 before assayed. The FVIII:Ag concentrations were extrapolated from a standard curve.

FVIII immunodetection of F8BDD variants (continuation)

For Western blot analysis, the protein concentration of the cell homogenates was measured using the DC protein assay (Bio-Rad Laboratories). Equal amounts of protein lysates were diluted in NuPAGE 4× sample buffer containing 1× NuPAGE sample reducing agent and electrophoresed at 95 V on midi NuPAGE Novex Bis-Tris 4-12% gels using MOPS-SDS running buffer containing NuPAGE antioxidant. The gels were blotted onto nitrocellulose membranes using the iBlot2 device and immunodetection was performed using the iBind system (all from Thermo Fisher Scientific). Qualitative estimation of Western blot data was performed using the ImageJ software (NIH, https://imagej.nih.gov/ij/).

For immunofluorescent detection, the cells were grown in the presence or absence of RTAs for 48 h, rinsed twice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature (RT) followed by three washes with PBS. Fixed cells were permeabilized with 0.1% Triton X-100 for 15 min and blocked for 1 h with 3% BSA in PBS. Primary antibodies were incubated overnight at 4ºC with 1% BSA in PBS. After three washes with PBS-BSA-Tween, the cells were incubated with a 1:1000 dilution of secondary antibodies in the dark with agitation for 1 hour at RT. Nuclei were stained with 0.5 µg DAPI (4,6-diamidino-2-phenylindole) mL^{-1} (all reagents were purchased from Sigma-Aldrich) and then mounted in Faramount aqueous mounting medium (Dako/Agilent Technologies, Santa Clara, CA, USA). Images were captured using an Olympus U-RFL-T epi-fluorescent microscope. Merged channels images were obtained using the ImageJ software.

FVIII activity of F8BDD variants (continuation)

In brief, the cells were treated with increasing doses of RTAs for 48 h, after which culture supernatants were collected and diluted 1:30 in assay buffer to determine relative FVIII:C activity compared to the untreated cells and the *F8*BDD WT variant. This model of FVIII production and analysis was reliable, since FVIII:C measured in the supernatants of the CHO cells transfected with the WT *F8*BDD plasmid reached up to 130% of the FVIII:C values found in the plasma of healthy individuals or a Human Standard Plasma.

In silico analysis

The functional consequences of missense changes generated in the different *F8*BDD variants were predicted *in silico* using Polyphen-2 (Polymorphism Phenotyping v2; http://genetics.bwh.harvard.edu/pph2/) and SIFT (https://sift.bii.a_star.edu.sg) algorithms. [3] Because nucleotides adjacent to PTCs were reported to be determinants of gentamicin readthrough responsiveness, they were annotated and compared for each of the patient fibroblasts (Table 1) and for each *F8*BDD variant (Tables 2 and S2).

Statistical analysis

The statistical significance of the data was assessed by a one-way ANOVA using the GraphPad Prism software. All data are presented as the mean ± SD. p values <0.05 (* $p<0.05$, ** $p<0.01$ and *** <0.001) were considered to indicate statistical significance.

Supplemental tables and figures

Supplementary Table S1. Primary and secondary antibodies used in FVIII immunodetection

Antibody	Raised against	Commercial source	Ref.	Dilution Western blot‡	Dilution immunofluorescence	Host
FVIII heavy chain (H-140)	N-terminal*	Santa Cruz	sc-33583	1:250	1:100	Rabbit
FVIII light chain (H-100)	C-terminal†	Santa Cruz	sc-33584	1:100	1:50	Rabbit
Anti-rabbit IgG –HRP	IgG	Sigma	A0545	1:1500	N.A	Goat
Anti-rabbit IgG, Alexa Fluor® 488 conjugate	IgG (H+L)	Thermo Fisher	A-21206	N.A.	1:1000	Donkey

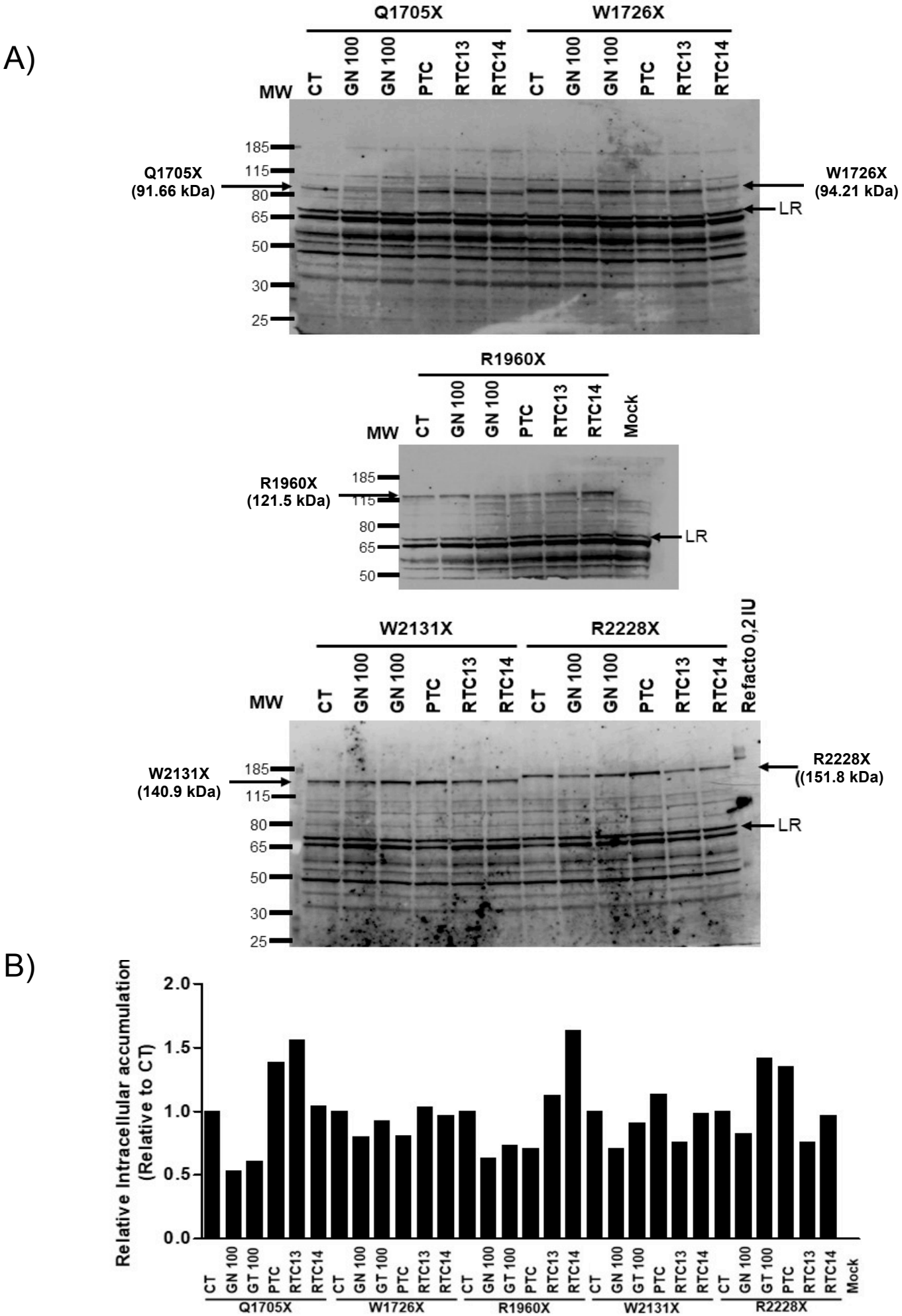
*Epitope corresponding to amino acids 261_400, mapping within an internal region of FVIII
† Epitope corresponding to amino acids 2252_2351, mapping at the C-terminus of FVIII
‡ Dilution used with the IBind2 system
N.A.: not applicable

Supplementary Table S2. Summary of readthrough effects according to the PTC molecular environment.

Mutation HGVS*	Mutation Legacy†	Seq. Context (mRNA) -5, -1, Stop, +4, +8	RT efficiency#	Expected effect on protein ‡	FVIII:C (fold increase)	FVIII:Ag (fold increase)
p.Q462X	p.Q443X	***C***TAT***T***-***TAG***-***C***ATG***A***	High	Mainly WT variant	2.6	5.12
p.Q1705X	p.Q1686X	***A***AAA***T***-***TAG***-***A***GCC***C***	Low	Mainly WT variant	1	3.6
p.Q1764X	p.Q1745X	***T***TAC***T***-***TAG***-***C***CCT***T***	High	Mainly WT variant	3.3	8.37
p.R1715X	p.R1696X	***A***AAC***A***-***TGA***-***C***ACT***A***	High	Mainly nonfunctional variant	1.2	11.17
p.R1822X	p.R1803X	***A***ACC***T***-***TGA***-***A***AAA***A***	Medium	Mainly nonfunctional variant	1.3	13.25
p.R1960X	p.R1941X	***G***GAT***T***-***TGA***-***T***GGT***A***	Medium	Mainly nonfunctional variant	1	8.68
p.R2071X	p.R2052X	***T***GGC***C***-***TGA***-***C***TTC***A***	High	Mainly nonfunctional variant	1.2	20
p.R2228X	p.R2209X	***A***AGC***T***-***TGA***-***C***TTC***A***	Very high	Mainly nonfunctional variant	1.1	5
p.W274X	p.W255X	***T***CTA***T***-***TGA***-***C***ATG***T***	Very high	WT variant	3.7	15.87
p.W1726X	p.W1707X	***G***GCT***C***-***TGA***-***G***ATT***A***	Low	WT variant	1	6.67
p.W2015X	p.W1996X	***G***AAT***T***-***TGA***-***C***GGG***T***	Very high	WT variant	2.9	31.3
p.W2131X	p.W2112X	***A***GAA***G***-***TGA***-***C***AGA***C***	High	WT variant	2.7	4.56

*HGVS: Human Genome Variation Society numbering, where codons are numbered with codon +1 coding for the first residue (Met) of the 19-residue signal peptide (this is -19 in Legacy numbering). †Legacy numbering, where codon +1 refers to that coding for the first amino acid of the mature FVIII protein (in HGVS numbering, this is codon +20). #According to nucleotide context rule (-1-**Stop**-+4). ‡According to Roy *et al.* [15]

Supplementary Figure S1



Supplementary Figure 1. RTA effects on intracellular accumulation of FVIII. Representative images of the immunodetection of FVIII heavy chain in the cell homogenates of transiently transfected CHO cells. A) Western blot of CHO cells transfected with the p.Q1705X (91.66 kDa), p.W1726X (94.21 kDa) (upper panel), p.R1960X (121.5 kDa) (middle panel) and p.W2131X (140.99 kDa), p.R2228X (151.85 kDa) variants (lower panel). The intracellular accumulation of the truncated proteins before and after RTA-treatment is shown. B) Densitometry of the bands in (A). CT: Untreated cells; GN100: 100 μg geneticin/mL; GT100: 100 μg gentamicin/mL; PTC: PTC124 10 μM; RTC13: RTC13 10 μM and RTC14: RTC14 10 μM; MOCK: Untransfected cells; MW: Pageruler Plus prestained protein ladder; Refacto®: recombinant human BDD-FVIII (Pfizer, New York, NY, USA); LR: loading reference used for densitometry normalization.

References

1. Schmittgen TD, Zakrajsek BA, Mills AG, et al. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal. Biochem.* 2000;285(2):194–204.

2. Martorell L, Corrales I, Ramirez L, et al. Molecular characterization of ten F8 splicing mutations in RNA isolated from patient's leucocytes: assessment of in silico prediction tools accuracy. *Haemophilia*. 2015;21(2):249–57.

3. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat. Methods*. 2010;7(4):248–9.