SUPPLEMENTARY APPENDIX

Cryptic non-canonical splice site activation is part of the mechanism that abolishes multimer organization in the c.2269_2270del von Willebrand factor

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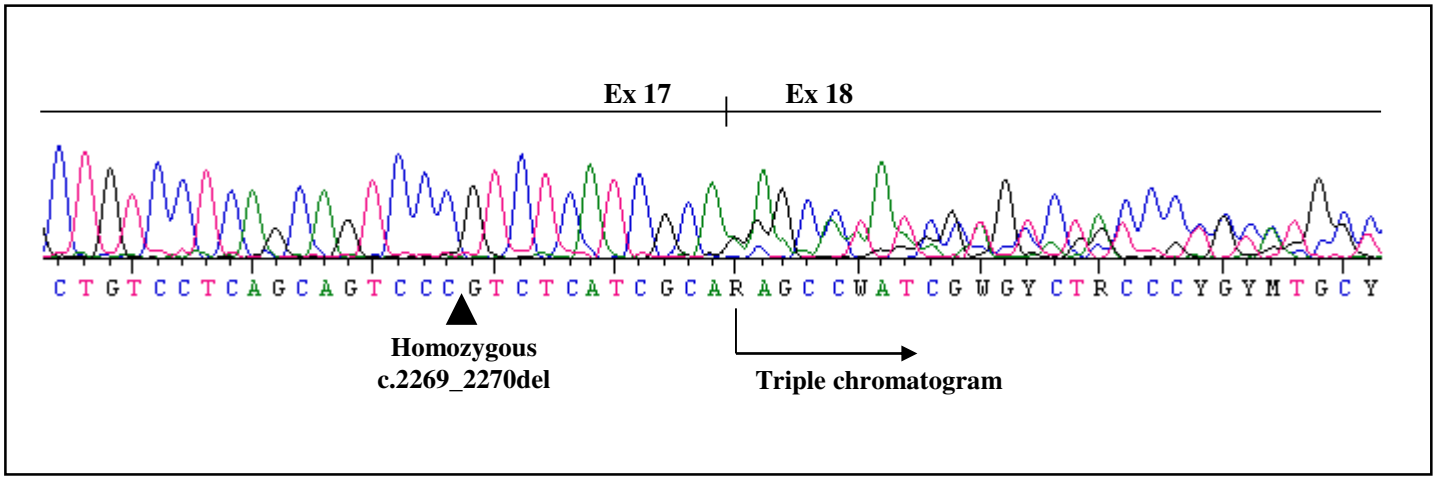

Supplemental Figure 1

Supplemental Figure 1. **The proband's VWF cDNA.** The sequence was obtained using primers VWF cDNA 4 F and VWF cDNA 4 R. Note the homozygous c.2269_2270del mutation in exon17, and the triple chromatogram starting at the beginning of exon 18.

Supplemental Methods

Hemostatic analysis. Basic hemostatic tests, activated partial thromboplastin time (aPTT), platelet function analysis (by PFA100), ristocetin-induced platelet aggregation (RIPA), plasma VWF antigen (VWF:Ag), VWF ristocetin cofactor (VWF:RCo), VWF collagen binding (VWF:CB), VWF to FVIII binding capacity (VWF:FVIIIB) and FVIII activity (FVIII:C), were performed as explained elsewhere (22-23). Platelet VWF content was measured as previously reported (24). The proband's samples were diluted less than the control plasma to adjust for his markedly reduced VWF levels. VWF multimers were analyzed under low-resolution conditions (1.6% low gelling temperature agarose), and detected by autoradiography using a ^{125}I-labelled anti-VWF antibody, according to an already-reported procedure (25). DDAVP was administered subcutaneously at a dose of 0.3 µg/kg, and the time course of VWF and FVIII levels was charted at 0, 15, 30, 60, 120 and 240 minutes after injection (26). Anti-VWF antibody detection was carried out by ELISA, as described elsewhere (27). ADAMTS13 activity was ascertained using FRETS-VWF73 (Peptide International, Lexington, KY, USA) as a substrate. Fluorescence readings were acquired with Victor3 1420 Perkin Elmer, Boston, MA, USA. Anti-ADAMTS13 IgGs were measured using the ADAMTS13-INH ELISA kit (Technoclone, Vienna, Austria). A negative test was defined as a titer ≤16 IU/mL.

Genetic analysis. The patients' genomic DNA was obtained from whole blood, using the Maxwell® 16 automated DNA extractor, and the Maxwell® 16 Blood DNA Purification kit (Promega, Wisconsin, USA). Total RNA was extracted from the platelets, starting from 40 mL of blood anticoagulated with sodium citrate and centrifuged at 180 x g for 10 minutes. The resulting platelet-rich plasma (PRP) supernatant was collected, leaving at least 2 cm of PRP above the interface with the red blood cells to avoid any significant contamination with white blood cells. Platelets were then pelleted in a second centrifugation step, at 1000 x g for 15 minutes, and resuspended in Trizol reagent (Thermo Fisher Scientific, Carlsbad, California, USA). RNA was extracted from Trizol-lysed platelets according to the standard protocol. cDNA was generated using the Superscript III kit (Thermo Fisher Scientific) with random primers, according to the manufacturer's protocol. PCR reactions were performed using the Qiagen HotStartTaq Master Mix and an AB2720 Thermal Cycler (Applied Biosystems, AB, Foster City, CA, USA). For Sanger sequencing, the Big Dye Terminator Sequencing kit v.1.1 (Perkin Elmer, Wellesley, MA, USA), and an ABI3130 XL Genetic Analyzer (AB) were used. The pGEM®-T Easy Vector (Promega, Madison, Wisconsin, USA) was used to clone the PCR product found altered in the proband (17).

Digital droplet PCR (ddPCR). The digital droplet PCR (ddPCR) was used to quantify the three aberrant mRNA species found in the proband. The assays were performed in the QX200 ddPCR Instrument (Bio-Rad, Hercules, California, USA) using the ddPCR^{TM} Supermix for Probes (Bio-Rad) with different primer pairs and probes, specifically designed for aberrant mRNA sequences, according to the manufacturer's instructions. Primers 5'cctgacgctgtcctc3' and 5'ggggccgacaggata3', together with the HEX-labelled 5'ccgtctcatcgcagcaa3' probe identified mRNA r.2269_2270del (RNAI); 5'cctgacgctgtcctc3' and 5'tgaccatgggggggc3', together with the FAM-labeled 5'ccgtctcatcgcagagc3' probe identified mRNA r.[2269_2270del;2282_2288del] (RNAII); 5'gtgaggatggcttca3' and 5'gccgacaggatagg3' together with FAM-labelled 5'ccgtctcatcgcaagg3' probe, identified mRNA r.[2269_2270del;2281_2282insAG] (RNAIII). The assay was performed in multiplex, starting from 25 ng of the proband's cDNA, at T=56°C and adding 2% DMSO to the PCR mix. cDNA was obtained by retrotranscribing RNA extracted from the proband's platelets, as explained in the previous paragraph.

***In vitro* expression experiments.** The pRc/CMV-VWF vector containing the wild-type (WT) human full-length VWF cDNA was mutated by site-directed mutagenesis using the QUIKCHANGE II XL kit (Stratagene, La Jolla, CA, USA). Primer pairs 5'cctcagcagtcccgtctcatcgcagc3' and 5'gctgcgatgagacgggactgctgagg3' were used to obtain the r.2269_2270del mutated vector (pRc/CMV-VWF-RNAI). pRc/CMV-VWF-RNAI was subsequently used as a template for the production of the r.[2269_2270del;2282_2288del] and r.[2269_2270del;2281_2282insAG] vectors (identified as pRc/CMV-VWF-RNAII and pRc/CMV-VWF-RNAIII, respectively), using primers 5'cagcagtcccgtctcatcgcagagcctatcctgtcggcccccc3' and 5'gggggccgacaggataggctctgcgatgagacgggactgctg3' for the former, and 5'gcagtcccgtctcatcgcaaggcaaaaggagcctatcctgtcg3' and 5'cgacaggataggctccttttgccttgcgatgagacgg gactgc3' for the latter. The vectors were used to transiently transfect Human Embryonic Kidney 293T (HEK293T) cells. Transfections were performed in T25 flasks using the Lipofectamine 2000 transfection reagent (Life Biotechnology, Carlsbad, CA, USA) and 4 µg of vector/T25. After 48 hours of transfection, the media containing the recombinant VWF (rVWF) were collected, and the rVWF was quantified by ELISA. The rVWF intracellular content was also measured after lysing cells with a 2% Triton-X-100 solution, as already reported (17). The results of each transfection experiment were calculated as the mean of three replicates.

SDS-PAGE and Western blot. rVWF samples were run in reducing conditions using the XCell SureLock Mini electrophoresis system (Invitrogen, California, USA) on a Novex Tris-Glycine 3-8% precast gel (Invitrogen). Samples were diluted 1:12 with the NuPage LDS Sample buffer and the NuPage reducing agent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and then denatured for 10 minutes at 100°C. The gel was run at 100 V at room temperature in the Novex

Tris-Glycine running buffer (Invitrogen). The Bio-Rad Semi-Dry System was used to blot samples on a Immobilon-FL PVDF membrane (Millipore, Burlington, Massachusetts, USA) for 30 minutes. After blotting, the membrane was blocked with TBS-T with 10% skimmed milk, and incubated overnight at 4°C with a polyclonal rabbit anti-VWF antibody (DAKO, Glostrup, Denmark) diluted 1:1,000 in TBS-T 2% BSA. The membrane was then washed with TBS-T and incubated for 1 hour at room temperature with a polyclonal anti-rabbit-HRP antibody diluted 1:2000 (DAKO). The chemiluminescent signal was revealed using the Euroclone LiteAblot Turbo ECL (Euroclone, Milano, Italy) in a Uvitec Alliance imaging system (Uvitec, Cambridge, UK).

Supplemental table 1. *Primers used for patients' mutation characterization*

Primer	**Sequence 5'-3'**
VWF ex17F	aggaggggttatgggactgg
VWF ex17R	atgccttggcctgaaagtca
VWF ex18F	agactctaggggaccaaaggaca
VWF ex18R	tgcctacaagaaaactgaagggca
VWF cDNA 4F	tcaacccgcgcatgacca
VWF cDNA 4R	cgtcgaaggtgaggtagtgg