haematologica
Journal of the Ferrata Storti Foundation

Whole-blood RNA-seq facilitates splice variant classification in platelet disorders

by Tiziana Lischetti, Andreas Ørslev Rasmussen, Majbritt Busk Madsen, Maria Rossing and Eva Birgitte Leinoe

Received: August 25, 2026.
Accepted: November 12, 2026.

Citation: Tiziana Lischetti, Andreas Ørslev Rasmussen, Majbritt Busk Madsen, Maria Rossing and Eva Birgitte Leinoe. Whole-blood RNA-seq facilitates splice variant classification in platelet disorders. Haematologica. 2026 Nov 19. doi: 10.3324/haematol.2026.301887 [Epub ahead of print]

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication.
E-publishing of this PDF file has been approved by the authors.
After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval, the final version of the manuscript will then appear in a regular issue of the journal.
All legal disclaimers that apply to the journal also pertain to this production process.

Whole-blood RNA-seq facilitates splice variant classification in platelet disorders

Tiziana Lischetti[1], Andreas Ørslev Rasmussen[1], Majbritt Busk Madsen[1], [#]Maria Rossing[1,2], [#]Eva Leinøe[1,3]

[1] Center for Genomic Medicine, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark

[2] Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

[3] Department of Haematology, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark

[#] MR and EL contributed equally as co-last authors.

Running Title: RNA-seq Platelet Disorders

Disclosures: EL: Consulting/advisory role for Sanofi. Honoraria for Sobi and Sanofi

Author Contributions: TL, EL and MR designed the study, collected data, performed analysis and wrote the paper. AØR and MBM performed analysis and wrote the paper.

Corresponding author: Eva Leinøe (ORCID 0000-0003-0739-7293)
eva.birgitte.leinoee@regionh.dk

Acknowledgment: None.

Data-sharing statement: Transcriptomic profiles used in this study can be made available from the corresponding author on request.

Declaration of AI use: During the preparation of this manuscript, the authors used AI solely for language editing and improvement of readability. The authors reviewed and edited the

suggested changes and take full responsibility for the accuracy, integrity, and final content of the manuscript.

Whole genome sequencing (WGS) has proven effective in diagnosing inherited thrombocytopenias and rare inherited disorders in general[1]. Nonetheless, its diagnostic utility in rare inherited bleeding and platelet disorders (IBPD) remains limited, due to both the incomplete identification of genes responsible for the full spectrum of bleeding phenotypes and to the frequent identification of variants of uncertain significance (VUS), which cannot inform clinical decision-making without functional validation and/or family co-segregation studies[2,3]. Although up to 25% of germline variants identified by WGS are predicted by *in silico* tools to affect mRNA splicing, these predictions alone generally provide insufficient evidence for clinical variant classification[4]. While such algorithms provide predictions, RNA-sequencing (RNA-seq) can address this uncertainty by providing direct evidence of altered splicing patterns.

RNA-seq studies of platelet-specific transcripts typically require RNA isolated from purified platelets or platelet-derived cultures[5–7]. In a clinical setting, however, whole blood (WB) RNA-seq is more readily applicable. We therefore investigated whether WB RNA-seq could provide clinically informative transcriptomic evidence in unresolved IBPD, with a particular focus on aberrant splicing and the functional interpretation of genomic variants.

We studied 62 unresolved IBPD cases from the Minibridge Study ("Functional and Genetic Characterization of Inherited Platelet Disorders")[3]. All patients provided informed consent for the use and publication of their data in accordance with the Helsinki Declaration. The study was approved by the Regional Ethics Committee, Copenhagen, Denmark (H-15011677). Among the included patients, ten fulfilled the clinical diagnostic criteria for hypermobile Ehlers-Danlos syndrome (EDS), 26 had laboratory indications of a platelet function disorder, and three had thrombocytopenia (Supplementary Table 1). All patients had previously undergone WGS analysis as part of the diagnostic evaluation, initially focusing on a panel of 121 genes associated with inherited platelet and bleeding disorders, including International Society on Thrombosis and Hemostasis Tier 1 genes[8] and genes associated with classical and vascular EDS (Supplementary Table 2). Reassessment of WGS data led to identification of a pathogenic non-spliceogenic missense *PTPN11* variant, resulting in a diagnosis of Noonan syndrome[9], while a patient with laboratory diagnosed grey platelet syndrome (GPS) (thrombocytopenia, large pale platelets, lack of alpha granules on transmission electron microscopy examination, and low CD62P expression) was found to carry a homozygous intronic *NBEAL2* variant, which was initially interpreted as a VUS according to ACMG/AMP guidelines. Among the remaining patients, six carried VUS-high, while four harbored heterozygous class 4 variants in *F5*, *F10*,

or *VWF* that were not considered sufficient to explain their bleeding phenotype (Supplementary Table 1). To investigate the genetic basis of the initial unresolved IBPD cohort, RNA-seq was performed on WB samples from the 62 patients (collected in PAXgene Blood RNA tubes), and total RNA isolated using standard protocols (Figure 1A). RNA-seq data were analyzed using FRASER (version 1.14.1) to identify aberrant splicing events[10]. As FRASER estimates splicing variation based on the samples included in the analysis, RNA-seq data from 15 additional patients with an established molecular diagnosis of IBPD were processed using the same workflow and included to provide a larger reference set and thus improve the robustness of model estimation (Supplementary Table 2). These 15 additional patients were independent of the 62 unresolved IBPD cases and contributed to the estimation of splicing variation used for outlier detection but were not included in the assessment of diagnostic yield (Figure 1A).

We first assessed whether the expression levels of genes associated with IBPD were sufficient in WB RNA to support downstream transcriptome analysis. Of the 121 genes included in our IBPD diagnostic panel, 99 (82%) were expressed in WB, of which 58 (48%) reached a mean expression of 450 normalized reads, a level previously associated with sufficient sensitivity to detect approximately 50% under-expression[11] (Figure 1B, Supplementary Table 3). Most IBPD genes with low or undetectable expression in WB were associated with coagulation factors, connective tissue, or hereditary hemorrhagic telangiectasia and are primarily expressed in liver cells, fibroblasts, or endothelial colony-forming cells[12,13]. However, 73 out of 81 platelet-associated genes (90%) were expressed in WB, with 49/81 (60%) showing a mean expression of at least 450 normalized reads (Figure 1B).

Subsequently, the RNA-seq data were used to investigate aberrant splicing. Candidate splicing events were filtered and manually curated to exclude technical artefacts. High-confidence aberrant splicing events were subsequently evaluated by integrating information from public databases, published literature, gene function, gene-disease association, inheritance patterns, and clinical phenotypes. For genes considered plausible candidates, genomic data was revisited to identify variants potentially explaining the observed splicing abnormalities. Candidate variants were interpreted according to standard criteria, including ACMG classification. Transcriptomic and genomic findings were then jointly assessed to determine their potential contribution to the bleeding phenotype (Figure 1A).

In the patient with suspected GPS (Patient 1), a homozygous intronic VUS was identified in *NBEAL2* (NM_015175.3:c.925+5del). Five *in silico* splice prediction tools (SpliceAI,

SpliceSiteFinder-like, MaxEntScan, GeneSplicer, and NNSplice) consistently predicted disruption of the canonical donor site of exon 8 and activation of a cryptic donor site three nucleotides downstream (NM_015175.3:c.925+3), suggesting only a subtle in-frame transcript alteration. Based on the available evidence, the variant had initially been classified as a VUS according to ACMG guidelines (ACMG criteria applied: PM2_Supporting, PP3, PP4). RNA-seq instead demonstrated complete skipping of exon 8, resulting in a frameshift (NP_055990.1:p.(Glu215Thrfs*5)) and introduction of a premature termination codon (Figure 2A). The same aberrant transcript was detected in the patient's mother and two siblings, consistent with their heterozygous carrier status (Figure 2B-C). Together, these findings provided functional evidence for reclassification of the variant as pathogenic (ACMG criteria applied: PVS1_RNA, PM2_Supporting, PP4), illustrating the value of transcriptome-level analyses for resolving the clinical impact of VUS. Importantly, although *in silico* tools consistently predicted an effect on splicing, RNA-seq revealed a different and more severe transcript consequence than predicted, highlighting the value of transcriptome sequencing for refining variant interpretation beyond *in silico* predictions. Of note, a definitive genetic diagnosis can have important clinical and social implications, providing diagnostic certainty and enabling appropriate long-term follow-up and genetic counselling.

In a second patient (Patient 2), a 59-year-old woman with ISTH BAT score 11, we identified an in-frame exon-skipping event involving exons 4-6 of *PSMC4* (Figure 3A). WGS revealed a heterozygous deletion of approximately 2.8 kb, comprising exon 4, 5 and 6 of *PSMC4* and absent in gnomAD v4.1, thus providing a potential explanation for the aberrant splicing event (Figure 3B). The resulting exon 3-7 junction was not detected in GTEx junction data from healthy individuals, either in the WB subset or across the full GTEx dataset (GTEx analysis 2025-08-22 v11 STARv2.7.11b junctions), supporting its interpretation as an aberrant transcript (Figure 3C). The aberrant transcript affected the highly conserved AAA ATPase domain, which is essential for proteasome function. PSMC4 is a component of the 26S proteasome, and accumulating evidence suggests that platelet proteasomes contribute to platelet activation and aggregation, with proteasome inhibition resulting in impaired platelet function[14,15]. Consistent with this biological role, the patient showed laboratory evidence of a platelet function defect, including reduced ADP- and TRAP-induced PAC-1 activation and impaired TRAP- and collagen-induced lactadherin expression. *PSMC4* has a pLI score of 1.0 in gnomAD v4.1, indicating strong constraint against loss-of-function variation, and was expressed in WB in our cohort and in the GTEx RNA-seq dataset, supporting its assessment by WB RNA-seq. Thus,

PSMC4 represents a biologically plausible candidate gene for IBPD; however, there is currently no direct evidence that germline *PSMC4* variants cause an inherited platelet disorder or platelet dysfunction in humans. As only the patient`s asymptomatic mother was available for co-segregation analysis, and she did not carry the deletion (Figure 3D) we don't have positive segregation evidence for this case.

Our findings demonstrate that WB RNA-seq can provide clinically relevant transcriptomic evidence in unresolved IBPD, complementing genomic analysis by directly revealing aberrant splicing that would require a specific transcript to be predicted *a priori*. RNA-seq provided functional evidence that enabled reclassification of an intronic *NBEAL2* VUS to pathogenic in one patient, corresponding to a definitive diagnostic yield of 1/62 (1.6%). In another patient, RNA-seq identified aberrant splicing in *PSMC4*, a gene not currently associated with IBPD. While this finding requires further evidence to establish a novel disease-gene association, it highlights the potential of transcriptomic analysis to extend beyond established disease genes.

Patients with bleeding tendency of unknown cause may harbor variants in unknown platelet genes that confer defects in interaction with the vasculature; a function that was not examined in this study. Moreover, variation in blood cell composition and the dependence on gene expression in blood may limit the detection of platelet-associated transcripts and the sensitivity of WB RNA-seq for genes with restricted tissue or platelet expression. Thus, WB RNA-seq should not be considered a replacement for platelet- or tissue-specific transcriptomic approaches, but rather a clinically accessible complement to WGS that can provide functional evidence for variant interpretation and uncover candidate disease mechanisms in unresolved IBPD. Integrating transcriptomic and genomic data may therefore represent a practical strategy to increase the diagnostic resolution of unresolved IBPD while simultaneously generating hypotheses for the discovery of new disease genes.

References

1. Ramanan R, Verstraete A, Van Laer C, Freson K. Implementation and clinical utility of multigene panels for bleeding, platelet, and thrombotic disorders. J Throm Haemost. 2025;23(8):2371-2387.

2. Downes K, Megy K, Duarte D, et al. Diagnostic high-throughput sequencing of 2396 patients with bleeding, thrombotic, and platelet disorders. Blood. 2019;134(23):2082-2091.

3. Leinøe E, Zetterberg E, Kinalis S, et al. Application of whole-exome sequencing to direct the specific functional testing and diagnosis of rare inherited bleeding disorders in patients from the Öresund Region, Scandinavia. Br J Haematol. 2017;179(2):308-322.

4. Sha Y, Ortiz JB, Bristow SL, et al. Refining the interpretation of variants of uncertain significance in hereditary cancer screening through integrated RNA sequencing. Genet Med Open. 2025;3:101914.

5. De Wispelaere K, Freson K. The analysis of the human megakaryocyte and platelet coding transcriptome in healthy and diseased subjects. Int J Mol Sci. 2022;23(14):7647.

6. Noetzli L, Lo RW, Lee-Sherick AB, et al. Germline mutations in ETV6 are associated with thrombocytopenia, red cell macrocytosis and predisposition to lymphoblastic leukemia. Nat Genet. 2015;47(5):535-538.

7. Kahr WHA, Hinckley J, Li L, et al. Mutations in NBEAL2, encoding a BEACH protein, cause gray platelet syndrome. Nat Genet. 2011;43(8):738-740.

8. Megy K, Downes K, Simeoni I, et al. Curated disease-causing genes for bleeding, thrombotic, and platelet disorders: communication from the SSC of the ISTH. J Throm Haemost. 2019;17(8):1253-1260.

9. Leinøe E, Fridriksdottir H, Rasmussen AØ, et al. Low vitamin C status and hypermobility-related disorders in patients with bleeding disorder of unknown cause. Haemophilia. 2024;30(6):1366-1372.

10. Mertes C, Scheller IF, Yépez VA, et al. Detection of aberrant splicing events in RNA-seq data using FRASER. Nat Commun. 2021;12(1):529.

11. Yépez VA, Gusic M, Kopajtich R, et al. Clinical implementation of RNA sequencing for Mendelian disease diagnostics. Genome Med. 2022;14(1):38.

12. Hough C, Notley C, Mo A, Videl B, Lillicrap D. Heterogeneity and reciprocity of FVIII and VWF expression, and the response to shear stress in cultured human endothelial cells. J Thromb Haemost. 2022;20(11):2507-2518.

13. Ritelli M, Chiarelli N, Cinquina V, et al. RNA-Seq of dermal fibroblasts from patients with hypermobile ehlers-danlos syndrome and hypermobility spectrum disorders supports their categorization as a single entity with involvement of extracellular matrix degrading and proinflammatory pathomechanisms. Cells. 2022;11(24):4040.

14. El-Kadiry AEH, Merhi Y. The role of the proteasome in platelet function. Int J Mol Sci. 2021;22(8):3999.

15. Colberg L, Cammann C, Greinacher A, Seifert U. Structure and function of the ubiquitin-proteasome system in platelets. J Thromb Haemost. 2020;18(4):771-780.

Figure 1. Study design and RNA-seq analysis of patients with inherited bleeding and platelet disorders.

A. Peripheral whole-blood (WB) samples from 62 patients with inherited bleeding and platelet disorders (IBPD) and negative whole genome sequencing (WGS) results were collected in PaxGene tubes. Total RNA was isolated using the AllPrep RNA mini kit (Qiagen) according to manufacturer's instructions and subsequently treated with DNase using the Rneasy mini kit (Qiagen). RNA-library prep was performed with Stranded Total RNA Prep (Illumina) with an input of 100 ng RNA. Sequencing was performed on the Illumina NovaSeq 6000 platform, generating a median of 104.0 million reads per sample (range: 58.3–194.5 million reads). FASTQ files were mapped to the hg38 human reference genome with STAR v.2.7.10a with the parameter twopassMode set to “Basic” to allow the detection of novel splice junctions as described by Yépez and colleagues[11]. RNA-seq data from these 62 patients were combined with data from 15 additional cases with known diagnoses, which were included to improve FRASER model estimation. Splicing outliers were identified using FRASER v.1.14.1, applying the default thresholds of FDR-adjusted $p < 0.05$ and $|\Delta\psi| > 0.3$. The resulting outliers were then filtered to retain only protein-coding genes and exclude HLA genes, with at least 50 reads in one sample or a mean read count of at least 50 across samples. Downstream analyses, including manual curation of splicing outliers through literature review and visualization of sequencing reads, were restricted to the 62 WGS-negative IBPD cases. Candidate genes were subsequently evaluated in the WGS data to identify plausible causative genotypes. **B.** Mean expression levels of the 121 genes included in our IBPD diagnostic panel. The dotted red line indicates the threshold of 450 normalized reads, previously associated with sufficient sensitivity to detect approximately 50% under-expression. Genes are divided into five subcategories: platelet, coagulation factors (CF), hemorrhagic telangiectasia (HT), Ehlers-Danlos syndrome (EDS), and other.

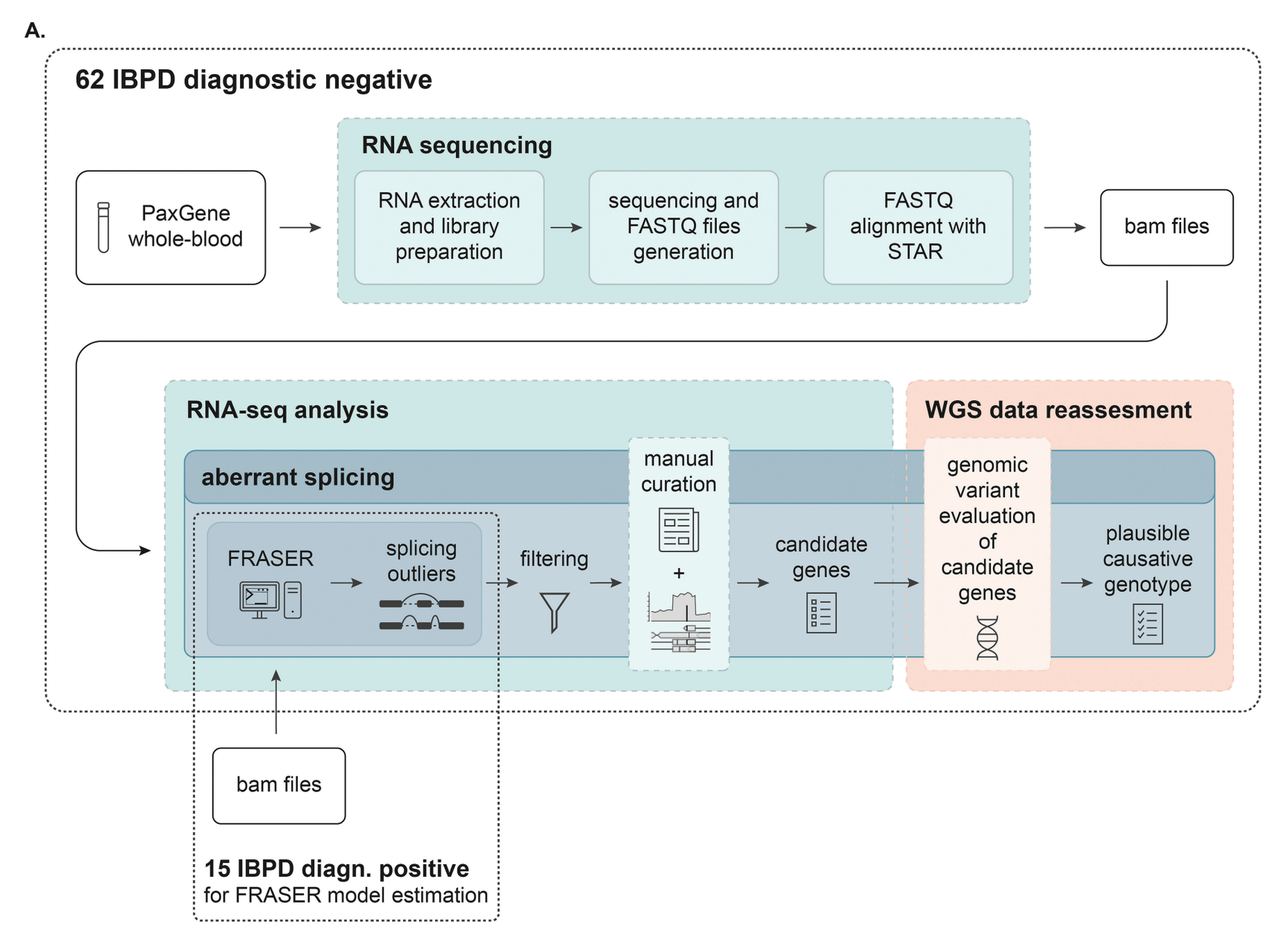
Figure 2. Exon skipping in NBEAL2 transcript caused by the homozygous intronic variant NM_015175.3:c.925+5del.

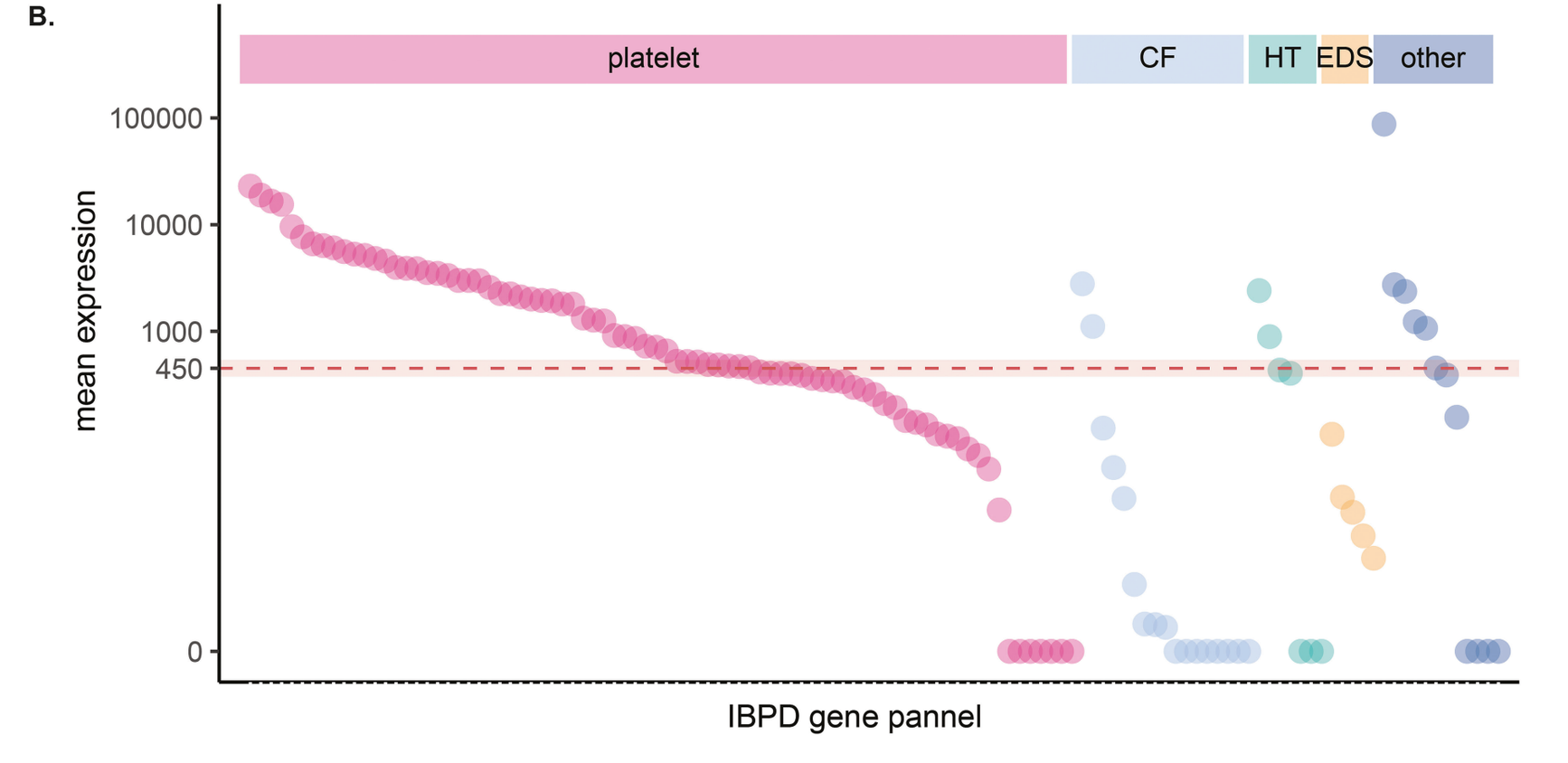
A. Schematic representation of the aberrant splicing event in patient 1 leading to a premature stop codon (top). Coverage and sashimi plot of the proband RNA sample of the involved region in NBEAL2 are shown. Only the novel splicing event between exon 7 and 9 is detected, with no reads supporting the canonical splicing. The position of variant NM_015175.3:c.925+5del is indicated by the black arrow. The genomic

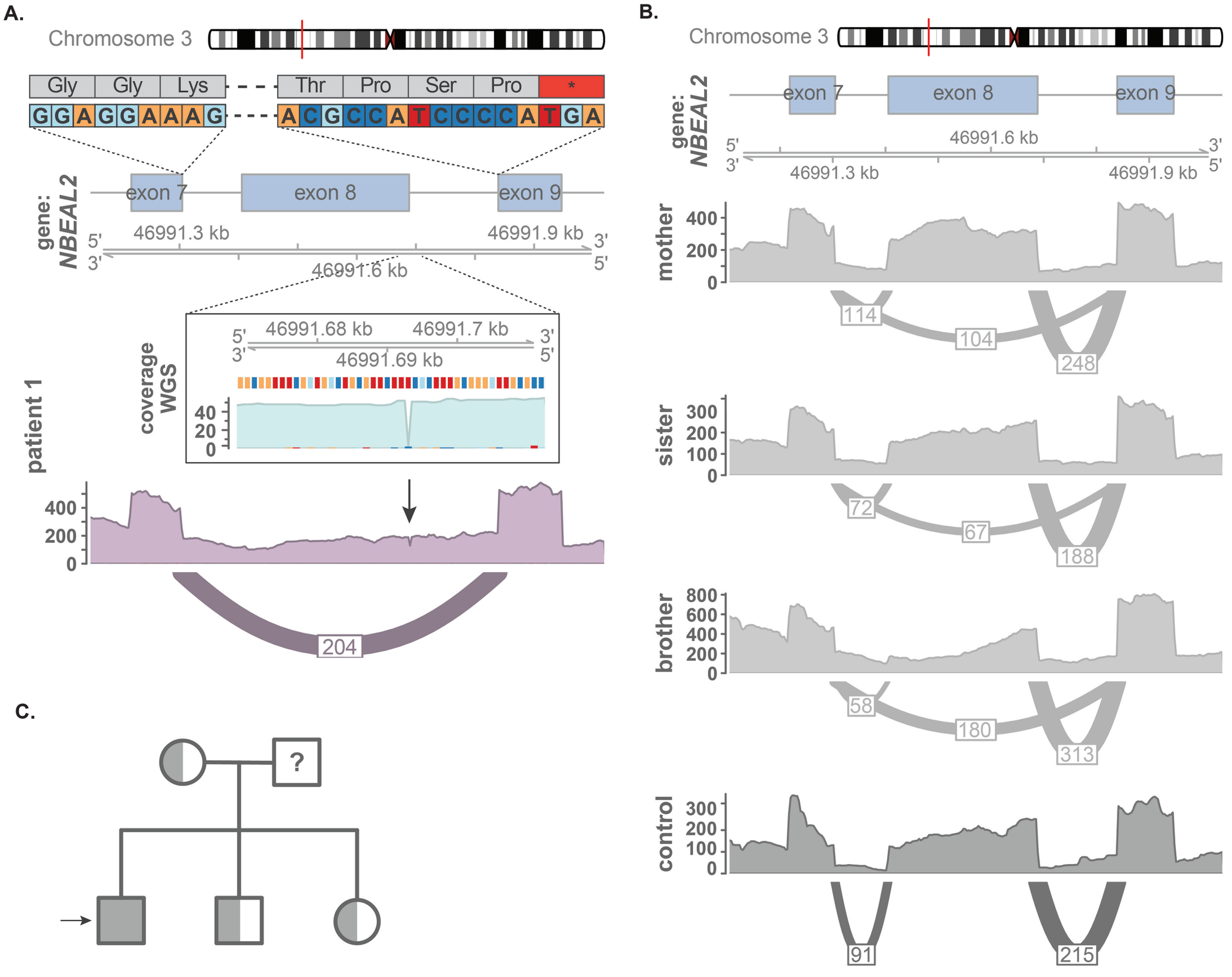
coverage of the region encompassing the deletion is shown in the box above. **B.** Coverage and sashimi plot of the region of interest in NBEAL2 of 3 relatives of the proband (from top to bottom: mother, sister and brother, light grey) and one unrelated control (grey). All three relatives carry the NM_015175.3:c.925+5del variant in the heterozygous state and show both canonical splicing and the aberrant exon skipping events, consistent with their heterozygous genotype. The number of split reads between each donor-acceptor couple are indicated on the arches. **C.** Pedigree of the family; the arrow indicates the proband. Filled and half-filled symbols indicate affected and carrier individuals, respectively; the father was not tested.

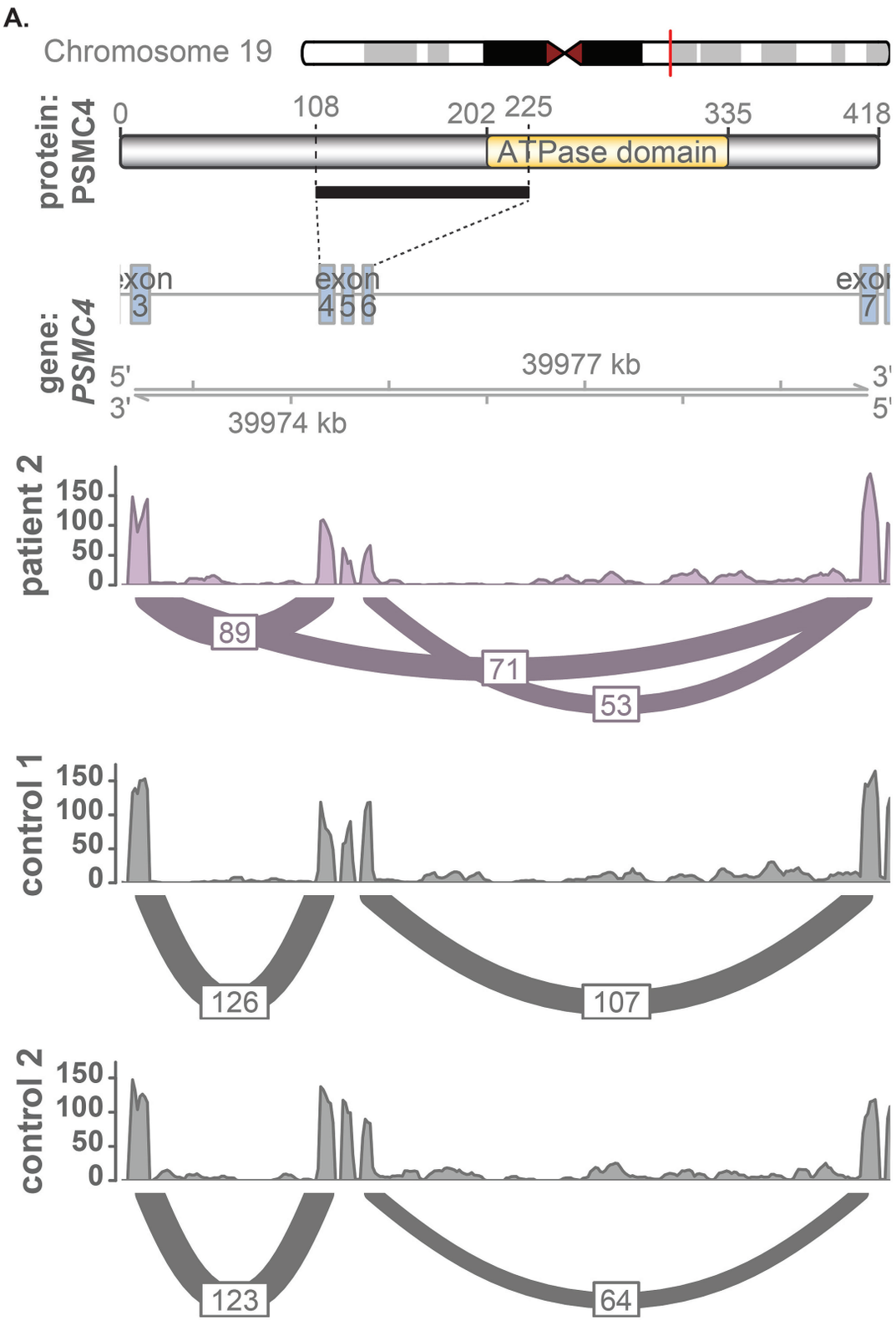
Figure 3. Exon skipping in PSMC4 resulting in the in-frame skipping of exons 4-6.

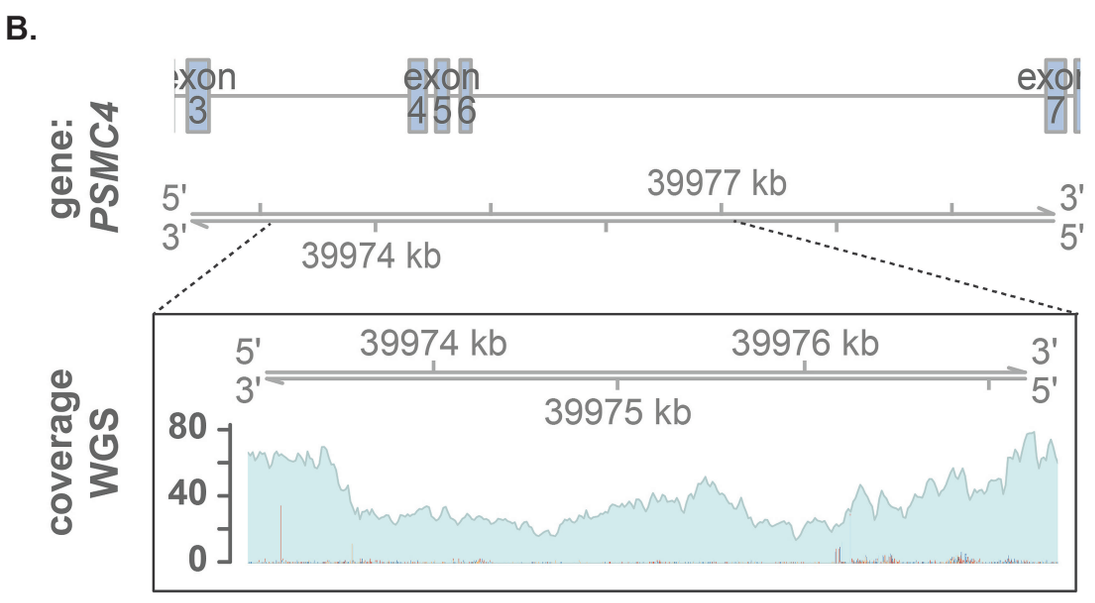
A. Linear representation of the PSMC4 protein (top). The region affected by the exon skipping event, including the first 22 amino acids of the ATPase domain, is indicated by the black line underneath. Coverage and sashimi plot of the RNA reads of the proband (purple), and two unrelated controls (grey) aligned to the region of interest in *PSMC4* (bottom). In the proband, the alternative splicing event resulting in the skipping of exons 4-6 is supported by approximately half of the reads spanning the region; the number of split reads supporting each donor-acceptor junction pair are indicated on the arches. The event is not observed in either control. **B.** View of the *PSMC4* region encompassing exon 4, 5 and 6 in the proband. The genomic coverage of the region is shown in the inset. **C.** Number of GTEx samples in which the canonical exon 3-4 and exon 6-7 junctions, and the aberrant exon 3-7 junction identified in the patient, were detected. Results are shown for the complete GTEx dataset and for the WB subset. The exon 3-7 junction was not detected in any GTEx sample. **D.** Pedigree of the family of the affected individual; the arrow indicates the proband. The proband is shown as half-filled to indicate heterozygosity for the deletion.

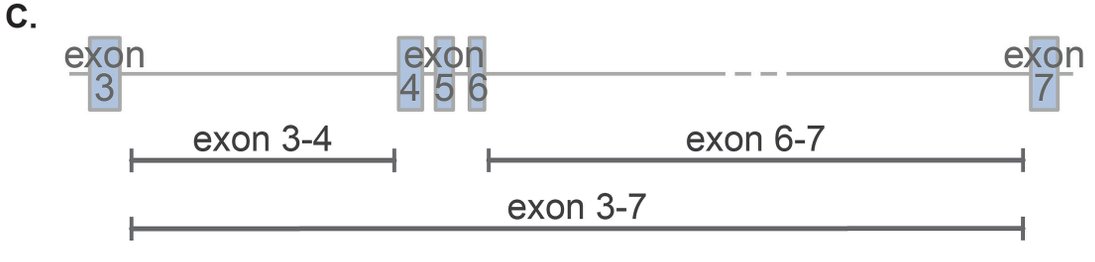

A.
62 IBPD diagnostic negative
PaxGene whole-blood
RNA sequencing
RNA extraction and library preparation
sequencing and FASTQ files generation
FASTQ alignment with STAR
bam files
RNA-seq analysis
aberrant splicing
FRASER
splicing outliers
filtering
manual curation
+
candidate genes
WGS data reassesment
genomic variant evaluation of candidate genes
plausible causative genotype
bam files
15 IBPD diagn. positive
for FRASER model estimation


B.
platelet
CF
HT
EDS
other
100000
10000
1000
450
0
mean expression
IBPD gene pannel

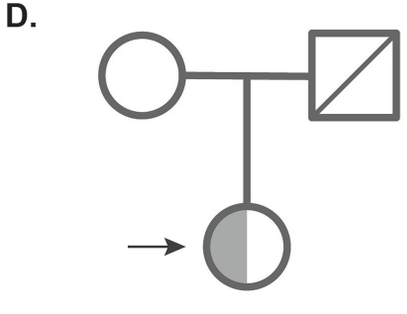

A.
Chromosome 3
Gly Gly Lys Thr Pro Ser Pro *
GGAGGAAAG ACGCCATCCCCATGA
gene: NBEAL2
exon 7
exon 8
exon 9
5'
3'
46991.3 kb
46991.9 kb
46991.6 kb
patient 1
coverage WGS
46991.68 kb
46991.7 kb
46991.69 kb
40
20
0
400
200
0
204
B.
Chromosome 3
gene: NBEAL2
exon 7
exon 8
exon 9
46991.6 kb
46991.3 kb
46991.9 kb
mother
400
200
0
114
104
248
sister
300
200
100
0
72
67
188
brother
800
600
400
200
0
58
180
313
control
300
200
100
0
91
215
C.
?







Junction	GTEx tot	GTEx WB
exon 3-4	19788/19788	803/803
exon 6-7	19788/19788	803/803
exon 3-7	0/19788	0/803



Online Supplementary Information (see Supplementary Excel File)

Online Supplementary Table 1. Clinical characteristics and plausible candidate genes. ASD: atrium septum defect. ADHD: attention deficit hyperactivity disorder. FC: Flow cytometry. HEDS: hypermobile Ehlers-Danlos syndrome. IF: Immunofluorescens microscopy. IBD: inflammatory bowel disease. IHD: ischemic heart disease. KOL: chronic obstructive lung disease. LTA: Light transmission aggregometry. MPV: mean platelet volume. ND: not done. TEM: transmission electron microscopy.

Online Supplementary Table 2. Supplementary dataset with an established genetic diagnosis of an inherited bleeding or platelet disorder.

Online Supplementary Table 3. Gene panel used for WGS analysis. HT: hemorrhagic telangiectasia. EDS: Ehlers-Danlos syndrome. CF: coagulation factors.