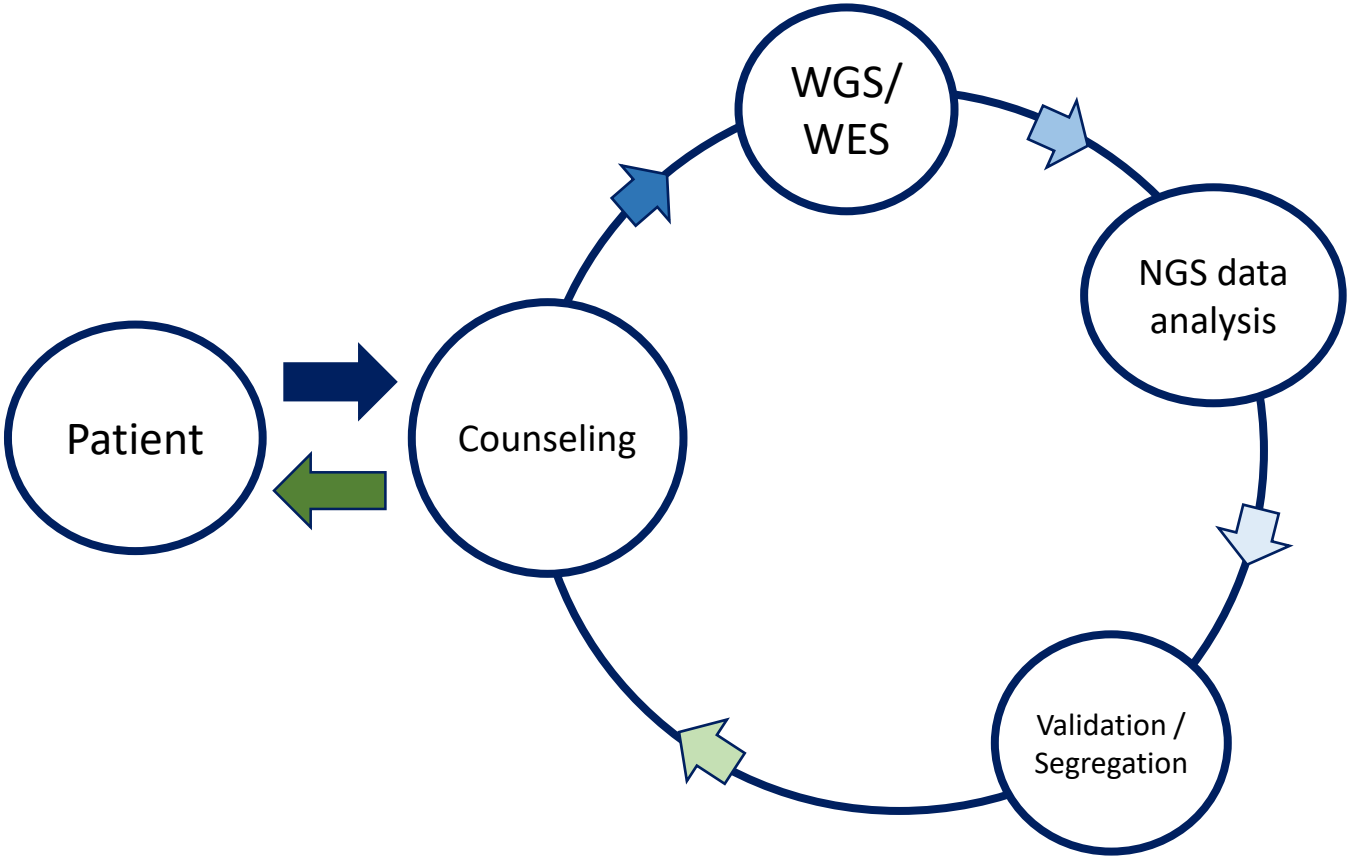
Functional Analysis of Splice Affecting Variants

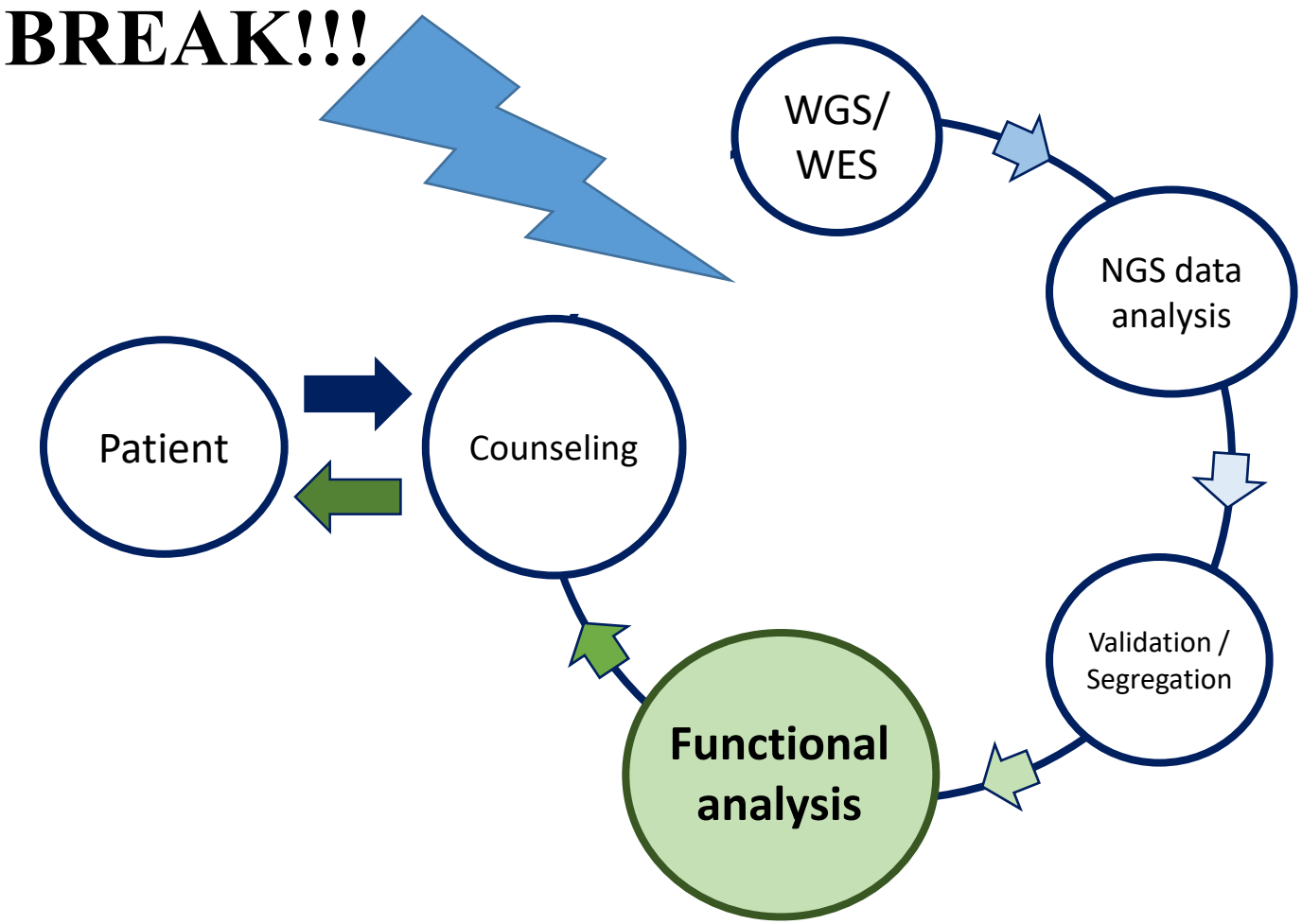
Mikhail Skoblov

VEPTC Moscow, 2019

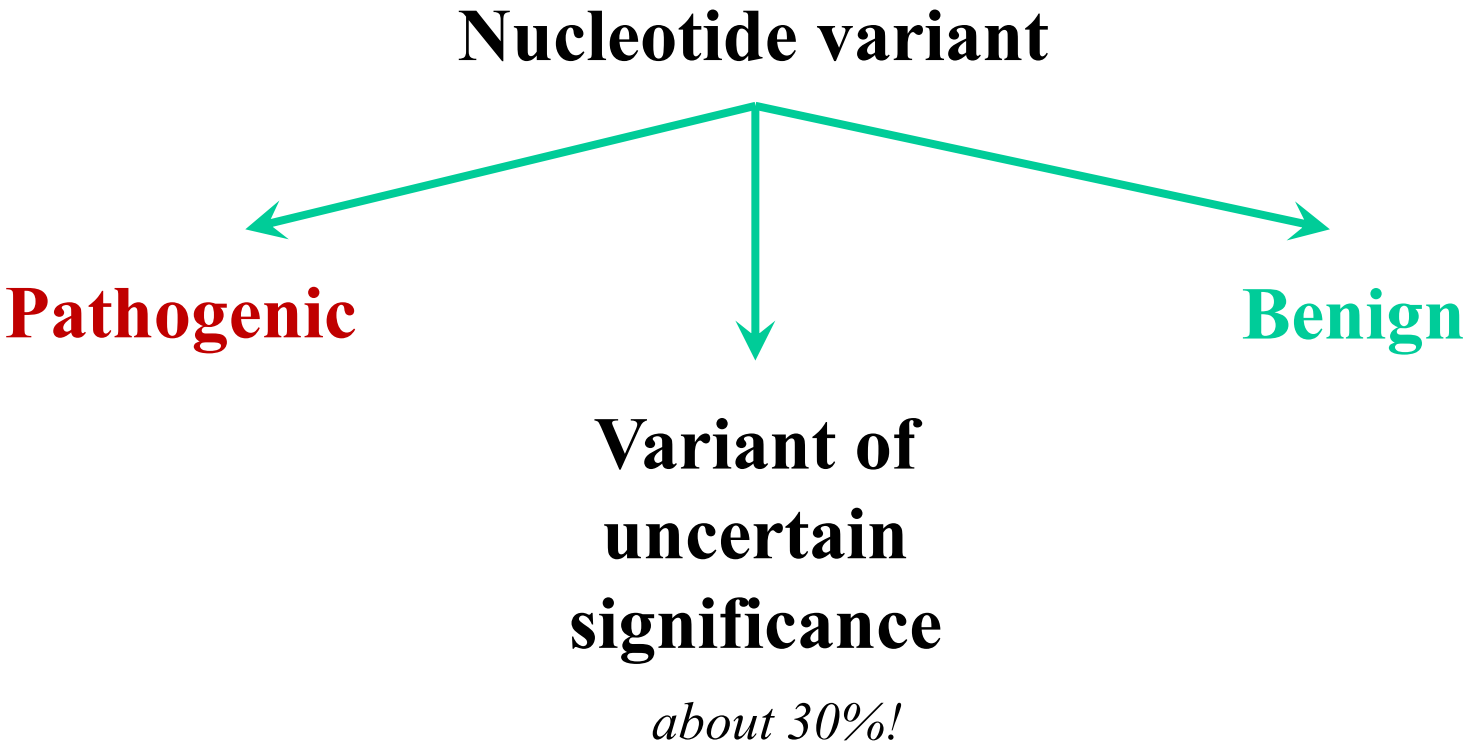
Vicious circle



Vicious circle



Evaluation of the pathogenicity of the nucleotide variant



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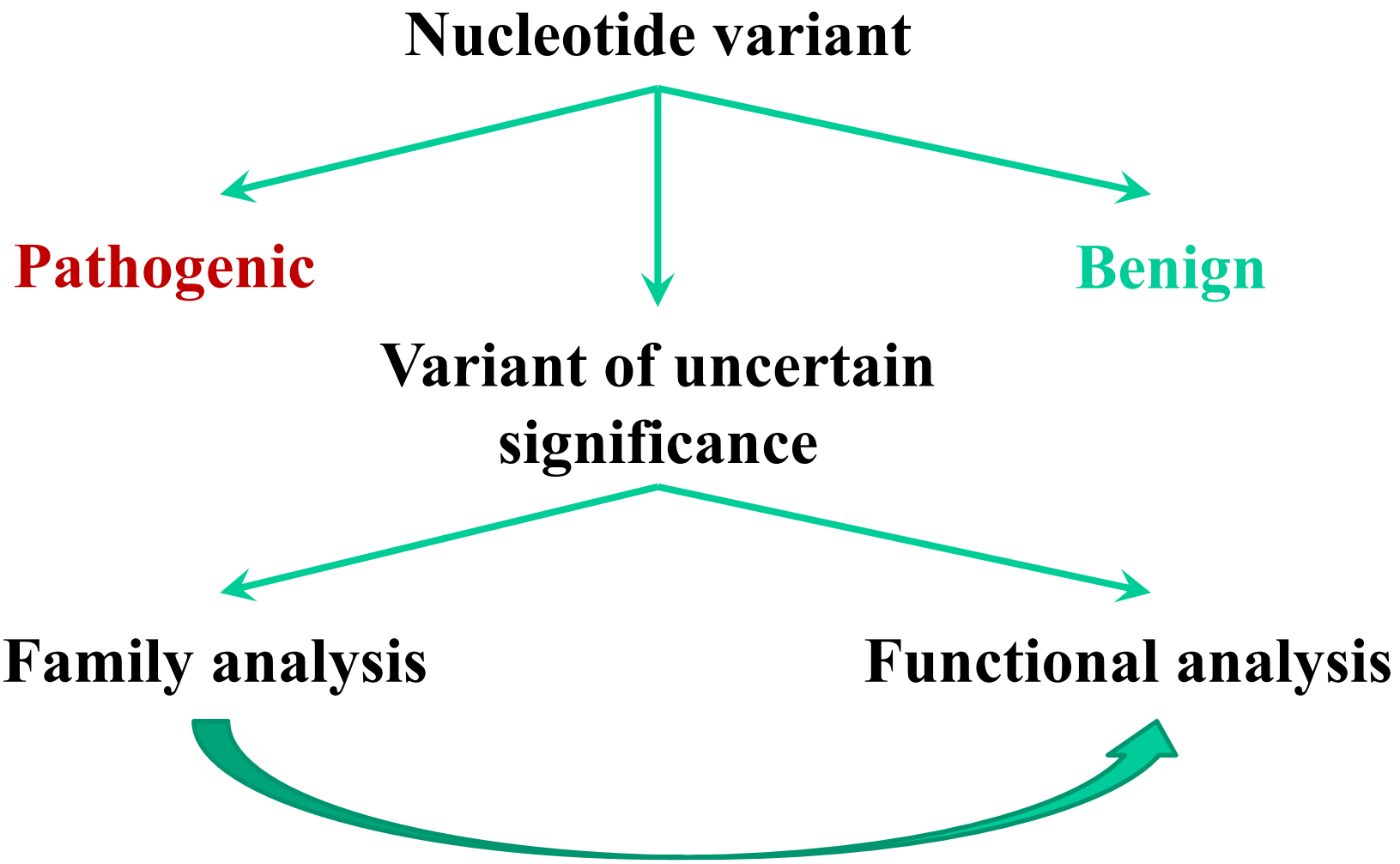
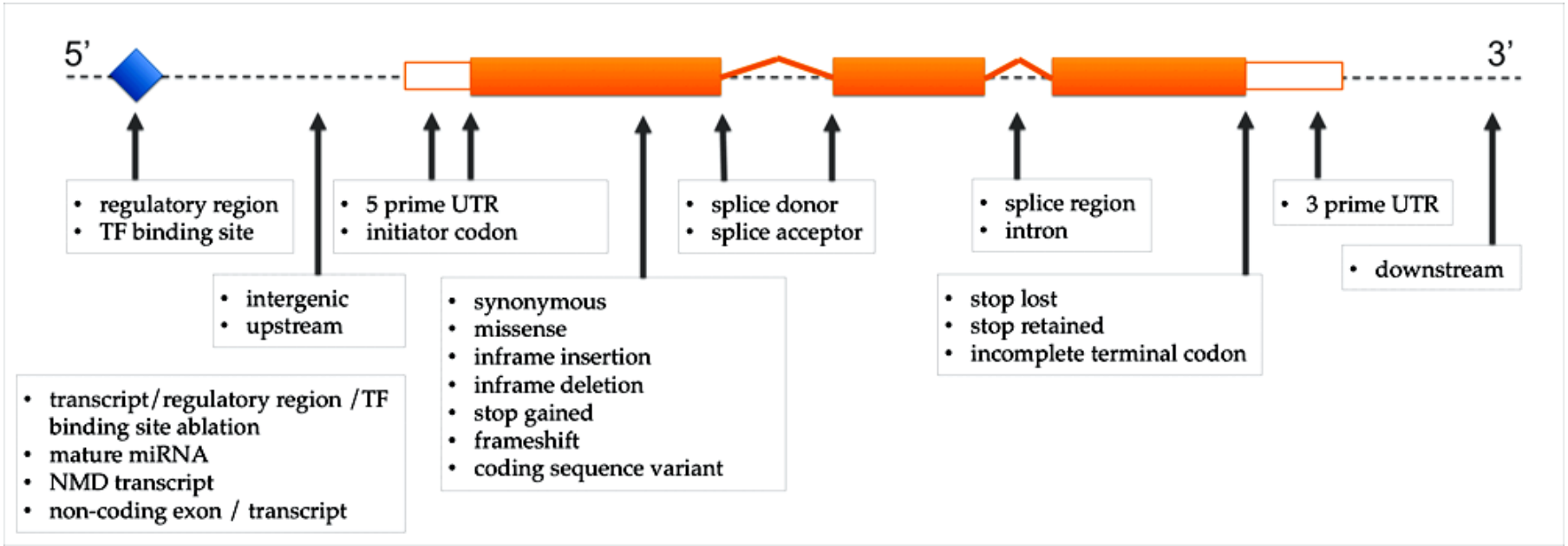


Where there may be pathogenic variants?



Variant classification

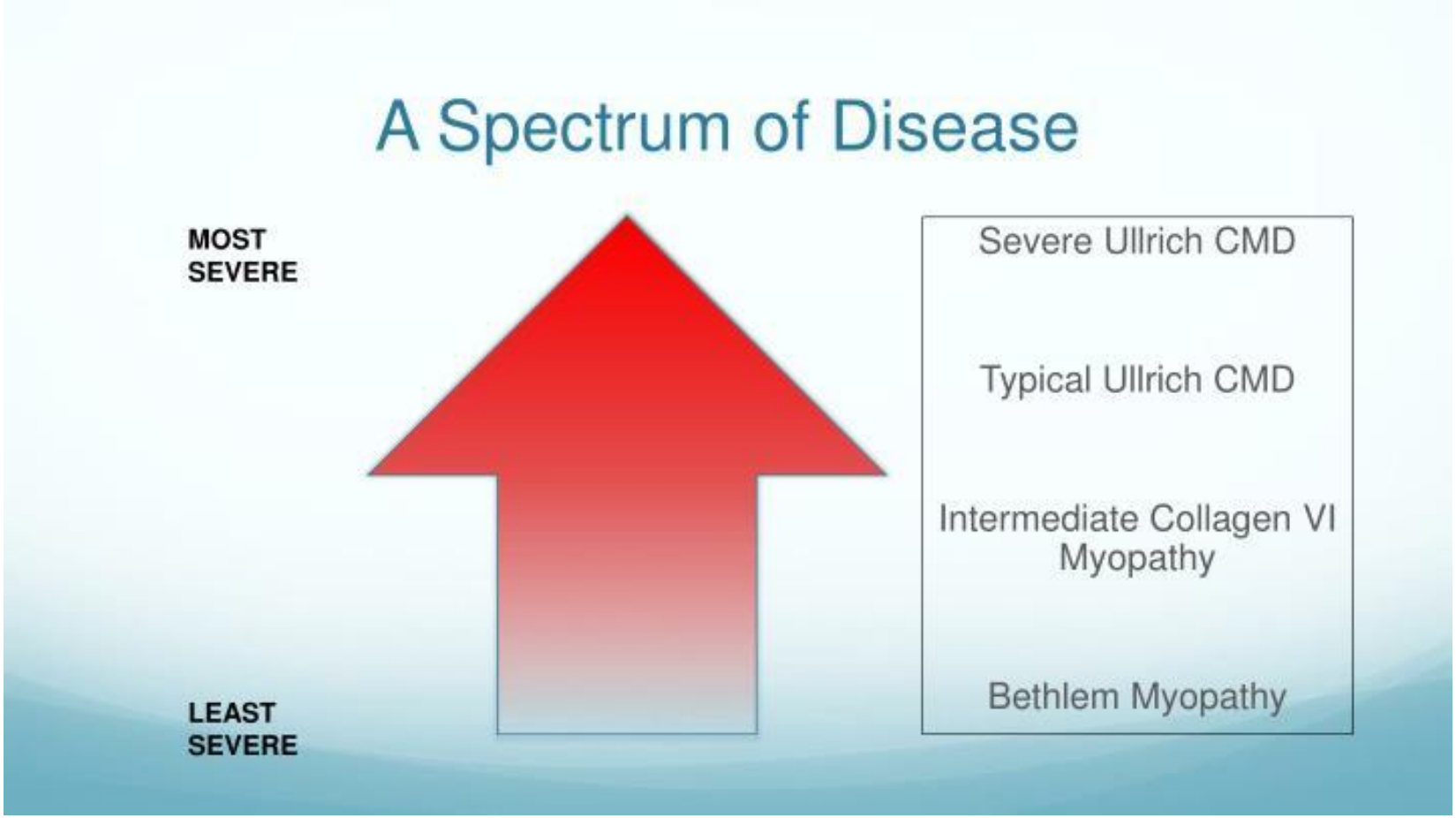
- LOF variants
- Missense variants
- In-frame indel
- Synonymous variants
- Splice variants
- Regulatory variants

Two ways for functional analysis

- Analysis of primary cultures obtained from patients

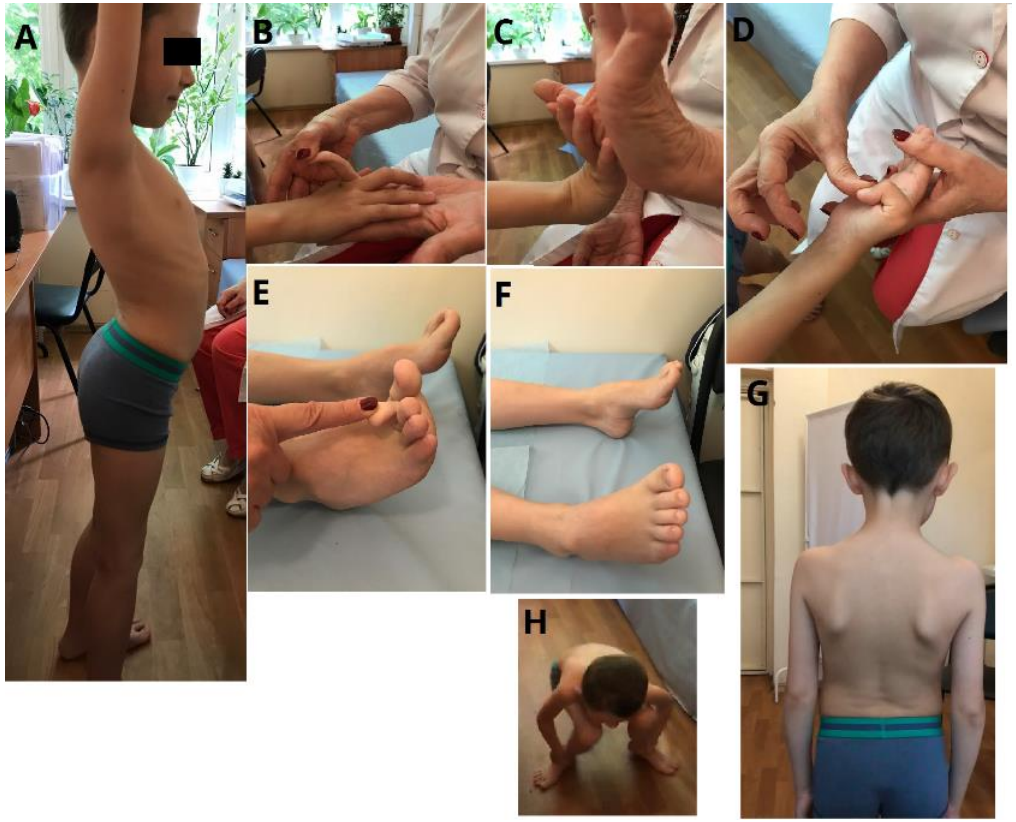
- Research with expression vectors in *suitable cell line or model organism:*
  - *Cloning*
  - *Transfection*
  - *Analysis of transfected culture*

One gene – several phenotypes

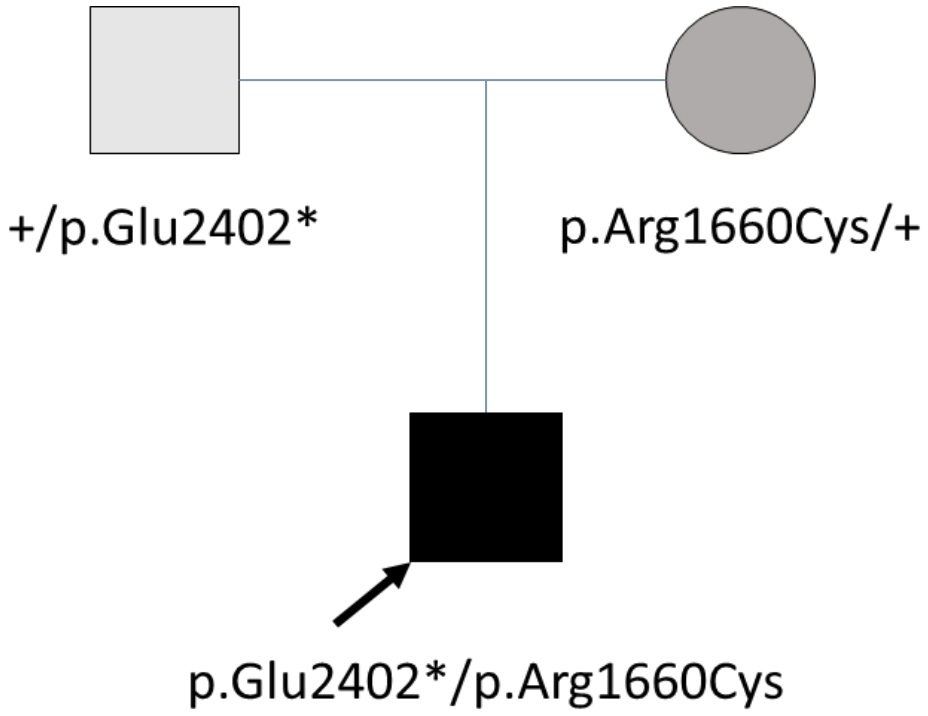


Clinical picture

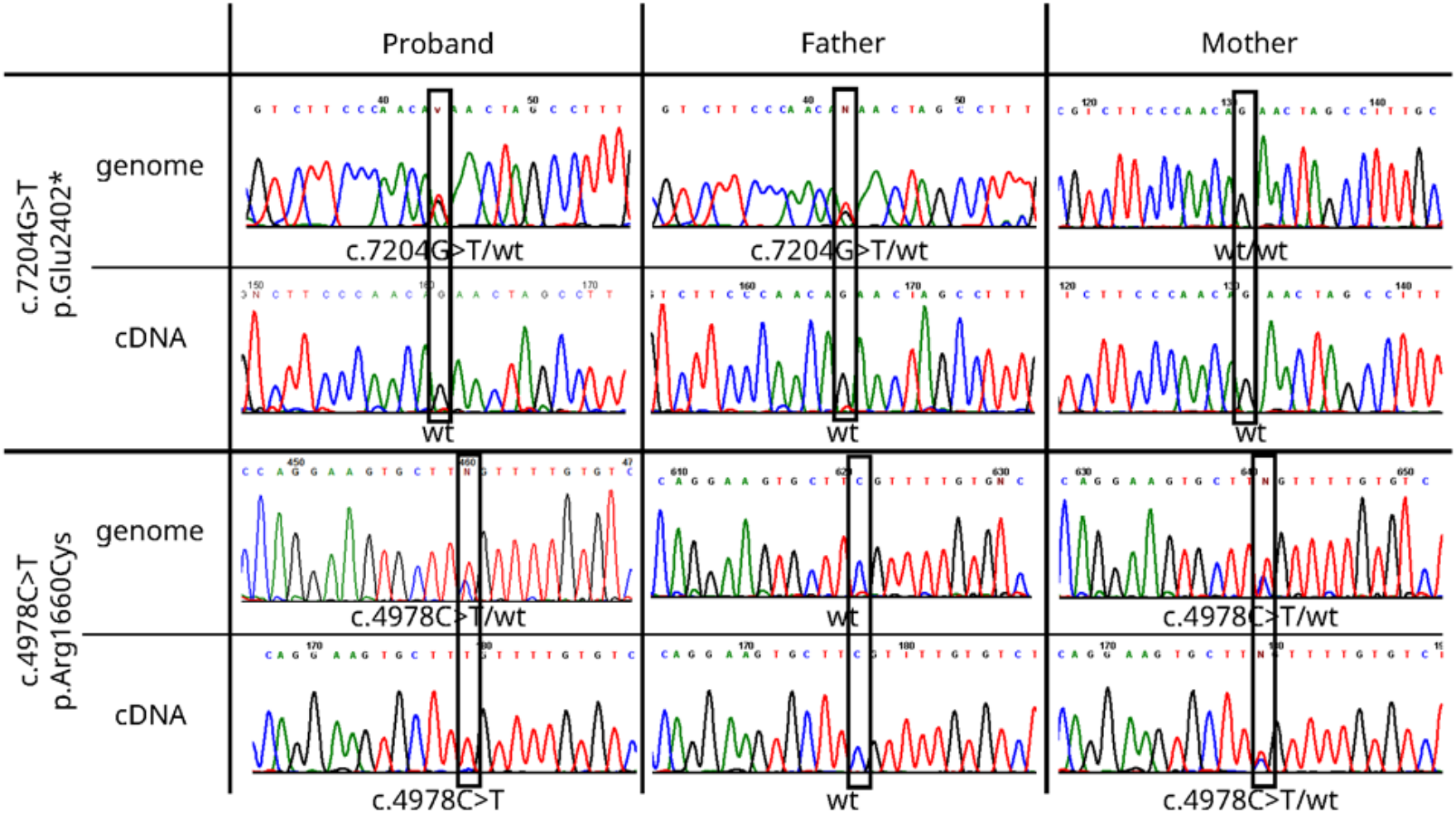
- Proband, a boy of 8 years, was examined for complaints of clumsiness, weakness of feet, gait disturbance, a tendency to walking on the toes, quick fatigue, difficulty with stair climbing, insufficiency of fine motor skills, over-extension in interphalangeal joints of hands and feet.
- The arches of the foot were tall, but when walking, the planovalgus deformity of the left foot acquired a varus position, with a marked pulling forward in the step. Hypermobility is present mainly in interphalangeal joints while ankle joints demonstrate stiffness with early signs of retraction of Achilles tendons. Walking on the heels is not possible. Walking on the toes is well. Standing from the crouched position without the Hoover's sign. Tendon reflexes are brisk.



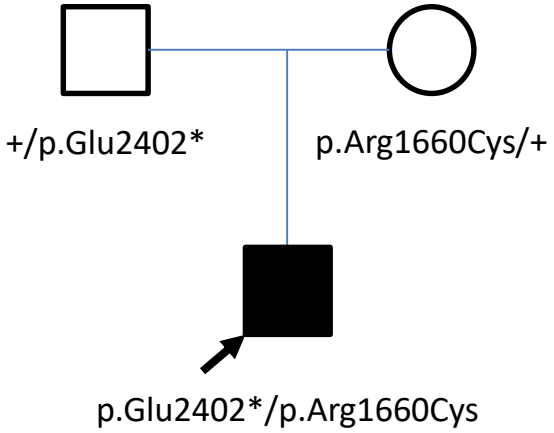
Analysis of variants in the COL6A3 gene

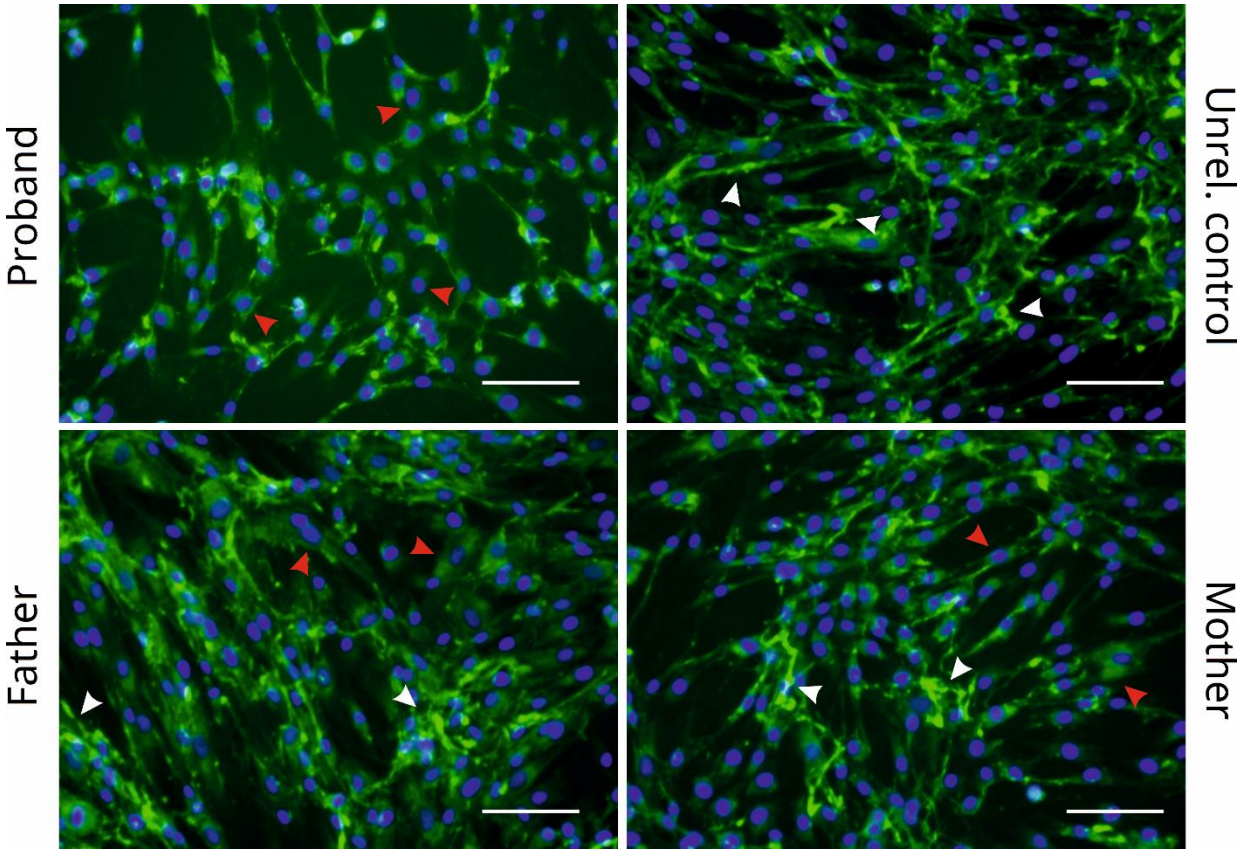


Family analysis of COL6A3 variants in DNA and RNA



Immunofluorescent staining of fibroblasts cultures

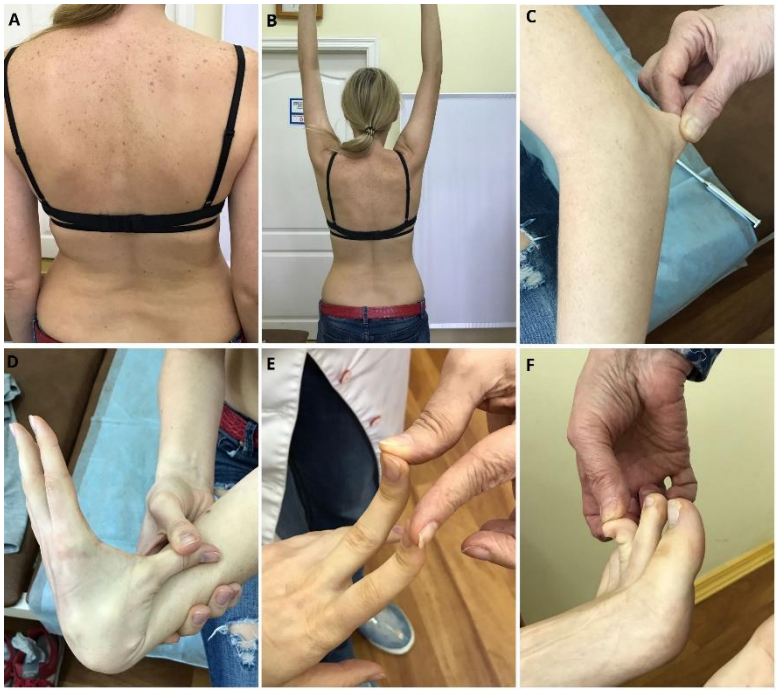


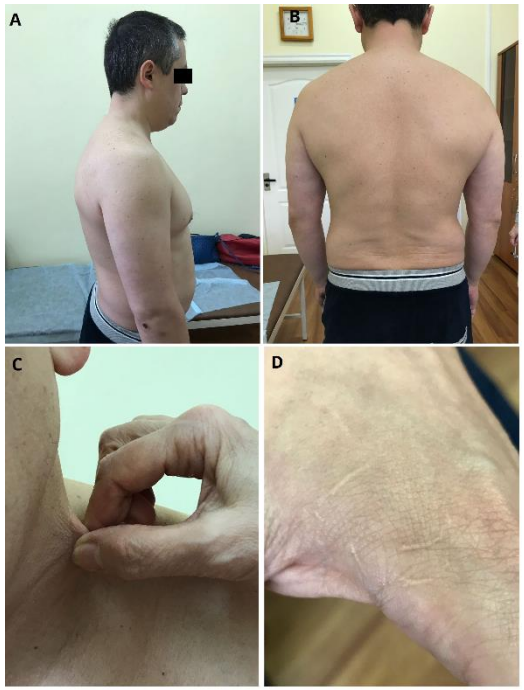


Clinical examination of parents

Despite the fact that the parents of the proband are clinically healthy, according to the anamnesis they display certain subclinical signs of **undifferentiated connective tissue dysplasia:**

- The mother of the proband has hyperlaxity in the elbow joint area, hypermobility in the interphalangeal joints of the hands and feet, the Orshansky sign, the habitual subluxation of the wrist joint, and soft scoliosis of the thoracolumbar region.
- The father has increased the CK activity level up to 556 u/L (normal is under 190), increased skin velvety and the formation of parchment scars on the site of microtraumas, hyperlaxity in the elbow joint and neck, hypermobility of interphalangeal joints of the hands, soft chest scoliosis, marked lumbar lordosis and thoracic kyphosis.





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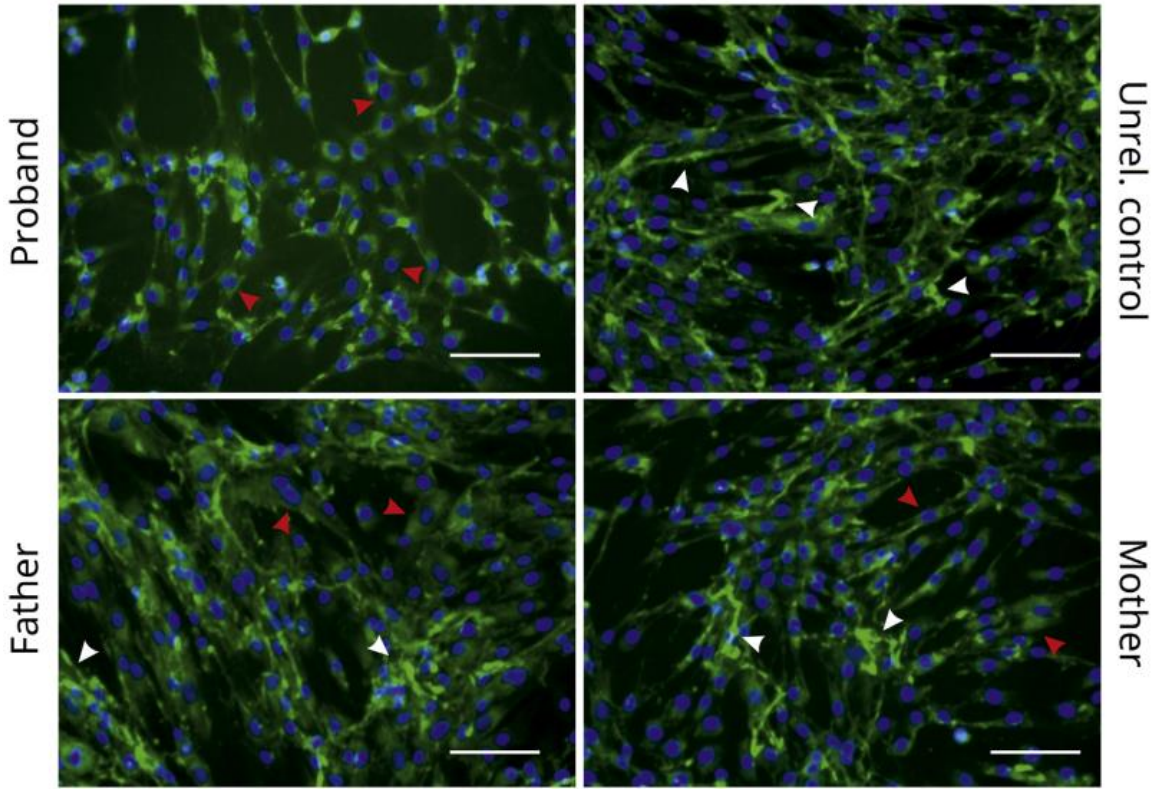
journal homepage: www.elsevier.com/locate/gene

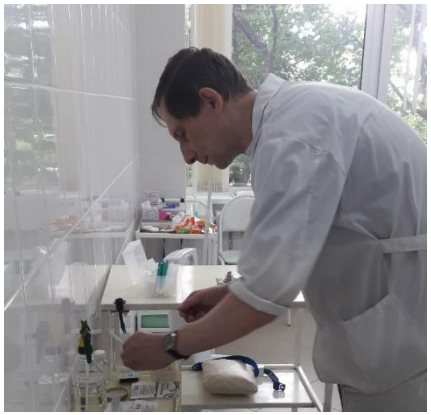
Research paper

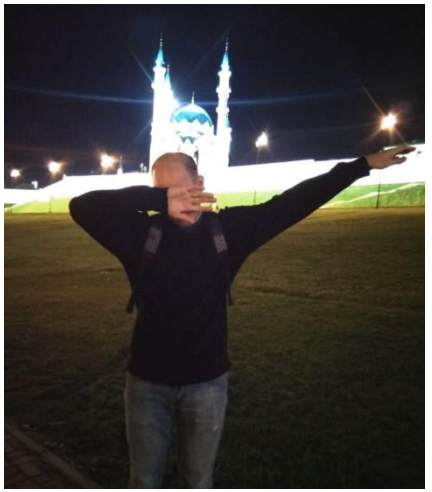
Two novel *COL6A3* mutations disrupt extracellular matrix formation and lead to myopathy from Ullrich congenital muscular dystrophy and Bethlem myopathy spectrum

Andrey V. Marakhonov[a,b], Vyacheslav Yu. Tabakov[a], Nikolay V. Zernov[a], Elena L. Dadali[a], Inna V. Sharkova[a], Mikhail Yu. Skoblov[a,b,*]

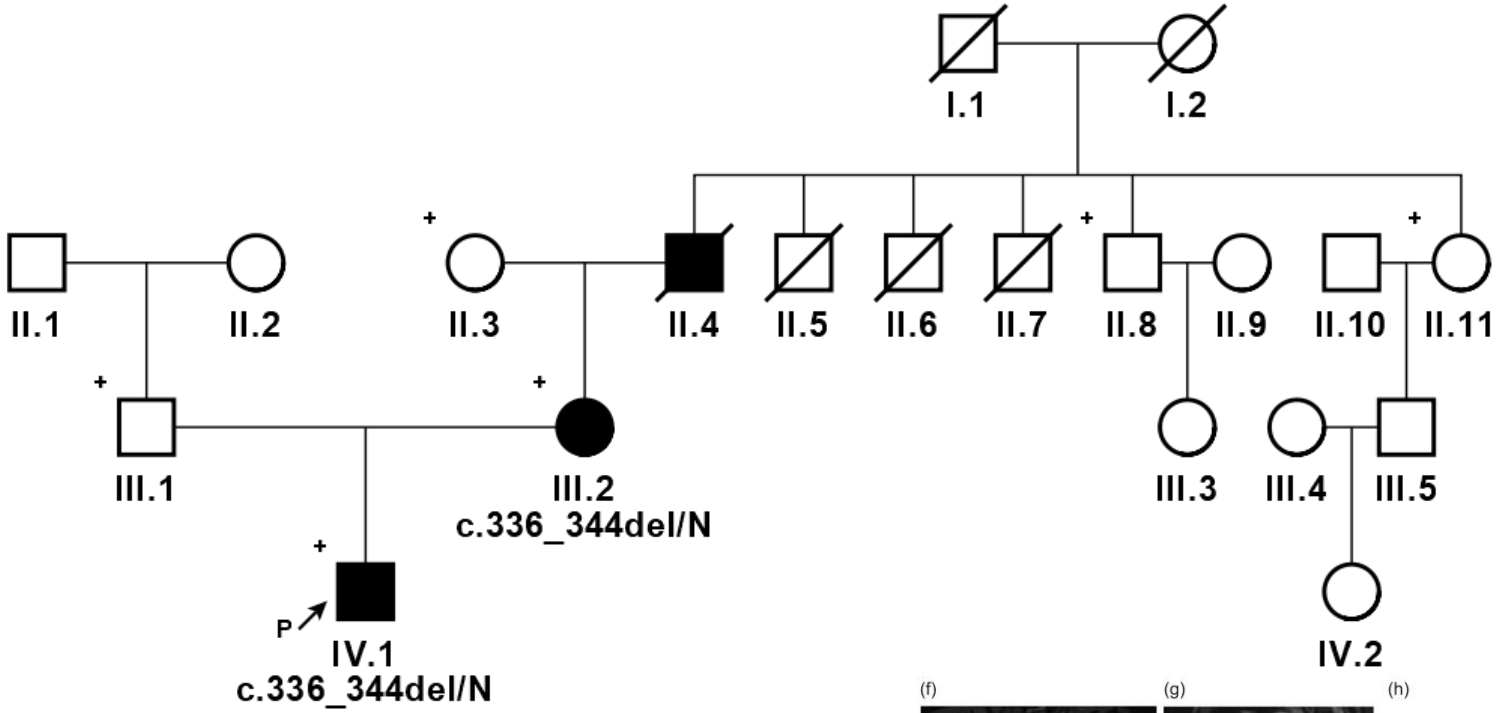
[a] Research Centre for Medical Genetics, Moscow 115478, Russia
[b] Moscow Institute of Physics and Technology, Dolgoprudny 141701, Russia



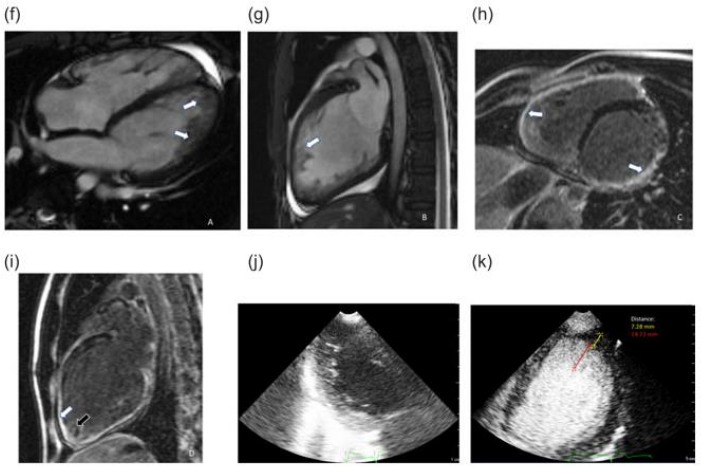




Case with noncompaction cardiomyopathy

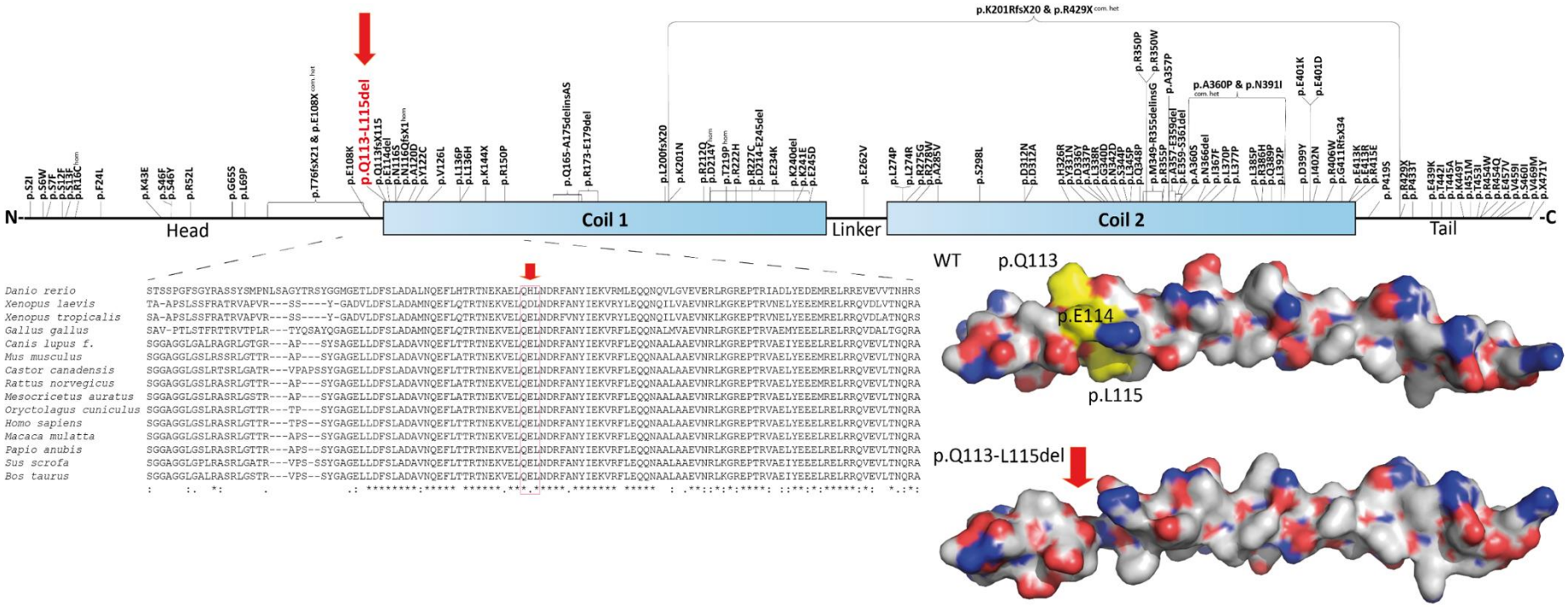


- Left ventricular noncompaction cardiomyopathy is a rare structural abnormality of the left ventricular myocardium of uncertain etiology and with an unknown molecular pathogenetic mechanism.



Case with noncompaction cardiomyopathy

- DES encoding the muscle-specific intermediate filament (IF) protein wchich form a three-dimensional scaffold that connects different cell organelles like the desmosomes, costameres, Z-bands and presumably also the nuclei.
- DES mutations might cause either myopathies, for example, myofibrillar myopathy type 1 (MIM# 601419), Kaiser-type neurogenic scapuloperoneal syndrome (SCPNK; MIM# 181400) or different cardiomyopathies including DCM (MIM# 604765), HCM, RCM, or arrhythmogenic right ventricular cardiomyopathy (ARVC).
- It is unknown, whether DES mutations are associated with left ventricular hypertrabeculation.



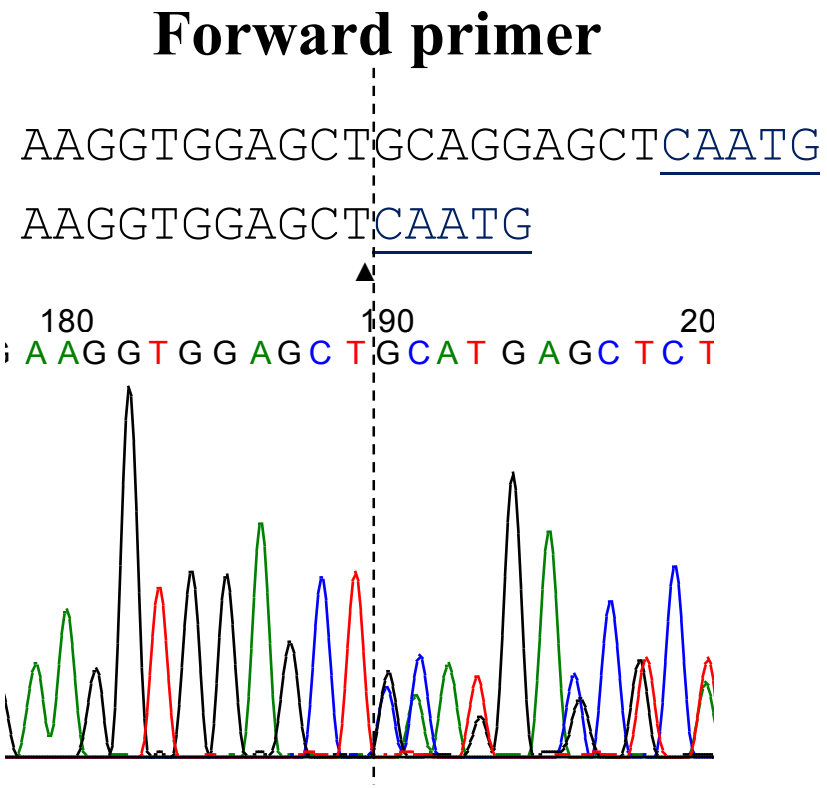
Case with noncompaction cardiomyopathy

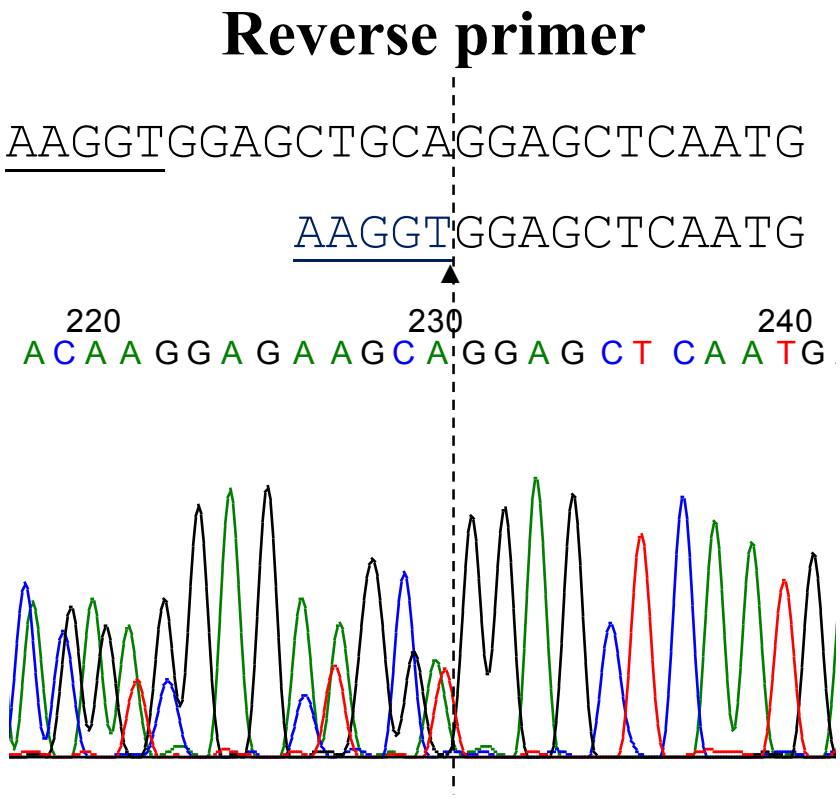
NM_001927.3(DES_v001):c.300_308del

NM_001927.3(DES_v001):c.336_344del

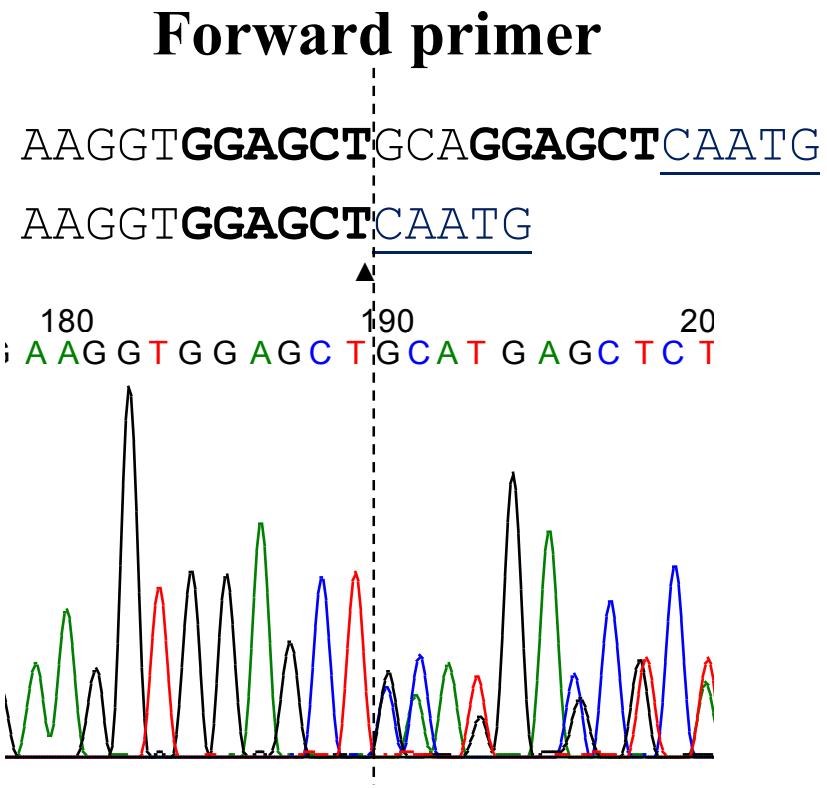
NM_001927.3(DES_v001):c.330_338del

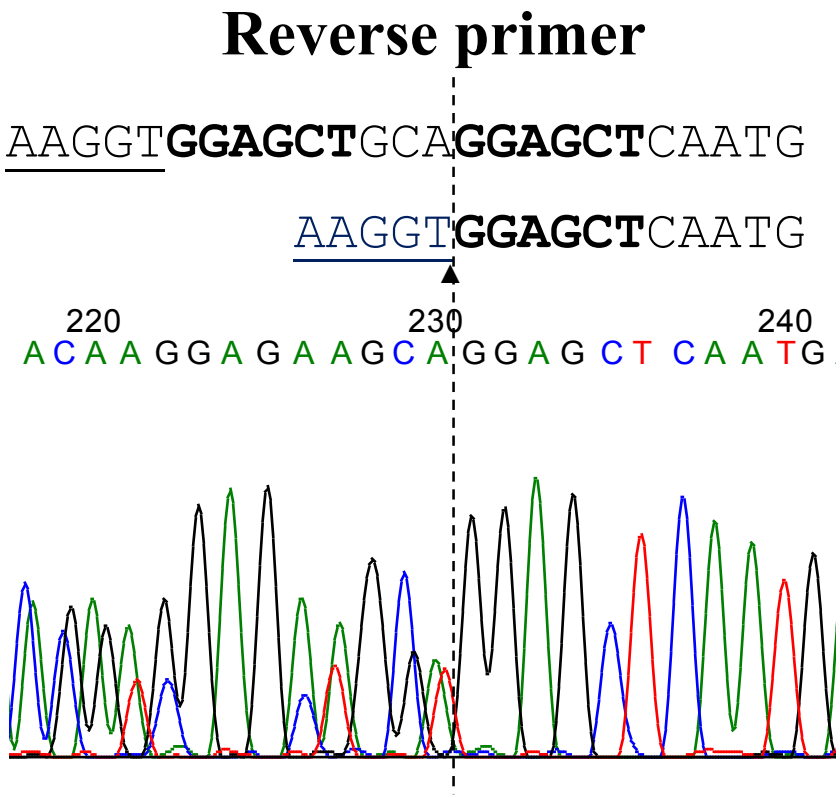
Problem of sequence interpretation



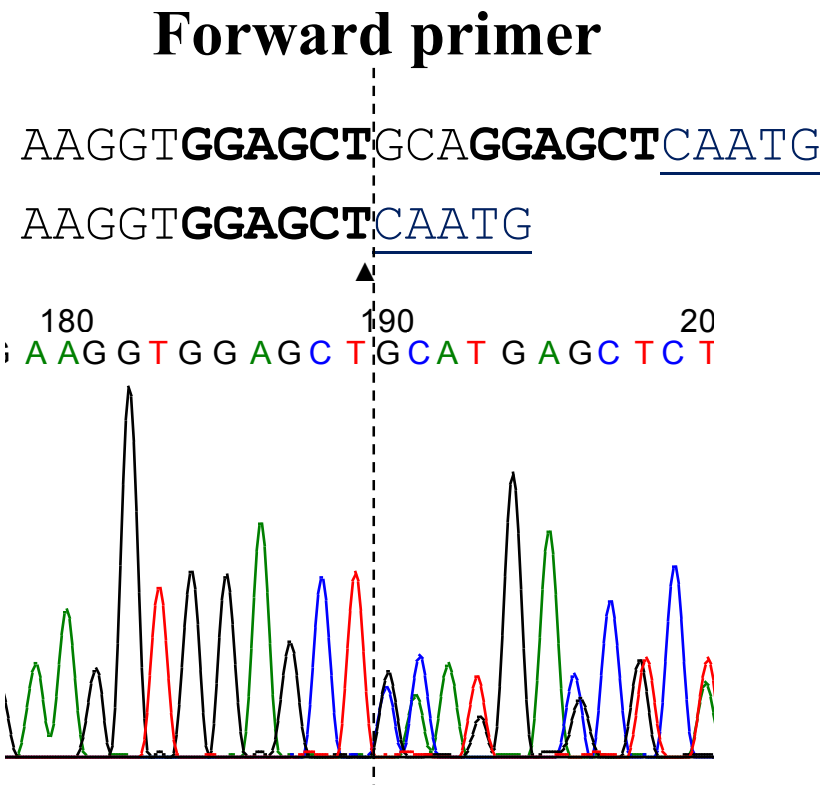


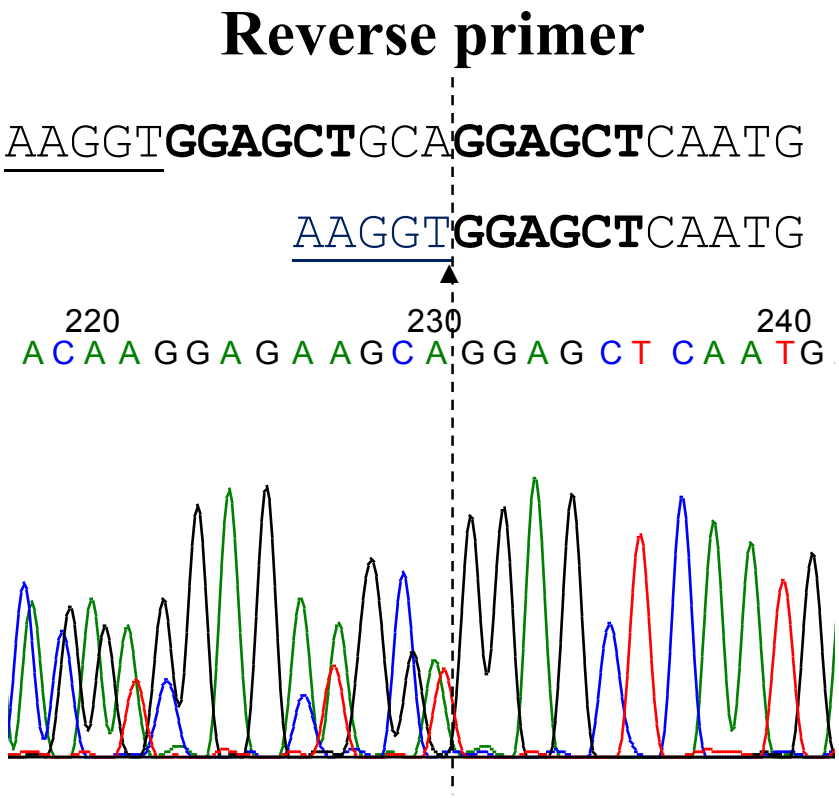
Problem of sequence interpretation

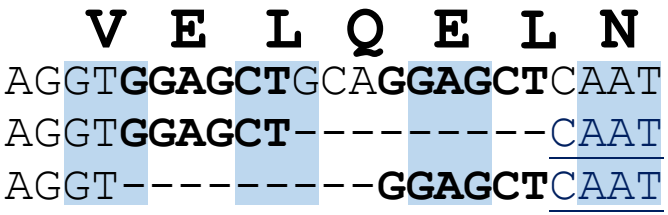




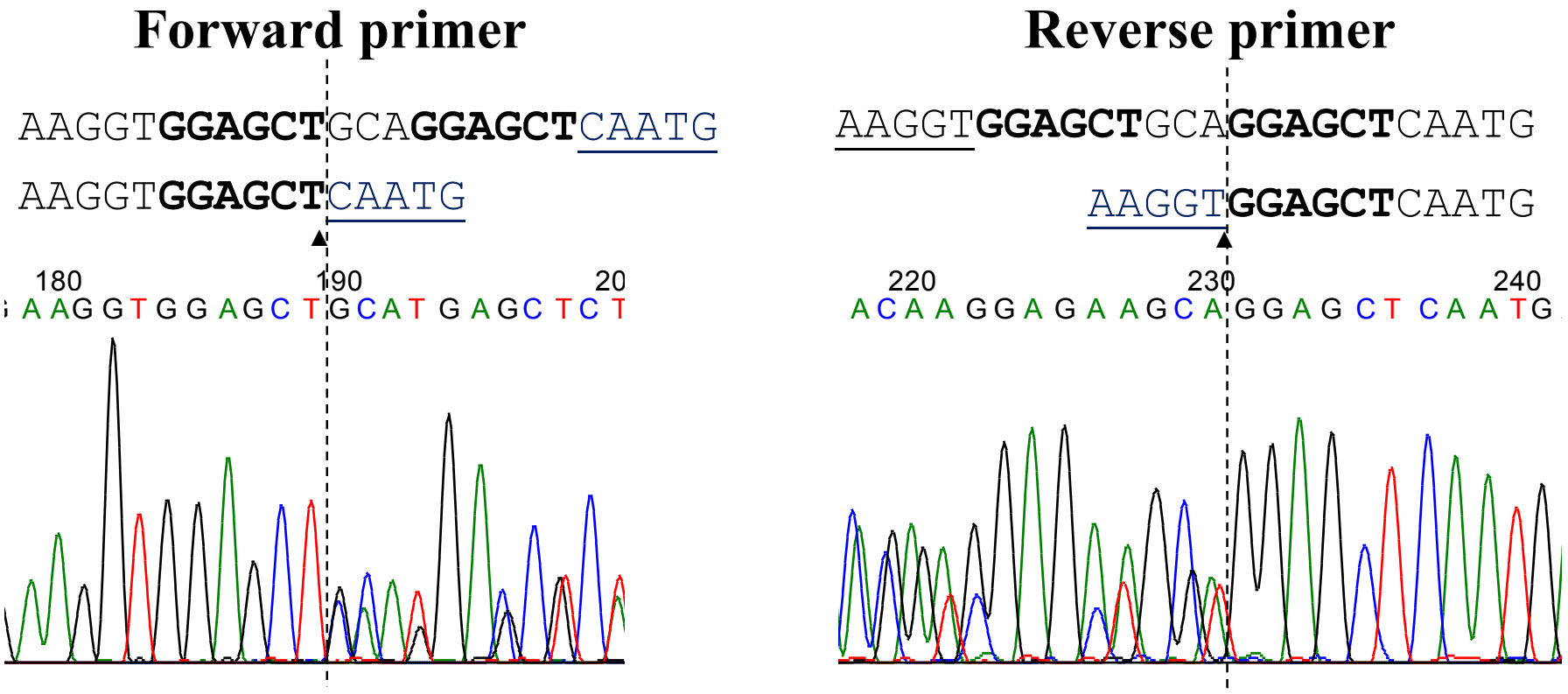
Problem of sequence interpretation







Problem of sequence interpretation

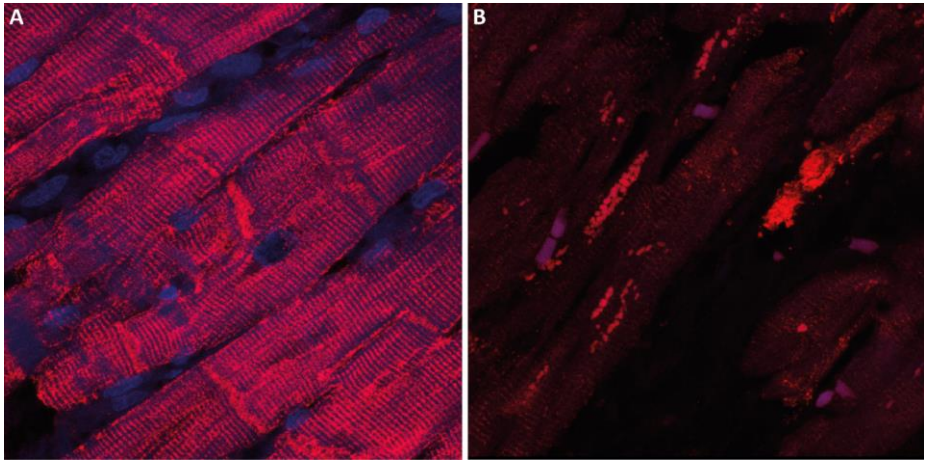


V E L Q E L N

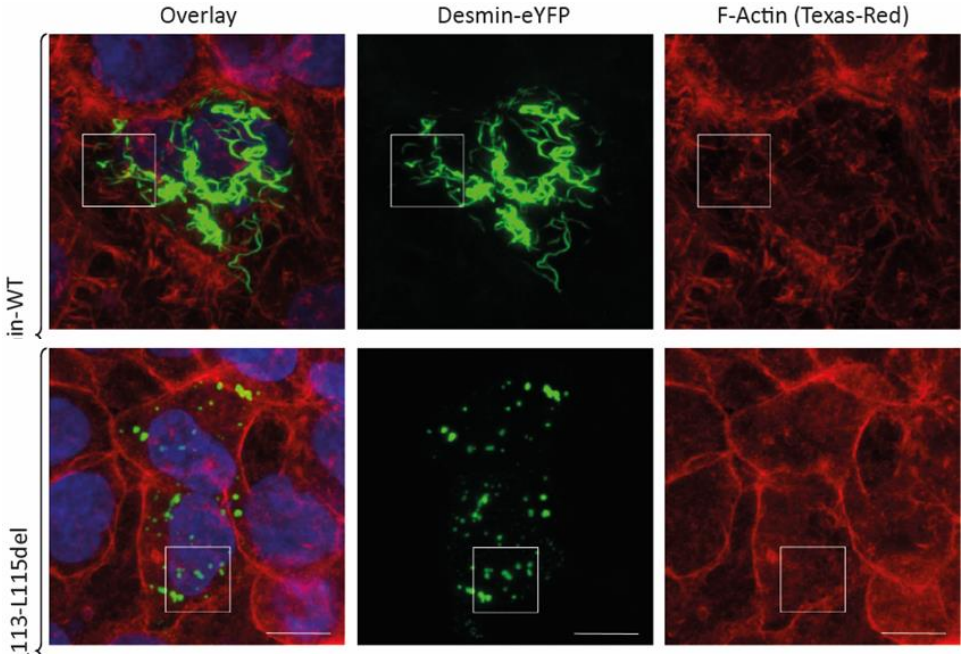
AGGTGGAGCTGCAGGAGCTCAAT
AGGTGGAGCT---------CAAT → **c.336_344**delGCAGGAGCT (p.Q113_L115del)
AGGT---------GGAGCTCAAT → **c.330_338**delGGAGCTGCA (p.E111_Q113del)

Functional study

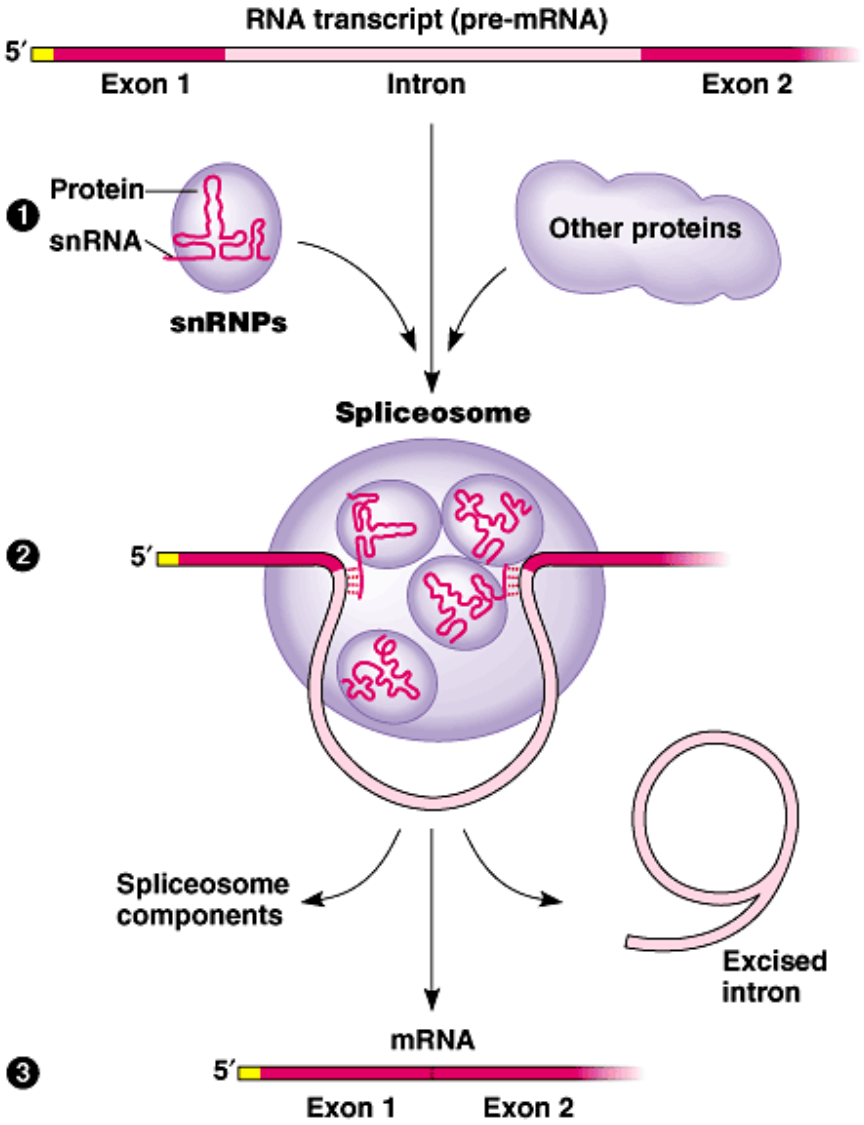
- Immunohistochemistry analysis of explanted myocardial tissue of a non-failing heart **(A)** and of the index patient IV.1 **(B)** using anti-desmin antibodies (red) and 4',6-Diamidin-2-phenylindol (DAPI, blue).



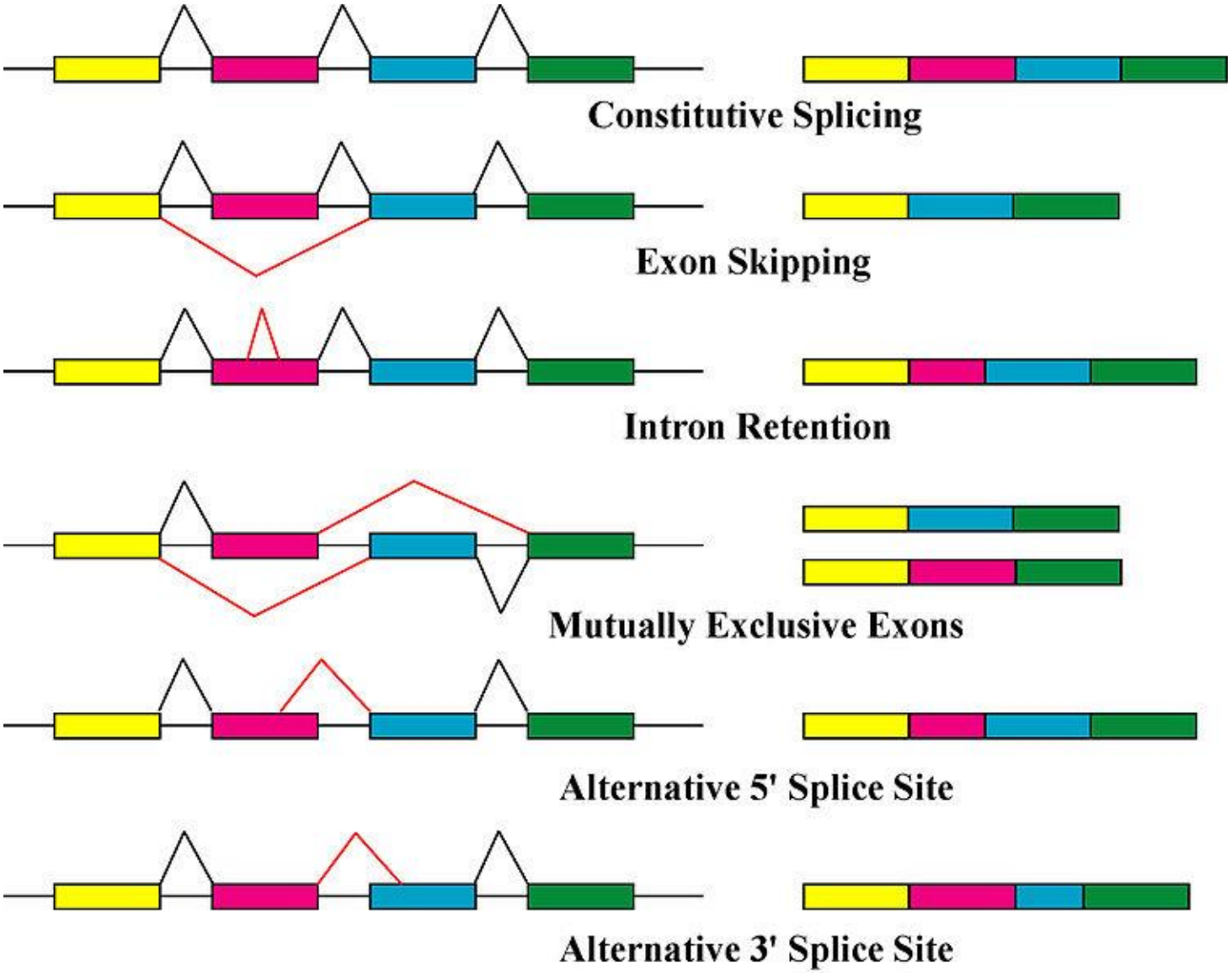
- Cell transfection studies. Wild-type desmin (green) forms typical intermediate filaments, whereas the mutation DES-c.336_344del forms cytoplasmic aggregates of different shape and size. F-actin was labeled with phalloidin conjugated with Texas Red (red) and the nuclei were labelled with DAPI (blue).



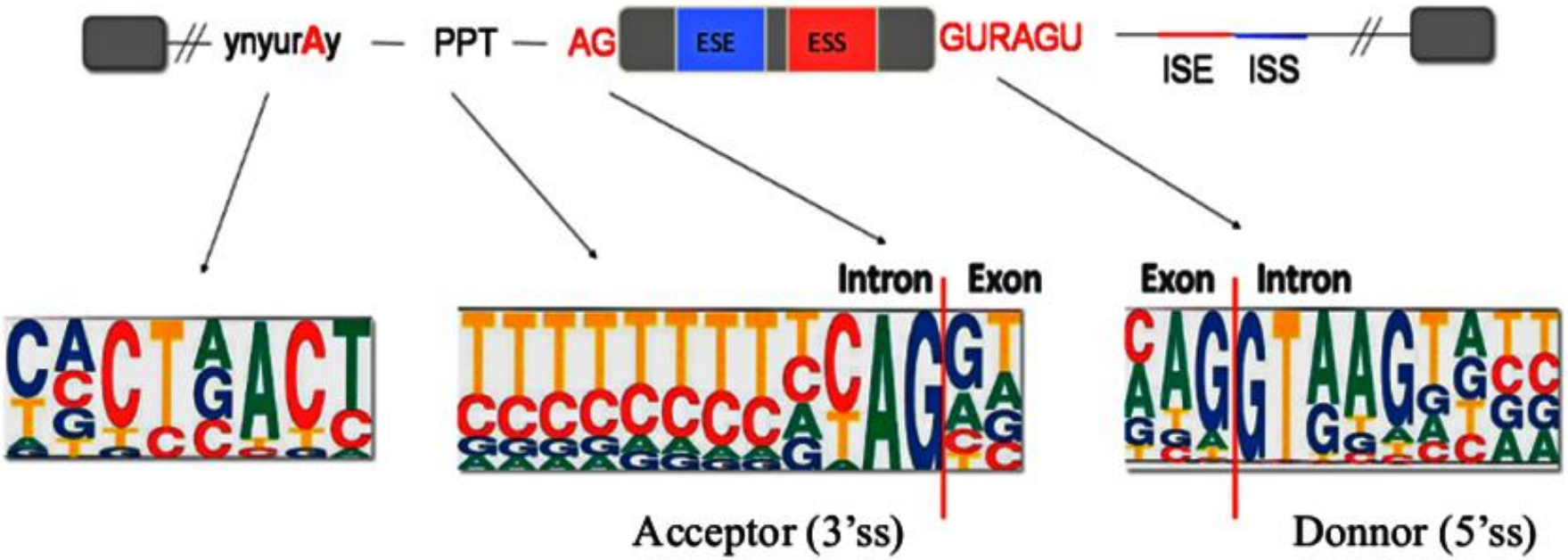
Splicing mechanism



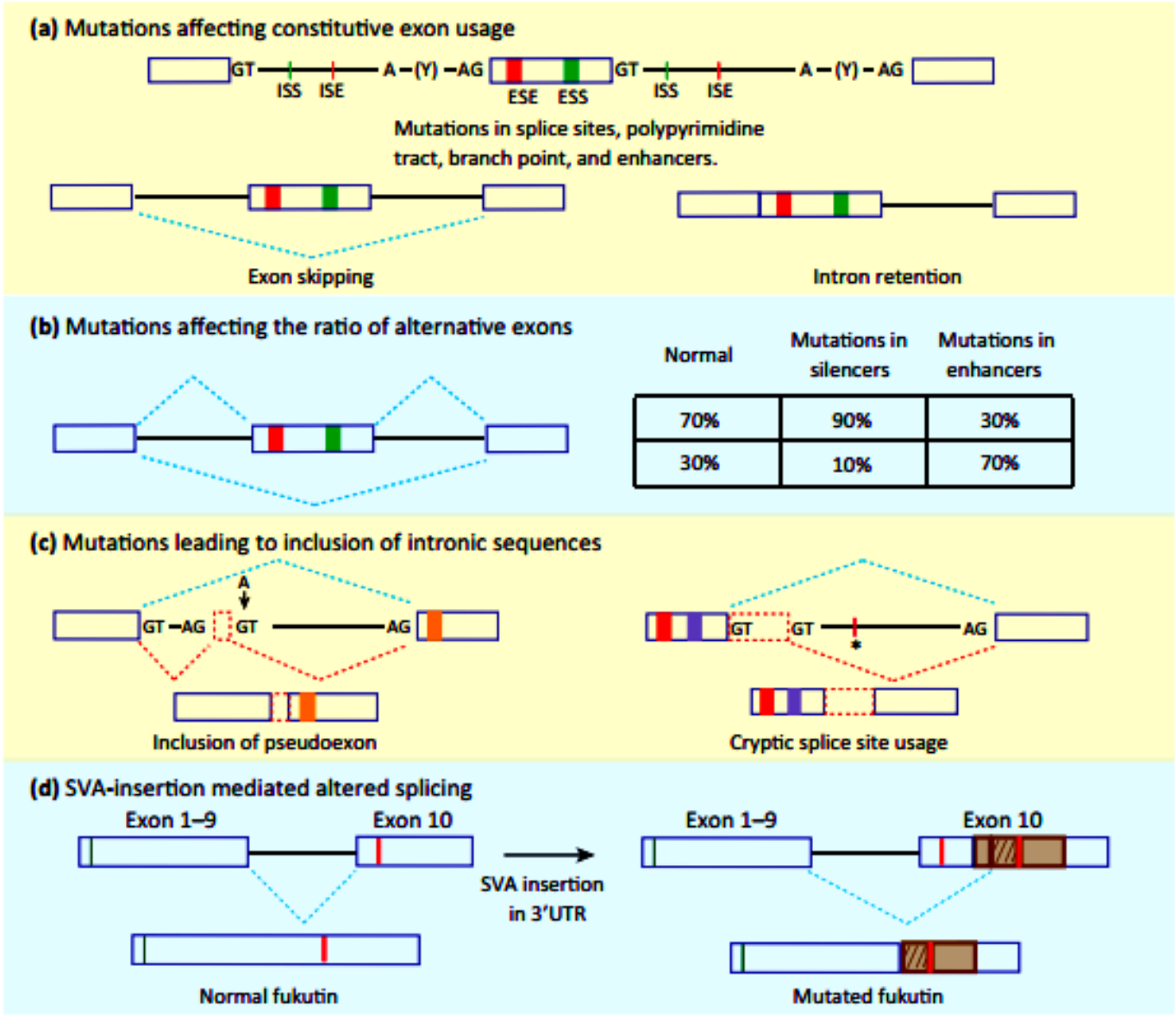
Alternative splicing



Important regions for splicing

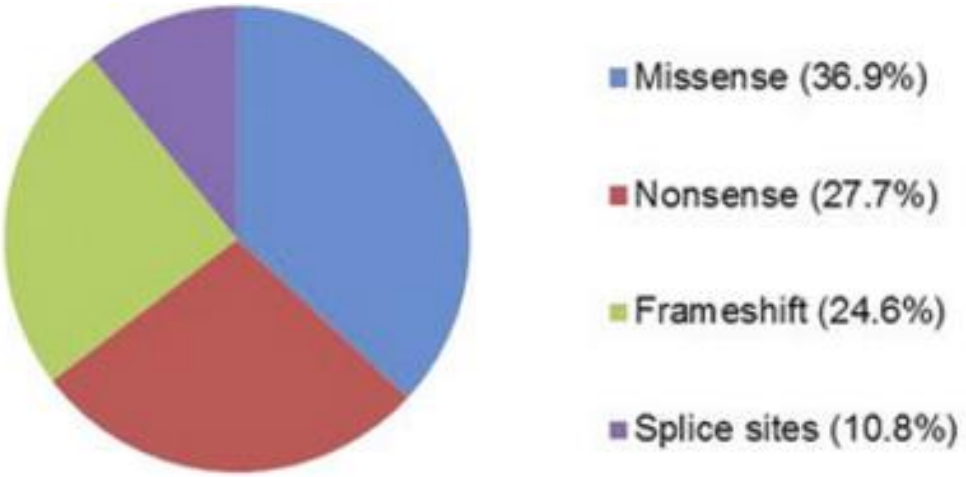


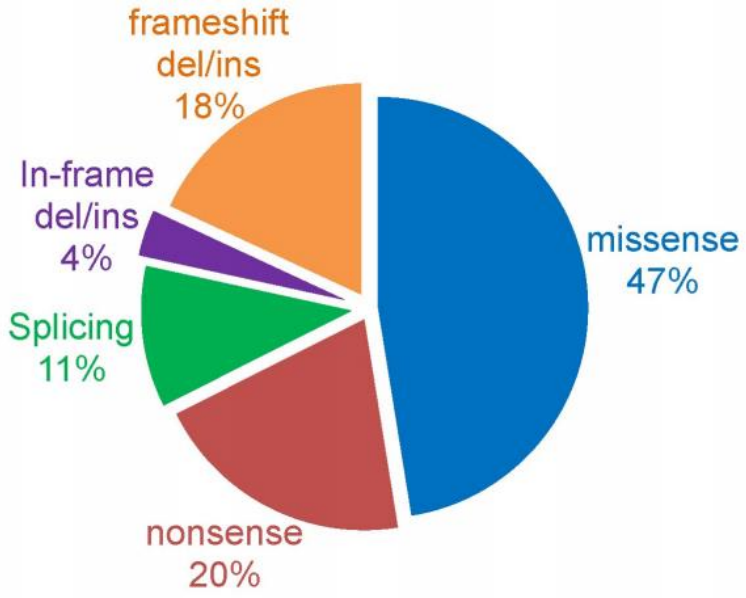
How mutations can affect splicing



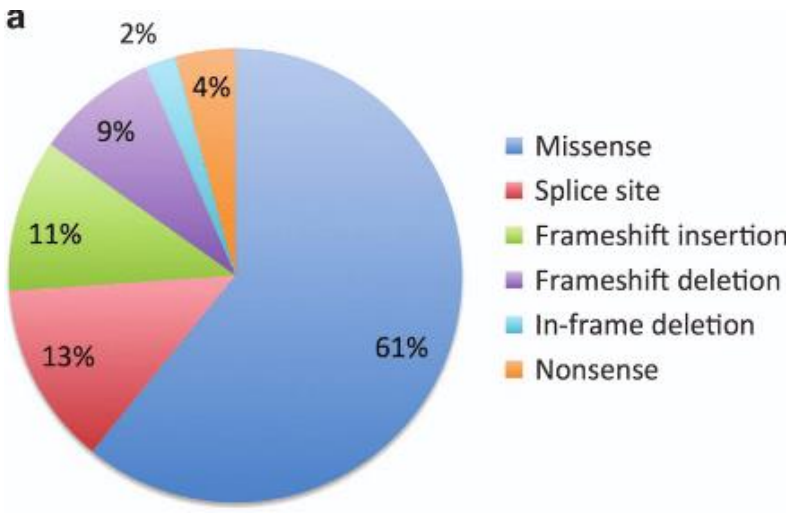
doi.org/10.1016/j.molmed.2012.06.006

Splicing mutations identified by WES

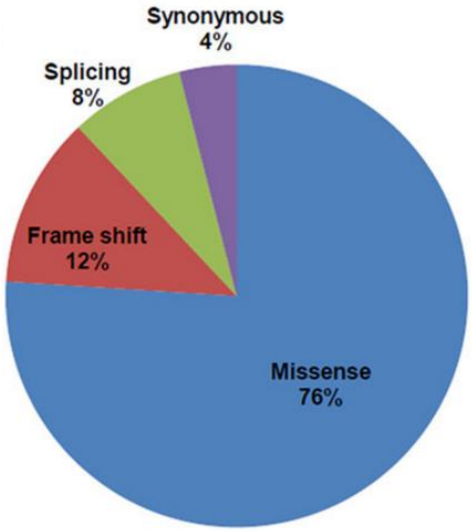




doi:10.1038/nbt.3514



doi: 10.1038/modpathol.2017.21

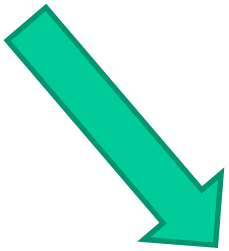


doi:10.1038/srep29088

Splicing mutations

- Current estimates of disease causing single nucleotide polymorphisms (SNPs) from the Human Genome Mutation Database (HGMD) predict that **15% of mutations are located within splice sites** and **more than 20% of missense mutations lie within predicted splicing elements**, such that more than one-third of the disease-causing SNPs have the potential to disrupt splicing (doi.org/10.1016/j.molmed.2012.06.006).
- It has been estimated that **one third** of the hereditary genetic diseases as well as many forms of cancer are caused by mutations resulting in the generation of altered transcript (doi: 10.1002/mgg3.23).
- about **50%** of the mutations result in aberrant splicing (doi:10.1016/j.febslet.2005.02.047).
- It is estimated that approximately **60%** of disease mutations in the human genome are splicing mutations (https://doi.org/10.1371/journal.pone.0004732).

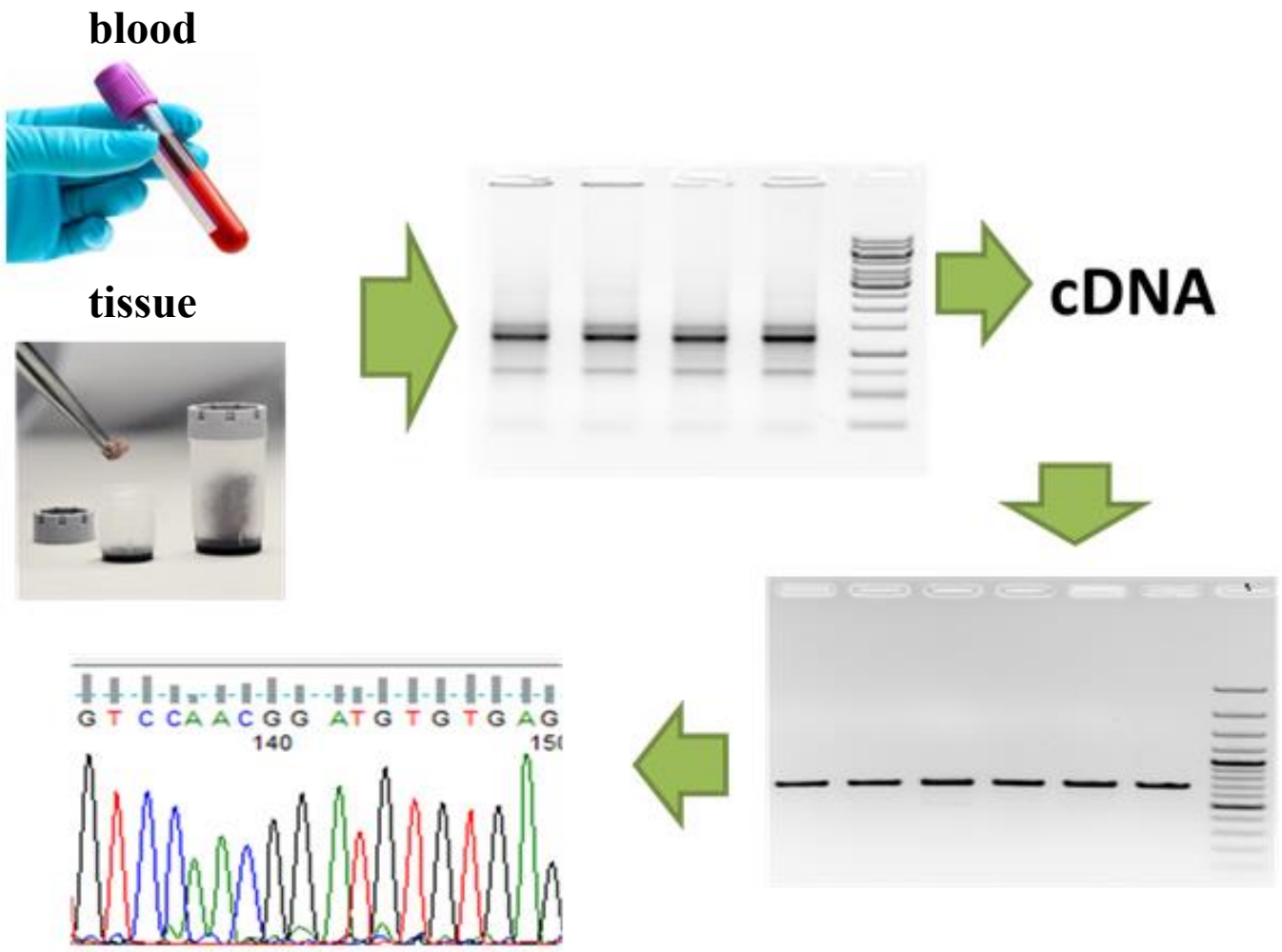
Functional analysis of splicing mutations



Investigation of mRNA structure in a patient sample

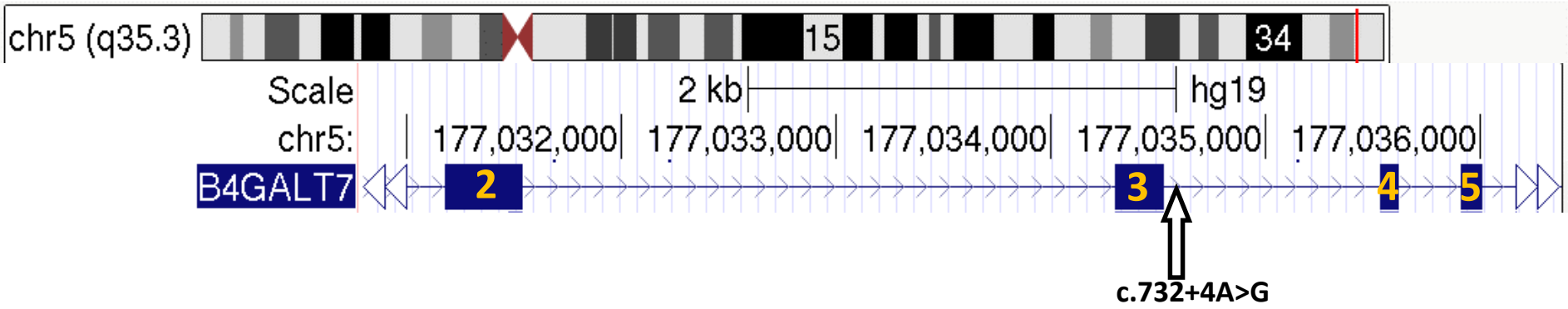
An in vitro analysis with minigene systems

Investigation of mRNA structure in a patient sample



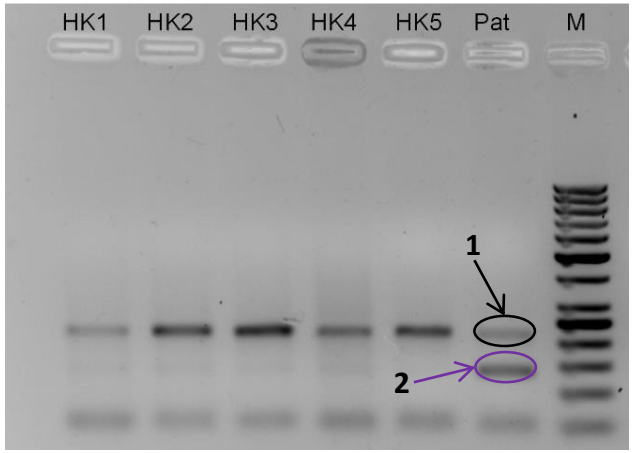
Functional analysis of mutation in B4GALT7 gene (c.723+4A>G)

- Fetus with multiple congenital abnormalities
- Sanger sequencing identified homozygous variant c.723+4A>G in B4GALT7



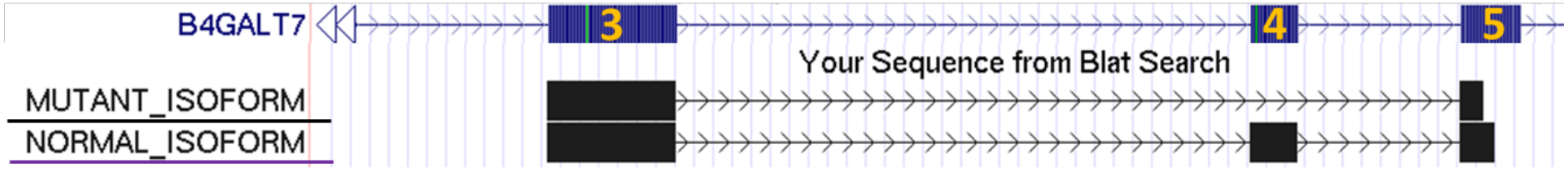
- Good expression level of B4GALT7 in blood

Results of RT-PCR



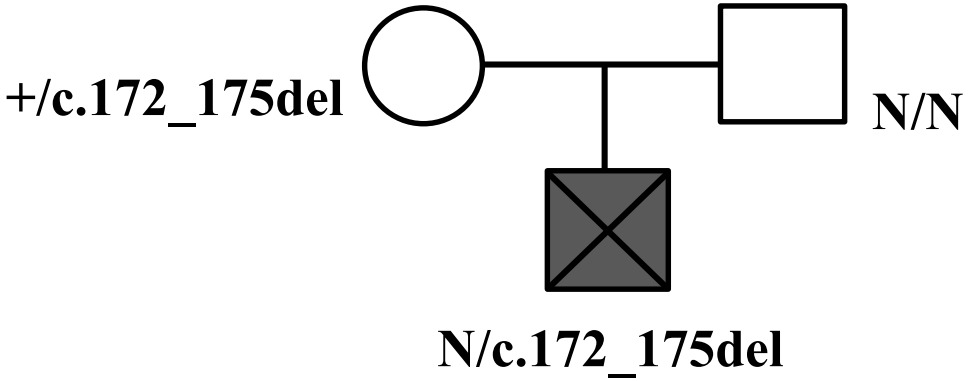
- 1 matches normal isoform
- 2 matches abnormal transcript

Sanger sequencing



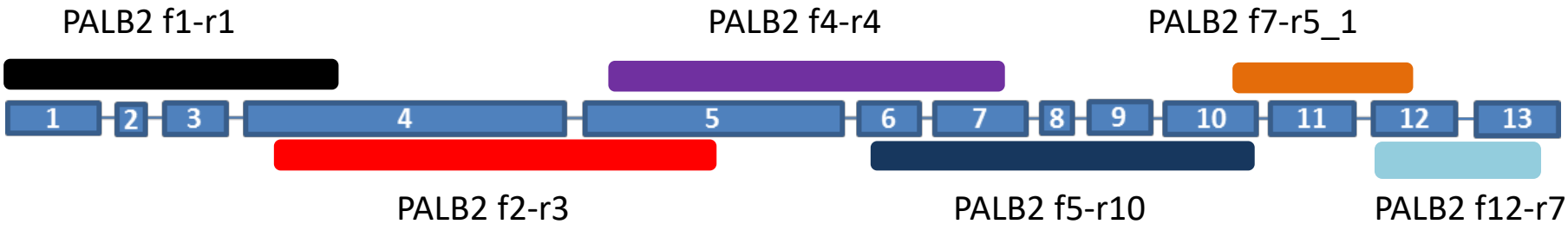
Case of Fanconi anemia

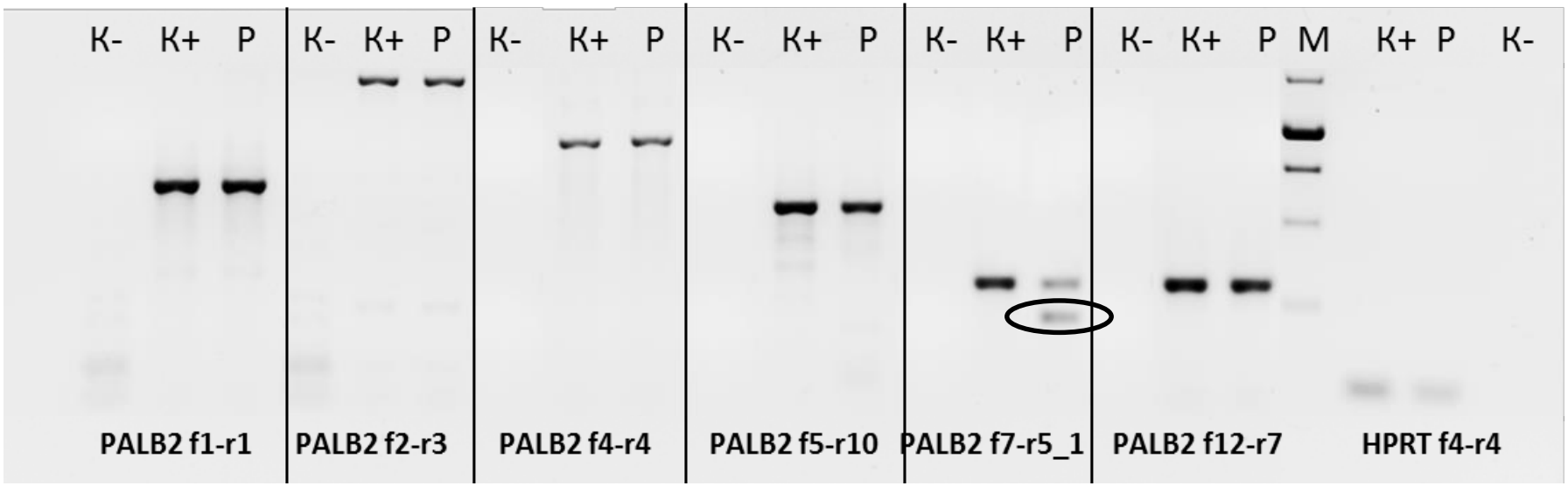
- The boy, from the 1st pregnancy, the diagnosis of Fanconi anemia is set at the 1st year of life.
- Exome sequencing identified only one mutation, PALB2: c.172_175delTTGT, inherited from the mother.
- No PALB2 copy-number changes were seen in MLPA neither in proband, nor in his father.



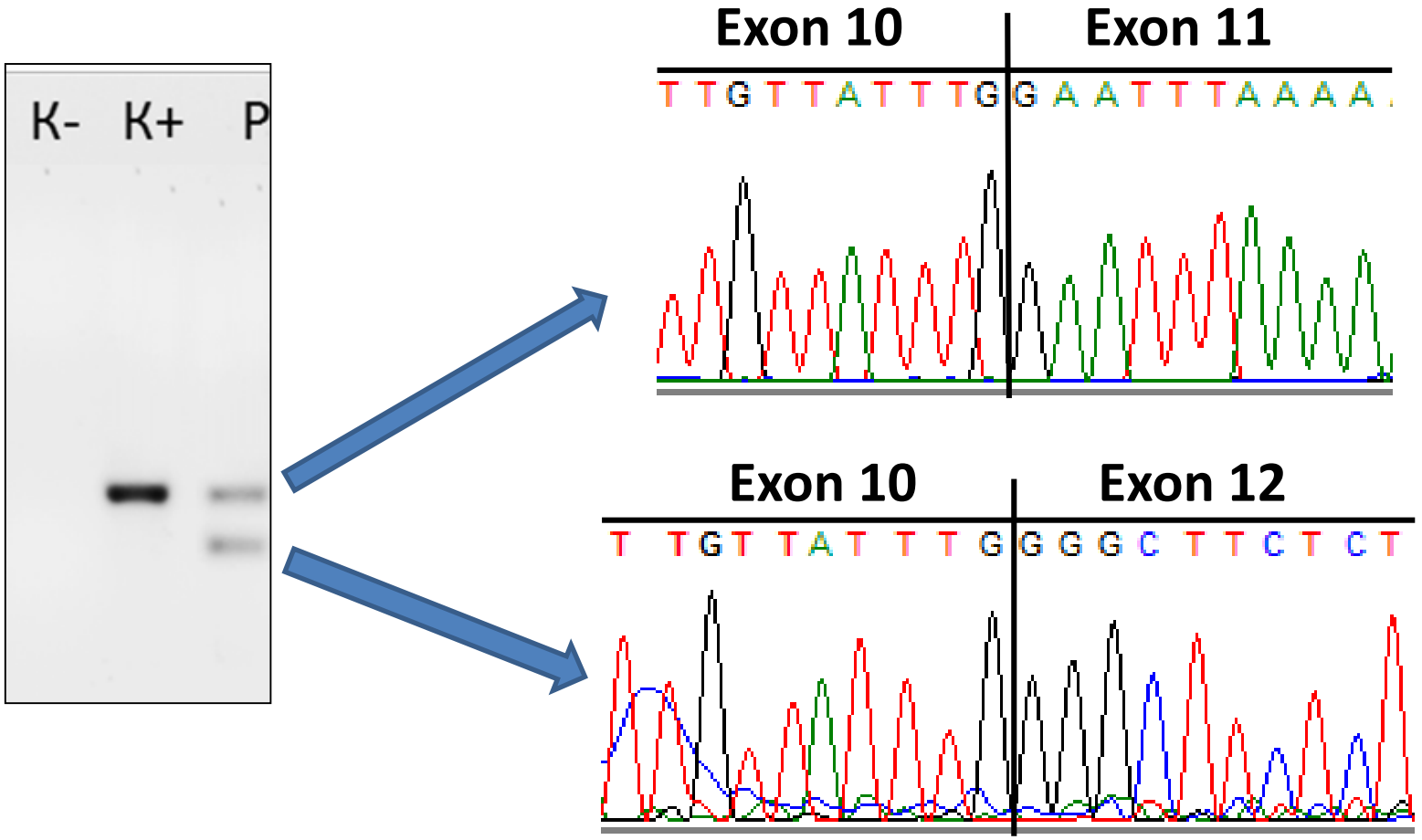
- Good expression level of PALB2 in blood

System for studying the structure of the mRNA of the PALB2 gene

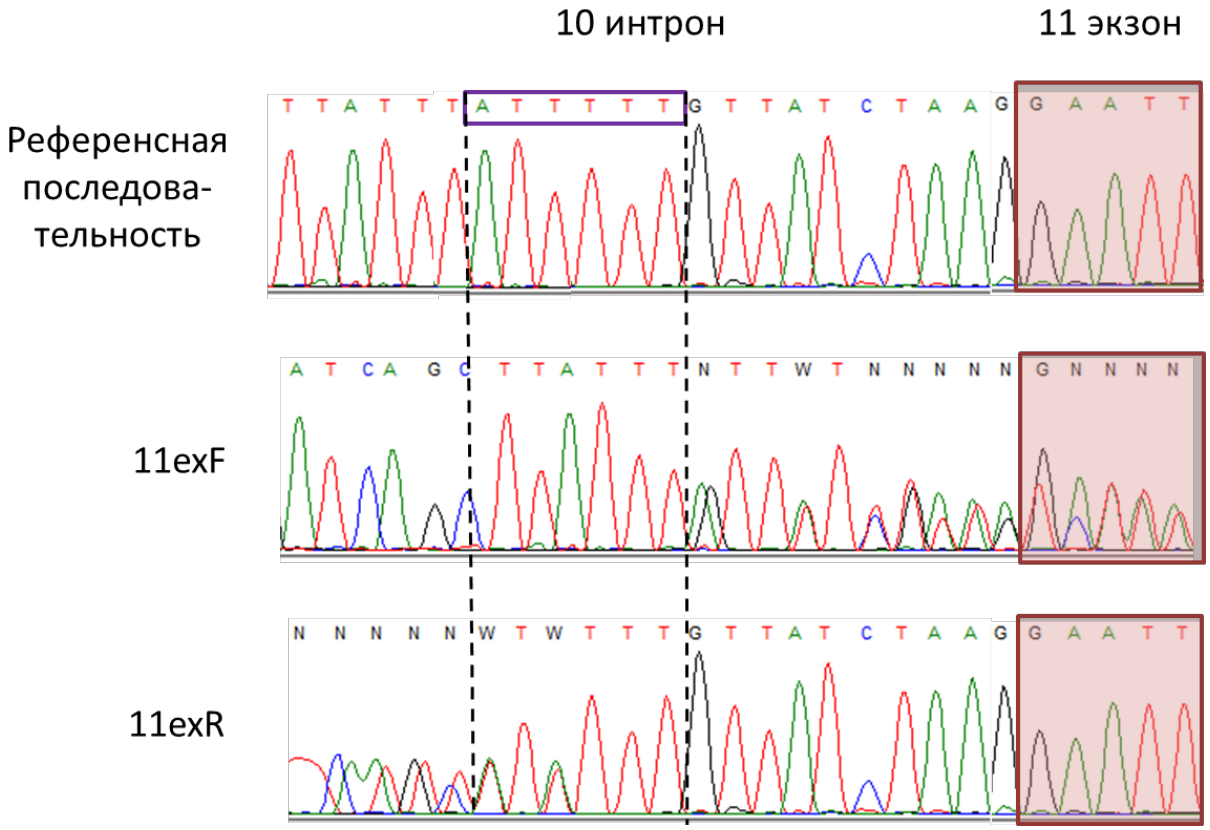




The results of sequencing of the abnormal product of the mRNA of the PALB2 gene

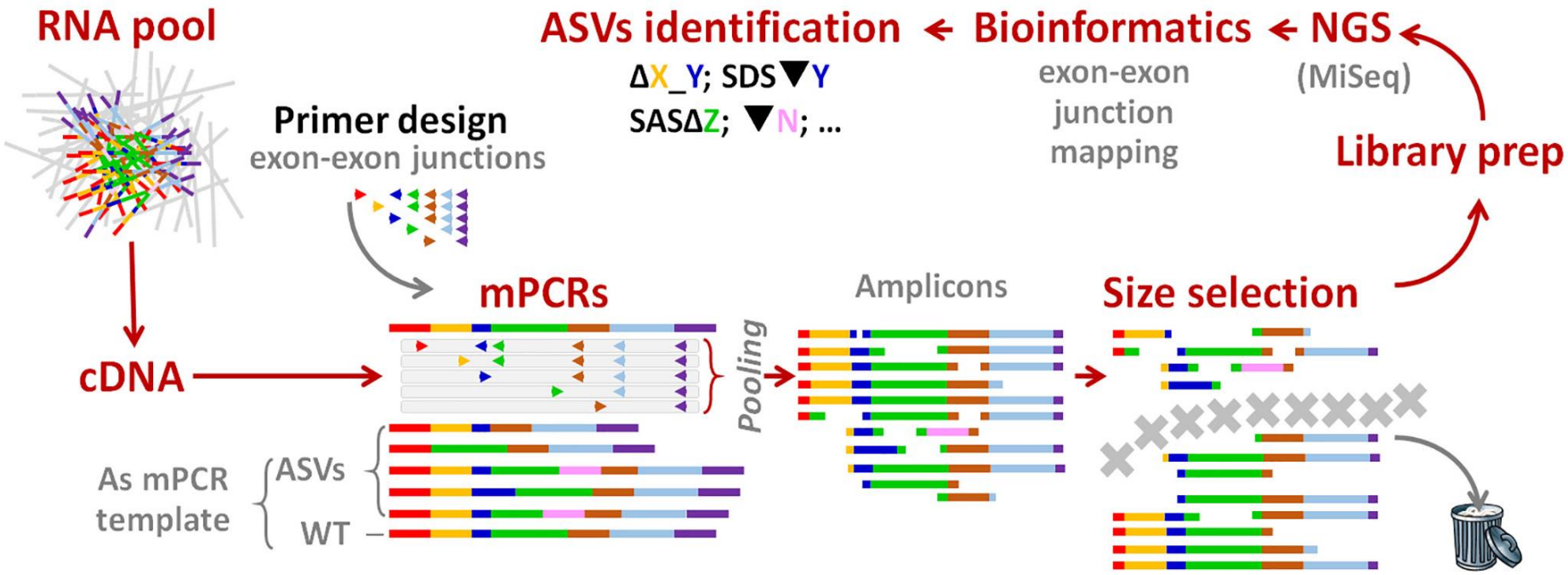


Search for the variant of the nucleotide sequence responsible for missing 11 exon

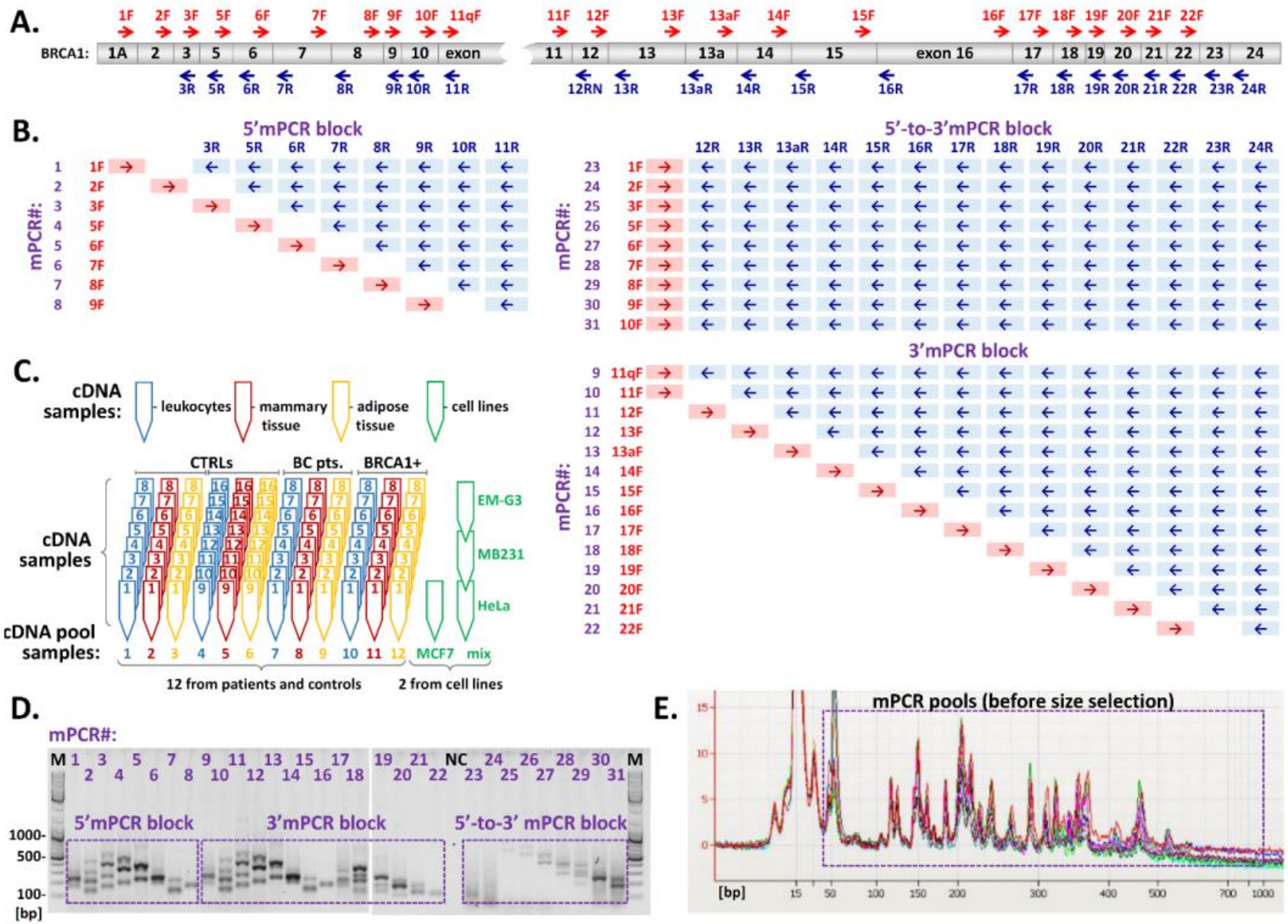


- Sanger sequencing of *PALB2* exon 11 and its intronic vicinity showed a deletion NC_000016.9: g.23625423delAAAAATA in one of the paternal chromosomes.
- Aberrant mRNA isoform of PALB2 translates into a C-terminus truncated version with 142 amino acids deletion p.Asn1039Glyfs*7 residing within WD40-repeat domain indispensable for correct cellular localization of the protein.

Analysis of BRCA1 splicing pattern

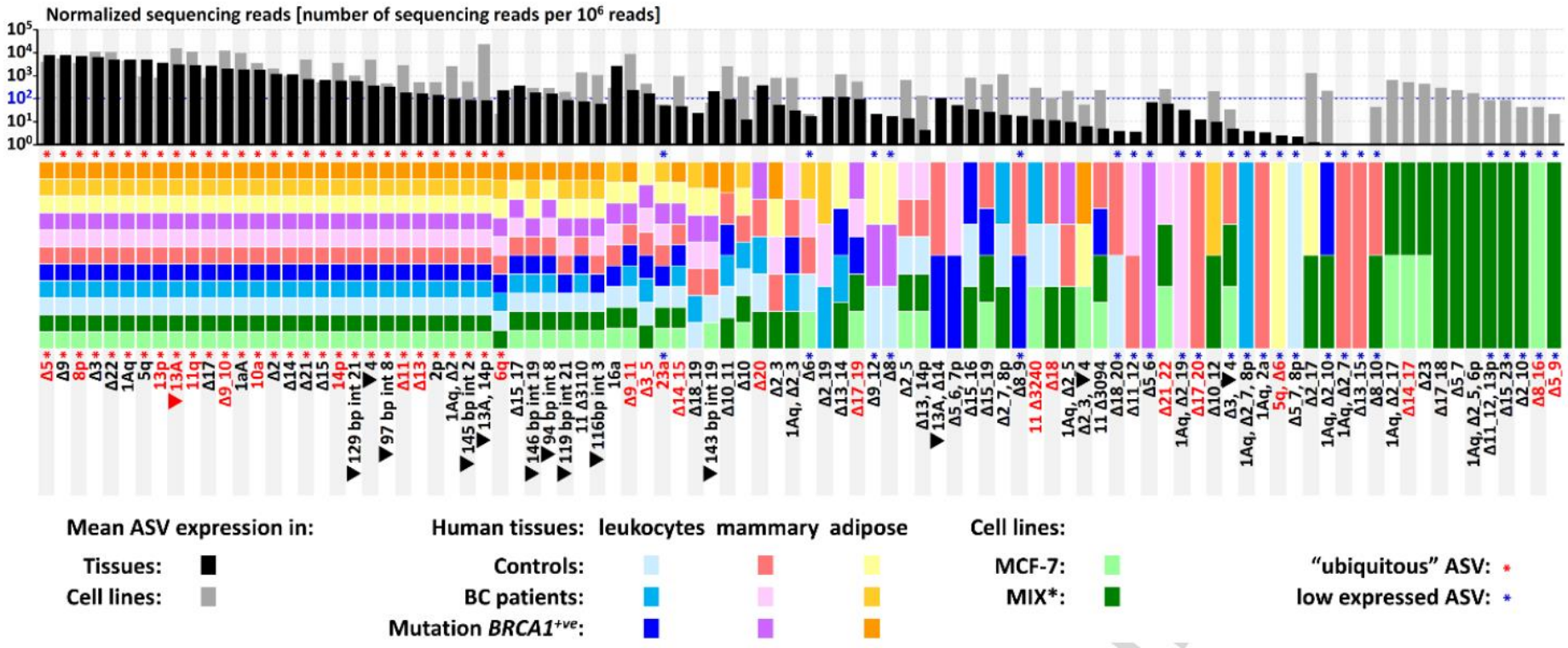


Analysis of BRCA1 splicing pattern



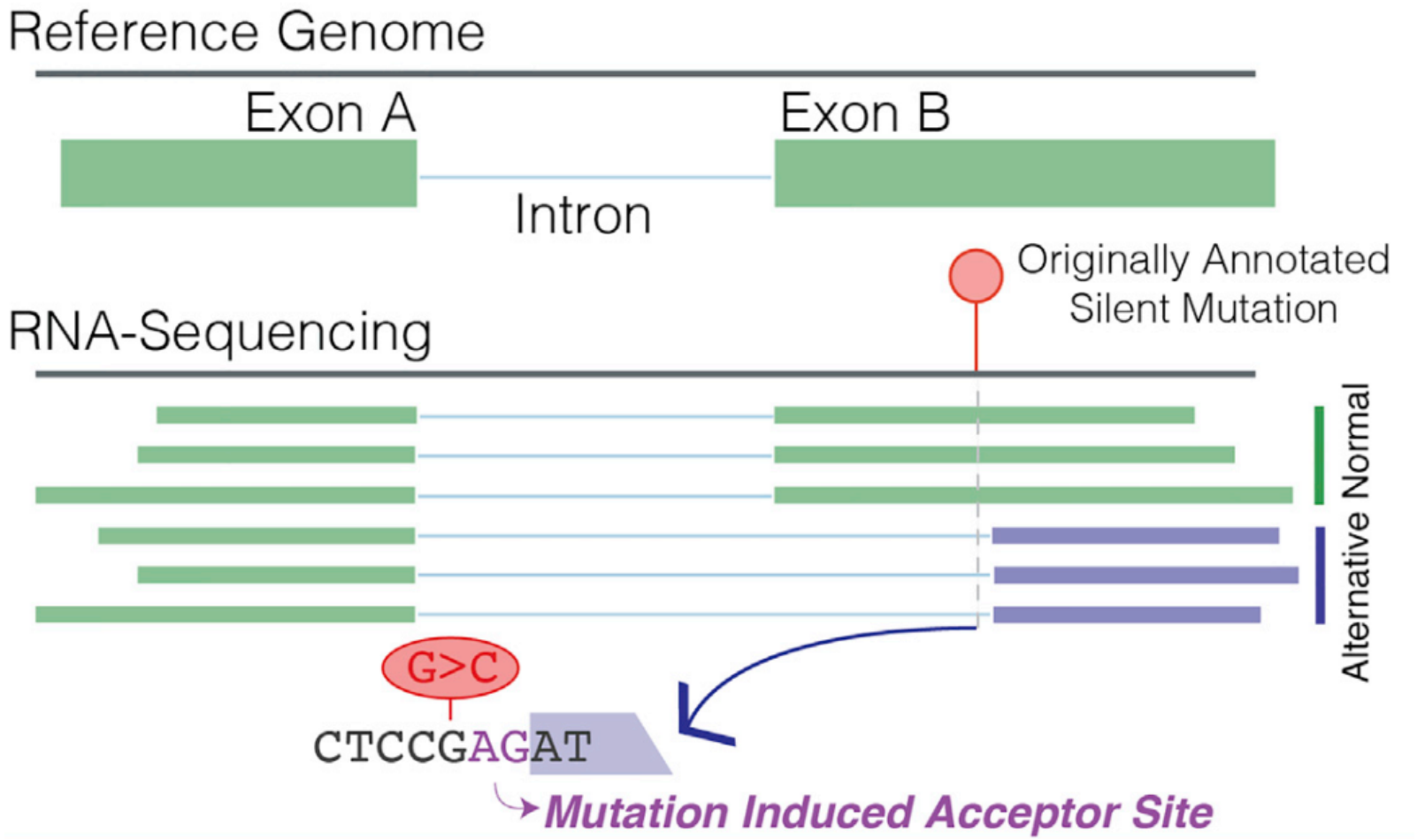
Analysis of BRCA1 splicing pattern

- We revealed 94 BRCA1 ASVs, including 29 variants present in all tested samples.



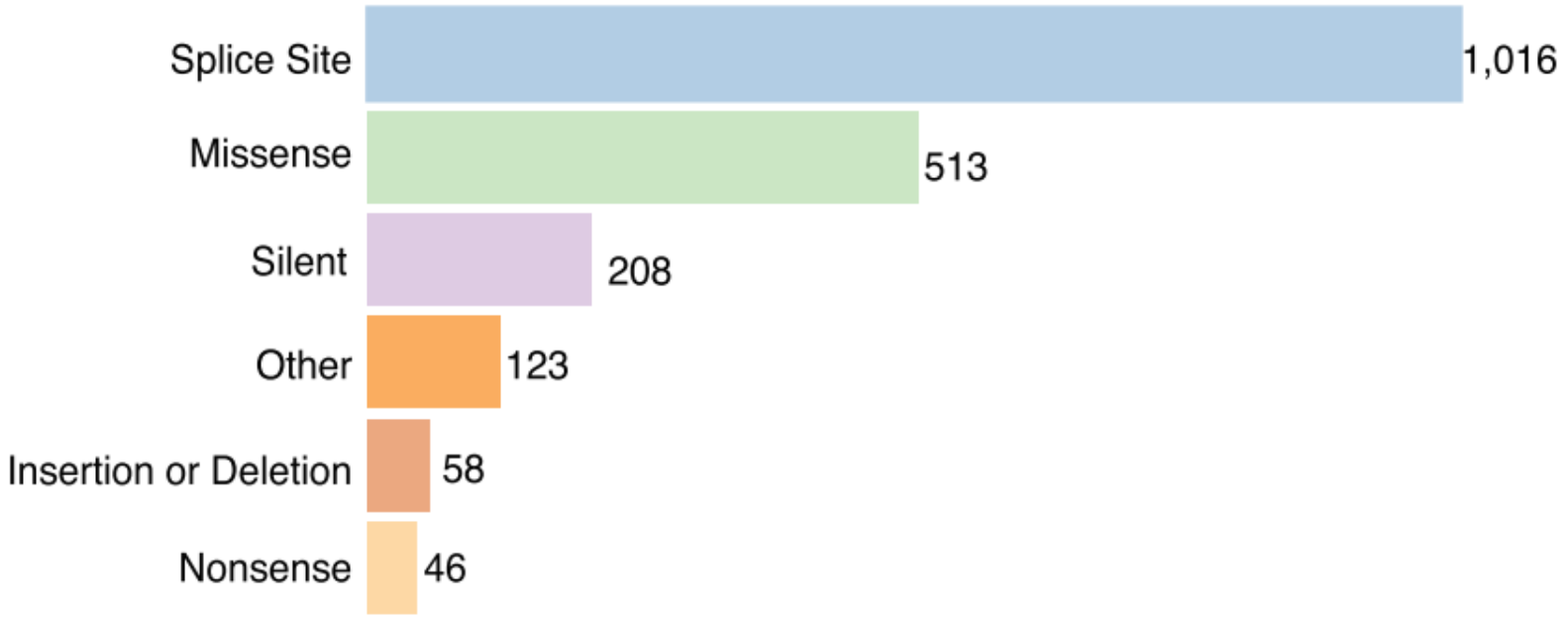
- While differences in the qualitative expression of BRCA1 ASVs among the analyzed human tissues were minor, larger differences were detected between tissue and cell line samples.

Analysis of Splice Site-Creating Mutations in Cancer



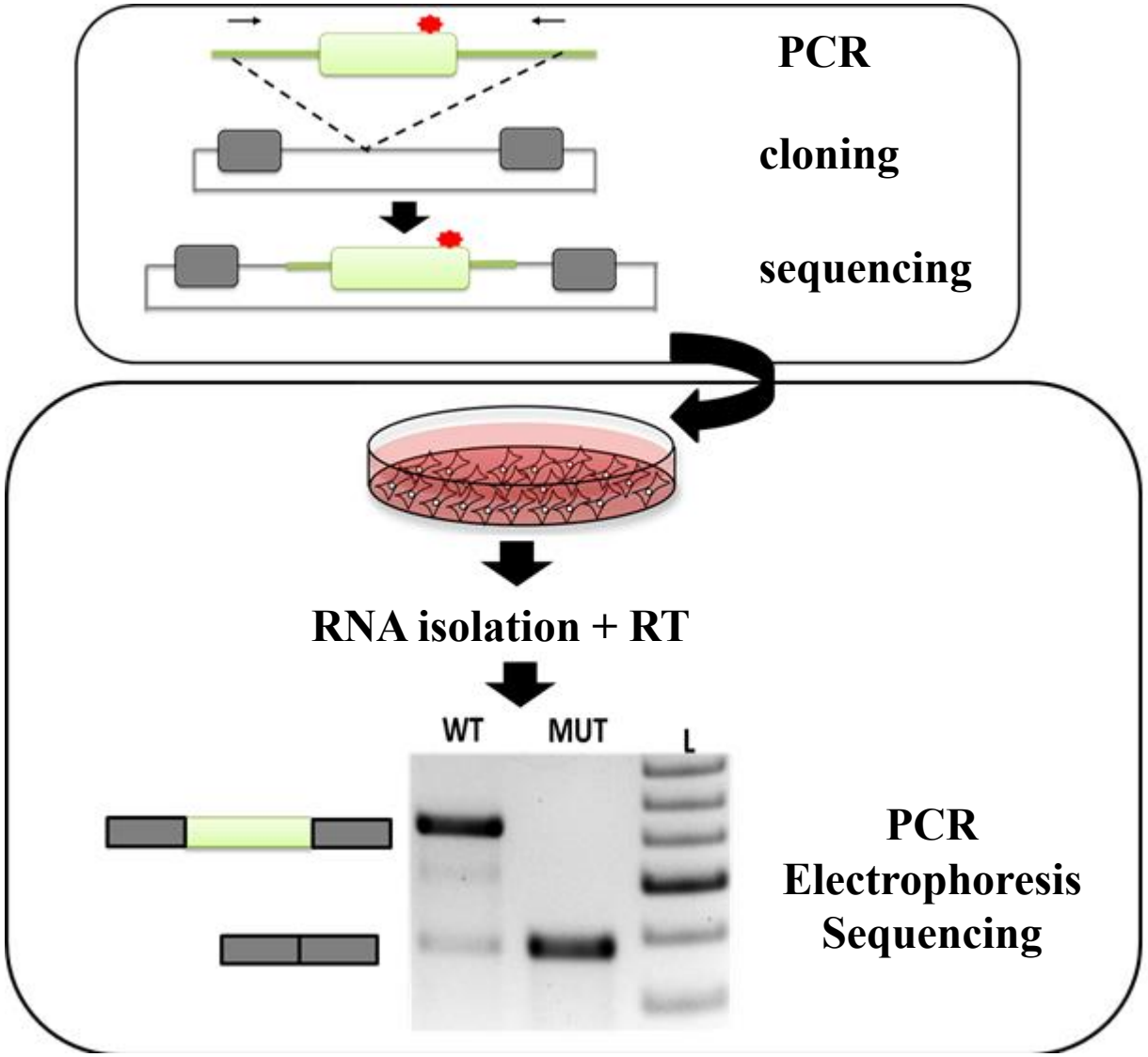
Analysis of Splice Site-Creating Mutations in Cancer

- 8,656 tumors across 33 cancer types derived from The Cancer Genome Atlas having available TCGA RNA-seq data
- MiSplice search for alternative splice junctions within windows of ±20 bp from the mutation of interest.
- 1,416,566 candidate mutations examined, 4,448 had five or more unique RNA-seq reads supporting the predicted alternative junction in proximity to the mutation.



- Importantly, 26% (513) and 11% (208) of the SCMs had previously been **mis-annotated** as missense and silent mutations, respectively.

An in vitro analysis with minigene systems



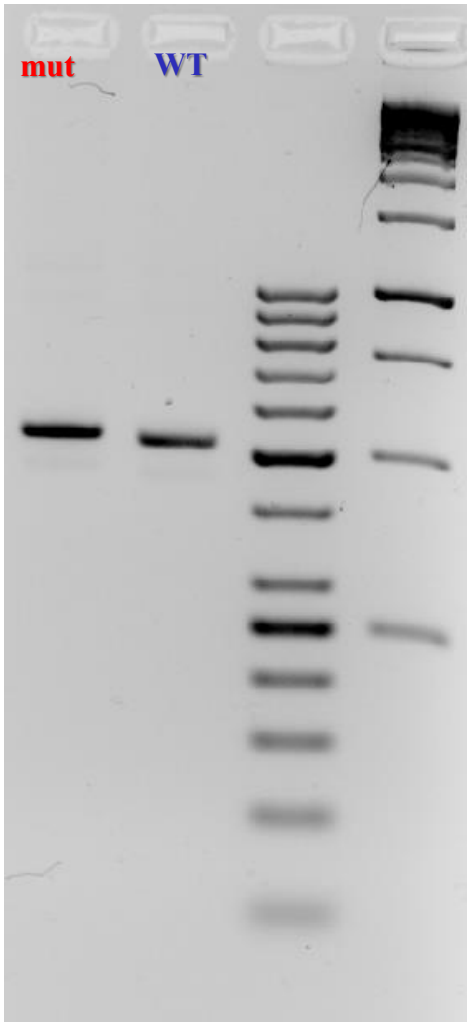
Functional analysis of mutation in PAX6 gene (c.142-14C>G)

- Patient with congenital aniridia
- The genomic variant de novo c.142-14C> G in the 5 intron of the PAX6 gene

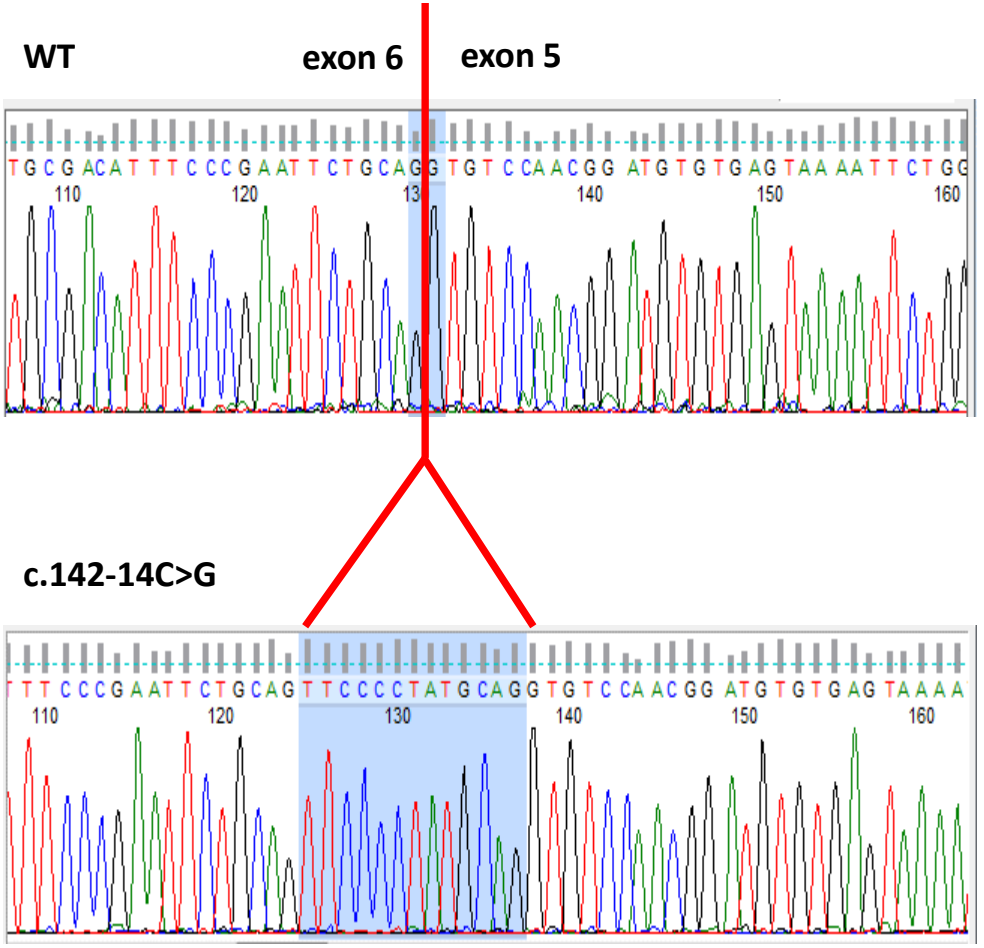
- ACMG: Variant of unknown significance!
- Genomic variant:
  - Absence in healthy parents
  - Cosegregate with phenotype in family
  - Absence in Disease databases

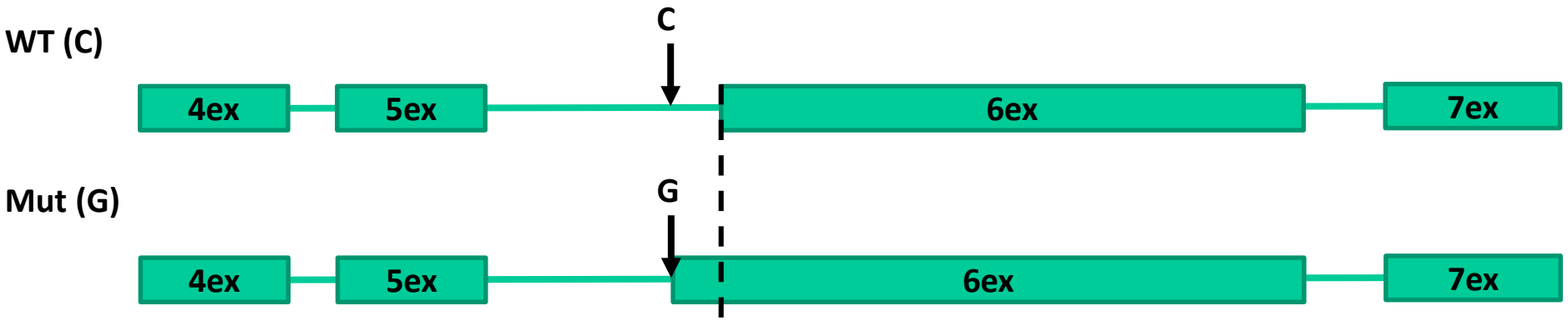
Scheme of functional analysis

- Design of plasmid
- Transfection of HEK293
- Isolation of RNA
- Synthesis of cDNA
- Amplification of splice products from the plasmid
- Sequencing



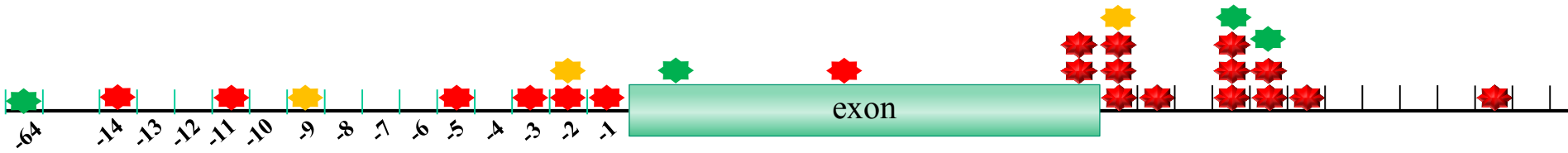
Analysis of sequencing results





Experience of the laboratory of functional genomics

- 27 variants of the nucleotide sequence



- the effect on splicing of pre-mRNA (20 variants) was revealed

- no effect on splicing of pre-mRNA (4 variants)

- in the process of analysis (3 options)

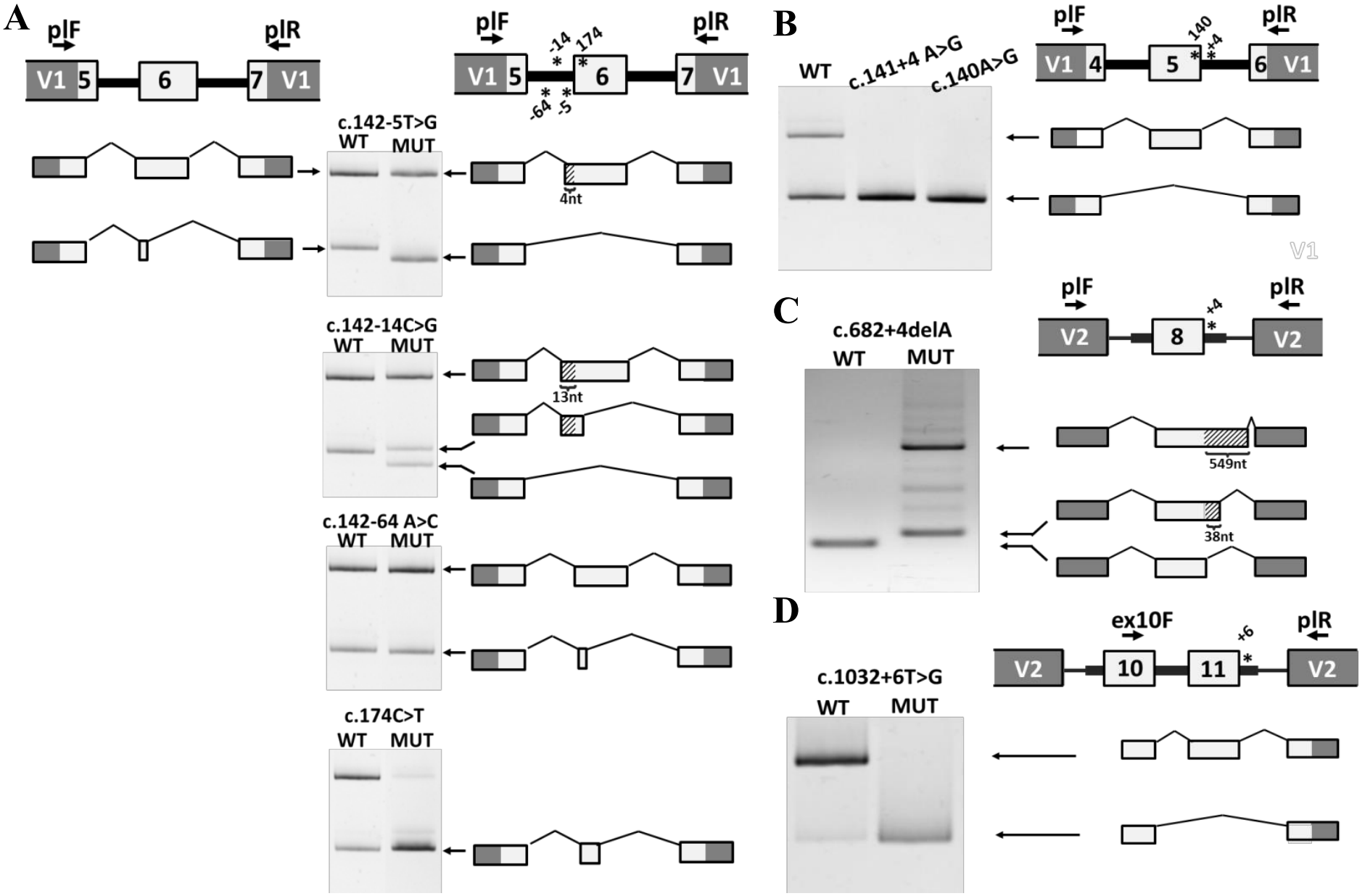
12 genes

- PAX6
- MYH7
- ABCA4
- SCN1A
- SPTB
- KIAA1109
- B2GALT7
- GAD1
- C19orf12
- SDHB
- PALB2
- NPC2

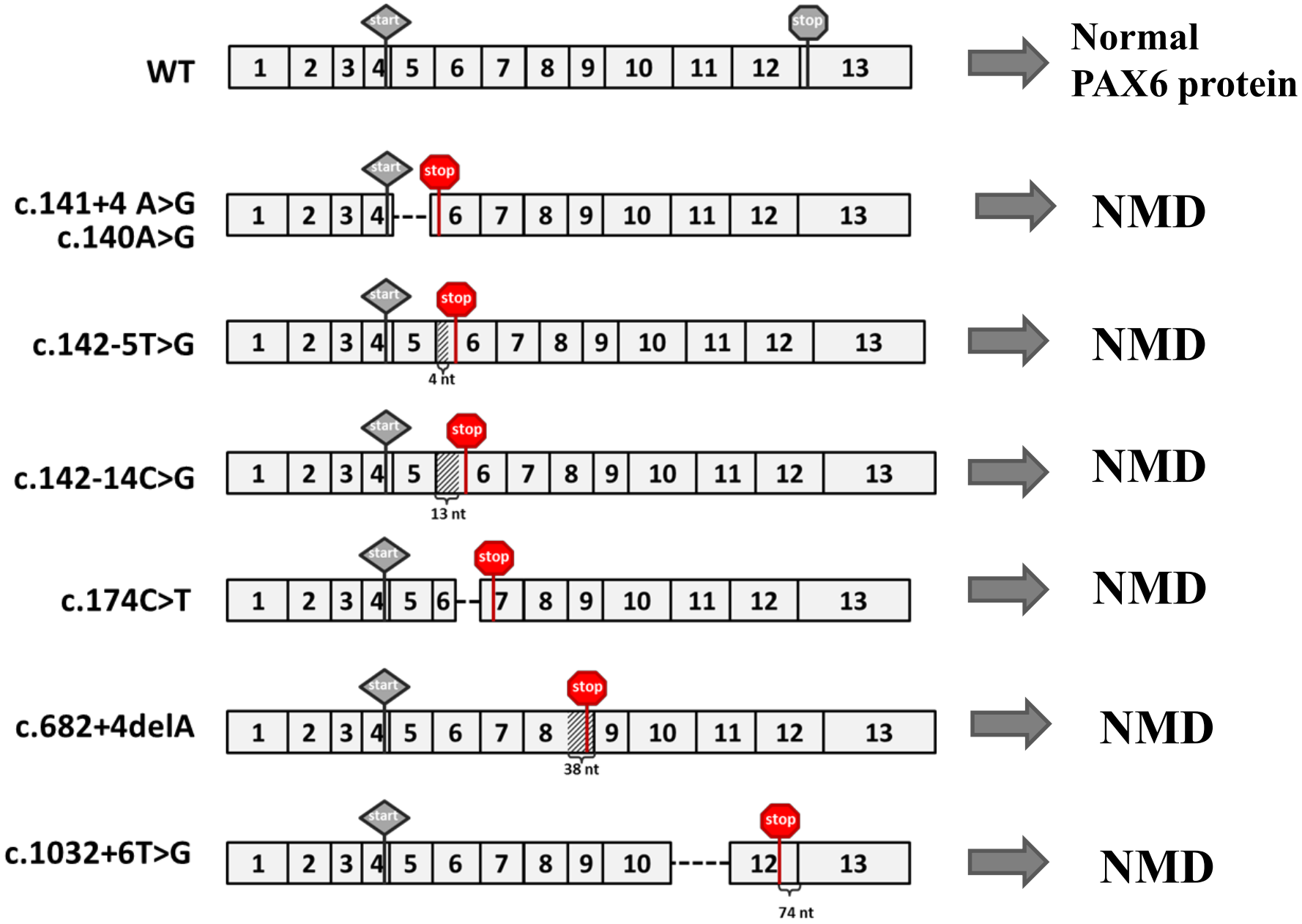
11 diseases

- Neurodegeneration with accumulation of iron in the brain
- Niman-Pisco disease, type C2
- Congenital dilated cardiomyopathy
- Spastic quadriplegic cerebral palsy
- Congenital aniridia
- Spherocytosis
- Ehlers-Danlos Syndrome
- Stargardt's disease
- Fanconi Anemia
- Alkurai-Kuchinskas Syndrome
- Hereditary epilepsy

Analysis of splice variants in the PAX6 gene

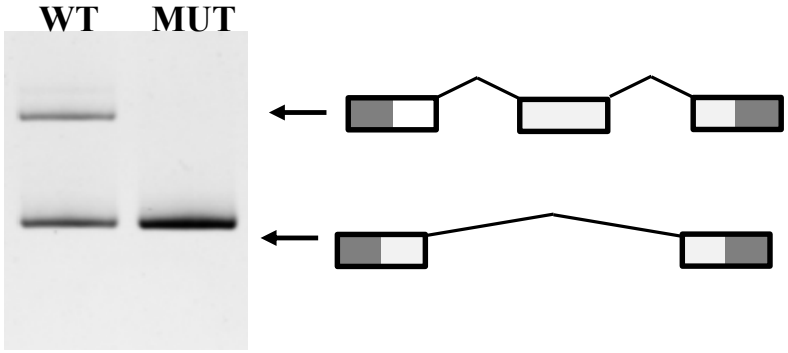


Analysis of splice variants in the PAX6 gene

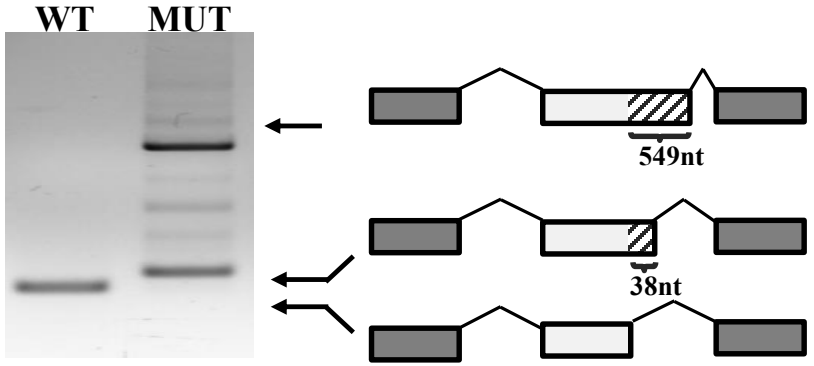


Different mechanisms with variations in one position of the splice site

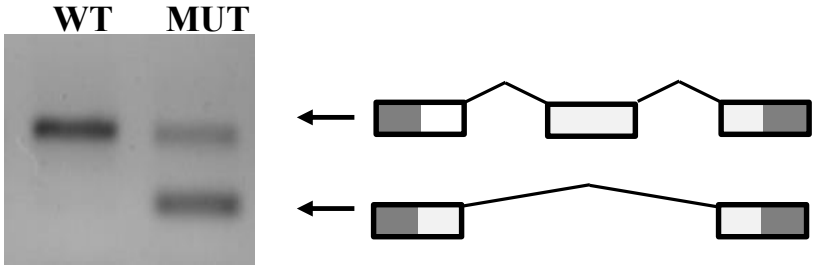
PAX6 c.141+4A>G



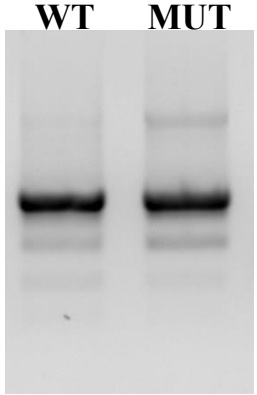
PAX6 c.682+4delA



B4GALT7 c.723+4A>G



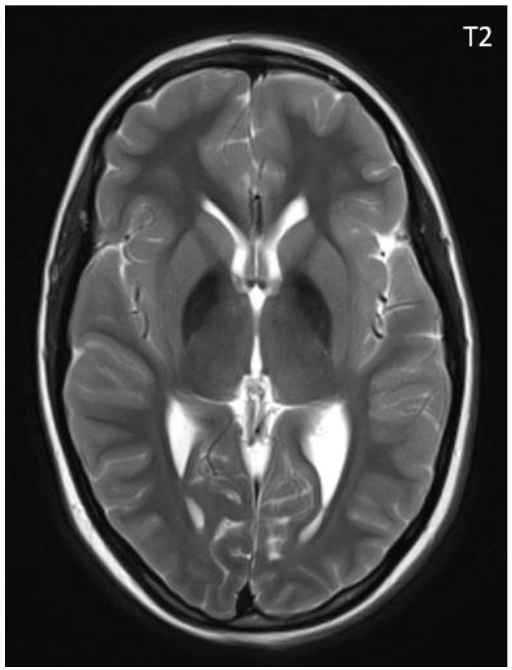
GAD1 c.1002+4T>G

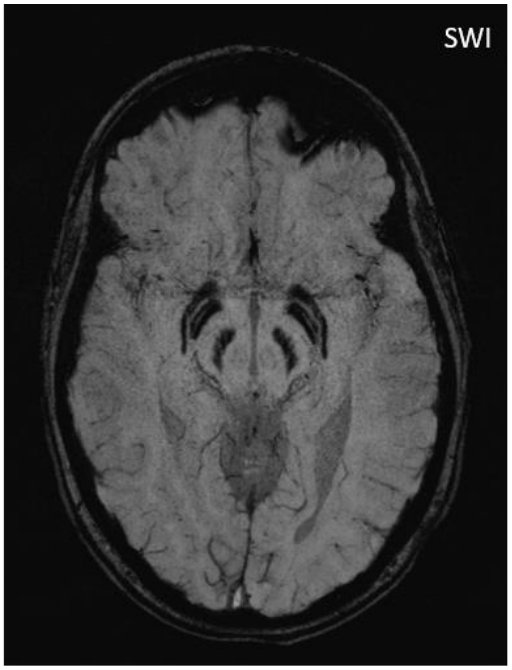


No effect

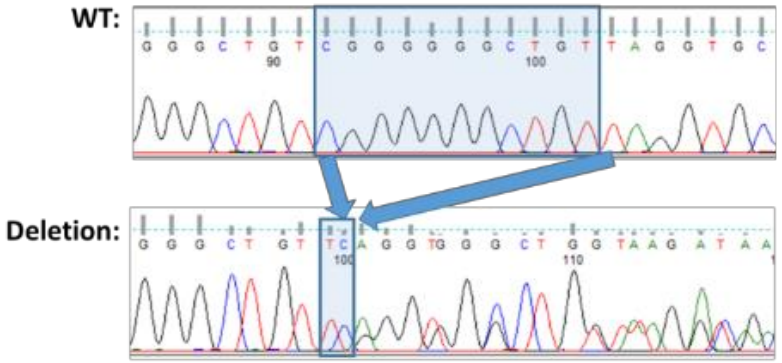
Clinical case of neurodegeneration with brain iron accumulation 4 (NBIA4)

- A family with a 12-year-old daughter with a bilateral pyramidal insufficiency, postural instability, partial atrophy of the optic nerve, signs of iron accumulation in the subcortical nuclei and stem structures

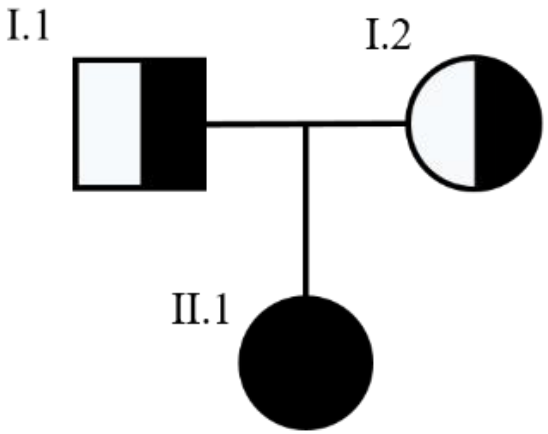


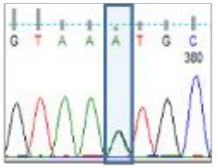


Whole exome sequencing revealed two variants



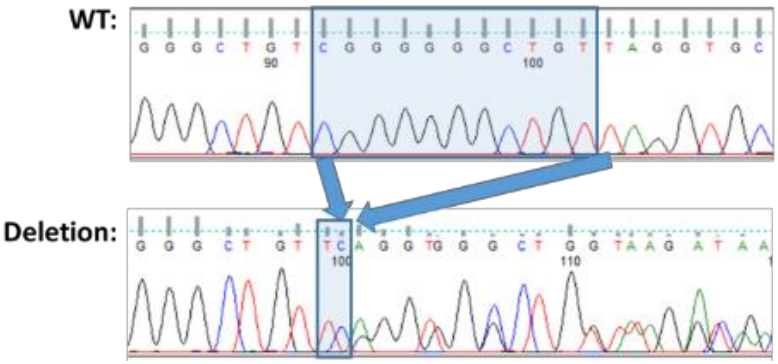
WT/c.204_214del11 (p.Gly69Argfs*10)

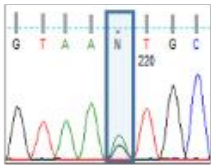




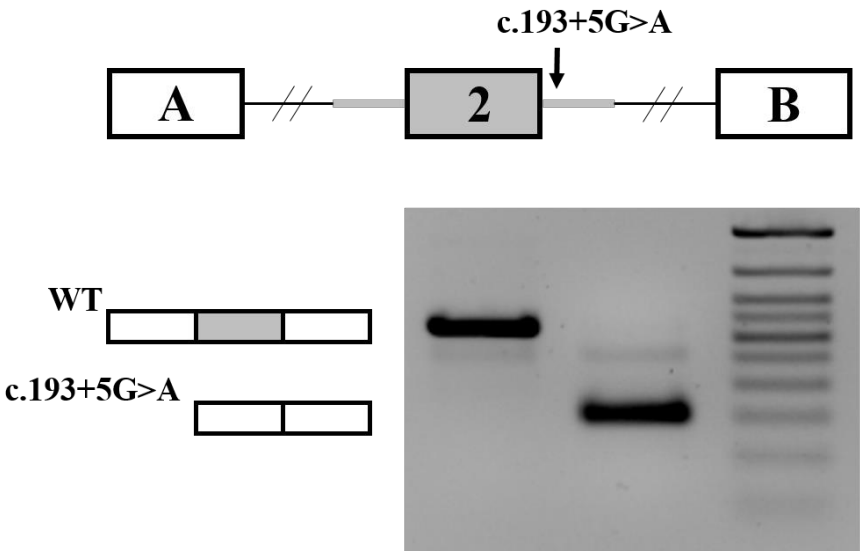
WT/c.193+5G>A

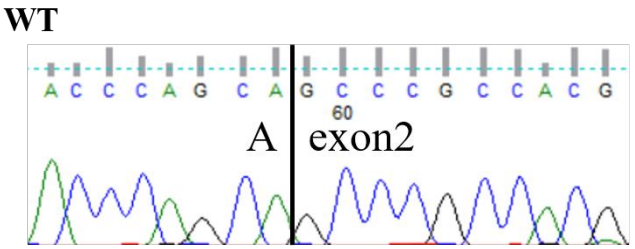
c.204_214del11 (p.Gly69Argfs*10)/c.193+5G>A

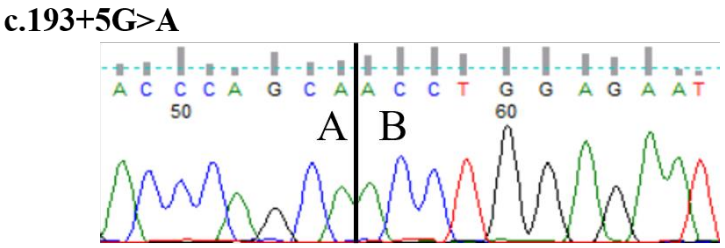




The results of minigene assay

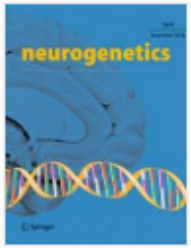






- New assessment: pathogenic variant (PM2, PVS1, PS3)

Launch of the NBIA4 therapy project



neurogenetics
December 2018, Volume 19, Issue 4, pp 257–260 | Cite as

Novel case of neurodegeneration with brain iron accumulation 4 (NBIA4) caused by a pathogenic variant affecting splicing

Authors | Authors and affiliations

Peter Sparber ✉, Andrey Marakhonov, Alexandra Filatova, Inna Sharkova, Mikhail Skoblov

Short Communication
First Online: 03 Novembe

NBIA Poland signs an agreement supporting the BRAINCURE Project.

Publicada en *6 abril, 2019*

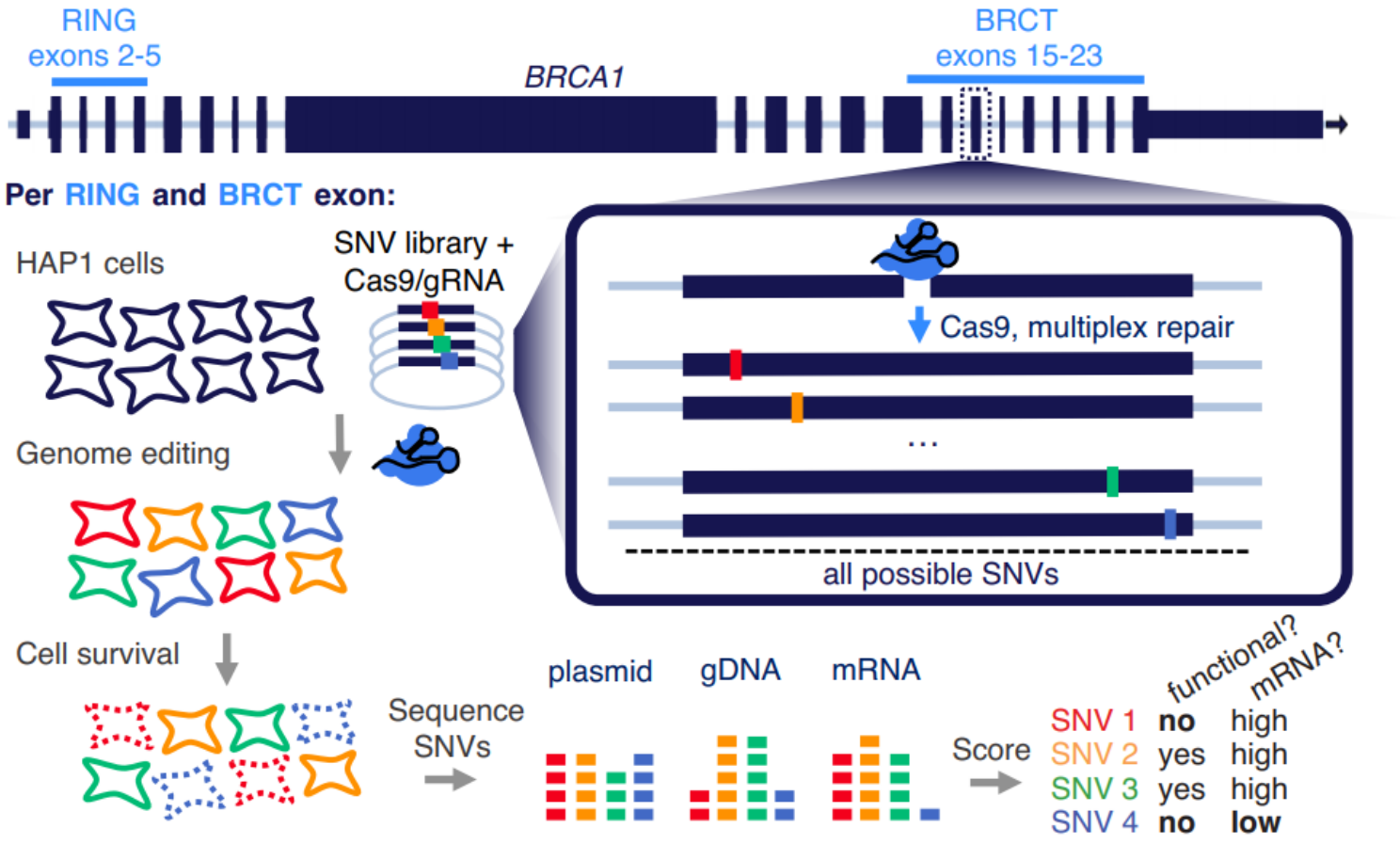
NBIA ALLIANCE

Worldwide Partners for a Cure

Massively parallel reporter assays (MPRAs) that combine next-generation sequencing with extensive variation have been applied to study splicing

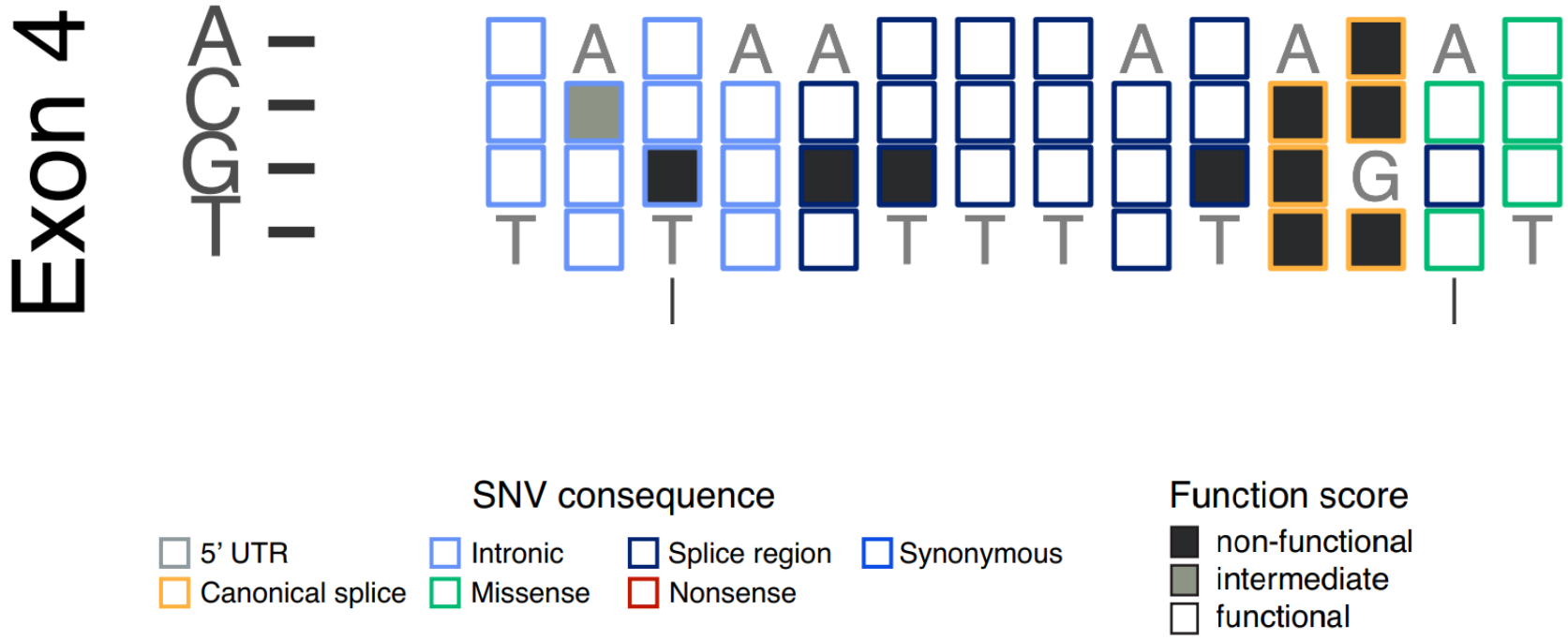
Functional classification of thousands of variants of the BRCA1 gene

- For 13 exons with domains RING and BRCT, 4'035 possible SNVs were created.



http://dx.doi.org/10.1101/294520

Functional classification of thousands of variants of the BRCA1 gene



http://dx.doi.org/10.1101/294520

Functional classification of thousands of variants of the BRCA1 gene

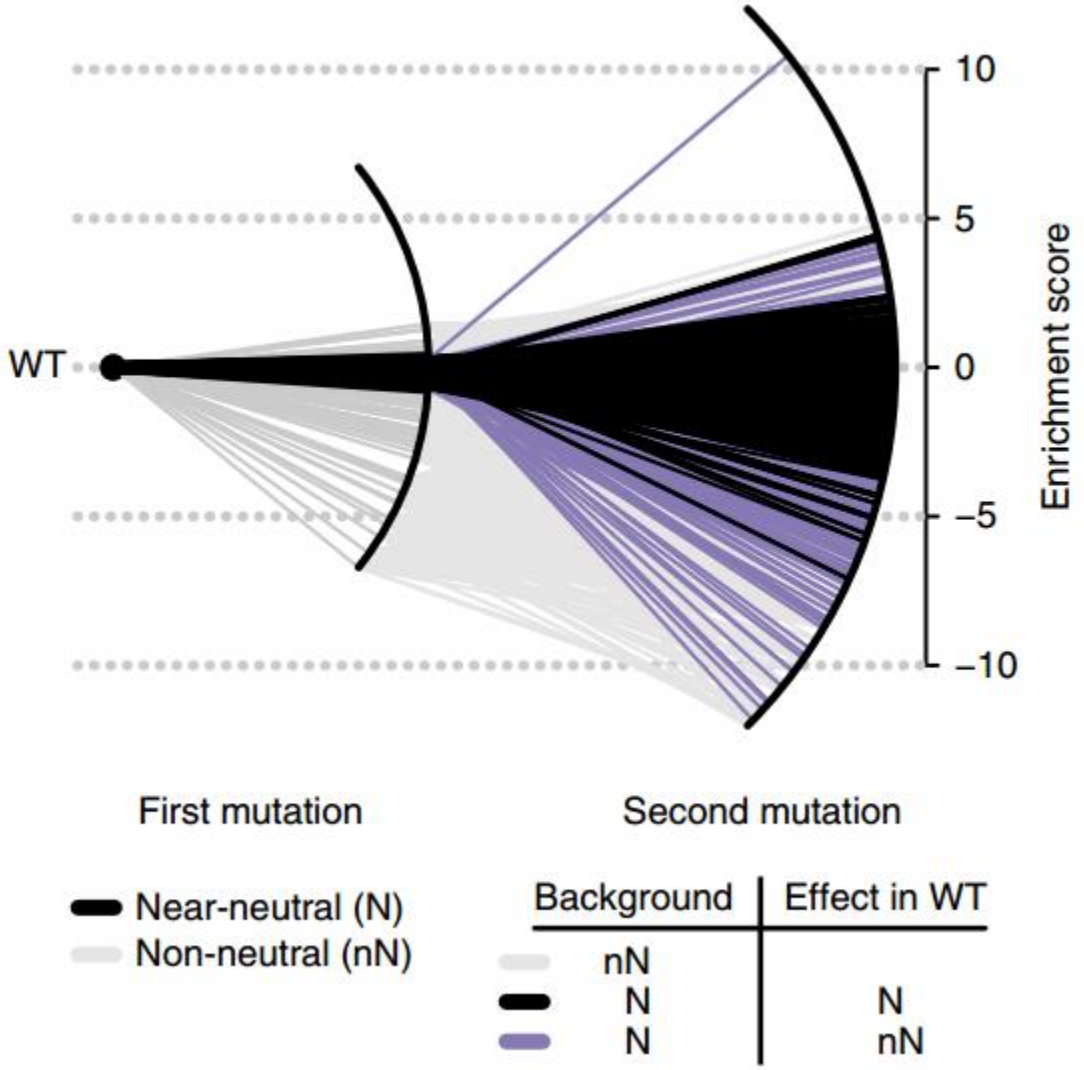
http://dx.doi.org/10.1101/294520

All possible single and double mutations in exon 6 of human FAS/CD95

- Out of the 189 possible single mutations, 74 (39%) significantly reduced exon inclusion, 41 (22%) promoted inclusion and 73 (39%) had no statistically distinguishable effect on splicing.
- The distributions of mutation effects were similar for synonymous and non-synonymous mutations: 49 synonymous and 140 non-synonymous mutations.
- In total, single nucleotide changes at 58/63 positions (92%) affected splicing, demonstrating that splicing regulatory sequences are distributed across nearly every nucleotide in the exon

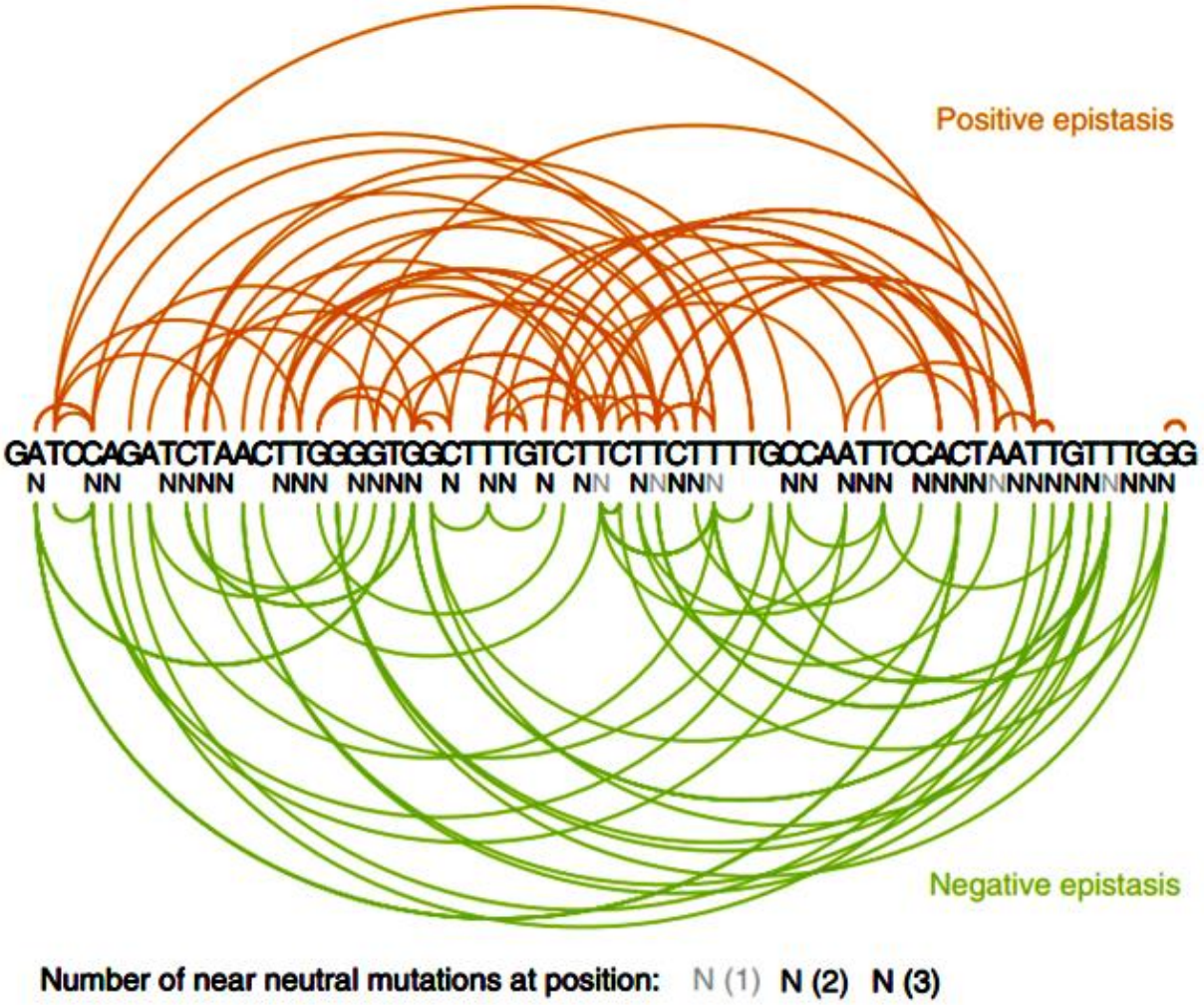
DOI: 10.1038/ncomms11558

Effects of double mutations on FAS exon 6 inclusion



DOI: 10.1038/ncomms11558

Visualization of positive and negative epistatic interactions between near-neutral mutations and any other mutation

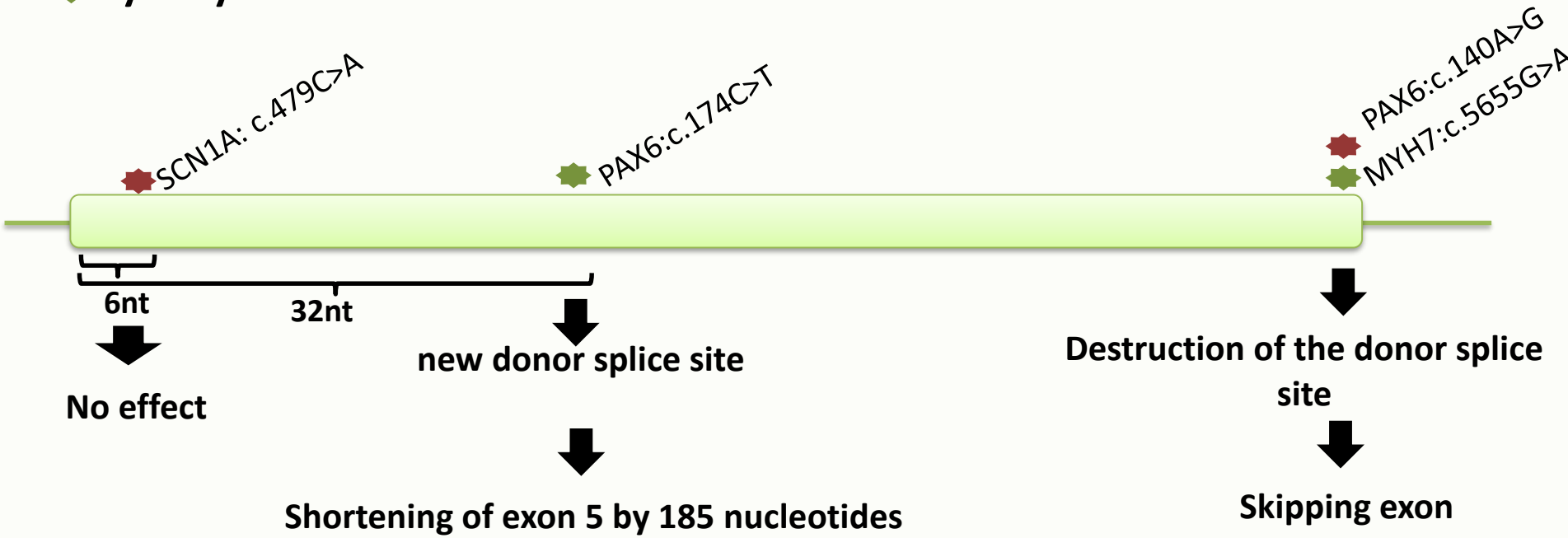


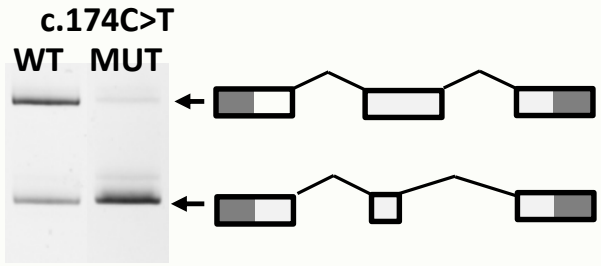
DOI: 10.1038/ncomms11558

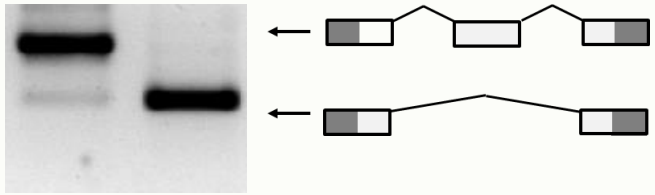
Our experience

missense variant

synonymous variant



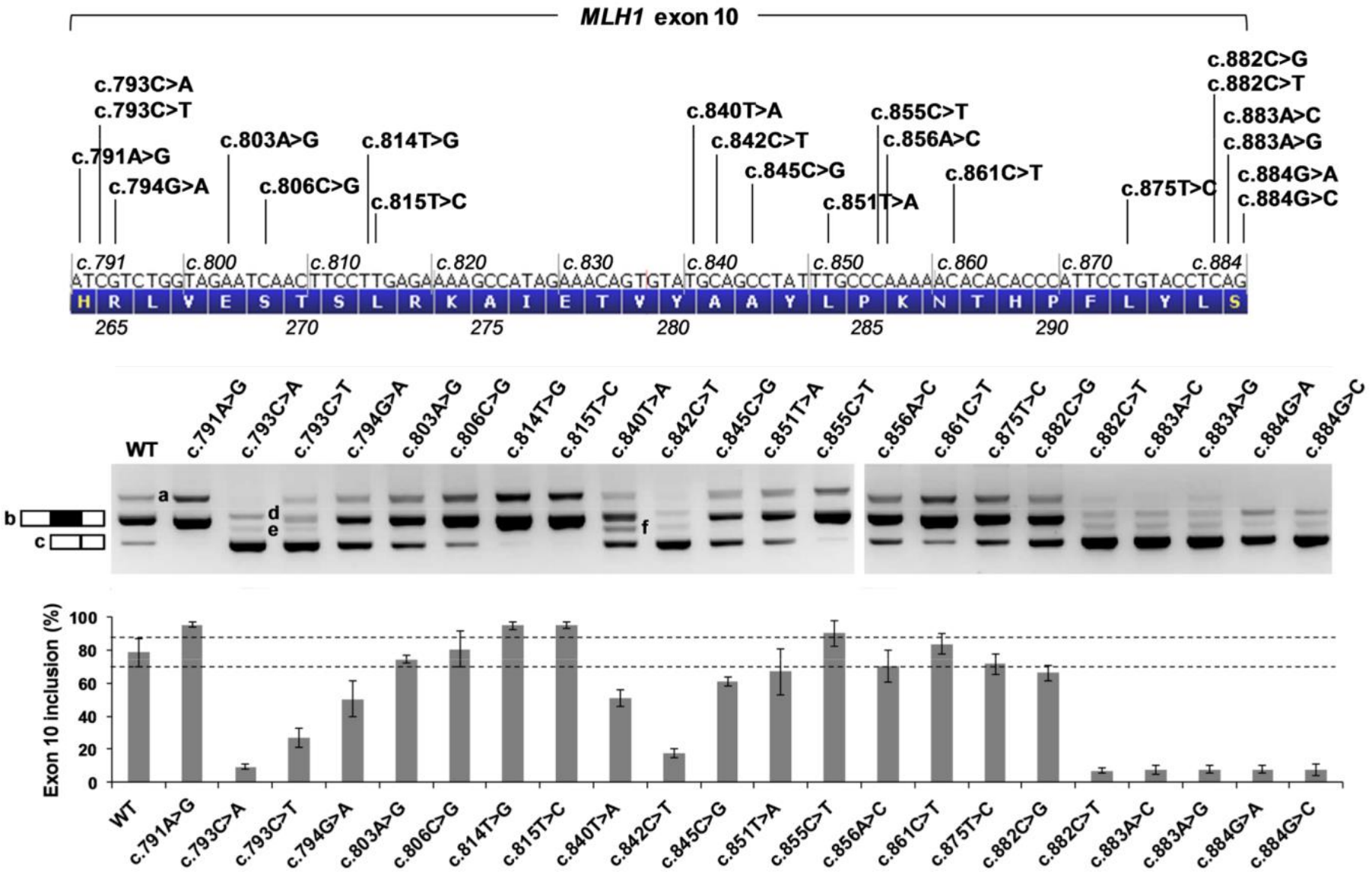




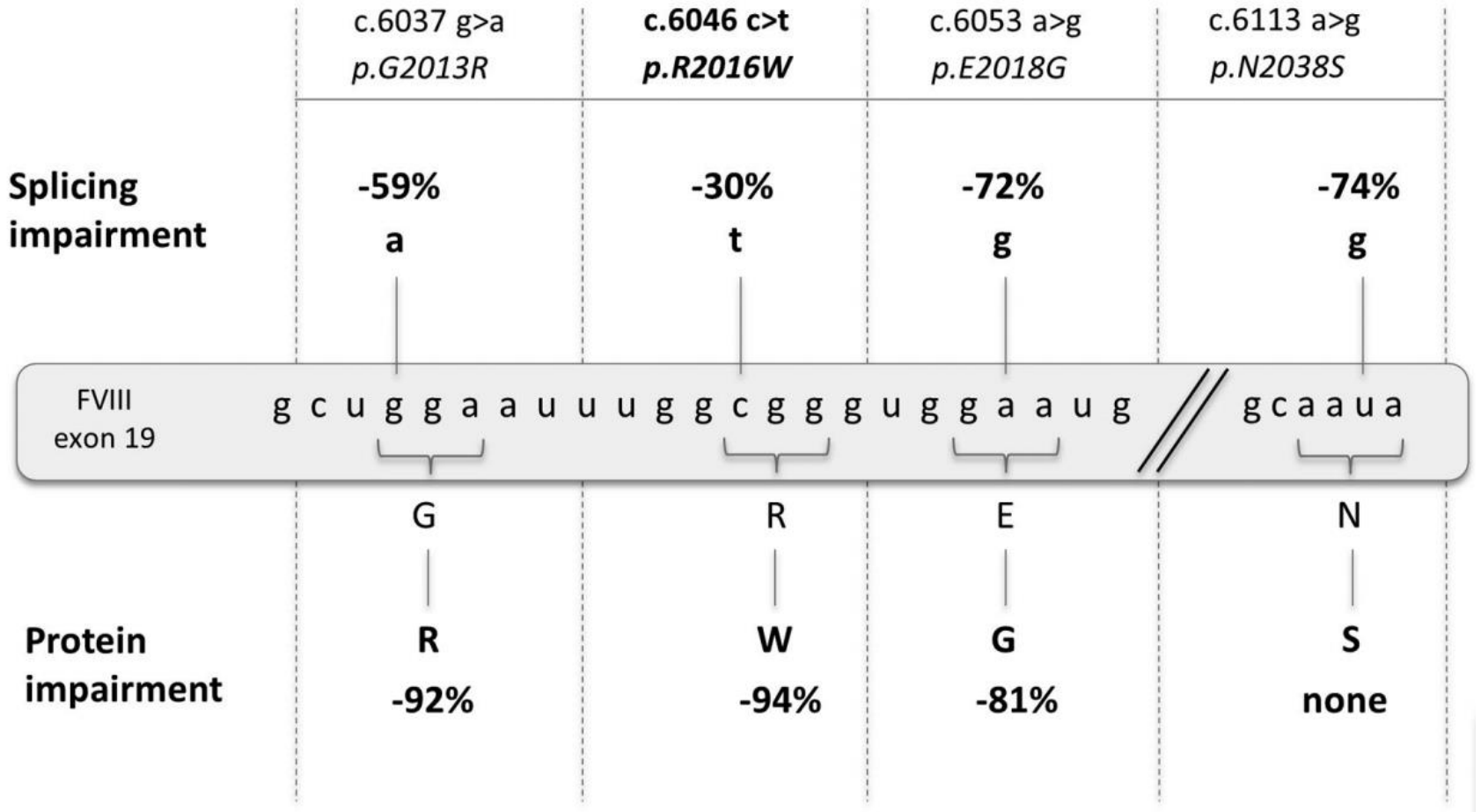
Identification of an unexpected high proportion of splicing mutations in the exon 10 of MLH1

Position in *MLH1* exon 10	Nucleotide variant	Predicted amino acid change	Databases	Variant classification
+1	c.791A>G	p.His264Arg	LOVD, dbSNP	3
+3	c.793C>A	p.Arg265Ser	LOVD, dbSNP	4
+3	c.793C>T	p.Arg265Cys	LOVD, dbSNP, UMD-MLH1	5
+4	c.794G>A	p.Arg265His	LOVD, dbSNP, UMD-MLH1	3
+13	c.803A>G	p.Glu268Gly	LOVD, dbSNP	1
+16	c.806C>G	p.Ser269*	LOVD, dbSNP, UMD-MLH1	5
+24	c.814T>G	p.Leu272Val	LOVD, dbSNP	3
+25	c.815T>C	p.Leu272Ser	LOVD, dbSNP	n/a
+50	c.840T>A	p.Tyr280*	LOVD, dbSNP	5
+52	c.842C>T	p.Ala281Val	LOVD, dbSNP, UMD-MLH1	5
+55	c.845C>G	p.Ala282Gly	LOVD, dbSNP, Swiss-Prot	2
+61	c.851T>A	p.Leu284*	LOVD, dbSNP, UMD-MLH1	5
+65	c.855C>T	p. = (p.Pro285Pro)	LOVD, dbSNP	n/a
+66	c.856A>C	p.Lys286Gln	LOVD, dbSNP	3
+71	c.861C>T	p. = (p.Asn287Asn)	LOVD, dbSNP	3
+85	c.875T>C	p.Leu292Pro	LOVD, dbSNP, Swiss-Prot	3
+92	c.882C>G	p. = (p.Leu294Leu)	LOVD	3
+92	c.882C>T	p. = (p.Leu294Leu)	LOVD, dbSNP, UMD-MLH1	5
+93	c.883A>C	p.Ser295Arg	LOVD, dbSNP	5
+93	c.883A>G	p.Ser295Gly	LOVD, dbSNP	5
+94	c.884G>A	p.Ser295Asn	LOVD, dbSNP	5
+94	c.884G>C	p.Ser295Thr	LOVD, dbSNP, Swiss-Prot	4

Identification of an unexpected high proportion of splicing mutations in the exon 10 of MLH1

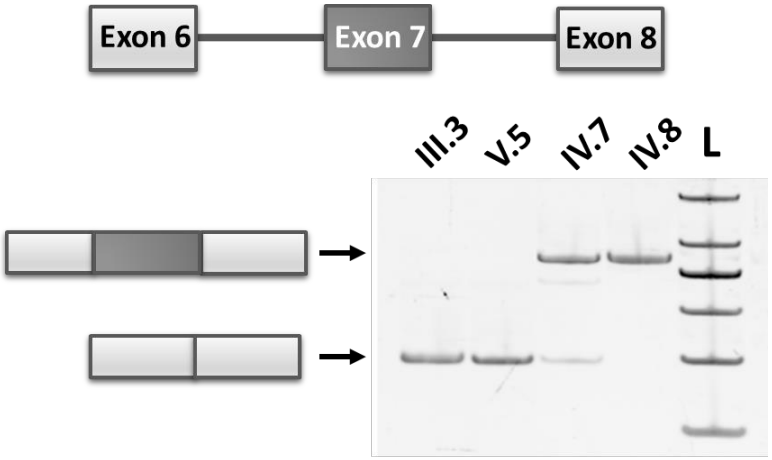


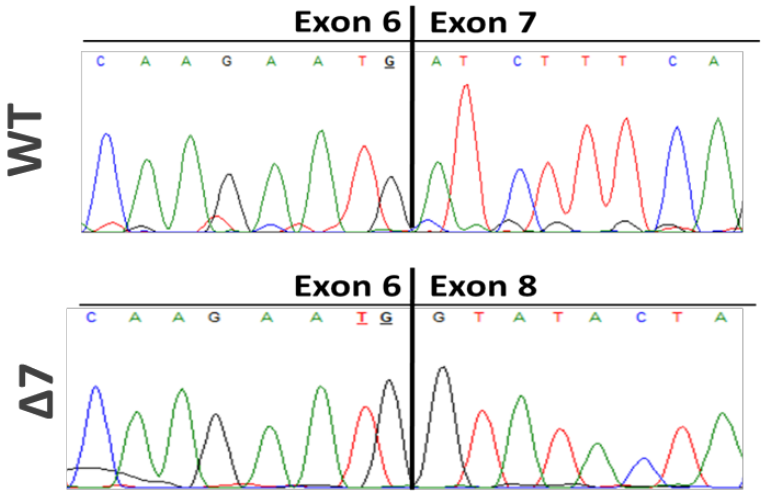
F8 missense mutations cause hemophilia A by combined alteration of splicing and protein biosynthesis and activity



- Detrimental effects of variants on *F8* splicing and on Factor VIII (FVIII) protein expressed as % reduction extent of wildtype (wt) of correct transcripts (top: splicing impairment) or of co-factor activity (bottom: protein impairment)

LoF variants

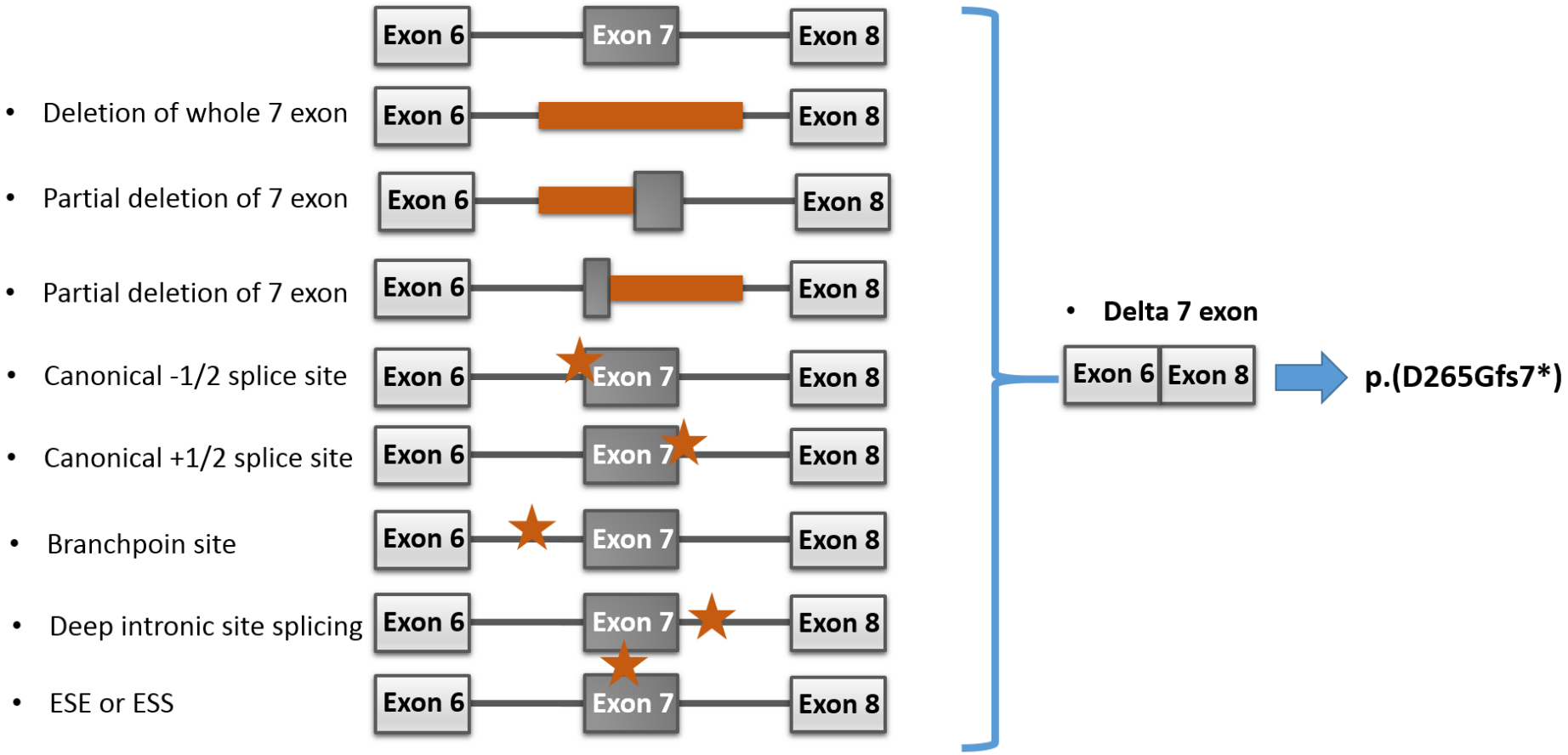




- The absence of the exon 7 in the structure of the mature mRNA leads to a frame shift resulted in truncated non-functional protein p.(D265G*fs*7*), lacking part of the conserved motifs in the TNF-like domains and the cysteine-rich C terminal domain

- loss-of-function (LoF) variants – genetic changes predicted to completely disrupt the function of protein-coding genes.

LoF variants



Clinical classification guidelines that apply to SRE-affecting variants

Class		Observation
5: pathogenic	•	assay on mRNA from patients tissue samples
	AND	no wt transcript detected from variant allele
	AND	aberrant transcripts introduce PTC or deletion disrupting functional domain
		OR deletion disrupting protein conformation
	OR	damaging effect on the gene or gene product (extent not specified)
	AND	other lines of evidence supporting variant pathogenicity [2] (stronger than for class 4)
	•	lab assays based on mRNA (e.g., minigenes)
	AND	variant-specific abrogated function (extent not specified)
	AND	additional frequency/co-segregation/clinical data, additional molecular/mechanistic evidences from other sources, supporting variant pathogenicity (stronger than for class 4)
4: probably pathogenic	•	assay on mRNA from patients tissue samples
	AND	damaging effect on the gene or gene product (extent not specified)
	AND	other lines of evidence supporting variant pathogenicity (milder than for class 5)
	•	lab assays based on mRNA (e.g., minigenes)
	AND	variant-specific abrogated function (extent not specified)
	AND	additional frequency/co-segregation/clinical data, additional molecular/mechanistic evidences from other sources, supporting variant pathogenicity (milder than for class 5)
	•	minigene assays
	AND	complete aberrant and frameshifting effect/ deletion of a functional domain effect

Thanks to everyone