Welcome and overview

3rd March, 2008

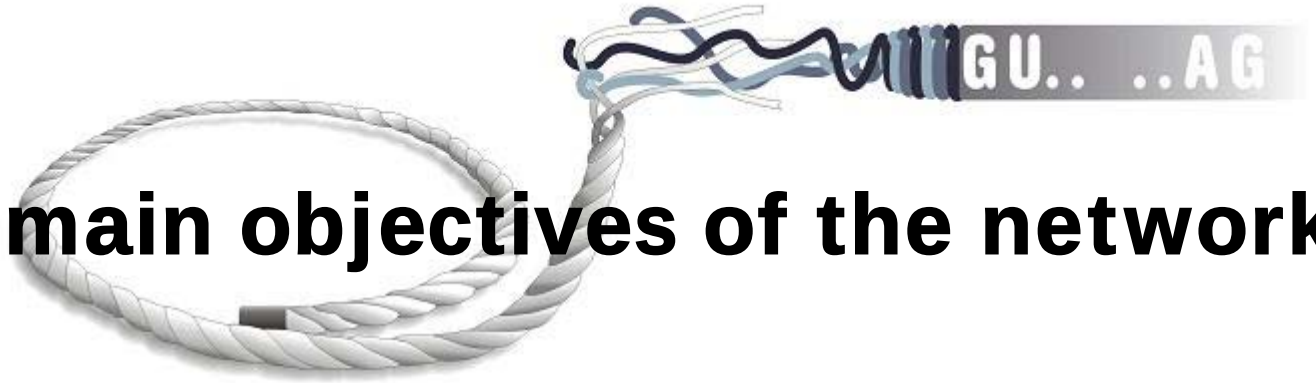
Diana Baralle

European alternative splicing network of excellence

- The Alternative Splicing Network of Excellence brings together 30 leading research groups and ten Young Investigators, from eleven European countries as well as Israel and Argentina.

- For a period of five years (2006 - 2010), this consortium has secured ten million Euros in funding within the Framework 6 Program (FP6) of the European Union, for Research in Alternative Splicing (starting January 1st, 2006).

- Coordination by Prof. Reinhard Lührmann of the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany



The main objectives of the network are:

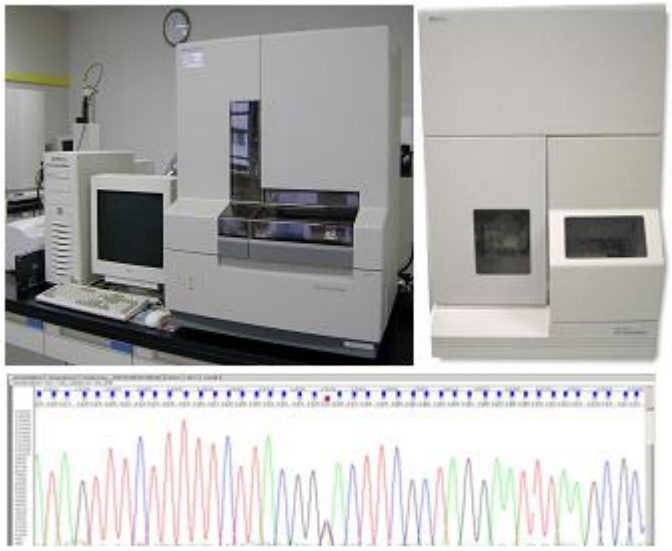
- elucidate the mechanisms of alternative splicing and the interference with other regulatory processes
- establish a communication platform for the exchange of information, methods and material among the network partners
- support ten “Young Investigators” to join EURASNET and establish new research groups
- raise awareness of the importance of alternative splicing among medical practitioners, policy makers and the general public

Objectives of today

Molecular Genetic testing

- Make the most use of the genetic test results.

- Distinguish pathogenic from polymorphic sequence changes.



Resolve diagnostic uncertainty.

Medical Genetics Service
Molecular Genetics Laboratory

Addenbrooke's Hospital NHS
Cambridge University Hospitals NHS Foundation Trust

Department of Clinical Genetics
Clinical Genetics, Box 134
Addenbrooke's Hospital
Cambridge
Cambridgeshire
CB2 2QQ

Copy to:
Department of Clinical Genetics

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MOLECULAR GENETIC ANALYSIS FOR BREAST CANCER

Name of Patient: Date of Birth:

REFERRAL REASON: BRCA2 sequence variant in family - plse screen for this.

REPORT SUMMARY: **Sequence variant of unknown clinical significance**

REPORT INTERPRETATION:

has a family history of breast/ovarian cancer and has had breast cancer herself. A sequence variant (c.3690C>T) of unknown clinical significance has been identified in 3 of affected relatives, including one case of male breast cancer.

Clinical Chemistry 50: 313-320 2004). This variant may be the causative mutation of breast cancer in this family or a tightly linked polymorphism. A mutation in the BRCA2 gene would be consistent with the male breast cancer that occurred in this family.

We will arrange for an affected member of this family to have the remainder of the BRCA2 gene screened for mutations. We anticipate that this will be completed by the end of June 2005. We recommend that DNA testing is not offered to unaffected relatives at present.

BASIS OF TEST:

Fluorescent sequence analysis of exon 11 of the BRCA2 gene.

Reported: SACumming Sally Cumming 07/02/2005

Checked: R. Treacy Becky Treacy 07/02/2005

Regional Medical Genetics Service
Molecular Genetics Laboratory
Kefford House, Maris Lane
Trumpington, Cambridge CB2 2FF

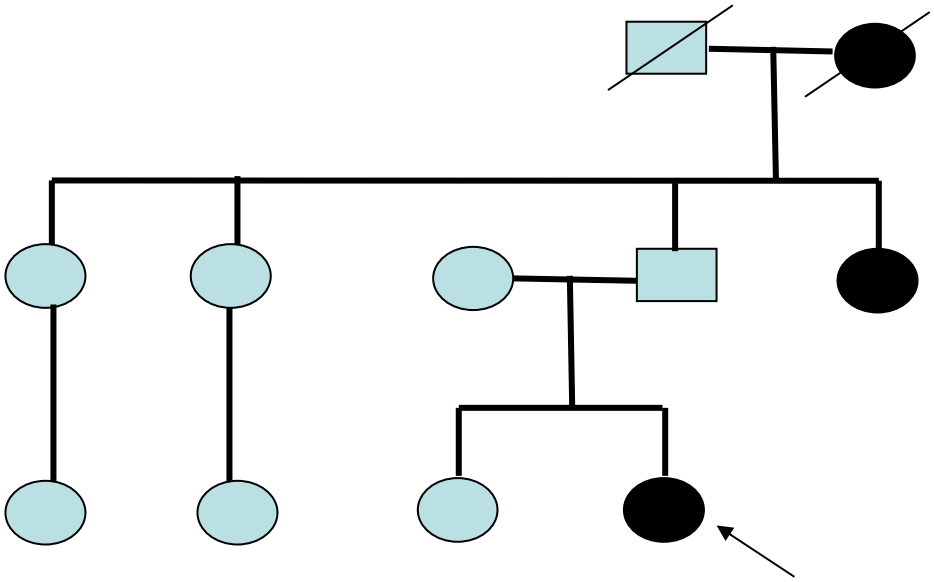
Please note:
The remainder of the DNA from this patient has been stored in the laboratory

Telephone: +44(0)1223 550700
Fax: +44(0)1223 550701

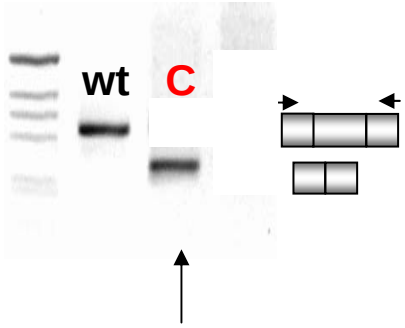
Wessex Regional Molecular Genetics Laboratory

- 1085 breast cancer screens
- 30% sequence variation pick up
- Half of these, 15%, unknown pathogenicity
- 160 families

Minigene Assay example



BRCA1 exon 18 unclassified sequence variant, 5077 G>C



CMGS CLINICAL MOLECULAR GENETICS SOCIETY

VKGL

Practice guidelines for the Interpretation and Reporting of Unclassified Variants (UVs) in Clinical Molecular Genetics.

Prepared and edited by Jennie Bell[1], Danielle Bodmer[2], Erik Sistermans[3] and Simon C Ramsden[4]

1. West Midlands Regional Genetics Laboratory, Birmingham Women's Hospital NHS Trust, Metchley Park Road, Edgbaston, Birmingham, B15 2TG, United Kingdom.
2. Dept of human genetics, Radboud University Nijmegen Medical Centre, PO box 9101, 6500 HB Nijmegen, The Netherlands
3. Dept of clinical genetics, VU University Medical Center, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands
4. National Genetics Reference Laboratory (Manchester), Dept of Medical Genetics, Saint Mary's Hospital, Hathersage Road, Manchester, M13 OJH, United Kingdom.

Guidelines ratified by the UK Clinical Molecular Genetics Society (11th January, 2008) and the Dutch Society of Clinical Genetic Laboratory Specialists (Vereniging Klinisch Genetische Laboratoriumspecialisten; VKGL) (22nd October, 2007).

1. INTRODUCTION

With the increased demand for molecular genetic testing over recent years there has been a marked change in the scale and sensitivity of molecular genetic analysis within the service environment. Inevitably this has resulted in a rapid increase in the detection of novel sequence variations of unknown pathogenicity. Whilst research laboratories may have large resources at their disposal to investigate individual variants, routine diagnostic service laboratories must undertake this analysis within a limited timescale and budget.

It is essential, therefore, that diagnostic laboratories have a set of agreed standards to assist in the determination of the clinical significance of variants identified in routine testing. In addition guidelines should be designed to educate referring clinicians as to possible testing outcomes so that they may inform their patients and families appropriately.

The standards outlined here have been drawn up as a guide to assess variants of unknown clinical significance for situations where there is likely to be a clinical benefit. It may not be appropriate to perform this analysis on all identified variants. The authors and the ratifying bodies (CMGS and VGKL) recognise that these guidelines are aspirational and the practicalities of implementation may lead to future revision.

2. SCOPE OF THE GUIDELINES.

boundaries. This document does not consider changes that alter the invariant AG/GT boundries nor nonsense mutations however we do recognize that these changes cannot be exclusively regarded as pathogenic.

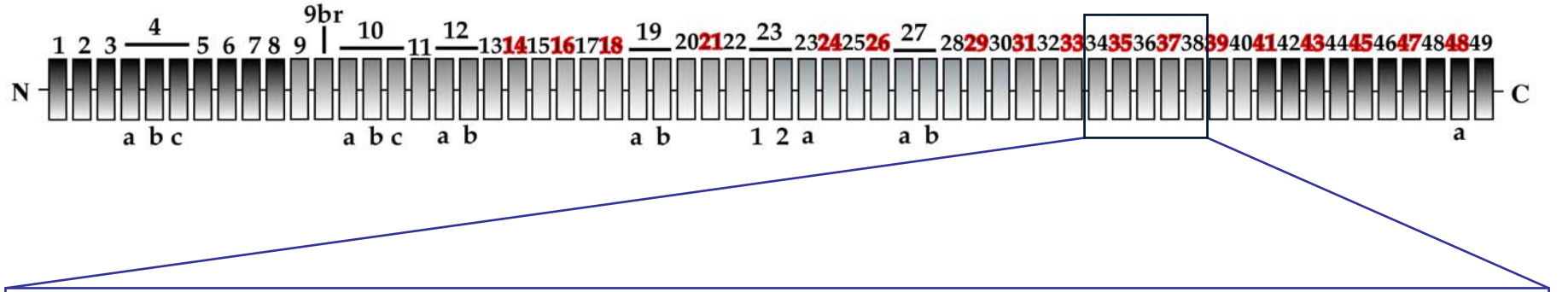
3. QUALITY STANDARDS.

3.1 Minimum quality standards for laboratories interpreting and reporting unclassified variants.

It is essential that the interpretation and reporting of variants of unknown pathogenicity is carried out by appropriately qualified and experienced staff working within certified laboratories that are working to recognized international quality standards (such as ISO 17025 and 15189).

3.2 Test Validation and External Quality Assessment/ Proficiency Testing

All technologies used to identify sequence variants must be appropriately validated to ensure that they meet acceptable performance standards and are fit for the purpose for which they will be used. Validation can be particularly difficult for genetic testing for rare disorders when it may be difficult to obtain suitable positive mutation controls. There is little guidance on the minimum requirements for validation. However the Clinical and Laboratory Standards Institute (www.clsi.org) has published guidance on the use of molecular diagnostic methods for genetic disease which includes a comprehensive section on test validation.

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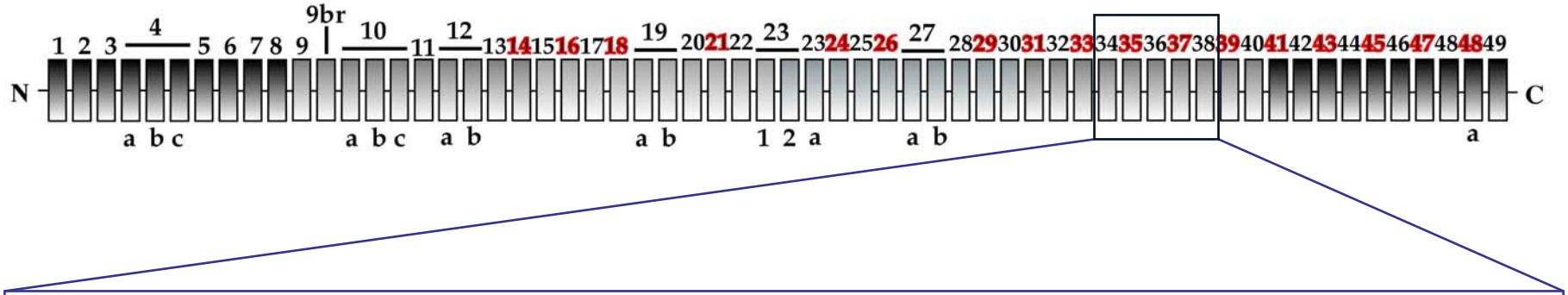

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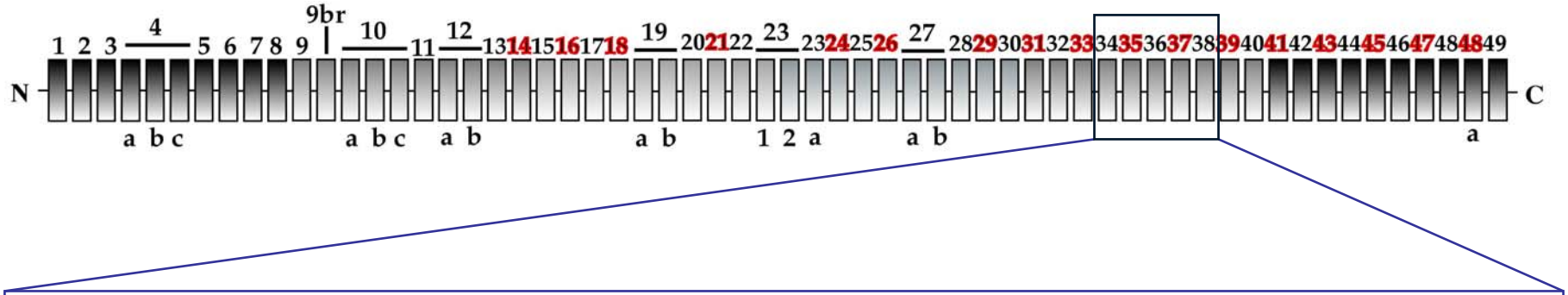

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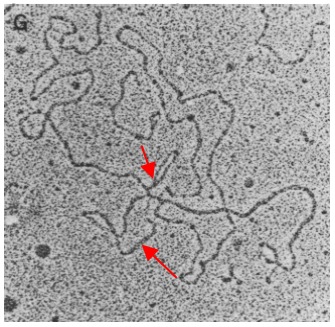
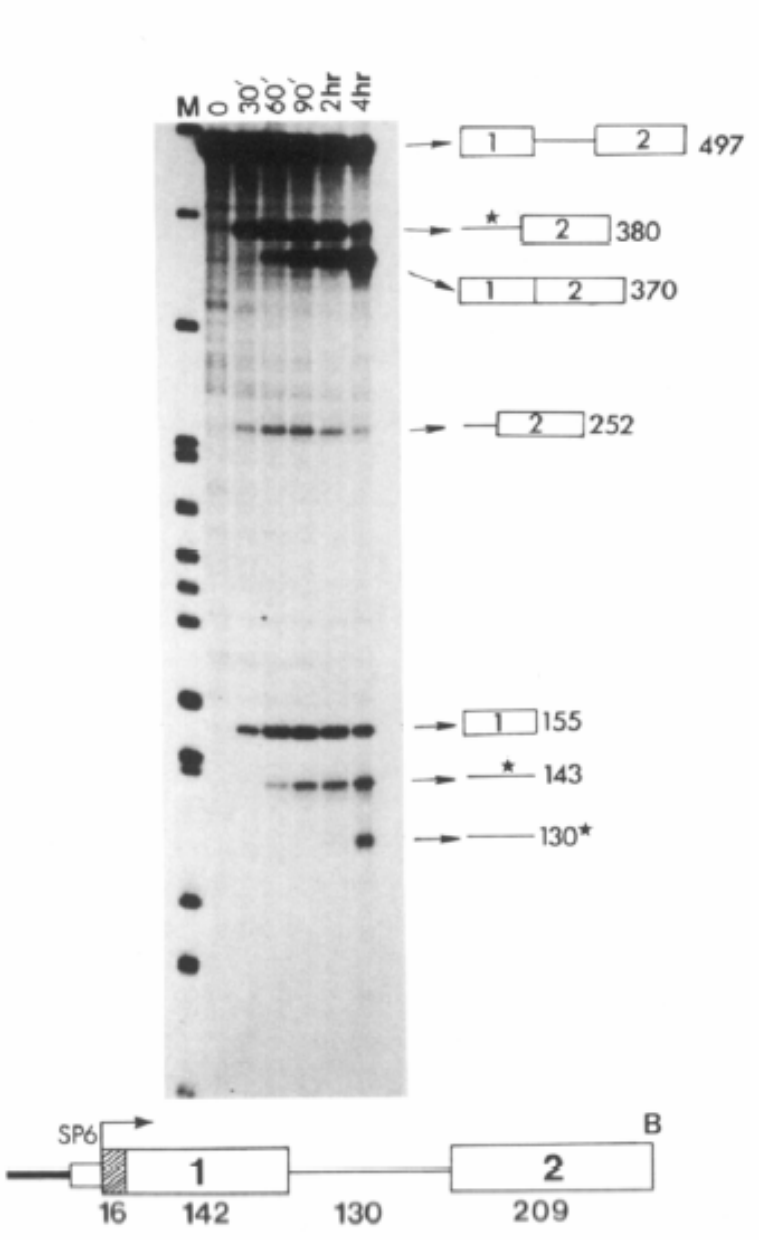
Discovery of intervening sequences (intron) in 1977

Nobel laureats 1993

R. Roberts

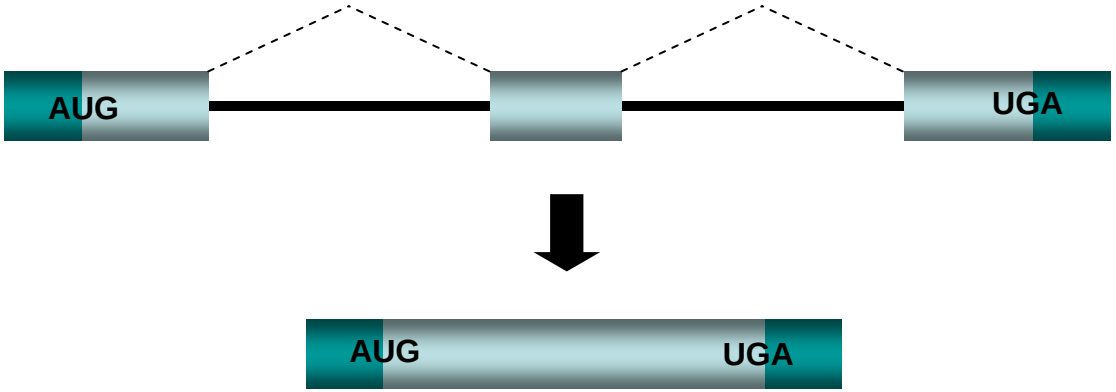
Ph. Sharp





Adapted from Ruskin et al. 1984 Cell 38: 317-331

Pre-mRNA splicing

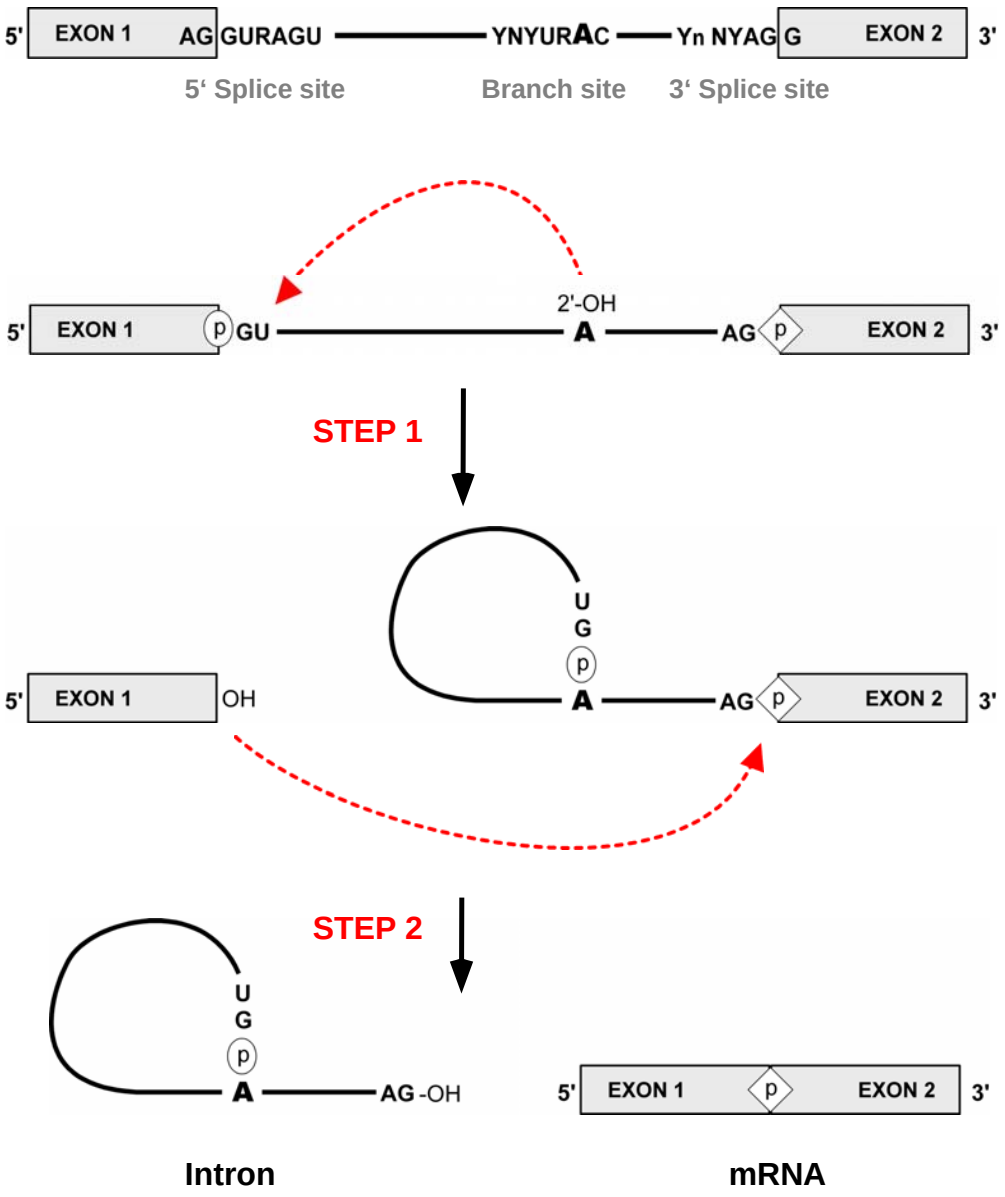


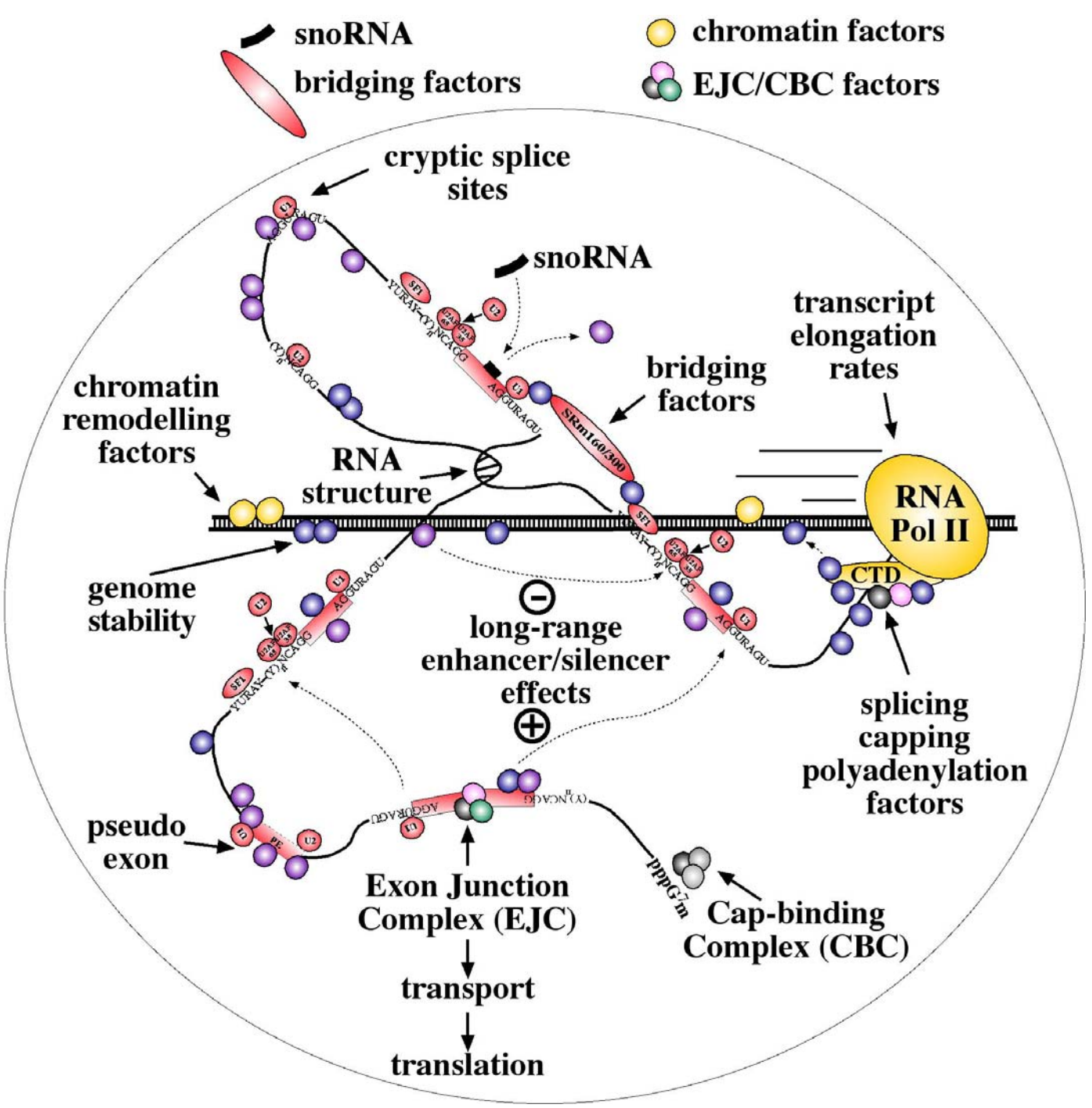
- Essential step in gene expression
- >15% of human genetic diseases involve splicing errors

Alternative splicing

- Important *regulatory* step in gene expression

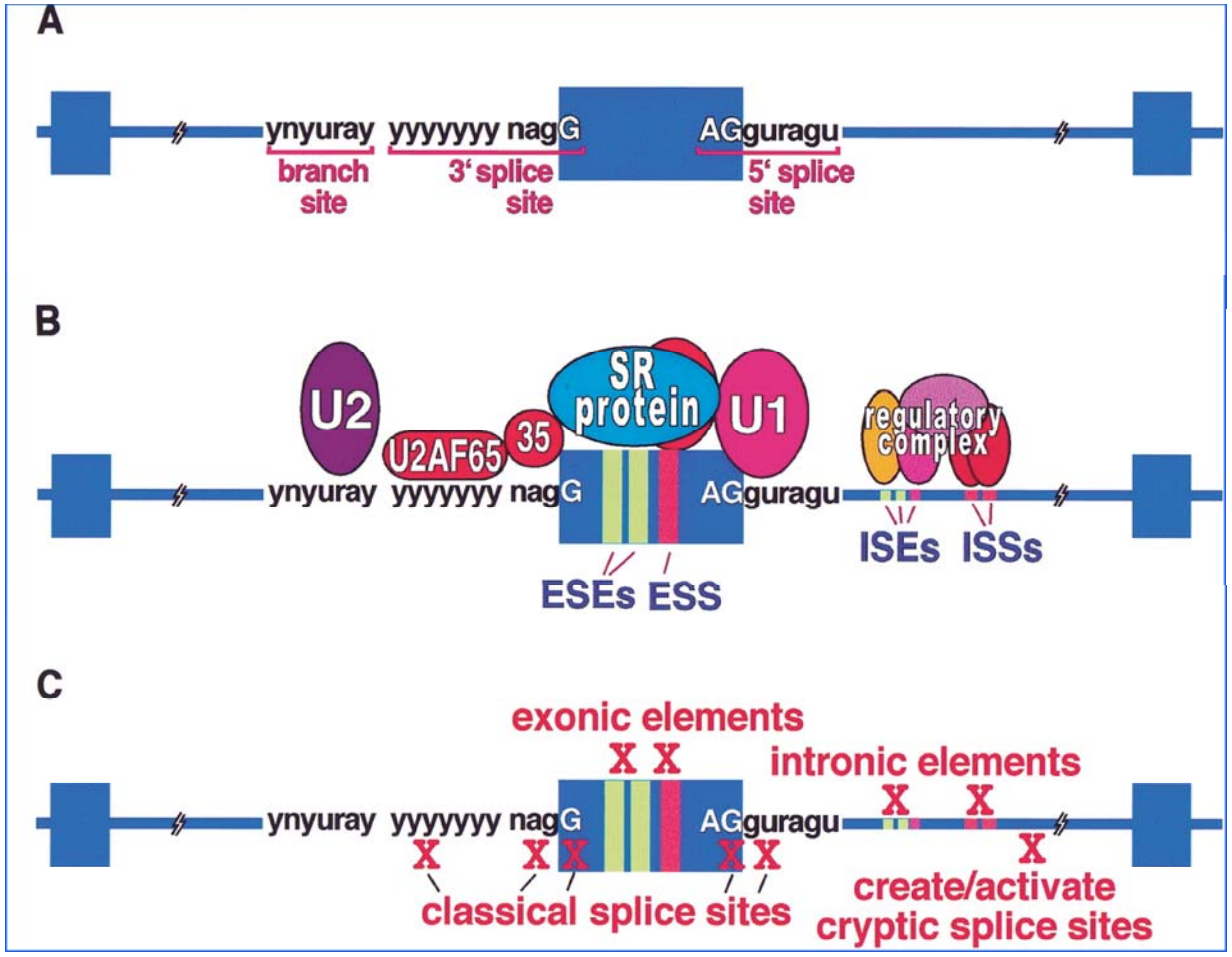
Two step mechanism of pre-mRNA splicing




snoRNA
bridging factors
chromatin factors
EJC/CBC factors
cryptic splice sites
snoRNA
transcript elongation rates
bridging factors
chromatin remodelling factors
RNA structure
RNA Pol II
CTD
genome stability
long-range enhancer/silencer effects
splicing capping polyadenylation factors
pseudo exon
Exon Junction Complex (EJC)
Cap-binding Complex (CBC)
transport
translation

Assembly and structural dynamics of the spliceosome, one of the most complex molecular machines in the cell

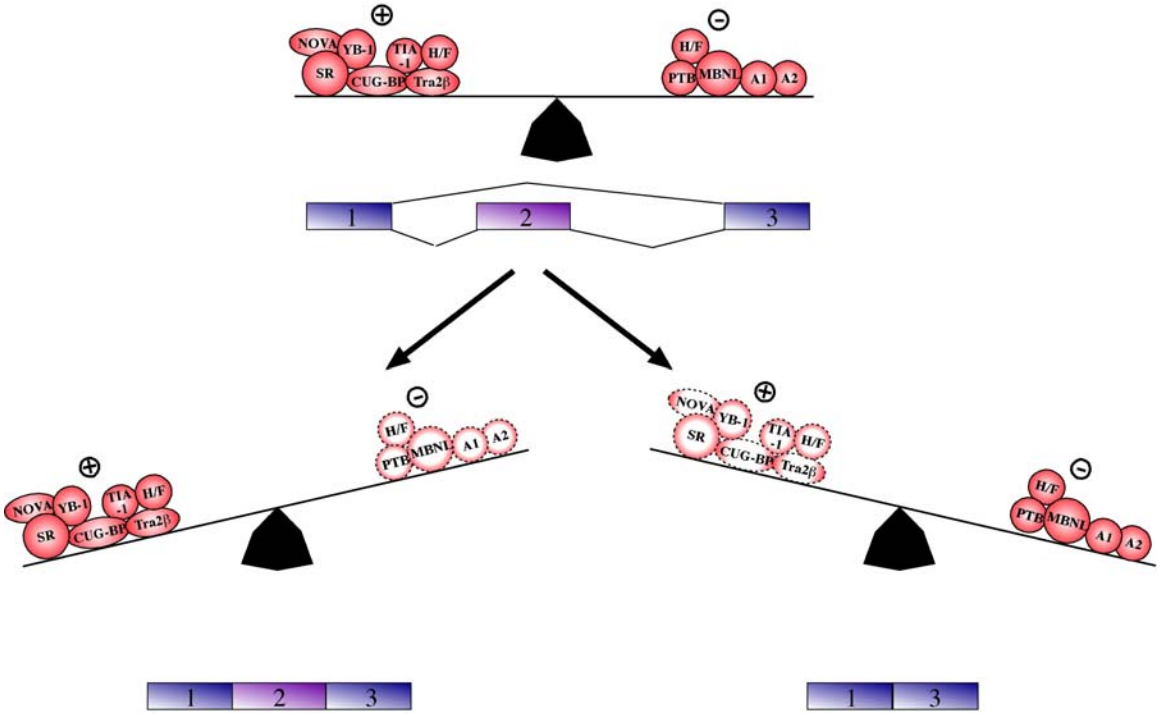
Disease causing disruption of pre-mRNA splicing regulatory elements



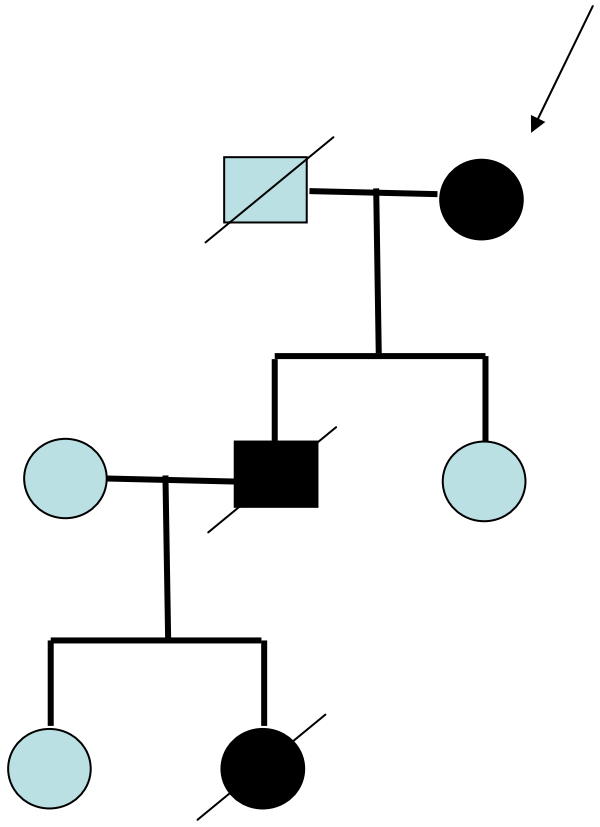
Over the years, a great number of Enhancer and Silencer factors have been identified:

Enhancers	Silencers
SR protein family	hnRNPs (such as A/B family, PTB,
CELF protein family	hnRNP H…etc).
hnRNP L	TDP-43
Tra2	
YB-1	
NOVA	

- **In many cases exclusion/inclusion may thus determined by the resulting balance of power**



NF1 intron 3 Mutation 288+5 G>C

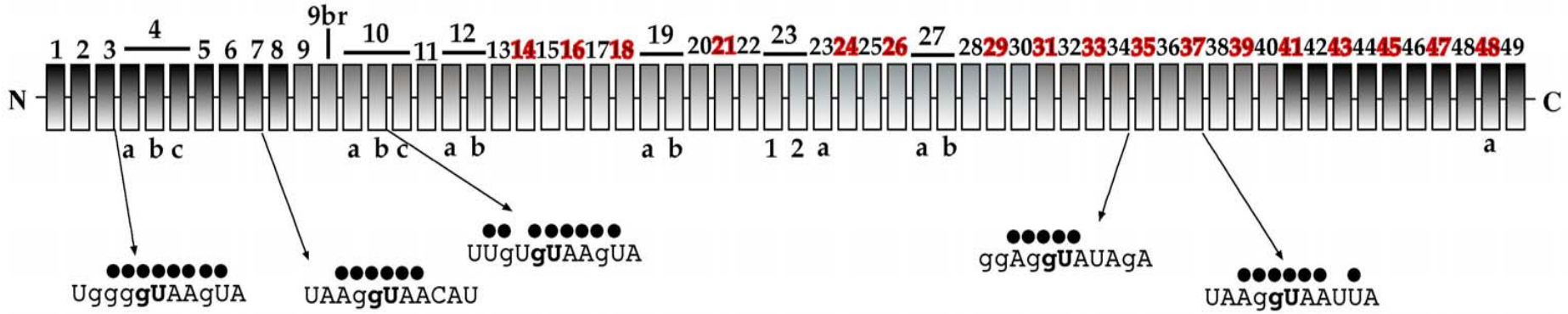


83y Numerous CAL, and neurofibromas. Axillary and inguinal freckling

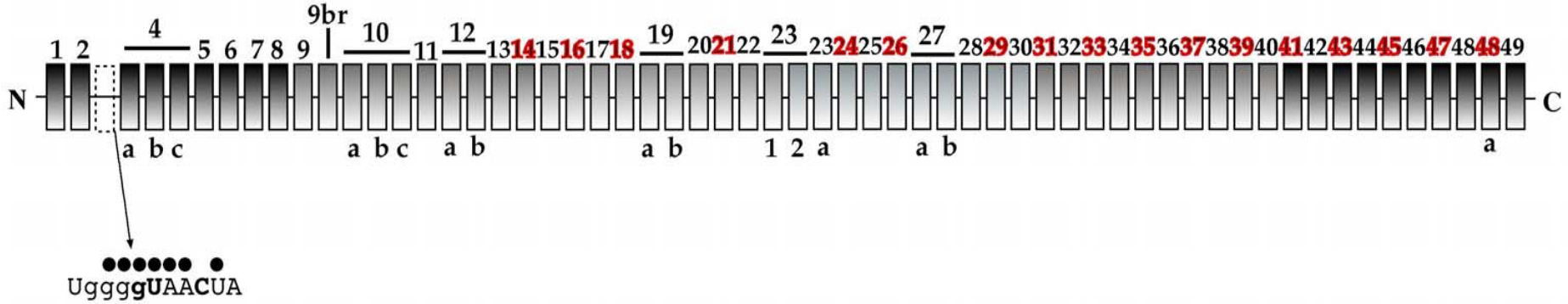
Died at 40y RTA. Mild NF1

Macrocephaly. Died 31y malignant peripheral nerve sheath tumour (MPNST) of coeliac axis.

NF-1 wt

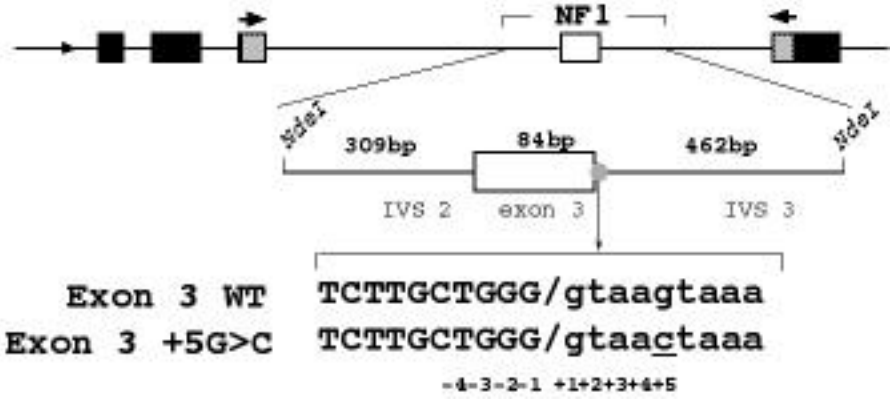


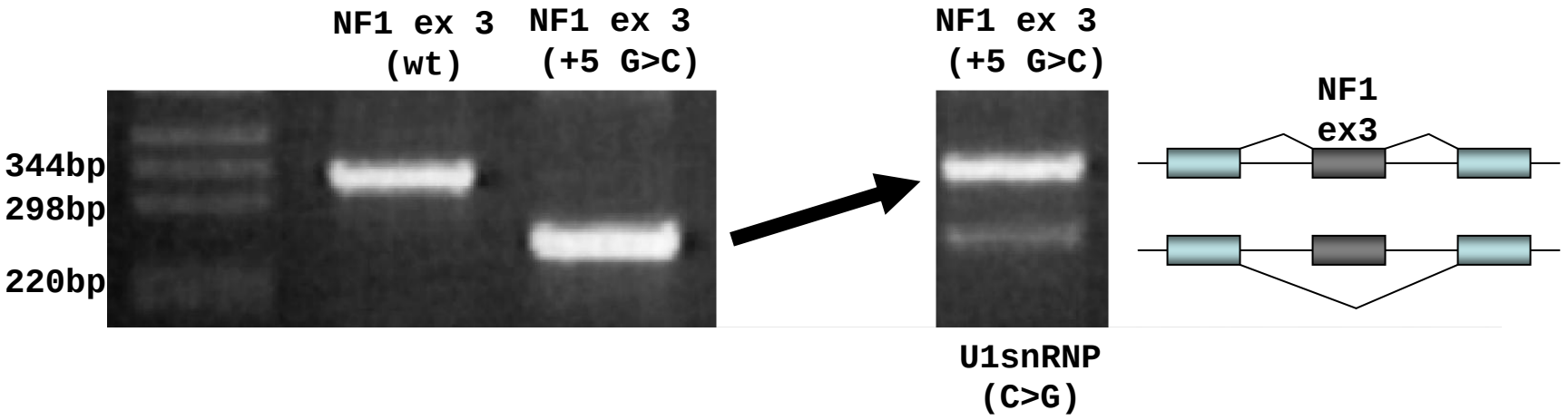
NF-1 ex3 +5G>C



U1snRNA ggUCCAUUCAUA

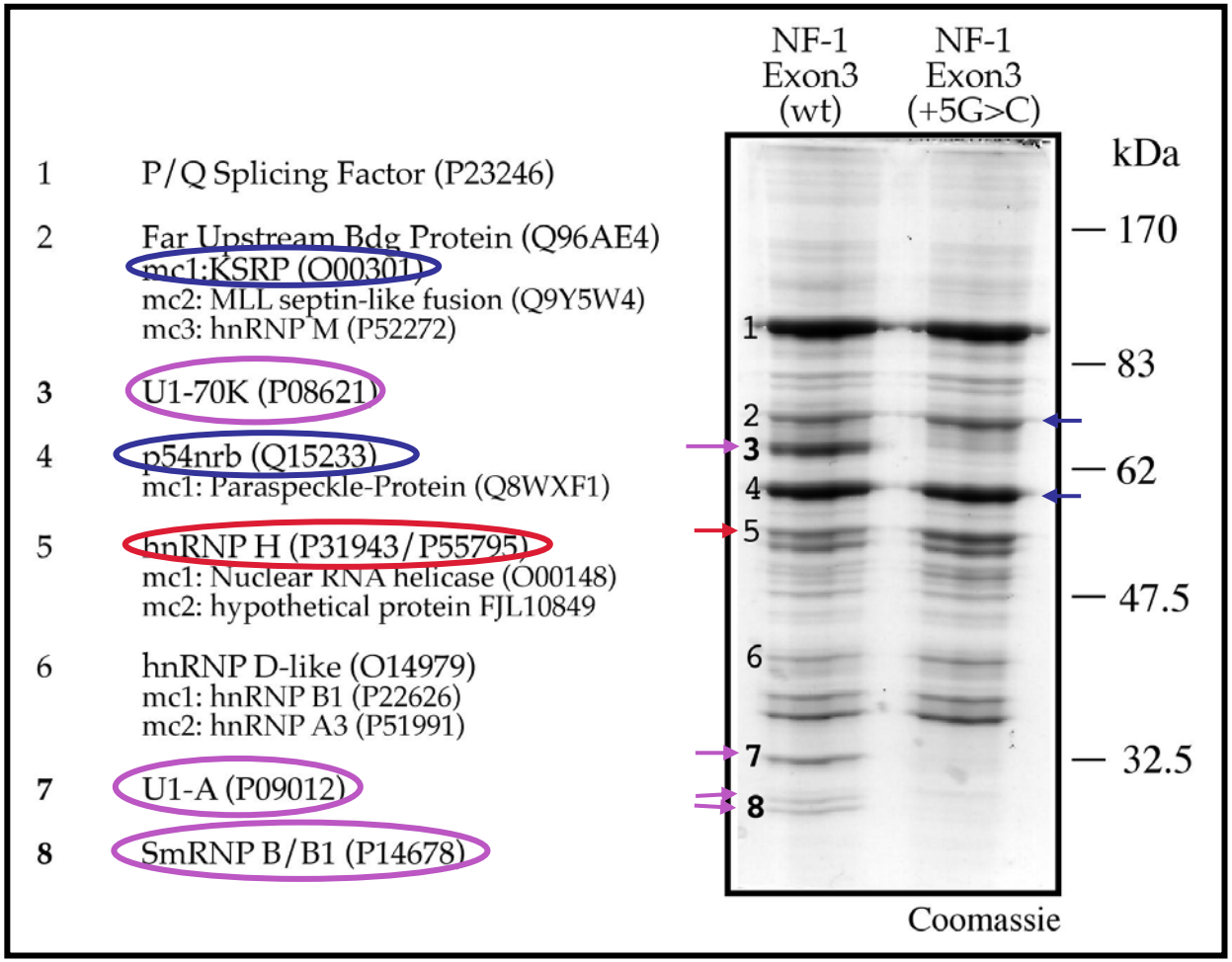
● base-pair match





Baralle et al, J.Med. Gen. 2003

Pull down analysis of the wt and mutated (+5G>C) exon 3 sequence.



1 P/Q Splicing Factor (P23246)

2 Far Upstream Bdg Protein (Q96AE4)
mc1:KSRP (O00301)
mc2: MLL septin-like fusion (Q9Y5W4)
mc3: hnRNP M (P52272)

3 U1-70K (P08621)

4 p54nrb (Q15233)
mc1: Paraspeckle-Protein (Q8WXF1)

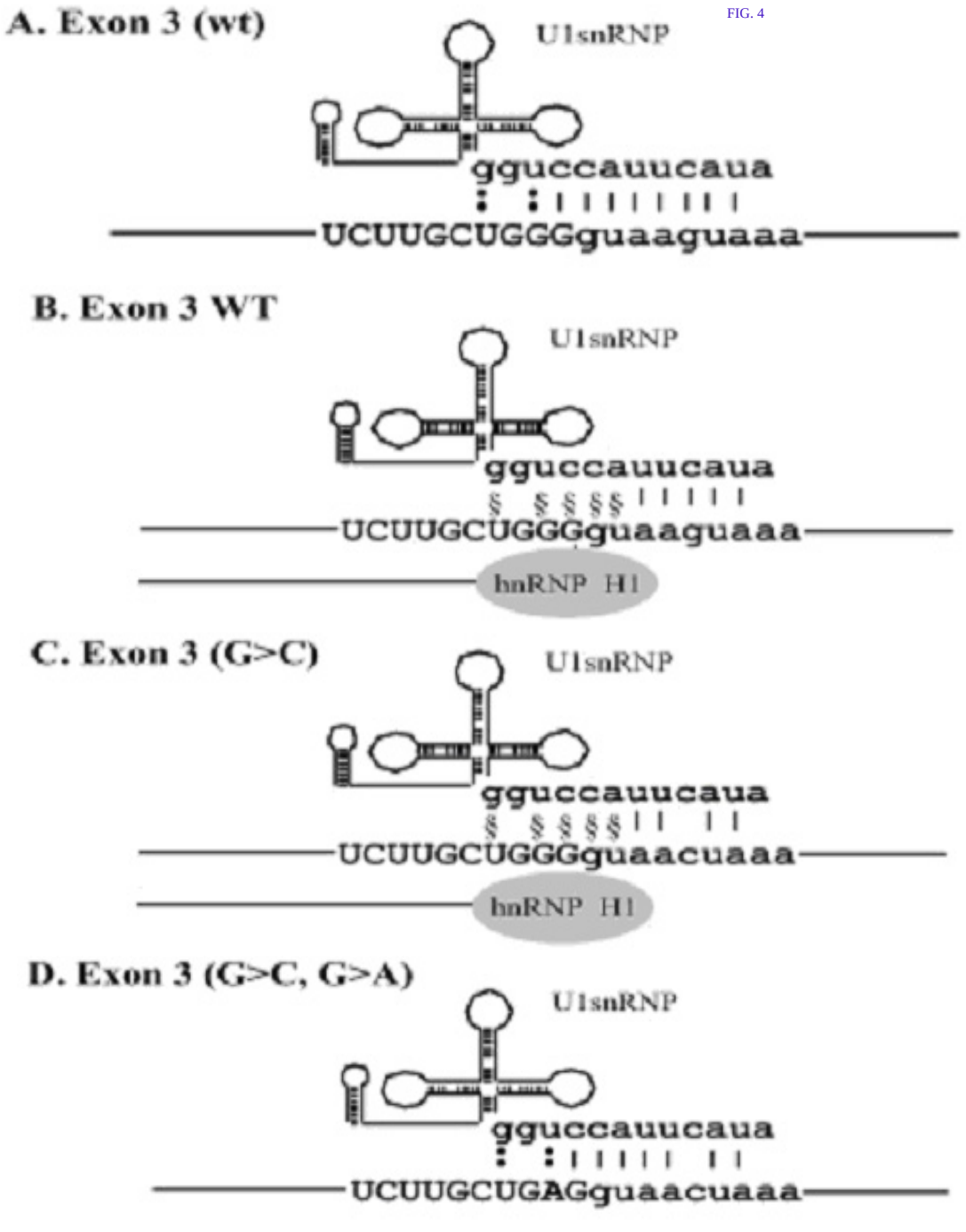
5 hnRNP H (P31943/P55795)
mc1: Nuclear RNA helicase (O00148)
mc2: hypothetical protein FJL10849

6 hnRNP D-like (O14979)
mc1: hnRNP B1 (P22626)
mc2: hnRNP A3 (P51991)

7 U1-A (P09012)

8 SmRNP B/B1 (P14678)

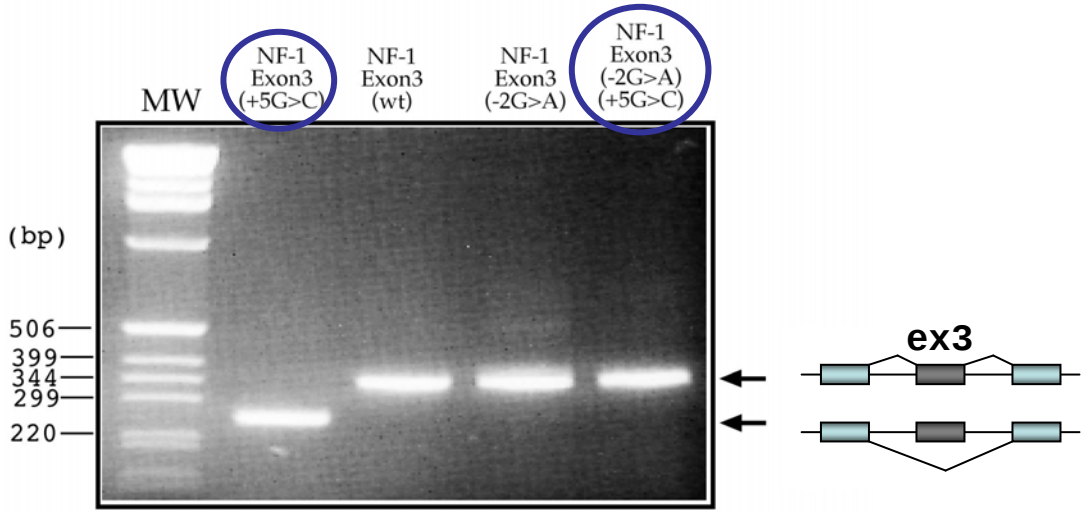
FIG. 4

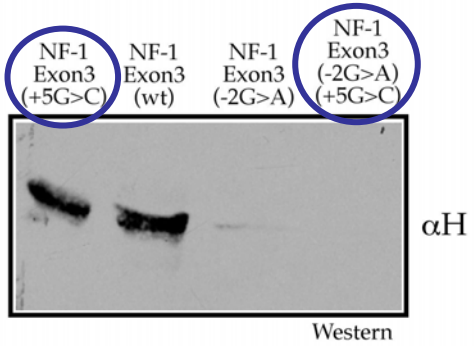

A. Exon 3 (wt)
U1snRNP
gguccauucaua
UCUUGCUGGGguaaguaaa
B. Exon 3 WT
U1snRNP
gguccauucaua
UCUUGCUGGGguaaguaaa
hnRNP H1
C. Exon 3 (G>C)
U1snRNP
gguccauucaua
UCUUGCUGGGguaacuaaa
hnRNP H1
D. Exon 3 (G>C, G>A)
U1snRNP
gguccauucaua
UCUUGCUGAGguaacuaaa

```
                                    ●●●●●●●●●
NF-1 exon 3 (wt)                    UCUUGCUGGGguaaguaaa

                                    ●●●●●● ●●
NF-1 exon 3 (+5G>C)                 UCUUGCUGGGguaacuaaa

                                    ●●●●●●●●●
NF-1 exon 3 (-2G>A)                 UCUUGCUGAGguaaguaaa

                                    ●●●●●● ●●
NF-1 exon 3 (-2G>A,+5G>C)           UCUUGCUGAGguaacuaaa
```

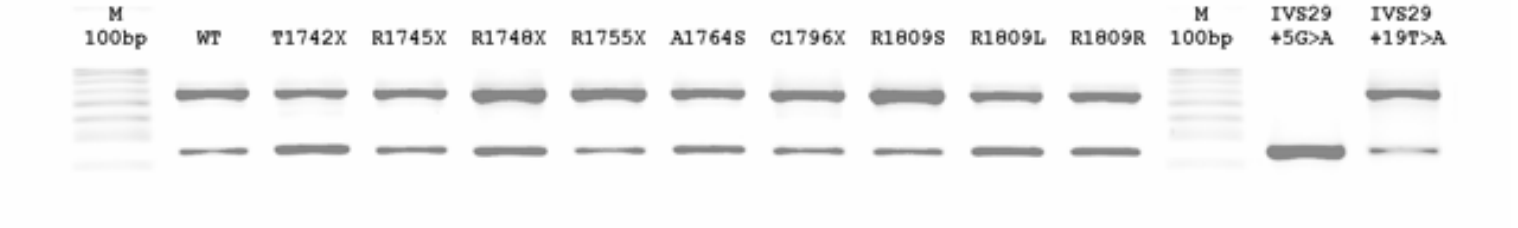
U1 SnRNA ggUCCAUUCAUA

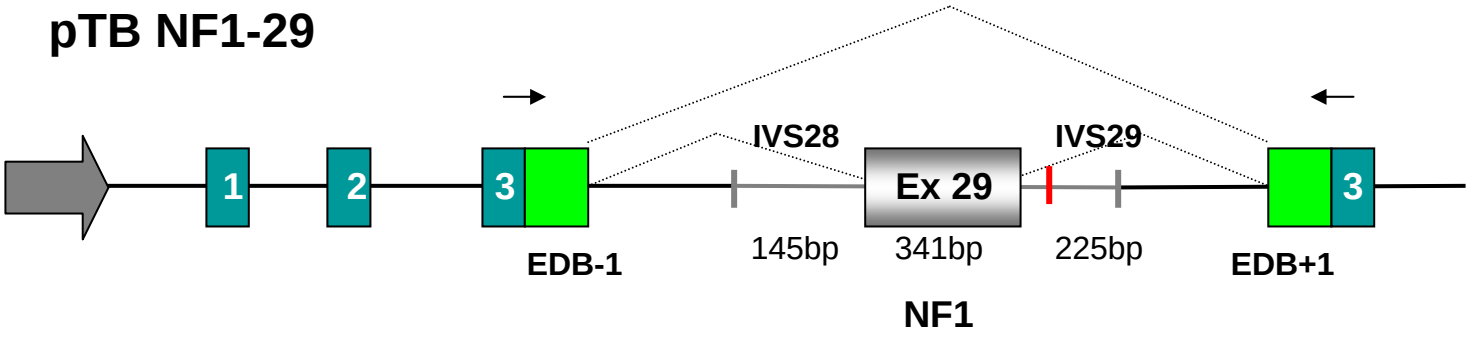
- Base-pair match





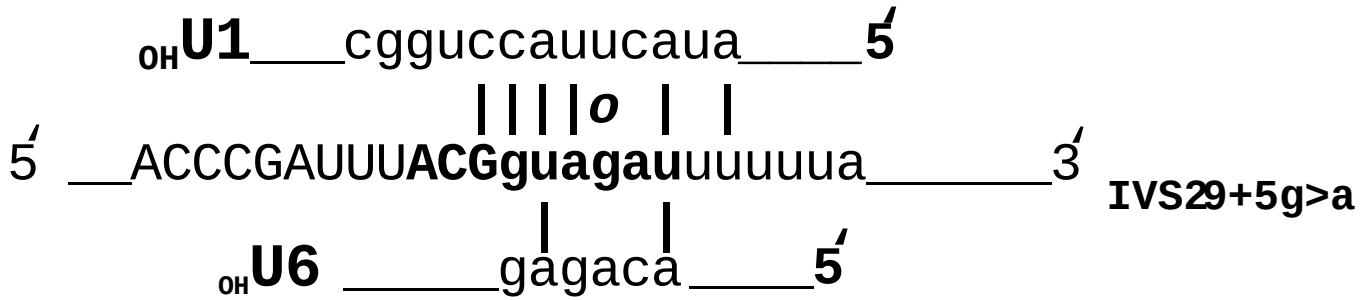
Nonsense, missense, synonymous mutations in NF1 exon 29



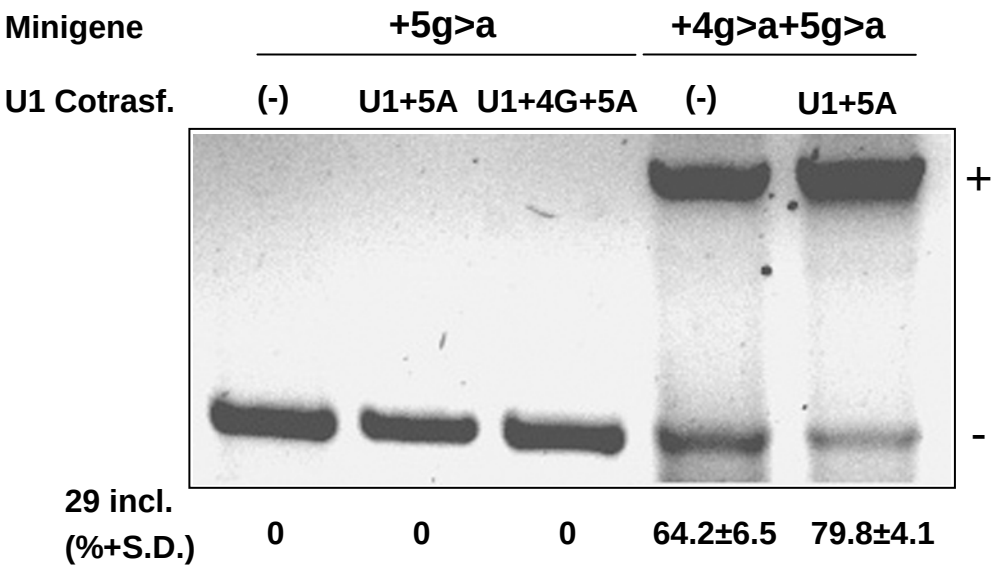



```
                      T(T1742X)    G(R1745X)  T(R1748X)                 G(R1755X)                          T(A1764S)
                      |            |          |                         |                                  |
GTT GGT TCT ACT GCT GTC CAA GTA ACT TCA GCA GAG CGA ACA AAA GTC CTA GGG CAA TCA GTC TTT CTA AAT GAC ATT TAT TAT GCT TCG GAA ATT

                                                                                                      A(C1796X)
                                                                                                      |
GAA GAA ATC TGC CTA GTA GAT GAG AAC CAG TTC ACC TTA ACC ATT GCA AAC CAG GGC ACG CCG CTC ACC TTC ATG CAC CAG GAG TGT GAA GCC ATT

                                 A(R1809S)
                                 |T(R1809L)
                                 ||T(R1809R)
                                 |||
GTC CAG TCT ATC ATT CAT ATC CGG ACC CGC TGG GAA CTG TCA CAG CCC GAC TCT ATC CCC CAA CAC ACC AAG ATT CGG CCA AAA GAT GTC CCT GGG


ACA CTG CTC AAT ATC GCA TTA CTT AAT TTA GGC AGT TCT GAC CCG AGT TTA CG
```

a)



b)



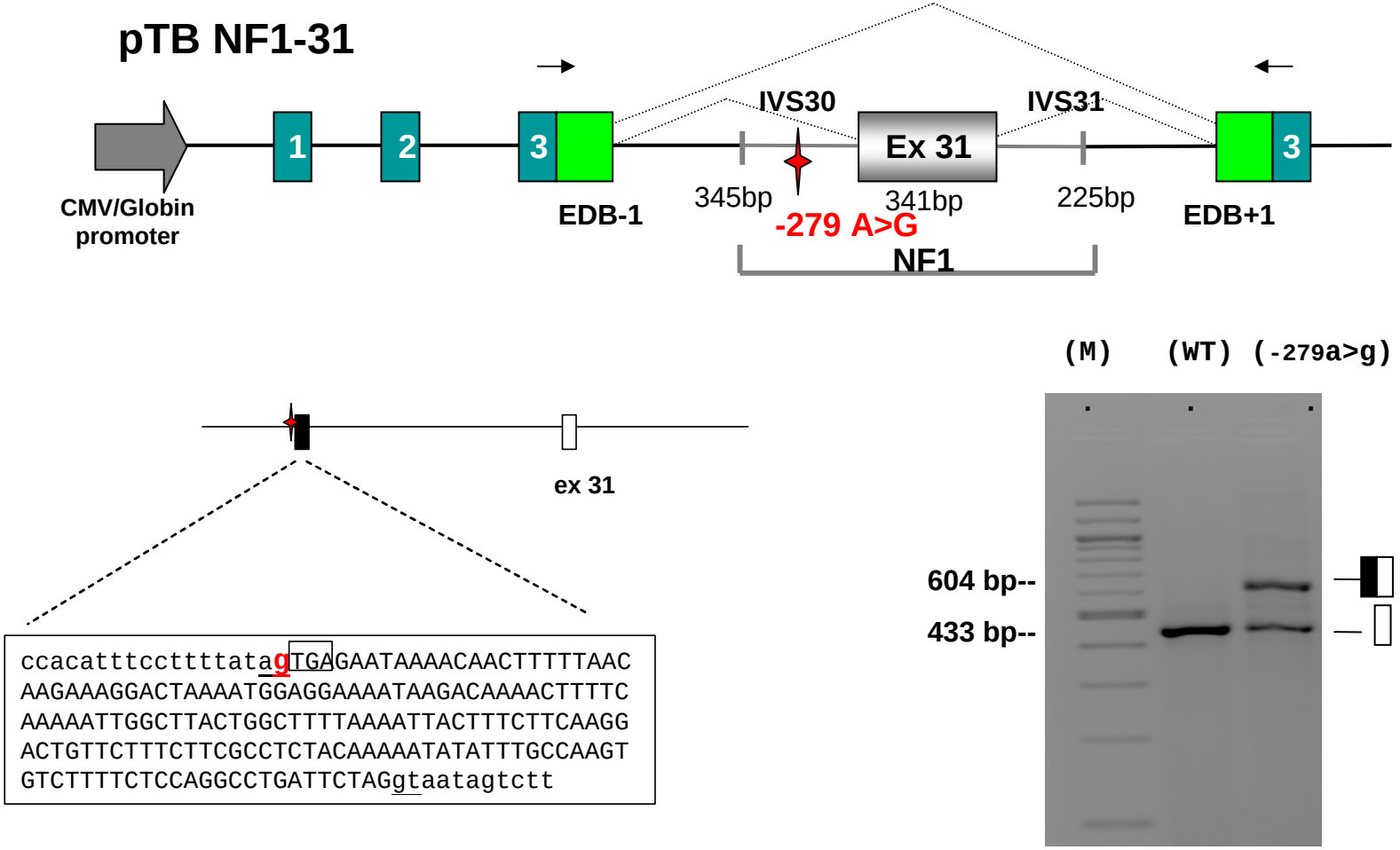
U1+5A
3′ cgguccauu**U**aua 5′

U1+4G+5A
3′ cgguccau**CU**aua 5′

Fig.4

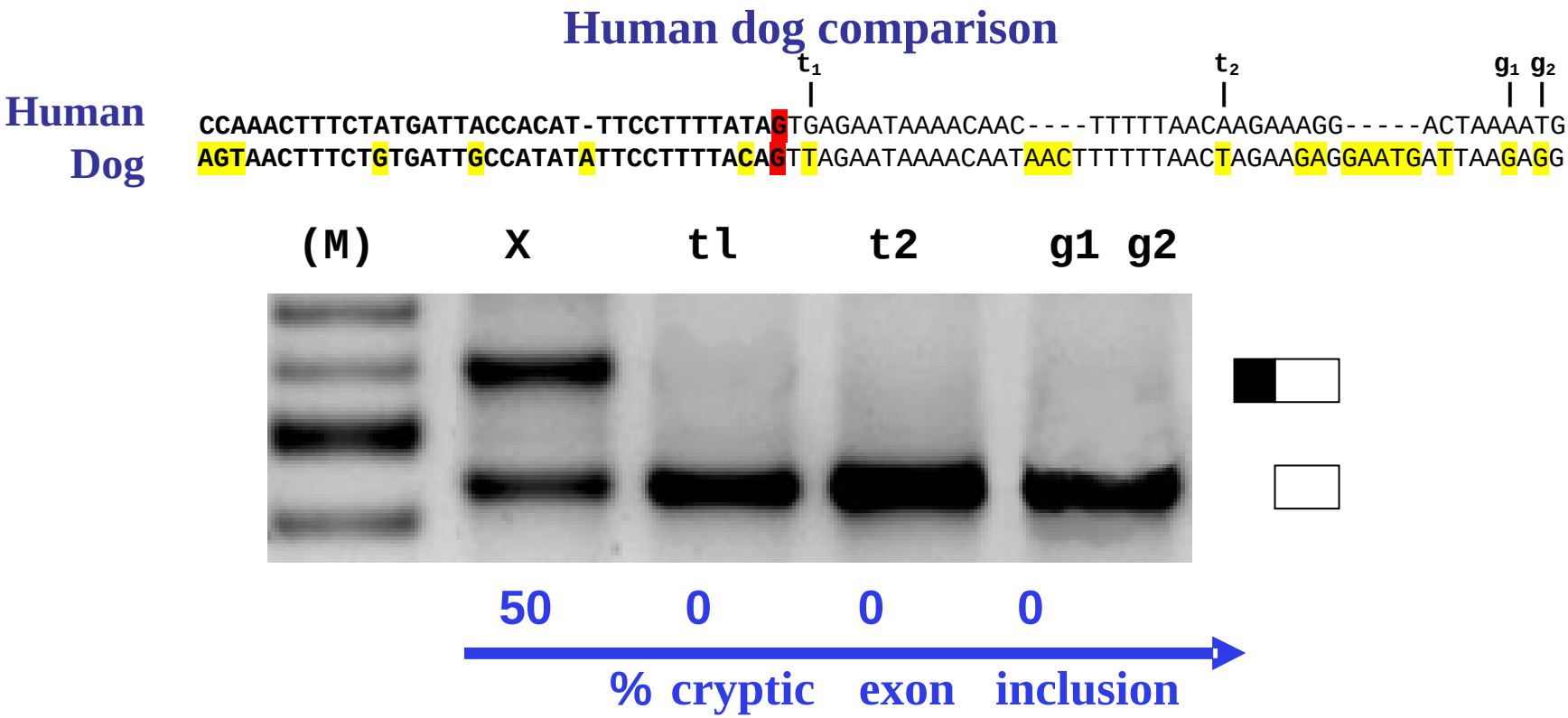
NF1 c.31-279A>G

- Multiple Café au Lait
- >100 neurofibromas
- Plexiform neurofibroma
- Spinal neurofibromas- quadriplegia



Raponi M, Upadhyaya M, Baralle D. **Functional splicing assay shows a pathogenic intronic mutation in neurofibromatosis type 1 (NF1) due to intronic sequence exonization.** Hum Mut. 2006 Mar;27(3):294-5.

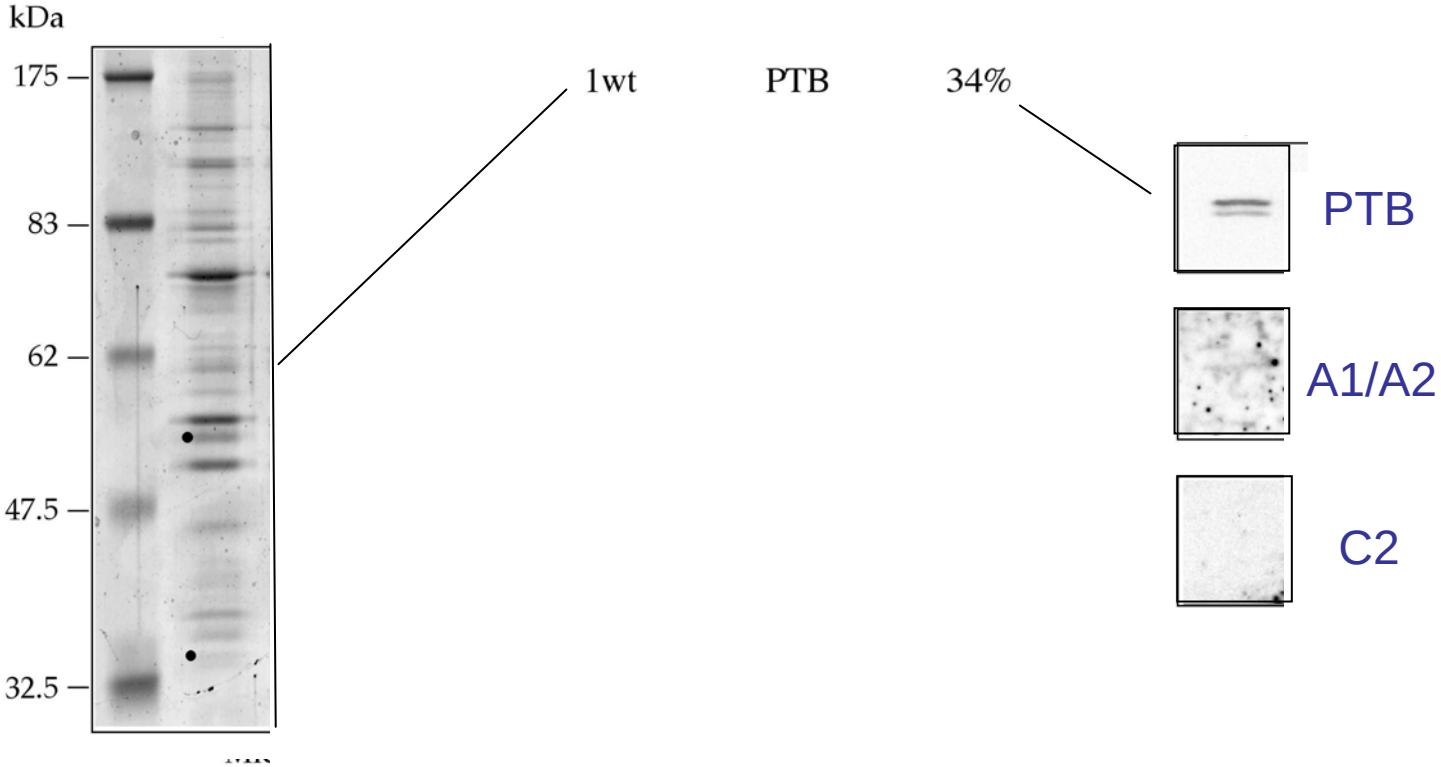
Point mutations in mutant sequence rescue the inclusion of the cryptic exon



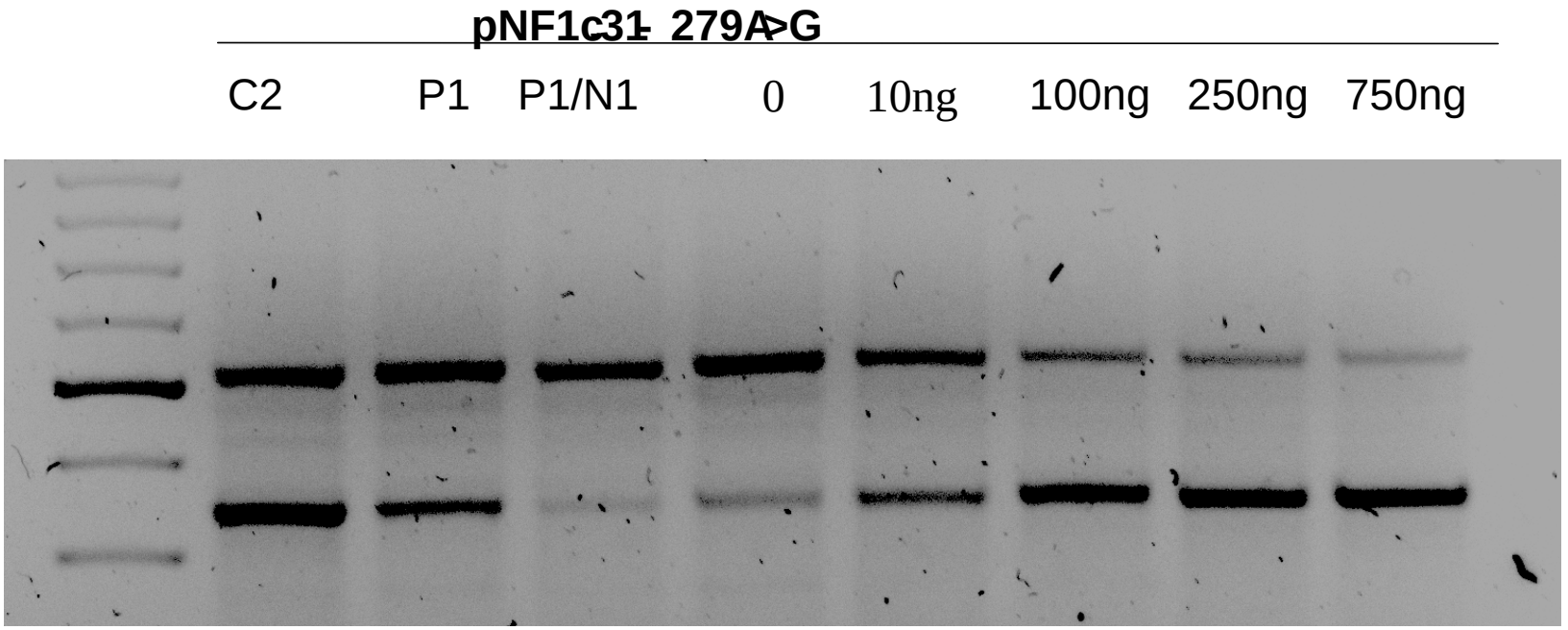
Pull down

Sequence analysis by mass spectrometry

Western against PTB, hnRNPA1 and hnRNPC2



RNA i



•PTB is preventing exonizatin of these seqs. Is this function more widespread than we think?

• In this case detected only because of the mutation observed in this patient.

How frequent?

Table 2 | **Missense and silent mutations associated with altered splicing**

Gene	Mutation	Exon	Ref.
Missense mutations			
ADA	A215T	7	116
ATM	E2032K	44	49
ATP7A	G1302R	4	117
BRCA1	E1694K	18	78
CFTR	G58E	9	118
	D565G	12	119
F8	R1997W	19	120
FAH	Q279R*	9	121
FBN2	D1114H‡	25	122
FECH	A155P‡	4	123
HEXB	P404L	11	124
HMBS	E29L‡	3	125
HPRT1	G40V	2	73
	R48H	3	73
	A161E	6	73
	G180E	8	73
	G180V	8	73
	E182K	8	73
	P184L	8	73
	D194Y	8	73
	E197K	8	73
	E197V	8	73
	D201V	8	73
IL2RG	R285Q‡	6	126
IVD	R21C	2	127
	R21P	2	127
	D20N	2	127
MAPT	N279K§	10	89
	S305N*§	10	89
MLH1	R659P	17	128
	R659L	17	128
PDHA1	A175T	6	129
PMM2	E139K	5	130
RHAG	G380V	9	131
Silent mutations			
APC	R623R	14	132
AR	S888S	8	133
ATM	S706S‡	16	49
	S1135S‡	26	49
CYP27A1	G112G	2	134
FAH	N232N	8	135
FBN1	I2118I	51	69
HEXA	L187L‡	5	136
HMBS	R28R	3	137
HPRT1	F199F	8	73
ITGB3	T420T	9	138
LIPA	Q277Q‡	8	139
MAPT	L284L§	10	89
	N296N§	10	89
	S305S‡§	10	89
MLH1	S577S‡	16	140
NF1	K354K	8	141
PAH	V399V	11	142
PDHA1	G185G‡	6	143
PKLR	A423A	9	144
PTPRC	P48P	4	145
PTS	E81E‡	4	146
RET	I647I	11	147
SMN1	F280F	7	84
TNFRSF5	T136T	5	148
UROD	E314E	9	149

Nature Review Genetics 2002

How frequent?

- Extent of splicing mutations has been underestimated.
- 50% for some genes, eg NF1, ATM

- Baralle *et al* 2005 J. Medical Genetics
- Buratti *et al* 2006 Nucleic Acids Research. Defective splicing, disease and therapy: searching for master checkpoints in exon definition.

Wessex pilot study

- National genetics reference lab, salisbury, wessex regional genetics lab. and EURASNET

- Which genes? How many? RNA, DNA assays? Which techniques?