EURASNET Workshop on RNA splicing and genetic diagnosis, London, UK

Medium-Chain Acyl-CoA Dehydrogenase (MCAD) splicing mutations identified in newborns with an abnormal MS/MS profile

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MCAD deficiency:

1. **M**edium-**C**hain **A**cyl-CoA **D**ehydrogenase (MCAD) deficiency is a potentially fatal defect in the mitochondrial fatty acid oxidation

2. Disease manifestation can be reduced/avoided by fasting and diet - Good outcome if patient is diagnosed before clinical presentation

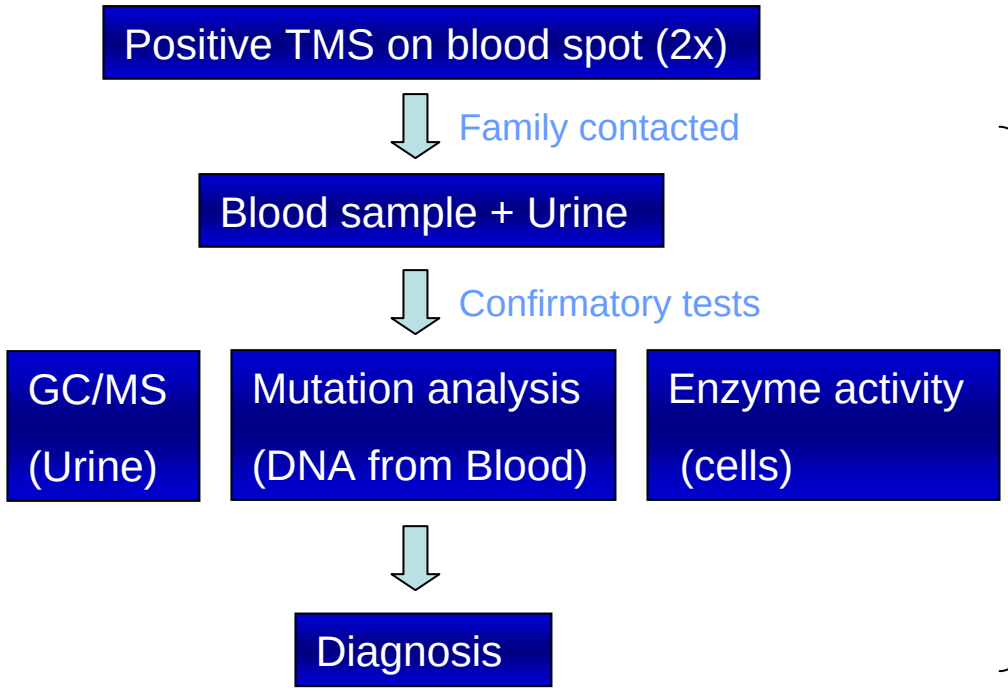
3. Can be detected by Tandem MS analysis for acylcarnitines in blood spots

Therefore: Included in many Tandem MS based newborn screening programs worldwide – Routine screening in UK since March 2007
More than 600.000 newborns screened pr. year.

We perform mutation analysis for many programs worldwide

Why mutation analysis?

Traditional diagnostic procedure:



Collection of new samples

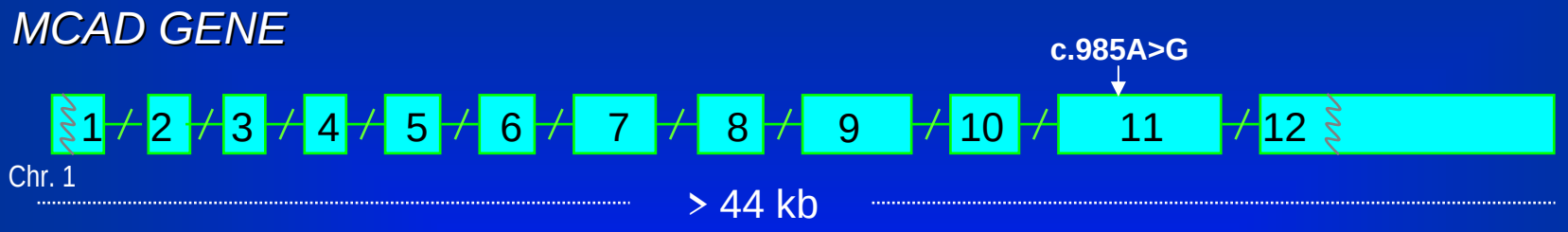
Borderline cases

Overlapping excretion patterns

DNA analysis on original blood spot is fast !!

It is crucial to distinguish between normal variations and deleterious variations. Consequently, knowledge about the functional consequences of detected mutations is fundamental for correct diagnosis

MCAD deficiency – Mutation spectrum



Prevalent mutation: c.985A>G / K304E - Carrier frequency 1/40-1/100

Clinically presenting patients:

Northern European descent:	**80%** Homozygous	**18%** Heterozygous	**2%** No c.985A>G
Denmark:	**74%** Homozygous	**16%** Heterozygous	**10%** No c.985A>G

Newborn screening:

UK:	**55%** Homozygous	**21%** Heterozygous	**24%** No c.985A>G
Denmark	**42%** Homozygous	**35%** Heterozygous	**23%** No c.985A>G

>100 different mutations known

Mutations in newborns not homozygous for c.985A>G

	UK: (>1 mill newborns screened)	Total:
Newborns with other genotypes:	69	>350
Different mutations:	35	>100
Splicing mutations:	10	29
Splicesite	7	15
Silent	2	3
Missense	1	3

Splicing mutations make up a significant proportion of total

How to evaluate:

1. **Absent in 100 chromosomes (Ethnicity? Rare variants?)**

2. **Bioinformatics (Splice site strength - ESE/ESS prediction)**

3. **Patient cells (Confirms defect – Linked or causative?)**

4. **Minigenes / *In vitro* RNA techniques (Independent of patient genetic background - Artificial effects)**

5. **Database of evaluated mutations**

How to evaluate:

Two examples:

1. **Splice site mutation (IVS10-6T>G)**

   Most prevalent splice mutation in UK

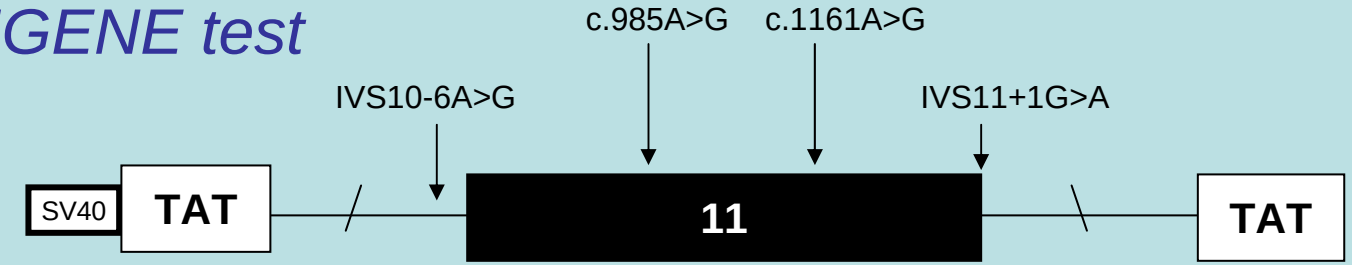
   8 newborns originating from Pakistan/India

   Phenotype????

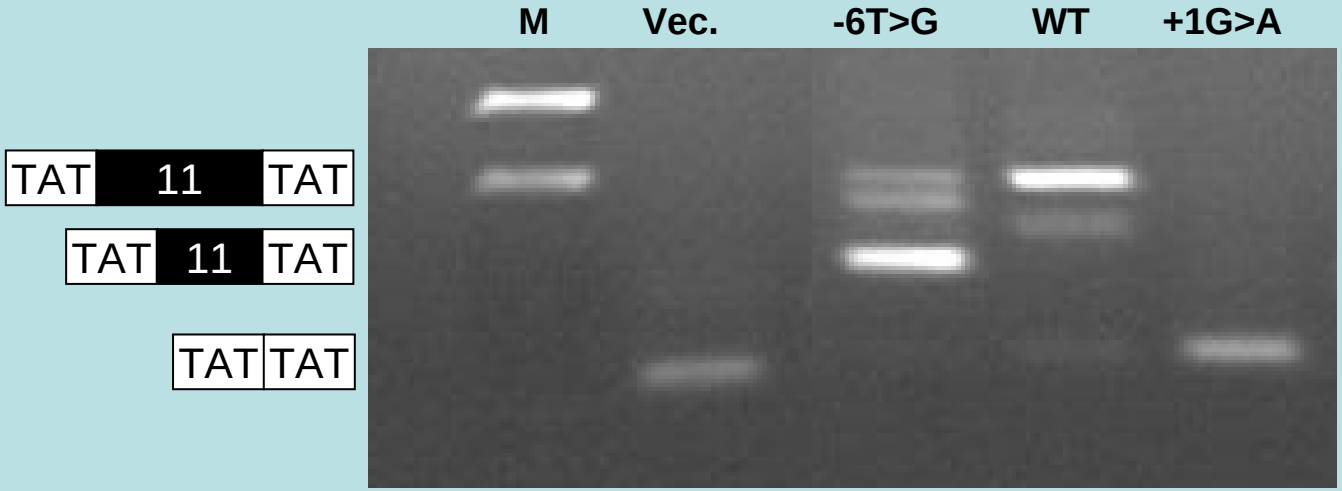
2. **Silent mutation in exon 2 (c.87A>G)**

Exon 11 missplicing – Caused by IVS10-6T>G

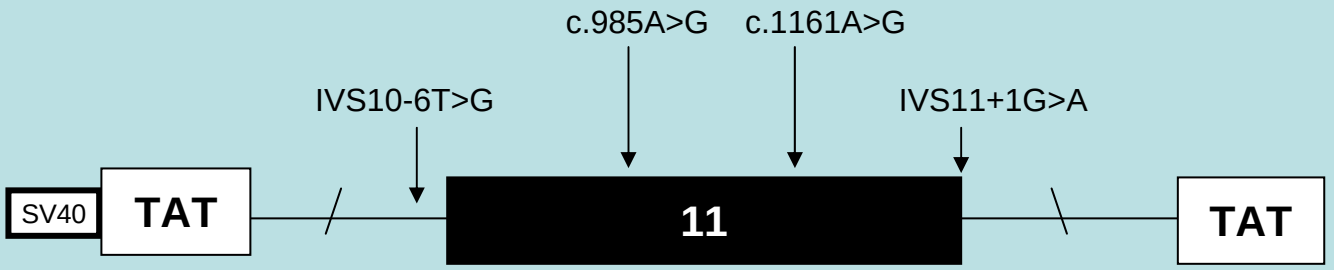
MINIGENE test



		Score			Score
WT	:tttaattct**ag**CAC	87	**WT**:	TCAG/gtaagg…	86
-6A>G:	ttta***ag***tct**ag**CAC	83 83	**+1G>A**:	TCAG/***a***taagg…	--



Exon 11 missplicing – Caused by IVS10-6T>G



		Score			Score
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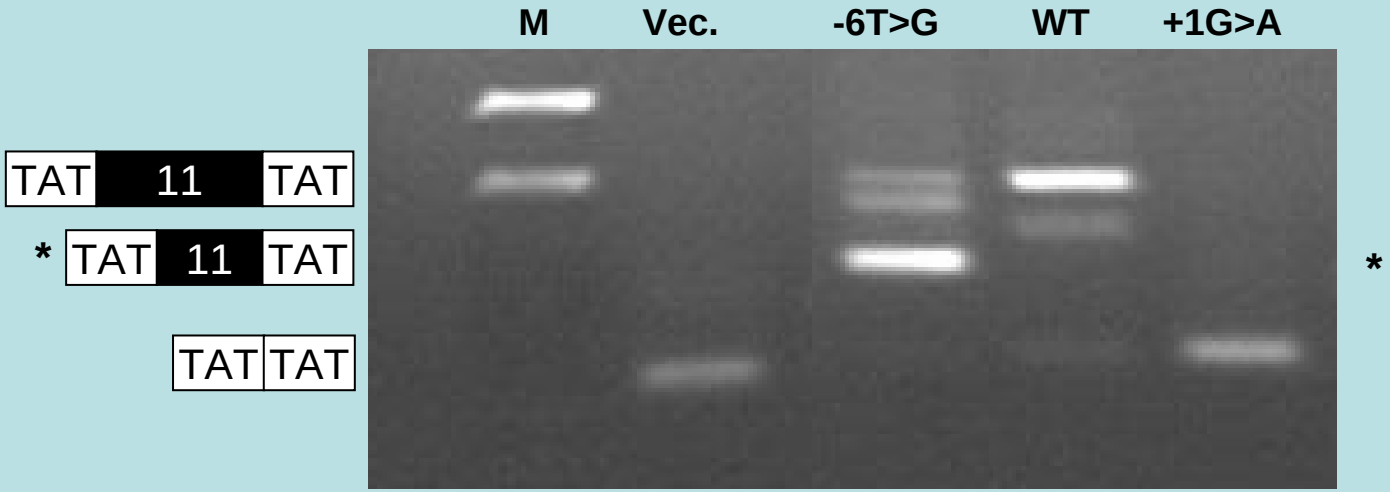
-6A>G:	ttta**ag**tct**ag**CAC	83 83	**+1G>A**:	TCAG/**a**taagg…	--
***Pseudo site** :	ctattgcaaagGCA	77			



How to evaluate:

Splice site mutation (IVS10-6T>G):

Prediction: New pseudosite and old acceptor equal score

Expect: Some normal splicing and some with 5 bp ins (new pseudo splice site) .

5bp ins -> FS -> premature stop codon -> NMD

Instead: New site with low score activated – No use of new site

No significance here as both scenarios results in loss of function

BUT: Other genes

A c.87A>G synonymous mutation in MCAD Exon 2 in two unrelated newborns

1. Two unrelated newborns with a positive tandem MS screening

2. Sequence analysis of the entire MCAD gene showed:

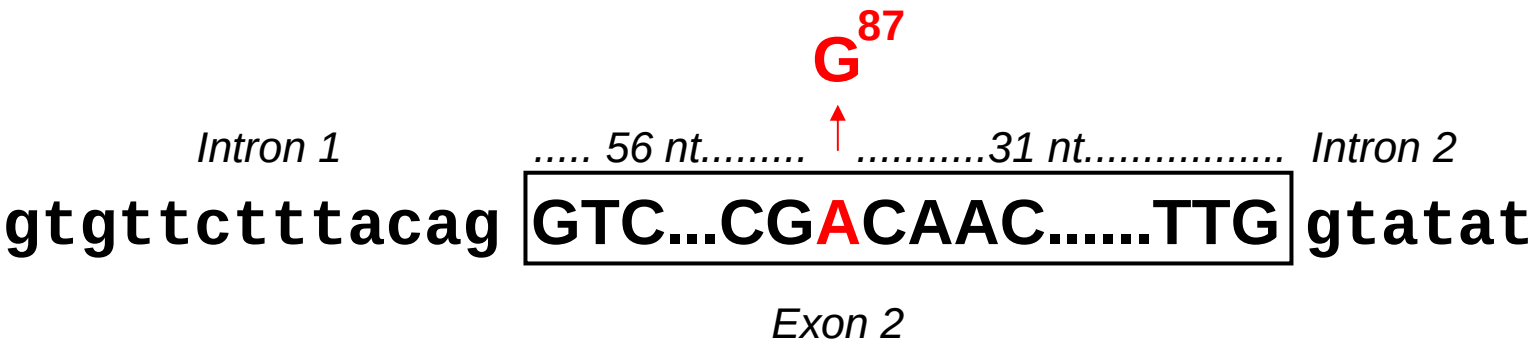
   Heterozygosity for c.985A>G (Prevalent – K304E)
   Heterozygosity for c.87A>G (CGA>CGG (Arg^4 > Arg^4) (Silent))

3. Analysis of parental DNA showed that the mutations are located in separate alleles

4. c.87A>G is not present in controls (>100 chromosomes).

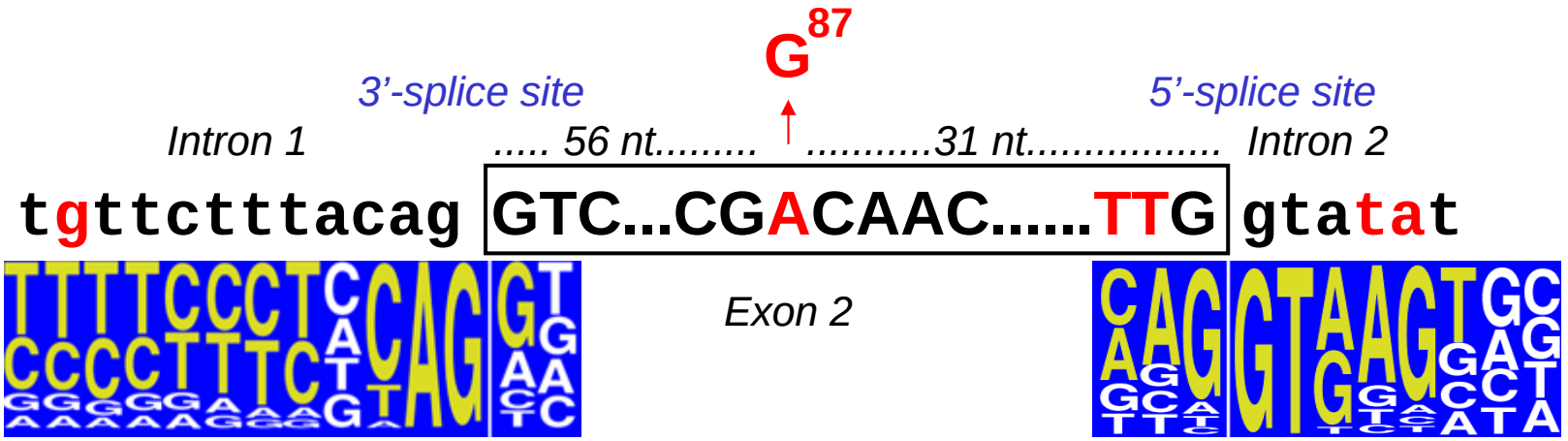
Is the c.87A>G mutation deleterious despite being a silent mutation? Splicing??

Does the c.87A>G mutation affect splicing?



1. c.87A>G is located in the middle of exon 2 – It does not affect splice sites directly, nor does it create a pseudo-splice site.

Does the c.87A>G mutation affect splicing?



1. c.87A>G is located in the middle of exon 2 – It does not affect splice sites directly, nor does it create a pseudosplice site.

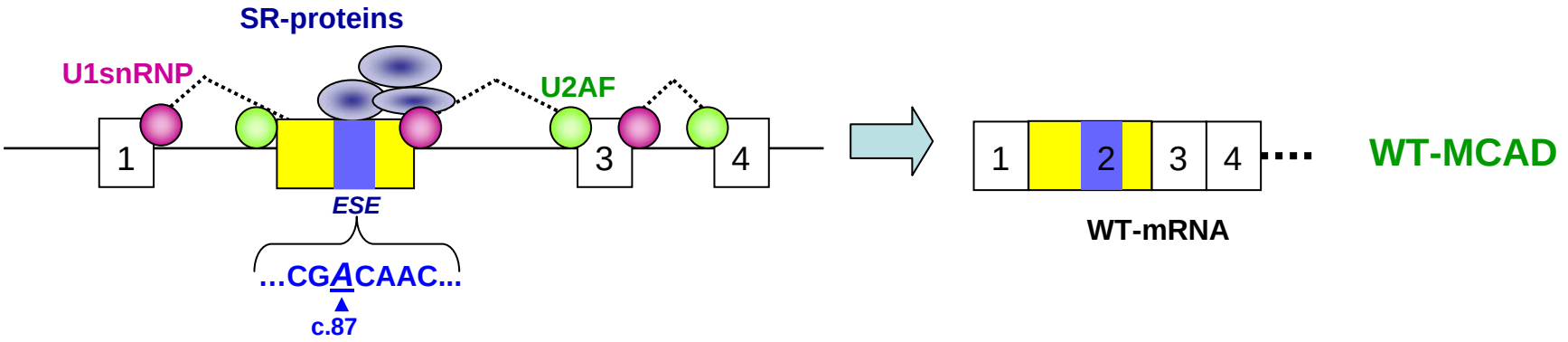
2. Exon 2 5'splice site is weak, with a score of only 1.76 (Threshold for functional sites is 6.67).

Exon 2 has a very weak 5' splice site, which is hard to recognize for splicing factors such as U1snRNP

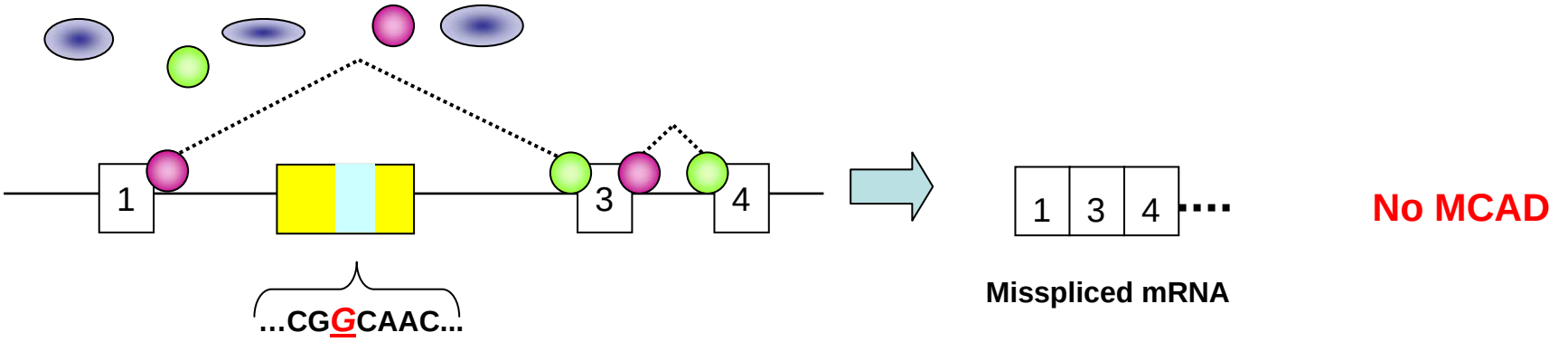
Does the c.87A>G mutation disrupt a splicing regulatory element which is necessary for recognition of the weak splice site ?

Hypothesis 1: Exon Splicing Enhancer (ESE) inactivation

Splicing of exon 2 with "c.87A" :

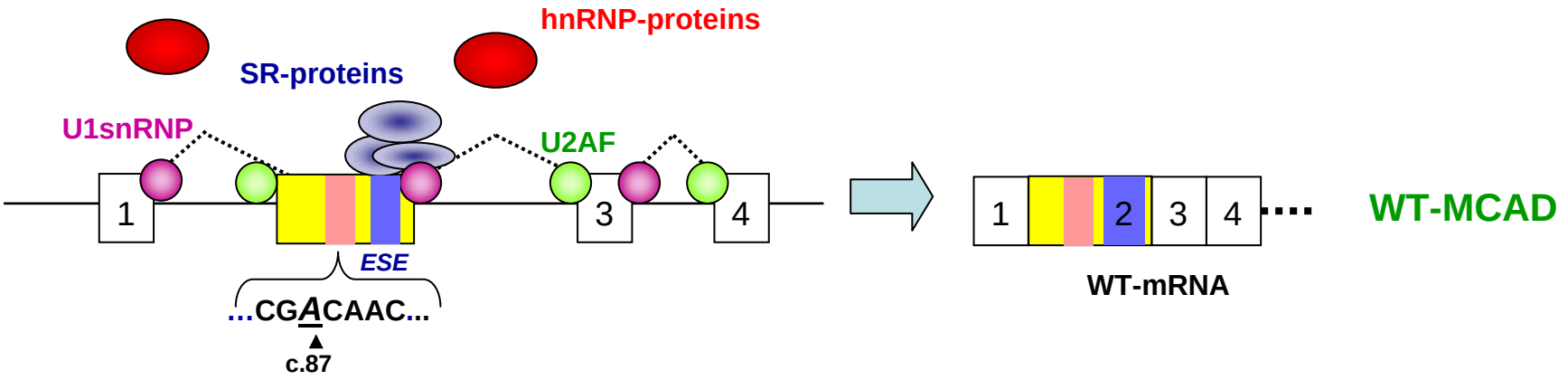


Splicing of exon 2 with "c.87A>G" :

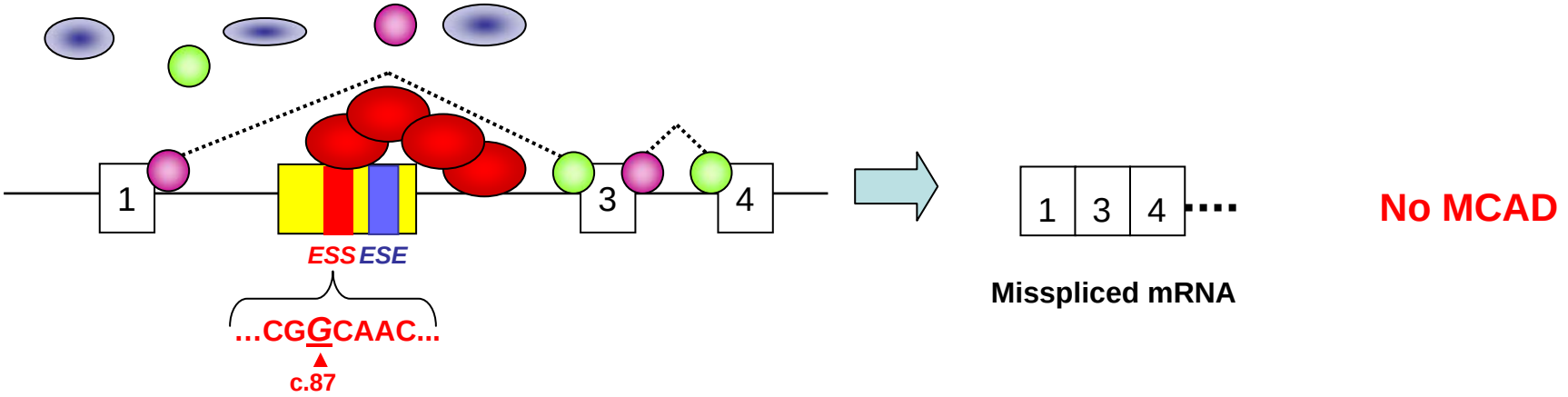


Hypothesis 2: Exon Splicing Silencer (ESS) activation

Splicing of exon 2 with "c.87A" :



Splicing of exon 2 with "c.87A>G" :



In silico analysis of MCAD exon 2: c.87A>G and c.85C>T

RESCUE ESE and FASS ESS analysis:

			RESCUE ESE:
	WT (85C-87A)	GCCAATCGACAACGT	**ACAACG ESE motif**
(“Control”)	**85C>T (85T-87A)**	GCCAATTGACAACGT	**ACAACG ESE motif**
	87C>G (85C-87G)	GCCAATCGGCAACGT	**No ESE motif**

No inhibitory motifs predicted by *FASS-ESS* prediction

This may suggest that the c.87A>G mutation disrupts an ESE, and that it does not create an ESS.

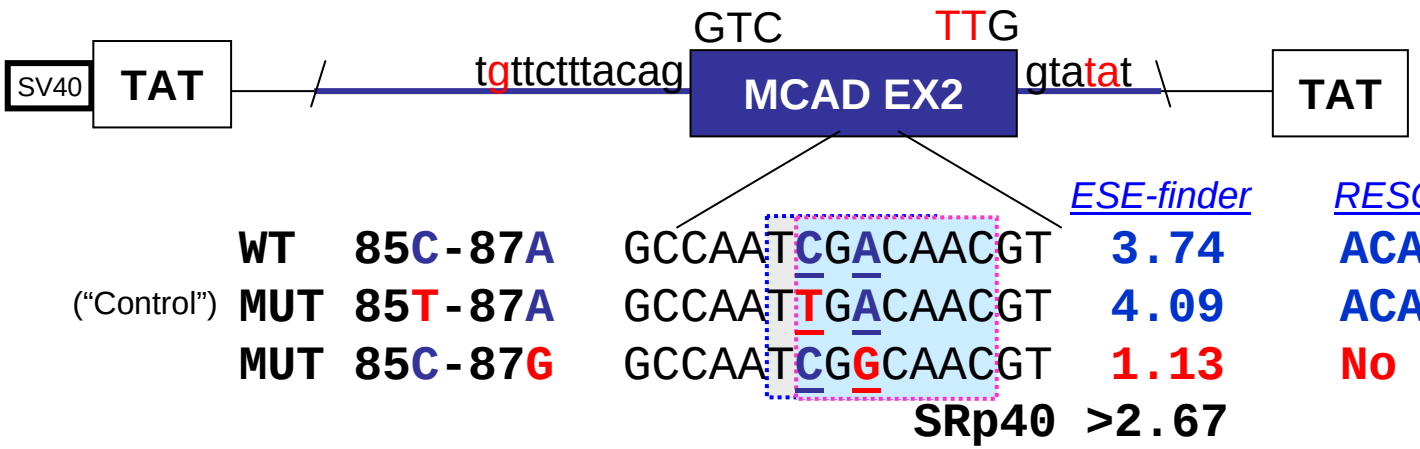
In silico analysis of MCAD exon 2: c.87A>G and c.85C>T

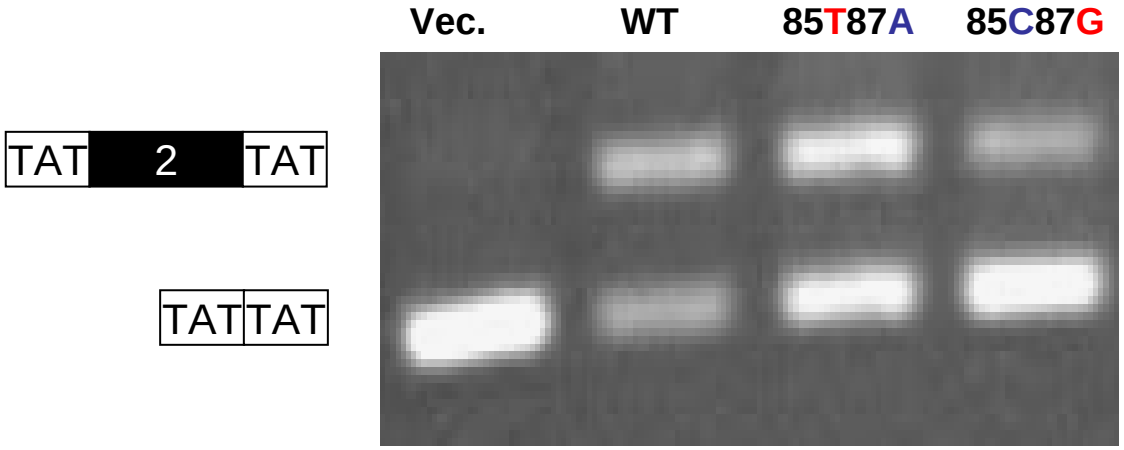
ESE finder analysis:

			ESE-finder
	WT	**(85C-87A)**	GCCAATCGACAACGT **3.74**
(“Control”)	**85C>T**	**(85T-87A)**	GCCAATTGACAACGT **4.09**
	87A>G	**(85C-87G)**	GCCAATCGGCAACGT **1.13**
			SRp40 >2.67

This may suggest that the c.87A>G mutation disrupts an SRp40 binding ESE

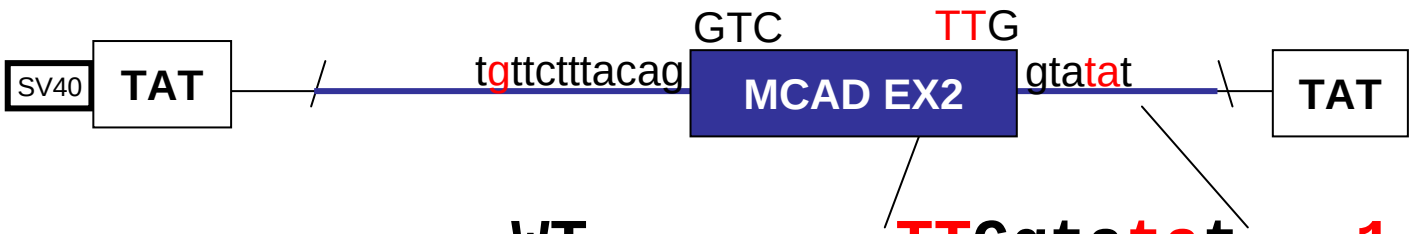
Minigene studies of MCAD exon 2: c.87A>G and c.85C>T



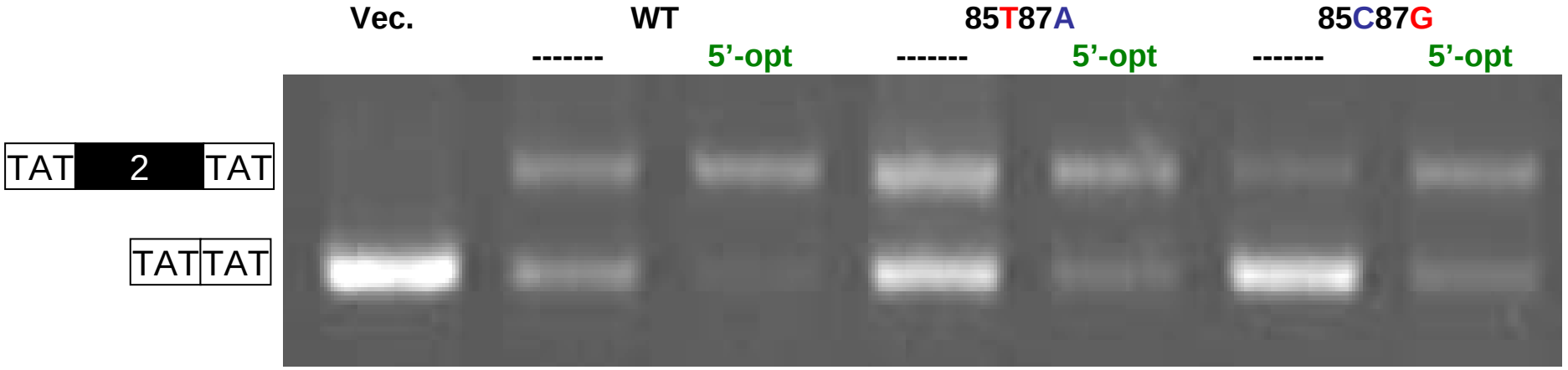


This shows that wild type exon 2 is difficult to splice. It confirms that c.87A>G causes missplicing and is consistent with disruption of an SRp40 binding ESE

Optimization of 5’-splice site of MCAD exon 2:



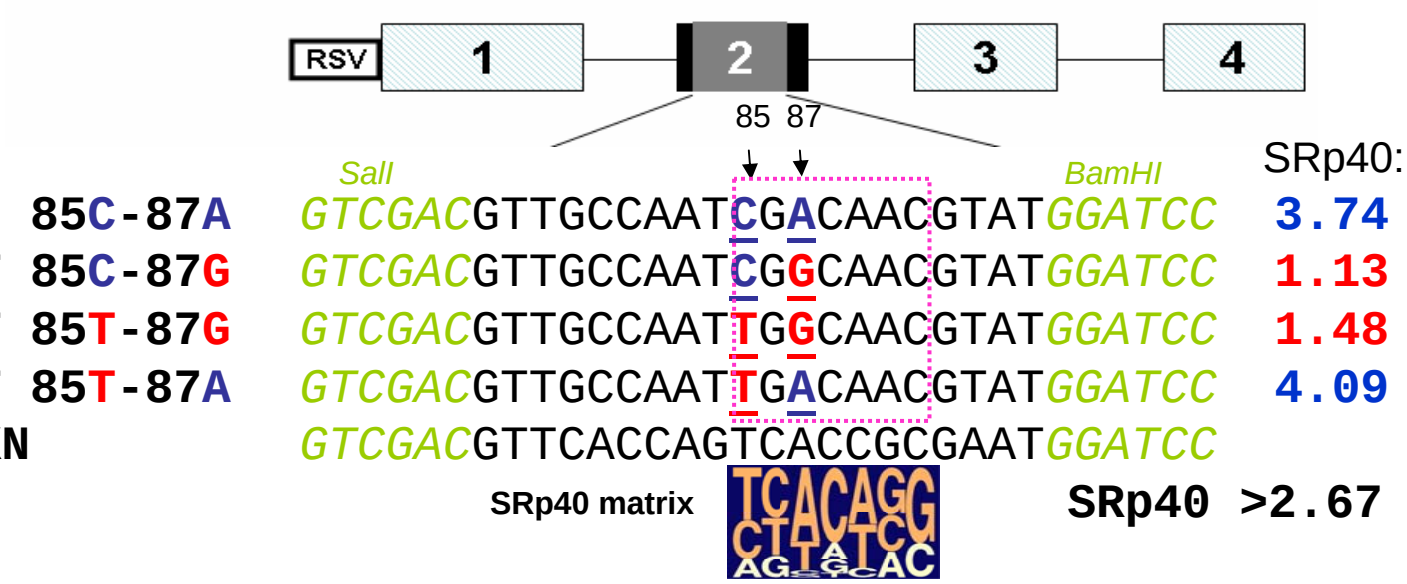
WT	TTGgtatat	1.76
5’-Opt	CAGgtaagt	12.39
Threshold		>6.67



This shows that exon 2 is difficult to splice, mainly due to the weak 5’-splice site. It suggests that the putative ESE is needed for recognition of this weak 5’ splice site.

pSXN Minigene studies of the putative ESE

1. Testing of target sequence in a heterologous context like the pSXN reporter can confirm if it can function as an ESE
2. Distinguish between ESE disruption and ESS creation by the c.87A>G mutation
3. Possible to explore the consensus for an ESE



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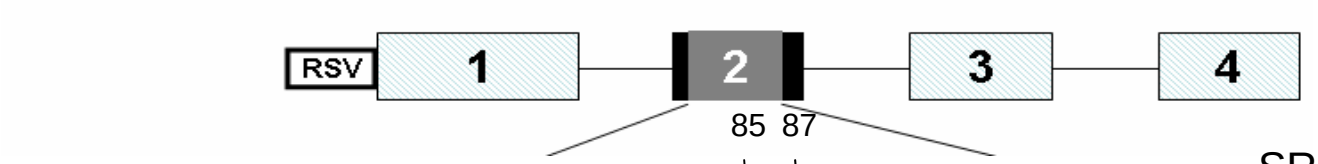
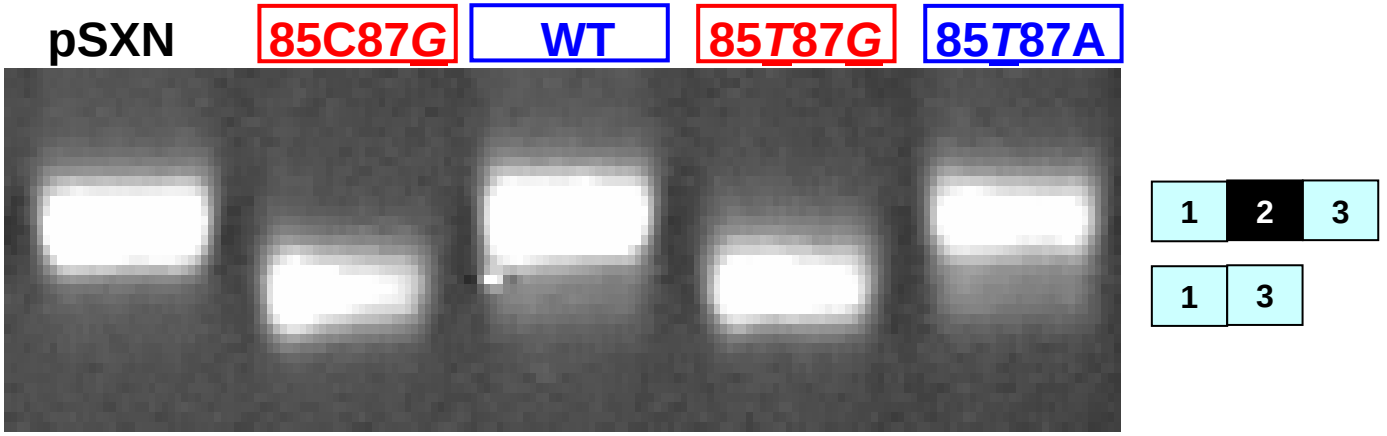


			SRp40:
		SalI ... BamHI	
WT	85C-87A	GTCGACGTTGCCAATCGACAACGTATGGATCC	3.74
MUT	85C-87G	GTCGACGTTGCCAATCGGCAACGTATGGATCC	1.13
MUT	85T-87G	GTCGACGTTGCCAATTGGCAACGTATGGATCC	1.48
MUT	85T-87A	GTCGACGTTGCCAATTGACAACGTATGGATCC	4.09
pSXN		GTCGACGTTCACCAGTCACCGCGAATGGATCC	

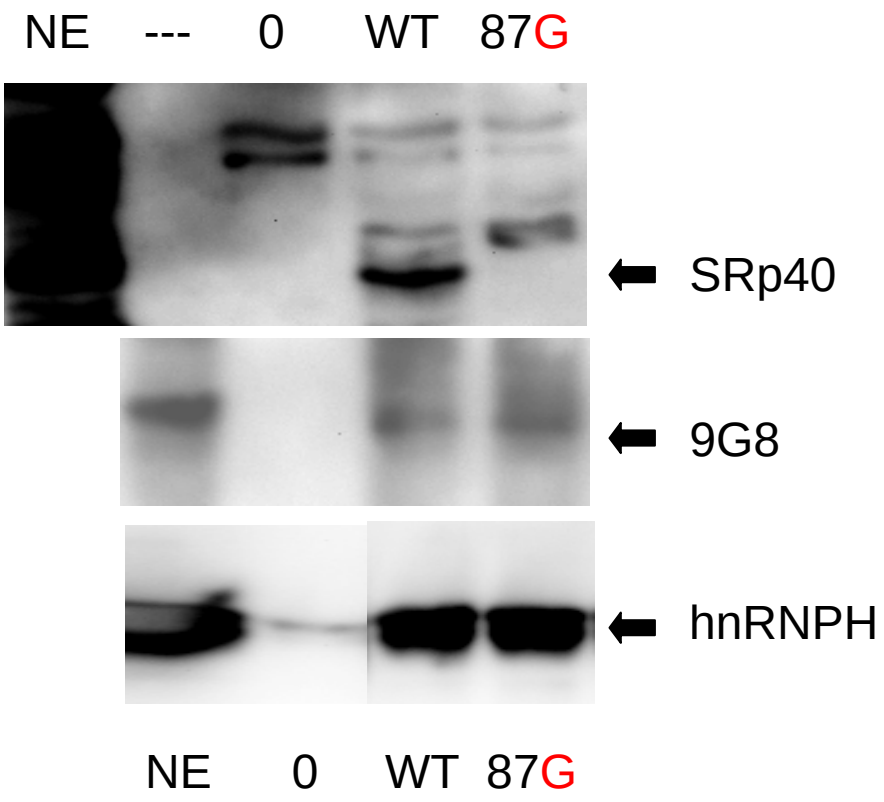
SRp40 >2.67



This confirms that the c.87A>G mutation disrupts an ESE, with a consensus sequence like the SRp40 binding motif

RNA-affinity purification

Western blot analysis of affinity purified proteins:



The c.87A>G mutation disrupts binding of SRp40

c.87A>G causes ESE inactivation by disruption of SRp40 binding

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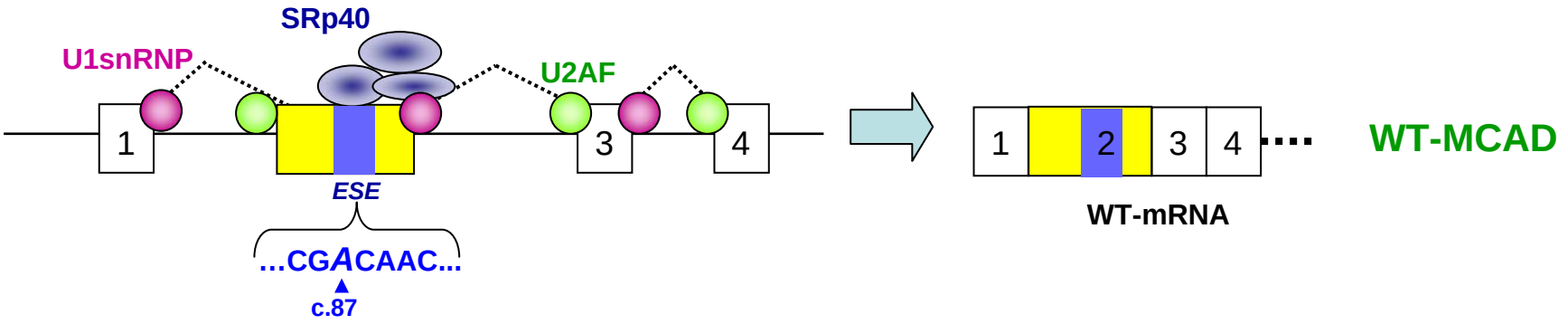

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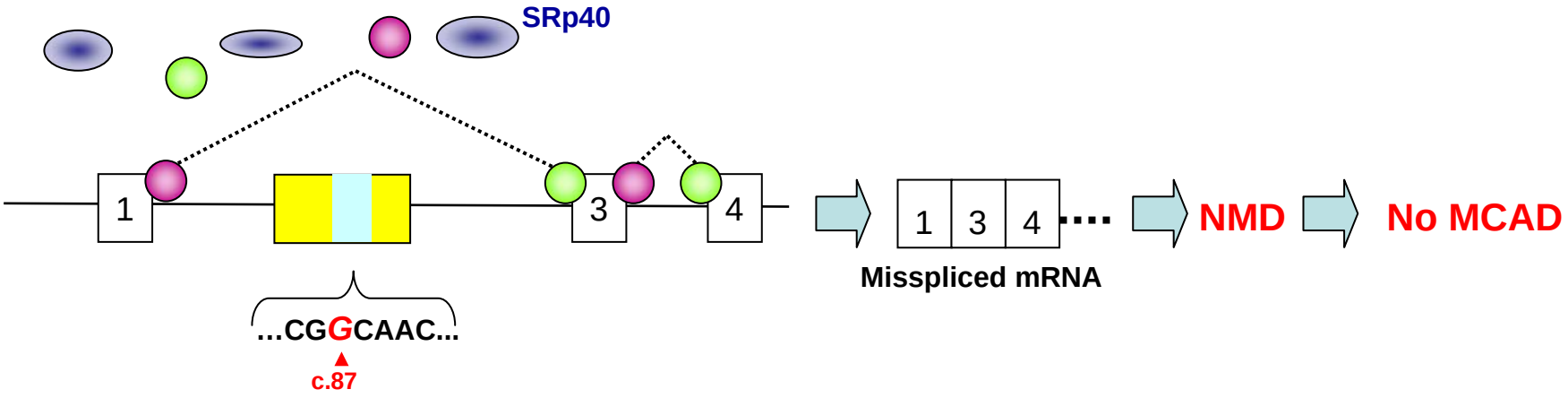


Confirmation of the splicing defect in patient cells

Cells from 2 controls, the patient and her father (Both heterozygous for c.87A>G) were cultured with and without cyclohexamide (Blocks NMD).

EXON 2 skipping

↓

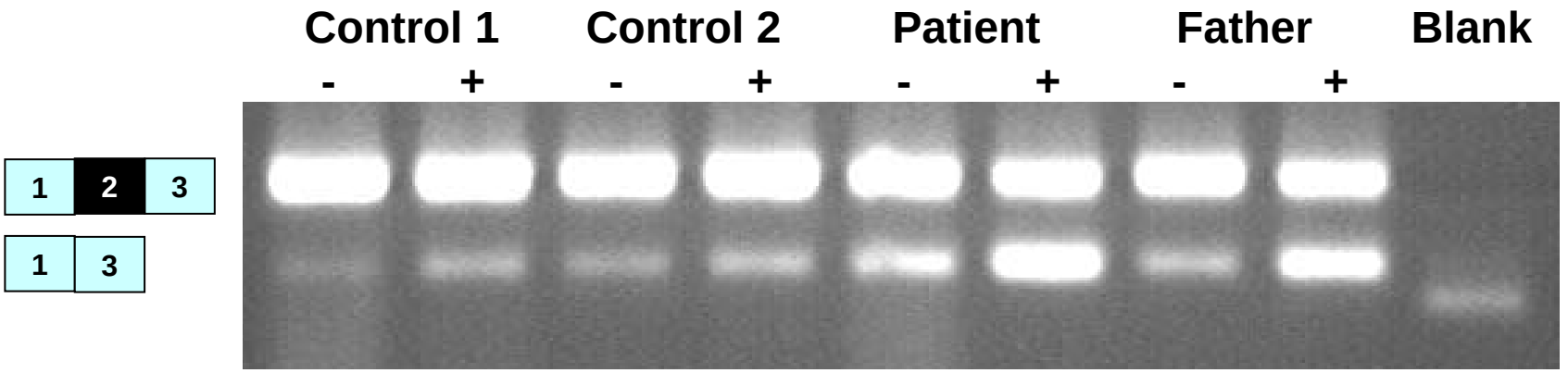
Shifted reading frame

↓

PTC (TAA stop) at c.230-32 in exon 4

↓

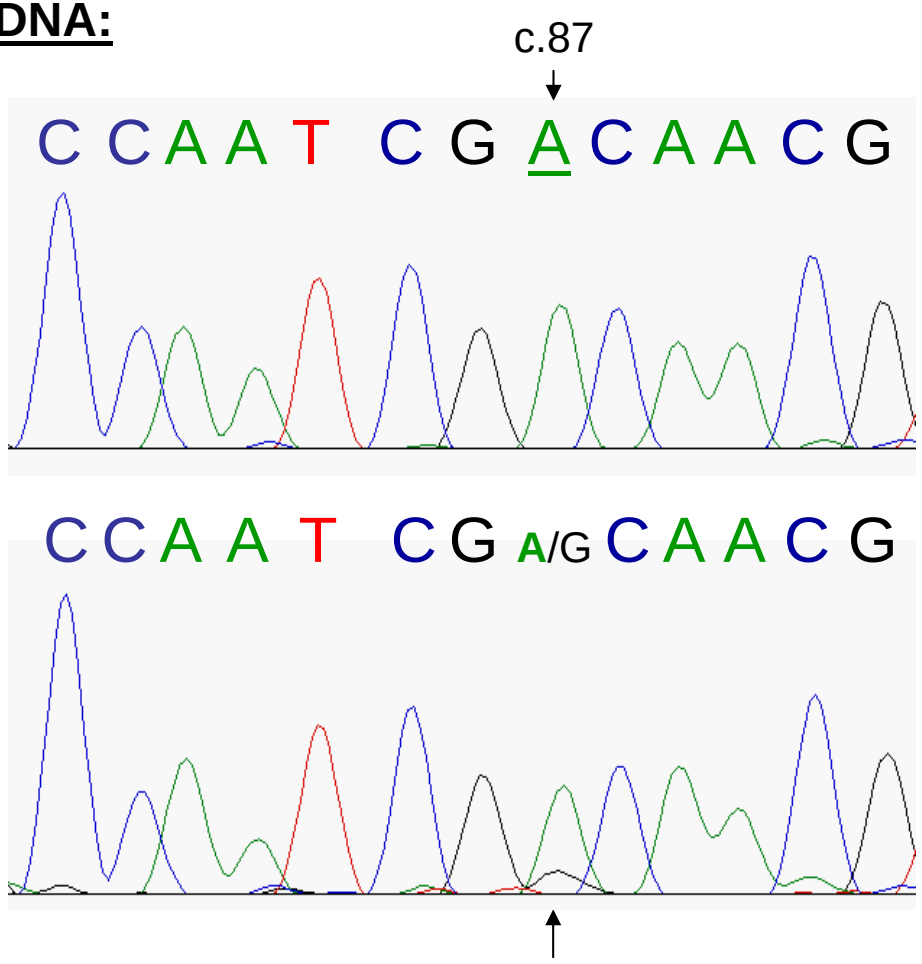
NMD (Degradation)



This confirms that the c.87A>G mutation causes exon 2 skipping and NMD in patient cells.

Confirmation of the splicing defect in patient cells

Sequence analysis of patient cDNA:



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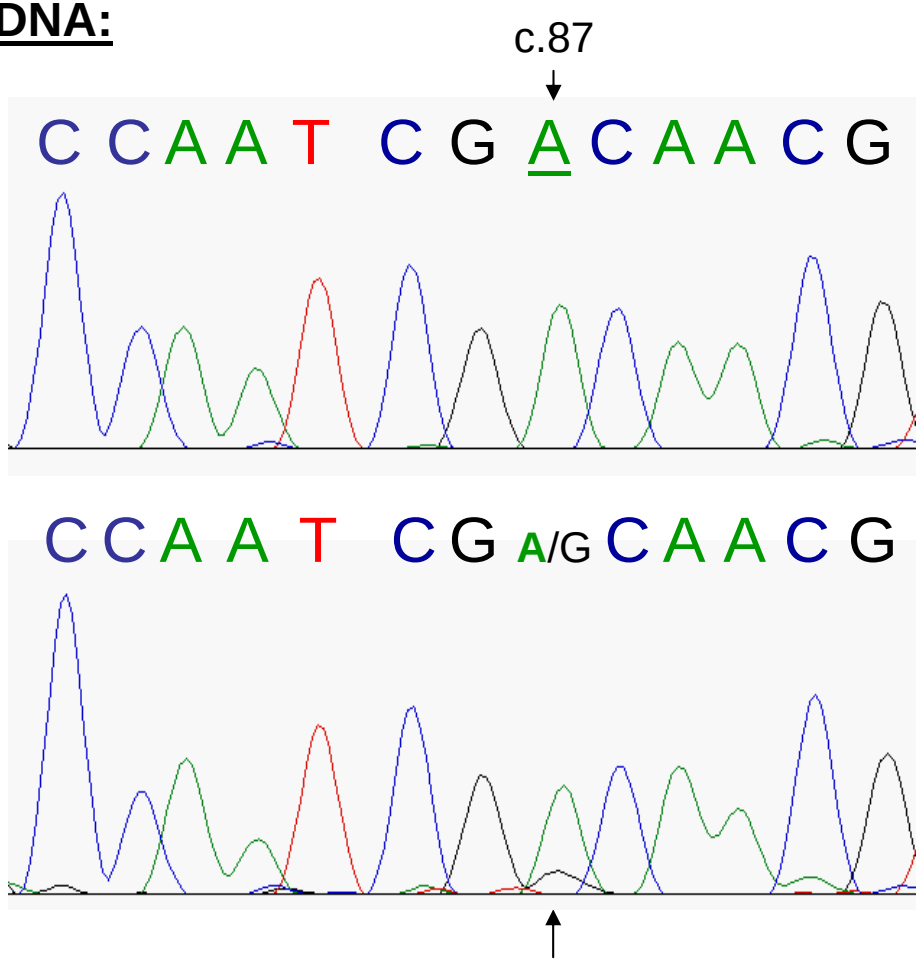

Amounts from c.87G allele are dramatically reduced in full length (exon 2 inclusion) product

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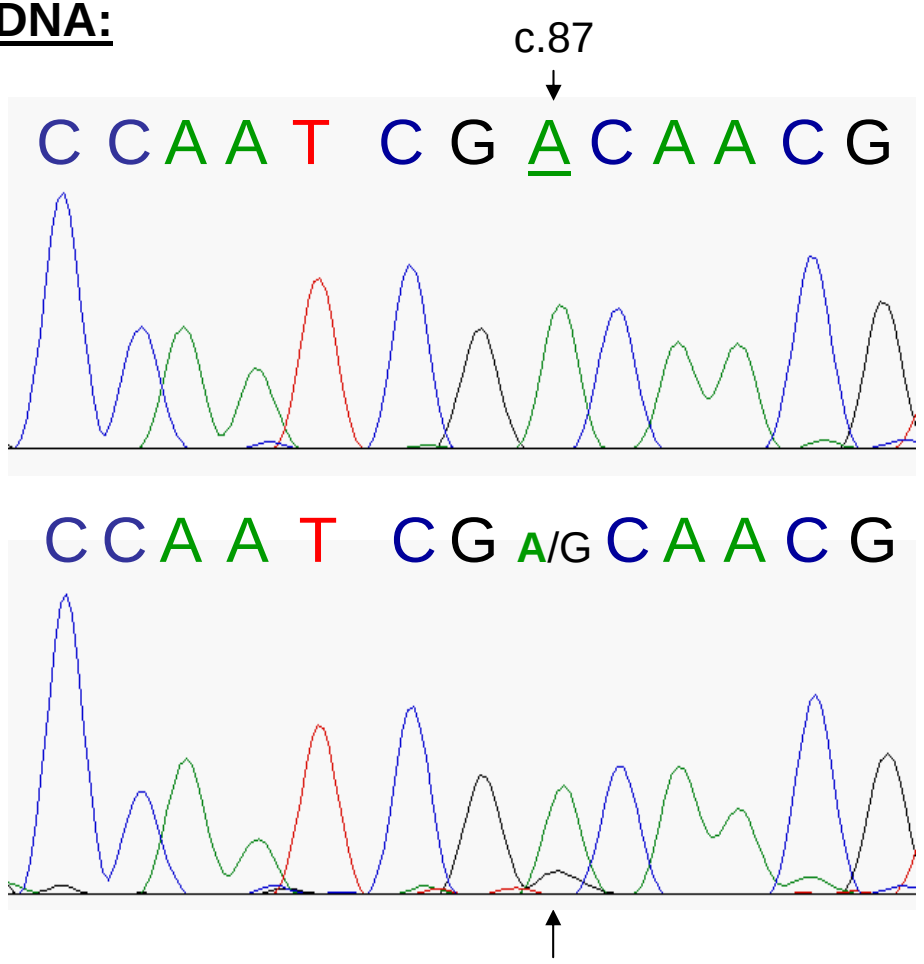

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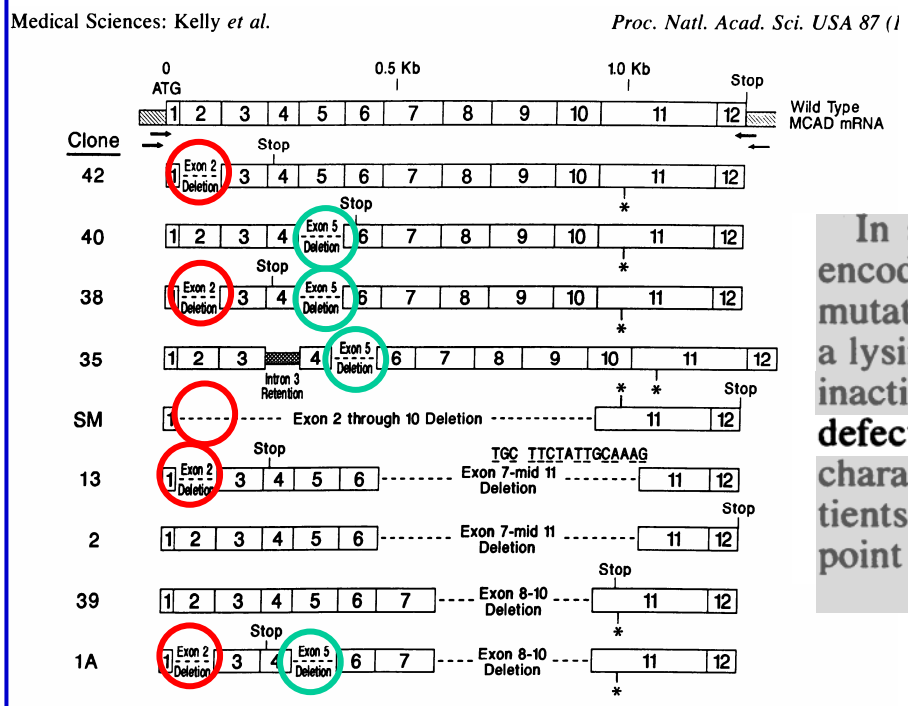
→ **This confirms that the c.87A>G mutation causes exon 2 skipping and NMD in patient cells.**

Unexplained mis-splicing in MCAD patients homozygous for c.985A>G - A mystery for more than 15 years



Kelly DP et al. (1990) *PNAS* 87:9236-9240:

In summary, we have shown that the gene and mRNA encoding the defective MCAD in this family contain a point mutation that results in the substitution of a glutamic acid for a lysine and most likely results in an unstable, catalytically inactive protein. **In addition, the patient has an unusual defect in the splicing of the MCAD pre-mRNA.** Molecular characterization of additional unrelated MCAD-deficient patients will allow us to determine the frequency of the G^{985} point mutation.

Gregersen, Andresen et al. (1991) *Hum Genet* 86(6):545-51:

"*.. It seems that the amount of cDNA containing deletions is higher in the patient than in normal persons. …… The deletions of exons 2, 5 and 8 can only be explained by mis-splicing…….This secondary pathophysiological phenomenon might seriously contribute to the course of life-threatening attacks".*

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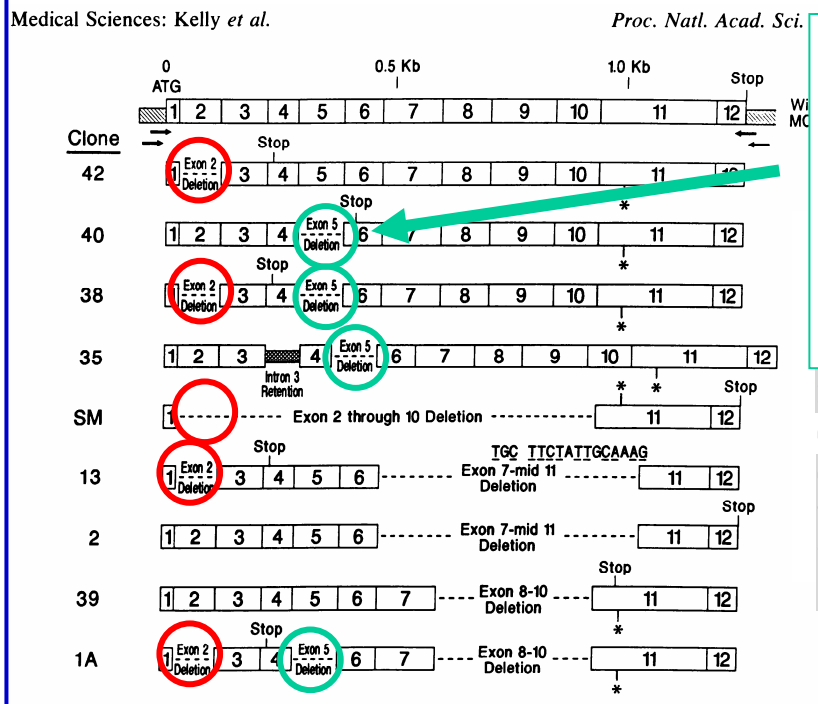

Nielsen et al. (2007) *Am J. Hum Genet* 80:416-32

ESE in Exon 5 needed for correct splicing

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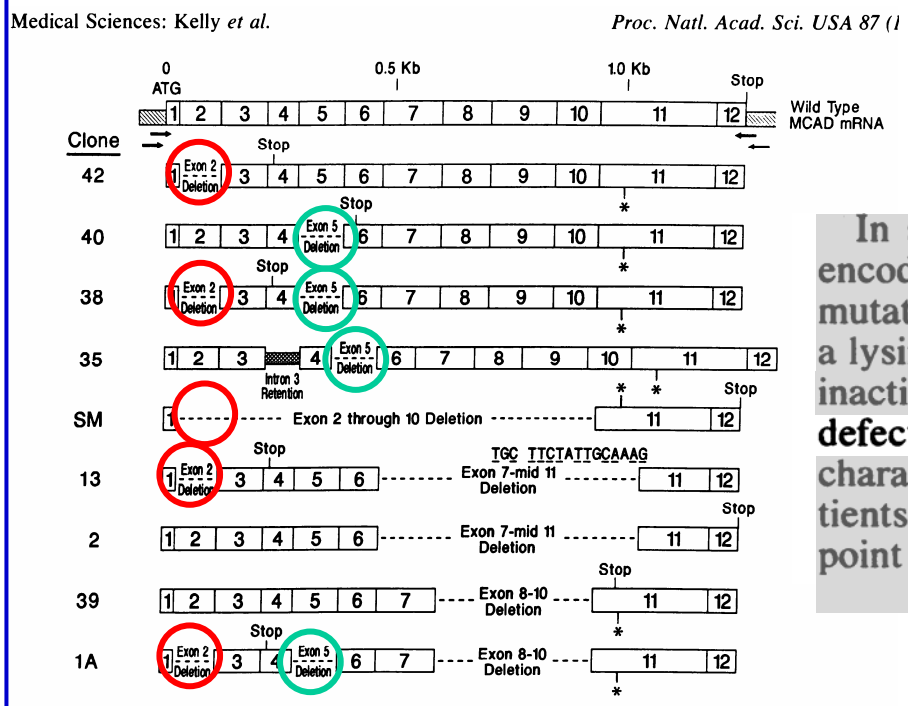

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Exon 2 and 5 are vulnerable and dependent on ESEs – Increased skipping of exon 2 and 5 in patients with other mutations

Conclusions

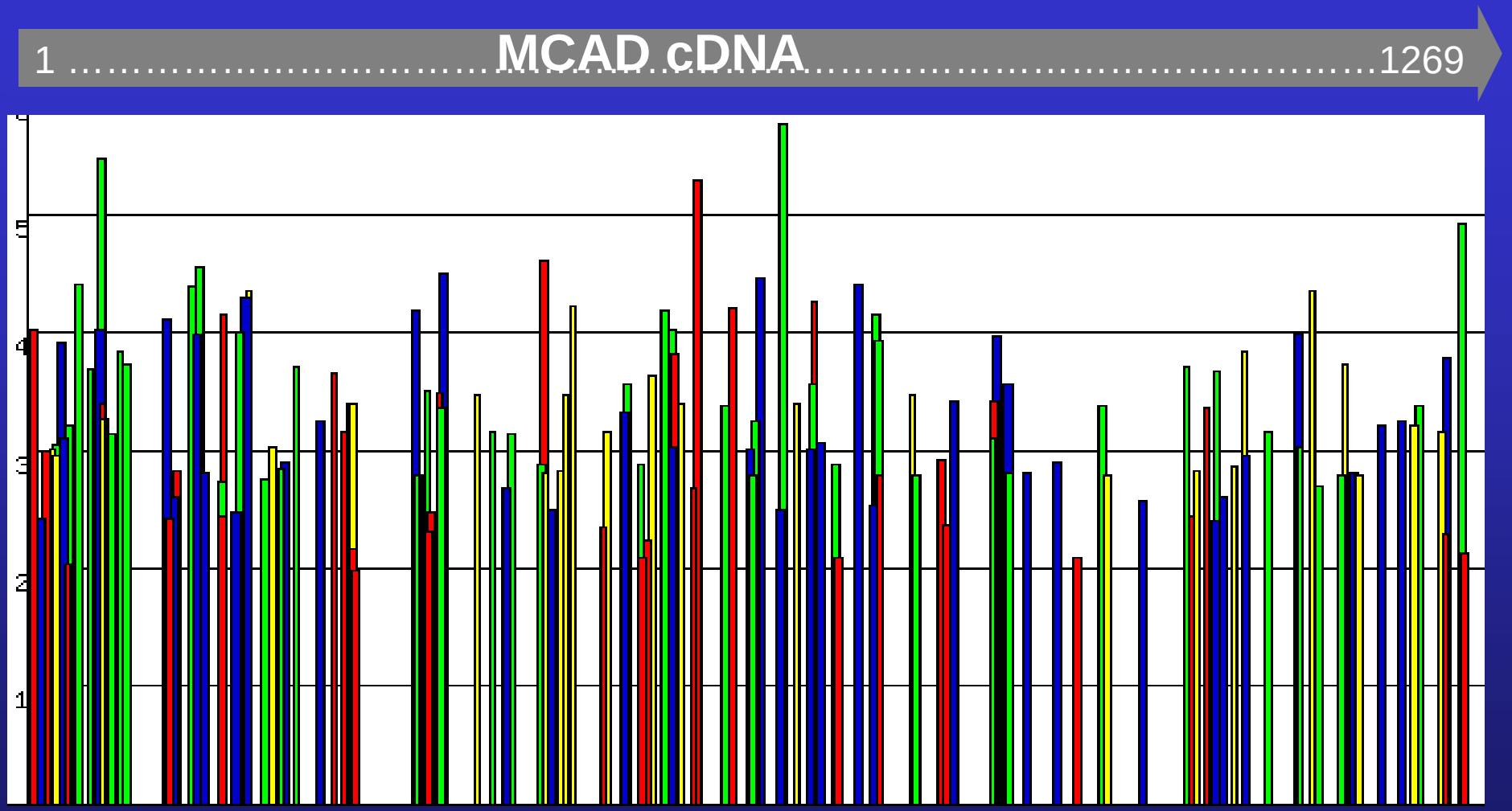
1. Splicing mutations are common
   Total 25-30% of these as many as 5-10% are silent/missense mutations

2. Prediction programs are pretty good, but functional testing and data collection vital for future

3. Effect is highly dependent on context (weak splice sites – flanking ESS/ESE etc.).

4. All genes have vulnerable exons, which may be misspliced if mutations hit exon splicing regulatory elements or compromise splice sites

SEARCH BY *ESE FINDER*



SF2/ASF
SRp40
SC35
SRp55

93 mutations in exons
42 mutations either create/abolish high score motifs
24 (26%) destroys high score motifs

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ACKNOWLEDGMENTS:

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Anne Vested Jensen

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Niels Gregersen

Lisbeth D. Schroeder

Karsten Bork Nielsen

Pia P Madsen

Birthe Gahrn

MUTATION ANALYSIS OF UK NEWBORNS PART OF THE UK COLLABORATIVE STUDY

Missplicing and pathophysiology – Increased MCAD exon 2 and 5 skipping when patients are metabolically stressed?

1. SR-proteins and SRp40 activity is regulated by phosphorylation by SR-kinases
2. Insulin activates these kinases (for instance Akt-2 kinase)
3. Insulin has been shown to regulated Glucose-6-Phosphate dehydrogenase and PKCbeta through SRp40 activation (by phosphorylation).

Hypothesis:

Fasting - Metabolic stress

↓

Low Insulin

↓

Low SR-protein activity – Lack of phosphorylation

↓

Skipping of ESE dependent exons like MCAD exon 2 and 5

↓

Loss of residual enzyme activity

↓

Crisis

So far purely speculative, but it might be a contributing factor!