

Spinální svalová atrofie (SMA)

- Onemocnění s autosomálně recesivním typem dědičnosti

frekvence onemocnění: 1/6000 - 8000

frekvence přenašečů onemocnění: 1/50 (1/36)

- Druhá nejčastější smrtelná porucha s autosomálně recesivním typem dědičnosti (1. cystická fibróza).
- Charakterizována degradací alfa-motorických neuronů míchy a atrofií svalů.



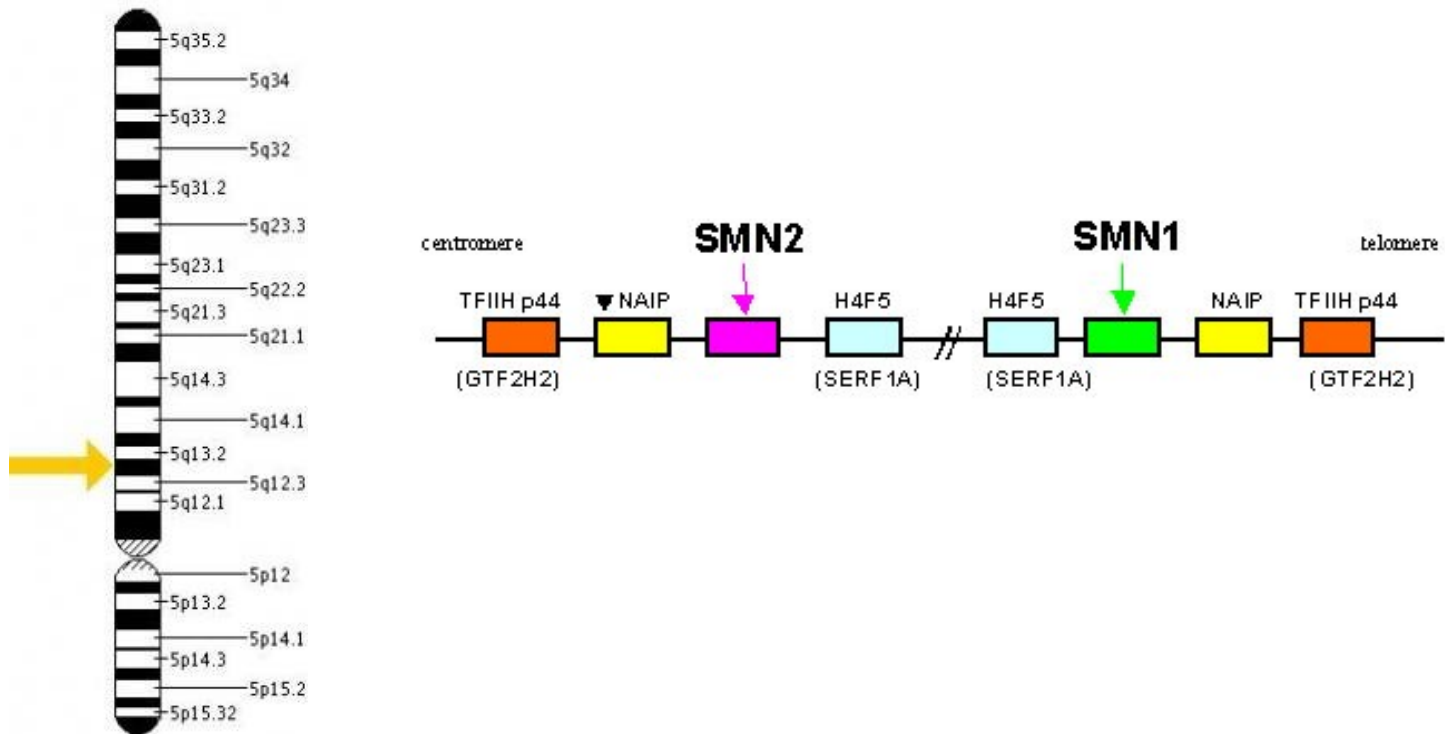
SMA is subdivided into 4 clinical groups on the basis of age of onset and clinical course.

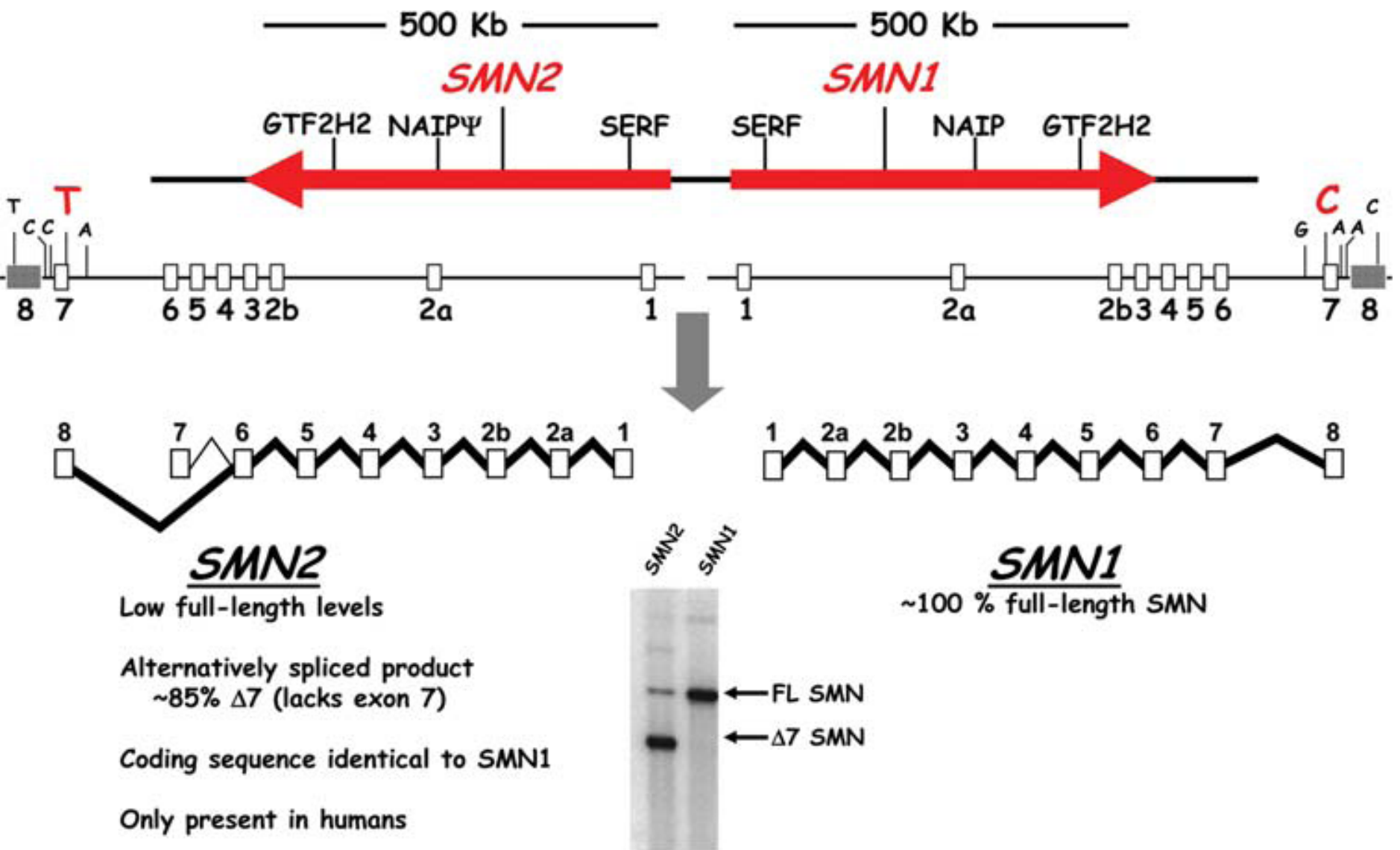
- **Type I SMA (Werdnig-Hoffmann)**, characterized by severe, generalized muscle weakness and hypotonia at birth or within the first three months. Death from respiratory failure usually occurs within the first two years.
- **Type II (intermediate form)**, with clinical manifestation starting 6–18 months after birth and life expectancy of 2–30 years; children with type II SMA are able to sit, although they cannot stand or walk unaided.
- **Type III (Kugelberg-Welander disease)**, first impacts are typically observed after the second year of life; patients often get wheelchair-bound within or after adolescence.

Two additional types are described in the literature, namely **the very severe type 0**, with prenatal onset and early neonatal death and **type IV**, a genetically heterogeneous appearance with comparatively mild consequences emerging only during adulthood.

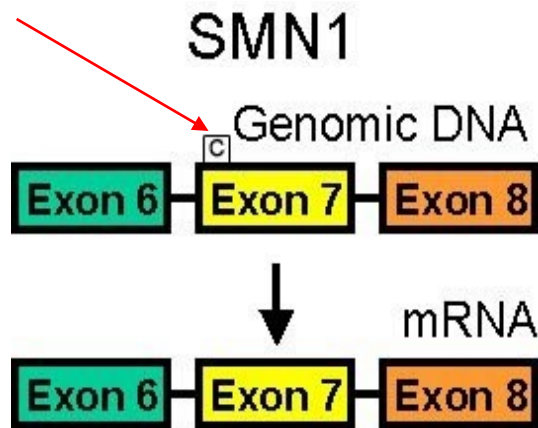


- More than 95% of all SMA cases are due to a homozygous deletion in **the SMN1 gene (Survival of Motor Neuron 1)** located on chromosome region 5q12-5q13.38. This region contains a 500-kb inverted duplication.
- **The SMN1 and SMN2 genes** differ in only a few nucleotides (none of which affect the encoded protein sequence).
- Due to gene conversion and duplication events, the number of SMN copies can vary.



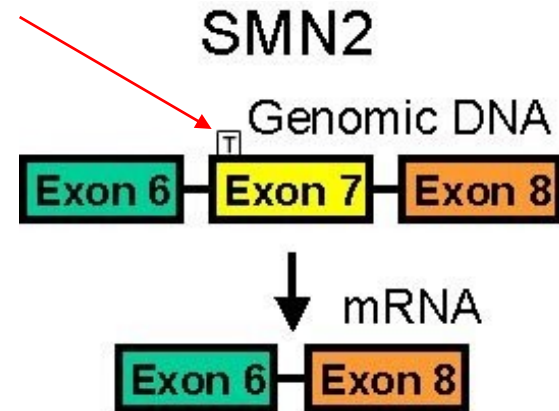


Schematic of the human SMN locus. The human SMN genes, SMN1 and SMN2, are located in close proximity on chromosome 5. The SMN2 locus is likely derived from a recent duplication event of a genomic region spanning 500 kb which contains additional genes and microsatellite markers. The SMN genes comprise nine exons and eight introns and encode an identical protein product. A silent C–T transition in exon 7 of SMN2 alters a critical exonic splice enhancer and results in a strong reduction of exon 7 inclusion during splicing. Consequently, 85% of the mature mRNA lacks exon 7 (D7), highlighted by the RT–PCR in the bottom panel. The truncated protein is defective in SMN self-association and is degraded rapidly.



SMN1: TTC (Phe)

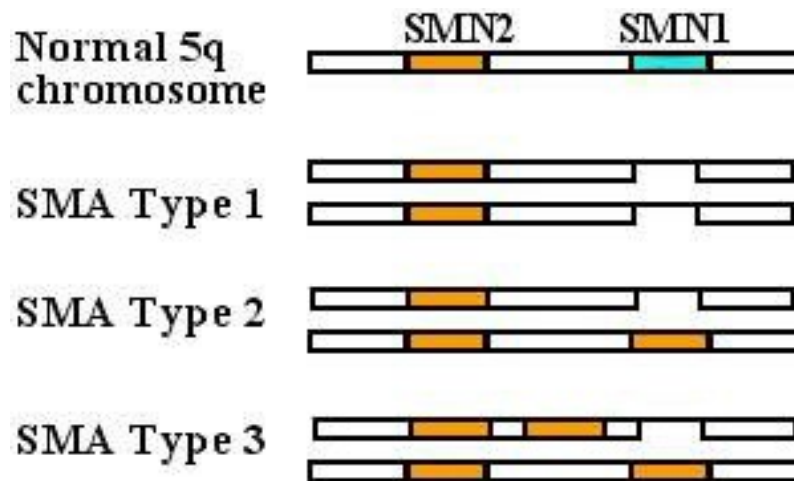
- v **95%** vzniká mRNA obsahující **všechny exony**

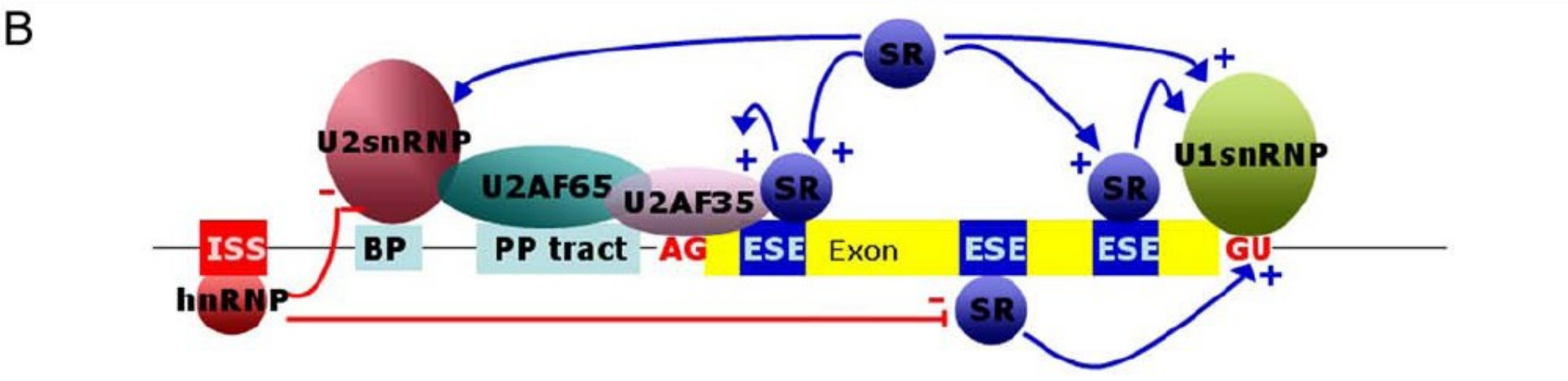
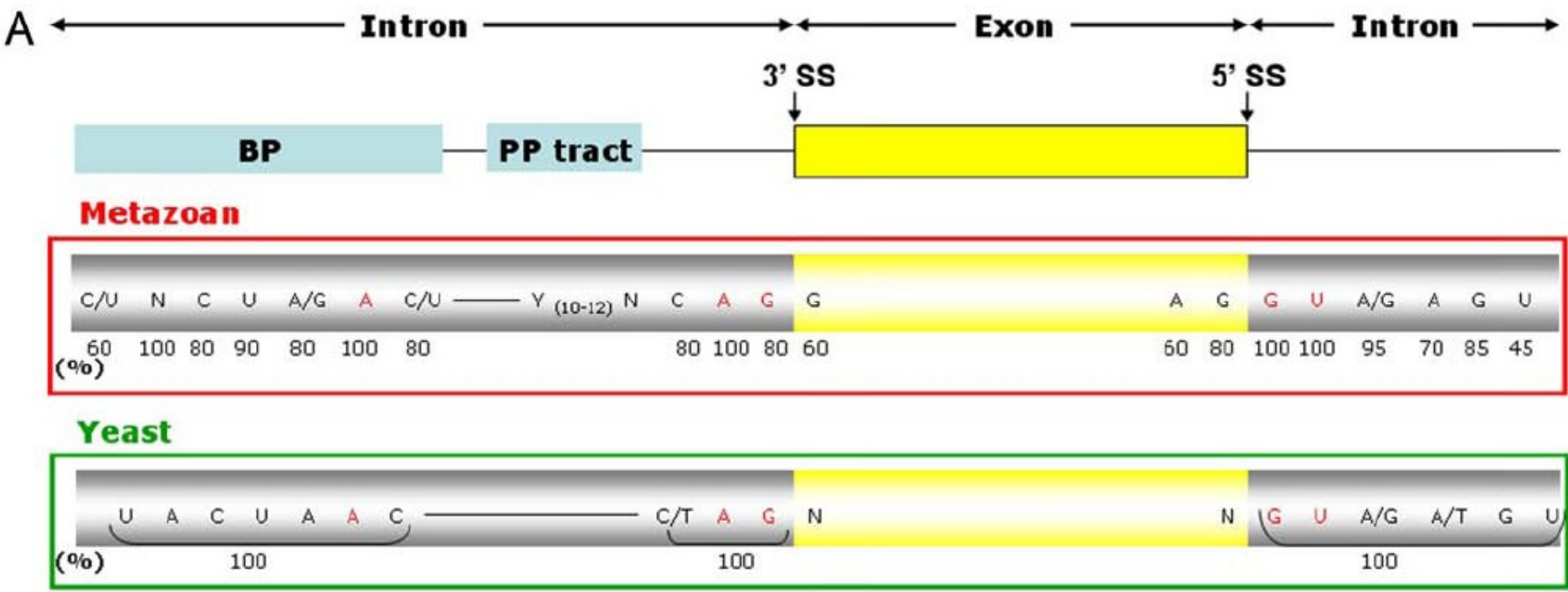


SMN2: TTT(Phe)

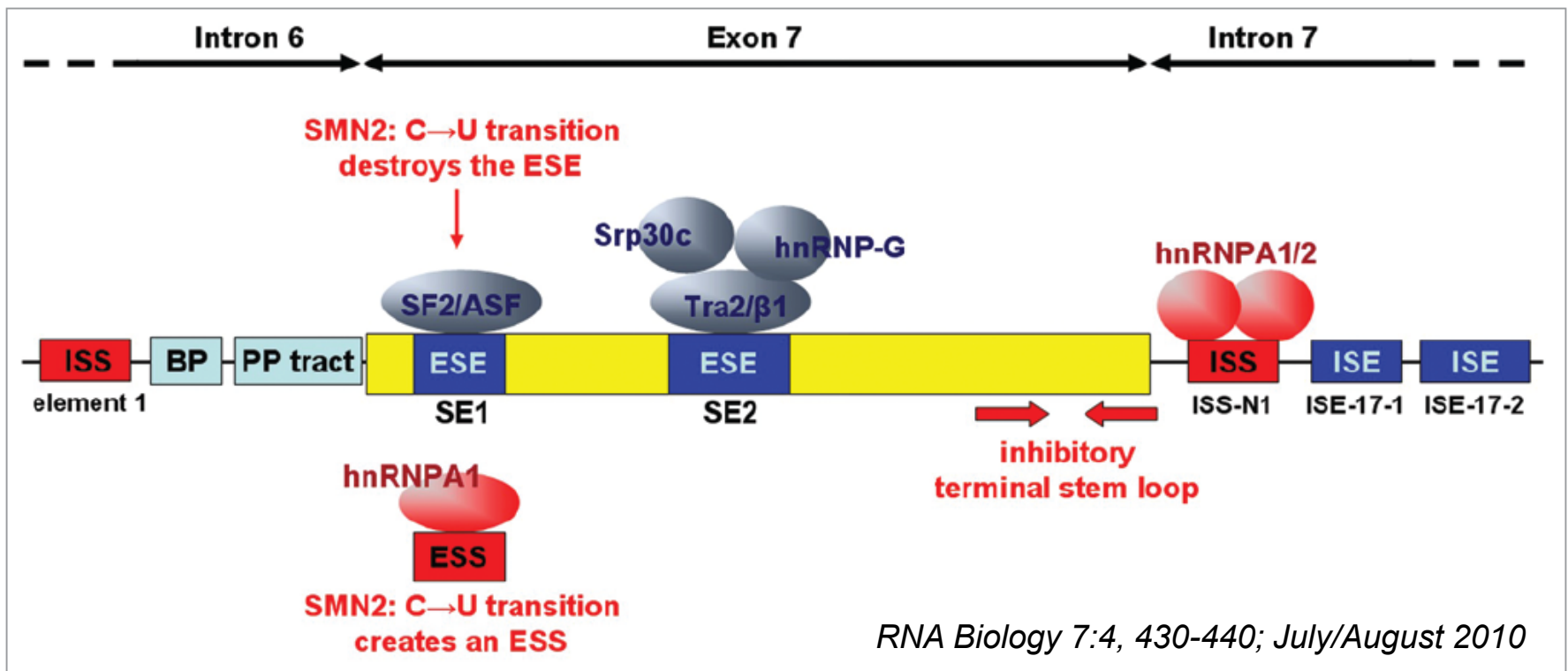
- v **85%** vzniká mRNA s **deleci exonu 7**
- v **10%** vzniká mRNA obsahující **všechny exony**

- No phenotype-genotype correlation was initially observed because **SMN1 deletion is absent in the majority of patients**, independent of the type of SMA.
- Several studies have shown that **the SMN2 copy number modifies the severity of the disease** - the SMN2 copy number varies from 0 to 3 copies in the normal population. However, patients with the milder type II or III SMA have been shown to have more copies of SMN2 than type I patients.
- The majority of patients with the severe type I form have one or two copies of SMN2; most patients with type II have three SMN2 copies; and most patients with type III have three or four SMN2 copies.

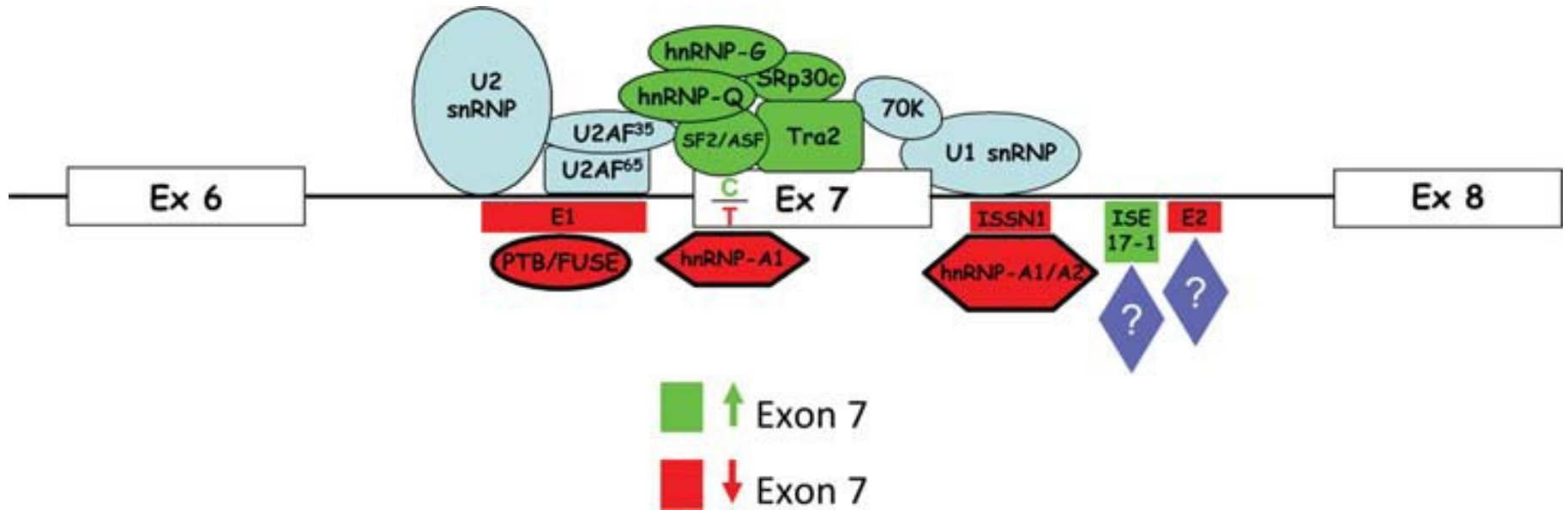




Cis acting sequence elements and trans-acting factors determining exon definition. (A) Conserved sequences flanking metazoan and yeast exons. The exon is symbolised by a yellow rectangle. The flanking introns are symbolised by lines in which the branch point (BP) sequence and polypyrimidine tract (PP tract) are highlighted as light blue boxes. The conserved nucleotides of the BP, PP tract, 3' splice site (3' SS) and 5' splice site (5' SS) are shown below with numbers indicating the percent prevalence of the most frequent nucleotides at each position. Shown in red are the branch point adenosine as well as the virtually invariant last two and first two nucleotides of the introns. (B) Role of splicing activators and repressors in splicing modulation. SR proteins bind to exonic splicing enhancers (ESE; dark blue) via their RNA recognition motifs and favour the recruitment of the splicing machinery (+) at the 5' and 3' SS, mainly by stabilising the interaction between snRNPs and the pre-mRNA. SR proteins also act by direct proteinprotein interactions with U1 snRNPs at the 5' SS, and with the U2 snRNP and U2AF co-factors at the 3' SS. In contrast, hnRNP proteins most frequently bind to intronic splicing silencers (ISS; red) and are involved in repression (-) of splicing, by exerting negative effects on U snRNPs or SR proteins. These different interactions are represented by arrows.



Splicing architecture of exon 7 of the human *SMN1* and *SMN2* genes. The diagram represents exon 7 (yellow box) and its flanking intronic regions (lines). Elements inhibiting exon 7 inclusion are shown in red, whereas the positive elements are represented in dark blue. The suboptimal branch point (BP) and polypyrimidine tract (PP tract) are indicated in light blue. SF2/ASF and Tra2/β1 bind to the exonic splicing enhancers SE1 and SE2, respectively. The recognition of SE1 by SF2/ASF is prevented in *SMN2*, due to the C → U transition. This sequence alteration also creates a hnRNP A1-dependent splicing silencer. Exon 7 is extremely short (only 54 bp).

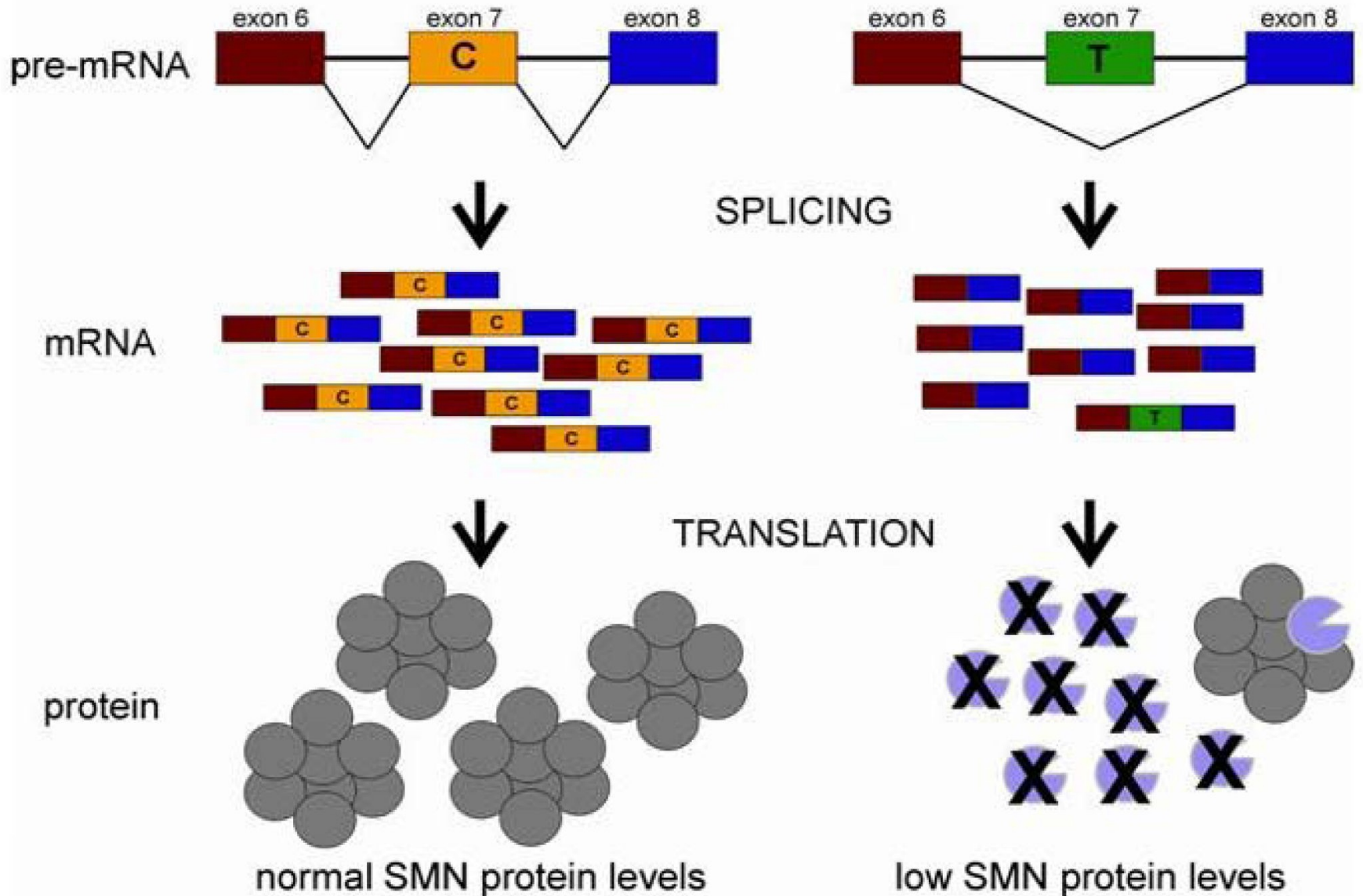


Human Molecular Genetics, 2010, Vol. 19, Review Issue 1, R111–R118

Schematic of the exon 7 region and the factors involved in the inclusion or exclusion of exon 7 within SMN pre-mRNA. Components of the machinery are shown in blue. The positively acting sequences and splicing factors are shown in green. The negatively acting sequences and splicing factors are shown in red. The C–T transition at +6 is indicated.

SMN1

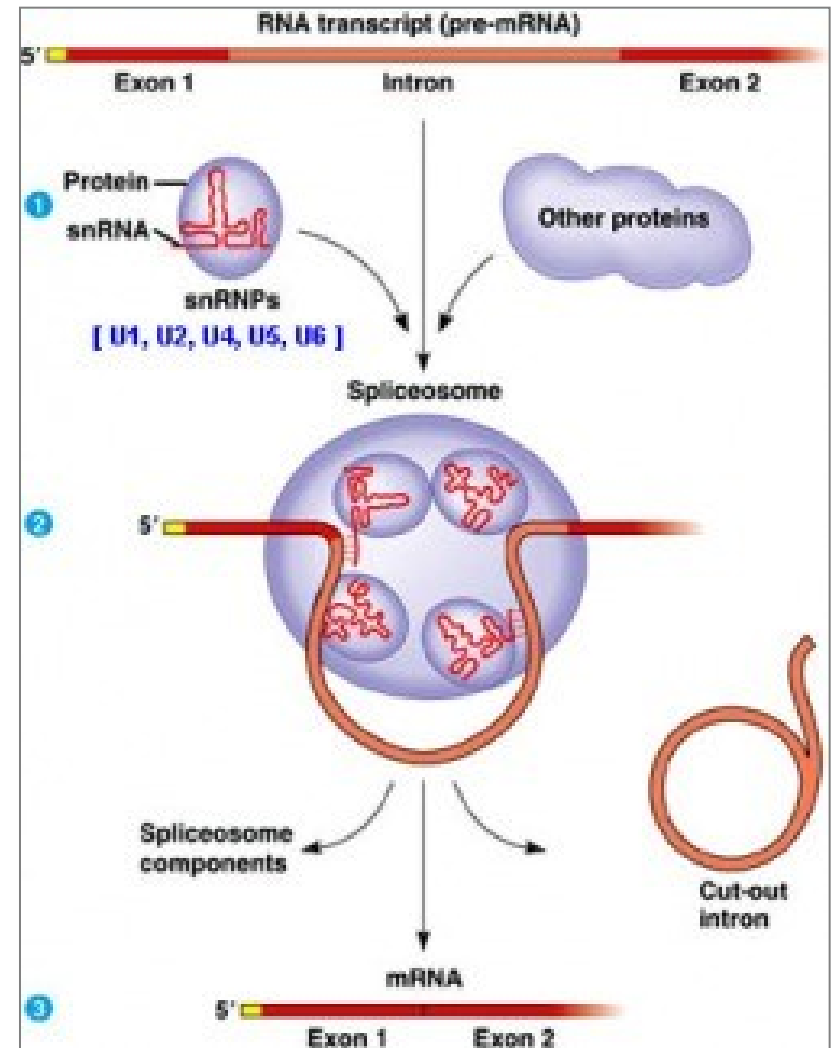
SMN2

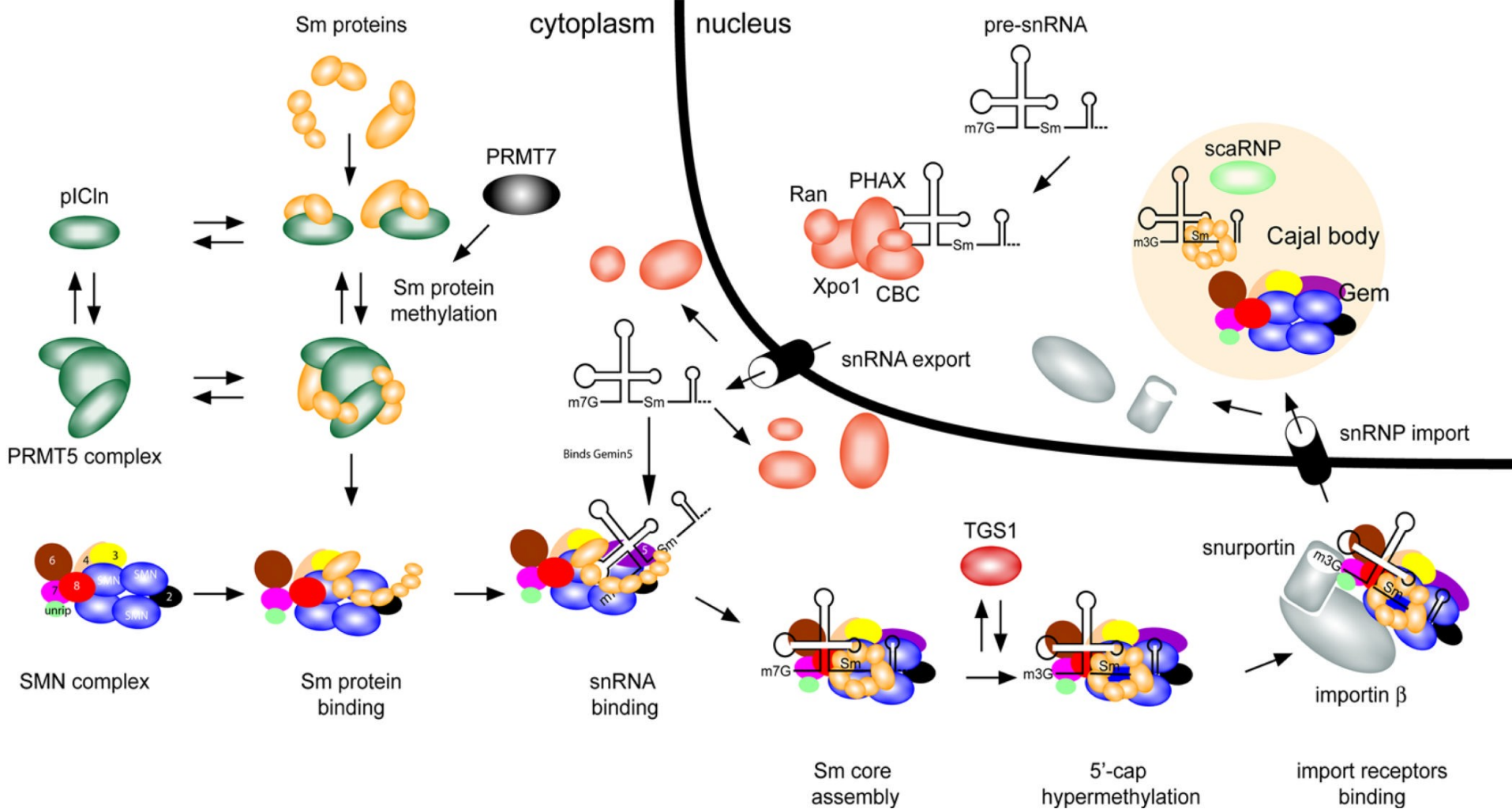


***SMN1* and *SMN2* genes: structure and splicing**

The *SMN1* and *SMN2* have identical gene structure and are 99.9% identical at the sequence level. The essential difference between the two genes is a single nucleotide change in exon 7 (C or T as indicated). This single nucleotide change affects the splicing of the gene. Thus the majority of SMN transcripts from *SMN2* lack exon 7 whereas those from *SMN1* contain exon 7. However, because *SMN2* does produce some full-length SMN it can be viewed as a gene with reduced function but not loss of function. The loss of amino acids that are encoded by exon 7 results in the production of SMN protein with severely decreased oligomerization efficiency and stability. The SMN monomers are rapidly degraded. Thus, loss of *SMN1* results in reduction of SMN levels in most tissues. The SMN oligomer is represented as an octomer based on gel filtration of SMN complexes formed *in vitro*.

- **SMN has a ubiquitous and essential function involving production of **small nuclear ribonucleoprotein complexes**.**
- Small nuclear ribonucleoproteins (snRNPs) are active in recognizing and removing introns from pre-mRNA in the nucleus. Each snRNP particle is composed of small nuclear RNA (snRNA) of approximately 150 nucleotides, several Sm proteins and a number of specific proteins that are unique for each snRNP. Survival motor neuron (SMN) functions in the cytoplasm to assemble Sm proteins onto the snRNAs to produce an active snRNP.
- **SMN's neuronal- or motoneuronal-specific function?????**





Function of SMN in snRNP assembly A) In the cytoplasm, Sm proteins bind to the chloride conductance regulatory protein (pICln). The protein arginine methyltransferase PRMT methylate Sm proteins. Sm proteins are released from pICln-PRMT complex and bind the SMN complex. B1) The SMN complex is composed of SMN, Gemins 2-8 and unrip. B2) snRNA is transcribed in the nucleus and then binds the export proteins PHAX, CBC, Xpo1, Ran, which transport it to the cytoplasm. C) The SMN complex places the Sm proteins onto the snRNA. The m7G cap of the snRNA is hypermethylated, allowing the SMN complex with the snRNA to bind snurportin and importin, which mediates transport of the SMN complex with an assembled snRNP into the nucleus. D) In the nucleus, the SMN complex and snRNPs localize to the Cajal body and snRNPs undergo further maturation.

Function of SMN in snRNP assembly

Small nuclear ribonucleoproteins (snRNPs) are active in recognizing and removing introns from pre-mRNA in the nucleus. Each snRNP particle is composed of small nuclear RNA (snRNA) of approximately 150 nucleotides, several Sm proteins and a number of specific proteins that are unique for each snRNP. Survival motor neuron (SMN) functions in the cytoplasm to assemble Sm proteins onto the snRNAs to produce an active snRNP.

A) In the cytoplasm the 7 Sm proteins bind to the chloride conductance regulatory protein (pICln). *In vitro* studies reveal that pICln first binds the Sm proteins as two separate complexes: SmB, SmD3, and SmD1, SmD2. The latter subsequently binds SmE, SmF and SmG44. The protein arginine methyltransferase (PRMT5 complex) and PRMT7 methylate the Sm proteins SmB, SmD1 and SmD3. Sm proteins are released from pICln-PRMT5 complex and bind the SMN complex.

B1) The SMN complex is composed of SMN, Gemins2-8 and unrip. SMN is shown in the figure as an oligomer as it has been shown to self-associate and it has been suggested that oligomerization is critical for SMN function. The exact numbers of SMN monomers in a SMN complex is unknown (it has been suggested to be an octomer). The Gemins are shown as single units for simplicity as the exact stoichiometry of the SMN complex has not been determined.

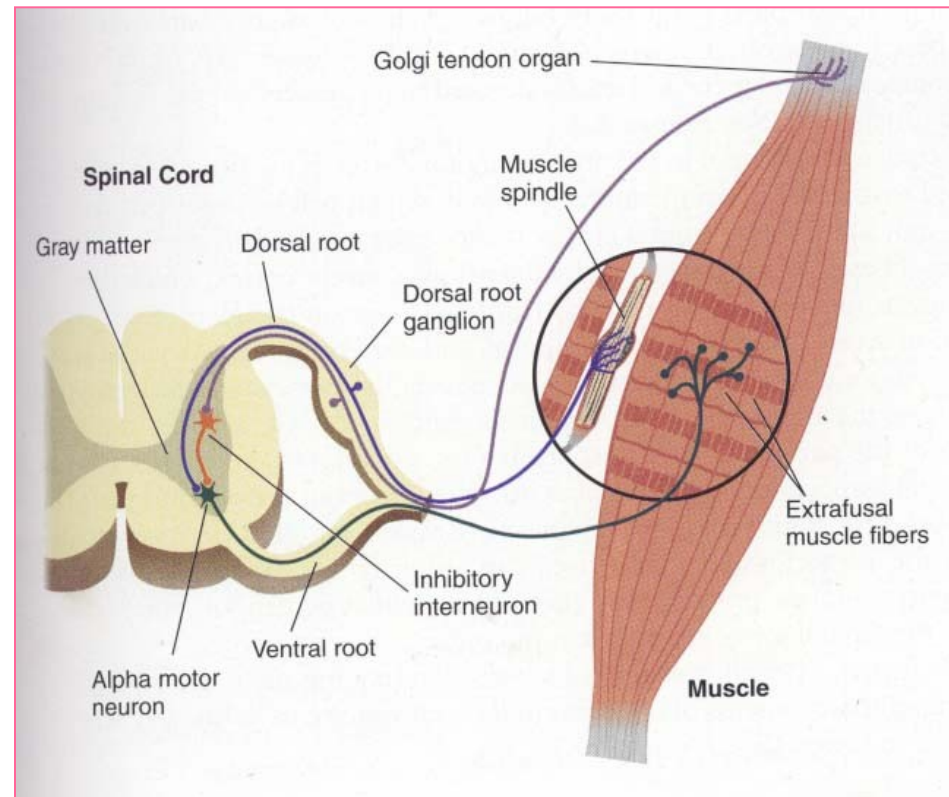
B2) snRNA is transcribed in the nucleus and then binds the export proteins phosphorylated adaptor for RNA export (PHAX), Cap-binding complex (CBC), exportin (Xpo1) and ras-related nuclear protein GTP (Ran), which transport it to the cytoplasm. In vertebrates, the snRNA is brought into the Sm protein-bound SMN complex by binding to Gemin5.

C) The SMN complex places the Sm proteins onto the snRNA. The m⁷G cap of the snRNA is hypermethylated by trimethylguanosine synthetase 1 (TGS), allowing the SMN complex with the snRNA to bind snurportin and importin, which mediates transport of the SMN complex with an assembled snRNP into the nucleus.

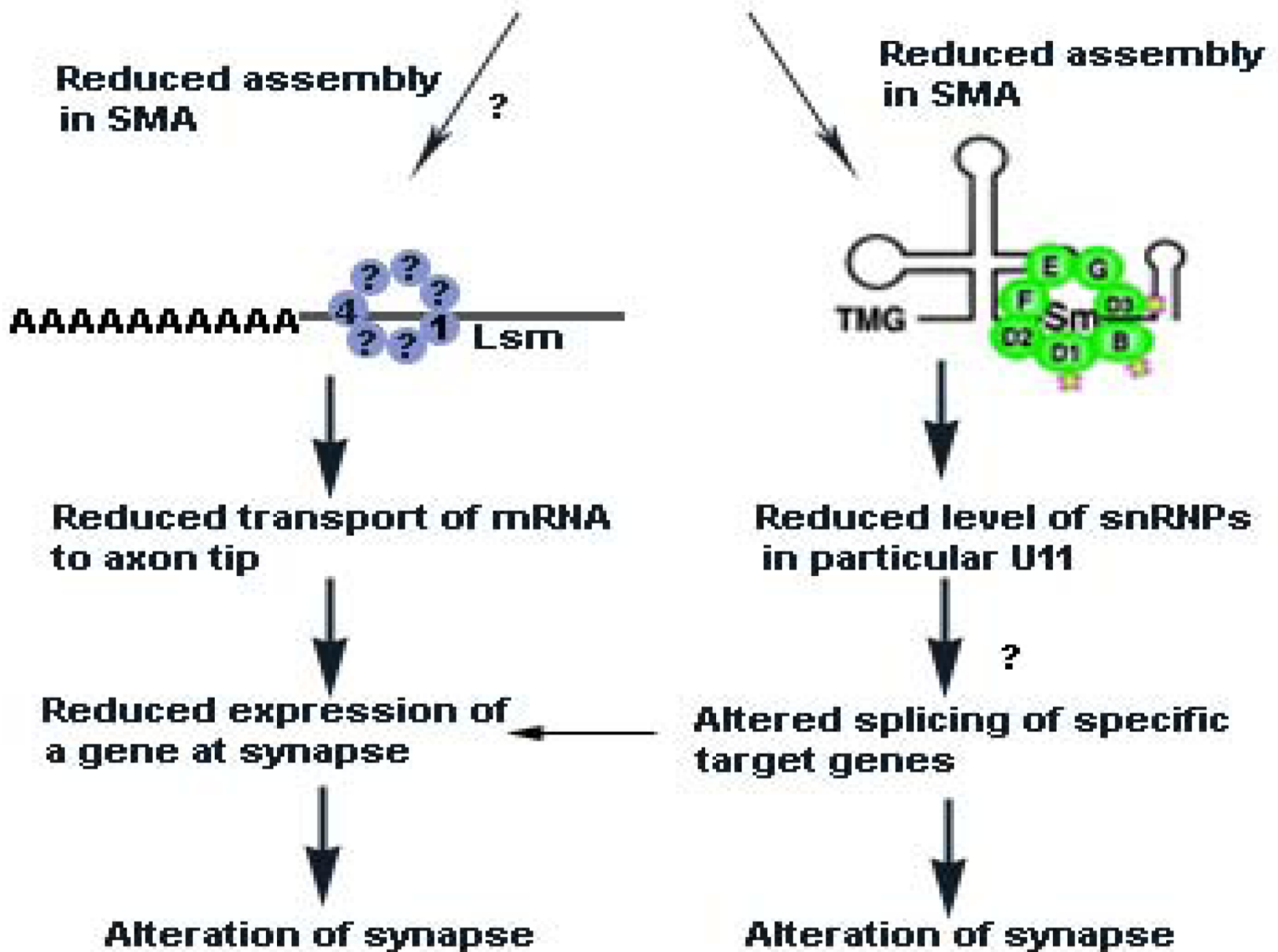
D) In the nucleus the SMN complex and snRNPs localize to the Cajal body and snRNPs undergo further maturation. Depending on the cell type and developmental stage, SMN can localize as a separate body adjacent to the Cajal body.

- It is still not clear if the primary defect (degradation of motoneurons) seen in SMA patients are related to SMN's role in snRNP metabolism (although partial and unequal deficiencies in snRNP concentrations have been observed).
- It is still unclear if the primary defect is a disturbance of splicing patterns that would be most pronounced or whose effects would be most damaging for motoneurons.

• ?????? Some additional interactions and functions of SMN could be vital to the maintenance of motoneuron functionality and viability. This view is supported by studies showing that the SMN protein is present in transport inside motoneurons. SMN is involved in the transport or translational regulation of actin mRNA or other so far not characterized mRNAs and thereby control the organisation of the axon terminal.



Assembly reactions of SMN



Mechanisms proposed to explain how reduced SMN levels cause SMA

According to one hypothesis, reduced SMN levels result in reduced assembly of Sm proteins onto snRNA. This unevenly alters the levels of specific endogenous snRNPs, such as those used to splice minor introns (particularly U11) from pre-mRNA. It remains to be determined what the downstream target genes of the affected snRNPs are and how this specifically affects motor neuron function (indicated by a question mark (?)). One possibility is that the critical target gene is specific to motor neuron system. Alternatively, a function of critical importance to motor neurons could be disrupted.

In addition, it has been suggested that reduced levels of β -actin mRNA or other mRNA occur at the axon tip or synapse due to SMN having a function in axon RNA transport at the growth cones of motor neurons cultured from SMA mice. It has been proposed that hRNPQ/R66, 67 and ZBP69 participate with SMN in this complex and that the reduced β -actin transport leads to alteration of calcium channel distribution at the axon terminal which in turn could affect neurotransmitter release.

Lsm proteins 1 and 4 have been found in axons in an RNP complex. We suggest that it is possible that reduced SMN levels affect the assembly of Lsm proteins required for axonal transport of mRNA, leading to reduced expression of specific genes at the synapse. However, a functional biochemical assay linking reduced SMN levels to an alteration in the formation of the required complex for transport of mRNA is lacking (indicated by ?). Whether other Lsm proteins, such as Lsm14, associate with this complex in neurons is not known.

We have not indicated other potential or known SMN dependent assembly pathways, such as assembly of U7 snRNA, as it is not clear how alteration of this pathway would give rise to SMA. However, we cannot eliminate the possibility that other RNP assembly reactions are affected by reduced SMN levels. Lastly, it is possible to unite the two hypotheses where reduced snRNP assembly causes reduced splicing of a target gene that is critical for transport of mRNA to the motor neuron synapse.

SMA

- 95% způsobeno homozygotní delecí *SMN1* genu
- 5% způsobeno delecí *SMN1* genu na jednom chromozomu a bodovou mutací na druhém

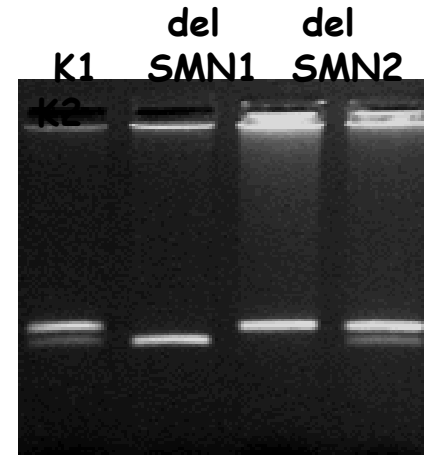
Real-time PCR nebo MLPA

- stanovení počtu kopií *SMN1* genu na genom
- stanovení přenašečství SMA v rodinách s výskytem SMA (1 kopie - přenašeč, 2 kopie - zdravý)
- vytipování pacientů u kterých se bude provádět sekvenční analýza *SMN1* genu (1 kopie *SMN1*)

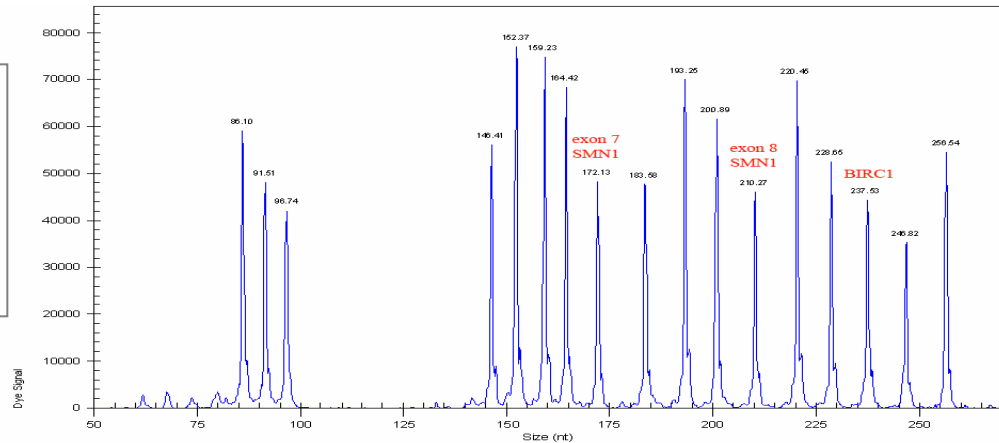
Stanovení delece genu SMN1

- **PCR a restrikční analýza** (stanovení homozygotní delece *SMN1* - sledování přítomnosti 7. exonu)

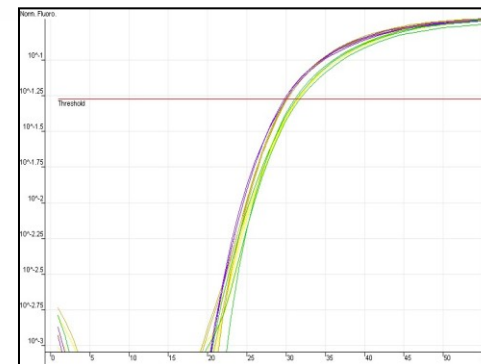
SMN1 gen: 188 pb,
SMN2 gen: 149 pb + 39 pb



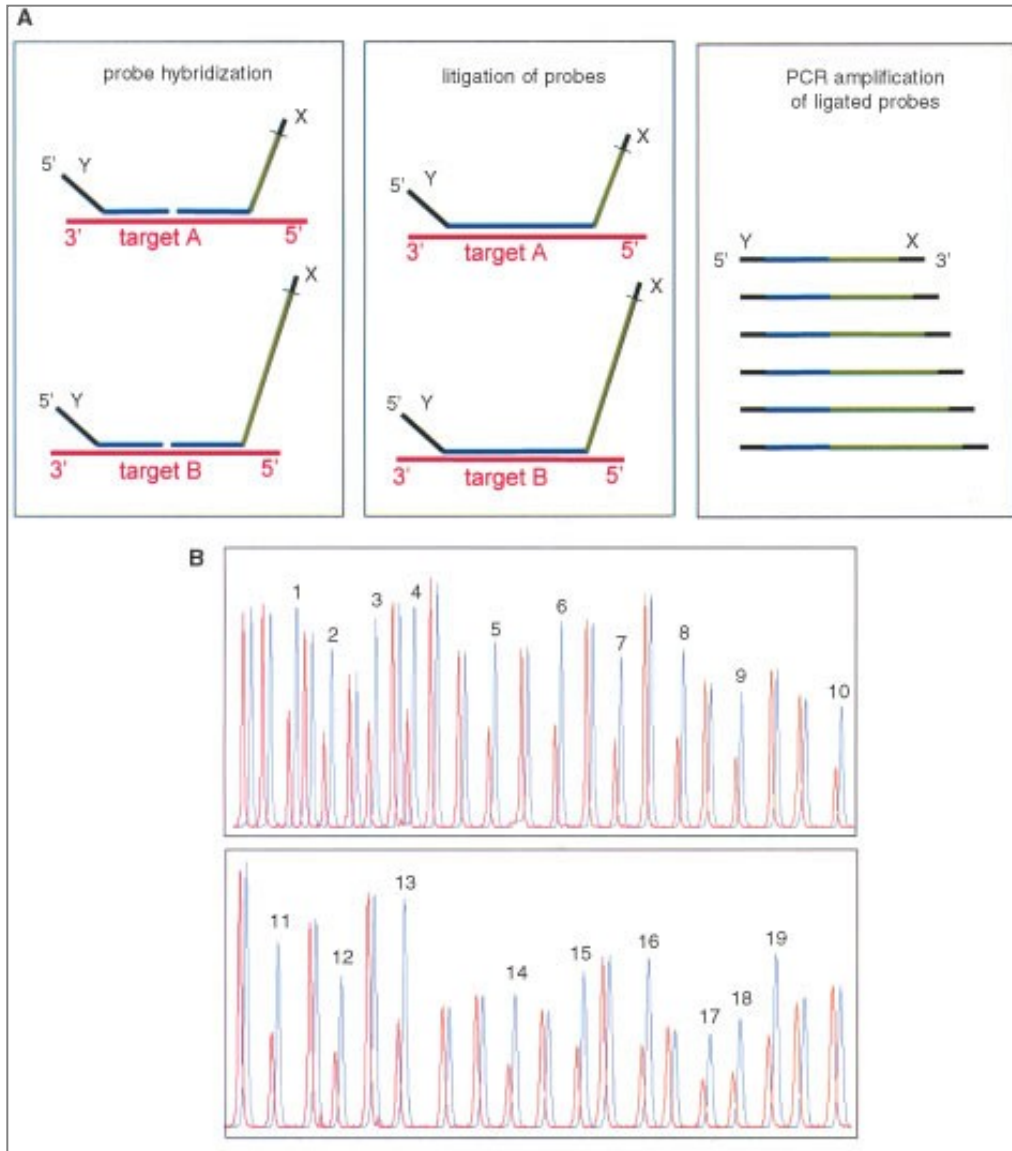
- **SALSA MLPA kit P060** (stanovení homozygotní delece *SMN1* + stanovení počtu kopií *SMN1*)



- **Real-time PCR** (stanovení homozygotní delece *SMN1* + stanovení počtu kopií *SMN1*)

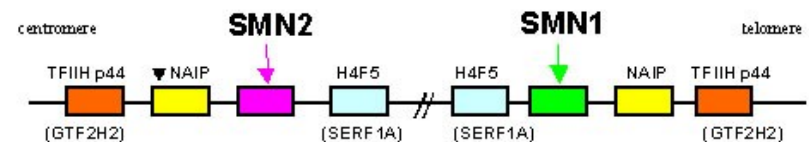


Multiplex Ligation-dependent Probe Amplification (MLPA)



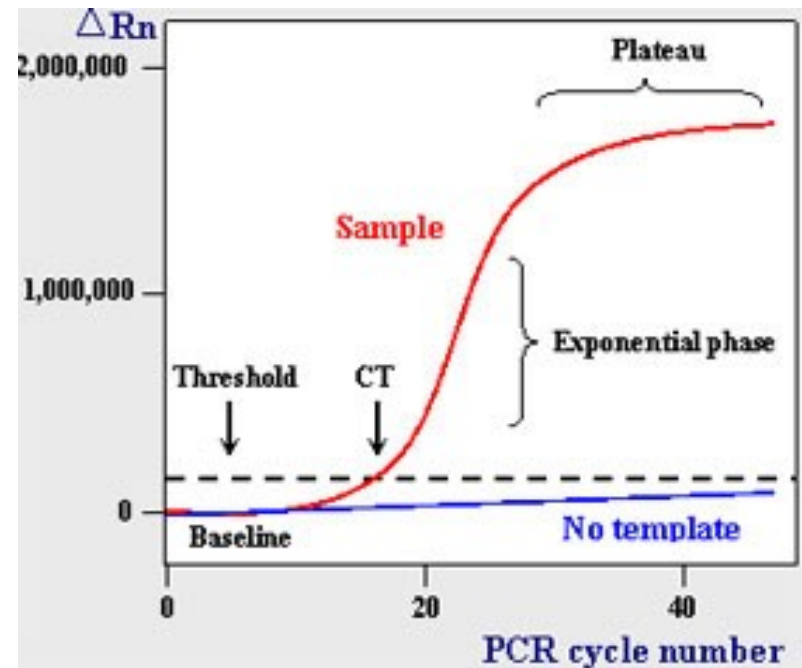
Denatured genomic DNA is hybridised with a mixture of probes. Each MLPA probe consists of two oligonucleotides. The two parts of each probe hybridise to adjacent target sequences and are ligated by a thermostable ligase. All probe ligation products are amplified simultaneously by PCR using a single primer pair labeled with 6-FAM. The amplification product of each probe has a unique length. Amplification products are separated by capillary electrophoresis. Relative amounts of probe amplification products reflect the relative copy number of target sequences.

The SMA probe mix contains 18 different control probes as well as exon 7 and 8 probes specific for SMN1 and SMN2.

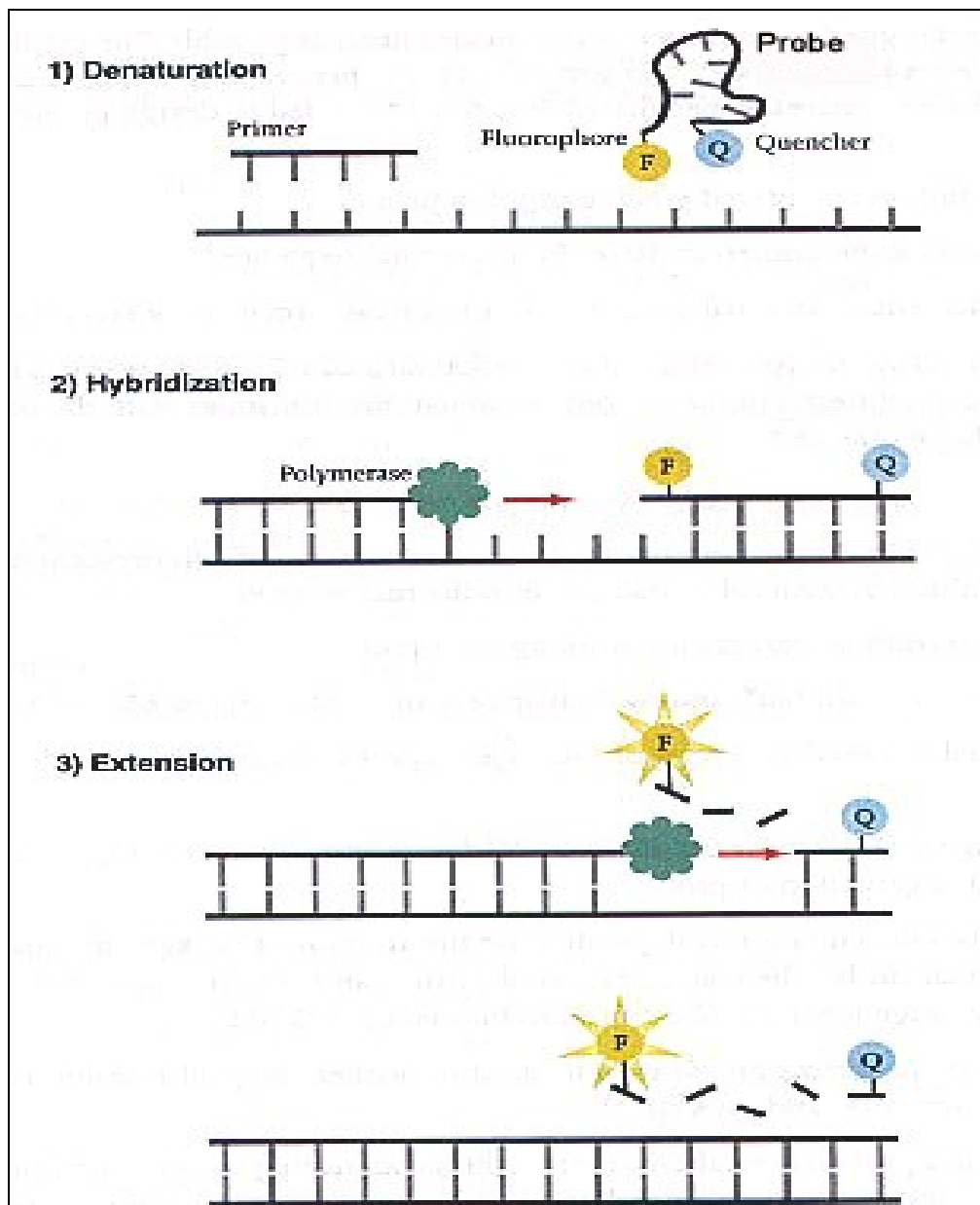


Real-time PCR pro stanovení počtu kopií SMN1 genu

- specifické primery a sonda (FAM) pro amplifikaci SMN1 genu,
- specifické primery a sonda (Cy5) pro amplifikaci referenčního genu,
- koamplifikace SMN1 genu a referenčního genu (referenční gen – normalizátor - umožňuje normalizaci výsledků, např. kompenzace rozdílů v množství DNA přidané do reakce),
- amplifikace dvou kontrolních DNA (DNA s 1 a 2 kopiemi SMN1 genu) - kalibrátory pro výpočet pomocí **komparativní C_T metody pro relativní kvantifikaci**



Princip *real-time* PCR využívající TaqMan hybridizační sondy



Komparativní C_T metoda pro relativní kvantifikaci

$$(1 + E_{SMN})^{-\Delta C_{T,SMN}}$$

$$(1 + E_{Ref})^{-\Delta C_{T,Ref}}$$

$$\Delta C_T = C_{T,vzorek} - C_{T,kalibrátor}$$

E - účinnost amplifikace

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$\Delta C_{T,SMN}$ - rozdíl *threshold* cyclu pro *SMN* gen u

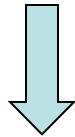
analyzovaného vzorku a kalibrátoru

$\Delta C_{T,Ref}$ - rozdíl *threshold* cyclu pro referenční gen u

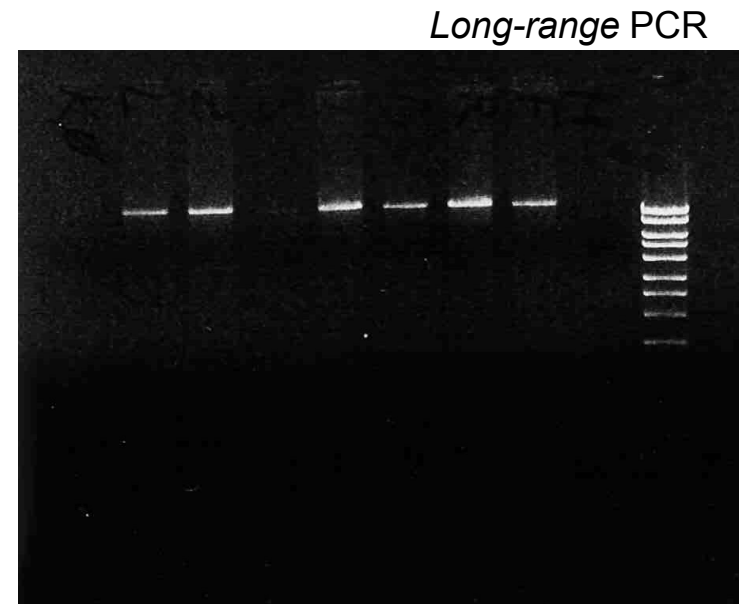
analyzovaného vzorku a kalibrátoru

Analýza bodových mutací v genu SMN1

- Amplifikace genu *SMN1* pomocí *long-range* PCR:
 - amplifikace exonů 2a – 6
 - velikost produktu 13,5 kb
 - izolace produktu PCR z gelu



- *Nested* PCR jednotlivých exonů
- Sekvenční analýza DNA



Výsledky analýzy bodových mutací v genu *SMN1*

- ❑ Počet pacientů s podezřením na SMA analyzovaných na počet kopií genu *SMN1*:

FN Brno: 156, FN Motol 145

- ❑ Celkový počet pacientů s jednou kopií genu *SMN1*: 14

- ❑ Analýza bodových mutací:

p.Y272C (3 pacienti), p.T274I, p.I33fsX6, p.A188S