La strategia dell’exon skipping nella terapia genica della Distrofia Muscolare di Duchenne

Why study RNA?

Post-transcriptional regulation

nucleus

splicing/processing sn-snoRNAs
polyadenylation/3' end formation snRNAs
RNA modifications (CH_3,ψU) snoRNAs

transport

cytoplasm

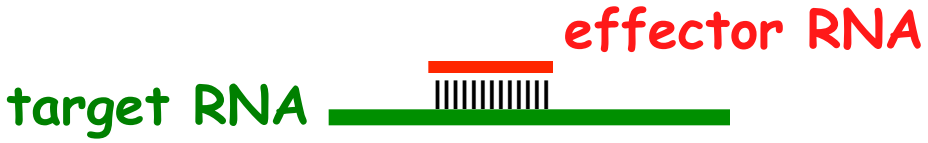
translation miRNAs
editing gRNAs
stability siRNAs

RNA/RNA interactions are at the base of all these processes
Specificity is provided by short base-pairing

Economist.com

The RNA revolution

Biology's Big Bang



One of the major goals of molecular biologists is to control, or modify, gene expression in a sequence-specific way (basic science and applied research)

Why select RNA?

- RNA molecules can interfere with gene expression in a sequence-specific way

- The specificity is extremely high and can be obtained with molecules of low complexity

- Non-immunogenic

Therapeutic RNAs

RNA functions that can be exploited for
controlling gene expression in a sequence-specific way:

Antisense
Aptamers
Ribozymes
Modifying RNAs
RNA interference
miRNA

Advantages of RNA-base gene therapy

- RNA molecules can interfere with gene expression in a sequence-specific way

- The specificity is extremely high and can be obtained with molecules of low complexity

Therapeutic RNAs should be stably expressed in order to obtain a long term activity

Requirements for an effective therapeutic RNA

- Efficient expression

polIII, polII promoters, LTR

- Stability

insert into stable RNAs

- Structure

recognition regions exposed

- Subcellular compartmentalization

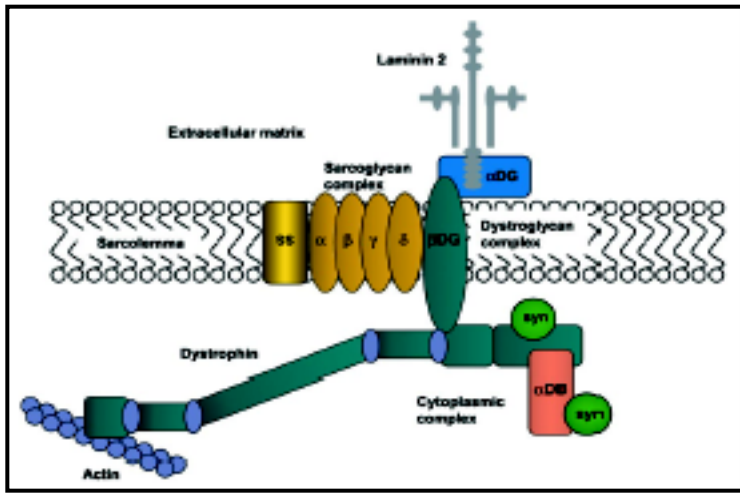
co-localization with the target

Genes for sncRNAs as vectors for the delivery of therapeutic RNAs

RNA	localization	function
U1	nucleoplasm	splicing
U2	nucleoplasm	splicing
U7	nucleoplasm	3’ processing of histone pre-mRNA
U16	nucleolus	site-specific methylation of pre-rRNA
VA1	cytoplasm	translation interference

Duchenne Muscular Dystrophy (DMD)

- X-linked recessive disorder
- affects 1 in 3500 live males
- DMD muscles degenerate with activity
- leads to ***death*** by the third decade of life



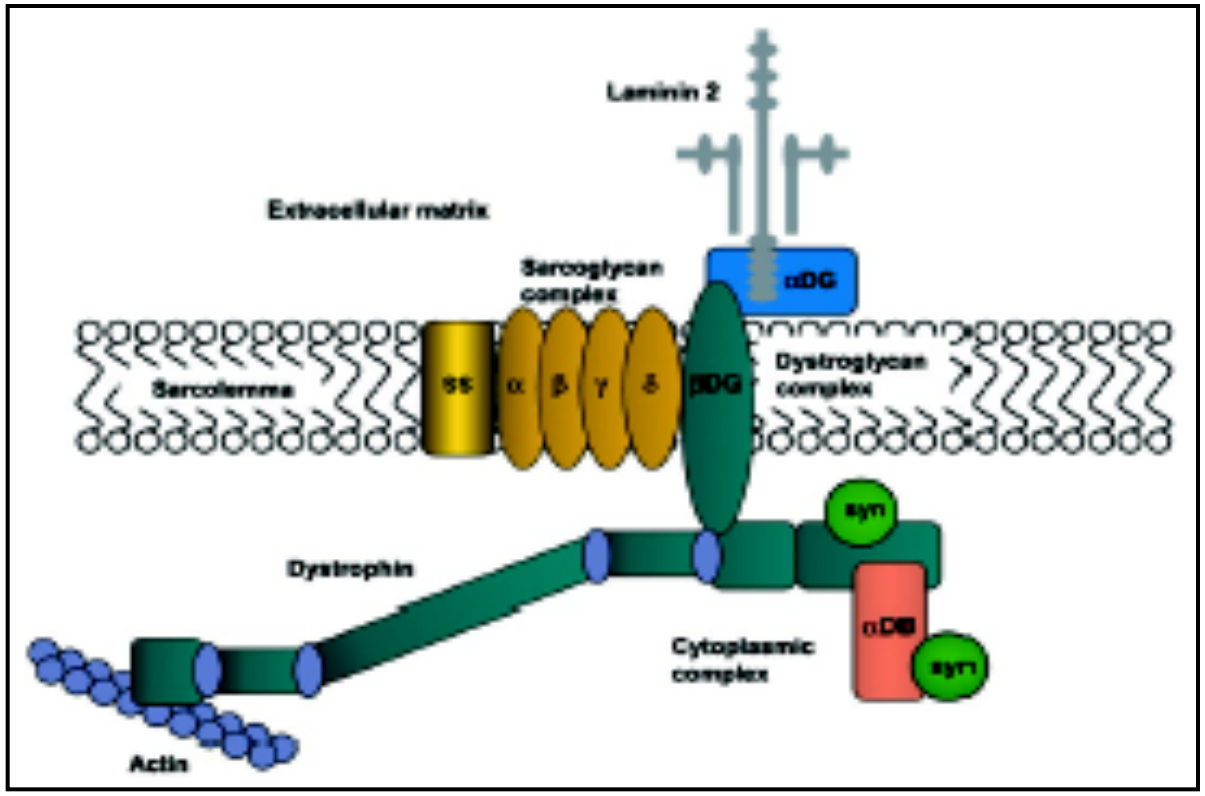
The gene is too big for a classical gene therapy intervention

Dystrophin

- **protein= 427 KDa**
- **DNA= 2,5 Mb**
- **cDNA= 14 Kb**

Duchenne Muscular Dystrophy (DMD)

- X-linked recessive disorder due to mutations in the dystrophin gene



Dystrophin

- **protein= 427 KDa**
- **DNA= 2,5 Mb**
- **cDNA= 14 Kb**

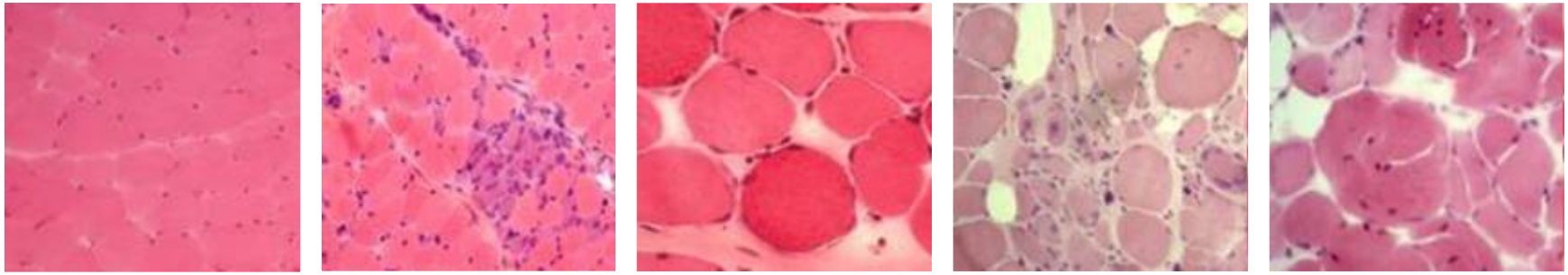
dystrophin - connects intracellular actin microfilaments to the extracellular matrix determining a **structural** stabilization of the sarcolemma

Duchenne Muscular Dystrophy (DMD)

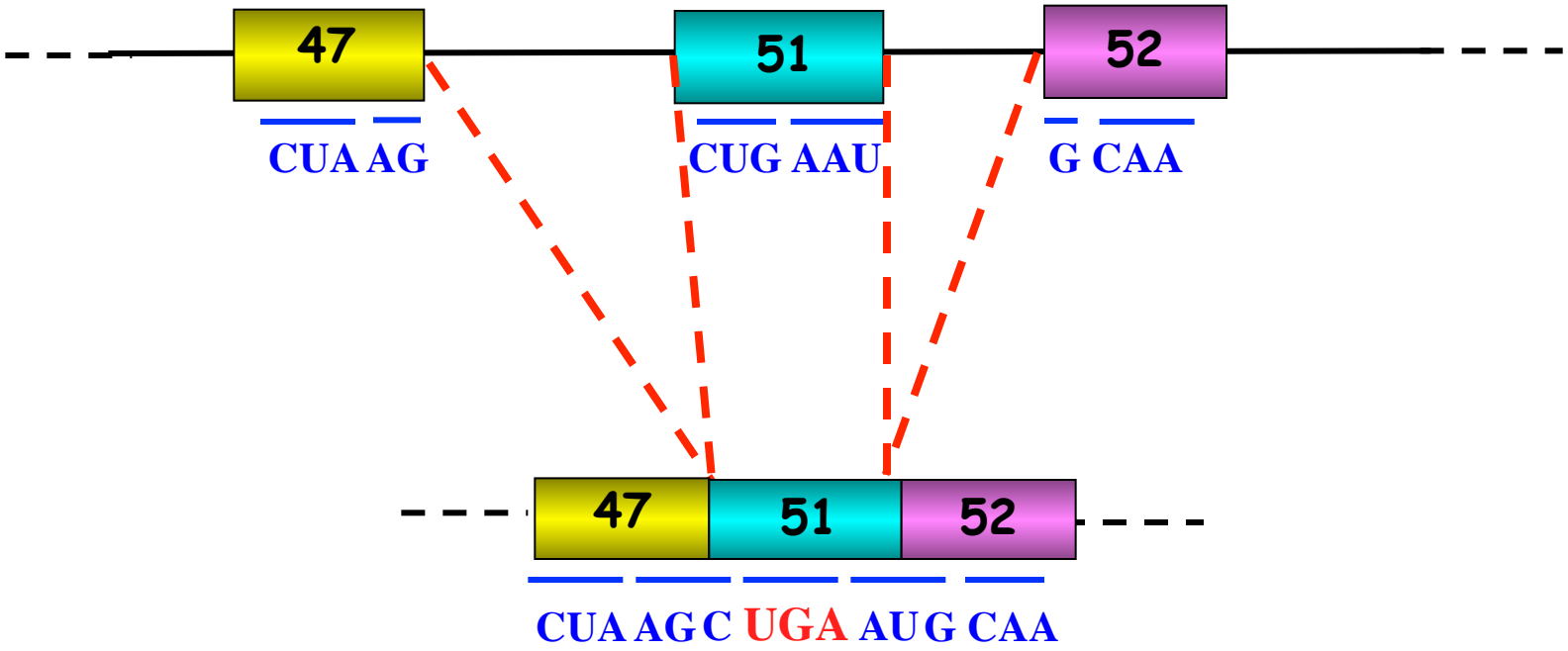


is a severe disorder characterized by rapid progression of muscle degeneration leading to loss of ambulation and death.

Histopathology of a Duchenne muscle



Duchenne Muscular Dystrophy
- the 48-50 deletion -



out-of-frame fusion → **UGA** (stop codon) → **premature translation termination**
mRNA degradation

Approcci alla terapia delle distrofie muscolari

Terapia genica – sviluppo di nuovi vettori capaci di trasferire il gene mancante ai nuclei delle fibre muscolari.
Limiti: gene troppo grande - difficoltà a raggiungere efficacemente tutti i distretti muscolari

Farmaci – recupero del registro di lettura (PTC), stimolare l' exon skipping o la produzione di proteine d' interesse come l' utrofina.
Limiti: somministrazione continua

Terapia cellulare – ricostituire un tessuto funzionale fornendo cellule satellite o cellule staminali (mesoangioblasti).
Limiti: limitata capacità proliferativa e migratoria delle cellule satelliti. Necessità di cellule autologhe.

A new strategy:

Modify the mutated dystrophin mRNA through the use of **antisense RNA molecules**

- RNA molecules can interfere with gene expression in a sequence-specific way

- The specificity is extremely high and can be obtained with molecules of low complexity

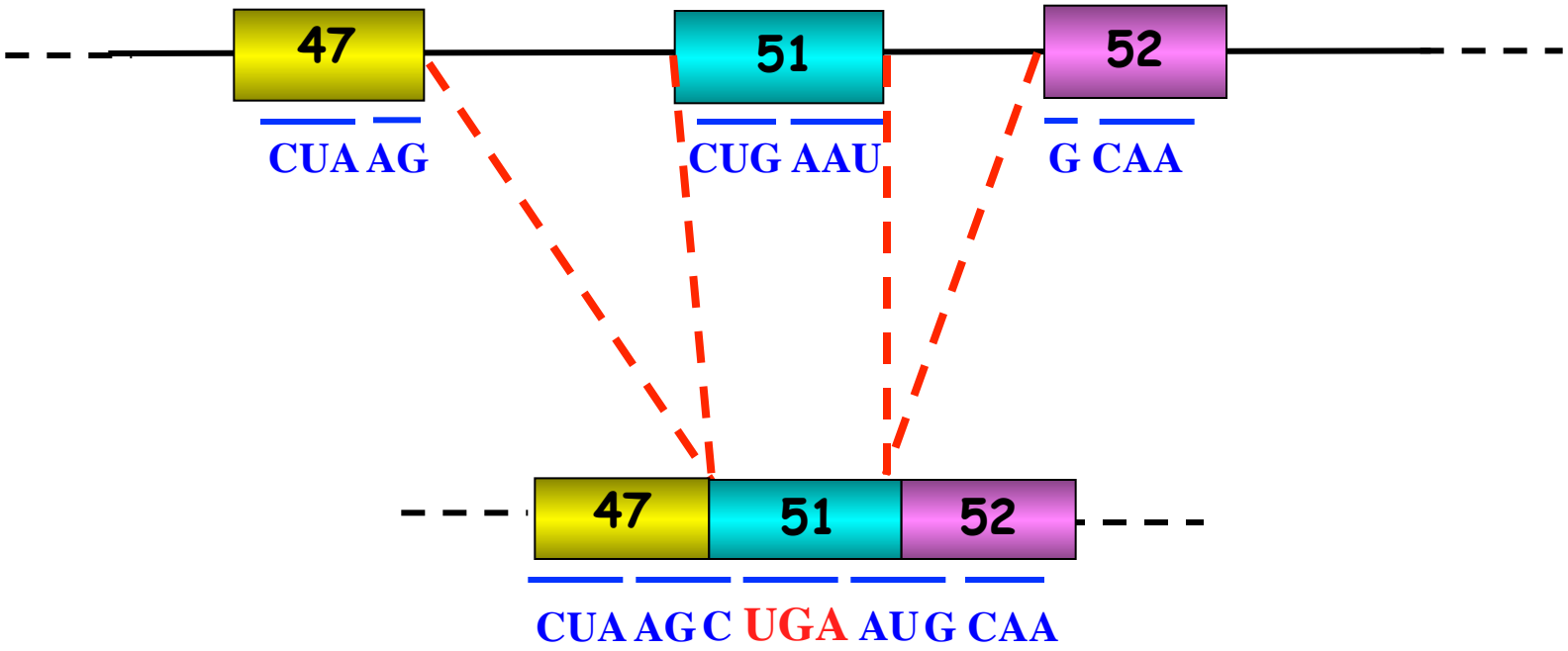
- Non-immunogenic

Economist.com

The RNA revolution

Biology's Big Bang

Duchenne Muscular Dystrophy
- the 48-50 deletion -



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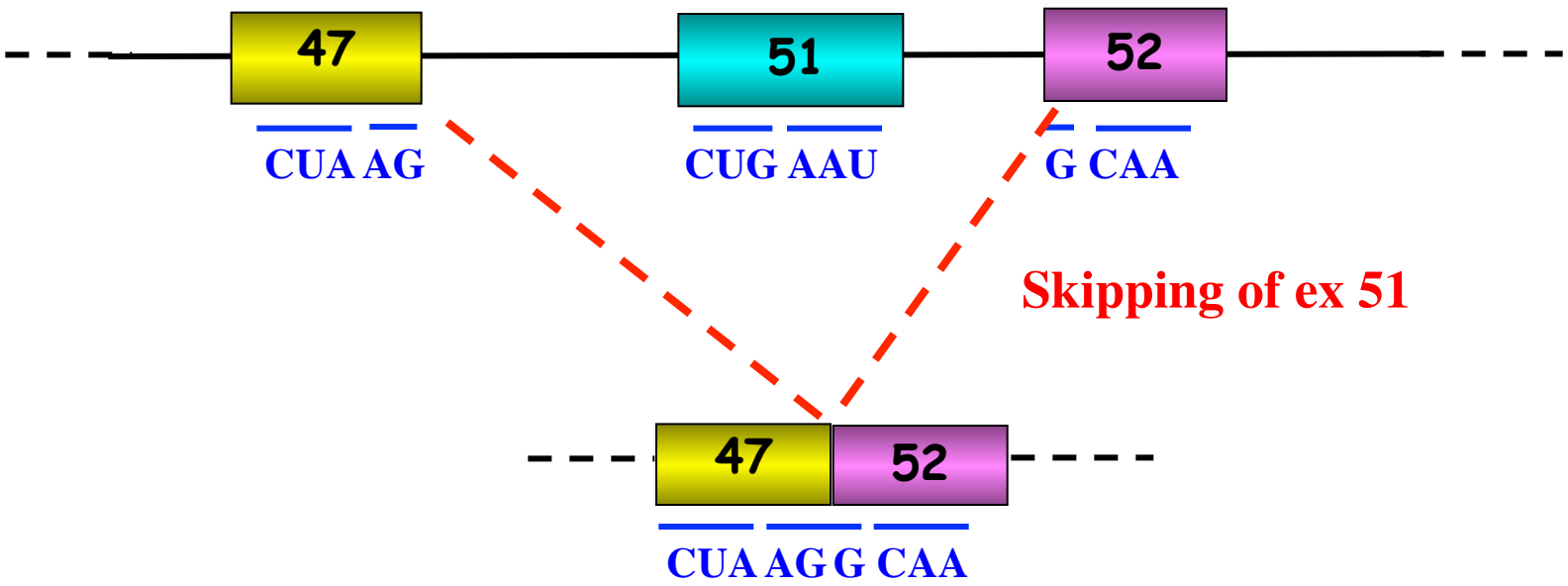
mRNA degradation

The exon skipping



In-frame mRNA → translation of a shorter but still functional protein

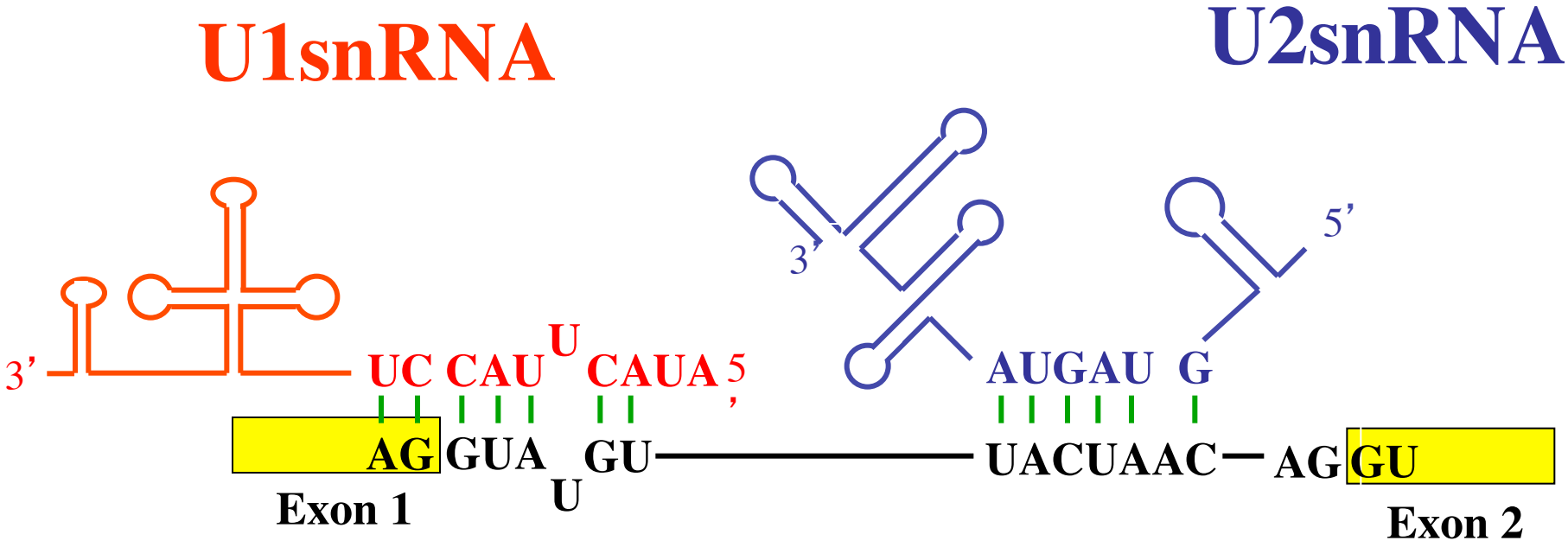
- Beker-type -

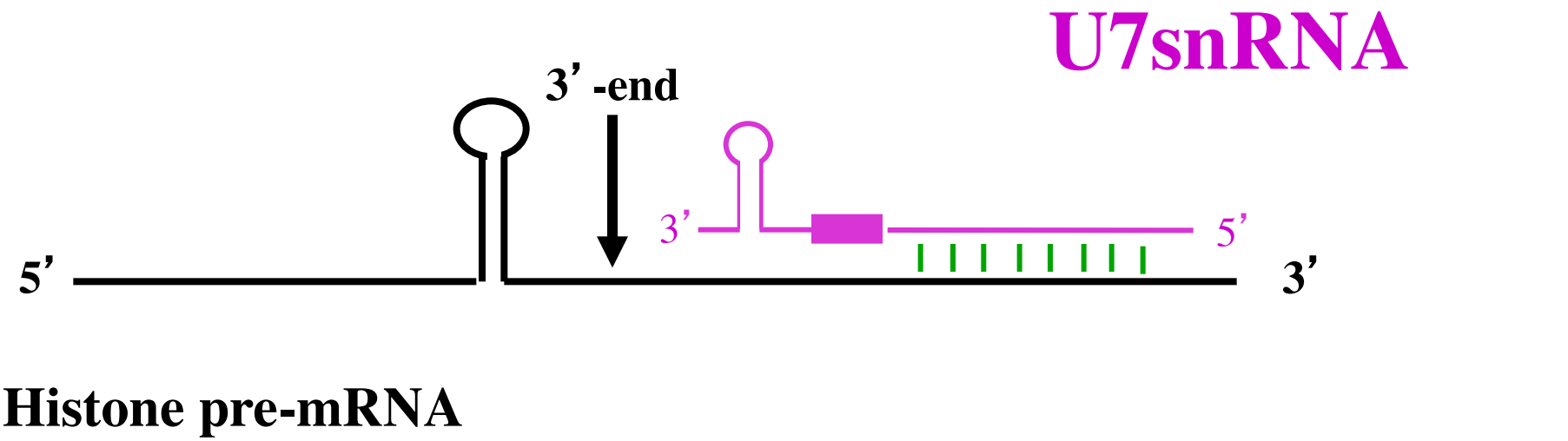
75% of all known dystrophin mutations can be cured by exon skipping

skipping of ex 51 - 15%

How to get long-term persistence of exon skipping?

1) Antisense sequences as part of stable cellular RNAs
2) Transcription driven by strong promoters
3) In vivo delivery through viral vectors

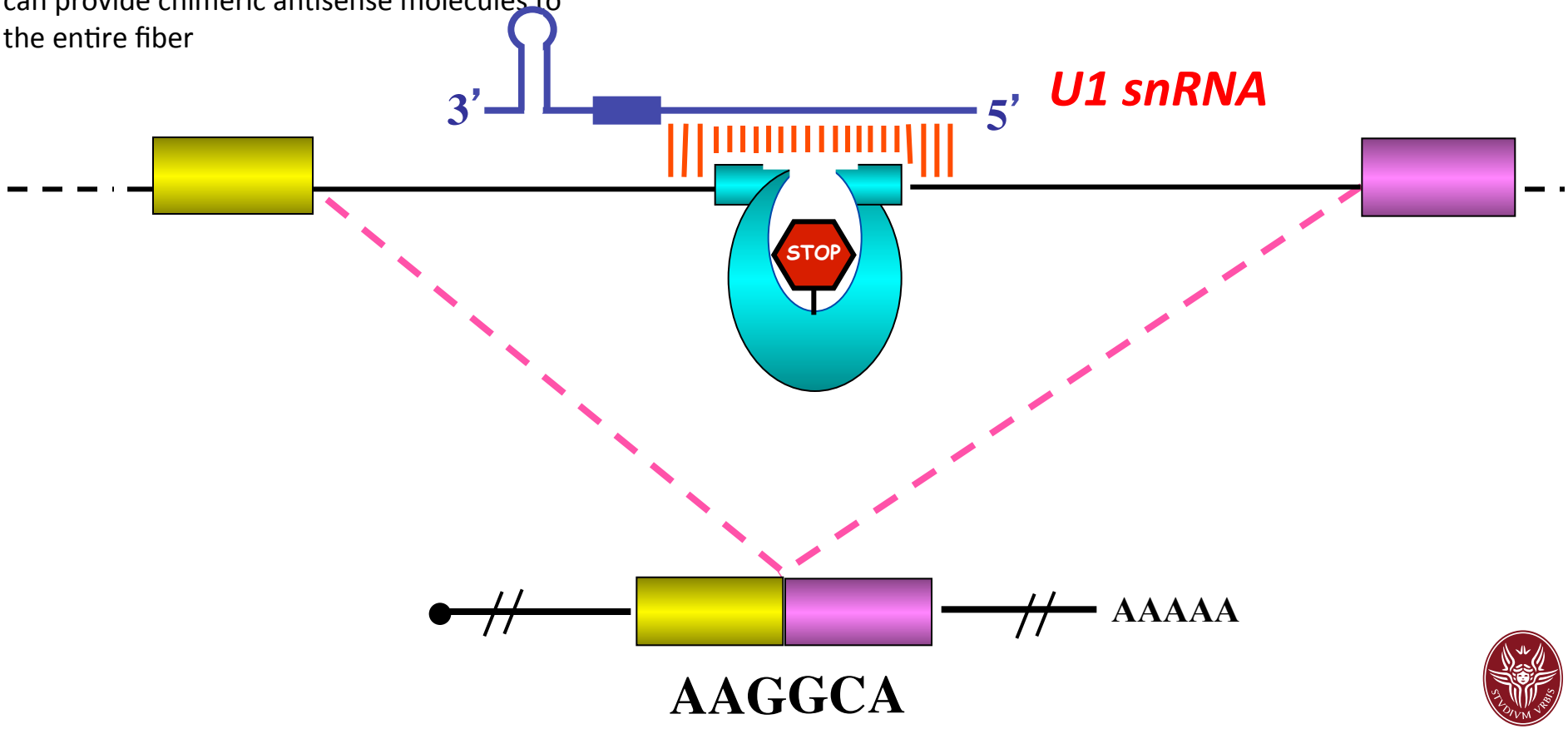

U1snRNA
U2snRNA
3'
5'
UC CAU U CAUA 5'
AUGAU G
AG GUA GU
U
UACUAAC
AG GU
Exon 1
Exon 2
pre-mRNA


U7snRNA
3' -end
3'
5'
5'
3'
Histone pre-mRNA

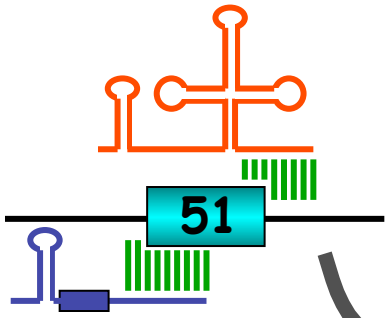
Antisense RNA technology applied to the correction of DMD mutations

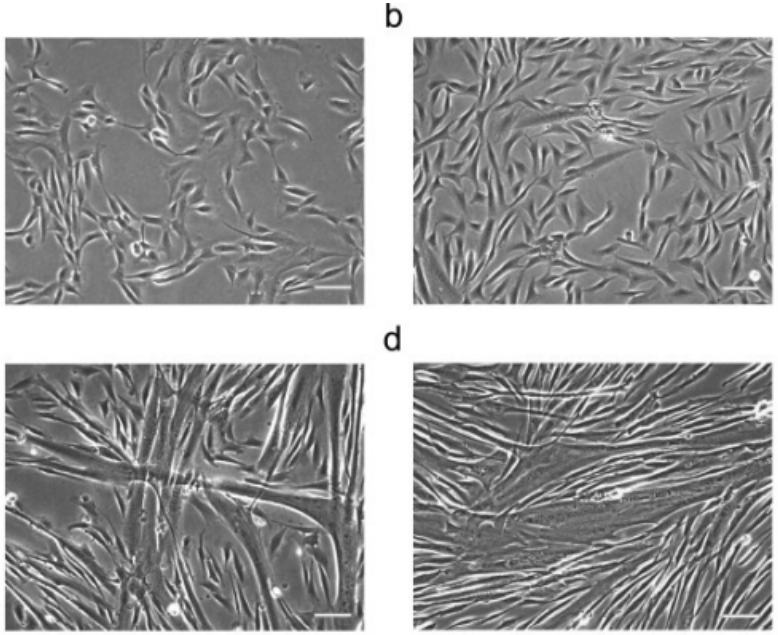
U1 snRNA

- nuclear RNA with specific recognition for splice junctions
- is matured in the cytoplasm an then reimported in the nucleus
- few transduced nuclei in the muscle fiber can provide chimeric antisense molecules to the entire fiber

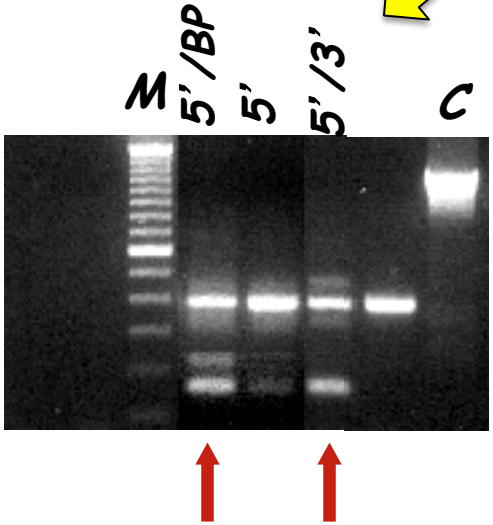


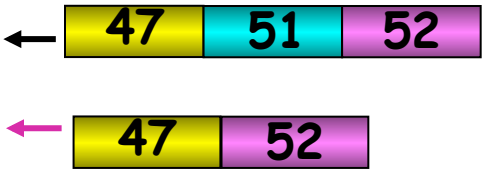
Human DMD myoblasts





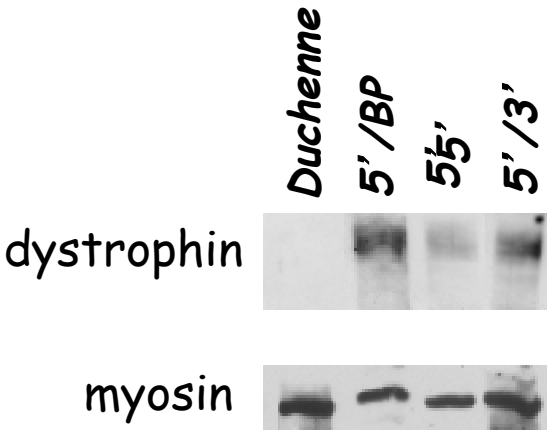
exon skipping





double antisense more efficient !!!!

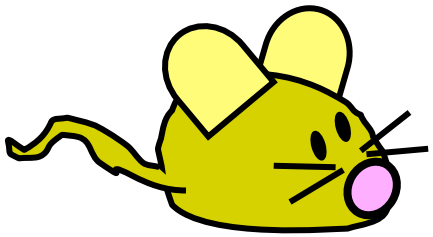
Rescue of dystrophin expression

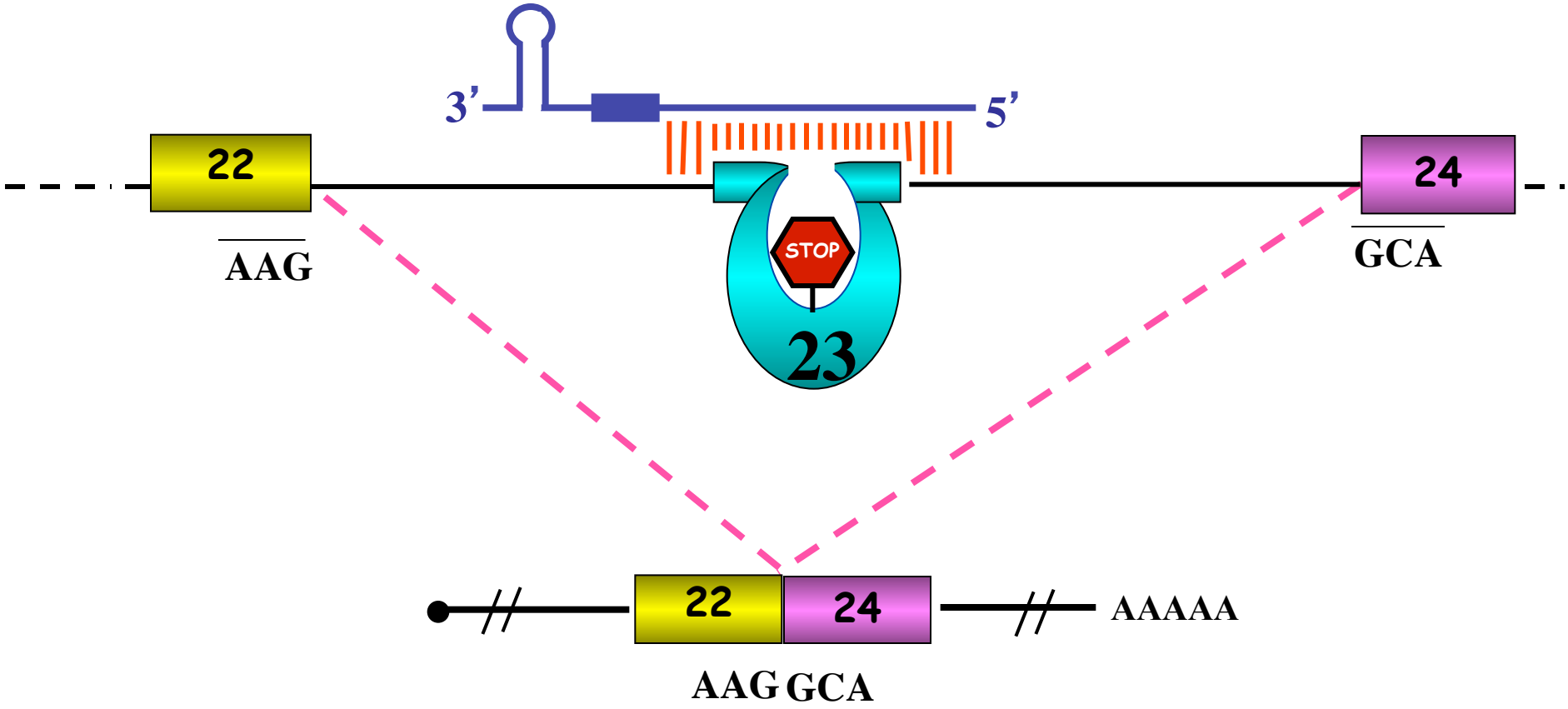


Patent n.:1333549

Towards an in vivo approach of gene therapy

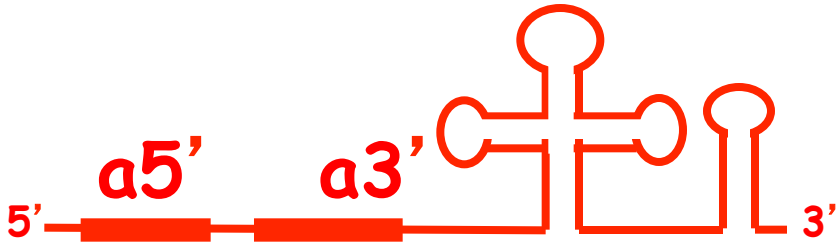
The mdx mouse



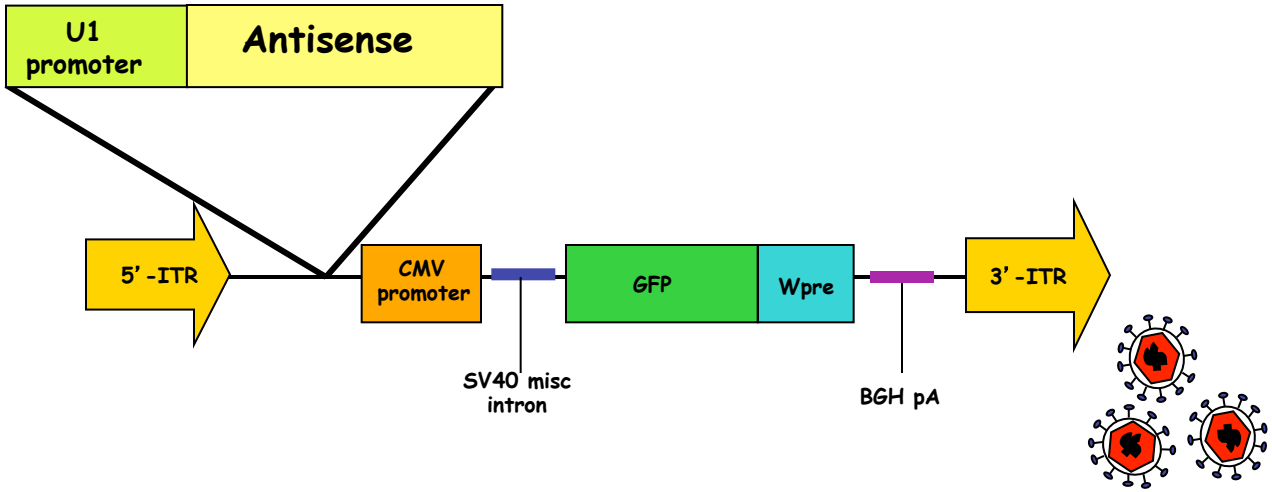


Antisense RNAs

mU1#23a5' 3'



How to deliver in vivo antisense molecules? use of AAV2/1 vectors



1) Intramuscular injection of antisense-AAV

2) Systemic delivery through vein injection

In vivo transduction of antisense molecules: use of AAV vectors

Advantages

Non-pathogenic, small genome able to accomodate short genes

Absence of viral genes whose expression may be responsible for causing an undesirable immune response

Efficient transduction of muscle cells (in particular the 1, 6, 8 and 9 serotypes)

The modified AAV genomes do not integrate in the host genome (multi-copies)

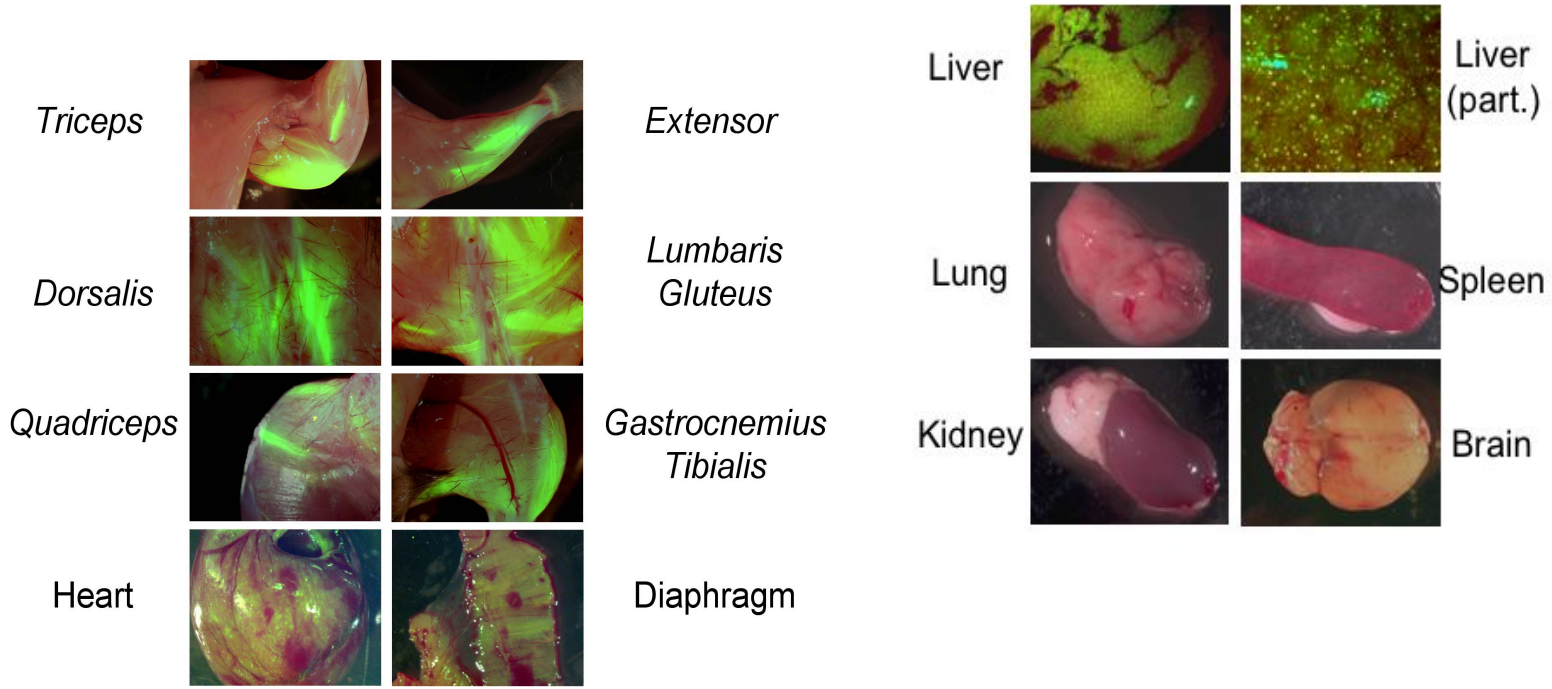
After a single injection the DNA remains for several years (5 years in monkeys)

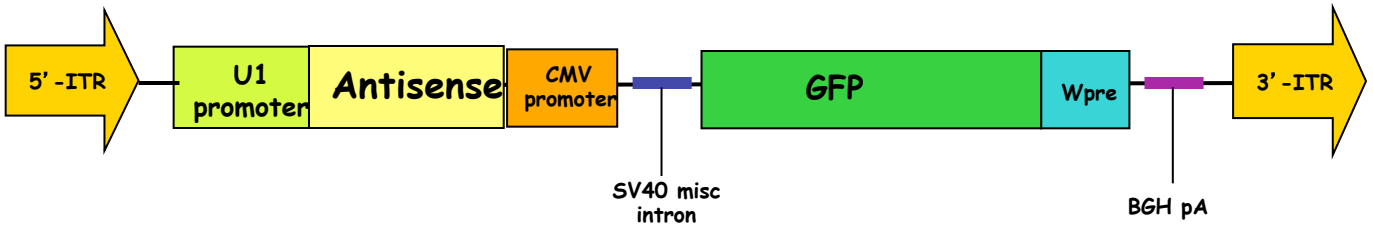
Disadvantages

Immune response at the second injection.

It can be overcome either by immunosuppression during the first injection or utilizing a different serotype in the second injection.

2. Systemic delivery through tail vein injection *(serotype 1)*





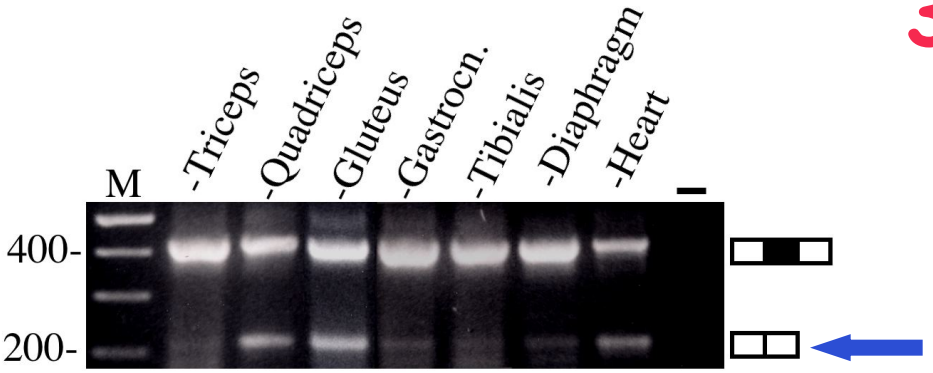
Detection of AAV-U1#23 distribution by GFP localization

Denti *et al.*, PNAS **2006** 10: 3758–3763

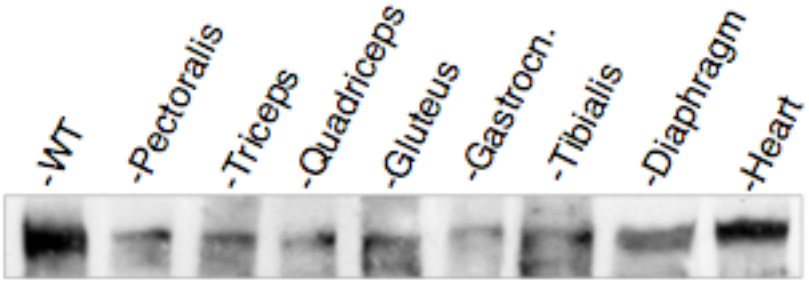
Systemic delivery of antisense-AAV:
Exon skipping and dystrophin rescue

3 months

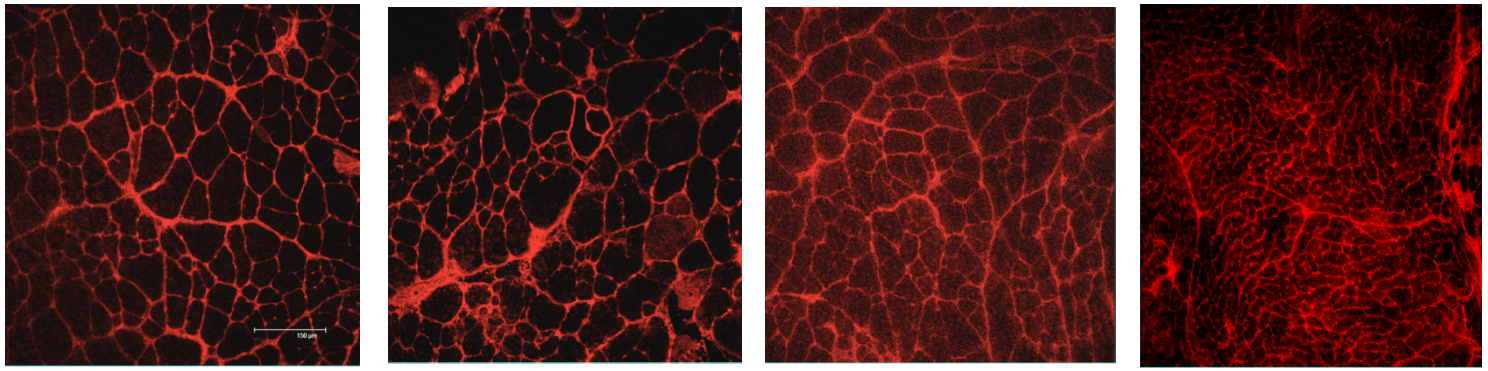
RT-PCR



Western blot

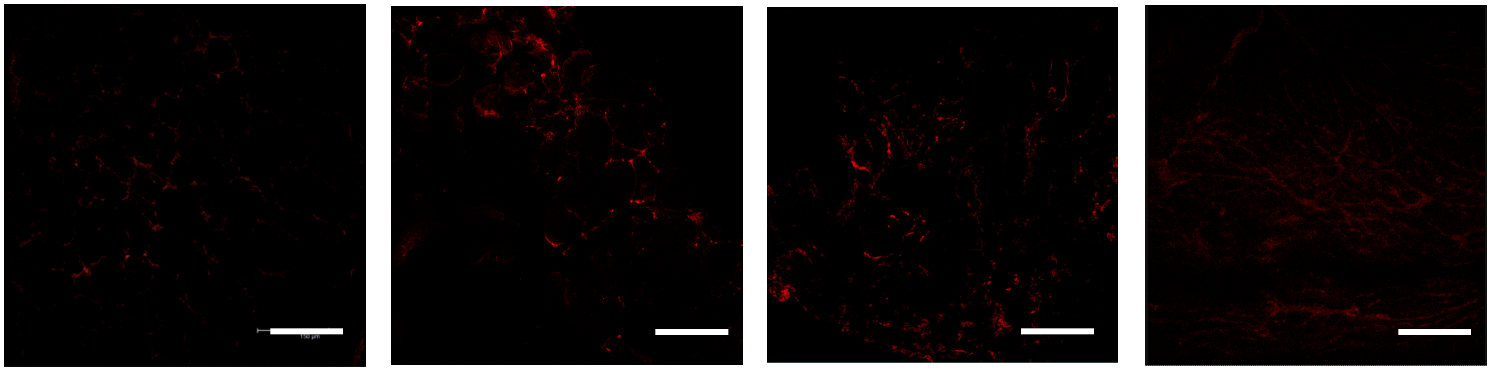


— Dys



tibialis *EDL* *gastroc.* *heart*

U1

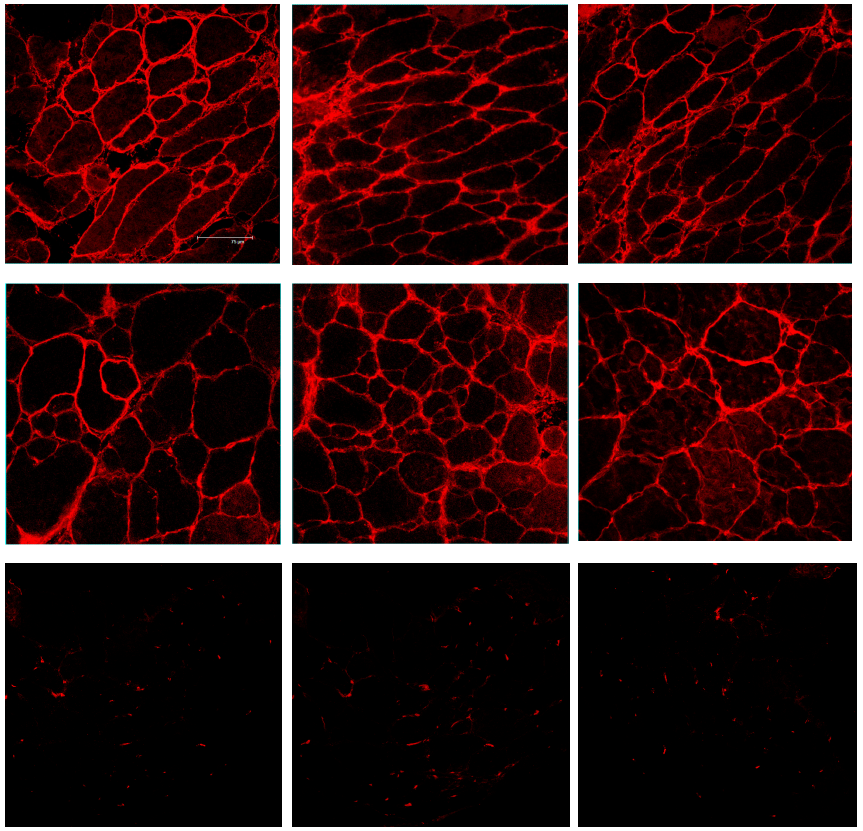


mdx

Dystrophin

Dystrophin and DAPC localization

dys α-sarc β-sarc

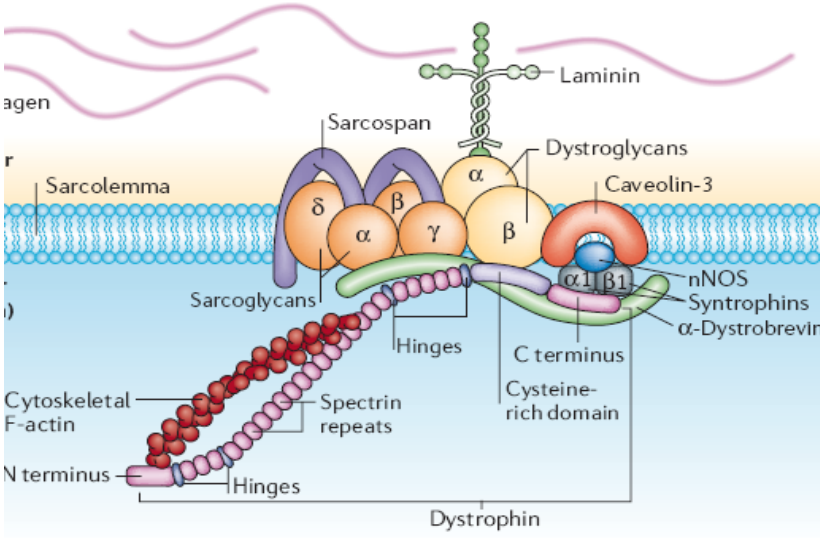


U1
(mouse F)

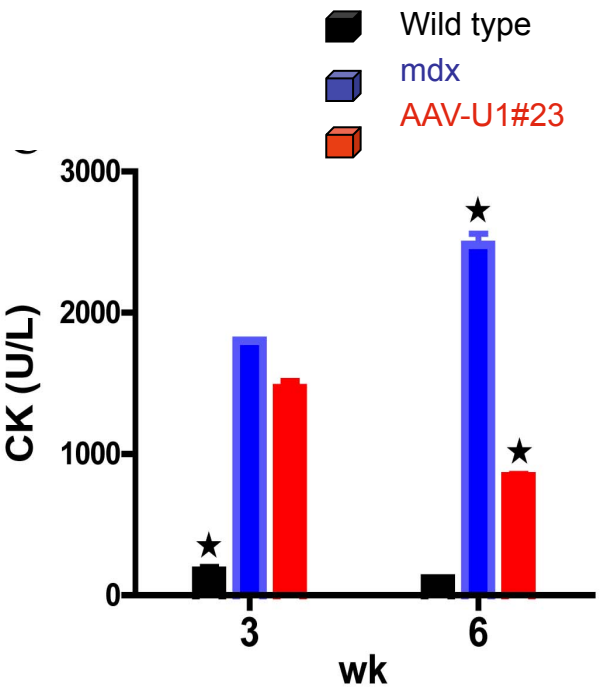
U1
(mouse F7)

mdx

EDL 40X



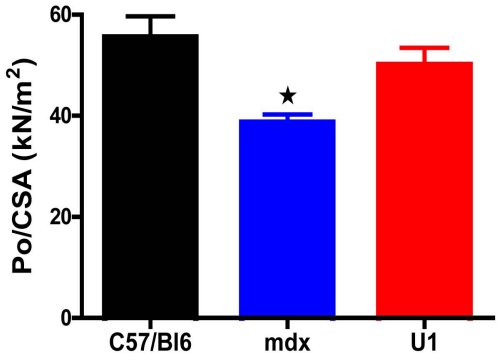
Injected mice have reduced levels of serum CK



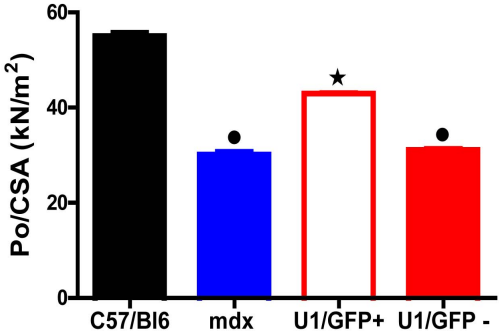
Creatine kinase (CK) concentration in the serum is an indicator of muscle damage extent.

Denti *et al.*, PNAS **2006** 10: 3758–3763

Increase in the specific force of single fibers from the gastrocnemius of injected mice

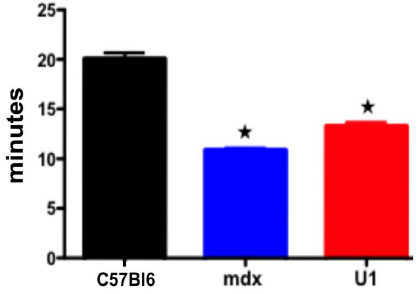


Increase in the specific force of GFP-positive fibers from the vastus of injected mice



Functional assays - Treadmill exhaustion test

injected mice show increased tolerance to exercise



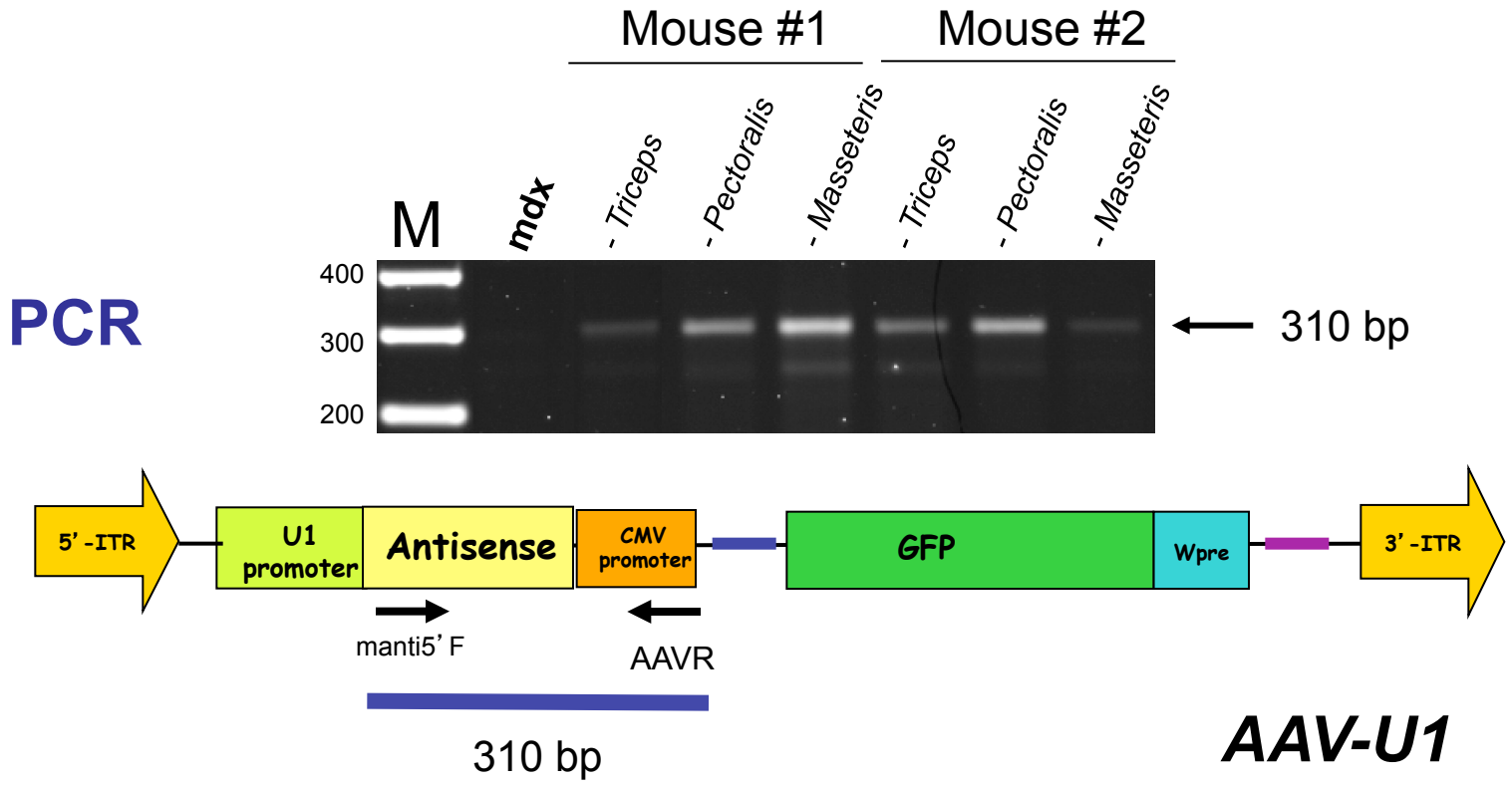
Denti *et al.*, PNAS **2006** 10: 3758–3763

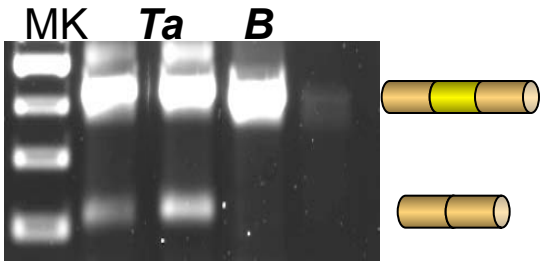
Long-term persistence of the therapeutic benefit

mdx mice injected at **6 weeks** and

sacrificed at **20 months**

20 months - Persistence of the transgene and…

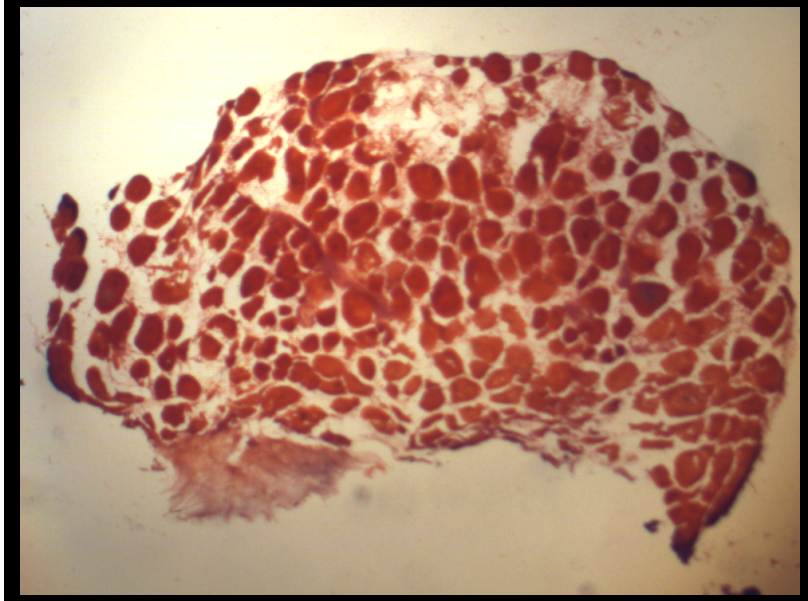


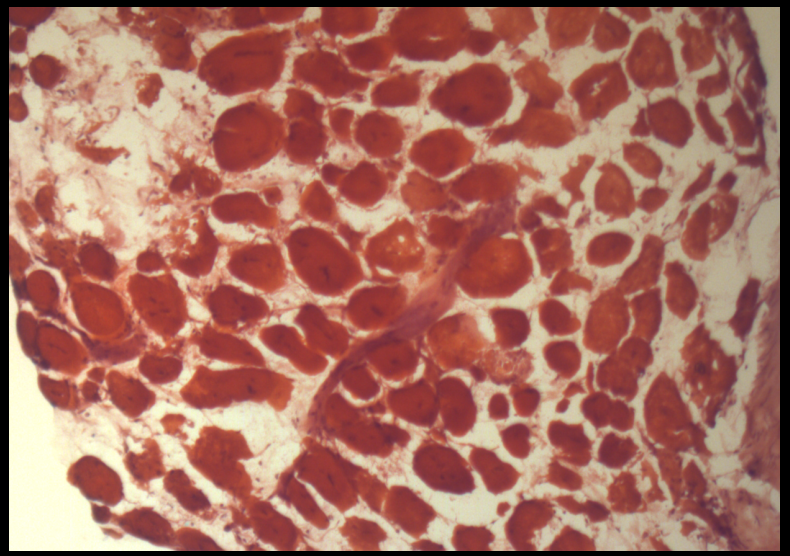


Persistence of exon skipping

Histology - edl - 20 months

mdx

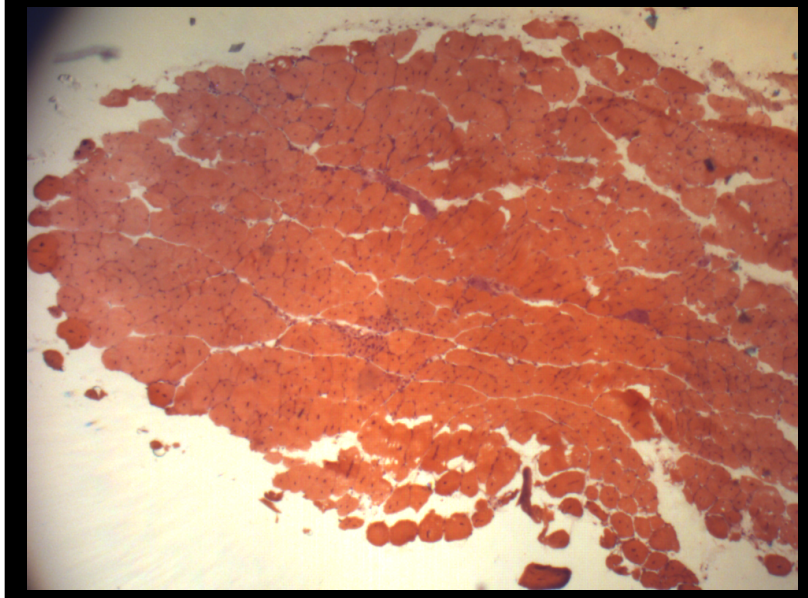


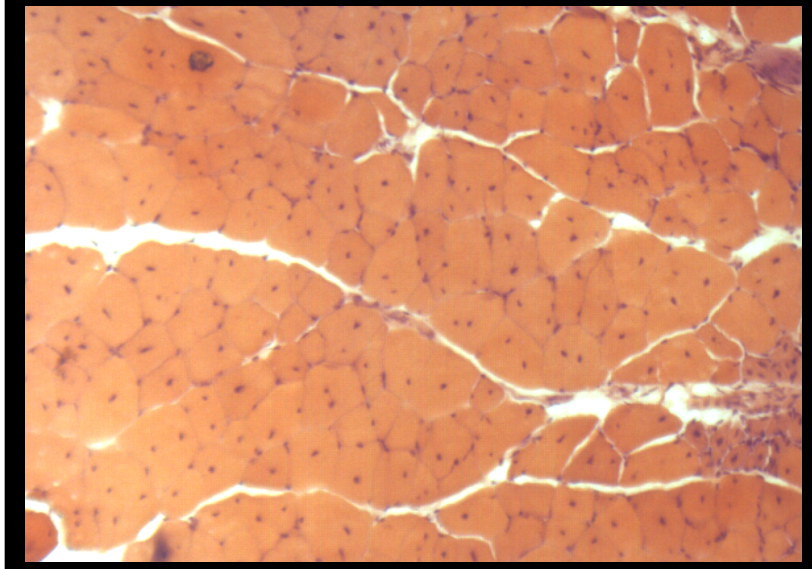


4X

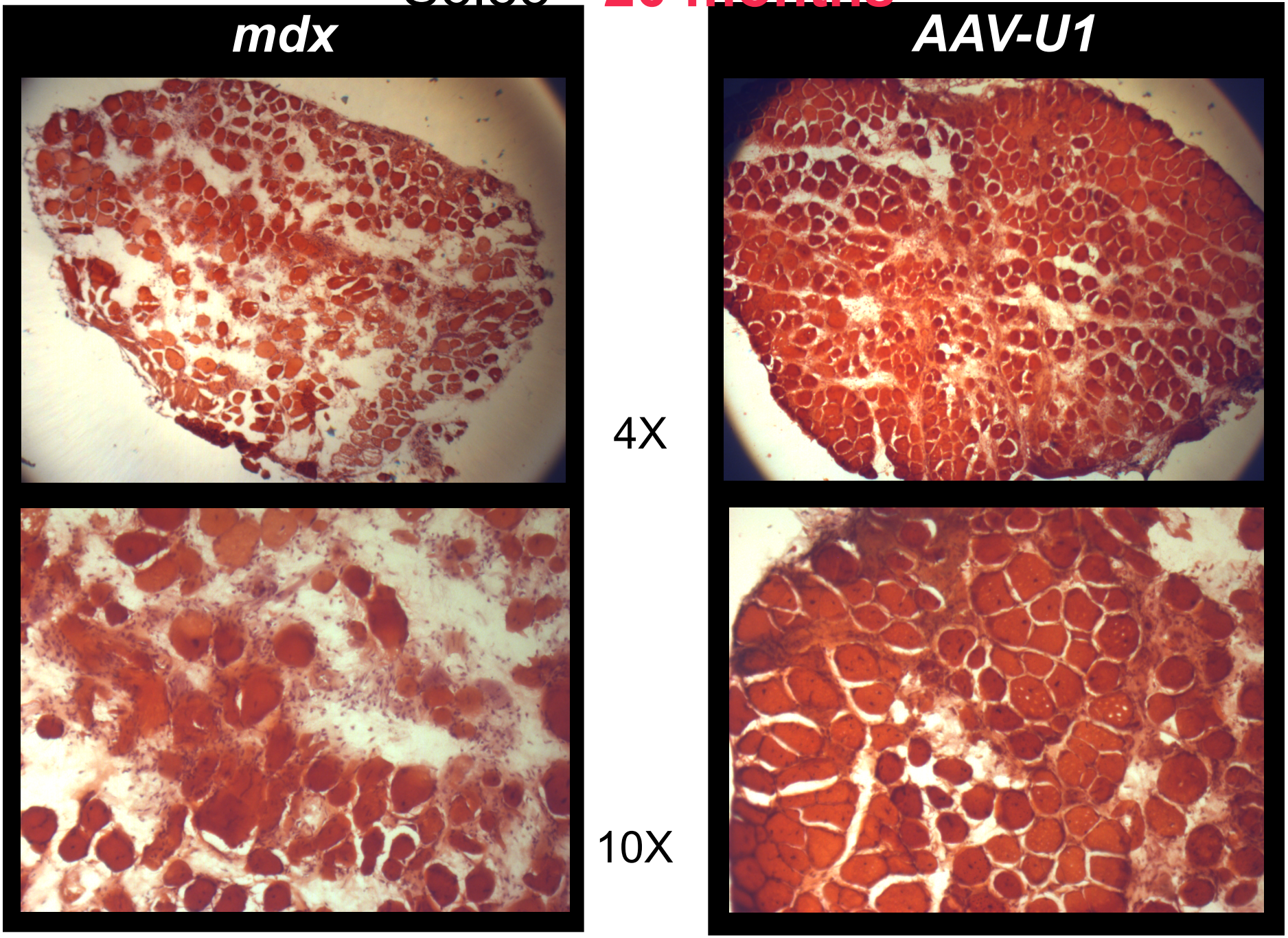
10X

AAV-U1

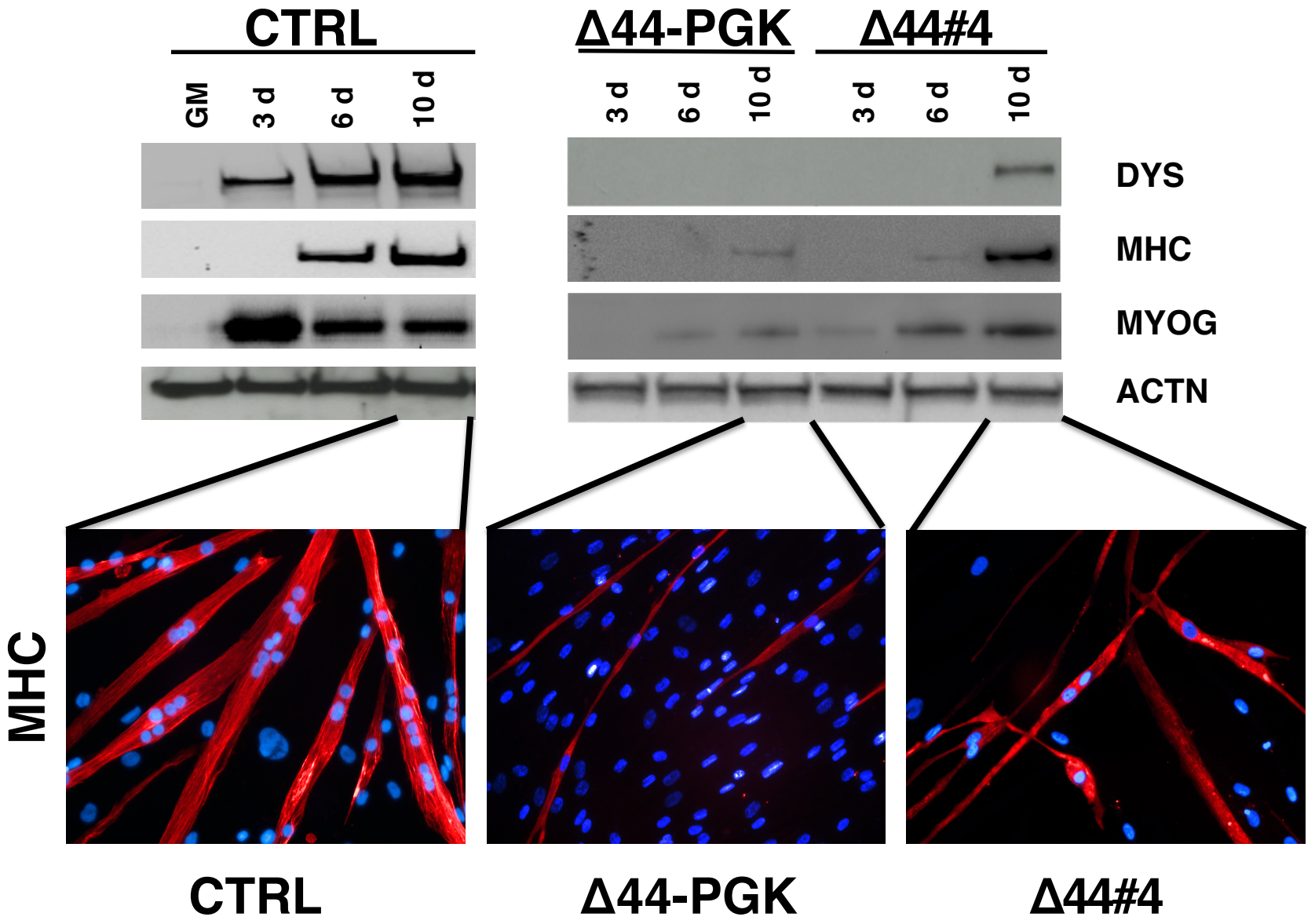




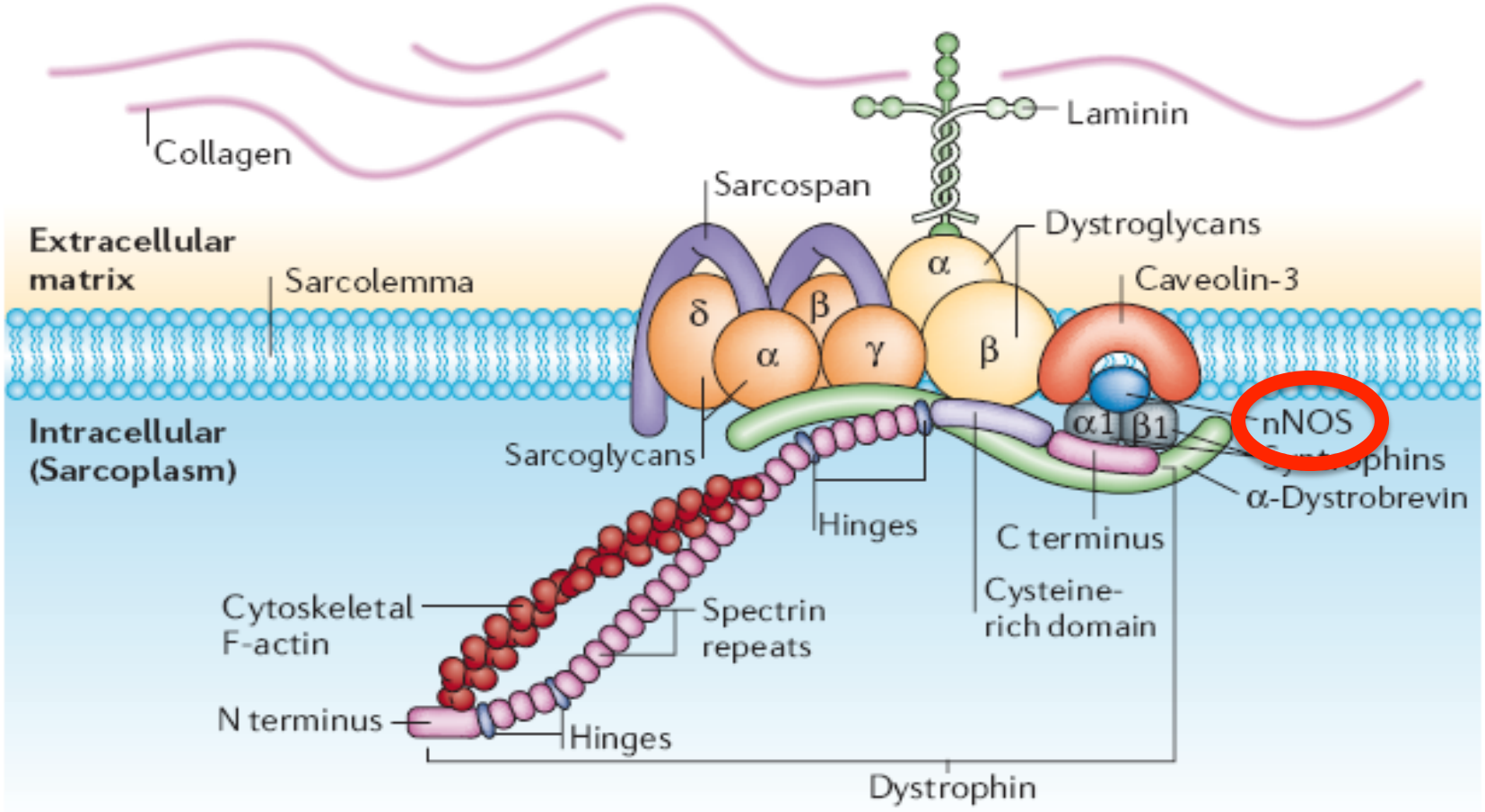
Soleo - 20 months



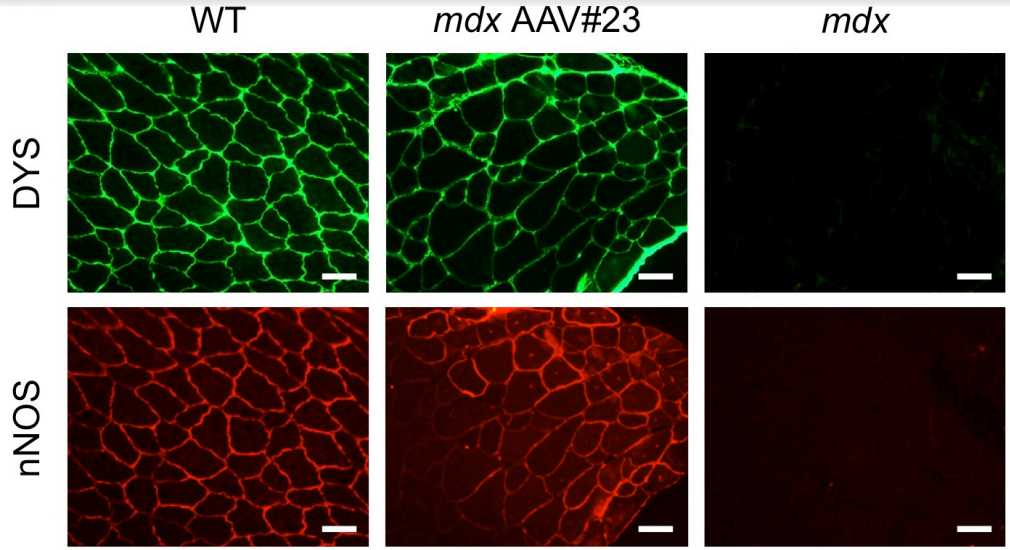
Exon skipping rescues dystrophin expression and correct timing of myogenic marker expression

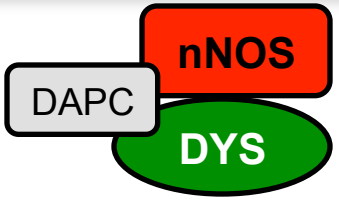


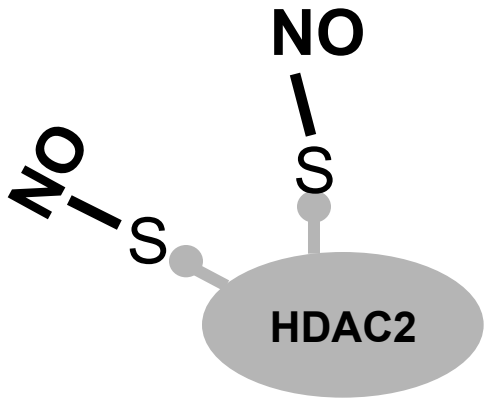
Dystrophin localizes and stabilizes nNOS



Dys stabilizes and activates nNOS

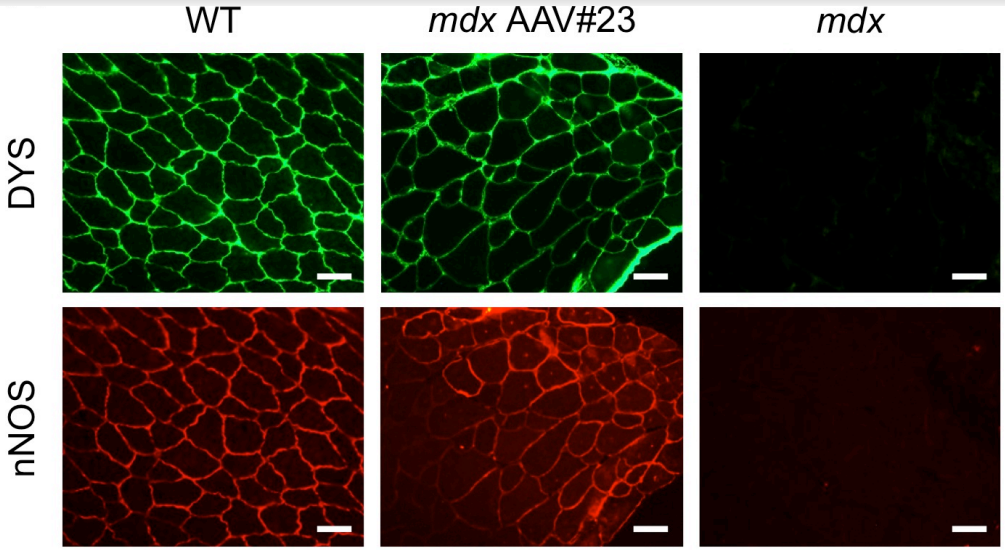


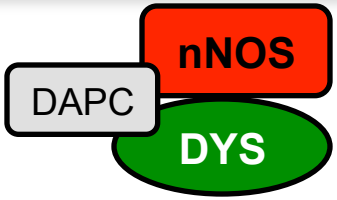


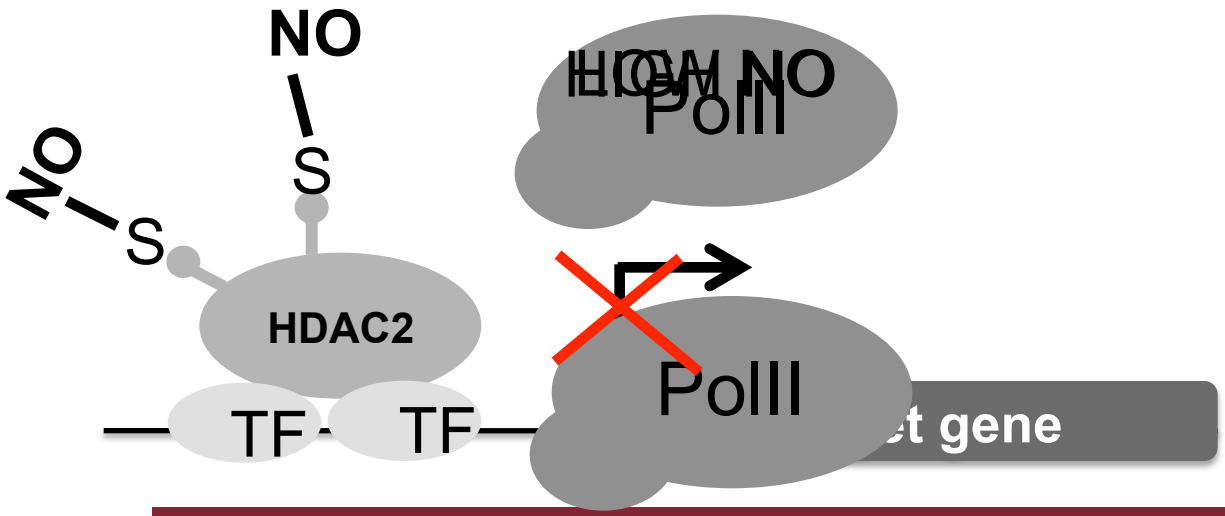


Minetti et al. 2006 Nat Med
Nott et al. 2008 Nature

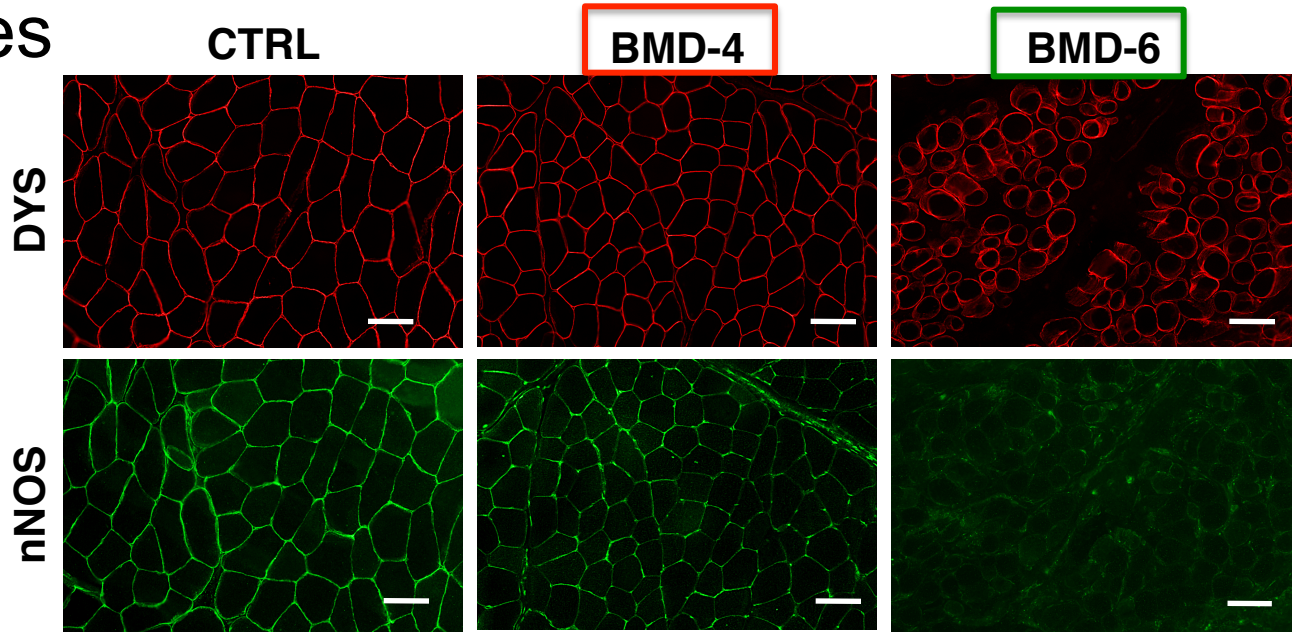
Dys controls NO-HDAC2 through the activation of nNOS



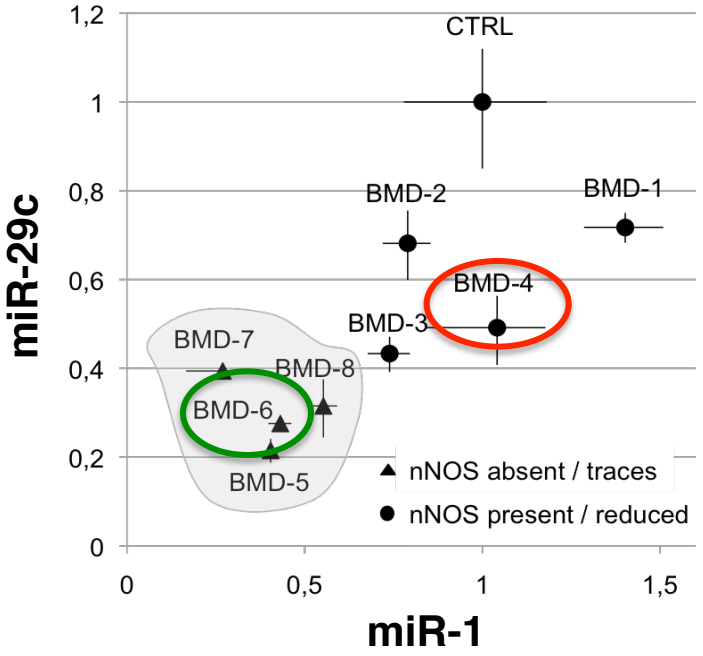




nNOS localization and miRNAs expression in Becker biopsies



ID	Deletion	nNOS
CTRL	/	present
BMD-1	39	present
BMD-2	74	reduced
BMD-3	48-49	present
BMD-4	45-51	present
BMD-5	45 - 47	absent
BMD-6	45 - 49	absent
BMD-7	45-49	absent
BMD-8	42 - 53	traces



Conclusions

- AAV-U1 allows the body wide rescue of dystrophin synthesis even in heart and diaphragm and improves muscle functionality

- AAV-U1 DNA is still present after 18 months from the first injection and maintains efficacy in terms of dystrophin expression, muscle strength and tissue integrity

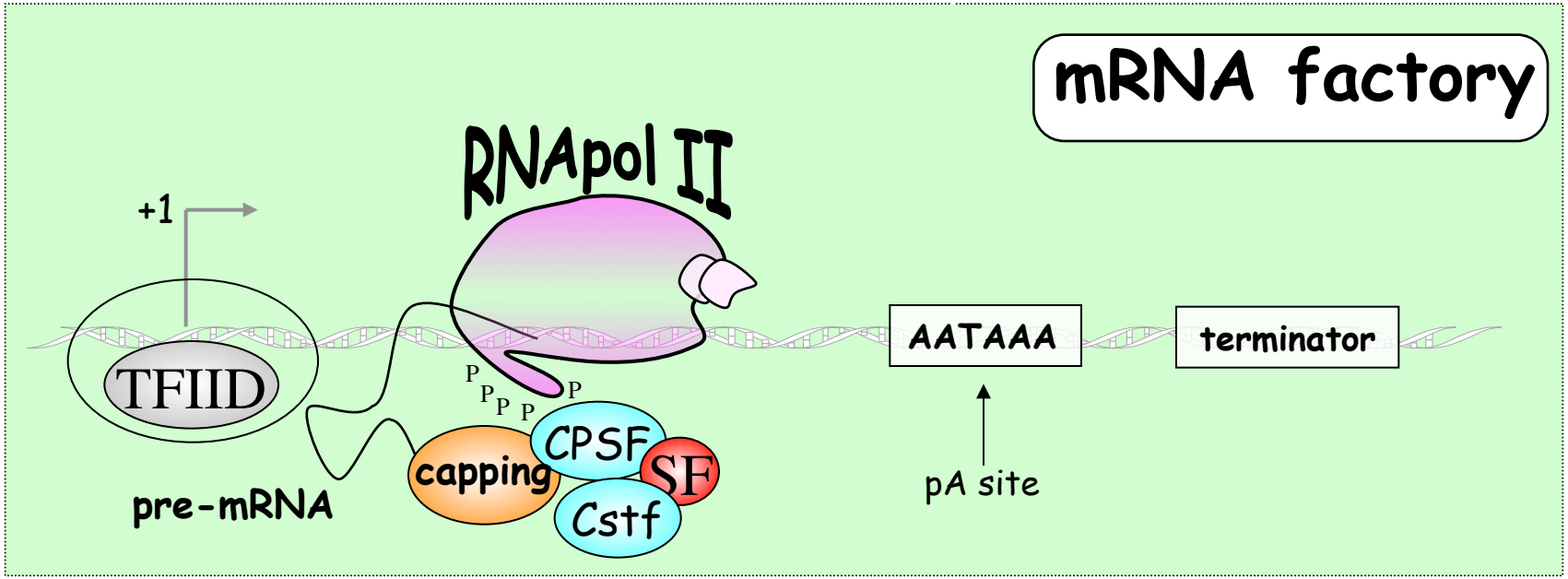
- One single injection is sufficient for producing a long-term benefit (Denti et al., 2008, ***Hum. Gene Ther.***, in press)

La “fabbrica” dell’ mRNA

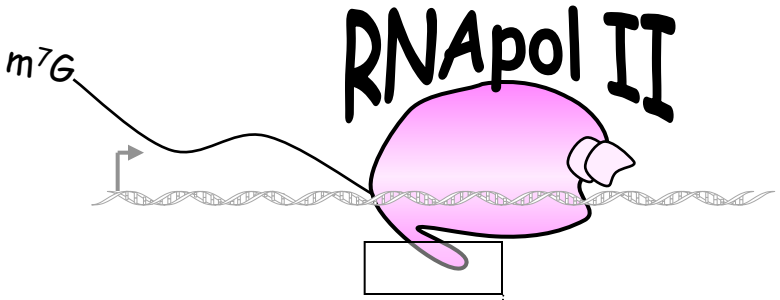
I processi di maturazione dell’ RNA sono accoppiati fisicamente e temporalmente

Gene expression regulation

Coupling plays a critical role in gene expression by **tethering** machines to each other and to their substrates, a mechanism that dramatically increases the specificity of enzymatic reactions. It has been shown that starting from the first steps of gene expression, transcription initiation, the binding of specific factors tags the nascent ribonucleoprotein complexes such as to direct them along specific pathways of maturation



The fate of a specific RNA is determined at the beginning of transcription



CarbossiTerminalDomain

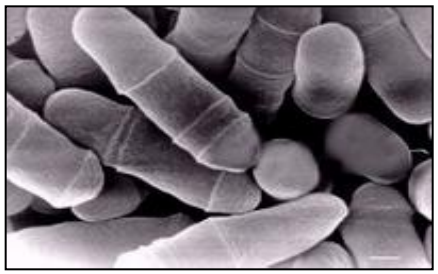
Il CTD è costituito dalla ripetizione di un eptapeptide
Contenente residui di Serina che possono essere fosforilati

	1	2	3	4	5	6	7
1.	Glu	Ala	Pro	Thr	Ser	Pro	Gly
2.	Phe	Gly	Val	Ser	Ser	Pro	Gly
3.	Phe	Ser	Pro	Thr	Ser	Pro	Thr
4.	Tyr	Ser	Pro	Thr	Ser	Pro	Ala
5.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
6.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
7.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
8.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
9.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
10.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
11.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
12.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
13.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
14.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
15.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
16.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
17.	Tyr	Ser	Pro	Thr	Ser	Pro	Ala
18.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
19.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
20.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
21.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
22.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
23.	Tyr	Ser	Pro	Thr	Ser	Pro	Asn
24.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
25.	Tyr	Ser	Pro	Thr	Ser	Pro	Gly
26.	Tyr	Ser	Pro	Thr	Ser	Pro	Ala

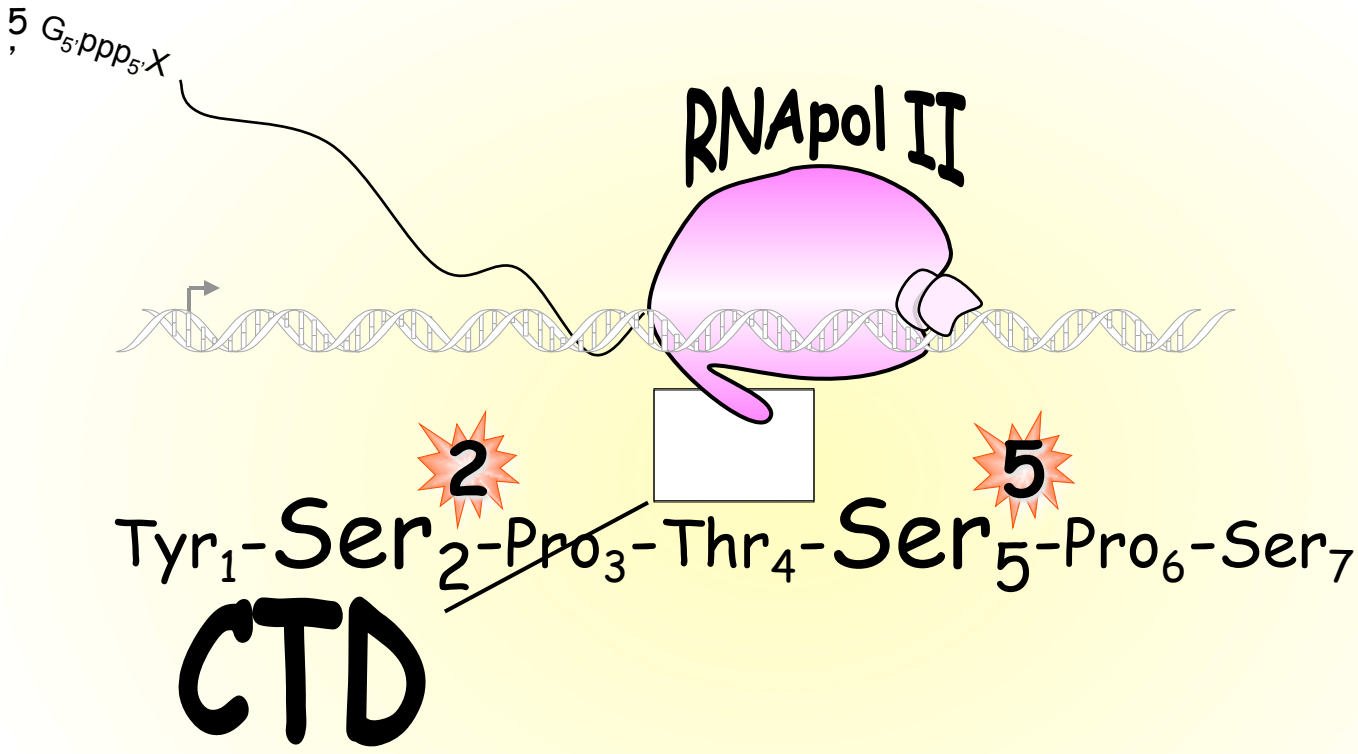
Tyr Ser Pro Lys Gln Asp Glu Gln Lys His Asn Glu Asn Glu Asn Ser Arg

Mouse

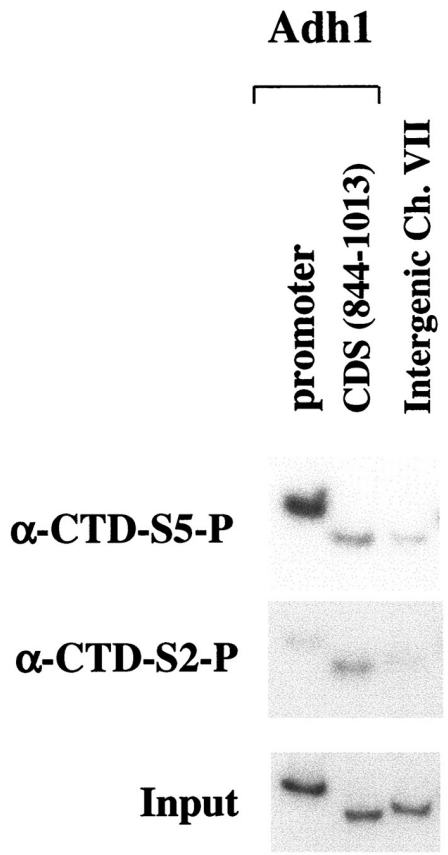
Yeast



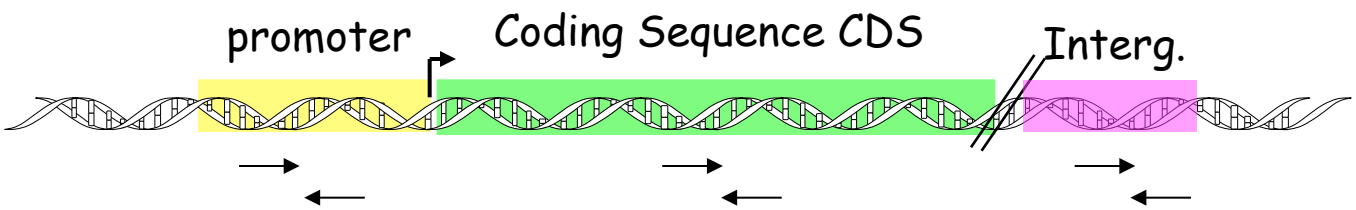
	1	2	3	4	5	6	7
	Glu	Gly	Ala	Met	Ser	Pro	Ser
1.	Tyr	Ser	Pro	Thr	Ser	Pro	Ala
2.	Tyr	Glu	Pro	Arg	Ser	Pro	Gly Gly
3.	Tyr	Thr	Pro	Gln	Ser	Pro	Ser
4.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
5.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
6.	Tyr	Ser	Pro	Thr	Ser	Pro	Asn
7.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
8.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
9.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
10.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
11.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
12.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
13.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
14.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
15.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
16.	Tyr	Ser	Pro	Thr	Ser	Pro	Ala
17.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
18.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
19.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
20.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
21.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
22.	Tyr	Ser	Pro	Thr	Ser	Pro	Asn
23.	Tyr	Ser	Pro	Thr	Ser	Pro	Asn
24.	Tyr	Thr	Pro	Thr	Ser	Pro	Ser
25.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
26.	Tyr	Ser	Pro	Thr	Ser	Pro	Asn
27.	Tyr	Ser	Pro	Thr	Ser	Pro	Asn
28.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
29.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
30.	Tyr	Ser	Pro	Thr	Ser	Pro	Ser
31.	Tyr	Ser	Pro	Ser	Ser	Pro	Arg
32.	Tyr	Thr	Pro	Gln	Ser	Pro	Thr
33.	Tyr	Thr	Pro	Ser	Ser	Pro	Ser
34.	Tyr	Ser	Pro	Ser	Ser	Pro	Ser
35.	Tyr	Ser	Pro	Thr	Ser	Pro	Lys
36.	Tyr	Thr	Pro	Thr	Ser	Pro	Ser
37.	Tyr	Ser	Pro	Ser	Ser	Pro	Glu
38.	Tyr	Thr	Pro	Ala	Ser	Pro	Lys
39.	Tyr	Ser	Pro	Thr	Ser	Pro	Lys
40.	Tyr	Ser	Pro	Thr	Ser	Pro	Lys
41.	Tyr	Ser	Pro	Thr	Ser	Pro	Thr
42.	Tyr	Ser	Pro	Thr	Thr	Pro	Lys
43.	Tyr	Ser	Pro	Thr	Ser	Pro	Thr
44.	Tyr	Ser	Pro	Thr	Ser	Pro	Val
45.	Tyr	Thr	Pro	Thr	Ser	Pro	Lys
46.	Tyr	Ser	Pro	Thr	Ser	Pro	Thr
47.	Tyr	Ser	Pro	Thr	Ser	Pro	Lys
48.	Tyr	Ser	Pro	Thr	Ser	Pro	Thr
49.	Tyr	Ser	Pro	Thr	Ser	Pro	Lys Gly Ser Thr
50.	Tyr	Ser	Pro	Thr	Ser	Pro	Gly
51.	Tyr	Ser	Pro	Thr	Ser	Pro	Thr
52.	Tyr	Ser	Leu	Thr	Ser	Pro	Ala
53.	Ile	Ser	Pro	Asp	Asp	Ser	Asp Glu Glu Asn


5' G5'ppp5'X
RNApol II
2
5
Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7
CTD

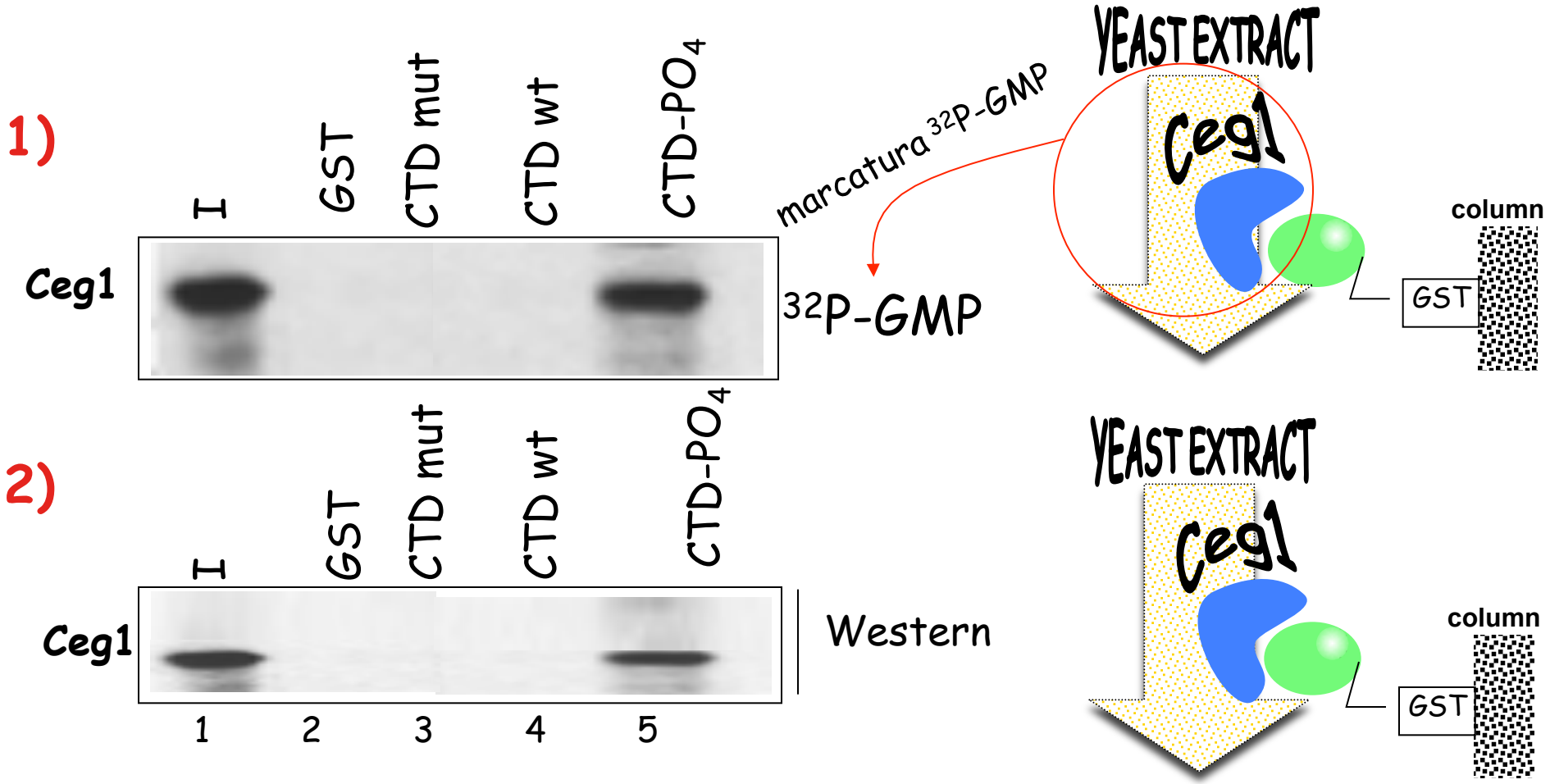
Stato di fosforilazione del CTD della RNA polII durante la trascrizione



Buratowski e colleghi eseguirono un'analisi mediante ChIP dell'associazione delle due forme fosforilate del CTD con la cromatina vicina o lontana ai promotori dei geni ADH1 e PMA1. Trascrizione del gene ADH1. La cromatina viene precipitata con anticorpi diretti contro il CTD fosforilato sulla Ser2 o sulla Ser5 dell'eptamero (αCTD-ser2, αCTD-ser5). Sul DNA estratto viene poi eseguita una amplificazione per PCR con primer specifici per regioni vicine o lontane dal promotore, e una regione intergenica, come indicato nello schema in basso. La cromatina di partenza non immunoprecipitata è indicata come INPUT

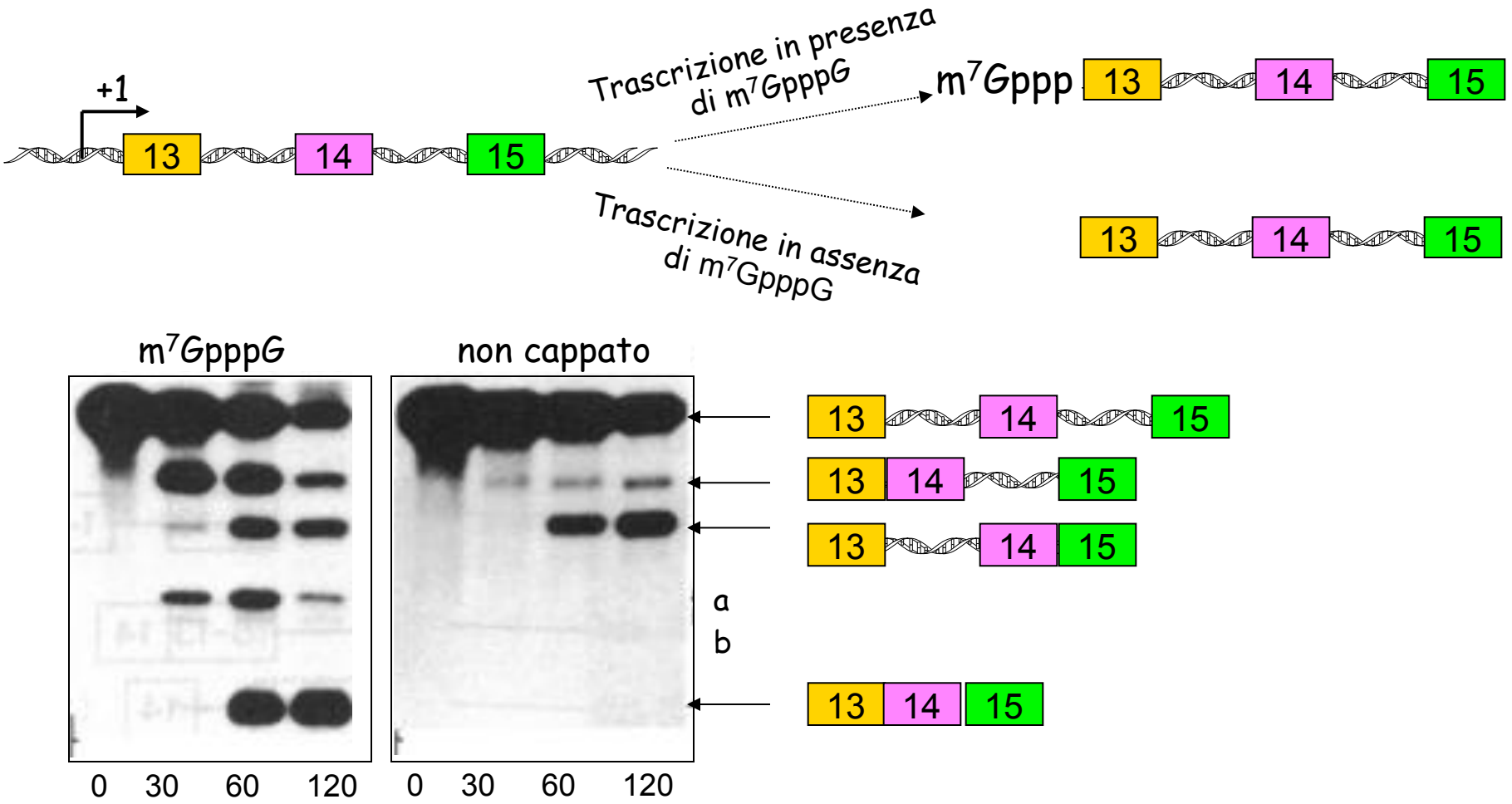


Gli enzimi di capping legano il CTD fosforilato



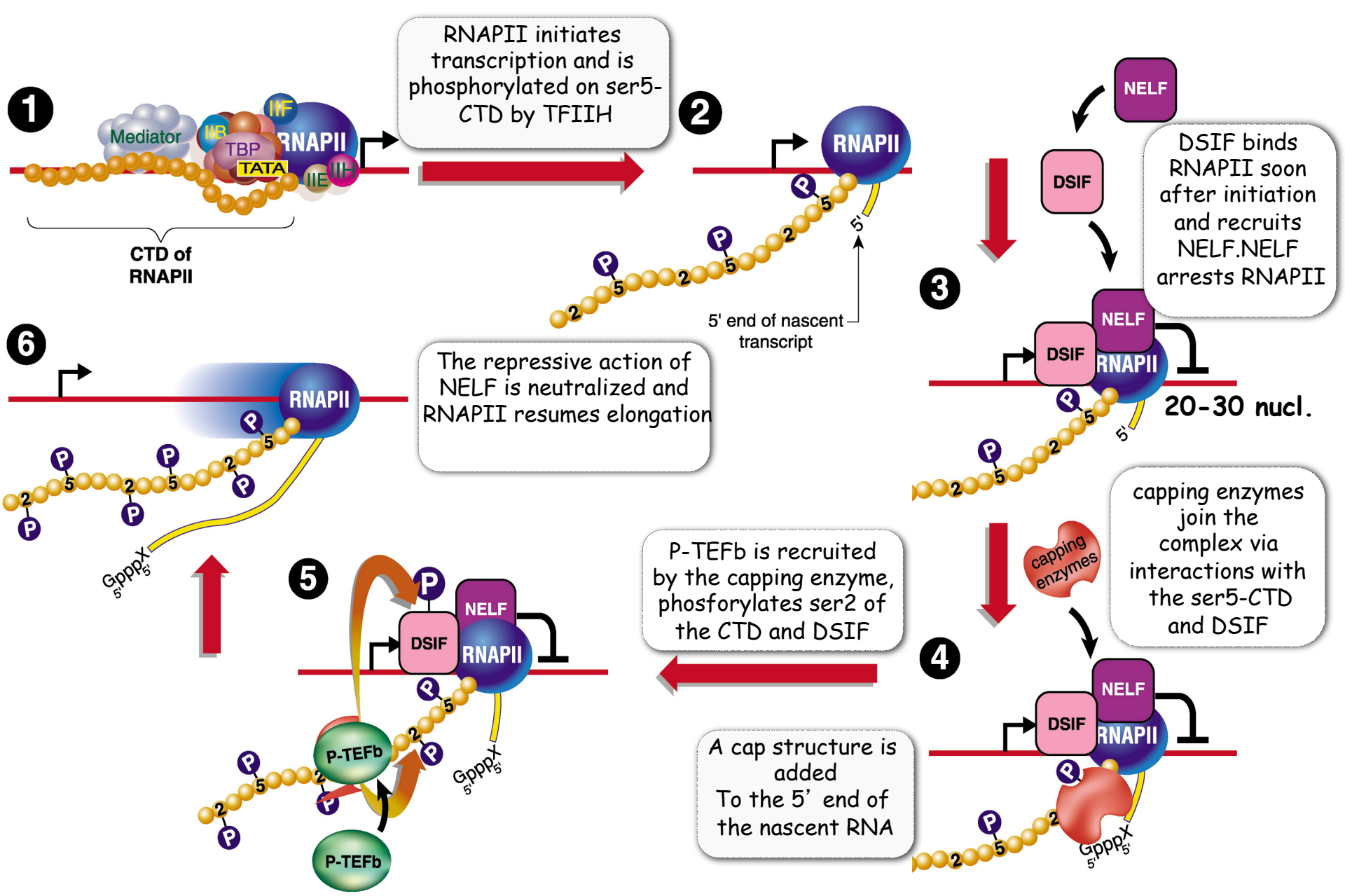
Bentley e colleghi sottoposero un estratto nucleare di cellule HeLa a cromatografia di affinità su resine contenenti le sostanze indicate in alto, quindi saggiarono gli eluati per la guanililtrasferasi misurando: 1) la formazione di un complesso con il ^{32}P(GMP) che può essere misurato mediante SDS-PAGE e autoradiografato; 2) con western blot. "I" indica l'estratto totale caricato su colonna.

Il Cap aumenta l'efficienza di splicing

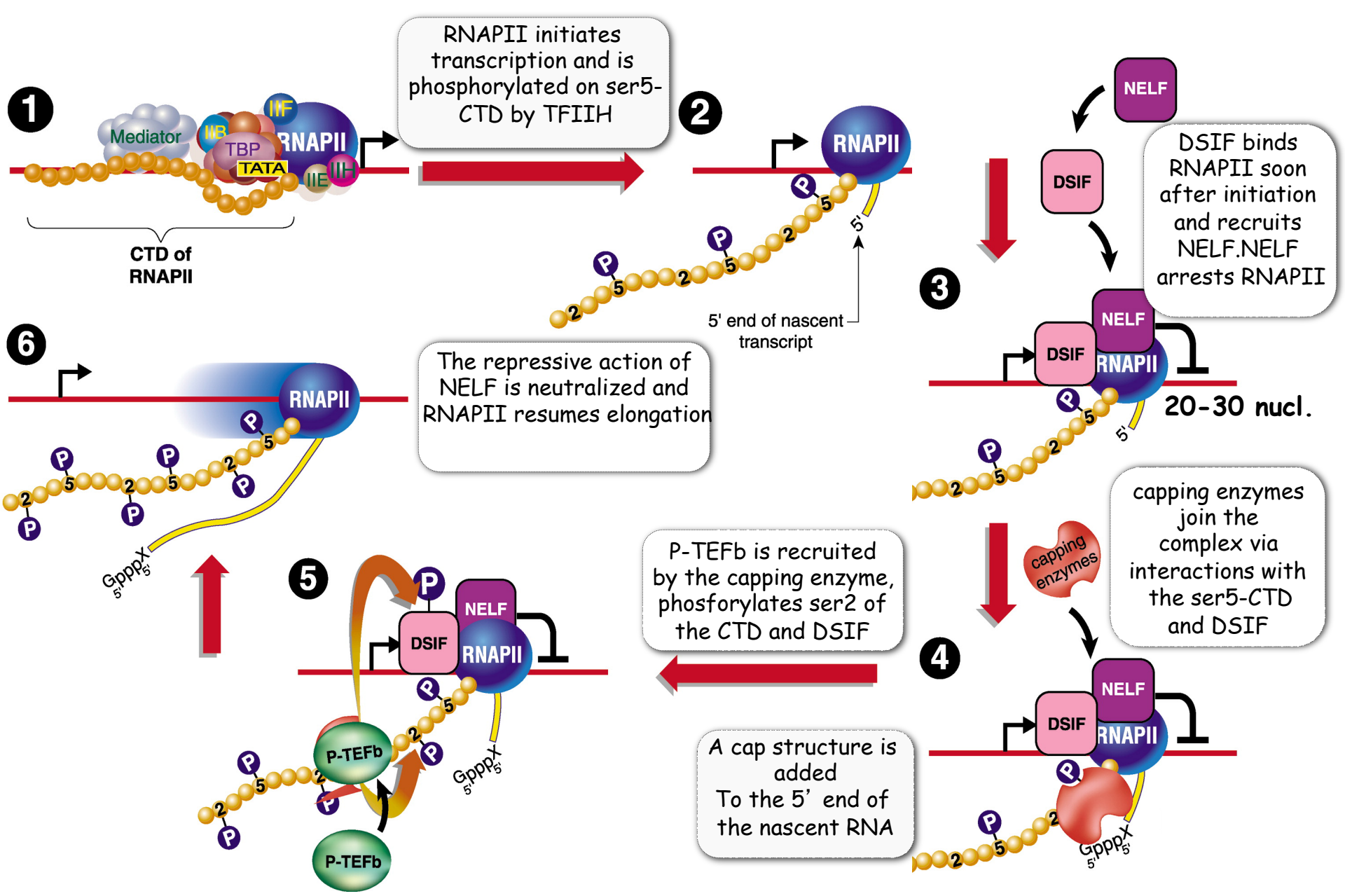


Shimura e colleghi costruirono substrati di splicing modello marcati con P^{32} e con e senza cap. Quindi li incubarono in estratti nucleari di cellule HeLa. Infine, sottoposero a elettroforesi e ad autoradiografia i gel per identificare i precursori di splicing, gli intermedi ed i prodotti finali. La posizione degli RNA più abbondanti è indicata schematicamente a destra. La banda "a" è il lariat del primo introne; la banda "b" è l'RNA lineare contenete gli esoni 13 e 14 con il primo introne in mezzo.

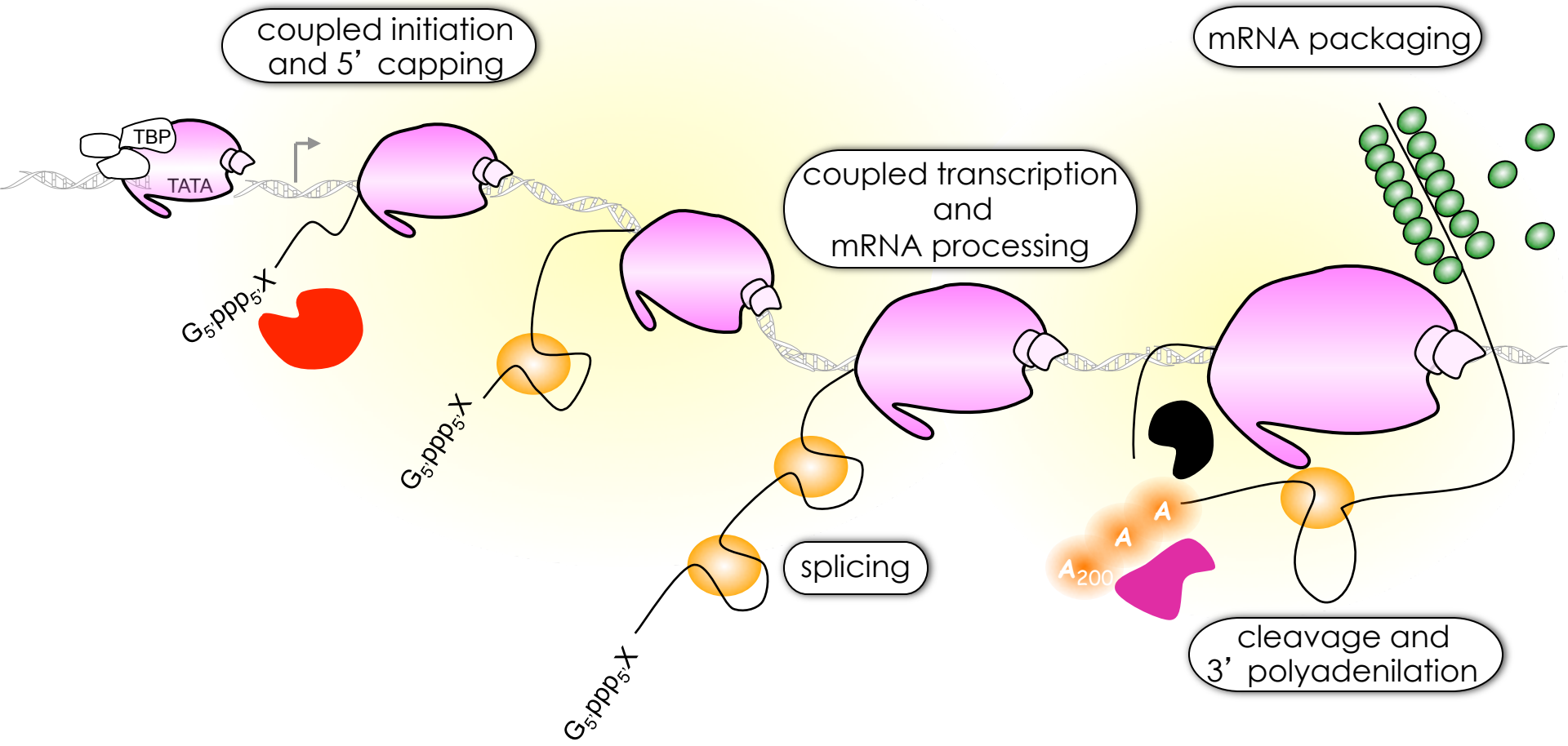
Checkpoint model for coupling the 5' pre-mRNA capping and transcription elongation



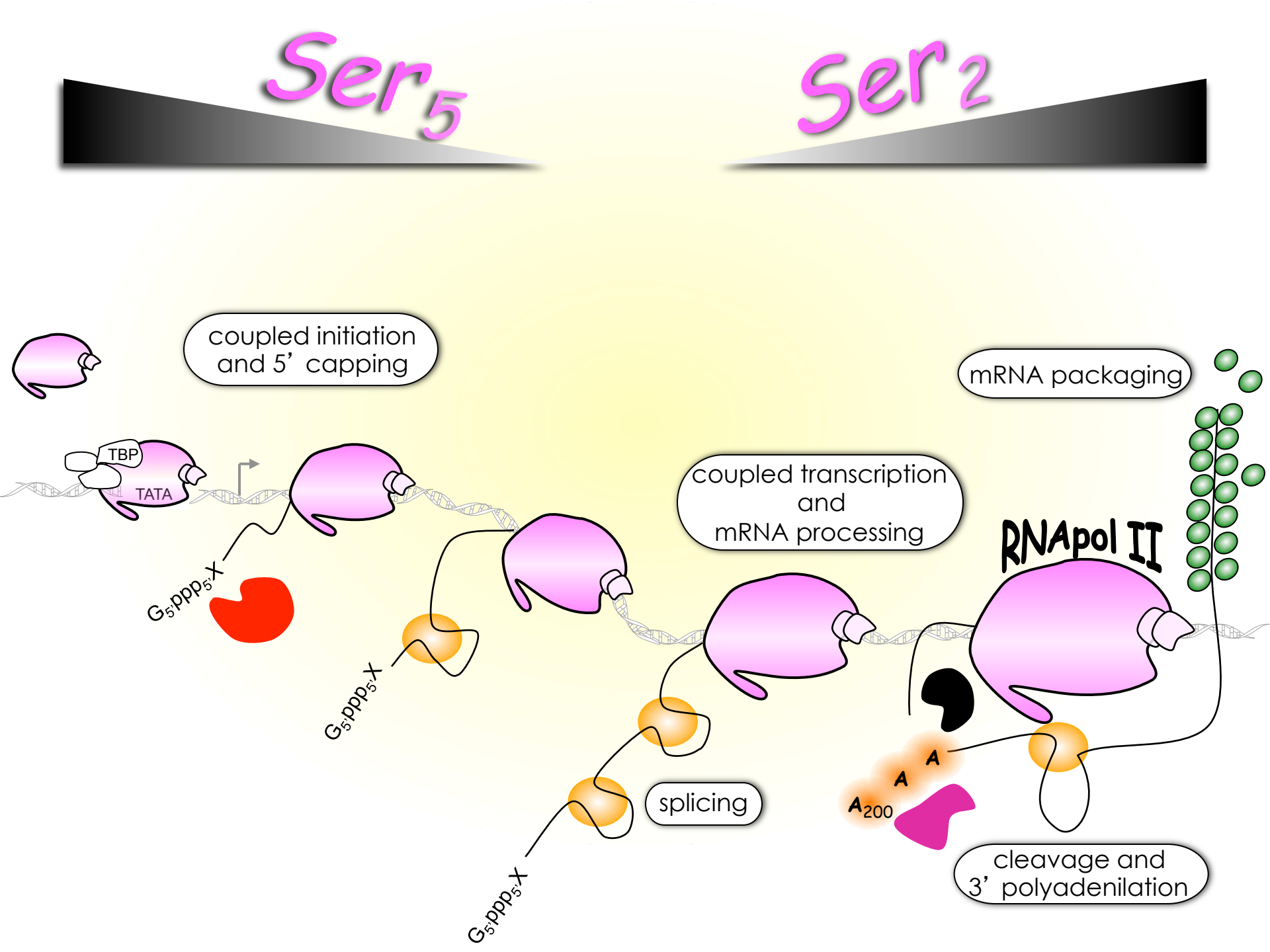
Checkpoint model for coupling the 5' pre-mRNA capping and transcription elongation



La fabbrica dell'RNA



Il destino di uno specifico trascritto è determinato all'inizio della trascrizione


Ser5
Ser2
coupled initiation and 5' capping
TBP
TATA
G5'ppp5'X
G5'ppp5'X
G5'ppp5'X
coupled transcription and mRNA processing
RNApol II
mRNA packaging
splicing
A
A
A200
cleavage and 3' polyadenilation