Signal dependency of Tra2-beta1 alternative splicing

Der Naturwissenschaftlichen Fakultät
der Friedrich-Alexander-Universität Erlangen-Nürnberg
zur
Erlangung des Doktorgrades

vorgelegt von
Natalya Benderska
aus Odessa, Ukraine

2008

Als Dissertation genehmigt von der Naturwissenschaftlichen Fakultät
der Universität Erlangen-Nürnberg.

Tag der mündlichen Prüfung:	23.07.2008
Vorsitzender der Prüfungskomission:	Prof. Dr. Eberhard Bänsch
Erstberichterstatter:	Prof. Dr. Andreas Burkovski
Zweitberichterstatter:	Prof. Dr. Michael Wegner

To my son

Acknowledgments

First, I would like to express my gratitude to my supervisor Prof. Dr. Stefan Stamm for giving me the opportunity to do my doctoral thesis at the Institute of Biochemistry, University of Erlangen-Nürnberg. I would like to thank him for his constant guidance, understanding and patience during the PhD course, and for his critical reading of this thesis.

I want sincerely to thank all my colleagues and friends from Stamm's lab Tatyana Novoyatleva, Bettina Heinrich, Zhaiyi Zhang, Shivendra Kishore, Yesheng Tang, Jingyi Hui, Amit Khanna, Dominique Olbert and Ilona Rafalska for their help and support in scientific and daily life.

I am grateful to Prof. Dr. Henning Urlaub and Dr. Mads Grönborg (Max-Planck Institute, Göttingen) for mass-spectrometry analysis, performed for my scientific project.

Also, I would like to thank Prof. Dr. Peter Gmeiner and his Diploma student Andrea Pinsker for the chemical synthesis of cantharidine derivatives (Institute of Pharmacy, Erlangen).

My gratitude goes to Dr. Horst Wolff from the Institute of Molecular Virology, Neuherberg, for performing protein shuttling assay.

I am very grateful to Dr. Denis Herve' and Dr. Jean-Antoine Girault (INSERM, France) for providing DNA plasmids of DARPP-32 and DARPP-antibody for my research.

I would like to thank Prof. Dr. Michael Wegner and his group members for the help with microscopic devices in my experimental work.

I would like to thank Prof. Dr. Cord-Michael Becker and Dr. Ralf Enz and their group members for sharing their resources with us.

I am very grateful to Dr. Said Hashemolhosseini's group, especially to Tatiana Cheusova, Amir Khan and Steffen Schubert.

I am indebted to Prof. Dr. Andreas Burkovski for undertaking the revision of this thesis.

I would like to thank all my Teachers of Biology from Odessa National University.

In the end, I wish to thank my family who has believed in me and for their unfailing support and encouragement during my work in Germany.

Большое спасибо моим родным за вашу веру в меня, поддержку и терпение!

PUBLICATIONS

Parts of this work are included in the following publications:

Benderska, N., Hui, J., Novoyatleva, T. and Stamm S. (2008) Regulation of alternative splicing through cAMP-dependent phosphorylation of Tra2-beta1 splicing factor. (manuscript in preparation)

Heinrich, B., Raitskin, O., Hiller, M., **Benderska, N.,** Hartmann, A., Bracco, L., Elliott, D., Ben Ari, S., Soreq, H., Sperling, J., Sperling, R. and Stamm S. (2008) hnRNP G regulates splice site selection by binding to CC(A/C) rich regions in pre mRNA. (submitted)

Novoyatleva, T., Heinrich, B., Tang, Y., **Benderska, N.,** Butchbach, M., Lorson, C., Lorson, M., Ben-Dov, C., Fehlbaum, P., Bracco, L., Burghes, A, Bollen, M. and Stamm, S. (2008) Protein phosphatase 1 binds to the RNA recognition motif of several splicing factors and regulates alternative pre-mRNA processing. *Hum Mol Genet* 17(1):52-70.

Tang, Y., Novoyatleva, T., **Benderska, N**., Kishore, S., Thanaraj, T.A. and Stamm S. (2004) Analysis of alternative splicing in vivo using minigenes. In Westhof, Bindereif, Schön and Hartmann (eds.), *Handbook of RNA Biochemistry*. Wiley-VCH:755-782.

Stoss, O., Novoyatleva, T., Gencheva, M., Olbrich, M., **Benderska**, **N**. and Stamm S**.** (2004) p59(fyn)-mediated phosphorylation regulates the activity of the tissue-specific splicing factor rSLM-1**.** *Mol Cell Neurosci* 27(1):8-21.

Rafalska, I., Zhang, Z., **Benderska, N**., Wollf, H., Hartmann, AM., Brack-Werner, R., Stamm, S. (2004) The intranuclear localization and function of YT521-B is regulated by tyrosine phosphorylation**.** *Hum Mol Genet* 13(15):1535-49.

CONTENTS

FIGURES AND TABLES

FIGURES

TABLES

ABBREVIATIONS

AKAP	Protein kinase A anchoring protein
AMP	adenosine monophosphate
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	amyloid precursor protein
ATP	adenosine 5'tri phosphate
ASD	alternative splicing database
ASF	alternative splicing factor
ATP	adenosine 5'-triphosphate
Bcl 2	B-cell leukemia/lymphoma 2
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CaMKII	calmodulin-dependent protein kinase II
CBs	Cajal bodies
CCK	cholecystokinin receptor
Cdk	cyclin dependent kinase (2 or 5)
Cdc2	cycline dependent kinase 2
CDC5	cell division cycle 5-like protein
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator ATP-binding cassette subfamily C member 7
CFP	cyan fluorescent protein
CGRP	calcitonin gene-related peptide
CLK	CDC2-like kinase
CLB	clathrin light chain B gene
CK1	casein kinase 1
CK2	casein kinase 2
CPI-17	protein kinase C (PKC)-dependent phosphatase inhibitor of 17 kDa
CTD	carboxyterminal domain (of RNA polymerase II)
CYP	cerebrotendinous xanthomatosis
DARRP-32	dopamine- and cAMP-regulated phosphoprotein 32 kDa
dH_2O	distilled water
DMEM	Dulbeco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotidtriphosphate
Dscam	Down syndrome cell adhesion molecule
dsx	doublesex
DTT	dithiothreitol
ECL	enhanced chemiluminiscence
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallocatechin gallate
EGFP	enhanced green fluorescent protein
ERK	extracellular receptor kinase
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
ESSENCE	exon-specific splicing enhancement by small chimeric effectors
EST	expressed sequence tag
FCS	fetal calf serum
FF domain	two phenylalanines domain
FHA	forkhead-associated domain,
FTDP-17	frontotemporal dementia with Parkinsonism linked to chromosome 17

9G8	splicing factor, arginine/serine-rich 7
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
G-Proteins	guanosine triphosphate binding protein
GSK-3	glycogen synthase kinase 3
GST	glutathione S-transferase
HDAC	histone deacetylase
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HLA	histocompatibility leukocyte antigens
hnRNP	heterogenous nuclear ribonucleoprotein
HRP	horseradish peroxidase
HU	hydroxyurea
Il-4	interleukin-4
IPTG	isopropyl-D-1-thiogalactopyranoside
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
kDa	kilodalton
KH	domain hnRNP K homology domain
MAPK	mitogen-activated protein kinase
mGluR7b	metabotropic glutamate receptor
MEL	murine erythroleukaemia kinase
mRNA	messenger RNA
NaB	sodium butyrate
NE	nuclear extract
Nek2	NIMA related protein kinase 2
Ni-NTA	nickel-nitrilotriacetic acid
NIPP1	nuclear inhibitor of protein phosphatase 1
NMD	nonsense-mediated decay
NMDAR1	N-methyl-D-aspartate receptor 1
NO	nitric oxide
NPC	nuclear pore complex
nt	nucleotide
NF-L	Neurofilament L
PABPC3	Polyadenylate binding protein 3
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHI	phopsphatase holoenzyme inhibitor
PICK1	protein interacting with PKC
p54nrb (NONO)	Non-POU domain-containing octamer-binding, 54kDa nuclear RNA- and DNA-binding protein
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PMSF	phenylmethanesulfonyl fluoride
PP	protein phosphatase
PPM	Mg^{2+}-dependent phosphatases
PSF1	polypyrimidine tract binding protein assotiated splicing factor
PRP31	pre-mRNA processings factor 31
PTP	protein tyrosine phosphatases
RBM6	RNA binding motif protein 6
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolutions per minute
RRM	RNA recognition motif
RT-PCR	reverse transcription followed by polymerase chain reaction

SAF	scaffold attachment factor (A or B)
Sam-68	Src associated in mitosis 68kDa protein
SAP155	spliceosome-associated protein 155
SF3b155	splicing factor 3B subunit 1/Spliceosome-associated protein 155
SFPQ	splicing factor proline/glutamine-rich
SC35	splicing component, 35 kDa; splicing factor, arginine/serine-rich 2
SDS	sodium dodecyl sulfate
SF	splicing factor (1 or 2)
SFRS14	splicing factor, arginine/serine-rich 14
SIP1	SMN Interacting Protein 1
SH	Src homology domain (2 or 3)
SLM	Sam-68 like mammalian protein (1 or 2)
SMA	Spinal Muscular Atrophy
SMN	Survival Motor Neuron gene (1 or 2)
SnoRNP	small nucleolar ribonucleoprotein
SnRNP	small nuclear ribonucleoprotein paricle
SIP	SMN interacting protein
Src	Rous Sarcoma virus kinase
SR-protein	serine-arginine- rich protein
SRm 160/300	SR-related nuclear matrix proteins of 160 and 300 kDa
STAR	signal transduction and activation of RNA
TBE	tris-borate-EDTA buffer
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
tRNA	transfer RNA
Tra2	transformer 2
tRNA	transfer RNA
TCA	Trichostatin
U1 70K	U1 snRNP 70 kDa protein
U2AF	U2 snRNP auxiliary factor (35 or 65 kDa)
UTR	untranslated region
WW domain	two highly conserved tryptophans
YTH domain	YT521-B homology domain

ZUSAMMENFASSUNG

Alternatives Spleißen ist einer der wichtigsten Mechanismus, um die Expression zellulärer und viraler Gene zu modulieren und erhöht die Anzahl der mRNA, die aus einem Gen gebildet wird. Dies ermöglicht die Synthese mehrerer strukturell und funktionell verschiedener Protein-Isoformen aus einem einzigen Gen. Die Faktoren, die an prä-mRNA Spleißen beteiligt sind und die Kontrolle ihrer Interaktion sind noch kaum verstanden.

Diese Arbeit konzentriert sich auf die Kontrolle der Spleißstellenauswahl durch unterschiedliche Signaltransduktionswege. Die Rolle der Proteine rSLM-1, Tra2-beta1 und DARPP-32 bei der Spleißstellenauswahl wurde untersucht.

Im ersten Teil der Arbeit konzentrierte ich mich auf die Sam68-ähnlichen Proteine rSLM-1 und rSLM-2, die zu der STAR Proteinfamilie gehören. Wir fanden auffällige Unterschiede in ihrer Expression in verschiedenen Gehirnregionen. Beide Spleißfaktoren können die Benutzung des alternativen Exons des SMN2 Reporter Minigens verändern. Die Untersuchung möglicher Regulationsmechanismen zeigt, dass rSLM-1 durch diverse Nicht-Rezeptor Tyrosinkinasen phosphoryliert wird, und dass $p59^{fyn}$-vermittelte Phosphorylierung die Fähigkeit von rSLM-1 aufhebt, die Spleißstellenauswahl zu regulieren.

Der zweite Teil dieser Untersuchung ist Tra2-beta1 gewidmet, einem SR-ähnlichen Protein, das ein Protein Phosphatase 1 Bindemotiv und eine evolutionär konservierte Region oberhalb des RVDF-Motivs enthält. Wir untersuchten die funktionelle Rolle dieses Peptids und fanden heraus, dass es entscheidend für den Einfluss der Spleißstellenauswahl von Tra2-beta1 ist. Hemmung der Tra2-beta1 Dephosphorylierung durch PP1 Inhibitoren oder erhöhte cAMP Level führt zur Verwendung des alternativen Exons 7 des SMN2 Gens. Wir zeigen, dass das Dopamin- und cAMP regulierte Phosphoprotein (DARPP-32) mit Tra2-beta1 in vivo und in vitro interagiert. DARPP-32 kann die Spleißstellenauswahl ändern. Es verursacht den Ausschluss von Exon 10 des tau Minigens. Die Modulierung einzelner Komponenten von Signaltransduktionssystemen beeinflusst alternatives Spleißen von prä-mRNA Transkripten durch Phosphorylierung und Dephosphorylierung des Tra2-beta1 Spleißfaktors.

Zusammengenommen weist die Interaktion zwischen humanem Tra2-beta1 und DARPP-32 auf eine bisher unbekannte Funktion von Tra2-beta1 in der cAMP-abhängigen Regulation der Spleißstellenauswahl hin. Unsere Ergebnisse legen ein neues Element in den zahlreichen Mechanismen, die alternatives Spleißen regulieren, nahe. Es erlaubt die Regulation von Spleißstellen durch zelluläre Signale.

ABSTRACT

Alternative splicing is a major mechanism for modulating the expression of cellular and viral genes and enables a single gene to increase its coding capacity, allowing the synthesis of several structurally and functionally distinct protein isoforms from a single gene. The factors involved in pre-RNA splicing and how the interaction of the various splicing constituents is controlled are still poorly understood.

This work focuses on control of splice site selection through different signal transduction pathways. The role of the proteins rSLM-1, Tra2-beta1 and DARPP-32 in splice site selection was studied.

In the first part of the work I focused on the Sam68-like mammalian proteins: rSLM-1 and rSLM-2 which belong to the STAR family of proteins. We found striking differences in their expression in various brain areas. Both splicing factors can change alternative exon usage of SMN2 reporter minigene. The study of possible regulatory mechanisms shows that rSLM-1 is phosphorylated by several non-receptor tyrosine kinases and $p59^{fyn}$-mediated phosphorylation abolishes the ability of rSLM-1 to regulate splice site selection.

The second part of this research is dedicated to Tra2-beta1, an SR-related protein which contains a protein phosphatase 1 binding motif and an evolutionary conserved region downstream of RVDF. We investigated the functional role of this peptide and found it to be crucial for the influence of splice site selection of Tra2-beta1. Blocking of Tra2-beta1 dephosphorylation by inhibitors of PP1 or an increasing cAMP level leads to promotion of the alternative exon 7 of SMN2 gene. We showed that the dopamine- and cAMP-regulated phosphoprotein (DARPP-32) interacts with Tra2-beta1 in vivo and in vitro. DARPP-32 can change splice site selection. It promotes skipping of exon 10 of the tau minigene. Modulation of certain components of signal transduction system influence alternative splicing of pre-mRNA transcripts by phosphorylation and dephosphorylation of the Tra2-beta1 splicing factor.

Taken together, the interaction between human Tra2-beta1 and DARPP-32 indicates a previously unknown function of Tra2-beta1 in the cAMP-dependent regulation of splice site selection. Our finding suggests a new element in the multiple mechanisms that regulate alternative splicing. It allows the convergence of their regulatory signals on splice site selection.

1. INTRODUCTION

Recent genomic analyses have revealed that increase in proteins is achieved by a limited number of 25 000-39 000 genes in humans (Venter et al., 2001). These data underscore thereby the importance of transcriptional and post-transcriptional mechanisms in the increase of protein diversity.

Among these mechanisms, which include the use of multiple transcription start sites, polyadenylation, pre-mRNA editing, and post-transcription protein modifications, alternative splicing is considered to be the most important source of different protein molecules in vertebrates (Black, 2003; Graveley et al., 2001; Maniatis et al., 2002). The most striking example is the Drosophila Dscam gene which contains 95 alternatively spliced exons and has the potential to generate over 38 000 different mRNA isoforms, which is 2-3 times the number of predicted drosophila's genes (Schmucker et al., 2000).

Human genes contain exons separated by introns. Introns are non-coding sequences that are removed during the splicing process. As soon as the primary transcript comes out of the transcriptional machinery, exon-intron and intron-exon boundaries (splice sites) are recognized by the splicing machinery (the spliceosome), which is composed of >300 proteins (Jurica and Moore, 2003). These proteins belong to major two distinct groups - heterogenous nuclear ribonucleoparticle protein families (hnRNPs) and serine-arginine rich (SR) proteins.

Alternative splicing can be regulated by both cis-acting RNA elements, such as splicing enhancers and splicing silencers, and trans-acting factors - members of the SR protein family, which most often bind enhancer elements to facilitate the recognition and activation of weak splice sites (Li et al., 2003). Phosphorylation-dephosphorylation of the arginine-serine dipeptides of SR proteins is also known to affect the activity and subnuclear localization (Caceres et al., 1998; Kanopka et al., 1996; Xiao et al., 1997; Xiao et al., 1998; Hartmann et al., 2001; Stamm, 2008).

The differential exon content of gene transcripts can change the nature of the encoded proteins and the biological function of gene products (Kriventseva et al., 2003; Stamm, 2005; Blencowe et al., 2006).

Wrong splice site usage has been observed in numerous human genetic diseases. Missplicing of cellular genes can be either a symptom of an underlying molecular defect or the actual cause of the disease (Stamm, 2008).

The factors involved in pre-RNA splicing and how the interaction of the various components is regulated is still poorly understood. Establishing new aspects of signal transduction pathways, which leads to changes of usage of alternative exons are necessary for developing therapeutic strategies against human disorders.

The aim of this research is to study the controlling mechanisms of alternative splice site selection by different splicing factors, like SLM-1 and Tra2-beta1.

1.1. Basic mechanism of splicing

Splicing is the inclusion of exons or introns from the pre-mRNAs into the mature RNA. The sequences, which are joined together and exported into the cytosol, are called exons. The intervening sequences, which are removed are named introns.

The average exon is small, approximately 150bp with 99% of known exons shorter than 400 nucleotides, however exons of more than 5000 nucleotides are known. By contrast, introns vary greatly in length averaging around 1500 nucleotides but can be up to a hundred thousand nucleotides. The narrow range of exon size has lead to the exon definition hypothesis whereby splicing factors bound to a 3' splice site interact with factors at the next downstream 5' splice site, across the exon, thereby defining the position of the exon before switching to interact with factors at the upstream 5' splice site, across the intron, to allow splicing of the introns (Figure 1).

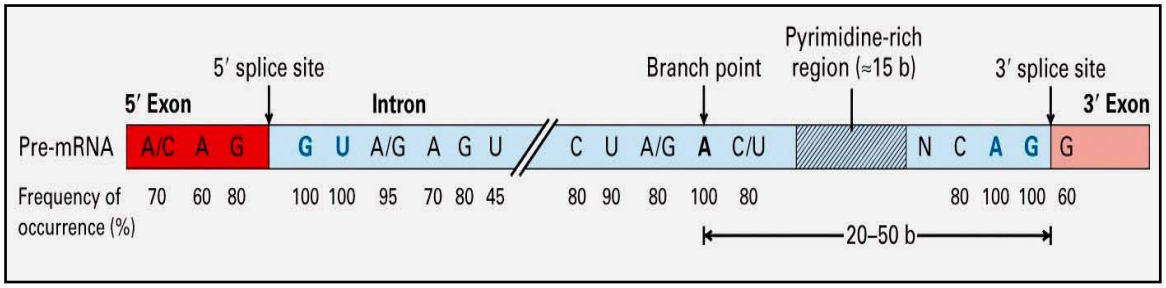


Figure 1. The classical and auxiliary splicing signals. The figure is adapted from Faustino N. and Cooper T., 2003.

In cases where the intron is short and the exon is longer than 300 nucleotides, the "intron bridging" model which proposes interactions across the intron is invoked to explain pairing of the 5' splice site with the correct 3' splice site (Robberson et al., 1990).

The typical human gene contains an average of 8.8 exons. Exons are defined by short and degenerate splice site sequences at the intron / exon borders (5' splice site, 3' splice site), and the branch-point (Figure 1).

The major class of human introns (>99%) contains highly conserved dinucleotides at the 5' and 3' termini (GT and AG respectively), shown in Table 1.

Table 1. Sequence elements indicating introns.

Element	Consensus sequence[1]
5' (donor) splice site	YRG/GURAGU
3' (acceptor) splice site preceded by a polypyrimidine stretch	Y_{12}NYAG
Branch point located 18-200 nucleotides upstream of the 3' splice site	YNYURAY

[1]Symbols used: Y – pyrimidine; R – purine; N – any nucleotide. Slash denotes the exon-intron border. Invariant nucleotides are underlined.

These introns with GT-AG termini are called U2-type introns. A novel class of eukaryotic nuclear pre-mRNA introns was found on the basis of their unusual splice sites (Hall et al., 1994; Padgett et al., 1994). These introns contain AT and AC at the 5' and 3' splice sites, respectively. This type of introns is named U12 introns. U12 introns were recognized by different nuclear RNA components (Hall S.L. and Padgett R.A., 1996; Tarn and Steitz, 1997). The U12 type of introns is present in the nuclei of vertebrates, insects, and plants (Wu et al., 1996). Analysis of splice junction pairs from GenBank annotated mammalian genes showed that 98.71% conformed to canonical GT-AG, 0.56% to non-canonical GC-AG and 0.73% to other non-canonical splice termini (Burset et al., 2001).

1.1.1. Biochemistry of general splicing

Pre-mRNA splicing occurs through two sequential trans-esterification reactions involving a dynamic multicomponent complex called spliceosome. The spliceosome consists of five subcomplexes called small nuclear ribonucleoprotein particles (snRNPs U1, U2, U4, U5 and U6 in major class U2 type spliceosome; snRNPs U11, U12, U4, U5

and U6 in minor class U12 type spliceosome). Junctions between an intron and its flanking exons, namely the 5' and the 3' splice sites on the pre-mRNA are precisely identified by the spliceosome. The splicing reaction proceeds by an orchestrated formation and disruption of RNA-RNA, RNA-protein and protein-protein interactions within the spliceosome, which leads to exon ligation and release of the intron lariat. In the first step, a nucleophilic attack by the 2' hydroxyl group of a conserved adenosine residue at the Branch point, cleaves the 5' exon-intron junction generating a free 3' hydroxyl group on the upstream exon as well as a branched lariat intermediate. In the second step, the 3' intron-exon junction is attacked by the 3' hydroxyl of the 5' exon, displacing a lariat intron and ligating the exons (Figure 2).

The function of the spliceosome is recognizing the splice site and performing the catalysis.

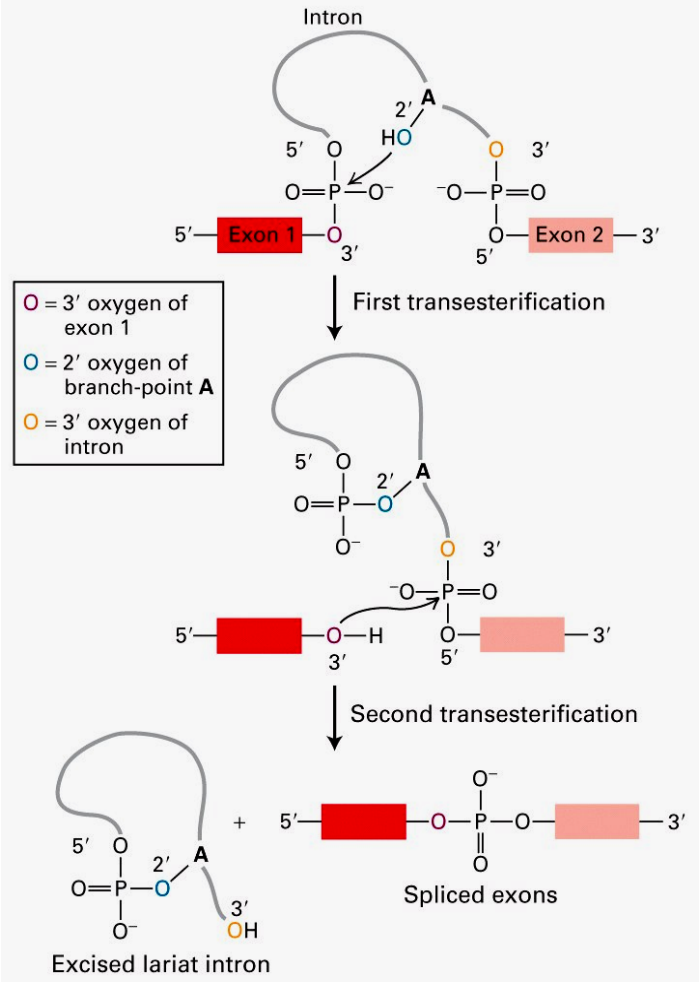


Figure 2. The pre-mRNA splicing reaction. The first step involves the cleavage at the 5' splice site to yield the excised 5' exon while the intron is still covalently attached to the distal (3') exon. This is acieved by exchange of a 3'→5' for a 2'→5' bond: the 5' P of the intron is attacted by the 2'-OH of the branch site adenosine, causing cleavage of a 3'-5'phosphodiester bond and formation of a 2'-5'phosphodiester bond (not hydrolysis followed by ligation). The second step includes exchange of one 3'→5' phosphodiester with another, that would lead to the releasing of intron by a cut at its 3' end and ligation of exons: the newly formed 3'-OH of exon 1 attacts the 5'P of exon 2, causing cleavage of a phosphodiester bond and formation of a new bond. (Moore et al., 1993; Guthrie et al., 1991; Ruby et al., 1991). Both the excised intron and the intron-exon intermediate are in the form of a lariat in which the 5' terminal nucleotide of the intron is joined through a 2'→5' phosphodiester bond with an adenosine residue 18 to 40 nucleotides upstream of the 3' splice site (Ruskin et al., 1984).

1.1.2. Creation of dynamic assembly complex

The splicing process starts with formation of the E complex. The assembly of the E complex involves recognition of 5' splice site, polypyrimidine tract and 3' splice site by U1 snRNA, heterodimeric splicing factor U2AF (U2 snRNP auxiliary factor), consisting of U2 auxiliary factor 65 (U2AF65) and U2 auxiliary factor 35 (U2AF35). The branch point is recognized by the splicing factor 1 (SF1).

Several non-snRNP splicing factors such as serine/arginine (SR) proteins and SR related proteins also associate to the pre-mRNA at this step. In addition, U4/U6*U5 tri-snRNP can associate with the first exon near the 5' splice site in the E-complex (Figure 3)

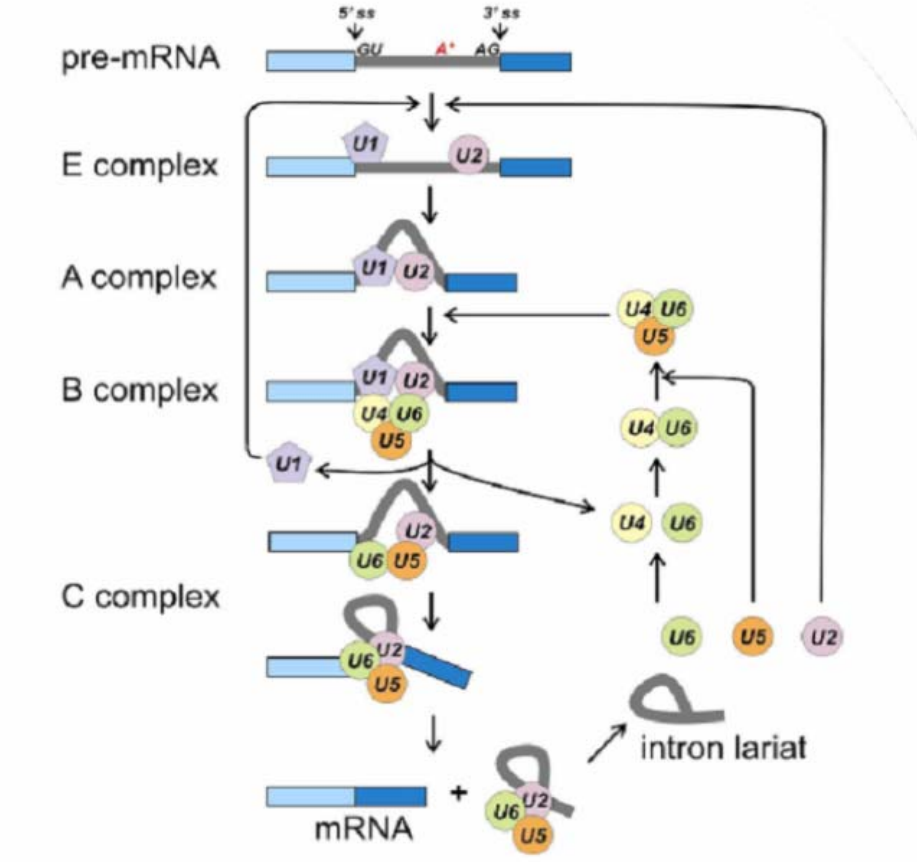


Figure 3. The spliceosome cycle. The U1, U2, U4, U5, and U6 snRNPs associate with the pre-mRNA and interact with each other in an ordered manner to form the spliceosome. Each formation of complexes A,B,C and E is marked. The two transesterification reactions take place in the catalytic core of the spliceosome. After splicing, the spliceosome dissociates, and is re-assembled to take part in a new round of splicing cycle (taken from Hui, 2003).

This association is ATP dependent. Then ATP dependent base pairing of U2 snRNP with the branch point leads to formation of the A complex. The B complex is formed by recruitment of the U4/U6•U5 tri-snRNP to the pre-spliceosome. The U6/U4 duplex is disrupted and a new duplex between U6 and the 5' splice site is formed, leading to displacing of the U1 snRNP.

The 5' splice site is brought close to the branch point and the 3' splice site through U6/U2 snRNA base pairing and interaction of U5 snRNP with both exons near splice sites. At this point, U4 snRNP leaves the complex and the first catalytic step of splicing occurs, creating the intron lariat. Finally, U5 snRNP base pairs with both 5' and 3' exons, thus positioning the ends of the two exons for the second step of splicing.

After the second step has been completed, the ligated exons and the lariat intron are released and the spliceosomal components dissociate to be recycled for further rounds of splicing. The nature and function of the splicing machinery and the biochemistry of splicing are well known. However, the answer to the question of how splice sites are selected in vivo still remains unclear. One possible answer is presence of the short sequences that serve to define exon-intron junctions. Consensus splice signals that are not normally used as splice sites (known as "cryptic" splice sites) occur frequently in a given pre-mRNA. Furthermore, non-consensus splice site sequences that contain mismatches at highly conserved positions are sometimes used as splice sites. These observations have lead to the discovery of auxiliary cis-acting sequences that can influence splice site recognition. These sequences are known as splicing enhancers and inhibitor sequences.

1.1.3. Alternative splicing

Alternative mRNA splicing is the term used to describe the regulated process of differential inclusion or exclusion of regions of the pre-mRNA. It is an important source of protein diversity in higher eukaryotes. Alternative splicing is often regulated in a temporal or tissue-specific fashion giving rise to different protein isoforms in different tissues or developmental states. At the organism level, specific isoforms are known to be produced at certain developmental stages or in specific tissues or as a consequence of regulation by extracellular signaling mechanisms (Matter et al., 2002).

Alternative splicing has been reported in almost all eukaryotic groups including plants, apicomplexans, diatoms, amoebae, animals and fungi. Ancient genes (shared between animals, fungi and plants) show high levels of alternative splicing. Genes with products expressed in the nucleus or plasma membrane are generally more alternatively spliced while those expressed in extracellular location show less alternative splicing (Irimia et al., 2007).

The types of alternative splicing alteration are different. For example, exons can be extended or shortened, skipped or included, and introns can be removed or retained in the mRNA (Cartegni et al., 2002). Almost all alternative splicing events can be classified into five basic splicing patterns: cassette exons; alternative 5' and 3' splice sites, mutually exclusive cassette exons, retained introns (summarized in Figure 4). Alternative poly A sites and alternative promoters have been identified separately.

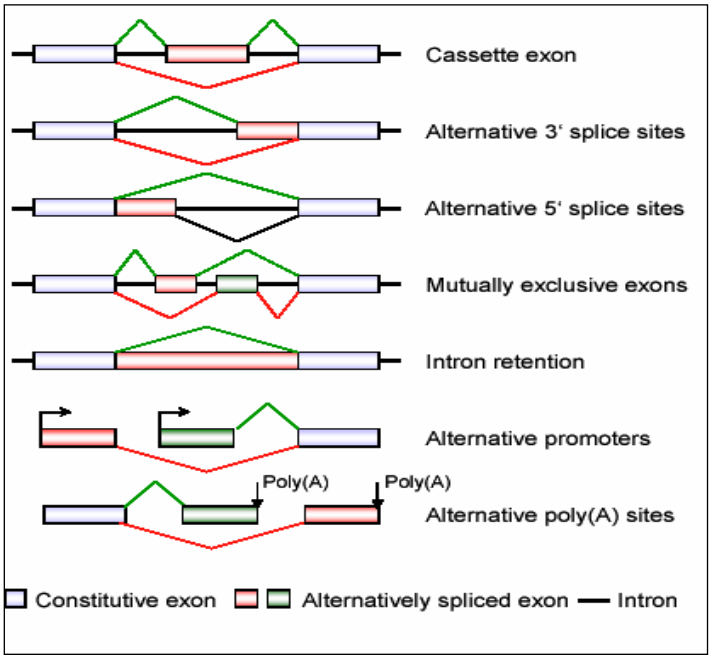


Figure 4. Types of Alternative splicing. Flanking constitutive exons are shown as grey boxes, alternatively spliced exons are shown as a green and red boxes. Introns indicated as horizontal lines.

Internal alternative cassette exons belong to the largest group and account for 38% of the alternative splicing events conserved between human and mouse genomes (Ast et al., 2004). However, more complicated patterns, such as multiple 5' or 3' splice sites, coordinated usage of internal exons, and combinations of the basic types are also

frequently observed. An estimated 75% of all alternative splicing patterns change the coding sequence (Kan et al., 2001; Okazaki et al., 2002) indicating that alternative splicing is a major mechanism enhancing protein diversity.

In the human genome more than 90% of the pre-mRNA is removed as introns and only about 10% of the pre-mRNA is retained as the final spliced transcript. EST based database analysis indicates that 35-65% of human genes are subjected to alternative splicing which contributes significantly to human proteome complexity and explains the numerical disparity between the low number of human protein coding genes (~26 000) and the number of human proteins (~90 000) (Modrek et al., 2001).

1.1.4. Intron bridging and exon recognition

Regulatory proteins interact with specific sequences within pre-mRNAs and stimulate or repress exon recognition. These proteins bind directly to 5' or 3' splice sites, or to other pre-mRNA sequences called exonic or intronic splicing enhancers (ESEs or ISEs) and silencers (ESSs or ISSs) (Ladd et al., 2002). These elements are generally short (8-10 nucleotides) and are even less conserved than those present at exon-intron junctions. They stimulate or repress splice-site selection, respectively.

Control of alternative splice site recognition is mediated by proteins which belong to two large groups: serine/arginin rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). A common feature of this proteins that regulate splicing is the presence of two functional domains: an RNA-binding domain and protein-protein interaction domain (Cartegni et al., 2002).

The interaction between individual splicing factor and regulatory sequence is weak to facilitate easy disloding of the factors after splicing. As the interaction is weak and not highly specific, different SR and SR like proteins can act through the same regulatory elements and influence the same splice sites (Figure 5). Higher specificity is also achieved by protein–protein interactions that allow simultaneous binding of multiple proteins to RNA. It is well known that SR or SR-like proteins can promote the formation of complexes containing U1 snRNP bound to the 5' splice site and U2 snRNP bound to the pre-mRNA branch site. They can also facilitate the recruitment of U4/U6 and U5

snRNPs. In addition, SR and SR-like proteins can bridge the introns by interacting with themselves and the core spliceosomal components.

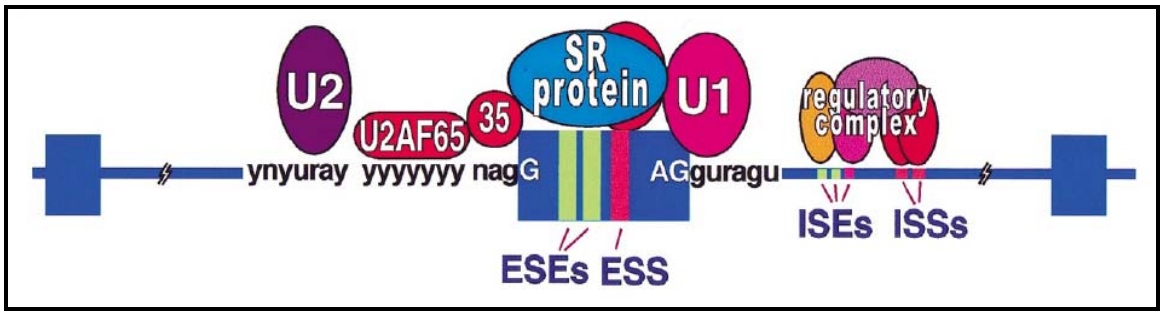


Figure 5. Classical and auxiliary splicing elements and binding factors. Factors that bind classical and auxiliary splicing elements are indicated as circles and ellipses. Exons are indicated as boxes, introns as thin lines. Auxiliary enhancer elements within exons or introns (ESEs and ISEs) are indicated with green lines, silencing elements within exons or introns are marked with red lines (ESSs and ISSs). Intronic elements also serve to modulate cell-specific use of alternative exons by binding multicomponent regulatory complexes. This figure is adapted from Faustino N. A. and Cooper T. A., 2003.

Splicing enhancers are located close to the splice sites that they activate. However, the action of splicing enhancers is position dependent. Changing the location of splicing enhancers alters their dependence on particular trans-acting factors (Tian et al., 1994), and determines whether they activate 5' or 3' splice sites (Heinrichs et al., 1998). The location can even transform them into negative regulatory elements (Kanopka et al., 1996). The relative concentration of trans-acting factors varies between cell types and tissues as well as during development. Therefore, patterns of splice site selection change depending on local concentrations of general splice factors and/or gene specific regulators. Due to this combinatorial control, a large number of alternative exons can be regulated by a limited number of regulatory proteins. This explains the importance of modulation of splice site selection, depending on the developmental stage, on tissue differentiation, or on metabolic changes of the cells (Black, 1995).

1.1.5. Role of SR/SR-related proteins in constitutive and alternative splicing

SR-proteins have a modular structure consisting of one or two copies of an N-terminal RNA recognition motif (RRM) and a C-terminal domain rich in alternating arginine and serine residues (RS domain). The RRMs mediate sequence-specific binding

to the RNA and thereby determine the substrate specificity, whereas the RS is mainly involved in protein-protein interactions that are thought to be essential for the recruitment of the splicing machinery and for splice-site pairing (Wu et al., 1993; Tacke et al., 1999). Recently, it has been proposed that the RS domain also mediates sequential recognition of splice sites during spliceosome assembly (Shen et al., 2004).

Mammals contain 10 canonical SR proteins, with size ranging from 20 to 75 kDa (Bourgeois, 2004). These proteins were initially identified and grouped into a family based on common biochemical and immunological properties (Zahler, 1992). SR proteins belong to a larger superfamily of SR-like proteins that are characterized by the presence of RS or RS-like domains (Fu, 1995). A bioinformatics approach identified about 50 proteins with RS domain in Homo sapiens, 80 in Caenorhabditis elegans and 110 in Drosophila melanogaster (Boucher et al., 2001).

The serines in an RS domain can be highly phosphorylated. At least eight members of the family including ASF/SF2, SC35, SRp20, SRp30c, 9G8, SRp40, SRp55, and Tra2-beta1 contain phosphopeptides that are recognized by the monoclonal antibody mAb104 (Mayeda et al., 1992; Graveley et al., 2000). The mAb104 epitope includes a phosphate indicating that these proteins are phosphorylated in vivo (Roth et al, 1990). A number of additional splicing factors containing RS domains are structurally and functionally related to SR proteins and are collectively referred to as SR-related proteins or SRrps. Although SR proteins or SR-related proteins do recognize specific RNA sequences, their consensus sequences are rather degenerate. As the interaction is weak and not highly specific between individual splicing factors and the regulatory sequences, different SR and SR-related proteins can act through the same regulatory elements and influence the same splice sites. Higher specificity is also achieved by protein–protein interactions that allow simultaneous binding of multiple proteins to RNA.

In constitutive splicing, SR proteins are known to promote cross-intron and cross-exon interactions and to influence the recruitment of the U1 snRNP and U2AF splicing factor into the spliceosome (Wu and Maniatis, 1993). In alternative splicing, SR proteins are known to interact with ESEs and to stimulate the splicing of adjusted introns (Graveley et al., 2000). The most characterized function of SR proteins in the regulation of alternative splicing is the activation of weak 3’ splice sites, mostly characterized by an

imperfect polypyrimidine tract poorly recognized by U2AF. Upon binding to ESEs, SR proteins improve the recruitment of U2AF to the polypyrimidine tract and, subsequently, that of U2 snRNP (Zuo and Maniatis 1996; Zhu and Krainer 2000; Graveley et al. 2001; Guth et al. 2001). Alternatively, binding of a SR protein to an ESE element can counteract the effect of a juxtaposed splicing silencer prior to the recruitment of U2AF and U2 snRNP (Kan et al., 1999; Zhu et al. 2001).

It is well known that SR or SR-related proteins can promote and stabilize the formation of E complexes containing U1 snRNP bound to the 5' splice site and U2 snRNP bound to the pre-mRNA branch site. They can also facilitate the recruitment of U4/U6 and U5 snRNPs. In addition, SR and SR-like proteins can bridge the introns by interacting with themselves and the core spliceosomal components (Figure 6).

Exon recognition and splice site selection are achieved by a coordinated action of both positive and negative regulation, provided by SR and SR-like proteins and hnRNP proteins, respectively. The factors that often oppose the action of SR family proteins are heterogeneous nuclear ribonucleoproteins (hnRNPs).

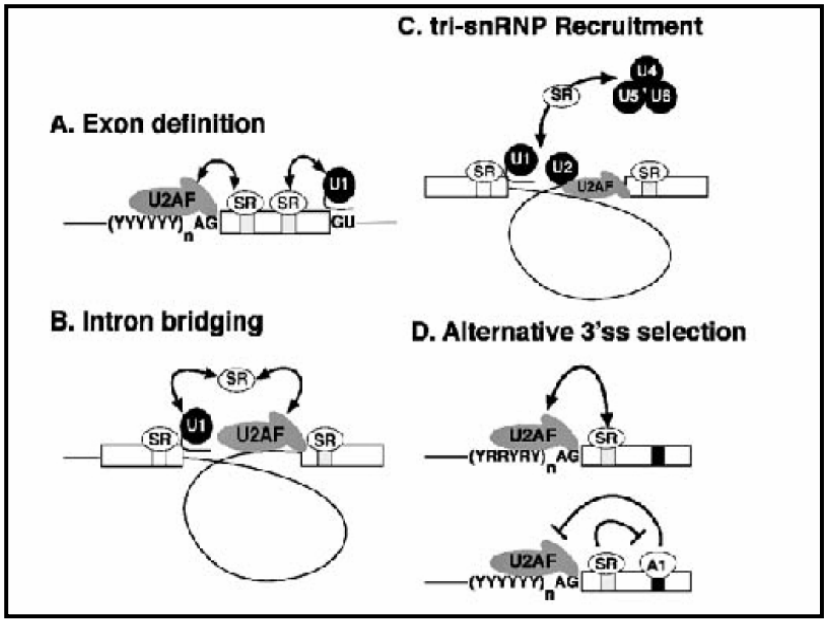


Figure 6. Roles of SR proteins in spliceosome assembly. (A) U2AF, marked as a grey oval, at an upstream 3' splice site and U1 snRNP, which is indicated as black circle, at a downstream 5' splice site. The binding to RNA is facilitated by SR proteins bound to ESEs (light grey boxes). The polypyrimidine tract (YYYYYY) is a part of 3' splice site. (B) The 5' and 3' splice sites can be juxtaposed early in the splicing reaction by intron bridging interactions between SR proteins and the RS domain containing subunits of U1 snRNP and U2AF. (C) SR proteins recruit the U4/U6•U5 tri-snRNP to the pre-spliceosome. (D) SR proteins bound to ESEs, promote alternative 3' splice site selection by recruiting U2AF to 3' splice site. Alternatively, exonic splicing silencers, marked as black boxes, can recruit splicing repressor proteins such as hnRNP A1 and block 3' splice site selection by U2AF (Adapted from Sanford J.R. et al., 2005).

1.1.6. Phosphorylation of SR proteins

Spliceosome assembly may be promoted by phosphorylation of SR proteins that facilitate specific protein interactions, while preventing SR proteins from binding randomly to RNA. Once a functional spliceosome has formed, dephosphorylation of SR proteins appears to be necessary to allow the transesterification reactions to occur (Cao et al., 1997). Therefore, the sequential phosphorylation and dephosphorylation of SR proteins may mark the transition between stages in each round of the splicing reaction.
It is clear that specific phosphorylation of serine residues within the RS domain of SR proteins may be one of key determinant regulating splicing events because it modulates homophilic and heterophilic interactions between RS domain-containing proteins (Soret et al., 2003; Stamm, 2008).

In as much as RNA–protein interactions can also be affected by phosphorylation of the RS domain, these observations raise the intriguing possibility that phosphorylation of SR proteins may act as a regulatory switch whereby initial interactions with constitutive splicing factors like U1 snRNP, U2AF, and specific RNA sequences are strengthened to allow selection of an authentic splice site. Conversely, dephosphorylation of SR proteins weakens them allowing subsequent steps of spliceosome assembly to proceed (Soret et al., 2003; Bourgeois et al., 2004). Accordingly, both hyper- and hypophosphorylation of SR proteins inhibit splicing, demonstrating the critical importance of SR protein phosphorylation.

To date, several kinases have been reported to phosphorylate SR proteins, including cytosolic SRPK1 and SRPK2 (SR protein kinases)-family kinases (Kuroyanagi et al., 1998), mammalian nuclear PRP4 (pre-mRNA processing mutant 4) (Kojima et al., 2001), and a family of kinases termed Clk (Cdc2-like kinase), or LAMMER kinases from the consensus motif, consisting of four members (Clk1-4) (Ben-David et al., 1991; Colwill et al., 1996; Nayler et al., 1997). In addition to these, some kinases, such a DNA topoisomerase I (Rossi et al., 1996) and Cdc2 kinase (Okamoto et al., 1998) have been reported to phosphorylate SR proteins. Although they have been directly implicated in splicing, it has also been shown that shuttling of SR proteins between the nucleus and cytoplasm is critically dependent on the phosphorylation status of the RS domain. Indeed, phosphorylation releases SR proteins from nuclear speckles and recruits them to the sites

of active transcription. Thus the differential effects of phosphorylation on SR protein interactions have the potential to affect both splicing activity and subcellular trafficking (Soret and Tazi, 2003).

1.1.7. Heterogeneous nuclear ribonucleoproteins (hnRNPs)

HnRNPs as a group of nuclear RNA-binding proteins were first described in 1997 by Nakielny et al. hnRNPs belong to a highly abundant family of proteins that lack an RS domain and associate with heterogeneous nuclear pre-mRNAs during transcription (Nakielny et al., 1997).

In addition these proteins are shown to be involved in the biogenesis and nucleocytoplasmic transport of mRNA (Dreyfuss et al., 1993). Members of the hnRNP A, B and C families associate with RNA to form a regular array of 20-25 nm particles. Several family members contain an arginine/glycine-rich domain that may be involved in both RNA binding and interactions with other proteins. hnRNP proteins are diffusely distributed throughout the nucleus (Dreyfuss et al., 1993), unlike SR proteins, which co-localize with other splicing factors in nuclear speckles (Hastings et al., 2001).

In antagonizing the positive effects of SR proteins, hnRNPs can influence splice site selection by binding to splicing silencer sequences. Two mechanisms of splicing repression have been proposed. First, binding of these regulatory factors to the silencer sequences could interfere directly with the assembly of spliceosomal components. It could block the exon bridging interactions that occur during exon recognition or it could block splicing activation by SR proteins binding to adjacent ESEs. Second, dimerization of inhibitory proteins surrounding the exon causes the alternative exon to loop out and to be skipped by the splicing machinery.

One well-studied protein belonging to the family of hnRNPs is hnRNP A1. hnRNP A1 protein was found to antagonize the action of SR proteins that promote distal 5’ splice site usage in E1A and ß-globin pre-mRNAs (Caceres et al., 1994; Mayeda and Krainer, 1992). In addition, hnRNPA1 controls inclusion of exon 7b of its own transcript (Blanchette and Chabot, 1999) and of exon 2 of the HIV Tat-pre-mRNA (Caputi M. et al., 1999).

Another protein providing an example of a negative regulation of splice site choice is the ubiquitously expressed polypyrimidine tract-binding protein (PTB), also called hnRNP I. This protein can repress exon inclusion by directly interfering with binding of general splicing factors to the pyrimidine tract (Garcia-Blanco et al., 1989).

It is important to note that some proteins containing an RS domain function as splicing repressors, whereas certain hnRNP proteins have been shown to function as splicing activators (Smith and Valcarcel, 2000).

1.1.8. Role of Tra splicing factor in the Drosophila sex-determination pathway

The best characterized examples of regulated alternative splicing derive from studies of the Drosophila sex-determination pathway (Schutt et al., 2000). The Drosophila female-specific protein Sex-lethal (SXL) represses male-specific 3' splice sites in transformer (tra) and SXL pre-mRNAs by two distinct mechanisms. As shown in Figure 7A, exon 2 of tra pre-mRNA is preceded by two alternative 3' splice sites. The proximal and distal 3' splice sites are used in males and females, respectively. Splicing to the male-specific 3' splice site produces an mRNA containing a premature translational stop codon. By contrast, splicing to the female-specific 3' splice site produces an mRNA that encodes functional TRA protein. In males, the splicing factor U2AF binds to the male-specific 3' splice site and initiates spliceosome assembly (Figure 7A) (Schutt et al., 2000). However, in females this splice site is bound by the female-specific splicing repressor SXL, thus blocking the binding of U2AF. Instead, U2AF binds to the female-specific 3' splice site, and functional tra mRNA is produced (Figure 7).

SXL also regulates the alternative splicing of its own pre-mRNA, albeit by an entirely different mechanism . Sxl exon 3 is excluded from the mRNA only in females. In males, inclusion of exon 3 introduces a premature translational stop codon. Exon 3 inclusion requires the protein SPF45, a second-step splicing factor that binds to the AG dinucleotide of the male-specific 3' splice site (Figure 7B). In females, SXL binds to a site adjacent to SPF45, and the two proteins interact. This interaction interferes with the activity of SPF45, and thus blocks the second step of the splicing reaction. As a consequence, exon 3 is skipped and exon 2 is spliced to exon 4, thus producing an mRNA

encoding functional SXL protein. Thus, SXL blocks the first step of the splicing reaction in tra pre-mRNA and the second step in sxl pre-mRNA. sxl autoregulation is the only known example in which alternative pre-mRNA splicing is regulated at the second step of the splicing reaction.

While the previous examples of regulated alternative splicing involve splice-site repression, the female-specific splicing of Drosophila doublesex (dsx) pre-mRNA is the best characterized example of splice-site activation. The 3' splice site immediately upstream from exon 4 of dsx pre-mRNA is not recognized by the splicing machinery in males, thus leading to the exclusion of this exon (Fiure 7C). The male-specific dsx mRNA encodes a transcriptional repressor of female-specific genes. In females, the regulatory protein TRA promotes the cooperative binding of an SR protein, RBP1, and an SR-like protein, Transformer 2 (TRA2), to individual ESEs within exon 4{Hertel, 1997 #83}.

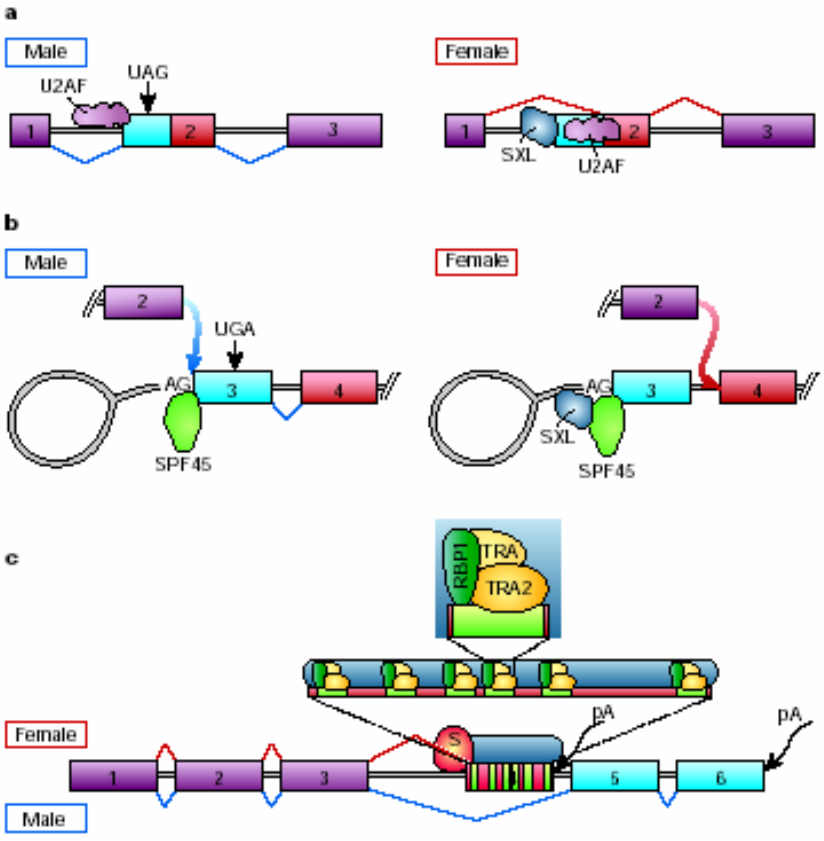


Figure 7. Regulation of alternative pre-mRNA splicing in the Drosophila sex-determination pathway. A. Alternative selection of 3' splice sites preceding exon 2 of tra pre-mRNA is regulated by the SXL protein. In males, the splicing factor U2AF binds to the proximal 3' splice site, leading to an mRNA containing a premature translational stop codon (UAG). In females, SXL binds to the proximal 3' splice site, thus preventing the binding of U2AF. Instead, U2AF binds to the distal 3' splice site, leading to an mRNA that encodes functional TRA protein. In all panels, the exons are indicated by coloured rectangles, while introns are shown as pale grey lines.

B. Alternative inclusion of exon 3 of sxl pre-mRNA is regulated by SXL protein. In both males and females, the first step of the splicing reaction results in lariat formation at the branchpoint sequence upstream from the 3' splice site preceding exon 3. Subsequently, the second-step splicing factor SPF45

binds to the AG dinucleotide of this splice site. In males, SPF45 promotes the second step of the splicing reaction, leading to the inclusion of exon 3. In females, SXL binds to a sequence upstream of the AG dinucleotide, interacts with SPF45 and inhibits its activity. This prevents the second step of the splicing reaction, leading to the exclusion of exon 3 and splicing of exon 2 to exon 4. Seven constitutively spliced exons are not shown. C. Alternative splicing of dsx pre-mRNA is regulated by the assembly of heterotrimeric protein complexes on female-specific ESEs. The first three exons are constitutively spliced in both sexes. In males, the 3' splice site preceding exon 4 is not recognized by the splicing machinery, resulting in the exclusion of this exon, and splicing of exon 3 to exon 5. In females, the female-specific TRA protein promotes the binding of the SR protein RBP1, and the SR-like protein TRA2 to six copies of an ESE (indicated by green rectangles). These splicing enhancer complexes then recruit the splicing machinery to the 3' splice site preceding exon 4, leading to its inclusion in the mRNA. In females, polyadenylation (pA) occurs downstream of exon 4, whereas in males it occurs downstream of exon 6. 'S' designates the splicing machinery (taken from Maniatis and Tasic, 2002).

This heterotrimeric protein complex recruits the splicing machinery to the upstream 3' splice site, leading to the inclusion of exon 4.

The female-specific dsx mRNA encodes a transcriptional repressor of male-specific genes. The basic mechanisms of alternative splicing established in Drosophila have been shown to function in mammals. Alternative splice-site selection in mammals is also controlled by differential binding of regulatory proteins to splice sites, or to enhancer or silencer sequences within the pre-mRNA (Smith and Valcarcel, 2000). The organization of these sequences and the interplay of different regulatory proteins determine the outcome of the splicing reaction. As in Drosophila, regulatory proteins can exert competing influences on the splicing of a pre-mRNA.

1.1.9. Human Transformer 2-beta1

Human transformer 2-beta1 is an SR-like protein that regulates alternative splice site selection and it was reported as a homologue of the Drosophila melanogaster sex determination factor Transformer-2. Together with Transformer, this protein regulates sex-determination in somatic cells through a cascade of alternative splicing events (Nayler et al., 1998; Dauwalder et al., 1996). Drosophila Tra2 has another mammalian homologue, Tra2-alpha (Beil et al., 1997; Dauwalder et al., 1996; Matsuo et al., 1995; Segade et al., 1996). All three proteins: Tra2, Tra2-beta and Tra2-alpha share a similar structure consisting of two RS domains flanking a central RNA recognition motif (RRM). This suggests that the tra-2 products of flies and humans have similar molecular functions.

Recently NMR investigations demonstrate the structure of RRM in human tra2-beta1 (www.pdb.org, Someya T., “Solution structure of the RNA recognition motif in

Arginine/serine-rich splicing factor 10"). The RRM domain of Tra2-beta is typical RRM, which consist of a beta1-alpha1-beta2-beta3-beta4 fold with a four-stranded antiparallel beta sheet forming a surface that displays two highly conserved RNP 1 and RNP 2 motifs (Figure 8).

Human Tra2-alpha has splicing regulatory functions that are conserved between drosophila and humans. When expressed in flies, hTra2-alpha can partially compensate for the loss of Drosophila Tra-2, which affects both female sexual differentiation and alternative splicing of doublesex dsx pre-mRNA.

In both mammalian and Drosophila systems, Tra2-beta was proposed to be part of a splicing regulatory complex conserved from Drosophila to human (Nayler et al., 1998; Daoud et al., 1999). Human Tra2-beta1 was isolated as a human cDNA bearing high homology to the Drosophila transformer-2 (Tra-2) protein. It was identified via its interaction with the splicing factor SC35, SF2/ASF and SRp30c (Beil et al., 1997; Amrein et al., 1994; Nayler et al., 1998).

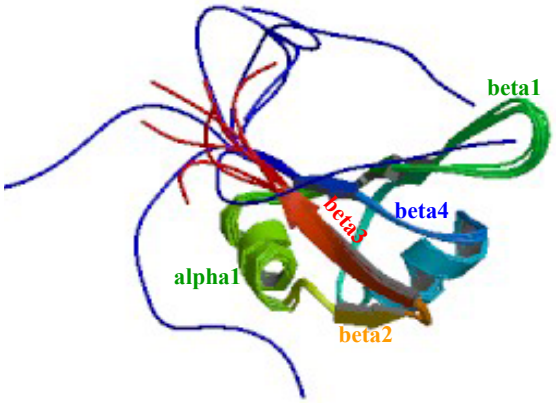


Figure 8. Three-dimensional structure of human Tra2 RNA recognition motif. The RRM consist of a four-stranded antiparallel beta-sheet and one alpha-helix (between beta1 and beta2 sheets). The second alpha-helix indicated in purpure is located after beta4 strand on the C-end of the RRM.

The gene gives rise to at least five RNA isoforms (tra2-beta1-beta5), which are generated through alternative splicing, alternative polyadenylation and alternative promoter usage of the human tra2-beta gene (Figure 9). They contain three different open reading frames. Exon 1 contains a start codon in a long open reading frame (ORF) encoding tra2-beta1, as well as a short ORF present in tra2-beta2. Both proceed in exon

1 (Daoud et al., 1999). In the beta3 and beta4 isoforms, this start codon is followed by frame stop codons. A start codon in exon 4 precedes an ORF encoding tra2-beta3. These mRNAs generate only two proteins hTra2-beta1 and hTra2–beta3 (Nayler et al., 1998). They differ in the presence of the first RS domain (Figure 9). The resulting short hTra2-beta3 protein is expressed in several tissues and has no influence on tra2-beta splice site selection. Two RNA isoforms, tra2-beta2 and -beta4 are not translated into protein (Daoud et al., 1999; Stoilov et al., 2004), but their generation through alternative splicing is regulated by external stimuli, such as T-cell stimulation (Beil et al., 1997) and neuronal activity (Daoud et al., 1999).

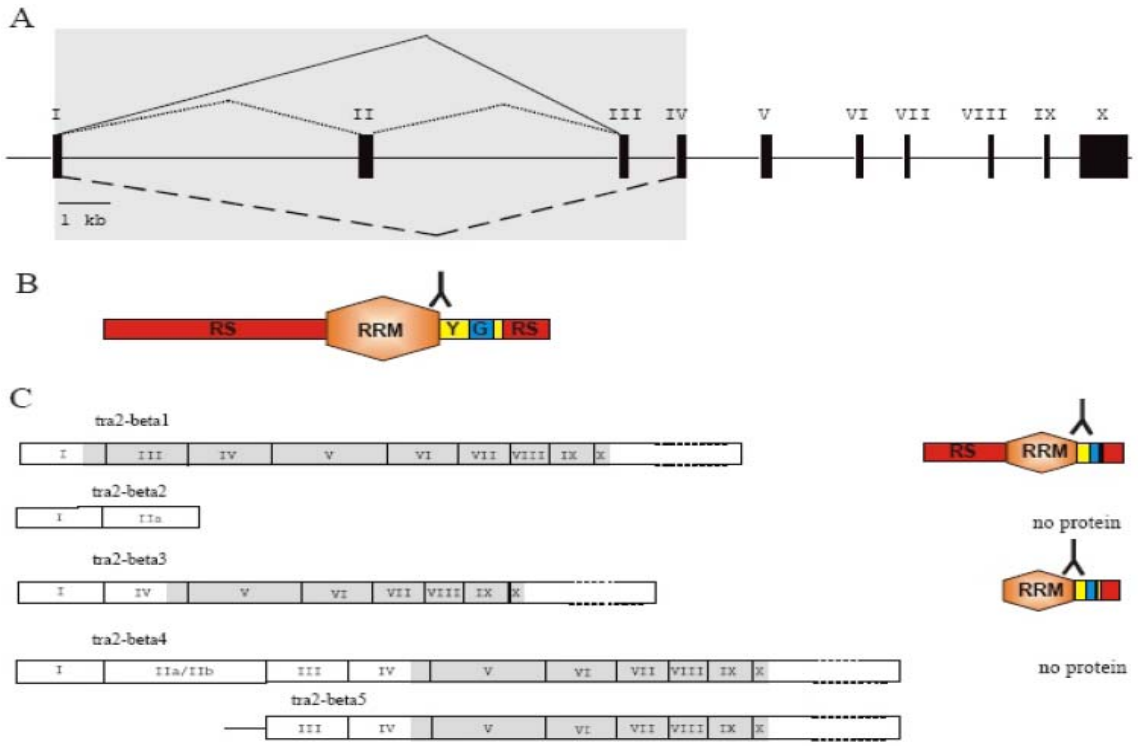


Figure 9. The tra2-beta gene structure. (A) The exon-intron structure is drawn to scale. Exons are shown as black boxes, introns as lines. The shaded region marks the sequence of Tra2-beta minigene. (B) Structure of the protein. Tra2-beta1 protein consists of a RNA recognition motif (RRM), flanked by two SR repeats (red). The protein also has a tyrosine rich (yellow) and glycine rich (blue) stretch, located between the C-terminal SR repeat and the RRM. The position of the epitope in Tra2-beta1 protein recognized by the pan-Tra2 antiserum is shown on top. (C) Transcripts derived from the tra2-beta gene. Boxes indicate the individual exons. The shading shows the open reading frame. On the right are the proteins that are encoded by each of the transcripts. The position of the epitope in the Tra2-beta1 protein recognized by the pan-Tra2 antiserum is shown on top. The picture is adapted from Stoilov et al., 2004.

The protein changes the splicing pattern of other genes (Daoud et al., 1999). This clearly indicates its role in splice site selection in vivo. Tra2-beta1 protein concentration

is autoregulated through a negative feedback regulation. The increased concentration of hTra2-beta1 changes the splicing of its own pre-mRNA towards an isoform that does not generate the protein. Hyperphosphorylated Tra2-beta1 has reduced ability to bind to RNA (Stoilov et al., 2004). It was demonstrated that presence of CLK2 kinase prevents the usage of exons 2 and 3, generating the htra2-beta3 mRNA. Recently it was established that hTra2-beta1 binds to the degenerate RNA sequence GHVVGANR. This motif was found more frequently in exons than in introns (Stoilov et al., 2004). This sequence is part of the splicing enhancer of SMN2 exon 7, where it mediates Tra2-beta1-dependent inclusion. Therefore, splicing factor Tra2-beta1 generally promotes inclusion of exons by recruiting or stabilizing an exon recognition complex after binding to a degenerate RNA element.

1.1.10. Sam68 like mammalian proteins

The SLM-1 and SLM-2 proteins belong to the STAR (Signal Transduction and Activation of RNA) family of proteins (Vernet and Artzt, 1997), also called GSG (GRP33, SAM68, GLD-1) proteins (Jones and Schedl, 1995; Chen et al., 1999). These are nuclear RNA-binding proteins that share an extended hnRNP K homology domain (KH domain), which was first identified in hnRNP K protein (Siomi et al., 1993), and C-terminal sequences typically involved in signal transduction. One of the best characterized members of this family is the SAM68 (Src-associated during mitosis) protein (Wong et al., 1992). SLM-1 and SLM-2 share a common KH-RNA binding domain and contain both proline- and tyrosine-rich stretches (Di Fruscio et al., 1999). They are highly related to the Sam68 protein. It was demonstrated that SLM-1 and SLM-2 heterodimerize with Sam68 (Di Fruscio et al., 1999). All the members of the STAR family are methylated in vivo (Cote et al., 2003).

Using the scaffold attachment factor B protein as bait in a yeast two hybrid screen with a rat brain library (Nayler et al., 1998; Weighardt et al., 1999), the Sam68-like mammalian protein rSLM-2 was isolated (Stoss et al., 2001). In addition, cDNAs bearing high homology to the previously reported SLM-1 (Di Fruscio et al., 1999) and Sam68 (Richard et al., 1995) were isolated and named rSLM-1 and rSam68, respectively. Previously, it was shown that SLM-2 interacts with SR-proteins and hnRNPs and

regulates alternative splice site selection in vivo (Stoss et al., 2001). It was demonstrated that Sam68 plays an important role in alternative splicing (Matter et al., 2002), cell cycle regulation (Barlat et al., 1996; Taylor et al., 1995) and RNA export (Reddy et al., 2000). The extended N-terminus of rSam68 contains several ERK (extracellular receptor kinase) phosphorylation sites (Matter et al., 2002). Its function is influenced by ERK-mediated threonine phosphorylation (Matter et al., 2002) and Sik/BRK-mediated tyrosine phosphorylation (Coyle et al., 2003). Figure 10 shows the domains of these three highly related proteins, rSam68, rSLM-1 and rSLM-2, which differ from each other only by the numbers of proline-rich regions.

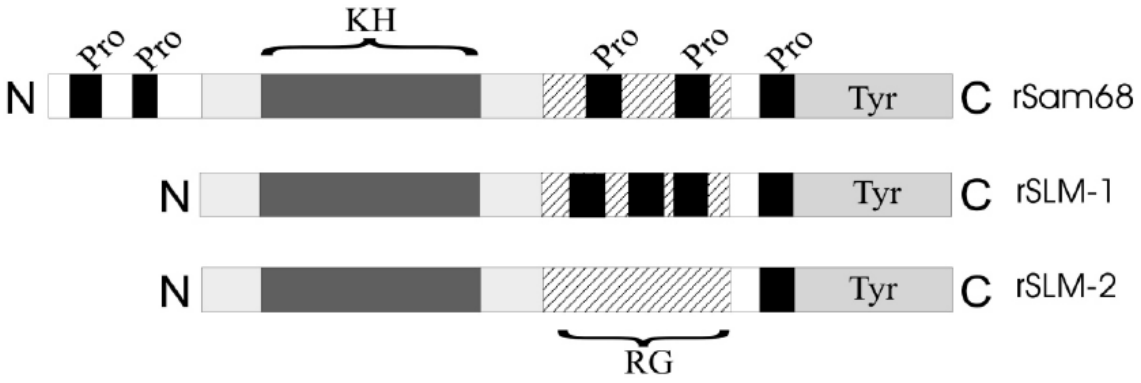


Figure 10. **The domain structure comparison of rSam68, rSLM-1 and rSLM-2.** Pro: proline-rich regions; KH: hnRNP K homology domain; RG: arginine/glycine-rich region; Tyr: tyrosine-rich region.

Therefore, these three highly related cDNAs exist, both in rat and humans, sharing a similar, but not identical domain structure.

1.2. Regulation of alternative splicing by signal transduction pathways

Pre-mRNA splicing is regulated in a tissue-specific or developmental stage-specific manner (Stamm, 2002). The selection of splice sites can be altered by numerous extracellular stimuli such as hormones, immune response, neuronal depolarization, and cellular stress, through changes in synthesis/degradation, complex formation, and intracellular localization of regulatory proteins.

1.2.1. Hormonal regulation

Cytokines, growth factors and hormones alter splicing patterns of several genes. Exon inclusion of a protein kinase C (PKC) isoform by insulin is a well characterized

example of the hormone-dependent regulation of alternative splicing. PKC βI and PKC βII are generated from one gene by alternative splicing, and differ only by C-terminal 50–52 amino acids (Chalfant et al., 1995). The binding motif of SRp40, one of the SR protein family members, is located downstream of the PKC βII specific exon. Activation of the insulin receptor by insulin in rat L6 skeletal myoblasts induces phosphorylation of SRp40 within 30 min. Pretreatment of L6 myotubes with LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), blocks both SRp40 phosphorylation and exon inclusion induced by insulin, suggesting that an unidentified kinase phosphorylates SRp40 and regulates alternative splicing downstream of the PI 3-kinase cascade.

1.2.2. Immune response

There are now numerous examples of alternatively spliced genes in the immune system, with some evidence for a functional role of alternative splicing (Lynch et al., 2004). The change of CD44 isoforms in response to T-cell activation is a well studied example. CD44 is a cell-adhesion molecule and widely expressed in most tissues. The CD44 gene has 10 variable cassette exons, and more than 20 isoforms are produced by various combinations of the variable exons (Blencowe et al., 2000). The variable exons (v1–v10) of CD44 encode portions of the membrane–proximal extracellular domain of the protein, and presence of the variable exons has been shown to increase the association of CD44 with various proteins or with the extracellular matrix polysaccharide hyaluronan (Dreyfuss et al., 2002). Native T cells mainly express the smallest CD44 isoform that lacks all variable exons, whereas T cells activated by injection of allogeneic lymphocytes, by TCR stimulation with an anti-CD3 antibody, or by phorbol-ester treatment, express alternatively spliced CD44 isoforms, with exon inclusion of v1, v3, v4, v5, v7, or v10 (Hanamura et al., 1998; Graveley et al., 2000). Antibodies against these larger molecular-weight variants of CD44 can block activation of T cells, indicating that the alternative splicing of CD44 is crucial for T cell function (Graveley et al., 2000). The mechanisms controlling activation-induced inclusion of exon v5 in T cells is best understood. In resting T cells, an ESS in this exon represses the inclusion and promotes exon skipping by binding of splicing repressor protein hnRNP A1 (Jensen et al., 2000). In activated T cells, Sam68 is phosphorylated by ERK (extracellular signal-regulated kinase) and

thought to bind to the ESS of exon v5 competing with hnRNP A1, thereby relieving the splicing repression (Markovtsov et al., 2000).

1.2.3. Neuronal depolarization

Pre-mRNA splicing of ion channels is allowed by electrical stimulation. The depolarization-dependent repression of the STREX (stress axis) exon of the large-conductance Ca^{2+} voltage-activated potassium (BK) channel in GH3 pituitary cells is a typical example of alternative splicing in response to neuronal depolarization (Sanford et al., 2003). A pyrimidine-rich element located in the 3'-splice site of the STREX exon is crucial for the repression of STREX exon inclusion by neuronal simulation and Ca^{2+}/calmodulin-dependent protein kinase IV (CaMK IV) activation. This element was named as CaRRE (CaMKIV-responsive RNA element). The factors binding to this element remain to be determined (Mahe et al., 2000). Another example is tra2-beta1, whose isoforms change within one hour in animal hippocampi after increased neuronal activity (Daoud et al., 1999).

1.2.4. Cellular stress

When cells are stressed by pH change, osmotic shock or lack of oxygen, cytoplasmic accumulation of splicing regulatory proteins such as hnRNP A1, tra2-beta, and SAM68 are observed, with alteration of splicing site selection in several genes (Nissim-Rafinia et al., 2002). When mice are forced to swimm during five minutes, its already enough to change the splicing pattern of acetylcholinesterase (Soreq and Seidman, 2001). Brain ischemia that occurs during a stroke causes hyperphosphorylation of tra2-beta1, leading to accumulation of this splice factor in the cytosol and changes in alternative splicing patterns (Daoud et al., 2002).

1.3. Phosphorylation-dependent regulation of alternative splicing

Phosphorylation is a ubiquitous cellular regulatory mechanism and one of the main regulators of alternative splicing. It is a reversible, covalent modification of a protein that serves to modify the activity of the phosphorylated molecule by inducing conformational changes within the molecule. This modification occurs either through the addition of phosphate groups via the transfer of the terminal phosphate from ATP to an

amino acid residue and/or by their removal. The function of these post-translational modifications is to alter the substrate's activity, subcellular localization, binding properties or association with other proteins. Families of specialized molecules catalyze the addition (kinases) or removal (phosphatases) of phosphate groups from proteins (Figure 11). Different classes of protein kinases and phosphatases act specifically on serine/threonine residues, or tyrosine residues. An important feature of kinases and phosphatases is that a single molecule is able to activate many substrate molecules, thus allowing for amplification of the initial signal.

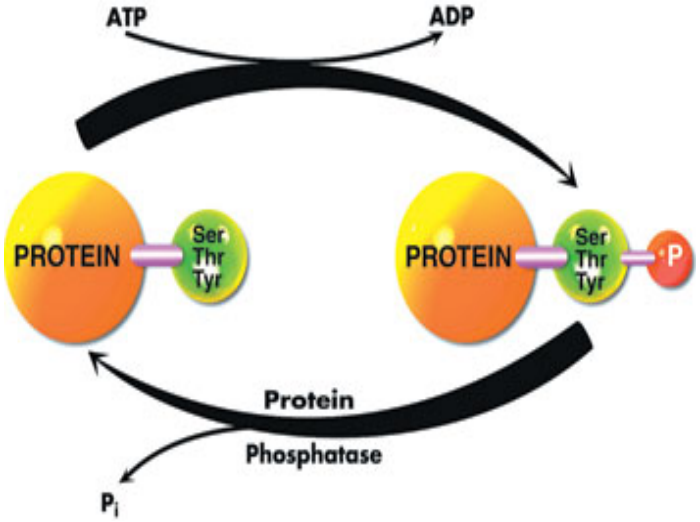


Figure 11. Model of reversible phosphorylation in the cell. The phosphorylation occurs through the addition of phosphate groups via the transfer of the terminal phosphate from ATP to an amino acid residue, which contain an OH-group in the structure; dephosphorylation occurs by removal of the terminal phosphate by special protein phosphatase. (Figure adapted from Invitrogen).

The sequencing of entire genomes has revealed that the total amount of protein kinase is 518, representing 1.7% of human genome (Plowman et al., 1999). Protein phosphatases, in turn, representing only 0.4% (Plowman et al., 1999). Surprisingly, there appear to be 2-5 times fewer protein phosphatases than protein kinases (Bollen, 2001). For example, the human genome encodes ~20 times lessr Ser/Thr protein phosphateses than Ser/Thr protein kinases. The diversity of Ser/Thr protein phosphateses is achieved not only by the evolution of new catalytic subunits but also by the ability of a single catalytic subunit to interact with multiple regulatory subunits (Bollen, 2001).

Up to 30% of all proteins may be modified by kinase activity, and as noted previously, the components of the pre-mRNA splicing machinery undergo

phosphorylation and dephosphorylation during the splicing process (DePaoli-Roach, 2003). The alteration in splice site selection occurs very fast, within an hour (Stamm, 2008) and this rapid cellular process does not require protein synthesis de novo. It is therefore not surprising that a change in alternative splice site selection was observed when the activity of kinases or phosphatases acting on splicing factors was manipulated (Hartmann et al., 2001; Allemand et al., 2007; Muraki et al., 2004). These phosphorylation changes are reversible. Early studies demonstrated that complete inhibition of phosphatases or absence of ATP completely blocked the splicing reaction (Mermoud et al., 1992; Krainer et al., 1984).

Phosphorylation alters the interaction between individual splicing regulatory proteins and between some of this proteins and RNA. One of the consequences of phosphorylation is a difference in the mRNP composition that alters splice site selection. The other consequence is a different subcellular localization of regulatory proteins, which alters their concentration in areas where splicing occurs and again result in a change in splice site selection (Stamm, 2008).

It was shown that the reversible phosphorylation of SR proteins is important in splicing reaction. The interactions between SF2/ASF (Splicing factor 2/Alternative Splicing Factor) with other RS domain containing splicing factors play an important role in the spliceosome assembly, such as U1-70K (Xiao and Manley, 1997) and cytoplasmic RNA, are regulated via phosphorylation. In fact, phosphorylated SF2/ASF is present in the cytoplasm and does not bind to RNA, whereas dephosphorylation enhances cytoplasmic mRNA binding to SF2/ASF (Sanford et al., 2005). Phosphorylation of SR proteins mediates their translocation from storage compartments, the nuclear speckles, to the sites of active transcription (Misteli et al., 1998; Wang et al., 1998). Therefore, the phosphorylation status of proteins play an important role in several processes, like assembly of the spliceosome, regulation of the splice site selection, and subcellular localization of splicing factors (Soret and Tazi, 2003).

Splicing dependent dephosphorylation of shuttling SR proteins was observed (Huang et al., 2004; Lai and Tarn, 2004). It was demonstrated that hypophosphorylated SF2/ASF, a shuttling SR protein, binds the mRNA export receptor TAP and associates with mature mRNPs (Lai and Tarn, 2004). Therefore, dephosphorylation of SR proteins

is possibly crucial for their nuclear export or post-splicing functions. In conclusion, differential and dynamic phosphorylation of SR proteins in their RS domain has an important role in modulating their splicing activity, subcellular localization and functions during mRNP maturation and/or export.

The tyrosine phosphorylation of non-SR proteins can also lead to changes of cellular localization like it was shown for splicing factor YT521-B. Phosphorylation of YT521-B by specific nuclear non-receptor tyrosine kinases causes dispersion from YT bodies to the nucleoplasm and forces the phosphorylated protein into insoluble nuclear fractions (Rafalska et al., 2004).

1.3.1. Protein Phosphatase 1

Type 1 protein phosphatases (PP-1) comprise a group of widely distributed enzymes that specifically dephosphorylate serine and threonine residues of certain phosphoproteins. They all contain a catalytic subunit, which has an extremely conserved primary structure (Bollen et al., 1992). One of the properties of PP-1 that allows one to distinguish them from other serine/threonine protein phosphatases is their sensitivity to inhibition by two proteins, termed inhibitor 1 and inhibitor 2. In the cell the type 1 catalytic subunit is associated with noncatalytic subunits that determine the activity, the substrate specificity, and the subcellular location of the phosphatase. PP-1 plays an essential role in glycogen metabolism, calcium transport, muscle contraction, intracellular transport, protein synthesis, and cell division. The activity of PP-1 is regulated by hormones like insulin, glucagon, alpha- and beta-adrenergic agonists, glucocorticoids, and thyroid hormones.

PP1 and PP2A holoenzymes represent the major protein phosphatases that dephosphorylate serine/threonine residues (Cohen, 1997). The PP1 family of holoenzymes is composed of oligomeric complexes comprising a core enzyme, the catalytic subunit PP1c, which can bind to and form complexes with a huge number of over 70 regulatory proteins that modulate the activity of the phosphatase. All the PP1 catalytic subunits in the cell are associated with various regulatory subunits forming heteromeric complexes (Wera and Hemmings, 1995). The regulatory subunits define the activity and specificity of catalytic subunit of PP1. They act as activity-modulators and

bring the phosphatase into close proximity with specific substrates. PP1 is also regulated by its interaction with a variety of protein subunits that target the catalytic subunit to specific subcellular compartments. These targeting subunits serve to localize PP1c in proximity to particular substrates, and also to reduce its activity towards other potential substrates (Feng et al., 1991; Stuart et al., 1994). Hormones, growth factors and metabolites control the function of PP1 holoenzymes mainly by modulating the interaction of the subunits.

Most regulators of PP1 contain an RVxF motif. This motif confirms to the consensus sequence [RK]x0–1[VI]{P}[FW], where x can be any residue and {P}refers to any residue other than proline (Ceulemans et al., 2002; Egloff et al.,1997; Zhao and Lee, 1997). Another type of PP1 binding motifs representing a new consensus sequence F-x-x-[RK]-x-[RK] was recently discovered. The second motif (RK) is required for recognition and binding of some Bcl-2 proteins (Ayllón et al., 2002) and Inhibitor 2 to PP1c (Helps and Cohen, 1999).

1.3.2. DARPP-32 is a specific inhibitor of PP1

The activity of protein phosphatases is regulated by several inhibitors, presented in Table 2.

The dopamine- and cAMP-regulated phosphoprotein of 32 kD (DARPP-32) is one important modulator of the cAMP pathway and predominant inhibitor of PP1, which is highly expressed in both striatonigral and striatopallidal neurons (Ouimet et al., 1998).

The signaling pathways occurring in the brain regulating the DARPP-32 and PP1 interaction was the subject of extensive studies during the last decade (Shenolikar and Nairn, 1991; Greengard et al., 1998; Price and Mumby, 1999).

Over the past 20 years, using a variety of molecular, cellular, and functional approaches, DARPP-32 has been established as a crucial mediator of the biochemical, electrophysiological, transcriptional, and behavioral effects of dopamine.

Table 2. Protein inhibitors of PP-1 subfamily members

1	Inhibitor 1 (I-1) DARPP-32 (brain homolog of I-1)	19 kD 32 kD	Heat stable Phosphorylation by cAMP-dependent protein kinase required for activity
2	CPI 17	17 kD	Heat stable Phosphorylated by protein kinase C Inhibits smooth muscle myosin-associated phosphatase
3	NIPP1	38 kD	Heat stable Nuclear localization No phosphorylation requirement
4	Inhibitor-2 PP1-I2	23 kD	Heat stable No phosphorylation requirement

In 2000, Paul Greengard was awarded by Nobel Prize for the understanding of dopamine-regulated signaling cascades, particularly the important role played by DARPP-32 in the neostriatum of the brain receiving high dopaminergic input. The neurotransmitter dopamine, acts on dopamine D1-like receptors, causing activation of Protein Kinase A (PKA) after activation of its receptor. Phosphorylation catalysed by PKA at Thr34 of phosphoprotein converts DARPP-32 into an inhibitor of protein phosphates-1 (Hemmings et al., 1984; Hemmings et al., 1989).

Phosphorylation of DARPP-32 occurs at several sites different from Thr34. For example the phosphorylation of DARPP-32 at Thr75 by Cdk5/p32 prevents the phosphorylation of this protein by PKA at Thr34 and as a result the inhibition of PP1 (Bibb et al., 1999). Therefore, in such a case DARPP-32 plays the role of a strong inhibitor of PKA, suggesting that DARPP-32 is a dual-function protein, acting either as an inhibitor of PP1 or of PKA (Figure 12).

Convertion of DARPP-32 to inhibitor of PKA is regulated, for example, by glutamate, which acts on NMDA receptors, that increases Ca^{2+} entry, and consequently stimulating PP2B/calcineurin. The activated protein phosphatase-2B (PP2B)

dephosphorylates DARPP-32 on S102 and S137 residues, releasing the active PP1 (Halpain et al., 1990). Released PP1 can dephosphorylate other proteins. Activation of dopamine D2-like receptors may also stimulate PP1 through dephosphorylation of DARPP-32 catalyzed by the inhibition of PKA (Nishi et al., 1999). In contrast, activation of adenosine A2 receptors leads to the phosphorylation of DARPP-32 and inhibition of PP1 (Svenningsson et al., 2000). Targets of PP1 activity in dopaminergic neurons include neurotransmitter receptors and ion channels such as the NR1 subunit of the NMDA glutamate receptor (Snyder et al., 1998), the AMPA-type glutamate receptor (Yan et al., 1999), the GABAA receptor ß1 subunit (Flores-Hernandez et al., 2000) and the Na^+/K^+-ATPase ion pump (Fiscone et al., 1998). The inhibition of PP1 by phosphorylated inhibitor-1 and DARPP-32 have all been attributed to the binding of the phosphorylated residue at or near the catalytic site as a pseudosubstrate. These studies show the important role played by DARPP-32 in integrating neuronal signaling cascades that modulate responses PP1 (Fienberg and Greengard, 2000).

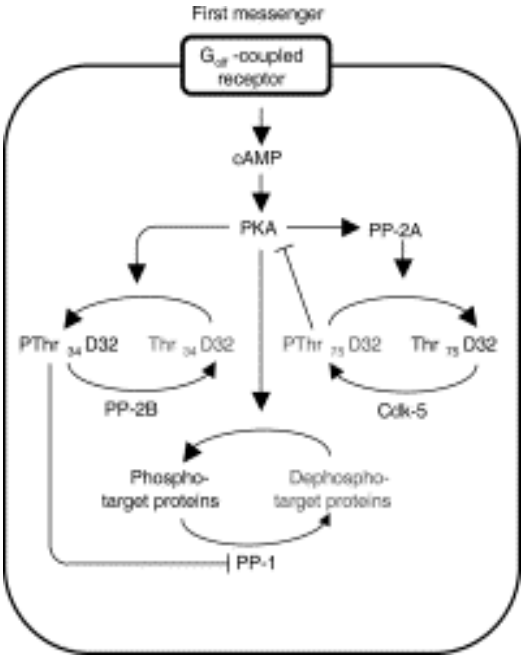


Figure 12. Scheme of dual action of DARPP-32. The neurotransmitter acts on G-coupled receptors, causes activation of Protein Kinase A (PKA) after activation of its receptor. Phosphorylation catalysed by PKA at Thr34 of phosphoprotein converts DARPP-32 into an inhibitor of PP1. Phosphorylation of DARPP-32 at Thr75 by Cdk5/p32 prevents the phosphorylation of this protein by PKA at Thr34 and as a result the inhibition of PP1.

PKA converts DARPP-32 into a potent high-affinity inhibitor of PP-1 with an IC_{50} of approximately 10^{9} M (Hemmings et al., 1984). DARPP-32 is expressed in very high concentration (50 μM) in virtually all strial neurons. The total concentration of all PP-1 isoforms in medium spiny neurons is less than 20 μM (da Cruze et al., 1995), and DARPP-32 can be phosphorylated at Thr34 following dopamine D1 receptor activation. Thus, a substantial proportion of PP-1 activity will be inhibited in response to dopaminergic regulation of medium spiny neurons (Svenningsson et al., 2005).

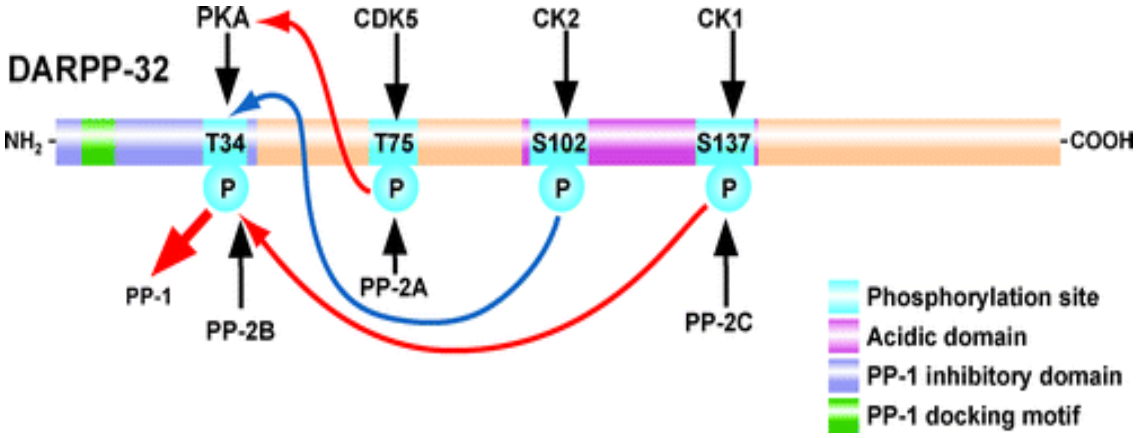


Figure 13. Multisite phosphorylation of DARPP-32. DARPP-32 is phosphorylated at Thr34 by PKA and protein kinase G (PKG), not shown], at Thr75 by cdk5, at Ser102 by CK2, and at Ser137 by CK1. Phospho-Thr34 is preferentially dephosphorylated by PP-2B (or calcineurin), although PP-2A also can dephosphorylate this site; phospho-Thr75 is preferentially dephosphorylated by PP-2A; phospho-Ser137 is preferentially dephosphorylated by PP-2C; the phosphatase for phospho-Ser102 is not yet fully characterized. Phosphorylation of Ser102 of DARPP-32 by CK2 increases the rate of phosphorylation of Thr34 by PKA (but not by PKG); phosphorylation of Ser137 of DARPP-32 by CK1 decreases the rate of dephosphorylation of phospho-Thr34 by PP-2B. Phosphorylation at Thr75 converts DARPP-32 into an inhibitor of PKA, reducing its ability to phosphorylate DARPP-32 and other substrates. (Blue arrow indicates positive effect; red arrows indicate negative effect). Phosphorylation of Thr34 converts DARPP-32 into a potent inhibitor of PP-1. The NH_2-terminal domain of DARPP-32, which contains the PP-1 docking motif and phospho-Thr34, is shown. In distinct ways, phosphorylation of Ser102 and Ser137 acts to increase phosphorylation of Thr34 and therefore potentiate dopaminergic signaling via the cAMP/PKA/DARPP-32/PP-1 pathway. In contrast, phosphorylation of Thr75 acts to inhibit dopaminergic signaling via this pathway (taken from Svenningsson, 2004).

A variety of structure-function studies have indicated that two domains of DARPP-32 (and also inhibitor-1) are involved in its interaction with PP-1 (Figure 13) (Hemmings et al., 1990; Desdouits et al., 1995). An inhibitory domain, consisting of phospho-Thr34 and the surrounding residues, is likely to occupy or bind close to the active site of the enzyme in a manner in which access to phosphorylated substrate is prevented. A second domain of DARPP-32, consisting of residues 7–11 (KKIQF),

interacts with PP-1 at a site removed from the active site. Studies of a number of PP-1 targeting subunits (Desdouits et al., 1995) have revealed that they contain a domain related to the KKIQF sequence and that this constitutes a common structural motif involved in binding of DARPP-32 and the various targeting proteins to PP-1. Identification of the PP-1 docking motif in DARPP-32 and other proteins has allowed the development of peptides that can antagonize the interaction of phospho-DARPP-32 or targeting subunits with PP-1 (Hsieh-Wilson et al., 1999; Yan et al., 1999). The determination of additional details of the interactions of PP-1 with phospho-DARPP-32 may prove useful in the development of nonpeptide inhibitors for treatment of disorders of dopamine signaling pathways.

1.3.3. Regulation of alternative splicing by snoRNA

Recently, it was shown by our lab (Kishore and Stamm, 2006) that small nucleolar RNAs can regulate processing of pre-mRNA. Small nucleolar RNAs (snoRNA) belong to the one class of non-coding RNAs and the main function of them is the postranslational modification of ribosomal RNA (Cavaille et al., 2000). Computational genomic searches have now identified more than 200 snoRNAs in vertebrates (Bachellerie et al., 2002), most of which have no known RNA targets.

One of the human brain specific snoRNAs that are not translated into proteins – HBII-52, present in 47 nearly identical copies, exhibits sequence complementarity to the alternatively spliced exon Vb of the serotonin receptor 5-HT2cR. Exon Vb inclusion is promoted by the Adenosine to Inosine editing at 5 different sites (A-E) by editing enzymes ADARs.

First, it was demonstrated that HBII-52 binds to a splicing silencing element in exon Vb and promotes its inclusion without RNA editing (Kishore and Stamm, 2006). Patients suffering from Prader-Willi syndrome (PWS), a congenital disease caused by the loss of paternal gene expression from the long arm of chromosome 15q11-q13, do not express HBII-52 snoRNA and as a result their splicing regulation of the serotonin receptor is disturbed. These results could explain why PWS patients respond positively to selective serotonine reuptake inhibitors. It is likely that a defect in the serotonergic system is a contributing cause of PWS.

This example suggests the role of small nuclear RNAs in regulating higher brain functions.

1.4. Function of alternative splicing

Gene regulation through alternative splicing is more versatile than regulation through promoter activity. Variant transcripts generated through alternative splicing, similar to those initiated from distinct promoters, are often tissue and/or development specific, resulting in effects seen only in certain cells or developmental stages. However, changes in promoter activity alter predominantly the expression levels of the mRNA. In contrast, changes in alternative splicing can modulate transcript expression levels by subjecting mRNAs to nonsense-mediated decay and alter the structure of the gene product by inserting or deleting novel protein parts. The structural changes fall into three categories: introduction of stop codons, changes of the protein structure and changes in the 5' or 3' untranslated region. The effects caused by alternative splicing range from a complete loss of function to subtle effects that are difficult to detect. Functional relevance of pre-mRNA alternative splicing with respect to encoded protein is summarized below.

1.4.1. Introduction of stop codons

mRNAs containing premature termination codons (PTCs) present more than 50-55 nt upstream of the last exon-exon junction can be degraded by Nonsense Mediated Decay (NMD). About 25-35% of alternative exons introduce frameshifts or stop codons into the pre-mRNA (Stamm et al., 2000; Lewis et al., 2003). Since approximately 75% of these exons are predicted to be subject to nonsense-mediated decay, an estimated 18-25% of transcripts are switched off by stop codons introduced in alternative splicing and nonsense mediated decay (Lewis et al., 2003). This process, which has been termed RUST for Regulated Introduction Unproductive Splicing and Translation, currently represents the function of alternative splicing with the most obvious biological consequences (Figure 14).

1.4.2. Addition of new protein parts

Approximately 75% of alternative splicing events occur in the protein coding regions of the mRNAs (Okazaki et al., 2002; Zavolan et al., 2003). Changes in the protein

primary structure can alter the binding properties of proteins, influence their intracellular localization and modify their enzymatic activity and/or protein stability by diverse mechanisms.

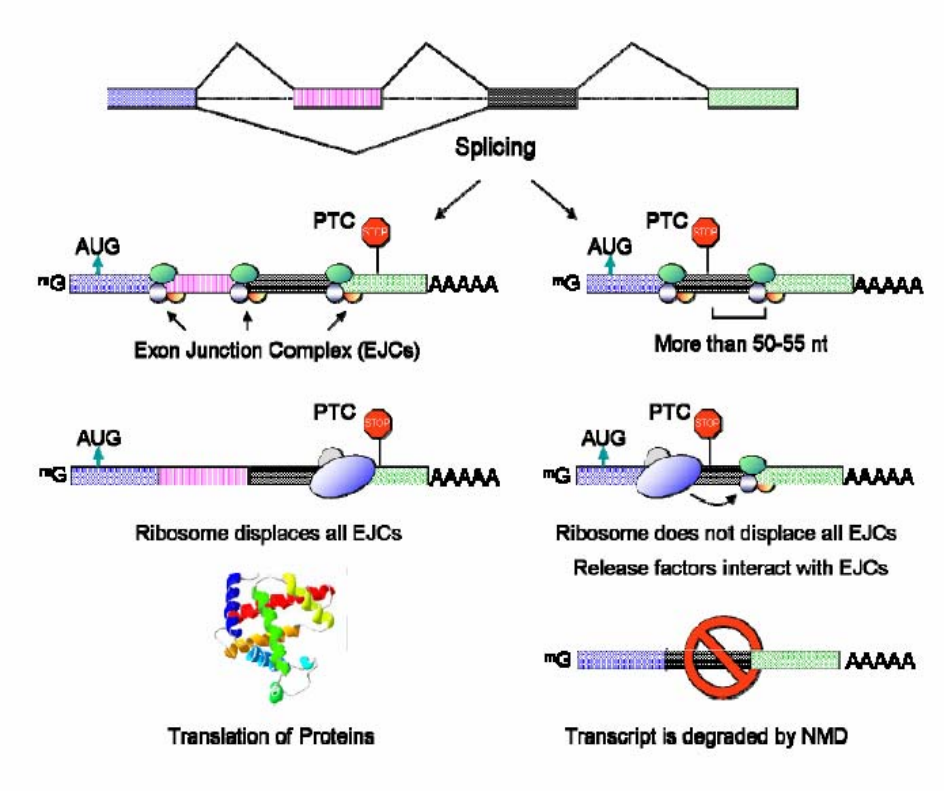


Figure 14. A current model for NMD in mammalian cells. A PTC 50 nucleotides (nt) upstream of the last exon-exon junction activates NMD. Translation causes the displacement of the EJCs deposited upstream of each splice junction. However, termination of translation upstream of one or more EJCs triggers NMD by recruitment and activation of factors released by Ribosomes. Termination in the last exon results in displacement of all EJCs and a stable mRNA. (Figure adapted from http://compbio.berkeley.edu/ people/ed/ rust/).

1.4.3. Binding properties

Alternative splicing can delete binding domains or introduce structural changes by inserting protein sequences, that can abolish binding activity of a protein. For instance, alternative splice variants of Thyroid Stimulating Hormone (TSH) receptor are unable to bind TSH (Ando et al., 2001). In some cases, alternative splicing can also create novel binding domains or control the number of multiple binding motifs to modulate protein binding affinities. For example, in tenascin C, alternative splicing can alter the number of fibronectin type III domains which regulates binding to fibronectin (Puente et al., 2001).

Similarly, alternative splicing could as well regulate transcription by abolishing the DNA binding domain of transcription factors.

1.4.4. Intracellular localization

Alternative splicing can influence the intracellular localization of numerous proteins, usually by influencing localization signals or regulating the interaction of proteins with membranes. Deletion or interruption of transmembrane domains of membranous proteins can cause them to accumulate in the cytoplasm or to be secreted in the extracellular space. Nonmembrane bound soluble isoforms can lose their ability to transduce signals (Tone et al., 2001) and become less stable (Garrison et al., 2001). Alternative splicing can regulate the localization of proteins in various subcellular sites and organelles. Proteins can be sequestered into compartments, where they perform no function. This mechanism is widely used for receptor molecules and alternative splicing can regulate their retention in membrane enclosed compartments. For example, the inclusion of an endoplasmatic reticulum retention signal in the metabotropic Glutamate Receptor 1B reduces the cell surface expression of this receptor and restricts its trafficking (Chan et al., 2001). Also, the localization of proteins within organelle can be regulated by alternative splicing. In the nucleus, proteins can be present in different nuclear substructures, such as in the nucleoplasm and speckles, due to alternative splicing (Nishizawa et al., 2001).

1.4.5. Protein and mRNA stability

Inclusion of alternate protein domains can regulate the half-life of proteins. Protein stability can be altered due to autophosphorylation that signals the degradation of receptor molecules. For the interleukin-1 receptor-associated kinase, this autophosphorylation-dependent degradation is isoform-specific, leading to a molecule that is not down-regulated by its ligand (Jensen and Whitehead, 2001). The effect of alternative splicing-dependent protein stability has been studied for the fosB gene, which generates a shorter isoform that is more stable than the full-length protein. This isoform accumulates in brain in a region-specific manner in response to many types of chronic behavioral changes (Nestler et al., 1999). Some examples have been described where alternative splicing changes the properties of the mRNA. Alternative splicing events

occuring in 5' and 3' UTRs may change the stability of the RNA. For example, alternative exons in the 5' UTR of the HIV-1 virus can either promote or inhibit the nuclear degradation of their mRNA, which regulates HIV-1 gene expression (Krummheuer et al., 2001).

1.4.6. Posttranslational modifications

Posttranslational modifications can be dictated by alternative splicing, usually by generating consensus sites for phosphorylation, glycosylation, palmitoylation or sulfatation. For instance, isoform-dependent phosphorylation of the potassium channel Kv4.3 allows the modulation of outward currents by the alpha-adrenergic system via protein kinase C (Po et al., 2001). In addition, binding between a kinase and its substrate can be regulated by alternative splicing, e.g. binding of the SR-protein kinase 1 to scaffold attachment factor B (Nikolakaki et al., 2001).

1.4.7. Influence on protein function

Alternative splicing can modulate affinity, substrate specificity and catalytic activity of enzymes. Inclusion of a stop codon prior to the active center or deletions of protein parts that are necessary for catalysis are frequent mechanisms to regulate enzymatic activity. Also, alternative splicing can either completely abolish or modulate the function of a protein by inserting novel sequences into intracellular domains, as in the case of CD46 (Purcell et al., 1991) or the prostaglandin receptor EP3 that differ in their coupling efficiencies to adenylatecyclase (Harazono et al., 1994). Similarly, interaction of transcription factors with DNA can be modified by alternative splicing, which contributes to transcriptional regulation.

1.5. Alternative splicing and disease

Mistakes in splice site usage have been observed in numerous diseases. Missplicing of cellular genes can either be a symptom of an underlying molecular defect, or the actual cause of the disease (Stamm, 2008). Changes in alternative splicing are frequently observed in cancer, where they are probably the result of the cellular transformation. In several genetic diseases, such a FDTP-17 or spinal muscular atrophy a

change in splicing is caused by mutations and is the actual cause of the disease (Jeanteur, 2006).

Approximately 15% of mutations that cause genetic diseases affect pre-mNA splicing (Krawczak et al., 1992). Mutations located in noncoding regions, such as those affecting 5' and 3' splic sites, branch sites or polyadenylation signals, are frequently the cause of hereditary disease (Caceres et al., 2002). Depending on what and how mutation affect pre-mRNA splicing, Faustino and Cooper grouped all pre-mRNAs defects that lead to diseases into four categories (Figure 15). (Faustino and Cooper, 2003; Garcia-Blanco et al., 2004; Caceres et al., 2002; Cartegni et al., 2002; Novoyatleva T. et al., 2006).

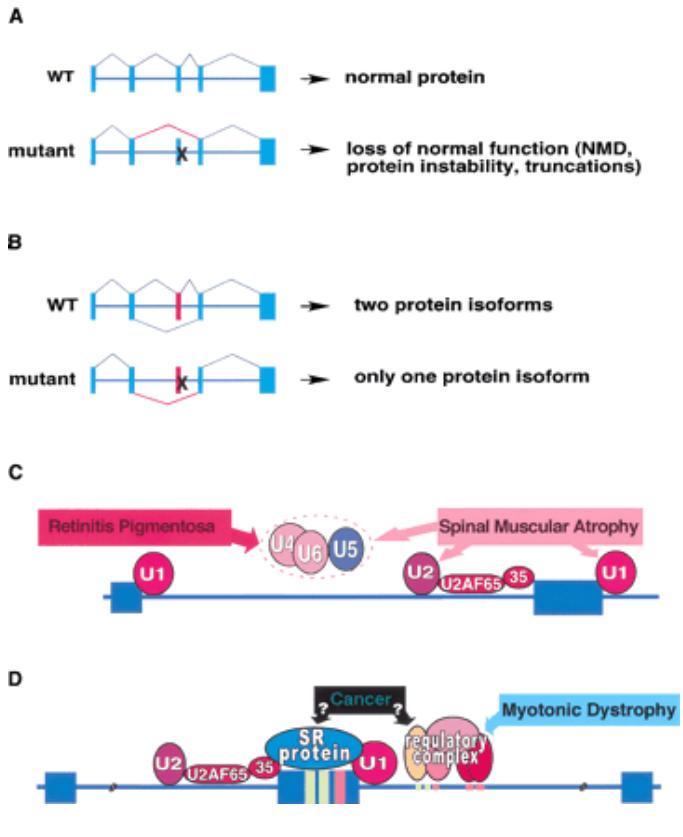


Figure 15. Four classes of pre-mRNA splicing defects that cause disease. A. Cis-acting mutations that disrupt use of constitutive splice sites: mutations that disrupt classical splicing signals of a constitutive exon. The result is expression of unnatural mRNAs, and most often loss of function of the mutated allele due to nonsense-mediated decay or expression of proteins containing internal deletions, a shift in the reading frame, or C-terminal truncations. B. Cis-acting mutations that disrupt use of alternative splice sites: Cis-acting mutations that cause disease by disrupting alternative splicing have been described for four different genes. C. Trans-acting mutations that disrupt the basal splicing machinery: two diseases are known to be caused by mutations that affect the function of the basal splicing machinery. D. Trans-acting mutations that disrupt splicing regulation: regulation of alternative splicing is disrupted in several forms of cancer and the trinucleotide repeat disorder, myotonic dystrophy. (Faustino and Cooper, 2003).

1.5.1. Mutation of cis-acting elements

Mutations of cis-acting elements can be classified according to their location and action. Type I mutations occur in the splice sites and destroy exon usage, type II mutations create novel splice sites that cause inclusion of a novel exon, type III and IV

mutations occur in exons or introns, respectively, and affect exon usage. Type I and II mutations are the simplest mutations to be recognized. Although bioinformatics resources such as the ESE finder (Cartegni et al., 2003), or the RNA workbench (Thanaraj et al., 2004) help to predict type III and IV mutations, the theoretical models often do not fit the predictions (Pagani et al., 2003). However, genotype screening in human diseases has identified numerous exonic and intronic variations. Their association with a disease phenotype is often unclear since apparently benign polymorphism, such as codon third position variations or conservative amino acid replacement, are difficult to assess. A list of well-studied mutations in splicing regulatory elements is given in Table 3 and is maintained at the alternative splicing database web site (http://www.ebi.ac.uk/asd/).

Table 3. A list of mutations in splicing regulatory elements (taken from Novoyatleva et al., 2006).

Disease	Gene	Mutation	Reference
FTDP-17 Frontotemporal Dementia with Parkinsonism linked to chromosome 17	Tau	T>G at pos 15 of Exon 10 (N279K) ATTAATAAGAAG ATTAAGAAGAAG	(Clark LN et al., 1998)
		AAG del at 16 of Exon10 (Δ280K) ATTAATAAGAAGCTG ATTAAT------AAGCTG	(Rizzu P.et al., 1999)
		T>C at pos 30 of Exon 10 (L284L) CTGGATCTTAGCAAC CTGGATCTCAGCAAC	(D'Souza I. et al., 1999)
		G>A at pos 92 of Exon10 (S305N) improves the splice site GGCAGTGTGA GGCAATGTGA	(Iijima M.et al., 1999)
Thrombasthenia of glanzmann and naegeli	Integrin gpiiia	ACGGTGAGgt ACAGTGAGgt at position 20624 of the GPIIIa gene G>A 6 bp upstream of the GPIIIa exon 9 splice donor site at pos. 134 of exon 9	(Jin Y. et al., 1996)
Menkes disease	Mnk	GATCTTCTGGA GATCT---GGAT Del 1339L - 4159 TCT of exon 21	(Gu Y.H et al., 2001)
Metachromatic	Arylsulfatase A	CAGACGAGGTC	(Hasegawa Y. et al.,1994)

leukodystrophy		CAGACAAGGTC 2330T C-to-T substitution, 22 nucleotides downstream from the exon 8 splice acceptor site	
Immunodeficiency	TNFRSF5, tumour-necrosis factor receptor superfamily, member 5 (CD40)	CTACAGGG CTACTGGG A to T substitution at nucleotide 455 is a silent mutation that occurs within a putative binding motif for the SF2/ASF protein.	(Ferrari S.et al., 2001)
Cerebrotendinous xanthomatosis	CYP27A1	CCTATGGGCCGTT CCTATGTGCCGTT T replaced G at the third position of codon 112, 13 bp upstream from the 3' terminus of exon 2	(Chen W. et al., 1998)
Marfan syndrome	Fibrillin 1	IVS51+41 (C>T) GGGATCATCGTGGGA GGGATCATTGTGGGA (I2118I) IVS51+26 (T>G) TGTCCTTATGGAAGT TGTCCTTAGGGAAGT (Y2113X)	(Liu Q.et al., 1997)
Acute intermittent porphyria	Porphobilinogen deaminase	IVS3-22 (C>G) GTGATTCGCGTGGGT GTGATTCGGGTGGGT (R21R)	(Llewellyn D.H. et al.,1996)
Hereditary tyrosinemia	Fumarylacetoac etat hydrolase	IVS8-11 (C>T) CTTATGAACGACTGG CTTATGAATGACTGG (N232N)	(Ploos van Amstel J.K. et al., 1996)
Leigh's encephalomyelopathy	Pyruvat dehydrogenase E1 alpha	628G→A GGGCGCTGG GGGCACTGG G to A substitution at nucleotide 13 of exon 6	(De Meirleir L. et al., 1994)
Homocystinuria	Methionine synthase	TCAGCCTGAGAGGA TCAGCCCGAGAGGA T to C transition within intron 6 of the mtrr gene	(Zavadakova P. et al., 2002; Zavadakova P. et al., 2005)

1.5.2. Mutations in trans-acting elements

There are several genetic diseases in which a mutation disrupts the machinery of splicing, either the constitutive components of the spliceosome (Figure 15C) or auxiliary factors that regulate alternative splicing (Figure 15D). Null mutations in spliceosome components are generally lethal or synthetic lethal in yeast and are most often lethal at

the cellular level in metazoans. Despite the expectation that dysfunction of the basal splicing machinery should be cell-lethal regardless of cell type, mutations that disrupt the assembly or function of spliceosomal snRNPs are responsible for two human diseases in which two different subsets of neurons are affected (Figure 15C).: retinitis pigmentosa and spinal muscular atrophy.

1.5.2.1. Spinal muscular atrophy (SMA)

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder, characterized by the loss of spinal cord alpha motor neurons, which results as proximal, symmetrical limb, and trunk muscle weakness with progressive paralysis ultimately leading to death (Pearn et al., 1980). The incidence is 1 to 6000 for live births and the carrier frequency is 1 in 40.

Using linkage analysis, all the clinical subtypes of SMA have been mapped to chromosome 5q11.2-13.3 (Brzustowicz et al., 1990). The gene responsible for the disease is the survival of motor neuron (SMN) gene (Lefebvre et al., 1980; Rochette et al., 2001). SMN is a ubiquitously expressed, 294-amino-acid (~38kDa) protein, which is localized both in cytoplasm (Lefebvre et al., 1995) and in nuclear bodies, called gems. Gems were shown to be involved in small ribonucleoprotein assembly and their recycling (Paushkin et al., 2002), where SMN plays a critical role in spliceosomal snRNP assembly. In addition, it was shown that SMN is required for the regeneration of spliceosomes (Fisher et al., 1997; Pellizoni et al., 1998).

SMN1 and SMN2 represent two nearly identical copies of the SMN gene in humans (Lefebvre et al., 1995). SMN1 predominantly produces full-length transcripts, whereas SMN2 mostly produces transcripts lacking exon 7, which encode truncated unstable protein (Figure 16).

Deletion of SMN1 coupled with the inability of SMN2 to compensate for the loss of SMN1 leads to spinal muscular atrophy. It is generally believed that correction of SMN2 exon splicing holds the promise for cure of SMA (Singh et al., 2004). However, this requires a full understanding of SMN2 exon splicing. Comparison between SMN1 and SMN2 revealed a critical single-nucleotide C to T mutation at the 6th position (C6U transition in transcript) in the exon of SMN2.

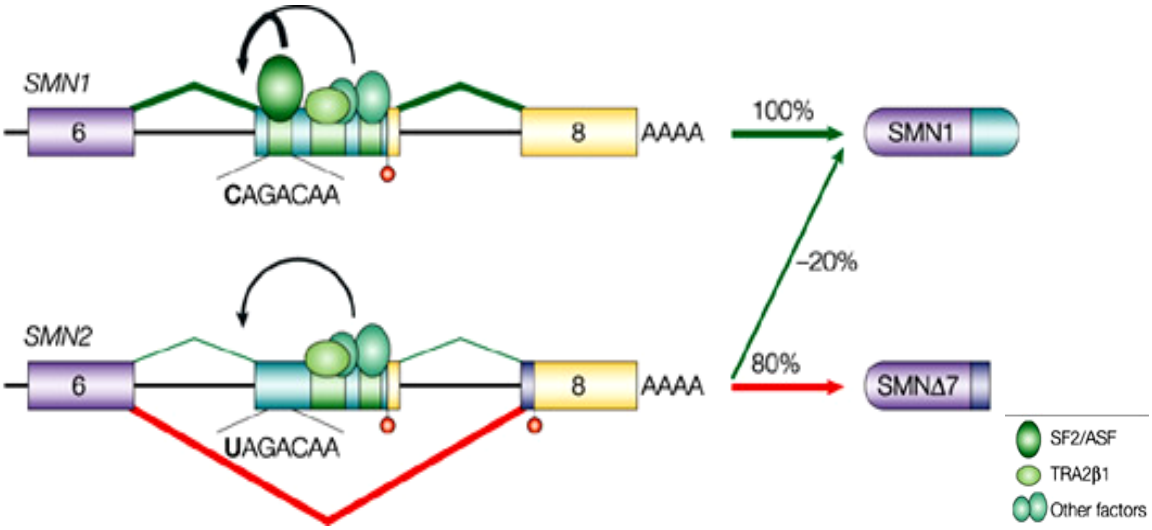


Figure 16. Splicing of pre-mRNAs from the human spinal muscular atrophy SMN1 and SMN2 genes. Binding of SF2/ASF to its cognate heptamer ESE in SMN1 exon 7 (top) promotes exon definition so that exon 7 is constitutively included, allowing for translation of full-length SMN protein. The C6T change in SMN2 exon 7 (bottom) prevents efficient SF2/ASF binding to the corresponding heptamer. Exon 7 is thus mostly skipped, resulting in the production of defective SMNdelta7 protein. Other ESEs in the exon can mediate weak exon inclusion even in the absence of the SF2/ASF motif, probably through binding of other SR or SR-like proteins, including hTra2-beta1. Partial inclusion of SMN2 exon 7 generates a small amount of full-length SMN protein, identical to that encoded by the SMN1 gene. Exons are represented as boxes and introns as lines. Curved arrows denote promotion of exon definition and chevrons indicate splicing patterns. Line thicknesses are indicative of relative splicing efficiency. The diagrams of SMN and SMNdelta7 proteins illustrate the different C-terminal domains. STOP codons are indicated in red dots. For simplicity, other SMN isoforms are not considered in this model. Drawings are not to scale. (Adapted from Cartegni, 2002)

A silent C→T mutation within exon 7 inhibits the exonic splicing enhancer (ESE), which ultimately leads to exon 7 skipping (Lorson et al., 1999). SMN2 exon seems to have a weak 3´ splice site (3´ss). Consistently, an improved polypyrimidine tract (PPT), which defines the 3´ss, restored inclusion of SMN2 exon (Lorson and Androphy, 2000). However, an improved PPT is not sufficient to compensate for the loss of a purine-rich ESE in the middle of the exon. This ESE was identified as the binding site for splicing factor Tra2-beta1. In addition to hTra2-beta1, other SR proteins, like ASF/SF2 and SRp30c can stimulate exon 7 inclusion and restore its inclusion (Young et al., 2002; Hofmann et al., 2000).

1.5.3. Tra2-beta1 and diseases

Tra2-beta1 was found to be involved in alteration of function of the ESE in exon 10 of tau pre-mRNA (Hartmann et al., 2001; Jiang et al., 2000). Tau is a microtubule-

associated protein expressed in the nervous system, where it plays a role in the polymerization and stabilization of microtubules (Delacourte et al., 1997). Mutation in the tau gene correlated with frontotemporal dementia and parkinsonism linked to chromosome 17 (Hutton et al., 1998; Spillantini et al., 1998). Human Tra2-beta1 directly and ASF/SF2 indirectly associated with the ESE of wild type tau exon 10 and promotes splicing of this exon (Kondo et al., 2004). Analysing of the splicing patterns of tau, htra2-beta, presenilin 2 and clk2 genes in affected brain areas of sporadic Alzheimer's disease patients shows that in these affected areas, the amount of mRNAs of tau isoforms including exon 10, the htra2-beta1 isoform and an inactive form of clk2 (kinase which phosphorylate tra) are significantly increased (Glatz , 2006).

Tra2-beta1 binds to the purine-rich exonic splicing enhancer in the middle of exon 7 of SMN2 and promote its inclusion (Young et al., 2002).

Recently, it was shown that Tra2-beta1 enhances the in vivo inclusion of CD44 exons v4 and v5. In a matched pair analysis of human breast cancers and non pathological tissue control, tra2-beta1 demonstrated significant induction in invasive breast cancer. This data suggest that Tra2-beta1 might be responsible for splicing of CD44 isoforms assocciated with tumor progression and metastasis (Watermann et al., 2006). It has been reported that concentration of Tra2-beta1 are altered in ovarian cancer as well (Fischer et al., 2004).

1.5.4. Manipulation of alternative splicing with compounds and their application for diseases

For therapeutic modulation of alternative splicing several trials with antisense oligonucleotide containing binding motifs for splicing activators (Sazani and Kole, 2003), peptide nucleic acid (PNA) oligonucleotide (Cartegni and Krainer, 2003), and RNAi (Celotto et al., 2005) have been reported. These approaches could be useful for manipulating a specific splice site selection of a known target sequence. These approach already was used for correction of β-globin of β–thalassemia (Sazani and Kole, 2003). Low molecular weight drugs were used in second diffusion for treatment of several disease. Histone deacetylase inhibitors, such as sodium butyrate and valproic acid, have been used to increase the exon 7 inclusion of the SMN2 gene (Chang et al., 2001; Brichta

et al., 2003). The main disadvantage against using such compounds is high toxity, low specifity and their broad mechanism of action, which is not restricted to pre-mRNA. The serious barrier against using these compounds is that most of them have side effects.

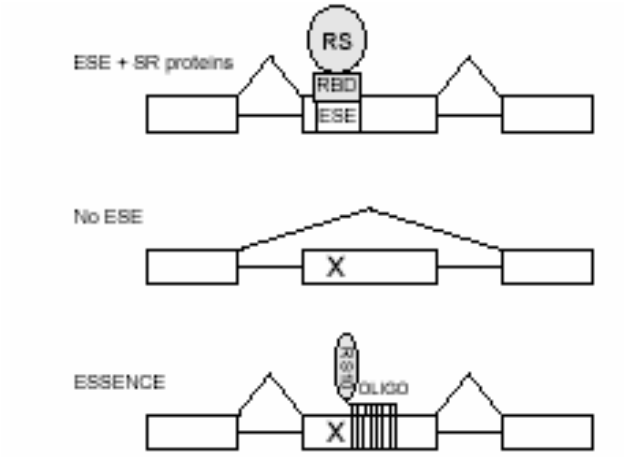


Figure 17. Design of synthetic compounds that specifically promote exon inclusion. ESSENCE emulates the ESE-dependent function of SR proteins. SR proteins (top) bind to an ESE through their RNA-binding domain (RBD) and promote exon inclusion by recruiting the splicing machinery through their RS domain (RS). In the absence of an ESE (middle), SR proteins cannot bind and the exon is skipped. An ESSENCE compound (bottom) tethers a minimal RS domain to the exon by Watson-Crick base pairing and rescues splicing. Any sequence along the exon can be chosen as the target site. (Figure adapted from Cartegni and Krainer, 2003).

Designing of molecules that promote exon inclusion is more promising. The approach, which is called ESSENCE (for Exon-Specific Splicing Enhancement by small Chimeric Effectors) is based on the assumption that the main function of SR proteins in ESE-dependent splicing reflects two features: a targeting domain which recognizes specific RNA sequences, and a recruitment domain which mediates interactions with the splicing machinery (Figure 17).

1.6. Modern technologies for analysis of alternative splicing

The alternative splicing process generates huge complexity and numerous databases have been developed to describe the splice variants that are produced. These databases are based on the analysis of either mRNA or expressed sequences tags (ESTs) in publicly available libraries, represented in the Table 4.

In addition, large-scale analysis of alternative splicing variants is possible following recent improvements in microarrays (Blencowe et al., 2006; Hughes et al., 2006; Pan et al., 2004). Three major kinds of microarrays are used for large-scale transcriptomic analyses. In ‘classical’ expression arrays, probes are selected from within the last exon of each gene. For example, Affymetrix 3′ Expression Arrays contain 11 pairs of probes within 600 bp of the 3′ end of each gene.

Table 4. Web resources on alternative splicing

Databases	Data	Organisms	URL
ASAP II [a]	ESTs and mRNAs	15 organisms	http://www.bioinformatics.ucla.edu/ASAP2/
ASD [b]	ESTs and mRNAs	Human and mouse	http://www.ebi.ac.uk/asd/
ASTRA [c]	mRNAs	Six organisms	http://alterna.cbrc.jp/
EASED [d]	ESTs and mRNAs	Nine organisms	http://eased.bioinf.mdc-berlin.de/
ECgene [e]	ESTs and mRNAs	Nine organisms	http://genome.ewha.ac.kr/ECgene/
fast DB [f]	ESTs and mRNAs	Human and mouse	http://www.fast-db.com/
Hollywood	ESTs and mRNAs	Human and mouse	http://hollywood.mit.edu/
MAASE [g]	Manual annotations	Three organisms	http://maase.genomics.purdue.edu/
PALS db [h]	ESTs and mRNAs	Human and mouse	http://ymbc.ym.edu.tw/palsdb/
ProSplicer	Proteins, mRNAs and ESTs	Human	http://prosplicer.mbc.nctu.edu.tw/
SpliceNest	ESTs and mRNAs	Four organisms	http://splicenest.molgen.mpg.de/

[a] Alternative splicing annotation project.
[b] Alternative splicing database.
[c] Alternative splicing and transcription archives.
[d] Extended alternatively spliced EST database.
[e] Gene modeling by EST clustering.
[f] Friendly alternative spicing and transcripts database.
[g] Manually annotated alternatively spliced events.
[h] Putative alternative splicing database.

This kind of array, which is the gene-level expression-profiling tool that has been used most often by the research community to investigate numerous biological processes, characterize regulatory pathways and validates drug targets. However, probing the 3′ end of the transcripts does not distinguish expression of splice variants. The Affymetrix ‘Exon Arrays’ contain >6 500 000 probes, which enables us to estimate the expression of >1 million known and predicted exons. Therefore, in addition to providing gene-level expression that is not biased at the 3′ end, expression profiles can be studied at the exon level, and provide information on skipping and inclusion of alternative exons. However, these arrays do not provide information on exon–exon junctions and exon combinations

in transcripts. Finally, ‘splicing arrays’ are designed with probes that match either exons or exon–exon junctions (Blencowe et al., 2006; Hughes et al., 2006; Relogio et al., 2005). This kind of array enables the estimation of the expression and expression ratios of many known splicing events. However, because of the many probes required, only well-known exons and splicing events are included on these custom chips.

It is expected that such large-scale studies of alternative splicing will enable us to better understand cell responses to stimuli in the future.

2. Research overview

A single pre-mRNA can give rise to two or more mature mRNAs depending on the combination of exons spliced together, due to a mechanism, which is called alternative splicing.

The factors involved in pre-RNA splicing and their interaction with various splicing components are still poorly understood. One of the regulatory mechanisms which was postulated during the last years is the reversible phosphorylation of splicing factors.

In the first part of this work I focused on the Sam68-like mammalian proteins: rSLM-1 and rSLM-2, which belong to the STAR family of proteins. We found striking differences in their localization in brain areas. Both splicing factors can change alternative exon usage of survival of motoneuron 2 gene (SMN2) reporter minigenes. The study of possible regulatory mechanisms shows that rSLM-1 is phosphorylated by several non-receptor tyrosine kinases and $p59^{fyn}$-mediated phosphorylation abolishes the ability of rSLM-1 to regulate splice site selection.

The second part of this work was dedicated to Tra2-beta1, an SR-related protein which contains a protein phosphatase 1 binding motif (RVDF) and an evolutionary conserved region downstream of this binding motif. We investigated the functional role of the downstream peptide and found it to be crucial for the ability of Tra2-beta1 to change splice site selection. Blocking of Tra2-beta1 dephosphorylation by inhibitors of PP1 or increasing the level of cAMP leads to promotion of the alternative exon 7 inclusion of the SMN2 gene. We showed that the dopamine- and cAMP-regulated phosphoprotein (DARPP-32) interacts with Tra2-beta1 in vivo and in vitro. DARPP-32 can change splice site selection. It promotes skipping of exon 10 of the tau minigene. Modulation of components of signal transduction systems influence alternative splicing of pre-mRNA transcripts by phosphorylation and dephosphorylation of the Tra2-beta1 splice factor.

Taken together, the interaction between human Tra2-beta1 and DARPP-32 indicates a previously unknown function of Tra2-beta1 in the cAMP-dependent regulation of splice site selection. Our finding suggests new mechanisms of regulation of alternative splicing that involves a combination of reversible phosphorylation events. The data show that multiple signal transduction pathways converge on the spliceosome.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Product	Supplier	Product	Supplier
30% Acrylamide/Bis solution	Sigma	β-Mercaptoethanol	Merck
Agar	GibcoBRL	Methanol	Roth
Ultra Pure agarose	Invitrogen	Microcystin	Axxora
Ampicilin	Sigma	Ni-NTA Agarose	Qiagen
Aprotinin	Sigma	Nodularin	Axxora
[γ-^{32}P]-ATP	Amersham	Nonidet P-40 / Igepal CA-630	Sigma
Benzonase	Sigma	dNTPs	Invitrogen
Boric acid	Roth	Paraformaldehyde	Merck
Bradford reagent (BioRad Protein Assay)	BioRad	PEG 3500	Sigma
Brilliant Blue R 250	Sigma	Perhydrol 30% H_2O_2	Merck
Bromophenol blue	Merck	Phenol: Chloroform: Isoamyl alcohol	Sigma
Calyculin	Upstate	PMSF	Sigma
Cantharidin	Sigma	Poly[C]/[U]/[G]/[A] Agarose Beads	Sigma
Cellfectin	Invitrogen	Potassium chloride	Merck
Chloramphenicol		2-Propanol	Roth
Chloroform: Isoamyl alcohol	Sigma	Protease Inhibitor Cocktail	Sigma
Deoxycholic acid	Sigma	Protein A Sepharose	Amersham
ssDNA/dsDNA Cellulose	Sigma	RNase Inhibitor	Roche
Dextrose	Sigma	SDS	Sigma
DMSO	Sigma	Sepharose CL-4B	Pharmacia
Dephostatin	Calbiochem	Silver Stain Plus	BioRad
DTT	Merck	Sodium acetate	Merck
EDTA	Merck	Sodium chloride	Roth
Ethanol	Roth	Sodium dihydrogen phosphate	Merck
Ethidium bromide	Sigma	Sodium fluoride	Sigma
Ficoll 400	Fluka	Sodium hydroxide	Merck
Gelatin	Sigma	Sodium orthovanadate	Sigma
Glutathione–Sepharose 4B	Amersham	Sodium pyrophosphate	Merck
Glycerol	Sigma	di-Sodiumhydrogen phosphate	Merck
Glycerol 2-phosphate	Sigma	Tautomycin	Calbiochem
Glycin	Roth	TEMED	Sigma
HiperFect	Qiagen	TNT Reticulocyte KIT	Promega
HEPES	Sigma	Tris base	Aldrich
Imidazole	Roth	TRIzol	Sigma
p-Iodophenol	Sigma	Triton X-100	Sigma

IPTG	Sigma	Tryptone	Sigma
Kanamycin	Sigma	Tween 20	Sigma
Luminol	Sigma	Valproic acid	
Lysozyme	Sigma	Yeast Extract	Sigma
Magnesium chloride	Merck	X-Gal	Sigma
Magnesium sulfate	Sigma	Xylene cyanole FF	Sigma

3.1.2. Enzymes

Product	Supplier	Product	Supplier
EcoRI	NEB	CaMKII	NEB
NotI	NEB	Erk2	NEB
SacII	NEB	T4 PNK	NEB
DpnI	NEB	T7 DNA Polymerase	NEB
NheI	NEB	FastLink T4 DNA Ligase	Biozym
Cdc2	NEB	EcoRI	NEB
Akt1/PKB	NEB	PP1	NEB
CK1	NEB	AmpliTaq DNA polymerase	Roche
GSK-3β	NEB	Platinum Pfx polymerase	Invitrogen
PKA	NEB	SuperScript II	Invitrogen

3.1.3. Cell lines and media

Cells were replated when reaching 80% confluence. Cells were detached by washing and subsequent incubation with trypsin/EDTA at 37 °C for 2-3 minutes until single cell suspension was formed. 1/5 to 1/10 of this suspension was transfered to a new dish and mixed with the corresponding growth medium. Cells were maintained in DMEM supplemented with 10% Fetal Calf Serum (GibcoBRL) in incubator at 37 °C, 5% CO2.

Name	Description	ATCC number
COS-7	African green monkey kidney SV40 transformed	CRL-1651
HEK293	Human embryonic kidney transformed with adenovirus 5 DNA	CRL-1573
BHK-21	Hamster Syrian kidney fibroblasts	CCL-10
Neuro-2a	Mouse neuroblastoma	CCL-131

3.1.4. Preparation of LB media

LB medium (1L)

10g NaCl

10g Tryptone

5g Yeast extract

20g Agar

LB Agar (1L)

10g NaCl

10g Tryptone

5g Yeast extract

3.1.5. Bacterial strains and media

Strain	Genotype	Reference
BL21 (DE3)-RIL	ompT hsdS(rB mB) dcm+ Tetr gal λ(DE3) endA Hte [argU ileY leuW Camr]	(Studier, F.W. et al., 1990)
XL1-Blue MRF'	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)]	(Bullock W.O. et al.,1987)
CJ236	F´ cat (pCJ105=pOX38::cat=FΔ(HindIII)::cat [Tra+ Pil+ CamR]/ ung-1 relA1 dut-1 thi-1 spoT1	(Kunkel T.A. et. al., 1987)
TOP10	F-mcrA Δ(mrr-hsdRMS-mcrBC) F80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 nupG	(Invitrogene, Instruction Manual 25-0276)
DH10Bac™	F-mcrAΔ(mrr-hsdRMS-mcrBC)Φ80lacZΔM15ΔlacX74deoRrecA1 endA1araD139Δ(ara,leu)7697galKλrpsLnupG/bMON14272/pMON7124	(Hanahan D., 1983)

3.1.6. Antibiotics

In research were used following antibiotics

Antibiotic	Working concentration	
	Liquid culture	Agar plates
Ampicilin	100µg/ml	100µg/ml
Chloramphenicol	15µg/ml	30µg/ml
Kanamycin	20µg/ml	20µg/ml
Gentamycin	10mg/ml	7 µg/ml
Tetracycline	10mg/ml	10µg/ml

Ampicilin and kanamycin were stored at 4ºC. Chloramphenicol and tetracyclin were stored at -20º. Gentamycin was stored at RT.

3.1.7. Antibodies

Antibody	Organism	Source
anti-actin (1:2000)	Mouse	Amersham
anti-GAPDH	Mouse	Sigma
anti-GFP (1:3000)	Mouse	Roche
anti-Flag M2 (1:1000)	Mouse	Sigma
anti-FCM (anti PP1) (1:5000)	Rabbit	Custom made (Gift from M. Bollen)
anti-SLM-1 (1:1000)	Rabbit	Custom made[1]
anti-Fyn (1:50) IHC	Mouse	Santa-Cruz Biotechnology
anti-SLM-2 (1:800)	Rabbit	Custom made[2]
anti-SMN	Mouse	Santa Cruz
anti-p(Tyr)PY20 (1:5000)	Mouse	Santa Cruz
anti-Tra2-beta1 (1:2000)	Rabbit	Custom made[3]
anti-Tra2-beta1+alpha (ps568)(1:1000)	Rabbit	Custom made[4]
anti-mouse Ig (1:10000)	Sheep	Amersham
anti-rabbit Ig (1:10000)	Rabbit	Amersham

anti-SF2/ASF (1:200)	Mouse	ZYMED Laboratories
anti-sc35 (1:2000)	Mouse	SIGMA
anti-PP1γ1 (1:100)	Goat	Santa Cruz Biotechnology
anti-DARPP (1:500)	Mouse	Gift from Dr. J.-A. Girault
anti-Tra-p-T203 (1:1000)	Rabbit	Custome made[5]
anti-YT521-B (PK2) (1:3000)	Mouse	Custom made[6]

1) anti-SLM-1 peptides were used after coupling to KLH: VNEDAYDSYAPEEWTTCG and DQTYEAYDNSYVTPTQSVPECG
2) anti-SLM-2 peptides: VVTGKSTLRTRGVTCG and PRARGVPPTGYRPCG
3) anti-Tra2-beta1 peptide:MSDSGEQNYGERVNVEEGKCGSRHLTSFINEYLKLRNK
4) anti-ps568/Tra peptide used : GC(StBu)SITKRPHTPTPGIYMGRPTY
5) anti-Tra-p-T203 peptide used: P**pT**PGIYMGC
6) anti-YT521-B was used against a mixture of two YT521-B peptides: P1 RSARSVILIFSVRESGKFQCG and P2 KDGELNVLDDILTEVPEQDDECG (Nayler O. et al., 2000

3.1.8. Plasmids

Clones from the Stamm's lab collection or outside sources:

Name	**Backbone**	**Description**	**Reference**
pEGFP-C2	pEGFP-C2	CMV promoter, Kan^r/Neo^r, f1 ori	Clontech
hTra-delRS1	pEGFP-C2	Deletion of RS1 domain from Tra2-beta1	(Stoilov P. et al., 2004)
hTra-delRS2	pEGFP-C2	Deletion of RS2 domain from Tra2-beta1	(Stoilov P. et al., 2004)
hTra-delRS1+2	pEGFP-C2	Deletion of both RS1 and RS2 domains from Tra2-beta1	(Stoilov P. et al., 2004)
hTra2-beta1 EGFP	p-EGFP-C2	human Tra2-beta1 in pEGFP C2	(Beil B. et al., 1997; Nayler O. et al., 1998a)
hTra2-beta1 Flag	pcDNA	Flag Tagged human Tra2-beta1 in pcDNA	(Nayler O. et al., 1998)
pRK5-fyn	pRK5	Fyn kinase	(Nayler O. et al., 1998)
pRK5-abl	pRK5	c-Abl kinase	(Nayler O. et al.,1998)
D32 WT	pEGFP-N2	Rat DARPP-32 cloned into p-EGFP-N2	(Nishi A. et al., 2002)
D32T34A	pEGFP-N2	Mutation of Threonine 34 to Alanin	(Nishi A. et al., 2002)
D32T75A	pEGFP-N2	Mutation of Threonine 75 to Alanin	(Nishi A. et al., 2002)
D32F11A	pEGFP-N2	Mutation of Phenilalanine 34 to Alanin	(Nishi A. et al., 2002)
D32 WT-pSVL	p-SVL	Rat DARPP-32 cloned into p-SVL	(Nishi A. et al., 2002)
D32 T34A-pSVL	p-SVL	Mutation for inactivation of phosphorylation site by PKA	(Nishi A. et al., 2002)
D32 T34E-pSVL	p-SVL	Mutation for hyperactivation of phosphorylation site by PKA	(Nishi A. et al., 2002) Gift from J.-A. Girault
D32 T75A-pSVL	p-SVL	Inactivation of phosphorylation site by Cdk5	(Nishi A. et al., 2002)
pCR3.1 MGTra	pCR3.1TA	Tra2-beta minigene	(Stoilov P. et al., 2004)
SV9/10L/11	Exontrap	Tau minigene	(Gao Q.-S. et al., 2000)
WT SMN MG	pCI	SMN2 minigene	(Lorson C. et al., 1999)
CD44 exon v5	pETv5	CD44 minigene	(Koenig, et al., 1998)
PP17-Gex (ATG)	pGEX	rat SLM-1 cloned into pGEX vector	(Stoss O. et al., 2001)
PP17 EGFP	pEGFP-C2	rat SLM-1 cloned in p-EGFP-C2	(Stoss O. et al., 2001)
ESAF EGFP	pEGFP-C2	rat SLM-2 cloned in pEGFP-C2	(Stoss O. et al., 2001)
ESAF-Gex (ATG)	pGEX	rat SLM-2 cloned into pGEX vector	(Stoss O. et al., 2001)

Name	Backbone	Description	Reference
rSAF-B EGFP	pEGFP-C2	rat SAF-B partial clone lacking the RRM, cloned in pEGFP-C2	(Nayler O. et al., 1998)
hnRNP-G EGFP	pEGFP-C2	rat hnRNP-G full length in pEGFP-C2	(Hartmann A.,)
SF2/ASF-EGFP	pEGFP-C2	SF2/ASF cloned in pEGFP-C2	(Nayler O. et al., 1998; Nayler O. et al., 1997)
SRp30c-EGFP	pEGFP-C2	human SRp30c cloned in pEGFP C2	None
Tra2beta1-HTa	pFastBac-HTa	human Tra2-beta1 cloned into *Drosophila* vector	None
PP1γ1 EGFP	pEGFP-C1	rat PP1gamma 1 introduced into pEGFP-C1 vector	(Lesage B. et al., 2004) Gift from M.Bollen
NIPP1-C2	pEGFP-C1	nuclear inhibitor of PP1	(Van Eynde A.S. et al., 1995) Gift from M. Bollen
HA-PP1γ	HA-pCMV5	PP1γ has HA Tag	(Maximov. A. et al., 1999) Gift from M. Bollen
hGSK3beta-myc565	p-SG5	Glycogen synthase kinase beta, myc tagged	Gift from Dr.Buee
GSK3beta S9A	p-XG73	Constituvely active mutant of Glycogene synthase beta isoform	Gift from Dr.Behrens
CK1δ-HA	pcDNA3.1/V5-His-TOPO	HA-tagged casein kinase 1 delta isoform	Gift from Dr. Knipschilld
pDEST22 Tra2-beta1	pDEST22	Tra2-beta1 in gateway destination vector for YTH (prey)	Tang Y. et al., 2008

Newly made clones:

Name	Backbone	Description	Tag
DARPP-pDEST32(bait)	pDEST 32	Mouse DARPP cloned into pDEST 32 for yeast two hybrid screening	none
rSLM1-YFP	pYFP Shutlle vector	Rat SLM-1 cloned into shuttle vector p-YFP	EYFP
EGFP Tra2-beta S(194)A, T(196)A	pEGFP-C2	Double mutant cloned into p-EGFP-c2	EGFP
EGFP-Tra2-beta1 S(194)E, T(196)E	pEGFP-C2	Double mutant cloned into p-EGFP-c2	EGFP
Tra S(194)A-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1 after RVDF motif	EGFP
Tra S(194)E-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1 after RVDF motif	EGFP
Tra T(196)A-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP
Tra T(201)A-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP
Tra T(201)E-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP
Tra T(203)A-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP
Tra T(203)E-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP
Tra H(200)A-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP

Tra H(200)E-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP
Tra Y(207)R-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP
Tra Y(207)F-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP
Tra G(209)I-pDEST53	pcDNA-pDEST53	Point mutation of Tra2-beta1	EGFP
Tra TPT/KAI-pDEST53	pcDNA-pDEST53	Three exchanges of amino acids of Tra2-beta1	EGFP
Tra4A-pDEST53	pcDNA-pDEST53	Four mutation of all phosphorylation sites (S194, T196, T201, T203) to Alanine of Tra2-beta1	EGFP
Tra4E-pDEST53	pcDNA-pDEST53	Four mutation of all phosphorylation sites (S194, T196, T201, T203) to Glutamate acid of Tra2-beta1	EGFP
IL-4R LNG MG	pET01	Interleukin - 4 Receptor minigene	none

3.1.9. Primers

Primers used for mutagenesis

Name	**Sequence 5'→3'**	**Introduced mutation**	**Name of the generated clone**
RATA SRp30c F	CGGCTTCGTGCGACGGCCCCCAGGACT	gtg(V)→gcg(A) gag(E) →acg(T) ttc(F) →gcc(A)	SRp30c-RATApDEST53
RATA SRp30c R	AGTCCTGGGGGCCGTCGCACGAAGCCG	gtg(V)→gcg(A) gag(E) →acg(T) ttc(F) →gcc(A)	SRp30c-RATApDEST53
RATA SF2 F	CGTCTGCGGGCGACGGCTCCTCGAAGC	gtg(V) →gcg(A) gag(E) →acg(T) ttt(F) →gct(A)	SF2-RATApDEST53
RATA SF2 R	GCTTCGAGGAGCCGTCGCCCGCAGACG	gtg(V) →gcg(A) gag(E) →acg(T) ttt(F) →gct(A)	SF2-RATApDEST53
Tra(S_A)/(T_A)	GATTTCGCTATAGCAAAAAGA	tct→gct, aca→gca	TraS194A/T196A-pEGFP-c2
Tra(S_E)/(T_E)	GATTTCGAAATAGAAAAAAGA	tct→gaa, aca→gaa	TraS194E/T196E-pEGFP-c2
Y207F For	CCAGGAATTTTTATGGGGAGAC	tac→ttt	TraY207F-pDEST53
Y207F Rev	GTCTCCCCATAAAAATTCCTGG	tac→ttt	TraY207F-pDEST53
Y207R For	ACCAGGAATTAGAATGGGGAGAC	tac→aga	TraY207R-pDEST53
Y207R Rev	GTCTCCCCATTCTAATCCTGGT	tac→aga	TraY207R-pDEST53
H200E For	AAAAAGACCAGAAACGCCAACAC	cat→gaa	TraH200E-pDEST53
H200E Rev	GTGTTGGCGTTTCTGGTCTTTTT	cat→gaa	TraH200E-pDEST53
H200A For	AAAAAGACCAGCTACGCCAACA	cat→gct	TraH200A-pDEST53

Name	Sequence 5'→3'	Introduced mutation	Name of the generated clone
H200A Rev	TGTTGGCGTAGCTGGTCTTTTT	cat→gct	TraH200A-pDEST53
G209I For	AATTTACATGATTAGACCTACCT	ggg→att	TraG209I-pDEST53
G209I Rev	AGGTAGGTCTAATCATGTAAATT	ggg→att	TraG209I-pDEST53
TraT/ELong For	GTTGATTTCTCTATAGAAAAAAGACCACATACG	aca→gaa	TraT196E pDEST53
TraT/ELong Rev	CGTATGTGGTCTTTTTTCTATAGAGAAATCAAC	aca→gaa	TraT196E pDEST53
TraS/E F	AGTTGATTTCGAAATAACAAAAA	tct→gaa	TraS194E pDEST53
TraS/E R	TTTTTGTTATTTCGAAATCAACT	tct→gaa	TraS194E pDEST53
TraHT201E Forv	AAGACCACATGAGCCAACACCA	acg→gag	TraT201E pDEST53
TraHT201E Rev	TGGTGTTGGCTCATGTGGTCTT	acg→gag	TraT201E pDEST53
TraPT203E Forv	ACATACGCCAGAACCAGGAATT	aca→gaa	TraT203E pDEST53
TraPT203E Rev	AATTCCTGGTTCTGGCGTATGT	aca→gaa	TraT203E pDEST53
Mut TRA 4A R	ATTCCTGGTCGTGGCGCATGTGGTCTTTTTGCTATAGCGAAATCAACTC	tct→gct, aca→gca, acg→gcg, aca→gca	Tra4A pDEST53
Mut TRA 4A F	AGTTGATTTCGCTATAGCAAAAAGACCACATGCGCCAGCACCAGGAAT	tct→gct, aca→gca, acg→gcg, aca→gca	Tra4A pDEST53
Mut TRA 4E F	AGTTGATTTCGAAATAGAAAAAAGACCACATAAGCCAGAACCAGGAATT	tct→gaa, aca→gaa, acg→gaa, aca→gaa	Tra4E pDEST53
Mut TRA 4E R	AATTCCTGGTTCTGGCTCATGTGGTCTTTTTTCTATTTCGAAATCAACT	tct→gaa, aca→gaa, acg→gaa, aca→gaa	Tra4E pDEST53
TPT/KAI F	AGACCACATAAAGCAATACCAGGAAAT	aca→aaa, cga→gca, aca→ata	TPT/KAI pDEST53
TPT/KAI R	AATTCCTGGTATTGCTTTATGTGGTCT	aca→aaa, cga→gca, aca→ata	TPT/KAI pDEST53

Primers used for RT-PCR:

Name	Orientation	Sequence	Target
N3Ins N5Ins	antisense sense	CTCCCGGGCCACCTCCAGTGCC GAGGGATCCGCTTCCTGCCCC	CD44v5, IL4R minigenes
X16R T7	antisense sense	CCTGGTCGACACTCTAGATTTCCTTTCATTTGACC TAATACGACTCACTATAGGG	SRp20 minigene
INS3 INS1	antisense sense	CACCTCCAGTGCCAAGGTCTGAAGGTCACC CAGCTACAGTCGGAAACCATCAGCAAGCAG	Tau minigene
pCR3.1-RT MGTra-Xho MGTra-Bam	antisense antisense sense	GCCCTCTAGACTCGAGCTCGA GGGCTCGAGTACCCGATTCCCAACATGACG GGGCCAGTTGGGCGACCGGCGCGTCGTGCG	Tra2-beta minigene
SMNex8rev pCIfor	antisense sense	GCCTCACCACCGTGCTGG GGTGTCCACTCCCAGTTCAA	SMN2 minigene
SMN rev	antisense	TCACATTGCATTTGGTTATTACA	SMN

SMN for	sense	ATAGGATCCACCTCCCATATG	endogenous
GAPDH Rev 790bp hum	antisense	ACCTGGTGCTCAGTGTAGCC	GAPDH endo-genous
For SFRS14 Rev SFRS14	sense antisense	CAAGGACTTGGACTTCGCC CTTCTAGGCTTTATCAAGGC	Splicing factor, arginine/serine -rich 14,endogenous
For Fyn Rev Fyn	sense antisense	GAGAGCTGCAGGTCTCTG CTCGGTGCGATGTAGATG,	Fyn oncogene related to Src, Fgr, Yes endogenous
For CLK4 Rev CLK4	sense antisense	GTCCGCAGCAGGAGAAGC CATGCCATGATCAATGCACTC	CDC-like kinase4, endogenous
SELEX N20	sense	GCGTCTCGAGAAGCTTCCNNNNNNNNNNNNNNNNNNAGTCGGGAATTCGGATCCCTATAG TGAGTCGTATTA	Initial PCR for SELEX
SELEX T7Pro	sense	TAATACGACTCACTATAGGGATCCGAATTCC CGACT	PCR for SELEX
SELEX RT	antisense	GCGTCTCGAGAAGCTTCC	RT-PCR for SELEX

Primers used for subcloning into pDONR 221 (Gateway system, Invitrogene):

All reverse primers are without stop codon for possible C-terminal cloning

Name	**Orientation**	**Sequence**
attB mDARPP32 for	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGACCCC AAGGACCGCAAGAAG
attb mDARPP32 rev	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGGGGGGCTG GGATGCTGAGGTTC
attB Tra F	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGAGCGAC AGCGGCGAG
attb Tra R	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTAATAGCGACGA GGTGAGTAT
SFRS14attB1F	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCATAACCTC CAGCAGGCA
SFRS14attB2R	antisense	GGGGACCACTTTGTACAAGAAGCTGGGTGTGAGGTTTGCT CCTTCAGC
FYNattB1F	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTGACCGGG AAGAAAGTT
FYNattB2R	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTAAATATGCCCC TTGTTCGTG
attB SF2 F	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGTCGGGA GGTGTGATT
attb SF2 R	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTATGTACGAGAG CGAGATCTG
attB Tra2short Rev	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCATAGCGACGA GG
attB hCK1delta for	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGAGCTG AGAGTCGGGAACAGG
attB hCK1delta rev	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCGGTGCACG ACAGACTGAAGACC
attB hGSK3b for	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGTCAGGG CGGCCCAGAACCACC
attB hGSK3b rev	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGTGGAGTTG GAAGCTGATGCAGA

attB hInhibitor-1 for	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGAGCAAGACAACAGC
attB hInhibitor-1 rev	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCGACCGAGTTGGCTCCCTTG
attB hPPP1CC for	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGCGGATTTAGATAAACTCAAC
attB hPPP1CC rev	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTCTTTGCTTGCTTTGTGATCAT
attB SRp30c F	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGTCGGGGTGGGCGGAC
attB SRp30c R	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTAGGGCCTGAAAGGAGAG

Primers used for subcloning for construction of minigenes and SLM-1 plasmids

Name	**Orientation**	**Sequence**
SLM1-YFP-NheI F	sense	TCCGCTAGCGCCGCCACCATGGGAGAAGAGAAATACTTGCC
SLM1-YFP-NheI R	antisense	CTAGCTAGCATATCTACCATAGGGGTGTTCCC
SLM1_left	sense	TCCGGGAGTTGTCTTACTTG
6exRNAIP_hum_for	sense	CCCAGGAAACCTGACAGTTC
12exRNAIP_hum_rev	antisense	CAGGGCAAGAGCTTGGTAAG
HumIL4RaF	sense	CGCGGATCCAGGCTGGTGGCTCTTAAACA
HumIL4RaR	antisense	ATTCCGCGGTGGGGTCCTTGTCGATATGT
IL4LngMgEcoRI	antisense	GGGGAATTCTTGGTAATGCTGAAGTAACAGAACA
IL4LngMgXhoIF	sense	CTACTCGAGTTCATAGTCTATAATGTGACCTACAAGGA
IL4Lngfor	sense	CGCGGATCCATCCCATTTCCAGCACTGAG
IL4Lngrev	antisense	TGCTCTAGATTCTCAGCCAGCACAATGAC

3.2. Methods

3.2.1. Molecular Biology Methods

3.2.1.1. Plasmid DNA isolation ("mini-prep" method)

The "mini-prep" method is useful for preparing partially purified plasmid DNA in small quantities from a number of transformants. It is based on alkaline lysis method using SDS (Birnboim H.C. and Doly J., 1979). A single colony was selected and put with a sterile toothpick into 3-5 ml of LB medium containing the appropriate antibiotic. Bacterial cells were cultured overnight at 37 °C while shaking. The cells were harvested by brief centrifugation for 30 sec-1 min at 14000 rpm in a microfuge. At first, the pellet was resuspended in 150 µl of P1 buffer by pipetting or short vortexing. Then equal volume of P2 lysis buffer was added. The lysis was performed for 5 min at RT. After lysis, 150 µl of neutralization buffer P3 was added. The mixture was centrifuged for 10 min at 14000 rpm and the resulting supernatant decanted. DNA was precipitated by adding 1 volume of 99% isopropanol. For best DNA precipitation, tubes were incubated on ice for 15-20 minutes with subsequent centrifugation for 10 min at 14000 rpm. Supernatant was carefully discarded. DNA pellet was washed with 70% ethanol, air-dried and dissolved in 30 µl of buffer TE. Large amounts of plasmid DNA were prepared using the Qiagen Plasmid Maxi Kit according to the manufacturer's protocol.

P1 Buffer	50 mM Tris-HCl, pH 8.0 10 mM EDTA, 100 µg/ml RNase A
P2 Buffer	200 mM NaOH, 1% SDS
P3 Buffer	3 M KAc pH 5.1
TE Buffer	10 mM Tris-HCl, pH 8.0 1 mM EDTA

Larger amounts of plasmid (20-2500 µg) were prepared with the use of Macherey-Nagel Plasmid DNA Purification Kit (Nucleobond AX) according the manufactures protocol, which is based on a modified alkaline lysis procedure followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins and low-molecular-weight impurities are removed by a medium-salt wash;

DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

3.2.1.2. Determination of nucleic acids concentration

The DNA and RNA concentrations in solution were estimated using a spectrophotometer (Eppendorf BioPhotometer 6131). Plastic cuvettes were used for visible spectrophotometry. The absorbance of the solution was measured at 260 nm and concentration was calculated using following formulas:

1 A_{260}=50 µg/ml for double stranded DNA
1 A_{260}=37 µg/ml for single stranded DNA
1 A_{260}=40 µg/ml for RNA

3.2.1.3. Electrophoresis of DNA

The DNA was resolved on 0.7-2% agarose gels prepared in 1 × TBE buffer, containing 90 mM Tris-borate and 20 mM EDTA. The electrophoresis was run for 80 min at 1XTBE buffer. The gels were stained for 30 min in 0.5 mg/ml ethidium bromide and visualized under UV light, λ=260 nm.

6xGEL–LOADING BUFFER:

0.25% bromophenol blue
0.25% xylene cyanol FF
15% Ficoll 400 in dH_2O

3.2.1.4. Amplification of DNA by PCR

For PCR amplifications, a standard PCR reaction was set up. 1-10 ng of highly pure plasmid DNA was used as a template for the reaction. Master Mix was prepared as described below:

Forward and reverse primers each -0.5 µM

dNTPs -200 µM

1 × Taq-polymerase buffer

$MgCl_2$ -1.5 mM

1 U Taq polymerase (AmpliTaq DNA polymerase, Perkin Elmer)

For cloning purposes, Platinum Pfx polymerase was used instead of AmpliTag DNA Polymerase. The amplification was carried out in a Perkin Elmer GeneAmp PCR

System 9700 thermocycler under the following conditions: initial denaturing for 2-4 min at 94 °C; 25-35 cycles of 15-30 sec at 94 °C, annealing at the Tm of the primers pair, extension of 1 min per 1 kb at 72 °C (or 68 °C for Pfx polymerase). After the last cycle the reaction was held for 5-10 min at the extension temperature to allow completion of amplification of all products.

3.2.1.5. Elution of DNA from agarose gels

The DNA was run on 0.7-2% agarose gels in 1 × TBE buffer where 6 × Crystal Violet Gel Loading Buffer (0,25% crystal violet and 15% Ficoll400 in dH2O) was added to a final concentration of 2 μg per ml. DNA (visible under normal light) was purified from agarose gels using the Qiagen Qiaex II gel extraction kit according to the manufacturer's protocol.

3.2.1.6. Preparation of competent E.coli cells

A single colony of E.coli strain was inoculated in LB medium and cultured overnight. 4 ml of grown culture was added into fresh 250 ml LB and grown to early logarithmic phase (OD600=0.3-0.6). The culture was centrifuged for 10 min at 2500 rpm at 4 °C. The bacterial pellet was resuspended in 1/10 volume of cold TSB buffer and incubated on ice for 10 min. Cells were aliquoted into cold Eppendorf tubes and frozen in liquid nitrogen. Competent bacterial cells were stored at -80 °C.

TSB BUFFER:
10% PEG 3500
5% DMSO
10 mM $MgCl_2$
10 mM $MgSO_4$
in LB medium, pH 6.1

3.2.1.7. Transformation of E.coli cells

1-10 ng of plasmid DNA or a ligation reaction were added to 20 μl of 5 × KCM buffer, containing 500 mM KCl, 150 mM CaCl2, 250 mM MgCl2 and afterwards the 100 μl of water was added. Equal volume of thawed competent cells was added to the reaction. The reaction mixture was incubated on ice for 20 min followed by incubation at RT for 10 min. Then 1 ml of LB medium was added and the bacteria were incubated for 1 h at 37 °C

with vigorous shaking. Finally cells were plated on LB Agar plates containing appropriate antibiotic. Plates were incubated at 37 °C until colonies were visible.

3.2.1.8. Site-directed mutagenesis of DNA

Site-directed mutagenesis was performed according to the method described by Kunkel (Kunkel T.A. et al., 1985). The DNA of interest was cloned into a vector carrying the f1 phage origin of replication and thus capable of existing in both single- and double-stranded forms. The recombinant plasmid was transformed into E.coli strain CJ236 deficient in dUTPase (dut) and uracil N-glycosylase (ung). These mutations result in a number of uracils being substituted for thymine in the nascent DNA. After transformation, bacteria were grown on plates containing chloramphenicol in addition to the plasmid specific antibiotic, to ensure the presence of the F' episome necessary for production of helper phage. To isolate single-stranded DNA from the plasmid of interest, colonies were grown in 5 ml of LB medium for 90 min and then 5108 × pfu of helper phage M13KO7 (New England BioLabs) was added. The culture was grown for overnight at 37 °C and single-stranded DNA was isolated with the Qiagen M13 kit according to the manufacturer's protocol. This uracil containing DNA was used as a template in the in vitro mutagenesis reaction. Phosphorylated oligonucleotides containing desired mutations were annealed to the template at a molar ratio of 20:1 in 10 µl of 1 × T7 DNA polymerase buffer. The DNA was denatured for 5 min at 94 °C and then the temperature was gradually decreased from 70 °C to 37 °C at a rate of 1 °C per minute. The extension of the annealed primer was carried out in 20 µl by adding to the same tube 1 µl of 10 × T7 DNA Polymerase buffer, 0.8 µl of 10 mM dNTPs, 1.5 µl of 10 mM ATP, 3 U T7 DNA Polymerase and 2 U FastLink T4 DNA Ligase. The reaction was incubated at 37 °C for 45 min. The ligase was inactivated by incubation at 65 °C for 20 min. The mutagenesis reaction was transformed into competent XL1Blue E.coli cells. Replication of the plasmid in this strain leads to repair of the template strand and consequently to production of plasmid carrying the desired mutation. All mutant plasmids were verified by sequencing.

3.2.1.9. Site directed mutagenesis by overlap extension

Four primers were designed to introduce mutations by this method which was first described by Higuchi et al., 1989. One set of forward F and reverse R primer was

complementary to the extreme ends of the DNA template (Figure 1). The other set of forward MF and reverse MR primer carrying the desired mutation, were complementary to each other and target the site where the mutation was desired. Mutant primers had 8-10 bases on either side of the mutation cassette to allow precise annealing. The first PCR was carried out with Proofreading polymerase to avoid any A-overhang. Individual PCRs were carried out to amplify fragments with primer sets F/ MR and with MF/R respectively.

The amplified fragments were gel eluted to free them from any contaminating DNA template. 200 ng of the individual purified fragments were pooled together and allowed to anneal and extend without any addition of primer with dNTPs (200 µM), 1 x Taq polymerase buffer, 1.5 mM MgCl2 and 1 U Taq polymerase in total volume of 25 µl. The amplification was carried out in a Perkin Elmer GeneAmp PCR System 9700 thermocycler under the following conditions: initial denaturing for 5 min at 94°C; 10 cycles of 30 sec at 94°C, annealing at 50°C, extension of 1 min per 1 kb at 72°C. After the last cycle the reaction was held for 5 min at the extension temperature to complete the amplification of all products. External primers (F and R) were then added and the reaction was again supplemented with 1 U of Taq polymerase. Final PCR was performed with the following conditions: initial denaturation for 5 min at 94°C; 30 cycles of 30 sec at 94°C, annealing at 60°C and extension of 1 min per 1 kb at 72°C. The last cycle was followed by another 5 min of extension at 72°C. A part of the amplified fragment was run on the Agarose gel and the other subcloned into pCR4 TOPO for sequencing.

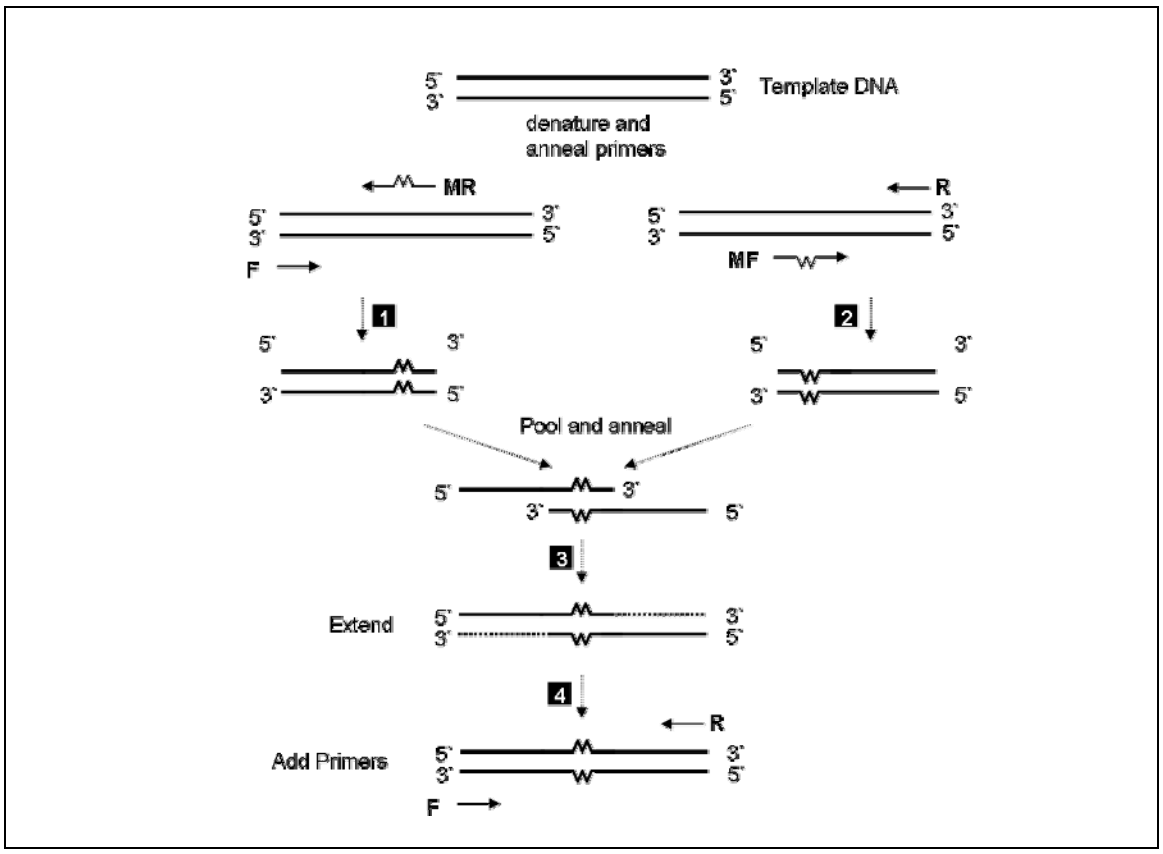


Figure 18. Site-directed Mutagenesis by Overlap Extension. In separate PCR amplification reactions 1 (Primers F and MR) and 2 (Primers MF and R), two partially overlapping fragments of the target gene containing the mutation are amplified. In PCR 3, the denatured products from PCR 1 and PCR 2 anneal at the region of overlap and extend to form full length double –stranded mutant DNA. In PCR 4, the full length mutant DNA is amplified using primers F and R. (Adapted from Molecular Cloning: A Laboratory Manual, Sambrook and Russel, third edition, 2001).

3.2.1.10. In vitro splicing assay

The experiment was performed as was previously described (Hui J., Bindereif A. EMBO, 2005) with minor modifications.

Splicing reaction included:

1 x splicing reaction setup mixture (25µl)

12.5 mM ATP	1µl
80 mM MgCl2	1µl
500 mM creatine phosphate	1µl
RNasin (40 U/µl)	1µl
RNA (10 ng/µl)	1µl
13.3%polyvinylalcohol (v/v)	5µl
HeLa cell nuclear extract	15µl

The mixture was incubated at 30ºC. Aliquots of 20 µl were removed at different time points, and stored at -20ºC.

3.2.1.11. In vivo splicing assay

To determine the influence of a protein on the splicing of selected minigenes, in vivo splicing was performed as described (Stoss O. et al., 1999; Tang Y. et al., 2005). 1 to 2 µg of the minigene plasmid were transfected in eukaryotic cells together with an expression construct for the protein. Usually a concentration dependent effect was assessed. The protein was transfected in increasing amounts, in the range of 0 to 3 µg. The 'empty' parental expression plasmid containing the promoter was added in decreasing amounts, to ensure a constant amount of transfected DNA. Cells were plated in 6-well plates and transfection was done 24 hours after plating. After incubation for 14-17 hours at 3% CO2 total RNA was isolated from the cells (see 3.2.26.).

400 ng of RNA were used in a reverse transcription reaction (see 3.4.6.). The reverse primer used for RT was specific for the vector in which the minigene was cloned, to suppress reverse transcription of the endogenous RNA. To avoid the problem of amplification from minigene DNA, DpnI restriction enzyme was added into the reverse transcription reaction. DpnI cuts GATC sequence in double-stranded DNA when the adenosine is methylated but does not cut non-methylated single-stranded DNA or cDNA. A control reaction with dH_2O instead of RNA was included. 1/8 of the reverse transcription reactions were used for PCR with minigene-specific primers. The primers were selected to amplify alternatively spliced minigene products. A control reaction with no template (RNA instead of cDNA) was included in the PCR. The PCR programs were optimized for each minigene in trial experiments. PCR reactions were resolved on a 0.3-0.4 cm thick 2% agarose TBE gel and the image was analyzed using ImageJ analysis software (http://rsb.info.nih.gov/ij/). The percentage inclusion is the percentage of spliced RNA that contains the exon (IOD of the peak of inclusion band/ (IOD of the peak of exclusion band + IOD of the peak of the inclusion band)) x 100. Mean values (± standard deviation) were calculated from three independent experiments each. Statistical analysis was performed by ANOVA followed by the Scheffe *F* test for comparison between samples. $P < 0.05$ was regarded as significant.

Name of the program	PCR conditions
Tra MG	94 °C 2 min; 33 cycles – 94 °C 20 sec, 65 °C 20 sec, 72 °C 40 sec; 72 °C 2 min
SMN2 MG	94 °C 4 min; 25 cycles – 94 °C 20 sec, 62 °C 20 sec, 72 °C 20 sec; 72 °C 5 min
Tau MG	94 °C 2 min; 30 cycles – 94 °C 1 min, 60 °C 1 min, 72 °C 48 sec; 72 °C 10 min
IL4R MG	94 °C 2 min; 35 cycles – 94 °C 30 sec, 72 °C 90 sec; 72 °C 20 min
SRp20 MG	94 °C 2 min ; 30 cycles - 94°C 50 sec; 55° C 50 sec; 72°C 1 min; 72 °C 5 min
CD44 MG	94 °C 4 min; 30 cycles - 94°C 20 sec; 68°C 30 sec; 72°C 40 sec;72 °C 5 min
CHIP	94 °C 4 min; 30 cycles – 94 °C 30 sec, 58 °C 30 sec, 72 °C 30 sec; 72 °C 5 min

3.2.1.12. Isolation of total RNA

Total RNA was isolated from eukaryotic cells after transfection for 16-20 hours in 6-well plates. Fifty micrograms of RNA was isolated using RNeasy Mini kit (Qiagen) accordingly to the manufacturer's protocol. RNA was eluted from the column in 30 µl of RNase-free dH2O. Alternatively, in RNA immunoprecipitation procedure RNA was isolated from Sepharose beads using TRIzol reagent according to the manufacturer's protocol. After ethanol precipitation the RNA pellet was dissolved in 20 µl of RNase-free dH2O.

3.2.1.13. Complementary DNA-synthesis (Reverse Transcription) – PCR

The reverse transcription (RT) allows the transcription of RNA in complementary DNA (cDNA) that can be subsequently used as template for PCR.

For cDNA-synthesis, 400 ng of total RNA (200 ng/µl), 5 pmol of reverse primer, 40 U of SuperScript II reverse transcriptase, and optionally 4 U of DpnI restriction endonuclease were mixed in 5 µl of RT buffer (300 µl of 5 × First strand synthesis buffer, 150 µl of 0.1 M DTT, 75 µl of 10 mM dNTPs, 475 µl of dH2O). To reverse transcribe the RNA, the reaction was incubated at 42 °C for 45 min-1 h. 1-3.5 µl of reverse transcription reaction was used to amplify cDNA. The reaction was held in 25 µl and contained 10 pmol of specific forward and reverse primers, 200 mM dNTPs, 1 × Taq polymerase buffer and 1 U of Taq DNA polymerase. The conditions of the PCR cycles were dependent on the template to be amplified (see 3.2.1.11. on conditions to amplify minigene products from *in vivo* splicing assays).

3.2.1.14. Construction of minigenes

Minigenes were constructed as described previously in Tang et al., 2004. In brief, most minigenes were amplified from genomic DNA and contained the alternatively spliced exon and its flanking constitutive exons. In majority of the cases, these parts could be amplified by long-range PCR. If the introns were too long, several kilo-bases flanking the exons were amplified and ligated together to the multiple cloning sites of exon trap vectors (Mobitec, Goettingen, Germany) between two constitutive rat insulin exons. This chimeric gene was then analyzed similar to a genomic construct.

3.2.1.15. Isolation of nuclear extract and RNA immunoprecipitation

To isolate the nuclear extract, cells were trypsinized 24-36 hours after the transfection (section 3.2.14.) and washed in 30 volumes of PBS. The pellet was then resuspended in one packed cell volume of buffer A and allowed to swell on ice for 15 minutes. Cells were lysed with a 23G hypodermic needle and nuclei were recovered by centrifugation for 20 sec at 12000*g* at RT. The crude nuclear pellet was resuspended in two-thirds of one packed cell volume of buffer C and incubated for 30 min at 4°C with stirring. The nuclear debris was pelleted by 5 min centrifugation at 12000*g*. Collected nuclei were then resuspended in 0.6 ml of NET-Triton, sonicated gently several times on ice and centrifuged. The supernatant contained the nuclear extract.

For immunoprecipitaion, on the day before anti-GFP was allowed to bind to Protein A sepharose in NET-Triton overnight at 4°C. Next day, BSA was added to a final concentration of 1 mg/ml and allowed to incubate at 4°C for another 2-3 hours to block the sepharose beads. Parallely, anti-IgG in NET-Triton was added to the sepharose beads and allowed to mix at 4°C for a few hours. The nuclear extract was precleared for 1 hour at 4°C with the anti-IgG bound Protein A sepharose. Immunoprecipitation of the desired GFP-Tagged protein from the precleared nuclear extract was done with Protein A Sepharose bound anti-GFP antibody overnight at 4°C, followed by five washes with cold RIPA buffer. RNA was isolated using the TRIzol reagent. After ethanol precipitation, the RNA pellet was dissolved in RNase-free water and DNAase treated as per manufacturer's protocol.

BUFFER A:	BUFFER C:	NET-TRITON:
10 mM HEPES, pH 8.0	20 mM HEPES, pH 8.0	150 mM NaCl
1.5 mM MgC_{12}	25% (v/v) Glycerol	50 mM Tris-HCl, pH 7.4

10 mM KCl	420 mM NaCl	0.1% Triton X-100
1 mM DTT	0.2 mM EDTA, pH 8.0	Protease inhibitors
	1 mM DTT	RNAse inhibitors
	0.5 mM PMSF	

All the three buffers were supplemented with Protease and RNAse inhibitors in appropriate concentrations. The buffers were always prepared fresh and kept on ice for subsequent use.

3.2.1.16. RNA electrophoretic mobility shift assay (gel shift)

Labeled RNA incubated with protein or nucelar extract (NE) in 30°C for 30 minutes. For positive control, add 0.1µl NE; for negative control, add H_2O; for protein, add 2-5.5µl purified Tra2-beta1. Add H_20 to 25µl. Incubate in 30°C for 30min. Then add 1µl Heparin (5mg/ml), RT for 5 min. Load samples to 5% Gel, 300V in 4°C for 2 hours (depends on instrument).

For modification of the protocol, [α -32P] CTP-labeled RNAs were incubated at 30°C under standard splicing conditions in HeLa cell nuclear extract or with different amount of recombinant Tra2-beta1 proteins. 5 µl of aliquot was removed at different time points and transferred to a new tube containing 1 µl of heparin (5 mg/ml). After 5 min of heparin-treatment at room temperature, 1 µl of native RNA gel loading buffer was added and the samples were kept on ice until all samples were loaded into a 5% native RNA gel.

5% native RNA gel setup mixture (30 ml)
DMPC-treated H_2O 21.03 ml
30% (w/v) acrylamide/bisacrylamide 4.95 ml
5x Tris/Glycine Buffer 3.0 ml
Glycerol 750 µl
10% APS (fresh prepared) 240 µl
TEMED 30 µl

5% Tris/Glycine Buffer (1L)
Tris-base 30.28 g
Glycine 142.7 g
EDTA 3.92 g

6× native RNA gel loading buffer
0.025% (w/v) bromophenol blue
30% (v/v) glycerol

2x Buffer D
40 mM HEPES pH8.0
40% glycerol
200 mM KCl
0.4 mM EDTA
2 mM DTT
1 mM PMSF

RNA with protein binding (in 25 µl)
10 mM ATP 1 µl
80 mM MgCl2 1 µl
[α -32P] UTP labeled RNA (10ng/ul) 4 µl
Rnasin or RNase Inhibitor 1 µl
2x Buffer D 12.5 µl

In vitro transcription

The sequences were amplified from TOPO vector by PCR using T7pro and RT primers for their flanking regions. T7pro contains the T7 promoter. After PCR amplification the cDNA was purified from a 2% Agarose gel using QIAquick Gel extraction Kit (Qiagen) following the manufacturer's protocol. The purified cDNA was eluted with 35 µl dH2O and used as template in the following transcription reaction:

5-10 µl	cDNA
2.5 µl	10 x transcription buffer
1.0 µl	10mM rATP
1.0 µl	10 mM rCTP
1.0 µl	10 mM rGTP
1.0 µl	10 mM rUTP
2-3 µl	α-[32P]-UTP (400Ci/mmol)
1 µl	RNase Inhibitor
1 µl	T7 RNA polymerase
up to 25 µl with dH2O	

The reaction was incubated for 1 hour at 37 °C. Afterwards 1 µl DNase was added and incubated for 30 min at 37 °C. Finally the reaction was purified with miniquick Spin RNA columns (Roche) following the manufacturer's protocol. The purified RNA product was checked on a poly acrylamide (SIGMA) gel and used for electrophoretic mobility shift assay.

3.2.1.17. In vitro kinases assay

The activity of protein kinases was determined as amount of radioactive phosphate incorporation into their substrates. Screening of predicted protein kinases that could potentially phosphorylate tra2beta1 shown several enzymes: cAMP-dependent protein

kinase A (PKA), Akt I/protein kinase B (PKB), Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII), Cyclin-dependent protein kinase 2 (Cdc2/Cdk1), Glycogen synthase kinase 3 beta (GSK-3β), casein kinase I (CKI), p42 MAP kinase (Ekr). Tra2-beta1 protein was isolated from Drosophila cells and binds to the Ni-NTA agarose beads. Beads with bound Tra2beta1 were resuspend in 25 µl of the kinase buffer in the presence of 1 Unit of enzyme and 10 mM ATP. The reaction was started by the addition of 1.0 µl γ-32 ATP (specific activity 60000 cpm/pmol) at 30ºC and stopped by the addition of 20µl 3 x Lammli after 15 min. After boiling for 5 min, the samples were resolved by 12% SDS-gel for radiography. For the positive control we used 1µl of nuclear extract instead of kinase in 10 x PTK4 Buffer.

As a variation of experiment for investigation of dephosphorylation effect on the substrate, after incubation with enzymes to parallel probes were added 1µl (2.5 Unit) of protein phospharase 1 and incubate for 20 min at 30ºC. Reaction was stopped by the addition of 3 x Lammli Buffer and analyzed by gel separation.

3.2.1.18. SELEX

Selective Evolution of Ligands by Exponential enrichment is typically a collection of synthetic, random-sequence nucleic acid molecules containing from 1014-1016 different sequences. Each molecule contains a segment of random sequence flanked by primer-binding sequences at each end to facilitate amplification. During each iteration of the SELEX cycle (Figure 2), the population is partitioned to allow preferential recovery of RNAs that display the biochemical property being sought.

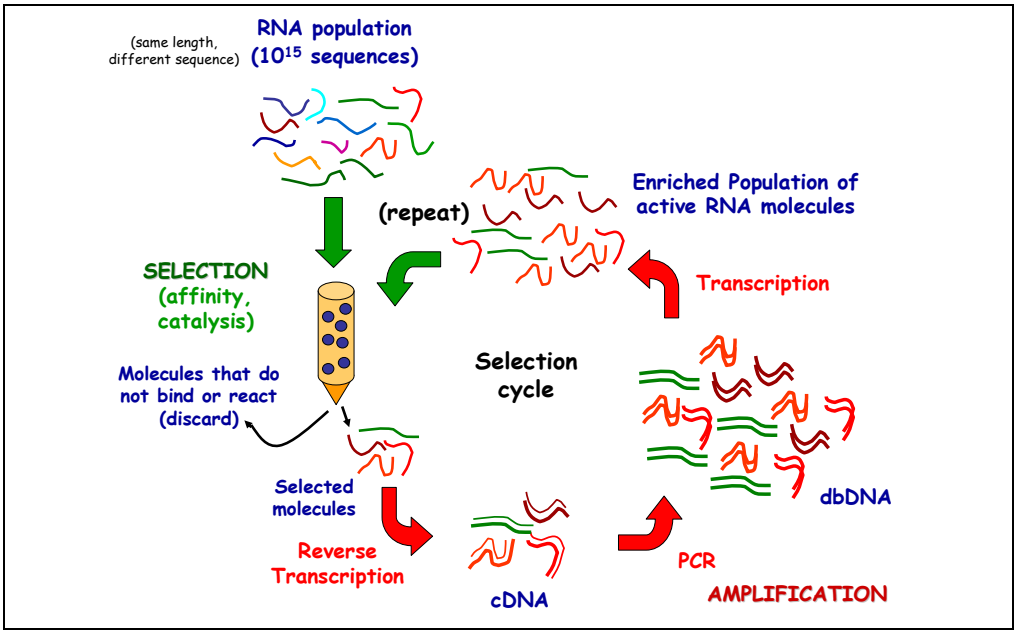


Figure 19. The schema of the SELEX experiment. The beginning of the round indicated as green arrow. (Adapted from Burge, 2002).

SELEX procedures were performed as described previously (White et al., 2001).

The DNA template for a random RNA pool carrying 20 nucleotides of degenerate sequence was prepared by PCR in a standart 50-µl PCR reaction. The RNA transcript was transcribed with T7 RNA polymerase followed by treatment with RQ1 DNase. The RNA pool was further purified by extraction with phenol/chloroform and ethanol precipitation.

The proteins were incubated with the RNA in Binding buffer (10mM Tris-HCl, pH 7.5; 100mM KCl; 2.5mM $MgCl_2$; 0.1%Triton X-100; 0.1mg/ml yeast tRNA) at 25ºC for 30 min in order to allow the binding of proteins to *in vitro* transcribed RNA pool. After we added 25µl Ni-NTA resin for His-tagged hnRNP-G and gluthatione-Sepharose beads for GST-tagged SLM-1 and SLM-2 proteins and allow binding to the resin for 30 min at 4ºC. To increase the specificity of binding, the concentration of proteins was reduced from 0.8µM in the first four rounds to 0.5µM in the fifth and sixth rounds of SELEX. After binding the beads were washed four times with 1 ml Washing buffer (10mM Tris-HCl, pH 7.5; 100mM KCl; 2.5mM $MgCl_2$; 0.1%Triton X-100;), followed by treatment with 50 µg of Proteinase K in 200 µl PK Buffer(100 mM Tris-HCl, pH 7.5; 12.5 mM EDTA; 150 mM NaCl; and 1% SDS) at 37ºC for 30 min. Selected RNA was extracted by phenol/chloroform followed by ethanol precipitation. For amplification, the selected RNA was annealed and reverse-transcribed by SuperScript RNase reverse trancriptase according to manufacturer's instructions. The RNA transcript for the next round of selection was prepared as described above. To enforce the selection of the full-length RNA, the DNA pool was purified after

each round of SELEX. After 6 rounds of selection, PCR products containing selected sequences were cleaved by BamHI and XhoI and cloned into pcDNA3 vector followed by DNA sequencing (AGOWA, Germany) of 15-25 individuals clones.These sequences were analyzed using the program MEME.

STEP of SELEX:	Components:	Conditions:
Initial PCR	In 10 ml: 204pmol of the template DNA (…N20…) 2 nmol T7P and RT promers 0.2nmol dNTPs 3mM $MgCl_2$	94 °C 2 min; 5 cycles – 96 °C 30 sec, 55 °C 30 sec, 72 °C 45 sec; 72 °C 5 min
In vitro transcription	1ug starting pool DNA 1xTrascription Buffer 5mM DTT 20mM$MgCl_2$ 4mM rNTPs 100 U RNAsin 725 U T7 RNA Polymerase 1.0 x 10^{-4}mM α-P^{32}-CTP To 250µl dH_2O	Incubate at 37ºC for 2 hours
Reverse transcription	A. 8µl of RNA 400ng RT primer 5µl 10mM dNTPs B. 5mM DTT 40 U RNAsin 400 U RTranscriptase	A: Incubate at 65ºC for 10 min B: Incubate at 42ºC for 2 hours, followed by 15 min at 72ºC
Following PCR	2mM dNTPs 3mM $MgCl_2$ 1.6 pmol T7P primer	94 °C 2 min; 30 cycles – 96 °C 30 sec, 55 °C 30 sec, 72 °C 45 sec; 72 °C 5 min

	1.5 pmol RT primer	
Cloning	5µl of the PCR DNA digested by BamHI and XhoI and cloned into pcDNA 3 vector	Sequenced by T7 primer and M13 reverse primer

3.2.2. Protein Biochemistry Methods

3.2.2.1. Immunoprecipitation of proteins

RIPA: 1% NP40 1% Sodium deoxycholate 0.1% SDS 150 mM NaCl 10 mM Na-phosphate, pH 7.2 2 mM EDTA 50 mM NaF 5 mM β-glycerophophate freshly added: 4 mM Sodium orthovanadate 1 mM DTT 1 mM PMSF 20 µg/ml Aprotinin 100 U/ml Benzonase	RIPA RESCUE: 20 mM NaCl 10 mM Na-phosphate, pH 7.2 1 mM NaF 5 mM β-glycerophosphate freshly added: 2 mM Sodium orthovanadate 1 mM DTT 1 mM PMSF 20 µg/ml Aprotinin HNTG WASH: 50 mM HEPES, pH 7.5 150 mM NaCl 1 mM EDTA 10% Glycerol 0.1% Triton X-100 freshly added: 2 mM Sodium orthovanadate 100 mM NaF 1 mM PMSF 20 µg/ml Aprotinin

Cells were transfected with plasmids of interest at 24 hours after plating them on 6-well plates. At 18 -24 h after transfection cells were washed with cold 1xPBS and lysed for 20-30 min at 4 °C or on ice in 200-210 µl of RIPA buffer. The lysates were collected by disposable polypropylene tips in Eppendorf tubes and cleared by centrifugation for a few seconds at 12000 rpm. 850-900 µl of RIPA rescue buffer was added to the decanted supernatant. The antibody recognizing the tag of the expressed protein was added for immunoprecipitation and incubated at 4°C on the rotating wheel.

For immunoprecipitations, either anti-Flag or anti-GFP antibodies were used, which had to be added to the buffer in the amount of 1.8-2 µl for anti-GFP and 1.5-1.7 µl for anti-Flag. After 90 min of shaking the 50 µl of Protein A Sepharose / Sepharose CL-4B (1:1) was added and the incubation continued overnight. The Sepharose beads were pelleted by centrifugation for 1 min at 1000 rpm in a microcentrifuge followed by 3 washes with 500 µl of 1 × HNTG buffer. At the end 20 µl of 3 × SDS sample buffer were added to

the washed pellet and boiled for 5 min at 95 °C. The beads were spin down and the supernatant loaded on SDS-polyacrylamide gel. The resolved proteins on the gel were transferred to nitrocellulose membrane. The membranes were equilibrated for 5 min in the Protein Transfer Buffer before the transfer. The analysis of Western blot was performed using ECL solutions.

Preparation of Protein A Sepharose / Sepharose CL-4B

Protein A Sepharose beads were washed in 15 ml of distilled H2O and pelleted at 500 rpm for 2 min at 4 °C. After a second wash with dH2O equal volume of Sepharose CL-4B was added and the beads were washed two more times in RIPA rescue buffer and kept at 4 °C.

3.2.2.2. Electrophoresis of proteins

Proteins bands were resolved on denaturing SDS polyacrylamide gels, using the BioRad gel electrophoresis system (with standards: 10 cm × 7.5 cm × 0.5 cm gels). The separating gel was 7.5-15%, depending on the molecular weight of the proteins, and the stacking gel was 4%. The proteins were mixed with sample loading buffer, denatured at 96 °C for 3-5 min and loaded on the gel. Electrophoresis was carried out at 100 V for 2 hours in SDS gel running buffer.

Separating gel (10ml)	7.5%	10%	12%
dH_2O	4.85 ml	4.1 ml	3.5ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml
10% SDS	100 µl	100 µl	100 µl
30% Acrylamide/Bis	2.5 ml	3.3 ml	4.0 ml
10% Ammonium Persulfate	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl

The stacking gel was always 4%: dH20 –6.1 ml, 30% Acrylamide Bis-4.0 ml.

3 × SDS SAMPLE BUFFER: 150 mM Tris-HCl, pH 6.8 6% SDS 30% Glycerol 3% β-Mercaptoethanol 0.3% Bromophenol blue	SDS GEL RUNNING BUFFER: 250 mM Glycine, pH 8.3 25 mM Tris 0.1% SDS

3.2.2.3. Expression of HIS-tagged protein in the Baculovirus system

For expression of HIS-tagged proteins the Bac-to-Bac® Baculovirus Expression System from Invitrogen was used. This method is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in E. coli (Luckow, Lee et al. 1993; Ciccarone, Polayes et al. 1997). pFastBac vector containing the gene desired for expression was transformed into E. coli DH10Bac™. Generating bacmid from these cells followed the Bac-to-Bac manual from Invitrogen. The isolated bacmid was tested by PCR and sequenced with the forward primers used for cloning into pFastBac-HTa (gene specific) and M13 reverse primers. Transfection of bacmid to generate virus was performed in 6 well plates according to the Bac-to-Bac manual from Invitrogen, using unsupplemented Grace's Medium and Cellfectin (both Invitrogen). When cells showed typical signs of infection (about 72 hours after transfection) the medium was collected from each well (about 2 ml) and transferred into sterile 15 ml falcons. The P1 viral stock was stored at 4 C, protected from light. For amplification of P1 viral stock SF9 cells were infected at a multiplicity of infection according to the manual. Cells were harvested about 48h after infection and expression of recombinant protein was analyzed by SDS-PAGE and Western Blot or protein staining.

3.2.2.4. Purification of HIS-tagged protein in insect cells

denaturing lysis buffer

6 M	Guanidine HCl
20 mM	$NaPO_4$, pH 7.8
500 mM	NaCl

denaturing binding /washing buffer

8 M	Urea
20 mM	$NaPO_4$, pH 7.8
500 mM	NaCl

denaturing washing buffer

8 M	Urea
20 mM	$NaPO_4$, pH 7.8 / pH 6.4
500 mM	NaCl
0.1%	Triton X-100
30 mM	Imidazol

native buffer (pH 8.0)

50 mM	NaH_2PO_4
300 mM	NaCl
30 mM	Imidazol

48 hours after infection, SF9 cells were centrifuged at 500 g for 10 min. The pellet was resuspended in 1 ml of denaturing lysis buffer. The suspension was lysed with a 19 G hypodermic needle and centrifuged at 14,000 rpm for 25 min in a 5417R centrifuge (Eppendorf). The supernatant was incubated for 1-2 hours at 4 °C with Ni-NTA agarose resin (Qiagen), equilibrated once with dH_2O and once with denaturing binding/washing buffer, pH 7.8. After incubation, the resin was washed twice with denaturing wash buffer,

pH 7.8, twice with denaturing wash buffer pH 6.4 and once with native buffer. Protein elution from the resin was performed with native buffer containing 250 mM Imidazol. Fractions of each step were run on SDS-PAGE.

3.2.2.5. Staining of protein gels

Coomassie staining was used to detect proteins in SDS polyacrylamide gels. After electrophoresis, the gel was placed in staining solution (2.5% Coomassie Brilliant Blue R250, 45% Methanol, 10% Acetic acid) for 2-3 h at RT. The gel was then washed 2-3 times for 30 min in 50% Methanol/10% Acetic acid and 2-3 more times in 20% Methanol/10% Acetic acid. Alternatively, polyacrylamide gels were stained with Silver Stain Plus solutions according to the manufacturer's protocol. The gel was dried on Whatman paper covered with a cellophane sheet on a Gel dryer SE1160 (Hoefer Scientific Instruments).

3.2.2.6. Western blotting

Proteins resolved on SDS polyacrylamide gels were transferred to nitrocellulose membrane (Schleicher and Schuell) in transfer buffer, for 45 min at 120 V. Before the transfer, membrane and the gel were equilibrated for 5 min in the protein transfer buffer. After transferring the membrane was blocked for 1 hour in 1 × NET-gelatine buffer at RT. Primary antibody was then added and the incubation was allowed to proceed overnight at 4 °C or at RT for 2 hours. The membrane was washed three times for 15-20 min in 1 × NET-gelatine and incubated with a secondary antibody coupled to horseradish peroxidase for 1 hour. The membrane was subsequently washed three times for 20 min in 1 × NET-gelatine and the bound antibodies were detected by the ECL system. Equal amounts of solutions ECL1 and ECL2 were mixed and added to the membrane for 5 min. The membrane was then exposed to an X-ray film (Fuji SuperRX) and developed in a Kodak developing machine.

TRANSFER BUFFER: 192 mM Glycine 25 mM Tris 20% Methanol	NET-GELATINE: 150 mM NaCl 5 mM EDTA 50 mM Tris-HCl, pH 7.5 0.05% Triton X-100 0.25% Gelatine

ECL1:	ECL2:
4.5 mM Luminol	0.003% H_2O_2
4.3 mM p-Iodophenol	100 mM Tris, pH 9.5
100 mM Tris, pH 9.5	

3.2.2.7. Expression and purification of GST-tagged proteins in bacteria

To overexpress GST-tagged rSLM-1 and rSLM-2 proteins, GEX-ESAF-ATG constructs were transformed into BL21 (DE3)-RIL E.coli strain. After the transformation, cells were plated on LB agar plate containing both kanamycin (to select plasmid containing bacteria) and chloramphenicol (to maintain pACYC plasmid coding for additional argU, ileY, and leuW tRNAs). Single colony was then inoculated into 5 ml of LB medium and grown overnight. The next day the culture was inoculated into 100 ml of fresh LB, containing 50 μg/ml of ampicillin. The induction of the culture was performed with 1 mM of IPTG (at OD600~0.5-0.7). The culture was grown for another 2 hr at 30°C with vigorous shaking. After the induction, cells were harvested by centrifugation for 30 min at 4000 rpm. The pellet was resuspended in 10 ml of lysis buffer and then lysozyme was added to a final concentration of 1 mg/ml. Cells were sonicated after 30 minutes of lysis. The supernatant was collected by centrifugation for 30 minutes at 14000 rpm and then filtered through 0.45-μm filter. Supernatant was then mixed top over top with Glutathione-Sepharose 4B l while rotating for 2 h at 4°C. The resin was subsequently washed 5 times with buffer A. The protein was finally eluted in buffer, containing 0.5 M Glutathione, dialyzed against 1xPBS overnight and concentrated using centricon concentrators (Amicon). The protein concentration was measured by Bradford method and monitored by Coomassie Staining SDS-PAGE.

BUFFER A:	ELUTION BUFFER:
PBS	PBS
500 mM NaCl	500 mM NaCl
1%Triton X-100	1% Triton X-100
	0.5M Glutathione

3.2.2.8. Determination of protein concentration

Protein concentration was estimated using BioRad Protein Assay Kit based on Bradford method. Protein in 800 µl of distilled water was mixed with 200 µl of 1 × Dye Reagent and incubated for 5 min at RT. Absorbance of the solution was measured in a spectrophotometer at λ=595 nm. Concentration of samples was read from the standard curve where OD595 was plotted versus concentration of BSA standards.

3.2.2.9. In vitro transcription / translation of DNA into radiolabelled protein and GST pull-down assay

This method allows identification of potentially translated products from partially purified DNA. It translates DNA into RNA and RNA into protein with subsequent analysis. The cDNA of potential proteins was cloned in pCR3.1 (Invitrogen) and used for an in vitro reticulocyte lysate transcription/translation (TNT, Coupled reticulocyte system, Promega), to obtain the corresponding 35S-labelled proteins. In vitro transcription/translation was performed accordingly to the manufacturer's protocol. For binding experiments 2 µl of the reactions were incubated with 1 µg of rSLM-1-GST coupled to Glutathione-Sepharose 4B in the presence of 200 µl of 1xPBS buffer/0.1%Triton X 100 for 2 h at 4 °C.

3.2.2.10. Microtiter assay of Protein Phosphatases by pNPP Hydrolysis

The activity of PP1 with p-nitrophenol phosphate as substrate was assayed essentially accordingly to the manufactures protocol. The initial rate of liberation of p-NPP was determinated spectrophotometrically at 405 nm. The A_{405} reflects phosphatase activity. One Unit of activity is equivalent to 1 nmol pNPP hydrolyzed per minute. To calculate phosphates activity we used the following equation:

$$\frac{\text{(Sample volume in liters) x } A_{405}}{(\varepsilon) \text{ x (Path length of light in cm)x(assay time in min)x(µg of enzyme)}}$$

ε = Extinction coefficient for pNPP at A_{405} = 1.78x104 $M^{-1}cm^{-1}$

3.2.2.11. Protein shuttling

Protein shuttling was essentially performed as described (Neumann et al., 2001). Briefly, HeLa cells were transfected with plasmids directing expression of EGFP-SLM1 and yellow fluorescent protein (YFP) which served as fusion control. Transfected HeLa

cells were cultured with an excess of untransfected HeLa cells and fusion induced by treatment with polyethylene glycol 1500 for 180 sec. Fluorescence was monitored by real-time imaging using the Zeiss "Cell Observer", a temperature-controlled Zeiss Axiovert 200M research microscopy with scanning stage, CO_2-incubation and Software AxioVision 4.1.

3.2.2.12. Mass spectrometry of Tra2-beta1 protein

Mass spectrometry analysis was performed by Dr. M.Grönborg and Dr. H.Urlaub from Max Planck Institute of Biophysical Chemistry (Göttingen, Germany) by standart protocol. Briefly, cross-linked samples were analysed on a Reflex IV mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with 2,5-dihydroxybenzoic acid (DHB) as MALDI matrix in the positive reflectron mode for the U1 snRNP and the [15.5K-61K-U4atac snRNA] particles and in the negative reflectron mode for the [p14/SF3b14a-SF3b155282–424-U2 snRNA BSiR] complex, both under standard conditions, by summing up to 1000 laser shots.

0.5 µl of sample were mixed with the same volume of DHB (10 mg/ml in 50% ACN or water, respectively) on an AnchorChipTM MALDI target plate (Bruker Daltonik GmbH, Bremen, Germany) with spot sizes of 600 or 400 µm. The preparation was allowed to dry at room temperature and subjected to MS. Close external calibration with peptide standards was performed.

3.2.2.13. Yeast Two hybrid

Two-Hybrid System is a genetic method for detecting interactions between proteins *in vivo* in the yeast *Saccharomyces cerevisiae* (Durfee et al., 1993; Vojtek et al., 1993). Two-hybrid system exploit the fact that transcription factors are comprised of two domains, a DNA binding domain ("bait") and an activation domain ("prey"). These two hybrids are encoded on separate yeast expression plasmids, with independent selectable markers.

For this experiment we used DARPP-32 pDEST32 expression construct as a bait plasmid and Tra2-beta1 pDEST 22 as a prey plasmid.

Based on protocol from Proquest two-hybrid system (Invitrogen):

1. Make overnight culture - vortex a single colony in 10 ml YPAD. Grow at 30^{o}C, 250 rpm, 16 hours (After ~ 16 h the OD600 should be between 0.2 and 0.3).
2. Take 10 ml of the overnight culture and transfer to 100 ml YPAD. Grow for ~ 4

h, 30°C, shaker. Check OD600 - should be between 0.4 - 0.6 (Clontech; our lab protocol - 0.6 - 0.9).

3. Transfer cells to 50 ml Falcons and centrifuge at 2000g for 5 min at room temperature.
4. Wash with 50 ml dH_2O. Resuspend by vortexing. Centrifuge 2000 rpm for 5 min at room temperature.
5. Resuspend the cells in freshly prepared 1 x LiAc /0.5x TE, 2ml. Incubate the cells at room temperature for 10 minutes.
6. Combine 1µg plasmid and 100 µg denatured sheared salmon sperm DNA and add to 100 µl yeast cells.
7. Add 700 µl 1 x LiAc/40%PEG-3350/1xTE. Vortex 10 sec. Incubate at 30°C for 30 min, 250 rpm
8. Add 88 µl DMSO. Mix (no vortex).
9. Heat shock 15 min at 42°C.
10. Chill on ice 10 min.
11. Centrifuge 2000 rpm, 1 min. Discard super, resuspend in 5 ml YPAD. Shaker, 30°C for 1 hour.
12. Centrifuge, resuspend in TE Buffer.
13. Plate on appropriate medium (-Leu-Trp-His). Colonies appear after 2-3 days.
14. Second plate on 5mM or 10mM 3AT added plate. 30°C for one week.

YPAD Medium and plate:

YPD (Clontech, cat.630409) 50g

Adenine sulphate (SIGMA, A3159) 100mg

Autoclaved, distilled water to 1 liter

For agar plates, add 20g bacteriological grade agar per liter of non autoclaved YPAD medium. Adjust the pH to 6.0 with HCl. Autoclave at 121°C for 25 minutes. Cool to 55°C and dispense into sterile Petri dishes. Store plates when solidified upside down at 4°C.

3.2.3. Cell Cultures Methods and Immunostaining

3.2.3.1. Freezing, thawing and subculturing of eukaryotic cells

For freezing, cells at first were grown to mid logarithmic phase (about 75% of confluence) in 10 cm Petri dishes. After subsequent washing and trypsinization with 1xTrypsin/EDTA, cell pellet had to be resuspended in 1ml of the freezing medium (90% of the growth medium and 10% of DMSO). Vials (Eppendorf tubes) were placed in Nalge Nunc Cooler giving a cooling rate of ~1 °C/min while at -80 °C. All the cell lines were stored later in liquid nitrogen. For thawing, tube with cells shortly was incubated at 37 °C. The entire content of the tube was transferred to a 10 cm Petri dish, where 10 ml of the growth medium were added. The dish was placed in the incubator at 37 °C and 5% CO2. When cells were attached to the plastic surface, the medium was removed and replaced with fresh one. The cells were maintained in the incubator until ready for subculturing. Subculturing of cells normally was done when cells had reached confluence. The cell monolayer was detached by adding 1 × Trypsin /EDTA and incubating at 37 °C until single cell suspension was formed. 1/5 – 1/10 of this suspension was transferred to a new dish and mixed with the growth medium. Cells were maintained in the incubator at 37 °C and 5% CO2.

3.2.3.2. Transfection of eukaryotic cells

Transfection of HEK293 cells was based on procedure published by Chen C. and Okayama H., in 1987. Cells were plated at a density of about 3 × 105 cells / 8 cm2 with growth medium DMEM +10% FCS. After splitting cells were incubated at 37 °C, 5% CO2 for about 24 h to reach 60-80% of confluence. For most applications, cells were grown in 6-well plates, with 2 ml of growth medium per well. The transfection reaction was performed by Calcium-phosphate method: 1 to 5 μg of expression construct were mixed with 25 μl of 1 M CaCl2 in final volume of 100 μl for one well The equal volume of 2 × HBS buffer (280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4 × 2 H2O, 12 mM Dextrose, 50 mM Hepes, pH 6.95) was added drop wise with constant mixing. The solution was allowed to stay at RT for 20 min for formation of precipitants and later on added to the growth medium by swirling. To express the transfected plasmid, cells were grown for additional 17- 24 h at 37 °C, 3% CO2.

3.2.3.3. Fixing attached eukaryotic cells on cover slips

Cells grown on cover slips and transfected with pEGFP-C2 constructs were fixed with 4% formaldehyde in 1 × PBS, pH 7.4 for 20 min at 4 °C. Cells were washed 3 times in PBS prior to mounting on microscope slides with Gel/Mount (Biomeda). Cells were examined by confocal laser scanning microscopy (Leica).

3.2.3.4. Immunostaining

Cells grown on cover slips were fixed in 4% paraformaldehyde in 1x PBS, pH 7.4 for 20 min at 4 °C. After fixing, the cells were washed three times in PBS with 0.1% Triton X-100 and blocked in PBS containing 0.1% Triton X-100 and 3% NGS (or BSA) at room temperature. Cells were incubated with the desired antibody (diluted on PBS with 0.1% Triton X-100 and 3% BSA) overnight at 4 °C. After washing three times in PBS with 0.1% Triton X-100, cells were incubated with CY3 or CY5 coupled antibody, diluted 1:500 in PBS with 0.1% Triton X-100 for 2 hours at room temperature. After washing for another three times in PBS with 0.1% Triton X-100, the cover slips were mounted on microscope slides with Gel/Mount. Finally, stained cells were examinated by confocal laser scanning microscopy.

3.2.4. Bioinformatical Support

3.2.4.1. Databases and computational tool

Database/ software	URL	Description	Reference
ASD	http://www.ebi.ac.uk/asd	The alternative splicing database	(Thanaraj et al., 2004)
ClustalW	http://www.ebi.ac.uk/clustalw/index.html	Multiple sequence alignment program for DNA or proteins	(Thompson et al, 1994)
ESE	http://rulai.cshl.edu/tools/ESE/	Finds putative binding regions for several splice factors	(Cartegni et al., 2003)
Human BLAT Search	http://www.genome.ucsc.edu/cgi-bin/hgBlat	Sequence alignment tool similar to BLAST	(Kent, 2002)
NCBI BLAST and PSI-BLAST	http://www3.ncbi.nlm.nih.gov/BLAST/	Finds regions of sequence similarity	(Altschul et al., 1990); (Altschul et al., 1997)
MEME	http://bioweb.pasteur.fr/seqanal/motif/meme/	Discover motifs (highly conserved regions) in groups of DNA or protein sequences	(Bailey T.L, Elkan C, 1994)

4. RESULTS

4.1. Regulation of alternative splicing by tyrosine phosphorylation

Alternative splicing is one of the most important mechanisms to generate a large number of mRNA and protein isoforms. The detailed mechanisms, which are involved in the control of splice site selection, are poorly understood. The phosphorylation of splicing regulatory proteins is one of the general regulatory mechanisms to change alternative splice site usage. The rSLM-1 and rSLM-2 proteins belong to the family of signal transduction and activation of RNA proteins (STAR) and are involved in alternative splice site selection. They are potential substrates of non-receptor tyrosine kinases. Studies presented in this chapter show that tyrosine phosphorylation emanating from non-receptor tyrosine kinases can alter the function of rSLM-1 and rSLM-2 and as a result modulate splice site selection.

4.1.1. rSLM-1 and rSLM-2 have a similar domain organization and different tissue-specific expression

The first paper which described the cloning and properties of Sam68 (the 68-kDa Src substrate associated during mitosis)-like mammalian proteins 1 and 2 was published almost one decade back (Di Fruscio et al., 1999). Later, using scaffold attachment factor B (SAF-B) in a yeast two-hybrid system, the rSLM-1 and rSLM-2 proteins were isolated from a rat brain cDNA library (Stoss et al., 2001).

All these proteins contain an hnRNP K homology RNA-binding domain (KH), several arginine-glycine dipeptides and a tyrosine-rich carboxy terminus. SLM-1 and SLM-2 have an ≈70% sequence identity with Sam68 in their GSG domain. The three highly related proteins, rSam68, rSLM-1, and rSLM-2, differ by the numbers of proline-rich stretches. The sequence of rSLM-1 is shown in Figure 20. The protein has a maxi-KH RNA binding domain, which harbours the RNA binding activity (gray box).

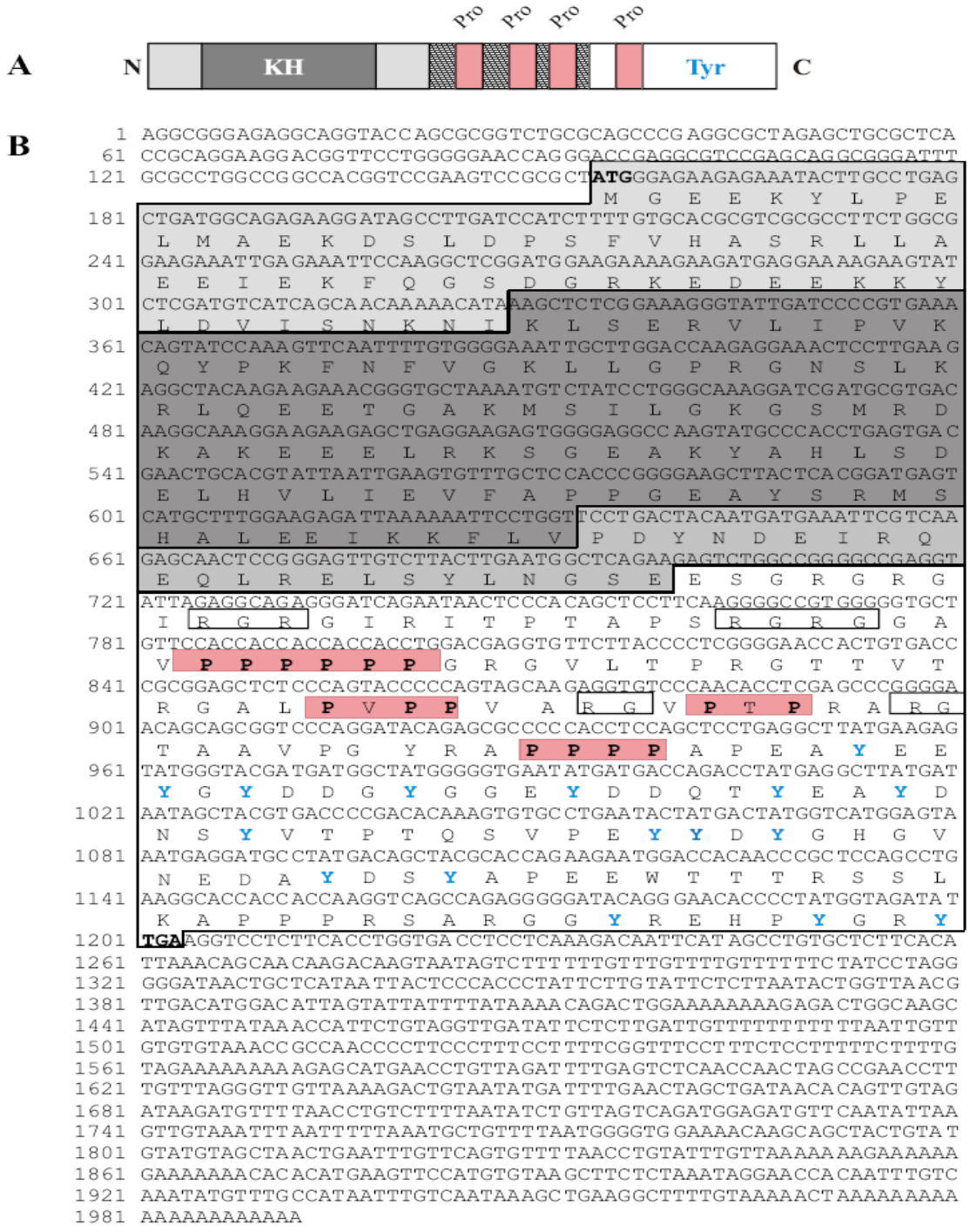


Figure 20. Sequence analysis of the rSLM-1 protein. (A) Domain structure of rSLM-1. Pro: proline-rich regions, marked in pink; KH: hnRNP K homology domain-grey; RG: arginine/glycine-rich region; Tyr: tyrosine-rich region. (B) cDNA and protein-sequence of rat rSLM-1 are shown. Start and stop codons are shown in bold. The protein sequence is shown underneath the cDNA sequence. The KH RNA binding domain is shown as a shadowed dark grey box and is flanked by the QUA1 and QUA2 regions, indicated as light grey boxes. The arginine and glycine dipeptides clustered in the central part of the protein are boxed. Proline residues are boxed and marked in pink. Tyrosine residues in the carboxy terminal part of the protein are indicated in blue.

SLM-1 contains several arginine-glycine rich regions, which could be methylated by arginine methyl transferases (Cote et al., 2003). The proline rich sequences can bind to SH3 (Src homology) and WW (domain with two conserved tryptophan) domains. The C-terminus contains tyrosine-rich residues, potential sites for action of tyrosine kinases.

Both in rat and human, three highly related cDNAs exist that shares a similar, but not identical domain structure, what can reflect their specific functions.

We found that from these three related proteins (Sam68, rSLM-1 and rSLM-2), rSLM-1 shows the most restricted expression pattern. It has the least abundant RNA distribution, compared to others, suggesting a specialized function predominantly in the brain.

It was shown, that rSLM-1 and rSLM-2 proteins can be detected in all brain areas, as well as in testis (Stoss et al., 2004). We then investigated their cellular expression patterns in the forebrain to find out whether the proteins differ in localization. We observed no colocalizations of the proteins in the cells in cortical layers, and in brain sections or peripheral nervous system. In the hippocampus, we found a striking difference in the expression of rSLM-1 and rSLM-2. There, rSLM-1 is predominantly expressed in the dentate gyrus (Stoss, et al. 2004). The cells expressing rSLM-1- and rSLM-2 are neurons.

4.1.2. rSLM-1 is phosphorylated by the p59^{fyn} kinase

The domain structure of rSLM-1 and rSLM-2 suggested that these proteins can bind to proteins containing SH3 and SH2 (Src homology) domains, respectively. Therefore, these two proteins could be substrates of tyrosine kinases. First, it was tested whether the non-receptor tyrosine kinases c-src, p59^{fyn} and hFer phosphorylate rSLM-1 and rSLM-2 *in vivo*.

EGFP-rSLM-1 and EGFP-rSLM-2 were cotransfected with equal amounts of expression constructs of these kinases. The overexpressed protein was immunoprecipitated using the specific GFP tag. Immunoprecipitates were analyzed using an anti-phosphotyrosine antibody, PY20.

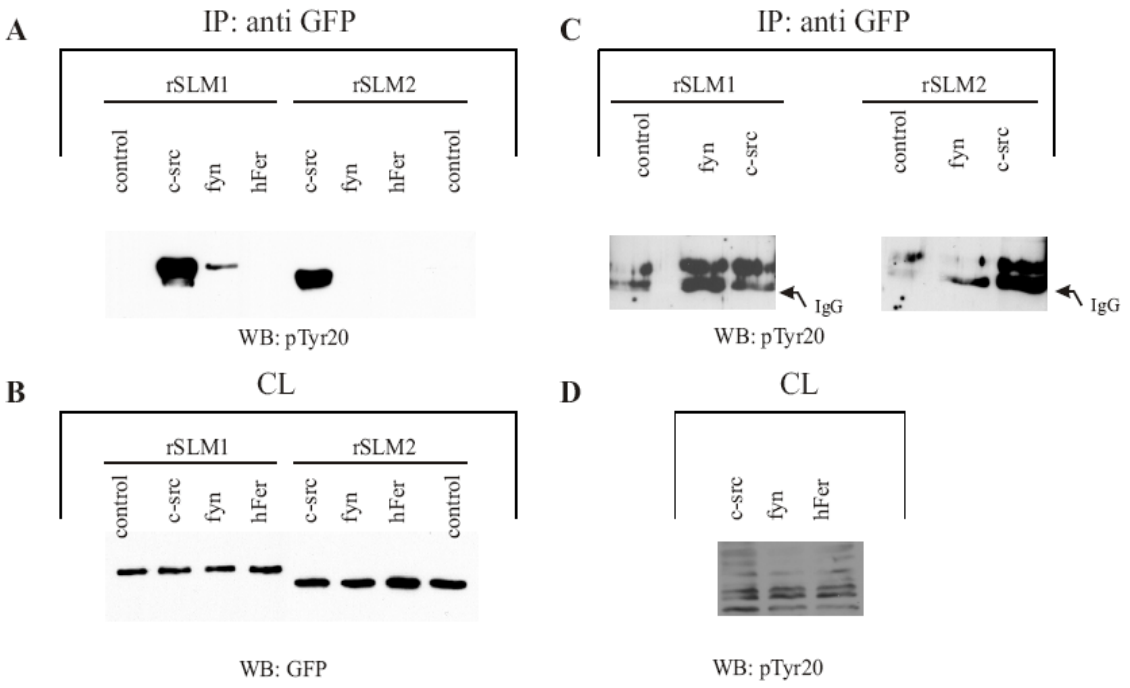


Figure 21. Tyrosine phosphorylation of rSLM-1 and rSLM-2 by non-receptor tyrosine kinases. (A) HEK293 cells were transfected with EGFP-SLM-1 or EGFP-SLM-2 and the kinases indicated. Protein was immunoprecipitated with anti-EGFP and tyrosine phosphorylation was detected with pTyr20. (B) The reblot was performed with anti-GFP to demonstrate the successful immunoprecipitation and the equal loading. (C) Three micrograms of EGFP-rSLM-1 and EGFP-rSLM-2 were coexpressed with one microgramm c-src, $p59^{fyn}$, and hFer in Neuro2A cells and immunoprecipitated with an anti-GFP antibody. The precipitates were analysed using the anti-phosphotyrosine antibody PY20 using Western-Blot. (D) Crude cellular lysates transfected with the kinases were analysed with PY20 and are shown on the lower panel on right.

As it is shown in Figure 21A-C, both proteins were strongly phosphorylated by the c-src kinase. In contrast to c-src, the $p59^{fyn}$ phosphorylated rSLM-1, but not rSLM-2, although equal amounts of rSLM-1 and rSLM-2 were present (Figure 21B, GFP reblot). The experiment was performed both in HEK293 (Figure 21A) and in Neuro 2A (Figure 21B) cells. Overexpressed hFer phosphorylated none of the proteins. All the kinases tested were active, as demonstrated by their ability to phosphorylate different proteins in cell lysates (Figure 21D).

4.1.3. rSLM-1 is colocalized with the p59fyn kinase in neurons

We were the first to analyze the expression of the $p59^{fyn}$ protein in neurons. However it was shown earlier by RNA in situ hybridization that $p59^{fyn}$ is widely expressed in neurons and oligodendrocytes in the adult brain (Umemori et al., 1992). To test whether rSLM-1 could be phosphorylated by $p59^{fyn}$ we performed colocalization experiments. The phosphorylation could only happen when both proteins are expressed in the same cell. Therefore, we determined the rSLM-1 and $p59^{fyn}$ protein expression by

immunohistochemistry. We detected $p59^{fyn}$ protein expression in all neurons of the hippocampal formation. In addition, we determined that rSLM-1 and $p59^{fyn}$ are expressed in the same cells within the dentate gyrus, as shown in Figure 22.

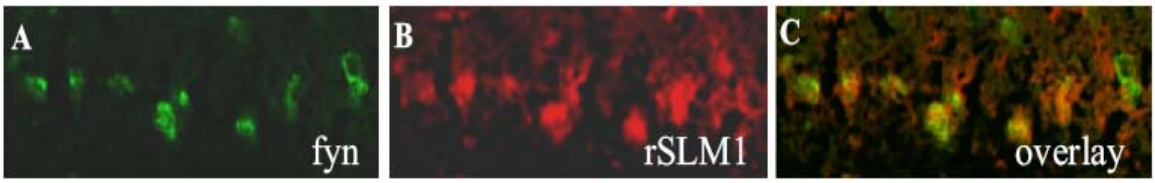


Figure 22. rSLM-1 and p59fyn are colocalized together in the hippocampal cells. Staining of hippocampal sections was performed with specific anti-fyn (in green) and anti-rSLM-1(in red) antibodies. The colocalization between $p59^{fyn}$ (A) and rSLM-1 (B). The superimposition (C) between images (A) and (B).

This provides strong evidence that rSLM-1, but not rSLM-2 is a substrate for p59fyn phosphorylation in vivo and that p59fyn and rSLM-1 are expressed in the same neuronal cells. Taken together, this suggests that p59fyn may regulate rSLM-1 function in the dentate gyrus.

4.1.4. The phosphorylation of rSLM-1 influences its binding properties

The SLM-1-GST protein was produced in bacteria to study the biochemical properties of protein:protein interactions. Previously (Stoss et al., 2001), it was demonstrated that rSLM-1 acts as a splicing regulatory protein and interacts with several SR proteins; SR related proteins and several hnRNPs. To test whether interactions between splice factors are mediated through phosphorylation, we performed GST-pull down assays of purified protein together with its interactors in the presence and absence of c-abl and radioactive ATP. Recombinant GST-rSLM-1 was induced in bacteria and incubated with *in vitro* translated, radioactively labeled interacting proteins in the presence of glutathione sepharose. Half of the recombinant protein was phosphorylated by recombinant c-abl in presence of ATP.

As it is shown in Figure 23, the phosphorylation of SLM-1 splicing factor by c-abl changes protein:protein interactions.

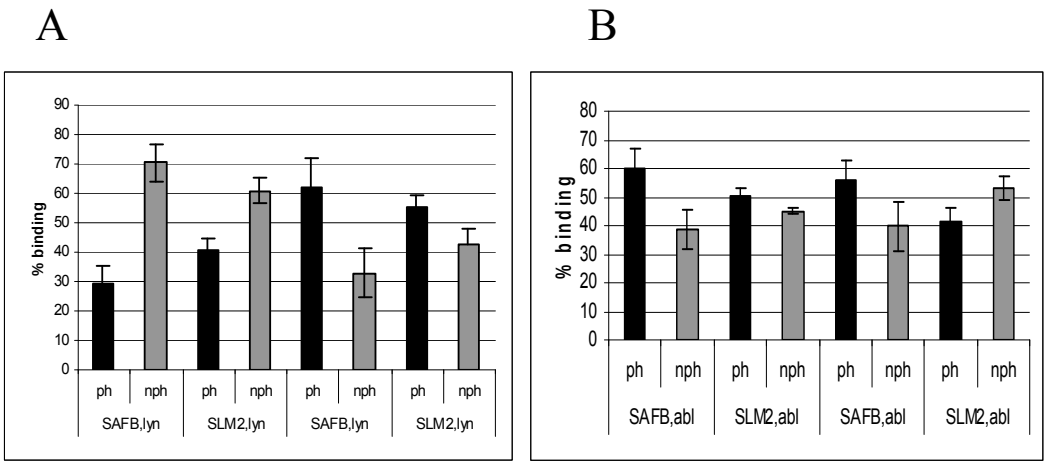


Figure 23. Phosphorylation dependent protein:protein interactions are influenced by the presence of RNA. rSLM-1-GST-pull down assay was implemented with either phosphorylated (black), or not phosphorylated (grey) recombinant protein. Binding was performed in the presence of lyn kinase (A) or presence of c-abl kinase (B).

In order to investigate the possible role of RNA in interactions between these proteins, and to eliminate possible role of the RNA in the assay, RNAse (benzonase) was used for *in vitro* binding assay with Glutathione Sepharose.

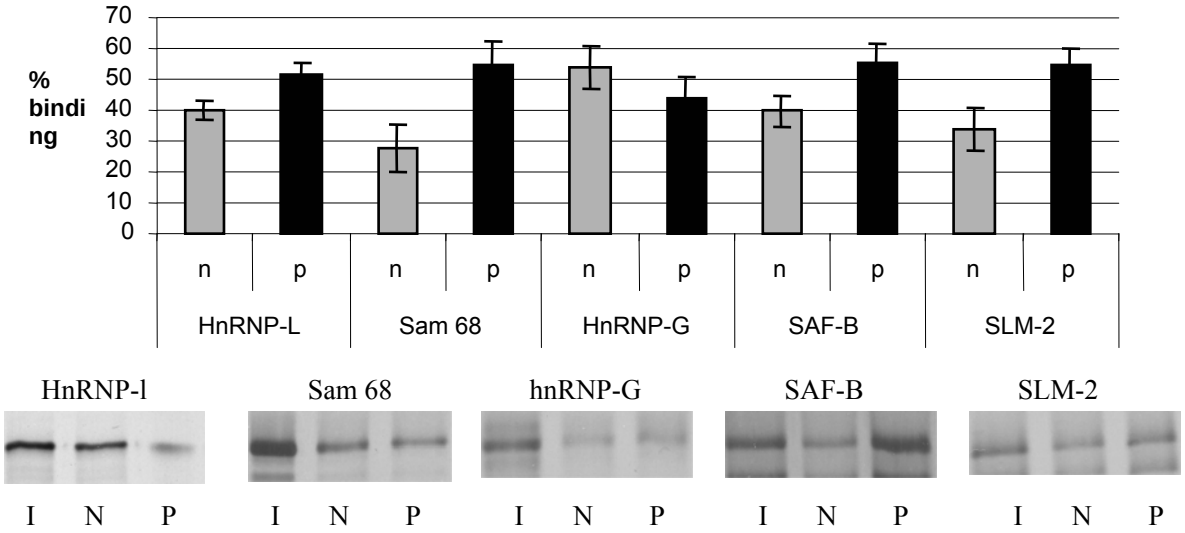


Figure 24. Phosphorylation mediated interaction of recombinant rSLM1 protein with its partners. Recombinant rSLM1-GST tag protein was incubated with several in vitro translated proteins. The load of in vitro translated protein marked as i, n-indicates the binding of specific in vitro translated interactor to recombinant rSLM1-GST tag protein, p-shows the binding of phosphorylated recombinant protein by c-abl to its interactors. The percentage % shows the affinity of binding.

As it is shown in Figure 24 the binding properties of the rSLM-1 protein are influenced by the presence of RNA. Interestingly, for some proteins like for Sam 68 the

presence of RNA in the reaction is a necessary component of binding of these proteins to SLM-1. This experiment clearly demonstrates that the phosphorylation of rSLM-1 protein can regulate a number of protein:protein interactions. The RNA can serve as an important mediator in this process.

4.1.5. The p59fyn kinase regulates the ability of rSLM-1 to influence splice site selection

Using minigene reporter system we determined that both rSLM-1 and rSLM-2 can regulate splice site selection of the SMN2 pre-mRNA in a concentration-dependent manner: the increasing amount of either EGFP-rSLM-1 or EFGP-rSLM-2 expression plasmids promotes skipping of alternative spliced exon 7 of SMN2 minigene (Stoss et al., 2004). The SMN2 minigene consists of alternative exon 7, which contains a purine-rich enhancer. The exon 7 is flanked by two constitutive exons 6 and 8.

Since the phosphorylation by $p59^{fyn}$ was a major difference between rSLM-1 and rSLM-2, we asked whether $p59^{fyn}$-mediated phosphorylation would influence the ability of rSLM-1 to change splice site selection. We used again the SMN2 minigene as a reporter. Both proteins were overexpressed with a reporter gene in the presence of parental vector pEGFP-C2, in order to balance the amount of the cDNA. The cotransfection assays were performed on HEK293 and Neuro2A cell lines. We found that the skipping of the alternative exon 7 was promoted by increasing the amount of either EGFP-rSLM-1 or EGFP-rSLM-2 expression constructs (Figure 25).

In presence of $p59^{fyn}$ we also observed a reduction of exon 7 inclusion of SMN2 from about 60% to 35%. Furthermore, $p59^{fyn}$ abolished the concentration-dependent ability of rSLM-1 to promote skipping of alternative exon 7. There was no statistical significant difference between the transfection results of various rSLM-1 concentrations when $p59^{fyn}$ was present. In contrast, the ability of rSLM-2 to promote skipping of the same exon was unchanged. Higher rSLM-2 concentrations increased exon skipping from 35% to 12%, a statistical significant change ($p=0.001$, students t-test, $t=8.23$).

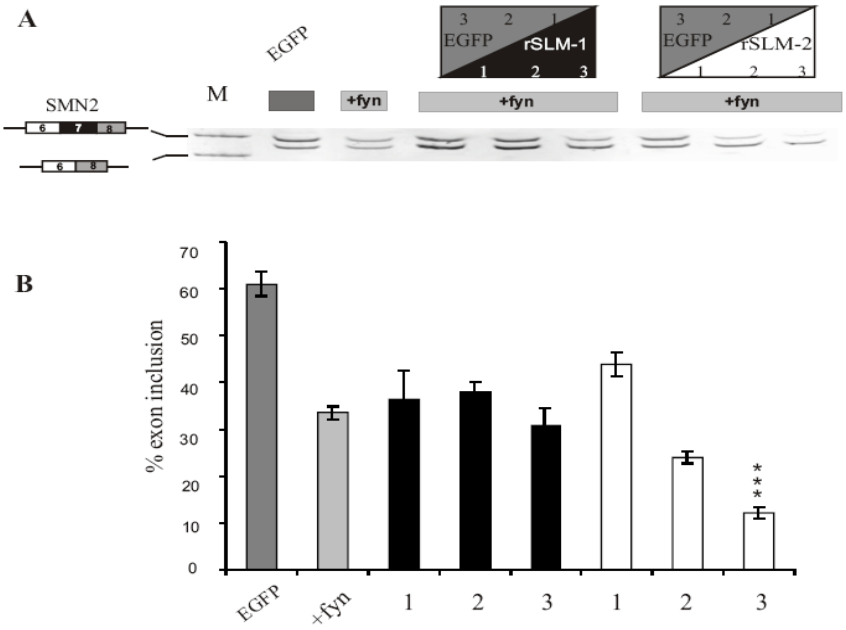


Figure 25. rSLM-1, but not rSLM-2 promotes skipping of exon 7, in the presence of p59^{fyn}. (A) Neuro2A cells were transiently transfected with increasing amounts of EGFP-rSLM-1 and EGFP-rSLM-2 and constant amount of the p59^{fyn} kinase in presence of SMN2 reporter construct. The amount of transfected rSLM-1 and rSLM-2 are normalized with the pEGFP-C2 constract. The RNA was analysed by RT-PCR.The strucutre of amplified products is indicated on the left. (B) The statistical evaluation of RT-PCR results. The ratio between the signal corresponding to exon exclusion and all products was determined from at least three different independent experiments. Stars indicate statistical significant differences with $p<0.001$.

Therefore rSLM-2 is most likely not affected by the kinase because it is not phosphorylated by p59^{fyn} (Figure 25). Similar results were obtained when we used HEK293 cells. Together, these data show that the p59^{fyn}-mediated phosphorylation of rSLM-1 abolishes the ability of rSLM-1 to regulate splice site selection.

4.1.6. rSLM-1 acts on IL-4 Receptor minigene

rSLM-1 contains a KH domain that binds to RNA and was found as binding partner of several splicing factors. Thus, we were interested whether a direct interaction between rSLM-1 and RNA could be detected. We designed an interleukin 4 receptor minigene, which consist of alternative exon 8, flanked by constitutive exons 7 and 9. EGFP-rSLM-1 alone and together with abl kinase expression clone were immunoprecipitated in the presence of the IL-4 Receptor minigene. The RNA was detected in the precipitates by RT-PCR (Buckanovich and Darnell, 1997) using IL-4R minigene specific primers. The amplification of IL-4R isoforms demonstrates that rSLM-1 associates with this mRNA and

thus can possibly regulate it directly (Figure 26). The protein immunoprecipitates were tested having equal amounts of each protein.

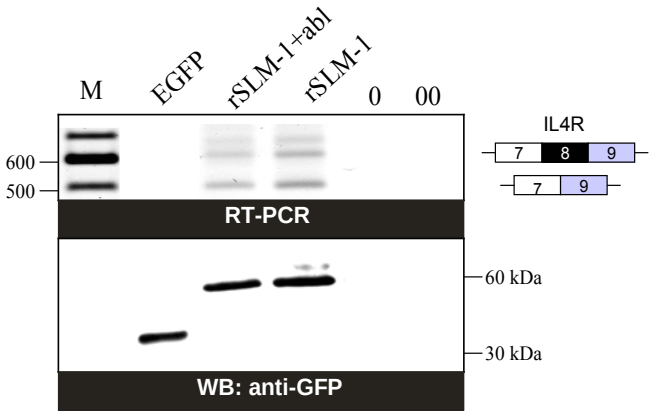


Figure 26. Association of rSLM-1 with interleukin-4 receptor pre-mRNA in vivo. The IL-4R minigene was cotransfected with EGFP-SLM-1 (third line) or together with c-abl (line two). As control the minigene was cotransfected with the parental vector EGFP-C2 (first line). Protein-RNA complexes were isolated by immunoprecipitation with anti-GFP in the absence of RNases. IL-4R RNA was detected by RT-PCR using minigene specific primers. 0-immunoprecipitate without reverse transcriptase, 00-RT-PCR control (no template).

These data show that rSLM-1 and rSLM-1 phosphorylated by the abl kinase associate with IL-4R minigene and can possibly regulate it.

4.1.7. rSLM-1 regulates splice site selection via purine-rich enhancer

Next we wanted to determine whether rSLM-1 acts through the purine-rich RNA element that acts as a splicing enhancer in CD44 exon v5 (König, 1998; Stoss, 2001). For this experiment we employed linker-scanning mutants that replace the enhancer. We found that replacing the purine-rich element ATGAAGAGGA by CGACGCGTCG in ls9 reduced the ability of rSLM-1 to promote exon v5 inclusion (Figure 27). Replacing the adjacent sequences in ls8 and ls10 had no statistical significant effects. These data show that similar to rSLM2 (Stoss et al., 2001), rSLM-1 acts on a purine-rich element present in exon v5.

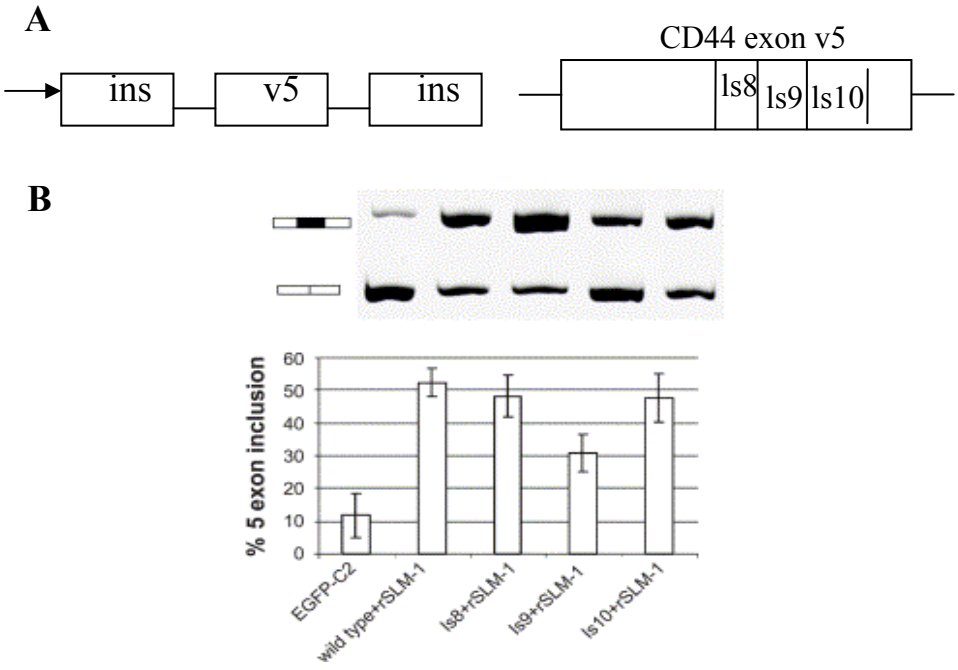


Figure 27. rSLM1 regulate splice site selection through purine-rich enhancer. A. Structure of the minigene. The CD44v5 minigene containing the alternative-spliced exon v5 and flanking constitutive insulin exons is schematically indicated on the left. The arrow presents the CMV promoter. The location of the linker scanning mutations (Konig et al., 1998) is schematically indicated on the right. B. The purine-rich element in exon v5 is necessary for rSLM-1 function. Two micrograms of EGFP-rSLM-1 were cotransfected with the wild type and linker scanning mutations indicated.

4.1.8. Candidate RNA targets binding to rSLM-1 and rSLM-2

To identify the sequences requirements of the RNA motif that bind to Sam68-like mammalian proteins 1 and 2, in vitro SELEX experiment were performed. Systematic evolution of ligands by exponential enrichment (SELEX) is a technique that allows the simultaneous screening of highly diverse pools of different RNA or DNA molecules for a particular feature. In order to determine the nucleotide feature of rSLM-1 and rSLM-2 proteins, the bacterial generated recombinant proteins and a random 20-mer RNA pool were used. After five rounds of SELEX, RNA sequences that bind to recombinant proteins were selected and cloned into pCR-TOPO vector and sequenced (AGOWA, Berlin). The results are listed in Table 5.

Table 5 List of possible targets sequences for rSLM-1 and rSLM-2 proteins founded by SELEX. Nucleotide sequences given in sense orientation. Forward primer, flanked the SELEX target is T7Pro: UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU; reverse primer is RT: GGAAGCUUCUCGAGACGC.

SLM-1	SLM-2
GGGCCACGAAGCGCGUUGCA	GGGCUCGUUCGGGUGUGCGG
GCAGUCAGGAGUGGCGGCAG	GGCCGGGGAGGAUAGUGGGC
GACCCACGUCCCUGGCAGGU	UGCUGUGCGGCGUGUCGAGG
UCAACGAGACAGGCAUCGUG	GGGGACUGCAGGGCAAGGGC
GGCGAUAAUGUGGAAUUGCC	GACAAGACAUGGGCAGGAGA
GGUGAUGGUGGCGUUAGCUA	GGGCACGCAUCGGGGGUAGG
AGCGAUUACUUAACACGUCA	AGGCAGCAGAGGUGAUCGGC
UACUAUAGACCUGUCCCCCU	CAACGGGCGAUGUCCAUCGU
GGUCAGAUCGUAUCUCCCCG	CAGGUGGAACCAGUCCAAGA
UGGGACCUAUUGCCGUAGCG	CCCCCGAUCCACGCCACUGG
AUAGCGCCAAGACGCACAGG	CGUACGCACCUAGCCCCCCA
GGGGGUGUAUCGACCGGCAU	UCCGCCCCUUCCCCUCCCCC
ACCUCGAGACGGGUCAGGCC	UUACGCGACUACGAUGACCA
GCACAUUGAACCGCCCCCGG	GGCGUCCCCUUCACAGUGCG
UUGUUGUCGUGACCUGUGUGG	CUCGGUGCCCGCCAGCUGUG
AUAGGGUACCGAAUUCCGACU	
GGCGACUGGGGGGTCAGGGU	
GGGCGGGACAGACGCGAGCC	

Similar to other RNA binding proteins, rSLM-1 and rSLM-2 binds to highly degenerate sequences that can be described by a weight matrix, which is shown in Figure 28.

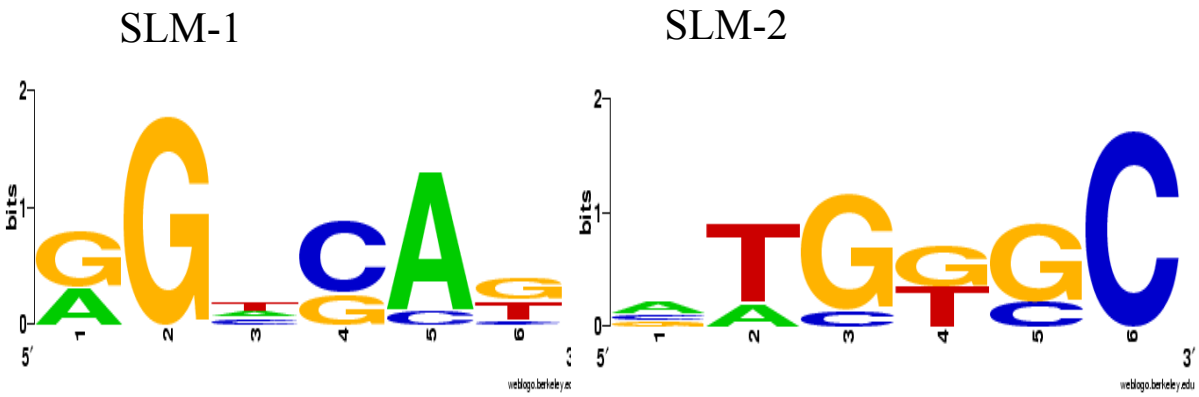


Figure 28. Weigth matrix, generated by WebLogo program (http://weblogo.berkeley.edu) describes the degenerate sequence element that present in SELEX sequences.

In order to obtain the constitutive motif of rSLM-1 and rSLM-2 proteins we used the MEME software. After screening of all candidates, presented in Table 5, we found the consensus motif is (AAGU) with an E-value=4.2e for rSLM-1 protein. The consensus motif is (CA x UC) with an E-value=3.1e corresponds to rSLM-2.

4.1.9. rSLM-1 shuttles between nucleus and cytosol

rSLM-1 was phosphorylated by the membrane bound kinases c-Src (Stoss, 2004) and $p59^{fyn}$ to which it binds in immunoprecipitation (Stoss, 2004). Since rSLM-1 is detected only in the nucleus of cells, a phosphorylation by membrane bound kinases would only be possible during mitosis when the nuclear structure disintegrates or would require shuttling of rSLM-1 between nucleus and cytosol that has been reported for several proteins implicated in splice site selection (Caceres et al., 1998). To test these possibilities, a cell fusion assay was employed to assess the capability of EYFP-SLM-1 to shuttle between the nucleus and cytoplasma.

The assay monitors accumulation of a fluorescent protein in acceptor nuclei of a newly formed polykaryon (Lee et al., 1999; Neumann et al., 2001). This experiment was performed in collaboration with Dr. Ruth Brack-Werner (GSF Institute of Molecular Virology in Neuherberger, Germany). Transfected HeLa cells expressing EYFP-SLM-1 were fused with an excess of untransfected HeLa cells and accumulation of EYFP-SLM-1 in acceptor nuclei monitored by time-lapse imaging (Figure 29).

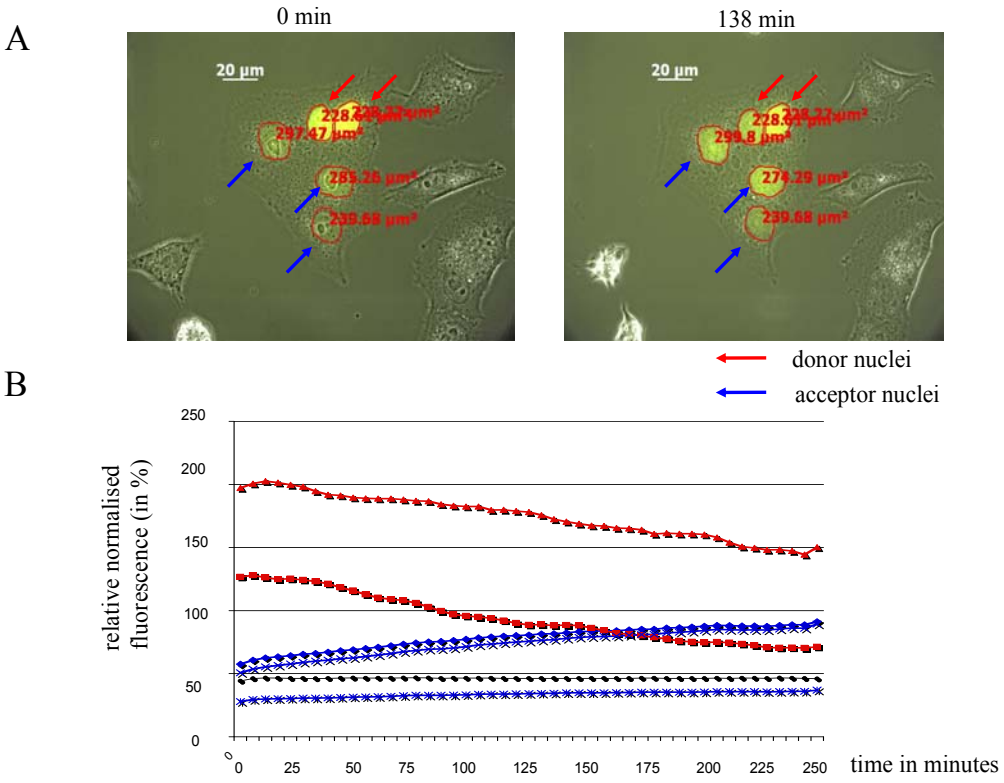


Figure 29. rSLM-1 shuttles between nucleus and cytosol. A. Hela cells transfected with EYFP-SLM-1 were fused to an excess of untransfected cells. Fluorescence after 2 hours 18 minutes is shown on right side of cell picture. Red arrows indicate donor nuclei, blue arrows indicate acceptor nuclei showing accumulation of rSLM-1-YFP. B. Quantitative analysis of fluorescence. Graphs show the loss in donor nucleus fluorescence (red curve) and increase in acceptor s fluorescence (blue curve) in rSLM-1**.**

The donor cells were marked with red fluorescent protein to allow distinction from acceptor cells. As shown in Figure 29, EYFP-SLM-1 fluorescence is visible in the acceptor nuclei after cell fusion and increases after 138 min. Concomitantly, the fluorescence intensity of the donor nucleus decreases. These results indicate that SLM-1 shuttles between nucleus and cytosol, where it could interact with membrane bound src-family kinases.

To summarize, we characterized the splicing factors rSLM-1 and rSLM-2 and determined the difference of these proteins on splice site selection in the presence of p59fyn kinase. Tyrosine phosphorylation plays an important role in splice site selection and splicing factors (particularly rSLM-1) could serve as an important links between signal transduction emantating from kinase $p59^{fyn}$ and alternative splicing.

.

4.2. Regulation of alternative splicing by cAMP transduction pathway

Human transformer Tra2-beta1 is a member of SR protein family of splice factors that regulate splice site selection by recruiting regulatory proteins to exon sequences. Our previous work demonstrate that the catalytic subunit of protein phosphatase 1 (PP1) binds to and dephosphorylates Tra2-beta1 via a phylogenetically conserved RVDF sequence located on the RNA recognition motif of Tra2-beta1 (Novoyatleva, 2008). Dephosphorylation of tra2-beta1 causes skipping of Tra2-beta1-dependent alternative exons. Inhibition of PP1 causes exon inclusion, notably that of SMN2 exon 7 and promotes the formation of the SMA protein in patient cell lines and mice models of spinal muscular atrophy. Our analysis also revealed that binding to PP1 is an intrinsic property of the RNA recognition motif (RRM) of at least nine other splicing regulatory proteins and where the PP1 binding motif is always located in the beta4 strand of the RRM. cAMP-dependent activation of PKA results in the activation of inhibitors of PP1, in particular, dopamine and cAMP-phosphoprotein of 32 KDa (DARPP-32). Therefore, the influence of cAMP-dependent PP1 activition tra2-beta1-mediated splicing provides now a model to mechanistically understand the drugs action for SMA treatment, since most of them change cAMP level.

4.2.1. Phylogenetic alignment of Tra2-beta1 protein sequence reveals a conserved PP1 binding motif

The human transformer splice factor Tra2-beta1 is known as one of the proteins that regulate splice site selection by recruiting regulatory proteins to exon sequences. The structure of the Tra2-beta1 protein is shown in Figure 30A. The protein is composed of two RS domains (in grey), which are flanking the central RNA recognition motif (in pink). The RRM motif is followed by a glycine-rich region (green). The RS domains allow the homomultimerisation and heteromultimerisation of the protein.

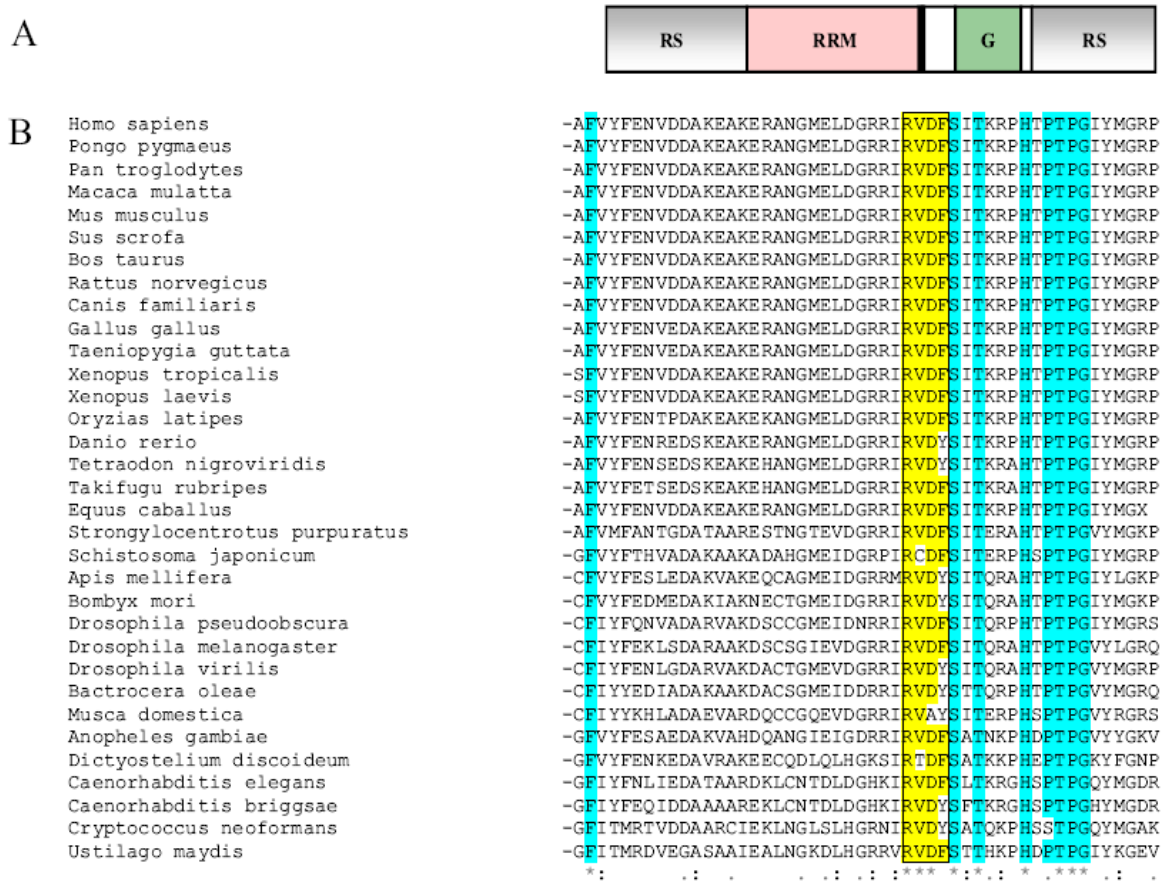


Figure 30. Tra2-beta1 protein sequence alignment. Alignment of Tra2-beta1 sequences from different species. The cartoon on the top (A) shows the domain structure of Tra2-beta1, RS: arginine-serine-rich domain, RRM: RNA recognition motif, G: glycine-rich region. The black line shows the conserved RVDF motif. (B) All available sequences corresponding to human Tra2-beta1 residues are aligned. Residues that are conserved in all species are marked in blue. A box indicates the putative RVDF motif implicated in PP1 binding and its conserved residues are marked in yellow.

Previous work demonstrated that Tra2-beta1, which is detected in all metazoans is a member of the SR-protein family, consisting of central RNA recognition motif and two serine/arginine-rich domains. By protein sequence alignment of the splicing factor human Tra2-beta1 we identified a conserved RVDF sequence downstream of the RRM (marked yellow) (Figure 30B). The RVxF motif was previously shown to be a PP1 (Protein Phosphatase 1) binding motif. The motif is present in most PP1 binding partners. Comparison with solved RRM structures and the NMR structure of the Tra2-beta1 RRM (protein database code: 2CQC) shows that this motif is located in the beta4 strand of the RRM.

This docking motif interacts with a hydrophobic channel of PP1 that is remote from the catalytic site (Bollen, 2001; Egloff et al., 1997). As in other PP1 interactors, the RVDF motif of Tra2-beta1 is N-terminally flanked by basic residues at position –2 and/or –3 that

promote the initial binding to PP1 (Meiselbach et al., 2006). The RVDF is followed by three evolutionary fully conserved amino acids: S/nonpolar/T (marked blue). It is fully conserved in all Tra2-beta1 sequences from vertebrates, except some fish species and non-vertebrate species, where it is changed to RVDY. However, a tyrosine at the last position is present in other PP1 interactors (Yang et al., 2000).

4.2.2. Several SR proteins bind to PP1 via a phylogenetically conserved RVXF motif

Next we investigated whether PP1 could also influence other splicing factors. From the sequence analysis of Tra2-beta1 protein we found that the PP1 binding site of Tra2-beta1 is located within the RRM. We investigated whether other RRMs could function in PP1 binding. We examined all known proteins containing an RRM for the presence of the RVXF binding consensus sequence.

From 497 proteins analyzed, twelve contain the RVXF binding motif. In two proteins, poly adenylate binding protein 3, PABPC3 and RNA binding motif protein 6, RBM6 the RVXF motif is located outside of the RRM. In the remaining cases, the motif is located in the beta4 sheet of the RRM (Figure 31). These proteins can be subdivided into SR-proteins and hnRNPs. In addition to Tra2-beta1, the group of SR-proteins contains SF2/ASF, SRp30c and the related SRp54. The remaining hnRNPs p54nrb (NONO), PSF (SFPQ), nPTB and ROD1 are all structurally related to the polypyrimidine tract binding protein (PTB, hnRNP I).

The presence of a PP1 binding motif in PSF explains earlier findings that PSF binds to PP1 in yeast assays (Hirano et al., 1996).

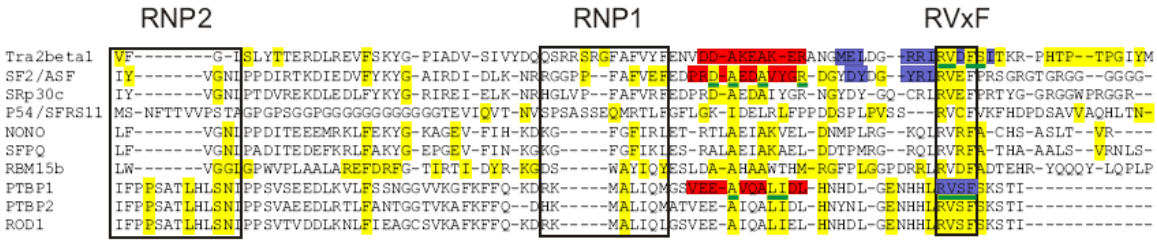


Figure 31. The alignment of RRMs of the human SR and SR-like proteins. RNP2, RNP1 and the PP1 binding motif RVxF are boxed. The proteins Tra2-beta1, SF2/ASF, SRp30c, SRp54, p54nrb(NONO) (Brown S.A. et al., 2005; Dong B. et al., 1993), SFPQ, (Clark J. et al., 1997), RBM15 (Ma Z. et al., 2001), PTB (Chan R.C. and Black D.L., 1997), nPTB (Markovtsov V. et al., 2000) and ROD1 (Yamamoto H. et al., 1999). Amino acids that are fully evolutionary conserved in each protein are marked in yellow. The known structural elements of Tra2-beta1, SF2/ASF and PTB are indicated (red: alpha helix, blue: beta strand). The conserved residues in alpha helixes or beta sheets are underlined with green line.

Since an interaction between SR-proteins and PP1 has not been reported so far, we tested binding of PP1 to SF2/ASF and SRp30c by coimmunoprecipitation. We found that PP1 coimmunoprecipitates with SF2/ASF and SRp30c (Figure 32A). However, when the PP1 binding motif RVEF present in both proteins is mutated to RATA the coimmunoprecipitation is completely abolished. These data strongly suggest that PP1 binds to the beta4 sheet of the RRM of SF2/ASF and SRp30c via the RVxF motif.

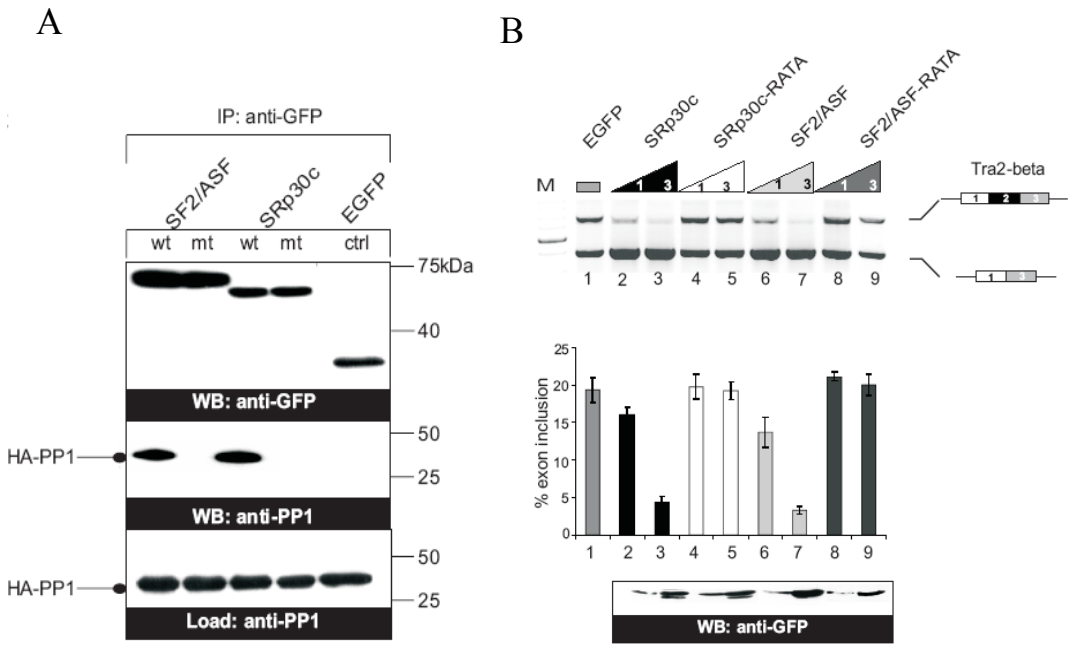


Figure 32. PP1 binding depends on the RVEF motif present in SF2/ASF and SRp30c. (A) EGFP-tagged SF2/ASF and SRp30c were expressed in HEK293 cells together with HA-PP1 and immunoprecipitated with anti-GFP antibodies. Wt: RVEF, mt: RVEF changed to RATA. The presence of HA-PP1 in the immunoprecipitates was determined by Western blot. Load: Western Blot using material from the cellular lysates. (B) The ability of SF2/ASF and SRp30c to influence Tra2-beta1 exon 2 inclusion depends on the RVEF motif. The gel shows the RT-PCR analysis of a transfection assays using a tra2-beta reporter gene and expression constructs of the EGFP-tagged proteins indicated. The Western Blot detects expression of the transfected proteins.

Finally, we used the alternative spliced exon 2 of the tra2-beta pre-mRNA to test whether the interaction with PP1 is functional important for SF2/ASF and SRp30c. We showed previously that SRp30c and SF2/ASF abolish exon 2 inclusion by sequestering Tra2-beta1 (Stoilov et al., 2004).

As shown in Figure 32B, SRp30c-RATA and SF2/ASF-RATA have no effect on exon 2 inclusion in transfection assays, whereas the wild type sequences promote exon 2 skipping, demonstrating that binding of PP1 is important for the function of these splicing

factors. These data show that PP1 regulates the activity of several splicing factors after binding to a phylogenetically conserved motif located on RRM.

4.2.3. Mutational analysis of the traCR

Among SR proteins, there is only in Tra2-beta1 a PP1-binding site that is followed by the highly evolutionary conserved region, suggesting they have an important role (Figure 30B). We called this region TraCR (Tra2beta1 Conserved Region). Next we would like to know, which amino acid or amino acids are crucial for function of tra2-beta1 in its conserve region. We mutated all conserved residues in the traCR (Figure 33) and tested the effect of the mutants on exon 7 inclusion in cotransfection experiments as well as binding properties to PP1 by immunoprecipitation analysis.

As shown in Figure 34, every single mutation destroyed the ability of tra2-beta1 to promote exon inclusion. There was no difference when serine/threonine residues were mutated to either glutamic acid or alanine, indicating that these residues need either be reversibly phosphorylated or function without phosphorylation.

A

188
…RIRVDFSITKRPH T PTPGIYMGRP…

B

S194E	S194A
T196E	T196A
T201E	T201A
T203E	T203A
H200E	H200A
S(194),T(196)/E	S(194),T(196)/A
Y207F	TPT(201,202,203)/KAI
Y207R	G209I
S(194),T(196),T(201),T(203)/E=4E	S(194),T(196),T(201),T(203)/A=4A

Figure 33. Sequences of Tra CR. A. The evolutionary highly conserved peptide. The PP1 binding site is marked in blue and underlined. All residues which were mutated indicated in red. Amino acid R corresponds to position 188 in the total sequences of tra2-beta1. B. List of mutation in TraCR.

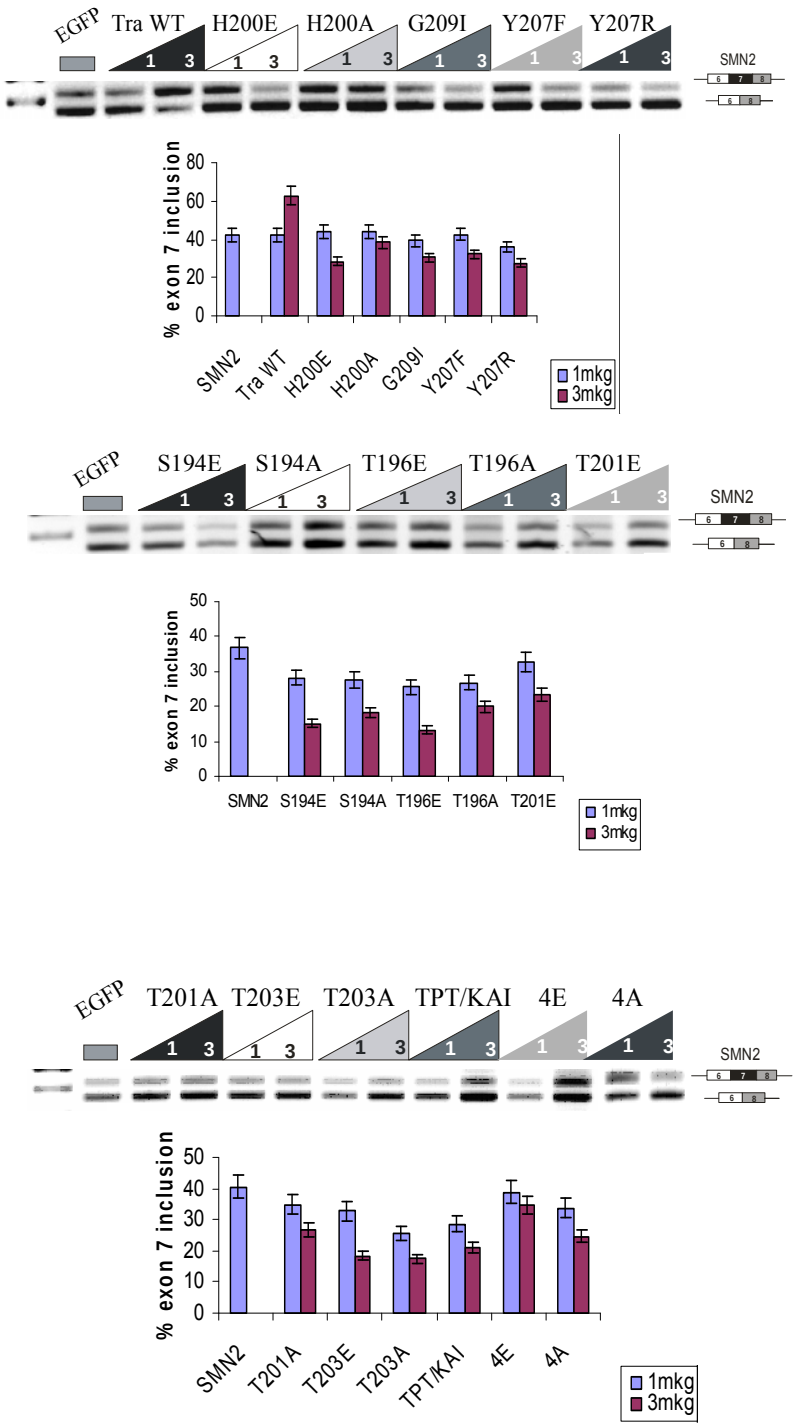


Figure 34. Effect of mutation of the traCR. The graph shows a statistical evaluation of cotransfection experiments using a SMN2 reporter minigene. The number indicate micrograms transfected into HEK293 cell lines. Five independent experiments were analyzed.

When we employed double mutants, where we replaced two aminoacids close to RVDF motif to alanine (S194A,T196A) or glutamic acid (S194E,T196E), in *in vivo* splicing analysis, we found a significant difference. Increasing the amount of EGFP

expression construct from 1 to 4 µg, mimicking the hyperphosphorylation form, promotes skipping of alternative exon 7 of SMN2 minigene from 36% to 3,8% (Figure 35). The opposite effect was observed in case of increasing amount of EGFP expression construct, mimic notphosphorylated form, which leads to increasing exon 7 inclusion. These data show, that each unique residue is very important for generation of splicing isoform and the phosphorylation events could leads to cooperative effect on SMN2 minigene.

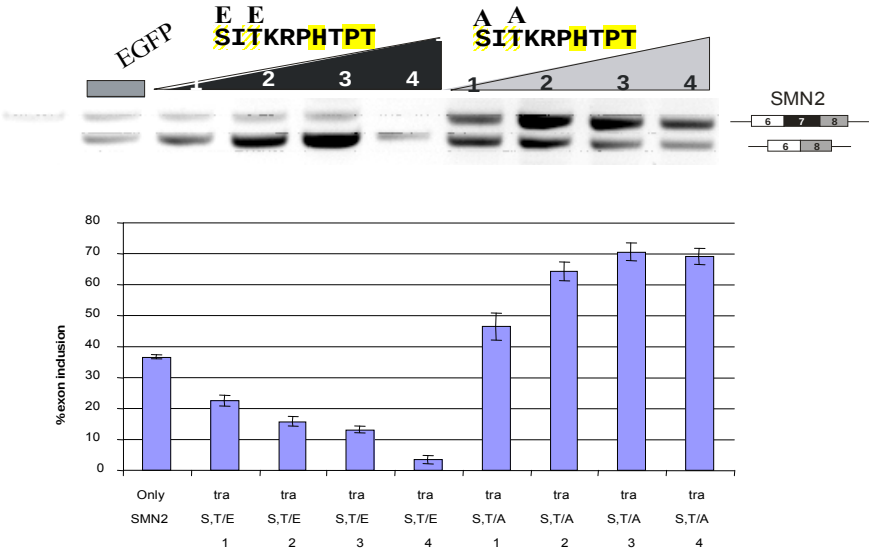


Figure 35. Effect of double mutation of the traCR. The graph shows a statistical evaluation of cotransfection experiments using a SMN2 reporter minigene. The number indicate micrograms transfected into HEK293 cell lines. Three independent experiments were analyzed.

We next tested the cellular localization of tra2-beta1 mutants and their ability to binds to PP1. As shown in Figure 36 all obtained mutants with point mutation downstream of the RVDF motif had similar to wild type a nuclear localization and formed speckles.

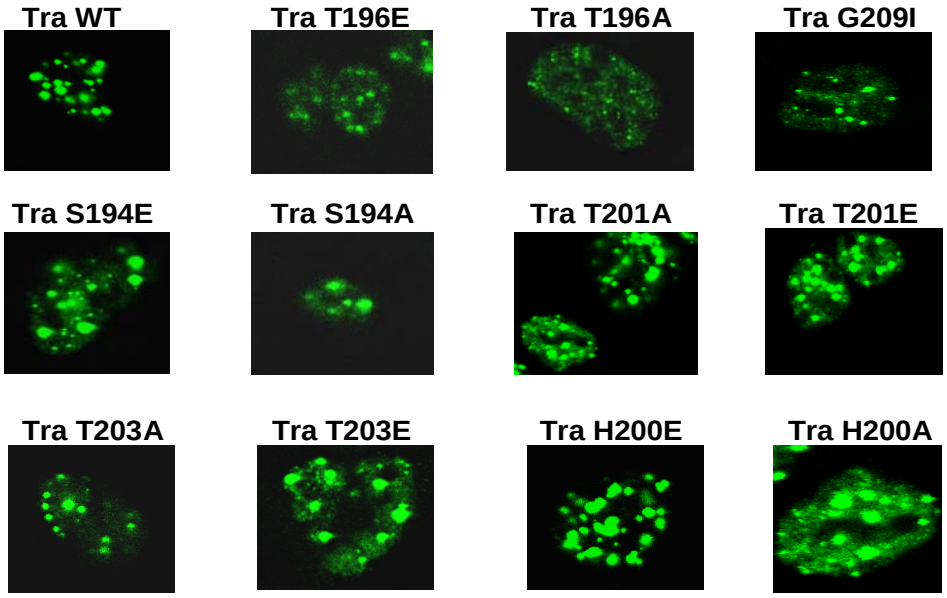


Figure 36. Nuclear localization of Tra2-beta1 mutants. Proteins with carboxy-terminal tagged EGFP were expressed in HEK293 cells. The pictures show representative cells.

Next we identified whether the mutants can still binds to PP1. We overexpressed mutants clones tagged with green fluorescence protein together with protein phosphatase 1 expression plasmid, tagged with hemagglutinin (Figure 37). Precipitates were tested by Western Blot analysis with a PP1 specific antiserum. The result obtained by Western blot show that all mutants are able to binds to PP1. The signals were almost identical (upper panel). The equal amount of expressed proteins were used for analyzing (Load, lower panel).

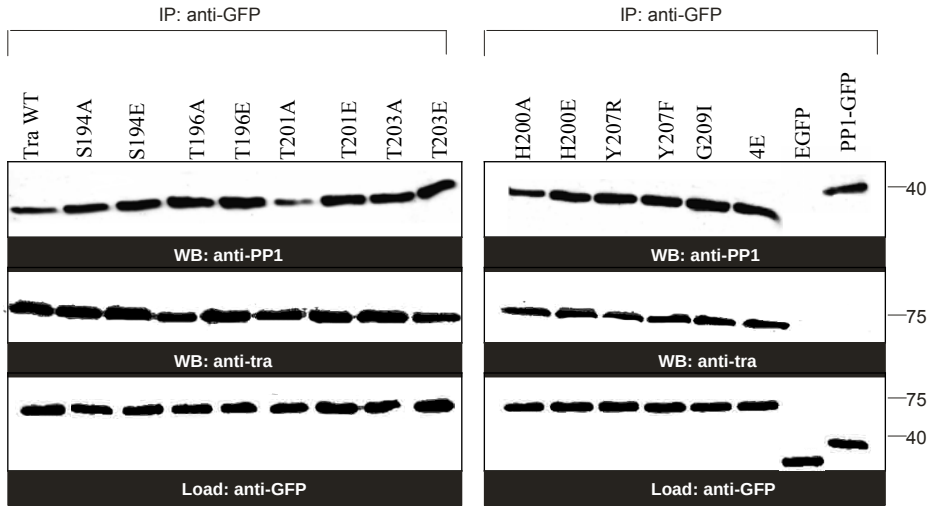


Figure 37. Tra2-beta1 mutants binds to PP1. EGFP-tagged Tra mutants were expressed in HEK293 cells together with HA-PP1 and immunoprecipiteted with anti-GFP antibodies. The presence of HA-PP1 in the immunoprecipitates was determined by Western Blot. Load: Western Blot using material from the cellular lysates.

4.2.4. Predicted kinases which could phosphorylate Tra2-beta1

It was previously shown that CLK1-4 kinases and the SR-protein kinases SRPK1/2 phosphorylate tra2-beta1 (Yun et al., 2003; Nayler et al., 1998). To get a full picture, we tested all kinases having putative (including weak) consensus phosphorylation sites on tra2-beta1. From available on-line sources as www.phosida.com, www.kinasphos.com, www.phosphosite.org we created a list of possible kinases: cAMP-dependent protein kinase (PKA), protein kinase B (PKB), casein kinase I (CKI), calmodulim-dependent protein kinase II (CaMK II), glycogen synthase kinase 3 (GSK 3), mitogen-activated protein kinase or extracellular signal-regulated kinase (MAPK, Erk) and cyclin dependent kinase 2 (Cdc2 protein kinase). Tra2-beta1 protein and radioactive-labeled γ-ATP were incubated with kinase for 20 min and analyzed by radiography. As shown in Figure 38A, three of tested kinases, GSK3-beta1, Cdc2 and CKI phosphorylated tra2-beta1 (upper panel). After 48 hours of exposure a signal from MAPK was observed. The recognition motif of MAPK contains amino acids that are located in TraCR (..PHTP..). Since the signal appears only after long exposure time, it is likely that it is not a specific phosphorylation event.

Table 6. **Recognition motifs of protein kinase, which phosphorylates Tra2-beta1**. Context adapted from www.invitrogen.com/kinase/table.

Protein Kinase	Recognition Motifs	Phosphorylation sites	Protein substrate
Casein Kinase I (CK1)	S(P)-X-X-S/T	R_4TLS(P)VSSLPGL D_{43}IGS(P)ES(P)TEDQ	Glycogen synthase, rabbit muscle, α-casein
Glycogen Synthase Kinase 3 (GSK-3)	S-X-X-X-S(P)	S_{641}VPPSPSLS(P) S_{641}VPPS(P)PSLS(P)	Glycogen synthase, human (site 3a and 3b)
Mitogen-activated Protein Kinase (MAPK)	P-X-S/T-P X-X-S/T-P	P_{244}LSP P_{92}SSP V_{420}LSP	c-Jun, cuclin B, Elk-1
Cdc2 Protein Kinase; CDK2-cyclin A	S/T-P-X-R/K	P_{13}AKTPVK H_{122}STPPKKKRK	Histone H1,calf thymus, large T antigen
cAMP-dependent Protein Kinase (PKA)	R-X-S/T R-R/K-X-S/T	Y_7LRRASLAQLT F_1RRLSIST A_{29}GARRKASGPP	Pyruvate kinase, phosphorylasi kinase, α-chain,histone H1, bovine
Calmodulin-dependent Protein Kinase (CaMK II)	R-X-X-S/T R-X-X-S/T-V	N_2YLRRRLSDSN K_{191}MARVFSVLR	Synapsin (site 1), calcineurin
Protein Kinase B (PKB, Akt 1)	R-X-R-X-X-S/T	S_{124}PSPNS T_{450}PPDQDDS	Transcription factor FKHR1, Bad-caspase-9, GSK 3 ß

The equal presence of Tra2-beta1 on the affinity resin was confirmed by Western blot using specific antiserum (lower panel). Half of each sample was stained in Comassie blue dye for identification of protein (Figure 38B). Surprisingly, first we found that GSK3-beta kinase can bind to tra2-beta1 almost stochiometrically *in vitro*.

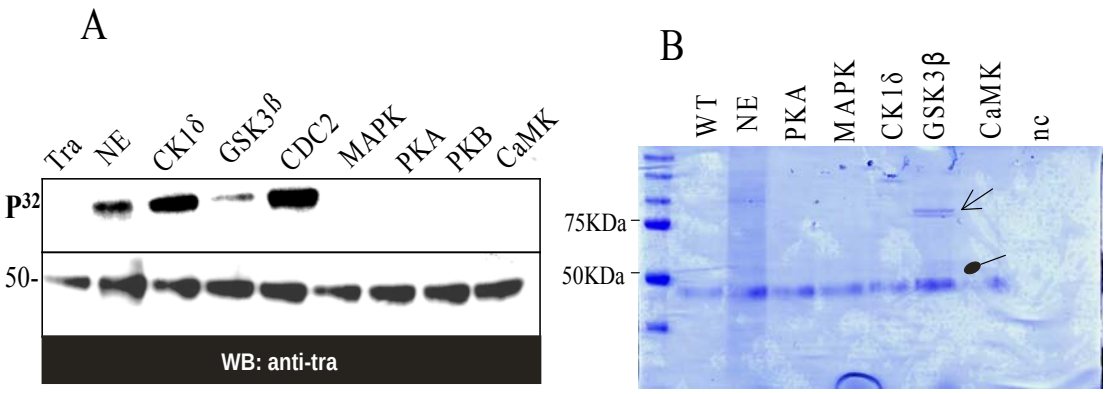


Figure 38. In vitro phosphorylation assay. A) Recombinant tra2-beta1 was incubated with the kinases indicated in the presence of gamma P^{32}-ATP. The top panel shows the result of autoradiography, the lower panel is a Western Blot with anti-tra2-beta1 as loading control. B) GSK3-beta binds to tra2-beta1 in vitro. The reactions from panel A were resolved on PAGE and stained with coomassie blue. The pointed arrow indicates GSK3-beta, the round arrow tra2-beta1. nc – as a negative control we used empty Ni-NTA agarose beads.

We also observed binding between tra2-beta1 and these kinase by immunoprecipitation after cotransfection in HEK293 cells (Figure 39).

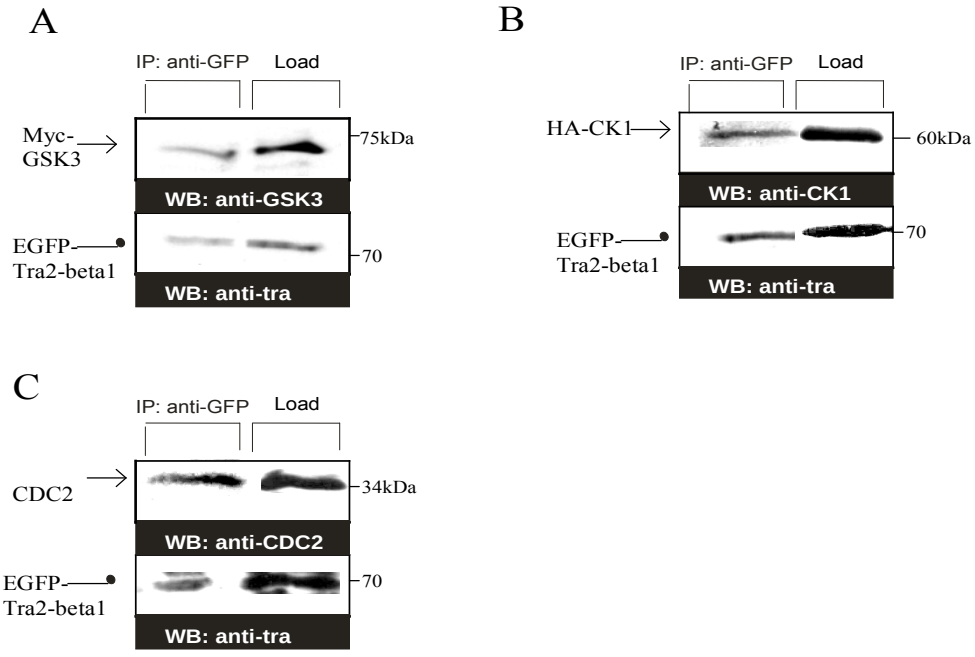


Figure 39. Interaction of Tra2-beta1 with kinases. EGFP-Tra2-beta1 and Myc-GSK3 (A), HA-CK1(B) and CDC2 (C) were co-expressed in HEK293 cells and precipitated with anti-GFP antibody. Co-

immunoprecipitated Tra2-beta1 and kinases in the precipitates and the lysates (load) were detected by Western Blot using their specific antibodies (upper panels). The EGFP-fused wild type Tra2-beta1 was detected by anti-Tra in the co-immunoprecipitates and lysates (lower panel).

Next we analysed the phosphorylation pattern of the traCR using a mass-spectroscopic approach that employed bacculo-virus generated tra2-beta1 and recombinant kinases, as well as the kinases and GFP-tagged tra2-beta1 overexpressed in HEK293 cells. This experiment was done in collaboration with Dr. Henning Urlaub and Dr. Mads Grönborg from Max-Plank Institute in Göttingen (Germany).

We could clearly identify residues T201, S264, S266 as being phosphorlyated. GSK3-beta1, Cdc2 and CKI caused increased phosphorylation at T201, which is located in the middle of evolutionary conserved region of Tra2-beta1.

To further analyse phosphorylation, rabbit polyclonal antisera were raised against peptide CIRVDF**pS**I (anti-phospho-S194) and P**pT**PGIYMGC (anti-phospho-T203), as they are fully evolutionary conserved. Although the antisera technically worked, we could not detect phosphorylation of tra2-beta1 protein at S194 sites. The phospho-T203 antiserum can detect not only the phophorylated threonine at 203, but also at position 201, what could indicate a cascade mechanism of phosphorylation (Figure 40).

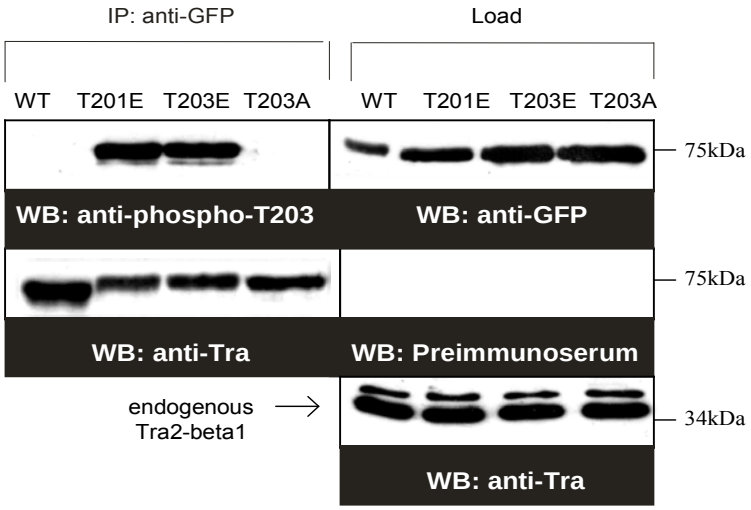


Figure 40. Antisera against phospho T203 do not specifically recognize the protein. Left: EGFP-Tra2-beta1 WT, EGFP-Tra (T201E), EGFP-Tra (T203E) and EGFP-Tra (T203A) were overexpressed in HEK 293 cells. Protein was precipitated with anti-GFP and detected with anti-phosphoT203 (upper panel). A reblot showed that anti-Tra recognizes both wild type and mutated Tra2-beta1. Rigth: crude cell lysate were analysed for proteins expression with anti-GFP antibody (upper panel). A reblot with preimmunoserum showed no detection of the protein (medium panel). Endogenous tra2-beta1 is shown on lower panel.

We suggest that most of the traCR is not structured; yet the mutational analysis clearly shows that each residue is important for tra2-beta1 function. Together the data indicate that the traCR is crucial for the ability of tra2-beta1 to promote exon 7 inclusion.

4.2.5. Tra2-beta1 dependent exons are regulated through cAMP

It is well established that PP1 can be inactivated by cytosolic inhibitors. The best studied inhibitors are DARPP-32 and Inhibitor-2. Both proteins are highly similar, but DARPP-32 is predominantly expressed in neurons: 96,4% of the medium-sized neurons were found immunopositive for DARPP-32 (Ouimet, 1984). cAMP activated protein kinase (PKA), phosphorylates threonine T34 of DARPP-32. This phosphorylated protein binds to the active center of PP1 and inactivates it.

We suggest that activation of PKA will lead to the changes in splice site selection of SMN2 minigene and promotes inclusion of exon 7 by repression of PP1 via cAMP/PKA/DARPP-32 transduction pathway. In order to test our hypothesis, we cotransfected PP1 and DARPP-32 expression constructs together with SMN2 reporter minigene. In our experiment we elevated cAMP level by forskolin, a known drug that activate adenylate cyclase. As shown in Figure 41, forskolin enhances exon 7. The construct was driven by a CMV promoter which lacks cAMP response element. DARPP-

32 alone slightly promotes exon 7 inclusion and forskolin completely abolishes the action of transfected PP1, that alone strongly promotes exon 7 skipping (Figure 41A).

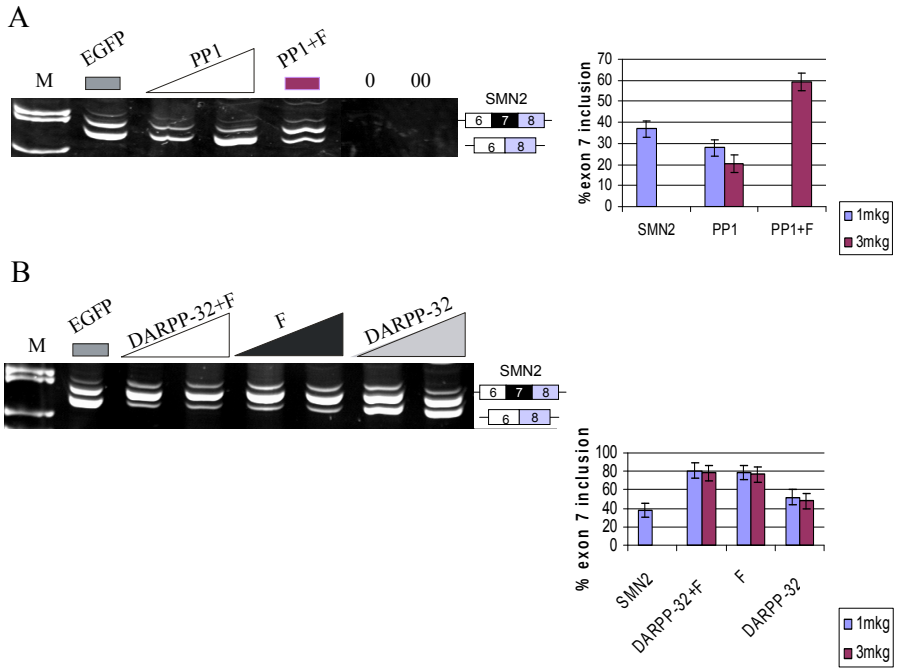


Figure 41. Forskolin influences SMN2 exon 7. EGFP-PP1 (A) and EGFP-DARPP-32 (B) were co-transfected with 1µg SMN2 minigene in HEK293 cells. Forskolin treatment was overnight, at a concentration 30µM. The cDNAs was amplified using minigene specific primers.

4.2.6. DARPP-32 interacts with Tra2-beta1 in vivo

DARPP-32 (Dopamine and cAMP regulated phosphoprotein, 32 kDa) is a major physiological regulator of PP1. In response to an increasing concentration of cAMP, Protein Kinase A is activated and phosphorylates DARPP32 on residue T34. This phosphorylated DARPP32 is now a strong inhibitor of PP1 (Bibb et al., 1999; Svenningsson et al., 2004), which dephosphorylates Tra2-beta1 and changes splice site selection, for example, on the SMN2 minigene. These data suggest that proteins regulating PP1 activity bind directly to tra2-beta1, leading to the hypothesis that PP1 activity is regulated while the protein is attached to tra2-beta1.

To investigate whether Tra2-beta1 can bind directly to DARPP-32, we transfected the DARPP-32 expression clone and EGFP-Tra2-beta1 (wild type) in HEK293 cells. Immunoprecipitation with anti-GFP antibody and the following Western blot analysis using specific anti-DARPP-32 antibody showed, that this two proteins can strongly bind to each other (Figure 42B, lane1).

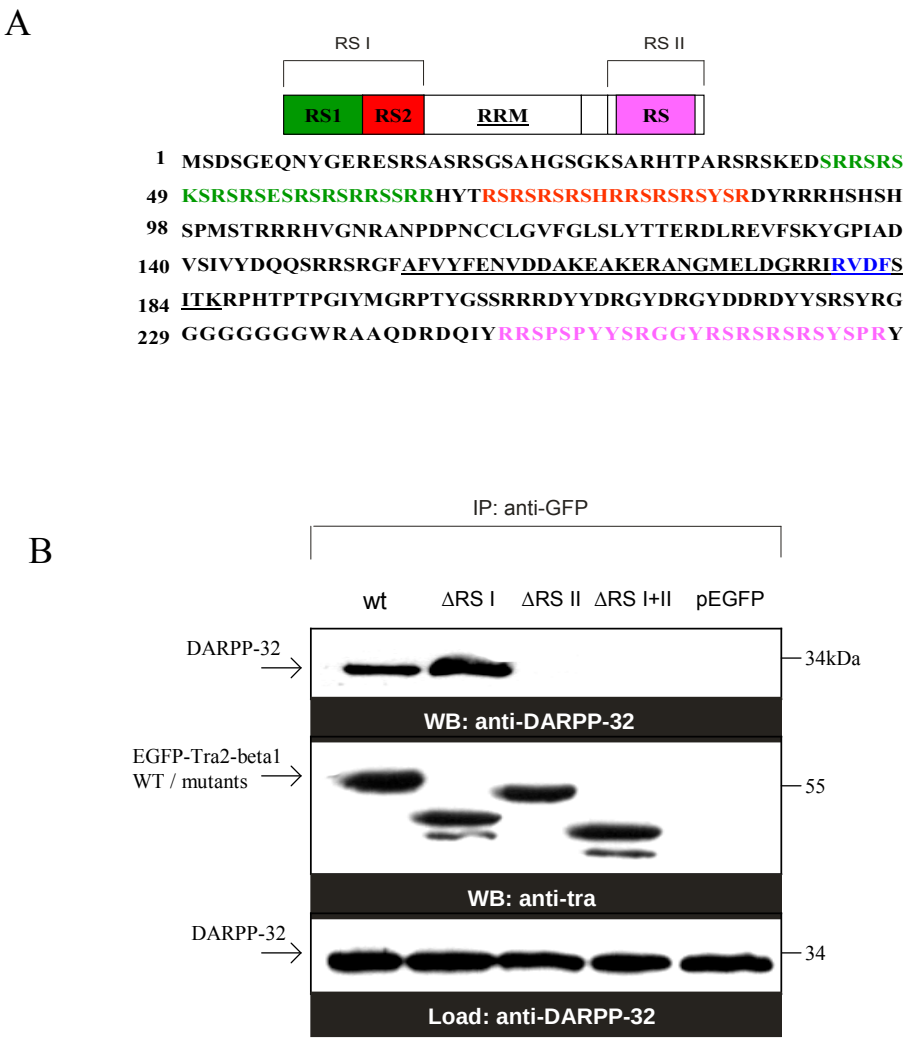


Figure 42. Binding of DARPP-32 to Tra2-beta1 and its mutants. A.The Tra2-beta1 structure reveals two RS domains which are flanking the RRM domain, containing the PP1 binding site (in blue). B. EGFP-tagged Tra2-beta1 wild type and mutants were expressed in HEK293 cells together with DARPP-32 and immunoprecipiteted with anti-GFP antibodies. The presence of DARPP-32 in the immunoprecipitates was determined by Western Blot. Load: Western Blot using material from the cellular lysates.

The Tra2-beta1 protein consists of two RS domains (RSI and RSII), which flank the RRM (Figure 42A). It was shown before (Cao et al., 1997; Stoilov et al., 2004) that serine residues located within the RS domains could be phosphorylated. The RS domain phosphorylation of SR proteins can have an important impact on the activity of SR proteins required to complete spliceosomal assembly and to regulate splicing events (Graveley et al., 2000). In order to identify which part is necessary for interaction with DARPP-32 we used mutants which lacks RSI or RSII domains, as well as mutant with a deletion of both RS

domains. As shown on Figure 42B (lanes 2-4), we can still detect signal corresponding DARPP-32 in case of deletion of RSI domain, but not RSII. The absence of a DARPP-32 signal when both domains were removed (RS I+II) confirmed that the RSII motif of Tra2-beta1 is responsible for binding to DARPP-32 protein.

4.2.7. DARPP-32 binds to Tra2-beta1 in vitro

The binding between DARPP-32 and Tra2-beta1 *in vitro* was analysed using *in vitro* translated DARPP-32 (GST-tagged) and baculovirus generated Tra2-beta1 (His-tagged) immobilized on Ni-NTA agarose. Figure 43A shows an *in vitro* translation experiment, where DARPP-32–GST was translated (lane 1). Figure 43B clearly showed, that after incubation of S^{35}-labeled *in vitro* translated products together with Tra2-beta1, a strong interaction were found only in case of incubation of DARPP-32. *In vitro* translated Tra-Flag was used as a positive control, since it is known, that Tra2-beta1 interact with itself. The YT521-B splicing factor was used as a negative control (Hartmann et al., 1999).

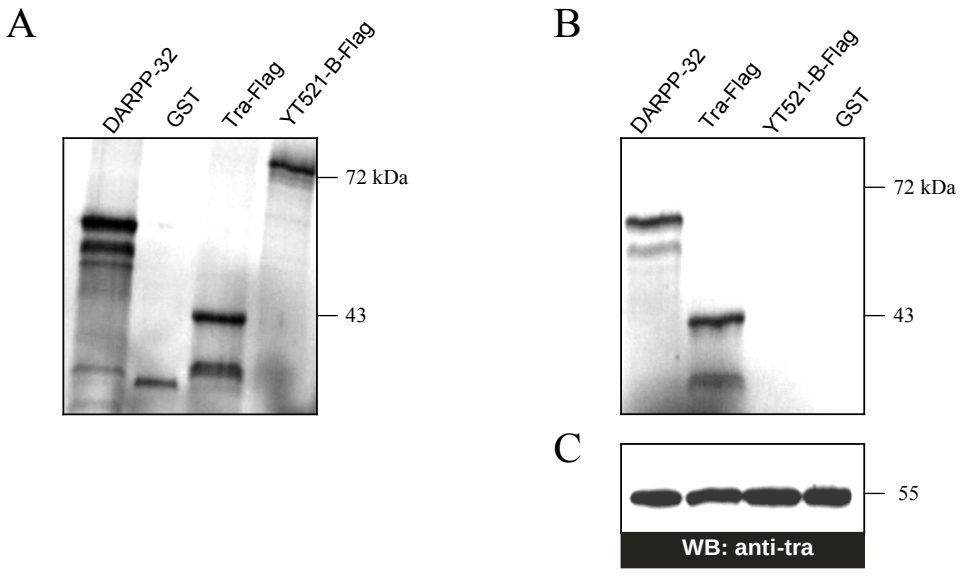


Figure 43. DARPP-32 binds to Tra2-beta1 in vitro. A. In vitro translation: 1µg of DNA templates were in vitro translated with reticulo lysates in the presence of S^{35}. The reaction product was tested by radiography. B. In vitro binding: recombinant His-Tra2-beta1 was coupled to a Ni^{2+}-agarose affinity column. This column was incubated with in vitro translated DARPP-32-GST (lane 1), Tra-Flag (lane 2) as a positive control, YT521-B-Flag (lane 3) and GST (lane 4) as a negative controls in Native Buffer at 4ºC for 2 hours and separated by 12% SDS-PAGE. Bound DARPP-32 and Tra-Flag were detected by radiography. C. The presence of equal amount of protein was detected using western blot and an antiserum against Tra2-beta1.

4.2.8. DARPP-32 competes with PP1 for Tra2-beta1 binding in vitro

As we already noted, PP1 binds to Tra2-beta1 directly via RVDF motif (Novoyatleva et al., 2008, p.147). DARPP-32 acts as an amplifier of PKA-mediated signaling when it is phosphorylated at Thr ^{34} and interacts with PP1 using a second domain, consisting of residues KKIQF (Svenningsson et al., 2004). The next questions was therefore: could these three proteins binds together and form one complex? For those stable or dynamic interactions?

Using recombinant proteins – DARPP-32, purified from rat brain, which is partially phosphorylated and bacculovirus generated tra2-beta1 bound on the Ni-NTA beads and the PP1 α isoform, which is commercially available, we tested these hypothesis. We found that DARPP-32 competes with Tra2-beta1 for PP1 binding.

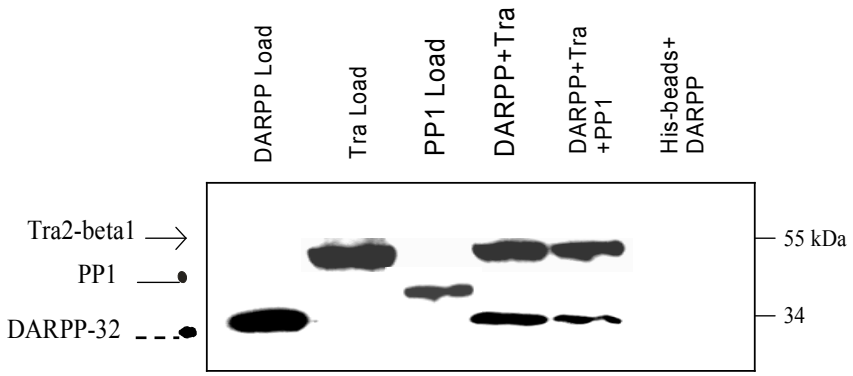


Figure 44. DARPP-32 competes with PP1 for binding to Tra2-beta1. Recombinant His-Tra2-beta1 was coupled to a Ni^{2+}-agarose affinity column. Equal molar concentration of DARPP-32, Tra2-beta1, PP1 proteins were used in the experiment and loaded in first three lanes. The column was incubated with recombinant DARPP-32 (lane 4) and in combination with PP1 (lane 5) at 4ºC for 2 hours. The presence of bound protein was detected using Western Blot and specific antibodies.

Equal molar concentration of each protein were used for the investigation. In this case, a tri-component assay shows much weaker DARPP-32 signal (Figure 44, lane 5), indicated by DARPP-32 antibody, comperative to the same probes, obtained without adding of PP1 (Figure 44, lane 4). A reblot with an anti-PP1 antibody can detect one band only from load control (Figure 44, lane 3), but not from the tri-components lane: Tra+DARPP-32+PP1 (lane 5). This indicated that DARPP-32 covered all possible binding to the Tra2-beta1 molecules and free, unbinding PP1 was removed on the washing step of experiment.

Using different concentration of PP1 we repeated the experiment. PP1 competes for binding of DARPP32 to tra2-beta1, as the amount of bound DARPP32 decreases when the PP1 concentration increases, as indicated by the large triangle in Figure 45.

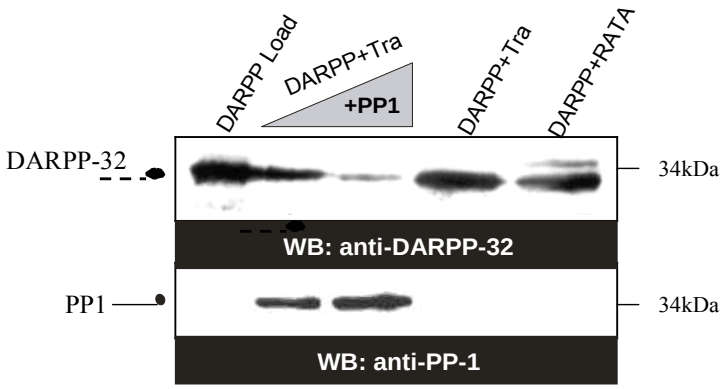


Figure 45. DARPP-32 competes with PP1 in concentration dependent manner for binding to Tra2-beta1. Recombinant His-tra2-beta1 was coupled to a Ni^{2+}-agarose affinity column. This column was incubated with recombinant DARPP-32 (lane 4), and DARPP-32 in the presence of PP1. PP1 was present in half and equal molar amounts to DARPP-32. (lane 2, 3). In lane 5, tra2-beta1-RATA was coupled to the affinity column and incubated with DARPP-32. Bound DARPP-32 was detected using Western blot and an antiserum against DARPP-32. A reblot with anti-PP1 antibody is shown on lower panel.

Finally, DARPP-32 also binds to the tra2-beta1 mutant (RVDF->RATA) that cannot bind to PP1 (Figure 45, lane 5), indicating that the two proteins have distinct binding requirements.

Together, these data show, that DARPP-32, naturally phosphorylated at Thr 75 and dephosphorylated at Thr 34, *in vitro* binds to Tra2-beta1 and competes with it for bindig to PP1.

4.2.9. Functional effect of DARPP-32 and its mutants on reporter minigene systems in vivo

Activation of PKA or PKG stimulates DARPP-32 phosphorylation at Thr^{34} and converts DARPP-32 into a potent inhibitor of PP1. The state of phosphorylation of DARPP-32 at these residues depends on the phosphorylation state of two serine residues, Ser^{102} and Ser^{137}, which are phosphorylated by CK2 and CK1, respectively. DARPP-32 is also phosphorylated at Thr^{75} by Cdk5 and it converts DARPP-32 into an inhibitor of PKA. Thus, DARPP-32 has the unique property of being a dual-function protein, acting either as an inhibitor of PP1 or of PKA.

Previous work has demonstrated that Tra2-beta1 regulates alternative exons by binding to purine-rich sequences that often act as exonic enhancers (Stoilov et al., 2004). Since DARPP-32 and Tra2-beta1 can interact with each other, this raised the question whether DARPP-32 also could regulate splice site selection of alternative exons and if the phospho/dephosphorylation status of DARPP-32 could influence exon usage. We therefore performed transfection experiments using established minigene assays, in which the alternative exon is flanked by its constitutive exons in a reporter gene construct. Expression clones of the wild type of DARPP-32 and two mutants carried either alanine (T34A) instead of threonine 34, mimicking the non-phosphorylation state or glutamic acid (T34E), resembling the hyperphosphorylated state were used. Increasing amount of these plasmids were transfected into HEK293 cells. The influence of DARPP-32 and its mutants on alternative splicing was determined by RT-PCR.

As shown in Figure 46A, an increase of DARPP-32, as well as mutated forms T34A and T34E, promoted the skipping of alternative exon 10 on tau minigene. At the same time no difference was found between expression of wild type and mutants. On the tra reporter minigene (Figure 46C) increasing the amount of DARPP-32 together with mutants promoted inclusion of tra2beta3 isoform, which was found only in brain (Stoilov, 2004). No significant changes were observed in *in vivo* splicing assay of the SMN2 minigene (Figure 46B).

Testing the influence of DARPP-32 and its mutants co-transfected together with CK1 and CK2 expression constructs did not show any significant difference on splice site selection (data not shown).

Our data suggest that DARPP-32 can change splice site selection on tau minigene independently on its phosphorylation status.

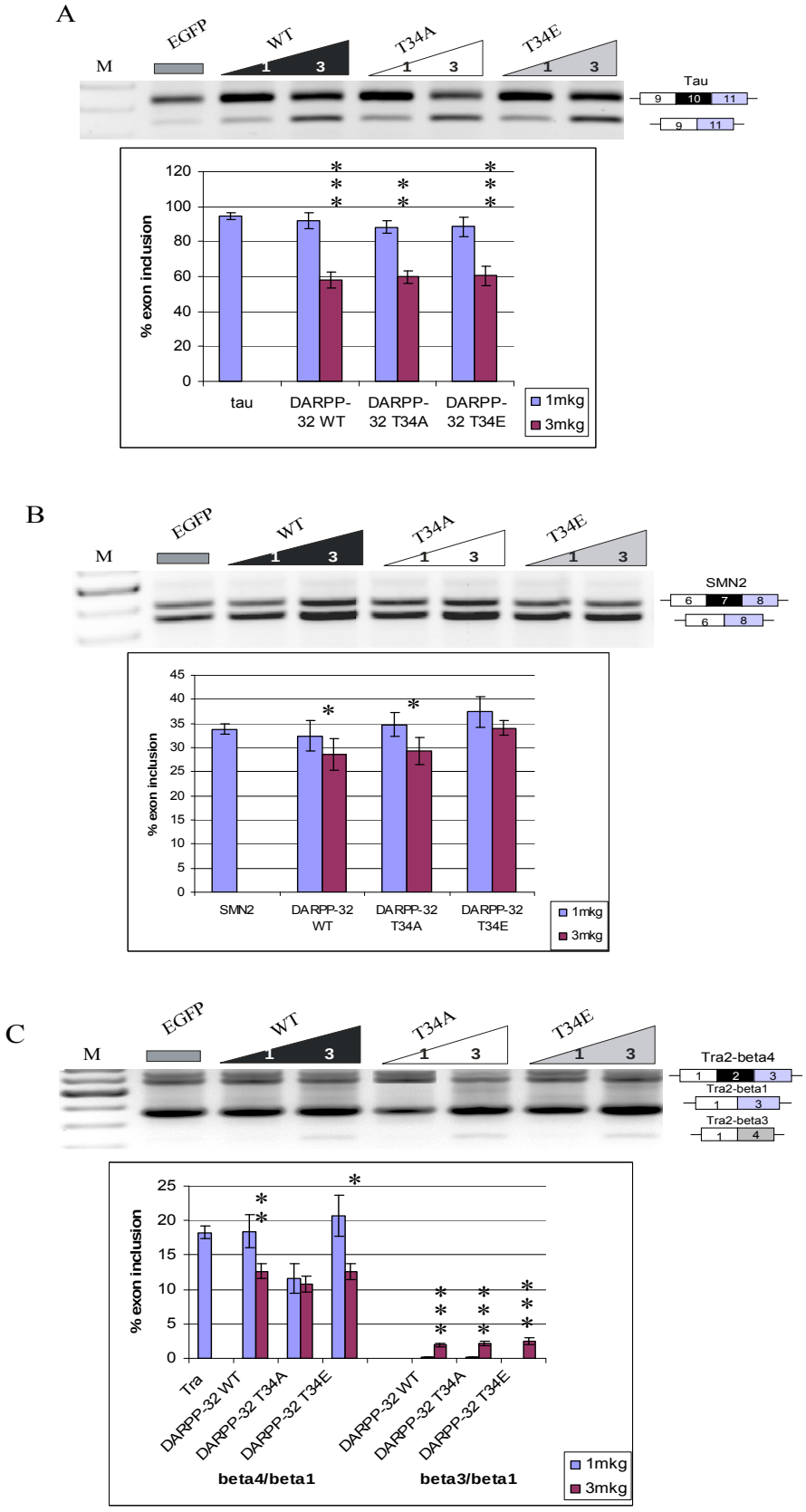


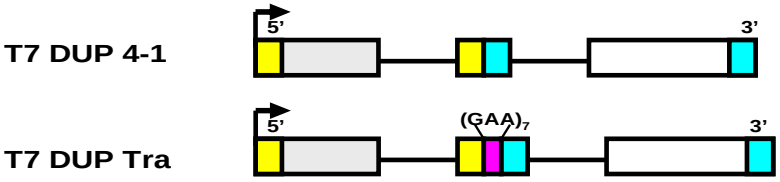
Figure 46. Functional effect of DARPP-32 and its mutants on reporter minigenes. An increasing amount of expression clones for DARPP-32 and its mutants T34A mimicking the nonphosphorylated status of the protein and T34E, mimicking the phosphorylated status of DARPP-32 was cotransfecred with the indicated reporter minigenes containing Tra2-beta1 dependent exons. The alternative splicing of each reporter

minigene was determined by RT-PCR. A representive ethidium-bromide stained gel of each experiment is shown. Under each experiment the statistical evaluation of at least four independent experiments is shown. (A) Tau, exon10; (B) SMN2, exon7; (C) Tra2-beta1, exon2. Statistical evaluation of the RT-PCR results is marked as stars. Stars indicated p-values from student's test. The ratio between the signal corresponding to exon inclusion and all products was determined from at least three different experiments.

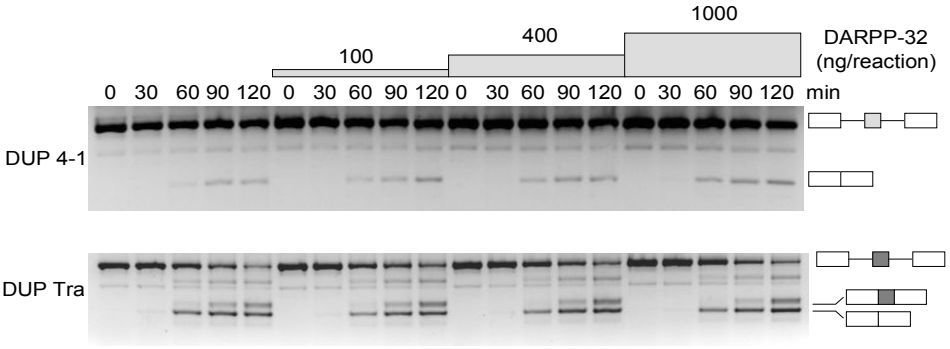
4.2.10. Functional effect of DARPP-32 in vitro

To study the influence of DARPP-32 on splicing activity *in vitro* we employed *in vitro* splicing technique in HeLa nuclear extract using T7 DUP's constructs.

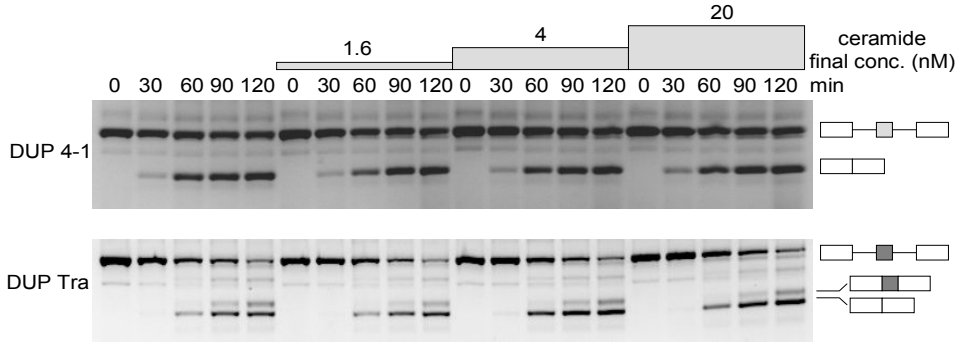
A



B



C



D

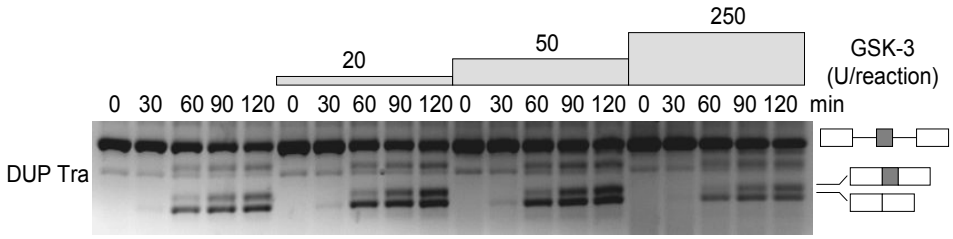


Figure 47. In vitro splicing assay of DUP constructs. A. Schematic representation of heterologous constructs: DUP4-1 is derived from exons 1 and 2 of the human ß-globin gene, with an artificial short exon (33 nt) between two identical introns. DUP Tra construct contains in the hybrid exon $(GAA)_7$ sequence cloned by substitution of 21 nt of the middle exon. In vitro splicing of DUP minigenes in Hela nuclear extract supplemented with DARPP-32 (B), ceramide (C), GSK3ß (D). Each of this two pre-mRNAs was spliced in vitro and aliquots were assays by RT-PCR at time points between 0 and 120 min, using minigene specific primers. Position of products corresponding to pre-mRNAs and spliced mRNAs are schematically shown on the right.

As shown in Figure 47A, the original construct T7 DUP 4-1 derived from the human ß-globin gene contained three exons and two introns. The first and last exons are ß-globin exons 1 and 2, respectively. An additional 33-nucleotide hybrid exon containing the duplicated 3'ss of ß-globin exon 2 and the duplicated 5'ss of ß-globin exon 1 have been inserted between the flanking exons. Thus, this clone contains two identical 5'ss, two identical 3'ss, and two identical introns. *In vivo*, the hybrid central exon is skipped due to its short length (Modaferri and Black, 1997).

A new clone T7 DUP Tra was derived from the DUP4-1 construct by insertion to hybrid exon Tra2-beta1-binding sequences which contains seven repeats of GAA.

Figure 47B shows, that in presence of increasing amounts of DARPP-32, in the original construct, T7 DUP 4-1 was spliced in vitro only inefficiently. Inserting the $(GAA)_7$ sequence greatly stimulated splicing activity. The same effect we observed in addition of ceramide -a drug known as a activator of PP1 (Figure 47C)

Earlier we found that GSK3 ß and DARPP-32 bind directly to Tra2-beta1 (Figures 38B and 39A). It is well known that both proteins are also interacting with PP1 (Ceulemans and Bollen, 2004). PP1 activates GSK3 ß by dephosphorylating it and phosphorylated DARPP-32 inhibits PP1 by tightly binding to it. We investigated the influence of GSK3 ß on splicing of DUP Tra construct and, as shown in Figure 47D, glycogen synthase kinase 3 beta promotes splicing activity in concentration dependent manner.

Taken together, these results confirm that DARPP-32, ceramide and GSK3 ß has the sufficient properties for activating splicing *in vitro* in HeLa extract.

4.2.11. DARPP-32 influence splice site selection on Tra-dependent exons

Recently we showed, that Tra2-beta1 regulates alternative splice site selection of numerous exons from human genes using micro array analysis (Novoyatleva, 2008). The experiment was performed in collaboration with ExonHit Therapeutics, Paris (France). In this study, 14 events were confirmed for their dependency on Tra2-beta1 by RT-PCR, using primers in the flanking constitutive exons.

As we found that DARPP-32 also can regulate splice site selection of some exons, we decided to test its action on the already known Tra-dependent targets. We overexpressed DARPP-32 wild type, its mutants D32 T34A and D32 F11A, as well as GSK3 beta and CK1 constructs in HEK293 cells. D32 T34A is a mutant, which can not be phosphorylated by PKA because of mutation of the T34 site. D32 F11A is a mutant, which can not binds to PP1 because of point mutation in the docking binding motif. As a control we used untransfected cells. RNA was obtained in 18 hours and analyzed by RT-PCR. We used specific primers for following genes: Srp75/SFRS4, Fyn, SFRS14, FE65, Clk4, hnRNP M, FUS, PPIL3, CPSF6, SRRM1, and PPIE. Only in three events we found the ability of DARPP-32 wild type to promotes exon inclusion: Clk4, Fyn, and SFRS14 genes (Figure 48A).

Based on this results, next we designed new minigenes of Fyn and SFRS14 (Figure 48 B,C). After overexpression of Tra2-beta1 and DARPP-32 in increasing amount we observed significant change in splice site selection only in case of Tra2-beta1. DARPP-32 can slightly influence on splicing selection in the minigene systems.

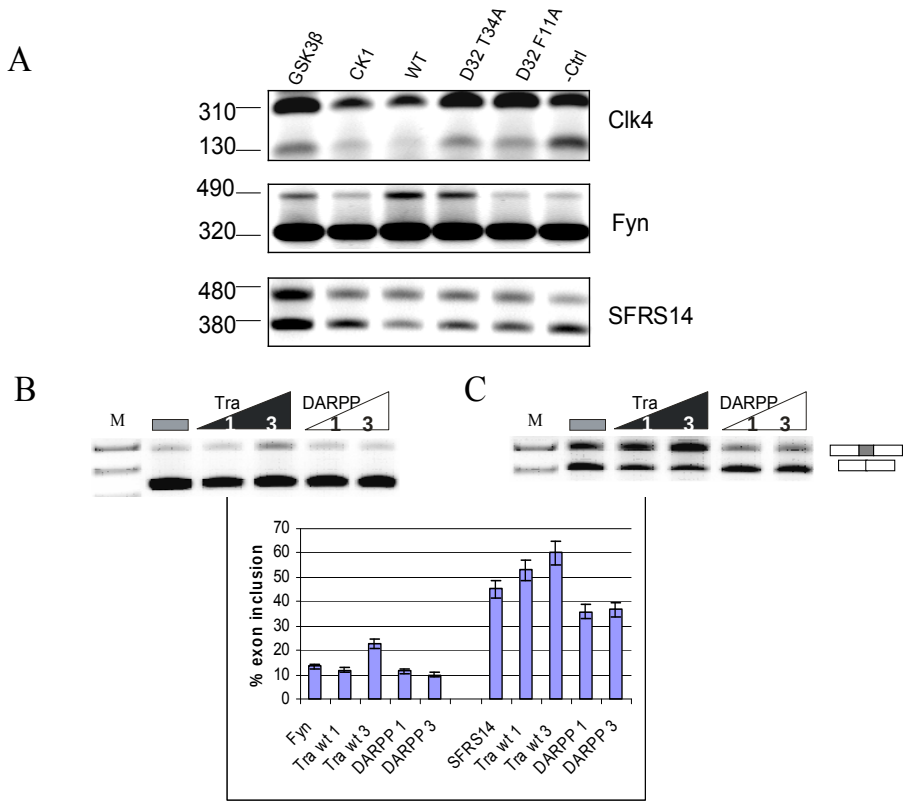


Figure 48. DARPP-32 influence splice site selection on Tra2-beta1 dependent exons. A. Expressions clones were transfected in HEK293 cells and the isolated RNA was amplified using specific primers. EGFP-Tra2-beta1 and DARPP-32 was cotransfected with the Fyn (B) and SFRS14 (C) reporter minigenes. The alternative splicing of each reporter minigene was determined by RT-PCR. A representive ethidium-bromide stained gel of each experiment is shown. Under each experiment the statistical evaluation of at least three independent experiments is shown. –Ctrl for untransfected HEK293 cells.

4.2.12. Interaction of DARPP-32 with Tra2-beta1 binding to RNA

Since we did not find a clear dependency of splice site selection on DARPP-32 binding and phosphorylation, we next tested its influence on Tra2-beta1/RNA binding.

Earlier *in vivo* cross linking and immunnoprecipitation (CLIP) experiments showed that Tra2-beta1 can binds to 18s and 28S rRNA (Tang et al., 2008). For our investigation we choose 28S rRNA. Recombinant DARPP-32 protein was used in 0.2µg/µl and 1.0µg/µl concentration.

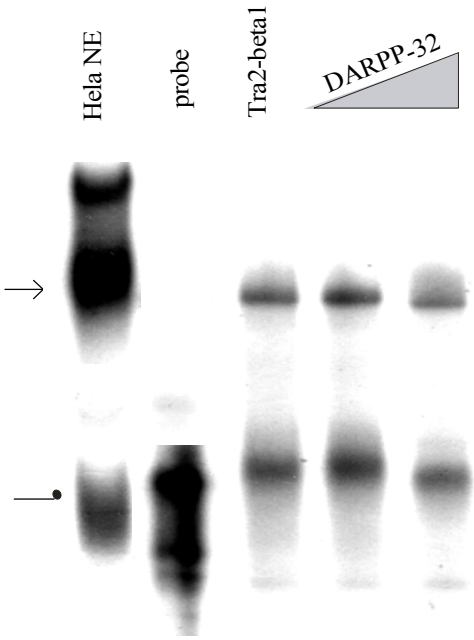


Figure 49. Interaction of Tra2-beta-1 with DARPP-32. Gel retardation assay: 1µg of nuclear extract (NE), recombinant Tra2-beta1 and DARPP-32 were incubated with radioactive probes of 28s rRNA (CGAAGCTACCATCTGTGGGATTATGACTGAACGCCTCTAAGTCAGAATCCCGCCCAGGCGGAA CGATACGGCAGCGCCGCGGAGCCTCGGTTGGCCTCGGATAGCCGGTCCCCCGCCTGTCC). The pointed arrow indicates the RNA-protein complexes, the round arrow the free probes.

As shown in Figure 49, a DARPP-32/RNA complex changes RNA mobility under increasing concentration of protein. In the last lane (lane 5) we observed that a band indicating RNA/protein complex was increased in intensity. These could mean that high concentration of DARPP-32 prevents the binding to RNA 28 Subunit.

These data suggest that DARPP-32 changes the ability of Tra2-beta1 to bind to RNA.

4.2.13. Synthesis of a cantharidine/PNA derivatives

Missplicing of the pre-mRNA is the cause of several diseases or is associated with these diseases. Among them are Alzheimer's disease (Glatz et al., 2006), breast cancer (Watermann et al., 2006) and spinal muscular atrophy (Hofmann et al., 2000). Therefore, the correction of improper splicing is an important goal in developing of molecular therapeutic strategies.

A silent, non-polymorphic C→T nucleotide transition in exon 7 of SMN2 gene leads to production of minor amount of full-length transcripts and high levels of a transcript that lacks exon 7 (Young et al., 2002). The latter transcript results in a truncated protein that is less stable and has a reduced ability to oligomerize (Lorson et al., 1998), explaining

why SMN2 cannot prevent SMA. However, detail studies of regulatory splicing elements in alternative exon 7, allowed the use of intact SMN2 gene in SMA patients that provides a natural target for therapeutic intervention.

One of possible therapeutic approaches is to increase the full length SMN level by activation of trans-acting factors (Sazani and Kole, 2003), RNAi (Celoto et al., 2005) or by antisense RNA (Cartegniet and Krainer, 2003).

Inhibition of PP1 has a strong effect on SMN production in vivo when applied to mice (Novoyatleva et al., 2008, Figure 7). However, DNA array analysis also clearly shows that PP1 inhibition will affect multiple aspects of RNA metabolism (Novoyatleva et al., 2008, Figure 6). This raises the obvious question: how can PP1 inhibitors be designed that are specifically acting on SMN2?

Currently, the only known specific part of the SMN2-tra2-beta1 complex is the SMN2 pre-mRNA. We therefore wanted to link oligonucleotides that bind to SMN2-pre mRNA to PP1 inhibiting molecules. This approach can be done, since one PP1 inhibitor, cantharidin, can be easily synthesized by a Diels-Alders reaction. Using modified educts for the reaction, we could generate cantharidin derivatives that block PP1 in vitro and slightly promote exon 7 usage *in vivo*.

Cantharidine (Figure 50-1) is a natural drug that can be isolated from Spanish fly. Cantharidine is a protein phosphatase inhibitor with broad biological activity and high toxicity, therefore, it is of little use in medicine. However, the toxicity could result from the unselective inhibition of cellular processes and development of more directed and selective drugs could reduce such side effect.

As a compound which has sequence-specific location we used peptide nucleic acid (PNA), which is artificially synthesized. Like its natural prototype, the binding of the PNAs occurs antiparallel via Watson-Crick pairing.

The effect of these new, nor-cantharidin, derivatives are weaker than of cantharidin, which is expected. By introducing an azide group, these components can be coupled to an alkine group via a “Click” reaction that occurs under mild conditions. PNA oligonucleotides with alkine linkers are commercially available. The design of the components is shown in Figure 50B and Figure 51.

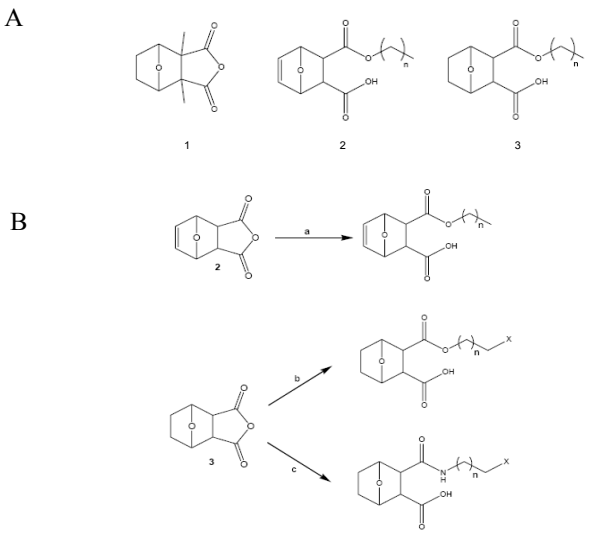


Figure 50. Synthesis of cantharidine compounds. A. General structure of the tested compounds: cantharidine (1), didehydronorcantharidin derivatives (2), norcantharidine derivatives (3). B. Ring Opening Reactions of dehydrogenated derivative (2) and norcantharidine (3) is shown. a) alcohol, TEA, CH_2Cl_2, 0C, over night; b) alcohol, TEA, CH_2Cl_2, RT, over night; c) amine, RT, over night

Click reactions are defined as reliable and easily perfomable reactions under mild conditions. Due to these advantages the Cu (I) catalyzed triazole formation was selected as linking reaction between the cantharidine and the PNA (Figure 51).

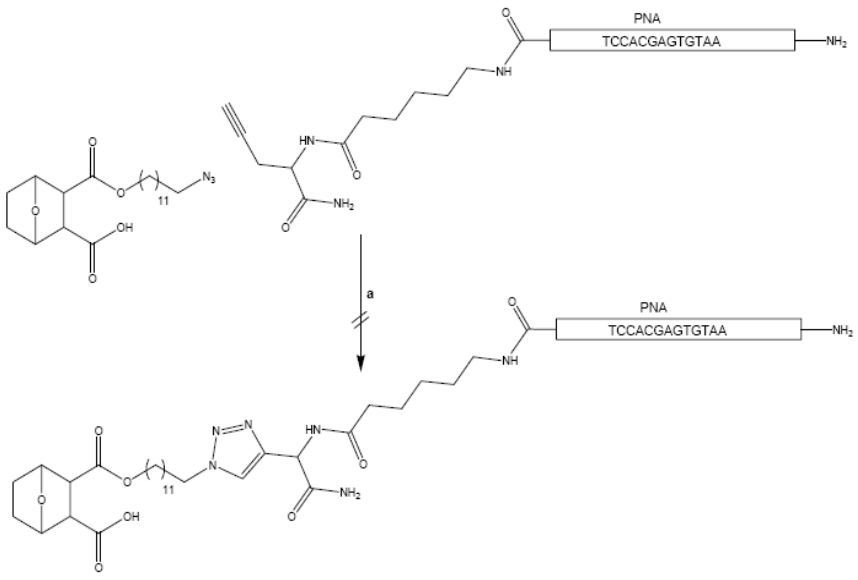


Figure 51. Synthesis of a cantharidin:PNA derivative using CLIK chemistry. a) $CuSO_4x5H_2O$, sodium ascorbate, CH_2Cl_2/BuOH/H_2O 1:2:1, RT, over night.

4.2.14. Screening of not-complete cantharidine derivatives to inhibit PP1 in vivo and in vitro

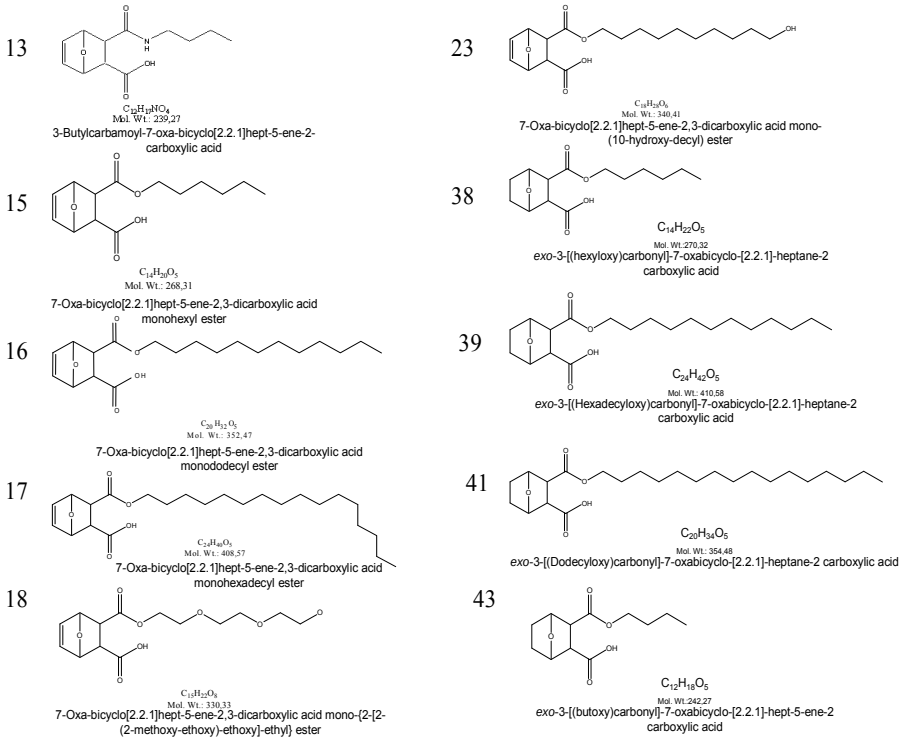
The chemical synthesis was done in collaboration with Dr. P.Gmeiner and Andrea Pinsker, Instute of Pharmacy, Erlangen (Germany). Figure 52A shows a list of compounds with different alcohol lengths that were generated.

We transfected the SMN2 reporter minigene to HEK293 cells for testing *in vivo* cantharidine derivatives for their ability to inhibits PP1. Treatment of compounds diluted in DMSO was done in 4 hours after transfection in test concentration at 1 and 6µM. RNA was isolated in 18 hours and analyzed by RT-PCR using minigene specific primers.

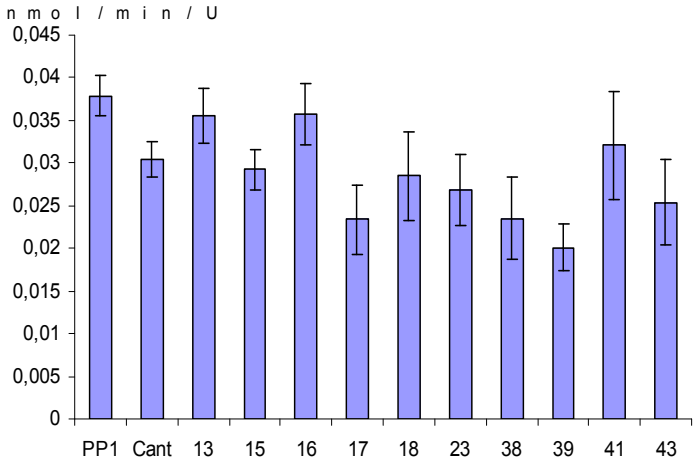
As shown in Figure 52C, new-synthesized compounds in general had a weaker effect compare to pure cantharidine itself. For example, compounds 13, 15, 16, 17, 18 and 23 belong to a didehydronorcantharidine group and these compounds tend to undergo a retro Diels-Alder reaction which leads to much smaller, presumably inactive compounds. As the norcantharidine derivatives 28, 39, 41 and 43 are lacking the double bond, they can not undergo this Diels-Alder reaction, and possess good activity against PP1.

Analysis by hydrolysis of para-nitrophenylphosphate (pNPP) (Figure 52B) detected that most all of them inhibit PP1 *in vitro* as well.

A



B



C

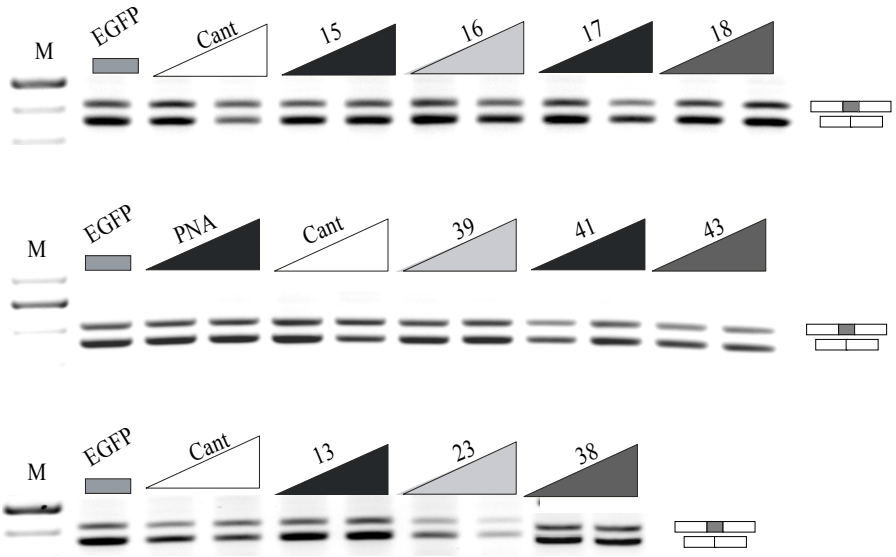


Figure 52. Protein Phosphatase 1 activity by pNPP hydrolysis. A. Chemical structure of cantharidine-based derivatives. B. In vitro assay was done accordingly to manufacture protocol. 2.5 Unit of enzyme was used per reaction. C. HEK293 cells were transfected with 1 µg SMN2 reporter minigene. In 3 hours after transfection cells were treated by 1µM and 6µM compounds diluted in DMSO. In 18 h RNA was isolated and analyzed by RT-PCR. Cntr-Cantharidine; PNA-peptide nucleic acid, used concentration 100µM and 1µM. The structure of the SMN2 products is schematically indicated.

Treatment of cells with the pure uncoupled PNA was performed to test whether it influences splicing, maybe due to antisense effects. PNAs normally are not able to cross cell membranes, therefore, we did not see significant changes of the exon 7 inclusion on SMN2 minigene even in very high concentration (100µM and 1µM) (Figure 52C).

Accordingly to *in vivo* and *in vitro* data, promising results have amides, since they are more stable and therefore cause more significant effect.

These experiments can be easily adapted to other PP1 binding areas. For example, if we obtain evidence that PP1 influences translation, we would couple the PP1 inhibitor to sequences in the 5' or 3' UTR. These components could serve as prototypes of newly rationally designed drugs against SMA and as tools to get further insights into the splicing mechanism.

Taken together, our data shows that multiple manipulation of separated units of signal transduction and pre-mRNA splicing processes influence protein transcription and, as a result, cell metabolism. Cooperative actions of protein kinases, protein phosphatases and their inhibitors represent a plastic network in one of the aspects of all regulatory system in the organism. Further investigation of this network and understanding of the role of each factor in this system is necessary for the correction of natural mutations, which cause diseases and developing new approaches to prevent existing disorder in the cell.

Table 7. Genes sequences from microarray data, which response to DARPP-32 overexpression.

Gene	Exon	Protein	Function
FYN NM_002037 Homo sapiens FYN oncogene related to SRC, FGR, YES 907.002.002	AAAGCTGATGGTTTGTGTTTTAACTTAACTG TGATTGCATCGAGTTGTACCCCACAAACTTC TGGATTGGCTAAAGATGCTTGGGAAGTTGCA CGTCGTTCGTTGTGTCTGGAGAAGAAGCTGG GTCAGGGGTGTTTCGCTGAAGTGTGGCTTG	KADGLCFNLTVIASSCTPQTSGLAKDAWEVARRSLCLEKKLG QGCFAEVWL	N-myristoylation N-glycosylation
SFRS14 NM_014884 Homo sapiens splicing factor, arginine/serine rich 14 179.1.1	GGGAACCCCCTCGGAAGGGGAAGGGTTGGGT GCTGACGGGCAGGAGCACAAAGAAGACACAT TCGATGTGTTCCGACAGAGGATGATGCAGAT GTACAGACACAAGCGGGCCAACAAATAG	GTPSEGEGLGADGQEHKEDTFDVFRQRMMQMYRHKRANK	Casein kinase II
CLK4 NM_020666 Homo sapiens CDC-like kinase 4 208.3.3	CCCCGCTGAATGAATTGCGTATTCTGCCCTG AATTCACTCTGATATATTGATTGGCTGGACG ATCTTGGTGCTGCCCACTTGCCGTTCCAGAA GAGCCACCGAAGGAAAAGATCCAGGAGTATA GAGGATGATGAGGAGGGTCACCTGATCTGTC AAAGTGGAGACGTTCTAAGAGCAAGATG	PAE*	Stop codon, NMD

5. DISCUSSION

In this work I investigated the regulation of alternative splicing by RNA-binding proteins via two distinct mechanisms.

First, we demonstrated that the rSLM-1 splice factor can change splice site selection through tyrosine phosphorylation mediated by the $p59^{fyn}$ kinase.

The second part of our research shows that splice site selection could also be regulated by reversible phosphorylation of the Tra2-beta1 splicing factor through the cAMP/PKA signaling cascade. These mechanisms involve protein phosphatase 1 and its inhibitor dopamine- and cAMP-regulated phosphoprotein, Mw 32 KDa (DARPP-32). Our results indicated a new function of DARPP-32 in influencing exon usage of pre-mRNA.

The pathological expression of pre-mRNA isoforms can be treated by transferring nucleic acid derivatives into cells that interfere with sequence elements on the pre-mRNA (Novoyatleva et al., 2006). In our work we demonstrated a possible approach to increase full-length product of the SMN2 gene by synthesis of cantharidine derivatives coupled to PNA. The SMN2 gene is a known target for treatment of spinal muscular atrophy.

5.1. Tyrosine phosphorylation of splicing factor rSLM-1 changes splice site selection

Alternative splicing is a major link between the estimated 25 000-30 000 protein coding genes and the ~1 000 000 of proteins that are necessary to build complex organisms (Leipzig et al., 2004). Previous studies reported that over half of all known human genes might be alternatively spliced (Mironov et al., 1999; Graveley et al., 2001). The splice sites are generally recognized by the splicing machinery, a ribonuclear-protein complex known as spliceosome. Spliceosome binding is determined by competing activities of various auxiliary regulatory proteins, such as members of SR protein or hnRNP families, which bind specific regulatory sequences and alter the binding of the spliceosome to a particular splice site (Black, 2003; Smith et al., 2000).

However, the molecular details of how alternative splicing is linked to signal transduction are still not clear (Stamm, 2002). For example, the alternative usage of numerous exons changes during brain development. This regulatory plasticity is not confined to the development, as external cues can regulate alternative splicing in the adult brain. Numerous stimuli have been shown to regulate alternative splice site selection. For

example, neuronal activity evoked by pilocarpin (Daoud et al., 1999), kindling (Kamphuis et al., 1992), cocaine treatment (Berke et al., 2001), or pavlovian fear memory (Stork et al., 2001) change alternative splicing patterns.

The first part of this study includes the characterization of rSLM-1 and the comparison regarding their relevance to alternative splice site selection between rSLM-1 and rSLM-2. These two proteins belong to the STAR (Signal Transduction and Activation of RNA) protein family and have similar sequences and posses almost identical biochemical properties. Both proteins regulate the same alternative exons, acting on purine-rich enhancer sequences present in the alternative exons of CD44 and SMN2 (Stoss et al., 1999; Stoss et al., 2004). It was demonstrated that both proteins interact with many other splicing factors (Stoss et al., 1999; Stoss et al., 2004), including scaffold attachment factor B, the protein which is known to be a component of the transcription apparatus. Among these proteins are SRp30c, YT521-B and hnRNP G. It was also determined that both proteins associate with SLM-1, SLM-2, and Sam68. This can be explained by the fact that the GSG domain which is necessary for RNA binding is necessary for oligomerization (Chen et al., 1997). The GSG domain contains a single KH domain flanked by two regions homologous to the murine quaking gene, Qua1 and Qua2 (Vernet and Artzt, 1997). The GSG domain was demonstrated to form an RNA interaction surface.

All three proteins bind RNA, which interacts with them through specific RNA binding domains. It is very likely that unique combinations of these proteins regulate alternative splicing.

We showed that rSLM-1 and rSLM-2 regulate alternative splice site selection of the SMN2 minigene. SMN2 exon 7 was also shown to be regulated by SRp30c (Young et al., 2002) and hnRNP G (Hofmann and Wirth, 2002) proteins. Considering that all these proteins heterodimerize, we concluded that binding properties of rSLM-1 and rSLM-2 are reflected in the formation of regulatory complexes *in vivo*. Interestingly, exon regulation by rSLM-1 and rSLM-2 is very specific, as several other alternative exons, such as clathrin light chain B, exon EN (Stamm et al., 1999), or neurofilament tau exon 10 (Wang et al., 2004) were not affected.

The identification of rSLM-1 and rSLM-2 proteins leads to the assumption that these proteins serve as potential candidates to link signaling pathways to RNA metabolism. We searched for functional differences between these two proteins based on $p59^{fyn}$ phosphorylation. We showed that rSLM-1 is tyrosine phosphorylated by $p59^{fyn}$ non-receptor tyrosine kinase, whereas this tyrosine kinase had no detectable effect on rSLM-2.

The $p59^{fyn}$-mediated phosphorylation of rSLM-1 was observed by cotransfection assays. Expression of $p59^{fyn}$ abolished the rSLM-1-mediated exon repression, but had no effect on the function of rSLM-2 under the same conditions. We observed this effect both in neuronal and fibroblast cell lines, which indicates that it is an intrinsic property of rSLM-1. This shows that alternative splicing can be influenced by tyrosine kinase mediated phosphorylation.

However, the exact mechanism by which tyrosine phosphorylation changes alternative splice site selection still remains to be determined. Our laboratory demonstrated that sequestration of splicing factors could change splice site selection (Stoilov et al., 2004). Since the protein phosphorylation could be one of the important mechanisms regulating splice site selection, we decided to test how phosphorylation of splice factors mediate protein:protein interactions. Particularly, we found that rSLM-1 phosphorylation by c-abl kinase modulates its interactions with other components of the spliceosome, suggesting a phosphorylation-dependent sequestration as a likely mechanism that remains to be tested.

The $p59^{fyn}$ kinase is membrane bound. One of the mechanisms that explains how this kinase can phosphorylate the nuclear protein rSLM-1, is that rSLM-1 shuttles between the nucleus and cytoplasm in hippocampal neurons. Indeed it was shown that rSLM-1 shuttles between the nucleus and cytoplasm in Hela cells. Previous work has demonstrated that Sam68 translocates to the nucleus (Paronettoet al., 2006). However we did not observe shuttling of this protein in fibroblasts (data not shown). Another mechanism, which could be involved is that different nuclear kinases are activated by p59fyn and direct nuclear translocation of plasma-membrane integral tyrosine kinases that has been observed in several cases (Lin et al., 2001; Marti and Wells, 2000; Ni et al., 2001; Offterdinger et al., 2002; Wells and Marti, 2002). In fact, after overexpression $p59^{fyn}$ can be detected in the nucleus and endogenous $p59^{fyn}$ was observed in the nuclei of T-lymphocytes (Ley et al., 1994; Shima et al., 2001). The present work demonstrates that rSLM-1 influences splice site selection depending on its phosphorylation status that could be changed in the nucleus.

In order to answer why an organism needs to have so many homological proteins, we performed an immunolocalization analysis. Our data of protein expression in the brain revealed that both rSLM-1 and rSLM-2 are expressed in neurons (Stoss et al., 2004). The main difference was observed in the hippocampus where rSLM-1 is typical for cells of the dentate gyrus, whereas rSLM-2 is expressed in the CA1, CA3, and CA4 region, where rSLM-1 is absent. Therefore rSLM1 could serve as a cellular marker for the dentate gyrus.

Based on the knowledge that both proteins show non-overlapping expression patterns, we hypothesised that they are regulating cell type-specific splicing in the brain.

To find possible target sequences of rSLM-1 and rSLM-2, SELEX was performed. Analyzing the obtained sequences, we could not find a possible consensus sequences. Work published by Lin, 1997 suggested that a target motif for Sam68 is UAAA. We also observed from our mutation analysis on *in vivo* CD44 splicing assays that rSLM-1 binds to a purine-rich region in the regulatory element of the alternative exon. Divergent sequences analysed by the MEME informatics tool showed short motifs with random nucleotides inside. Therefore, further experiments such as CLIP are needed to identify the binding sequence.

Probably rSLM-1 changes splicing patterns in a subset of neurons, which could nicely explain why in addition to rSLM-2, a highly related protein is expressed in the brain. We propose that SLM-1 is part of a signal transduction pathway linking extracellular cues to pre-mRNA processing. Tyrosine phosphorylation-dependent alternative splicing provides a model for the observation that activation of receptor tyrosine kinases by growth factors can change alternative splicing (McKay et al., 1994; Scotet and Houssaint, 1998; Sell et al., 1994; Wang et al., 1991) and that a change in alternative splicing is often correlated with cancer caused by tyrosine kinase misregulation.

5.2. The cAMP –dependent phosphorylation of the Tra2-beta1 protein regulates splice site selection

Previous studies have demonstrated that changes in splicing were observed after various cellular signals and that alteration on splice site selection will than occur fast, within 1 hour (Stamm, 2008). Studies in different systems demonstrated that this rapid cellular process does not require protein synthesis and is often concomitant with a change in phosphorylation of splicing factors (Stamm, 2002). Phosphorylation alters the interaction between individual splicing regulatory proteins and between some of these proteins and RNA.

We identified the presence of a Protein Phosphatase 1 binding site, RVDF, in the beta4 sheet of the RRM of SR-like protein splice factor Tra2-beta1. Sequence alignment of all available species of Tra2-beta1 reveals the presence of their PP1 binding motifs and followed a downstream peptide with high evolutionary conservation, suggesting they have an important role.

It is well known that RNA recognition motifs are generally arranged in a conserved structure containing four antiparallel beta sheets connected by two alpha helices. The RNA contacts directly only the central beta1 and beta3 sheets, whereas the beta4 and beta2 sheets increase the affinity and specificity by influencing the structure (Maris et al., 2005). The RRM functions both in binding single stranded RNA and also in binding proteins (Maris et al., 2005). Detailed computational analysis of all SR and SR-like proteins shows that RRMs from 10 proteins contain the characteristic RVxF motif that docks to PP1 in their predicted beta4 sheets.

We performed a computational analysis of SR and SR-like proteins and found nine other proteins containing a PP1 binding motif, RVxF. Figure 31 (section 4.2.2) shows all the SR proteins containing the conserved RVxF motif. We hypothesize that the full conservation of the RVxF motif is connected to its functional importance. We demonstrated a direct binding of PP1 to the RRM of SR-proteins SF2/ASF and SRp30c as well as Tra2-beta1 (Novoyatleva et al., 2008). Most RVxF motifs interacting with the binding pocket of PP1 are located in an extended beta sheet formation (Egloff et al., 1997; Meiselbach et al., 2006), which suggests that the structural arrangement of the RVxF motif in RRMs allows binding to PP1.

The RRM structure of Tra2-beta has been solved and is accessible from the protein database. However, the traCR was not in this structure. On the base of known structure, we generated in collaboration with Dr. Bujnicki (Warsaw, Poland) a computer model of the traCR.

As shown in Figure 53, the region corresponding to the traCR is predicted to be unstructured, but the amino acids H(200), T(201) and P(202) are predicted to be in alpha helical confirmation. The unstructured nature of the traCR is necessary for its function, as it allows the protein to adopt various conformations that will fit to each interacting protein. Threonine 201 seems to be targeted by several kinases and resides in the only structured part of the traCR. The phosphorylation could be a mean to determine specificity to traCR ligands by forcing the traCR into a certain confirmation or by changing the affinity to binding proteins.

A

…RIRVDFSITKRPH T PTPGIYMGRP…

B

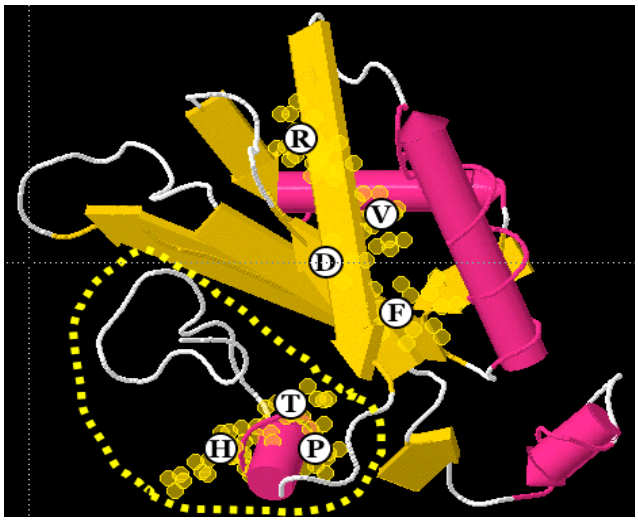


Figure 53. Sequence and structure of the traCR. A. Sequence of the traCR (residues 188-211). Phylogenetically fully conserved residues are indicated in orange. The RVDF motif is underlined and marked in blue. T201 that is phosphorylated is marked in red. B. Predicted structure of the traCR. The model shows amino acids 101-211 of Tra2-beta1 (MSTRR…TYGSS), which represent the RRM and its flanking regions. Residues of the RVDF (PP1 binding site) are indicated as halos and marked. The region corresponding to the traCR is predicted to be unstructured, however the residues HTP are predicted to be in an α-helical conformation and are indicated in the model. The threonine in this model is phosphorylated.

We tested therefore the traCR functionally by generating a series of mutants, in which each single amino acid was replaced. Since none of these mutants affected binding to PP1 it is unlikely that these region controls PP1 binding (section 4.2.3).

Previous work showed that Tra2-beta1 is activated by CLK2 kinase, which promotes the formation of Tra2-beta3 isoform in the brain. We screened the traCR for the possible action of multiple kinases. We found that Cdc2, CK1 and GSK3 beta can phosphorylate Tra2-beta1 in vitro (section 4.2.4.). GSK3-beta and, to a less degree, CK1 can directly bind to Tra2-beta1 (section 4.2.4, Figure 38B). The sequences S(P)-X-X-(S/T), located in the RS2 domain of Tra2-beta correspond to a CK1 phosphorylation site. It is also well known, that CK1 is first prephosphorylated by GSK3-beta. GSK3-beta phosphorylates the SR protein SC35, resulting in increased skipping of Tau exon 10 (Hernandez et al., 2004). The family of Cdc2-like nuclear kinases acts on already phosphorylated SR proteins (Misteli et al., 1998).

Previous work demonstrated that human Tra2-beta1 can stimulate exon 7 usage of the SMN gene. The homozygous loss of SMN1 gene is a cause of the neurodegenerative

diseases, spinal muscular atrophy. Reversible phosphorylation of Tra2-beta1 at serine/threonine residues have an important impact on the splice site selection: blocking the dephosphorylation with PP1 inhibitors (cantharidine, tautomycin) stimulates exon 7 inclusion and promotes SMN full length protein production in patient fibroblasts (Novoyatleva et al., 2008).

We therefore suggested a predicted mechanism of regulation of SMN exon 7 through PP1-dependent phosphorylation (Figure 54).

A central link in this model is DARPP-32, an inhibitor of PP1. Elevation of cAMP level by forskolin leads to activation of PKA, which phosphorylates DARPP-32 at T34.

In order to determine whether DARPP-32 and Tra2-beta1 can interact directly, *in vivo* and *in vitro* analyses were performed. Both systems demonstrated strong binding between DARPP-32 and Tra2-beta1 (section 4.2.6, 4.2.7). Moreover, *in vitro* binding assays showed competition between DARPP-32 and PP1 for binding to Tra2-beta1 (section 4.2.8., Figure 44).

SR proteins possess a C-terminal region rich in serine/arginine dipeptides that is target of extensive phosphorylation and has been linked to splicing activity (Graveley et al., 2000). Using mutants of Tra2-beta1 with deletion of different RS domains in the sequences, we could show that DARPP-32 binds to the second RS domain, which is located closer to the traCR.

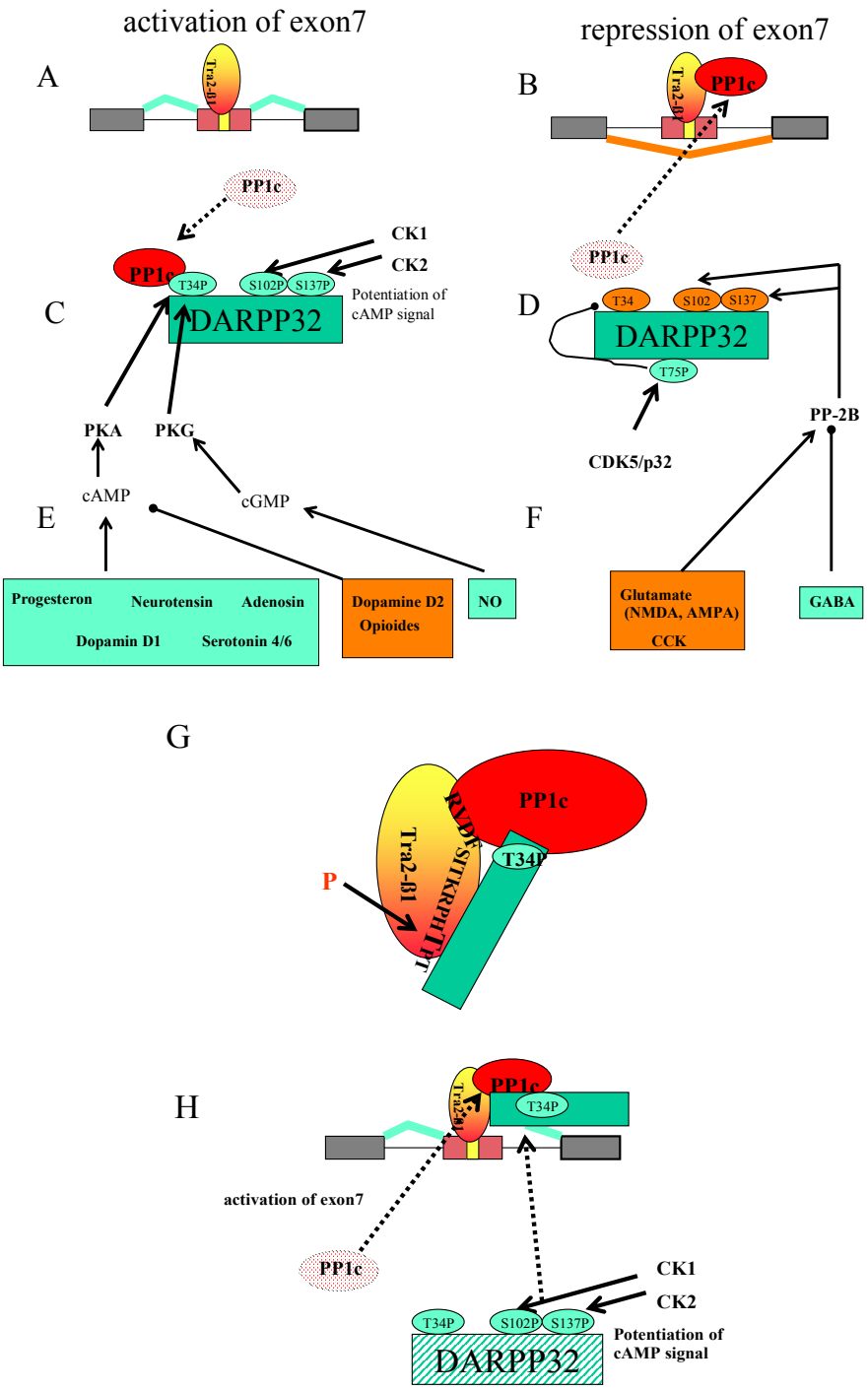


Figure 54. Working hypothesis of complex interaction between Tra2-beta1, DARPP-32 and PP1, and its action on SMN2 gene. A. Tra2-beta1 binds to the core splicing enhancer of SMN2 exon 7 and promotes its inclusion by recruiting or stabilizing an exon-recognition complex. B. Protein phosphatase 1 can bind to and dephosphorylate Tra2-beta1, leading to changes of the exon-recognition complex and causing exon 7 skipping. C. PP1 is inactivated through DARPP-32 after its phosphorylation of Threonine 34. T34 is phosphorylated by Protein Kinase A. This inhibition is potentiated by phosphorylation of Serine 102 and Serine 137 by Casein Kinase 1 and 2. D. Release of PP1 is achieved by blocking the phosphorylation of T34 by PKA. This blockage is mediated by phosphorylation of T75 through CDK5/p32, which converts DARPP-32 to a strong inhibitor of PKA. The binding between PP1 and DARPP-32 is further blocked by dephosphorylation of S102 and S 137 mediated by Protein phosphatase-2B. E. Activation of the receptors for progesterone, neurotensin, adenosine, dopamine and serotonine activate PKA through elevation of cAMP.

Similarly, NO activates Protein Kinase G through cGMP. Activation of these receptors is predicted to cause exon 7 inclusion. Activation of the Dopamine D2 and opioid receptors inhibits binding of DARPP-32 to PP1 through a decrease of the cAMP level, which is predicted to cause exon 7 skipping. F. Glutamate acts through NMDA and AMPA receptors and cholecystokinin (CCK) activates PP-2B, which dephosphorylates T34, S102 and S137. GABA inhibits PP-2B. G. A newly found trimeric complex, which can interact with each other when DARPP-32 is phosphorylated on T34 and Tra2-beta1 is phosphorylated on T201. H. The association of DARPP-32 with Tra2-beta1/PP1 complex influences splice site selection. Components, marked in green boxes would promote exon 7 inclusion; substances, marked in orange boxes would inhibit exon 7 inclusion. The phosphorylation states that activate exon 7 inclusion are indicated in green, the ones repressing are indicated in orange.

However, we could not identify binding between Tra2-beta1 and DARPP-32 using yeast two-hybrid screens. This could reflect different phosphorylation requirements in yeast and mammalian systems.

We observed a negative feedback when we tested DARPP-32 and its mutants in *in vivo* splicing assay, when it was co-transfected together with the CDK5 kinase. This could be due to phosphorylation of DARPP-32 by CDK5, which converts DARPP-32 to its intact form (Figure 54).

In addition, our gel shift analysis suggests, that DARPP-32 antagonize the binding of Tra2-beta1 on RNA (section 4.2.12).

In order to determine whether DARPP-32 activity can influence splice site selection, we performed a number of *in vivo* splicing assays. In most cases analyzed, we found that DARPP-32 changes alternative exon usage. In the experiments where DARPP-32 or its mutants were transfected (section 4.2.9), the promotion of alternative exon skipping was observed. In addition, DARPP-32 can activate splicing of Tra-dependent DUP construct in *in vitro* conditions (section 4.2.10).

Taken together, the interaction between human Tra2-beta1 and DARPP-32 indicates a previously unknown property of Tra2-beta1 in the cAMP-dependent regulation of splice site selection.

The existing diversity of regulatory mechanisms raised an obvious question: why did an organism develop so many routes to control exon inclusion. One possibility is that the most efficient mechanism for the regulation of any particular gene is dependent on the specific rate-limiting step in spliceosome assembly and catalysis for that gene (House, 2008).

5.3. Inhibitors of PP1 as a model approach for SMA treatment

Wrong splice site usage has been observed in numerous diseases, among them Alzheimer's disease (Glatz et al., 2006), breast cancer (Watermann et al., 2006) and spinal muscular atrophy (Hofmann et al., 2000). Therefore, the correction of improper splicing is an important goal in the developing of molecular therapeutic strategies.

One neurodegenerative disorder, in which the molecular pathogenesis is well characterized and that has a clear genetic basis involving loss of function is spinal muscular atrophy. One of the possible therapeutic strategies for treatment of SMA would be specific activation of exon 7 inclusion in SMN2 transcripts. Recently it was established that hTra2-beta1 binds to the degenerate RNA sequence GHVVGANR. This motif is found more frequently in exons than in introns (Stoilov et al., 2004). This sequence is a part of the splicing enhancer of SMN2 exon 7, where it mediates Tra2-beta1-dependent inclusion. The protein promotes the inclusion of SMN exon 7, which subsequently stimulates full-length SMN2 expression (Hofmann et al., 2000). Therefore, specific agents that could potentially release hTra2-beta1 from its nuclear storage sites and promote high levels of SMN protein expression might be a therapeutic approach for SMA. Thus we suggested that the modification of Tra2-beta1 reversible phosphorylation by PP1 would have impact on Tra2-beta1 dependent exon inclusion.

Earlier we tested the PP1 inhibitor tautomycin in patient cells and found that treatment with this inhibitor promoted inclusion of exon 7 and caused the formation of SMN protein (Novoyatleva et al., 2008). This proves that modulation of phosphatase activity can be used to alter alternative splice site selection. However, in this case, PP1 inhibition causes a change in numerous exons.

Some PP1 inhibitors (tautomycin and cantharidin) were tested on transgenic mice in collaboration with Prof. Dr. Arthur Burghes (USA). Figure 55 demonstrates the accumulation of SMN protein in the spinal cord of SMN2 transgenic mice after treatment.

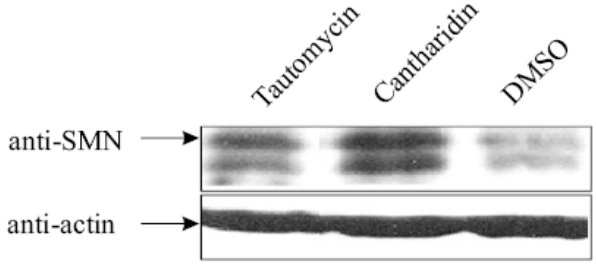


Figure 55. The PP1 inhibitors tautomycin and cantharidin promote the accumulation of SMN protein in transgenic mice. The mice (line 89) homozygous for Smn contain two copies of SMN2 (Smn–/–; SMN2) (Monani et al., 2000).

Similar results were observed in liver and forebrain extracts from these treated mice. Semiquantitative analysis of Western Blots showed that tautomycin increases SMN protein by ~65%, cantharidin by ~1.3-fold and forskolin by 2.3-fold (Novoyatleva et al., 2008).

Based on these results we suggest that these compounds could further be used for establishing new therapeutical approaches. The use of synthetic PNA allows the efficient delivery of targeting molecules to the appropriate target regions of the nervous system.

Using cantharidine as a precursor, we synthesized several classes of its derivatives. Preliminary data of *in vivo* and *in vitro* analysis of these compounds showed less effect comparative to the cantharidine itself. However, the more stable chemical structure of norcantharidine amide derivatives could be used for the next coupling of PP1 inhibitor to nucleic acids, similar to chimeras between RS domains and antisense oligonucleotides (Cartegni and Krainer, 2003).

It is known that placing mismatches or mutations within double-stranded siRNAs can attenuate or abolish their ability to silence their mRNA targets effectively. This direction also could be used for further investigation and development perspective approaches for disease treatment.

This research was performed to understand the mechanisms by which signal transduction pathways could be linked to change splice site selection.

The results of the first part of the dissertation provide evidence that tyrosine phosphorylation of the splicing regulatory protein rSLM-1 can change splice site selection, abolishing the normal 1.5- to 2-fold stimulate of SMN2 exon 7 usage. Effects of comparable magnitude (1.5 to 4 fold) were observed by many other researchers (Matter et al., 2002; Abdennebi et al., 2002; Holdiman et al., 2002; Meshorer et al., 2002; Stamm, 2002).

The data of the second part of the thesis demonstrate the novel interaction between Tra2-beta1 splicing factor and DARPP-32 indicating previously unknown properties of both proteins in the cAMP-dependent regulation of splice site selection.

Taken together, our findings suggest a new branch in the multiple mechanisms of alternative splicing regulation that involves a combination of reversible phosphorylation and dopaminergic signaling, allowing the convergence of multiple regulatory signals on splice site selection.

6. References

Amrein, H., Hedley, M.L., and Maniatis, T. (1994). The role of specific protein-RNA and protein-protein interactions in positive and negative control of pre-mRNA splicing by Transformer 2. Cell, 76, 735-746.

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W. and Lipman, D.J. (1990). Basic Local Alignment Search Tool. Journal of Molecular Biology, 215, 403-410.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res., 25, 3389-3402.

Andreassen, P.R., Lacroix, F.B., Villa-Moruzzi, E., and Margolis, R.L. (1998). Differential subcellular localization of protein phosphatase-1α, γ1 and δ isoforms during both interphase and mitosis in mammalian cells. J. Cell Biol., 141, 1207-1215.

Asparuhova, M., Kole, R., Schumperli, D. (2004). Antisense derivatives of U7 and other small nuclear RNAs as tools to modify pre-mRNA splicing patterns. Gene Ther. Regulation, 2, 321-349.

Ayllón, V., Martinez-A., C., Garcia, A., Cayla, X. and Rebollo, A. (2000). Protein phosphatase 1 is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. EMBO J., 19, 2237-2246.

Ayllón, V., Cayla, X., Garcia, A., Roncal, F., Fernandez, R., Albar, J. P.,Martinez-A., C. and Rebollo, A. (2001). Bcl-2 targets protein phosphatase 1 alpha to bad. J. Immunol., 166, 7345-7352.

Barlat, I., Maurier, F., Duchesne, M., Guitard, E., Tocque, B., Schweighoffer, F. (1996). A role for Sam68 in cell cycle progression antagonized by a spliced variant within the KH domain. J. Biol. Chem., 272, 3129-3132.

Beil, B., Screaton, G. and Stamm, S. (1997). Molecular cloning of htra2-beta-1 and htra2-beta-2, two human homologs of tra-2 generated by alternative splicing. DNA Cell Biol., 16, 679-690.

Berke, J.D., Sgambato, V., Zhu, P.P., Lavoie, B., Vincent, M., Krause, M., Hyman, S.E. (2001). Dopamine and glutamate induce distinct striatal splice forms of Ania-6, an RNA polymerase II-associated cyclin. Neuron, 32, 277-287.

Beullens, M. and Bollen, M. (2002). The protein phosphatase-1 regulator NIPP1 is also a splicing factor involved in a late step of spliceosome assembly. J. Biol. Chem., 277, 19855-60.

Bibb, J.A., Snyder, G.L., Nishi, A., Yan, Z., Meijer, L., Fienberg, A.A., Tsai, L.H., Kwon Y.T., Girault J.A., Czernik A.J. (1999). Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. Nature., 669-671.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res., 7, 1513-1523.

Black, D.L. (1995). Finding splice sites within a wilderness of RNA. RNA, 1, 763–771.

Black, D.L. (2003). Mechanisms of alternative pre-messenger RNA splicing. Annu. Rev. Biochem., 72, 291-336.

Blanchette, M. and Chabot, B. (1999). Modulation of exon skipping by high-affinity hnRNP A1-binding sites and by intron elements that repress splice site utilization. EMBO J., 1, 18(7), 1939-52.

Blencowe, B.J. (2006). Alternative splicing: new insights from global analyses. Cell, 126(1):37-47.

Blencowe, B.J., Nickerson, J.A., Issner, R., Penman, S., and Sharp, P.A. (1994). Association of nuclear matrix antigens with exon-containing splicing complexes. J. Cell Biol., 127(3), 593-607.

Bollen, M. Combinatorial control of protein phosphatase-1. (2001). TIBS., 26, 426-431.

Boukis, L.A., Liu, N., Furuyama, S. and Bruzik, J.P. (2004). Ser/Arg-rich protein-mediated communication between U1 and U2 small nuclear ribonucleoprotein particles. J. Biol. Chem., 279, 29647-29653.

Bullock, W.O., Fernandez, J.M. and Short, J.M. (1987). XL1-Blue: A high efficiency plasmid transforming recA Escherichia coli strain with beta-galactosidase selection. Biotechniques, 5, 376-378.

Burge, C.B., Tuschl, T., and Sharp, P.A. (1999). Splicing of precursors to mRNAs by the spliceosomes. In The RNA world (ed. R.F. Gesteland, et al.) Cold Spring Harbor Press, Cold Spring Harbor, NY., 525-560.

Burset, M., Seledtsov, I.A., Solovyev, V.V. (2001). SpliceDB: database of canonical and non-canonical mammalian splice sites. Nucleic Acids Res., 29(1), 255-259.

Bourgeois, C., Lejeune, F., Stevenin, J. (2004). Broad specificity of SR (serine/arginine) proteins in the regulation of alternative splicing of pre-messenger RNA. Prog Nucleic Acid Res Mol Biol. 78:37-88.

Boudrez, A., Buellens, M., Groenen, P., Van Eynde, A., Vulsteke, V., Jagiello, I., Murray, M., Krainer, A.R., Stalmans, W. and Bollen, M. (2000). NIPP1-mediated interaction of protein phosphatase-1 with CDC5L, a regulator of pre-mRNA splicing and mitotic entry. J. Biol. Chem., 275, 25411-25417.

Bordelon, J.R., Smith, Y., Nairn, A.C., Colbran, R.J., Greengard, P. and Muly, E.C. (2005). Differential localization of protein phosphatase-1alpha, beta and gamma1 isoforms in primate prefrontal cortex. Cereb. Cortex, 15, 1928-1937.

Blencowe, B.J. (2000). Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. Trends Biochem. Science, 25, 106-110.

Brichta, L., Hofmann, Y., Hahnen, E., Siebzehnrubl, F.A., Raschke, H., Blumcke, I., Eyupoglu, I.Y., Wirth, B. (2003). Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. Hum. Mol. Genet., 12, 2481-2489.

Brown, S.A., Ripperger, J., Kadener, S., Fleury-Olela, F., Vilbois, F., Rosbash, M., and Schibler, U. (2005). PERIOD1-associated proteins modulate the negative limb of the mammalian circadian oscillator. Science, 308, 693-696.

Brzustowicz, L.M., Lehner, T., Castilla, L.H., Penchaszadeh, G.K., Wilhelmsen, K.C., Daniels, R., Davies, K.E., Leppert, M., Ziter, F., Wood, D., et al. (1990). Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. Nature, 344(6266), 540-551.

Caceres, J.F., Misteli, T., Screaton, G.R., Spector, D.L. and Krainer, A.R. (1997). Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. J. Cell Biol., 138, 225-238.

Caceres, J., Stamm S., Helfman D.M., and Krainer, A.R. (1994). Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. Science, 265, 1706-1709.

Caceres, J.F., Screaton, G.R. and Krainer, A.R. (1998). A specific subset of SR proteins shuttles continuously between the nucleus and the cytoplasm. Genes Dev., 12, 55-66.

Cao, W., Jamison, S.F. and Garcia-Blanco, M.A. (1997). Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro. RNA, 3, 1456-1467.

Caceres, J.F. and Kornblihtt, A.R. (2002). Alternative splicing: multiple control mechanisms and involvement in human disease. Trends Genet., 18, 186-193.

Caputi, M., Mayeda, A., Krainer, A.R., and Zahler, A.M. (1999). hnRNP A/B proteins are required for inhibition of HIV-1 pre-mRNA splicing. EMBO J., 18(14), 4060-4067.

Carlo, T., Sterner, D.A., Berget, S.M. (1996). An intron splicing enhancer containing a G-rich repeat facilitates inclusion of a vertebrate micro-exon. RNA, 2, 342-53.

Cartegni, L., Chew, S.L. and Krainer, A.R. (2002). Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat. Rev. Genet., 3, 285-298.

Cartegni, L. and Krainer, A.R. (2002). Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. Nat. Genet., 30, 377-384.

Cartegni, L. and Krainer, A.R. (2003). Correction of disease-associated exon skipping by synthetic exon-specific activators. Nat. Struct. Biol., 10, 120-125.

Cartegni, L., Wang, J., Zhu, Z., Zhang, M.Q. and Krainer A.R. (2003). ESEfinder: a web resource to identify exonic splicing enhancers, Nucleic Acid Research, 31(13), 3568-7.

Cartegni, L., Hastings, M.L., Calarco, J.A., de Stanchina, E., Krainer, A.R. (2006). Determinants of exon 7 splicing in the spinal muscular atrophy genes, SMN1 and SMN2. Am. J. Hum.Genet., 78(1), 63-77.

Carty, S.M., Goldstrohm, A.C., Sune, C., Garcia-Blanco, M.A. and. Greenleaf, A.L. (2000). Protein-interaction modules that organize nuclear function: FF domains of CA150 bind the phosphoCTD of RNA polymerase II. PNAS USA, 97, 9015-9020.

Celotto, A., Lee, J., Graveley, B.(2005). Exon-specific RNA interference: a tool to determine the functional relevance of proteins encoded by alternatively spliced mRNAs. Methods Mol Biol. 309:273-82

Celotto, A.M. and Graveley, B.R. (2002). Exon-specific RNAi: a tool for dissecting the functional relevance of alternative splicing. RNA, 8, 718-724.

Ceulemans, H., Stalmans, W., and Bollen, M. (2002). Regulator-driven functional diversification of protein phosphatase-1 in eukaryotic evolution. Bioessays, 24, 371-381.

Chan, R.C., and Black, D.L. (1997). The polypyrimidine tract binding protein binds upstream of neural cell- specific c-src exon N1 to repress the splicing of the intron downstream. Mol. Cell Biol., 17, 4667-4676.

Chang, J.G., Hsieh-Li, H.M., Jong, Y.J., Wang, N.M., Tsai, C.H., Li, H. (2001). Treatment of spinal muscular atrophy by sodium butyrate. PNAS USA, 98, 9808-9813.

Chao, H., Mansfield, S.G., Bartel, R.C., Hiriyanna, S., Mitchell, L.G., Garcia-Blanco, M.A., Walsh, C.E. (2003). Phenotype correction of hemophilia A mice by spliceosome-mediated RNA trans-splicing. Nat. Med., 9, 1015-9.

Chalfant, C.E., Mischak, H., Watson, J.E., Winkler, B.C., Goodnight, J., Farese, R.V., and Cooper, D.R. (1995). Regulation of alternative splicing of protein kinase C beta by insulin. J. Biol. Chem., 270, 13326-13332.

Charroux B., Pellizzoni L., Perkinson R.A., Yong J. (2000). Gemin 4 A novel component of the SN complex that is found in both gems and nucleoli. J. Cell. Biol., 148, 1177-1186.

Chen, C. and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell Biol., 7, 2745-2752.

Chen, T., Boisvert, F-M., Bazett-Jones, D.P., Richard, S. (1999). A role for the GSG domain in localizing Sam68 to novel nuclear structures in cancer cell lines. Mol. Biol. Cell, 10, 3015-3033.

Chen, W., Kubota, S., Teramoto, T., Nishimura, Y., Yonemoto, K., Seyama, Y. (1998). Silent nucleotide substitution in the sterol 27-hydroxylase gene (CYP 27) leads to alternative pre-mRNA splicing by activating a cryptic 5' splice site at the mutant codon in cerebrotendinous xanthomatosis patients. Biochemistry, 37, 4420-4428.

Chen, T., Damaj, B., Herrerra, C., Lasko, P., Richard, S. (1997). Mol. Cell Biol., 17, 5707-5718.

Ciccarone, V.C., Polayes, D., and Luckow V.A. (1997). Generation of Recombinant Baculovirus DNA in E. coli Using Baculovirus Shuttle Vector, Volume 13, U. Reischt, ed. (Totowa, NJ: Humana Press Inc.).

Clark, L.N., Poorkaj, P., Wszolek, Z., Geschwind, D.H., Nasreddine, Z.S., Miller, B., Li, D., Payami, H., Awert, F., Markopoulou, K., Andreadis, A., D'Souza, I., Lee, V.M., Reed, L., Trojanowski, J.Q., Zhukareva, V., Bird, T., Schellenberg, G., Wilhelmsen, K.C. (1998). Pathogenic implications of mutations in the tau gene in pallido-ponto-nigral degeneration and related neurodegenerative disorders linked to chromosome 17. PNAS USA, 95, 13103-13107.

Clark J., Lu Y. J., Sidhar S. K., Parker C., Gill S., Smedley D., Hamoudi R., Linehan W. M., Shipley J., and Cooper C.S. (1997). Fusion of splicing factor genes PSF and NonO (p54nrb) to the TFE3 gene in papillary renal cell carcinoma. Oncogene, 15, 2233-2239.

Cline, T.W. and Meyer, B.J. (1996). Vive la diff´erence: males vs females in flies vs worms. Annu. Rev. Genet., 30, 637-702.

Cohen, P.T.W. (1988). Two isoforms of protein phosphatase 1 may be produced from the same gene. FEBS Lett., 232, 17-23.

Cohen, P.T.W. (1997). Novel protein serine/threonine phosphatases: variety is the spice of life Trends Biochem. Science, 22, 245-251.

Cote, J., Boisvert, F.M., Boulanger, M.C., Bedford, M.T., Richard, S. (2003). Sam68 RNA binding protein is an in vivo substrate for protein arginine N-methyltransferase 1. Mol. Biol. Cell., 14, 274-87.

Coovert, D.D., Le, T.T., McAndrew, P.E., Strasswimmer, J., Crawford, T.O., Mendell, J.R., Coulson, S.E., Androphy, E.J., Prior, T.W. and Burghes, A.H. (1997). The survival motor neuron protein in spinal muscular atrophy. Hum. Mol. Genet., 6, 1205-1214.

Coyle, J.H., Guzik, B.W., Bor, Y.C., Jin, L., Eisner-Smerage, L., Taylor, S.J., Rekosh, D., Hammarskjold, M.L. (2003). Sam68 enhances the cytoplasmic utilization of intron-containing RNA and is functionally regulated by the nuclear kinase Sik/BRK. Mol. Cell. Biol., 23, 92-103.

Daoud, R., Da Penha Berzaghi, M., Siedler, F., Hubener, M. and Stamm, S. (1999). Activity-dependent regulation of alternative splicing patterns in the rat brain. Eur. J. Neurosci., 11, 788-802.

D'Souza, I., Poorkaj, P., Hong, M., Nochlin, D., Lee, V.M.-Y., Bird, T.D., Schellenberg, G. D. (1999). Missense and silent tau gene mutations cause frontotemporal dementia with Parkinsonism-chromosome 17 type, by affecting multiple alternative RNA splicing regulatory elements. PNAS USA, 96, 5598-5603.

Dauwalder, B., Amaya-Manzanares, F. and Mattox, W. (1996). A human homologue of the Drosophila sex determination factor transformer-2 has conserved splicing regulatory functions. PNAS USA, 93, 9004-9009.

Debnath, J., Chamorro, M., Czar, M.J., Schaeffer, E.M., Lenardo, M.J., Varmus, H.E. and Schwartzberg, P.L. (1999). rlk/TXK Encodes Two Forms of a Novel Cysteine String Tyrosine Kinase Activated by Src Family Kinases. Mol. Cell. Biol., 19, 1498-1507.

De Meirleir, L., Lissens, W., Benelli, C., Ponsot, G., Desguerre, I., Marsac, C., Rodriguez, D., Saudubray, J.M., Poggi, F., Liebaers, I. (1994). Aberrant splicing of exon 6 in the pyruvate dehydrogenase-E1 alpha mRNA linked to a silent mutation in a large family with Leigh's encephalomyelopathy. Pediatr. Res., 36, 707-712.

Derry, J.J., Richard, S., Valderrama Carvajal, H., Ye, X., Vasioukhin, V., Cochrane, A.W., Chen, T. and Tyner, A.L. (2000) Sik (BRK) Phosphorylates Sam68 in the Nucleus and Negatively Regulates Its RNA Binding Ability. Mol. Cell. Biol., 20, 6114-6126.

Desdouits, F., Cohen, D., Nairn, A., Greengard, P., Girault, J.A. (1995). Phosphorylation of DARPP-32, a dopamine- and cAMP-regulated phosphoprotein, by casein kinase I in vitro and in vivo. J Biol Chem. 14;270(15):8772-8.

Desdouits, F., Siciliano, J., Greengard, P., Girault, J.A. (1995). Dopamine- and cAMP-regulated phosphoprotein DARPP-32: phosphorylation of Ser-137 by casein kinase I inhibits dephosphorylation of Thr-34 by calcineurin. Proc Natl Acad Sci U S A. 28;92(7):2682-5

Di Fruscio, M., Chen, T., Richard, S. (1999). Characterization of Sam68-like mammalian proteins SLM-1 and SLM-2: SLM-1 is a Src substrate during mitosis. PNAS USA, 96, 2710-2715.

Doherty, M. J., Moorhead, G., Morrice, N., Cohen, P. and Cohen, P.T.W. (1995). Amino acid sequence and expression of the hepatic glycogen binding (GL) subunit of protein phosphatase-1. FEBS Lett., 375, 284-298.

Dombrádi, V. (1997). Comparative analysis of Ser/Thr protein phosphatases. Trends Comparative Biochem. Physiol., 3, 23-48.

Dombrádi, V., Axton, J. M., Brewis, N. D., da Cruze Silva, E.F., Alphey, L. and Cohen, P. T. W. (1990). Drosophila contains three genes that encode distinct isoforms of protein phosphatase 1. Eur. J. Biochem., 194, 739-745.

Dong, B., Horowitz, D.S., Kobayashi, R., and Krainer, A.R. (1993). Purification and cDNA cloning of HeLa cell p54nrb, a nuclear protein with two RNA recognition motifs and extensive homology to human splicing factor PSF and Drosophila NONA/BJ6. Nucleic Acids Res., 21, 4085-4092.

Dreyfuss, G., Matunis, M.J., Pinol-Roma, S., Burd, C.G. (1993). hnRNP proteins and the biogenesis of mRNA. Annu. Rev. Biochem., 62, 289-321.

Du, C., McGuffin, E., Dauwalder, B., Rabinow, L. and Mattox, W. (1998). Protein phosphorylation plays an essential role in the regulation of alternative splicing and sex determination in drosophila. Mol. Cell, 2, 741-750.

Du, H. and Rosbash, M. (2002). The U1 snRNP protein U1C recognizes the 5' splice site in the absence of base pairing. Nature, 419, 86-90.

Duncan, P.I., Stojkl, D.F., Marius, R.M. and Bell, J.C. (1997). In vivo regulation of alternative premRNA splicing by the Clk1 protein kinase. Mol. Cell. Biol., 17, 5996-6001.

Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H. and Elledge, S.J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev., 7, 555-569.

Egloff, M.P., Johnson, D.F., Moorhead, G., Cohen, P.T., Cohen, P., and Barford, D. (1997). Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. EMBO J., 16, 1876-1887.

Eperon, I.C., Makarova, O.V., Mayeda, A., Munroe, S.H., Caceres, J.F., Hayward, D.G. and Krainer, A.R. (2000). Selection of Alternative 5' Splice Sites: Role of U1 snRNP and Models for the Antagonistic Effects of SF2/ASF and hnRNP A1. Mol. Cell. Biol., 20, 8303-8318.

Faustino, N.A. and Cooper, T.A. (2003). Pre-mRNA splicing and human disease. Genes Dev., 17(4), 419-37.

Fehlbaum, P., Guihal, C., Bracco, L. and Cochet, O. (2005). A microarray configuration to quantify expression levels and relative abundance of splice variants. Nucleic Acids Res., 33, 47.

Feng, Z.H., Wilson, S.E., Peng, Z.Y., Schlender, K.K., Reimann, E.M. and Trumbly, R.J. (1991). The yeast GLC7 gene required for glycogen accumulation encodes a type 1 protein phosphatase. J. Biol. Chem., 266, 23796-23801.

Ferrari, S., Giliani, S., Insalaco, A., Al-Ghonaium, A., Soresina, A.R., Loubse,r M., Avanzini, M.A., Marconi, M., Badolato, R., Ugazio, A.G., Levy Y., Catalan, N., Durandy, A., Tbakhi, A., Notarangelo L.D., Plebani A. (2001). Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. PNAS USA, 98, 12614-12619.

Fienberg, A.A. and Greengard, P. (2000). The DARP-32 knockout mouse. Brain Research Reviews, 31, 313-319.

Fisher, U., Liu, Q. and Dreyfuss, G. (1997). The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. Cell, 90(6), 1023-9.

Fiscone, G., Aperia, A., Snyder, G.L. and Greengard, P. (1998). ATPase phosphorylation in the choroid plexus: synergistic regulation by serotonin/protein kinase C and isproterenol/cAMP-PK/PP-1 pathways. Mol..Med., 4, 258-265.

Flores-Hernandez, J., Hernandez, S., Snyder, G.L., Yan, Z., Feinberg, A.A., Moss, S.J., Greengard, P. and Surmeier, D.J. (2000). D1 dopamine receptor activation reduces GABAA receptor currents in neostriatal neurons through a PKA/DARPP-32/PP1 signalling cascade. J. Neurophysiol., 83, 2996-3004.

Fu, X.D. (1995). The superfamily of arginine/serine-rich splicing factors. RNA, 1, 663-680.

Gallego, M.E., Gattoni, R., Stevenin, J., Marie, J. and Expert-Bezancon, A. (1997). The SR splicing factors ASF/SF2 and SC35 have antagonistic effects on intronic enhancer-dependent splicing of the beta-tropomyosin alternative exon 6A. EMBO J., 16, 1772-1784.

Gao, Q.S., Memmott, J., Lafyatis, R., Stamm, S., Screaton, G. and Andreadis A. (2000). Complex Regulation of Tau Exon 10, Whose Missplicing Causes Frontotemporal Dementia. J. Neurochem., 74, 490-500.

Garcia-Blanco, M.A., Baraniak, A.P., Lasda, E.L. (2004). Alternative splicing in disease and therapy. Nat. Biotechnol., 22, 535-546.

Garcia-Blanco, M.A., Jamison, S.F., Sharp, P.A. (1989). Identification and purification of a 62,000-dalton protein that binds specifically to the polypyrimidine tract of introns. Genes Dev., 3, 1874-1886.

Ge, H., Si, Y. and Wolffe, A.P. (1998). A novel transcriptional coactivator, p52, functionally interacts with the essential splicing factor ASF/SF2. Mol. Cell, 2, 751-759.

Gebauer, F., Merendino, L., Hentze, M., Valcarcel, J. (1998). The Drosophila splicing regulator Sex-lethal directly inhibits translation of male-specific lethal 2 mRNA. RNA, 4, 142-50.

Glatz, D., Rujescu, D. et al. (2006). The alternative splicing of tau exon 10 and its regulatory proteins CLK2 and TRA2-BETA1 changes in sporadic Alzheimer's disease. J Neurochem 96(3): 635-44.

Goldber, J., Huan, H.B., Kwon, Y.G., Greengard, P., Nairn, A.C., Kuriyan, J. (1995). Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature, 376, 745-753.

Goldstrohm, A.C., Albrecht, T.R., Sune, C., Bedford, M.T. and Garcia-Blanco, M.A. (2001). The transcription elongation factor CA150 interacts with RNA polymerase II and the pre-mRNA splicing factor SF1. Mol. Cell. Biol., 21**,** 7617-7628.

Goren, A., Ram, O., Amit, M., Keren, H., Lev-Maor, G., Vig, I., Pupko, T. and Ast, G. (2006). Comparative analysis identifies exonic splicing regulatory sequences-the complex definition of enhancers and silencers. Mol. Cell, 22(6), 769-81.

Graveley, B.R. (2000). Sorting out the complexity of SR protein functions. RNA, 6, 1197-1211.

Greengard, P., Nairn, A.C., Girault, J.A., Ouimet, C.C., Snyder, G.L., Fisone, G., Allen, P.B., Fienberg, A.A. and Nishi, A. (1998). The DARPP-32 protein phosphatase-1 cascade: a model for signal integration. Brain Research Reviews, 274-284.

Grossman, J.S., Meyer, M.I., Wang, Y.C., Mulligan, G.J., Kobayashi, R., Helfman, D.M., (1998). The use of antibodies to the polypyrimidine tract binding protein (PTB) to analyze the protein components that assemble on alternatively spliced pre-mRNAs that use distant branch points. RNA, 4, 613-625.

Guthrie, C. (1991). Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. Science, 253, 157-163.

Gu, Y.H., Kodama, H., Murata, Y., Mochizuki, D., Yanagawa, Y., Ushijima, H., Shiba, T., Lee, C.C. (2001). ATP7A gene mutations in 16 patients with Menkes disease and a patient with occipital horn syndrome. Am. J. Med. Genet., 99, 217-222.

Irimia, M., Rukov, J., Penny, D., Roy, S. (2007). Functional and evolutionary analysis of alternatively spliced genes is consistent with an early eukaryotic origin of alternative splicing. BMC Evol Biol. 7:188

Iijima, M., Tabira, T., Poorkaj, P., Schellenberg, G.D., Trojanowski, J.Q., Lee, V.M., Schmidt, M.L., Takahashi, K., Nabika, T., Matsumoto, T., Yamashita, Y., Yoshioka, S., Ishino, H. (1999). A distinct familial presenile dementia with a novel missense mutation in the tau gene. Neuroreport, 10, 497-501.

Haegebarth, A., Heap, D., Bie, W., Derry, J.J., Richard, S. and Tyner, A.L. (2004). The Nuclear Tyrosine Kinase BRK/Sik Phosphorylates and Inhibits the RNA-binding Activities of the Sam68-like Mammalian Proteins SLM-1 and SLM-2. J. Biol. Chem., 279, 54398-54404.

Hall, K.U., Collins, S.P., Gamm, D.M., Massa, E., DePaoli-Roach, A.A. and Uhler, M.D. (1999). Phosphorylation-dependent inhibition of protein-1 by G-substrate. J. Biol. Chem., 274, 3485-3495.

Halpain, S., Girault, J.-A. and Greengard, P. (1990). Activation of NMDA receptors induces dephosphorylation of DARPP-32 in rat striatal slices. Nature, 343, 369-372.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol., 166, 557.

Hao, Q.L., Ferris, D.K., White, G., Heisterkamp, N. and Groffen, J. (1991). Nuclear and cytoplasmic location of the FER tyrosine kinase. Mol. Cell Biol., 11, 1180-1183.

Hasegawa, Y., Kawame, H., Ida, H., Ohashi, T., Eto, Y. (1994). Single exon mutation in arylsulfatase A gene has two effects: loss of enzyme activity and aberrant splicing. Hum. Genet., 93, 415-420.

Hastings, M.L. and Krainer, A.R. (2001). Pre-mRNA splicing in the new millennium. Curr. Opin. Cell. Biol., 13, 302-309.

Heinrichs, V., Ryner, L.C., Baker, B.S. (1998). Regulation of sex-specific selection of fruitless 50 splice sites by transformer and transformer-2. Mol. Cell. Biol., 18, 450-458.

Helps, N.R., Luo, X., Barker, H.M. and Cohen, P.T.W. (2000). NIMA-related kinase 2(Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. Biochem. J., 349, 509-518.

Helps, N.R., Barker, H.M., Elledge, S.J. and Cohen, P.T.W. (1995). Protein phosphatase 1 interacts with p53BP2, a protein which binds to the tumour suppressor p53. FEBS Lett., 377, 295-300.

Helps, N.R., Cohen, P.T.W., Bahri, S.M., Chia, W. and Babu, K. (2001). Interaction with protein phosphatase 1 is essential for bifocal function during morphogenesis of the Drosophila compound eye. Mol. Cell. Biol., 21, 2154-2164.

Hemmings, H.C.J., Nairn, A.C., Elliott, J.,Greengard, P.(1990). Synthetic peptide analogs of DARPP-32 (Mr 32,000 dopamine- and cAMP-regulated phosphoprotein), an inhibitor of protein phosphatase-1. Phosphorylation, dephosphorylation, and inhibitory activity. J Biol Chem., 265(33):20369-76.

Hemmings, H.C., Greengard, P., Tung, H.Y.L. and Cohen, P. (1984). DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase1. Nature, 310, 503-508.

Hemmings, H.C.J., Nairn, A.C., McGuinness, T.L., Huganir, R.L. and Greengard, P. (1989). Role of protein phosphorylation in neuronal signal transduction. Faseb J., 3, 1583-1592.

den Hertog, J.(2003).Regulation of protein phosphatases in disease and behaviour. EMBO Rep., 4(11), 1027-32.

Hirano, K., Erdödi, F., Patton, J.G. and Hartshorne, D.J. (1996). Interaction of protein phosphatase type 1 with a splicing factor. FEBS Lett., 389, 191-194.

Hofmann, Y. Lorson, C.L., Stamm, S., Androphy, E.J., Wirth, B. (2000). Htra2-beta 1 stimulates an exonic splicing enhancer and can restore full-length SMN expression to survival motor neuron 2 (SMN2). PNAS USA, 97, 9618-9623.

Holdiman, A.J., Fergus, D.J., England, S.K. (2002). 17beta-Estradiol upregulates distinct maxi-K channel transcripts in mouse uterus. Mol. Cell. Endocrinol., 192, 1-6.

Huang, Y., Yario, T.A. and Steitz, J.A. (2004). A molecular link between SR protein dephosphorylation and mRNA export. PNAS USA, 101, 9666-9670.

Huang, Y. and Steitz, J.A. (2005). Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA. Mol. Cell, 17, 613-615.

Huang, F.L. and Glinsman, W.H. (1976). Separation and characterization of two phosphorylase phosphatase inhibitors from rabbit skeletal muscle. Eur. J. Biochem., 70, 419-426.

Hui, J. (2003) Identification and functional analysis of a novel intronic splicing enhancer in the human endothelial nitric oxide synthase gene. PhD thesis.

Hull, J., Shackleton, S., Harris, A. (1994). Analysis of mutations and alternative splicing patterns in the CFTR gene using mRNA derived from nasal epithelial cells. Hum. Mol. Genet., 3, 1141-1146.

Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell, 80, 225-236.

Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D.,Davies, P., Petersen, R.C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J.M., Nowotny, P., Che, L.K., Norton, J., Morris, J.C., Reed, L.A., Trojanowski, J., Basun, H., Lannfelt, L., Neystat, M., Fahn, S., Dark, F., Tannenberg, T., Dodd, P.R., Hayward, N., Kwok, J.B.J., Schofield, P.R., Andreadis, A., Snowden, J., Craufurd, D., Neary, D., Owen, F., Oostra, B.A., Hardy, J., Goate, A., van Swieten, J., Mann, D., Lynch, T. and Heutink, P. (1998). Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature, 393, 702-705.

Jagiello, I., Buellens, M., Stalmans, W. and Bollen, M. (1995). Subunit structure and regulation of protein phosphatase-1 in rat liver nuclei. J. Biol. Chem., 270, 17257-17263.

Johnson, J.M., Castle, J., Garret-Engele P., Kan, Z., Loerch, P.M., Armour, C.D., Santos, R., Shadt, E.E., Stoughton, R. and Shoemaker, D.D. (2003).Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science, 302, 2141-2144.

Jones, A.R., Schedl, T. (1995). Mutations in gld-1, a female germ-cell specific tumor suppressor gene in Caenorhabditis elegans, affect a conserved domain also found in Src-associated protein Sam68. Genes Dev., 9, 1491-1504.

Jones, A.R., Francis, R. and Schedl, T. (1996). GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage- and sex-specific expression during Caenorhabditis elegans germline development. Dev. Biol., 180, 165-183.

Jensen, K.B., Dredge, B.K., Stefani, G., Zhong, R., Buckanovich, R.J., Okano, H.J., Yang, Y.Y., Darnell, R.B. (2000). Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability. Neuron, 25, 359-371.

Jiang, Z., Tang, H., Havlioglu, N., Zhang, X., Stamm, S., Ya,n R. and Wu, J.Y. (2003). Mutations in tau gene exon 10 associated with FTDP-17 alter the activity of an exonic splicing enhancer to interact with Tra2-beta1. J. Biol. Chem., 278, 18997-19007.

Jin, Y., Dietz, H.C., Montgomery, R.A., Bell, W.R., McIntosh, I., Coller, B., Bray, P.F. (1996). Glanzmann thrombasthenia. Cooperation between sequence variants in cis during splice site selection. J. Clin. Invest., 98, 1745-54.

Jumaa, H., Guenet, J.-L., and Nielsen, P.J. (1997). Regulated expression and RNA processing of transcripts from the Srp20 splicing factor gene during the cell cycle. Mol. Cell. Biol., 17, 3116-3124.

Jumaa, H., Wei, G., Nielsen, P.J. (1999). Blastocyst formation is blocked in mouse embryos lacking the splicing factor SRp20. Curr. Biol., 9, 899-902.

Jurica, M.S. and Moore, M.J. (2003). Pre-mRNA splicing:awash in a sea of proteins. Molecular Cell, 12(1):5-14.

Kadener, S., Fededa, J.P., Rosbash, M., and Kornblihtt, A.R. (2002). Regulation of alternative splicing by a transcriptional enhancer through RNA pol II elongation. PNAS USA, 99(12), 8185-90.

Kamphuis, W., Monyer, H., De Rijk, T.C., Lopes da Silva, F.H. (1992). Hippocampal kindling increases the expression of glutamate receptorA Flip and -B Flip mRNA in dentate granule cells. Neurosci. Lett., 148, 51-54.

Kan, Z., Rouchka, E.C., Gish, W.R. and States, D.J. (2001). Gene Structure Prediction and Alternative Splicing Analysis Using Genomically Aligned ESTs. Genome Res., 11, 889-900.

Kanopka, A., Mühlemann, O. and Akusjarvi, G. (1996). SR proteins that are essential for generic pre-mRNA splicing inhibit splicing of a regulated adenovirus pre-mRNA. Nature, 381, 535-538.

Kashima, T. and Manley, J.L. (2003). A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. Nat. Genet., 34, 460-463.

Kataoka, N., Bachorik, J.L. and Dreyfuss, G. (1999). Transportin-SR, a nuclear import receptor for SR proteins. J. Cell Biol., 145, 1145-1152.

Kaufer, D., Friedman, A., Seidman, S., and Soreq, H. (1998). Acute stress facilitates longlasting changes in cholinergic gene expression. Nature, 393, 373-377.

Kawabe, T., Muslin, A.J. and Korsmeyer, S.J. (1997). Hox11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint. Nature, 385, 454-458.

Kent, W.J. (2002). BLAT - The BLAST-Like Alignment Tool. Genome Res., 12, 656-664.

Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M. and Haussler, D. (2002). The Human Genome Browser at UCSC. Genome Res., 12, 996-1006.

Kowalska, A., Hasegawa, M., Miyamoto, K., Akiguchi, I., Ikemoto, A., Takahashi, K., Araki, W. and Tabira, T. (2002). A novel mutation at position +11 in the intron following exon 10 of the tau gene in FTDP-17. J. Appl. Genet., 43, 535-543.

Kishore, S., Stamm, S. (2006). The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C. Science, 311(5758):230-2.

Kralovicova, J., Houngninou-Molango, S., Kramer, A., Vorechovsky, I. (2004). Branch site haplotypes that control alternative splicing. Hum. Mol. Genet., 13, 3189-3202.

Kramer, A. (1996). The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu. Rev. Biochem., 65, 367-409.

Kornblihtt, A.R., de la Mata, M., Fededa, J.P., Muñoz, M.J. and Nogués, G. (2004). Multiple links between transcription and splicing. RNA., 10, 1489-98.

Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. PNAS USA,., 82, 488-492.

Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol., 154, 367-382.

Kuroyanagi, N., Onogi, H., Wakabayashi, T., Hagiwara, M. (1998). Novel SR-protein-specific kinase, SRPK2, disassembles nuclear speckles. Biochem Biophys Res Commun. 1998 Jan 14;242(2):357-64.

Lacerra, G., Sierakowska, H., Carestia, C., Fucharoen, S., Summerton, J., Weller, D., Kole, R. (2000). Restoration of hemoglobin A synthesis in erythroid cells from peripheral blood of thalassemic patients. PNAS USA, 97, 9591-9596.

Ladd, A.N. and Cooper, T.A. (2002). Finding signals that regulate alternative splicing in the post-genomic era. Genome Biol., 23, 3-11.

Lai, M.C. and Tarn, W.Y. (2004). Hypophosphorylated ASF/SF2 binds TAP and is present in messenger ribonucleoproteins. J.Biol.Chem., 279, 31745-31749.

Lallena, M.J., Chalmers, K.J., Llamazares, S., Lamond, A.I., and Valcarcel, J. (2002). Splicing regulation at the second catalytic step by Sex-lethal involves 3' splice site recognition by SPF45. Cell, 109, 285-296.

Lan, N., Howrey, R.P., Lee, S.W., Smith, C.A., Sullenger, B.A. (1998). Ribozyme-mediated repair of sickle beta-globin mRNAs in erythrocyte precursors. Science, 280, 1593-1596.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. et al. (2001). International Human Genome Sequencing Consortium, Initial sequencing and analysis of the human genome. Nature, 409, 860-921.

Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., and Zeviani, M. (1995). Identification and characterization of a spinal muscular atrophy-determining gene. Cell, 80, 155-165.

Lesage, B., Beullens M., Nuytten M., Van Eynde A., Keppens S., Himpens B., and Bollen M. (2004). Interactor-mediated nuclear translocation and retention of protein phosphatase-1. J. Biol. Chem., 279, 55978-84.

Ley, S.C., Marsh, M., Bebbington, C.R., Proudfoot, K., and Jordan, P. (1994). Distinct intracellular localization of Lck and Fyn protein tyrosine kinases in human T lymphocytes. J. Cell. Biol., 125, 639-649.

Leytus, S.P., Melhado, L.L., and Mangel, W.F. (1983). Rhodamine-based compounds as fluorogenic substrates for serine proteinases. Biochem. J., 209, 299-307.

Liao, H., Li, Y., Brautigan, D.L. and Gundersen, G.G. (1998). Protein phosphatase 1 is targeted to microtubules by the microtubule-associated protein tau. J. Biol. Chem. 273, 21901-21908.

Li, J., Lee, G., Van Doren, S.R. and Walker, J.C. (2000). The FHA domain mediates phosphoprotein interactions. J. Cell Sci., 113, 4143-4149.

Li, J., Hawkins, I., Harvey, C., Jennings, J., et al. (2003). Regulation of alternative splicing by SRp86 and its interacting proteins. Mol Cell Biol. 23(21), 7437-47.

Lin, S.Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K.Y., Bour guignon, L., Hung, M.C. (2001). Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat. Cell Biol., 3, 802-808.

Liu,Q., Fischer, U., Wang, F., and Dreyfuss, G. (1997). The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP-1 are in a complex with spliceosomal snRNP proteins. Cell, 90, 1013-1021.

Liu, Z.R. (2002). p68 RNA helicase is an essential human splicing factor that acts at the U1 snRNA-5' splice site duplex. Mol. Cell. Biol., 22, 5443-5450.

Liu, S., Asparuhova, M., Brondani, V., Ziekau, I., Klimkait, T., Schumperli, D. (2004). Inhibition of HIV-1 multiplication by antisense U7 snRNAs and siRNAs targeting cyclophilin A. Nucleic Acids Res., 32, 3752-3759.

Llewellyn, D.H., Scobie, G.A., Urquhart, A.J., Whatley, S.D., Roberts, A.G., Harrison, P.R., Elder, G.H. (1996). Acute intermittent porphyria caused by defective splicing of porphobilinogen deaminase RNA: a synonymous codon mutation at 22 bp from the 5' splice site causes skipping of exon 3. J. Med. Genet., 33, 437-438.

Lopez-Bigas, N., Audita, B., Ouzounis, C., Parra, G., Guigo, R. (2005). Are splicing mutations the most frequent cause of hereditary disease? FEBS Lett., 579(9), 1900-3.

Lopez, A.J. (1998). Alaternative splicing of pre-mRNA: Developmental Consequences and Mechanisms of Regulation. Annu. Rev. Genet., 32, 279-305.

Lorson, C.L., Androphy E.J. and Zhou J. (2001). An in vivo reporter system for measuring increased inclusion of exon 7 in SMN2 mRNA potential therapy of SMA. 8(20), 1532-1538.

Lorson, C.L., and Androphy, E.J. (2000). An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. Hum. Mol. Genet., 9, 259-265.

Lorson, C.L., Strasswimmer, J., Yao, J.M., Baleja, J.D., Hahnen, E., Wirth, B., Le, T., Burghes, A.H., and Androphy, E.J. (1998). Nat. Genet., 19, 63-66.

Lorson, C.L., Hahnen, E., Androphy, E.J., and Wirth, B. (1999). PNAS USA, 97, 9618-9623.

Lu, Q.L. et al. (2003). Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. Nat. Med., 9, 1009-1014.

Lin, Q., Taylor, S., Shalloway, D. (1997) Specificity and determinants of Sam68 RNA binding. Implications for the biological function of K homology domains. J Biol Chem, 272(43):27274-80

Lunn, M.R., Root, D.E., Martino, A.M., Flaherty, S.P., Kelley, B.P., Coovert, D.D., Burghes, A.H., Man, N.T., Morris, G.E., Zhou, J., Androphy, E.J., Sumner, C.J., Stockwell, B.R. (2004). Indoprofen upregulates the survival motor neuron protein through a cyclooxygenase-independent mechanism. Chem. Biol., 11, 1489-1493.

Lynch, K.W. and Maniatis, T. (1996). Assembly of specific SR protein complexes on distinct regulatory elements of the Drosophila doublesex splicing enhancer. Genes Dev., 10, 2089-2101.

MacDougall, C., Harbison, D., Bownes, M. (1995). The developmental consequences of alternate splicing in sex determination and differentiation in Drosophila. Dev. Biol., 172, 353-76.

Maximov, A., Sudhof, T.C., Bezprozvanny, I. (1999). Association of neuronal calcium channels with modular adaptor proteins. J. Biol. Chem., 274, 24453-24456.

Maniatis, T. and Tasic B. (2002). Alternative pre-mRNA splicing and proteome expansion in metazoans. Nature, 418, 236-43

Maniatis, T. and Reed, R. (2002). An extensive network of coupling among gene expression machines. Nature, 416, 499-506.

Mann, C.J., Honeyman, K., Cheng, A.J., Ly, T., Lloyd, F., Fletcher, S., Morgan, J.E., Partridge, T.A., Wilton, S.D. (2001). Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. PNAS USA, 98, 42-47.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T. and Sudarsanam, S. (2002). The Protein Kinase Complement of the Human Genome. Science, 298 (5600), 1912-1934

Maris, C., Dominguez, C., and Allain, F.H. (2005). The RNA recognition motif, a plastic RNAbinding platform to regulate post-transcriptional gene expression. FEBS J., 272, 2118-2131.

Markovtsov, V., Nikolic, J.M., Goldman, J.A., Turck, C.W., Chou, M.Y.,Black, D.L., (2000). Cooperative assembly of an hnRNP complex induced by a tissue-specific homolog of polypyrimidine tract binding. protein. Mol. Cell. Biol., 20, 7463-7479.

Marti, U. and Wells, A. (2000). The nuclear accumulation of a variant epidermal growth factor receptor (EGFR) lacking the transmembrane domain requires coexpression of a full-length EGFR. Mol. Cell. Biol., 3, 8-14.

Matter, N., Herrlich, P. and Konig, H. (2002). Signal-dependent regulation of splicing via phosphorylation of Sam68. Nature, 420, 691-695.

Mattox, W., Palmer, M. J., and Baker, B. S. (1990). Alternative splicing of the sex determination gene transformer-2 is sex-specific in the germ line but not in the soma. Genes Dev., 4, 789-805.

Mayeda, A., and Krainer, A.R. (1992). Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. Cell, 68, 365-375.

Matsuo, N., Ogawa, S., Imai, Y., Takagi, T., Tohyama, M., Stern, D., and Wanaka, A.(1995). Cloning of a novel RNA binding polypeptide (RA301) induced by hypoxia/reoxygenation. J. Biol. Chem., 270, 28216-28222.

McKay, N.G., Hunter, D.J., Haites, N.E., Power, D.A. (1994). Regulation of alternative splicing of the fibronectin IIICS domain by cytokines. Biochem. Biophys. Res. Commun., 199, 1005-1011.

Meiselbach, H., Sticht, H. and Enz, R. (2006). Structural analysis of the protein phosphatase 1 docking motif, molecular description of binding specificities identifies interacting proteins. Chem. Biol., 13, 49-59.

Mermoud, J., Cohen, P., Lamond, A.I. (1992). Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing. Nucleic Acids Res, 20(20):5263-9.

Meshorer, E., Erb C., Gazit, R., Pavlovsky, L., Kaufer, D., Friedman, A., Glick, D., Ben-Arie, N., and Soreq, H. (2002). Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. Science, 295, 508-512.

Misteli, T. and Spector, D.L. (1999). RNA polymerase II targets pre-mRNA splicing factors to transcription sites in vivo. Mol. Cell., 3, 697-705.

Modrek, B., Resch, A, et al. (2002). Genome-wide detection of alternative splicing in expressed sequences of human genes. Nucl. Acids Res. 29(13), 2850-59.

Moller, L.B., Tumer, Z., Lund, C., Petersen, C., Cole, T., Hanusch, R., Seidel, J., Jensen, L.R. and Horn, N. (2000). Similar splice-site mutations of the ATP7A gene lead to different phenotypes: classical Menkes disease or occipital horn syndrome. Am. J. Hum. Genet., 66(4), 1211-20.

Monneret, C. (2001). Recent developments in the field of antitumour anthracyclines. Eur J Med. Chem., 36(6), 483-93.

Moore, M.J., Query, C.C., and Sharp, P.A. (1993). in The RNA World (Gesteland, R. F., and Atkins, J. F., eds) Cold Spring Harbor, NY, 13, 303-357.

Moorhead, G., MacKintosh, C., Morrice, N. and Cohen, P. (1995). Purification of the hepatic glycogen-associated form of protein phosphatase-1 by microcystin-Sepharose affinity chromatography. FEBS Lett., 362, 101-105.

Morrison, D.K., Murakami, M.S. and Cleghon, V. (2000). Protein kinases and phosphatases in the Drosophila genome. J. Cell Biol., 150, F57-F62.

Muraki, M., Ohkawara, B., Hosoya, T., Onogi, H., Koizumi, J., Koizumi, T., Sumi, K., Yomoda, J. I., Murray, M. V., Kimura, H., et al. (2004). Manipulation of alternative splicing by a newly developed inhibitor of Clks. J. Biol. Chem., 279(23), 24246-54.

Murray, M.V., Kobayashi, R., and Krainer, A.R. (1999). The type 2C ser/thr phosphatase PP2Cgamma is a pre-mRNA splicing factor. Genes Dev., 13, 87-97.

Nakielny, S., Fischer, U., Michael, W. M. and Dreyfuss, G. (1997). Annu. Rev. Neurosci. 20, 269-301.

Nayler, O., Stamm, S. and Ullrich, A. (1997).Characterisation and comparison of four SR protein kinases. Biochem. J., 326, 693-700.

Nayler, O., Stratling, W., Bourquin, J.-P., Stagljar, I., Lindemann, L., Jasper, H., Hartmann, A.M., Fackelmayer, F.O., Ullrich, A., Stamm, S. (1998). SAF-B protein couples transcription and pre-mRNA splicing to SAR/MAR elements. Nucleic Acids Res., 26, 3542-3549.

Nayler, O., Schnorrer, F., Stamm, S. and Ullrich, A. (1998). The Cellular Localization of the Murine Serine/Arginine-rich Protein Kinase CLK2 Is Regulated by Serine 141 Autophosphorylation. J. Biol. Chem., 273, 34341-34348.

Nayler, O., Cap, C. and Stamm, S. (1998). Human Transformer-2-beta Gene (SFRS10): Complete Nucleotide Sequence, Chromosomal Localization, and Generation of aTissue-Specific Isoform. Genomics, 53, 191-202.

Nayler, O., Hartmann, A.M. and Stamm, S. (2000). The ER Repeat Protein YT521-Localizes to a Novel Subnuclear Compartment. J. Cell Biol., 150, 949-962.

Nakai, K., Sakamoto, H. (1994). Construction of a novel database containing aberrant splicing mutations of mammalian genes. Gene, 141, 171-177.

Neek, K. and Hunter, T. (1996).Vertebrate non-receptor protein-tyrosine kinase families. Genes Cells, 2, 147-69.

Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A., and Mann, M. (1998). Nat. Genet., 20, 46-50.

Neumann, M., Afonina, E., Ceccherini-Silberstein, F., Schlicht, S., Erfle, V., Pavlakis, G.N. and Brack-Werner, R. (2001) Nucleocytoplasmic transport in human astrocytes: decreased nuclear uptake of the HIV Rev shuttle protein. J.Cell. Biol., 114, 1717-1729.

Neugebauer, K.M. (2002). On the importance of being co-transcriptional. J. Cell Sci., 115, 3865-3871.

Ni, C.Y., Murphy, M.P., Golde, T.E., Carpenter, G. (2001). Gamma-secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science, 294, 2179-2181.

Nishi, A., Snyder, G. L., Fienberg, A. A., Fisone, G., Aperia, A., Naim, A. C. and Greengard, P. (1999). Requirement for DARPP-32 in mediating effect of dopamine D2 receptor activation. Eur. J. Neurosci., 11, 2589-2592.

Nissim-Rafinia, M., Kerem, B. (2002). Splicing regulation as a potential genetic modifier. Trends Genet., 18, 123-127.

Nogues, G., Kadener, S., Cramer, P., de la Mata, M, Fededa, J.P., Blaustein, M., Srebrow, A. and Kornblihtt, A.R. (2003). Control of alternative pre-mRNA splicing by RNA Pol II elongation: faster is not always better. IUBMB Life, 55(4-5), 235-41.

Novoyatleva, T., Heinrich, B., Tang, Y., Benderska, N., Butchbach, ME., Lorson, CL., Lorson, MA., Ben-Dov, C., Fehlbaum, P., Bracco, L., Burghes, A., Bollen, M., Stamm, S. (2008) Protein phosphatase 1 binds to the RNA recognition motif of several splicing factors and regulates alternative pre-mRNA processing. Hum Mol Genet., 1(17), 52-70.

Novoyatleva, T., Rafalska, I., Tang, Y. and Stamm, S. (2006). Pre-mRNA missplicing as a cause of human disease. Prog. Mol. Subcell. Biol., 44, 27-46.

Offterdinger, M., Schofer, C., Weipoltshammer, K., Grunt, T.W. (2002). c-erbB-3: a nuclear protein in mammary epithelial cells. J. Cell Biol., 157, 929-939.

Okazaki, Y., Furuno, M., Kasukawa, T. et al. (2002). Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature, 420, 563-573.

Padgett, R.A., Konarska, P., Grabowski, S.F., Hardy S.F., and Sharp, P. A. (1984). Lariat RNAs as intermediates and products in the splicing of messenger RNA precursors. Science, 225, 898-903.

Pagani, F., Buratti, E., Stuani, C., Baralle, F.E. (2003a). Missense, nonsense, and neutral mutations define juxtaposed regulatory elements of splicing in cystic fibrosis transmembrane regulator exon 9. J. Biol. Chem., 278, 26580-26588.

Pagani, F., Stuani, C., Tzetis, M., Kanavakis, E., Efthymiadou, A., Doudounakis, S., Casals, T., Baralle, F.E. (2003b). New type of disease causing mutations: the example of the

composite exonic regulatory elements of splicing in CFTR exon 12. Hum. Mol. Genet. 12, 1111-1120.

Pagani, F., Stuani, C., Zuccato, E., Kornblihtt, A.R. and Baralle, F.E. (2003a). Promoter architecture modulates CFTR exon 9 skipping. J Biol Chem. Jan 17; 278(3), 1511-7.

Paronetto Maria Paola, Zalfa Francesca, Botti Flavia, Geremia Raffaele, Bagni Claudia and Sette Claudio (2006). The Nuclear RNA-binding Protein Sam68 Translocates to the Cytoplasm and Associates with the Polysomes in Mouse Spermatocytes. Mol Biol Cell., 17(1), 14-24.

Patel, A.A. and Steitz, J.A. (2003). Splicing double: insights from the second spliceosome. Nat Rev Mol Cell Biol., 4(12), 960-70.

Paushkin, S., Gubitz, A.K., Massenet, S., Dreyfuss, G. (2002). The SMN complex, an assemblyosome of ribonucleoproteins. Curr. Opin. Cell. Biol., 14 (3), 305-12.

Pearn, J.H. (1980). Classification of spinal musculat atrophies. Lancet, 1, 919-922.

Pinna, L.A. and Ruzzene, M. (1996). How do protein kinases recognize their substrates? Biochim. Biophys. Acta., 1314, 191-225.

Pinna, L.A. and Donella-Deana, A. (1994). Phosphorylated synthetic peptides as tools for studying protein phosphatases. Biochim. Biophys. Acta., 1222, 415-431.

Ploos van Amstel J.K., Bergman, A.J., van Beurden, E.A., Roijers, J.F., Peelen, T., van den Berg, I.E., Poll, B.T., Kvittingen, E.A., Berger, R. (1996). Hereditary tyrosinemia type 1: novel missense, nonsense and splice consensus mutations in the human fumarylacetoacetate hydrolase gene; variability of the genotype-phenotype relationship. Hum. Genet., 97, 51-59.

Poorkaj, P., Bird, T.D., Wijsman, E., Nemens, E., Garruto, R.M., Anderson, L., Andreadis, A., Wiederholt, W.C., Raskind, M. and Schellenberg, G.D. (1998). Tau is a candidate gene for chromosome 17 frontotemporal dementia. Ann. Neurol., 43, 815-825.

Price, N.E. and Mumby, M.C. (1999). Brain protein serine/threonine phosphatases. Curr. Opin. Neurobiol., 9, 336-342.

Rafalska, I., Zhang, Z., Benderska, N., Wolff, H., Hartmann, A.M., Brack-Werner, R. and Stamm, S. (2004). The intranuclear localization and function of YT521-B is regulated by tyrosine phosphorylation. Hum Mol Genet., 13(15), 1535-49.

Robberson, B.L., Cote, G.J. and Berget, S.M. (1990). Exon definition may facilitate splice site selection in RNAs with multiple exons. Mol. Cell. Biol., 10, 84-94.

Reed, R. (1996). Initial splice-site recognition and pairing during pre-mRNA splicing. Curr. Opin. Genet. Dev., 6, 215-220.

Relogio, A., Ben-Dov, C., Baum, M., Ruggiu, M., Gemund, C., Benes, V., Darnell, R.B., Valcarcel, J. (2005). Alternative splicing microarrays reveal functional expression of neuron-specific regulators in Hodgkin lymphoma cells. J. Biol. Chem., 280, 4779-4784.

Richard, S., Yu, D., Blumer, K.J., Hausladen, D., Olszowy, M.W., Connelly, P.A., Shaw, A.S. (1995). Association of p62, a multifunctional SH2- and SH3-domain-binding protein, with src family tyrosine kinases, Grb2, and phospholipase C gamma-1. Mol. Cell. Biol., 15, 186-197.

Rizzu, P., Van Swieten J.C., Joosse, M., Hasegawa, M., Stevens, M., Tibben, A., Niermeijer, M.F., Hillebrand, M., Ravid, R., Oostra, B.A., Goedert, M., van Duijn, C.M., Heutink, P. (1999). High prevalence of mutations in the microtubule-associated protein tau in a population study of frontotemporal dementia in the Netherlands. Am. J. Hum. Genet., 64, 414-421.

Reed, R. (2003). Coupling transcription, splicing and mRNA export Curr. Opin. Cell. Biol., 15(3), 326-31.

Rochette, C.T., Gilbert, N., and Simard, L.R. (2001). SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to Homo sapiens. Hum. Genet., 108, 255-266.

Roscigno, R.F. and Garcia-Blanco, M.A. (1995). SR proteins escort the U4/U6.U5 tri-snRNP to the spliceosome. RNA, 1, 692-706.

Reed, R. and Hurt, E. (2002). A conserved mRNA export machinery coupled to pre-mRNA splicing. Cell, 108, 523-531.

Reddy, T.R., Tang, H., Xu, W., Wong-Staal, F. (2000). Sam68, RNA helicase A and Tap cooperate in the post-transcriptional regulation of human immunodeficiency virus and type D retroviral mRNA. Oncogene, 19, 3570-3575.

Relogio, A., Ben-Dov, C., Baum, M., Ruggiu, M., Gemund, C., Benes, V., Darnell, R.B., Valcarcel, J. (2005). Alternative splicing microarrays reveal functional expression of neuron-specific regulators in Hodgkin lymphoma cells. J. Biol. Chem., 280, 4779-4784.

Ruby, S.W. and Abelson, J. (1991). Pre-mRNA splicing in yeast. Trends Genet., 7, 79-85.

Ruskin, B., Krainer A.R., Maniatis T. and Green R. (1984). Excision of an intact intron as a novel lariat structure during pre-mRNA splicing in vitro. Cell, 38, 317-331.

Sanford, J.R., Longman, D. and Caceres, J.F. (2003). Multiple roles of the SR protein family in splicing regulation. Prog. Mol. Subcell. Biol., 31, 33–58.

Sanford, J.R., Ellis, J. and Caceres, J.F. (2005). Multiple roles of arginine/serine-rich splicing factors in RNA processing. RNA structure and function. Biochemival Society Transactions, 33, 443-447.

Sanford, J.R., Ellis, J.D., Gazalla, D. and Caceres, J.F. (2005). Reversible phosphorylation differentially affects nuclear and cytoplasmic functions of splicing factor 2/alternative splicing factor. PNAS USA, 102, 15042-15047.

Sazani, P. and Kole, R. (2003) Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing. J. Clin. Invest., 112, 481-486.

Scotet, E. and Houssaint, E. (1998). Exon III splicing switch of fibroblast growth factor (FGF) receptor-2 and -3 can be induced by FGF-1 or FGF-2. Oncogene, 17, 67-76.

Screaton, G.R., Caceres, J.F., Mayeda, A., Bell, M.V., Plebanski, M., Jackson, D.G., Bell, J.I., Krainer, A.R. (1995). Identification and characterization of three members of the human SR family of pre-mRNA splicing factors. EMBO J., 14, 4336-4349.

Segade, F., Hurle, B., Claudio, E., Ramos, S., and Lazo, P.S. (1996). Molecular cloning of a mouse homologue for the Drosophila splicing regulator Tra2. FEBS Lett., 387, 152-156.

Sell, S.M., Reese, D., Ossowski, V. M. (1994). Insulin-inducible changes in insulin receptor mRNA splice variants. J. Biol. Chem., 269, 30769-30772.

Shatkin, A. J., and Manley, J. L. (2000). The ends of the affair: capping and polyadenylation. Nat. Struct. Biol., 7, 838-842.

Shen, H., Kan, J.L. and Green, M.R. (2004). Arginine-serine-rich domains bound at splicing enhancers contact the branchpoint to promote prespliceosome assembly. Mol. Cell, 13, 367–376.

Shenolikar, S. and Nairn, A.C. (1991) Protein Phosphatases: Recent Progress. In Greengard, P. and Robinson, G.A. (eds.) Advances in Second Messengers and Phosphoprotein Research. Raven Press, New York, 23, 1-121.

Shi, Y., Reddy, B., and Manley, J.L. (2006). PP1/PP2A Phosphatases Are Required for the Second Step of Pre-mRNA Splicing and Target Specific snRNP Proteins. Mol. Cell, 23, 819-829.

Shima, T., Okumura, N., Takao, T., Satomi, Y., Yagi, T., Okada, M., Nagai, K. (2001). Interaction of the SH2 domain of Fyn with a cytoskeletal protein, beta-adducin. J. Biol. Chem., 276, 42233-42240.

Singh, N.N., Androphy, E.J. and Singh, R.N. (2004a) An extended inhibitory context causes skipping of exon 7 of SMN2 in spinal muscular atrophy. Biochem. Biophys. Res. Commun., 315, 381-388.

Singh N.N., Androphy E.J. and Singh R.N. (2004b) In vivo selection reveals combinatorial controls that define a critical exon in the spinal muscular atrophy genes. RNA, 10, 1291-1305.

Singh, N.N., Androphy, E.J. and Singh, R.N. (2004c) The regulation and regulatory activities of alternative splicing of the SMN gene. Crit. Rev. Eukary. Gene. Exp., 14(4), 271-85.

Sitz, J.H., Tigges, M., Baumgärtel, K., Khaspekov, L.G. and Lutz, B. (2004). Dyrk1A Potentiates Steroid Hormone-Induced Transcription via the Chromatin Remodeling Factor Arip4. Mol. Cell. Biol., 24(13), 5821-5834.

Skordis, L.A., Dunckley, M.G., Yue, B., Eperon, I.C., Muntoni, F. (2003). Bifunctional antisense oligonucleotides provide a trans-acting splicing enhancer that stimulates SMN2 gene expression in patient fibroblasts. PNAS USA, 1, 100(7), 4114-9.

Smith, C. and Valcarcel, J. (2000). Alternative pre-mRNA splicing: the logic of combinatorial control. Trends in Biochemical Sciences, 25(8), 318-8.

Snyder, G.L., Fienberg, A.A., Huganir, R.L. and Greengard, P. (1998). A dopamine/D1 receptor/protein kinase A/dopamine- and cAMP-regulated phosphoprotein (Mr 32 kDa) /protein phosphatse-1 pathway regulated dephosphorylation of the NMDA receptor. J. Neurosci., 18, 10297-10303.

Someya, T. (2006). Solution structure of the RNA recognition motif in Arginine/serine rich splicing factor 10.

Soreq, H., Seidman, S (2001).Acetylcholinesterase--new roles for an old actor. Nat Rev Neurosci 2001 Apr;2(4):294-302.

Soret, J. and Tazi, J. (2003). Phosphorylation-dependent control of the pre-mRNA splicing machinery. In Jeanteur, P. (ed.), Regulation of alternative splicing. Springer, 31, 89-126.

Soret, J., Bakkour, N., Maire, S., Durand, S., Zekri, L., Gabut, M., Fic, W., Divita, G., Rivalle, C., Dauzonne, D., Nguyen, C.H., Jeanteur, P., Tazi, J. (2005). Selective modification of alternative splicing by indole derivatives that target SR protein splicing factors. PNAS USA, 102(24), 8764-9.

Soulard, M., Della Valle, V., Siomi, M.C., Pinol-Roma, S., Codogno, P., Bauvy, C., Bellini, M., Lacroix, J.C., Monod, G., Dreyfuss, G. (1993). hnRNP G: sequence and characterization of a glycosylated RNA- binding protein. Nucleic Acids Res., 21, 4210-4217.

Spillantini, M.G. and Goedert, M. (1998). Tau protein pathology in neurodegenerative diseases. Trends Neurosci., 21, 428-433.

Stamm, S. (2008). Regulation of alternative splicing by reversible protein phosphorylation. J Biol Chem. 283(3):1223-7

Stamm, S., Casper, D., Hanson, V. and Helfman, D.M. (1999). Regulation of the neuron-specific exon of clathrin light chain B. Mol. Brain Res., 64,108-118.

Stamm, S., Zhu, J., Nakai, K., Stoilov, P., Stoss, O., Zhang, M.Q., (2000). An alternative-exon database and its statistical analysis. DNA Cell. Biol., 19, 739-756.

Stark, M.J.R. (1996). Yeast protein serine/threonine phosphatases: multiple roles and diverse regulation. Yeast, 12, 1647-1675.

Steen, R.L. and Collas, P. (2001). Mistargeting of B-type lamins at the end of mitosis: implications on cell survival and regulation of lamins A/C expression. J. Cell Biol., 153, 621-626.

Steen, R.L., Martins, S.B., Tasken, K. and Collas, P. (2000). Recruitment of protein phosphatase 1 to the nuclear envelope by A-kinase anchoring protein AKAP149 is a prerequisite for nuclear lamina assembly. J. Cell Biol., 150, 1251-1261.

Stenson, P.D., Ball, E.V., Mort, M., Phillips, A.D., Shiel, J.A., Thomas, N.S., Abeysinghe, S., Krawczak, M., Cooper, D.N. (2003). Human Gene Mutation Database (HGMD), Update. Hum. Mutat., 21, 577-581.

Stoilov, P., Meshorer, E., Gencheva, M., Glick, D., Soreq, H., Stamm, S. (2002). Defects in pre-mRNA processing as causes of and predisposition to diseases. DNA Cell. Biol., 21, 803-818.

Stoilov, P., Rafalska, I. and Stamm, S. (2002). YTH: a new domain in nuclear proteins. Trends Biochem. Sci., 27, 495-496.

Stork, O., Stork, S., Pape, H.C., Obata, K. (2001). Identification of genes expressed in the amygdala during the formation of fear memory. Learn. Mem., 8, 209-219.

Stoss, O., Olbrich, M., Hartmann, A.M., Konig, H., Memmott, J., Andreadis, A., Stamm, S. (2001). The STAR/GSG family protein rSLM-2 regulates the selection of alternative splice sites. J. Biol. Chem., 276, 8665-8673.

Stoss, O., Novoyatleva, T., Gencheva, M., Olbrich, M., Benderska, N. and Stamm, S. (2004). $p59^{fyn}$-mediated phosphorylation regulates the activity of the tissue specific splicing factor rSLM-1. Mol. Cell. Neurosci., 27, 8-21.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol., 185, 60-89.

Svenningsson, P., Nairn, A., Greengard, P. (2005). DARPP-32 mediates the actions of multiple drugs of abuse. AAPS J.7(2):E353-60.

Svenningsson, P., Lindskog, M., Ledent, C., Parmentier, M., Greengard, P., Fredholm, B. B. and Fisone, G. (2000). Regulation of the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa in vivo by dopamine D-1, dopamine D-2, and adenosine A (2A) receptors. PNAS USA, 97, 1856-1860.

Sugimoto, Y., Morita, R., Amano, K., Shah, P.U., Pascual-Castroviejo, I., Khan, S., Delgado-Escueta, A.V., Yamakawa, K. (2001). T-STAR gene: fine mapping in the candidate region for childhood absence epilepsy on 8q24 and mutational analysis in patients. Epilepsy Res., 46, 139-144.

Tacke, R., Tohyama, M., Ogawa, S., Manley, J.L. (1998). Human Tra2 proteins are sequence-specific activators of pre-mRNA splicing. Cell, 93(1), 139-48.

Tacke, R. and Manley, J.L. (1999). Determinants of SR protein specificity. Curr. Opin. Cell Biol., 11, 358-362.

Tang, P.M., Bondor, J.A., Swiderek, K.M. and DePoali-Roach, P. (1991). Molecular cloning and expression of the regulatory (RGl) Subunit of the glycogen associated protein phosphatase. J. Biol. Chem., 266, 15782-15789.

Taylor, S.J., Anafi, M., Pawson, T., Shalloway, D. (1995). Functional interaction between c-src and its mitotic target, Sam68. J. Biol. Chem., 270, 10120-10124.

Tarn, W.Y. and Steitz, J.A. (1997). Pre-mRNA splicing: The discovery of a new spliceosome doubles the challenge. Trends Biochem. Science, 22, 132-137.

Terry-Lorenzo, R. T., Inoue, M., Conner, J. H., Haystead, T. A. J.,Armbruster, B. N., Gupta, R. P., Oliver, C. J. and Shenolikar, S. (2000). Neurofilament-L is a protein phosphatase-1 binding associated with neuronal plasma membrane and post-synaptic density. J. Biol. Chem., 275, 2439-2446.

Thanaraj, T.A., Stamm, S., Clark, F., Riethoven, J.J., Le Texier, V., Muilu, J. (2004). ASD: the Alternative Splicing Database. Nucleic Acids Res., 32, Database issue D64-69.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22, 4673-4680.

Tian, M. and Maniatis, T. (1994). A splicing enhancer exhibits both constitutive and regulated activities. Genes Dev., 8, 1703-12.

Trinkle-Mulcahy, L., Andrews, P.D. Wickramasinghe, S., Sleeman, J., Prescott, A., Lam, Y.W., Lyon, C., Swedlow, J.R. and Lamond, A.I. (2003).Time-lapse imaging reveals dynamic relocalization of PP1gamma throughout the mammalian cell cycle. Mol. Biol. Cell., 14, 107-17.

Umemori, H., Wanaka, A., Kato, H., Takeuchi, M., Tohyama, M., Yamamoto, T., 1992. Specific expressions of Fyn and Lyn, lymphocyte antigen receptor-associated tyrosine kinases, in the central nervous system. Brain Res. Mol. Brain Res., 16, 303-310.

Van Eynde, A., Wera, S., Beullens, M., Torrekens, S., Van Leuven, F., Stalmans, W. and Bollen, M. (1995). Molecular cloning of NIPP-1, a nuclear inhibitor of protein phosphatase-1, reveals homology with polypeptides involved in RNA processing. J. Biol. Chem., 270, 28068-28074.

Venables, J.P., Elliott, D.J., Makarova, O.V., Makarov, E.M., Cooke, H.J., Eperon, I.C. (2000). RBMY, a probable human spermatogenesis factor, and other hnRNP G proteins interact with Tra2beta and affect splicing. Hum. Mol. Genet., 9, 685-694.

Vernet, C. and Artzt, K. (1997). STAR, a gene family involved in signal transduction and activation of RNA. Trends Genet. 13, 479-484.

Venter, J., Adams, M., Myers, E., Li, P. et al. (2001). The sequence of the human genome. Science, 291 (5507), 1304-51.

Vithana, E.N., Abu-Safieh, L., Allen, M.J., Carey, A., Papaioannou, M., Chakarova, C., Al-Maghtheh, M., Ebenezer, N.D., Willis, C., Moore, A.T., Bird, A.C., Hunt, D.M., Bhattacharya, S.S. (2001). A human homolog of yeast pre-mRNA splicing gene, PRP31, underlies autosomal dominant retinitis pigmentosa on chromosome 19q13.4 (RP11). Mol. Cell, 8, 375-381.

Vulsteke, V., Beullens, M., Waelkens, E., Stalmans, W. and Bollen, M. (1997). Properties and phosphorylation sites of baculovirus-expressed nuclear inhibitor of protein phosphatase-1 (NIPP-1). J. Biol. Chem., 272, 32972-32978.

Wakula, P., Beullens, M., Ceulemans, H., Stalmans, W. and Bollen, M. (2003). Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. J. Biol. Chem., 278, 18817-23.

Wagner, E.J. and Garcia-Blanco, M.A. (2001). Polypyrimidine Tract Binding Protein Antagonizes Exon Definition. Molecular and Cellular Biology, 21(10), 3281-3288.

Wang, J., Takagaki, Y., Manley, J.L. (1996). Targeted disruption of an essential vertebrate gene: ASF/SF2 is required for cell viability. Genes Dev., 10(20), 2588-99.

Wang, J., Xiao, S.H., Manley, J.L. (1998). Genetic analysis of the SR protein ASF/SF2: interchangeability of RS domains and negative control of splicing. Genes Dev., 12(14), 2222-33.

Wang, H.Y., Xu, X., Ding, J.H., Bermingham, J.R. Jr. and Fu, X.D. (2001). SC35 plays a role in T cell development and alternative splicing of CD45. Mol. Cell, 7, 331-342.

Wang, J., Gao, Q.S., Wang, Y., Lafyatis, R., Stamm, S. and Andreadis, A. (1988). Tau exon 10, whose missplicing causes frontotemporal dementia, is regulated by an intricate interplay of cis elements and trans factors. Journal of Neurochemistry, 5, 1078-1090.

Watanabe, T., Sullenger, B.A. (2000). Induction of wild-type p53 activity in human cancer cells by ribozymes that repair mutant p53 transcripts. PNAS USA, 97, 8490-8494.

Watermann, D.O., Tang, Y., zur Hausen, A. , Jäger, M., Stamm, S. and Stickeler, E. (2006). Splicing factor Tra2-beta1 is specifically induced in breast cancer and regulates alternative splicing of the CD44 gene. Cancer Res., 66(9), 4774-80.

Wedekind, J.E., Dance, G.S.C., Sowden, M.P. and Smith, H.C. (2003). Messenger RNA editing in mammals: new members of the APOBEC family seeking roles in the family business. Trends Genet., 19, 207-216.

Weighardt, F., Cobianchi, F., Cartegni, L., Chiodi, I., Villa, A., Riva, S., Biamonti, G., (1999). A novel hnRNP protein (HAP/SAF-B) enters a subset of hnRNP complexes and relocates in nuclear granules in response to heat shock. J. Cell Sci., 112, 1465-1476.

White, E, Moore-Jarrett., T. and Ruley H. (2001). PUM2, a novel murine puf protein, and its consensus RNA-binding site. RNA 7:1855-1866.

Wera, S. and Hemmings, B.A. (1995). Serine/threonine protein phosphatases. Biochem. J., 311, 17-29.

Winder, D.G., and Sweatt, J.D. (2001). Roles of serine/threonine phosphatases in hippocampal-synaptic plasticity. Nat. Rev. Neurosci., 2, 461-474.

Wirth, B. (2000). An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy (SMA). Hum. Mutat., 15, 228-237.

Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M.F., Polakis, McCormick, F., (1992). Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. Cell, 69, 551-558.

Wu, J.Y. and Maniatis, T. (1993). Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell, 75, 1061-1070.

Xavier, K.A., Eder, P.S. and Giordano, T. (2000). RNA as a drug target: methods for biophysical characterization and screening. Trends Biotechnol., 18, 349-356.

Xiao, S.H. and Manley, J.L. (1997). Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. Genes Dev., 11, 334-344.

Xie, J., and Black, D.L. (2001). A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels. Nature, 410, 936-939.

Xie, J., and McCobb, D.P. (1998). Control of alternative splicing of potassium channels by stress hormones. Science, 280, 443-446.

Xu, X., Yang, D., Ding, J.H., Wang, W., Chu, P.H., Dalton, N.D., Wang, H.Y., Bermingham, J.R.Jr, Ye, Z., Liu, F., Rosenfeld, M.G., Manley, J.L., Ross, J., Chen, J., Xiao, R.P., Cheng, H., Fu, X.D. (2005). ASF/SF2-regulated CaMKIIdelta alternative splicing temporally reprograms excitation-contraction coupling in cardiac muscle. Cell, 120, 59-72.

Yamamoto, H., Tsukahara, K., Kanaoka, Y., Jinno, S., and Okayama, H. (1999). Isolation of a mammalian homologue of a fission yeast differentiation regulator. Mol. Cell. Biol., 19, 3829-3841.

Yan, Z., Hsieh-Wilson, L., Feng, J.A., Tomizawa, K., Allen, P.B., Fienberg, A.A., Nairn, A.C. and Greengard, P. (1999). Protein phosphatase 1 modulation of neostriatal AMPA channels: regulation by DARPP-32 and spinophilin. Nature Neurosci., 2, 13-17.

Yang, J., Hurley, T.D. and DePaoli-Roach, A.A. (2000). Interaction of inhibitor-2 with the catalytic subunit of type 1 protein phosphatase. Identification of a sequence analogous to the consensus type 1 protein phosphatase-binding motif. J. Biol. Chem., 275, 22635-44.

Yang, Y.Y., Yin, G.L., Darnell, R.B. (1998). The neuronal RNA-binding protein Nova-2 is implicated as the autoantigen targeted in POMA patients with dementia. PNAS USA, 95, 13254-13259.

Young, P.J., DiDonato, C.J., Hu, D., Kothary, R., Androphy, E.J., Lorson, C.L. (2002). SRp30c-dependent stimulation of survival motor neuron (SMN) exon 7 inclusion is facilitated by a direct interaction with hTra2-beta1. Hum. Mol. Genet., 11, 577-587.

Yoshida, K., Watanabe, M., Kato, H., Dutta, A. and Sugano, S. (1999). BH-protocadherin-c, a member of the cadherin superfamily, interacts with protein phosphatase 1 alpha through its intracellular domain. FEBS Lett., 460, 93-98.

Zavadakova, P., Fowler, B., Zeman, J., Suormala, T., Pristoupilova, K., Kozich, V. (2002). CblE type of homocystinuria due to methionine synthase reductase deficiency: clinical and molecular studies and prenatal diagnosis in two families. J. Inherit. Metab. Dis., 25, 461-476

Zavadakova, P., Fowler, B., Suormala, T., Novotna, Z., Mueller, P., Hennermann, J.B., Zeman, J., Vilaseca, M.A., Vilarinho, L., Gutsche, S., Wilichowski, E., Horneff, G., Kozich, V. (2005). cblE type of homocystinuria due to methionine synthase reductase deficiency: functional correction by minigene expression. Hum. Mutat., 25, 239-247.

Zhang, X.H.F. and Chasin, L.A. (2004). Computational definition of sequence motifs governing constitutive exon splicing. Genes Dev., 18(11), 1241-1250.

Zhang, M.L., Lorson, C.L., Androphy, E.J., Zhou, J. (2001). An in vivo reporter system for measuring increased inclusion of exon 7 in SMN2 mRNA: potential therapy of SMA. Gene Ther., 8, 1532-1538.

Zhao, S. and Lee, E.Y. (1997). A protein phosphatase-1-binding motif identified by the panning of a random peptide display library. J. Biol. Chem., 272, 28368-28372.

Zhu, J., and Krainer, A.R. (2000). Pre-mRNA splicing in the absence of an SR protein RS domain. Genes Dev., 14, 3166-3178.

Zhu, J., Mayeda, A., and Krainer, A.R. (2001). Exon identity established through differential antagonism between exonic splicing silencer-bound hnRNP A1 and enhancer-bound SR proteins.Mol.Cell,8,1351-1361

CURRICULUM VITAE

Name: **Benderska Natalya**
Date of Birth: **28.05.1977**
Place of Birth: **Odessa, Ukraine**

Education

2002-2008	Friedrich-Alexander-University, Erlangen, Germany Graduate Student in Prof. Dr. Stefan Stamm's lab
1998-1999	Odessa National University, Odessa, Ukraine Master of Biology (Diploma), Diploma thesis: "The activity of proteolytic enzymes at breaches of protein exchange in organism" Advisors: Dr. Irina Vovchuk, Prof. Dr. Leonid Karpov
1994-1998	Odessa National University, Odessa, Ukraine Bachelor of Science, Biology
1984-1994	Novosilska Middle School, Chmeljnucka obl., Ukraine

Research Experience

2002-2008	Friedrich-Alexander University Erlangen-Nürnberg, Germany Research for PhD thesis
1999-2002	Department of Biochemistry, Odessa National University, Ukraine Assistant engineer
1998-1999	National Center of Molecular Biology and Biotechnology, Ukraine Technician in Prof. Dr. Yurij Sivolap lab

Awards received

11/2001	Odessa National University, Odessa, Ukraine and Biological Research Center, Szeged, Hungary short-term fellowship

Publications

- **Benderska N.**, Hui J., Novoyatleva T., Stamm S. (2008) Regulation of alternative splicing through cAMP-dependent phosphorylation of Tra2-beta1 splice factor. (manuscript in preparation)
- Heinrich B., Raitskin O., Hiller M., **Benderska N.,** Hartmann A., Bracco L., Elliott D., Ben Ari S., Soreq H., Sperling J., Sperling R. and Stamm S. (2008) hnRNP G regulates splice site selection by binding to CC(A/C) rich regions in pre mRNA. (submitted)
- Novoyatleva, T., Heinrich, B., Tang, Y., **Benderska, N.,** Butchbach, M., Lorson, C., Lorson, M., Ben-Dov, C., Fehlbaum, P., Bracco, L., Burghes, A, Bollen, M. and Stamm, S. (2008) Protein phosphatase 1 binds to the RNA recognition motif of several splicing factors and regulates alternative pre-mRNA processing. *Hum Mol Genet* 17(1):52-70.

- Tang, Y., Novoyatleva, T., **Benderska, N**., Kishore, S., Thanaraj, T.A. and Stamm, S. (2004) Analysis of alternative splicing in vivo using minigenes. In Westhof, Bindereif, Schön and Hartmann (eds.), *Handbook of RNA Biochemistry*. Wiley-VCH:755-782.
- Stoss, O., Novoyatleva, T., Gencheva, M., Olbrich, M., **Benderska**, **N**., Stamm, S**.** (2004) p59(fyn)-mediated phosphorylation regulates the activity of the tissue-specific splicing factor rSLM-1. *Mol Cell Neurosci* 27(1):8-21.
- Rafalska, I., Zhang, Z., **Benderska, N**., Wollf, H., Hartmann, AM., Brack-Werner, R., Stamm, S. (2004) The intranuclear localization and function of YT521-B is regulated by tyrosine phosphorylation. *Hum Mol Genet* 13(15):1535-49.
- Vovchuk, IL., Dyzyk, F., Anufriev, S., Chernadchuk, S., **Benderskaia**, **N**., Motruk, N. (2001) The blood serum peptidhydrolase activity in women with neoplastic diseases of the endometrium. *The Questions of Medical Chemistry*. 47(1):98-102. (in Russian)
- Vovchuk, IL., **Benderskaia**, **N**., Chernadchuk, S., Motruk, N. (2001) The blood serum proteinases of women with malignant and nonmalignant tumors of reproductive organs. *Odessa National University Herald*. 6(1):5-10. (in Russian)
- **Benderska, N.,** Novoyatleva, T., Stamm, S. Regulation of alternative splicing through cAMP-dependent phosphorylation. 2-nd Symposium on Alternative Transcript Diversity II-Biology and Therapeutics. EMBL, Heidelberg, Germany, 21-23 March 2006, p.35 (conference materials)
- Novoyatleva, T., **Benderska, N.,** Heinrich, B., Stamm, S. Tra2-beta1 phosphorylation-dependent regulation of SMN2 exon 7. 10-th Annual International Spinal Muscular Atrophy Research Group Meeting. Montreal, Canada, June 10-12, 2006, p.37 (conference materials)
- Novoyatleva, T., **Benderska, N.,** Stoss, O., Gencheva, M., Stamm, S. Localisation and functional differences of selected STAR family proteins rSLM-1 and rSLM-2 and their varied roles in alternative splicing. Conferences Jacques-Monod: Regulation of pre-mRNA splicing and human disease, Aussois, France, 27 April-1 May 2005, p.101 (conference materials)
- Novoyatleva, T., Gencheva, M., **Benderska, N.,** Koecher T., Stamm, S. Tyrosin phosphorylation influences regulation of alternative splicing by Sam68, rSLM-1 and rSLM-2. RNA 8-th Annual Meeting of the RNA Society, Vienna, Austria, July 1-6, 2003, p.105 (conference materials)