Regulation of alternative splice site selection by reversible protein phosphorylation

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To my parents

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ABBREVIATIONS

AKAP	Protein kinase A anchoring protein
AMP	adenosine mono phosphate
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	cycline adenosine mono phosphate
CBs	Cajal bodies
Cdk	cyclin dependent kinase (2 or 5)
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
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
DARRP32	dopamine- and cAMP-regulated phosphoprotein 32 kDa
dH2O	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
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
G-Proteins	guanosine triphosphate binding protein
GST	glutathione S-transferase
HDAC	histone deacetylase
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HLA	histocompatibility leukocyte antigens
hnRNP	heterogenous nuclear ribonucleoprotein

HOX11	Homeodomain transcription factor
HU	Hydroxyurea
IPTG	isopropyl-D-1-thiogalactopyranoside
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
kDa	kilodalton
KH	domain hnRNP K homology domain
mGluR7b	metabotropic glutamate receptor
MEL	murine erythroleukaemia kinase
mRNA	messenger RNA
NaB	sodium butyrate
NE	nuclear extract
Nek2	NIMA related protein kinase 2
NIPP1	nuclear inhibitor of protein phosphatase 1
NMD	nonsense-mediated decay
NMDAR1	N-methyl-D-aspartate receptor 1
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
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)
Sam68	Src associated in mitosis 68kDa
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	Sam68 like molecule (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
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
TOES	targeted oligonucleotide enhancer of splicing
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
VPA	valproic acid
WW domain	two highly conserved tryptophans
YTH domain	YT521-B homology domain

ZUSAMMENFASSUNG

Spleissen ist der Prozess, bei dem aus einer prä-Boten-RNA (prä-messenger RNA, prä-mRNA) Introns entfernt und Exons verknüpft werden. Es ist ein wesentlicher Schritt bei der Prozessierung von prä-mRNA, um reife mRNA zu bilden. Microarray-Analysen deuten darauf hin, dass etwa 75 % der menschlichen Gene Transkripte bilden, die alternativ gespleißt sind. Alternatives Spleißen ist einer der Hauptmechanismen, um aus einer begrenzten Anzahl von Genen eine große Anzahl von Protein-Isoformen zu erzeugen. Die genaue Regulierung der Auswahl alternativer Spleißstellen wird durch Verknüpfung einer Reihe von RNA:RNA, RNA:Protein und Protein:Protein Interaktionen erreicht.

Aufgrund der Bedeutung von Spleißfaktoren haben wir die Rolle der zwei Faktoren rSLM-1 und Tra2-beta1 bei der Auswahl von Spleißstellen untersucht. Wir konzentrierten uns auf deren Regulation durch reversible Phosphorylierung, die als Grundlage für die Auswahl alternativer Spleißstellen über Signaltransduktionswege dient.

Der erste Teil der Arbeit behandelt rSLM-1 und rSLM-2, Mitglieder der STAR (Signal Transduction and Activation of RNA) Familie. Diese Proteine interagieren mit einigen Spleißfaktoren und ändern die Auswahl von Spleißstellen konzentrationsabhängig. Die Proteine zeigen unterschiedliche gewebespezifische Expression und charakterisieren sich durch nicht-überlappende Expression im Gehirn.
Beide Proteine sind Substrate für die Phosphorylierung durch einige Nicht-Rezeptor Tyrosin-Kinasen. Wir zeigen, dass Tyrosinphosphorylierung durch die Kinase $p59^{fyn}$ die Aktivierung des Spleißfaktors rSLM-1 reguliert. Dies weist darauf hin, dass der Spleißfaktor rSLM-1 als wichtiges Bindeglied zwischen alternativem Spleißen und Signaltransduktion dienen könnte.

Im zweiten Teil wurde das humane SR-ähnliche Protein Tra2-beta1 untersucht. Durch ein Proteinsequenz-Alignment wurde ein in der Evolution konserviertes Bindemotiv für Protein Phosphatase 1 im beta4-Faltblatt des RRM erkennbar, was auf eine neue Funktion dieses Motivs in neun RRMs spleißregulierender Proteine hinweist. Wir zeigen, dass Tra2-beta1 durch dieses Motiv direkt an PP1 bindet. PP1 dephosphoryliert Tra2-beta1 sowohl *in vivo* als auch *in vitro*. Mutationen in dem Motiv heben die Fähigkeit von Tra2-beta1 auf, die Spleißstellen-Auswahl konzentrationsabhängig zu beeinflussen.

Vor Kurzem wurde gezeigt, dass die Überexpression des SR-ähnlichen Spleißfaktors Tra2-beta1 den Einbau von Exon 7 im Gen SMN (survival of motor neuron)

anregt. Der homozygote Verlust des SMN1 Gens ist die Ursache von Spinaler Muskelatrophie (SMA), einer fortschreitenden Degenaration der Motoneuronen. Reversible Phosphorylierung von Tra2-beta1 an Serin/Threonin-Resten könnte eine wichtige Auswirkung auf die Auswahl von Spleißstellen haben. Tatsächlich regt die Hemmung der Dephosphorylierung mit PP1-Inhibitoren wie z. B. Cantharidin oder Tautomyzin den Einbau von Exon 7 an und fördert die Bildung von SMN Protein in Patienten-Fibroblasten und Maus-Modellen. Demzufolge könnte die Anwendung von PP1-Inhibitoren als ein nützlicher Ansatz dienen, neue therapeutische Strategien zur Behandlung von SMA zu entwickeln.

ABSTRACT

Splicing is the process that removes introns and joins exons from pre-mesenger RNA (pre-mRNA). It is an essential step in pre-mRNA processing that form the mature RNA. Microarray data indicates that approximately 75% of human genes produce transcripts that are alternatively spliced. Alternative splicing is one of the major mechanisms that ultimately generate high number of protein isoforms from a limited number of genes. The proper catalysis and regulation of alternative splice site selection is achieved by coordinated associations of a number of RNA: RNA, RNA: protein and protein: protein interactions.

Given the importance of splicing factors, we investigated the role of rSLM-1, rSLM-2 and Tra2-beta1 in splice site selection. We focused on their regulation by reversible phosphorylation, which is the basis of alternative splice site selection by signal transduction pathways.

This study focusses on control of splice site selection through a number of signal transduction pathways. The first part of this work concentrates on members of STAR (Signal Transduction and Activation of RNA) family, rSLM-1 and rSLM-2. These proteins were found to interact with several splicing factors and changed splice site selection in a concentration dependent manner. The proteins show different tissue specific expression and are characterized by non-overlapping expression in the brain. Both proteins are substrates of phosphorylation by several non-receptor tyrosine kinases. We demonstrate that tyrosine phosphorylation by $p59^{fyn}$ kinase regulates the activity of rSLM-1. This suggests that splicing factor rSLM-1 could serve as an important link between alternative splicing and signal transduction.

In the second part the human SR-like protein, Tra2-beta1 was investigated. Protein sequence alignment revealed a evolutionary conserved Protein Phosphatase1 binding motif in the beta4 sheet of the RRM, pointing out the new function of this motif in nine RRMs of splicing regulatory proteins. We demonstrated that Tra2-beta1 directly binds to PP1 through this motif. PP1 dephosphorylates Tra2-beta1 both *in vivo* and *in vitro*. Mutations in this motif abolish the ability of Tra2-beta1 to influence splice site selection in a concentration dependent manner.

It was previously shown that overexpression of SR-like splicing factor Tra2-beta1 can stimulate exon 7 usage of SMN (survival of motor neuron) gene. The

homozygous loss of SMN1 gene is a cause of degenerative motor neuron disorder, Spinal Muscular Atrophy (SMA). Reversible phosphorylation of Tra2-beta1 at serine/threonine residues could have an important impact on the splice site selection. In fact, blocking the dephosphorylation with PP1 inhibitors such as cantharidin or tautomycin stimulates exon 7 inclusion and promotes SMN protein formation in patient fibroblasts. As a consequence, the usage of PP1 inhibitors could serve as one of the useful approaches in developing new therapeutical strategies for the treatment of SMA.

1. INTRODUCTION

The sequencing of several genomes showed that the large proteomic complexity is achieved with a limited number of protein-coding genes. These findings reveal the importance of post-transcriptional mechanisms. Eukaryotic messenger RNA undergoes a series of processing events and all primary transcripts and are altered by one or more processing steps. These events include capping of the 5' ends, polyadenylation of the 3'ends (Shatkin A.J. and Manley J.L., 2000), splicing, excision the intervening non-coding sequences (introns) from the pre- mRNA and joining the flanking coding regions (exons) (Blencowe B.J. et al., 1994; Neugebauer K.M., 2002) and editing of the nucleotide sequence-covalent modification of the bases (Benne R., 1996; Wedekind J.E. et al., 2003). Mature messenger RNA (mRNA) is then exported through nuclear pore complex (NPC) to the cytoplasm for translation. These processes are crucial for the eukaryotic gene expression as they remove the intervening noncoding sequences from the pre-mRNA and stabilize it.

One of the most important roles in the generation of protein isoforms from a limited number of genes is fullfiled with splicing, which significantly increases the amount of the mRNAs. Alternative splicing is the inclusion of alternative exons or introns from the pre-mRNAs into the mature RNA. Alternative splicing switches gene expression on and off by the introduction of premature stop codons. A recent study of the human genome, which used exon-exon junction microarrays to analyze RNA samples from more than 50 tissues and cell lines, concluded that transcripts from at least 74% of all multi-exon genes are alternatively spliced (Jonson J.M. et al., 2003). Therefore, alternative splicing allows the existence of large proteomics complexity from a limited number of genes.

The usage of alternative exons often changes during development or in response to outside stimuli. However, the pathways that transduce the signal to the splicing machinery remain to be established. To develope therapeutic strategies against human disorders caused by wrong splice site selection, an understanding of these signal transduction pathways is necessary.

The aim of this study to understand the mechanisms of alternative splice site selection by focusing on reversible phosphorylation of splicing factors involved in this process.

1.1. Constitutive splicing and the basal splicing machinery

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 removed are named introns.

The typical human gene contains an average of 8.8 exons. Exons average 145 nucleotides (nt) in length, whereas intron measure more than 10 times this size and can be even much larger (Lander E.S. et al., 2001). 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).

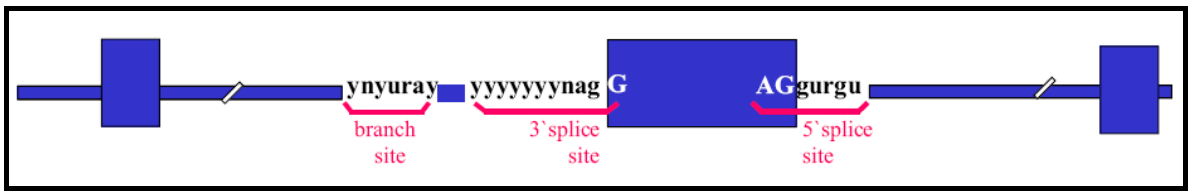

Figure 1. The classical and auxiliary splicing signals. (n=G, A, U or C; y=pyrimidine; r=purine). Introns are indicated as thin lines, exons as boxes. Only the sites around the central cassette exon are shown. The figure is adapted from Faustino N. and Cooper T., 2003.

The major class of human introns (>99%) contains the classical GT-AG splicing signals 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.

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 (Jackson I.J. in 1991 and Hall S.L. and Padgett R.A. in 1994). These introns contain AT and AC at the 5' and 3' splice sites, respectively. This type of introns was named U12 introns. U12 introns were recognized by different spliceosome nuclear RNA components (Hall S.L. and

Padgett R.A., 1996; Tarn W.Y. and Steitz J.A., 1996a, 1996b; Tarn W.Y. and Steitz J.A., 1997). The U12 type of introns is present in the nuclei of vertebrates, insects, and plants (Wu J.Y. 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 M. et al., 2001).

1.1.2. Mode of alternative splicing

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 and retained introns (summarized in Figure 2).

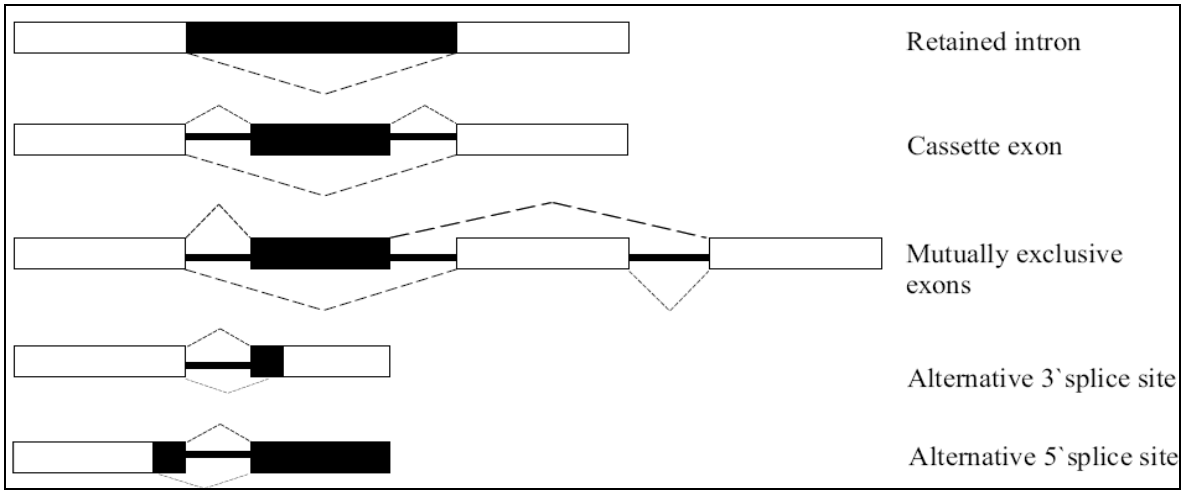

Figure 2. Types of alternative exons. Exons are indicated as boxes, introns as horizontal lines. Black color indicates alternatively spliced exons, flanking constitutive exons are shown in white.

Internal alternative cassette exons belong to the largest group. 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 Z. et al., 2001; Okazaki Y. et al., 2002).

1.1.3. Spliceosome commitment

One of the most intriguing questions is how the 5' and 3' splice sites are selected and paired together within large pre-mRNA sequences. The 5', 3' splice site and the branch point are characterized by short degenerate regulatory sequences that determine

an alternative exon. The formation of the spliceosome assembly is a highly dynamic process, which includes formation and disruption of RNA: RNA, RNA: protein and protein: protein interactions within the spliceosome. Recognition of both splice sites occurs during the spliceosome assembly by specific interactions with different components of the assembly (Burge C.B. et al., 1999; Du H. and Rosbash M., 2002; Lallena M.J. et al., 2002; Liu S. et al., 2004). These include interactions with cis-acting regulatory elements (cis-elements) located on the pre-mRNA, which are necessary for proper recognition of the exon and also a number of protein factors named trans-acting factors, that control the alternative splicing of the specific pre-mRNAs.

Trans-acting factors contain RNA binding domains and various protein: protein interaction domains, allowing the interaction of individual members of these protein families. As a result, complex protein networks on the pre-mRNA form around exons or introns and aid in their recognition by binding to components of the spliceosome. The individual interactions between cis- and trans-elements involved in splice site selection are weak. Only through several such interactions forming either across an intron or an exon the recognition is achieved. 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 D.L., 1995).

The pattern of alternative splicing can be regulated upon external stimuli or stress. For example, serum deprivation alters usage of the serine/arginine-rich protein 20 (SRp20) exon 4 (Jumaa H. et al., 1997). Neuronal activity changes the alternative splicing pattern of (CLB) clathrin light chain B, the NMDAR1 (N-methyl-D-aspartate receptor 1) receptor, and c-fos (Daoud R. et al., 1999). Stress causes changes in splicing patterns of potassium channels (Xie J. and Black D.L., 2001; Xie J. and McCobb D.P., 1998) and of acetylcholine esterase (Kaufer D. et al., 1998; Meshorer E. et al., 2002).

Many aspects of protein functions like ligand affinity, signaling capabilities of receptors, intracellular localisation of proteins, ion channel properties and many others are regulated by alternative splicing.

Much of the splicing regulation seems to occur at an early step of pre-mRNA recruitment to the spliceosomal assembly pathway (see Figure 3).

1.1.4. Action of splicing factors

Modulation of splicing reactions is achieved by the action of splicing trans-acting factors that recognize an arrangement of positive (splicing enhancers) and/or negative (splicing silencers) cis-acting sequence elements. These elements can be either exonic (ESEs/ESSs, exonic splicing enhancers/silencers) or intronic (ISE/ISS, intronic splicing enhancers/silencers).

They are short (5-8 nt) and degenerate, i.e. they follow only loose consensus sequences. This degeneracy prevents them from interfering with the coding capacity of genes.

These auxiliary elements are commonly required for efficient splicing of constitutive and alternative exons (Ladd A.N. and Cooper T.A., 2002; Novoyatleva T. et al., 2006). The cis-acting enhancers can help recruit the essential splicing factors, in case when the distances between splice sites are not equal, or when splice sites are weak (Black D.L., 1995).

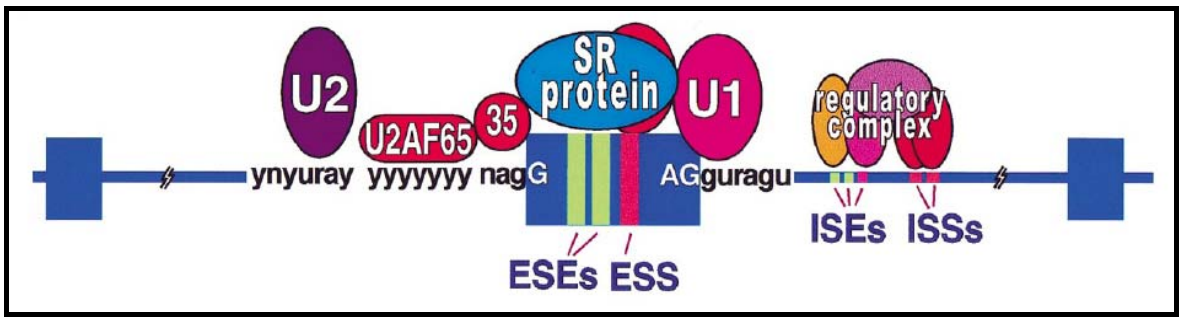

Figure 3. Classical and auxiliary splicing elements and binding factors. Factors that bind classical and auxiliary splicing elements are indicated as circles and ellipses. Exons are indicated as boxes, introns as thin lines. Auxiliary enhancer elements within exons or introns (ESEs and ISEs) are indicated 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 M. et al.,

1994), and determines whether they activate 5' or 3' splice sites (Heinrichs V. et al., 1998). It can even transform them into negative regulatory elements (Kanopka A. et al., 1996). A number of special web-based programs allow for searching ESEs on sequences. On the Table 2 there are some available tools for searching of ESE/ESS motifs.

Table 2. Tools for searching of ESEs and ESS motifs.

Name	URL	Reference
ESE finder	http://rulai.cshl.edu/tools/ESE/	Cartegni L. et al., 2003
Rescue ESE	http://genes.mit.edu/burgelab/rescue-ese/	Fairbrother W. G. et al., 2004
PESXs server	http://cubweb.biology.columbia.edu/pesx/	Zhang X. H. and Chasin L. A., 2004
ESR search	http://ast.bioinfo.tau.ac.il/ESR.htm	Goren A. et al., 2006

There also existing many intronic elements which serve to modulate cell-specific use of alternative exons by binding multicomponent regulatory complexes.

Proteins binding to enhancer or silencer sequences and modulating the alternative splice site selection can be subdivided into two major groups: members of the SR family of proteins (Manley J. and Tacke R., 1996) and hnRNPs (Weighardt F. et al., 1996).

1.1.5. The SR and SR-related family of proteins

SR group of proteins are essential splicing factors (Manley J. and Tacke R., 1996). They belong to a family of highly conserved proteins in metazoans. These proteins are required for constitutive splicing and also for the regulation of alternative splice site selection (Fu X.D. et al., 1995; Graveley B.R. et al, 2000). SR proteins have a modular structure, consisting of one or two copies of N-terminal RNA-binding domains (RNA-recognition motif, RRM), with RNA binding functions and a C-terminal RS domain. The RS domain of these proteins is rich in alternating serine and arginine residues. This helps in mediating the protein: protein interactions in the spliceosome. The RS domain contains multiple serine phosphorylation sites. Serine phosphorylation is important in the regulation of activities and the localization of SR proteins (Sanford J. et al., 2003). The RS domain of some SR proteins acts further as a nuclear localization signal that mediates the interaction between SR protein and nuclear import receptor (transportin-SR), which defines

nucleocytoplasmic shuttling of SR proteins (Caceres J.F. et al., 1997; Kataoka N. et al., 1999; Lai M.C. et al., 2000). The SR-related proteins (SRrps) belong to another class of RS domain containing proteins. Most of these proteins contain RRMs. Among them are U1-70K protein, both subunits of U2AF, SRm 160/300 (two SR-related nuclear matrix proteins of 160 and 300 kDa), as well as alternative splicing regulators such as Tra and Tra2, which are involved in splice site selection.

1.1.6. Role of SR and SR related proteins in constitutive and alternative splicing

SR and SR-related proteins help in splice site selection and spliceosome assembly by interacting with other splicing factors, via their RS domain. These proteins are recruiting components of the core splicing apparatus to promote splice site pairing (Tacke R., 1999; Wu J.Y., 1993). Therefore these proteins function as adapters between the pre-mRNA and the basal splicing machinery. In addition to pre-mRNA processing, these sequence-specific RNA binding proteins play a significant role in mRNA transport, stability, and translation. Serine phosphorylation of the RS domain regulates the activities and localization of SR proteins (reviewed by Sanford J.R. et al., 2003). It was also shown that RS domain could directly interact both with the pre-mRNA branch point and the 5' splice site (Shen H. et al., 2004). One of the function of SR family and SR-related proteins is to activate suboptimal adjacent splice sites (Blencowe B.J. et al., 2000) in alternative splicing. It has been proposed that the function of SR proteins is to stimulate the recognition of weak upstream 3' splice sites, by recruiting U2AF, or to facilitate U1 snRNP binding to the 5' splice site (reviewed by Black D.L., 2003). In addition, certain SR proteins have antagonistic effects on alternative splicing, which was shown for the regulation of β-tropomyosin by opposing action SF2/ASF and SC35 (Gallego M.E. et al., 1997).

SR and SR-related proteins interact with multiple cis-acting elements located within exonic or intronic sequences. The selection of splice sites therefore relies on the interaction of SR proteins with these elements, and subsequent participation at multiple steps in the assembly of the spliceosome. Remarkably, the effects of cis-acting elements are position-dependent. SR-protein-binding sites within ESEs exert a positive effect on splice site selection. Interactions between SR proteins and ESEs lead to recruitment and stabilization of the binding of U1 snRNP and U2AF to the 5' and 3' splice sites. This

process is known as exon definition (Berget S. et al., 1990; Boukis L.A. et al., 2004). It is schematically depicted in Figure 4A.

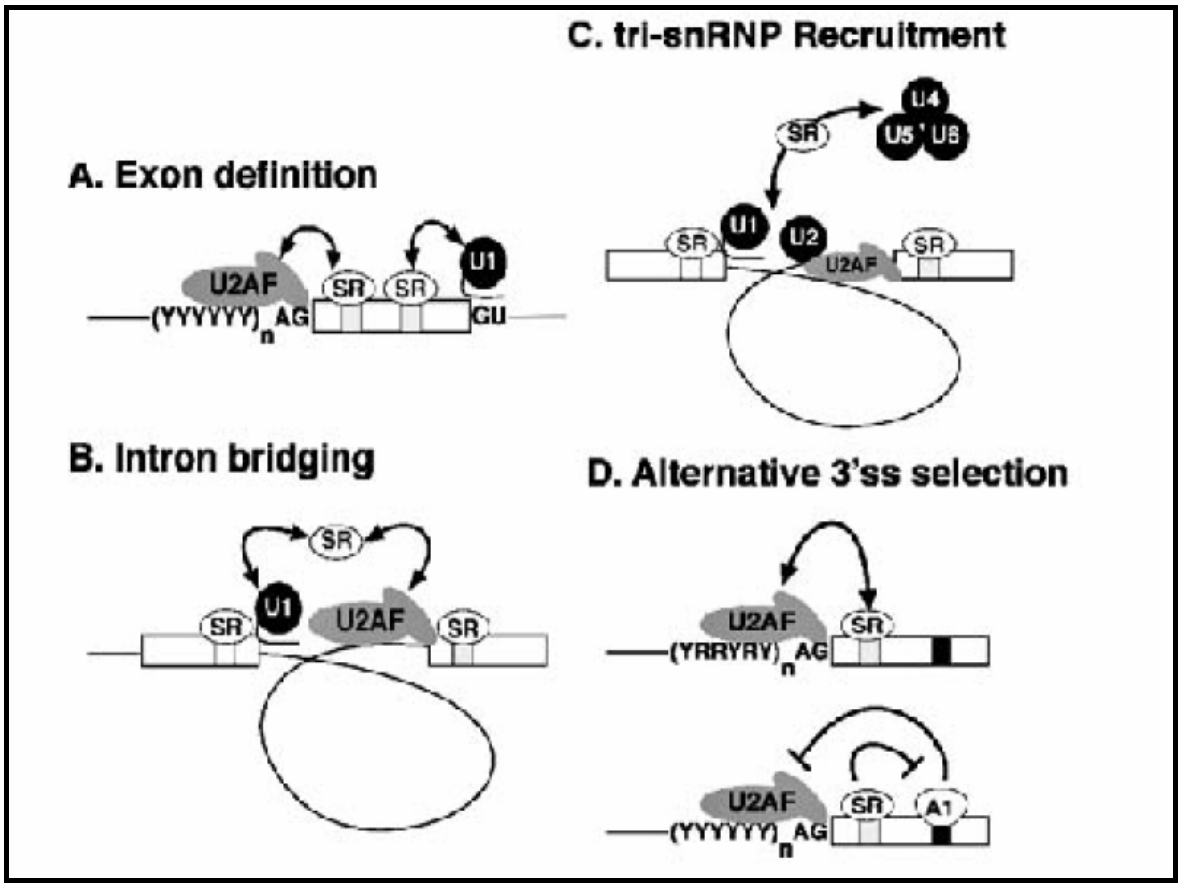

Figure 4. Roles of SR proteins in spliceosome assembly. (A) U2AF, marked as a grey oval, at an upstream 3' splice site and U1 snRNP , which is indicated as black circle, at a downstream 5' splice site. The binding to RNA is facilitated by SR proteins bound to ESEs (light grey boxes). The polypyrimidine 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).

SR proteins form a number of protein: protein interactions across introns to juxtapose the 5' and 3' splice sites early in spliceosome assembly (Figure 4B). This results in the formation of protein complexes across introns. The formation of these intron bridging complexes is mediated by simultaneous interactions of SR proteins with the U1 snRNP-associated 70kDa protein (U1-70K) at the 5' splice site, and with the 35kDa

subunit of U2AF (U2AF35) at the 3' splice site. The interactions occur via the RS domains of SR proteins. After the formation of the E complex, SR proteins help in the recruitment of the U4/U6·U5 tri-snRNP to the pre-spliceosome (Roscigno R.F. and Garcia-Blanco M.A., 1995). This step is shown in Figure 4C. SR proteins can compensate for a weak polypyrimidine tract by recruiting U2AF (Figure 4D, upper panel), while interacting with ESEs. However, SR proteins bound to ESEs promote alternative exon inclusion by antagonizing the negative activity of hnRNPs (heterogeneous nuclear RNPs), such as hnRNP A1 (Blencowe B.J. 2000; Hastings M.L. and Krainer A.R. 2001), (shown in Figure 5D, lower panel).

In addition SR and SR related proteins can displace factors from the pre-mRNA that inhibit splicing by competing for binding sites on the target RNA (Eperon I.C. et al., 2000; Zhu J. et al., 2001). This was demonstrated *in vitro* on substrates that have relatively strong 3' splice sites which do not depend on the SR repeat (Zhu J. and Krainer A., 2000).

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 of proteins are heterogenous nuclear ribonucleoprotein proteins (hnRNPs).

1.1.7. hnRNPs

hnRNPs were first described as a group of nuclear RNA-binding proteins. hnRNPs belong to a highly abundant family of proteins that associate with heterogeneous nuclear pre-mRNAs during transcription and remain associated with mRNAs after splicing is completed (Nakielny S. et al., 1997).

In addition these proteins are shown to be involved in the biogenesis and nucleocytoplasmic transport of mRNA (reviewed by Dreyfuss G. 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.

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 β-globin pre-mRNAs (Caceres J. et al., 1994; Mayeda A. and Krainer A., 1992). In addition, hnRNPA1 controls inclusion of exon 7b of its own

transcript (Blanchette M. and Chabot B., 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). PTB was discovered as a protein that bound the U-rich polypyrimidine tract of several introns (Garcia-Blanco M.A et al., 1989). PTB mediates silencing of exons, by binding to a huge number of intronic splicing silencers of alternatively spliced pre-mRNAs (reviewed by Wagner E.J. and Garcia-Blanco M. A., 2001). This suggests that the protein functions as a global repressor of regulated exons.

1.1.8. Human Transformer-2 beta

The human Transformer-2 beta1 protein is a homologue of the *Drosophila melanogaster* sex determination factor Transformer-2. Together with Transformer, this protein regulates sex-determination in somatic cells though a cascade of alternative splicing events (Nayler O. et al., 1998; Dauwalder B. et al., 1996). Drosophila Tra2 has another mammalian homologue, Tra2-alpha (Beil B. et al., 1997; Dauwalder B. et al., 1996; Matsuo N. et al., 1995; Segade F. et al., 1996). All three proteins: Tra2, Tra2-beta and Tra2-alpha share a similar structure consisting of two RS domains flanking a central RRM. This suggests that the tra-2 products of flies and humans have similar molecular functions.

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 O. et al., 1998; Daoud R. 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 B. et al., 1997; Amrein H. et al., 1994; Nayler O. et al., 1998).

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 5). 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 R. 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 O. et al., 1998). They differ in the presence of the first RS domain (Figure 5). 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 R. et al., 1999; Stoilov P. et al., 2004), but their generation through alternative splicing is regulated by external stimuli, such as T-cell stimulation (Beil B. et al., 1997) and neuronal activity (Daoud R. et al., 1999).

The hTra-2beta1 protein is a member of the SR related family of proteins. The protein was extensively characterized. Human Tra2-beta1 is a nuclear protein. It localizes in speckles, and interacts with chromatin organizing proteins. The splicing factor Tra2-beta1 is upregulated in breast cancer and regulates alternative splicing of the CD44 gene (Watermann D.O., et al., 2006).

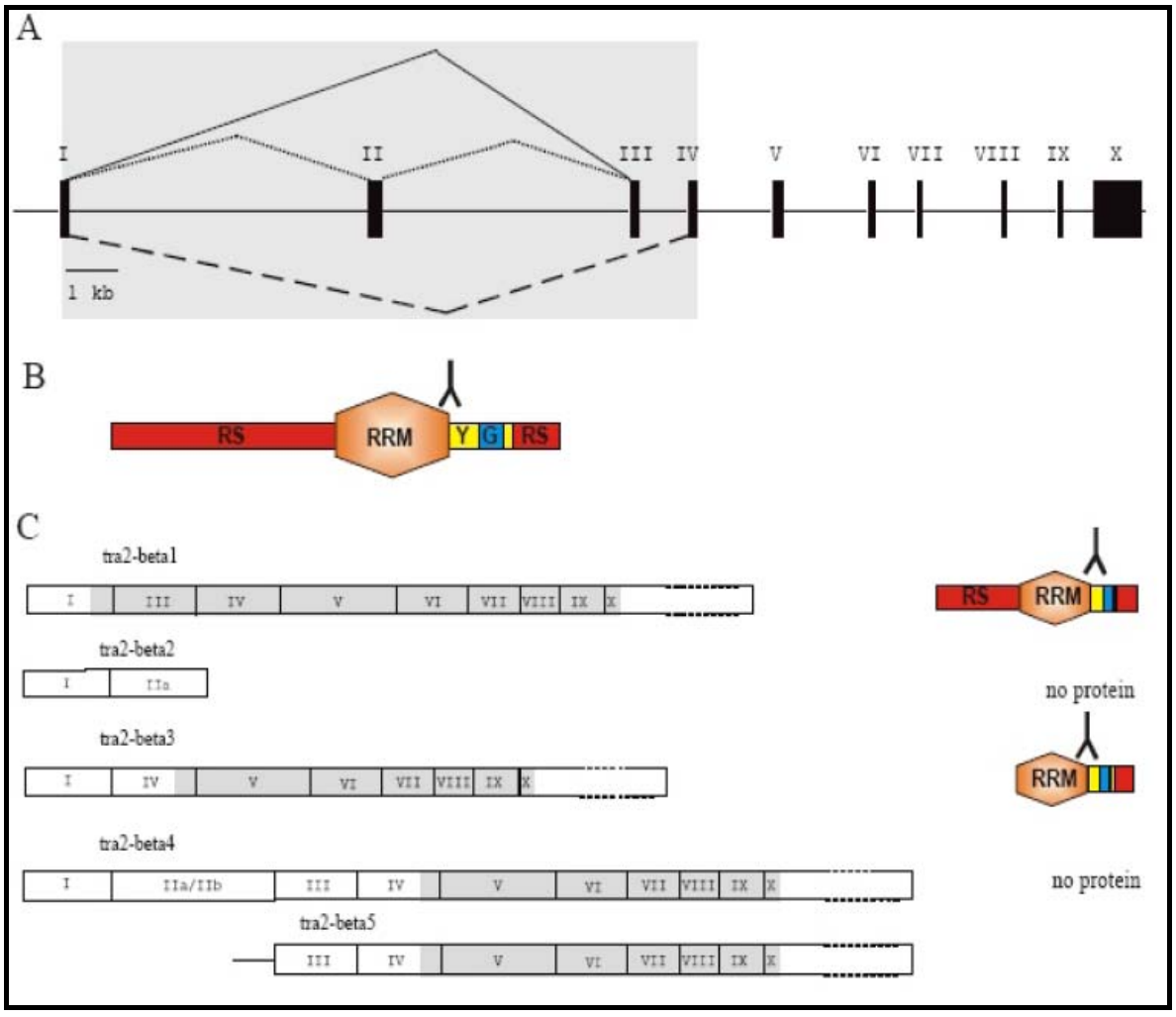

Figure 5. The tra2-beta gene structure. (A) The exon-intron structure is drawn to scale. Exons are shown as black boxes, introns as lines. The shaded region marks the sequence of Tra2-beta minigene. (B) Structure of the protein. Tra2-beta1 protein consists of a RNA recognition motif (RRM), flanked by 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 P. et al., 2004.

The protein changes splicing pattern of other genes (Daoud R. 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 P. et al., 2004). It was demonstrated that presence of the 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 P. 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.

1.1.9. SLM-1 and SLM-2 are Sam68 like mammalian proteins

The SLM-1 and SLM-2 proteins belong to STAR (Signal Transduction and Activation of RNA) family of proteins (Vernet C. and Artzt K. 1997), also called GSG (GRP33, SAM68, GLD-1) proteins (Jones A.R. and Schedl T. 1995; Chen T. et al., 1999). These are nuclear RNA-binding proteins that share an extended hnRNP K homology domain (KH domain), which was firstly 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 G. 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 M. 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 M. et al., 1999). All the members of STAR family are methylated *in vivo* (Cote J. et al., 2003).

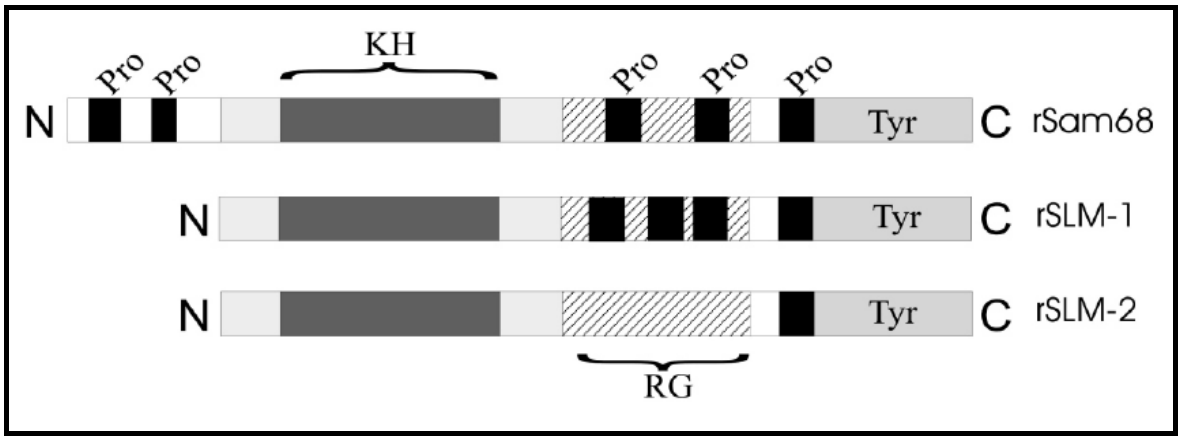

Figure 6. The domain structure comparison of rSam68, rSLM-1 and rSLM-2. Pro: proline-rich regions; KH: hnRNP K homology domain; RG: arginine/glycine-rich region; Tyr: tyrosine-rich region.

Using the scaffold attachment factor B protein, as bait in a yeast two hybrid screen with a rat brain library (Nayler O. et al., 1998; Weighardt F. et al., 1999) the Sam68-like mammalian protein rSLM-2 was isolated (Stoss O. et al., 2001). In addition cDNAs bearing high homology to the previously reported SLM-1 (Di Fruscio M. et al., 1999) and Sam68 (Richard S. 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 O. et al., 2001). It was demonstrated that Sam68 plays an important role in alternative splicing (Matter N. et al., 2002), cell cycle regulation (Barlat I. et al., 1996; Taylor S.J. et al., 1995) and RNA export (Reddy P. et al., 2000). The extended N-terminus of rSam68 contains several ERK (extracellular receptor kinase) phosphorylation sites (Matter N. et al., 2002). Its function is influenced by ERK-mediated threonine phosphorylation (Matter N. et al., 2002) and Sik/BRK-mediated tyrosine phosphorylation (Coyle J.H. et al., 2003). Figure 6 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.

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

1.1.10. Coupling of splicing and transcription

Many studies published in recent years provide evidence for a coupling of transcription and pre-mRNA processing. It was first shown in 2002 that splicing is linked to mRNA export in metazoans, where spliced mRNAs are assembled into a distinct 'spliced mRNP' complex that targets the mRNA for export (see review Reed R. and Hurt E. 2002).

Soon it was described that the promoter of the fibronectin gene controls the alternative splice site selection (Kadener S. et al., 2002). Further studies with cystic fibrosis, CD44 and CGRP genes strongly support that transcription is coupled to pre-mRNA splicing (Pagani F. et al., 2003). The model explaining these results is that the promoter itself is responsible for recruiting splicing factors to the sites of transcription, possibly through interaction of transcription factors with promoter or the transcriptional enhancers. The finding that p52, a transcriptional co-activator, interacts with SF2/ASF stimulating pre-mRNA splicing is consistent with this model (Ge H. et al., 1998). Furthermore, some proteins could have a dual function, acting in both processes.

Another model describes the involvement of modulation of RNA polymerase II elongation rate (Nogues G. et al., 2003). The model proposes that the responsiveness of exon skipping to the elongation correlates inversely with 3' splice site strength. Mainly, when RNA polymerase II elongates poorly, the splicing machinery recognizes alternative exon better. When RNA polymerase II is highly processive, the strong splice site emerges sooner thus enhancing exon skipping. The model is supported by findings that cis- and trans-acting factors that modulate RNA polymerase II elongation on a particular template also provoke changes in the alternative splicing balance of the encoded mRNAs (Kornblihtt A.R. et al., 2004).

Transcriptional activation of RNA polymerase II genes causes an association of SR proteins such as SF2/ASF to sites of transcription. If RNA polymerase II has a truncated C-terminal domain (CTD), relocalisation of SR proteins to sites of transcription does not occur (Misteli T. and Spector D. L., 1998). The CTD has a central role in linking mRNA synthesis with the splicing machinery.

Some other proteins, such as SAF-B, which mediate chromatin attachment to the nuclear matrix, have been implicated in the coupling of transcription and pre-mRNA splicing (Nayler O. et al., 1998). The RNA polymerase itself could be responsible for recruiting these proteins, perhaps through its CTD. Some proteins which possibly couple

transcription and splicing, bind to phosphorylated CTD through their WW or FF domains. WW domains contain two conserved tryptophan residues for binding proline-rich peptides, whereas FF domains contain two conserved phenylalanine residues which bind to acidic or phosphorylated peptide motifs. For example, the human transcription factor CA150, a regulator of RNA polymerase II activity, consists of three WW and six FF domains, which can associate with the pre-mRNA splicing factor SF1 and RNA polymerase II, respectively (Carty S.M. et al., 2000; Goldstrohm A.C., et al., 2001). Such interactions bridge splicing complexes to actively transcribing RNA polymerase II. However there are many other strong evidences supporting transcription being coupled to the spliceosome. For example, three elongation factors: RNA polymerase II transcriptional elongation factor (cyclin-dependent kinase 9/cyclin T), P-TEFb, HIV-1 Tat cellular coactivator, TAT-SF1 and transcriptional factor S-II, TFIIS are found to link splicing and transcription (reviewed by Maniatis T. and Reed R., 2002).

In summary, the tight and spatial coordinated connection between transcription by RNA Polymerase II and pre-mRNA splicing provide the proper processing of nascent pre-mRNAs.

1.2. Phosphorylation dependent control of the pre-mRNA splicing machinery

The components of the pre-mRNA splicing machinery undergo phosphorylation and dephosphorylation during the splicing process. Previously (Cao W. et al., 1997; Xiao S.H. and Manley J., 1997; Shi Y. et al., 2006) 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 S.H. and Manley J., 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 J.R. et al., 2005). Phosphorylation of SR proteins mediates their translocation from storage compartments, the nuclear speckles, to the sites of active transcription (Misteli T. et al., 1998; Wang J. et al., 1998). Therefore, the phosphorylation status of proteins play an important a role in several processes, like assembly of the

spliceosome, regulation of the splice site selection, and subcellular localization of splicing factors (reviewed by Soret J. and Tazi J., 2003).

The splicing dependent dephosphorylation of shuttling SR proteins was observed (Huang Y. et al., 2004; Lai M.C. and Tarn W.Y., 2004). It was demonstrated that hypophosphorylated SF2/ASF, a shuttling SR protein, binds the mRNA export receptor TAP and associates with mature mRNPs (Lai M.C. and Tarn W.Y., 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 could also lead to the 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 fraction (Rafalska I. et al., 2004).

Many examples demonstrated that phosphorylation of specific splicing factors changes splice site selection. One of the examples is phosphorylation of specific splicing factors by CDC2-Like Kinases (CLK1-4), which promotes exclusion of human Tau exon 10 (Hartmann A. et al., 2001). Exon 10 of human Tau microtubule-associated protein is known to be associated with frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) (Hutton M. et al., 1998; Poorkaj P. et al., 1998; Spillantini M.G. and Goedert M., 1998; Kowalska A. et al., 2002).

Another well studied example of phosphorylation-dependent regulation of splice site selection is the formation of variant CD44 isoforms during immune response (Weg-Remers et al., 2001). Activation of the Ras-Raf-MEK-ERK signaling pathway stimulates the exon v5 inclusion upon T-cell receptor stimulation. The similar effect of the enhanced ERK-mediated exon v5 inclusion could be observed after forced expression of Sam68 and phorbol-ester stimulation (Matter N. et al., 2002). Tyrosine phosphorylation of SLM-1 and SLM-2 (Sam68-like mammalian proteins) by BRK/Sik kinase led to inhibition of their RNA-binding activities (Haegebarth A. et al., 2004).

1.2.1. Protein Phosphatase 1

Recent research uncovered a large number of proteins in eukaryotic cells that undergo reversible phosphorylation mediated by multiple protein kinases and

phosphatases, which in turn modulate their biological activity (Hunter T., 1995). Protein phosphatases are classified into two major functional groups, protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases (PPs). Serine/threonine protein phosphorylation regulates numerous diverse functions. Among them neurotransmission, muscle contraction, glycogen synthesis, T-cell activation, neuronal plasticity and cell proliferation.

The group of serine/threonine phosphatases is divided into three families designated PPP, PPM and FCP.

Table 3. Classification of serine/threonine protein phosphatases.

Serine/Threonine Protein Phosphatases		
PPP	PPM	FCP
PP1, PP2A, PP4, PP2B, PP5, PP7	Mg^{2+} dependent PP2C	Mg^{2+} dependent FCP1

Multiple isoforms of PP1c (Protein Phosphatase 1 catalytic subunit) are encoded in most eukaryotes by multiple genes with the exception of *S. cerevisiae*, where only one gene Glc7 encodes PP1c (Stark M.J.R., 1996; Dombrádi V., 1997). GeneCards reveal that 21 isoforms of PP1 catalytic subunit are generated by alternative splicing (http://www.genecards.org/). So far only four mammalian isoforms of the PP1c gene have been extensively described. There are two alternatively spliced isofoms of PP1α: PP1α: and PP1α2 (Durfee T. et al., 1993; Yoshida K. et al., 1999), PP1β (which is known also as PP1δ) and two alternatively spliced forms of PP1γ: PP1γ1 and PP1γ2 (Cohen P., 1988; Dombradi V. et al., 1990; Sasaki K. et al., 1990).

Indeed PP1 and PP2A holoenzymes represent the major protein phosphatases both in number and importance that dephosphorylate serine/threonine residues (reviewed by Cohen P.T.W., 1997). The near-completion of the human genome sequence now allows the identification of almost all human protein kinases, which is about 1.7% of all human genes (Manning G. et al., 2002). Mammalian genomes encode in total approximately 100 protein tyrosine kinases and protein tyrosine phosphatases (Ceullemans H. and Bollen M., 2003) However, the number of protein serine/threonine phosphatases (~25) is much fewer than protein serine/threonine kinases (~400) (Plowman G.D. *et al.*, 1999). This is because 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. The catalytic core of PP1c is nearly identical for all isoforms

and very similar to catalytic subunits of PP2 A and PP2B (Andreassen P.R. et al., 1998; Goldberg J. et al., 1995). These enzymes, sharing a common catalytic core of 280 residues are most divergent within their noncatalytic N- and C-termini and are distinguished by their associated regulatory subunits to form a diverse variety of holoenzymes.

This is the reason why unlike serine/threonine kinases (Pinna L.A. and Ruzzene M., 1996) Protein Phosphatase 1 does not display obvious consensus sequence selectivity and dephosphorylates multiple substrates *in vivo* and *in vitro* (Pinna L.A. and Donella-Deana A., 1994). All the PP1 catalytic subunits in the cell are associated with various regulatory subunits forming heteromeric complexes (Wera S. and Hemmings B.A., 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 Z.H. et al., 1991; Stuart J.S. 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 PP1c contain an RVxF motif. This motif confirms to the consensus sequence [RK]x_{0-1}[VI]{P}[FW], where *x* could be any residue and {P}refers to any residue other than proline (Ceulemans H. et al., 2002; Egloff MP. et al.,1997; Zhao S. and Lee E.Y. 1997). Table 4 shows the classification of regulatory proteins possessing the RVxF binding site. Another type of PP1 binding motifs representing a new consensus sequence F-x-x-[RK]-x-[RK] was recently discovered. The second motif is required for recognition and binding of some Bcl-2 proteins (Ayllón V. et al., 2002) and Inhibitor 2 to PP1c (Helps N. R. and Cohen P. T. W., 1999).

The metabotropic glutamate receptor mGluR7b contains a binding motif KSVTW that is similar to previously identified motifs, but not identical. The motif is located at the N-terminus of the binding domains which are necessary for interactions with PICK1 and syntenin (Enz R. and Croci C., 2003).

Recently, a new web-based tool (http://pp1signature.pasteur.fr/) has been developed to identify putative PP1 binding proteins.

1.2.2. Combinatorial control of PP1c

The binding of regulatory subunits to PP1c, which occurs in a mutually exclusive manner, is mediated by multiple, degenerate, short sequence motifs. Regulatory subunits bind to different subsets of a limited number of binding sites, sharing interaction sites. Currently, it is impossible to predict the number of binding pockets for the R subunits on the surface of PP1c.

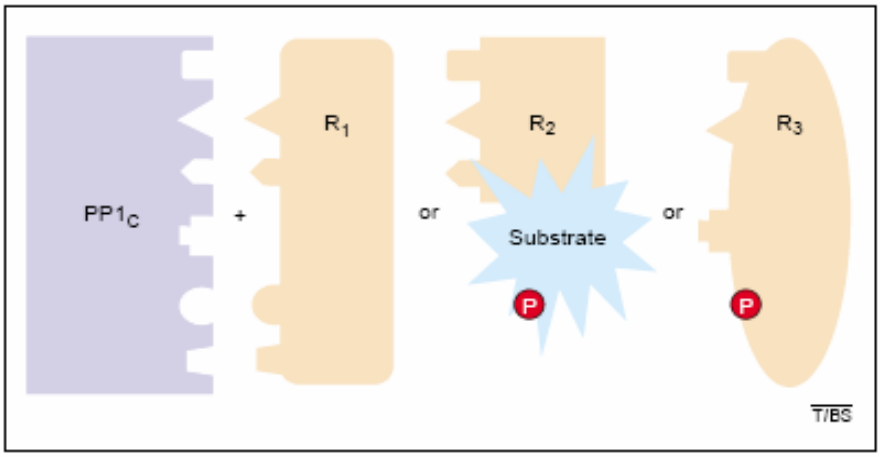

Figure 7. The model of combinatorial control of PP1c. The picture is adapted from Bollen M., 2001

However, it can be calculated that with only six different binding sites for the R subunits, the latter could theoretically interact in more than 60 different ways with the catalytic subunit of PP1 if the number of interaction sites varies between one and six. The model of combinatorial control of PP1c (shown in Figure 7) does not exclude that some R subunits might have unique binding sites on PP1c and there is a possibility of cooperativity between binding sites, increasing activity or substrate specificity of PP1c (Bollen M., 2001).

1.2.3. Regulation of PP1 by diverse mechanisms

As mentioned above (section 1.2.2.), present studies describe more than 70 proteins interacting with PP1. The cell-cycle dependent phosphorylation of Ser/Thr residues regulates the activity of the PP1 catalytic subunit on its C-terminus (Helps N.R. et al., 2001). In contrast, the regulation of the PP1 holoenzymes is mediated by extracellular signals acting through regulatory subunits. This anchors the holoenzyme in close proximity to its substrates, which determines the activity of the enzyme. Various cellular signaling pathways regulate phosphorylation of targeting subunits and inhibitor proteins, which in turn controls the activity of PP1c.

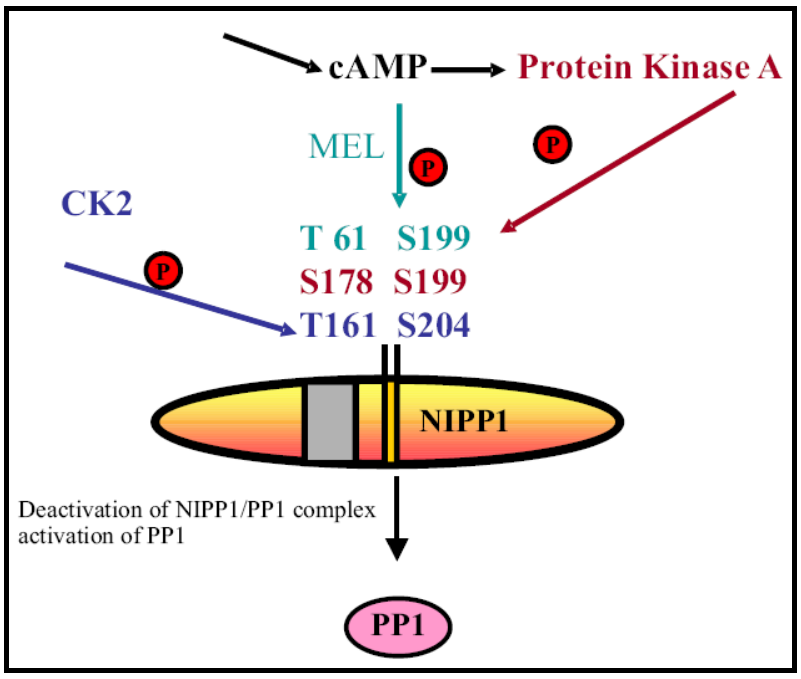

Figure 8. Regulation of PP1c activity by NIPP1 phosphorylation. Grey box indicates the FHA, Forkhead associated domain, the yellow box stands for RVxF, the PP1c binding motif; S stands for Serine, T for threonine; MEL represents murine erythroleukaemia kinase; CK2 represents casein kinase 2.

In several cases the effect of phosphorylation on these complexes is extensively studied. As an example, three unrelated targeting subunits (muscle-specific Protein Phosphatase PP1G/RGL (GM), neurabin I and Nuclear Inhibitor of PP1 (NIPP1)) are phosphorylated within or close to the RVxF motif. The phosphorylation of these residues eventually decreases binding to PP1c. One of them is the highly selective nuclear inhibitor of PP1 (NIPP1), which is activated by phosphorylation of the central domain of NIPP1 by PKA and CKII at sites within and close to the RVxF motif, which is shown in Figure 8 (Vulsteke V. et al., 1997). In nuclear extracts, NIPP1 is present with PP1c as an inactive complex. However, the native hepatic NIPP1 was shown to have a reduced affinity for PP1c after phosphorylation by PKA *in vitro* and after glucagon-induced phosphorylation *in vivo*. These findings suggested that the complex NIPP1-PP1c could be deactivated by phosphorylation (Jagiello I. et al., 1995). NIPP1 is localized to subnuclear speckles and binds to two splice factors. These are splicing factor 3B subunit 1/spliceosome-associated protein 155 (SAP155/SF3b155), a component of the U2 snRNP and CDC5 cell division cycle 5-like protein (CDC5L). It also interacts with MEL kinase, involved in splicing process. Further regulation of NIPP1 occurs through its N-terminal region, which consists of a forkhead-associated (FHA) domain, a known phosphopeptide-interaction module (Li J.

et al., 2000), through which SAP155 and CDC5L are interacting. CDC5L is a human homologue of *S. pombe* Cdc5p, which regulates pre-mRNA splicing, and this interaction depends on the phosphorylation of CDC5L by kinases such as cyclin-E-Cdk2 (Boudrez A. et al., 2000). MEL kinase phosphorylates NIPP1 at Thr61 in the FHA domain or at Ser 199 in the PP1-binding site, abolishing its interaction with ligands and at the same times its localization to nuclear speckles (reviewed by den Hertog J., 2003). This shows that NIPP1 plays an important role as a specific adaptor protein that is regulated by phosphorylation emanating from diverse signal transduction pathways.

Phosphorylation can also occur at other PP1c inhibitors sites. Inhibitors like Inhibitor-1, DARPP-32, protein kinase C-dependent phosphatase inhibitor of 17 kDa, CPI-17 and phopsphatase holoenzyme inhibitor, PHI function to decrease the phosphatase activity whereas others, like Inhibitor-2, increase it. This works by phosphorylation mediated strengthening or weakening of the inhibitory properties of proteins. Similar to NIPP1 the activity of other protein inhibitors, such as I-1 (Inhibitor 1) and DARPP-32 (dopamine- and cAMP-regulated phosphoprotein 32 kDa), is also regulated by PKA.

The predominant inhibitor of PP1c in neurons is DARPP-32, which is highly expressed in brain. The signaling pathways occurring in the brain regulating the DARPP-32 and PP1c interaction was the subject of extensive studies during last decade (Shenolikar S. and Nairn A.C., 1991; Greengard P. et al., 1998; Price N.E. and Mumby M.C., 1999). In 2000, Nobel Prize was awarded 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, causes activation of Protein Kinase A (PKA) after activation of its receptor. PKA phosphorylates DARPP-32 on Thr34, which causes the inhibition of PP1c (Hemmings H.C.J. et al., 1984; Hemmings H.C.J. et al., 1989). When phosphorylated on Thr34, the DARPP-32 protein binds to and inhibits PP1c. The 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 J.A. et al., 1999). Therefore, in such a case DARPP-32 plays a 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. PP1c is activated in neurons by inhibiting its binding to DARPP-32. This is regulated by another neurotransmitter, 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, which occurs on S102 and S137 residues, releasing the active PP1c (Halpain S. et al., 1990). Released PP1c 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 A. et al., 1999). In contrast, activation of adenosine A2 receptors leads to the phosphorylation of DARPP-32 and inhibition of PP1 (Svenningsson P. 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 G.L. et al., 1998), the AMPA-type glutamate receptor (Yan Z. et al., 1999), the GABAA receptor ß1 subunit (Flores-Hernandez J. et al., 2000) and the Na+/K+ATPase ion pump (Fiscone G. et al., 1998). These studies show the important role played by DARPP-32 in integrating neuronal signaling cascades that modulate responses PP1c (Fienberg A.A. and Greengard P., 2000).

Table 4 demonstrates some of regulatory subunits containing of RVxF motif, their phosiological functions and subcellular distributions.

Table 4. Regulatory subinits of PP1c, containing the RVxF PP1 binding motif. Table is modified from Cohen P.T.W., 1997

Regulatory subunits	Mammalian proteins, consisting of RVxF motif	Physiological function regulated	Tissue/ subcellular distribution	Reference
Activity modulators	(I-1) Inhibitor –1 – PPP1R1A	Inhibition of PP1c	Cytosol	Huang F.L. and Glinsmann W.H., 1976;
	DARPP-32 (dopamine and cAMP regulated phosphoprotein Mr 32000)	Inhibition of PP1c, integration of neurotransmitter signals in the neostriatum	Brain, kidney, cytosol	Hemmings H.C. et al., 1984, Shenolikar S. and Nairn C.A., 1991;
	(I-2) Inhibitor-2 – PPP1R1B	Molecular chaperone, inhibition of PP1c	Cytosol and nucleus	Huang F.L. and Glinsmann W.H., 1976;
	CPI-17 (PKC potentiated inhibitor) – PPP1R14A	Inhibition of PP1c	Smooth muscle	Eto M. et al., 1997;
	PHI-2 (phopsphatase holoenzyme inhibitor)	Inhibition of PP1 holoenzymes	Widely distributed	Eto M. et al., 1999;
	G-substrate (cGMP-dependent protein kinase substrate)	Inhibition of PP1c	Brain	Aitken A. et al., 1981, Hall K.U. et al., 1999
Targeting proteins	AKAPs (AKAP149)	A- kinase anchoring protein 149	Everywhere, nuclear envelope	Steen R.L. et al., 2003, Steen R.L. and Collas P., 2001;
	NF-L (neurofilament L)	Nuclear envelope reassembly	Neuronal , plasma membrane and cytoskeleton	Terry-Lorenzo R.T. et al., 2000;
	G subunits (R_{GL}, R3) PPP1R3A, G_M	Synaptic transmission	Skeletal muscle, heart, liver, glycogen particles	Stralfors P. et al., 1985, Tang P.M. et al., 1991;
	M subunits (R4) PPP1R3B, G_L	Glycogen metabolism	Liver, glycogen particles	Moorhead G. et al., 1995, Doherty M.J. et al., 1995;
	NIPP1 (Nuclear inhibitor of PP1, Ard1-fragment) PPP1R8	Transcription and pre-mRNA processing, essential for early embryonic development and cell proliferation	Widely distributed, Nucleus	Van Eynde A. et al., 1995, Van Eynde A. et al., 2004;
	Tau Microtubule targeting	Microtubule stability and function	Neuronal microtubules	Liao H. et al., 1998;
	Bcl 2 (B-cell leukemia/lymphoma 2)	Apoptosis (dephosphorylation of Bad)	Widely distributed mitochondrial membrane	Ayllon V. et al., 2000, Ayllon V.et al., 2001;
	PSF1 (polypyrimidine tract-binding protein associated splicing factor)	Pre-mRNA splicing	Widely distributed, nucleus	Hirano K. et al., 1996;
Substrates	Nek2 (NIMA related protein kinase 2)	Centrosome targeting-centrosome separation	Widely distributed, centrosome and cytosol	Helps N.R. et al., 2000;
	53BP2(TP53BP2, p53 binding protein 2)	Cell cycle check point (dephosphorylation of p53)	Widely distributed, cytosol	Helps N.R. et al., 1995;
Unclassified	PSF1 (polypyrimidine tract binding protein assotiated splicing factor)	Pre-mRNA splicing	Widely distributed, nucleus	Hirano K. et al., 1996;
	Host-cell-factor (Human factor C1, host cell factor)	Transcription, cell cycle	Widely distributed	Ajuh P.M. et al., 2000;
	HOX11 (Homeodomain transcription factor)	Cell cycle checkpoint	Hematopoetic cells nucleus	Kawabe T. et al., 1997;

1.3. Alternative splicing and human disease

Since alternative splicing plays such an important role in gene expression and serves as a major source of proteome diversity in humans, it is not surprising that it is highly relevant to disease and therapy. Therefore, abnormal splicing is now recognized as a source of an increasing number of diseases (Faustino N. and Cooper T., 2003; Garcia-Blanco M. et al., 2004; Caceres J.F. and Kornblihtt A.R., 2002; Cartegni L. et al., 2002; Faustino N.A. and Cooper T.A., 2003; Novoyatleva T. et al., 2006).

From all the mutations which were annotated on the human genome, about 10% affect canonical splice site sequences. Statistics provided by the Human Gene Mutation Database (update of January 20, 2004) reveal that out of 38,177 mutations annotated, 3,659 mutations impinge on splice sites (Lu Q.L. et al., 2003; Cartegni L. and Krainer A.R., 2003). They have been compiled in that database (Stenson P.D et al., 2003) and in other specialized databases (Nakai K. and Sakamoto H., 1994).

However, these numbers could be underestimated since they did not contain the mutations affecting the intronic and exonic enhancer and silencer elements and also mutations associated with disruptions in trans-acting factors. This suggests that a large fraction of all human mutations affect splicing.

1.3.1. Human diseases that are caused by mutation in splicing signals

The disease-causing mechanism connected with alternative splicing can be subdivided into changes in cis- and trans-factors. Changes in cis-factors are caused by mutation in splice sites and in splicing regulatory elements, such as silencer and enhancer sequences, and through generation of novel binding sites for proteins in triplet repeat extensions. Alterations in trans-acting factors are frequently observed in tumor development, where the concentration and ratio of individual trans-acting factors change.
Mutations can be seen as new sources for alternative splicing regulation. For example, the alternative splicing patterns of different histocompatibility leukocyte antigens (HLA) are regulated by allele-specific mutations in the branch point sequences. Since the variability of HLAs is the basis of the adaptive immune response, these mutations strengthen the immunity by enlarging the number of potential HLA molecules (Kralovicova J. et al., 2004).

1.3.2. 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 mutation to be recognized. Although bioinformatics resources such as the ESE finder (Cartegni L. et al., 2003), or the RNA workbench (Thanaraj A. et al., 2004) help to predict type III and IV mutations, the theoretical models often do not fit the predictions (Pagani F. et al., 2003a). 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 5 and is maintained at the alternative splicing database web site (http://www.ebi.ac.uk/asd/).

Table 5. A list of mutations in splicing regulatory elements.

FTDP-17 Frontotemporal Dementia with Parkinsonism linked to chromosome 17	*Tau*	T>G at pos 15 of Exon 10 (N279K) ATTAA**T**AAGAAG ATTAA**G**AAGAAG	(Clark LN et al., 1998)
		AAG del at 16 of Exon10 (Δ280K) ATTAAT**AAG**AAGCTG ATTAAT------AAGCTG	(Rizzu P.et al. 1999)
		T>C at pos 30 of Exon 10 (L284L) CTGGATCT**T**AGCAAC CTGGATCT**C**AGCAAC	(D'Souza I. et al., 1999)
		G>A at pos 92 of Exon10 (S305N) improves the splice site GGCA**G**TGTGA GGCA**A**TGTGA	(Iijima M.et al., 1999)
Thrombasthenia of glanzmann and naegeli	*Integrin gpiiia*	AC**G**GTGAGgt AC**A**GTGAGgt at position 20624 of the GPIIIa gene G>A	(Jin Y. et al., 1996)

		6 bp upstream of the GPIIIa exon 9 splice donor site at pos. 134 of exon 9	
Menkes disease	*Mnk*	GATCT**T**C**T**GGA GATCT---GGAT Del 1339L - 4159 TCT of exon 21	(Gu Y.H et al., 2001)
Metachromatic leukodystrophy	*Arylsulfatase A*	CAGAC**G**AGGTC CAGAC**A**AGGTC 2330T C-to-T substitution, 22 nucleotides downstream from the exon 8 splice acceptor site	(Hasegawa Y. et al., 1994)
Immunodeficiency	*TNFRSF5, tumour-necrosis factor receptor superfamily, member 5 (CD40)*	CTAC**A**GGG CTAC**T**GGG 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*	CCTATG**G**GCCGTT CCTATG**T**GCCGTT 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) GGGATC**A**T**C**GTGGGA GGGATC**A**T**T**GTGGGA (I2118I) IVS51+26 (T>G) TGTCCT**TA**TGGAAGT TGTCCT**TAG**GGAAGT (Y2113X)	(Liu Q.et al., 1997)
Acute intermittent porphyria	*Porphobilinogen deaminase*	IVS3-22 (C>G) GTGATT**CG**CGTGGGT GTGATT**CGG**GTGGGT (R21R)	(Llewellyn D.H. et al., 1996)
Hereditary tyrosinemia	*Fumarylacetoacetat hydrolase*	IVS8-11 (C>T) CTTATG**AA**CGACTGG CTTATG**AAT**GACTGG (N232N)	(Ploos van Amstel J.K. et al., 1996)
Leigh's encephalomyelopathy	*Pyruvat dehydrogenase E1 alpha*	628G→A GGGC**G**CTGG GGGC**A**CTGG G to A substitution at nucleotide 13 of exon 6	(De Meirleir L. et al., 1994)
Homocystinuria	*Methionine synthase*	TCAGCC**T**GAGAGGA TCAGCC**C**GAGAGGA Tto C transition within intron 6 of the mtrr gene	(Zavadakova P. et al., 2002; Zavadakova P. et al., 2005)

1.3.3. Spinal muscular atrophy (SMA)

Spinal muscular atrophy (SMA) is a common 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 J.H., 1980). The incidence is 1 to 6000 for live births and the carrier frequency is 1 in 40. This makes SMA the second most common fatal autosomal recessive disorder and the most frequent genetic cause of infantile death. The disease can manifest in four phenotypes (type I to IV) that differ in age of onset and severity of the phenotype.

Using linkage analysis, all the clinical subtypes of SMA have been mapped to chromosome 5q11.2-13.3 (Brzustowicz L.M., et al., 1990). The gene responsible for the disease is the survival of the motor neuron (SMN) gene (Lefebvre S. et al., 1980; Rochette C.T. et al., 2001). The SMN gene encodes a 294-amino acid (~38kDa) protein, which is localized both in cytoplasm (Lefebvre S. et al., 1995) and in nuclear bodies, called gems. Gems were shown to be involved in small ribonucleoprotein assembly and their recycling (Paushkin S. et al., 2002). In both cytoplasm and gems, SMN is part of a large complex containing several proteins. In gems, the SMN protein complex includes several tightly associated proteins: the SMN protein, Gemin2 (previously called SIP1), the DEAD box protein Gemin3 and Gemin4 (Charroux B.et al., 2000). In the cytoplasm, it associates with Gemin4 and small spliceosomal nuclear ribonucleoproteins (snRNP) including B/B′, D, E, F, and G and Sm core proteins (Liu Q. et al., 1997). This data suggests that SMN plays a critical role in spliceosomal snRNP assembly. In addition, it was shown that SMN is required for the regeneration of spliceosomes (Fisher U. et al., 1997; Pellizoni L. et al., 1998).

Humans posses two nearly identical copies of the SMN gene, SMN1 and SMN2. SMA is caused by the homozygous loss or mutations of SMN1 gene (Brahe C. et al, 1996; Wirth B., 2000). Although both genes are almost identical in sequence, due to a translationally silent C→T change at position 6 in exon 7, they have different splicing patterns and exon 7 is predominantly excluded in SMN2. SMN2 transcripts could also lack exons 3, 5 but most frequently exon 7, with only a small amount of full length of mRNA generated (Wang J. et al., 1996; Wang J. et al., 1998). The exon 7 skipping event in SMN2 generates a truncated, less stable protein (Lorson C. et al., 2000) with a reduced ability to

oligomerize, explaining why SMN2 cannot compensate the loss of SMN1 and prevent the disease (Lefebvre S. et al., 1980; Lorson C. et al., 1999; Pellizoni L. et al., 1999).

Mutations of the Survival Motor Neuron gene (SMN1) are responsible for 95% of the cases of SMA. The highest level of SMN2 was found among patients of type I, and the lowest among the patients of mildest type III (Moller L.B. et al., 2000). This suggests that SMN2 protein level correlates with disease severity (Tacke R., 1998) and phenotypes correlate with the number of SMN2 copies in the genome.

A silent C→T mutation within exon 7 inhibits exonic splicing enhancer (ESE), which ultimately leads to exon 7 skipping (Lorson C.L. et al., 1999). It was shown that this ESE is recognized by hTra2-beta1. In addition to hTra2-beta1 other SR proteins, like SRp30c can stimulate exon 7 inclusion (Young P.J. et al., 2002).

It was suggested by Kashima T. and Manley J. in 2003, that the C→T transition creates an exonic splicing silencer (ESS) in SMN2. They have demonstrated that ESS functions as a binding site for a known repressor protein, hnRNPA1, which binds to SMN2, but not to SMN1, exon 7 RNA. This is in contrast to a previous finding that the C→T transition disrupts exonic splicing enhancer (ESE) in SMN1 (Cartegni L. and Krainer A. R., 2002). Recent analysis of the enhancer-loss and silencer-gain models performed by Cartegni L. et al., demonstrates that hnRNP A/B proteins antagonize SF2/ASF–dependent ESE activity and promote exon 7 skipping. The hnRNP A/B proteins act by a mechanism that is independent of the C→T transition and is, therefore, common to both SMN1 and SMN2 (Cartegni L. et al., 2006).

Since stimulation of SMN2 exon 7 usage would increase SMN protein levels and potentially cure the disease, work has concentrated on understanding the regulation of exon 7. As for CFTR (cystic fibrosis transmembrane conductance regulator ATP-binding cassette subfamily C member 7) exon 9 and 12, multiple factors determine the regulation, including a suboptimal polypyrimidine tract (Singh N. et al., 2004c), a central Tra2-beta1-dependent enhancer (Hofmann Y. et al., 2000) and the sequence around the C→T change at position 6 that can bind to SF2/ASF (Cartegni L. and Krainer A., 2002; Cartegni L. et al., 2006).

1.3.4. Current Cellular Models for Evaluating SMA Therapeutics

As it was mentioned above (section 1.3.3.), SMA disease severity correlates inverserly with the copy number of SMN2 gene. Therefore, increasing levels of SMN gene can compensate for SMN1 deficiency. Two distinct approaches for treatment of SMA are

known. These are either increasing the transcription from the SMN2 gene or preventing exon 7 skipping in SMN2 transcripts. There is a number of demonstrations that levels of full-length SMN2 transcript and/or functional SMN protein were found to increase following treatment with small molecular weight drugs or a tailed antisense oligonucleotide containing binding motifs for splicing activators (Table 6).

Histone deacetylase inhibitors, such as sodium butyrate and valproic acid, have been used to increase the exon 7 inclusion (Chang J.G. et al. 2001; Brichta L. et al., 2003). Sodium butyrate was used in one of the first treatment trials of SMA. This compound effectively increased the amount of SMN protein in SMA lymphoid cell lines by changing the alternative splicing pattern of exon 7 in SMN2 gene (Chang J.G. et al., 2001).

Another recently found drug, which effectively increased SMN expression, is valproic acid (VPA) (Monneret C. et al., 2001). This compound is currently in clinical use against epilepsy. VPA, like sodium butyrate, and the nonsteroidal anti-inflammatory drug indoprofen (Lunn M.R. et al., 2004) are histone deacetylase inhibitors, leading to the stimulation of transcription of many genes. This is the main difficulty in using these compounds.

SMN2 splicing was also influenced by the phosphatase inhibitor sodium vanadate (Zhang M.L. et al., 2001), which could stimulate exon 7 inclusion of SMN2 (Lorson C.L. et al., 2001). This drug is toxic.

The cytotoxic anthracycline antibiotic aclarubicin (Andreassi C. et al., 2001) effectively induced incorporation of exon 7 into SMN2 transcripts from the endogenous gene in type I SMA fibroblasts as well as into transcripts from a SMN2 minigene in the motor neuron cell line NSC34. The treatment in type I fibroblasts resulted in an increase in SMN protein and gems to normal levels (Andreassi C. et al., 2001). A serious barrier against use of this drug is that it is associated with side affects including cardiomyopathy (Charlotte J. et al., 2003).

The main disadvantage against of using of above mentioned compounds is their broad mechanism of action, which is not restricted to pre-mRNA. On the table 6 there is a list of compounds that are potential candidates for treatment of SMA (listing according to last Annual Meeting of Families of Spinal Muscular Atrophy, June, 2006, Montreal, Canada). The serious barrier against using these compounds is that most of them have side effects.

Table 6. Potential drugs for treatment of SMA.

Drug	Function	Clinical use
Indoprofen	HDAC inhibition-stimulation of transcription	Not in the market from 1980s due to reports of serious gastrointestinal reactions
Valproic acid	HDAC inhibition-stimulation of transcription	Treatment of epilepsy and bipolar disorder. It is also used to treat migraine headaches and schizophrenia
NaB	HDAC inhibition stimulation of transcription	Used against sickle cell disease
HU	HDAC inhibition stimulation of transcription	Antineoplastic drug used sickle-cell anemia and some other hematological disorders. It is also used as an antiretroviral agent (e.g., against HIV).
Salbutamol	β-adrenergic receptor agonist- cAMP increase	Asthma
Aclarubicin	Catalytic inhibitor of topoisomerase II-subcellular redistribution of SR proteins	Anticancer agent used against acute non-lymphocytic leukaemia
Benzamidin M344	HDAC inhibition stimulation of transcription	
TCA	HDAC inhibition stimulation of transcription	Serves as an antifungal antibiotic

Abbreviations: HDAC, histone deacetylase; NaB, Sodium butyrate; TCA, Trichostatin A; HU, Hydroxyurea; HIV, Human immunodeficiency virus.

1.3.5. Changes of trans-factors associated with diseases

Knock-out experiments indicate that the complete loss of splicing factors NOVA-1, SRp20, SC35, and SF2/ASF causes early embryonic lethality (Jensen KB. et al., 2000; Jumaa H. et al., 1999; Wang H.Y. et al., 2001; Xu X. et al., 2005). Up to now, knock-outs of splicing regulatory factors are largely absent in libraries of ES cells where one allele was silenced through gene trapping. This indicates that the proper concentration of regulatory factors is necessary for cell survival. However, the loss of some splicing factors in differentiated cells can be tolerated and leads to specific phenotypes (Xu X. et al., 2005).

Mutations in proteins implicated in splicing have been observed in retinitis pigmentosa, a progressive loss of photoreceptor cells during childhood, where PRP31 (pre-mRNA processings factor 31) is mutated (Vithana E.N. et al., 2001) and forms of azospermia, where RBMY (RNA-binding motif Y chromosome protein) has been deleted (Venables J.P. et al., 2000). Changes in the concentration or localization of splicing factors are frequently observed in tumor genesis. For example, the concentration of SC35, SF2/ASF, and Tra2-beta1 are altered in ovarian cancer (Fischer U. et al., 2004). An array-based study of changes in Hodgkin's lymphoma revealed 2-5 fold changes in seven general

splicing factors as well as the ectopic expression of the neuron-specific splicing factor NOVA-1 and NOVA-2 (Relogio A. et al., 2005). In addition, numerous splicing events were altered, but it is not possible to explain how these changes are related to alterations of trans-acting factors.

1.3.6. Treatment of diseases caused by missplicing

1.3.6.1. Gene Transfer Methods

Type I and II mutations either destroy splice sites or activate cryptic splice sites. Antisense nucleic acids can suppress point mutations and promote the formation of normal gene products. Special chemistries were devised to prevent RNAseH-mediated cleavage of the RNA and to lower the toxicity (Sazani P. and Kole R., 2003). Oligonucleotides have been used to target cryptic splice sites that are activated in beta thalassemias (Lacerra G. et al., 2000), to suppress exon usage in Duchenne muscular dystrophy (Mann C.J. et al., 2001) and block HIV replication (Liu S. et al., 2004). The antisense approach was further developed in ESSENCE (exon-specific splicing enhancement by small chimeric effectors). ESSENCE uses bifunctional reagents that contain a peptide effector domain and an antisense-targeting domain. The effector domains of these proteins-nucleic acids were arginine/serine (RS) repeats that mimic the effect of SR proteins (Cartegni L. and Krainer A., 2003).

Related to ESSENCE is the use of bifunctional oligonucleotides in TOES (targeted oligonucleotide enhancer of splicing), where a part of the oligonucleotide binds to an SR protein, which promotes exon inclusion (Skordis L.A. et al., 2003). Several RNA based approaches have been tested in cell culture. They include use of RNAi to suppress unwanted isoforms (Celotto A.M. and Graveley B.R., 2002), spliceosome-mediated RNA trans-splicing (SmaRT) to correct factor VIII deficiency in a mouse model (Chao H. et al., 2003) and ribozymes that use trans-splicing to replace defective p53, beta-globin mRNA and a chloride channel in cell culture (Lan N. et al., 1998; Watanabe T. and Sullenger B.A., 2000; Rogers C.S. et al., 2002). Finally, antisense oligonucleotides have been used to modify U7 snRNA, which results in the nuclear accumulation of the oligonucleotide sequences in stable U7 snRNP complexes (Asparuhova M. et al., 2005) that interact with the mutant target gene.

1.3.6.2. Low molecular weight drugs

It is well known that small molecules can interact with RNA, and this principle is used by several RNA-binding antibiotics, such as gentamicin, chloramphenicol, and tetracycline (Xavier K.A. et al., 2000). Therefore, several chemical screens were performed to identify small-molecular-weight molecules that interfere with splice site selection. It was found that epigallocatechin gallate (EGCG), a polyphenol and component of green tea (Anderson S.L. et al., 2003), as well as kinetin and the related benzyladenine, a plant hormone (Slaugenhaupt S.A. et al., 2004), promotes correct splice site usage in the IKAP gene, involved in familial dysautonomia. The compounds used for treatment of SMA are described in section 1.3.4., and listed in Table 6. Major disadvantage of most of these inhibitors is their low specificity. However, surprisingly indole derivatives were found to act on specific SR proteins that regulate specific ESE sequences (Soret H. et al., 2005). Since these substances block HIV replication by interfering with early viral splicing events, they open the intriguing possibility of a specific pharmacological treatment for splicing disorders.

1.3.7. Diagnostics

Up to now, the majority of studies analyzing splice site selection were done by RT-PCR (Stamm S. et al., 2000). Recently, microarray formats have successfully been used to detect changes in splice site selection associated with diseases (Fehlbaum P. et al., 2005; Relogio A. et al., 2005). These microarrays use several oligonucleotides located within the exon and on the exon-exon junctions to infer the presence and connections of alternative exons. The arrays detect the usage of a single exon, and it is currently not possible to infer the composition of complete mRNAs using microarrays. One important finding of microarray analysis is that diseases can be associated with a large number of small changes in alternative splice site selection, rather than with a few large changes. It will therefore be necessary to analyze data obtained with exon-specific microarrays with different software tools that use gene ontologies to detect coordinated small changes in groups of exons (Ben-Shaul Y. et al., 2005).

1.4. Mechanism of splicing

Pre-mRNA splicing is a two-step enzymatic transesterification 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 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. (Figure 9) (Moore M.J. et al., 1993; Guthrie C., 1991; Ruby S.W. 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 (Padgett R.A. et al., 1984; Ruskin B. et al., 1984). The components of the basal splicing machinery form the multicomponent splicing complex, which is known as the spliceosome. There are two functions of spliceosome. First function is the recognition of the intron/exon boundaries and the second one is the removal of the introns and joining of the exons.

The spliceosome is a large and highly dynamic molecular complex. It is composed of five small ribonucleoprotein particles, snRNPs: U1, U2, U4, U5 and U6 in major class U2 type spliceosome (Moore M.J. et al., 1993; Kramer A. et al., 1996) and five other snRNPs: U11, U12, U4, U5 and U6 in minor class U12 type spliceosome and approximately 50–100 non-snRNP splicing factors (Moore M.J. et al., 1993; Neubauer G. et al., 1998; Kramer A. et. al., 1996). Each snRNP is composed of a single small nuclear RNA (snRNA) and multiple proteins.

The binding sites and functions of these particles are very specific. For example, U1 snRNP binds the 5' splice site, whereas U2 snRNP binds the branch site via RNA: RNA interactions between the snRNA and the pre-mRNA (Figure 9).

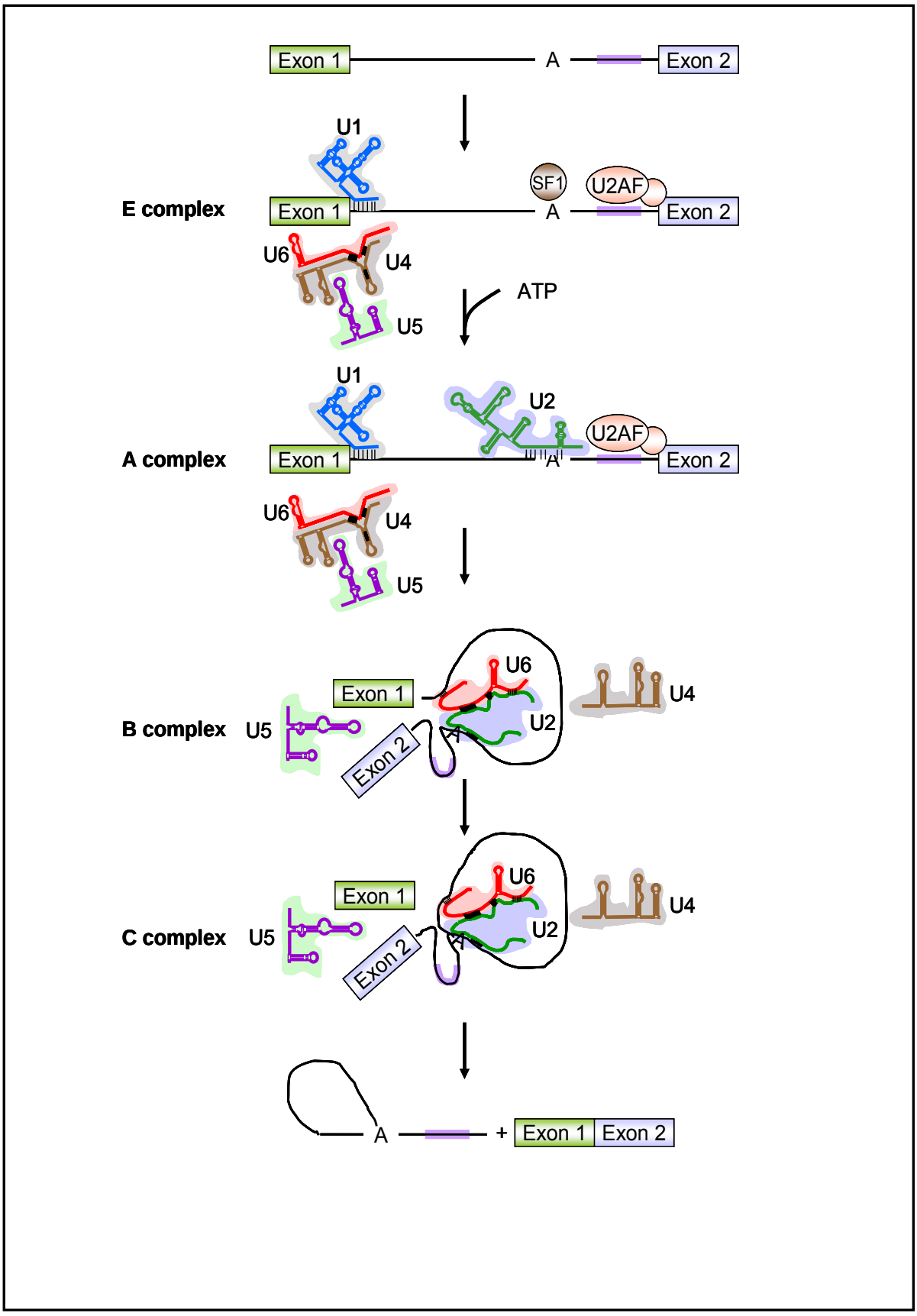

Figure 9. Spliceosome formation and rearrangement during the splicing reaction. Figure adapted from Patel A.A. et al., 2003.

The splicing process starts with the formation of the E complex. Assembly of the E complex involves the recognition of 5' splice site, the 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 factor1 (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. This association is ATP dependent. Then the ATP dependent base pairing of U2 snRNP with the branch point, leads to the formation of A complex. The B complex is formed by the 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 the 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. Figure 9 schematically depicts the spliceosomal assembly, the formation of catalytic spliceosome and the excision of the intron from the pre-mRNA.

2. RESEARCH OVERVIEW

Alternative splicing is one of the most important steps in the process of eukaryotic gene expression. A large number of protein isoforms are generated from a relatively small number of genes by alternative splicing. Alternative splice site selection requires the coordinated action of a number of protein: RNA and protein: protein complexes. Proteins involved in splice site selection are named splicing factors.

In first part of this research, we focused on two members of STAR family, rSLM-1 and rSLM-2 splicing factors. We characterized these proteins and studied their roles in certain signaling cascades upon tyrosine phosphorylation. Proteins were found to interact with several splicing factors including hnRNP G, Sam68, SRp30c, SAF-B and YT521-B. rSLM-1 and rSLM-2 exhibited striking difference in their localization in various brain regions. The proteins were tested for phosphorylation by several tyrosine kinases. We showed that they are phosphorylated by several non-receptor tyrosine kinases. Both splice factors change exon 7 usage of SMN2 reporter minigene. $p59^{fyn}$-mediated phosphorylation abolishes the ability of rSLM-1 to regulate splice site selection, but has no effect on rSLM-2 activity, suggesting that rSLM-1 is a tissue-specific splicing factor whose activity is regulated by tyrosine phosphorylation signals emanating from $p59^{fyn}$. Hence, phosphorylation by $p59^{fyn}$ kinase is an important feature discriminating these proteins, suggesting that they may perform different functions in the organism.

The second part of this study concentrates on a SR-like protein, Tra2-beta1. The protein belongs to a group of RRM containing RNA binding proteins. We examined the protein by sequence computational analysis and found a PP1 (Protein Phosphatase 1) binding site in the beta4 sheet of the RRM, which was also discovered in nine other proteins. We determined that PP1 interacts with Tra2-beta1 and also with its interactors SF2/ASF and SRp30c. Protein phosphatase1 dephosphorylates Tra2-beta1. Dephosphorylation of Tra2-beta1 protein influences the formation of homo and heterodimers. Reducing PP1 activity by either cell permeable inhibitor tautomycin or by its nuclear inhibitor NIPP1, promotes usage of numerous alternative exons, including exon 7 of the survival of motoneuron 2 (SMN2) gene, demonstrating a role of PP1 in splice site selection. The data summarizes that PP1c dephosphorylation plays a role in the regulation of splice site selection and that binding to PP1c is a new function of RRMs.

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	AmpliTaq DNA polymerase	Roche
NotI	NEB	Platinum Pfx polymerase	Invitrogen
SacII	NEB	T4 PNK	NEB
DpnI	NEB	T7 DNA Polymerase	NEB
SuperScript II	Invitrogen	FastLink T4 DNA Ligase	Biozym
NheI	NEB	EcoRI	NEB
PP1	NEB		

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 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% CO_2.

Name	**Description**	**ATCC number**
Human fibroblasts 95-2806	Patient human cell fibroblasts of SMA I: 2 copies of SMN^C and 0 copies of SMN^T	Coovert D.D. et al., 1997
Human fibroblasts 95-2827	Patient human cell fibroblasts of SMA II: 2 copies of SMN^C and 0 copies of SMN^T	Coovert D.D. et al., 1997
Human fibroblasts 95-2873	Patient human cell fibroblasts of SMA III: 2 copies of SMN^C and 0 copies of SMN^T	Coovert D.D. et al., 1997
Human fibroblasts 96-2842	Normal human cell fibroblasts have 1 copy of SMN^C and 2 copies of SMN^T	Coovert D.D. et al., 1997
COS-7	African green monkey kidney SV40 transformed	CRL-1651
HEK293	Human embryonic kidney transformed with adenovirus 5 DNA	CRL-1573

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*(r_B m_B) *dcm*$^+$ Tet^r *gal* λ(DE3) endA Hte [*argU ileY leuW* Cam^r]	(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 lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]	(Bullock W.O. et al.,1987)
CJ236	F′ *cat (pCJ105=pOX38::cat=FΔ(HindIII)::cat [Tra*$^+$ *Pil*$^+$ *Cam*^{R}*]/ ung-1 relA1 dut-1 thi-1 spoT1*	(Kunkel T.A. et. al., 1987)
DH10Bac^{TM}	F$^-$*mcr*AΔ(*mrr-hsd*RMS-*mcr*BC)Φ80*lac*ZΔM15ΔlacX74*deo*R*rec*A1 *end*A1*ara*D139Δ(*ara,leu*)7697*gal*Kλ*rps*L*nup*G/bMON14272/pMON 7124	(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-YT521-B (PK2) (1:3000)	Mouse	Custom made[5]

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-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
pht6-Fl-FLAG	pcDNA	YT521-B FLAG-tagged	(Nayler O. et al., 1998)

Name	Backbone	Description	Reference
c-src wt	pcDNA3.1	c-Src kinase	(Wong B.R. et al., 1999)
pRK5-abl	pRK5	c-Abl kinase	(Nayler O. et al.,1998)
pRK5-fyn	pRK5	Fyn kinase	(Nayler O. et al., 1998)
pRK5-fyn-KA	pRK5	Catalytic inactive Fyn kinase	(Nayler O. et al., 1998)
pUHG10-3(FER)	pUHD10-3	FerH kinase	(Hao Q.L. et al., 1991)
pEGFP-DYRK1A	pEGFP-C2	Dual-specificity tyrosine phosphorylated and regulated kinase DYRK1A	(Sitz J.H. et al., 2004)
Sik YF pRK5-fyn	pcDNA3	Constitutively active Sik kinase	(Derry J.J. et al., 2000)
pSVL-Syk	pSVL	pSVL Syk kinase	(Zhang J. et al., 1996)
CSK	pcDNA3	CSK kinase	(Nayler O. et al., 1998)
AUG1(pcDNA3-Rlk)	pcDNA3	Rlk kinase	(Debnath J. et al., 1999)
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)
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)
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)
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	None
SF2/ASF-EGFP	pEGFP-C2	SF2/ASF cloned in pEGFP-C2	(Nayler O. et al., 1998b; 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
PP1γ1(F257A)EGFP	pEGFP-C1	mutation of RVXF – binding chanel of rat PP1γ1	(Lesage B. et al., 2004) Gift from M. Bollen
PP1γ1(H125A)EGFP	pEGFP-C1	Catalytically inactive mutant of rat PP1γ1	(Zhang J. et al.,1996) Gift from M. Bollen
HA-PP1γ	HA-pCMV5	PP1γ has HA Tag	(Maximov. A. et al., 1999) Gift from M. Bollen
PP1 γ1-CFP	pECFP	PP1γ1	None Gift from M. Bollen
S MN2 SE MG	pCI	Exonic enhancer AG rich region is mutated	(Hofmann Y. et al., 2000) Gift from C.Lorson
YT521-B	pFastBac-HTa	YT521-B cloned into *Drosophila* vector	None

Newly made clones

Name	Backbone	Description	Tag
EGFP Tra2-beta1-NES	pEGFP-C2	NES signal exported between human Tra2-beta1 and EGFP(located instead of nucleus in the cytoplasm, nucleus is dark)	EGFP
EGFP Tra2-beta1-RATA	pEGFP-C2	human Tra2-beta1 PP1 binding site RVDF was mutated into RATA	EGFP
EGFP Tra2-beta 1-RATA–NES	pEGFP-C2	NES inserted to Tra2-beta1-RATA-C2 mutant	EGFP
EGFP Tra2-beta1 RS1A	pEGFP-C2	Tra2-beta1 all the S residues mutated to A in the first part of the first RS domain	EGFP
EGFP Tra2-beta1 RS1E	pEGFP-C2	Tra2-beta1 all the S residues mutated to E in the first part of the first RS domain	EGFP
EGFP Tra2-beta1 RS2A	pEGFP-C2	Tra2-beta1 all the S residues mutated to A in the second part of the first RS domain	EGFP
EGFP Tra2-beta1 RS2E	pEGFP-C2	Tra2-beta1 all the S residues mutated to A in the second part of the first RS domain	EGFP
HIS-Tra2-beta1-RATA	pFastBac-HTa	human Tra2-beta1-RVDF-RATA mutant cloned into *Drosophila* vector	HIS
EGFP SF2/ASF-RVEF-RATA	pDEST53	human SF2/ASF with RVEF-RATA mutation was cloned into pDEST53	EGFP
EGFP SRp30c-RVEF-RATA	pDEST53	human SRp30c with RVEF-RATA mutation cloned into pDEST53	EGFP

3.1.9. Primers

Primers used for mutagenesis

Following Tra RVDF-RATA primer was used for mutagenesis

Name	Sequence	Introduced mutation	Name of the generated clone
Tra RVDF-RATA	CGTAGGATCAGAGCTACTGCCTCTATAACAAAA	RVDF-RATA	RATA

Primers used for RT-PCR

Name	Orientation	Sequence	Target
N3Ins N5Ins	antisense sense	CTCCCGGGCCACCTCCAGTGCC GAGGGATCCGCTTCCTGCCCC	CD44v5 minigene
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 SMN for	antisense sense	TCACATTGCATTTGGTTATTACA ATAGGATCCACCTCCCATATG	SMN endogenous

Primers used for verifying the microarray data

Name	Orientation	Sequence	Exon	Target
For SRp75/SFRS4 Rev SRp75/SFRS4	sense antisense	CAGCCATCACTGCCGTTGCC GCGTCCAGAACCGTAACTGC	104.9.3 Novel exon Ref: NM_005626 Var: BX447499	Splicing factor, arginine/serine-rich 4
For PPIL3 Rev PPIL3	sense antisense	GAGCTCGCTGTAAGACTGAG GATATTCACTGTATTCATC	236.10.1 Alternative splice acceptor Ref: NM_032472 Var: BU195819	Peptidyl prolylisomerase like protein 3
For hnM Rev hnM	sense antisense	GATGAGAGGGCCTTACCAA CCTGCCCATGTTCATCCCA	114.1.1 Exon skipping Ref: NM_005968 Var: AL516884	Heterogeneous nuclear ribonucleoprotein M
For SFRS14 Rev SFRS14	sense antisense	CAAGGACTTGGACTTCGCC CTTCTAGGCTTTATCAAGGC	179.1.1 Exon skipping Ref: NM_014884 Var: AI089022	Splicing factor, arginine/serine-rich 14
For Fyn Rev Fyn	sense antisense	GAGAGCTGCAGGTCTCTG CTCGGTGCGATGTAGATG	907.002.002 Novel exon Ref:NM_002037.3 Var:NM_153047.1	Fyn oncogene related to Src, Fgr, Yes
For DEAD Rev DEAD	sense antisense	GAACGTCGGGAACGCAGG GTAGTCAATGGATGTGTCCT	91.1.1 Intron retention Ref:NM_004818 Var:BU174750	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23 (U5-100KD)
For Fus Rev Fus	sense antisense	GACAACAACACCATCTTTGG CCTCCACGACCATTGCCAC	93.1.1 Exon skipping Ref: NM_004960 Var: AJ549096	Fusion (involved in t(12;16) in malignant liposarcoma)
For IEBF3 Rev IEBF3	sense antisense	GTCAGTTCTACAGCAACGG CACCTCGCGACCAGCAAC	159.5.2 Exon skipping Ref: NM_012218 Var: BM876556	Interleukin enhancer binding factor 3, 90kDa
For MTMPR1 Rev MTMPR1	sense antisense	GAGACTGAGCGGAAGAAGC CTGACACTGTCATGAAGAGC	137.1.1 Novel exon Ref: NM_006697 Var: U78556	Myotubularin related protein 11
For Nuc 5A Rev Nuc 5A	sense antisense	GTGGAGGAGTCTGTGCTCA GCAGGTGGGTCTCCAAGAG	127.3.1 Novel exon Ref:NM_006392 Var: BE253695	Nucleolar protein 5A (56kDa with KKE/D repeat)

For CLK4 Rev CLK4	sense antisense	GTCCGCAGCAGGAGAAGC CATGCCATGATCAATGCACTC	208.8.3 Intron retention Ref: NM_020666 Var: BX491417	CDC-like kinase 4
For FE65 Rev FE65	sense antisense	CAGAGCCGTTGCCCCAAG CACTGTCCCGCCCGAC	909.039.002 Exons skipping Ref: NM_001164 Var: BX420711.1	Amyloid beta (A4) precursor protein-binding, family B, member1 (Fe65)
For SFRS5 Rev SFRS5	sense antisense	GAGGATCCAAGGGATGCAGAT G GCCAGCTGACTCTTGAGGA	144.1.3 Partial internal exon deletion Ref: NM_006925 Var: BC018823	Splicing factor, arginine/serine-rich 5
For CPSF6 Rev CPSF 6	sense antisense	CAATCAGGACAAATGTCTGGG CACTGGCATCAGACACAGC	148.1.1 Novel exn Ref: NM_007007 Var: AL557975	Homo sapiens cleavage and polyadenylation specific factor 6
For SRRM1 Rev SRRM1	sense antisense	GATGAACGACCCAAGAGATC GGAGACCGTCGCCTTCTG	110.3.1 Novel exon Ref: NM_005839 Var: BE931442	Serine/arginine repetitive matrix 1
For PPIE Rev PPIE	sense antisense	GTCAGATGATGACTGGTTGAA CATGAGTGCACAGGCAGCG	117.8.1 Exon skipping Ref: NM_006112 Var: BI821836	Peptidylprolyl isomerase E (cyclophilin E)

3.2. Methods

3.2.1. 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 denaturation 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.

3.2.2. 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 centriuged 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.

BUFFER P1:
50 mM Tris-HCl, pH 8.0
10 mM EDTA
100 µg/ml RNase A

BUFFER P3:
3 M Potassium acetate, pH 5.5

BUFFER P2:
200 mM NaOH
1% SDS

BUFFER TE:
10 mM Tris-HCl, pH 8.0
1 mM EDTA

3.2.3. 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

3.2.4. 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

3.2.5. 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 dH_2O) 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.

3.2.6. 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.

3.2.7. 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 aliquot 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

3.2.8. 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 $CaCl_2$, 250 mM $MgCl_2$ and afterwards the 100 µl of water was added. Equal volume of defrozen 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.

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

To overexpress GST-tagged rSLM-2 protein, GEX-ESAF-ATG construct was 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

3.2.10. Expression and purification of HIS-tagged proteins in Baculovirus Expression System

The Bac-to-Bac®Baculovirus Expression System facilitates rapid and efficient generation of recombinant baculoviruses (Ciccarone V.C. et al., 1997). Based on the method developed by Luckow V.A. et al., in 1993, the Bac-to-Bac®Baculovirus Expression System takes advantage of the site-specific transposition properties of the Tn7 transposon to simplify and enhance the process of generating the recombinant bacmid DNA. Before the transfection and expression experiments, SF9 cells were thawed and cultured under serum free conditions (using TNM-FH medium, Becton-Dickenson).

The cells were cultured at optimal 28°C, at pH range 6.1-6.4 and at 10% to 50% of air saturation. After generating the pFastBac™ construct, the purified plasmid DNA was transformed into DH10 Bac™, for transposition into the bacmid. 48 hours after transfection blue/white screening selection was used to identify colonies containing the recombinant bacmid.

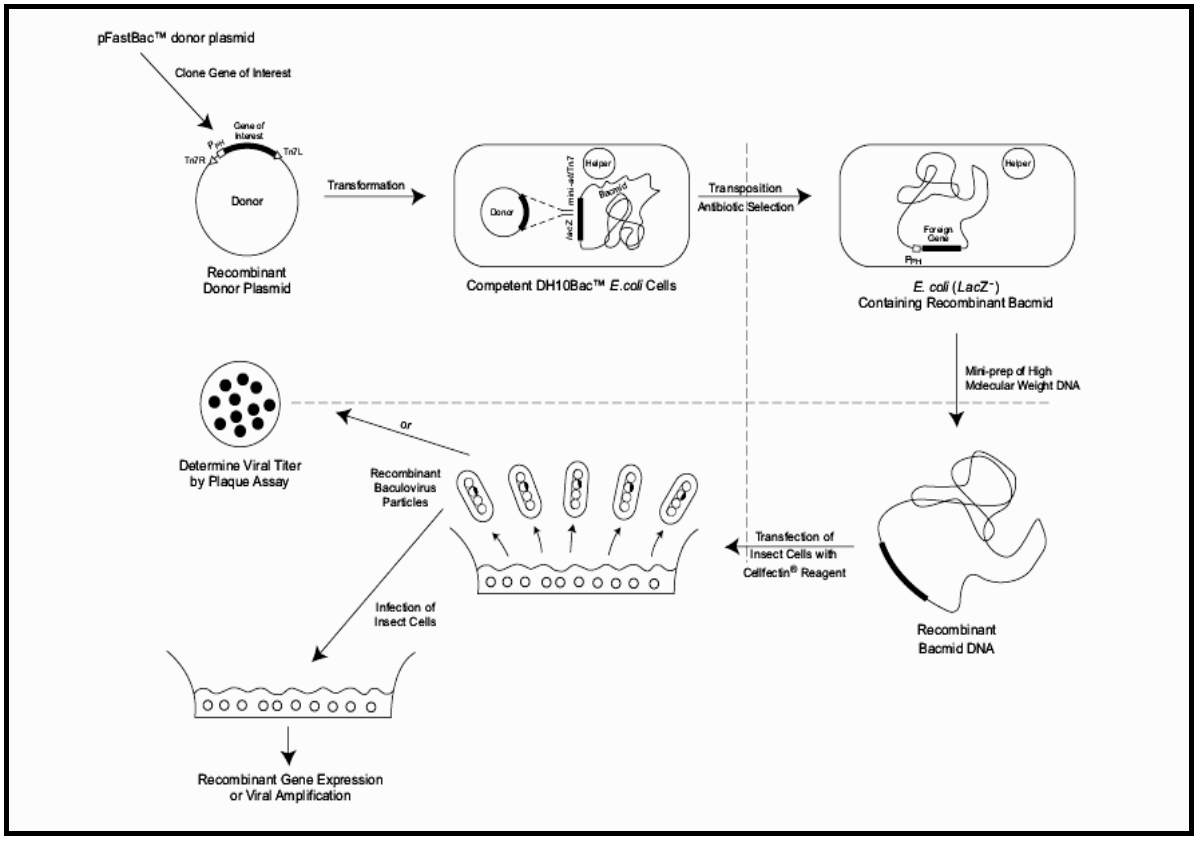

Figure 10. The process of generation of His-tagged protein in Bac to Bac system. The picture is from Invitrogen catalog.

Successful DNA transposition into the Bacmid was verified by PCR analysis with Forward specific Tra2-beta1 and M13 Reverse primers. 1 µg of the purified recombinant bacmid DNA (500 ng/µl in TE Buffer, pH 8.0) from pFastBac^{TM} construct was diluted in 100 µl of unsuplemented Grace´s Medium and combined together with mixture of 6 µl Cellfectin ® in 100 µl of unsuplemented Grace´s Medium. The mixture of DNA-lipid complexes was transfected into SF9 cells in a 6-well format in 45 minutes of incubation. After 72 hours of post-transfection cells displayed typical sites of late stage infection (increasing of cell in diameter, size of nuclei, detachment and lysis). The medium was collected from each well (~2 ml) and transferred into sterile 15 ml snap-cap tubes. The P1 viral stock was stored at 4 °C, protected from light. For amplification of P1 viral stock SF9 cells growing on a monolayer were infected at a multiplicity of infection (MOI) ranging from 0.05 to 0.1. MOI is defined as a number of virus particles per cell.

Following formula was used to calculate the amount of viral stock needed to obtain a specific MOI:

$$\textit{Inoculum required (ml)} = \frac{\text{MOI(pfu/cell) x number of cells}}{\text{titer of viral stock (pfu/ml)}}$$

Cells were harvested at appropriate time (48 hours postinfection) and expression of recombinant protein was analyzed by lysing the cell pellet in 1 × SDS-PAGE Buffer (62.5 mM Tri-HCl, pH 6.8, 2% SDS), boiling the samples for 3 minutes at 95ºC and separating proteins by SDS-PAGE. Protein bands were monitered by Coomassie Blue Staining.

Buffers used for purification of HIS-tagged recombinant proteins from insect cells:

Guanidinium Lysis Buffer, 6M	6 M Guanidine HCl 20 mM $NaP0_4$ pH 7.8 500 mM NaCl
Denaturing Binding/Wash Buffer	8M Urea 20 mM $NaPO_4$, pH 7.8 500 mM NaCl 0.1% Triton
Native Wash Buffer	500 mM NaH_2PO_4 300 mM NaCl pH 8.0 20 mM Imidazol 0.1% Triton
Native Elution Buffer	50-750 mM Imidazol in Native Wash Buffer pH 8.0

The pH was adjusted with NaOH.

48h after infection, SF9 cells were centrifuged at 500 *g* for 10 min and the pellet was resuspended in 1ml of lysis buffer pH 7.8 (6 M Guanidine HCl, 20 mM Na_3P04 and 500 mM NaCl). The suspension was lysed with a 19 G hypodermic needle and centrifuged at 14000 rpm in a 5417R (Eppendorf) for 25 min. The supernatant was then incubated for 1 hour at 4°C with Ninitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen) equilibrated with denaturing bind/wash buffer (8 M Urea, 20 mM NaPO4, 500 mM NaCl, 0.1% Triton buffer pH 7.8). After incubation, the beads were loaded onto a column, washed with denaturing bind/wash buffer 2 times and with native wash buffer (500 mM NaH_2PO4, 300 mM NaCl, 20 mM Imidazol, 0.1% Triton, pH 8.0) three times.

Beads were eluted with native elution buffer (50-750 mM Imidazol) at 4ºC. From each step the fraction was run on SDS-PAGE.

3.2.11. 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 OD_{595} was plotted versus concentration of BSA standards.

3.2.12. Dephosphorylation assay of HIS Tra2-beta1 recombinant protein

The His-Tra2-beta1 protein at concentrations of 1 mg/ml bound to a Ni-NTA resin was incubated in a typical reaction mixture of 60 µl containing HeLa nuclear extract, 0.3 µl, [gamma-^{32}P] ATP, (250 mCi/ml) (Hartmann Analytic), 25 mM $MgCl_2$, 3. 3 mM Tris-acetate (pH 7.8), 6.6 mM potassium acetate, 1 mM magnesium acetate and 0.5 mM DTT for 30 min at 30 °C. The samples were then washed once in cold 1 × PBS and 2 times in native wash buffer containing 500 mM NaH_2PO_4, 300 mM NaCl, 30 mM Imidazol, 0.1% Triton (pH 8.0). Half of the protein then was dephosphorylated with PP1, purified from rabbit skeletal muscle with a final concentration of 40 ng/µl in PP1 buffer (NEB). After collecting the resin by centrifugation, the supernatants were boiled in SDS sample buffer and subjected to electrophoresis in 12% polyacrylamide gels followed by Coomassie Staining and Western Blotting.

1 × PP1 buffer:

50 mM HEPES

0.1 mM Na_2EDTA

5 mM DTT

0.025 Tween 20%

3.2.13. 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 ^{35}S-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-2-GST or GST proteins coupled to Glutathione-Sepharose 4B in the presence of 200 µl of 1XPBS buffer/0.1%Triton X100 for 2 h at 4 °C.

3.2.14. 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% CO_2. 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% CO_2.

3.2.15. Subculturing of primary human fibroblasts and treatment by phosphatase 1 inhibitors

The human primary foreskin fibroblasts were split with DMEM+10% FCS each third day. Cells were plated in an amount of 5- 8 × 10^5. On the next day cells were treated with appropriate amount of inhibitors in a dosage response manner from 1 to 5 days. After treatment cells were lysed by RIPA buffer 30 min at 4°C. The lysates were cleared by centrifugation for a few seconds at 10000 rpm. The expression of the SMN protein was analyzed by SDS-PAGE with subsequent Western Blotting analysis with the specific antibody.

3.2.16. 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 × 10^5 cells / 8 cm^2 with growth medium DMEM +10% FCS. After splitting cells were incubated at 37 °C, 5% CO_2 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 $CaCl_2$ 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 Na_2HPO_4 × 2 H_2O, 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% CO_2.

3.2.17. 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).

3.2.18. Immunohistochemistry

Cos7 cells were grown on coverslips, transfected with pEGFP-Tra2-beta1 and pECFP PP1cgamma constructs overnight, washed in PBS at pH 7.4 and fixed in 4% formaldehyde for 20 min at 4°C. Fixed cells were washed 3 times in 1xPBS prior to mounting on microscope slides with Gel/Mount (Biomeda). The cells were examined by confocal laser scanning microscopy (Leica DMIRE2) using a HCX Plan Appochromat 100 X 1.4 CS oil immersion objective. For the simultaneous imaging of EGFP and ECFP fluorescence, both labels were exited with the 438-nm line of an argon laser and 488-nm line of a helium–neon laser, respectively. The emission from each flurochrome was detected using 465-514nm (CFP) and 543-633nm (GFP). The mean distribution for each signal was determined. Colocalisation was defined when signals from both flurochromes were larger than the mean minus the standard deviation. The area of the cell was manually outlined and the degree of colocalisation was determined using the Leica confocal software LCS v2.5 1347.

3.2.19. Immunostaining

Adult rat brain cryosate sections (10 μm) or cells grown on coverslips were fixed for 30 min in 4% paraformaldehyde in PBS at 4 °C. Permeabilization and blocking for rat brain sections were performed for 2 h with 0.5% Triton X-100 and 3% NGS in PBS. Cells fixed with paraformaldehyde were washed three times in PBS and 0.1% Triton X-100, and blocked in PBS, 0.1% Triton X-100, and 3% NGS for 2 hours at room temperature. The incubation with primary antibody (diluted in PBS, 0.1% Triton X-100, and 3% BSA, bovine serum albumin) was performed overnight at 4 °C with subsequent washing (three times) with PBS and 0.1% Triton X-100. Secondary immunofluorescent antibody (diluted in PBS and 0.1% Triton X-100) was added for 2 hours at room temperature. Finally, the sections or the cells on coverslips were washed three times in PBS and 0.1% Triton X-100 and later examined by confocal laser scanning microscopy (Leica, DMIRE2).

3.2.20. Quantification of colocalisations in cells

Cells were analyzed with confocal microscopy and single images were analyzed using Photoshop.

3.2.21. Immunoprecipitation of proteins

RIPA:	RIPA RESCUE:
1% NP40	20 mM NaCl
1% Sodium deoxycholate	10 mM Na-phosphate, pH 7.2
0.1% SDS	1 mM NaF
150 mM NaCl	5 mM β-glycerophosphate
10 mM Na-phosphate, pH 7.2	freshly added:
2 mM EDTA	2 mM Sodium orthovanadate
50 mM NaF	1 mM DTT
5 mM β-glycerophophate	1 mM PMSF
freshly added:	20 μg/ml Aprotinin
4 mM Sodium orthovanadate	
1 mM DTT	HNTG WASH:
1 mM PMSF	50 mM HEPES, pH 7.5
20 μg/ml Aprotinin	150 mM NaCl
100 U/ml Benzonase	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 dH_2O 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.

3.2.22. 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%: dH_20 –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

3.2.23. 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.

3.2.24. 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:	NET-GELATINE:
192 mM Glycine	150 mM NaCl
25 mM Tris	5 mM EDTA
20% Methanol	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	

3.2.25. 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% CO_2 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 (see 3.2.24.). 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/).

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
CHIP	94 °C 4 min; 30 cycles – 94 °C 30 sec, 58 °C 30 sec, 72 °C 30 sec; 72 °C 5 min

3.2.26. 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 dH_2O. 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 dH_2O.

3.2.27. RT–PCR

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 3.2.22. on conditions to amplify minigene products from *in vivo* splicing assays).

3.2.28. Array analysis

5 µg of total RNA was reverse transcribed in 30 µl total reaction volume with 200U Superscript II (Life Technologies), 4 µg random primers (Life Technologies) and 250 µM final concentrations each of dTTP and aa-dUTP, in 1xfirst strand buffer (Life Technologies) at 42 °C for 2 hours. RNA strands were hydrolysed with 10 µl NaOH (1 N) during 10 min at 65 °C. The reaction mixtures were neutralised with 10 µl HCl (1 N). The resulting aa-cDNAs were precipitated using 6 µl of NaAc, $3H_2O$ (3M) pH 5.2, 132 µl absolute ethanol, 0.5 µl glycogen and incubated over night at -20 °C. aa-cDNA pellets were resuspended in 2.5 µl RNAse free water and labelled with 5 µl sodium bicarbonate (0.1 M), pH 9.0 and 2.5 µl Cyanine 3 or Cyanine 5 (Amersham Biosciences) in DMSO. Each sample was incubated in the dark for 1 hour. The reactions were completed by adding 4.5 µl hydroxylamine (4M) and incubated for 15 minutes. Labelled materials were purified using JetQuick PCR purification columns (GenoMed) as per manufacturer's instructions and eluted twice with 50 µl each of RNase free water preheated at 65 °C. The cDNA yields and dye incorporation was quantified by spectrophotometry. Hybridizations were performed using 2.5 µg of Cy3 and Cy5 labelled targets (for 11K and 22K slides respectively) along with 20 µg of herring sperm DNA (Invitrogen).

3.3. Databases and computational tool

Database/ software	URL	Description	Reference
ASD	http://www.ebi.ac.uk/asd	The alternative splicing database	(Thanaraj A. et al., 2004)
ASePCR	http://genome.ewha.ac.kr/ASePCR/	Web-based application emulating the RT-PCR in various tissues	(Thanaraj A. et al., 2004)
ClustalW	http://www.ebi.ac.uk/clustalw/index.html	Multiple sequence alignment program for DNA or proteins	(Thompson J.D. et al, 1994)
ESE	http://rulai.cshl.edu/tools/ESE/	Finds putative binding regions for several splice factors	(Cartegni L. et al., 2003)
Human BLAT Search	http://www.genome.ucsc.edu/cgi-bin/hgBlat	Sequence alignment tool similar to BLAST	(Kent W.J., 2002)
NCBI BLAST and PSI-BLAST	http://www3.ncbi.nlm.nih.gov/BLAST/	Finds regions of sequence similarity	(Altschul S.F. et al., 1990); (Altschul S.F. et al., 1997)

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 (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 exhibit strong sequence identity

Two related sequences of Sam68 exist in rat. Using scaffold attachment factor B SAF-B (Nayler O. et al., 1998; Weighardt F. et al., 1999) in a yeast two hybrid assay, the Sam68-like mammalian protein, rSLM-2 (Stoss O. et al., 2001) was isolated from a rat brain cDNA library. In addition, cDNAs bearing high homology to the previously reported SLM-1 (Di Fruscio M. et al., 1999) and Sam68 (Richard S. et al., 1995) were isolated. We named them therefore rSLM-1 and rSam68, respectively. rSam68, rSLM-1, and rSLM-2 are members of the STAR protein family (Vernet C. and Artzt K., 1997), also called GSG family (Grp33, Sam68, Gls-1) (Jones A.R. and Schedl T. 1995; Chen W. et al., 1999). All these three proteins contain a hnRNP K homology RNA-binding domain KH, several arginine-glycine dipeptides and a tyrosine-rich carboxy terminus. The three highly related proteins, rSam68, rSLM-1, and rSLM-2, differ by the numbers of proline-rich stretches. Thus, both in rat and human, three highly related cDNAs exist that shares a similar, but not identical domain structure. The sequence of rSLM-1 is shown in Figure 11. The protein has a maxi-KH RNA binding domain, which harbours the RNA binding activity (gray box).

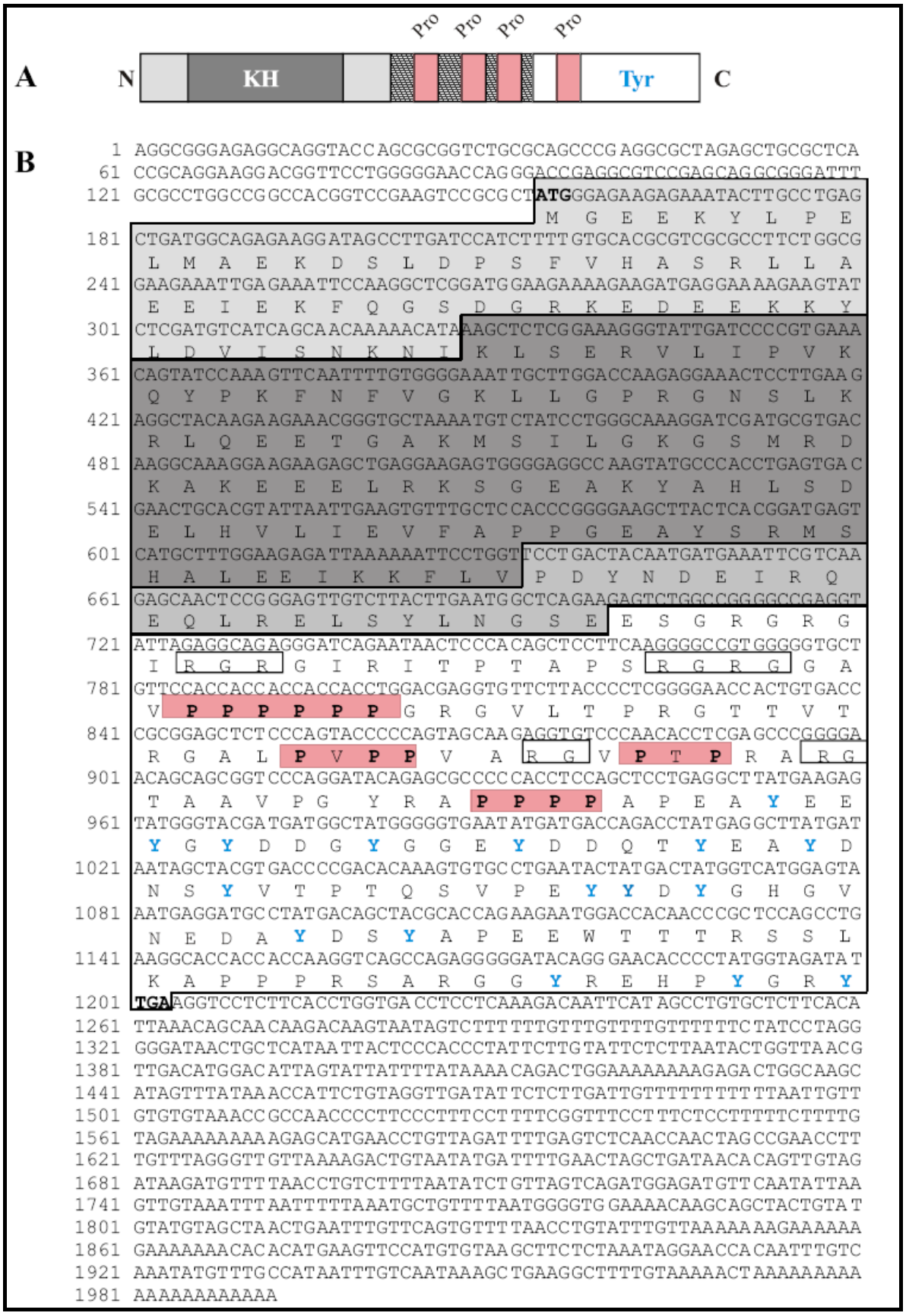

Figure 11. Sequence analysis of the rSLM-1 protein. (A) Domain structure of rSLM-1. Pro: proline-rich regions, marked in pink; KH: hnRNP K homology domain-grey; RG: arginine/glycine-rich region; Tyr: tyrosine-rich region. (B) cDNA and protein-sequence of rat rSLM-1 are shown. Start and

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.

The protein, like other members of GSG family: SLM-2, QKI-5, GRP33 and heteronuclear ribonucleoprotein K contains several arginine glycine rich regions, which could be methylated by arginine methyl transferases (Côté J. et al., 2003). The protein also contains proline residues, located downstream of the GSG domain (marked in pink). Proline rich sequences can bind to SH3 (Src homology) and WW (domain with two conserved tryptophan) domains. The C-terminus contains tyrosine-rich residues (marked blue), potential sites for tyrosine kinases.

4.1.2 rSLM-1 interacts with proteins that function in splice site selection

It was demonstrated earlier that rSLM-2 acts as a splicing regulatory protein (Stoss O. et al., 2001). We have tested proteins involved in splice site selection for their interaction with rSLM-1 in yeast two hybrid screens and showed that rSLM-1 multimerises with itself and with the related proteins rSLM-2 and rSam68. We also detected an interaction of rSLM-1 with the hnRNPs, SAF-B, hnRNP G and YT521-B - a nuclear protein implicated in splice site selection (Nayler O. et al., 1998; Soulard M. et al., 1993; Weighardt F. et al., 1999; Hartmann A. et al., 1999; Nayler O. et al., 2000; Stoilov O. et al., 2002). We did not observe any interaction between rSLM-1 and the SF1 protein (Rain et al., 1998). The only SR-protein binding to rSLM-1 in yeast was SRp30c (Screaton G.R. et al., 1995), whereas all other SR-proteins tested (SF2/ASF, SC35, SRp40, SRp55, SRp75) did not interact with rSLM-1.

We performed therefore GST-pull down assay to study whether the proteins directly interact with each other. Recombinant GST-rSLM-1 was produced in bacteria and incubated with *in vitro* translated, radioactively labeled interacting proteins in the presence of glutathione sepharose. The splicing regulatory proteins YT521-B, hnRNP G, SAF-B, and SRp30c bound to recombinant GST-rSLM-1, whereas the SR protein SF2/ASF and hnRNP L failed to bind, which confirmed the yeast two hybrid data. None of the proteins bound to recombinant GST (Figure 12A). We then performed coimmunoprecipitation experiments to study the interactions of the proteins *in vivo*. EGFP-rSLM-1 was transiently

expressed in HEK293 cells and immunoprecipitated with an anti-GFP antibody (Figure 12B).

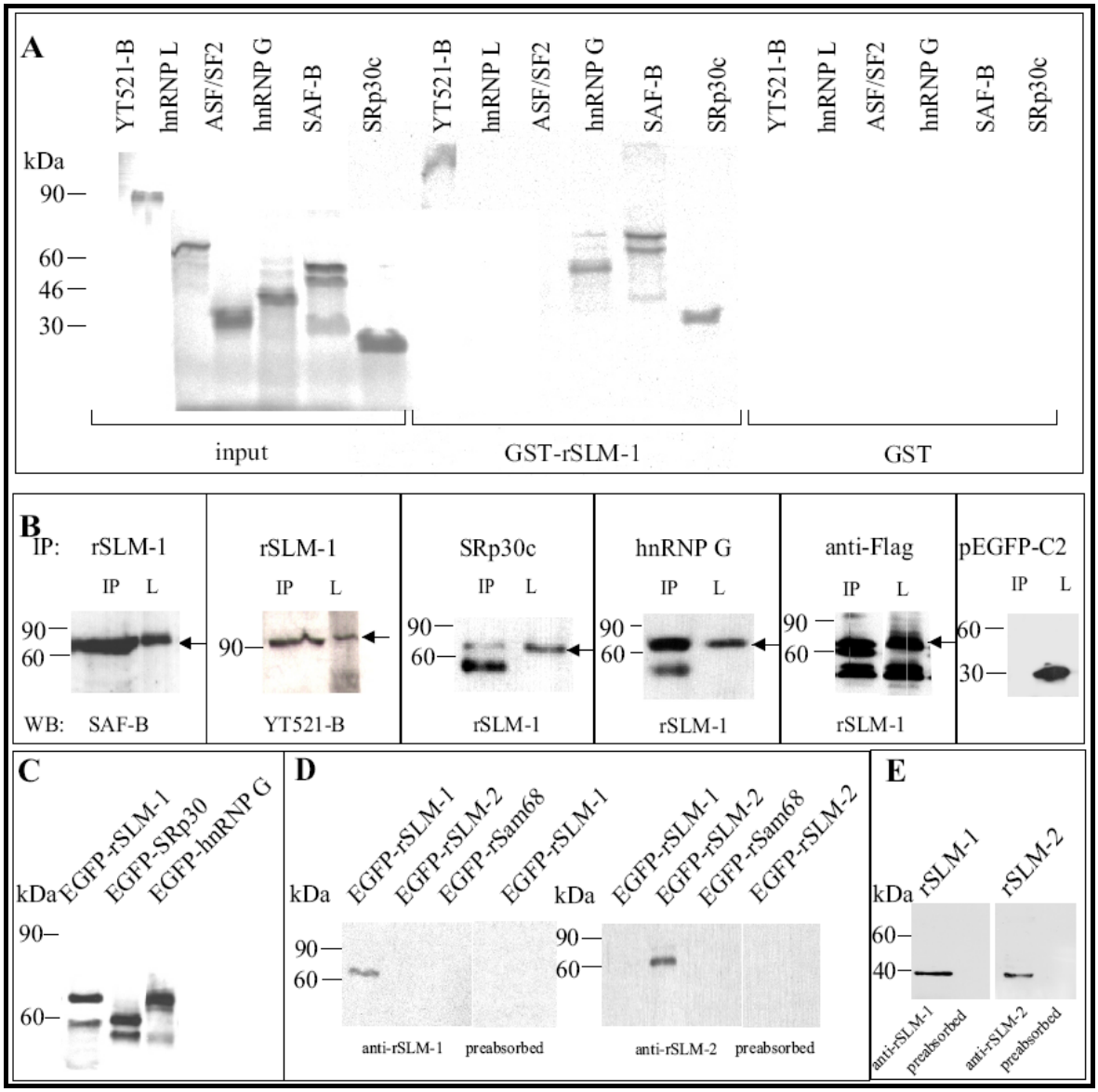

Figure 12. The rSLM-1-GST-tagged protein interacts with several splicing factors. (A) Recombinant GST or GST-rSLM-1 was incubated with interacting proteins indicated in the presence of glutathione-Sepharose 4B. The inputs of the radioactive proteins are shown in the first six rows. GST-rSLM1 (but not GST) interacts with YT521-B, hnRNP G, SAF-B, and SRp30c and does not interact with hnRNP L and ASF/SF2. (B) Coimmunoprecipitations. IP: immunoprecipitate, L: lysate. EGFP-rSLM-1 was expressed in HEK293 cells and precipitated with an anti-GFP antibody. Coimmunoprecipitated endogenous SAF-B or YT521-B (IP) and the corresponding lysates (L) were detected on Western blots using their specific antibodies. The interaction of rSLM-1 with SRp30c and hnRNP G and itself: FLAG-rSLM-1 was coexpressed with EGFP-SRp30c or EGFP-hnRNP G. The EGFP constructs were immunoprecipitated using an anti-GFP antibody and the FLAG-rSLM-1 was detected by the specific antibody in Western blots. No coimmunoprecipitation was seen between EGFP and FLAG-rSLM-1. The immunoprecipitated EGFP-fused proteins were detected by Western blot using an anti-GFP

antibody to show their integrity. (D) Analysis of the antisera against rSLM-1 and rSLM-2. EGFP-rSLM-1, EGFP-rSLM-2, and EGFP-rSam68 were overexpressed in HEK293 cells and lysates were analysed with affinity purified anti-rSLM-1 or ant-rSLM-2 antisera. Both antisera are specific for either rSLM-1 or rSLM-2 and do not recognize the related proteins rSam68 or rSLM-2 and rSLM-1, respectively. Preabsorbtion of the antisera with the peptides used to generate them abolish the signal. (E) Detection of endogenous protein using the rSLM-1 and rSLM-2 antisera. Lysates from Neuro2A cells were analyzed by Western blot using the rSLM-1 and rSLM-2 antisera. Preabsorbtion of the antisera abolished the signal.

The endogenous SAF-B and YT521-B proteins could be identified with the specific antibodies (Figure 12C). EGFP-tagged SRp30c and hnRNP G were coexpressed together with FLAG-rSLM-1 in HEK293 cells. The EGFP-tagged proteins were immunoprecipitated and coprecipitated FLAG-rSLM-1 was identified using the anti-rSLM-1 antibody. Using this assay, we could confirm binding of rSLM-1 to SRp30c, hnRNP G, and to itself (Figure 12B). No interactions were observed with EGFP (Figure 12B), SF1, or SF2/ASF (data not shown).

Finally, we raised peptide antisera against rSLM-1 and rSLM-2. The peptides were chosen in parts specific to rSLM-1 and rSLM-2 and no cross-reactivity between the STAR family members was observed after affinity purification (Figure 12D). The antisera detect proteins of the predicted size in lysates of Neuro2A cells. The signal from both the overexpressed (Figure 12D) and endogenous (Figure 12E) protein disappears after preabsorbtion, demonstrating the specificity of the antisera. In summary our data show that rSLM-1 directly interacts with itself and several proteins functioning in RNA processing like SAF-B, hnRNP G, SRp30c, and YT521-B.

4.1.3 rSLM-1 and rSLM-2 show different tissue-specific expression

rSLM-1 and rSLM-2 are two highly related proteins, sharing almost identical molecular properties and having similar molecular binding partners. The tissue distribution of their RNAs was compared by Northern Blot analysis.

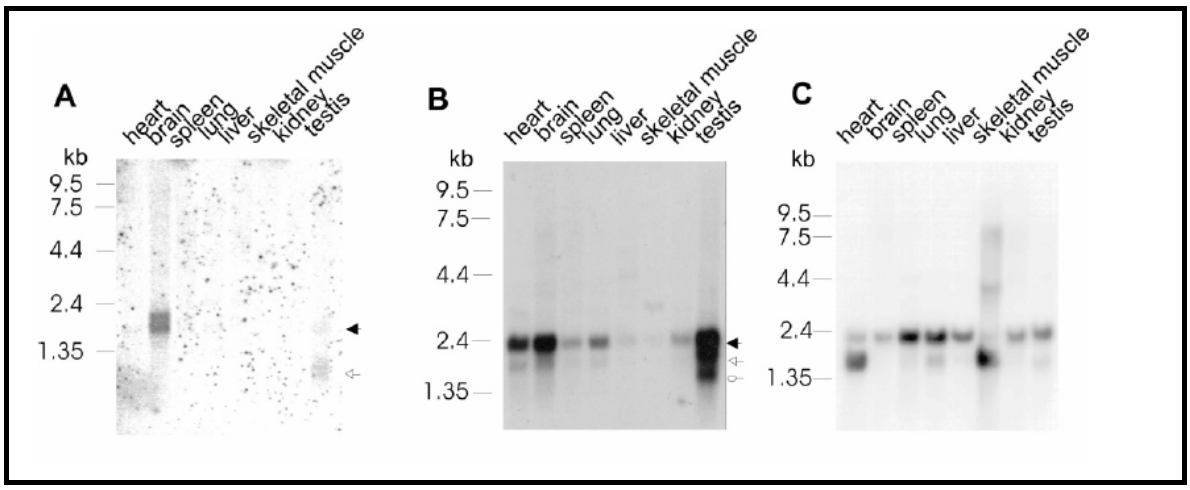

Figure 13. rSLM-1 and rSLM-2 have a different tissue expression. (A) Nothern Blot analysis of different rat tissues was probed with SLM-1. (B) The same blot was probed with SLM-2. (C) In order to demonstate the equal loading the same blot was probed with beta-actin.

cDNAs that lack the first 311 nucleotides which contain the most conserved part of KH domain were used as hybridization probes. This allows the discrimination of the related STAR proteins. Northern Blot analysis has shown that rSLM-1 is expressed only in brain and testis. In testis, two weak signals can be detected. One signal corresponds to a faster migrating mRNA, which could represent a shorter rSLM-1 variant or a cross-hybridization to another mRNA. Another faint signal of the expected size is visible (Figure 13A), corresponding to rSLM-1. rSLM-2 which was detected in all tissues examined was expressed mostly in testis, brain and heart (Figure 13B).

Previously it was observed in several systems that no protein was generated from mRNAs, most likely due to translational control. Therefore, the protein expression was investigated by Western Blot using lysates from different brain areas and testis.

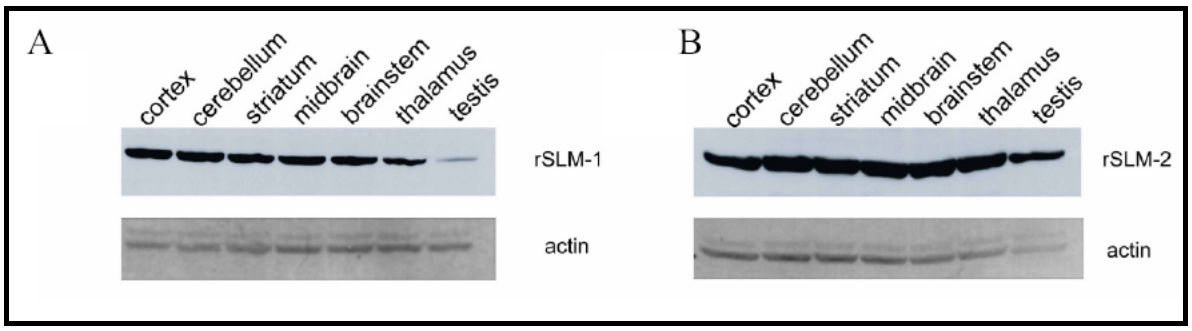

Figure 14. rSLM-1 and rSLM-2 expression in the brain regions and testis. Western blot analysis of protein from different brain regoins of rabbit was probed with anti-rSLM-1, 2 (top panel) and anti-actin (lower panel).

Figure 14 shows that rSLM-1 and rSLM-2 proteins can be detected in all brain areas, as well as in testis. However, in agreement with the mRNA expression, rSLM-1 is less abundant in testis than in brain. So far, our analysis did not allow for a direct comparison of the expression levels, as different probes had to be used. We wanted to compare the expression of these three highly related proteins. We used DNA array analysis to test the mRNA expression of SAM68, rSLM-1, and rSLM-2 in various tissues. The DNA array analysis was performed with reverse transcribed RNA. As it is shown in Figure 15, SAM68 is the most abundant form in all the tissues, except brain and testis. Interestingly, rSLM-1 mRNA is far less abundant than rSLM- 2 or SAM68 mRNAs in all the tissues.

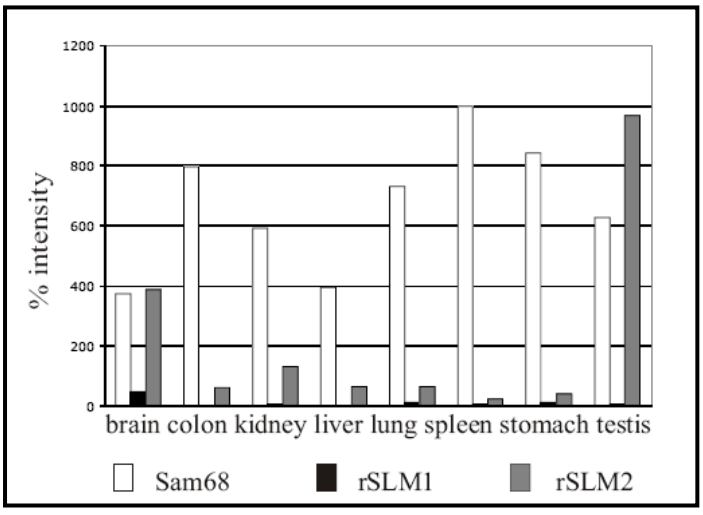

Figure 15. The expression pattern of three highly related proteins rSLM-1, rSLM-2 and Sam68. Total isolated RNA was used for DNA array analysis. Fifteen micrograms of fragmented labeled cRNA was hybridized to the human genome U133A and U133B arrays. These arrays contain 11 oligonucleotides per gene that bind to the 3' UTR. Signal intensities were amplified using a bio-tinylated anti-streptavidin antibody and a second SAPE staining step. Data were analyzed using the Microarray Suite software (MAS5, Affymetrics).

In summary 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.

4.1.4 rSLM-1 and rSLM-2 show non-overlapping neuronal expression in the brain

Both rSLM-1 and rSLM-2 are expressed in the brain. We then investigated their cellular expression patterns in the forebrain to found 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 (data not shown). 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 (Figure 16B).

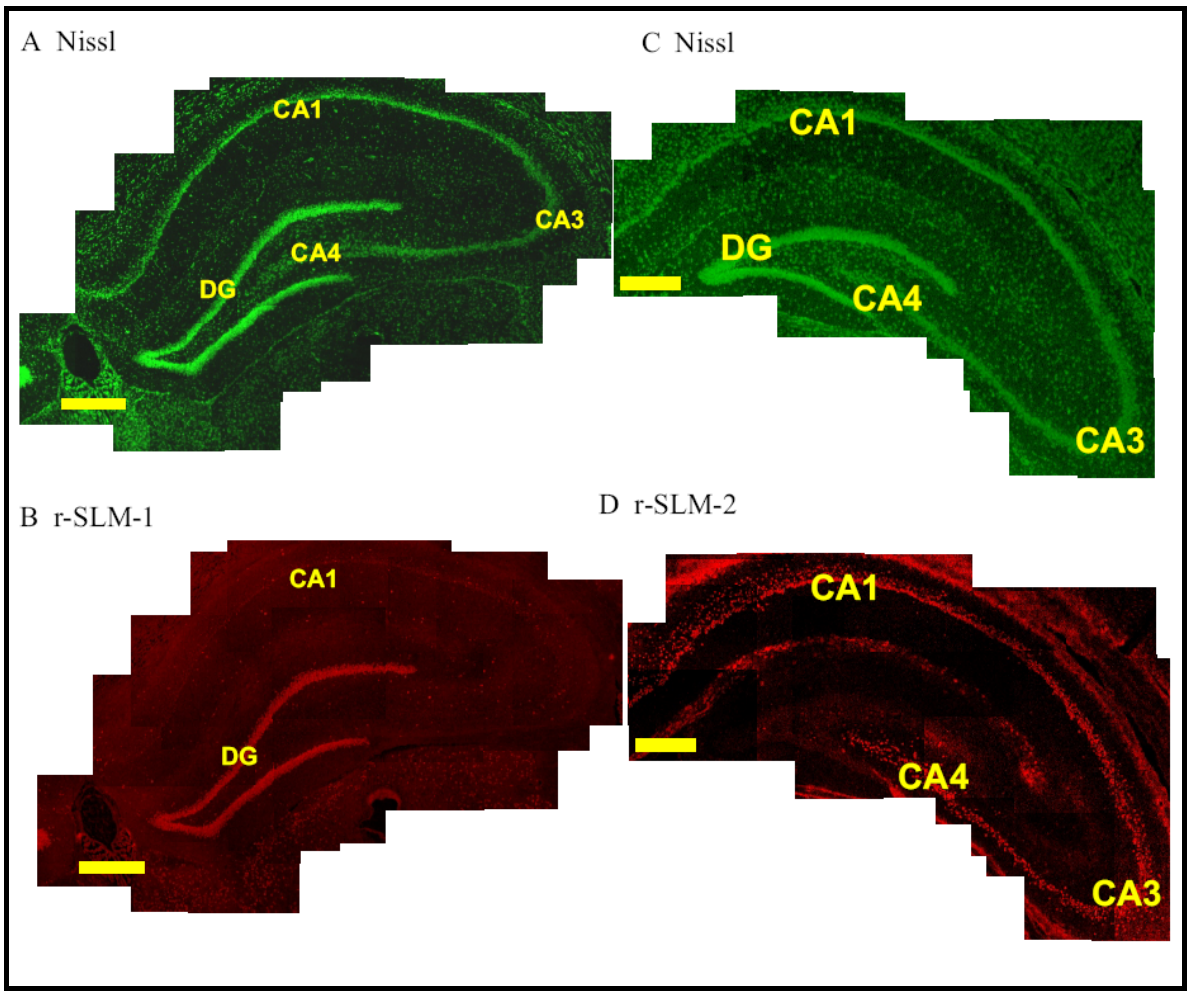

Figure 16. rSLM-1 and rSLM-2 show different expression in the hippocampus. Sections of hippocampus 10 microns from rat brain were analyzed with affinity purified specific antisera for rSLM-1 and rSLM-2. CA1, CA3, and CA4 regions and the granule cells of the dentate gyrus (DG) are

indicated. The scale bar represents 500 Am. (A, C) All nuclei of the brain cells were stained with neuro-trace green fluorescent Nissl staining. (B, D) Anti-rSLM-1, 2 staining with specific affinity purified antisera.

In contrast to rSLM-1, rSLM-2 was highly expressed in the CA1 to CA4 regions (Figure 16D). We then tested the expression of proteins in the dentate gyrus and CA4 region in detail (Figure 17).

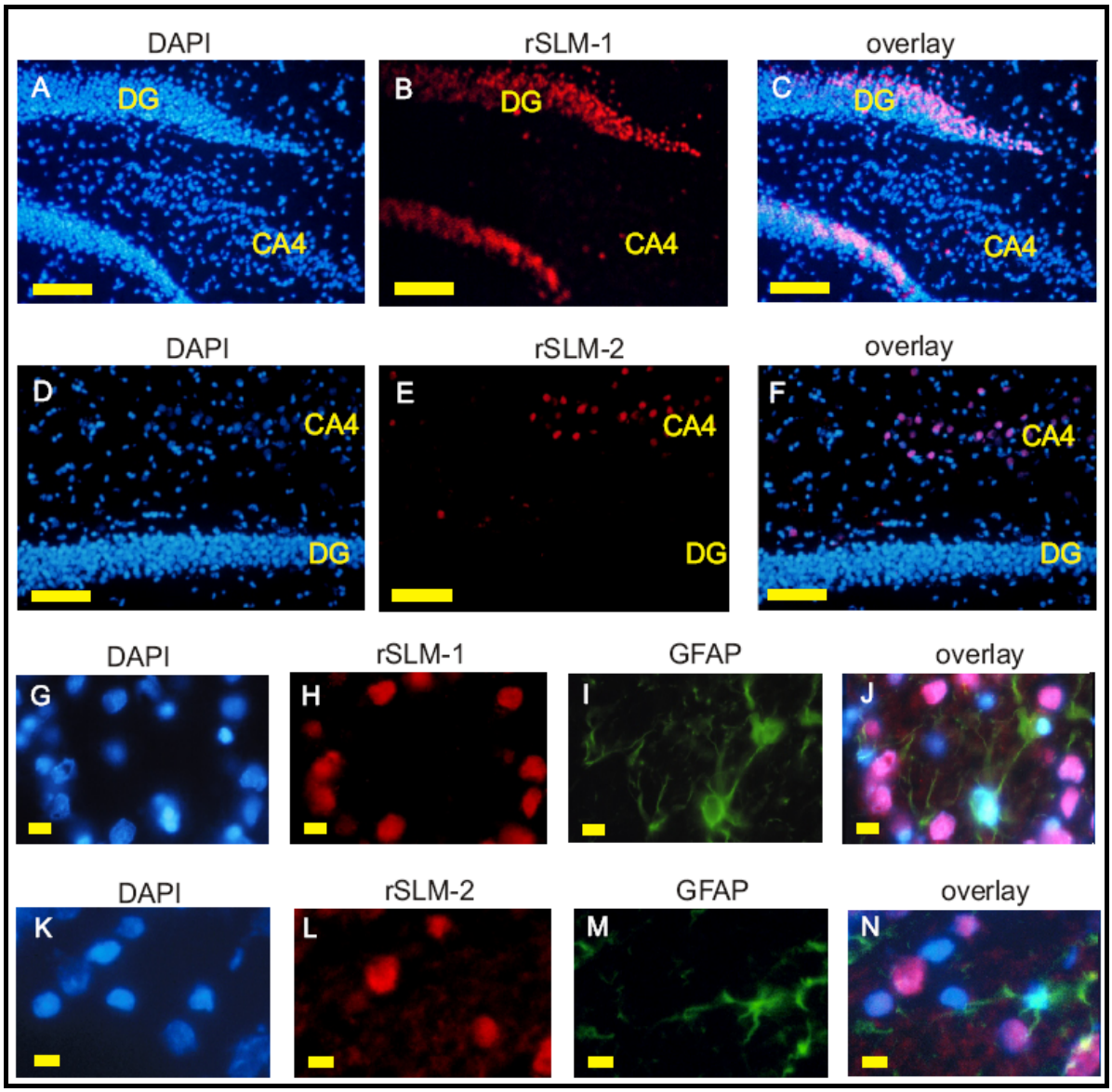

Figure 17. Comparison of rSLM-1 and rSLM-2 expression in the CA4 region and in the dentate gyrus. Ten micron sections from adult rat brain were stained with the anti-rSLM-1 and rSLM-2 antisera. Nuclear staining with DAPI is shown in blue; the rSLM signals are shown in red; GFAP staining is shown in green. (A, D) DAPI staining of the hippocampal region. The CA3 and CA4 regions (CA3, CA4) and the granule cells of the dentate gyrus (DG) are indicated. The scale bar represents 100µm. (E) rSLM-2 is present in the pyramidal cell nuclei of the CA4 region but is absent from the dentate gyrus. (C-F) Overlay of the corresponding DAPI and rSLM-1 or rSLM-2 stainings. (G-N) Triple staining from the neocortex with DAPI (G, K), anti-rSLM-1 antibody (H) or anti-rSLM-2 (L) and anti-

GFAP (I, M). The corresponding overlays are shown in (J) and (N). rSLM-1 and rSLM-2 are not expressed GFAP-positive cells. The scale bar represents 10µm.

We observed using higher magnification that most cells in the dentate gyrus express rSLM-1, whereas most cells in the CA4 region express rSLM-2. This shows a non-overlapping expression pattern of both proteins. Only few rSLM-1 positive cells in the CA4 region were rSLM-2-negative (Figure 17A–F). The morphology and location of the cells expressing rSLM-1- and rSLM-2 suggested that they were neurons.

4.1.5 rSLM-1 and rSLM-2 are expressed in neurons

The hypothesis that rSLM-1- and rSLM-2 are expressed in neurons was further investigated by double staining with the neuronal marker NeuN.

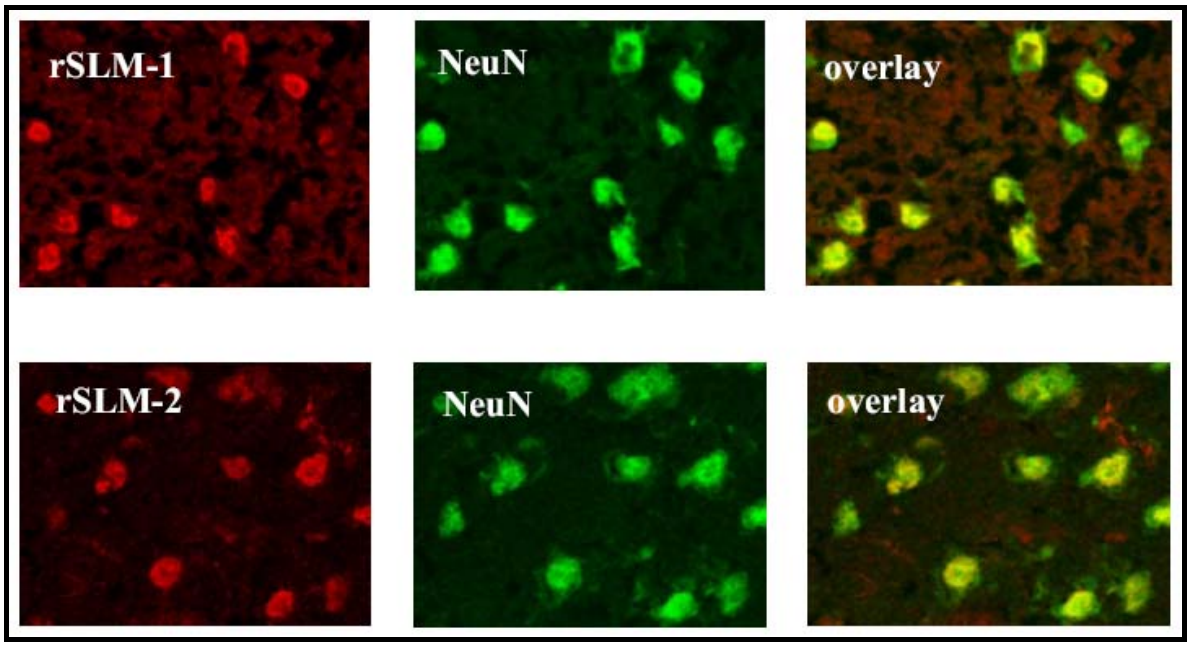

Figure 18. rSLM-1 and rSLM-2 proteins are localized in neurons. Double staining of hypocampus region with anti-rSLM-1 (upper panel) and anti-rSLM-2 (lower panel) specific antibodies and neuronal marker NeuN.

We found that SLM-1-positive (Figure 18, upper panel) and SLM-2-positive (Figure 18, lower panel) cells express NeuN. However, not all NeuN-positive cells express either SLM-1 or SLM-2, demonstrating a specific neuronal expression pattern. No specific signal was observed using preabsorbed antibodies. These experiments clearly showed that rSLM-1 and rSLM-2 have non-overlapping expression patterns in the brain and are expressed in neurons. Therefore the proteins will not heteromultimerise to each other in the hippocampus.

4.1.6 rSLM-1, but not rSLM-2 is phosphorylated by the p59^{fyn} kinase

It is known from the domain structure of rSLM-1 and rSLM-2 that these proteins could 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*.

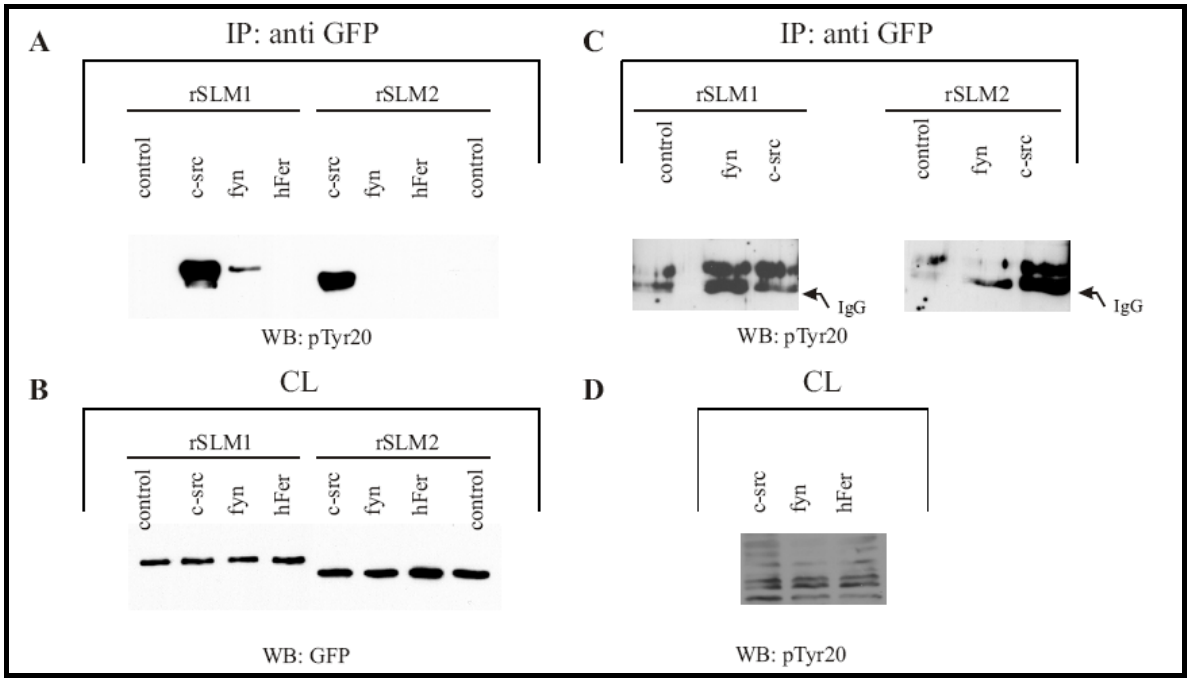

Figure 19. Tyrosine phosphorylation of rSLM-1 and rSLM-2 by non-receptor tyrosine kinases. (A) HEK293 cells were transfected with EGFP-SLM-1 or EGFP-SLM-2 and the kinases indicated. Protein was immunoprecipitated with anti-EGFP and tyrosine phosphorylation was detected with pTyr20. (B) The reblot was 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.

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.

As it is shown in Figure 19A-C, both proteins were strongly phosphorylated by c-src kinase. In contrast to c-src, the $p59^{fyn}$ phosphorylated rSLM-1, but not rSLM-1, although equal amounts of rSLM-1 and rSLM-2 were present (Figure 19B, GFP reblot). The experiment was performed both in HEK293 (Figure 19A) and in Neuro 2A (Figure 19B) 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 19D).

4.1.7 rSLM-1 is colocalized with the $p59^{fyn}$ 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 H. 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 20.

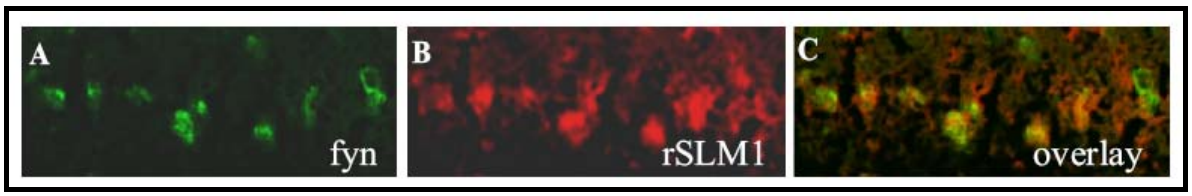

Figure 20. rSLM-1 and $p59^{fyn}$ are colocalized together in the hippocampal cells. Staining of hippocampal sections was performed with specific anti-fyn (in green) and anti-rSLM-1(in red) antibodies. The colocalization between $p59^{fyn}$ (A) and rSLM-1 (B). The superimposition (C) between images (A) and (B).

This provides strong evidence that rSLM-1, but not rSLM-2 is a substrate for $p59^{fyn}$ phosphorylation *in vivo* and that $p59^{fyn}$ and rSLM-1 are expressed in the same neuronal cells. Taken together, this suggests that $p59^{fyn}$ may regulate rSLM-1 function in the dentate gyrus.

4.1.8 rSLM-2 is phosphorylated by several non-receptor tyrosine kinases

In order to further investigate the potential candidate kinases for phosphorylation of rSLM-2, we tested the action of several non-receptor tyrosine kinases on the protein. The non receptor tyrosine kinases Syk, Csk, and FerH (Zhang J. et al., 1996; Nayler O. et al., 1998; Hao Q.L. et al., 1991) can be subdivived in to the SYK, CSK, and FES families (reveiew by Neek K. and Hunter T., 1996), had no effect at all. The strongest phosphorylation was observed with c-abl kinase (Figure 21A).

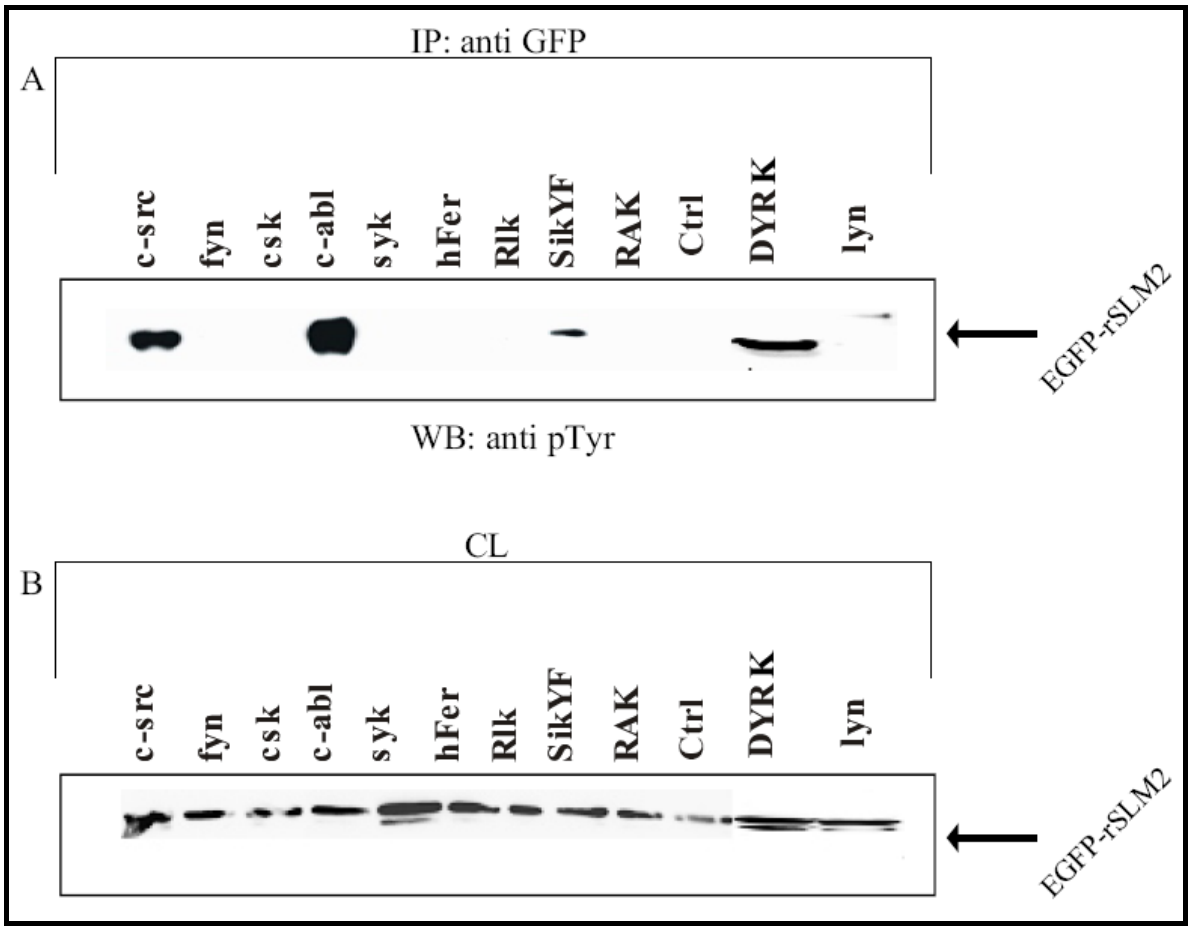

Figure 21. Several non-receptor tyrosine kinases phosphorylate rSLM-2. (A) EGFP-rSLM-2 was coexpressed together with constant amount of several indicated tyrosine kinases in HEK293 cells. Protein was immunoprecipitated with anti-GFP antibodies and the phosphorylation status of the protein was detected by the PY20, phosphotyrosine antibody. (B) CL: Crude lysatesCells crude lysates were analysed for overexpressed protein with anti-GFP antibodies. (C) IP: Immunoprecipitation.

4.1.9 rSLM-2 colocalizes with c-abl in the nucleus

In order to investigate whether c-abl and rSLM-2 are expressed in the same cell, we performed immunohistochemistry assay using the specific antibodies for both proteins.

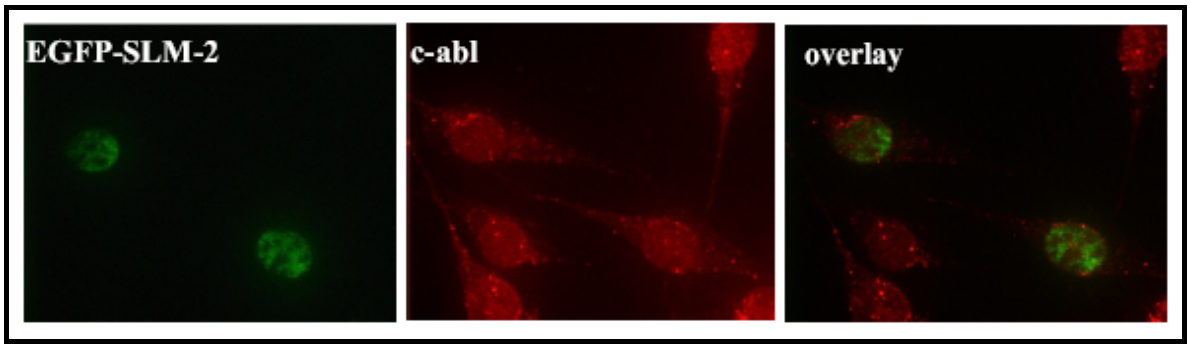

Figure 22. EGFP-rSLM-2 is colocalizes together with c-abl in the nucleus. (A) EGFP-rSLM2 was overexpressed together with c-abl expression construct in BHK cells. (B) Overexpressed c-abl was stained in red and detected both in the cytozol and in the nucleus (B). Superimposition between the images (A) and (B) shows the nuclear colocalization between EGFP-rSLM-2 and c-abl (C).

As shown in Figure 22, both proteins are colocalized in the nucleus of BHK cells. This experiment demonstrates that the $p59^{fyn}$ kinase, could interact with rSLM-2 protein in the same cells.

4.1.10 The phosphorylation of SLM-2 recombinant protein influences its binding properties

The SLM-2-GST protein was produced in bacteria to studying the biochemical properties of protein: protein interactions. Previously (Stoss O. et al., 2001), it was demonstrated that rSLM-2 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-2 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 demonstrated in Figure 23, the phosphorylation of SLM-2 splicing factor by c-abl changes protein: protein interactions.

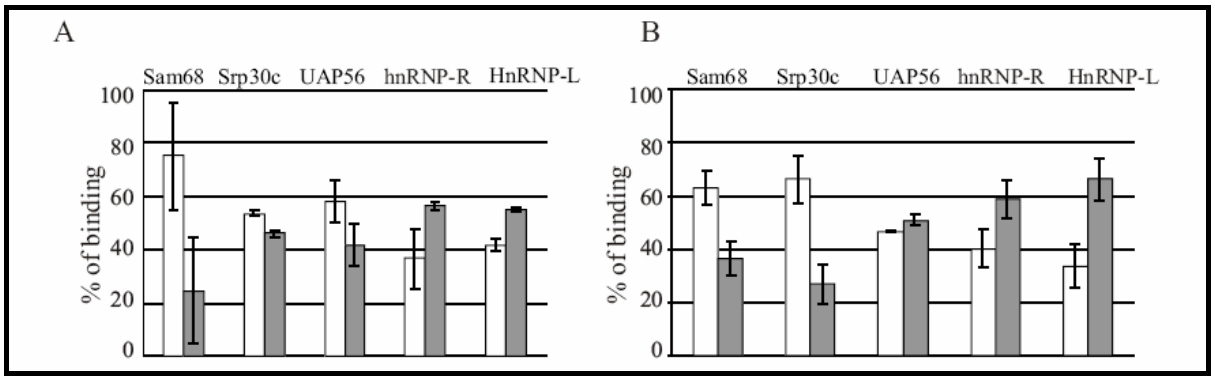

Figure 23. Phosphorylation dependent protein: protein interactions are influenced by the presence of RNA. rSLM-2-GST-pull down assay was implemented with either phosphorylated (white), or not phosphorylated (grey) recombinant protein. Binding was performed in the absence (A) or presence (B) of RNAse.

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 (benzonaze) was used for *in vitro* binding assay with Glutathione Sepharose.

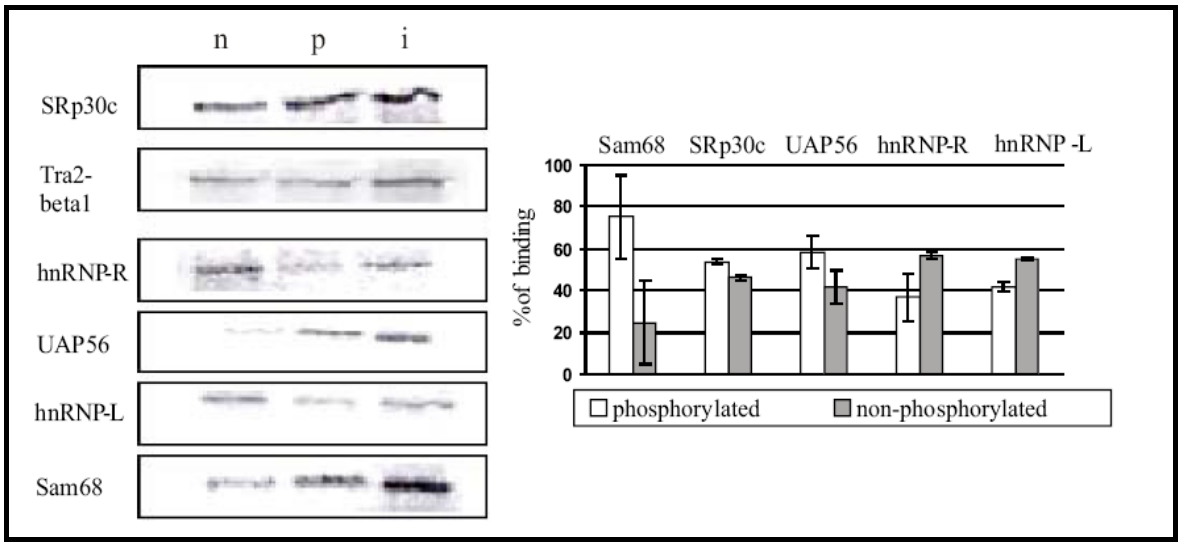

Figure 24. Phosphorylation mediated interaction of recombinant rSLM2 protein with its partners. Recombinant rSLM2-GST tag protein was incubated with several in vitro translated proteins. The load of in vitro translated protein marked as i, n-indicates the binding of specific in vitro translated interactor to recombinant rSLM2-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 23 the binding properties of the rSLM-2 protein are influenced by the presence of RNA. Interestingly, that for some proteins like for UAP56 or SRp30c the presence of RNA in the reaction is a necessary component of binding of these proteins to SLM-2. This experiment clearly demonstrates that the phosphorylation of rSLM-2 protein can regulate a number of protein: protein interactions. The RNA can serve as an important mediator in this process.

4.1.11. rSLM-1 and rSLM-2 regulate splice site selection of the SMN2 reporter minigene

Both splicing factors rSLM-1 and rSLM-2 bind to SAF-B (scaffold attachment factor B), SRp30c, YT521-B, hnRNP G, Sam68 and to themselves. All these proteins contain RNA binding domains. hnRNP G, SRp30c, and SAF-B have RRMs (RNA recognition motif) (Screaton G.R. et al., 1995; Soulard M. et al., 1993; Weighardt F. et al., 1999), the STAR proteins contain a KH (hnRNP K homology) domain and YT521-B contains a putative nucleic acid binding domain, the YTH (Stoilov P. et al., 2002).

It is therefore very likely that in combination these proteins regulate alternative splicing by acting similar to the RBP1/ tra2/tra and SF2/tra2/tra complexes (Lynch K.W. et al., 1996), hnRNP F, H /KSRP complexes (Markovtsov V. et al., 2000) and FBP/SAM68/PTB complexes (Grossman J.S. et al., 1998) that regulate alternative splicing of doublesex exon 4, the src N1 exon, and beta-tropomyosin exon 7, respectively. Therefore we decided to test this hypothesis experimentally.

Both proteins were overexpressed with a reporter gene in the present of parental empty vector pEGFP-C2, in order to balance the amount of the cDNA. As a reporter minigene we chose the SMN2 minigene, which consists of alternative exon 7, flanked by constitutive exons. Exon 7 has a purine-rich exonic enhancer (Lorson C. and Androphy E., 2000; Stoss O. et al., 2001), which is flanked by two constitutive exons 6 and 8. Since rSLM-1 and rSLM-2 were expressed in neurons, we performed the cotransfection assays in Neuro2A cells. We determined that rSLM-1 and rSLM-2 can regulate splice site selection of the SMN2 pre-mRNA in a concentration-dependent manner (Figure 25B). The quantification analysis showed that the effect was comparable for both proteins. The increase of the corresponding proteins was verified by Western Blot analysis (Figure 25 C, D).

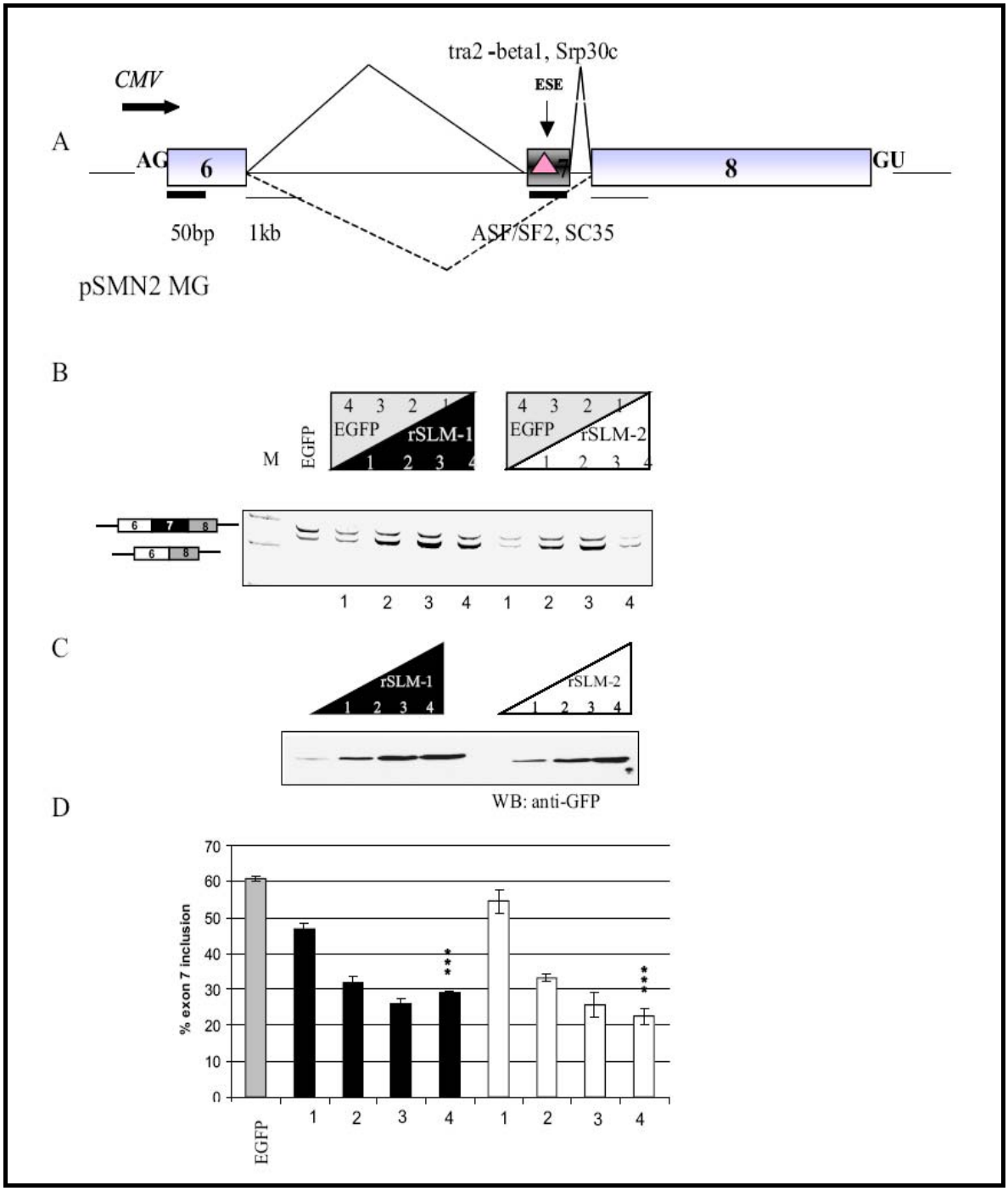

Figure 25. rSLM-1 and rSLM-2 regulate splice site selection on SMN2 reporter minigene. (A) Structure of the pSMN2 minigene (Lorson C. et al., 1999). Exons are showns as boxes, introns as lines. The sizes for exons are and indicated in thick and thin lines, respectively. The alternative exon 7 is indicated in grey. CMV promoter is marked as arrrow. The exonic splice enhancer located in the middle of exon 7 is marked as pink triangle. Splicing factors promoting inclusion or skipping of exon 7 are marked up or down relatively. (B) Neuro2A cells were transiently transfected with incresing amounts of EGFP-rSLM-1 and EGFP-rSLM-2 and constant amount of the pSMN2 minigene. The amount of transfected rSLM-1 and rSLM-2 are normalized with pEGFP-C2 contract. The RNA was analysed by RT-PCR.The strucutre of amplified products is indicated on the left. (C) Western Blot analysis of EGFP-rSLM-1 and EGFP-rSLM-2 proteins with specific antibodies. (D) Statistical

evaluation of RT-PCR results. The ratio between the signal correspondingto exon exclusion and all products was determined from at least three different independent experiments. The differences are statistically significant according to student´s t-test and indicated by stars, for rSLM-1: p=0.002, t=6.98, for rSLM-2: p=0.001, t=7.83.

4.1.12. The p59^{fyn} kinase regulates the ability of rSLM-1 to influence splice site selection

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. 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 26).

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). Therefore rSLM-2 is most likely not affected by kinase because it is not phosphorylated by p59^{fyn} (Figure 26). 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.

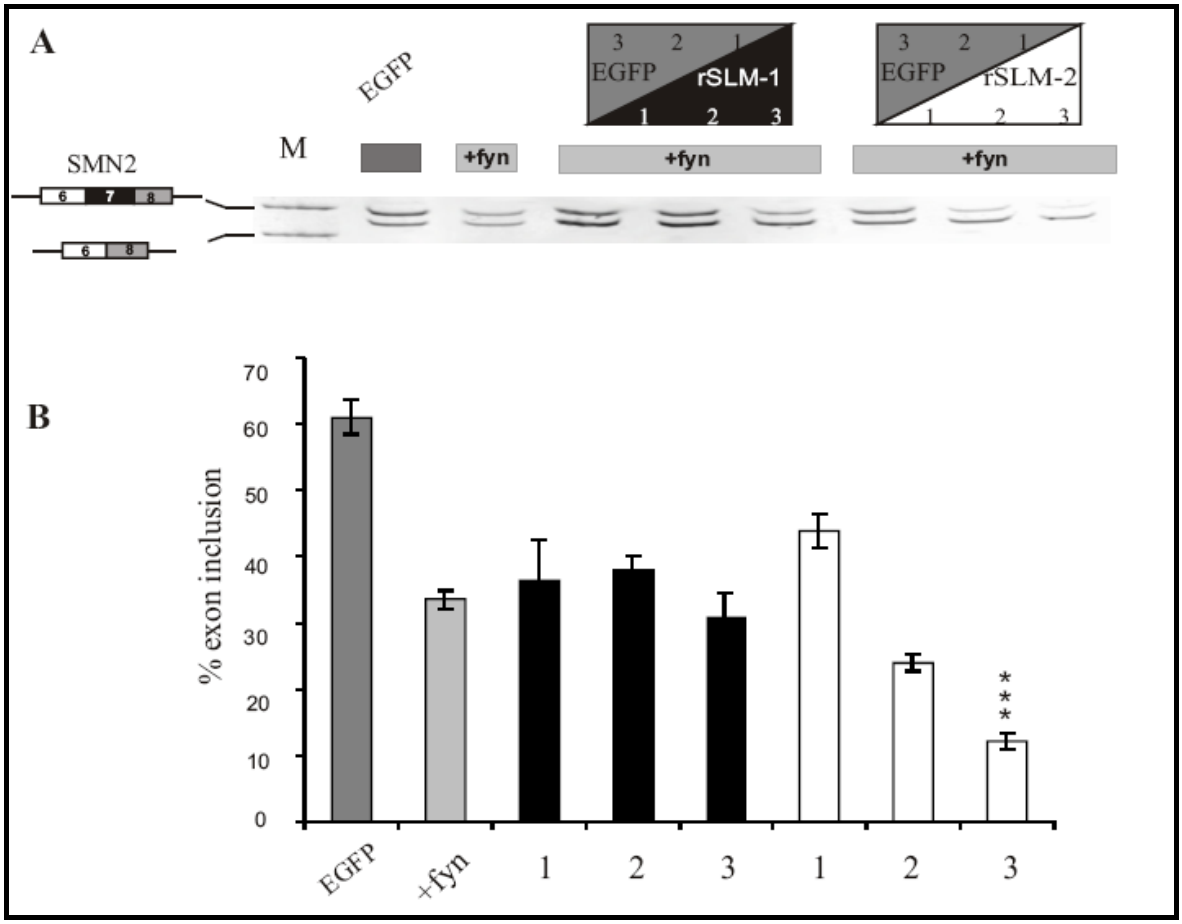

Figure 26. rSLM-1, but not rSLM-2 promotes skipping of exon 7, in the presence of p59^{fyn}. (A) Neuro2A cells were transiently transfected with increasing amounts of EGFP-rSLM-1 and EGFP-rSLM-2 and constant amount of the p59^{fyn} kinase in presence of SMN2 reporter construct. The amount of transfected 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.

To summarize, we characterized splicing factors rSLM-1 and rSLM-2 and determined the difference of these proteins on splice site selection in the presence of p59^{fyn} kinase. Therefore, 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 p59^{fyn} kinase and alternative splicing.

In the next chapter we studied, whether reversible protein phosphorylation of splicing factors can function in alternative splicing.

4.2. Regulation of alternative splicing by reversible phosphorylation

Reversible serine/threonine protein phosphorylation is an important regulatory mechanism of intracellular signal transduction. The protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), PP2B (calcineurin) and PP2C are four major serine/threonine phosphatases, mediating signaling cascades in eukaryotes. Previous research demonstrated that PP1 is involved in the overall organization of pre-mRNA splicing factors in the mammalian cell nucleus (Misteli T. and Spector D.L., 1996). Recently it was established that PP1/PP2A family phosphatases dephosphorylate U2 and U5 snRNP components facilitating overall structural rearrangements in the spliceosome during the transition from the first to the second step of splicing (Shi Y. et al., 2006). The reversible protein phosphorylation differentially regulates the subcellular localization and activity of shuttling SR proteins (Sanford J.R., 2005), whereas the dephosphorylation of the RS domain of SR proteins plays a regulatory role in a mechanism for mRNP transition from splicing to export (Shengrong L. et al., 2005). Human transformer Tra2-beta1 is a member of SR protein family splice factors that regulate splice site selection by recruiting regulatory proteins to exon sequences. In this part we demonstrate that protein phosphatase 1 (PP1) binds to Tra2-beta1 via a phylogenetically conserved RVDF sequence located on the RNA recognition motif of Tra2-beta1 and dephosphorylates the splicing factor. The PP1 binding motif is present in other RRMs of SR proteins as well.

We highlight in this studies that the binding to PP1 is a novel function of some RNA recognition motifs. Our findings further suggest that the known signal transduction pathways regulating PP1 activity influence alternative splicing events, which could be a new therapeutic strategy for some diseases that caused by missplicing.

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, which regulate splice site selection by recruiting regulatory proteins to exon sequences. The structure of the Tra2-beta1 protein is shown in Figure 27A. 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.

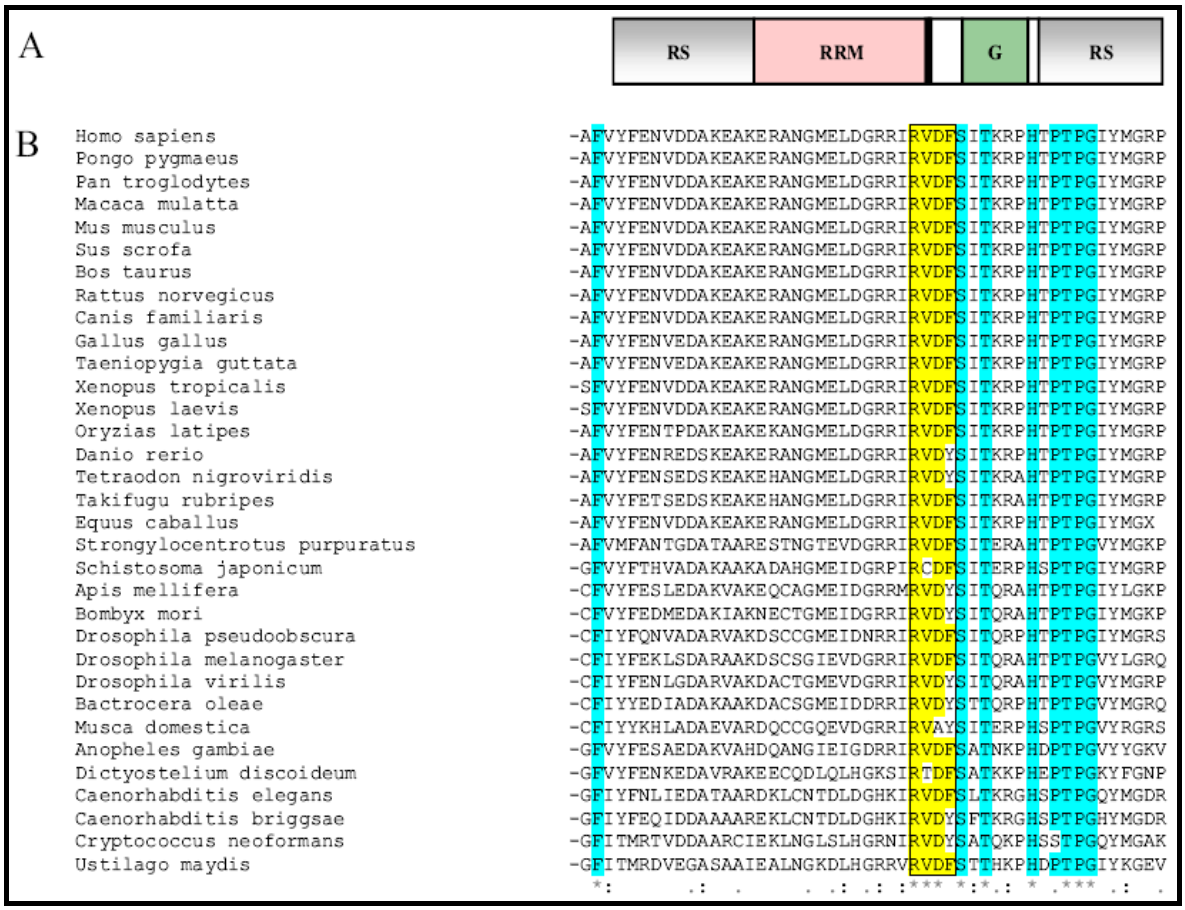

Figure 27. Tra2-beta1 protein sequence alignment. Alignment of Tra2-beta1 sequences from different species. The cartoon on the top (A) shows the domain structure of Tra2-beta1, RS: arginine-serine-rich domain, RRM: RNA recognition motif, G: glycine-rich region. The black line shows the conserved RVDF 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, except plants, 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 27B). The RVxF motif was previously demonstrated 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. The motif corresponds to the consensus RVDF sequence that is present in most binding partners of PP1.

This docking motif interacts with a hydrophobic channel of PP1 that is remote from the catalytic site (Bollen M., 2001; Egloff M.P. 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 H. 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 J. et al., 2000).

4.2.2. Interaction of Tra2-beta1 with PP1

Since there is a phylogenetically conserved putative PP1 binding site in the Tra2-beta1 protein, we performed immunoprecipitations to test whether PP1 binds to human Tra2-beta1. To determine whether the interaction is direct, we analyzed the binding between purified recombinant proteins. Tra2-beta1 was generated using the baculovirus expression system as a HIS-tagged protein. Figure 10 (see Materials and Methods, page 50) shows the experimental procedure to overexpress the HIS-tagged proteins in *Drosophila* SF9 cells. Full length HIS-tagged Tra2-beta1 was purified from SF9 insect cells with Ni-NTA resin. Purified Tra2-beta1 was immobilized on Ni^{2+}-agarose for HIS tag pull down assay and used as an affinity matrix for the binding of PP1, purified from rabbit skeletal muscle. In order to determine the dependency on the RVDF motif we used the HIS-Tra2-beta1-RATA recombinant protein. In this mutant, the RVDF motif was substituted by RATA, which is known to destroy or severely weakens the ability of this protein sequence to bind PP1 (Wakula P. et al., 2003).

As shown in Figure 28A, PP1 bound to Tra2-beta1, but not to the protein mutated in the PP1 binding site and the unrelated splicing factor YT521-B (Rafalska I. et al., 2004). This demonstrates a direct protein: protein interaction between Tra2-beta1 and PP1. This experiment demonstrated the direct interaction between Tra2-beta1 and PP1, which occurs via the conserved RVDF motif. We then performed analysis of the interaction of two proteins *in vivo*. As a negative control we used again a mutant, in which the PP1 binding site was mutated. As shown in Figure 28B, PP1 coimmunoprecipitates with Tra2-

beta1 (marked as a wild type), but not with Tra2-beta1-RATA (marked as a mutant). This indicates that both proteins are part of the same complex *in vivo.*

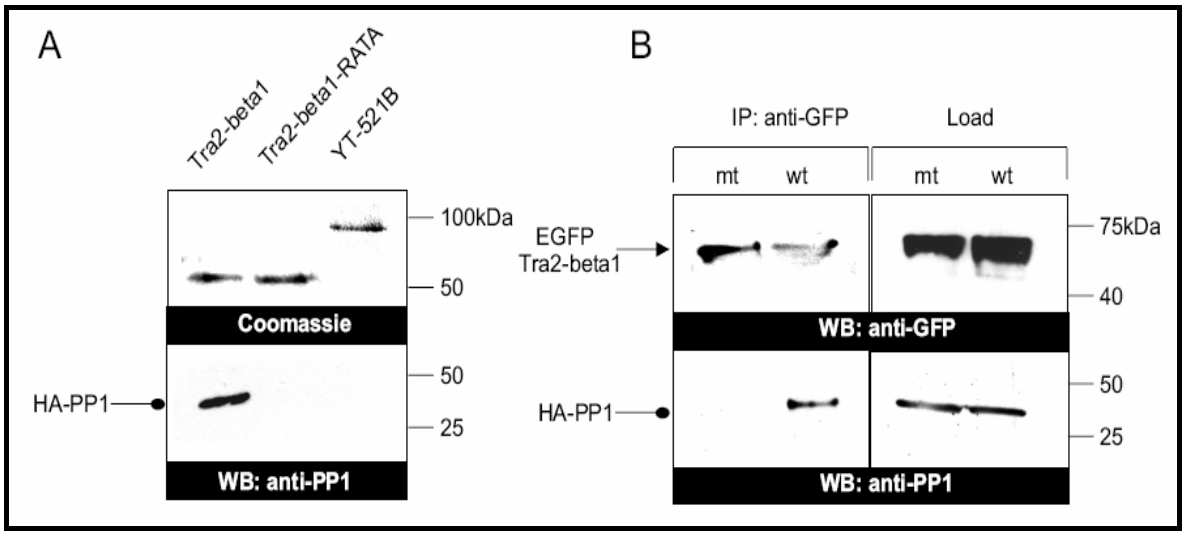

Figure 28. Tra2-beta1, but not its Tra2-beta1-RATA mutant interacts with PP1. (A) Direct interaction between Tra2-beta1 and PP1 protein. Recombinant His-tagged Tra2-beta1 was immobilized on Ni^{2+}-agarose and incubated with recombinant PP1. As negative controls Tra2-beta1-RATA mutated protein and the non- related YT-521B were used. After washing, proteins were detected by Coomassie Staining on PAGE gels and by Western Blot. (B) Immunoprecipitations were performed in HEK293 cells. EGFP-Tra2-beta1 wild type (wt), EGFP-Tra2-beta1 with a RVDF to RATA mutation (mt) and HA-PP1c gamma were expressed in HEK293 cells. Protein complexes were precipitated with anti-GFP antisera and identified by subsequent Western-blot analysis with anti-GFP and anti-PP1. A pointed arrow indicates Tra2-beta1 protein, round arrow indicate PP1 protein.

4.2.3. Tra2-beta1 and PP1cgamma partially colocalize in COS cells

In order to study the colocalization of both proteins, we investigated whether Tra2-beta1 and PP1 are colocalized in the same cell. It was shown previously that both proteins exhibit a dynamic localization in cells. Tra2-beta1 is mainly localized in nuclear speckles, but shuttles between nucleoplasma and cytosol where it accumulates under stress conditions, such as hypoxia (Stoilov P. et al., 2004; Daoud R. et al., 2002). PP1 is found in all cellular compartments. In cultured cells it is mostly expressed throughout the cell, but enriched in the nucleoli (Lesage B. et al., 2004; Trinkle-Mulcahy L. et al., 2003), whereas in neurons it is found in the soma, dendrites and presynaptic buttons (Bordelon J.R. et al., 2005; Winder D.G. and Sweatt J.D., 2001). Cotransfection assays were performed to study the colocalization of both proteins *in vivo*. In order to determine whether PP1 would influence this altered cellular localization we compared the Tra2-beta1-RATA mutant that does not bind PP1 with the Tra2-beta1 wild type.

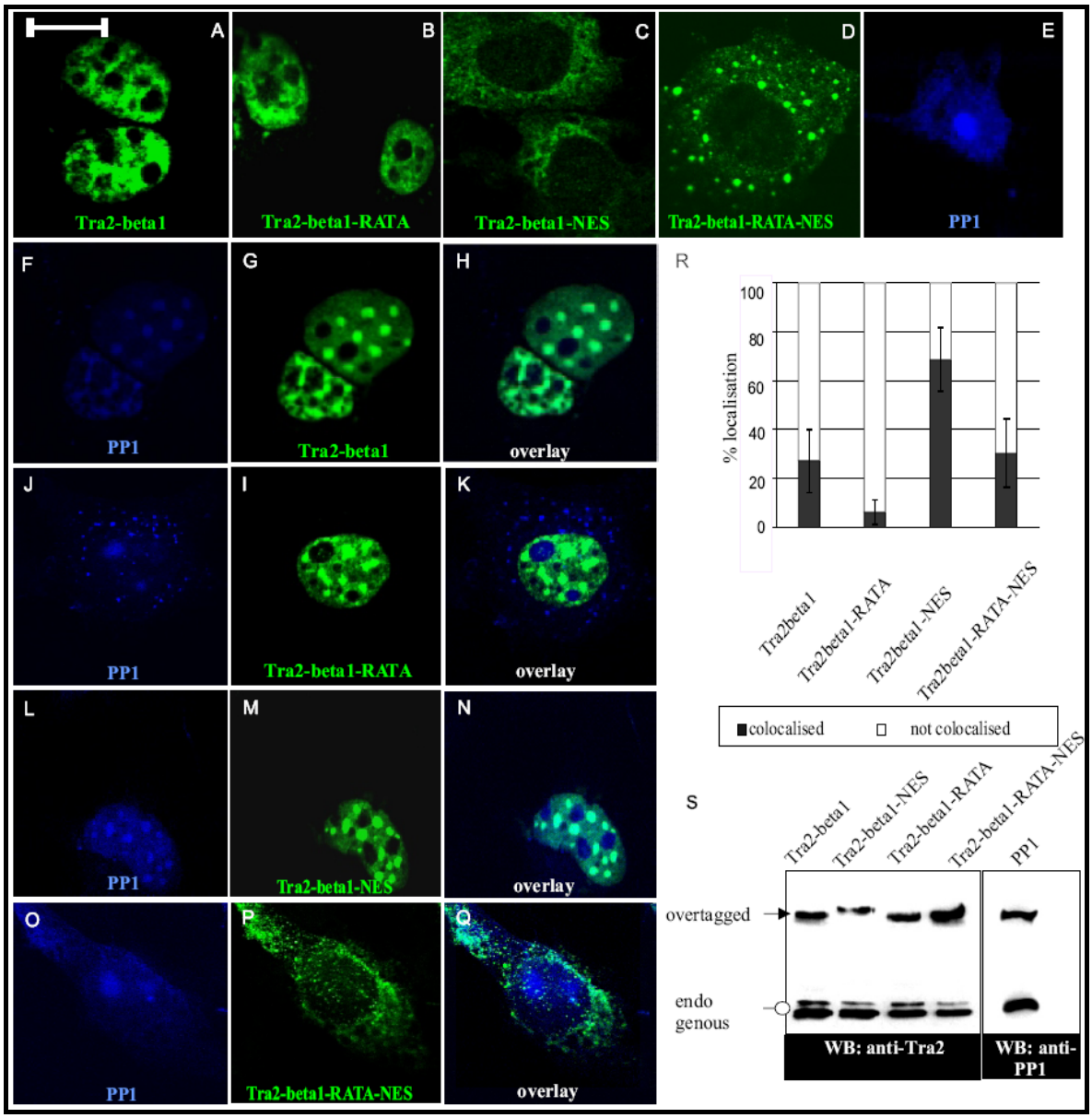

Figure 29. Tra2-beta1 and PP1cgamma partially colocalize in Cos 7 cells. Panels A to Q shows representative cells. (A) staining pattern obtained with Tra2-beta1 wild type, (B) Tra2-beta1 lacking the PP1 binding site, (C) Tra2-beta1 with a nuclear export signal (NES), (C) Tra2-beta1 lacking the PP1 binding site, and containing the NES (D) and CFP-PP1cgamma. (F-H) Coexpression of Tra2-beta1 and PP1cgamma J-K Coexpression of Tra2-beta1-RATA and PP1cgamma. (L-N) Coexpression of Tra2-beta1-NES and PP1cgamma. (O-Q) Coexpression of Tra2-beta1-RATA-NES and PP1cgamma. (R) Evaluation of the colocalisation of at least 200 cells. Errror bars indicate the standard deviation, p values of the student's test are indicated. (S) Western Blots showing the expression of the EGFP-tagged Tra2-beta1 mutants and CFP-tagged PP1gamma. Cell lysates were analyzed by Western Blot using antisera against Tra2-beta1 and PP1.

As shown in Figure 29A-B, Tra2-beta1 and Tra2-beta1-RATA are localized in the nucleoplasm and are enriched in a speckled structure. PP1cgamma is predominantly localized in the nucleoli. We performed confocal microscopy to determine the colocalization of the proteins and found about 30% of the fluorescence signal from the two proteins to be present in the same space (Figure 29F-H, R). When we employed the Tra2-beta1-RATA mutant that does not bind to PP1, this colocalization was reduced threefold (Figure 29J-K). After introducing a REV nuclear export signal in the Tra2-beta1-NES and Tra2-beta1-RATA-NES mutants both proteins localized in the cytosol, where the Tra2-beta1-RATA-NES protein accumulates in cytosolic bodies. We then tested whether PP1 would interfere with the cytosolic localization of the Tra2-beta1 mutants containing an NES. When CFP-PP1cgamma was coexpressed with EGFP-Tra2-beta1-NES, the Tra2-beta1-NES signal was no longer detected in the cytosol (Figure 29C), but was found to strongly colocalize with the PP1 signal in the nucleus (Figure 29L-N). The proteins showed strong colocalization in the nucleus (Figure 29L-N, R), indicating that the binding to PP1 is strong enough to antagonize the REV-NES. This colocalization was abolished when an EGFP-Tra2-beta1-RATA-NES mutant was used that is localized in the cytosol, but cannot bind to PP1cgamma. Now, PP1 is located in the nucleus and EGFP-Tra2-beta1-RATA-NES is located in the cytosol.

Together, the data show that although both proteins have a dynamic localization, they partially colocalize in cells and this colocalization is dependent on the ability of Tra2-beta1 to bind PP1.

4.2.4. PP1 dephosphorylates Tra2-beta1 protein

Since Tra2-beta1 can interact with PP1, we asked whether this enzyme dephosphorylates the protein. We investigated the effect of PP1 purified from rabbit skeletal muscle on recombinant Tra2-beta1. Recombinant Tra2-beta1 was phosphorylated *in vitro* by incubation with nuclear extract and ^{32}P gamma-ATP. The nuclear extract contains Ckl/Sty kinases that phosphorylate SR-proteins (Nayler O. et al., 1997), which can be detected by autoradiography of the labeled protein. After removing the nuclear extract and performing stringent wash steps, the phosphorylated Tra2-beta1 was incubated with PP1 (Figure 30). In order to demonstrate the specificity of protein dephosphorylation by PP1, we used the Tra2-beta1-RATA recombinant protein as a negative control in this assay.

Interestingly, comparable amounts of the mutated protein Tra2-beta1-RATA protein to Tra2-beta1 shows stronger labeling with ^{32}P, most probably due to the lower phosphatase activity of the protein. In order to eliminate a possible role of other protein phosphatases in the reaction we employed another negative control. Phosphorylated Tra2-beta1 was left with nuclear extract for 1 hour. The addition of PP1 inhibitor, tautomycin was another control (MacKintosh C. and Klumpp S., 1990). It showed no inhibition of phosphorylation.

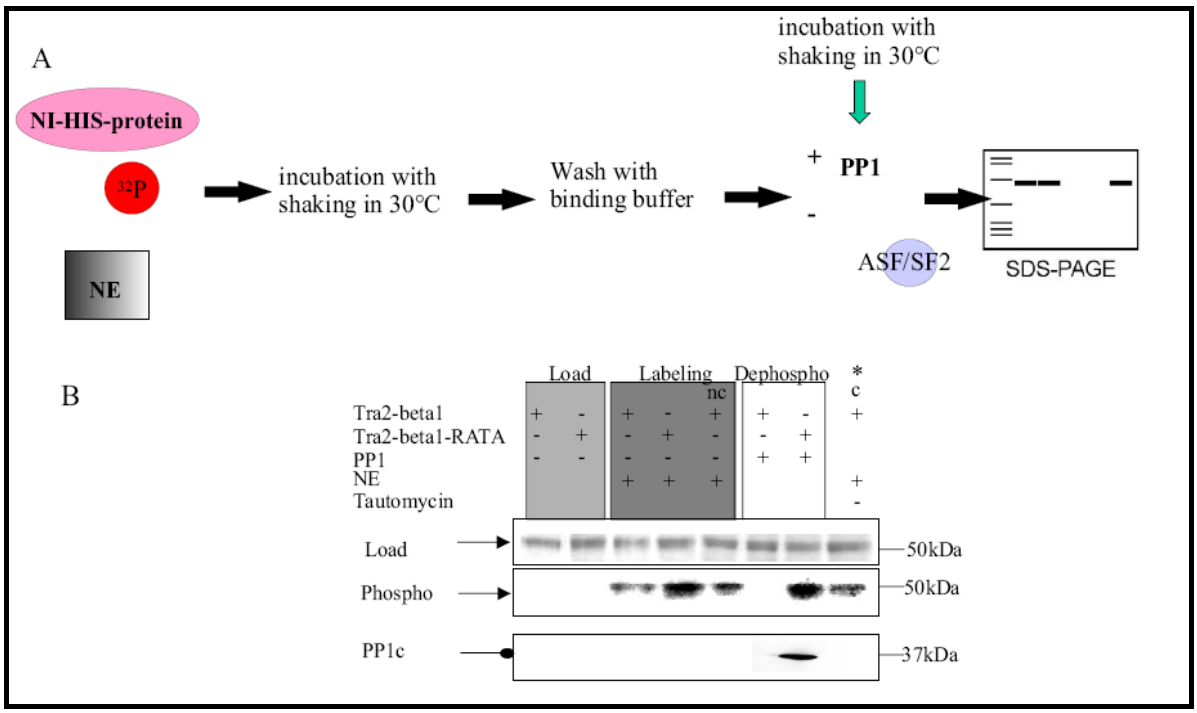

Figure 30. PP1 dephosphorylates Tra2-beta1 protein in vitro. (A) Schematic representation of dephosphorylation experiment. (B) Dephosphorylation in vitro: Recombinant His-Tra2-beta1 protein and the mutated HIS-Tra2-beta1-RATA protein were immobilized on Ni2+-agarose and incubated with nuclear extract in the presence of 32P-gamma ATP. The nuclear extract was then removed by stringent washing and purified, 32P-labeled Tra2-beta1 and its mutant were incubated with PP1 in presence of equimolar amount of ASF/SF2 protein. Nc: negative control indicates the Tra2-beta1 phosphorylated protein left for 1 hour with nuclear extract (NE). A star indicates the presence of tautomycin added to the phosphorylation mixture. Tra2-beta1 was detected by Coomassie staining (top row). The incorporation of 32P was detected by phospho-imaging (middle row) and PP1 was identified by Western Blot (lower row).

Dephosphorylation without strong binding is frequently observed with PP1 acting on purified proteins (Leytus S.P. et al., 1983). In agreement with these studies we observed the dephosphorylation of the Tra2-beta1 protein and Tra2-beta1-RATA protein

by PP1c. However, we did not see the binding of PP1 to the dephosphorylated Tra2-beta1-RATA protein (Figure 30B).

We therefore performed a dephosphorylation in the presence of SF2/ASF that can bind to PP1. SF2/ASF was used as a competitor to Tra2-beta1-RATA in the assay. We used equimolar amounts of SF2/ASF to Tra2-beta1 and Tra2-beta1-RATA. As it can be seen in Figure 30B, PP1 action removed the ^{32}P-labeling of Tra2-beta1, demonstrating that PP1 dephosphorylated Tra2-beta1 *in vitro*. The mutated protein Tra2-beta1-RATA did not reveal any dephosphorylation at the same conditions. The negative control with phosphorylated Tra2-beta1 left with nuclear extract did not reveal any dephosphorylation of the protein after 1 hour, showing that other protein phosphatases present in the mixture did not dephosphorylate the protein. Finally, we used stringent wash conditions to remove proteins present in nuclear extract and did not detect additional proteins after the washing steps by Coomassie Staining. Therefore, the dephosphorylation of Tra2-beta1 by PP1 occurs most likely through direct bimolecular interaction that is dependent on the RVDF motif.

4.2.5. Dephosphorylation of Tra2-beta1 protein influences homo/heterodimerozation and protein–protein interactions

Then we wanted to know whether the protein interactions of Tra2-beta1 are controlled by PP1 mediated dephosphorylation. We have determined that PP1 dephoshorylated Tra2-beta1 *in vivo* (Section 4.2.3). We analyzed the phosphorylation-dependent mobility of Tra2-beta1 on high concentration PAGE gels. Similar to other SR-proteins, Tra2-beta1 migrates as a hyper and hypophosphorylated form (Daoud R. et al., 1999). The activity of PP1 in HEK293 cells was blocked by tautomycin, a cell permeable phosphatase inhibitor that blocks PP1 about four times more potently than protein phosphatase 2A and 10,000 fold more potently than protein phosphatase 2B (MacKintosh C. et al., 1990). As shown in Figure 31A, treatment of cells with tautomycin caused a significant increase of the hyperphosphorylated form of Tra2-beta1. As a negative control we used the specific inhibitor of PP2A, fostriecin. Fostriecin had no effect, showing that the dephosphorylation of the protein is not caused by PP2A.

To investigate whether the change in the PP1 activity in cells directly changes the phosphorylation of Tra2-beta1 protein, we transfected the PP1c expression clone directly in cells. We observed a decrease in the hyperphosphorylation of Tra2-beta1

protein (Figure 31 A). Together the data (in section 4.2.4.) show that PP1 can dephosphorylate Tra2-beta1 both *in vivo* and *in vitro*.

Previous work on SR-protein kinases demonstrated that an increase in phosphorylation influences protein: protein interaction of SR-proteins (Xiao S.H. and Manley J.L., 1997). We therefore investigated whether dephosphorylation regulates the interaction between Tra2-beta1 and other proteins. Tra2-beta1 forms homodimers and can also heterodimerize with other SR-proteins, such as SF2/ASF (Beil B. et al., 1997; Nayler O. et al., 1998). We thus asked whether the dimerization is influenced by PP1 action. We expressed EGFP-tagged wild type Tra2-beta1 and Tra2-beta1-RATA in HEK293 cells. The immunoprecipitates were analyzed with an antiserum specific for Tra2-beta1, allowing the detection of endogenous protein. We found that in contrast to wild type Tra2-beta1, the Tra2-beta1-RATA mutant no longer homomultimerises with the endogenous Tra2-beta1 (Figure 31B).

We then determined whether the known interaction between Tra2-beta1 and SF2/ASF is dependent on the PP1 binding site of Tra2-beta1. Again, we immunoprecipitated wild-type Tra2-beta1 and Tra2-beta1-RATA mutant with anti-GFP and detected SF2/ASF present in the immunoprecipitates by Western blot. As shown in Figure 31C, no multimerization between SF2/ASF and Tra2-beta1-RATA could be observed.

Finally, we analyzed the phosphorylation-dependency of the binding between SF2/ASF and Tra2-beta1 using recombinant proteins. As described above, baculovirus generated His-Tra2-beta1 was phosphorylated in nuclear extract, immobilized on Ni^{2+}-agarose and after removal of the extract aliquots were dephosphorylated by PP1 from rabbit skeletal muscle. This affinity matrix was then used to determine binding of bacterially expressed GST SF2/ASF. As shown in Figure 31D, the dephosphorylated Tra2-beta1 protein exhibited a higher affinity towards PP1. This reflects the *in vivo* situation where Tra2-beta1 wild type binds to SF2/ASF and Tra2-beta1 whereas the Tra2-beta1-RATA mutant shows no interaction. These data indicate that the dephosphorylation of Tra2-beta1 by PP1 promotes the multimerisation between Tra2-beta1 and its interacting proteins.

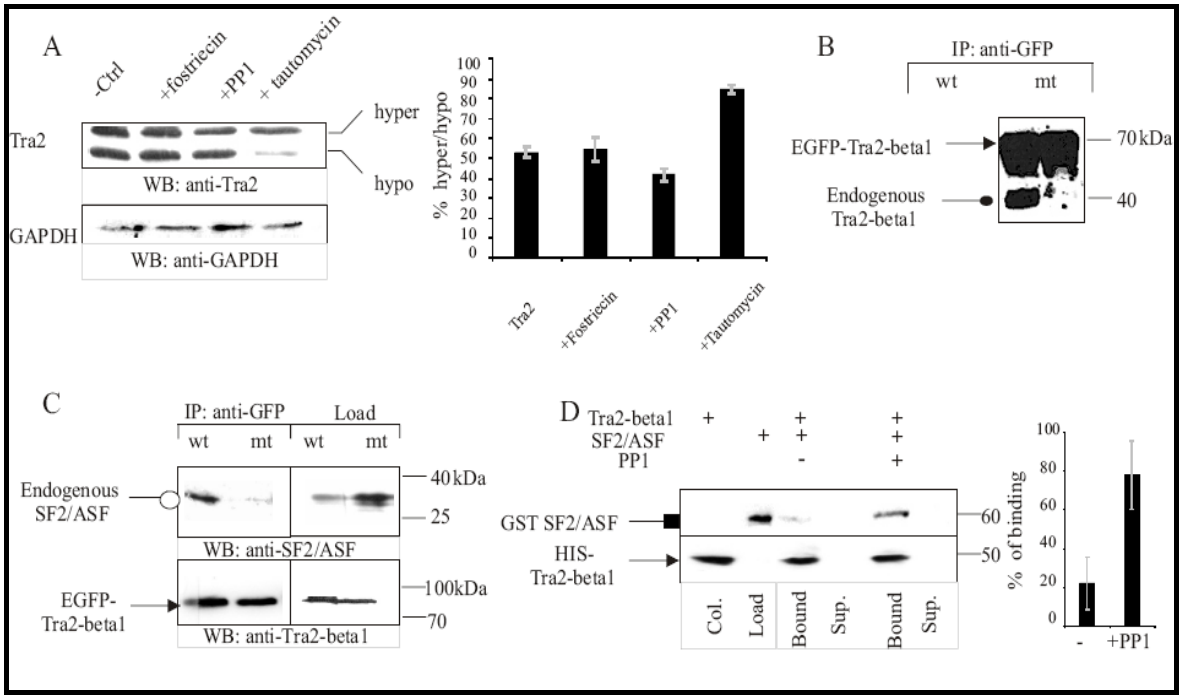

Figure 31. Change of Tra2-beta1 hyperphosphorylation in vivo. (A) HEK293 cells were treated with fostriecin (50nM) or tautomycin (40nM) overnight, or a PP1c expression clone was transfected into the cells. The mobility of Tra2-beta1 was determined by Western blot using Tra2-beta1 antisera and 15% PAGE gels (Daoud et al., 1999). The graph shows the quantification of five independent experiment demonstrating a statistical significant difference (p=0.013) between tautomycin treatment and the other conditions. GAPDH was used as a loading control. (B) PP1 dependent homomultimerisation of Tra2-beta1. EGFP-tagged wild-type and EGFP-Tra2-beta1-RATA was expressed in HEK293 cells. Protein complexes were recovered by immunoprecipitation with anti-GFP antisera. Endogenous Tra2-beta1 binding to these proteins was detected with a pan-Tra2-beta1 antiserum. Round arrow: endogenous Tra2-beta1, pointed arrow: EGFP-tagged Tra2-beta1. (C) PP1 dependent heteromultimerisation of Tra2-beta1. EGFP-tagged wild-type and EGFP-Tra2-beta1-RATA containing protein complexes were isolated as in (B). Top row: endogenous SF2/ASF was identified with anti-SF2/ASF; bottom row: reblot with anti-Tra2-beta1. (D) Phosphorylation-dependent interaction between recombinant Tra2-beta1 and SF2/ASF. His-Tra2-beta1 was immobilized on Ni2+-agarose and the binding of recombinant GST-SF2/ASF was determined without or after PP1 treatment, as indicated on top. The lower row shows that equal amount of His-Tra2-beta1 were present on the agarose (col: column, load: SF2/ASF loaded on column, sup: protein found in supernatant).

4.2.6. PP1 regulates usage of Tra2-beta1 dependent alternative exons

It was shown previously that Tra2-beta1 is one of the SR proteins that play an important role in constitutive and alternative splicing. Previous work has demonstrated

that Tra2-beta1 is able to bind to highly degenerate purine sequences in *vivo* (Stoilov P. et al., 2004). Tra2-beta1 regulates alternative exons by binding to characteristic, degenerate purine-rich sequences that often act as exonic enhancers (Tacke R. et al., 1998 and Stoilov P. et al., 2004). It is well known that increased concentration of Tra2-beta1 influences in addition to its own exon 2 several exons, like SMN2 exon 7 (Hofmann Y. et al., 2000), CLB (Clathrin Light Chain B gene) exon EN (Stamm S. et al., 1999), tau exon 10 (Jiang Z. et al., 2003) and APP (Amyloid Precursor Protein) exon 8 (Yamada T. et al., 1993). Tra2-beta1 regulates alternative splicing of a number of exons. This raised the question whether PP1 also could regulate the same exons and if the dephosphorylation of Tra2-beta1 by PP1 could influence the alternative splice site selection. We therefore performed transfection assays by using established minigenes, in which the alternative exon is flanked by its constitutive exons in a reporter gene construct.

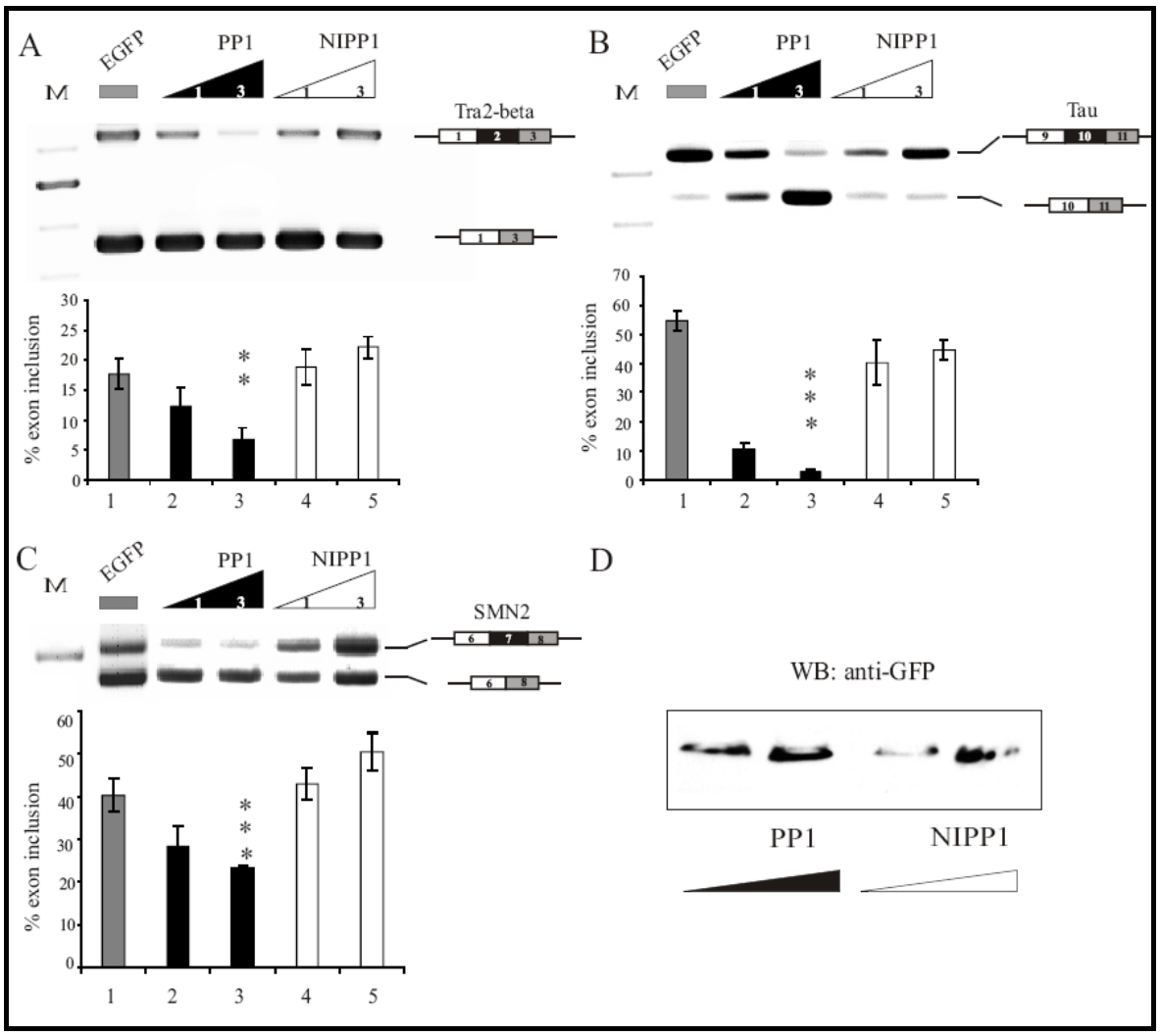

Figure 32. PP1 regulates the usage of Tra2-beta1 dependent alternative exons. An increasing amount of expression clones for Tra2-beta1 and the nuclear inhibitor of PP1 NIPP1 was cotransfected with the

indicated reporter minigenes containing Tra2-beta1 dependent exons. The alternative splicing of each reporter gene was determined by RT-PCR. A representative ethidium-bromide stained gel of each experiment is shown. Under each experiment, the statistical evaluation of at least four independent experiments is shown. (A)Tra2-beta, exon 2; (B) tau, exon 10; (C) SMN2, exon 7; (D) representative Western blot showing the expression of the transfected EGFP-PP1cgamma and EGFP-NIPP1. Stars indicate p-values from student's test (Tra2-beta: p=0.004; SMN: p=0.001; tau: p=0.0005).

The influence of trans-acting factors on alternative splicing was determined by RT-PCR. The nuclear inhibitor of protein phosphatase 1 (NIPP1) is a highly specific inhibitor of PP1 (Beullens M. and Bollen M., 2002). It was used in these cotransfection assays to specifically decrease the cellular activity of PP1. Transfections were performed in HEK293 cells with increasing concentration of PP1 or NIPP1, which increased or decreased cellular activity of PP1 (Beullens M. and Bollen M., 2002).

As shown in Figure 32A-C, an increase of PP1 expression promoted the exclusion of alternative exons. In contrast, the inhibition of nuclear PP1 by the expression of NIPP1 promoted exon inclusion. The effect on exon usage was proportional to the increase of the level of PP1cgamma and NIPP1 protein that were detected by Western-Blot from the transfected cells (Figure 32D).

4.2.7. The effect of PP1cgamma on splice site selection of SMN2 reporter minigene is dependent on Tra2-beta1 binding

Concentrating on exon 7 of SMN2, we then asked whether the effects seen in the cotransfection assay on splice site selection of SMN2 are dependent on the action of PP1 on Tra2-beta1. Tra2-beta1 binds to the SE element in SMN exon 7 (Lorson C.L. et al., 1999; Lorson C.L. and Androphy E.J. 2000).

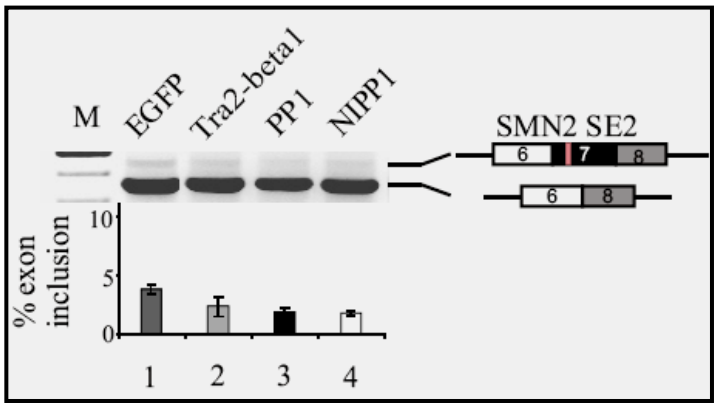

Figure 33. The effect of PP1cgamma on SMN2 minigene is dependent on Tra2-beta1 binding. SMN2 minigene with a mutated Tra2-beta1 enhancer sequence (SMN2-SE2) was cotransfected with three microgramms expression clones for a parental cloning vector (EGFP), Tra2-beta1, PP1cgamma, and NIPP1.

We used a SMN2 minigene, where the Tra2-beta1 binding site was mutated (SMN2-SE2) (Lorson C.L. and Androphy E.J., 2000). The AG-rich element in SMN2 SE minigene is disrupted so that the minigene shows reduced levels of exon 7 inclusion, which are not changed by increasing concentration of Tra2-beta1 (Figure 33). Neither did an increase in the concentration of PP1cgamma, nor NIPP1 significantly affect exon 7 inclusion, suggesting that their effect is mediated by Tra2-beta1. We concluded that the effect of PP1cgamma on splice site selection of SMN2 minigene is mediated through Tra2-beta1 action.

4.2.8. The Protein Phosphatase 1 effect on the selection of some splice sites is mediated by direct interaction of PP1 to Tra2-beta1

The next question, which arose in our research, was whether the effect of PP1 on splice site selection is dependent on its direct interaction with Tra2-beta1 or whether the effect is achieved through sequestration of Tra2-beta1 protein to the common spliceosomal complex. The action of PP1 was compared with its binding mutant PP1-F257A, which is mutated in the hydrophobic channel that mediates the binding to RVxF-motifs and with the catalytically inactive PP1-H125A mutant (Lesage B. et al., 2004). As it was expected, in contrast to the wild type, both mutants had no effect, demonstrating that binding and subsequent dephosphorylation is necessary for the effect of PP1 on splice site selection (Figure 34A).

Finally we compared the influence of Tra2-beta1 and Tra2-beta1-RATA on exon 7 inclusion. Whereas Tra2-beta1 increases exon 7 inclusion in a concentration dependent manner, comparable amounts of the Tra2-beta1-RATA mutated protein lead to exon skipping (Figure 34B). This demonstrates that the exact control of Tra2-beta1 dephosphorylation by PP1 is necessary for proper action of Tra2-beta1. In summary, these data indicate that PP1 regulates the inclusion of Tra2-beta1 dependent exons by controlling the phosphorylation of Tra2-beta1.

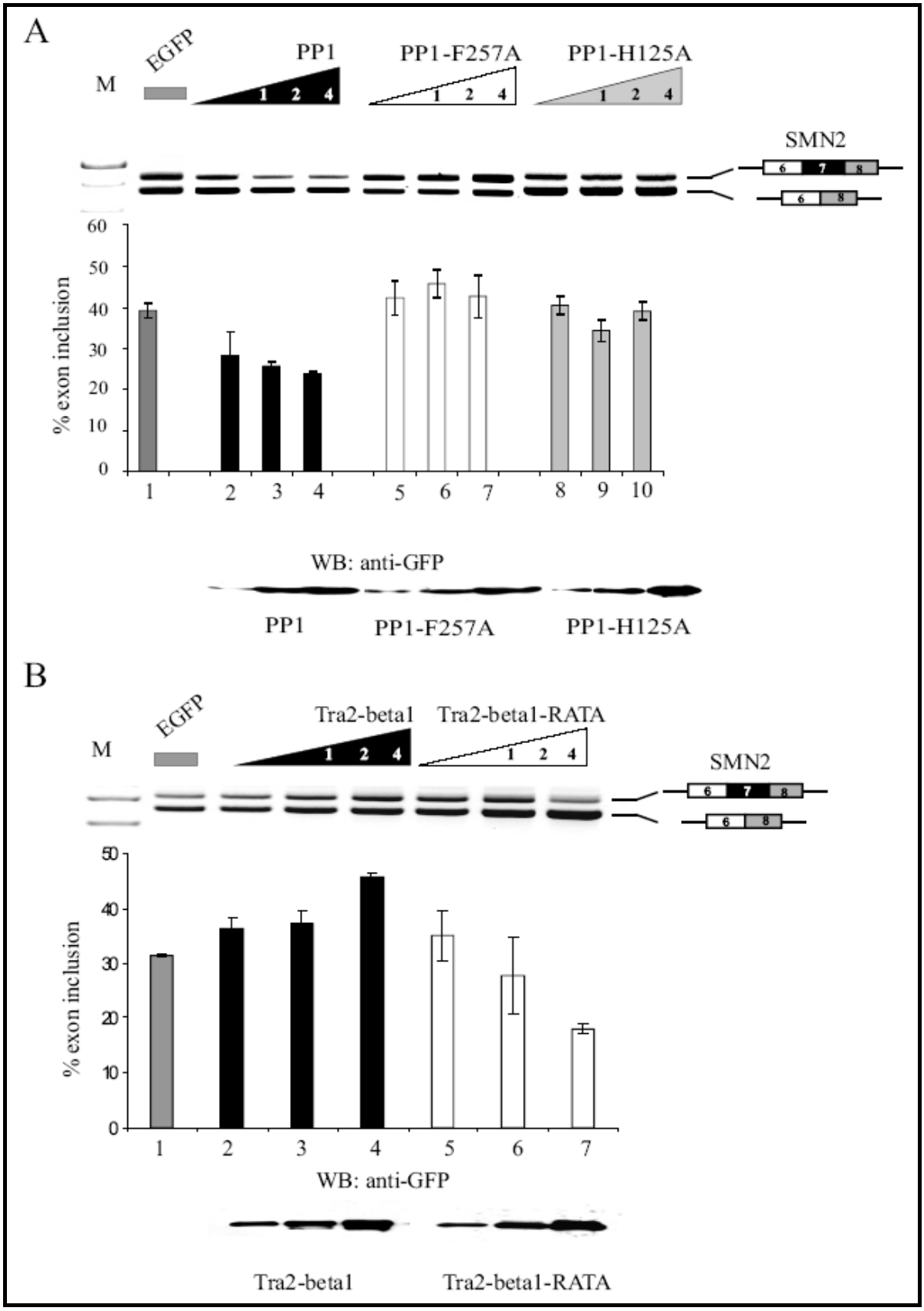

Figure 34. The effect of PP1 on some splice site selection is mediated by direct interaction of PP1 to Tra2-beta1. (A) The SMN2 reporter minigene was cotransfected with an increasing amount of PP1cgamma expression constructs, an expression construct encoding the binding mutant F257A, or the

catalytically dead H125A mutant. The Western blot below the statistical evaluation shows the accumulation of transfected PP1cgamma and PP1-F257A, and PP1-H125A in theHEK293 cells. (B) The SMN2 reporter minigene was cotransfected with an increasing amount of Tra2-beta1 expression constructs or an expression construct encoding the Tra2-beta1-RATA mutant. The Western blot shows the accumulation of transfected Tra2-beta1 and Tra2-beta1-RATA.

4.2.9. Tra2-beta1 mutant with S→E change in the first RS domain differ in the ability to change splice site selection from the wild type.

The Tra2-beta1 protein consists of two RS domains (RS1 and RS2), which are flanking the RRM. Previous work (Cao W. et al., 1997; Xiao S.H. and Manley J.L., 1998) demonstrated 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 B.R., 2000). In order to study which parts of the RS1 domain are important for the phosphorylation state of the Tra2-beta1 protein we performed mutation analysis of the first and second parts of the first RS domain. The phosphorylation of serines located within the RS1 and RS2 domains can be eliminated by mutation of the serine residues to alanine. Mutations of the same residues to glutamines mimic the phosphorylation.

All the serine residues in these regions were mutated to alanines or glutamines. The first part of the RS1 domain marked in blue and the second part in yellow in Figure 35. The mutants were analyzed by *in vivo* splicing assay with the SMN2 reporter minigene. Figure 35 shows a change in splice site selection for those mutants where all the serine where mutated to glutamines in the first part of the RS1 domain. At the same time the S→A mutants did not exhibit a difference in splice site selection. *In vivo* splicing analysis of the mutants from the second part of the RS1 domain did not show any significant change in splice site selection, pointing that only the first part of the RS1 is responsible for influencing the activityof the protein. This result indicates that phosphorlyation of the serine residues in the first part of the RS1 could play an important role in regulation of splice site selection.

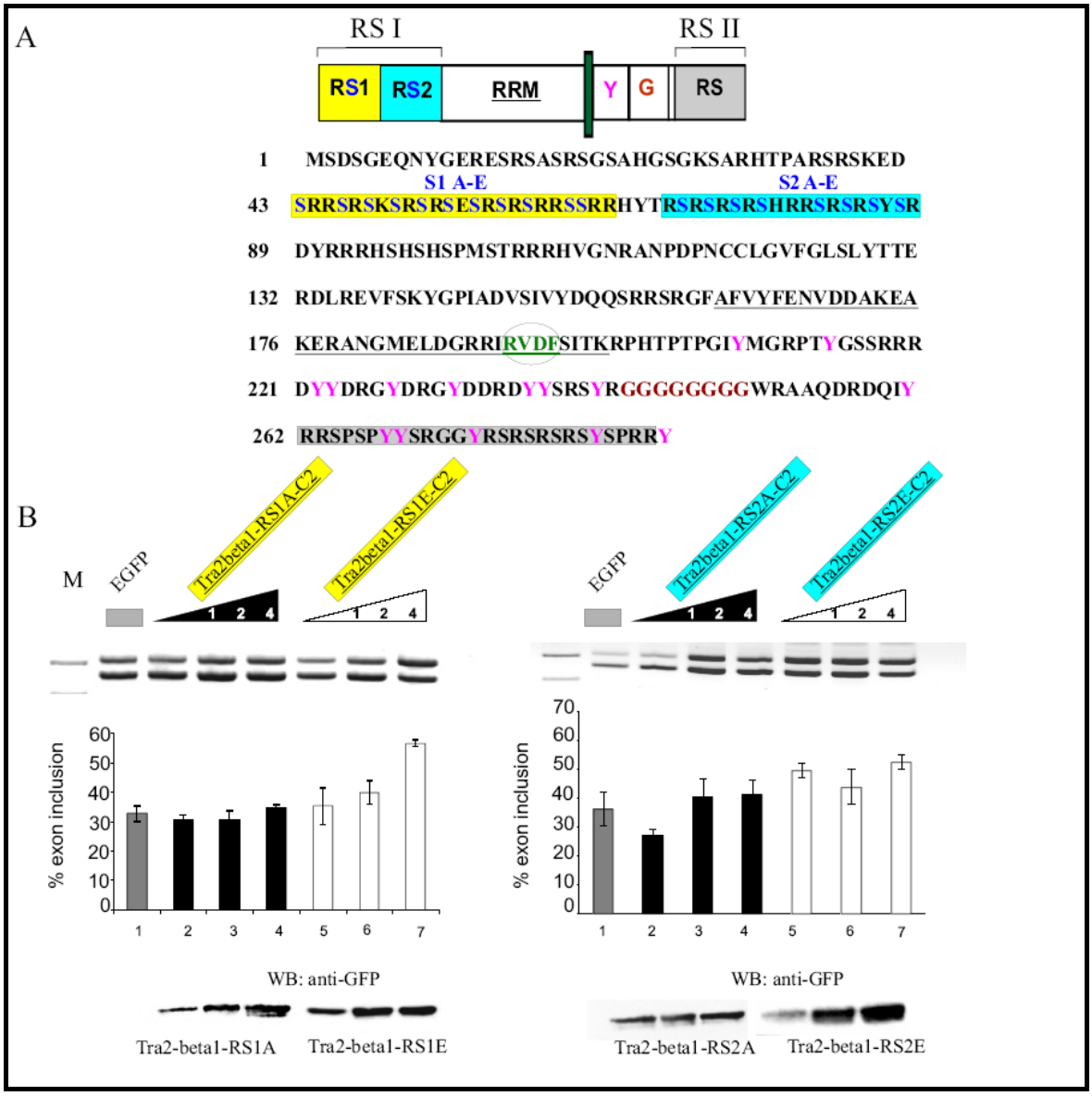

Figure 35. Influence of Tra2-beta1 protein on splice site selection. (A) The Tra2-beta1 structure reveals two RS domains which are flanking the RRM domain, containing PP1 binding site (in green). Mutations of each serine residue to alanine and glutamic acid in the first (in yellow) and second (in blue) part of the RS1 domain of Tra2-beta1 were analysed. The Tra2-beta1-RS1A, RS2A mutants mimick the nonphosphorylated status of the protein. The Tra2-beta1-RS1E, RS2E mutants (all S residues substituted with E in then same positions) mimick phosphorylated status (B). The phosphorylation, like substitutions in the first part of the RS1 domain changes alternative splice site selection on SMN2 reporter minigene. The SMN2 minigene was cotransfected with an increasing amount of Tra2-beta1-RS1A, RS2A and Tra2-beta1-RS1E, RS2E expression constructs. The Western blot shows the accumulation of transfected Tra2-beta1 constructs.

4.2.10. Protein Phosphatase I inhibitors promote exon 7 inclusion of SMN2 minigene

A number of cell permeable protein phosphatase inhibitors including tautomycin, microcystin, calyculin A and cantharidin can be used to study the role of protein phosphatases 1 and 2 in intact cells. We thus investigated whether these components would interfere with splice site selection. We tested these inhibitors in HEK293 cells that were transfected with the SMN2 reporter minigene. We observed a significant inclusion of exon 7 with all these inhibitors, but the effect was most pronounced with tautomycin, which inhibits PP1 more potently than PP2A and other phosphatases. In contrast, we did not see an effect with the protein tyrosine phosphatase inhibitor dephostatin. We also did not observe any effect when we used the specific PP2A inhibitor fostriecin and nodularin, inhibitors showing higher potency to PP2A than to PP1.

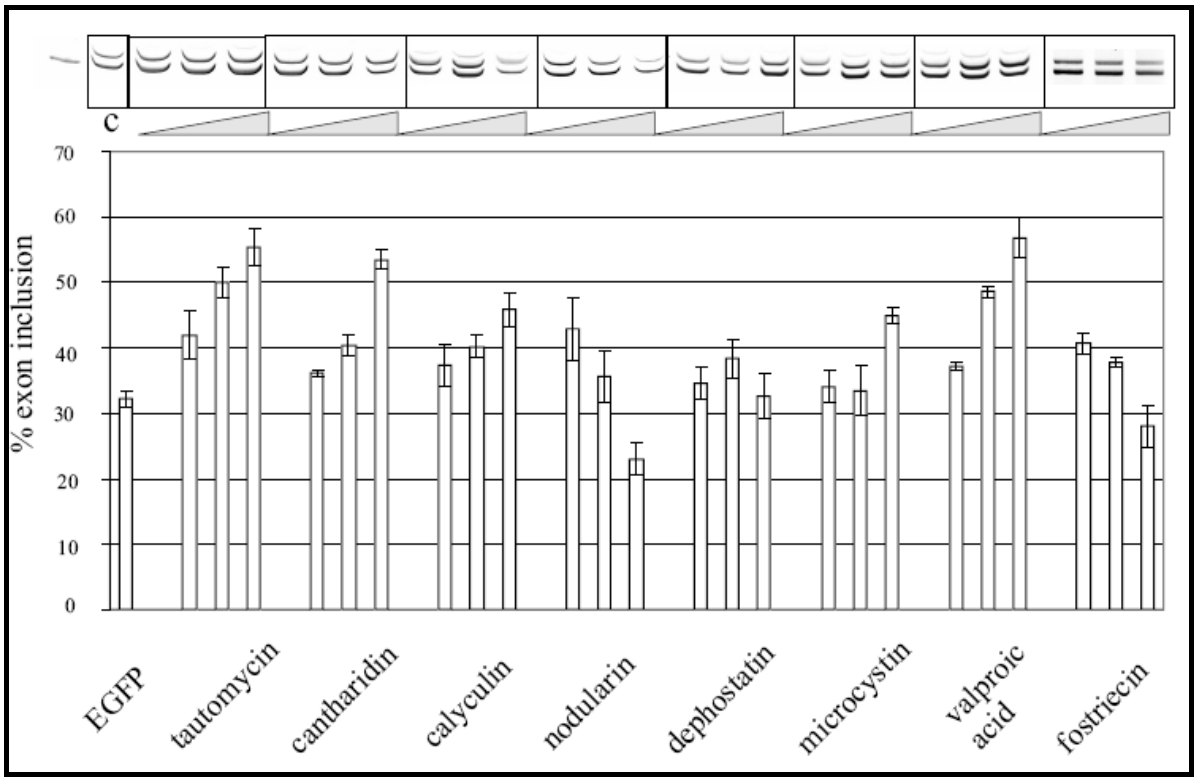

Figure 36. Effect of different PP1 inhibitors on SMN exon 7 usage. HEK293 cells were transiently transfected with the SMN2 reporter minigene and treated with an increasing concentration of the protein phosphatase inhibitors. Tautomycin has the highest specificity to block PP1, dephostatin is a tyrosine phosphatase inhibitor with low affinity to PP1, was used as a negative control. Fostriecin was used as another negative control for discriminating the action of PP2A from PP1 on exon 7 inclusion of SMN2 MG. The inhibitors were used in the following concentrations: tautomycin (5-50-100nM);

cantharidin (0.1-1-6µM); calyculin (0.1-1-2nM); nodularin (2-6-10nM); dephostatin (1-3-7.7µM); microcystin (2-6-10nM); valproic acid (0.5-25-50µM); fostriecin (5-50-100nM).

This experiment indicated that blocking of endogenous PP1 activity can influence splice site selection of SMN2 exon 7 *in vivo* (Figure 36).

4.2.11. Inhibition of PP1 induces the inclusion of exon 7 of SMN in vivo

The effect of PP1 on splice site selection was studied in the previous experiment in systems using overexpression of proteins and reporter genes. To analyze the effect of PP1 in an endogenous system, we employed primary fibroblast cell lines from persons with SMA type I to type III. In these cells, the SMN1 gene is deleted from both allels, but the SMN2 gene is still present. The number of SMN2 gene copies increases from type I to type III SMA. The SMN2 gene in these cells is alternatively spliced and exon 7 is predominantly skipped, resulting in low levels of SMN protein. These cells were treated with 10 nM of the PP1 inhibitor tautomycin and the exon 7 containing SMN mRNA was amplified using primers in exon 6 and 8. GAPDH was coamplified in the same reaction and served as a loading control.

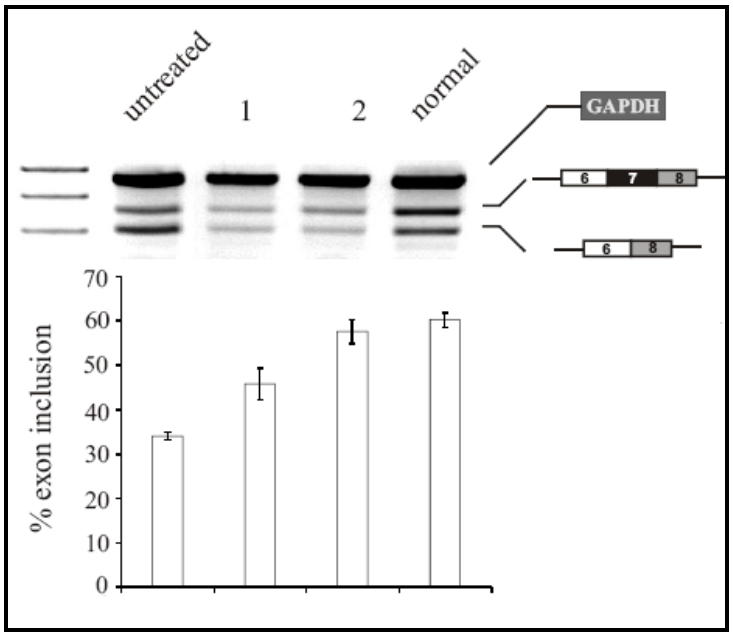

Figure 37. Tautomycin causes accumulation of SMN2 mRNA containing exon 7 in fibroblasts from children with SMA type I. Fibroblasts from a child with type I SMA that lack SMN1 were treated with tautomycin and the resulting RNA was analyzed by RT-PCR after one and two days. GAPDH was co-amplified in the same reaction as an internal loading control. The graph underneath the representative ethidium bromide stained gel shows the statistical evaluation of four independent experiments.

Figure 37 shows that this treatment resulted in an increase of mRNA containing exon 7. Since the SMN1 gene is absent in these cells, this increase in exon 7 containing mRNA can be only explained by a change in alternative splice site selection.

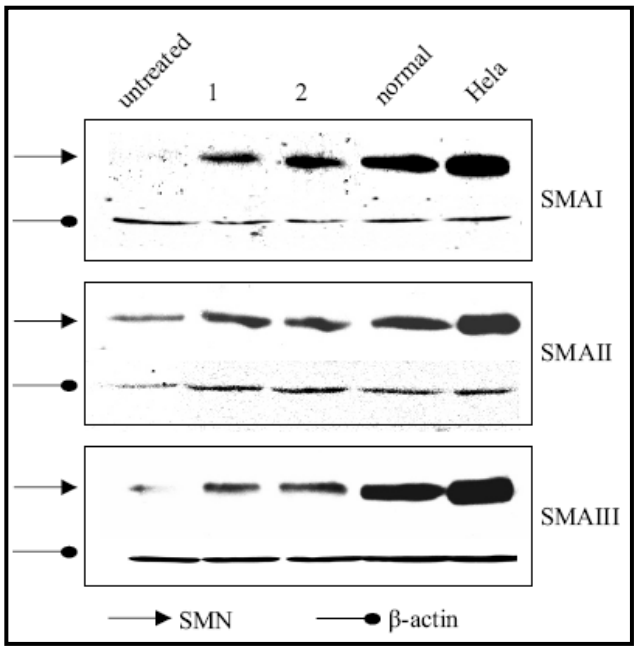

Figure 38. Tautomycin causes accumulation of SMN protein in SMA fibroblasts from type I-III patients. Cells were treated with 10 nM tautomycin from one to two days and cell lysates were analyzed using an antisera against SMN,.which detects the signal indicated by the pointed arrow. Round arrow: signal from beta-actin used as a loading control. Normal: lysates from nontransformed human control fibroblast, HeLa: lysates from HeLa cells.

In agreement with the change in alternative splicing we saw an effect on the SMN protein. The endogenous SMN protein was detected by Western Blot analysis with a specific antibody (anti-SMN). We observed an accumulation of SMN protein after tautomycin treatment for all types of SMA patients (Figure 38). These data show that reducing the activity of endogenous PP1 influences splice site selection of the endogenous SMN exon 7.

4.2.12. Tra2-beta1 regulates alternative splice site selectionof numerous exons

To test the hypothesis that the regulation of splice site selection by the Tra2-beta1 protein is similar to the blocking of PP1 activity we created an alternative exon specific oligonucleotide array for all known alternative splice events of splicing factors. For this study an oligonucleotide array that contained all known alternative splice variants from splicing factors, as well as some well-studied model systems was used. The experiment was performed in collaboration with Dr Laurent Bracco from ExonHit Therapeutics, Paris. The total RNA from HEK293 cells transfected with expression constructs for Tra2-beta1 and NIPP1. In addition cells were treated with 40 nM of specific PP1 inhibitor, tautomycin. As a control we used untreated RNA. RNA was obtained 24 hours after transfection.

				Tra2-beta1 to 293 cells	
Exon ID	**Gene name**	**Splicing event**	**Reference**	**CHIP**	**RT-PCR**
114.1.1	hnRNP M	Exon Skipping	NM_005968	UP	UP
148.1.1	CPSF6	Novel Exon	NM_007007	UP	Down
104.9.3	SFRS4	Novel Exon	NM_005626	Down	UP
236.10.1	PPIL3	Alternative Splice Acceptor	NM_032472	UP	Down
159.5.2	IEBF3	Novel Exon	NM_012218	UP	UP
110.3.1	SRRM1	Novel Exon	NM_005839	UP	UP
909.039.002	FE65	Exon Skipping	NM_001164	Down	UP
907.002.002	FYN	Novel Exon	NM_002037	Down	UP
179.1.1	SFRS14	Exon Skipping	NM_014884	UP	UP
93.1.1	FIML	Exon Skipping	NM_004960	UP	UP
137.1.1	MTMR 11	Novel Exon	NM_006697	Down	Down
91.1.1	DEAD	Intron Retention	NM_004818	Down	UP
117.8.1	PPIE	Exon Skipping	NM_006112	Down	Down
208.3.3	CLK4	Intron Retention	NM_020666	Down	UP

Figure 39. The references for alternative exons and description of splicing events. Exon ID indicates the number of the exon from the splicearray, the reference indicates the numbers of corresponding sequences from PubMed. Upregulated genes are marked in yellow, downregulated in blue.

The alternative splicing events were detected by a combination of exon-junction and exon body probes, as previously described (Fehlbaum P. et al., 2005). On the base of standard average and vectorial sum of fold changes we picked up the regulated candidate genes. From the whole 942 events we picked up 65 events, which are strongly influenced by Tra2-beta1 concentration. The genes and the corresponding exons for them are indicated on Table 7.

4.2.13. Validation of DNA-array results by RT-PCR

16 of these events we picked up for subsequent verification of CHIP data by RT-PCR. From these 16 events, 14 of them were confirmed for their dependency on Tra2-beta1 by RT-PCR, using primers in the flanking constitutive exons. In most cases (with the exception of four cases: CPSF6, PPIL3 and PPIE) Tra2-beta1 overexpression promotes exon inclusion, which correlates with earlier findings that the protein acts as a general activator of exons (Tacke R. et al., 1998; Stoilov P. et al., 2004). We then compared overexpression of Tra2-beta1 with inhibition of PP1 by NIPP1 in HEK293 cells. In most cases where we could confirm the array data by RT-PCR, we found that the blocking of PP1 activity has a similar effect on alternative splice site selection as an increasing Tra2-

beta1 concentration (Figure 40). However, in some cases (Clk4, FUS), NIPP1 expression had a stronger effect on exon inclusion than Tra2-beta1 overexpression. At the same time we observed the opposite effect for two cases, PPIL3 and PPIE. In summary, many, but not all Tra2-beta1 dependent exons are influenced by PP1 mediated dephosphorylation, which changes the activity of Tra2-beta1.

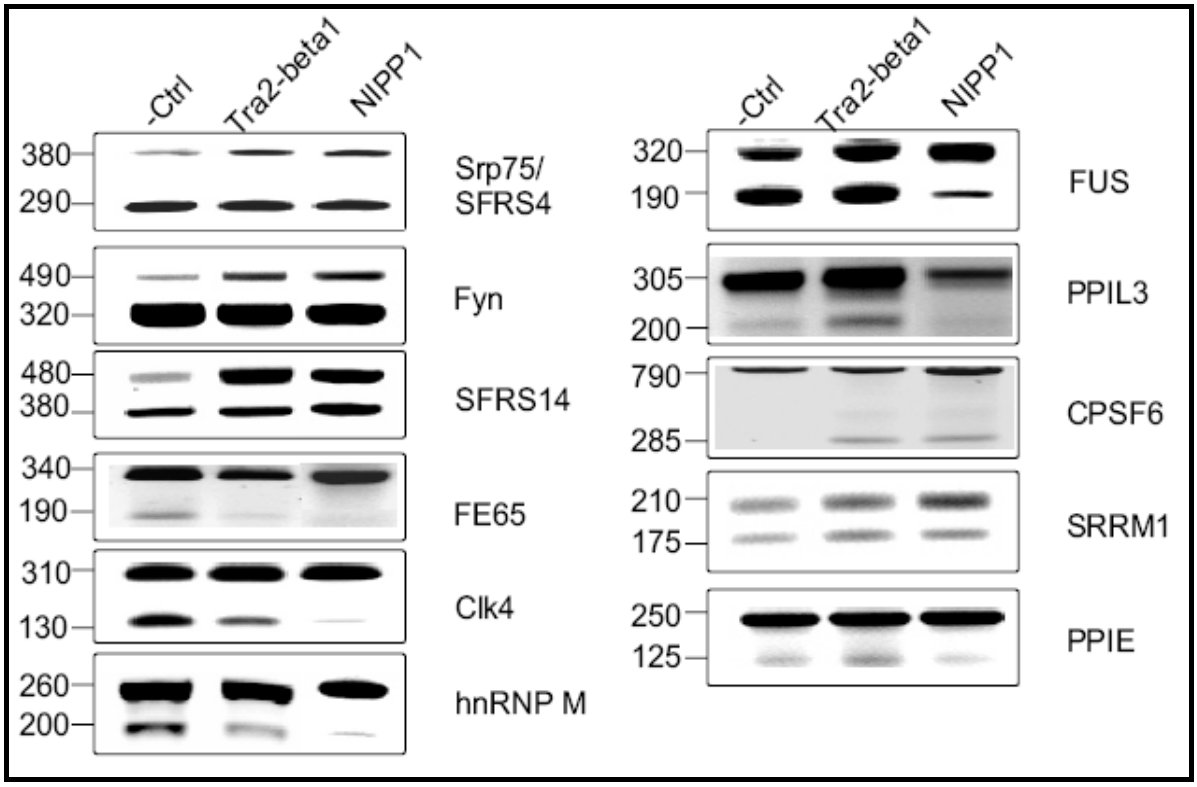

Figure 40. NIPP1 changes the usage of Tra2-beta1 dependent exons. **EGFP-Tra2-beta1 and EGFP-NIPP1 were transfected in HEK293 cells and the isolated RNA was amplified using specific primers. -Ctrl for untransfected HEK293 cells.**

Table 7. The names of the candidate's genes and sequences of corresponding exons chosen for validation of microarray data. The change of genes, whose sequences marked in grey, could not be verified by RT-PCR.

Gene	Exon	Protein	Function
Srp75/SFRS4 NM_005626 Homo sapiens splicing factor, arginine/serine-rich 4 104.9.3	ACATCCCTAATCCTGACCAGCCATGGAGAGG TTCGGTTCTTACAAGGAAAATGAGTGAATGT GGGAATGGCTCACCACATTGTGAAAACAAAA CTCACATCCTGCTGATGGTTGGCTGAGGACT GTCATGTCCACATACAAAGAAAAGCATGTGG CACAGTTTTAGGACAAAG	TSLILTSHGEVRFLQGK*	Stop codon, NMD
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
FE65 NM_001164 Homo sapiens amyloid beta (A4) precursor protein-binding, family B, member 1 (APBB1) 909.039.002	TGTTTCGCCGTGCGCTCCCTAGGCTGGGTAG AGATGACCGAGGAGGAGCTGGCCCCTGGACG CAGCAGTGTGGCAGTCAACAATTGCATCCGT CAGCTCTCTTACCACAAAAACAACCTGCATG ACCCCATGTCTGGGGGCTGGGGGGAA	CFAVRSLGWVEMTEEELAPGRSSVAVNNCIRQLSYHKNNLHD PMSGGWGE	Thiolases active site N-myristoylation
CLK4 NM_020666 Homo sapiens CDC-like kinase 4 208.3.3	CCCCGCTGAATGAATTGCGTATTCTGCCCTG AATTCACTCTGATATATTGATTGGCTGGACG ATCTTGGTGCTGCCCACTTGCCGTTCCAGAA GAGCCACCGAAGGAAAAGATCCAGGAGTATA GAGGATGATGAGGAGGGTCACCTGATCTGTC AAAGTGGAGACGTTCTAAGAGCAAGATG	PAE*	Stop codon, NMD
hnRNP M NM_005968 Homo sapiens heterogenous nuclear ribonucleoprotein M 114.1.1	GATGAGAGGGCCTTACCAAAAGGAGATTTCT TCCCTCCTGAGCGTCCACAACAACTTCCCC	DERALPKGDFFPPERPQQLP	Unknown
FUS NM_004960 Homo sapiens fusion (involved in t(12;16) in malignant	ACAAACAAGAAAACGGGACAGCCCATGATTA ATTTGTACACAGACAGGGAAACTGGCAAGCT GAAGGGAGAGGCAACGGTCTCTTTTGATGAC CCACCTTCAGCTAAAGCAGCTATTGACTGGT	TNKKTGQPMINLYTDRETGKLKGEATVSFDDPPSAKAAIDWF D	Protein kinase C Casein kinase II

liposarcoma) 93.1.1	TTGATG		
MTMR11 NM_006697 Homo sapiens myotubularin related protein 11 137.1.1	AGGTCTTTGTTTTCTCTTCACTTCTATGTGC TTTCTCAATCCTCCTACCCCAAACCTTTCCC CTCTGTGCCTCTCACTTCTCCTTGCCGTATT TCTCTTCTCATTCTCTTCTGCCCTAGTTTCT TGGACCCCTCTTCTTTGTCCCTTCTTCCTCT TTATCACCCAGAGCGCGGTGATCGTGATCTC AATGGCCTCCTCTCTTCACTCGTCCAGCTGC TTTCAGCCCCCGAAGCCCGAACACTGTTTGG CTTCCAATCACTAGTACAGCGAGAGTGGGTG GCAGCTGGACATCCCTTCCTGACTCGGCTTG GGGGAACTGGGGCCAGTGAAGAG	RSLFSLHFYVLSQSSYPKPFPSVPLTSPCRISLLILFCPSFL DPSSLSLLPLYHPERGDRDLNGLLSSLVQLLSAPEARTLFGF QSLVQREWVAAGHPFLTRLGGTGASEE	Casein kinase II
PPIL3 NM_032472 Homo sapiens peptidylprolyl isomerase (cyclophilin)-like 3 236.10.1	ATGGAGTCTCGCTGTGTCCCCCAGGCTGGAG TACAATGGCGCGATCTCGGCTCACTGCAACC TCCGCCTCCTGGGTTCAAGCAAGTCTTCTGC CTCAGCCTCCCGA	MESRCVPQAGVQWRDLGSLQPPPPGFKQVFCLSLP	Unknown
CPSF6 NM_007007 Homo sapiens cleavage and polyadenylation specific factor 6, 68kDa 148.1.1	CTGGACAGACTCCACCACGTCCACCCTTAGG TCCTCCAGGCCCACCTGGTCCACCAGGTCCT CCACCTCCTGGTCAGGTTCTGCCTCCTCCTC TAGCTGGGCCTCCTAATCGAGGAGATCGCCC TCCACCACCAGTTCTTTTTCCTGGACAACCT TTTGGGCAGCCTCCATTGGGTCCACTTCCTC CTGGCCCTCCACCTCCAGTTCCAGGCTACGG CCCCCCTCCTGGCCCACCACCTCCACAACAG GGACCACCTCCACCTCCAGGCCCCTTTCCAC CTCGTCCACCCGGTCCACTTGGGCCACCCCT TACACTAGCTCCTCCTCCGCATCTTCCTGGA CCACCTCCAGGTGCCCCACCGCCAGCTCCGC ATGTGAACCCAGCTTTCTTTCCTCCACCAAC TAACAGTGGCATGCCTACATCAGATAGCCGA GGTCCACCACCAACAGATCCATATGGGCGAC CTCCACCATATGATAGGGGTGACTATGGCCC CCCTGGAAG	GQTPPRPPLGPPGPPGPPGPPPPGQVLPPPLAGPPNRGDRPP PPVLFPGQPFGQPPLGPLPPGPPPPVPGYGPPPGPPPPQQGP PPPPGPFPPRPPGPLGPPLTLAPPPHLPGPPPGAPPPAPHVN PAFFPPPTNSGMPTSDSRGPPPTDPYGRPPPYDRGDYGPPG	N-myristoylation
DEAD NM_004818 Homo sapiens (Asp-Glu-Ala-Asp) box polypeptide23(U5-100KD) 91.1.1	GTGCAGGCCTTGGCTCCATCTTCACTTCTCT CCCACACAAAAATGAGGTTTAGTTATTTGGC CATTGTGATCTCTGCATGTGAGAATTGGGCC TCAAAGCTCATGTCTTTGTGTTCTGTCCCTG GGTAG	VQALAPSSLLSHTKMRFSYLAIVISACENWASKLMSLCSVP G	Casein kinase II

IEBF3 NM_012218 Interleukin enhancer binding factor 3 159.5.2	ACTACAGTGGTAGTGGAGGCCGAAGCGGCGG GAACAGCTACGGCTCAGGCGGGGCATCCTAC AACCCAGGGTCACACGGGGGCTACGGCGGAG GTTCTGGGGGCGGCTCCTCATACCAAGGCAA ACAAG	TTVVVEAEAAGTATAQAGHPTTQGHTGATAEVLGAAPHTKAN K	N-myristoylation
SRRM1 NM_005839 Serine/arginine repetitive matrix 1 110.3.1	GATGCAGATGGGAAAGCGATGGCAATCGCCA GTGACT	DADGKAMAIASD	N-myristoylation site
PPIE NM_006112 Peptidylprolyl isomerase E (cyclophilin E) 117.8.1	GGAGAGCCCATTGCTAAAAAGGCCCGCTCAA ATCCTCAGGTGTACATGGACATCAAGATTGG GAACAAGCCGGCTGGCCGCATCCAGATGCTC CTGCGTTCTGATGTCGTGCCCATGACAGCAG	GEPIAKKARSNPQVYMDIKIGNKPAGRIQMLLRSDVVPMTA	N-myristoylation Glycine-rich Alanine-rich Cysteine-rich regions
ETIF2B NM_012218 Eukaryotic translation initation factor 2B 14.1.1	ATTGGAACCAACCAGATGGCTGTGTGTGCCA AAGCACAGAACAAACCTTTCTATGTGGTTGC AGAAAGTTTCAAGTTTGTCCGGCTCTTTCCA CTAAACCAGCAAGACGTCCCAGATAAGTTTA AG	IGTNQMAVCAKAQNKPFYVVAESFKFVRLFPLNQQDVPDKFK	Protein kinase C phosphorylation
SFRS5 NM_006925 Splicing factor arginine/serine-rich 5 144.1.3	GTTACTATTGAACATGCTAGGGCTCGGTCAC GAGGTGGAAGAGGTAGAGGACGATACTCTGA CCGTTTTAGTAGTCGCAGACCTCGAAATGAT AGACGGTATGTGAAGGGTGGATGGCTGCATT GAACAATTATTGTAGGGGTAGCATTTAAGAT TCAGGAGTCATTAGCAGTGATGATTTTGGGT CCTGCCGTATAATCTGTTCTTCTATTCCCAC GTTAGCCAgTTGTTCTTGATGAATCTATATG AGTCATAGAACACAAATCTATTGACGGAAGT CATTAGAATGGCTTGTGATATCTGATGGCTT GAACTTGCCCACAGTTGAACACAAGTGCTGT CATTGCATTTCTTCCATTGTGAATACGAATT TTCTTCCTCAGAAATGCTCCACCTGTAAGAA CAGAAAATCGTCTTATAGTTGAGAATTTATC CTCAAGAGTCAGCTGGCAGGTTTGTTGAAAT ACAGTTTTGAGTTATTTTGATGTGGCTTTTT AAAAAAGTTAATGGGTAGCTAATGTTGTATT GTTTTATTAAAAGTAGTTTTAAATTAATGGA TTTAATGGAATTGTAATTTTAAACATTTAAT TAAATGTAATTTGGGGGATATTTTGAACTTT AATATTAGTGATCAAATGTAAATGTTTTGAG GATATTTTTCTTGTTTTTTAAAATCAATTTT	VTIEHARARSRGGRGRGRYSDRFSSRRPRNDRRYVKGGWLH*	Protein kinase C phosphorylation Tyrosine kinase phosphorylation Arginine-rich region profile Stop codon

	TAACCAAATATTAAACCCTTTATTTGCCAGC CTGTCTGTGTCGTTGGCCTTATGACGAGGAG TGCCTGTGGGTTATCCTAATCGTTGTCTTGG TCACTCTTGGTTGGGCCTGGTTGACTTTGCC AGTCAGCTCCTACAGTGATCAGTTGGCCACC TCTAGATCTGTGTTTGCCGGTCTAGACGTTA TCGGTGCACTTCCTTGAAGTGCCAGGTTGCA GCGATCCCAGAGCGTTGATAACGTGATCTGA TCCCTAAGTTGAGACCTGTCTTCCTGTAACG CTTCCGAATAAGAGGTCCGGTCGAAAAGAGT TGAAAGATACGAAATTAGGTGGTCGTTGGAA TCCTGATCGCAGTAAAGTGGTCCTTGGTAAC TGCTCGAGTAGAACATGTCTGCATCCGCCAa TAGTCCGCTAATAGGGAGGGCTGTGCGATTA AAGGCTTCCATCGATTGGGTAGTGTCCTTCA AGTGGGTGGCGAAGAGCCAGTCGGGCATATG TCATGAAGGTTTCTCCGACCGATTAATGGTT TGCTGCTTGACTAGCCTAGGGAAATAATAAT AAAGGTAAAGTAAACTTTTTTAATGACATAT GGGGCACAAAATAACTGGTGTCTTGTAAATG TTCTGTTTTTTTAAATAAAACTTCTCTACTT TAGTCTTTACTTTAAAAATATGTACTGTTTC CTTTTTTGTTGTTTTTTTGTTTGTTTTTTTT TCTTTTGTACTGAGCTCAGCATAGACTAATA CTACCTTAATGTTAAAATCTGAATTTCTTTT AGCATTTTGCTTAAAAGCAATATGCTATTTG CTTATTCCGTGCCCTGACATAGATAATTTTT GAATTCTGATAGAATACAAGTGTGGTAAATG CCATGTTTGTACTTTATCTAACATCTTCTCT TTCAGTAGTCTTGTTTTTTTTATATCATGTG ATTGTTTGTGTGTCCCCTTTCCTCTTCTTTG CTTAACACAATTATCTTGTGTTAAGGATCTC AAAGATTTCATGAGACAAGCTGGGGAAGTAA CGTTTGCGGATGCACACCGACCTAAATTAAA TGAAGG		

4.2.14. PP1 inhibitors regulate alternatively spliced exons

We next compared the overexpression of Tra2-beta1 with the inhibition of PP1 by tautomycin in HEK293 cells.

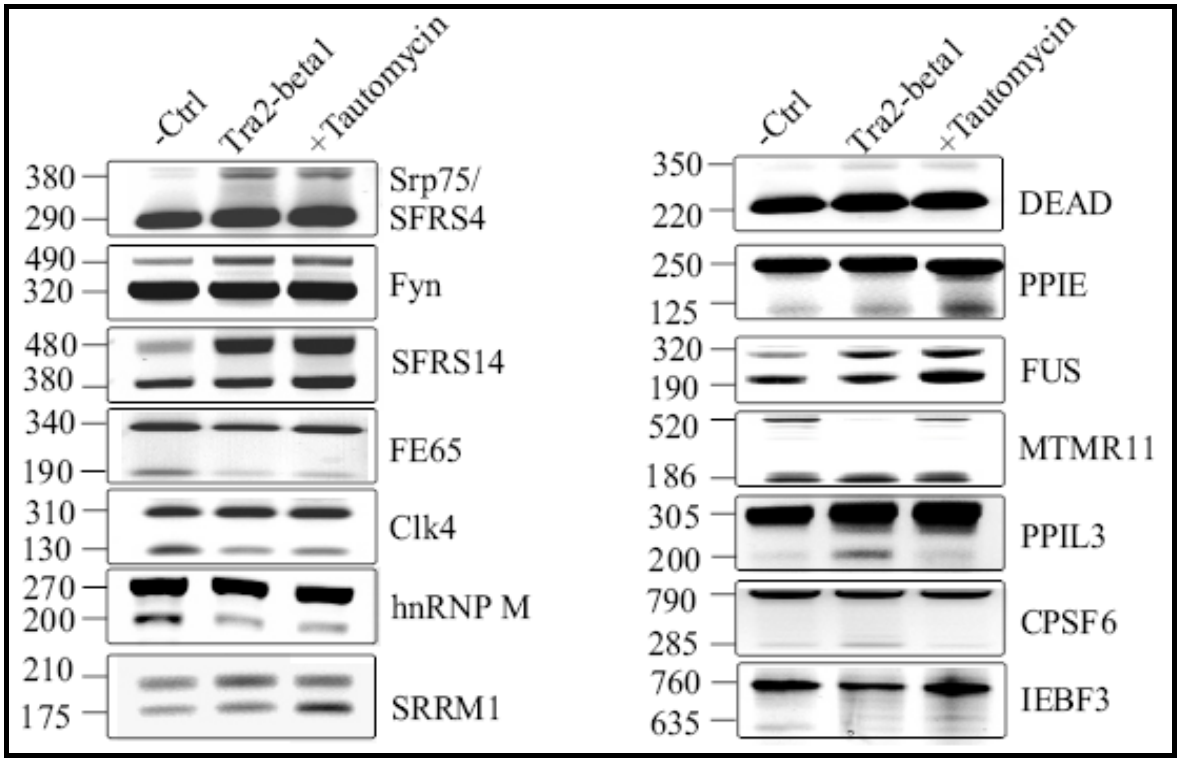

Figure 41. Tautomycin treatment changes usage of Tra2-beta1 dependent exons. EGFP-Tra2-beta1 was transiently overexpressed in HEK293 cells or cells were treated with 40 nM tautomycin. The exons were amplified by RT-PCR with specific primers using RNA from untreated cells, from cells overexpressing Tra2-beta1 and from cells treated with 40 nM tautomycin.

HEK293 cells were treated with 40nM of the PP1 inhibitor tautomycin, and the RT-PCR was performed with the same primers. In most of cases (12/16), we found that blocking PP1 activity has a similar effect on alternative splice site selection, as increasing Tra2-beta1 concentration (Figure 41). However, similarly to the inhibiton by NIPP1, in several cases we found difference between Tra2-beta1 and NIPP1.

4.2.15. The phosphorylation of Tra2-beta1 protein does not correlate with Tra2-beta1 regulation of splice site selection in mouse tissues

Next we wanted to determine whether the phosphorylation status of Tra2-beta1 protein *in vivo* correlates with the regulation of splice site selection by the same protein. For this aim RNA was isolated from mouse tissues and the RT-PCR was

performed with the specific primers for genes, which were strongly influenced by Tra2-beta1 concentration. At the same time the protein lysates from the same mouse tissues were run on a high percentage gel (15%), to determine the phosphorylation state of Tra2-beta1 protein.

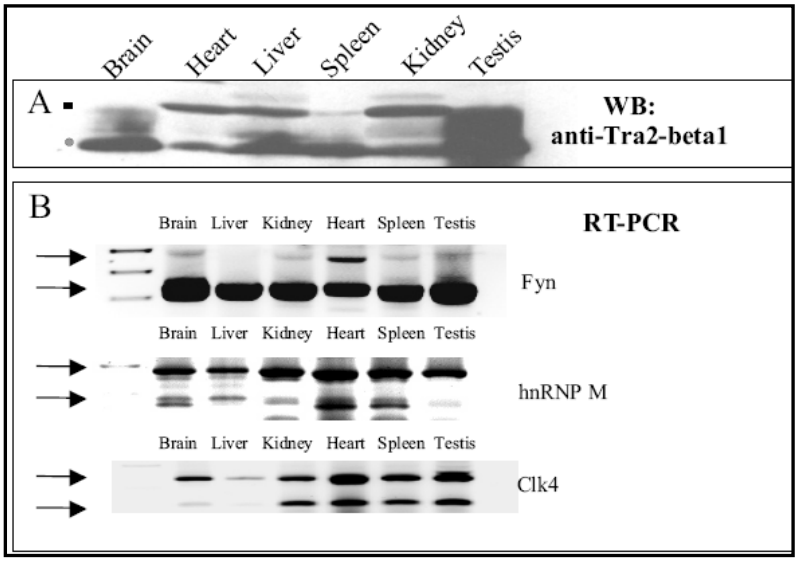

Figure 42. The phosphorylation status of Tra2-beta1 protein does not correlate with Tra2-beta1 dependent exon splice site selection from mouse tissues. (A) Western Blot demonstrates the hyperphosphorylation of Tra2-beta1 from mouse tissues. Ten microgramms of protein in lysates from different tissues were analysed in Western Blot with the pan-Tra2 antibody. The grey dot and the black square indicate the hypo- and hyperphosphorylated forms of the Tra2-beta proteins, respectively. (B) RT-PCR analysis of specific Tra2-beta1 dependent exons.

As shown in Figure 42, Tra2-beta1 hyperphosphorylation is predominant in heart, liver and kidney, whereas brain and spleen tissues show highest hypophosphorylation. The RT-PCR analysis from the same RNA amplified with specific primers shows that inclusion of Tra2-beta1 dependent exons does not change according to the phosphorylation of the protein. This indicates that Tra2-beta1 protein regulates splice site selection of a number of genes and this regulation does not depend on phosphorylation statue of the protein.

4.2.16. Several splicing factors bind to PP1 via a phylogenetically conserved RVXF motif located on the beta4 sheet of the RRM

The microarray data and their RT-PCR validation demonstrated that blocking the activity of PP1 by NIPP1 had sometimes a different effect on exon usage than Tra2-beta1 overexpression (Figure 40) and the phosphorylation of Tra2-beta1 and exon usage do not correlate (Figure 42). Therefore, we investigated whether PP1 could also influence other splicing factors. From the sequence analysis of Tra2-beta1 protein we

found out, 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 43). 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).

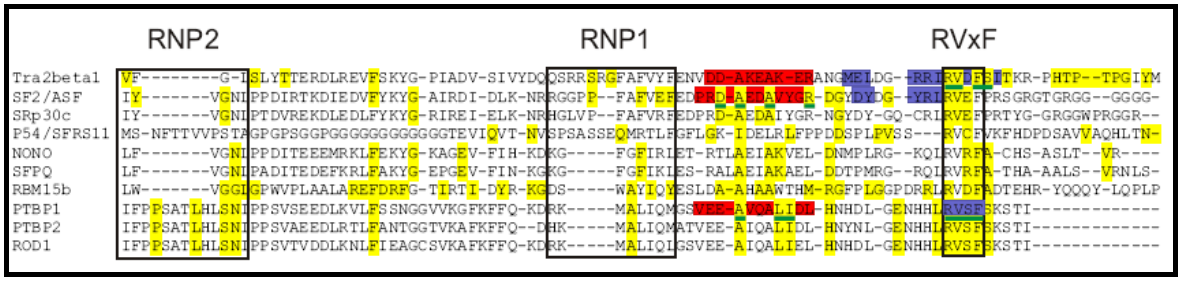

Figure 43. The alignment of RRMs of the human SR and SR-like proteins. RNP2, RNP1 and the PP1 binding motif RVxF are boxed. The proteins Tra2-beta1, SF2/ASF, SRp30c, SRp54, p54nrb(NONO) (Brown S.A. et al., 2005; Dong B. et al., 1993), SFPQ, (Clark J. et al., 1997), RBM15 (Ma Z. et al., 2001), PTB (Chan 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.

A further phylogenic analysis demonstrates that the motif is conserved throughout evolution in each protein (Figure 44).

ASF/SF2

```
Homo sapiens                    DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Pongo pygmaeus                  DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Pan troglodytes                 DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Mus musculus                    DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Sus scrofa                      DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Bos taurus                      DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Rattus norvegicus               DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Canis familiaris                DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Gallus gallus                   DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Xenopus tropicalis              DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Xenopus laevis                  DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Danio rerio                     DIDLKN-RRGGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSG
Tetraodon nigroviridis          DIDLKN-RRGGPPFAFIEFEDPRDADDAVYGRDGYDYDGYRLRVEFPRSG
Tribolium castaneum             FVDLKN-RRG-PPFAFVEFEDPRDADDAVHARDGYDYDGYRLRVEFPRGG
Schistosoma japonicum           AIDLKAGQRRGPPFAFVEFEDELDASDAVRGRDGYNFDGYALRVELPRTG
Apis mellifera                  FVDLKN-RRG-PPFAFVEFDDPRDAEDAVHARDGYDYDGYRLRVEFPRGG
Bombyx mori                     NVWVAR---NPPGFAFVEFEDPRDAEDAVRGLDGRTICGRRARVEMSNGG
Drosophila pseudoobscura        FVDLKN-RRG-PPFAFVEFEDARDADDAVKARDGYDYDGYRLRVEFPRGG
Drosophila melanogaster         FVDLKN-RRG-PPFAFVEFEDARDADDAVKARDGYDYDGYRLRVEFPRGG
Anopheles gambiae               FVDLKN-RRG-PPFAFVEFEDARDADDAVKARDGYDYDGYRLRVEFPRGG
Caenorhabditis elegans          YVDIKS-GRG-PAFAFVEFEDHRDAEDAVRARDGYEFDGRRIRVEFTRGV
Caenorhabditis briggsae         YIDVKS-GRG-PAFAFIEFEDNRDAEDAVRARDGYEFDGRRIRVEFTRGV
Oryza sativa                    DIDLKIPPRP-PGFAFVEFEDPRDAEDAIRGRDGYNFDGNRLRVELAHGG
Zea mays                        DIDLKVPPRP-PGYAFVEFEDPRDAEEAIAGRDGYNFDGHRLRVEAAHGG
Arabidopsis thaliana            QIDLKVPPRP-PGYAFVEFDDARDAEDAIHGRDGYDFDGHRLRVELAHGG
Triticum aestivum               EIDLKVPPRP-PGFAFVEFEDPRDAEDAIHGRDGYNFDGNRLRVELAHGG
Hordeum vulgare                 EIDLKVPPRP-PGFAFVEFEDPRDAEDAIQGRDGYNFDGNRLRVELAHGG
Solanum tuberosum               DIELKIPPRP-PCYCFVEFESSRDAEDAIRGRDGYNFDGCRLRVELAHGG
Plasmodium yoelii               KCDVKKTVSG-AAFAFIEFEDARDAADAIKEKDGCDFGGNKLRVEVPFNA
Theileria parva                 YCELKKSYSG-SPFAFIEFSDSRDARDAIRDKDGYEFHGKKLRVELPFRY
Theileria annulata              YCDLKKSYSG-SPFAFIEFSDSRDARDAIRDKDGYEFHGKKLRVELPFRY
Plasmodium berghei              KCDVKKTVSG-AAFAFIEFEDARDAADAIKEKDGCDFGGNKLRVEVPFNA
                                   :       . :.*:**..  ** :*:   **    *   *** .
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SRp30c

```
Homo sapiens                    -RHGL--VPFAFVRFEDPRDAEDAIYGRNGYDYGQCRLRVEFPRT-----
Mus musculus                    -RHGL--VPFAFVRFEDPRDAEDAIYGRNGYDYGQCRLRVEFPRT-----
Bos taurus                      -RHGL--VPFAFVRFEDPRDAEDAIYGRNGYDYGQCRLRVEFPRT-----
Rattus norvegicus               -RHGL--VPFAFVRFEDPRDAEDAIYGRNGYDYGQCRLRVEFPRA-----
Canis familiaris                -RHGL--VPFAFVRFEDPRDAEDAIYGRNGYDYGQCRLRVEFPRT-----
Gallus gallus                   -RRGG--PPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSGRGTG
Xenopus tropicalis              -RGGSSAAPFAFISFQDPRDAEDAVFARNGYEFGSCRLRVEFPRS-----
Xenopus laevis                  -RGGS-SAPFAFISYQDPRDAEDAVFGRNGYDFGSCRLRVEFPRS-----
Danio rerio                     -NRST--IPFAFVRFEDPRDAEDAVFGRNGYGFGDCKLRVEYPRSS--GS
Tetraodon nigroviridis          -RRGG--PPFAFIEFEDPRDADDAVYGRDGYDYDGYRLRVEFPRSGRGSR
Strongylocentrotus purpuratus PSEGP---EKSETLFIK--DADDAVLARDGYNYDGYRIRVEFPRGT----
Schistosoma japonicum           QRRGP---PFAFVEFEDELDASDAVRGRDGYNFDGYALRVELPRTG-SYN
Apis mellifera                  -RRGP---PFAFVEFDDPRDAEDAVHARDGYDYDGYRLRVEFPRGGGPSN
Bombyx mori                     -PPG-----FAFVEFEDPRDAEDAVRGLDGRTICGRRARVEMSN------
Caenorhabditis elegans          -GRGP---AFAFVEFEDHRDAEDAVRARDGYEFDGRRIRVEFTRGVGPRG
Zea mays                        -PRPP---GYAFVEFEDPRDADDAIYGRDGYNFDGYRLRVELAHGGRGQS
Arabidopsis thaliana            -PRPP---GYAFVEFEDPRDADDAIYGRDGYDFDGCRLRVEIAHGGRRFS
Plasmodium falciparum           -VSGA---AFAFIEFEDARDAADAIKEKDGCDFEGNKLRVEVPFNARENG
                                          :   : .  ** **:   :*        *** .
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p54/SFRS11

```
Homo sapiens                    SPSASSEQMRTLFGFLGKIDELRLFPPD-DSPLPVSSRVCFVKFHDPDSA
Pan troglodytes                 SPSASSEQMRTLFGFLGKIDELRLFPPD-DSPLPVSSRVCFVKFHDPDSA
Mus musculus                    SPSASSEQMRTLFGFLGKIDELRLFPPD-DSPLPVSSRVCFVKFHDPDSA
Bos taurus                      SPSASSEQMRTLFGFLGKIDELRLFPPD-DSPLPVSSRVCFVKFHDPDSA
Rattus norvegicus               SPSASSEQMRTLFGFLGKIDELRLFPPD-DSPLPVSSRVCFVKFHDPDSA
Gallus gallus                   SPSASSEQMRTLFGFLGKIEELRLFPPD-DSPLPVSSRVCFVKFHDPDSA
Xenopus tropicalis              SPSASSEQMKTLFGFLGKIEELRLFPPD-DSPLPVTSRVCFVKFQDPDSA
Xenopus laevis                  SPSASSEQMITLFGFLGKIEELRLFPPD-DSPLPVTSRVCFVKFQDPDSA
Danio rerio                     SPSSTAEQMRTLFGFIGSIDELRLFPPD-DSPLPVTSRVCFVKFHEPESV
Tribolium castaneum             APQATKDQMQTLFGYLGKIEDIRLYPTIRDVSCPVQSRICYVKFVDSSTV
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```
Tetraodon nigroviridis         SPSATSEQMRTLFGFLGTIEELKLFPPD-DSPMPVTSRVCFVKFQEPESV
Strongylocentrotus purpuratus SSAATLEQVRSLFSFLGKIEDIRLFPKE-DSVLPVTARICFILFEDPTSV
Apis mellifera                 APQATKDQMQTLFGYLGKIEDIRLYPTIRDVAVPVQSRICYIKFHDQGCV
Drosophila pseudoobscura       APQATKDQMQTLFGNIGKIEEIRLYPTIRDVSCPVQSRICYVKYTETTSV
Drosophila melanogaster        APQATKDQMQTLFGNIGKIEEIRLYPTIRDVSCPVQSRICYVKYTDTTSV
Anopheles gambiae              APQATKDQMQTFLGTVGKIDEIRLYPTIRDVSCPVVSRICYVKYFESSCV
                               :. :: :*: :::. :*.*::::*:*   *   ** :*:*:: : :
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NONO

```
Homo sapiens                   DNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAVV
SFPQ  Homo sapiens             DDTPMRGRQLRVRFATHAAALSVRNLSPYVSNELLEEAFSQFGPIERAVV
Pan troglodytes                DNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAVV
Macaca mulatta                 DNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAVV
Macaca fascicularis            DNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAVV
Mus musculus                   DNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAVV
Rattus norvegicus              DNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAVV
Bos taurus                     DNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAVV
Canis familiaris               DNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAVV
Gallus gallus                  DNMPLRGKQLRVRFACHSASLTVRNLPQFVSNELLEEAFSVFGQVERAVV
Danio rerio                    DDTIFRGRQIRVRFATHGAALTVKNLPQFVSNELLEEAFSMFGPIERAIV
Tetraodon nigroviridis         DDTPMKGRPLRVRFATHSAALSVKNLSPFVSNELLEEAFSQFGMVERAVV
Tribolium castaneum            DGILFKGKNLKIRFAPNGSTVKVKNLTPFVSNELLYYSFSVFGEVERAYV
Purple urchin                  DGTQQKNRTIRVRFATHGAALRVKNIPPSTSNELLEQAFSMFGEVERAIV
Apis mellifera                 DGTMRKGRALKVRFAPHSSTIKVKNLTPWISNELLERAFSVFGEIERAIV
Drosophila melanogaster        DGSMRKGRQLRVRFAPNATILRVSNLTPFVSNELLYKSFEIFGPIERASI
Chironomus tentans             DGRMRKNKPIRIRFAPNATTIRVKNLTPFVSNELLFKSFEVFGQVERAVI
Drosophila littoralis          DGSMRKGRQLRVRFAPNATILRVSNLTPFVSNELLYKSFEIFGPIERASI
Drosophila virilis             DGSMRKGRHVRVRFAPNATILRVSNLTPFVSNELLYKSFEIFGPIERASI
Anopheles gambiae              DGTTRKNRVLRVRFAPNATAIRVKNLTPYVTNELLYKAFEVFGPLERAVV
Xenopus tropicalis             DNLPLRGKQLRVRFACHSASLSVKNIPQFVSNELLEEAFSIFGQVERTVV
Xenopus laevis                 DNLPLRGKQLRVRFACHSAALSVKNIPQFVSNELLEEAFSMFGQVERAVV
Caenorhabditis elegans         DGRIIHGRQVRVRFAVHGAAIRVKELSPTVSNEMLYHAFSHFGDVERAVH
Caenorhabditis briggsae        DGRIIHGRQVRVRFAVHGAAIRVKELSPTVSNEMLYHAFSHFGDVERAVH
Bombyx mori                    DGKLRNSRTLRVRFAPHNSAVRVKNLPPFVSNELLYRSFEIFGKIERAYV
                               *.   ..: :::*** : : : * ::.   :**:*  :*. ** :**:
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PTBP1

```
Homo sapiens                   KDRKMALIQMGSVEEAVQALIDLHNHDLGENHHLRVSFSKSTI-------
PTBP2 Homo sapiens             -DHKMALLQMATVEEAIQALIDLHNYNLGENHHLRVSFSKSTI-------
ROD1  Homo sapiens             KDRKMALIQLGSVEEAIQALIELHNHDLGENHHLRVSFSKSTI-------
Pan troglodytes                KDRKMALIQLGSVEEAIQALIELHNHDLGENHHLRVSFSKSTI-------
Macaca mulatta                 KDRKMALIQMGSVEEAVQALIDLHNHDLGENHHLRVSFSKSTI-------
Mus musculus                   KDRKMALIQMGSVEEAVQALIELHNHDLGENHHLRVSFSKSTI-------
Rattus norvegicus              KDRKMALIQMGSVEEAVQALIELHNHDLGENHHLRVSFSKSTI-------
Sus scrofa                     KDRKMALIQMGSVEEAIQALIDLHNHDLGENHHLRVSFSKSTI-------
Bos taurus                     KDRKMALIQMGSVEEAIQALIDLHNHDLGENHHLRVSFSKSTI-------
Canis familiaris               KDRKMALIQMGSVEEAIQALIDLHNHDLGENHHLRVSFSKSTI-------
Gallus gallus                  KDRKMALIQMGSVEEAIQSLIDLHNHDLGENHHLRVSFSKSTI-------
Danio rerio                    KDRKMALIQLASVEEAIESLIKFHNHDLGENHHLRVSFSKSTI-------
Strongylocentrotus purpuratus KDRKMALLQMGSVEEAIHALIAMHNYQLAESNHLRVSFSKAQI-------
Paracentrotus lividus          KDRKMALLQMGSVEEAIHALIAMHNYQLAESNHLRVSFSKAQI-------
Tribolium castaneum            KDKKMALIQLPSMEEAVEALIRMHNYQLSESNHLRVSFSKSSI-------
Apis mellifera                 KDRKMALIQMPSMDDAVAALIKMHNYQLSESNHLRVSFSKSNI-------
Drosophila melanogaster        KDRKMALLQLLSVEEAVLALIKMHNHQLSESNHLRVSFSKSNI-------
Aedes aegypti                  KDHKMALIQLSSIEEAVCALIKMHNYQLSESNHLRVSFSKSNI-------
Anopheles gambiae              KDHKMALIQLSSIEEAVCALIKMHNYQLSESNHLRVSFSKSNI-------
Drosophila pseudoobscura       KDRKMALLQLSSVEEAVLALIKMHNHQLSESNHLRVSFSKSNI-------
Xenopus tropicalis             KDRKMALIQMGSVEEAIESLIELHNHDMGENHHLRVSFSKSTI-------
Xenopus laevis                 KDRKMALIQMGSVEEAIESLIELHNHDMGENHHLRVSFSKSTI-------
Caenorhabditis elegans         KDHKMALCQLEDIETAIDALIAMHNHKLAENAHLRVSFSKSGI-------
Caenorhabditis briggsae        KDHKMALCQLEDIETAIDALIKMHNHKLAENAHLRVSFSKSGI-------
Trypanosoma brucei             KSPTVAIVSLKDVETAVNALIAVHSHQLKER-FLRVTFSHFPPAPRSGTE
Trypanosoma cruzi              KNPSVAIVSLRDIETAVQALIVVHSQQLKER-FLRVTFSHFPPGPRSNGE
                                 . .:*: .:  :: *: :** .*. .: *  .***:**:
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RBM15

```
Homo sapiens                  DAAHAAWTHMRGFPLGGPDRRLRVDFADTEHRYQQQYLQPLPLT---HYE
Pan troglodytes               DAAHAAWTHMRGFPLGGPDRRLRVDFADTEHRYQQQYLQPLPLT---HYE
Macaca mulatta                DAAHAAWTHMRGFPLGGPDRRLRVDFADTEHRYQQQYLQPLPLT---HYE
Mus musculus                  DAAHAAWTHMRGFPLGGPDRRLRVDFADTEHRYQQQYLQPLPLT---HYE
Rattus norvegicus             DAAHAAWTHMRGFPLGGPDRRLRVDFADTEHRYQQQYLQPLPLT---HYE
Bos taurus                    DAAHAAWTHMRGFPLGGPDRRLRVDFADTEHRYQQQYLQPLPLT---HYE
Canis familiaris              DAAHAAWTHMRGFPLGGPDRRLRVDFADTEHRYQQQYLQPLPLT---HYE
Gallus gallus                 DAAQAACTHMRGFPLGGPDRRLRVDFADTEHRYQQPYLQPLPLPPPAHYE
Tetraodon nigroviridis        DAAQAACTHMRGFPLGGPERRLRVDFADNEHRYQQQFLQPLPIP---PFD
Strongylocentrotus purpuratus EAAQAAASHMRGVPLGGPDKRLRVDFAEHGPGLQTTKKQFKPYQG----
Apis mellifera                DAAQAAVKEMRGFPLGGPDRRLRVDFADVT------PGFGFKPR------
Tribolium castaneum           DAAQAAVKEMRGAPLGGPDRRLRTDFADVT---PGVVYRPKPPY------
Drosophila melanogaster       EAATAAVKEMRGFPLGGPERRLRTDFAELPGATPAAP-FKSSKP------
Drosophila pseudoobscura      EAATAAVKEMRGFPLGGPERRLRTDFAELPGATPAASSFKTSKP------
Xenopus laevis                DAAQAACTQMRGFPLGGDHRRLRVDFAEADVRFPHPYVQPLPIP---YID
Anopheles gambiae             DAATAAVKEMRGFALGGPDRRIRIDFADNG----TVPPFAKR--------
Aedes aegypti                 DAATAAVKEMRGFPLGAPDRRIRIDFADNG----TTPPFPKRGG------
                              :** ** .:*** .**. .:*:* ***:
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Figure 44. Phylogenetic comparison of proteins containing an RVXF motif in the beta4 sheet of their RRMs. Amino acids conserved in all species are highlighted in yellow.

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 45A). 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.

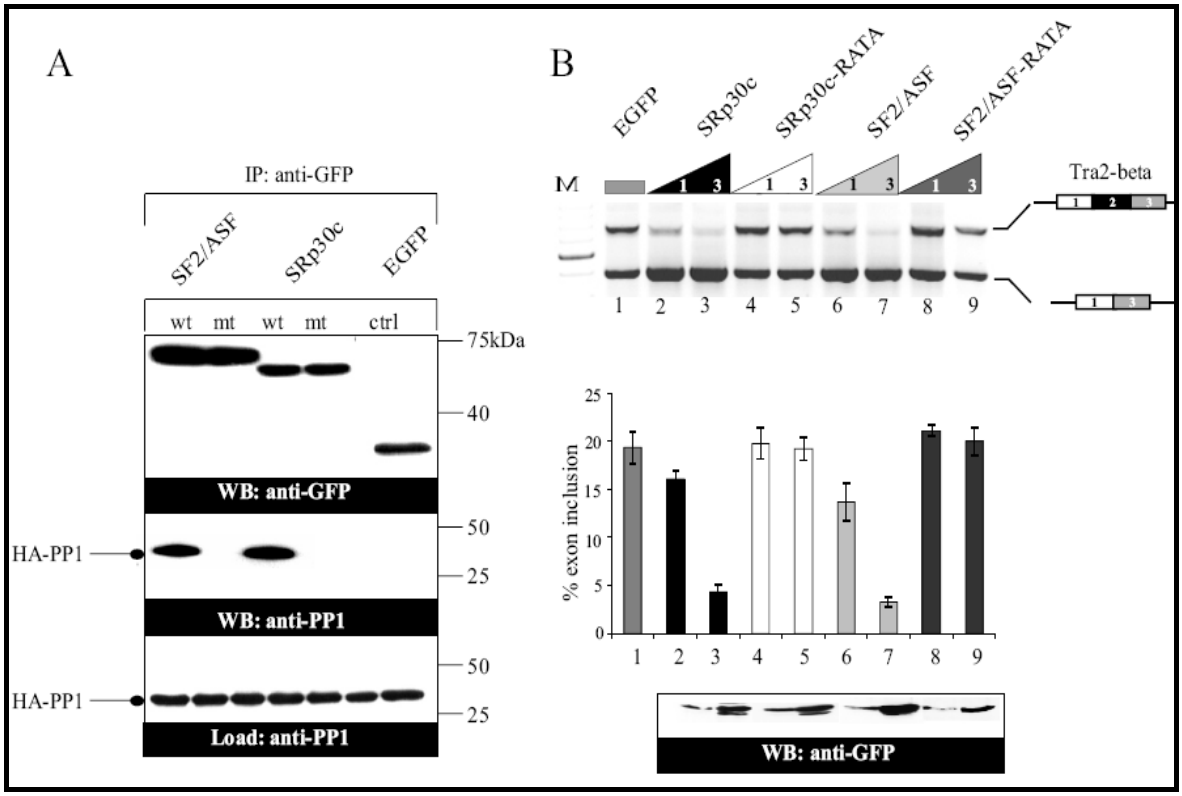

Figure 45. PP1 binding depends on the RVEF motif present in SF2/ASF and SRp30c. (A) EGFP-tagged SF2/ASF and SRp30c were expressed in HEK293 cells together with HA-PP1 and immunoprecipitated with anti-GFP antibodies. Wt: RVEF, mt: RVEF changed to RATA. The presence of HA-PP1 in the immunoprecipitates 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 P. et al., 2004).

As shown in Figure 45B, 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.

5. DISCUSSION

5.1. Tyrosine phosphorylation of splicing factors rSLM-1 and rSLM-2 changes splice site selection.

Alternative splicing is a process that generates different mRNAs. They often encode distinct diverse protein products from one gene. It therefore drastically increases the coding capacity of genes. Splice site choice is be strictly regulated. Alternative splicing is often tightly regulated in a tissue-specific or developmental stage-specific manner. Since the 5' and 3' splice sites of naturally occurring mammalian exons often deviate from the consensus (Stamm S. et al., 2000), it would require the additional elements on the sequence. These elements locate within intronic or exonic sequences and can act as either enhancers or silencers (Hastings M.L. and Krainer A.R., 2001). They are recognized by RNA binding proteins. The proper recognition of splice sites and the regulation of alternative exons involve crosstalk between proteins mediated by multiple, intrinsically weak interactions of (Maniatis T. and Reed R., 2002). These multiple interactions are important for establishing complexes that commit the pre-mRNA to splicing. The proper recognition is leading to the formation of a commitment complex that is recognized by the spliceosome (Reed R., 1996).

The first part of this study includes the characterization of two very similar splicing factors, rSLM-1 and rSLM-2 and the comparison of their relevance to alternative splice site selection. rSLM-1 and rSLM-2 members of the STAR (Signal Transduction and Activation of RNA) protein family. They were shown to 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 O. et al., 1999; Stoss O. et al., 2004). It was demonstrated that both proteins interact with many other splicing factors (Stoss O. et al., 1999; Stoss O. 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 fact that GSG domain which is necessary for RNA binding of these proteins is necessary for oligomerization (Chen T. et al., 1997). GSG domain contains single KH domain flanked by two regions homologous to the murine quaking gene, Qua1

and Qua2 (Vernet C. and Artzt K., 1997). The GSG domain was demonstrated to form high RNA interaction surface.

All of them are RNA binding proteins, which interact with RNA through specific RNA binding domains. It is very likely that unique combination of these proteins regulate alternative splicing.

The expression studies of two highly related proteins rSLM-1 and rSLM-2 revealed the difference between them. The previous work on humans demonstrated that rSLM-2 ortholog T-STAR is expressed in testis, brain and muscle (Sugimoto Y. et al., 2001). The expression pattern of the rSLM-2 ortholog in humans and mouse was similar. The expression of rat SLM-2 protein, analysed here reveals that it expresses in all the examined tissues, however it was mostly expressed in brain, heart and testis (Stoss O. et al., 2004). The Nothern Blot analysis of mouse tissues showed that the SLM-1 transcript is ubiquitously expressed (Di Fruscio M. et al., 1999). However, in our research we demonstrated that the protein is restricted to brain and testis (Stoss O. et al., 2004).

Analysis of protein expression in more detail in the brain revealed that both rSLM-1 and rSLM-2 are expressed in neurons. Detection revealed both protein expression in the cortex, but most likely not in the same cells (data not shown). Staining of adjacent sections revealed that the proteins are expressed in different neurons. 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 started to speculate about different mechanisms of cell type-specific splicing in the brain.

The regulation of alternative splicing by rSLM-1 and rSLM-2 proteins and their partners is achieved by acting similar to the RBP1/tra2/tra and SF2/tra2/tra complexes (Lynch K.W. and Maniatis T. 1996), hnRNP F, H/KSRP complexes (Markovtsov V. et al., 2000) and FBP/SAM68/PTB complexes (Grossman J.S. et al., 1998) that influence doublesex exon 4, the src N1 exon, and beta-tropomyosin exon 7, respectively. rSLM-1 and rSLM-2 regulate alternative splice site selection of the SMN2. SMN2 exon 7 was also shown to be regulated by SRp30c (Young P.L. et al., 2002) and hnRNPG (Hofmann Y. and Wirth B., 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 S. et al.,

1999), or neurofilament tau exon 10 (Wang J. et al., 2004) are not affected (data not shown).

The identification of rSLM-1 and rSLM-2 proteins leads to 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 proves that alternative splicing can be influenced by tyrosine kinase mediated phosphorylation.

Present studies do not show how alternative splicing links the signal transduction pathways. 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 R. et al., 1999), kindling (Kamphuis W. et al., 1992), cocaine treatment (Berke J.D. et al., 2001), or pavlovian fear memory (Stork O. et al., 2001) change alternative splicing patterns. However, the molecular details how alternative splicing is linked to signal transduction were still not clear (Stamm S., 2002). This research was performed to understand the mechanisms by which the signal transduction pathways could be linked to change on splice site selection. Particularly, the results of the first part of the dissertation provide the evidence that tyrosine phosphorylation of a 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 N. et al., 2002; Abdennebi L. et al., 2002; Holdiman A.J. et al., 2002; Meshorer E. et al., 2002; Stamm S., 2002).

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 P. 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 have found out that rSLM-2 phosphorylation by c-abl kinase modulate 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 explaines how this kinase can phosphorylate the nuclear protein rSLM-1, is that rSLM1 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 in contrast to rSLM-2, which does not shuttle. Previous work has demonstrated that Sam68 translocates to the nucleus (Paronetto M.P. et 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 $p59^{fyn}$ and direct nuclear translocation of plasma-membrane integral tyrosine kinases that has been observed in several cases (Lin S.Y. et al., 2001; Marti U. and Wells A., 2000; Ni C.Y. et al., 2001; Offterdinger M. et al., 2002; Wells A. and Marti U., 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 S.C. et al., 1994; Shima T. et al., 2001). The present work demonstrates that rSLM-1 influences splice site selection depending on its phosphorylation status.

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 N.G. et al., 1994; Scotet E.and Houssaint E., 1998; Sell S.M. et al., 1994; Wang J. et al., 1991) and that a change in alternative splicing is often correlated with cancer caused by tyrosine kinase misregulation.

5.2. The reversible phosphorylationof Tra2-beta1 protein regulates splice site selection

Previous studies have demonstrated that reversible phosphorylation of SR proteins is an important mediator for splice site selection (Chalfant C.E. et al., 1995; Du C. et al., 1998; Duncan P.I. et al., 1997; Muraki M. et al., 2004). For example, reversible

phosphorylation of SF2/ASF is necessary for the splicing reaction, which occurs after spliceosome assembly (Cao W. et al., 1997; Mermoud J.E. et al., 1994; Murray M.V. et al., 1999). Phosphorylation of SR proteins is an important step for formation protein: protein and protein: RNA interactions during spliceosome assembly. We identified the presence of a Protein Phosphatase 1 binding site, RVxF in the RRM of SR-like protein splice factor Tra2-beta1. The sequence alignment of all available species for Tra2-beta1 reveals the presence of a PP1 binding motif with high evolutionary conservation.

It is well known that RNA recognition motifs are generally arranged in a conserved structure containing four antiparallel beta strands 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 C. et al., 2005). The RRM functions both in binding single stranded RNA and also in binding proteins (Maris C. 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. The NMR structures of SF2/ASF and Tra2-beta1 (PDB:1X4A, 2CQC) and the crystal structure of the RRM of PTB (Petoukhov M.V. et al., 2006) proved the predicted structure. Figure 46 shows structural representation of RRM domain with the PP1 binding motif RVDF located in beta4 sheet of Tra2-beta1.

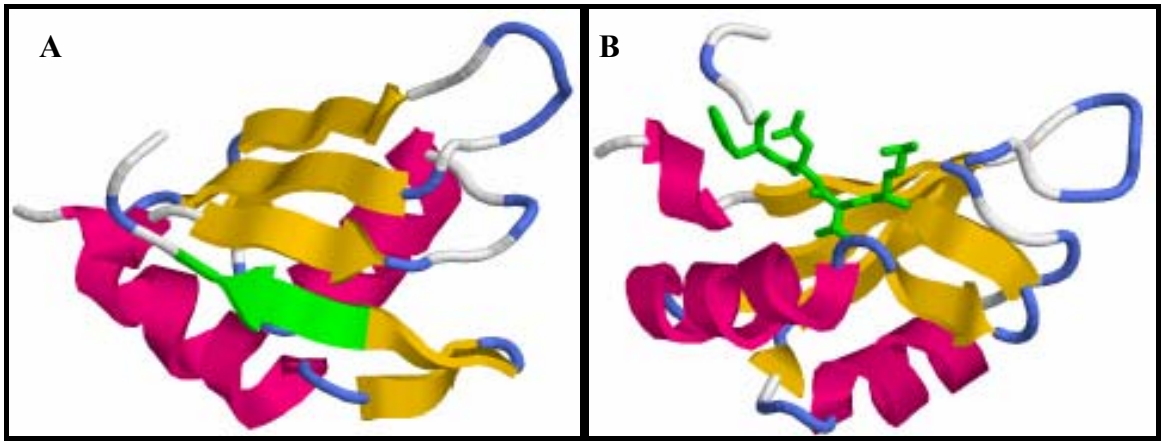

Figure 46. Structural representation of RRM domain with RVDF motif. The model comprises the RRM (RNA recognition motif) and is based on the experimentally determined NMR structure of Tra2-beta1 (PDB: 2CQC) for residues D115-R190 and homology model for the C-terminally adjacent residues (V191-K197) based on domain structure templates from Cold-Inducible RNA binding protein (PDB: 1x5s) and RNA binding protein Ubp1 (PDB: 1u6f). (A) Overall backbone model for the RRM domain architecture. Color code: pink, α-helices; brown, β-sheets; blue, random coil; green, RVDF motif residues. (B) Backbone model for the RRM domain architecture with side chains dispositions of RVDF motif residues (sticks model, green color).

We performed a computational analysis of SR and SR-like proteins and found nine other proteins containing a PP1 binding motif, RVxF. Figure 43 (section 4.2.16) 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 the Tra2-beta1. In addition to Tra2-beta1, two other SR-proteins SF2/ASF and SRp30c also bound to PP1. Most RVxF motifs interacting with the binding pocket of PP1 are located in an extended beta sheet formation (Egloff M.P. et al., 1997; Meiselbach H. et al., 2006), which suggests that the structural arrangement of the RVxF motif in RRMs allows binding to PP1.

Therefore, binding of RRM containing proteins to protein phosphatase is an evolutionary conserved function of these proteins. This evolutionary conservation of the sequence and structure of the PP1 docking motif in splicing factors could further suggest a conserved interaction with the spliceosome. Recently it was identified that U2 and U5 snRNPs are regulated by PP1 action (Shi Y. et al., 2006). However none of the particle components have known PP1 binding properties. Probably, the effect of PP1 acting on snRNPs in the spliceosome is due to the binding of PP1 catalyitic subunit to PP1 docking motif of RNA binding proteins during the splicing process.

As it is shown in this work PP1 dephosphorylates Tra2-beta1 after binding to it. The dephosphorylation of Tra2-beta1 changes the properties of the protein, for example binding to interacting proteins. Dephosphorylation of Tra2-beta1 promotes the RS domain mediated homomultimerization and binding to SF2/ASF (section 4.2.5.). After the destruction of the RVDF docking motif interactions between the proteins are severely abolished, indicating that PP1 dephosphorylates sites in the RS domains of Tra2-beta1.

In order to determine whether PP1 activity can influence splice site selection, we performed a number of *in vivo* splicing assays. The activity of PP1 in cells was either increased by transfecting PP1c expression constructs or decreased by transfecting NIPP1. NIPP1 is a specific nuclear protein of Protein Phosphatase 1. In most cases analyzed, we found that a decrease of PP1 activity on alternative exon usage, which we achieved by transfection of NIPP1 (section 4.2.13.) or treatment of cells with specific PP1 inhibitor tautomycin (section 4.2.14.), was similar, as did an increase of the Tra2-beta1 concentration. However, in several cases alternative exons responded much stronger to NIPP1 than to Tra2-beta1. This is probably due to the fact that other splicing factors, such as SF2/ASF or SRp30c are dephosphorylated by PP1. These factors are part of the protein complexes regulating these exons.

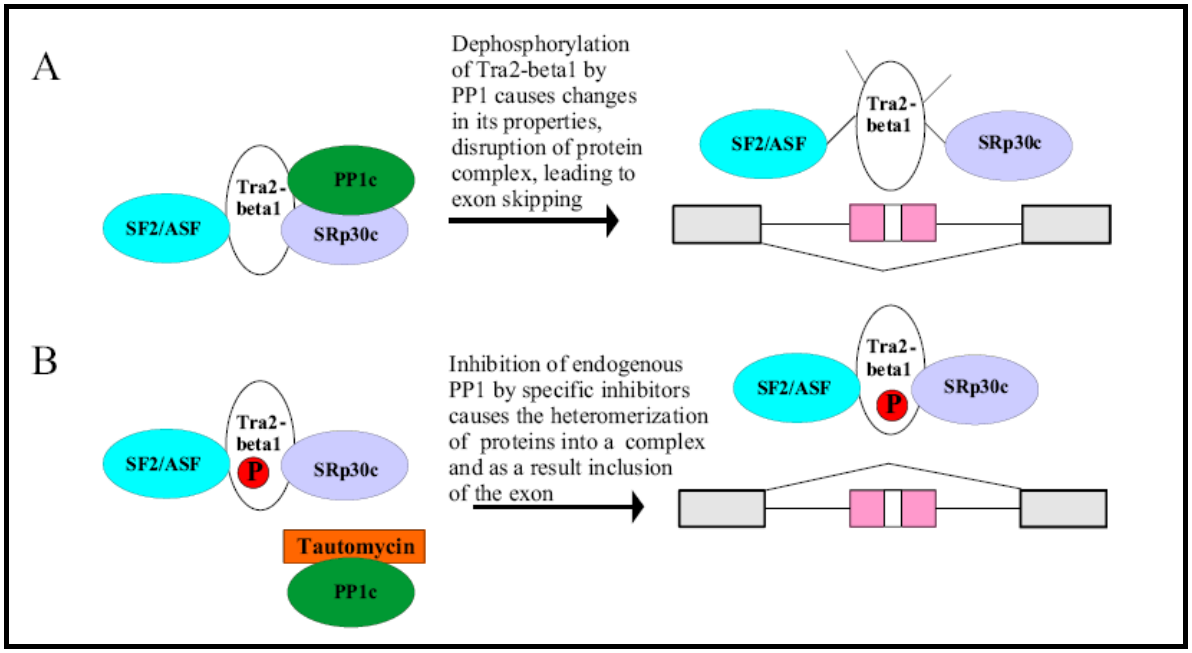

Figure 47. Dephosphorylation of Tra2-beta1 by PP1 changes the alternative splice site selection. Dephosphorylation of Tra2-beta1 protein causes disruption of Tra2-beta1:SF2/ASF:SRp30c protein complex which results in exon skipping (upper panel). Inhibition of endogenous PP1 by specific inhibitors leads to heterodimerization of Tra2-beta1, which results in exon inclusion (lower panel).

Figure 47 shows a schematic representation of how dephosphorylation of Tra2-beta1 and probably its interacting proteins, modulate binding properties of these proteins and as a result modify complex formation that affect splice site selection. The effect on splice site selection was abolished by using PP1 mutants, where phosphatase activity and binding properties were inhibited, showing that PP1 exerts its effect on splice site selection not just by binding to an RRM, but also by dephosphorylation (section 4.2.8).

The catalytic subunit of PP1 associates with a number different proteins that can function as targeting proteins, substrate-specifiers or inhibitors. Cellular signal transduction pathways regulate the binding of PP1 to these proteins. The association of PP1 with regulatory proteins will affect its ability to dephosphorylate splicing factors with the PP1 docking motif. This could explain why alternative splice site usage in various cell types is often very different, although the concentration of regulatory factors is quite similar. Our data suggest that the phosphorylation state of splicing regulatory factors is as important as their relative concentration and that PP1 activity is a key component in this regulation.

5.3. PP1 inhibitors are potentially beneficial for treating diseases caused by pathophysiological splice site selection

Missplicing of the pre-mRNA is one of the causes of several diseases, among them Alzheimer's disease (Glatz D. et al., 2006), breast cancer (Watermann D.O. et al., 2006) and spinal muscular atrophy (Hofmann Y. et al., 2000). Therefore, the correction of improper splicing is an important goal in developing of molecular therapeutic strategies.

One well-studied autosomal recessive disorder, caused by the single mutation in ESE is spinal muscular atrophy. We focused on this disease, since it was genetically clearly defined. 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 P. 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 would stimulate full-length SMN2 expression (Hofmann Y. et al., 2000). Therefore, specific agents that could potentially release hTra2-beta1 from its nuclear storage sites and promote high levels of full levels expression of SMN2 might be of therapeutic use for SMA. Therefore, we suggested that the modification of Tra2-beta1 reversible phosphorylation by PP1 would have impact on Tra2-beta1 dependent exon inclusion.

We thus 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 (section 4.2.11.). This proves that modulation of phosphatase activity can be used to alter alternative splice site selection. However, our array and RT-PCR analysis shows that PP1 inhibition causes a change in numerous exons (section 4.2.14.).

Some PP1 inhibitors (tautomycin and cantharidin) were tested on transgenic mice in collaboration with Prof. Dr. Arthur Burghes. The experiments were performed by Dr. Matthew E. R. Butchbach. The Western Blot demonstrates the accumulation of SMN protein in the spinal cord of SMN2 transgenic mice.

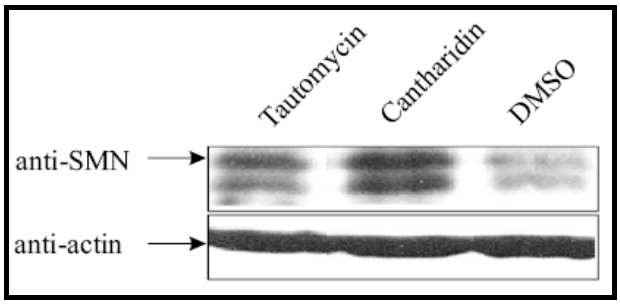

Figure 48. The PP1 inhibitors tautomycin and cantharidin promote the accumulation of SMN protein in transgenic mice. The mice (line 89) homozygous for the *Smn*, contains two copies of SMN2 (*Smn*$^{-/-}$;*SMN2*) (Monani U. R. et al., 2000).

Similar results were observed in the liver and forebrain extracts from these treated mice. Semiquantitation analysis of Western Blot showed that tautomycin increases SMN protein by ~65%, cantharidin by ~1.3-fold and forskolin by 2.3-fold.

Based on these preliminary data we suggest that these compounds could further be used for establishing new therapeutical approaches. Specificity for this new class of drugs could be achieved by combining PP1 inhibitors with other drugs that affect exon 7 inclusion, e.g valproic acid (Brichta L. et al., 2003), by finding substances specific for the Tra2-beta1: PP1 complex, or by coupling PP1 inhibitors to nucleic acids, similar to chimeras between RS domains and antisense oligonucleotides (Cartegni L. and Krainer A., 2003).

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