The role of tra2-beta1 in regulation of alternative splicing and translation

Den Naturwissenschaftlichen Fakultäten
Der Friedriech-Alexander-Universität Erlangen- Nürnberg
zur
Erlangung des Doktorgrades

vorgelegt von

Olga Kelemen

aus Budaörs, Hungary

2012

PUBLICATIONS

Kelemen, O., Convertini, P., Zhang, Z., Wen, Y., Shen, M., Falaleeva, M., Stamm, S. (2012). Function of alternative splicing. Gene (in press).

Sumanasekera, C., **Kelemen, O.,** Beullens, M., Aubol, B.E., Adams, J.A., Sunkara, M., Morris, A., Bollen, M., Andreadis, A., and Stamm, S. (2012). C6 pyridinium ceramide influences alternative pre-mRNA splicing by inhibiting protein phosphatase-1. Nucleic Acids Res 40, 4025-4039.

Zhang, Z., **Kelemen, O.,** van Santen, M.A., Yelton, S.M., Wendlandt, A.E., Sviripa, V.M., Bollen, M., Beullens, M., Urlaub, H., Luhrmann, R., Watt, D.S., and Stamm, S. (2011). Synthesis and characterization of pseudocantharidins, novel phosphatase modulators that promote the inclusion of exon 7 into the SMN (survival of motoneuron) pre-mRNA. J Biol Chem 286, 10126-10136.

Fishman J. B., **Kelemen O.,** Berg E. A. (2012) Modification State-Specific Antibodies. Alternative pre-mRNA splicing. Wiley-VCH Verlag GmbH & Co. KGaA

ACKNOWLEDGMENTS

This work presented here was performed in the Molecular and Cellular Biochemistry Department at University of Kentucky. There are many people to whom I owe the fulfillment of this dissertation. This work would not have been possible without the love and support of friends and family.

I would like to give my sincerest thanks to Prof. Dr. Stefan Stamm for his support and guidance.

I would also like to thank the present and former members of the Stamms lab, Dr. Zhaiyi Zhang, Dr. Manli Shen, Dr. Chiranthani Sumanasekera, Dr. Marina Falaleeva, Traci Bock, Samira Samtleben, Dr. Paolo Converitni, Dr. Amit Khanna and David Oliphant for their help and support.

I am also thankful to all members of the Molecular and Cellular Biochemistry Department of University of Kentucky for the help in particular, Dr. Jeffrey Rush.

I appreciate all my friends for their cheerful company and mental support.
In the end, I would like to thank my parents, my siblings and my family for their unconditional love, for their patience and for their encouragement in times when I needed most. Without their support, I couldn't be able to accomplish this work.
Many, many thanks!

Szeretném megköszönni mindenkinek a szeretetét és hogy mindig lehetett rátok számítani. Különösen Annának és Istvánnak hogy mindig felvették a telefont és végig hallgattak újra és újra! Emeskének és Kingának a hivatalos ügyeim intezését, Anyunak, Apunak és Istinek a támogatást!

Table of Contents

KURZFASSUNG

Alternatives mRNA-Splicing ist ein Schlüsselmechanismus der zellulären und funktionellen Komplexität in Eukaryoten. So werden geschätzte 95% der menschlichen Multiexontranskripte alternativ gespliced. Fehlregulierte Splicingereignisse resultieren in einer Vielzahl von menschlichen Krankheiten. Das humane tra2-beta1 wurde ursprünglich als Splicingfaktor isoliert; es spielt eine wichtige Rolle in konstitutivem und alternativem, Splicing. Tra2-beta1 gehört zur Gruppe der Serin-Arginin reichen Proteine (SR-like) und weist ein RNA-Erkennungsmotiv (RRM) auf, welches von zwei Serin-Arginin reichen Domänen (RS) flankiert wird. Tra2-beta1 bindet an purinreiche Sequencen und fördert die Einbeziehung alternativer Exons, die seine Erkennungssequenz beinhalten. Der Phosphorylierungsstatus der SR Domänen moduliert die Funtion von Tra2-beta1. Ein RVxF-artiges PP1 Andockmotiv befindet sich auf dem RRM von Tra2-beta1 und erlaubt die Interaktion von Tra2-beta1 mit PP1. PP1 moduliert die Splicingaktivität von Tra2-beta1; seine Inhibierung fördert Exon-Einbeziehung verschiedener Tra2-beta1 abhängiger Exons. Wir zeigen das Tra2-beta1 in katalytisch unabhängiger Art und Weise von PP1 reguliert wird. Gel Shift Ergebnisse weisen darauf hin, dass PP1 die Bindungsaffinität von Tra2-beta1 an spezifische RNAs stimuliert.

Tra2-beta1 ist vorwiegend im Nucleus lokalisiert. Unter Stressbedingungen verändert es seine subzelluläre Lokalisierung und akkumuliert im Cytoplasma was darauf hindeutet, dass seine Funktion nicht auf nukleäres pre-mRNA Splicing beschränkt ist. In dieser Arbeit zeigen wir, dass Tra2-beta1 an die 18S rRNA bindet und die Translation eines Reporterskonstrukts aktiviert, welches sein Bindemotiv enthält. Darüber hinaus wurde gezeigt, dass Tra2-beta1 in verschiedenen Typen von Krebszellen überexprimiert ist und unsere Hypoxiestudien zeigen weiterhin das Hypoxie die Translation von Tra2-beta1 abhängigen Reporter-mRNAs verstärkt. Folglich deutet die Assoziation von Tra2-

beta1 mit 18S rRNA sowie die *in vivo* Stimulation der Translation auf eine Rolle von Tra2-beta1 im Translationsprozess hin.

ABSTRACT

Alternative pre-mRNA splicing is one of the key mechanisms to increase cellular and functional complexity in eukaryotes. An estimated 95% of human multiexon transcripts undergo alternative splicing. Mis-regulated splicing events result in a wide range of human diseases. Human tra2-beta1 originally was characterized as a splicing factor; it plays an important role in constitutive and alternative splicing. Tra2-beta1 belongs to the Serine-arginine rich-like (SR-like) proteins and contains an RNA recognition motif (RRM) flanked by two serine-arginine rich (RS) domains. Tra2-beta1 binds to purine-rich sequences and promotes the inclusion of alternative exons that contain its recognition motif. The phosphorylation status of the SR domains modulates the function of tra2-beta1. An RVxF-type PP1 docking motif located on the RRM of tra2-beta1, that allows the interaction between tra2-beta1 and PP1. PP1 modulates the splicing activity of tra2-beta1; inhibition of PP1 promotes exon inclusion in several tra2-beta1 dependent exons. We demonstrate that PP1 regulates tra2-beta1 in a catalytically independent manner. Gel shift assays suggest that PP1 stimulates tra2-beta1 binding affinity to specific RNAs.

Tra2-beta1 is predominantly located in the nucleus but under stress conditions it changes its localization and accumulates in the cytoplasm, suggesting that its function may not be limited to nuclear pre-mRNA splicing. Here, we show that tra2-beta1 binds to the 18S rRNA and activates the translation of a reporter construct containing its binding motif. Moreover, it has been shown that tra2-beta1 is overexpressed in several types of cancer and our hypoxia studies indicates that hypoxia enhances translation of tra2-beta1-dependent reporter mRNAs. Thus, the association of tra2-beta1 with 18S rRNA and the stimulation of translation *in vivo* indicate a role for tra2-beta1 in translation.

ABBREVIATIONS

A alanine

ATP denosine 5`-triphosphate

BP branch point

BSA bovine serum albumnin

cDNA complementary DNA

CTD carboxyl-terminal domain

dH_2O distilled H_2O

CLIP Cross-linking and immunoprecipitation

CLK2 CDC2-like kinase

DMEM dulbeco's modified eagle medium

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

dNTP deoxyribonucleotidtriphosphate

Dscam Down syndrome cell adhesion molecule

DTT dithiothreitol

DRF1 DBF4 homolog B (S. cerevisiae)

E glutamic acid

ECL enhanced chemiluminiscence

EDTA ethylenediaminetetraacetic acid

EGFP enhanced green fluorescence protein

EJC exon-exon junction complex

EMSA electrophoretic mobility shift assay

ES6 expansion segment 6

ESE exonic splicing enhancer

HEK human embryonic kidney

hnRNP heterogenous nuclear ribonucleoprotein

IPTG isopropyl β-D-1-thiogalactopyranoside

kDa kilodalton

mRNA messenger RNA

mRNP messenger ribonucleoprotein

ncRNA noncoding RNA

NMD nonsense-mediated decay

nPTB polypyrimidine tract binding protein 2

PAA polyacrylamide

PAP Poly(A)- Polymerase

PBS phosphate buffered saline

PCR polymerase chain reaction

PMSF phenylmethanesulfonyl fluoride

POL-β DNA polymerase beta

PP1 Protein phosphatase 1

PP2A Protein phosphatase 2 A

PSF/SFPQ splicing factor proline/glutamine-rich

PTM Post-translational modification

P54nrb/NONO non-POU domain-containing octamer-binding protein

R arginine

RNA ribonucleic acid

RNase ribonuclease

ROD1 polypyrimidine tract binding protein 3

rpm revolutions per minute

RRM RNA recognition motif

rRNA ribosomal RNA

RS domain arginine-serine rich domain

RT-PCR reverse transcription followed by polymerase chain reaction

S serine

SAP155 splicing factor 3b, subunit 1

SDS sodium dodecyl sulfate

SF2/ASF serine/arginine-rich splicing factor 1

SMA Spinal Muscular Atrophy

SMN1 survival of motor neuron gene 1

SMN2 survival of motor neuron gene 2

snRNA small nuclear RNA

SR-protein serine-arginine rich protein

SRp30c serine/arginine-rich splicing factor 9

SRp54 signal recognition particle 54kDa

SYK spleen tyrosine kinase

TAU microtubule associated protein tau

TBE tris-borate-EDTA buffer

TEMED N,N,N',N'-tetramethylethylenediamine

TIAF1 TGFB1-induced antiapoptotic factor, 1

Tra2 transformer 2

UAP56 DEAD (Asp-Glu-Ala-Asp) box polypeptide 39B

U2AF U2 auxiliary factor

UTR untranslated region

1 Introduction

Genetic information is stored in DNA that is transcribed to RNA. RNA functions as an intermediary between DNA and proteins. The human genome contains only 20,000-25,000 protein-encoding genes (2004). Transcriptional and post-transcriptional mechanisms play an important role to increase the size of the proteome, including the usage of multiple transcription start sites, alternative pre-mRNA splicing, polyadenylation, pre-mRNA editing and post-translational protein modifications. Alternative splicing is one of the most important sources of protein diversity in metazoans (Maniatis and Tasic, 2002). Almost all human RNA polymerase II transcripts undergo pre-mRNA splicing. Through this process pre-mRNA sequences are joined together and exported into the cytoplasm (exons); the removed intervening sequences (introns) remain in the nucleus where they are eventually degraded.

The comparison of mRNA with genomic sequences in the late 1970s revealed that prior to export into the cytosol, viral sequences are removed from the pre-mRNA and the remaining sequences are joined together (Berget et al., 1977; Chow et al., 1977). It was quickly found that almost all mammalian RNA polymerase II transcripts undergo this process, called pre-mRNA splicing. Due to splicing, a relatively small fraction of sequences from the primary transcripts are joined together and exported as exons to the cytosol, forming the mature mRNA. The majority of intervening sequences (introns) remain in the nucleus where they are subsequently degraded (reviewed in (Sharp, 2005). It is now clear that the vast majority of pre-mRNAs contain exons that can be alternatively included in the mature mRNA, or removed from it, via this process called alternative splicing. Frequently, transcripts contain several alternative exons and their usage can be combined, which largely increases the diversity of the mRNA expressed from the

genome, giving alternative splicing a central role in forming complex organisms. Alternative splicing patterns constantly change under physiological conditions, allowing an organism the respond to changes in the environment by determining which part of the genome it expresses. Most of the changes in alternative splicing are studied in artificial experimental systems, but alternative exon usage changes in real-life scenarios: the stress of exams put onto medical students causes a change in phosphatidylinositol 3-kinase-related protein kinase alternative pre-mRNA splicing. This change in alternative splicing has later effects on nonsense-mediated RNA decay and the p53 pathway (Kurokawa et al., 2010).

1.1 mRNA processing is coupled with transcription

Eukaryotic mRNAs are extensively processed in prior to export into the cytoplasm where translation into protein occurs. Pre-mRNA transcripts undergo a series of processing steps that includes transcription, addition a 7-methyl-guanosine cap to the 5′ end of the RNA (Shatkin and Manley, 2000), editing that includes nucleoside modifications, nucleotide additions and insertions resulted in an altered information content of the mRNA (Maas and Rich, 2000), splicing, endonucleolytic cleavage of the 3`end followed polyadenylation (Shatkin and Manley, 2000) and degradation. It has been shown that many of these events are functionally coupled and occur co-transcriptionally (reviewed in (Hocine et al., 2010; Maniatis and Reed, 2002; Neugebauer, 2002). The carboxyl-terminal domain (CTD) of the RNA polymerase II plays a central role in coupling transcription to mRNA processing (Figure 1.1). It provides an interaction platform for regulatory proteins and it is required for efficient transcription, capping and 3` end modification (McCracken et al., 1997).

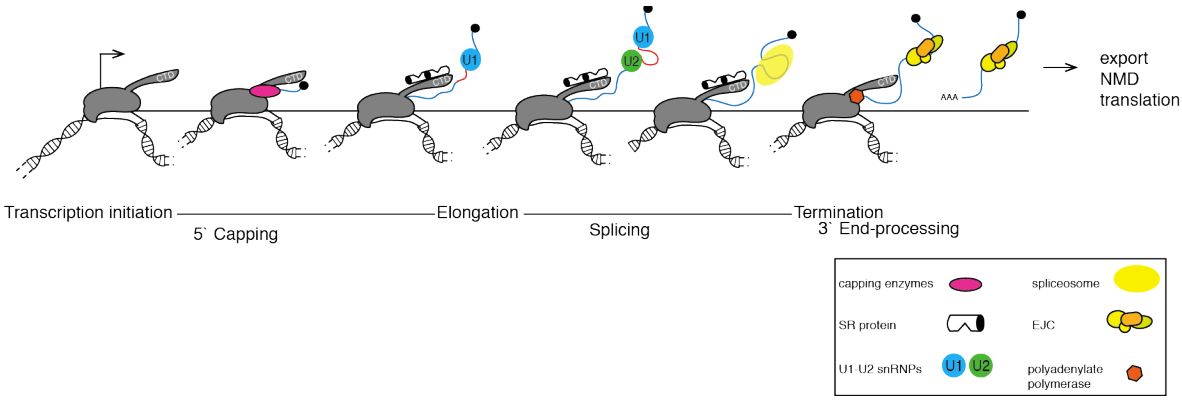


Figure 1.1. Co-transcriptional pre-mRNA processing. Schematic representation of transcription and pre-mRNA processing. RNA polymerase II (grey) initiates transcription at the promoter (arrow) and proceeds during elongation phase, terminating and releasing from the DNA template following passage through the polyadenilation signal. Capping enzymes (magenta oval) bind to its CTD as it enters the elongation phase. The 5` cap added by capping enzymes symbolized by black ball. Splicing factors recognizing the 5` and 3` splice sites and additional splicing factors bind to the CTD of Pol II followed by the spliceosome assembly. Polyadenilation factors are recruited to downstream regions (orange ball). At termination, Pol II and the mRNP are released from the template and subjected to nuclear transport. The figure was adapted from (Neugebauer, 2002).

Splicing factors can associate with the CTD as well and influence alternative splicing events. Two models have been suggested for this mechanism, the recruitment model and the kinetic model. The recruitment model assumes that splicing factors assemble at the CTD of RNA polymerase II and are released onto the nascent pre-mRNA during transcription (Figure 1.2). As these factors influence splice sites in a concentration dependent manner, the pre-loading of the CTD influences alternative exon usage (Das et al., 2006; Das et al., 2007).

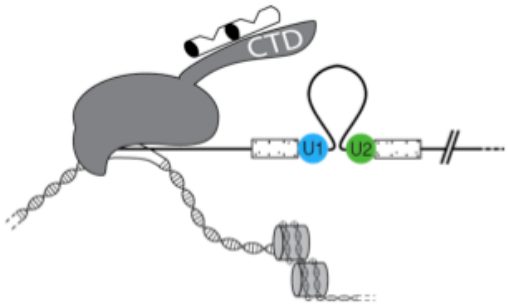


Figure 1.2. The recruitment model of alternative splicing. Splicing regulatory factors assemble at the CTD of RNA polymerase II and influences alternative splicing.

The kinetic model postulates that protein complexes need time to assemble on an exon, which leads to its recognition (Figure 1.3). Everything that slows down a polymerase would give more time for the recruitment of the regulatory complexes and favor alternative exon usage, as these exons usually depend more strongly on auxiliary factors (reviewed in(Kornblihtt, 2006, 2007). It has been previously shown that cap-binding proteins interact with U1 snRNP and help to recognize the 5` splice site (Lewis et al., 1996). The CTD domain of Poly(A)- Polymerase (PAP) interacts with U2AF65 and stimulates splicing (Vagner et al., 2000).

Post- transcriptional events occurring in the nucleus affect the downstream recruitment of proteins bound to mRNA, forming a messenger ribonucleoprotein (mRNP) complex (Figure 1.4). It has been shown that mRNA generated by splicing are more efficiently exported than the corresponding mRNA containing introns, suggesting that splicing factors bound to mRNA facilitate efficient export from the nucleus (Luo and Reed, 1999). This specific mRNP complex recruits Aly, an mRNA export factor, through a direct interaction between the splicing factor UAP56 and Aly (Luo et al., 2001; Zhou et al., 2000). It was previously reported that the splicing factor SR proteins, 9G8 and SRp20 promote export of intronless mRNAs (Huang and Steitz, 2001). 9G8, SRp20 and SF2/ASF bind to

TAP/NXF1 (Nuclear RNA export factor 1), which is the major receptor for mRNA export, (Rodriguez et al., 2004), facilitating mRNP export (Huang et al., 2003).

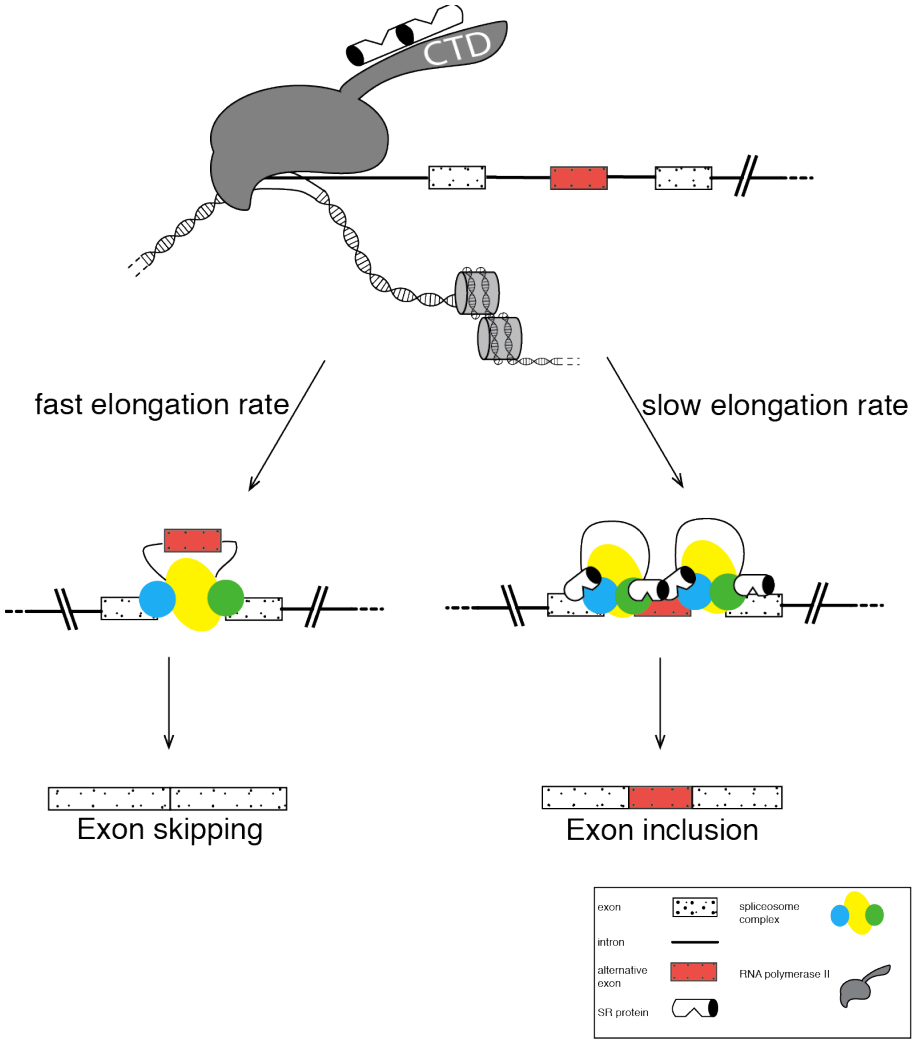


Figure 1.3. RNA polymerase II kinetic model of alternative splicing. Fast elongation rate of RNA polymerase II is resulted of skipping an alternative exon. Slow elongation favors the recruitment of splicing factors leading to exon inclusion.

The exon-exon junction complex (EJC) found exclusively on spliced mRNAs, and many other RNP components, remain bound after splicing is completed and provide a platform for factors involved in mRNA export and nonsense-mediated decay (NMD), which process targets aberrant mRNAs with premature stop codons for degradation (Le Hir et al., 2001). It was observed that the EJC

stimulates mRNA-polysome association and translation *in vivo* (Le Hir and Seraphin, 2008; Nott et al., 2004). Furthermore, it was published that shuttling SR proteins are associated with ribosomal particles and are capable of stimulating translation both *in vivo* and *in vitro* (Bedard et al., 2007; Sanford et al., 2005; Sanford et al., 2004). Accumulating evidences show that mRNA processing occurs co-transcriptionally and these steps are functionally linked to increase the efficiency and specificity of the maturation process of mRNA.

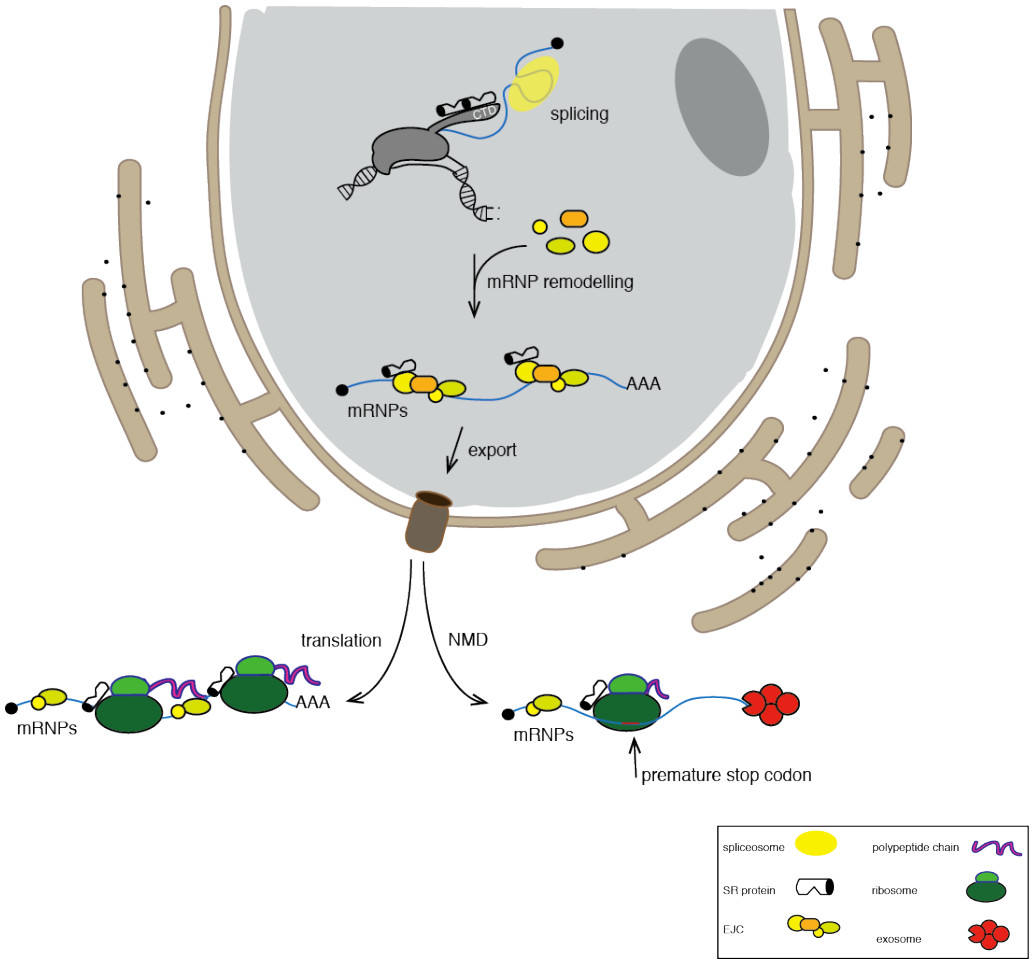


Figure 1.4. Splicing and translation. After pre-mRNA splicing completed EJCs deposited upstream of exon-exon boundaries on the spliced RNA. mRNP subjected to nuclear export. Translation occurs in the cytoplasm after mRNP remodeling and the RNAs that contain premature STOP codons subjected to nonsense-mediated decay (NMD).

1.2 Pre-mRNA splicing

Human exons are between 50 and 300 nt, with an average of about 137 nt in length (Berget, 1995). Thus, they represent short sequences of the pre-mRNA that are surrounded by larger introns, which have an average length of 3400 nt (Deutsch and Long, 1999). A large macromolecular complex, the spliceosome, recognizes exons and removes the intervening sequences (introns) while the pre-mRNA is synthesized by RNA polymerase II in the nucleus. The spliceosome is composed of at least 170 proteins and five small nuclear RNAs (snRNAs) (Behzadnia et al., 2007). Exons are defined by three major sequence elements, the 5' splice site, 3' splice site and the branch point (BP) (Figure 1.5). The spliceosome recognizes these elements and assembles in a stepwise manner on the nascent pre-mRNA and removes the introns and joins the exons in two trans-esterification reactions (Reed, 1996).

1.2.1 *Cis*-acting regulatory pre-mRNA elements

The first step in pre-mRNA splicing is the selections of splice sites by the splicing machinery, the spliceosome. The 5` splice site contains a GU dinucleotide flanked by a degenerate consensus sequence (Figure 1.5) that base pairs with U1 snRNA. The 3` splice site is composed of two sequence elements: the polypyrimidine tract (PPT) that is rich in pyrimidines and interacts with the U2 auxiliary factor (U2AF) and the exon/3`-intron junction. The branch point (BP) sequence contains a highly conserved adenosine that is used in the first step of splicing. These sequence elements determine the strength of a splice site. Generally, increased complementarity of the 5` splice site to the U1 snRNA and a longer polypyrimidine tract promotes higher-affinity binding and more efficient splice site recognition (Hertel, 2008).

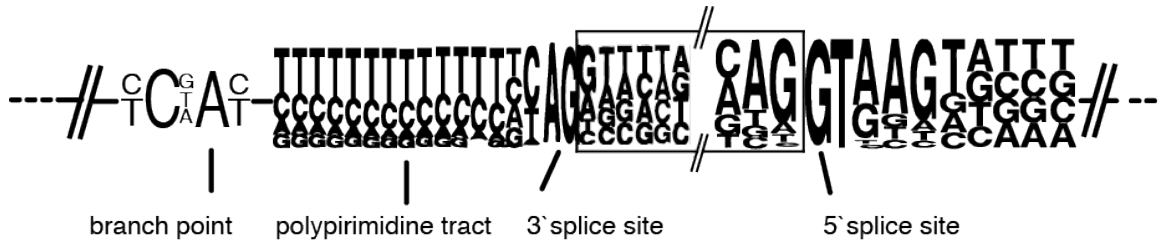


Figure 1.5. Classical splice site sequences. An exon is defined by three crucial elements the branchpoint, the 3' splice site and the 5' splice site.

The major class of introns (U2-dependent) contains highly conserved dinucleotides at the 5` and 3` splice site, these are GT and AG respectively (Table 1). The minor class of introns (U12-dependent), which is found only in a subset of eukaryotes, plants and metazoans, contain AT and AC dinucleotides at their 5' and 3' splice sites respectively. Here I will discuss only the U2-type spliceosome catalyzed splicing.

Elements	**Consensus Sequence***
5` donor splice site	YRG/GURAGU
3` acceptor splice site preceded by a polypyrimidine strech	Y_{12}NYAG/
Branch point located 18-200 nt upstream of the 3`splice site	NYURAY

Table 1.1. Sequence elements marking major class (GT-AG) intron. *Symbols used: Y-pyrimidine, R-purine, N-any nucleotide, slash denotes the exon-intron border, invariant nucleotides are underlined.

Since most splice sites are short and degenerate, additional *cis*-acting pre-mRNA elements modulate the splice site recognition and help in the correct splice site selection by recruiting regulatory proteins. These regulatory sequences include exonic and intronic splicing enhancers (ESEs and ISEs) and exonic and intronic splicing silencers (ESSs and ISSs). They are typically short and diverse sequences and either promote or repress splice-site usage by tethering regulatory proteins (Singh and Valcarcel, 2005) (Figure 1.6. A-B).

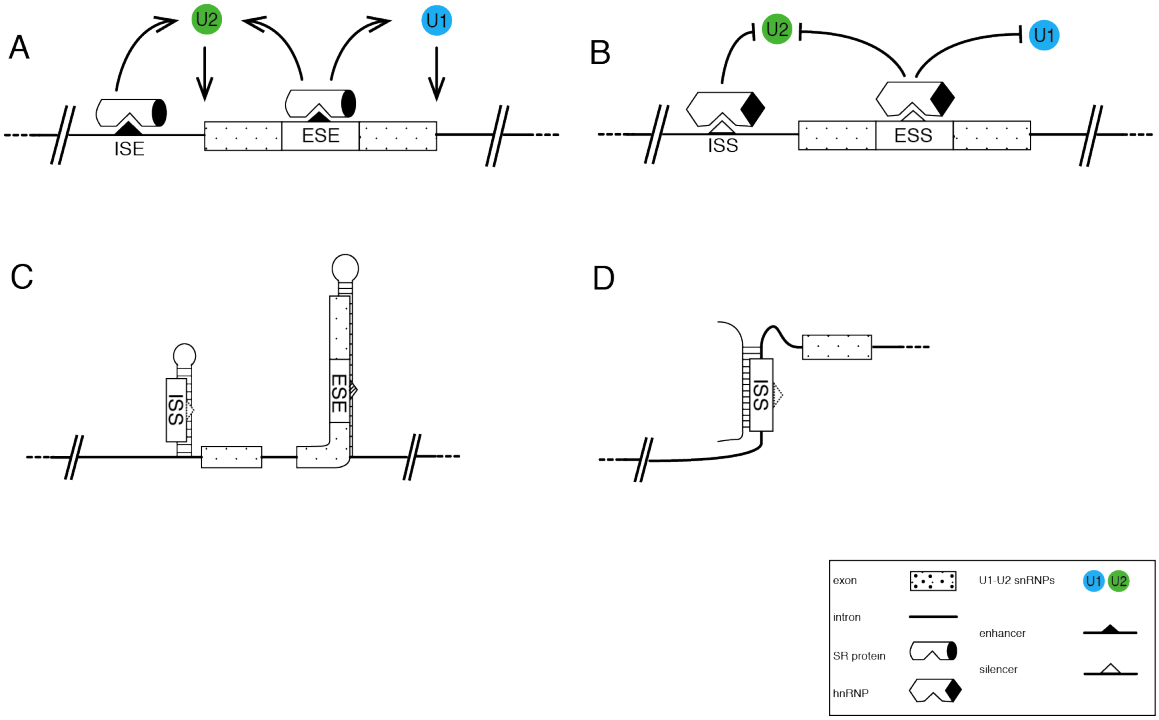


Figure 1.6. Exons need additional splicing regulatory elements to be recognized. Schematic representation of the splicing regulatory elements. *A*, Splicing factors are recruited to exonic splicing enhancer (ESE) and intronic splicing enhancer (ISE) facilitating spliceosome assembly and splicing. *B*, Splicing regulatory factors bind to exonic splicing silencers (ESS) and intronic splicing silencers (ISS) blocking the recruitment of the spliceosome. *C*, RNA secondary structures can inhibit or activate splicing by concealing *cis*-regulatory elements. *D,* Regulation of splice site selection by non-coding RNAs.

RNA itself could be involved in this regulation and there is emerging evidence of a larger role of non-coding RNA in exon selection (Figure 1.6. C-D). These RNA elements can be located on the pre-mRNA and regulate the binding of U1 snRNP by stabilizing the interaction of U1 with competing 5' splice sites (Yu et al., 2008). The RNAs can also be generated from other transcripts and then regulate pre-mRNAs (Khanna and Stamm, 2010). RNA elements within a pre-mRNA influence splice site selection. This is exemplified by the DSCAM (Down syndrome cell adhesion molecule) pre-mRNA, where the structure of the

pre-mRNA regulates alternative exon usage. In the DSCAM pre-mRNA, one alternative exon is chosen from 48 alternative exons by the formation of a double stranded RNA structure between a conserved sequence in the pre-mRNA and the alternative exon (Graveley, 2005). The 5' splice site of tau exon 10 is determined by its sequestration in a secondary structure (Varani et al., 1999).

1.2.2 *Trans*-acting regulatory factors

The 5' splice site, 3' splice site and branch point sequences are followed by only loose consensus sequences. Therefore, additional factors are required for exon recognition, which are brought into play by RNA sequence elements that can be either exonic or intronic. These short sequences bind to proteins that stabilize the binding of U1, U2 or SF1 to form the A complex. Likewise, binding of proteins to splicing silencers that block A complex formation inhibit exon recognition. The prototypical exon enhancing proteins are the serine-arginine-rich proteins (SR-proteins). These proteins contain a C-terminal domain rich in arginine and serine, termed as RS-domain, which mediates protein-protein interactions. SR-proteins N-terminal RNA recognition motifs (RRM) recognize and bind to specific RNA sequences. In contrast, most heterogeneous nuclear ribonucleoproteins (hnRNPs) bind to splicing silencers and inhibit exon recognition. These proteins are a diverse group of nuclear RNA-binding proteins and they contain one, or more, types of RNA-binding domains, such as RRM, hnRNP K homology (KH) or arginine-glycine-glycine (RGG). Many hnRNPs have additional domains that mediate protein-protein interactions. As a general rule, SR-proteins and SR-domain containing proteins promote exon inclusion and hnRNPs antagonize exon inclusion. However, there are numerous exceptions to this rule. These proteins play multiple roles in both constitutive and alternative splicing. Exon recognition and splice site selection is under combinatorial control of activating and inhibitory signals (Figure 1.7). Interplay between *cis*-acting RNA

sequence elements and *trans*-acting factors leads to the generation of differentially spliced mRNA isoforms (Hertel, 2008).

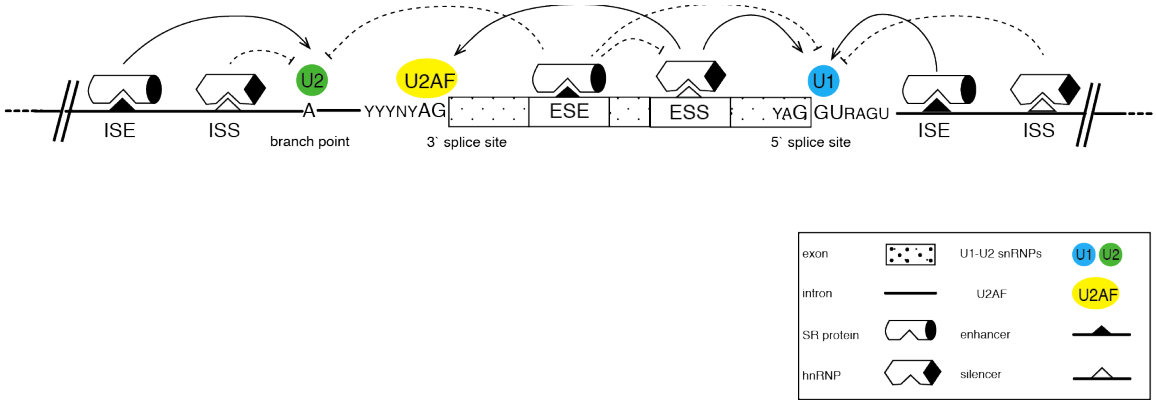


Figure 1.7. pre-mRNA splicing is controlled by interplay between *cis*-acting elements and *trans*-acting regulatory factors. Symbols used: y = Pyrimidine, r = Purine, n= any nucleotide. 3` splice site, 5` splice site and branch point are indicated. Auxiliary splicing regulatory elements within exons and introns are indicated (ESE: exonic splicing enhancer, ESS: exonic splicing silencer, ISE: intronic splicing enhancer, ISS: intronic splicing silencer). These elements are recognized by *trans*-acting splicing factors that positively or negatively regulate splicing.

1.2.3 Spliceosome assembly and the splicing reaction

The removal of introns and ligation of exons from the pre-mRNA proceeds through a two trans-esterification reaction (Figure 1.8). In the first reaction the 2` hydroxyl group of the BP adenosine attacks the 5` splice site phosphodiester bond, leading to a free 3` hydroxyl group and a branched lariat intermediate. In the second reaction, nucleophilic attack by the 3` hydroxyl group of the 5` exon cleaves the 3` splice site phosphodiester bond, generating ligated exons and an excised intron in a lariat structure.

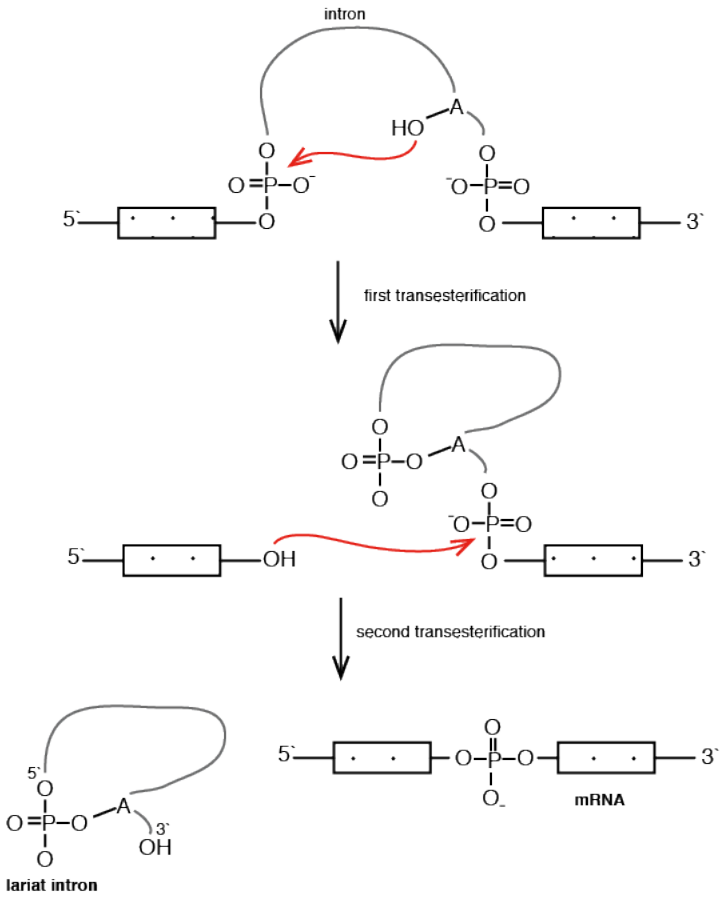


Figure 1.8. pre-mRNA splicing reaction. Schematic representation of the two-step splicing reaction. Removal of the intron between its flanking exons (boxes) proceeds via two trans-esterification reactions.

These two catalytic steps are carried out by a large and highly dynamic ribonucleoprotein (RNP) complex, the spliceosome. During the splicing reaction the spliceosome undergoes a series of coordinated assembly steps and conformational changes (Figure 1.9). The main building blocks of the major spliceosome are the U1, U2, U4/U6 and U5 uridine-rich small nuclear ribonucleoproteins (snRNPs) which are composed of U1, U2, U4, U6 and U5 snRNAs, seven Sm proteins and a variable number of particle-specific proteins (Wahl et al., 2009). Proteomic analysis of the human spliceosome indicated that between 150 (Zhou et al., 2002) and 300 (Rappsilber et al., 2002) distinct non-snRNP proteins associate with the spliceosomal complex. The assembly of the

spliceosome occurs in a well-orchestrated stepwise fashion forming protein-protein, RNA-protein and RNA-RNA interactions.

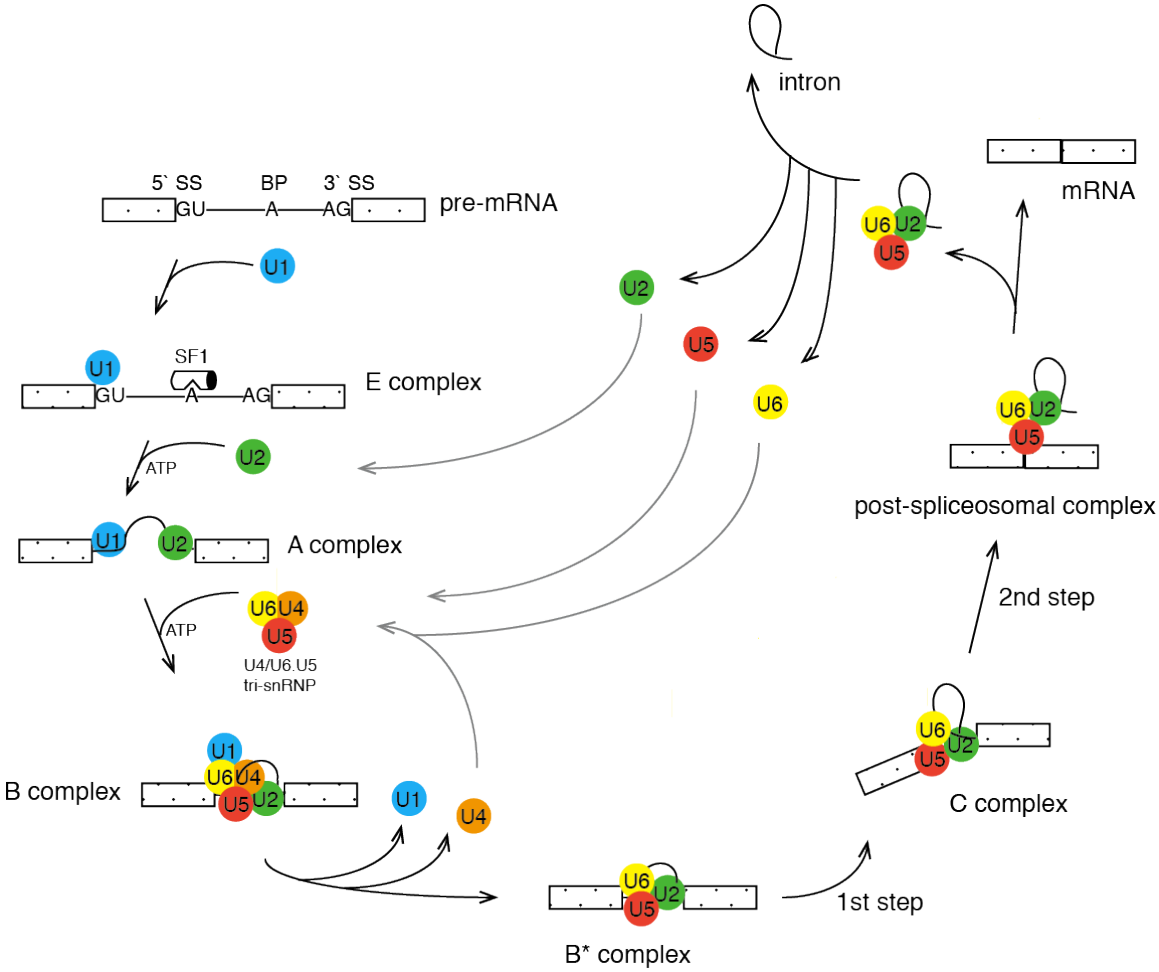


Figure 1.9. Spliceosome assembly. The stepwise interaction of the spliceosomal snRNPs (colored circles) during pre-mRNA splicing. This large ribonucleoprotein (RNP) complex catalyzes the two trans-esterification reactions that result in splicing of the exons (boxes) and excision of the intron (black line) as a lariat structure through formation of E, B, B* and C spliceosomal complexes. The figure was adapted from (Will et.al., 2011).

Spliceosomal assembly starts with the initial recognition of the 5` splice site and 3` splice site of an intron forming the ATP-independent early E complex on the nascent RNA. U1 snRNA base pairs to the 5` splice site of the intron and

splicing factor 1/branch point binding protein (SF1/BBP) and U2AF are recruited to the BP and PPT, respectively. The 35 kDa subunit of U2AF (U2AF35) binds to the AG dinucleotide of the 3` splice site and the SF1/BBP (splicing factor 1) in a cooperative interaction with the 65 kDa subunit of U2AF (U2AF65) recognizes the BP. In a subsequent step, U2 snRNP base pairs with the BP in an ATP-dependent manner resulting in the displacement of SF1/BBP from the BPS and formation of the A complex. Recruitment of the preassembled U4/U6.U5 tri-snRNP creates the pre-catalytic B complex. To yield the catalytically active B* spliceosome rearrangements in RNA-RNA and RNA-protein interactions are required. After destabilization of the U1 and U4 snRNPs the activated B* complex catalyzes the first step of splicing. This generates additional conformational and compositional rearrangements that lead to the formation of the C complex, which catalyzes the second reaction step of splicing. After the splicing reaction is completed, the spliceosome dissociates releasing the snRNPs and the mRNA associated with proteins (Will and Luhrmann, 2011).

1.3 Alternative pre-mRNA splicing

Almost all polymerase II transcripts undergo alternative pre-mRNA splicing; an estimated 95% of multiexon transcripts undergo alternative splicing (Pan et al., 2008). The overall function of alternative splicing is to increase diversity of the mRNAs expressed from the genome. Alternative splicing can have a profound functional effect on the synthesis of a protein. Experimental analysis of protein isoforms generated by alternative splicing showed that this mechanism regulates binding to other proteins, to nucleic acids and membranes. Alternative splicing regulates the localization of proteins, their enzymatic properties and their interaction with ligands. In most cases, changes caused by individual splicing isoforms are small. However, cells typically coordinate numerous changes in the ‘splicing program’, which can have strong effects on

cell proliferation, cell survival and properties of the nervous system. Alternative splicing is often regulated in a tissue or developmental-stage-specific manner. Due to its widespread usage and molecular versatility, alternative splicing emerges as a central element in gene regulation.

Alternatively spliced exons share the sequence features of constitutively used exons, but in general, these sequences deviate more from the consensus sequences, (i.e. are weaker), which implies a reduced affinity to the spliceosome resulting in reduced recognition. The BP of constitutive exons is typically within 40 nt of the 3' splice site, but in alternative exons this distance can be much larger, up to 400 nt. In these cases, there are no AG nucleotides between the branch-point and the 3' splice site, called the AG dinucleotide exclusion zone (Gooding et al., 2006). A difference of BP between constitutive and alternative exons is also reflected by the higher phylogenetic conservation of branch points proceeding alternative exons, which indicates that the *cis*-elements marking alternative exons are so weak that they cannot tolerate more perturbations (Kol et al., 2005).

Similarly, splice sites and exonic enhancers are generally weaker in alternative exons (Wang et al., 2005a), a feature that is evolutionarily conserved (Garg and Green, 2007). This conservation indicates that it is evolutionarily advantageous for an exon to be used alternatively. An important finding of genome wide analysis was the discovery of evolutionarily conserved intronic regions, which suggests that deep intronic regions contribute to splicing regulation (Sugnet et al., 2006; Yeo et al., 2007). These motifs are typically not discovered by *in vitro* methods that can analyze only shorter sequences and could explain the mechanism of action by disease-causing deep intronic sequences (Davis et al., 2009; Yeo et al., 2007).

Alternative splicing events fall into 5 basic types (Figure 1.10): (a) cassette exons, where the exon can be excluded together with its flanking introns (b) alternative 5` splice sites, (c) alternative 3` splice sites, (d) intron retention and

(c) mutually exclusive events. Furthermore, usage of alternative transcription sites or multiple polyadenylation sites give rise to alternative transcript variants. Exon skipping, which is the most prevalent event in vertebrates, accounts for 38% of the alternative splicing events conserved between human and mouse genomes, while other types was observed less frequently (Ast, 2004).

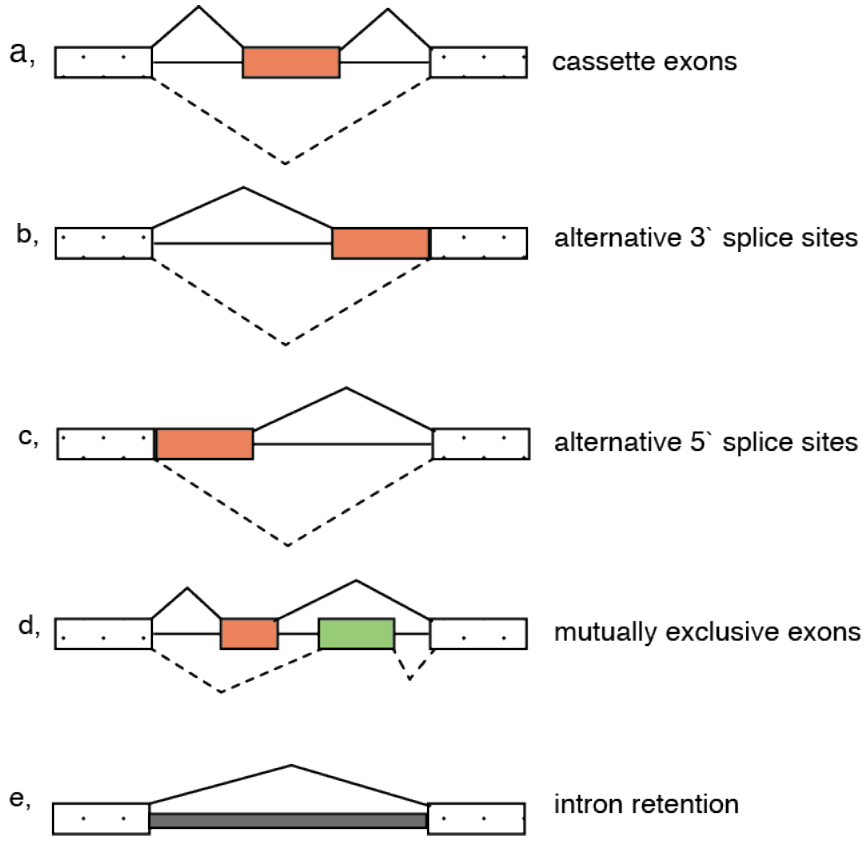


Figure 1.10. Types of alternative splicing. In all five examples of alternative splicing, flanking constitutive exons are indicated as white boxes, and alternative spliced regions in red or green, introns are presented by solid lines, dashed lines and solid lines indicate splicing activities. Figure was adapted (Ast, 2004).

1.3.1 Regulation of alternative splicing

Alternative splicing is controlled by the combination of various *cis*-acting elements and *trans*-acting factors that are often tightly regulated in a tissue- or developmental-specific manner or in response to external stimuli.

The active concentration of splicing regulatory proteins can be influenced by the cell through different expression levels or through sequestration in cellular organelles, which resulted in the concept that alternative splicing is regulated by the ratios of antagonistic splicing factors. Polypyrimidine tract binding protein (PTB) is an hnRNPA and a well-studied example of regulation by antagonistic proteins. For example, PTB competes with RNA binding motif protein 4 (RBM4) for a CU-rich regulatory element in alpha-tropomyosin (Lin and Tarn, 2005), with TIA1 cytotoxic granule-associated RNA binding protein (TIA1) for a U rich *cis*-element in myosin phosphatase (Shukla et al., 2005) and in Fas (TNF receptor superfamily, member 6) (FAS) (Izquierdo et al., 2005); with U2AF for regulation in the src gene (Sharma et al., 2005); and with SRp30c (serine/arginine-rich splicing factor 9) for regulation of the hnRNPA1 gene (Paradis et al., 2007). Antagonism can also occur between SR-proteins, for example between SRp20 (serine/arginine-rich splicing factor 3) and SF2/ASF (serine/arginine-rich splicing factor 1) in the rac1b gene (Goncalves et al., 2009). Splicing regulatory proteins cannot only antagonize each other, but can also enhance their action on an exon, where they act like coactivators. This is exemplified by Fox-3 (RNA binding protein fox-1 homolog 3) and PSF (splicing factor proline/glutamine-rich) that together promote usage of a neuron-specific exon in myosin heavy chain (Kim et al., 2011).

The importance of the concentration of splicing factors is further highlighted by the fact that most splicing factors autoregulate their expression levels, often by generating inactive variants as seen in fox proteins (Damianov and Black, 2010), SF2/ASF (Sun et al., 2010) and tra2-beta1(Stoilov et al., 2004).

As the splicing factors work within protein:RNA complexes, their action depends on the sequence context of their binding site.

Global analysis of drosophila cells showed that different splicing factors control a variable number of pre-mRNAs and that many alternative exons are regulated by multiple *trans*-acting factors, suggesting that the relative ratios of these factors control exon usage (Blanchette et al., 2005).

1.3.1.1 Signal-induced alternative pre-mRNA splicing

Little is known about the regulation of alternative splicing by signal transduction pathways although it is known that alternative splicing events are extensively regulated by signaling cascades. Reversible phosphorylation is the best studied among Post-translational modifications (PTM) and it occurs predominantly on serine residues (~90%) (Nita-Lazar et al., 2008). These reversible changes on the protein can modify the properties of a splicing factor altering its activity, localization and binding affinity. Members of the SR protein family are important splicing factors. They are composed of a C-terminal RS domain that is rich in serine and arginine residues and an N-terminal RRM that provides the RNA-binding specificity. (Reviewed in: (Long and Caceres, 2009; Shepard and Hertel, 2009). Reversible phosphorylation of the RS domain alters their activity and localization (Koizumi et al., 1999; Xiao and Manley, 1998). During spliceosomal assembly the RS domains are hyperphosphorylated and they become partially dephosphorylated during the splicing process (Colwill et al., 1996; Wang et al., 1998) Phosphorylation of the RS domain is necessary for the interaction with transportin-SR2 protein that plays role in the nuclear import of SR proteins (Lai et al., 2000; Lai et al., 2001) and dephosphorylation of the RS domain is required for TAP/NXF1 (nuclear RNA export factor)-dependent export) (Huang et al., 2004). In response tor growth factors, the extra domain A (EDA) of the fibronectin gene is included, which contains an ESE that recruits SR proteins

(Lavigueur et al., 1993). Changes in the phosphorylation status of SF2/ASF and 9G8 (serine/arginine-rich splicing factor 7), via a Ras- phosphatidylinositol 3-kinase (PI 3-kinase)- protein kinase B (AKT) pathway, alter the activity of these proteins that leads to inclusion of EDA and stimulates translation of a reporter mRNA (Blaustein et al., 2005). SR proteins are substrates of Cdc2-like kinases (Clk/Sty); their phosphorylation activity modulates alternative splicing (Prasad et al., 1999). Insulin promotes the inclusion of protein kinase C βII (PKC) exon through PI3K/Akt2 kinase cascade. Clk/Sty phosphorylated by Akt2 in response to insulin, results in a change in the phosphorylation status of SR proteins that leads to the inclusion of PKC βII (Jiang et al., 2009).

1.3.1.2 Roles of protein phosphatases in splicing

Reversible phosphorylation is required for pre-mRNA splicing, is coordinated by the activity of kinases and phosphatases. Changes in the phosphorylation status of splicing factors modulates the interaction between proteins and RNA that leads to a rearrangement of the spliceosome and alters the splice site selection. The phosphorylation status of a regulatory protein can determine its subcellular localization and therefore change the concentration of the splicing factors in the nucleus that again results in a change in splice site selection.

It was previously shown that PP2Cγ (protein phosphatase, Mg2+/Mn2+ dependent, 1G) is associated with the spliceosome and its phosphatase activity is required for A complex formation (Murray et al., 1999). Previous studies with phosphatase inhibitors indicated that PP1 (protein phosphatase 1) and PP2A (protein phosphatase 2A) enzymatic activity is necessary for pre-mRNA splicing, but not for spliceosome assembly (Mermoud et al., 1992; Tazi et al., 1992). Recently it was reported that PP1 and PP2A family phosphatases play essential but redundant roles in pre-mRNA splicing and their phosphatase activities are

required for the second trans-esterification reaction. Components of U2 and U5 snRNPs, SAP155 (splicing factor 3b, subunit 1, 155kDa) and U5-116 kDa, are key substrates of these phosphatases and their dephosphorylation is essential for the second catalytic step of splicing (Shi et al., 2006). Dephosphorylation of SF2/ASF is necessary for the first catalytic step in splicing but not for spliceosome assembly (Cao et al., 1997). Using mass spectrometry, it has been found that a PP1α isoform is present in the spliceosome-associated CDC5L complex (Ajuh et al., 2000).

The human genome is estimated to contain 428 serine/threonine kinases and less than 40 genes encode serine/threonine phosphatases (Johnson and Hunter, 2005; Shi, 2009). This disparity can be explained by the combinatorial formation of protein phosphatase holoenzymes from a shared catalytic subunit and a large number of regulatory subunits.

1.3.1.3 Protein Phosphatase 1

PP1 belongs to the PhosphoProtein Phosphatase (PPP) family, which also includes PP2A, PP3 (PP2B/Calcineurin), PP4, PP5, PP6 and PP7 (Shi, 2009). PP1 is a well-characterized protein serine/threonine phosphatase which regulates a wide range of cellular processes, including cell division and meiosis, membrane receptors, apoptosis and metabolism (Ceulemans and Bollen, 2004). In humans, three highly related genes encode PP1 that together give rise to four distinct catalytic subunits, PP1α, PP1β/δ and the splice variants PP1γ1 and PP1γ2 (Cohen, 2002). PP1 interacts with nearly 200 known regulatory proteins that modulate its activity, substrate specificity and subcellular localization (Bollen et al., 2010). The PP1 interacting proteins (PIP) contain short and degenerate PP1-docking motifs that bind to substrate-binding grooves of PP1. The primary PP1-docking motif, the RVxF motif, is present in ~70% of all PIPs (Bollen et al., 2010) and it binds to a hydrophobic groove that is remote from the catalytic site

of PP1 (Egloff et al., 1997; Wakula et al., 2003). This interaction does not change the conformation of PP1, rather functions as an anchor for PP1 recruitment. Previously it has been reported that several splicing factors contain a phylogenetically conserved RVxF-type motif on their RRM, including tra2-beta1, the SR proteins SF2/ASF, SRp30c and SRp54 (signal recognition particle 54kDa), and members of the hnRNP family, including p54nrb/NONO (non-POU domain-containing octamer-binding protein), PSF/SFPQ, nPTB (polypyrimidine tract binding protein 2) and ROD1 (polypyrimidine tract binding protein 3) (Novoyatleva et al., 2008). PP1 plays an important role in regulating splicing by dephosphorylating SR-proteins and other components of the spliceosome (Chen and Manley, 2009; Wahl et al., 2009).

1.3.2 Function of alternative splicing

The overall function of alternative splicing is to increase diversity of the mRNAs expressed from the genome. Due to the combinatorial control mechanisms that regulate alternative exon recognition, splicing programs coordinate the generation of mRNA isoforms from multiple genes. Evolution can select some of these isoforms to fulfill defined functions. Other isoforms could simply represent co-regulated exons without any direct function. It is also possible that a specific isoform shows only a functional effect when expressed with other isoforms generated by a coordinated change in splicing. The magnitude of alternative splicing regulation ranges from subtle modifications of protein functions, for example in ion channels to making binary on/off switches, observed in apoptosis genes.

Genome wide studies indicate that protein regions encoded by alternative exons are predominantly located in peripheral regions of the protein. Therefore, alternative exons do not change the general structure of the protein, but influence mainly local structures on the protein surface (Wang et al., 2005b). Alternative

exons are often located in unstructured regions allowing introduction of protein domains without disrupting the overall protein structure (Romero et al., 2006). Together, this indicates that the major function of alternative splicing is to modify, but not radically change the function of a protein (Figure 1.11).

Evolutionary analysis supports this notion, as human-mouse comparisons show that short alternative exons preserving the reading frame are conserved between human and mouse, indicating that small, incremental changes are caused by alternative splicing (Zhang et al., 2007).

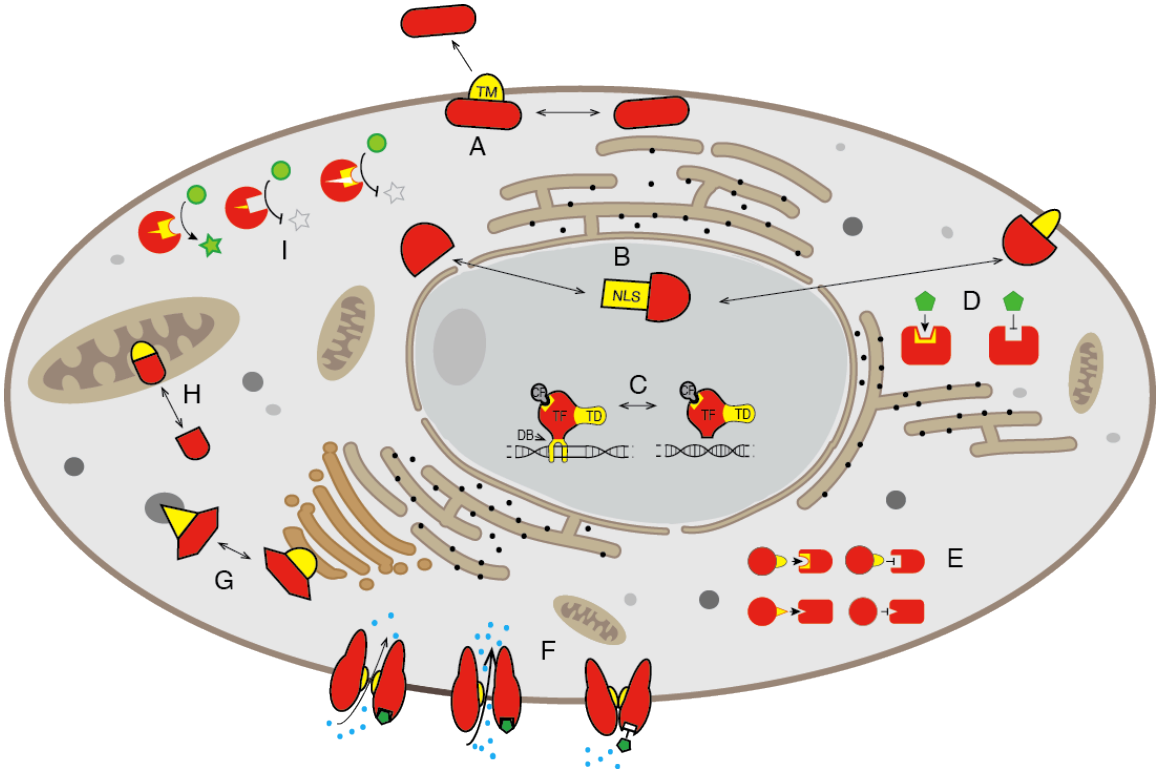


Figure 1.11. Function of alternative splicing. An eukaryotic cell is schematically shown. Proteins that undergo alternative splicing are shown in red, parts encoded by alternative exons are shown in yellow. *A*, Change between plasma membrane and cytosol *B*, Change between cytosol and nucleus by altering a nuclear localization signal (NLS) and between nucleus and membrane associated forms. *C*, Change in transcription factor. *D*, Alternative splicing changes a binding of a ligand (green) to a protein (red). *E*, Regulation of protein-protein interactions *F*, Regulation of ion channels by alternative splicing *G*, Change of localization between internal membranes. *H*, Change of localization in mitochondria. *I,* Regulation of enzymatic activity by alternative splicing. Alternative splicing influences the substrate binding, which prevents substrate formation (striped star). Alternative splicing changes the catalytic center (short triangle), which prevents substrate formation.

Analysis of the mouse transcriptome estimated that approximately 75% of the protein-coding splice variants likely affect the protein product (Okazaki et al., 2002; Zavolan et al., 2003) and computational analysis of human the transcriptome suggests that 67% of the alternatively spliced isoforms change the protein structural core and likely alter their conformation (Yura et al., 2006). Changes in the protein structure can affect protein`s binding properties, alter the intracellular localization of a protein, or modify enzyme activity by diverse mechanisms.

Proteins often acquire new functions in different compartments. For example, the lipin gene generates a nuclear and cytoplasmic variant due to inclusion of an alternative exon. The nuclear form acts as a transcription factor, whereas the cytosolic form has phosphatidate (PA) phosphatase activity (Han and Carman, 2010; Zhang et al., 2012) reviewed in (Csaki and Reue, 2010). By deleting membrane-binding domains, alternative splicing can control membrane association of proteins. The soluble proteins can either accumulate in the cytosol or be secreted, depending on their membrane topology. This results mostly in a loss of function of the normally membrane bound protein. However, the soluble proteins can have dominant effects, as shown for the MET (hepatocyte growth factor receptor) oncogene, where a soluble, secreted form inhibited the signaling of the membrane bound form (Tiran et al., 2008).

Alternative splicing frequently occurs in a cell-type or tissue-specific way, as illustrated by the MYB (transcriptional activator Myb) gene. The MYB gene modulates transcriptional activities through at least six alternative exons, which are differentially used in lymphomas and during hematopoietic differentiation. This example shows how cell-type specific splice site selection can contribute to cell-type specific transcriptional programs (O'Rourke and Ness, 2008).

Alternative splicing is an evolutionarily ancient process and likely appeared in the common ancestor of eukaryotes (Irimia et al., 2007). The

function of individual exons can be determined by evolutionary comparison (reviewed in (Keren et al., 2010)). All comparisons rely on the hypothesis that functionally important alternative exons will be conserved in evolution. In contrast, non-functional exons will be eliminated by selection. A frequently used property of alternative exons is the symmetry of an exon. An exon is symmetric, when it can be divided by three, which indicates that it keeps the reading frame. In general, conserved alternative exons in reading frames are symmetrical, whereas exons in non-protein coding regions and species-specific exons show a higher fraction of non-symmetrical exons (Magen and Ast, 2005; Xing and Lee, 2005). The level of alternative splicing increases from invertebrates to vertebrates (Kim et al., 2007), suggesting that the generation of new alternative exons could be a driving force in evolution. A characteristic feature of primates is the occurrence of Alu elements, which comprise about 10% of the human genome (Hasler et al., 2007). These Alu insertions can evolve into exons that contribute to generating a more complex transcriptome in primates (Dagan et al., 2004; Lev-Maor et al., 2003).

Individuals belonging to the same species show differences in alternative splicing. When 250 exons from 22 human individuals were compared, 6 out of 70 alternative exons showed consistent differences between individuals (Hull et al., 2007).

1.3.3 Splicing in disease

Given the widespread functions of alternative splicing, it is not surprising that aberrant regulation of alternative splicing leads to human disease. This connection to human health is increasingly recognized and has been covered in numerous reviews (Hallegger et al., 2010; Wang and Cooper, 2007; Ward and Cooper, 2010). In most cases, changes in alternative splicing are caused by point mutations, which have been collected in databases (Bechtel et al., 2008a, b).

Changes of alternative splicing in cancer cells are well studied, as a deregulation of alternative splicing is a hallmark of cancer (David and Manley, 2010; Kim et al., 2008). Several protein isoforms generated by alternative splicing are crucial for cancer progression and are subject of experimental therapeutically intervention. For example, the RON tyrosine kinase gene can generate a constitutively active kinase due to skipping of an alternative exon (Collesi et al., 1996). The exon is controlled by the splicing factor SF2/ASF and determines the epithelial to mesenchymal transition, that is the invasiveness of the cancer cells (Ghigna et al., 2005). The lower pH of the tumor microenvironment influences alternative splicing by changing the intracellular localization of numerous splicing factors. Most splicing factors are nuclear under steady state conditions, but they shuttle between nucleus and cytosol, which is typically driven by phosphorylation (Stamm, 2008). For example under hypoxia (Daoud et al., 2002) and chemically induced acidic conditions (Hirschfeld et al., 2011), the splicing factor tra2-beta1 accumulates in the cytosol and is depleted in the nucleus. As a result, the splicing of its target genes is altered. Important for cancer, the matricellular protein Cysteine rich 61 (Cyr61) that promotes metastasis changes its splicing pattern to an isoform supporting metastasis (Hirschfeld et al., 2011). Therefore, changes in the tumor microenvironment could contribute to the deregulation of alternative splicing in cancer.

Several neurological diseases are characterized by changes in alternative splicing. One of the best-investigated diseases is Myotonic Dystrophy type 1 (DM1), in which a CTG expansion in the 3' untranslated region of the dystrophia myotonica-protein kinase (DMPK) gene causes a sequestration of two splicing regulatory proteins: CUG RNA-binding protein (CUGBP1) and muscleblind-like splicing regulator 1 (MBNL1). As a result, a network of alternative splicing events is altered, which causes abnormalities in heart development and skeletal muscle (reviewed (Ranum and Cooper, 2006).

Spinal Muscular Atrophy (SMA) is an autosomal recessive disease and

currently is the leading genetic cause of death in infants (Lunn and Wang, 2008). Humans possess multiple copies of the SMN gene: one copy of the SMN1 gene encodes the survival of motor neuron (SMN) protein and several copies of the nearly identical SMN2 gene. The homozygous disruption of SMN1 causes the disorder that manifests in the degeneration of motor neurons of the spinal cord (Burglen et al., 1996). The nearly identical SMN2 sequence contains a C-to-T nucleotide transition on exon 7 that leads to an alternatively spliced isoform where exon 7 is being skipped and produces a truncated protein (Lorson et al., 1999). It was previously suggested that this C-to-T mutation disrupts an SF2/ASF-dependent ESE sequence (Lorson et al., 1999). Tra2-beta1 promotes the inclusion of exon 7 and stimulates full-length SMN protein expression (Hofmann et al., 2000). However, deletion of tra2-beta1 in murine embryonic fibroblast cells did not affect full-length SMN protein or transcript levels (Mende et al., 2010). Kashima and Manley suggested that the C-to-T transition creates an ESS in SMN2 that favors the exclusion of exon 7 by recruiting hnRNPA1 (Kashima and Manley, 2003). It has been shown that hnRNPA1 can antagonize SF2/ASF–dependent ESE activity and promote exon 7 skipping, however this activity of hnRNPA1 is not specific to SMN2 (Cartegni et al., 2006). These studies show that regulation of alternative splicing is a complex process controlled by combinatorial interplays between splicing regulatory factors.

1.3.4 Compounds that can change alternative splice-site selection

Aberrant regulation of alternative splicing leads to human diseases as was discussed previously. Compounds that modulate alternative splicing can be used to treat a variety of pathological conditions caused by improper splice site selection (Sumanasekera et al., 2008). Substances that target the splicing

machinery are also invaluable research tools providing information about the mechanism and regulation of alternative splicing.

Cantharidin is a toxic compound produced by many species of blister beetle. It was found to inhibit the growth of some tumor cell lines including HeLa cells, murine ascites hepatoma, and reticulocell sarcoma (Huang et al., 1997; Xu, 1981). However, the mechanism of cell death promotion by cantharidin remained unknown. It has been shown that cantharidin inhibits the phosphatase activity of PP1 and PP2A (Eldridge and Casida, 1995; Li et al., 1993) and modifies alternative splicing events (Novoyatleva et al., 2008). Cantharidin promoted the usage of exon 7 *in vivo* splicing assays and caused accumulation of the SMN protein in transgenic mice model (Novoyatleva et al., 2008). Pseudocantharidin A, B, C and D (Figure 1.12.A) derivatives of cantharidin, stimulated exon 7 inclusion in reporter gene assays and promoted production of full length SMN protein in patient fibroblast cell lines (Zhang et al., 2011). They change the alternative splicing of SMN2 mRNA through modulation of specific protein phosphatases. Pseudocantharidin A inhibits both PP1 and PP2A activity and Pseudocantharidin B and C are activators of PP2A.

Ceramides belong to a class of sphingolipids that are composed of shingosine and a fatty acid moiety (Figure 1.12.B). It has been previously shown that water-insoluble ceramides alter alternative splicing and decrease SR-protein phosphorylation by activating PP1 (Chalfant et al., 1999; Chalfant et al., 2001; Massiello et al., 2004). Water-soluble ceramide analogs, synthesized to improve delivery of these ceramide derivatives containing pyridinium moieties are able to induce apoptosis and were tested as anti-cancer drugs. D-e-C_6 ceramide, a mediator of apoptosis induced the level of the pro-apoptotic splice variant Bcl-x(s) (Apoptosis regulator Bcl-X) and Caspase-9 (apoptosis-related cysteine peptidase) in lung adenocarcinoma cells (Chalfant et al., 2002). C6 pyridinium ceramide is an anti-cancer drug candidate that is currently being tested for the treatment of squamous cell carcinomas (Karahatay et al., 2007). C6 pyridinium

ceramide, unlike the water-insoluble, natural C6 ceramide inhibits PP1 activity and therefore prevents the dephosphorylation of splicing regulatory proteins. C6 pyridinium ceramide binds directly to PP1 and inhibits its phosphatase activity. C6 pyridinium ceramide changes alternative splicing of several pre-mRNAs to promote both inclusion and skipping of the target exons (Sumanasekera et al., 2012). These studies suggest that conventional (synthetic and natural) ceramides may regulate alternative splicing through PP1 activation and the subsequent dephosphorylation of SR-proteins.

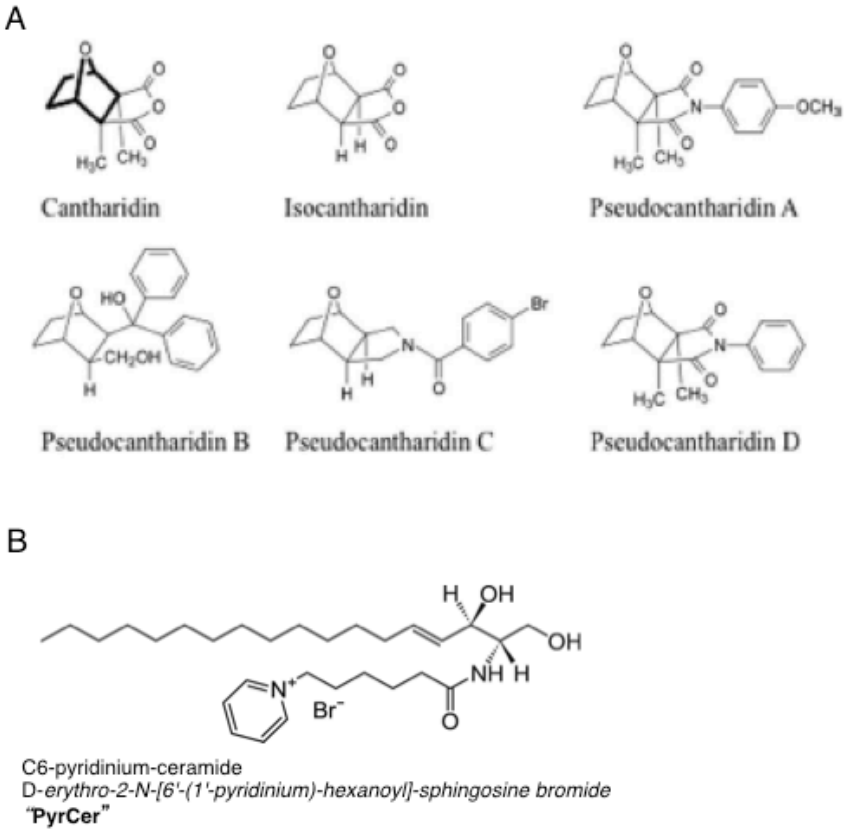


Figure 1.12. *A,* Structure of cantharidin and pseudocantharidins (Zhang et al., 2011). *B,* The structure of C6-pyridinium ceramide (Sumanasekera et al., 2012).

1.4 Human Transformer-2 beta1

Human Transformer-2 beta1 (tra2-beta1) belongs to the SR-related proteins. It has a unique modular organization, which is conserved in vertebrates and invertebrates and differs from the classical SR-protein family that have a single carboxyl-terminal RS domain (Shepard and Hertel, 2009). Tra2-beta1 comprises a single central RNA recognition motif (RRM), which recognizes and binds to specific RNA sequences, flanked by RS domains (Beil et al., 1997; Dauwalder et al., 1996) (Figure 1.13.C) Tra2-beta1 is a homologue of the Drosophila melanogaster Tra2 protein that is an important regulator of sex determination in fruit flies. Tra2 regulates the female specific splicing of doublesex (dsx) pre-mRNA cooperating with Tra through a cascade of alternative splicing events (Burtis, 1993).

Tra2-beta1 is a sequence specific activator of pre-mRNA splicing it promotes exon inclusion in a concentration-dependent manner. However, splicing assays with Hela S100 extracts indicated that it is not required for constitutive splicing (Tacke et al., 1998). The Sfrs10 gene encodes tra2-beta1 and its transcript is alternatively spliced generating five mRNA isoforms (Nayler et al., 1998). The major isoform encodes full-length tra2-beta1 that self-regulates its concentration by regulating the alternative splicing of its own exon2. Inclusion of exon 2 results in mRNAs that are not translated into protein (Stoilov et al., 2004). The expression of the tra2-beta3 isoform is developmentally regulated and is present predominantly in brain, liver and testis (Nayler et al., 1998). This isoform lacks the N-terminal RS domain and specifically represses inclusion of exons that are activated by the full-length isoform, tra2-beta1 (Grellscheid et al., 2011).

The central RRM recognizes and binds to specific RNA sequences and the flanking RS domains mediating protein-protein interactions, thereby facilitating the binding of regulatory proteins that result in the inclusion of an

exon. Tra2-beta1 binds to degenerate purine rich RNA sequences that are found more frequently in exons that in introns (Stoilov et al., 2004; Tacke et al., 1998).

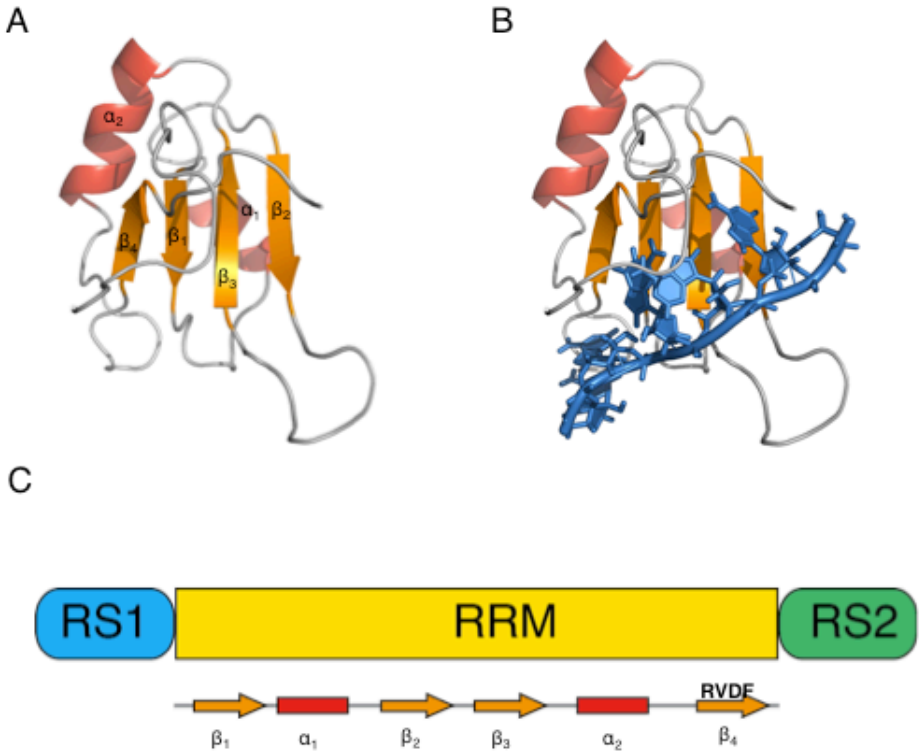


Figure 1.13. **Solution structure of the tra2-beta1 RRM** (Cléry et.al., 2011). *A,* Ribbon representation of the tra2-beta1 RRM. The β-strands and α-helices are colored orange and red, respectively. *B,* Solution structure of tra2-beta1 RRM -5`-AAGAAC-3` RNA complex. The β-strands and α-helices are colored orange and red, respectively. RNA sequence is shown in blue. All of the structural representations were prepared with PyMol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA). *C,* Schematic representation of the tra2-beta1 domains. The RVDF PP1 docking motif indicated on β_4-strand of RRM domain.

The high affinity binding motif of tra2-beta1 has been recently published, tra2-beta1 RRM binds to the 5` AGAA 3` sequence with 2.25 μM affinity and to the 5`GGAA 3` motif with 4.5 μM affinity (Figure 1.13.B). Tra2-beta1 has a canonical RRM that folds into a typical $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology (Figure 1.13.A). Mainly the β-sheet surface is involved in RNA binding, however the N- and C-terminal regions of the RRM also interact with the RNA sequence (Clery et al., 2011). A longer RNA sequence containing CAA motif is also recognized by tra2-beta1 when it is integrated in a stem-loop structure (Tsuda et al., 2011).

Tra2-beta1 contains an RVxF-type docking motif of PP1, which is the most frequent binding motif of PP1 (Egloff et al., 1997). The phylogenetically conserved motif located on the β_4 strand of tra2-beta1 RRM (Figure 1.13. C) (Novoyatleva et al., 2008). The RVxF motif interacts with PP1 through a hydrophobic channel that is remote from the catalytic site (Bollen et al., 2010). PP1 modulates the splicing activity of tra2-beta1 by dephosphorylating its RS domains and promotes the skipping of tra2-beta1-dependent exons (Novoyatleva et al., 2008).

Tra2-beta1 is predominantly localized in nuclear speckles, which is characteristic of other splicing factors. However, under stress conditions it changes localization and switches to an increased presence in the cytoplasm (Daoud et al., 2002; Hirschfeld et al., 2011). The expression of tra2-beta1 is significantly increased in breast and ovarian cancer (Fischer et al., 2004; Watermann et al., 2006) and regulates alternative splicing of genes linked to tumor progression, such as the CD44 gene and Cyr61 (Hirschfeld et al., 2011; Watermann et al., 2006).

2 Research overview

Tra2-beta1 is a crucial splicing factor it plays role in the regulation of constitutive and alternative splicing. The activity of tra2-beta1 is regulated by reversible phosphorylation. In the first part of this study custom-made tra2-beta1 phosphorylation-specific antibodies were characterized. SMA is the leading genetic cause of death in infants. Pseudocantharidins, derivative of cantharidin, promote the inclusion of SMN2 exon 7. Employing the phosphorylation-specific antibodies we demonstrated that pseudochantharidin B and C treatment in HEK293T cells leads to the dephophorylation of the T33 residue of tra2-beta1 and the phosphorylation status of this residue play a role in exon 7 inclusion.

C6 pyridinium ceramide is an anti-cancer drug candidate and inhibits the activity of PP1. C6 pyridinium ceramide changes alternative splicing of several pre-mRNAs to promote both inclusion and skipping of the target exons. C6 pyridinium ceramide responsive exons are unusually short and share suboptimal splice sites and they contain binding sequences of splicing regulatory factors. Here we show that ASF/SF2, tra2-beta1 and PSF/SFQ binds directly to these exons to regulate their splicing.

Tra2-beta1 predominantly localizes in the nucleus but under stress conditions it accumulates in the cytoplasm. We demonstrate that tra2-beta1 binds to the 18S rRNA and stimulates the translation of a firefly Luciferase reporter. We also found that hypoxia induces the translation of tra2-beta1 dependent reporter mRNA.

PP1 is a major protein phosphatase that binds to tra2-beta1 through an RVxF-type docking motif and regulates its activity in translation. We found that PP1 enhances the binding affinity of tra2-beta1 to specific RNAs, and this function of PP1 is independent of its catalytic activity.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals

Product	Supplier	Product	Supplier
ATP	Sigma	L-glutamic acid	Sigma
30% Acrylamide/Bis solution	Sigma	Luminol	Sigma
Agar	GibcoBRL	Lysosime	Sigma
Ultra Pure agarose	Invitrogen	Luciferin	Goldbio
Ampicilin	Sigma	Magnesium chloride	Merck
Aprotinin	Sigma	Magnesium sulfate	Sigma
[γ-32P]-ATP	PerkinElmer	β-Mercaptoethanol	Merck
Benzonase	Sigma	Methanol	Roth
Boric acid	Roth	MOPS	Sigma
BSA	NEB	Na_2EDTA	Sigma
Bradford reagent (BioRad Protein Assay)	BioRad	NaN_3	Sigma
Brilliant Blue R 250	Sigma	Ni-NTA Agarose	Qiagen
Bromophenol blue	Merck	Nonidet P-40 / Igepal CA-630	Sigma
Calyculin	Upstate	dNTPs	Invitrogen
Cantharidin	Sigma	Paraformaldehyde	Merck
Cellfectin	Invitrogen	PEG 3500	Sigma
Chloramphenicol	Sigma	Perhydrol 30% H2O2	Merck
Chloroform: Isoamyl alcohol	Sigma	Phenol: Chloroform: Isoamyl alcohol	Sigma
Coelentrazin	Avidity	PMSF	Sigma
Coenzyme A	Sigma	Potassium chloride	Merck
C6-pyridinium ceramide	Avanti Polar Lipids	Potassium phosphate monobasic	Fisher
DharmaFECT1	Dharmacon	Potassium phosphate dibasic	Fisher
DMSO	Sigma	2-propanol	

EDTA	Merck	Protease Inhibitor Cocktail	Sigma
EGTA	Sigma	RNase Inhibitor	Roche
Ethanol	Roth	SDS	Sigma
Ethidium bromide	Sigma	Sepharose CL-4B	Pharmacia
Ficoll 400	Fluka	Sodium acetate	Merck
Gelatin	Sigma	Sodium chloride	Roth
GelGreen	Biotium	Sodium dihydrogen phosphate	Merck
Glutathione–Sepharose 4B	Amersham	Sodium fluoride	Sigma
Glycerol	Sigma	Sodium hydroxide	Merck
Glycerol 2-phosphate	Sigma	Tautomycin	Calbiochem
Glycin	Roth	Sodium fluoride	Sigma
Glycylglycine	Sigma	Sodium hydroxide	Merck
GlycoBlue	ambion	Tautomycin	Calbiochem
Guanidium-HCl	Sigma	TEMED	Sigma
HiperFect	Qiagen	Tris base	Aldrich
HEPES	Sigma	TRIzol	Sigma
Imidazole	Roth	Triton-X100	Sigma
p-Idophenol	Sigma	Tryptone	Sigma
IPTG	Sigma	Tween 20	Sigma
Kanamycin	Sigma	Yest Extract	Sigma
L-Arginin	Sigma	Xylene cyanole	Sigma

3.1.1 Enzymes

Product	Supplier	Product	Supplier
BamHI	NEB	Platinum Pfx polymerase	Invitrogen
EcoRI	NEB	Proteinase K	Sigma
DpnI	NEB	SuperScriptII	Invitrogen
NotI	NEB	T4 DNA ligase	NEB
SalI	NEB	T4 Polynucleotide kinase	NEB
PP1	NEB	λ phosphatase	NEB

3.1.2 Cell lines and media

Cell line	Description	Supplier
HEK293	Human embryonic kidney transformed with adenovirus 5 DNA	ATCC CRL-1573
HEK293T	Human embryonic kidney cell line expressing the large T antigen	GeneHunter Q401
HeLa	Homo sapiens cervical cancer cells	ATCC CCL-2

3.1.3 Bacterial strains and media

Strain	Genotype	Reference
BL21(DE3)-RIL	*omp*T *hsdS*(r_B m_B) *dcm*$^+$ Tet^r *gal* λ(DE3) *end*A Hte [*arg*U i*le*Y *leu*W Cam^r]	(Studier, F.W. et. al., 1990)
XL1-Blue MRF`	Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*) 173 *end*A1 *sup*E44 *thi-1 rec*A1 *gyr*A96 *rel*A1 *lac* [F´ *pro*AB *lac*I^q ZΔ M*15* T*n10* (Tet^r)]	(Bullock et. al., 1987)
TOP10 cells	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *Φ80lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (*Str*^{R}) *end*A1 *nup*G	Invitrogen

LB MEDIUM (1L)

10 g NaCl

10 g tryptone

5 g yeast extract

LB AGAR (1L)

10 g NaCl

10 g tryptone

5 g yeast extract

20 g agar

3.1.4 Antibiotics

Antibiotic	**Working concentration** Liquid culture	Agar plates
Ampicilin	100 µg/ml	100 µg/ml
Chloramphenicol	15 µg/ml	30 µg/ml
Kanamycin	20 µg/ml	20 µg/ml
Tetracyline	10 mg/ml	10 µg/ml

3.1.5 Antibodies

Antibody	**Organism**	**Source**
anti-actin (1:2000)	Mouse	Amersham
anti-GFP (1:3000)	Mouse	Roche
anti-tra2-beta1+alpha (ps568)(1:1000)	Rabbit	Custom-made[1]
anti-tra2-beta1 pS95-97-99 (1:1000)	Rabbit	Custom-made[2]
anti-tra2-beta1 pT33 (1:1000)	Rabbit	Custom-made[3]
anti-tra2-beta1 pT201 (1:1000)	Rabbit	Custom-made[4]
anti-mouse Ig (1:10000)	Sheep	Amersham
anti-rabbit Ig (1:10000)	Rabbit	Amersham
anti-PP1γ (1:1000)	Goat	Santa Cruz Biotechnology
Anti- PP1 (1:1000)	Mouse	Santa Cruz Biotechnology

1: anti-ps568/tra peptide used : GC(StBu)SITKRPHTPTPGIYMGRPTY

2:anti-tra2-beta1 pS95-97-99:YRRH[pS]H[pS]H[pS]PM

3: anti-tra2-beta1 pT33: CKSARH[pT]PARSR

4:anti-tra2-beta1 pT201: SITKRPH[pT]PTPGI

3.1.6 Plasmids

Clones from the lab collection

Name	**Backbone**	**Description**	**Reference**
pEGFP-C2	pEGFP-C2	CMV promoter,	Clontech

		Kanr/Neor, f1 ori	
hTra-delRS1	pEGFP-C2	Deletion of RS1 domain from tra2-beta1	(Stoilov P. et al., 2004)
hTra-delRS2	pEGFP-C2	Deletion of RS2 domain from tra2-beta1	(Stoilov P. et al., 2004)
hTra-delRS1+2	pEGFP-C2	Deletion of both RS1 and RS2 domains from tra2-beta1	(Stoilov P. et al., 2004)
hTra2-beta1 EGFP	p-EGFP-C2	human tra2-beta1 in pEGFP C2	(Beil B. et al., 1997; Nayler O. et al., 1998a)
hTra2-beta1 Flag	pcDNA	Flag Tagged human tra2-beta1 in pcDNA	(Nayler O. et al., 1998)
hTra2-beta1 ΔSR1 Flag	pcDNA	Deletion of RS1 domain	
hTra2-beta1 ΔSR2 Flag	pcDNA	Deletion of RS2 domain from tra2-beta1	
hTra2-beta1 ΔSR1-2 Flag	pcDNA	Deletion of both RS1 and RS2 domains from tra2-beta1	
SV9/10L/11	Exontrap	Tau minigene	(Gao Q.-S. et al., 2000)
WT SMN MG	pCI	SMN2 minigene	(Lorson C. et al., 1999)
CD44 exon v5	pETv5	CD44 minigene	(Koenig, et al., 1998)
SF2/ASF-EGFP	pEGFP-C2	SF2/ASF cloned in pEGFP-C2	(Nayler O. et al., 1998; Nayler O. et al., 1997)
SMN MG	pCI	SMN2 minigene	(Lorson et al., 1999)
pLCS traESE3	pBPLUGA	tra2-beta1 motif to luciferase vector	
pLCS MT	pBPLUGA	mutated motif	(Sanford et al., 2004)
pLCS STOP	pBPLUGA	STOP codon inserted in the linker	(Sanford et al., 2004)
pRL-TK *Renilla*	pRL	Renilla luciferase reporte	Promega
PP1γ1 EGFP	pEGFP-C1	rat PP1gamma 1 introduced into pEGFP-C1 vector	(Lesage B. et al., 2004) Gift from M.Bollen

PP1γ1(F257A) EGFP	pEGFP-C1	mutation of RVXF – binding chanel of rat PP1 γ 1	(Lesage et al., 2004)
PP1γ1(H125A) EGFP	pEGFP-C1	Catalytically inactive mutant of rat PP1 γ 1	(Zhang et al., 1996)
PP1γ1(D64N) EGFP	pEGFP-C1	Catalytically inactive mutant of rat PP1 γ 1	(Zhang et al., 1996)
NIPP1-C2	pEGFP-C1	nuclear inhibitor of PP1	(Van Eynde A.S. et al., 1995) Gift from M. Bollen
HA-PP1γ	HA-pCMV5	PP1γ has HA Tag	(Maximov. A. et al., 1999) Gift from M. Bollen

Newly made clones

Name	Backbone	Description
hTra2-beta1 S22-29A	pEGFP-C2	S22, 26, 29 was mutated to A
hTra2-beta1 S22-29E	pEGFP-C2	S22, 26, 29 was mutated to E
hTra2-beta1 S26-29A	pEGFP-C2	S26, 29 was mutated to A
hTra2-beta1 S26-29E	pEGFP-C2	S26, 29 was mutated to E
hTra2-beta1 S26A	pEGFP-C2	S26 was mutated to A
hTra2-beta1 S26E	pEGFP-C2	S26 was mutated to E
hTra2-beta1 S29A	pEGFP-C2	S29 was mutated to A
hTra2-beta1 S29E	pEGFP-C2	S29 was mutated to E
hTra2-beta1 S29-T33A	pEGFP-C2	S26 and T33 was mutated to A
hTra2-beta1 S29-T33E	pEGFP-C2	S26 and T33 was mutated to E
hTra2-beta1 T33A	pEGFP-C2	T33 was mutated to A
hTra2-beta1 T33E	pEGFP-C2	T33 was mutated to E
hTra2-beta1 T37A	pEGFP-C2	T37 was mutated to A
hTra2-beta1 T37E	pEGFP-C2	T37 was mutated to E
hTra2-beta1 T39A	pEGFP-C2	T39 was mutated to A

hTra2-beta1 T39E	pEGFP-C2	T39 was mutated to E
hTra2-beta1 S37-39A	pEGFP-C2	S37, 39 and T33 was mutated to A
hTra2-beta1 S37-39E	pEGFP-C2	S37, 39 and T33 was mutated to E
hTra2-beta1 S95-97-99A	pEGFP-C2	S95, 97, 99 was mutated to A
hTra2-beta1 S95-97-99E	pEGFP-C2	S95, 97, 99 was mutated to E

3.1.7 Oligonucleotides

Primers used for mutagenesis

Name	Sequence 5`→3`	Target
S20Arev	GCTTCCAGAGCTGGAAGTGCTCACGGATCGGGG	tra2-beta1
S20Afw	GCACTTCCAGCTCTGGAAGCAGAACGGGATTCC	tra2-beta1
S20Efw	GCTTCCAGAGAAGGAAGTGCTCACGGATCGGGG	tra2-beta1
S20Erev	GCACTTCCTTCTCTGGAAGCAGAACGGGATTCC	tra2-beta1
S22Afw	GATCCGTGAGCAGCTCCACTTCTGGAAGCAGAAC	tra2-beta1
S22Arev	CCAGAAGTGGAGCTGCTCACGGATCGGGGAAATC	tra2-beta1
S22Efw	CCAGAAGTGGAGAGGCTCACGGATCGGGGAAATC	tra2-beta1
S22Erev	GATCCGTGAGCCTCTCCACTTCTGGAAGCAGAAC	tra2-beta1
S26Afw	GCTCACGGAGCGGGGAAATCTGCAAGGC	tra2-

		beta1
S26Arev	GATTTCCCCGCTCCGTGAGCACTTCCAC	tra2-beta1
S26Efw	GCTCACGGAGAGGGGAAATCTGCAAGGC	tra2-beta1
S26Erev	GATTTCCCCTCTCCGTGAGCACTTCCAC	tra2-beta1
S29Afw	CGGATCGGGGAAAGCTGCAAGGCATACCCC	tra2-beta1
S29Arev	GCCTTGCAGCTTTCCCCGATCCGTGAGCAC	tra2-beta1
S29Efw	GATCGGGGAAAGAAGCAAGGCATACCCCTG	tra2-beta1
S29Erev	GTATGCCTTGCTTCTTTCCCCGATCCGTGAGC	tra2-beta1
T33Afw	GCAAGGCATGCCCCTGCAAGGTCTCGCTCCAAGG	tra2-beta1
T33Arev	CTTGCAGGGGCATGCCTTGCAGATTTCCCCGATC	tra2-beta1
T33Efw	GCAAGGCATGAACCTGCAAGGTCTCGCTCCAAGG	tra2-beta1
T33Erev	CTTGCAGGTTCATGCCTTGCAGATTTCCCCGATC	tra2-beta1
S37Afw	CCTGCAAGGGCTCGCTCCAAGGAAG	tra2-beta1
S37Arev	GGAGCGAGCCCTTGCAGGGGTATGC	tra2-beta1
S37Afw	CCTGCAAGGGAACGCTCCAAGGAAGATTCC	tra2-beta1

S37Erev	CCTTGGAGCGTTCCCTTGCAGGGGTATGCC	tra2-beta1
S95Afw	AGACGGCACGCCCACAGCCATTCTCCCATGTCT	tra2-beta1
S95Arev	ATGGCTGTGGGCGTGCCGTCTACGATAATCTCG	tra2-beta1
S95Efw	AGACGGCACGAGCACAGCCATTCTCCCATGTCT	tra2-beta1
S95Erev	ATGGCTGTGCTCGTGCCGTCTACGATAATCTCG	tra2-beta1
S97Afw	CGGCACAGCCACGCCCATTCTCCCATGTCTACTCGC	tra2-beta1
S97Arev	GGGAGAATGGGCGTGGCTGTGCCGTCTACGATAATC	tra2-beta1
S97Efw	CGGCACAGCCACGAACATTCTCCCATGTCTACTCGC	tra2-beta1
S97Erev	GGGAGAATGTTCGTGGCTGTGCCGTCTACGATAATC	tra2-beta1
S99Afw	CACAGCCATGCTCCCATGTCTACTCGCAGGCGTC	tra2-beta1
S99Arev	ACATGGGAGCATGGCTGTGGCTGTGCCGTCTACG	tra2-beta1
S99Efw	CACAGCCATGAGCCCATGTCTACTCGCAGGCGTC	tra2-beta1
S99Erev	ACATGGGCTCATGGCTGTGGCTGTGCCGTCTACG	tra2-beta1

Primers used for minigene analysis

Name	Orientation	Sequence	Minigene

pCIfor	sense	GGTGTCCACTCCCAGTTCAA	SMN2
SMNex8rev	antisense	GCCTCACCACCGTGCTGG	SMN2

Primer used for microarray validation

Name	Sequence 5`→3`	Target
ANKfw	ACAAGCCCAGCAAAGAAGAA	ANKRD13C
ANKrev	ACACTTTCCCTGGATTGCTG	ANKRD13C
BRfw	TCCAGAACCAACACCAGTGA	BAZ1A
BRrev	GGATTGCTCAAACGAAGCTC	BAZ1A
BCLfw	CCTCCGGCGTGTTCATGC	BBC3
BCLrev	GCTGCTGCTCCTCTTGTC	BBC3
BSDfw	GATGTCATGCAGGCAAGATG	BSDC1
BSDrev	ATCGCAGTCGATGGTTTTGT	BSDC1
DERfw	GTGAAAAGCCAGGGAATGAA	DMKN
DERrev	CGGGACCTGGTTCTTGTTTA	DMKN
LIMfw	GCTGAAAAGCCAGGAGTCAA	FHL2
LIMrev	CTCATAGCAGGGCACACAGA	FHL2
HDACfw	CTGGTCCTGCATTACGGTCT	HDAC3
HDACrev	CCCGGTCAGTGAGGTAGAAA	HDAC3
TRfw	CAAATGTCCTCCATGGTC	TXNRD2
TRrev	CGTGGGAACATTGTCGTA	TXNRD2
EIFfw	CCAGAACCTCCACTCTTTCG	EIF1
EIFrev	TGATCGTCCTTAGCCAGTCC	EIF1
PP2fw	ACTACTGGGGCCTATTTGAT	PPM1J
PP2rev	GCAGGTAGATCACAACCAGT	PPM1J
RPL3fw	GTGTTTGGGCAGGATGAGAT	RPL3
RPL3rev	TCTCAGTGCGGTGATGGTAG	RPL3
RPL4fw	GAAGATCCATCGCAGAGTCC	RPL4

RPL4rev	TTGGTAGCTGCTGCCTTTTT	RPL4
RPL6fw	AAAAAGGGTAACCTCAAAGC	RPL6
RPL6rev	TAGAGGAACTCGATTGAGGA	RPL6
RPL7fw	AAGAAAAAGCGAAGGAATTT	RPL7
RPL7rev	ATTTACCAAGAGATCGAGCA	RPL7
RPL9fw	ATGCTCACTTCCCCATCAAC	RPL9
RPL9rev	TGTGGCTTGCTGAATCAAAG	RPL9
RPL13fw	CTGGTGCTTGATGGTCGAG	RPL13A
RPL13rev	GTTGGTGTTCATCCGCTTG	RPL13A
RPL18fw	GTCCCGCTTCTGGTACTTTG	RPL18A
RPL18rev	ACCCATGTCTCGGTAGCACT	RPL18A
RPL19fw	GATCCGGAAGCTCATCAAAG	RPL19
RPL19rev	CCATGAGAATCCGCTTGTTT	RPL19
RPL29fw	ACCCCGATCACAAAGATACG	RPL29
RPL29rev	GGCAATGTAGGCAAGTCGAT	RPL29
SCFfw	ACACCAGTGGTCTCTCTCTG	SLC38A3
SCFrev	AAGGTGAGGTATCCAAAGGT	SLC38A3
TTK2fw	TGGATGCCTGTGTAGCTGTT	TTBK2
TTKrev	AACGAACTGTCCCTCGAAAA	TTBK2
ZNFfw	CAAGTGATCCTCTCTCCTTG	ZNF613
ZNFrev	CTCGGTACAGGTCCTTCTG	ZNF613

Oligos used for siRNA knockdown

Name	Target Sequence	Supplier
SFRS10 ON-TARGETplus SMARTpool	UCACGUAGCAGGUCUUACA	Dharmacon
SFRS10 ON-TARGETplus SMARTpool	GCCGAUGUGUCUAUUGUAU	Dharmacon
SFRS10 ON-TARGETplus	AUACUCACCUCGUCGCUAU	Dharmacon

SMARTpool		
SFRS10 ON-TARGETplus SMARTpool	UAACUGUUGUCUUGGAGUA	Dharmacon

Oligos used in EMSA

Name	Sequence	Description
ES6	UAGGAAUAAUGGAAU	Human 18S rRNA ES6
h12	UGACGGGGAAUCAGG	Human 18S rRNA h12
Pol-B	ACUCGCAAACUUUGAGAAGAACGUGAG	DNA polymerase beta
smn	CAAAAAGAAGGAGG	SMN2 exon 7
TIAF	GGUGACAAGUAUCAGAAAAGAAAGAAT	TGFB1-induced antiapoptotic factor 1
Control1	CAAAAUCUUGGAAGG	
Control2	GGUGAGUUCUAUCACUUUACUUUGAAT	

3.2 Methods

3.2.1 Plasmid DNA isolation

For large-scale purification of plasmid DNA QIAGEN Plasmid Maxi kit was used. The procedure was carried out according to the manufacturer`s protocol. The dry pellet was diluted in dH_2O. Smaller amounts of plasmid DNA was isolated from the alkaline lysis method first described by Birnboim and Doly (Birnboim and Doly, 1979). In brief, bacterial cells carrying the desired plasmid were cultured overnight at 37°C in 5ml LB medium containing the appropriate antibiotics. The cells were harvested by centrifugation for 5 minutes at 12,000 rpm. The pellet was resuspended in 250µl buffer P1. Equal volume of lysis buffer P2 was then

added and the solution mixed gently by inverting. The cells were allowed to lyse for 5 minutes, followed by addition of the neutralization buffer P3. The tube was mixed gently by inversion and the solution was maintained on ice for 15 minutes. After centrifugation for 10 minutes at 12,000 rpm, the resulting supernatant was precipitated by adding 1 volume of isopropanol. Plasmid DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes, washed with 70 % ethanol, air-dried and dissolved in 30µl of TE buffer. All the steps were carried out at room temperature in a conventional tabletop microfuge.

BUFFER P1	**BUFFER P2**	**BUFFER P3**	**BUFFER TE**
50 mM Tris-HCl	200 mM NaOH	3M Potassium acetate	10 mM Tris-HCl
10 mM EDTA	1% SDS	pH 5.5	1 mM EDTA
100 µg/ml RNase A			pH 8.0
pH 8.0			

3.2.2 Electrophoresis of DNA

DNA was resolved on 0.8-2% agarose gels prepared in 1 x TBE buffer. The electrophoresis was run for 80 min at 100 V. The gels were stained for 30 min in 0.5 mg/ml ethidium bromide and visualized under UV light, λ = 260 nm.

1X TBE	**6X GEL-LOADING BUFFER**
90 mM Tris-borate	0.25% bromophenol blue
20 mM EDTA	0.25% xylene cyanol FF
	15% Ficoll 400 in dH_2O

3.2.3 Elution of DNA from agarose gels

DNA was purified from agarose gels where 1X GelGreen (Biotium Inc.) was added, DNA bands were visualized using UV transilluminator Individual bands were excised and DNA was extracted using the Qiagen QIAEX II gel extraction kit according to the manufacturer's protocol.

3.2.4 Determination of nucleic acid 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 A260=40 **µ**g/ml for RNA

1 A260=50 **µ**g/ml for double stranded DNA

1 A260=37 **µ**g/ml for single stranded DNA

3.2.5 PCR amplification of DNA

A standard PCR reaction to amplify DNA from a plasmid template contained 1-80 ng of plasmid DNA, forward and reverse primers (0.4 µM each), dNTPs (200 µM), 1 x Taq polymerase buffer, 1.5 mM MgCl2 and 1 U Taq polymerase in total volume of 25 µl. When the amplification was made for cloning purposes, a high-fidelity polymerase, i.e. Platinum Pfx polymerase was used instead of Taq 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 min at the extension temperature to complete the amplification of all products.

3.2.6 DNA ligation

When the vector ends were blunt or compatible with each other, the vector was dephosphorylated prior to ligation to prevent self-ligation. To remove 5' phosphates from the vector, 2 U of Calf intestinal phosphatase (CIP, Boehringer) was added to 5 µg of linearized vector in 1 x CIP buffer in 20 µl. The reaction was incubated for 1 hour at 37°C. CIP was subsequently inactivated by heating the reaction to 68°C for 20 min. A typical ligation reaction contained vector and insert at a ratio of about 1:3 (500-1000 ng total DNA), 1 x ligase buffer, 1mM ATP and 200-400 U T4 DNA Ligase (New England Biolabs) in 10 µl. The incubation was carried out for 1 hour at room temperature for cohesive-end ligation or overnight at 16°C for blunt-end ligation. After that, one third to one half of the ligation mixture was then transformed in E coli cells.

3.2.7 Site-directed mutagenesis of DNA

The following protocol was used for generating mutation:

1X Pfx amplification buffer
0.3 mM dNTP mix
1 mM $MgSO_4$
0.3 µM each primer
~80 ng DNA template
1 unit Platinum Pfx DNA polymerase
In dH_2O

The amplification was carried out in a Perkin Elmer GeneAmp PCR System 9700 thermocycler under the following conditions: initial denaturation for 4 min at 94°C; 12 cycles of 30 sec at 94°C, annealing for 30 sec at 55°C, extension of 1 min per 1 kb at 68°C. After the last cycle the reaction was held for 12 min at the extension temperature to complete the amplification of all products.

3.2.8 Preparation of competent E. coli cells

5 ml of LB medium were inoculated with a single bacterial colony and grown overnight at 37°C with vigorous shaking. 4 ml of this culture were transferred to 250 ml LB and grown to early logarithmic phase (OD600 = 0.3-0.6). The culture was transferred into a sterile 50 ml falcon tube and centrifuged for 10 min at 2500 rpm at 4°C. The bacterial pellet was sequentially resuspended in 1/10 volume of cold TSB buffer and incubated on ice for 10 min. Cells were aliquoted into cold Eppendorf tubes and frozen in liquid nitrogen. Competent bacterial cells could then be stored at -80°C for several months.

TSB BUFFER

10% PEG 3500
5% DMSO
10 mM $MgCl_2$
10 mM $MgSO_4$
in LB medium
pH 6.1

3.2.9 Transformation of E. coli cells

1-10 ng of plasmid DNA or a ligation reaction was added to 20 µl of 5 x KCM buffer and then the volume was brought with water up to 100 µl. Equal volume of

competent cells was added. The reaction mixture was incubated on ice for 20 min followed by incubation at room temperature 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.

5X KCM BUFFER

500 mM KCl

150 mM $CaCl_2$

250 mM $MgCl_2$

3.2.10 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 et al., 1999) Tang 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. To avoid 'squelching' effects, the 'empty' parental expression plasmid containing the promoter was added in decreasing amounts, to ensure a constant amount of transfected DNA. Cells were seeded in 6-well plates and transfection was done 24 hours after plating. After incubation for 14-17 hours at 3% CO2 total RNA was isolated from the cells.

400 ng of RNA were used in a reverse transcription reaction. 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. A control reaction with dH2O instead of RNA was included.

1/8 of the reverse transcription reactions were used for PCR with minigene-

specific primers. The primers were selected to amplify alternatively spliced minigene products. A control reaction with no template (RNA instead of cDNA) was included in the PCR. The PCR programs were optimized for each minigene in trial experiments. PCR reactions were resolved on a 0.3-0.4 cm thick 2 % agarose TBE gel and the image was analyzed using ImageJ analysis software (http://rsb.info.nih.gov/ij/).

3.2.11 Isolation of total RNA

Total RNA was isolated from eukaryotic cells grown in 6-well plates. Cells were washed with 1 x PBS and the RNeasy Mini kit was used according to the manufacturer's protocol. RNA was eluted from the column in 30 µl of RNase-free dH_2O. However this procedure was applied only when the RNA of interest was greater than 200 bases.

3.2.12 Reverse-transcription PCR

400 ng of total RNA (200 ng/µl), 5 pmol of reverse primer, 40 U of SuperScript II reverse transcriptase were mixed in 5 µl of RT buffer. To reverse transcribe the RNA, the reaction was incubated at 50°C for 50 min. 1/8 of a typical 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 x Taq polymerase buffer and 1 U of Taq DNA polymerase. The conditions of the PCR cycles were dependent on the template to be amplified.

RT BUFFER

300 µl 5X First strand synthesis buffer (Invitrogen)

150 µl 0.1 M DTT (Invitrogen)

75 µl 10mM dNTPs

475 µl dH_2O

3.2.13 Radiolabeling and purification of RNA oligonucleotides

RNA oligonucleotides were radiolabeled in the following reaction:

2 µl 10X PNK buffer
1 µl RNase inhibitor
2 µl 50pmol/µl RNA oligo
1 µl T4 PNK
4 µl [γ-^{32}P]-ATP (6000Ci/mmol)
10 µl RNase free H_2O

Labeling reaction was incubated for 30min at 37°C and purified using sequential Roche mini Quick Spin Oligo columns. To check the labeling reaction and probe purity, radiolabeled RNA oligonucleotides were run on a 15% native PAA gel for 20min at 120V.

3.2.13.1 RNA electrophoretic mobility shift assay

Labeled RNA incubated with recombinant protein at room temperature for 15 minutes. Samples were load on 5% polyacrylamide-RE gel and electrophoresis was performed at 4°C at 110V for 65 minutes.

5% PAA-RE Gel	**Gel shift buffer**
20 mM Na-phosphate buffer, pH 6.5	20 mM Na-phosphate buffer, pH 6.5
75 mM L-Arginin	75 mM L-Arginin
75 mM L-Glutamic acid	75 mM L-Glutamic acid
5% Acrylamide/Bis	8 mM MgCl
2.5% glycerol	0.01% Triton-X-100

0.05% TEMED
1% APS
in dH_2O

5% glycerol
in dH_2O

3.2.14 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 95 °C for 5 min and loaded on the gel. Electrophoresis was carried out at 100 V for 2 hours in SDS gel running buffer.
Solution used in SDS PAGE:

Separating gel (10 ml)	**10%**	**Stacking gel** (3 ml)	**4%**
30% Acrylamide/Bis	3.33 ml	30% Acrylamide/Bis	0.4 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	0.5 M Tris-HCl, pH 6.8	0.75 ml
10% SDS	100 µl	10% SDS	30 µl
10% Ammonium Persulfate	100 µl	10% Ammonium Persulfate	30 µl
dH_2O	3.96 ml	dH_2O	1.79 ml
TEMED	10 µl	TEMED	3 µl

3X SDS SAMPLE BUFFER
150 mM Tris-HCl, pH 6.8
6% SDS
30% Glycerol
3% β-Mercatoethanol
0.3% Bromophenol blue

1X SDS GEL RUNNING BUFFER
250 mM Glycine, pH 8.3
25 mM Tris
0.1% SDS

3.2.15 Staining of protein gels

After disassembling SDS-PAGE, the gel was incubated in Commassie blue staining solution (2.5% Coomassie Brilliant Blue R250, 45% Methanol, and 10% Acetic acid) for 2-3 h at RT with shaking. The gel was then destained for 20 min in 50% Methanol/10% Acetic acid and 2-3 more times in 20% Methanol/10% Acetic acid until the background became clear. The gel was dried using a gel dryer (Biorad).

3.2.16 Expression of HIS-tagged protein in bacteria

To overexpress His-tagged protein, the protein expressing construct was transformed into BL21(DE3)-RIL E.coli cells. After the transformation, cells were plated on LB agar plate containing the appropriate antibiotic. Single colony was then inoculated into 5 ml of LB medium and grown overnight. The next day the culture was inoculated into 500 ml of fresh LB containing the appropriate antibiotic. When the OD600 reached 0.4-0.6, 0.4 mM IPTG was added to induce protein expression. The culture was grown for another 4 hours at room temperature with vigorous shaking. After the incubation, cells were harvested by centrifugation for 30 min at 4000 rpm. The pellet was resuspended in 30 ml of lysis buffer and then lysozyme, protease inhibitor cocktail, DNase and RNase A was added to the lysates. Cells were lysed by using French press. The supernatant was collected by centrifugation for 30 minutes at 10000 rpm at 4°C. The supernatant was incubated with Ni-resin for 1 hour at room temperature. The mixture of Ni-resin and supernatant was poured to a column. The resin was washed with buffer 2 and with the refolding buffers. Refolding buffers were prepared by titrating buffer 3 and 4 (4M, 3M, 2M, 1M and 0M Gu-HCl). The protein was finally eluted in buffer 5 and concentrated using centricon

concentrators (Amicon). The protein concentration was measured by Bradford method and monitored by Coomassie Staining SDS-PAGE.

MASTER BUFFER

20 mM Tris

300 mM NaCl

100 mM MOPS

15% glycerol

Buffer 1: Master buffer + 4M guanidine-HCl, pH 7.5

Buffer 2: Master buffer+ 4M guanidine-HCl + 20 mM imidazole pH 7.5

Buffer 3: Master buffer + 4M guanidine-HCl, pH 6.5

Buffer 4: Master buffer, pH 6.5

Buffer 5: Master buffer, pH 2.2

3.2.17 Expression and purification of GST-tagged proteins in bacteria

To overexpress GST-tagged protein, the protein expressing construct was transformed into BL21 (DE3)-RIL E.coli strain. After the transformation, cells were plated on LB agar plate containing the appropriate antibiotic. 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 the appropriate antibiotic. The induction of the culture was performed with 1 mM of IPTG (at OD600~0.4-0.6). The culture was grown for another 2 hr at 30°C with vigorous shaking. After the incubation, 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 lysed using French press. 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
	0.5 M Gluthation

3.2.18 Western blotting

20-24 hours after plating transfection, cells were washed with 1X cold PBS and lysed for 20 min on ice in 200 µl of RIPA buffer. The lysates were collected and cleared by centrifugation for about 1 min at 12800 rpm. The supernatant was transferred to a fresh Eppendorf tube and diluted with 1X SDS sample buffer. 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 x 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 x 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 x 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.

Buffer used in Western blot:

RIPA BUFFER

1% NP40

1% Sodium deoxycholate

0.1% SDS

150 mM NaCl

10 mM $NaPO_4$, pH 7.2

2 mm EDTA

50 mM NaF

Freshly added

4 mM Sodium orthovanadate

1mM DTT

1mM PMSF

20 µg/ml Aprotinin

100 U/ml Benzonase

1mM NaF

TRANSFER BUFFER

192 mM Glycine

25 mM Tris

20% Methanol

1X NET-GELATINE

150 mM NaCl

5 mM EDTA

50 mM Tris-HCl, pH 7.5

0.05% Triton-X-100

0.25% Gelatine

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

3.2.19 Determination of protein concentration

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

3.2.20 Freezing, thawing and subculturing of eukaryotic cells

To freeze, cells were grown to mid logarithmic phase (about 75% of confluence) in 10 cm Petri dishes. They were collected by trypsinization with 1 x Trypsin/EDTA, resuspended in 1 ml of the freezing medium (90% of the growth medium and 10% of DMSO). Vials were placed in Nalge Nunc Cooler giving a cooling rate of ~1°C/min while at -80°C. Cells were stored later in liquid nitrogen.
To thaw, cells were incubated at 37°C. The entire content of the tube was transferred to a 10 cm Petri dish and 10 ml of the growth medium were added. The dish was placed in the incubator at 37°C and 5% CO2. When cells were attached to the plastic surface, the medium was removed and replaced with fresh one. The cells were maintained in the incubator until ready for the subculturing.
Cells were subcultured after reaching confluence. The monolayer was detached by adding 1 X Trypsin /EDTA and incubating at 37°C until single cell suspension was formed. 1/5 – 1/10 of this suspension was transferred to a new dish and

mixed with the growth medium. Cells were maintained in the incubator at 37°C and 5% CO2. For hypoxia experiments, cultures were transferred to hypoxic culture conditions (1% O2) using a HeraCell150i incubator. Cells were cultured under hypoxic conditions for 10 hours.

3.2.21 Transfection of eukaryotic cells

3.2.21.1 Plasmid transfection

The procedure used for HEK293 cells was based on the one published by Chen and Okayama (Chen and Okayama, 1987). Exponentially growing cells were replated at a density of about 3 x 105 cells / 8 cm2. Growth medium was added and the cells were incubated at 37°C, 5% CO2 for about 24 h, to reach 60-70% of confluence. For most applications cells were grown in 6- well plates, with 2 ml of growth medium per well. The transfection reaction for one well was made the following way. 1 to 5 µg of expression construct were mixed with 25 µl of 1 M CaCl2 in final volume of 100 µl. Equal volumes of 2 x HBS buffer was added drop by drop, with constant mixing. In order to form a precipitate, the solution was allowed to stay at room temperature for 20 min. After that, it was added to the growth medium. To express the transfected plasmid, cells were grown for additional 24 h at 37°C, 3 % CO2.

2X HBS BUFFER

280 mM NaCl

10 mM KCl

1.5 mM $Na_2HPO_4 \cdot 2H_2O$

12 mM Dextrose

5 mM HEPES

pH 6.95

3.2.21.2 siRNA transfection

HEK293 cells were cultured in DMEM supplemented with 10% fetal calf serum and the optimal confluency of adherent cells should be 50-80% on the day of transfection. On the day before transfection cells were seeded into a single well of a 24-well plate in 0.5ml of medium. On the day of transfection, 100 nM siRNA were diluted in 50 µl of DMEM medium without serum. In a second tube1µl of Dharmafect transfection reagent 1 was diluted in 50 µl serum-free DMEM medium. The contents of each tube were mixed gently and incubated at room temperature for 20 minutes. 400 µl complete medium was added to the mixture. Medium was removed from the wells and and the transfection mixture diluted in complete medium was added to the wells. The same procedure was repeated 24 hours later. The cells with the transfection complexes were maintained under normal growth condition. After an appropriate time, the cells were harvested and used for experiment.

3.2.22 Dual Luciferase enzyme assay

HEK293T cells were grown to 90% confluence in 24-well plates. A mixture of plasmid DNA consisting of 400 ng pLCS reporter, 200 ng TK-Renilla (Promega) and 0.5 µg of the pcDNA expression vector was used to transfect cells. For dual luciferase assays cells were lysed using passive lysis buffer (Promega) or Triton/glycylglycin lysis buffer. The levels of Firefly and Renilla luciferase were assayed using Promega`s Dual Luciferase Assay Kit according to the manufacturer`s instruction, or noncommercial dual luciferase enzyme assay system (Dyer et al., 2000). Light emission was measured in an Lmax Microplate Luminometer (Molecular Devices). The luminometer was set for a 1s delay followed by a 10s reading for luciferase activity, followed by a 5s delay and 10s reading for Renilla activity. 50 ul of cell lysate was plated in white 96-well plates. 100 µl of Luciferase buffer and 100 µl Renilla buffer was injected into the wells.

TRITON/GLYCYLGLYCINE LYSIS BUFFER	**LUCIFERASE BUFFER**	**RENILLA BUFFER**
1% Triton-X-100	25 mM glycylglycine	1.1 M NaCl
25 mM glycylglycin, pH 7.8	15 mM KxPO4, pH 8.0	2.2 M Na_2EDTA
15 mM $MgSO_4$	4 mM EGTA	220 mM KxPO4, pH 5.1
4 mM EGTA	15 mM $MgSO_4$	1.3 mM NaN_3
1 mM DTT		
	Freshly added	Freshly added
	2 mM ATP	0.44 mg/ml BSA
	1 mM DTT	1.43 µM coelentrazine
	0,1 mM CoA	
	75 µM luciferin	

3.2.23 Immunocytochemistry

HEK293T cells grown on chamber slides (LabTek) and were fixed in 4% paraformaldehyde and PBS for 15 min at 4°C, washed three times in PBS and 0.1% Triton X-100, and blocked in PBS, 0.1% Triton X-100, and 1% BSA for 1 hours at room temperature. Cells were then incubated with primary antibody (diluted in PBS, 0.1%Triton X-100, and 1% bovine serum albumin) overnight at 4°C and washed three times in PBS. Cells were then incubated with fluorescent molecule -coupled secondary antibody (Jackson ImmunoResearch), diluted in PBS and 0.1% Triton X-100, for 2 hours at room temperature, washed three times in PBS, and mounted on glass slides with Gel/Mount. Finally the cells were examined by fluorescence microscopy (Zeiss Axiovert 200M).

3.2.24 Affymetrix Exon Junction Array analysis

Each 5 µg of total RNA from three independent experiments were provided to the company Affymetrix for Human Exon Junction Array hybridization.

3.2.25 Bioinformatical Support

Database/software	URL	Description	Reference
ClustalW	http://www.ebi.ac.uk/clustalw/index.html	Multiple sequence alignment program for DNA or proteins	(Thompson et al., 1994)
DISOPRED 2	http://bioinf.cs.ucl.ac.uk/disopred	Prediction of disordered protein regions	(Ward et al., 2004)
ESE	http://rulai.cshl.edu/tools/ESE	Finds putative binding regions for several splice factors	(Cartegni et al., 2003)
FoldIndex	http://bip.weizmann.ac.il/fldbin/findex	Estimates the local and general probability for the provided sequence, under specified conditions, to fold.	(Prilusky et al., 2005)
Human BLAT search	http://www.genome.ucsc.edu/cgi-bin/hgBlat	Sequence alignment tool similar to BLAST	(Kent, 2002)
NCBI BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	Finds regions of sequence similarity	(Altschul et al., 1990; Altschul et al., 1997)
PyMOL	http://pymol.org/	Molecular visualization system.	(DeLano, W.L. 2002)

4 Results

4.1 PP1 regulates tra2-beta1 through dephosphorylation

Protein Phosphatase 1 (PP1) is a protein phosphatase with broad substrate specificity and plays an important role in the regulation of diverse cellular functions. The catalytic subunit of PP1 creates complexes with many different PP1-interacting proteins (Cohen, 2002), binding through the RVxF-type docking motif. PP1 dephosphorylates phospho-serine and phospho-threonine residues changing the activity and the function of its substrate proteins. PP1 regulates the activity of several splicing factors through its phosphatase activity, including tra2-beta1. PP1 inhibition promotes the inclusion of the tra2-beta1 dependent SMN exon 7 (Novoyatleva et al., 2008). Cantharidin and its derivatives are PP1 and PP2A inhibitors and they promote exon 7 inclusion via changing the phosphorylation status of tra2-beta1 (Zhang et al., 2011).

4.1.1 Validation and characterization of custom-made phospho-specific antibodies

Post-translational modifications (PTM) such as methylation, acetylation and phosphorylation can modify the properties of a protein altering its activity, conformation and binding affinities. These reversible changes on the protein can regulate cellular processes modulate protein-protein interactions, signal transduction and cell-cycle events (Deribe et al., 2010). A single protein can have many PTMs. The usage of antibodies that are specific for the modifications can give information about the changes in the PTM profile and help to understand the

mechanisms that lead to these changes. Among PTMs phosphorylation is the best studied, it occurs predominantly on serine residues (~90%) and with a less frequency on threonine (~10%) and tyrosine residues (~<0.05%) (Nita-Lazar et al., 2008).

The activity of tra2-beta1 is regulated by its phosphorylation profile. The N- and C-terminal serine-arginine rich domains contain several serine residues. The phosphorylation status of these residues modulates the function of tra2-beta1. To understand better how the phosphorylation status of tra2-beta1 affects its activity in alternative splicing we designed a series of phosphorylation-selective polyclonal antisera. Three sets of phosphorylation-specific antibodies were raised against specific amino acid residues of tra2-beta1. The selected residues were previously identified by mass spectrometric analysis and found to undergo reversible phosphorylation during the splicing process (Bessonov et al., 2008). Phosphorylation-specific antisera were generated against phospho-threonine33 (pT33), phospho-serine95, 97 and 99 (pS95-97-99) and phospho-threonine201 (pT201).

To validate the specificity of these antisera mutations were introduced at these specific positions of tra2-beta1. Selected serine (S) or threonine (T) residues of tra2-beta1 were changed to alanine (A), to prevent phosphorylation, or to glutamic acid (E), to mimic the constitutively phosphorylated form of tra2-beta1. HEK293T cells were transfected with EGFP-C2 constructs expressing wild type tra2-beta1 or point mutants. Cell extracts were prepared as described in 'Methods', separated on SDS-PAGE and transferred to nitrocellulose membrane followed by Western-blot analysis employing anti-pT33, anti-pS95-97-99 and anti-pT201 antibodies. As shown in Figure 4.1, cell extracts from cells expressing tra2-beta1 alone (tra2-beta1, Figure 4.1.A) show two prominent bands when western-blotted with anti-pT33. The lower molecular weight band at 37 kDa represents endogenous tra2-beta1; the band at approximately 65 kDa represents the EGFP-tagged wild type protein. This band is completely abolished when T33

was changed to either A (tra2-beta1 T33A) or E (tra2-beta1 T33E) demonstrating that this antibody is highly specific for phosphorylation of T33.

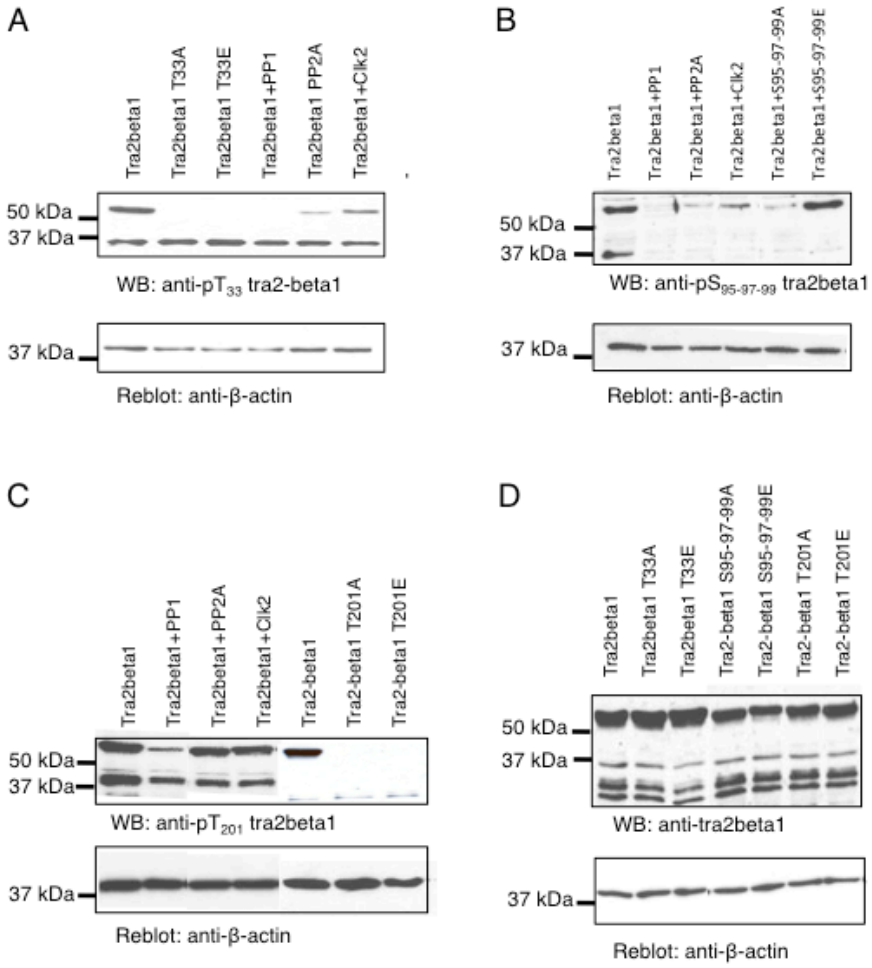


Figure 4.1. Characterization of phosphorylation-specific antibodies. HEK293T cells were transfected with cDNAs expressing the proteins indicated and analyzed with affinity-purified antisera that detects different phosphorylated residues of tra2-beta1. The membrane was reblot with anti-beta-actin as a loading control. *A*, Western-blot with anti-pT33-tra2-beta1 antisera. *B,* Western-blot with anti-pS95-97-99-tra2-beta1 antisera. *C*, Immunoblot analysis with anti-pT201-tra2-beta1 antisera. D, Western-blot with anti-tra2-beta1 antisera.

Similarly, anti-p95-97-99 antiserum recognizes endogenous tra2-beta1 and the EGFP-tagged wild-type protein (tra2-beta1, Figure 4.1.B) and did not detect tra2-beta1 mutant where serine residues were changed to A (tra2-beta1 S95-97-99A).

However anti-p95-97-99 detects the mutant protein, expressing the glutamic acid replacements (tra2-beta1 S95-97-99E), indicating the sensitivity of this antiserum.

Anti-pT201 antiserum detects the two bands representing endogenous and overexpressed protein when cells were transfected with wild type tra2-beta1 (tra2-beta1, Figure 4.1.C). It did not recognize the higher molecular weight band corresponding to EGFP-tagged tra2-beta1 when T201 was changed to either A or E. As an additional control wild-type EGFP-tagged tra2-beta1 and mutated tra2-beta1 were overexpressed in HEK293T cells and cell extracts were subjected to Western-blot analysis with anti-tra2-beta1 antibody, which was described previously (Daoud et al., 1999). As shown in Figure 4.1. D, each tra2-beta1 point mutants were overexpressed approximately at the same level compared to wild type. However, the bands representing overexpressed tra2-beta1 were slightly prominent when wild type protein either T33A or T33E mutants were overexpressed.

Since tra2-beta1 binds directly to PP1 and Protein Phosphatase 2A (PP2A) is involved in splicing reaction, we tested the effects of PP1 and PP2A on the phosphorylation of these specific sites. The SR protein kinase Clk2 was included as a positive control since it phosphorylates SR proteins. HEK293T cells were cotransfected with EGFP-tagged wild type tra2-beta1 and either EGFP-tagged PP1, EGFP-tagged PP2A or EGFP-tagged Clk2 and analyzed for tra2-beta1 phosphorylation as described above. The overexpression of PP1 (tra2-beta1+PP1 Figure 4.1.) completely abolished the signal at T33 (Figure 4.1.A) and S95-97-99 (Figure 4.1.B) positions and reduced it at T201 (Figure 4.1.C) position indicating that PP1 dephosphorylates T33, S95-97-99 and T201.

As shown in Figure 4.1, overexpression of PP2A (tra2-beta1+PP2A) reduced the signal of pT33 (Figure 4.1. A) and completely abolished at S95-97-99 (Figure 4.1.B). Anti-pT201 recognized EGFP-tagged tra2-beta1 when PP2A was overexpressed demonstrating that pT201 was not dephosphorylated by

PP2A. Overexpression of Clk2 did not show any effect when the phosphorylation of tra2-beta1 was analyzed by anti-pT33 or anti-pS95-97-99 antisera (Figure 4.1.A and C, tra2-beta1+Clk2). We observed a reduced signal when anti-pS95-97-99 was employed in the Western-blot analysis, suggesting that these resides are not target of Clk2 (Figure 4.1.B, tra2-beta1+Clk2).

These data demonstrate that these antisera are highly specific for phosphorylation of tra2-beta1 at T33, S95-97-99 and T201. Furthermore, the results indicate that the T33 and the S95-97-99 positions are dephosphorylated by PP1. Taken together, these antisera allow the investigation of the phosphorylation profile of tra2-beta1 providing information about the phosphorylation status of these specific positions.

4.1.2 Phosphatase modulator pseudocantharidins change the phosphorylation profile of tra2-beta1

Pseudocantharidins are derivatives of cantharidin, a toxic compound produced by many species of blister beetle. It inhibits the activity of PP1 and PP2A and it changes alternative splicing events (Novoyatleva et al., 2008). Pseudocantharidin A, B, C and D promote the inclusion of SMN2 exon 7. Pseudocantharidin A inhibits PP1 and PP2A, pseudocantharidin B-C stimulates PP2A activity and pseudocantharidin D does not affect the phosphatase activites of PP1 or PP2A. PP1 and PP2A play essential roles in constitutive splicing (Shi et al., 2006). Isocantharidin and pseudocantharidin A blocks C complex formation during the splicosomal assembly (Zhang et al., 2011).

Tra2-beta1 promotes the inclusion of SMN2 exon 7 in a concentration dependent manner and PP1 antagonizes its effect and causes the skipping of the exon (Novoyatleva et al., 2008). Since pseudocantharidin A, B and C have been shown to modulate the enzymatic activity of phosphatases we investigated whether these compounds influence the phosphorylation profile of tra2-beta1. By

taking advantage of the previously characterized phosphorylation selective antisera generated against specific phospho-tra2-beta1 isoforms, we tested the effect of the drugs on tra2-beta1.

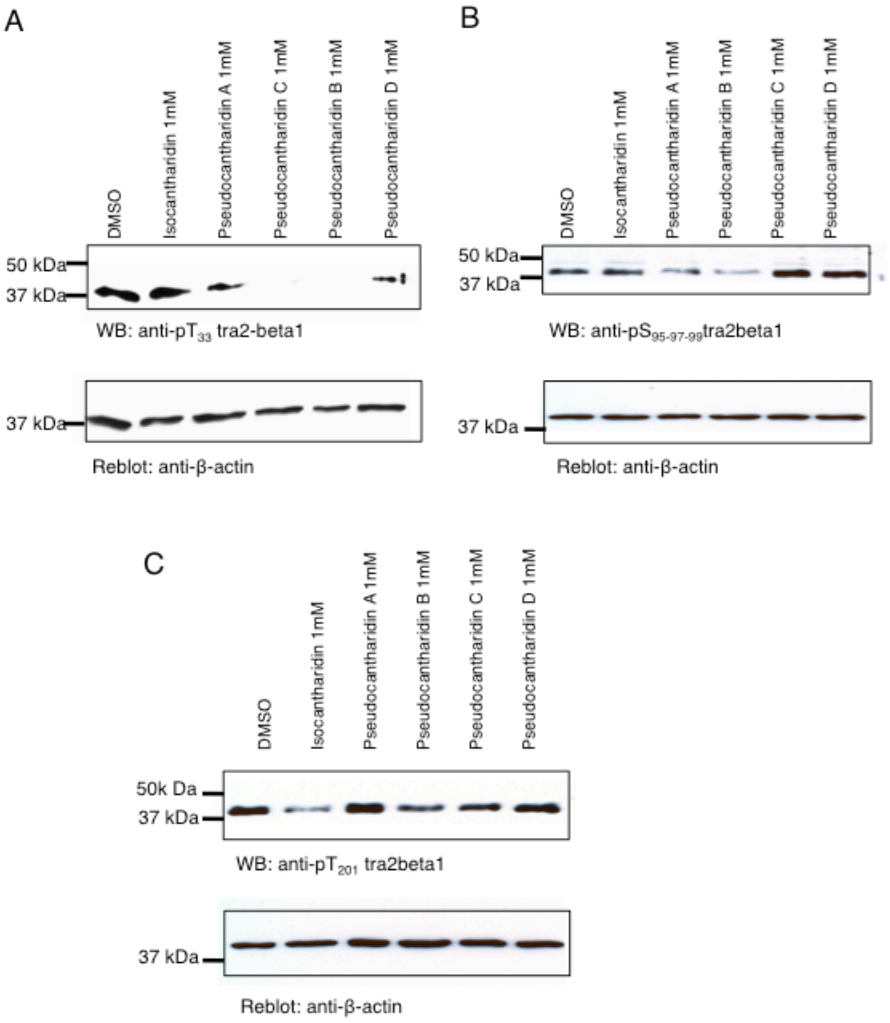


Figure 4.2. Pseudocantharidins change the phosphorylation profile of tra2-beta1. HEK293 cells were treated with the pseudocantharidins indicated, and the affect of the phosphorylation in different residues on tra2-beta1 was determined by Western blot analysis. Membranes were reblot with anti-β-actin as loading control. *A,* Western blot with the pT33 antisera. *B,* Western blot with the pS95-97-99 antisera. *C,* Western blot with the pT201 antisera.

HEK293T cells were treated with 1mM pseudocantharidins or isocantharidin. Cell extracts were separated on SDS-PAGE, transferred to nitrocellulose membrane and analysed by Western-blot employing pT33, pS95-97-99 and pT201 phosphorylation specific antisera. A prominent signal was observed at 37 kDa corresponding to the endogenous tra2-beta1 when cells were treated with isocantharidin, the signal was less pronounced after pseudocantharidin A and D treatment indicating that these compounds did not change the phosphorylation of tra2-beta1 at T33 (Figure 4.2.).

This result was expected since both isocantharidin and psedudocantharidin A inhibits the activity of PP1. Pseudocantharidin B and C caused dephosphorylation of T33 that is in agreement with their ability to activate PP2A.

We observed a decreased signal when cells were treated with pseudocantharidin A or B and cell extracts were analyzed with anti-pS95-97-99-tra2-beta1 antisera (Figure 4.2.B). This is in agreement with their effect on phosphatases, pseudocantharidin A inhibits the activity of PP1 and pseudocantharidin B activates PP2A. However, a prominent band was observed when cells were treated with pseudocantharidin C that activates PP2A.

The signal, which represents tra2-beta1 phosphorylated at T201, was decreased when cells were treated with pseudocantharidin B and C (Figure 4.2.C). The PP1 inhibitor isocantharidin caused dephosphorylation at this position; however, pseudocantharidin A, which inhibits the activity of PP1, did not show any affect on this site.

These data indicate that pseudocantharidins alter the phosphorylation profile of tra2-beta1. However, this effect is different at various positions indicating that the phosphorylation of these residues is regulated in different ways. Phosphorylation/dephosphorylation events are highly dynamic, finely tuned processes. The phosphorylation profile of a protein can change its activity and regulate its function. The phosphorylation/dephosphorylation of certain residues

can play an important role in this regulation while the phosphorylation status of other sites is not crucial in changing the activity of the protein. The phosphorylation of eukaryotic translation initiation factor-2α (eIF2α) at serine 51by stress-responsive kinases results in inhibition of translation (Holcik and Sonenberg, 2005).

4.1.3 Pseudocantharidin B and C changes phosphorylation of tra2-beta1 at position T33 and promotes SMN exon 7 inclusion

Pseudocantharidin A-D promotes the inclusion of exon 7 in splicing assays using SMN2 minigene (Zhang et al., 2011) and blocking the activity of PP1 leads to the same result (Novoyatleva et al., 2008). It has been previously described that tra2-beta1 stimulates the inclusion of SMN2 exon 7 (Hofmann et al., 2000). Therefore, we wanted to investigate whether individual phosphorylation sites regulate the alternative splicing of SMN2. Previously described *in vivo* splicing assays were performed using pCISMN2 minigene, which contains exon 6-8 (Lorson et al., 1999).

Point mutations were introduced in tra2-beta1 sequence mimicking phosphorylated or dephosphorylated forms of the protein. Mutants where amino acid residues were changed to A mimic dephosphorylated form of the protein and mutants contains E instead of S or T mimic phosphorylated tra2-beta1. HEK293T cells were cotransfected with the SMN2 minigene and either EGFP-tagged wild type tra2-beta1 or EGFP-tagged tra2-beta1 mutants. EGFP-C2 vector lacking tra2-beta1 was served as a control. In all experiments 1µg of expression vector with 1µg of SMN2 minigene construct were cotransfected. Total RNA was isolated 18 hours post-transfection and used for RT-PCR with minigene specific primers. As shown in Figure 4.3.A, tra2-beta1T33A mutants induced the inclusion

of exon 7 stronger than wild type tra2-beta1. The T33E mutant had an effect similar to wild type. No significant changes in the splicing pattern were observed when S95-97-99 was mutated to either A or E (Figure 4.3. B).

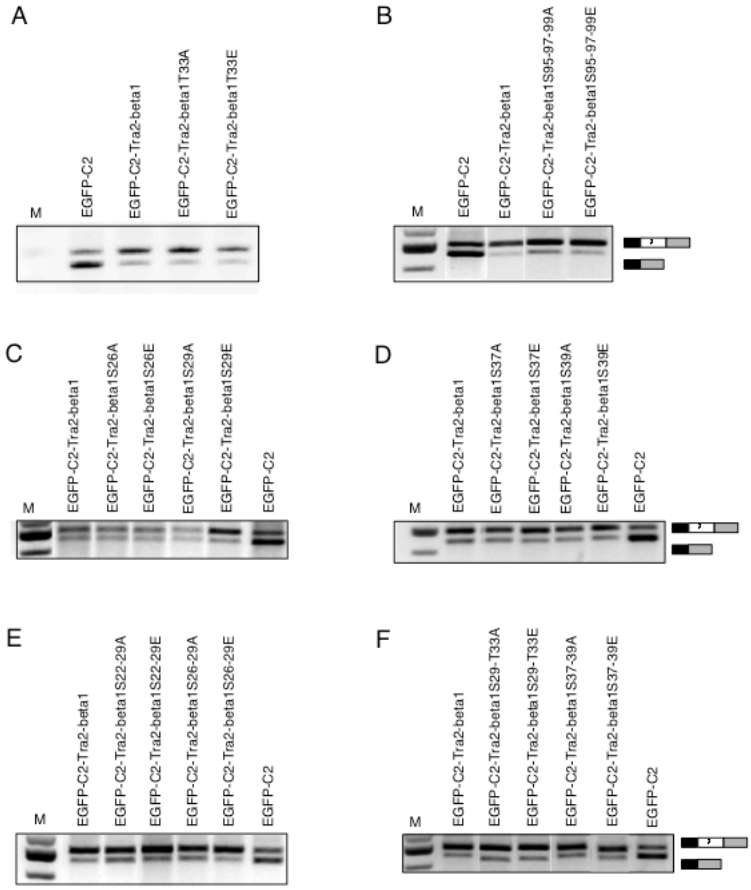


Figure 4.3. Mutation of tra2-beta1 residue T33 to alanine promotes exon 7 inclusion. *A-F,* the SMN2 reporter minigene, schematically shown on the right side of the figure, was transfected with cDNAs expressing tra2-beta1 wild type and the mutants indicated. The RNAs were analyzed by end point RT-PCR. M: marker.

Similarly, no effect could be detected when residues S26, S29, S37 and S39 were mutated to either A or E (Figure 4.3. C-D). No effect was observed when double or triple mutants were overexpressed (tra2-beta1 S22-29A or E, tra2-beta1 S26-29 A or E, tra2-beta1 S29-T33 A or E and tra2-beta1 S37-39 A or E, Figure 4.3. E-F). These data suggest that the phosphorylation status of T33 plays

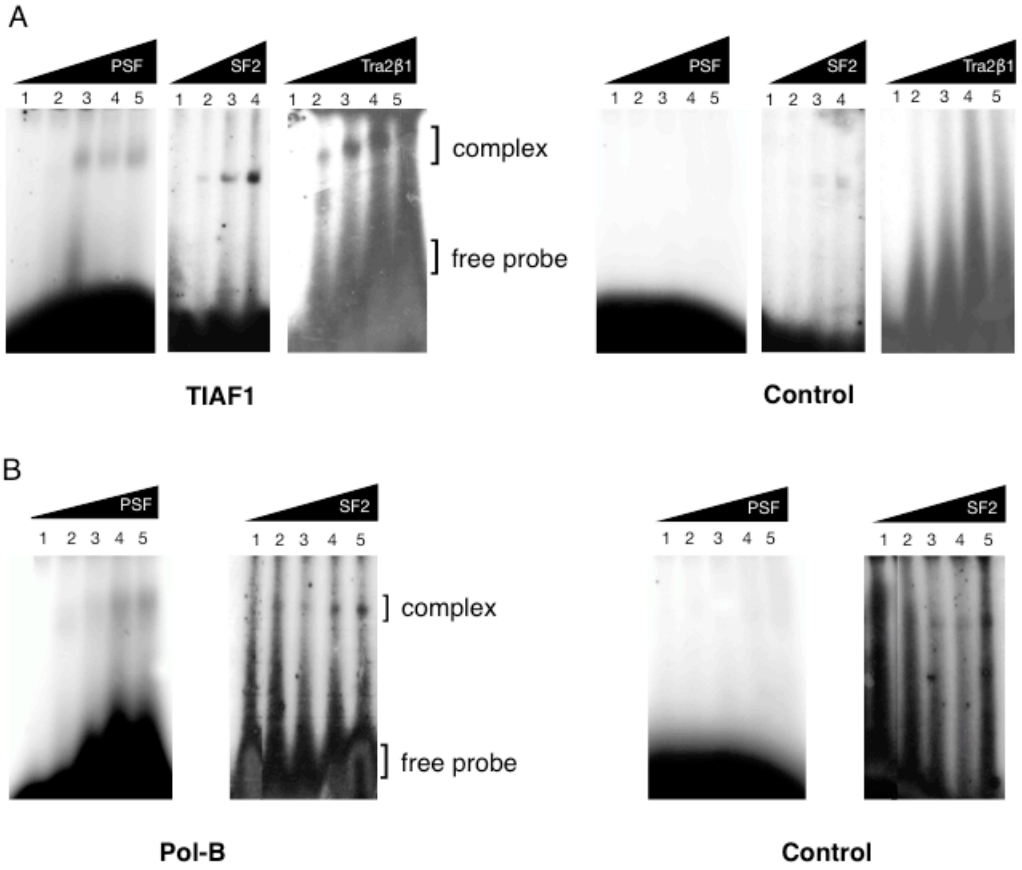
a role in the regulation of splicing activity of tra2-beta1 and dephosphorylation of this reside stimulates the inclusion of SMN2 exon 7. Mimicking the phosphorylation status of the other positions that were tested in these splicing assays did not change the splicing pattern of the minigene suggesting that phosphorylation/dephosphorylation of these residues is not sufficient to modulate the splicing activity of tra2-beta1.

4.1.4 Tra2-beta1 binds to ceramide responsive exons

Ceramides belong to a class of sphingolipids that are composed of shingosine and a fatty acid moiety. It has been previously shown that water-insoluble ceramides alter alternative splicing and decrease SR-protein phosphorylation by activating PP1 (Chalfant et al., 1999; Chalfant et al., 2001; Massiello et al., 2004). Water-soluble ceramide analogs were synthesized to improve delivery; these ceramide derivatives containing pyridinium moieties are able to induce apoptosis and are tested as anti-cancer drugs. C6 pyridinium ceramide is an anti-cancer drug candidate that is currently being tested for the treatment of squamous cell carcinomas (Karahatay et al., 2007). C6 pyridinium ceramide, unlike the water-insoluble, natural C6 ceramide inhibits PP1 activity and therefore prevents the dephosphorylation of splicing regulatory proteins. C6 pyridinium ceramide binds directly to PP1 and inhibits its phosphatase activity. C6 pyridinium ceramide changes alternative splicing of several pre-mRNAs to promote both inclusion and skipping of the target exons. C6 pyridinium ceramide regulated exons are unusually short and share suboptimal splice sites and two 4-nucleotide motifs, GAAR and CAAR (Sumanasekera et al., 2012). To increase the specificity of the alternative splicing events the usage of these exons depend on enhancer/silencer sequences.

To investigate the direct interaction between C6 pyridinium ceramide-responsive exons and splicing factors *in vitro* RNA electrophoretic mobility shift

assay (RNA gel shift) were performed. PSF/SFPQ, SF2/ASF and tra2-beta1 were tested in these assays. Almost all of the C6 pyridinium ceramide responsive exons contain the high affinity tra2-beta1 binding site, the AGAA motif (Clery et al., 2011). SELEX experiments showed that PSF binds to sequences containing GAA motifs (Peng et al., 2002) that are enriched in C6 pyridinium ceramide-responsive exons. Bioinformatics analysis using ESE finder indicates SF2/ASF binding sites in the regulated exons (Smith et al., 2006). Two sequences were used in the gel shift experiments that are from C6 pyridinium responsive exons and were radioactively labeled. 27 nucleotides long probes were synthetized the sequences are shown in Figure 4.4.C. Pol-B (DNA polymerase beta) probe contains one SF2/ASF binding site and three high affinity binding motifs of tra2-beta1. The TIAF1 (TGFB1-induced antiapoptotic factor 1) probe contains one binding site of SF2/ASF and two high affinity binding motifs of tra2-beta1. One artificial oligonucleotide that lacks binding motif of tra2-beta1 and SF2/ASF and does not contain GAAR or CAAR motifs either was also labeled or used as a control. These probes were incubated either with recombinant PSF/SFPQ, SF2/ASF or tra2-beta1. The change in the probes mobility was detected using non-denaturing polyacrylamide (PAA) gel electrophoresis. As shown in Figure 4.4.A, all three proteins bound to TIAF1 sequences. No changes in shift were observed when increasing concentration of PSF was added to the reaction (Figure 4.4 A, PSF). Addition of SF2/ASF in increasing concentration enhanced the signal of the shift (Figure 4.4.A, SF2/ASF). Tra2-beta1 forms increasing molecular weight complexes when its concentration was increased suggesting multimer formation (Figure 4.4 A, trab2β1). Similarly, RNA-protein complex formation was observed between the Pol-B probe, PSF/SFPQ and SF2/ASF (Figure 4.4.B). However tra2-beta1 did not bind to the POL-B-exon RNA. This was surprising, since the exon contains the AGAA binding motif that is the high affinity binding site for tra2-beta1 (Clery et al., 2011). This suggests that an unknown sequence context contributes to tra2-beta1 binding.



C

TIAF1	GGUGACAAGUAUCAGAAAAGAAAGAAT
Pol-B	ACUCGCAAACUUUGAGAAGAACGUGAG
Control	GGUGAGUUCUAUCACUUUACUUUGAAT

Figure 4.4. PSF, SF2/ASF and Tra2-beta1 bind to motifs enriched in PyrCer dependent exons. *A,* Gel retardation assay with TIAF1 and control probes. *B*, Gel retardation assay with POL-B and control probes. The concentration of all oligonucleotides was 0.4μM in all reactions in a total volume of 10μl. The protein concentrations for Tra2-beta1 were 0, 2.4, 3.6, 4.8 and 7.2μM in lanes 1–5, respectively. The concentrations for PSF/SFPQ were 0, 0.5, 1.1, 2.1 and 3.2μM in lanes 1–5, respectively. The concentration for SF2/ASF was 0, 1.1, 2.8, 4.0 and 5.7μM and in lanes 1–5, respectively. *C*, Sequence of the RNA probes. The GAAR motif is underlined; the CAAG motif is underlined with zigzag. The SF2/ASF site is indicated by a dotted superscript.

C6 pyridinium ceramide responsive alternative exons have suboptimal length, suboptimal splice sites and contain binding sites characteristic for splicing enhancers. Our data indicates that splicing factors bind directly to these sequences to increase the specificity of alternative splicing events.

4.2 The role of tra2-beta1 in the cytoplasm

Tra2-beta1 is predominantly a nuclear protein and its function in the nucleus has been extensively studied. However, the expression level of tra2-beta1 is increased in breast, cervical and ovarian cancer (Fischer et al., 2004; Gabriel et al., 2009; Watermann et al., 2006) and previous research has shown that tra2-beta1 changes localization under stress conditions and it accumulates in the cytosol (Daoud et al., 2002), the cytosolic role of tra2-beta has not been elucidated. It has been recently published that tra2-beta1 binds to non-coding RNA (ncRNA) sequences in vivo, including the 18S ribosomal RNA (18S rRNA) (Grellscheid et al., 2011). Furthermore, previous studies performed in our laboratory showed that tar2-beta1 cosediments with ribosomal fraction from cellular lysates (Tang.,doctoral thesis 2008). The roles of SR proteins in the cytoplasm have been extensively studied and several reviews have been published recently. Shuttling SR proteins regulate constitutive and alternative splicing and they are involved in diverse cytoplasmic processes and coordinate different steps in mRNA metabolism (Twyffels et al., 2011; Zhong et al., 2009). It has been shown that SF2/ASF stimulates the initiation of cap-dependent translation (Michlewski et al., 2008) and enhance the translation of a reporter in HeLa cells (Sanford et al., 2004). Therefore, we wanted to investigate whether tra2-beta1 is involved in regulation of translation.

4.2.1 Tra2-beta1 binds to the 18S rRNA

We analyzed tra2-beta1 cross-linking and immunoprecipitation (CLIP) targets from previously performed experiment (Tang.,doctoral thesis 2008) and compared the results to recently published CLIP RNA target sequences from mouse (Grellscheid et al., 2011). CLIP targets from both experiments were found from rRNA. Previously different binding sites of tra2-beta1 have been identified using NMR experiments (Clery et al., 2011; Tsuda et al., 2011). AGAA is the high affinity binding site of tra2-beta1 it binds to this motif with 2.2 µM affinity, tra2-beta1 binds to the GGAA motif with a lower, 4 µM affinity. We analyzed the sequence tags that were located within the small subunit rRNA coding region. 60 CLIP target sequence were found within the rs45sn gene containing either high affinity or low affinity binding sites of tra2-beta1. 7 tags contained the AGAA high affinity binding motif of tra2-beta1 and 53 were found with the GGAA lower affinity binding site. The sequences clustered in 9 region and contained 14 binding motif (Table 4.1). Next, the CLIP targets were mapped to the known secondary structure of human 18S rRNA (Watkins and Bohnsack, 2012) and the 3 dimensional crystal structure of the eukaryotic 18S rRNA obtained from *Saccharomyces cerevisiae* (Ben-Shem et al., 2011). We used the PyMol software to map the CLIP sequences to the 3 dimensional structure of 18s rRNA (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA.).

Sequence 5`→3`
ATCAACTTTCGATGGTAGTCGCCGTGCCTACCATGGTGACCACGGGTGACGGGGAA TCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAAC
GTGACGAAAAATAACAATACAGGACTCTTTCGAGGCCCTGTAATTGGAATGAGTCCA CTTT
AAAGCAGGCCCGAGCCGCCTGGATACCGCAGCTAGGAATAATGGAATAGGACCGC GGTTCTATTTTGTTGGTTTTCGGAACTGAGGCCATGATT
ATTCCCATGACCCGCCGGGCAGCTTCCGGGAAACCAAAGTCTTTGGGTTCCGGGG

GGAGTATGGTTGC
GTTCCGGGGGGAGTATGGTTGCAAAGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGAC
AACACGGGAAACCTCACCCGGCCCGGACACGGACAGGATTGACAGATTGAT
AATTATTCCCCATGAACGAGGAATTCCCAGTAAGTGCGGGCCATAAGCTTGCGTTGATT
GGCCCACGGCCCTGGTGGAGCGCTGAGAAGACGGTCGAACTTGACTATCTAGAGGAAGTAAAAGTC
AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGGGAGACTGTGGAGGAGCGGCGGC

Table 4.1. CLIP 18S rRNA targets from mouse. The high affinity tra2-beta1 binding site underlined with dots, the lower affinity tra2-beta1 binding site underlined.

The 9 tags containing tra2-beta1 binding site were located in regions of the 18S rRNA that are interacting with snoRNAs (Watkins and Bohnsack, 2012) and/or are single stranded (Figure 4.5.A). Since snoRNAs can base pairing to this double-stranded regions probably tra2-beta1 is able to interact with them. Mapping the CLIP tags to the three dimensional structure revealed a clustering on the region so-called expansion segment 6 (ES6). Eukaryotic rRNS contain several additional nucleotide elements inserted in the conserved core sequence called expansion segments (ES). These expansion segments can provide a platform for interaction with regulator proteins (Ben-Shem et al., 2011; Klinge et al., 2012).

We next wanted to see whether tra2-beta1 binds directly to these sequences *in vitro*. Tra2-beta1 RRM was purified from bacteria and gel retardation assays were performed using probes corresponding to the ribosomal CLIP targets (Figure 4.5.A-B, orange arrows). Two RNA oligonucleotide sequence were synthetized: ES6 that was mapped to the ES6 region of 18S rRNA and h12, which mapped to the helix 12 region. Artificial RNA oligonucleotide lacking binding motif of tra2-beta1 was used as a control. The ES6 probe contains 2 and the h12 probe contains one GGAA low affinity binding site of tra2-beta1 (Figure 4.5 B).

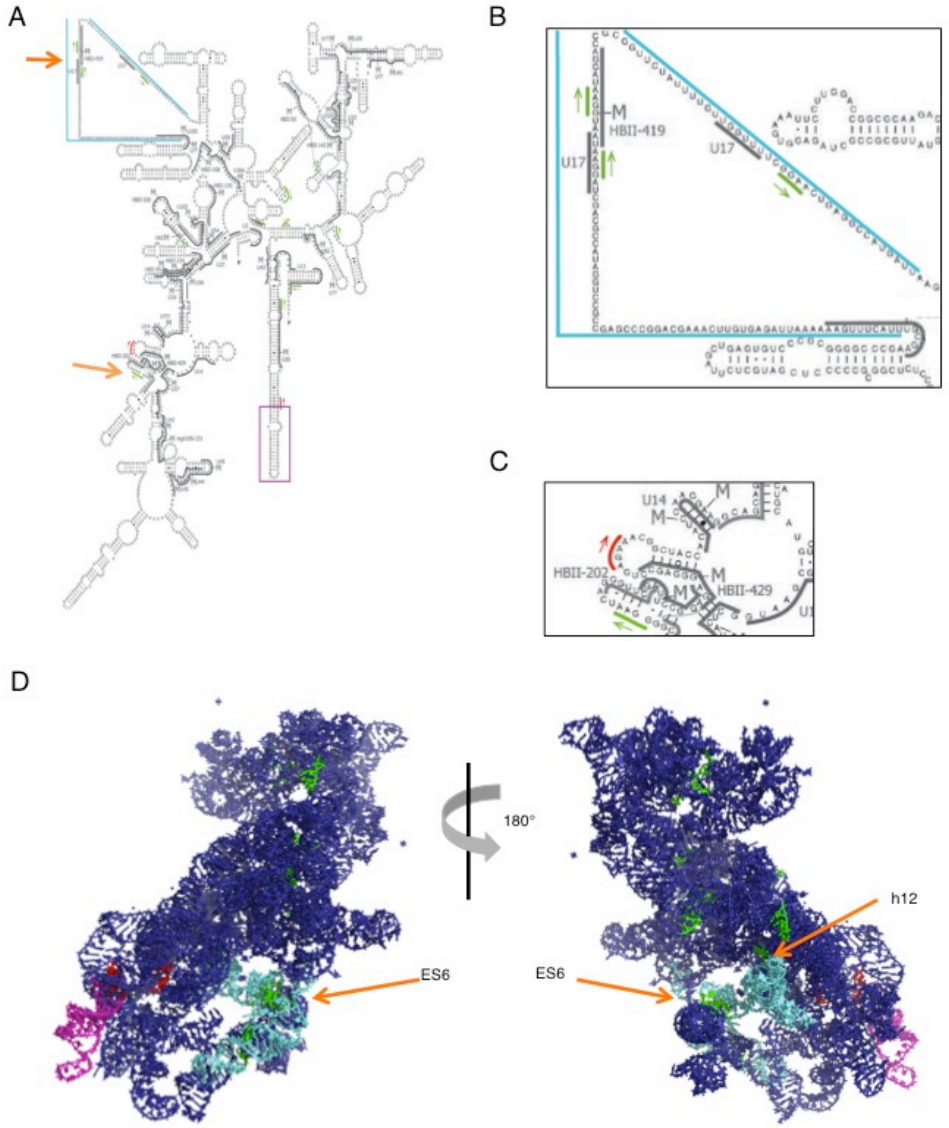
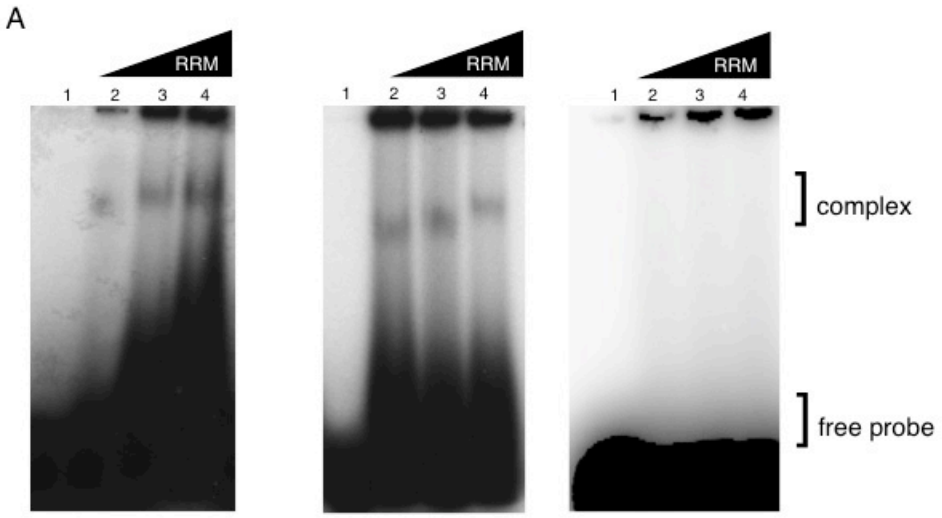


Figure 4.5. Localization of CLIP targets on 18S rRNA. *A*, The secondary structure of the human 18S rRNA. Tra2-beta1 binding sites found in CLIP tags are indicated. The high affinity AGAA binding motif underlined with red, the lower affinity GGAA binding site underlined with green color. ES6 framed with cyan and the ES12 framed with magenta. *B,* Enlargement of ES6. *C,* Enlargement of h12 area. The figure was modified from Watkins and Bohnsack, 2012. *D*, The localization of CLIP targets on the three dimensional structure of yeast 18S rRNA. High affinity binding sites are indicated in red, the lower affinity binding sites indicated in green. ES6 indicated in cyan and ES12 indicated in magenta. Figure was generated by using PyMol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA). RNA probes for gel shift assay indicated with orange arrows.

The probes were radioactively labeled and incubated in vitro with tra2-beta1 RRM. Changes in the shift were detected by non-denaturing PAA gel electrophoresis. As shown in Figure 4.6, tra2-beta1 RRM binds to ES6 sequence and to the h12 region as well under *in vitro* conditions. We observed increasing molecular weight RNA-protein complexes adding increasing concentration of tra2-beta1 protein indicating formation of RNA-protein multimers. The interaction between the protein and the probe derived from the h12 region is more pronounced. Less efficient binding was observed when the ES6 RNA probe was used however this probe contains two predicted low affinity binding site of tra2-beta1. These data suggests that tra2-beta1 can bind directly to the 18S rRNA.



B

ES6	UAGGAAUAAUGGAAU
h12	UGACGGGGAAUCAGG
control	AGUAUUGAGCAUCAUAGCCGC

Figure 4.6. Tra2-beta1 binds to the 18S rRNA. *A,* Gel retardation assay with the probes derived from 18S rRNA and with the control probe. The concentration of all oligonucleotides was 0.3 μM in all reactions in a total volume of 10μl. The protein concentrations for Tra2-beta1 RRM were 0, 4, 8 and 16 μM in lanes 1–4, respectively. *B*, Sequence of the RNA probes.

4.2.2 Tra2-beta1 stimulates translation of a luciferase reporter in HEK293T cells

Previous data from our lab and the gel retardation assays were performed using RNA probes derived from the 18S rRNA indicate that tra2-beta1 binds to ribosomal parts. Next we asked whether tra2-beta1 is involved in translation. To study its role in translation dual luciferase assays were performed in HEK293T cells. The luciferase reporter constructs were used in this study derived from a reporter that was published previously (Sanford et al., 2004) where the role of SF2/ASF in translation was studied. The reporter contains a gene expresses firefly luciferase controlled by SV40 promoter. Between a unique start codon and the firefly luciferase gene a linker sequence located that contains the binding site of tra2-beta1 (Figure 4.7 A). The third exonic-splicing enhancer sequence (ESE) from exon II of tra2-beta1 was cloned into this reporter, which is known to recruit tra2-beta1 (Stoilov et al., 2004). The control reporter vector contains a sequence that lacks the binding motif of tra2-beta1. An additional control was used in which a stop codon was introduced within the linker region (Sanford et al., 2004). This system allows testing the effect of tra2-beta1 on translation. *Renilla* luciferase reporter was cotransfected for normalizing the transfection efficiency.

4.2.2.1 Optimizations of a noncommercial dual luciferase enzyme assay system

For assaying the luciferase enzyme activity commercially kits are available. However, these assays are proprietary and can be expensive. We used a nonproprietary assay system for our studies (Dyer et al., 2000). HEK293T cells were cotransfected with the firefly luciferase reporter and the *Renilla* construct. To optimize the enzyme assay commercially available kit (Promega) was used as a control according to the manufacturer instructions.

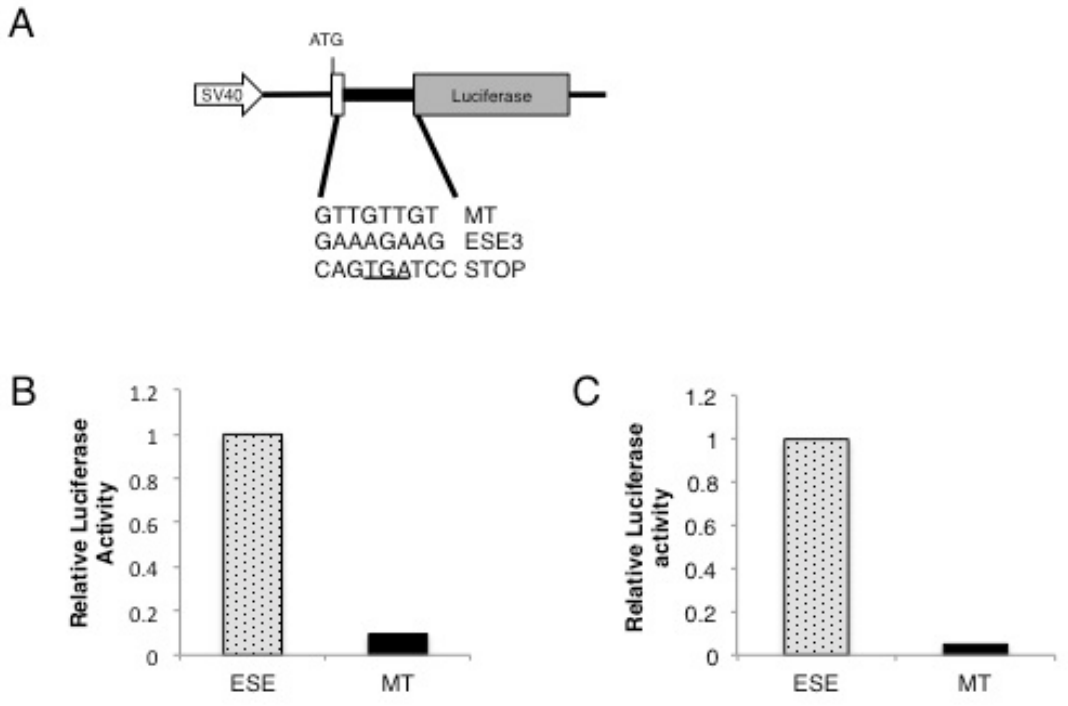


Figure 4.7. Optimization of the non-commercial dual luciferase enzyme assay system. HEK293T cells were cotransfected either with the pLCS-ESE, or the pLCS-MT reporter constructs and as an internal control with pRL-TK-*Renilla*. Cell extracts were assayed for firefly luciferase activity followed by *Renilla* luciferase activity. Light emission was measured in Lmax Microplate Luminometer. Relative Luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of ESE. *A*, Schematic structure of the luciferase construct based on previously published construct (Sanford et. al.; 2004). *B*, Dual luciferase assay was performed employee the noncommercial assay. *C*, Dual luciferase assay was performed utilizing the commercial assay.

We tested several set up with different delay and reading times. To determine the optimal delay and reading time for measuring enzyme activity firefly luciferase and *Renilla* luciferase reporters were cotransfected in HEK293T cells. 24 hour post-transfection dual luciferase assay were performed from the cell extracts. We found that setting the luminometer for a 2s delay followed by a 10s reading for luciferase activity, followed by a 5s delay and 10s reading for Renilla activity gave nearly the same result as the commercial kit. Relative

luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of ESE. Schematic structure of the firefly luciferase constructs shown in Figure 4.7 A. As can be seen in Figure 4.7 B the noncommercial *Renilla* buffer quench the firefly luciferase activity within 5s nearly as efficiently as the commercial buffer. As shown in Figure 4.7 B-C, the homemade enzyme activity assay system is comparable to the commercial kit that allows using a most cost-effective alternative.

4.2.2.2 Tra2-beta1 activates the translation of a reporter

Eukaryotic pre-mRNA processing occurs through several highly coordinated steps that are associated with complex cellular machines. These events are often functionally coupled (Moore and Proudfoot, 2009). It has been shown that shuttling SR proteins are involved in translation besides several other post splicing events (Twyffels et al., 2011; Zhong et al., 2009). Previously it has been reported that SF2/ASF stimulates the translation of a reporter (Sanford et al., 2004). Therefore we wanted to know whether tra2-beta1 is involved in translation.

To investigate the role of tra2-beta1 in translation in mammalian cells dual luciferase enzyme assays were performed. We used a luciferase reporter system described above for this purpose. The schematic illustration of the luciferase reporter used in this study shown in Figure 4.7 A. We cotransfected HEK293T cells with the pLCS reporter, a *Renilla* reporter and either Flag-tagged tra2-beta1 or, as a control the expression vector lacking tra2-beta1 sequence. 34 hour post transfection cell extracts were assayed for firefly and *Renilla* luciferase activity. Dual luciferase assays were preformed in the same reaction well. Noncommercial dual luciferase enzyme assay system was used to assay the levels of firefly and *Renilla* luciferase levels. Light emission was measured with

an Lmax Microplate Luminometer. The luciferase activity was normalized for transfection efficiency of each well by dividing the firefly luciferase activity by *Renilla* luciferase activity. Relative Luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of pLCS-ESE+ mock.

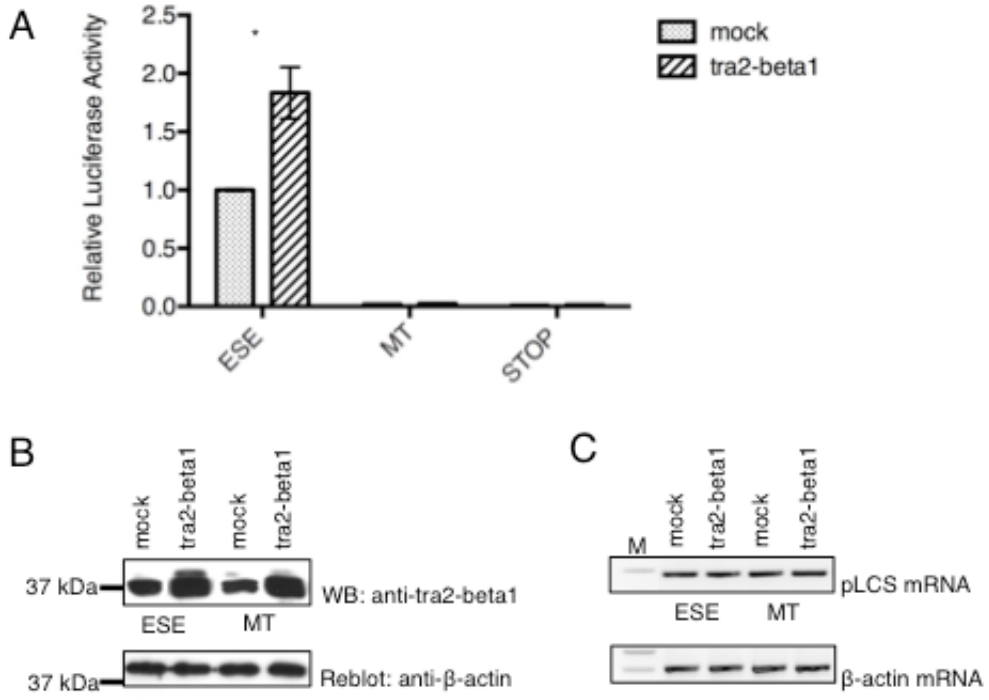


Figure 4.8. Overexpression of tra2-beta1 stimulates the translational activity of the luciferase reporter. *A,* HEK293T cells were cotransfected with the luciferase reporter constructs, *Renilla* luciferase reporter and either Flag-tagged tra2-beta1 or empty expression vector. Dual luciferase assay system was used to assay the level of firefly (translational reporter) and the *Renilla* (control the transfection efficiency) luciferase level. Luciferase activity was normalized to *Renilla* luciferase. Relative Luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of ESE mock. *p< 0.01,according to Student`s t-test n= 3 independent experiments. *B,* Cell extracts were analyzed by Western blot. Expression tra2-beta1 detected by anti-tra2-beta1 antibody. Membranes were reblot with anti-β-actin as loading control. *C,* Analysis of the reporter mRNAs by RT-PCR. β-actin was used as a loading control.

We observed that the presence of the tra2-beta1 binding site within the reporter stimulated the luciferase activity (Figure 4.8. A, ESE mock) compared to the control that lacks tra2-beta1 binding sequence (Figure 4.8.A, MT mock). This suggests that endogenous tra2-beta1 binds to the reporter vector containing the ESE sequence and activates the translation of the reporter mRNA. This hypothesis is supported with the observation that overexpression of tra2-beta1 caused a further increase in luciferase activity (Figure 4.8.A, ESE tra2-beta1) but did not stimulated significantly the luciferase activity of the reporter lacking its sequence (Figure 4.8.A, MT tra2-beta1). As a negative control luciferase reporter was used that contains a STOP codon (Figure 4.8.A, STOP) theretofore no translation occurs from the luciferase reporter. As a control, cell extracts from the luciferase assay experiments were load on SDS-PAGE and transferred to nitrocellulose membrane. To visualize the overexpression of tra2-beta1 Western-blot was performed employing anti-tra2-beta1 antibody. As a loading control membrane was reblotted with anti-β-actin antibody. As Figure 4.8.B shown, the overexpression of Flag-tagged tra2-beta1 was nearly equal in cells cotransfected with either pLCS-ESE or pLCS-MT. To exclude positive effect of tra2-beta1 on the reporter pre-mRNA level we assayed the pre-mRNA level of the reporters performing RT-PCR analysis with specific primers. HEK293T cells were cotransfected with either pLCS-ESE or pLCS-MT luciferase reporter constructs and Flag-tagged tra2-beta1 or empty expression vector. We did not observe any significant differences in the level of the reporter pre-mRNAs (Figure 4.8.C), the mRNA levels of the reporters remained constant when tra2-beta1 was overexpressed comparing to the control. This experiment suggests that overexpression of tra2-beta1 did not affect the stability of the reporter mRNAs supporting the hypothesis hat tra2-beta1 stimulates the translation of the luciferase reporter containing its binding sequence.

4.2.2.3 Hypoxia induces the translation of the tra2-beta1 dependent reporter

It has been shown that in human breast, cervical and ovarian cancer tra2-beta1 is overexpressed (Fischer et al., 2004; Gabriel et al., 2009; Watermann et al., 2006). The increased level of tra2-beta1 has an effect on the alternative splicing of several cancer-related mRNAs (Gabriel et al., 2009). Furthermore it was reported previously that tra2-beta1 accumulates in the cytoplasm under ischemia, hypoxia and acidosis (Daoud et al., 2002; Hirschfeld et al., 2011). Although the role of tra2-beta1 in nuclear pre-mRNA splicing has been characterized extensively, its cytoplasmic function is still unknown.

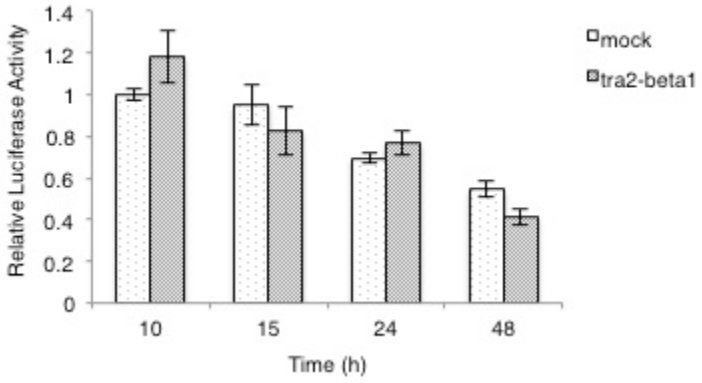


Figure 4.9. Time course of the effect of hypoxia on luciferase activity. HEK293T cells were cotransfected with the pLCS-ESE luciferase reporter construct, *Renilla* luciferase reporter and either Flag-tagged tra2-beta1 (tra2-beta1) or empty expression vector (mock). Cell extracts were assayed for firefly luciferase activity followed by *Renilla* luciferase activity. Light emission was measured in Lmax Microplate Luminometer. Relative Luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of ESE+mock at 10 h.

To investigate the effect of hypoxia on tra2-beta1-dependent translation, we cotransfected HEK293T cells with pLCS-ESE firefly luciferase reporter, *Renilla* luciferase reporter as an internal control and either Flag-tagged tra2-beta1 or the empty vector. 24 hours post-transfection the cells were placed under hypoxic condition (1% O2). Cells were cultivated for 10, 15, 24 and 48 hours under hypoxia. Cell extracts were assayed for firefly and *Renilla* luciferase activity as described above. The luciferase activity was normalized for transfection efficiency of each well by dividing the firefly luciferase activity by *Renilla* luciferase activity. Relative Luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of pLCS-ESE+ mock at 10 hours. We found the highest luciferase activity after 10-hour treatment under hypoxia (Figure 4.9.A, first two column). After 10-hour treatment the luciferase enzyme activity was gradually decreasing. In order to support this observation the intracellular localization of tra2-beta1 was investigated by immunocytochemical analyses employing the previously described tra2-beta1 antibody (Daoud et al., 1999). Previously it has been reported that tra2-beta1 changes its localization under acidic condition (Hirschfeld et al., 2011). Therefore we cultivated HEK293T cells under acidic condition and as a control under normal condition. Culture media was supplemented with 0.2% lactic acid and cells were incubated for different times. After incubation cells were prepared as described in Methods. We observed that under normal condition tra2-beta1 displayed a predominantly nuclear localization that switched to an increased cytoplasmic presence when cells were treated with lactic acid. It was found that the most pronounced changes in the localization of tra2-beta1 occurred after 10-15 hours of lactic acid treatment (data not shown). We performed the same experiment and determined the intracellular localization of tra2-beta1 protein immunocytochemically when HEK293T cells were cultivated under hypoxia for 10 hours (Figure 4.10.B). Under normal condition tra2-beta1 localized mainly in the nucleus (Figure 4.10.A, tra2-beta1) in contrast when cells were grown under

hypoxia the intracellular localization of tra2-beta1 markedly changed and tra2-beta1 shifted to an increased presence in the cytoplasm (Figure 4.10.B, tra2-beta1). The quantification of the cells showed that tra2-beta1 localizes in the cytoplasm in 37% of the cells under normal conditions (Figure 4.10.C. normoxia). The cytoplasmic presence of tra2-beta1 is more pronounced when cells were cultivated under hypoxia, tra2-beta1 changed its localization in 96% of the cells and accumulated in the cytoplasm (Figure 4.10.C. hypoxia).

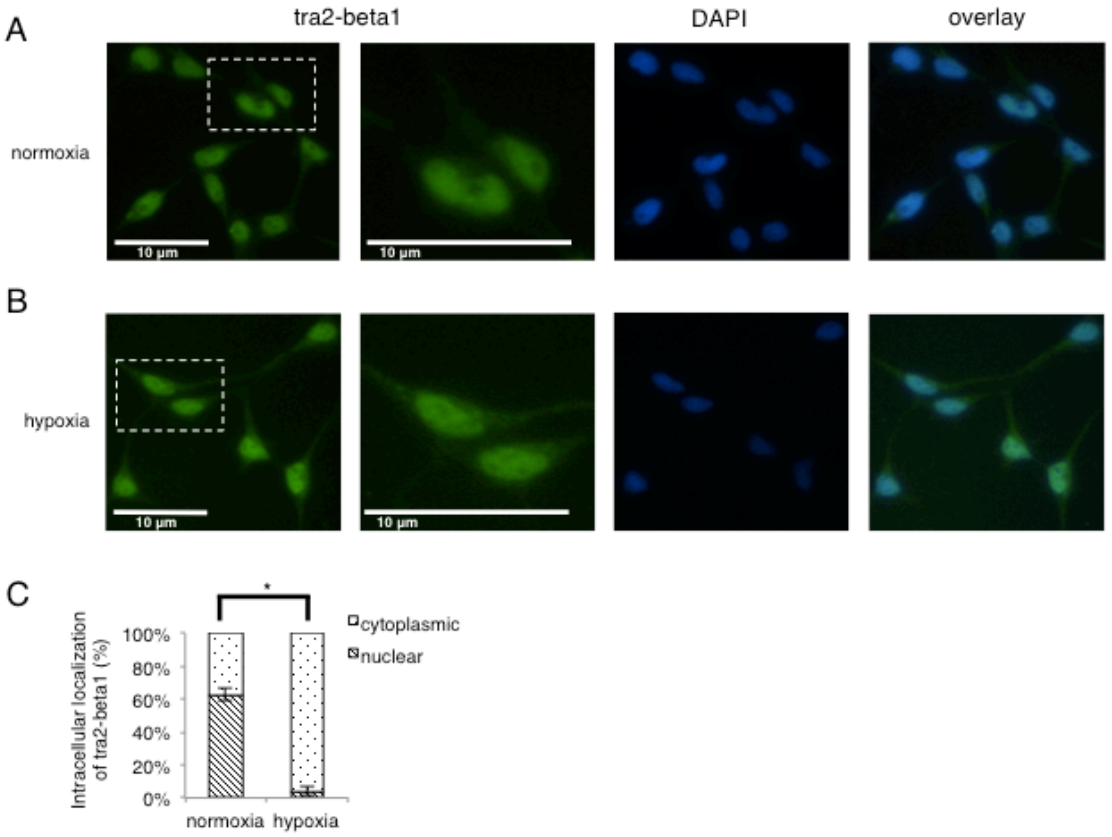


Figure 4.10. The intracellular localization of tra2-beta1 changes under hypoxia. Immunocytochemistry was used to detect the intracellular localization of endogenously expressed tra2-beta1 under normal (21% O2) and hypoxic (1% O2) conditions in HEK293T cells. Tra2-beta1 was detected by ant-tra2-beta1 antibody and visualized by FITC-conjugated secondary antisera. Nuclei were stained with DAPI. *A*, Immunostaining of tra2-beta1 under normoxia. *B,* Immunostaining for tra2-beta1 after 10 h hypoxia. *C*, The intracellular distribution of tra2-beta1 under normoxia and hypoxia. *p< 0.01, according to Student`s t-test. n (normoxia)=105, n (hypoxia)= 98.

Next, we used the dual luciferase reporter assays to assess the effect of oxygen deprivation on translation in our system. HEK293T cells were transiently cotransfected with the previously described luciferase reporters (pLCS-ESE, pLCS-MT, pLCS-STOP), *Renilla* luciferase construct and either flagged-tagged tra2-beta1 or the expression vector lacking the tra2-beta1 sequence. 24 hours post-transfection, the transfected cells were cultured for an additional 10 hours under hypoxic (1% O2) or under normoxic (21% O2) conditions. Cell extracts were assayed for firefly and *Renilla* luciferase activity as described previously.

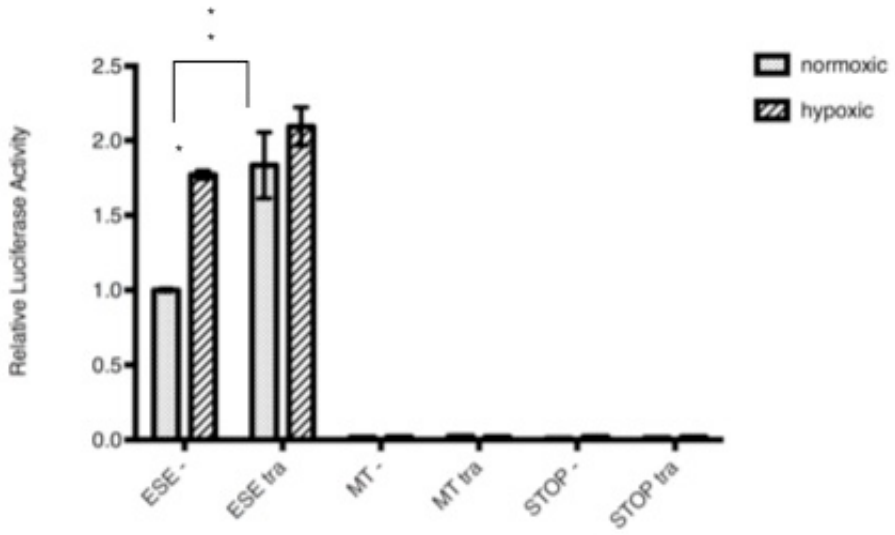


Figure 4.11. Hypoxia enhances the translational activity of the luciferase reporter containing tra2-beta1 specific ESE. HEK293T cells were cotransfected with the luciferase reporter constructs, *Renilla* luciferase reporter and either Flag-tagged tra2-beta1 or empty expression vector. Dual luciferase assay system was used to assay the level of firefly (translational reporter) and the *Renilla* (control the transfection efficiency) luciferase level. Cells were cultured under normoxia or hypoxia for 10 hours. Luciferase activity was normalized to *Renilla* luciferase. Relative Luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of ESE mock. *p< 0.01, **p<0.05, according to Student`s t-test n= 3 independent experiments.

As shown in Figure 4.11, hypoxia stimulated the translation of the reporter containing the tra2-beta1 binding site (Figure 4.11, ESE mock, hypoxic)

suggesting that endogenous tra2-beta1 changed its intracellular localization and activated the translation of the reporter. This effect of the endogenous protein is approximately the same when tra2-beta1 was overexpressed under normoxia (Figure 4.11, ESE tra normoxic). Overexpression of tra2-beta1 under hypoxia did not stimulate significantly the translation of the reporter compared to the effect on translation either of endogenous tra2-beta1 under hypoxia or overexpression under normoxia, indicating that this effect is not synergistic. No stimulation on the effect of tra2-beta1 or hypoxia was observed when cells were cotransfected with the reporter lacking the binding site of tra2-beta1 (Figure 4.11, MT mock and MT tra under hypoxia and normoxia). As a negative control, the pLCS-STOP construct was used, which contains a STOP codon within the ESE sequence.

These results indicate that tra2-beta1 stimulates translation of mRNA containing binding site of tra2-beta1 under hypoxic condition that is a novel role of tra2-beta1.

4.2.2.4 Tra2-beta1 ΔRS1, a naturally occurring isoform enhances the translation of the reporter

Phosphorylation/dephosphorylation of the serine residues modulate the conformation, activity and function of the SR proteins. The RS domains play an important role in creating protein-protein interactions. Tra2-beta1 contains two RS domains that flank the central RRM domain, which is necessary for recognition and binding to specific RNA molecules and can interact with other proteins as well. Tra2-beta3 is a naturally occurred isoform of tra2-beta1 that lacks the N-terminal RS domain. It is expressed in several tissues, predominantly in brain, liver and testis (Nayler et al., 1998). Tra2-beta3 specifically repressed inclusion of exons that are activated by the full-length isoform, tra2-beta1 (Grellscheid et al., 2011). It is known that the RS domain is

not required for the activity of SF2/ASF in translation but its second RRM domain plays an important role in stimulate translation of a reporter (Sanford et al., 2005).

Therefore we wanted to study whether the RS domains of tra2-beta1 influence its activity in translation. To determine the role of the RS domains in translation of the reporter construct dual luciferase assays were performed as described above. We transiently cotransfected HEK293T cells with the pLCS-ESE firefly luciferase reporter, *Renilla* luciferase reporter and either Flagged-tagged wild-type tra2-beta1 or mutant lacking the N-terminal RS domain (ΔRS1), mutant lacking the C-terminal RS domain (ΔRS2) or mutant lacking both RS domains (ΔRS1-2). Empty vector was used as a control (mock). Then, 34 hours post-transfection, cell extracts were assayed for luciferase activity. Relative Luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of pLCS-ESE+ control plasmid (mock).

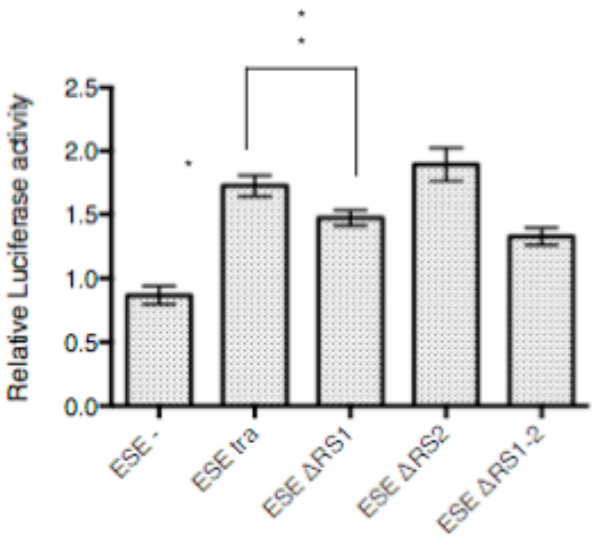


Figure 4.12. The deletion of the RS1 domain of tra2-beta1 decreases the translational activity of the tra2-beta1. HEK293T cells were cotransfected with pLCS-ESE, *Renilla* luciferase reporter, pcDNA expressing wild type tra2-beta1, or ΔRS1, ΔRS2 and ΔRS1-2. Empty vector was used as a control. Dual luciferase enzyme assay system was used to assay the level of Firefly (translational reporter) and the *Renilla* (control the transfection efficiency) luciferase activity. Relative Luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of ESE mock. *p< 0.01, **p<0.05, according to Student`s t-test n= 3 independent experiments.

As shown in Figure 4.12, the deletion of the first RS domain resulted in a decreased stimulation effect on translation (ΔRS1) compared to the wild type. Meanwhile, deletion of the second RS domain (ΔRS2) did not have any effect on the luciferase activity demonstrating that the second RS domain is not required for stimulating translation *in vivo*. The mutant lacking both RS domains (ΔRS1-2) enhanced the luciferase activity approximately at the same level as ΔRS1, suggesting that the RRM domain of tra2-beta1 is necessary and sufficient for translation stimulation.

Taken together, these results suggest that the C-terminal RS domain does not play a regulation role in translation and the RRM domain itself is sufficient to activate translation in vivo. Furthermore, these data indicate that the naturally occurring isoform of tra2-beta1, tra2-beta3 that lacks the N-terminal RS domain might paly a role in regulation of translation.

4.2.2.5 PP1 stimulates the translational activity of tra2-beta1

Tra2-beta1 is a PP1-interacting-protein (Bollen et al., 2010). PP1 interacts with tra2-beta1 through a conserved, short and degenerate RVxF-type motif that is located on its RRM domain (Hendrickx et al., 2009; Novoyatleva et al., 2008) and regulates its mRNA splicing activity through dephosphorylation. The formations of PIP-PP1 complexes modulates the substrate specificity, activity and subcellular localization of PP1 that leads to regulation of diverse cellular processes by PP1 (Cohen, 2002). It has been reported that PP1 binds to ribosomal protein L5 (RPL5) and cosediments with ribosomal particles containing RPL5 (Hirano et al., 1995). It has been previously proposed that PP1 plays a role in regulation of translation initiation by forming a complex with human inhibitor 1 (I1) and Growth Arrest and DNA Damage-inducible protein 34 (GADD34) (Connor et al., 2001). It was previously reported that hypophosphorylated SF2/ASF associated with the translation machinery and

hyperphosphorylation of its RS domain decreased its activity in translation (Sanford et al., 2005).

To investigate whether PP1 regulates the function of tra2-beta1 in translation, we utilized the dual luciferase assay as described previously. HEK293T cells were co-transfected with the luciferase reporter constructs, Flag-tagged tra2-beta1 and either EGFP-tagged PP1 or three site-specific PP1 mutants. We tested two catalytically less active point mutants, H125A and D64N (Zhang et al., 1996) and F257A a mutant that has decreased affinity for the RVxF-binding motif (Lesage et al., 2004). Overexpression of PP1 with tra2-beta1 significantly increased the luciferase activity (Figure 4.13.A, tra2-beta1/PP1). Meanwhile, PP1 H125A, the catalytically less active mutant, and F257A, the binding mutant, decreased the translational activity of tra2-beta1 (Figure 4.13.A, tra2-beta1/H125A and tra2-beta1/F257A). The PP1 D64N mutant, which has a decreased enzymatic activity, did not influence the activity of tra2-beta1 in translation of the luciferase reporter.

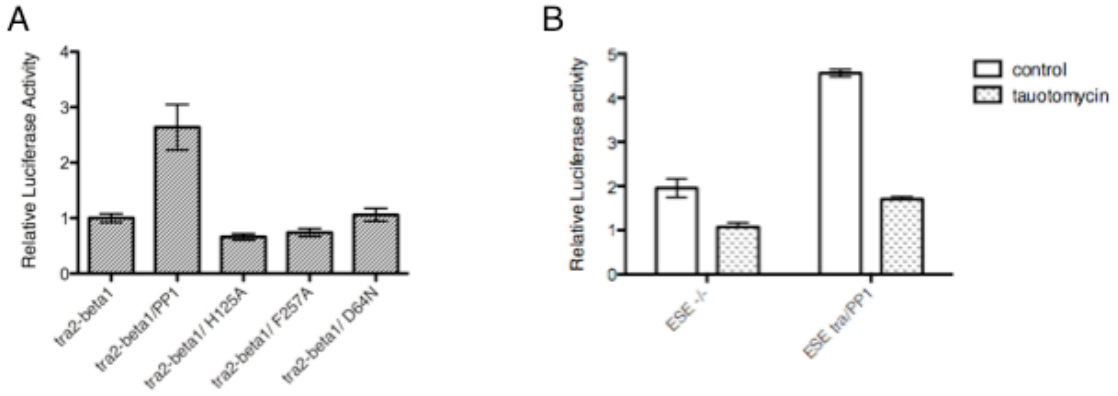


Figure 4.13. PP1 stimulates the activity of tra2-beta1 in translation. Dual luciferase enzyme assay system was used to assay the level of Firefly (translational reporter) and the *Renilla* (control the transfection efficiency) luciferase activity. Relative Luciferase activity was calculated as the normalized luciferase value divided by the normalized luciferase value of ESE mock. *A*, HEK293T cells were transiently transfected with pLCS-ESE, wild type tra2-beta1 and wild type PP1 or its mutant forms (phosphatase inactive mutants: H125A and D64N, or its binding mutant F257A). *B*, HEK293T cells were transiently transfected with pLCS-ESE, wild-type tra2-beta1 and wild type PP1. Empty vectors were used as controls. 24 hour post-transfection, cells were treated with tautomycin.

To confirm these observations we co-transfected HEK293T cells with the reporter constructs, Flag-tagged tra2-beta1 and EGFP-tagged PP1. 24 hours after transfection the cells were treated for an additional 10 hours with 10 nM tautomycin, which inhibits the phosphatase activity of PP1 (MacKintosh and Klumpp, 1990). Then, firefly and *Renilla* luciferase activities of the cell extracts were measured. As shown in Figure 4.13.B, inhibition of PP1 by tautomycin resulted in a decrease of tra2-beta1 mediated stimulation of translation. We observed a decreased luciferase activity after tautomycin treatment either in the control samples (Figure 4.13.B, ESE-/-) or when tra2-beta1 was overexpressed with PP1 (Figure 4.13.B, tra2-beta1/PP1). These results are consistent with our previous finding (Tang, doctoral thesis, 2009) when tra2-beta1-RATA-NES, a tra2-beta1 mutant, was overexpressed. In this mutant, the PP1 binding motif was interrupted and it contains a nuclear export signal (NES) forcing the protein into the cytosol (Novoyatleva et al., 2008). This mutant was less active in the translation assays than wild-type tra2-beta1 containing NES. These data suggest that PP1 modulates the function of tra2-beta1 in the cytoplasm and dephosphorylation of tra2-beta1 increases its activity in translation.

4.3 Knock down of tra2-beta1

To gain global insights into the role of tra2-beta1 we used a genome-wide approach and performed microarray analysis. To investigate the consequences of tra2-beta1 depletion in HEK293T cells we performed short-interfering-RNA (siRNA)-mediated knockdown of tra2-beta1 using commercially available siRNAs (ON-TARGETplus SMARTpool) followed by Affymetrix Human Exon Junction Array, a high density exon microarray for profiling alternative splicing events and gene expression.

HEK293T cells were transfected with siRNA (ON-TARGETplus

SMARTpool) according to the manufacturer instruction and after the incubation total RNA and protein was isolated. To confirm the efficiency of tra2-beta1 depletion on protein level cell extracts were loaded on SDS-PAGE and transferred to nitrocellulose membrane followed by Western-blot analysis employing anti-tra2-beta1 antisera (Figure 4.14.A). 70% of tra2-beta1 protein was successfully depleted (siRNA) compared to the control (mock). We analyzed the total RNA samples by RT-PCR analysis using primers against tra2-beta1 and as a loading control against β-actin (Figure 4.14. B).

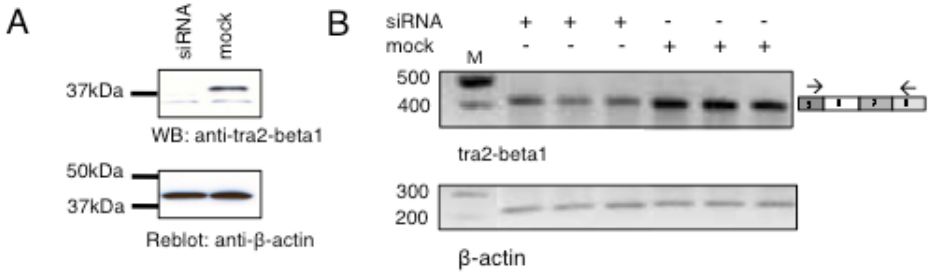


Figure 4.14. Knock down of tra2-beta1. *A*, Knock down of tra2-beta1 was analyzed by Western blot using anti-tra2-beta1 antisera. Samples were reblot with anti-β-actin as a loading control. *B,* Knock down of tra2-beta1 was analyzed by RT-PCR, β-actin were used as a loading control.

The depletion of tra2-beta1 on mRNA level was not as prominent as on the protein level, the mRNA of tra2-beta1 was reduced approximately 60%. RNA was purified on Qiagen columns and the integrity of the RNA was verified using Agilent bioanalyzer. The mRNAs isolated from tra2-beta1 depleted or from control cells were labeled and hybridized to the chip. The chip result was analyzed by commercial software and validated by RT-PCR. Surprisingly low number of regulated exons was found when tra2-beta1 was depleted. Four splicing events were found with high confidence, 55 events with medium confidence and 73 events with low confidence. This result was not expected

since tra2-beta1 is an important splicing factor and changes in its concentration have been shown to affect splice site selection (Tacke et al., 1998). Conformation of array predictions was carried out by RT-PCR analysis. Eight cassette exon with the highest fold changes were tested in the RT-PCR reaction (Table 4.2.A). As shown in Figure 4.15. A, none of the splicing events was validated. Next we wanted to analyze the changes found in gene expression. Again, the validation of the predicted regulated genes was performed by RT-PCR. The list of the validated genes is shown in Table 4.2 B.

***A*, Regulated cassette exons**

Gene symbol	Gene description	Regulation	Fold-change
BBC3	BCL2 binding component 3	Down	1.32
FHL2	Four and a half LIM domains 2	Down	1.30
BSDC1	BSD domain containing 1	Down	1.33
TXNRD2	Thioredoxin reductase 2	Down	1.27
BAZ1A	Bromodomain adjacent to zinc finger domain protein 1A	Up	1.31
HDAC3	Histone deacetylase 3	Up	1.06
ANKRD13C	Ankyrin repeat domain 13C	Up	1.30
DMKN	Dermokine	Up	1.28

***B*, Regulated genes**

Gene symbol	Gene description	Regulation	Fold-change
EIF1	Eukaryotic translation initiation factor 1	Down	1.56
RPL6	60S ribosomal protein L6	Down	1.61
RPL7	60S ribosomal protein L7	Down	1.61
RPL13A	60S ribosomal protein L13a	Down	1.59
TTBK2	Tau tubulin kinase 2	Up	1.55
PPM1J	Protein phosphatase 1J	Up	2.17

SLC38A3	Solute carrier family 38	Up	1.54
ZNF613	Zinc finger protein 613	Up	3.19

Table 4.2. *A*, Regulated cassette exons. *B*, Regulated genes.

We did not observed changes in the expression level when RNA samples were subjected to RT-PCR analysis. Only RPL6 was down regulated (Figure 4.15.B) when tra2-beta1 was depleted.

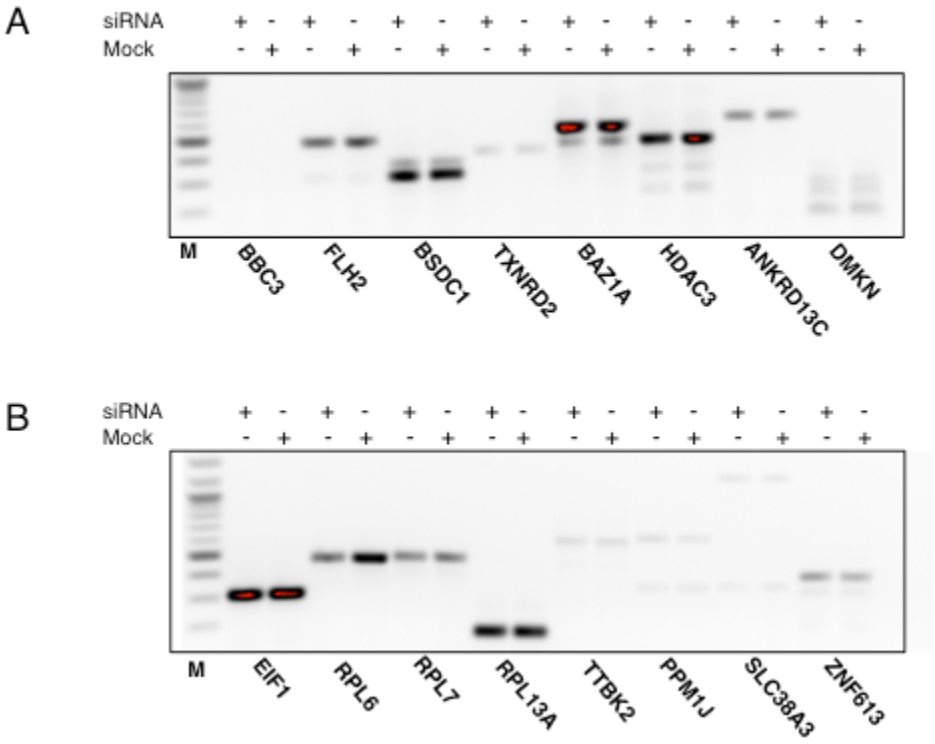


Figure 4.15. Validation of tra2-beta1 knock-down microarray. *A*, Validation of cassette exons. RT-PCR was performed to validate the usage of cassette exons of the indicated pre-mRNAs. *B*, Validation of the regulated genes. RT-PCR was performed using primers against the indicated genes.

Functional analysis was performed using GenoSplice technology and we found that 51 ribosomal protein genes were regulated out of 92, 51 was found down regulated and 1 gene was found up regulated (Figure 4.16.A). To confirm the pathway analysis RT-PCR was performed (Figure 4.16.B). Again, just one

gene showed changes in its expression level, RPL4 was found down regulated when tra-beta1 was depleted.

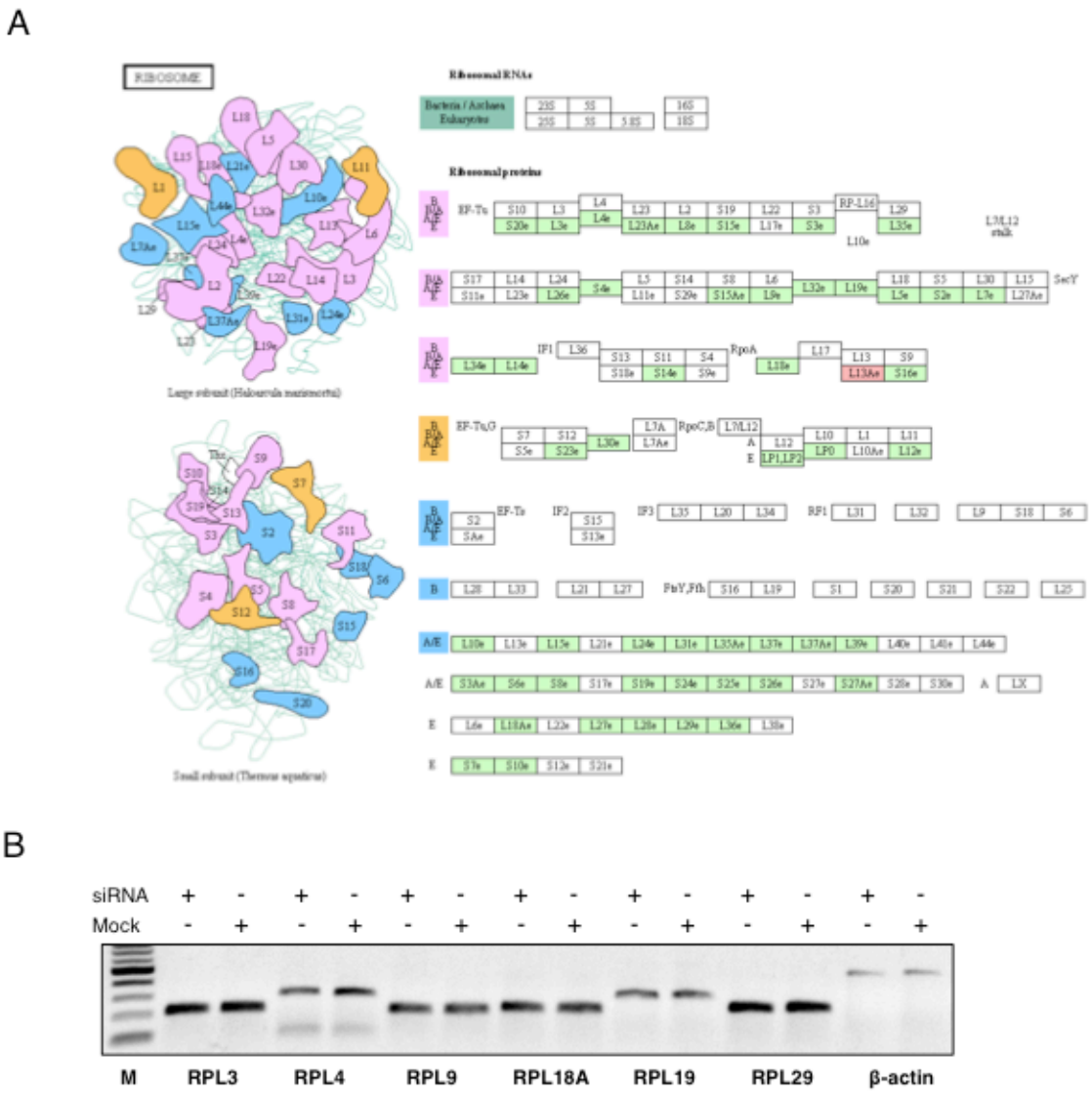


Figure 4.16. Knock down of tra2-beta1 causes down regulation of ribosomal protein encoding genes. *A,* Pathway analysis of the tra2-beta1 microarray. *B*, Validation of the regulated ribosomal protein encoding genes. RT-PCR was performed using primers against the indicated genes.

Taken together these data, the reproducibility of the array analysis was very poor. Tra2-beta1 may be involved in the regulation of ribosomal genes to elucidate its role a more sensitive analysis necessary to use such as quantitative Real-Time PCR analysis.

4.4 PP1 enhances the binding affinity of tra2-beta1 to RNA

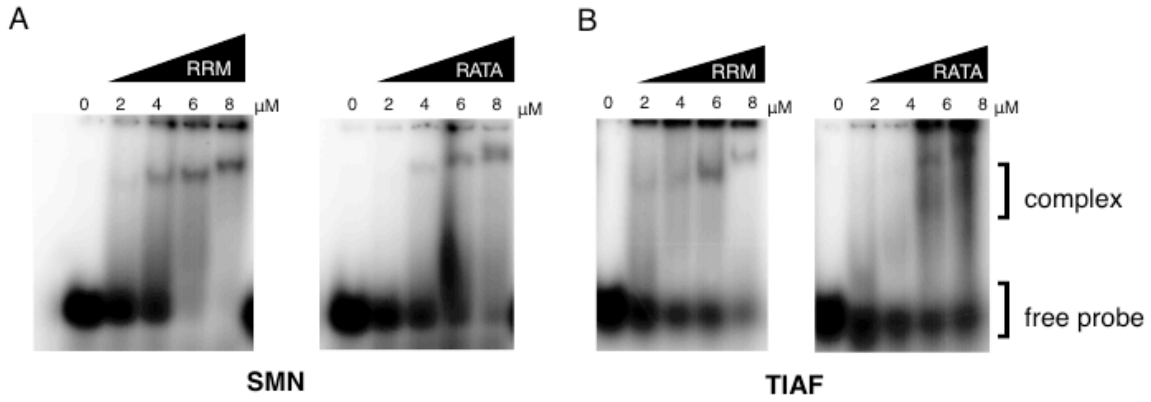
PP1 regulates diverse cellular processes through dephosphorylating its interacting protein (Cohen, 2002). The catalytic core of the enzyme interacts with a very diverse set of distinct PP1-interacting proteins creating a holoenzyme (Bollen et al., 2010). Formation of these complexes with PP1-interacting proteins mediates the function and substrate specificity of PP1. PP1 binds to tra2-beta1 via a conserved PP1 docking motif. This short motif the RVxF motif located on the RRM domain of tra2-beta1. The interaction modulates the activity of tra2-beta1 in splicing. Inhibiting the enzymatic activity of PP1 promotes exon 7 inclusion of the SMN2 minigene (Novoyatleva et al., 2008). Spinal muscular atrophy is a genetic disease causing death of children. Therefore, it is important to understand better the mechanism of this interaction and the regulation of exon 7 alternative splicing.

4.4.1 Tra2-beta1 forms multimers on its target sequences *in vitro*

Tra2-beta1 stimulates the inclusion of SMN2 exon 7 binding directly to an AG-rich ESE sequence located on exon 7 pre-mRNA (Hofmann et al., 2000). The RRM domain of tra2-beta1 determines the recognition and the binding specificity to the RNA and it is involved in protein-protein interactions as well. To investigate

in vitro the interaction between tra2-beta1 and the SMN2 exon 7 pre-mRNA we synthetized a 15 nucleotide long RNA oligonucleotide derived from SMN2 exon 7 that contains this ESE sequence (Figure 4.17. C).

His-tagged recombinant tra2-beta1 protein lacking the RS domains, referred as tra2-beta1 RRM (spanning residues: M101-Y206) and its mutated form where the RVxF-type docking motif of PP1 was disrupted, referred as tra2-beta1 RATA were overexpressed in *E. coli* and purified under denaturing condition in the presence of Ni-NTA agarose. The SMN RNA oligonucleotide and TIAF probe used as a control were radioactively labeled and incubated *in vitro* with tra2-beta1 RRM or RATA. To visualize RNA probe-protein interactions the samples were subjected to non-denaturing polyacrylamide gel electrophoresis.



C

SMN	CAAAAAGAAGGAGG
TIAF	GGUGACAAGUAUCAGAAAAGAAAGAAT

Figure 4.17. Tra2-beta1 forms multimers in the RNA binding complex. Recombinant tra2-beta1 RRM or RATA were incubated with radioactively labelled RNA probes. RNA–protein complexes were then resolved from free probe by electrophoresis on a 5% non-denaturing polyacrylamide gel. Indicated concentration of tra2-beta1 RRM and RATA were added to the reaction. The concentration of all oligonucleotides was 30 pM in all reactions in a total volume of 10µl. *A,* Gel retardation assay with SMN probes. *B*, Gel retardation assay with TIAF probes. *C,* Sequence of the RNA probes.

We observed formation of increasing molecular weight RNA-protein complexes when increasing concentration of tra2-beta1 RRM or RATA was adding to the reaction (Figure 4.17.A-B) indicating multimer formation. The retarded bands were more pronounced in the presence of tra2-beta1 RRM (Figure 4.17. A, left) compared to the RATA mutant (Figure 4.17.A right). The arginine190 part of the RVxF motif plays role in the stabilization of the RRM-RNA complex (Clery et al., 2011). Mutations in its surrounding area could influence the RRM-RNA interaction and decrease the binding affinity towards the RNA. Using TIAF probes we found a similar increasing molecular-weight complex formation in the presence of increasing concentration of tra2-beta1 RRM (Figure 4.17.B, left). TIAF oligonucleotide bound to the RATA mutant with lower affinity and we did not observed the formation of multimers (Figure 4.17.B, right). These results indicate that tra2-beta1 RRM forms multimers on SMN2 exon 7 and TIAF *in vitro*.

4.4.2 PP1 stimulates the formation of the RRM-RNA complex

Tra2-beta1 is a PP1-interacting-protein the direct binding is mediated by the RVxF motif that is located on the beta-4 strand of the tra2-beta1 RRM (Novoyatleva et al., 2008). PP1 binds to several splicing factors and mediate their splicing activity.

To get a further insight to the interaction between tra2-beta1 and PP1, gel retardation assays were performed. Recombinant tra2-beta RRM or RATA was used in the reaction. SMN, TIAF RNA probes were labeled radioactively and as a control RNA oligonucleotide was used lacking tra2-beta1 binding site. Tra2-beta1 RRM or RATA was added to increasing concentration of PP1 (NEB), then RNA probes were mixed to the reaction. Changes in the probes mobility was detected by non-denaturing polyacrylamide gel electrophoresis. Surprisingly, addition of PP1 increased the binding affinity of tra2-beta1 to the RNA oligonucleotides

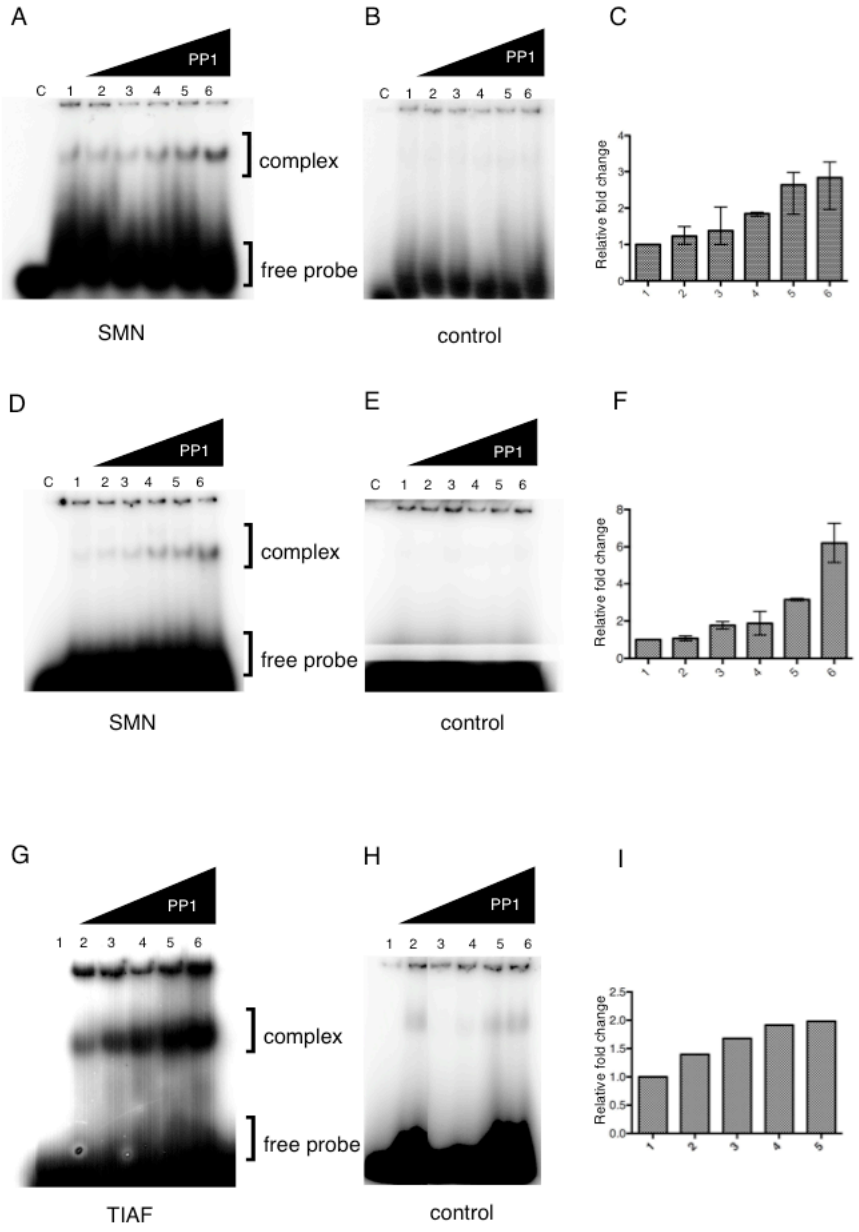


Figure 4.18. PP1 enhances the binding affinity of tra2-beta1 to RNA. *A-B*, Gel retardation assay with smn probe or the control oligo and RRM. Increasing concentration of PP1 was added to the reaction. The protein concentrations for PP1 were 0.13, 0.26, 0.52, 0.65 and 1.3μM in lanes 3-7, respectively. The concentration of all oligonucleotides was 30 pM in all reactions in a total volume of 10*μ*l. The concentration of tra2-beta1 RRM was 4 μM in lanes 2-7. *C*, The fold change of the intensity of the RRM-oligo comlexes. Data was analyzed by Imagequant programme.

D-E, Gel retardation assay with smn probe or the control probe and tra2-beta1 RRM RATA mutant. Increasing concentration of PP1 was added to the reaction. The protein concentrations for PP1 were 0.13, 0.26, 0.52, 0.65 and 1.3µM in lanes 3-7, respectively. The concentration of all oligonucleotides was 30 pM in all reactions in a total volume of 10*µ*l. The concentration of tra2-beta1 RATA was 4 µM in lanes 2-7. *F*, The fold change of the intensity of the RATA-oligo comlexes. Data was analyzed by Imagequant programme. *G-H*, Gel retardation assay with the TIAF probe or the control probe and RRM. Increasing concentration of PP1 was added to the reaction. The protein concentrations for PP1 were 0.26, 0.52, 0.65 and 1.3µM in lanes 3-6, respectively. The concentration of all oligonucleotides was 30 pM in all reactions in a total volume of 10*µ*l. The concentration of tra2-beta1 RRM was 4 µM in lanes 2-6.

(Figure 4.18.A-H). This was not expected, since the arginine from the RVDF PP1 binding motif contributes in the stabilization of the RNA-protein complex. Furthermore, it is known from NMR experiments that the arginine makes an intermolecular stacking interaction with the A2 in complex with the 5`-AAGAAC-3` oligonucleotide (Clery et al., 2011). As show in Figure 4.18.A, the binding affinity of RRM increased in a stepwise fashion towards the SMN probe (Figure 4.18.A), meanwhile PP1 did not increase the formation of RNA-RRM complexes when control probe was used. Similarly, we observed an increase in the binding affinity when RATA mutant was added to the reaction (Figure 4.18.D), however the intensity of the shifted bands was lower compared to the wild type protein, on the other hand its fold change was more dramatic adding increasing concentration of PP1 to the reaction (Figure 4.18. F). PP1 did not stimulate the formation of RNA-RATA complexes when the control probe was used lacking tra2-beta1 binding site (Figure 4.18.E). Increased binding affinity was observed when TIAF probe was tested (Figure 4.18.G), however we found a moderate increase in the intensity of relative fold change (Figure 4.18.I). Taken together, these data suggest that PP1 could stimulate the binding affinity of tra2-beta1 RRM towards RNAs containing its binding site.

4.4.3 The effect of PP1 to stimulate the binding affinity of tra2-beta1 to RNA is independent of its catalytic activity

Next we tested whether the function of PP1 to stimulate the tra2-beta1-RNA complex formation is depending on its catalytic activity. To answer on this question gel retardation assay were performed as described above and the reaction was substituted with PP1 inhibitors. We add 10µM cantharidin or C6 pyridinium ceramide to the reactions. Cantharidin is a compound produced by blister beetle and it inhibits the phosphatase activity of PP1 (Novoyatleva et al., 2008). C6 pyridinium ceramide is a water-soluble ceramide analog and it binds directly to PP1 and inhibits its catalytic activity (Sumanasekera et al., 2012).

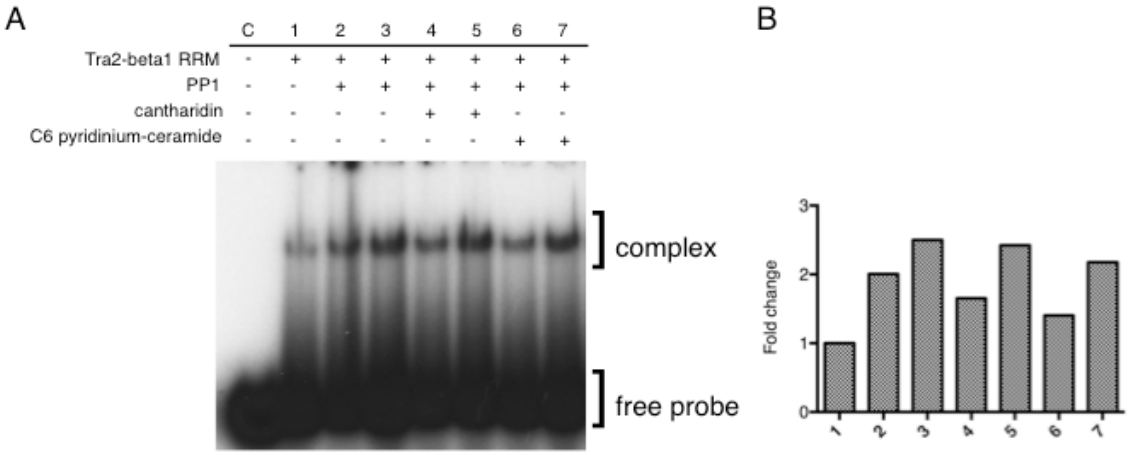


Figure 4.19. PP1 stimulates the binding affinity of tra2-beta1 to RNA that is independent of its catalytic activity. Gel retardation assay with the SMN probe. The concentration of all oligonucleotides was 30 pM in all reactions in a total volume of 10µl. The concentration of tra2-beta1 RRM was 4 µM in lanes 1-7. The protein concentrations for PP1 were 0.65 in lanes 2, 4 and 6 and 1.3µM in lanes 3, 5 and 7, respectively. 10 µM cantharidin was added to lanes 4-5, 10 µM pyridinium ceramide was added to lanes 6-7. B, The relative fold change of the intensity of the RRM-oligo comlexes. Data was analyzed by Imagequant programme.

As shown in Figure 4.19, this enhancer function of PP1 was not inhibited by cantharidin or C6 pyridinium ceramide. PP1 enhanced the binding between the RNA and the protein in the presence of the compounds.

Next, we asked whether this effect is PP1 specific, to answer this question we used recombinant λ Protein Phosphatase (λPP) in gel retardation assay with tra2-beta1 RRM and the SMN probe. As shown in Figure 4.20, we did not observed any effect on the RNA binding affinity of tra2-beta1 RRM when increasing concentration of λPP was added to the reaction.

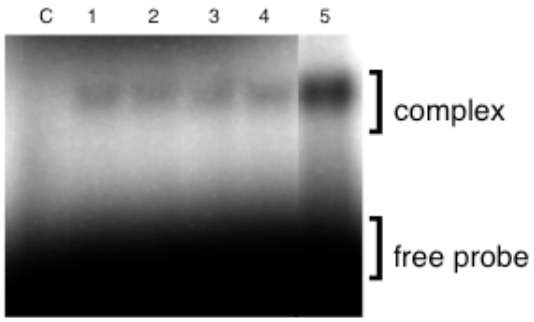


Figure 4.20. PP1 specifically stimulates the binding affinity of tra2-beta1 to RNA. Gel retardation assay with the SMN probe. The concentration of all oligonucleotides was 30 pM in all reactions in a total volume of 10μl. The concentration of tra2-beta1 RRM was 4 µM in lanes 1-5. The protein concentrations for λPP1 were 0.26, 0.52 and 0.65 µM in lanes 2, 3 and 4, respectively. The protein consentration of PP1 was 0.65 µM in lane 5.

These data suggests that he function of PP1 to stabilize the RNA-tra2-beta1 RRM complexes is specific and independent of its enzymatic activity. A noncatalityc function of PP2A has been reported recently. PP2A acts as a recruiter localizing condensing II and KIF4a to chromosomes (Takemoto et al., 2009).

4.4.4 PP1 is not part of the RNA-protein complex

Next we wanted to know whether PP1 is part of the RNA-tra2-beta1 complex and its function of stimulating the binding affinity of tra2-beta1 RRM towards the RNA regulated by a direct interaction with the complex. Supershift assays were performed to answer to this question. We used in our gel retardation assay a tra2-beta1 antiserum that was developed previously against it (Daoud et al., 1999) and PP1α antibody from Santa Cruz to detect PP1 in the complex.

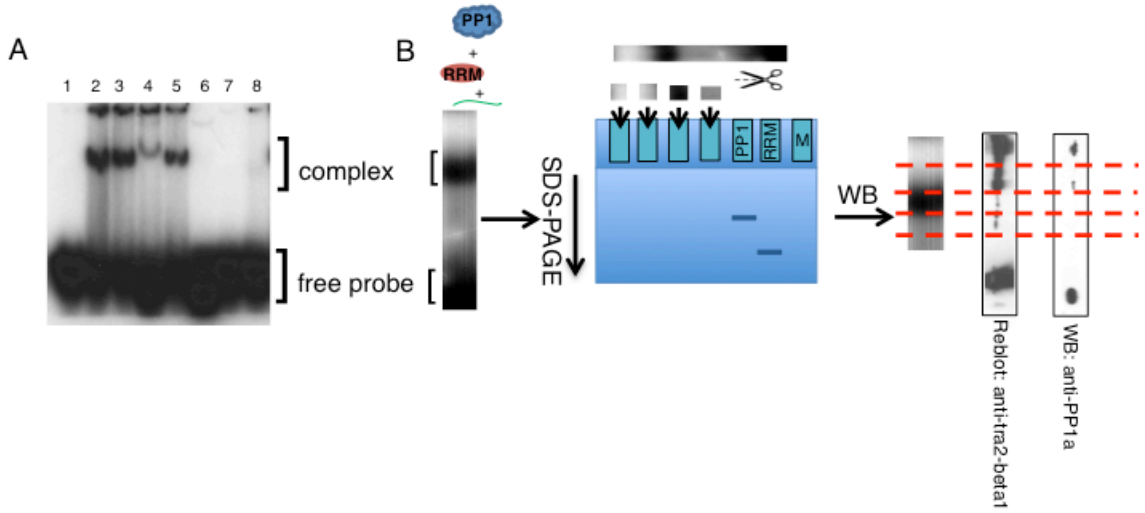


Figure 4.21. PP1 is not present in the RNA-RRM binding complex. *A,* Gel retardation assay with the SMN probe. The concentration of all oligonucleotides was 30 pM in all reactions in a total volume of 10*μ*l. The concentration of tra2-beta1 RRM was 4 μM in lanes 2-5 and lane 8. The concentration of PP1 was 0.65 μM in lanes 3-5. In lane 4 anti-tra2-beta1, in lane 5 anti-PP1α was added in 1:100. As a control in lanes 6-7 the antiserums were added (anti-tra2-beta1 and anti-PP1α, respectively) without the proteins. In lane 8 cold RNA oligonucleotide was added in 3 μM concentration. *B*, Gel shift assay were performed, after the electrophoresis proteins were transferred on membrane and Western blot was performed to identify the proteins in the complex. As controls purified tra2-beta1 RRM and PP1 was loaded to the gel.

We observed an increased shift in the presence of anti-tra2-beta1 but not anti-PP1α (Figure 4.21.A). However the binding affinity of tra2-beta1 toward the RNA decreased in the presence of the antiserum because the antibody epitope is located right next to the RVDF motif therefore the interaction between the antiserum and tra2-beta1 can partially cover the residues that plays role in the RNA recognition.

To confirm the observation that PP1 is not par of the tra2-beta1 RRM-RNA complex we performed Western-blot analysis. After gel shift assay was performed the lane, which contained the RNA probe, tra2-beta1 RRM and PP1, was cut to four pieces. The gel pieces were loaded on SDS-PAGE gel and after the electrophoresis it was transferred in to nitrocellulose membrane. The membrane was analyzed by Western-blot using tra2-beta1 antisera and anti-PP1α antibody to detect the proteins in the complex. As Figure 4.21.B shown, PP1 was not present in the tra2-beta1-RNA complex. It formed higher molecular weight complex with tra2-beta1 at the top of the gel. These data indicates that PP1 is not directly part of the tra2-beta1-RNA complex. PP1 could play a similar role like PP2A to localize condensing to the chromatin (Takemoto et al., 2009).

About two-thirds of the interaction proteins that contains The RVxF-type binding sequence predicted to have disordered region. The motif is flanked by these disordered regions in almost half of these PP1 interacting proteins, including tra2-beta1 (Bollen et al., 2010). To determine the disordered regions of tra2-beta1 we used bioinformatics analysis. We preformed the analysis using FoldIndex (Prilusky et al., 2005) and DISOPRED (http://bioinf.cs.ucl.ac.uk/disopred) two freely available graphic web servers that were designed to predict intrinsically disordered proteins.

Comparing the results generated by these computational tools tra2-beta1 contains two distinct unstructured regions. The first predicted region contains amino acid residues from 1 to 120. This region covers the N terminal RS domain

of tra2-beta1. The second region contains the amino acid residues from 193-288, which residues compose the C-terminal RS domain (Figure 4.22.A-B,).

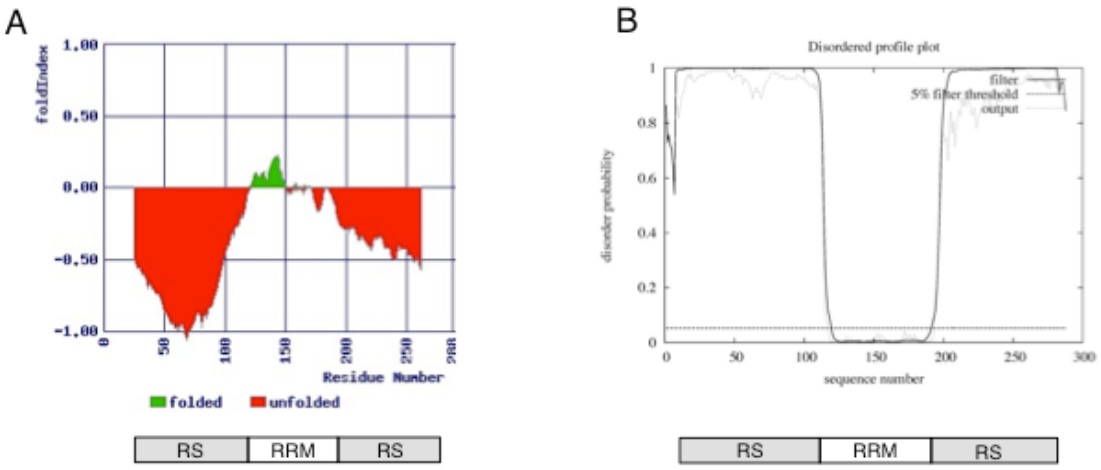


Figure 4.22. Tra2-beta1 contains two predicted intrinsically disordered region. *A,* Tra2-beta1 sequence generated by FoldIndex (Prilusky et al., 2005). The predicted disordered segments are shown in red. *B*, Disordered profile plot of tra2-beta1 generated by DISOPRED2 (http://bioinf.cs.ucl.ac.uk/disopred). Predicted disordered residues are marked with asterisk and highlighted in red. The schematic structure of tra2-beta1 showed at the bottom.

Intrinsically disordered proteins are unstructured or contain unstructured segments. These segments play an important role in regulating the function of the protein (Dunker et al., 2001). An increasing number of PP1 interacting proteins belong to the intrinsically disordered proteins. The highly dynamic nature of intrinsically disordered proteins provide an increased binding specificity despite the short, degenerate PP1 docking motifs. Formation of a stable PP1-interacting protein complex is associated with the folding of these proteins. It was recently proposed that PP1 might have a chaperone function that is not depend on its enzymatic activity (Heroes et al., 2012).

The interaction of tra2-beta1 with PP1 can change the conformation of tra2-beta1. This conformational change can lead to an increased binding affinity

of tra2-beta1 to RNA. This function of PP1 is independent of its phosphatase activity.

5 Discussion

5.1 Pseudocantharidin B and C change phosphorylation of tra2-beta1 at position T33 and promotes SMN exon 7 inclusion

Post-translational modifications (PTM) such as methylation, acetylation and phosphorylation play important roles in the regulation of cellular processes by modifying the properties of proteins altering their activities, conformations and binding affinities. Among PTMs phosphorylation is the best studied, occurring predominantly on serine residues (~90%) and with a less frequency on threonine (~10%) and tyrosine residues (~<0.05%) (Nita-Lazar et al., 2008).These reversible changes on the protein can modulate protein-protein interactions, signal transduction and cell-cycle events (Deribe et al., 2010). The use of antibodies that are specific for the modifications can give information about the changes in the PTM profile and help to understand the mechanisms that lead to these changes. The activity of tra2-beta1 in splicing is regulated by reversible phosphorylation (Novoyatleva et al., 2008). The N- and C-terminal serine/arginine rich domains contain several serine residues. The phosphorylation status of these residues modulates the function of tra2-beta1.

Three sets of phosphorylation-specific antibodies were raised against specific amino acid residues of tra2-beta1. The selected residues were previously identified by mass spectrometric analysis and found to undergo reversible phosphorylation during the splicing process (Bessonov et al., 2008). Phosphorylation-specific antisera were generated against phospho-threonine33 (pT33), phospho-serine95, 97 and 99 (pS95-97-99) and phospho-threonine201

(pT201). We validated the specificity of these antisera using point mutants either mimicking the phosphorylated form, or the non-phosphorylated from of tra2-beta1. We found that these antisera are highly specific for phosphorylation of tra2-beta1 at T33, S95-97-99 and T201.

Pseudocantharidins are generated from the well-established PP1 and PP2A inhibitor cantharidin. These compounds promote exon 7 inclusion when used in reporter gene assays and promote production of full-length SMN protein in patient fibroblasts, demonstrating their effect in a physiological system. Pseudocantharidin inhibits both PP1 and PP2A activity, while Pseudocantharidin B and C activate PP2A and have no effect on PP1, Pseudocantharidin D does not show any phosphatase inhibition (Zhang et al., 2011).

We used a site-specific anti-phosphotyrosine antisera against residue T-33 of tra2-beta1 to show that pseudocantharidin B and C promotes dephosphorylation of this amino acid. Tra2-beta1 binds to an enhancer sequence element of exon 7 and strongly promotes exon inclusion (Hofmann et al., 2000). The role of T-33 in exon 7 inclusion was supported by mutational analysis, because an alanine replacement mutant promoted exon 7 inclusion more effectively than wild type tra2-beta1 or the glutamic acid mutant. The exact molecular role of this phosphorylation remains to be determined. It is likely that T-33 indicates the dephosphorylation of other, as yet unidentified residues. Collectively, these changes could influence splice site selection. Although the composition of the complexes forming on exon 7 has not been determined, it is likely that similar to other SR proteins (Wahl et al., 2009), tra2-beta1 is present in spliceosomal A, B, and C complexes. Dephosphorylation of this and possibly other sites could increase the affinity of the spliceosome to exon 7, resulting in improved exon recognition. Our results demonstrate that modification state-specific antibodies can be powerful tools in research.

5.2 Tra2-beta1 binds to ceramide responsive exons

All polymerase-II transcripts undergo pre-mRNA processing and at least 95% of the transcriptional units are alternatively spliced (Pan et al., 2008). Regulation of alternative splicing is physiologically important, as an increasing number of human diseases are caused by improper splice site selection (Tazi et al., 2009). The regulation of alternative splicing is controlled by combinatorial interactions between *cis*-acting elements and *trans*-acting regulatory factors (Hertel, 2008). Various hnRNPs and SR-proteins bind to degenerate sequence elements to increase the specificity of splice site selection. The interaction between the proteins is regulated in part by reversible phosphorylation. For example, PP1-mediated dephosphorylation promotes the interaction between tra2-beta1 and SF2/ASF in vitro (Novoyatleva et al., 2008). Reversible phosphorylation of SR proteins and hnRNPs is regulated by interplay between protein kinases and phosphatases. Several SR-proteins contain an evolutionarily conserved RVxF-type PP1 docking-motif in their RRM, which allows PP1 to modulate their activity (Novoyatleva et al., 2008). A change in PP1 activity has been shown to alter splice site selection *in vivo* (Chalfant et al., 2002; Novoyatleva et al., 2008).

Ceramides are a class of sphingolipids that are composed of shingosine and a fatty acid moiety. Previously, it was found that short chain d-erythro-C6 ceramide (Chalfant et al., 2001) and long-chain d-erythro-C18-ceramide activate PP1 (Chalfant et al., 1999) and cause a decrease in SR-protein phosphorylation. C6 pyridinium ceramide is an anti-cancer drug candidate that is currently being tested for the treatment of squamous cell carcinomas (Karahatay et al., 2007). C6 pyridinium ceramide, unlike the water-insoluble, natural C6 ceramide inhibits PP1 activity and therefore prevents the dephosphorylation of splicing regulatory proteins including, SF2/ASF, tra2-beta1, PSF/SFPQ, UAP56 and SAP155. C6 pyridinium ceramide changes alternative splicing of several pre-mRNAs to

promote both inclusion and skipping of the target exons. The most significant effects were observed on the pre-mRNAs of DRF1, TIAF1, POL-β, TAU and SYK. Bioinformatic analysis of the affected exons showed that C6 pyridinium ceramide regulated exons are unusually short and share suboptimal splice sites and two 4-nucleotide motifs, GAAR and CAAR (Sumanasekera et al., 2012). C6 pyridinium-dependent alternative exons contain binding sites characteristic for splicing enhancers. To increase the specificity of the alternative splicing events the usage of these exons depends on these enhancer/silencer sequences. Bioinformatic analysis using ESE finder (Smith et al., 2006) predicted SF2/ASF binding motifs in all the tested exons. Furthermore, almost all of the C6 pyridinium ceramide responsive exons contained the high affinity tra2-beta1 binding motif, AGAA (Clery et al., 2011). SELEX experiments showed that PSF binds to sequences containing GAA motifs (Peng et al., 2002) that are enriched in C6 pyridinium ceramide-responsive exons. We confirmed that tra2-beta1, SF2/ASF and PSF/SFPQ bind directly to the tested ceramide-responsive exons using gel retardation assays.

C6 pyridinium ceramide responsive alternative exons have suboptimal length, suboptimal splice sites and contain binding sites characteristic for splicing enhancers. Our data indicates that splicing factors bind directly to these sequences to increase the specificity of alternative splicing events.

5.3 The role of tra2-beta1 in the cytoplasm

Human tra2-beta1 was first identified using a yeast two-hybrid screen, and was found to colocalized in the nucleus with SC35 (serine/arginine-rich splicing factor 2) in a speckled pattern (Beil et al., 1997). Tra2-beta1 belongs to the SR-like proteins and activates pre-mRNA splicing in a sequence-specific and concentration-dependent manner (Tacke et al., 1998). Tra2-beta1 has a unique composition, containing a central RRM flanked by C- and N-terminal RS

domains. The RRM domain recognizes and binds to specific RNA sequences that are rich in purines (Stoilov et al., 2004; Tacke et al., 1998). The molecular structure of tra2-beta1 RRM in complex with 5`AAGAAC-3` RNA has been solved recently (Clery et al., 2011). Under steady state conditions the protein predominantly localizes in the nucleus, however it shuttles between nucleus and cytoplasm (Nayler et al., 1998; Stoilov et al., 2004). It has been shown that tra2-beta1 is overexpressed in breast and ovarian cancer (Fischer et al., 2004; Watermann et al., 2006) and under stress conditions its intracellular localization switches towards the cytoplasm and change alternative splicing (Daoud et al., 2002; Hirschfeld et al., 2011). Although regulation of alternative pre-mRNA splicing in the nucleus by tra2-beta1 has been extensively studied its role in the cytoplasm has not been elucidated yet.

Previous studies reported that tra2-beta1 binds directly to RPL3 (ribosomal protein 3), which protein part of the large ribosomal subunit that plays a role in peptidyltransferase center formation. Furthermore, it was shown that in tra2-beta1 co-sediments with ribosomes and polysome fractions during sucrose gradient fractionation (Tang, doctoral thesis, 2008).

Endogenous cellular RNA targets for tra2-beta1 were identified from mouse testis cells employing high throughput sequencing cross-linking immuno-precipitation (HITS-CLIP) studies (Grellscheid et al., 2011). A portion of the RNA targets (2.54%) were found in regions located within ncRNAs, including the small subunit rRNA. We analyzed the target sequences mapped to the rn45s gene that sequence codes rRNAs as rDNA repeating units. The 45S rRNA serves as the precursor for the 18S, 5.8S and 28S rRNA, and it is transcribed from each rDNA unit by RNA polymerase I. The CLIP tag sequences clustered in 9 regions and contained 14 binding sites for tra2-beta1. Two of them contained the high affinity AGAA binding site and seven the lower affinity GGAA binding site of tra2-beta1. Mapping the CLIP tags to the three dimensional structure revealed a clustering in the so-called ES6 region. Eukaryotic rRNS contain several additional nucleotide

elements inserted in the conserved core sequence called expansion segments (ES). These expansion segments can provide a platform for interaction with regulator proteins (Ben-Shem et al., 2011; Klinge et al., 2012). We confirmed the direct interaction between tra2-beta1 and the 18S rRNA using gel retardation assays with recombinant proteins and commercially synthetised RNA oligonucleotides.

Recently, more and more shuttling SR proteins are shown to play roles in translation. It was reported that SF2/ASF stimulates the translation of a reporter both in vivo and in vitro (Sanford et al., 2004). SRp20 participates in the IRES-dependent translation of a viral RNA (Bedard et al., 2007). 9G8 stimulates the translation of unspliced RNA containing a constitutive transport element (CTE) (Swartz et al., 2007).

To investigate the activity of tra2-beta1 in the regulation of translation, we devised a luciferase translation reporter construct containing the tra2-beta1 binding motif. We found that the expression of the luciferase reporter construct containing a tra2-beta1 binding site in HEK293T cells increased the luciferase activity demonstrating that tra2-beta1 stimulated in vivo the translation of the construct containing its binding-site. We have shown that overexpression of tra2-beta1 significantly induced the translation of the tra2-beta1-dependent reporter mRNA.

Since tra2-beta1 accumulates in the cytoplasm under stress conditions (Daoud et al., 2002; Hirschfeld et al., 2011), we investigated its intracellular localization under hypoxic and acidic conditions. Our results have shown that after ten hours incubation under hypoxia there is a significant shift in the intracellular localization of tra2-beta1 to a pronounced cytoplasmic presence. We observed a markedly increased luciferase activity after oxygen deprivation indicating that tra2-beta1 activates the translation of the reporter mRNA containing its binding site under hypoxia. The microenvironment in malignant tumor is characterized by low pH and oxygen deficiency (Harris, 2002; Izumi et

al., 2003). In response to hypoxia, global protein synthesis is reduced to conserve ATP (Liu and Simon, 2004). Meanwhile, the translation of specific transcripts, which contribute to cancer cell progression, are induced in malignant cells (Brugarolas et al., 2004; Connolly et al., 2006). Changes in protein synthesis regulation, can facilitate expression of proteins required for cellular transformation, promotion of angiogenesis, and inhibition of apoptosis (Rosenwald, 2004). hnRNP A18 belongs to the heterogeneous nuclear ribonucleoproteins that are involved in a variety of processes occurring in the nucleus, including pre-mRNA splicing. hnRNP A18 is up regulated in several human cancers, including breast and colon cancers (Artero-Castro et al., 2009). Under normal physiologic conditions it is expressed in the nucleus but in response to cellular stress it accumulates in the cytoplasm (Wellmann et al., 2004) and regulates the translation of specific mRNA transcripts (Yang and Carrier, 2001). These studies show that tra2-beta1 can play an important role not just in pre-mRNA splicing but also in the regulation of translation of specific transcripts during cancer progression. This novel function of tra2-beta1 should be further studied.

The Sfrs10 gene encodes tra2-beta1 and its primary transcript is alternatively spliced, generating five mRNA isoforms (Nayler et al., 1998). The major isoform encodes full-length tra2-beta1 that auto-regulates its own cellular concentration via the alternative splicing of its own exon2. Inclusion of exon 2 results in mRNAs that are not translated into protein (Stoilov et al., 2004). The expression of the tra2-beta3 isoform is developmentally regulated and is present predominantly in brain, liver and testis (Nayler et al., 1998). This isoform lacks the N-terminal RS domain and functions as splicing repressor (Grellscheid et al., 2011; Nayler et al., 1998; Stoilov et al., 2004). We found that the C-terminal RS domain of tra2-beta1 does not play a regulatory role in translation in our system and the RRM domain itself is sufficient to activate translation *in vivo*. Furthermore, our data indicate that tra2-beta3, the naturally occurring isoform of

tra2-beta1, that lacks the N-terminal RS domain, might play a role in regulation of translation.

Phosphorylation/dephosphorylation plays an important role in regulation of distinct cellular processes. It has been shown in several studies that the phosphorylation status of the RS domain of SR proteins modulate their intracellular localization, interactions with other proteins and RNA (Twyffels et al., 2011). During spliceosomal assembly, the RS domains are hyperphosphorylated and become partially dephosphorylated during the splicing process (Colwill et al., 1996; Wang et al., 1998). Phosphorylation of the RS domain is necessary for interaction with the transportin-SR2 protein, which plays a role in the nuclear import of SR proteins (Lai et al., 2000; Lai et al., 2001) and dephosphorylation of the RS domain is required for the TAP/NXF1 (nuclear RNA export factor)-dependent export (Huang et al., 2004). It has been reported that hypophosphorylated SF2/ASF is bound to cytoplasmic mRNA and activates its translation (Sanford et al., 2005). To investigate the function of reversible phosphorylation of tra2-beta1 in translation, we coexpressed PP1 with tra2-beta1 in our luciferase system. PP1 interacts with tra2-beta1 through a conserved, short and degenerate RVxF-type motif that is located on its RRM domain (Hendrickx et al., 2009; Novoyatleva et al., 2008) and regulates its mRNA splicing activity through dephosphorylation. We observed a further increase in the translation of a luciferase reporter construct when PP1 was coexpressed with tra2-beta1 suggesting that dephosphorylation of the RS domains stimulates the translation activator function of tra2-beta1. We confirmed this observation by inhibiting PP1 enzymatic activity by tautomycin.

In summary, we have shown that tra2-beta1 binds to the 18s rRNA, stimulates translation in HEK293T cells during hypoxia and its activity in regulating translation is modulated by reversible phosphorylation. Although the precise role of tra2-beta1 in translation has not been determined, these results

suggest that tra2-beta1 can play an important role in cancer progression by regulating translation of specific RNA transcripts.

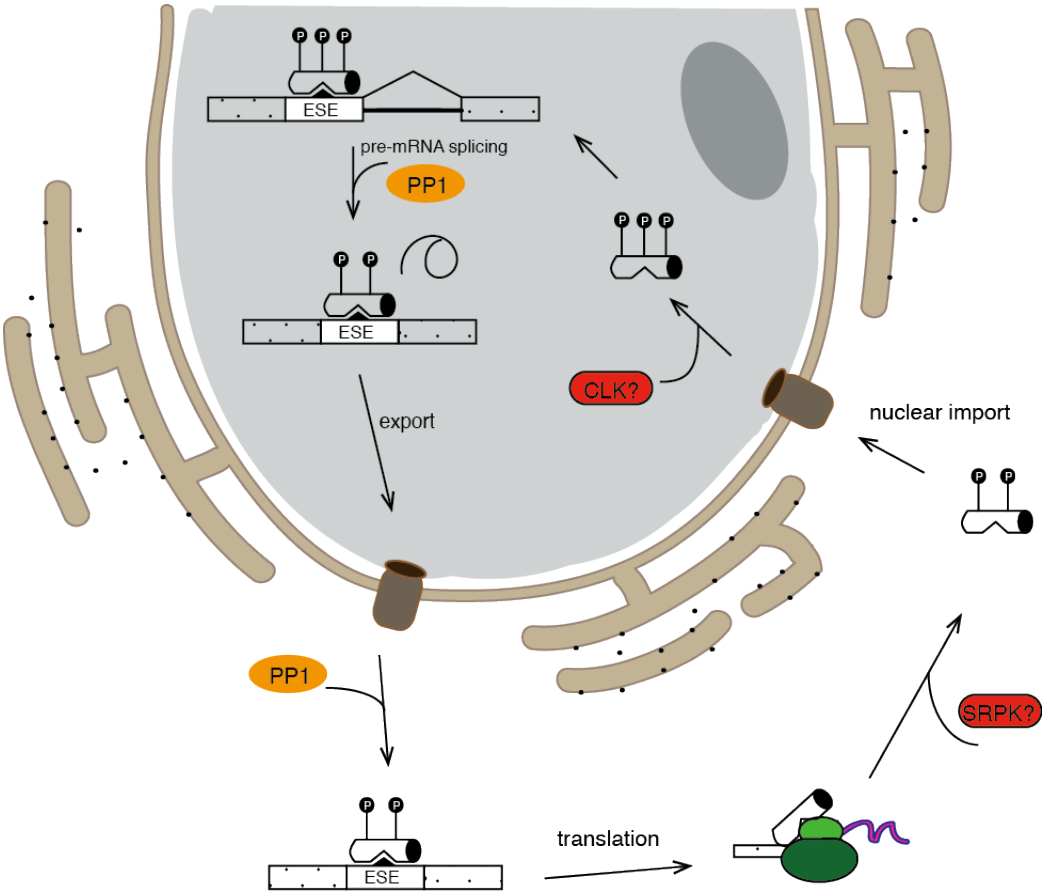


Figure 5.1. Model of the role of tra2-beta1 in translation. In the nucleus, CLK2 and other kinases are involved in tra2-beta1 phosphorylation. Hyperphosphorylated tra2-beta1 binds to specific pre-mRNAs and associates with the spliceosome to stimulate exon inclusion. During the splicing tra2-beta1 is dephosphorylated by PP1 and stay bound to the m-RNA forming mRNP complex with other factors that is exported to the cytoplasm through nuclear pore. In the cytosol, tra2-beta1 is dephosphorylated and recruited to the ribosome to stimulate translation of specific mRNA. Tra2-beta1 exists in several phosphorylation states that are not clearly defined. In the cytoplasm, tra2-beta1 is phosphorylated that facilitates its nuclear import.

5.1 Knock-down of tra2-beta1 down-regulates the expression of ribosomal protein genes

Microarray analysis has proven to be a powerful tool for profiling the expression levels of many genes in a single experiment and to monitoring splicing events (Clark et al., 2002; Johnson et al., 2003). To characterize the effect of tra2-beta1 depletion in HEK293T cells, we utilized a high-density exon array analysis (Affymetrix HJAY). The *Drosophila* homolog of human tra2-beta1, Transformer2 (tra2) is involved in sexual differentiation and spermatogenesis (Baker, 1989). Deficiency of tra2 in *Drosophila* leads to sterility, but otherwise, the loss-of function flies develop normally (Baker and Ridge, 1980) suggesting that it is not essential. In contrast, deletion of tra2-beta1 in mice leads to early embryonic lethality showing that tra2-beta1 has a crucial role during embryonic development (Mende et al., 2010).

siRNA-mediated knock-down of tra2-beta1 did not result in many significant changes of alternative splicing events and the confirmation of the results by RT-PCR was very poor. The low number of affected exons could be a result of functional redundancy between different splicing factors. Depletion of tra2-beta1 in *C. elegans* (rsp-8) resulted in slow growing and stunted worms (Longman et al., 2000). Furthermore, silencing of individual SR genes in *C. elegans* did not lead to any observable phenotypes, except SF2/ASF which caused late embryonic lethality, and no changes in constitutive or alternative splicing were detected when individual SR genes were depleted. These data suggests functional redundancy of SR-proteins in *C. elegans*.

Functional analysis of the microarray results has shown that 51 ribosomal genes were down regulated when tra2-beta1 was depleted. The validation of the pathway analysis by RT-PCR was very poor. It has been reported that depletion of the RNA-binding protein, fox-1 homolog (RBFOX1), which is a neuron specific splicing factor, leads to widespread transcriptional changes (Fogel et al., 2012).

Elucidation of the function of tra2-beta1 in the regulation of the expression of ribosomal protein genes requires further investigation.

5.2 PP1 facilitates the formation of the tra2-beta1 RRM-RNA complex *in vitro*

PP1 regulates diverse cellular processes through dephosphorylation of its interacting proteins (Cohen, 2002). The catalytic core of the enzyme interacts with a diverse set of distinct PP1-interacting proteins creating a holoenzyme (Bollen et al., 2010). Formation of these complexes with PP1-interacting proteins mediates the function and substrate specificity of PP1. PP1 binds to tra2-beta1 via the conserved RVDF PP1 docking motif located on the RRM domain of tra2-beta1. The interaction modulates the activity of tra2-beta1 in splicing.

Tra2-beta1 belongs to the SR-like family of proteins; it contains an N- and C-terminal RS domain and a central RRM. The RRM domain of tra2-beta1 determines the recognition and the binding specificity to the RNA and it is involved in protein-protein interactions as well.

To investigate the interplay between tra2-beta1, a specific RNA oligonucleotide and PP1 we used gel retardation assays. Tra2-beta1 RRM forms increasing molecular weight RNA-protein complexes when increasing concentration of tra2-beta1 RRM is present in the reaction, indicating multimer formation. Surprisingly, when PP1 was added to the reaction in competition with RNA we observed an increased binding affinity of tra2-beta1 RRM to RNA. This observation was not expected since the arginine from the RVDF PP1 binding motif on tra2-beta1 RRM contributes in the stabilization of the RNA-protein complex. Furthermore, it is known from NMR experiments that the arginine makes an intermolecular stacking interaction with the A2 in complex with the 5`-AAGAAC-3` oligonucleotide (Clery et al., 2011).

This novel function of PP1 is independent of its enzymatic activity since this activity was not affected by PP1 inhibitors. In the presence of cantharidin, or pyridinium ceramide PP1-enhanced tra2-beta1 binding was unaffected. Supershift assays indicated that PP1 is not bound to the tra2-beta1-RNA complex. To confirm this observation we performed Western-blot analysis of the complex. Considering our data together, it seems that PP1 can function as a chaperone; it mediates the association of tra2-beta1 to specific RNAs. Recently, a similar function of PP2A was reported. PP2A acts as a recruiter and localizes condensing II and KIF4A to chromosomes, this function of PP2A is independent from its enzymatic activity (Takemoto et al., 2009).

PP1 has nearly 200 validated interactors in vertebrates that modulate its enzymatic activity and intracellular localization. It has been predicted that about two-thirds of all known RVxF-type PP1-interacting proteins contain disordered regions, including tra2-beta1 (Bollen et al., 2010). We defined the predicted disordered regions of tra2-beta1 using computational analysis. Tra2-beta1 contains two predicted unstructured regions that flank its RRM. Intrinsically disordered proteins lack a well-defined tertiary structure or posses long disordered segments even under physiological conditions. Many intrinsically unstructured proteins function via binding to a structured partner and undergo transitions to more ordered states (Dyson and Wright, 2005). The unstructured regions provide functional flexibility to these proteins. They often bind to more than one biological target and these regions are frequently accessible for post-translational modification, allowing regulation of the protein activity and localization. For example, the majority of known post-translational modifications in p53 occur within the disordered N- and C-terminal domains modulating the function, localization and turnover of p53 (Bode and Dong, 2004).

Our data suggests that interaction between tra2-beta1 and PP1 causes structural changes in tra2-beta1 that lead to an increased binding affinity of tra2-beta1 for RNA. A recently proposed binding model of tra2-beta1 supports this

observation, suggesting that the association of tra2-beta1 with RNA changes its conformation, providing a binding platform for other regulatory factors, thereby increasing the binding affinity and specificity for the RNA (Clery et al., 2011). Recently it has been proposed that some PP1 functions may be independent of its phosphatase activity (Heroes et al., 2012). However this chaperone activity of PP1 has not been reported. To our knowledge, our findings are the first demonstration of a chaperone function for PP1 that is independent of its enzymatic activity, although the precise mechanism has yet to be elucidated.

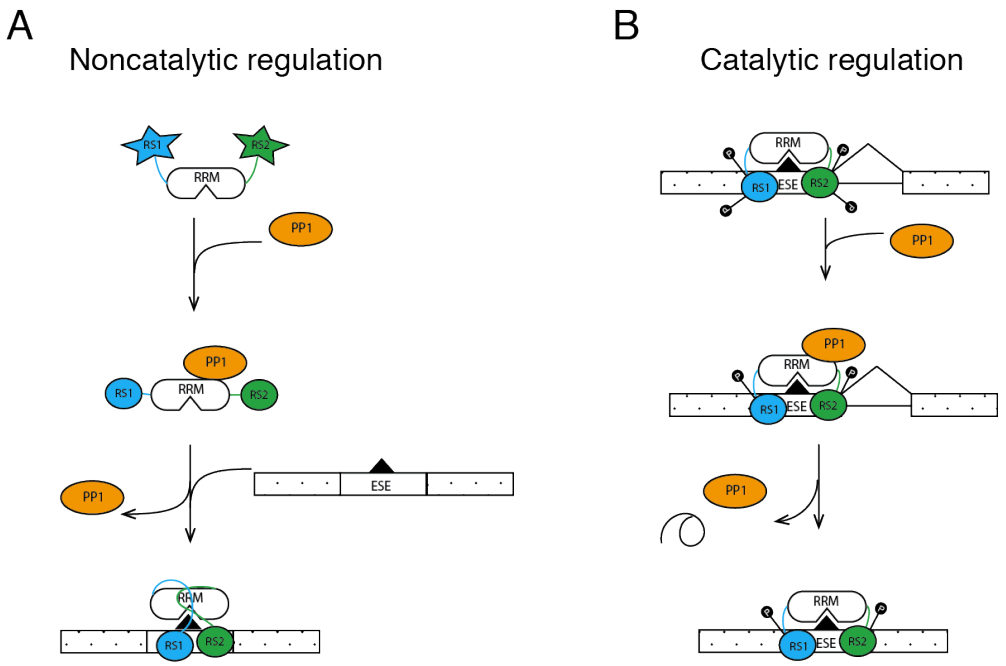


Figure 5.2. Model of the regulation of tra2-beta1 by PP1. PP1 noncatalytically (A) or catalytically regulates tra2-beta1 activity (B). *A*, PP1 binds to tra2-beta1 that leads to conformational changes resulted in a increased binding affinity of tra2-beta1 to RNA. *B*, PP1 regulates the activity of tra2-beta1 through dephosphorylating its RS domains.

6 References

(2004). Finishing the euchromatic sequence of the human genome. Nature *431*, 931-945.
Ajuh, P., Kuster, B., Panov, K., Zomerdijk, J.C., Mann, M., and Lamond, A.I. (2000). Functional analysis of the human CDC5L complex and identification of its components by mass spectrometry. The EMBO journal *19*, 6569-6581.
Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. Journal of molecular biology *215*, 403-410.
Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research *25*, 3389-3402.
Artero-Castro, A., Callejas, F.B., Castellvi, J., Kondoh, H., Carnero, A., Fernandez-Marcos, P.J., Serrano, M., Ramon y Cajal, S., and Lleonart, M.E. (2009). Cold-inducible RNA-binding protein bypasses replicative senescence in primary cells through extracellular signal-regulated kinase 1 and 2 activation. Molecular and cellular biology *29*, 1855-1868.
Ast, G. (2004). How did alternative splicing evolve? Nature reviews Genetics *5*, 773-782.
Baker, B.S. (1989). Sex in flies: the splice of life. Nature *340*, 521-524.
Baker, B.S., and Ridge, K.A. (1980). Sex and the single cell. I. On the action of major loci affecting sex determination in Drosophila melanogaster. Genetics *94*, 383-423.
Bechtel, J.M., Rajesh, P., Ilikchyan, I., Deng, Y., Mishra, P.K., Wang, Q., Wu, X., Afonin, K.A., Grose, W.E., Wang, Y.*, et al.* (2008a). The Alternative Splicing Mutation Database: a hub for investigations of alternative splicing using mutational evidence. BMC research notes *1*, 3.
Bechtel, J.M., Rajesh, P., Ilikchyan, I., Deng, Y., Mishra, P.K., Wang, Q., Wu, X., Afonin, K.A., Grose, W.E., Wang, Y.*, et al.* (2008b). Calculation of splicing potential from the Alternative Splicing Mutation Database. BMC research notes *1*, 4.

Bedard, K.M., Daijogo, S., and Semler, B.L. (2007). A nucleo-cytoplasmic SR protein functions in viral IRES-mediated translation initiation. The EMBO journal *26*, 459-467.
Behzadnia, N., Golas, M.M., Hartmuth, K., Sander, B., Kastner, B., Deckert, J., Dube, P., Will, C.L., Urlaub, H., Stark, H.*, et al.* (2007). Composition and three-dimensional EM structure of double affinity-purified, human prespliceosomal A complexes. The EMBO journal *26*, 1737-1748.
Beil, B., Screaton, G., and Stamm, S. (1997). Molecular cloning of htra2-beta-1 and htra2-beta-2, two human homologs of tra-2 generated by alternative splicing. DNA and cell biology *16*, 679-690.
Ben-Shem, A., Garreau de Loubresse, N., Melnikov, S., Jenner, L., Yusupova, G., and Yusupov, M. (2011). The structure of the eukaryotic ribosome at 3.0 A resolution. Science *334*, 1524-1529.
Berget, S.M. (1995). Exon recognition in vertebrate splicing. The Journal of biological chemistry *270*, 2411-2414.
Berget, S.M., Moore, C., and Sharp, P.A. (1977). Spliced segments at the 5' terminus of adenovirus 2 late mRNA. Proceedings of the National Academy of Sciences of the United States of America *74*, 3171-3175.
Bessonov, S., Anokhina, M., Will, C.L., Urlaub, H., and Luhrmann, R. (2008). Isolation of an active step I spliceosome and composition of its RNP core. Nature *452*, 846-850.
Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic acids research *7*, 1513-1523.
Blanchette, M., Green, R.E., Brenner, S.E., and Rio, D.C. (2005). Global analysis of positive and negative pre-mRNA splicing regulators in Drosophila. Genes & development *19*, 1306-1314.
Blaustein, M., Pelisch, F., Tanos, T., Munoz, M.J., Wengier, D., Quadrana, L., Sanford, J.R., Muschietti, J.P., Kornblihtt, A.R., Caceres, J.F.*, et al.* (2005). Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. Nature structural & molecular biology *12*, 1037-1044.
Bode, A.M., and Dong, Z. (2004). Post-translational modification of p53 in tumorigenesis. Nature reviews Cancer *4*, 793-805.
Bollen, M., Peti, W., Ragusa, M.J., and Beullens, M. (2010). The extended PP1 toolkit: designed to create specificity. Trends in biochemical sciences *35*, 450-458.
Brugarolas, J., Lei, K., Hurley, R.L., Manning, B.D., Reiling, J.H., Hafen, E., Witters, L.A., Ellisen, L.W., and Kaelin, W.G., Jr. (2004). Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes & development *18*, 2893-2904.

Burglen, L., Lefebvre, S., Clermont, O., Burlet, P., Viollet, L., Cruaud, C., Munnich, A., and Melki, J. (1996). Structure and organization of the human survival motor neurone (SMN) gene. Genomics *32*, 479-482.
Burtis, K.C. (1993). The regulation of sex determination and sexually dimorphic differentiation in Drosophila. Current opinion in cell biology *5*, 1006-1014.
Cao, W., Jamison, S.F., and Garcia-Blanco, M.A. (1997). Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro. RNA *3*, 1456-1467.
Cartegni, L., Hastings, M.L., Calarco, J.A., de Stanchina, E., and Krainer, A.R. (2006). Determinants of exon 7 splicing in the spinal muscular atrophy genes, SMN1 and SMN2. American journal of human genetics *78*, 63-77.
Cartegni, L., Wang, J., Zhu, Z., Zhang, M.Q., and Krainer, A.R. (2003). ESEfinder: A web resource to identify exonic splicing enhancers. Nucleic acids research *31*, 3568-3571.
Ceulemans, H., and Bollen, M. (2004). Functional diversity of protein phosphatase-1, a cellular economizer and reset button. Physiological reviews *84*, 1-39.
Chalfant, C.E., Kishikawa, K., Mumby, M.C., Kamibayashi, C., Bielawska, A., and Hannun, Y.A. (1999). Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. The Journal of biological chemistry *274*, 20313-20317.
Chalfant, C.E., Ogretmen, B., Galadari, S., Kroesen, B.J., Pettus, B.J., and Hannun, Y.A. (2001). FAS activation induces dephosphorylation of SR proteins; dependence on the de novo generation of ceramide and activation of protein phosphatase 1. The Journal of biological chemistry *276*, 44848-44855.
Chalfant, C.E., Rathman, K., Pinkerman, R.L., Wood, R.E., Obeid, L.M., Ogretmen, B., and Hannun, Y.A. (2002). De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1. The Journal of biological chemistry *277*, 12587-12595.
Chen, C., and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. Molecular and cellular biology *7*, 2745-2752.
Chen, M., and Manley, J.L. (2009). Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. Nature reviews Molecular cell biology *10*, 741-754.

Chow, L.T., Gelinas, R.E., Broker, T.R., and Roberts, R.J. (1977). An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. Cell *12*, 1-8.
Clark, T.A., Sugnet, C.W., and Ares, M., Jr. (2002). Genomewide analysis of mRNA processing in yeast using splicing-specific microarrays. Science *296*, 907-910.
Clery, A., Jayne, S., Benderska, N., Dominguez, C., Stamm, S., and Allain, F.H. (2011). Molecular basis of purine-rich RNA recognition by the human SR-like protein Tra2-beta1. Nature structural & molecular biology *18*, 443-450.
Cohen, P.T. (2002). Protein phosphatase 1--targeted in many directions. Journal of cell science *115*, 241-256.
Collesi, C., Santoro, M.M., Gaudino, G., and Comoglio, P.M. (1996). A splicing variant of the RON transcript induces constitutive tyrosine kinase activity and an invasive phenotype. Molecular and cellular biology *16*, 5518-5526.
Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J.L., Bell, J.C., and Duncan, P.I. (1996). The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. The EMBO journal *15*, 265-275.
Connolly, E., Braunstein, S., Formenti, S., and Schneider, R.J. (2006). Hypoxia inhibits protein synthesis through a 4E-BP1 and elongation factor 2 kinase pathway controlled by mTOR and uncoupled in breast cancer cells. Molecular and cellular biology *26*, 3955-3965.
Connor, J.H., Weiser, D.C., Li, S., Hallenbeck, J.M., and Shenolikar, S. (2001). Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. Molecular and cellular biology *21*, 6841-6850.
Csaki, L.S., and Reue, K. (2010). Lipins: multifunctional lipid metabolism proteins. Annual review of nutrition *30*, 257-272.
Dagan, T., Sorek, R., Sharon, E., Ast, G., and Graur, D. (2004). AluGene: a database of Alu elements incorporated within protein-coding genes. Nucleic acids research *32*, D489-492.
Damianov, A., and Black, D.L. (2010). Autoregulation of Fox protein expression to produce dominant negative splicing factors. RNA *16*, 405-416.
Daoud, R., Da Penha Berzaghi, M., Siedler, F., Hubener, M., and Stamm, S. (1999). Activity-dependent regulation of alternative splicing patterns in the rat brain. The European journal of neuroscience *11*, 788-802.
Daoud, R., Mies, G., Smialowska, A., Olah, L., Hossmann, K.A., and Stamm, S. (2002). Ischemia induces a translocation of the splicing factor

tra2-beta 1 and changes alternative splicing patterns in the brain. The Journal of neuroscience : the official journal of the Society for Neuroscience *22*, 5889-5899.
Das, R., Dufu, K., Romney, B., Feldt, M., Elenko, M., and Reed, R. (2006). Functional coupling of RNAP II transcription to spliceosome assembly. Genes & development *20*, 1100-1109.
Das, R., Yu, J., Zhang, Z., Gygi, M.P., Krainer, A.R., Gygi, S.P., and Reed, R. (2007). SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. Molecular cell *26*, 867-881.
Dauwalder, B., Amaya-Manzanares, F., and Mattox, W. (1996). A human homologue of the Drosophila sex determination factor transformer-2 has conserved splicing regulatory functions. Proceedings of the National Academy of Sciences of the United States of America *93*, 9004-9009.
David, C.J., and Manley, J.L. (2010). Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. Genes & development *24*, 2343-2364.
Davis, R.L., Homer, V.M., George, P.M., and Brennan, S.O. (2009). A deep intronic mutation in FGB creates a consensus exonic splicing enhancer motif that results in afibrinogenemia caused by aberrant mRNA splicing, which can be corrected in vitro with antisense oligonucleotide treatment. Human mutation *30*, 221-227.
Deribe, Y.L., Pawson, T., and Dikic, I. (2010). Post-translational modifications in signal integration. Nature structural & molecular biology *17*, 666-672.
Deutsch, M., and Long, M. (1999). Intron-exon structures of eukaryotic model organisms. Nucleic acids research *27*, 3219-3228.
Dunker, A.K., Lawson, J.D., Brown, C.J., Williams, R.M., Romero, P., Oh, J.S., Oldfield, C.J., Campen, A.M., Ratliff, C.M., Hipps, K.W.*, et al.* (2001). Intrinsically disordered protein. Journal of molecular graphics & modelling *19*, 26-59.
Dyer, B.W., Ferrer, F.A., Klinedinst, D.K., and Rodriguez, R. (2000). A noncommercial dual luciferase enzyme assay system for reporter gene analysis. Analytical biochemistry *282*, 158-161.
Dyson, H.J., and Wright, P.E. (2005). Intrinsically unstructured proteins and their functions. Nature reviews Molecular cell biology *6*, 197-208.
Egloff, M.P., Johnson, D.F., Moorhead, G., Cohen, P.T., Cohen, P., and Barford, D. (1997). Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. The EMBO journal *16*, 1876-1887.

Eldridge, R., and Casida, J.E. (1995). Cantharidin effects on protein phosphatases and the phosphorylation state of phosphoproteins in mice. Toxicology and applied pharmacology *130*, 95-100.
Fischer, D.C., Noack, K., Runnebaum, I.B., Watermann, D.O., Kieback, D.G., Stamm, S., and Stickeler, E. (2004). Expression of splicing factors in human ovarian cancer. Oncology reports *11*, 1085-1090.
Fogel, B.L., Wexler, E., Wahnich, A., Friedrich, T., Vijayendran, C., Gao, F., Parikshak, N., Konopka, G., and Geschwind, D.H. (2012). RBFOX1 regulates both splicing and transcriptional networks in human neuronal development. Human molecular genetics.
Gabriel, B., Zur Hausen, A., Bouda, J., Boudova, L., Koprivova, M., Hirschfeld, M., Jager, M., and Stickeler, E. (2009). Significance of nuclear hTra2-beta1 expression in cervical cancer. Acta obstetricia et gynecologica Scandinavica *88*, 216-221.
Garg, K., and Green, P. (2007). Differing patterns of selection in alternative and constitutive splice sites. Genome research *17*, 1015-1022.
Ghigna, C., Giordano, S., Shen, H., Benvenuto, F., Castiglioni, F., Comoglio, P.M., Green, M.R., Riva, S., and Biamonti, G. (2005). Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. Molecular cell *20*, 881-890.
Goncalves, V., Matos, P., and Jordan, P. (2009). Antagonistic SR proteins regulate alternative splicing of tumor-related Rac1b downstream of the PI3-kinase and Wnt pathways. Human molecular genetics *18*, 3696-3707.
Gooding, C., Clark, F., Wollerton, M.C., Grellscheid, S.N., Groom, H., and Smith, C.W. (2006). A class of human exons with predicted distant branch points revealed by analysis of AG dinucleotide exclusion zones. Genome biology *7*, R1.
Graveley, B.R. (2005). Mutually exclusive splicing of the insect Dscam pre-mRNA directed by competing intronic RNA secondary structures. Cell *123*, 65-73.
Grellscheid, S., Dalgliesh, C., Storbeck, M., Best, A., Liu, Y., Jakubik, M., Mende, Y., Ehrmann, I., Curk, T., Rossbach, K.*, et al.* (2011). Identification of evolutionarily conserved exons as regulated targets for the splicing activator tra2beta in development. PLoS genetics *7*, e1002390.
Hallegger, M., Llorian, M., and Smith, C.W. (2010). Alternative splicing: global insights. The FEBS journal *277*, 856-866.
Han, G.S., and Carman, G.M. (2010). Characterization of the human LPIN1-encoded phosphatidate phosphatase isoforms. The Journal of biological chemistry *285*, 14628-14638.
Harris, A.L. (2002). Hypoxia--a key regulatory factor in tumour growth. Nature reviews Cancer *2*, 38-47.

Hasler, J., Samuelsson, T., and Strub, K. (2007). Useful 'junk': Alu RNAs in the human transcriptome. Cellular and molecular life sciences : CMLS *64*, 1793-1800.
Hendrickx, A., Beullens, M., Ceulemans, H., Den Abt, T., Van Eynde, A., Nicolaescu, E., Lesage, B., and Bollen, M. (2009). Docking motif-guided mapping of the interactome of protein phosphatase-1. Chemistry & biology *16*, 365-371.
Heroes, E., Lesage, B., Gornemann, J., Beullens, M., Van Meervelt, L., and Bollen, M. (2012). The PP1 binding code: a molecular-lego strategy that governs specificity. The FEBS journal.
Hertel, K.J. (2008). Combinatorial control of exon recognition. The Journal of biological chemistry *283*, 1211-1215.
Hirano, K., Ito, M., and Hartshorne, D.J. (1995). Interaction of the ribosomal protein, L5, with protein phosphatase type 1. The Journal of biological chemistry *270*, 19786-19790.
Hirschfeld, M., Jaeger, M., Buratti, E., Stuani, C., Grueneisen, J., Gitsch, G., and Stickeler, E. (2011). Expression of tumor-promoting Cyr61 is regulated by hTRA2-beta1 and acidosis. Human molecular genetics *20*, 2356-2365.
Hocine, S., Singer, R.H., and Grunwald, D. (2010). RNA processing and export. Cold Spring Harbor perspectives in biology *2*, a000752.
Hofmann, Y., Lorson, C.L., Stamm, S., Androphy, E.J., and Wirth, B. (2000). Htra2-beta 1 stimulates an exonic splicing enhancer and can restore full-length SMN expression to survival motor neuron 2 (SMN2). Proceedings of the National Academy of Sciences of the United States of America *97*, 9618-9623.
Holcik, M., and Sonenberg, N. (2005). Translational control in stress and apoptosis. Nature reviews Molecular cell biology *6*, 318-327.
Huang, Y., Gattoni, R., Stevenin, J., and Steitz, J.A. (2003). SR splicing factors serve as adapter proteins for TAP-dependent mRNA export. Molecular cell *11*, 837-843.
Huang, Y., and Steitz, J.A. (2001). Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA. Molecular cell *7*, 899-905.
Huang, Y., Yario, T.A., and Steitz, J.A. (2004). A molecular link between SR protein dephosphorylation and mRNA export. Proceedings of the National Academy of Sciences of the United States of America *101*, 9666-9670.
Huang, Y.G., Kang, J.K., Liu, R.S., Oh, K.W., Nam, C.J., and Kim, H.S. (1997). Cytotoxic activities of various fractions extracted from some pharmaceutical insect relatives. Archives of pharmacal research *20*, 110-114.

Hull, J., Campino, S., Rowlands, K., Chan, M.S., Copley, R.R., Taylor, M.S., Rockett, K., Elvidge, G., Keating, B., Knight, J.*, et al.* (2007). Identification of common genetic variation that modulates alternative splicing. PLoS genetics *3*, e99.
Irimia, M., Rukov, J.L., Penny, D., and Roy, S.W. (2007). Functional and evolutionary analysis of alternatively spliced genes is consistent with an early eukaryotic origin of alternative splicing. BMC evolutionary biology *7*, 188.
Izquierdo, J.M., Majos, N., Bonnal, S., Martinez, C., Castelo, R., Guigo, R., Bilbao, D., and Valcarcel, J. (2005). Regulation of Fas alternative splicing by antagonistic effects of TIA-1 and PTB on exon definition. Molecular cell *19*, 475-484.
Izumi, H., Torigoe, T., Ishiguchi, H., Uramoto, H., Yoshida, Y., Tanabe, M., Ise, T., Murakami, T., Yoshida, T., Nomoto, M.*, et al.* (2003). Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. Cancer treatment reviews *29*, 541-549.
Jiang, K., Patel, N.A., Watson, J.E., Apostolatos, H., Kleiman, E., Hanson, O., Hagiwara, M., and Cooper, D.R. (2009). Akt2 regulation of Cdc2-like kinases (Clk/Sty), serine/arginine-rich (SR) protein phosphorylation, and insulin-induced alternative splicing of PKCbetaII messenger ribonucleic acid. Endocrinology *150*, 2087-2097.
Johnson, J.M., Castle, J., Garrett-Engele, P., Kan, Z., Loerch, P.M., Armour, C.D., Santos, R., Schadt, E.E., Stoughton, R., and Shoemaker, D.D. (2003). Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science *302*, 2141-2144.
Johnson, S.A., and Hunter, T. (2005). Kinomics: methods for deciphering the kinome. Nature methods *2*, 17-25.
Karahatay, S., Thomas, K., Koybasi, S., Senkal, C.E., Elojeimy, S., Liu, X., Bielawski, J., Day, T.A., Gillespie, M.B., Sinha, D.*, et al.* (2007). Clinical relevance of ceramide metabolism in the pathogenesis of human head and neck squamous cell carcinoma (HNSCC): attenuation of C(18)-ceramide in HNSCC tumors correlates with lymphovascular invasion and nodal metastasis. Cancer letters *256*, 101-111.
Kashima, T., and Manley, J.L. (2003). A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. Nature genetics *34*, 460-463.
Kent, W.J. (2002). BLAT--the BLAST-like alignment tool. Genome research *12*, 656-664.
Keren, H., Lev-Maor, G., and Ast, G. (2010). Alternative splicing and evolution: diversification, exon definition and function. Nature reviews Genetics *11*, 345-355.

Khanna, A., and Stamm, S. (2010). Regulation of alternative splicing by short non-coding nuclear RNAs. RNA biology *7*, 480-485.
Kim, E., Goren, A., and Ast, G. (2008). Insights into the connection between cancer and alternative splicing. Trends in genetics : TIG *24*, 7-10.
Kim, E., Magen, A., and Ast, G. (2007). Different levels of alternative splicing among eukaryotes. Nucleic acids research *35*, 125-131.
Kim, K.K., Kim, Y.C., Adelstein, R.S., and Kawamoto, S. (2011). Fox-3 and PSF interact to activate neural cell-specific alternative splicing. Nucleic acids research *39*, 3064-3078.
Klinge, S., Voigts-Hoffmann, F., Leibundgut, M., and Ban, N. (2012). Atomic structures of the eukaryotic ribosome. Trends in biochemical sciences *37*, 189-198.
Koizumi, J., Okamoto, Y., Onogi, H., Mayeda, A., Krainer, A.R., and Hagiwara, M. (1999). The subcellular localization of SF2/ASF is regulated by direct interaction with SR protein kinases (SRPKs). The Journal of biological chemistry *274*, 11125-11131.
Kol, G., Lev-Maor, G., and Ast, G. (2005). Human-mouse comparative analysis reveals that branch-site plasticity contributes to splicing regulation. Human molecular genetics *14*, 1559-1568.
Kornblihtt, A.R. (2006). Chromatin, transcript elongation and alternative splicing. Nature structural & molecular biology *13*, 5-7.
Kornblihtt, A.R. (2007). Coupling transcription and alternative splicing. Advances in experimental medicine and biology *623*, 175-189.
Kurokawa, K., Kuwano, Y., Tominaga, K., Kawai, T., Katsuura, S., Yamagishi, N., Satake, Y., Kajita, K., Tanahashi, T., and Rokutan, K. (2010). Brief naturalistic stress induces an alternative splice variant of SMG-1 lacking exon 63 in peripheral leukocytes. Neuroscience letters *484*, 128-132.
Lai, M.C., Lin, R.I., Huang, S.Y., Tsai, C.W., and Tarn, W.Y. (2000). A human importin-beta family protein, transportin-SR2, interacts with the phosphorylated RS domain of SR proteins. The Journal of biological chemistry *275*, 7950-7957.
Lai, M.C., Lin, R.I., and Tarn, W.Y. (2001). Transportin-SR2 mediates nuclear import of phosphorylated SR proteins. Proceedings of the National Academy of Sciences of the United States of America *98*, 10154-10159.
Lavigueur, A., La Branche, H., Kornblihtt, A.R., and Chabot, B. (1993). A splicing enhancer in the human fibronectin alternate ED1 exon interacts with SR proteins and stimulates U2 snRNP binding. Genes & development *7*, 2405-2417.
Le Hir, H., Gatfield, D., Izaurralde, E., and Moore, M.J. (2001). The exon-exon junction complex provides a binding platform for factors involved in

mRNA export and nonsense-mediated mRNA decay. The EMBO journal *20*, 4987-4997.
Le Hir, H., and Seraphin, B. (2008). EJCs at the heart of translational control. Cell *133*, 213-216.
Lesage, B., Beullens, M., Nuytten, M., Van Eynde, A., Keppens, S., Himpens, B., and Bollen, M. (2004). Interactor-mediated nuclear translocation and retention of protein phosphatase-1. The Journal of biological chemistry *279*, 55978-55984.
Lev-Maor, G., Sorek, R., Shomron, N., and Ast, G. (2003). The birth of an alternatively spliced exon: 3' splice-site selection in Alu exons. Science *300*, 1288-1291.
Lewis, J.D., Izaurralde, E., Jarmolowski, A., McGuigan, C., and Mattaj, I.W. (1996). A nuclear cap-binding complex facilitates association of U1 snRNP with the cap-proximal 5' splice site. Genes & development *10*, 1683-1698.
Li, Y.M., Mackintosh, C., and Casida, J.E. (1993). Protein phosphatase 2A and its [3H]cantharidin/[3H]endothall thioanhydride binding site. Inhibitor specificity of cantharidin and ATP analogues. Biochemical pharmacology *46*, 1435-1443.
Lin, J.C., and Tarn, W.Y. (2005). Exon selection in alpha-tropomyosin mRNA is regulated by the antagonistic action of RBM4 and PTB. Molecular and cellular biology *25*, 10111-10121.
Liu, L., and Simon, M.C. (2004). Regulation of transcription and translation by hypoxia. Cancer biology & therapy *3*, 492-497.
Long, J.C., and Caceres, J.F. (2009). The SR protein family of splicing factors: master regulators of gene expression. The Biochemical journal *417*, 15-27.
Longman, D., Johnstone, I.L., and Caceres, J.F. (2000). Functional characterization of SR and SR-related genes in Caenorhabditis elegans. The EMBO journal *19*, 1625-1637.
Lorson, C.L., Hahnen, E., Androphy, E.J., and Wirth, B. (1999). A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proceedings of the National Academy of Sciences of the United States of America *96*, 6307-6311.
Lunn, M.R., and Wang, C.H. (2008). Spinal muscular atrophy. Lancet *371*, 2120-2133.
Luo, M.J., and Reed, R. (1999). Splicing is required for rapid and efficient mRNA export in metazoans. Proceedings of the National Academy of Sciences of the United States of America *96*, 14937-14942.
Luo, M.L., Zhou, Z., Magni, K., Christoforides, C., Rappsilber, J., Mann, M., and Reed, R. (2001). Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly. Nature *413*, 644-647.

Maas, S., and Rich, A. (2000). Changing genetic information through RNA editing. BioEssays : news and reviews in molecular, cellular and developmental biology *22*, 790-802.
MacKintosh, C., and Klumpp, S. (1990). Tautomycin from the bacterium Streptomyces verticillatus. Another potent and specific inhibitor of protein phosphatases 1 and 2A. FEBS letters *277*, 137-140.
Magen, A., and Ast, G. (2005). The importance of being divisible by three in alternative splicing. Nucleic acids research *33*, 5574-5582.
Maniatis, T., and Reed, R. (2002). An extensive network of coupling among gene expression machines. Nature *416*, 499-506.
Maniatis, T., and Tasic, B. (2002). Alternative pre-mRNA splicing and proteome expansion in metazoans. Nature *418*, 236-243.
Massiello, A., Salas, A., Pinkerman, R.L., Roddy, P., Roesser, J.R., and Chalfant, C.E. (2004). Identification of two RNA cis-elements that function to regulate the 5' splice site selection of Bcl-x pre-mRNA in response to ceramide. The Journal of biological chemistry *279*, 15799-15804.
McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S.D., Wickens, M., and Bentley, D.L. (1997). The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. Nature *385*, 357-361.
Mende, Y., Jakubik, M., Riessland, M., Schoenen, F., Rossbach, K., Kleinridders, A., Kohler, C., Buch, T., and Wirth, B. (2010). Deficiency of the splicing factor Sfrs10 results in early embryonic lethality in mice and has no impact on full-length SMN/Smn splicing. Human molecular genetics *19*, 2154-2167.
Mermoud, J.E., Cohen, P., and Lamond, A.I. (1992). Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing. Nucleic acids research *20*, 5263-5269.
Michlewski, G., Sanford, J.R., and Caceres, J.F. (2008). The splicing factor SF2/ASF regulates translation initiation by enhancing phosphorylation of 4E-BP1. Molecular cell *30*, 179-189.
Moore, M.J., and Proudfoot, N.J. (2009). Pre-mRNA processing reaches back to transcription and ahead to translation. Cell *136*, 688-700.
Murray, M.V., Kobayashi, R., and Krainer, A.R. (1999). The type 2C Ser/Thr phosphatase PP2Cgamma is a pre-mRNA splicing factor. Genes & development *13*, 87-97.
Nayler, O., Cap, C., and Stamm, S. (1998). Human transformer-2-beta gene (SFRS10): complete nucleotide sequence, chromosomal localization, and generation of a tissue-specific isoform. Genomics *53*, 191-202.
Neugebauer, K.M. (2002). On the importance of being co-transcriptional. Journal of cell science *115*, 3865-3871.

Nita-Lazar, A., Saito-Benz, H., and White, F.M. (2008). Quantitative phosphoproteomics by mass spectrometry: past, present, and future. Proteomics *8*, 4433-4443.
Nott, A., Le Hir, H., and Moore, M.J. (2004). Splicing enhances translation in mammalian cells: an additional function of the exon junction complex. Genes & development *18*, 210-222.
Novoyatleva, T., Heinrich, B., Tang, Y., Benderska, N., Butchbach, M.E., Lorson, C.L., Lorson, M.A., Ben-Dov, C., Fehlbaum, P., Bracco, L.*, et al.* (2008). Protein phosphatase 1 binds to the RNA recognition motif of several splicing factors and regulates alternative pre-mRNA processing. Human molecular genetics *17*, 52-70.
O'Rourke, J.P., and Ness, S.A. (2008). Alternative RNA splicing produces multiple forms of c-Myb with unique transcriptional activities. Molecular and cellular biology *28*, 2091-2101.
Okazaki, Y., Furuno, M., Kasukawa, T., Adachi, J., Bono, H., Kondo, S., Nikaido, I., Osato, N., Saito, R., Suzuki, H.*, et al.* (2002). Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature *420*, 563-573.
Pan, Q., Shai, O., Lee, L.J., Frey, B.J., and Blencowe, B.J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. Nature genetics *40*, 1413-1415.
Paradis, C., Cloutier, P., Shkreta, L., Toutant, J., Klarskov, K., and Chabot, B. (2007). hnRNP I/PTB can antagonize the splicing repressor activity of SRp30c. RNA *13*, 1287-1300.
Peng, R., Dye, B.T., Perez, I., Barnard, D.C., Thompson, A.B., and Patton, J.G. (2002). PSF and p54nrb bind a conserved stem in U5 snRNA. RNA *8*, 1334-1347.
Prasad, J., Colwill, K., Pawson, T., and Manley, J.L. (1999). The protein kinase Clk/Sty directly modulates SR protein activity: both hyper- and hypophosphorylation inhibit splicing. Molecular and cellular biology *19*, 6991-7000.
Prilusky, J., Felder, C.E., Zeev-Ben-Mordehai, T., Rydberg, E.H., Man, O., Beckmann, J.S., Silman, I., and Sussman, J.L. (2005). FoldIndex: a simple tool to predict whether a given protein sequence is intrinsically unfolded. Bioinformatics *21*, 3435-3438.
Ranum, L.P., and Cooper, T.A. (2006). RNA-mediated neuromuscular disorders. Annual review of neuroscience *29*, 259-277.
Rappsilber, J., Ryder, U., Lamond, A.I., and Mann, M. (2002). Large-scale proteomic analysis of the human spliceosome. Genome research *12*, 1231-1245.

Reed, R. (1996). Initial splice-site recognition and pairing during pre-mRNA splicing. Current opinion in genetics & development *6*, 215-220.
Rodriguez, M.S., Dargemont, C., and Stutz, F. (2004). Nuclear export of RNA. Biology of the cell / under the auspices of the European Cell Biology Organization *96*, 639-655.
Romero, P.R., Zaidi, S., Fang, Y.Y., Uversky, V.N., Radivojac, P., Oldfield, C.J., Cortese, M.S., Sickmeier, M., LeGall, T., Obradovic, Z.*, et al.* (2006). Alternative splicing in concert with protein intrinsic disorder enables increased functional diversity in multicellular organisms. Proceedings of the National Academy of Sciences of the United States of America *103*, 8390-8395.
Rosenwald, I.B. (2004). The role of translation in neoplastic transformation from a pathologist's point of view. Oncogene *23*, 3230-3247.
Sanford, J.R., Ellis, J.D., Cazalla, D., and Caceres, J.F. (2005). Reversible phosphorylation differentially affects nuclear and cytoplasmic functions of splicing factor 2/alternative splicing factor. Proceedings of the National Academy of Sciences of the United States of America *102*, 15042-15047.
Sanford, J.R., Gray, N.K., Beckmann, K., and Caceres, J.F. (2004). A novel role for shuttling SR proteins in mRNA translation. Genes & development *18*, 755-768.
Sharma, S., Falick, A.M., and Black, D.L. (2005). Polypyrimidine tract binding protein blocks the 5' splice site-dependent assembly of U2AF and the prespliceosomal E complex. Molecular cell *19*, 485-496.
Sharp, P.A. (2005). The discovery of split genes and RNA splicing. Trends in biochemical sciences *30*, 279-281.
Shatkin, A.J., and Manley, J.L. (2000). The ends of the affair: capping and polyadenylation. Nature structural biology *7*, 838-842.
Shepard, P.J., and Hertel, K.J. (2009). The SR protein family. Genome biology *10*, 242.
Shi, Y. (2009). Serine/threonine phosphatases: mechanism through structure. Cell *139*, 468-484.
Shi, Y., Reddy, B., and Manley, J.L. (2006). PP1/PP2A phosphatases are required for the second step of Pre-mRNA splicing and target specific snRNP proteins. Molecular cell *23*, 819-829.
Shukla, S., Del Gatto-Konczak, F., Breathnach, R., and Fisher, S.A. (2005). Competition of PTB with TIA proteins for binding to a U-rich cis-element determines tissue-specific splicing of the myosin phosphatase targeting subunit 1. RNA *11*, 1725-1736.
Singh, R., and Valcarcel, J. (2005). Building specificity with nonspecific RNA-binding proteins. Nature structural & molecular biology *12*, 645-653.

Smith, P.J., Zhang, C., Wang, J., Chew, S.L., Zhang, M.Q., and Krainer, A.R. (2006). An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. Human molecular genetics *15*, 2490-2508.
Stamm, S. (2008). Regulation of alternative splicing by reversible protein phosphorylation. The Journal of biological chemistry *283*, 1223-1227.
Stoilov, P., Daoud, R., Nayler, O., and Stamm, S. (2004). Human tra2-beta1 autoregulates its protein concentration by influencing alternative splicing of its pre-mRNA. Human molecular genetics *13*, 509-524.
Stoss, O., Stoilov, P., Hartmann, A.M., Nayler, O., and Stamm, S. (1999). The in vivo minigene approach to analyze tissue-specific splicing. Brain research Brain research protocols *4*, 383-394.
Sugnet, C.W., Srinivasan, K., Clark, T.A., O'Brien, G., Cline, M.S., Wang, H., Williams, A., Kulp, D., Blume, J.E., Haussler, D.*, et al.* (2006). Unusual intron conservation near tissue-regulated exons found by splicing microarrays. PLoS computational biology *2*, e4.
Sumanasekera, C., Kelemen, O., Beullens, M., Aubol, B.E., Adams, J.A., Sunkara, M., Morris, A., Bollen, M., Andreadis, A., and Stamm, S. (2012). C6 pyridinium ceramide influences alternative pre-mRNA splicing by inhibiting protein phosphatase-1. Nucleic acids research *40*, 4025-4039.
Sumanasekera, C., Watt, D.S., and Stamm, S. (2008). Substances that can change alternative splice-site selection. Biochemical Society transactions *36*, 483-490.
Sun, S., Zhang, Z., Sinha, R., Karni, R., and Krainer, A.R. (2010). SF2/ASF autoregulation involves multiple layers of post-transcriptional and translational control. Nature structural & molecular biology *17*, 306-312.
Swartz, J.E., Bor, Y.C., Misawa, Y., Rekosh, D., and Hammarskjold, M.L. (2007). The shuttling SR protein 9G8 plays a role in translation of unspliced mRNA containing a constitutive transport element. The Journal of biological chemistry *282*, 19844-19853.
Tacke, R., Tohyama, M., Ogawa, S., and Manley, J.L. (1998). Human Tra2 proteins are sequence-specific activators of pre-mRNA splicing. Cell *93*, 139-148.
Takemoto, A., Maeshima, K., Ikehara, T., Yamaguchi, K., Murayama, A., Imamura, S., Imamoto, N., Yokoyama, S., Hirano, T., Watanabe, Y.*, et al.* (2009). The chromosomal association of condensin II is regulated by a noncatalytic function of PP2A. Nature structural & molecular biology *16*, 1302-1308.
Tazi, J., Bakkour, N., and Stamm, S. (2009). Alternative splicing and disease. Biochimica et biophysica acta *1792*, 14-26.

Tazi, J., Daugeron, M.C., Cathala, G., Brunel, C., and Jeanteur, P. (1992). Adenosine phosphorothioates (ATP alpha S and ATP tau S) differentially affect the two steps of mammalian pre-mRNA splicing. The Journal of biological chemistry *267*, 4322-4326.
Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research *22*, 4673-4680.
Tiran, Z., Oren, A., Hermesh, C., Rotman, G., Levine, Z., Amitai, H., Handelsman, T., Beiman, M., Chen, A., Landesman-Milo, D.*, et al.* (2008). A novel recombinant soluble splice variant of Met is a potent antagonist of the hepatocyte growth factor/scatter factor-Met pathway. Clinical cancer research : an official journal of the American Association for Cancer Research *14*, 4612-4621.
Tsuda, K., Someya, T., Kuwasako, K., Takahashi, M., He, F., Unzai, S., Inoue, M., Harada, T., Watanabe, S., Terada, T.*, et al.* (2011). Structural basis for the dual RNA-recognition modes of human Tra2-beta RRM. Nucleic acids research *39*, 1538-1553.
Twyffels, L., Gueydan, C., and Kruys, V. (2011). Shuttling SR proteins: more than splicing factors. The FEBS journal *278*, 3246-3255.
Vagner, S., Vagner, C., and Mattaj, I.W. (2000). The carboxyl terminus of vertebrate poly(A) polymerase interacts with U2AF 65 to couple 3'-end processing and splicing. Genes & development *14*, 403-413.
Varani, L., Hasegawa, M., Spillantini, M.G., Smith, M.J., Murrell, J.R., Ghetti, B., Klug, A., Goedert, M., and Varani, G. (1999). Structure of tau exon 10 splicing regulatory element RNA and destabilization by mutations of frontotemporal dementia and parkinsonism linked to chromosome 17. Proceedings of the National Academy of Sciences of the United States of America *96*, 8229-8234.
Wahl, M.C., Will, C.L., and Luhrmann, R. (2009). The spliceosome: design principles of a dynamic RNP machine. Cell *136*, 701-718.
Wakula, P., Beullens, M., Ceulemans, H., Stalmans, W., and Bollen, M. (2003). Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. The Journal of biological chemistry *278*, 18817-18823.
Wang, G.S., and Cooper, T.A. (2007). Splicing in disease: disruption of the splicing code and the decoding machinery. Nature reviews Genetics *8*, 749-761.
Wang, H.Y., Lin, W., Dyck, J.A., Yeakley, J.M., Songyang, Z., Cantley, L.C., and Fu, X.D. (1998). SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-

mRNA splicing factors in mammalian cells. The Journal of cell biology *140*, 737-750.
Wang, J., Smith, P.J., Krainer, A.R., and Zhang, M.Q. (2005a). Distribution of SR protein exonic splicing enhancer motifs in human protein-coding genes. Nucleic acids research *33*, 5053-5062.
Wang, L., Li, G., and Sugita, S. (2005b). A central kinase domain of type I phosphatidylinositol phosphate kinases is sufficient to prime exocytosis: isoform specificity and its underlying mechanism. The Journal of biological chemistry *280*, 16522-16527.
Ward, A.J., and Cooper, T.A. (2010). The pathobiology of splicing. The Journal of pathology *220*, 152-163.
Ward, J.J., Sodhi, J.S., McGuffin, L.J., Buxton, B.F., and Jones, D.T. (2004). Prediction and functional analysis of native disorder in proteins from the three kingdoms of life. Journal of molecular biology *337*, 635-645.
Watermann, D.O., Tang, Y., Zur Hausen, A., Jager, M., Stamm, S., and Stickeler, E. (2006). Splicing factor Tra2-beta1 is specifically induced in breast cancer and regulates alternative splicing of the CD44 gene. Cancer research *66*, 4774-4780.
Watkins, N.J., and Bohnsack, M.T. (2012). The box C/D and H/ACA snoRNPs: key players in the modification, processing and the dynamic folding of ribosomal RNA. Wiley interdisciplinary reviews RNA *3*, 397-414.
Wellmann, S., Buhrer, C., Moderegger, E., Zelmer, A., Kirschner, R., Koehne, P., Fujita, J., and Seeger, K. (2004). Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism. Journal of cell science *117*, 1785-1794.
Will, C.L., and Luhrmann, R. (2011). Spliceosome structure and function. Cold Spring Harbor perspectives in biology *3*.
Xiao, S.H., and Manley, J.L. (1998). Phosphorylation-dephosphorylation differentially affects activities of splicing factor ASF/SF2. The EMBO journal *17*, 6359-6367.
Xing, Y., and Lee, C.J. (2005). Protein modularity of alternatively spliced exons is associated with tissue-specific regulation of alternative splicing. PLoS genetics *1*, e34.
Xu, B. (1981). The influence of several anticancer agents on cell proliferation, differentiation and the cell cycle of murine erythroleukemia cells. The American journal of Chinese medicine *9*, 268-276.
Yang, C., and Carrier, F. (2001). The UV-inducible RNA-binding protein A18 (A18 hnRNP) plays a protective role in the genotoxic stress response. The Journal of biological chemistry *276*, 47277-47284.

Yeo, G.W., Van Nostrand, E.L., and Liang, T.Y. (2007). Discovery and analysis of evolutionarily conserved intronic splicing regulatory elements. PLoS genetics *3*, e85.
Yu, Y., Maroney, P.A., Denker, J.A., Zhang, X.H., Dybkov, O., Luhrmann, R., Jankowsky, E., Chasin, L.A., and Nilsen, T.W. (2008). Dynamic regulation of alternative splicing by silencers that modulate 5' splice site competition. Cell *135*, 1224-1236.
Yura, K., Shionyu, M., Hagino, K., Hijikata, A., Hirashima, Y., Nakahara, T., Eguchi, T., Shinoda, K., Yamaguchi, A., Takahashi, K.*, et al.* (2006). Alternative splicing in human transcriptome: functional and structural influence on proteins. Gene *380*, 63-71.
Zavolan, M., Kondo, S., Schonbach, C., Adachi, J., Hume, D.A., Hayashizaki, Y., and Gaasterland, T. (2003). Impact of alternative initiation, splicing, and termination on the diversity of the mRNA transcripts encoded by the mouse transcriptome. Genome research *13*, 1290-1300.
Zhang, C., Krainer, A.R., and Zhang, M.Q. (2007). Evolutionary impact of limited splicing fidelity in mammalian genes. Trends in genetics : TIG *23*, 484-488.
Zhang, J., Zhang, Z., Brew, K., and Lee, E.Y. (1996). Mutational analysis of the catalytic subunit of muscle protein phosphatase-1. Biochemistry *35*, 6276-6282.
Zhang, P., Takeuchi, K., Csaki, L.S., and Reue, K. (2012). Lipin-1 phosphatidic phosphatase activity modulates phosphatidate levels to promote peroxisome proliferator-activated receptor gamma (PPARgamma) gene expression during adipogenesis. The Journal of biological chemistry *287*, 3485-3494.
Zhang, Z., Kelemen, O., van Santen, M.A., Yelton, S.M., Wendlandt, A.E., Sviripa, V.M., Bollen, M., Beullens, M., Urlaub, H., Luhrmann, R.*, et al.* (2011). Synthesis and characterization of pseudocantharidins, novel phosphatase modulators that promote the inclusion of exon 7 into the SMN (survival of motoneuron) pre-mRNA. The Journal of biological chemistry *286*, 10126-10136.
Zhong, X.Y., Wang, P., Han, J., Rosenfeld, M.G., and Fu, X.D. (2009). SR proteins in vertical integration of gene expression from transcription to RNA processing to translation. Molecular cell *35*, 1-10.
Zhou, Z., Licklider, L.J., Gygi, S.P., and Reed, R. (2002). Comprehensive proteomic analysis of the human spliceosome. Nature *419*, 182-185.
Zhou, Z., Luo, M.J., Straesser, K., Katahira, J., Hurt, E., and Reed, R. (2000). The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans. Nature *407*, 401-405.