Characterisation of heterogeneous nuclear ribonucleoprotein G (hnRNP G) and its role in splice site selection.

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Bettina Heinrich

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ABBREVIATIONS

9G8	splicing factor, arginine/serine-rich 7
Abl	Ableson protein tyrosine kinase
Ack2	activated Cdc42-associated tyrosine kinase 2
AMP	adenosine mono phosphate
APP	amyloid precursor protein
APS	ammonium persulphate
ASD	alternative splicing database
ASF	alternative splicing factor
ATP	adenosine triphosphate
Bcl 2	B-cell leukaemia/lymphoma 2
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclin adenosine mono phosphate
CDC2	cell division cycle 2 protein
Cdk	cyclin dependent kinase
cds	coding sequence
CFP	cyan fluorescent protein
CFTR	cystic fibrosis transmembrane conductance regulator ATP-binding cassette subfamily C member 7
Clk	CDC2-like kinase
Cy3	cyanine 3 (green dye)
Cy5	cyanine 5 (red dye)
dH$_2$O	distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
Dscam	Down syndrome cell adhesion molecule
dsx	doublesex
DTT	dithiothreitol
DYRK1A	dual specificity tyrosine phosphorylated and regulated kinase
ECL	enhanced chemiluminiscence
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular receptor kinase
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
EST	expressed sequence tag
EtOH	ethanol
exinct	extended inhibitory context
FBS	foetal bovine serum
FGFR-2	fibroblast growth factor receptor
FTDP-17	frontotemporal dementia with Parkinsonism linked to chromosome 17
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GFP	green fluorescent protein
GnRH	gonadotrophin releasing hormone
GST	glutathione S-transferase
HEK	human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIV	human immunodeficiency virus
hnRNP	heterogeneous nuclear ribonucleoprotein
HRP	horseradish peroxidase
Il-4	interleukin-4

IPTG	isopropyl-D-1-thiogalactopyranoside
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
kb	kilo base pairs
kDa	kilodalton
KH domain	hnRNP K homology domain
mGluR7b	metabotropic glutamate receptor
mRNA	messenger RNA
NE	nuclear extract
Ni-NTA agarose	nickel-nitrilotriacetic acid agarose
NIPP1	nuclear inhibitor of protein phosphatase 1
NMD	nonsense mediated decay
NMDAR1	N-methyl-D-aspartate receptor 1
NPC	nuclear pore complex
nt	nucleotide
PABPC3	polyadenylate binding protein 3
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
PIC	protease inhibitor cocktail
PK	protein kinase
PMSF	phenylmethanesulfonyl fluoride
PP	protein phosphatase
PPM	Mg^{2+}-dependent phosphatases
PRP31	pre-mRNA processing factor 31
PSF1	polypyrimidine tract binding protein associated splicing factor
PTP	protein tyrosine phosphatases
RBM	RNA binding motif protein
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RRM	RNA recognition motif
RT-PCR	reverse transcription followed by polymerase chain reaction
RTZF	retroviral-type zinc finger
RUST	regulated unproductive splicing and translation
SAF	scaffold attachment factor
Sam68	Src associated in mitosis 68kDa
SAP155	spliceosome-associated protein 155
SF3b155	splicing factor 3B subunit 1/spliceosome-associated protein 155
SFPQ	splicing factor proline/glutamine-rich
SC35	splicing component, 35 kDa (splicing factor, arginine/serine-rich 2)
SDS	sodium dodecyl sulphate
SF	splicing factor
SFRS	splicing factor, arginine/serine-rich
SH	Src homology domain
SHP	Src homology 2 domain-containing tyrosine phosphatase 1
SLM	Sam68 like molecule
SMA	spinal muscular atrophy
SMN	survival of motor neuron
snoRNP	small nucleolar ribonucleoprotein
snRNP	small nuclear ribonucleoprotein particle
SRm 160/300	SR-related nuclear matrix proteins of 160 and 300 kDa
SR-protein	serine/arginine rich protein
STAR	signal transduction and activation of RNA
TBE	tris-borate-EDTA buffer
TCA	trichloroacetic acid

TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tm	melting temperature
Tra2	transformer 2
tRNA	transfer RNA
TSH	thyroid stimulating hormone
U1-70K	U1 snRNP 70 kDa protein
U2AF	U2 snRNP auxiliary factor (35 or 65 kDa)
UTP	uridine triphosphate
UTR	untranslated region
VPA	valproic acid
WT1	Wilms' tumour
YFP	yellow fluorescent protein
YTH domain	YT521-B homology domain

ZUSAMMENFASSUNG

Alternatives Spleißen ist einer der Hauptvorgänge, wodurch aus einer relativ geringen Anzahl an Genen eine große Proteinvielfalt hergestellt werden kann. Microarray Daten zeigen, dass etwa 74% der menschlichen Gene alternativ gespleißte Transkripte herstellen. Somit ist die Regulation des Spleißens ein sehr wichtiger Teil der Genregulation. Die Auswahl alternativer Spleißstellen wird durch eine Reihe koordinierter RNA:RNA, RNA:Protein und Protein:Protein Interaktionen exakt reguliert. Die Mechanismen, vor allem die Signaltransduktionswege, die Spleißen regulieren, sowie die meisten daran beteiligten Faktoren, sind bisher wenig verstanden.

Diese Arbeit konzentriert sich auf die Untersuchung verschiedener Spleißfaktoren und der Spleißstellenauswahl durch Signaltransduktionswege. Die Rolle der Proteine RBM4, Tra2-beta1 und hnRNP G bei der Auswahl von Spleißstellen wurde untersucht.

Im ersten Teil der Arbeit wurde RBM4 (RNA binding motif protein 4) studiert. RBM4 ist ein RNA bindendes Protein, das ein RNA Erkennungs-Motiv (RRM) enthält. Es wird gezeigt, dass RBM4 die Spleißstellenauswahl durch Sequestrierung ändern kann, was durch Interaktion mit Wilms Tumor1 (WT1) verhindert wird.

Eines der ersten Proteine, für das Sequestrierung durch andere Spleißfaktoren gezeigt wurde, ist das SR-ähnliche Protein Transformer2-beta1 (Tra2-beta1), das im zweiten Teil der Arbeit untersucht wurde. Es enthält das Protein Phosphatase 1 (PP1) Bindemotiv RVDF. Es wird gezeigt, dass Dephosphorylierung durch PP1 die Bindung von Tra2-beta1 und dem Spleißfaktor SF2/ASF *in vivo* und *in vitro* beeinflusst. Zudem wird dargestellt, dass eine Reduktion der PP1 Aktivität durch spezifische Inhibitoren oder RNA Interferenz den Gebrauch alternativer Tra2-beta1 abhängiger Exons begünstigt.

Der größte Teil der Arbeit beschäftigt sich mit heterogenem nukleärem Ribonukleoprotein G (hnRNP G). hnRNP G bindet an einige Spleißfaktoren und ist ein Bindepartner von Tra2-beta1. Es wird gezeigt, dass hnRNP G durch mehrere Kinasen tyrosinphosphoryliert wird und durch die Tyrosinphosphatase PTP1B dephosphoryliert wird. Wir haben herausgefunden, dass die Fähigkeit von hnRNP G, sich zwischen Zellkern und Cytosol zu bewegen („shuttling"), durch Dephosphorylierung signifikant reduziert wird. Es wird dargelegt, dass hnRNP G an RNA mit CCA-Motiven bindet und Spleißmuster einiger Exons reguliert, die diese Motive enthalten. In einigen dieser Exons kann hnRNP G die Spleißstellenauswahl phophorylierungsabhängig ändern.

Zusammenfassend wird gezeigt, dass RBM4 und hnRNP G Spleißfaktoren sind. Für Tra2-beta1 und hnRNP G wird dargestellt, dass reversible Phosphorylierung verschiedene Proteineigenschaften sowie den Effekt dieser Proteine auf die Spleißstellenauswahl ändert.

ABSTRACT

Alternative splicing is one of the main mechanisms to generate a large number of protein isoforms from a relatively small amount of genes. Microarray data indicate that approximately 74% of the human genes produce transcripts that are alternatively spliced. Consequently, the regulation of splicing is a very important part of gene regulation. The exact recognition and connection of alternative splice site selection is achieved by a number of coordinated RNA:RNA, RNA:protein and protein:protein interactions. The mechanisms for splicing regulation, especially the associated signal transduction pathways and a number of splicing factors, are still not well understood.

This work focuses on control of splice site selection through signal transduction pathways. The role of the proteins RBM4, Tra2-beta1 and hnRNP G in splice site selection was studied.

In the first part of this work, the role of RBM4 (RNA binding motif protein 4) in splice site selection was investigated. RBM4 is an RNA binding protein containing an RNA recognition motif (RRM). It was shown that RBM4 can change splice site selection of several minigenes by sequestration, which is inhibited by interaction with WT1 (Wilms' tumour).

One of the first proteins for which sequestration through other factors was shown is the human SR-related protein Tra2-beta1 (Transformer2-beta1). In the second part of this study Tra2-beta1, which contains the Protein phosphatase 1 (PP1) binding motif RVDF, was investigated. It is shown that dephosphorylation by PP1 affects binding of Tra2-beta1 with its interacting protein SF2/ASF. Furthermore, it is shown that reducing PP1 activity by its inhibitors or by RNA interference promotes usage of alternative Tra2-beta1 dependent exons.

The most part of this work concentrates on the heterogeneous nuclear ribonucleoprotein G (hnRNP G). hnRNP G was found to bind to several splicing factors and is a main interactor of Tra2-beta1. It is shown that hnRNP G is tyrosine phosphorylated by several kinases and dephosphorylated by the tyrosine phosphatase PTP1B. We found that hnRNP G has the ability to shuttle between cytoplasm and nucleus. This shuttling is significantly decreased by dephosphorylation. hnRNP G is demonstrated to bind to CCA motifs and is regulating the splicing pattern of several exons that contain these motifs. In a number of these exons hnRNP G can phosphorylation dependently change splice site selection.

In conclusion RBM4 and hnRNP G were shown to be splicing factors. For Tra2-beta1 and hnRNP G it is demonstrated that they are reversibly phosphorylated which influences different properties of these proteins and their effect on splice site selection.

1 INTRODUCTION

After transcription, eukaryotic precursor messenger RNA (pre-mRNA) is converted by several posttranscriptional modifications into mature mRNA. This processing of pre-mRNA is essential for the export of mRNA from the nucleus to the cytoplasm and accurate and efficient protein expression. The major processes involved are 5' capping, 3' polyadenylation (Shatkin and Manley, 2000), splicing (Blencowe, Nickerson *et al.,* 1994; Neugebauer, 2002; Kornblihtt, De La Mata *et al.,* 2004) and RNA editing (Benne, 1996; Wedekind, Dance *et al.,* 2003).

Alternative splicing is one of the most important mechanisms to generate a comparatively large amount of proteins (about 250,000) from a small number of genes (20,000-25,000) by significantly increasing the number of mRNAs. The majority of human protein-encoding genes undergo alternative splicing (Consortium, 2004).

The aim of this work was to better understand the mechanism of splice site selection by different splicing factors, like Tra2-beta1 and hnRNP G.

1.1 Basic pre-mRNA splicing

The eukaryotic genome is composed of non-coding sequences (introns) and flanking coding regions (exons). 98% of the chromosomal DNA consists of introns, but only 2% are exons. In the splicing reaction, introns are cut out and removed from the pre-mRNA and exons are spliced together to yield functional mRNA. This takes place in the spliceosome complex of the nucleus co- or posttranscriptionally.

In higher eukaryotes the average number of exons is 8.8, while the number of introns can vary between none and more than 50. The average exon contains 145 nucleotides, while the average intron length is about 3,300 nucleotides, some introns having more than 200,000 nucleotides. With the 5' and 3' UTRs consisting of 300 bp and 770 bp, respectively, the average gene spans about 27 kb. The coding sequence of processed mRNA in the cytosol is 1,340 bp, 1,070 bp are untranslated regions and the poly (A) tail (Lander, Linton *et al.,* 2001) on average. These numbers show that more than 90% of the pre-mRNA is removed as introns and only about 10% of the average pre-mRNA is joined by pre-mRNA splicing.

Exon/intron borders are defined by three weakly conserved degenerate sequence elements that are essential for intron recognition and their subsequent removal. The major

class introns (U2-type introns) have highly conserved dinucleotides at their 5' and 3' sites (GT and AG, respectively) (Table 1-1).

element	sequence
5' (donor) splice site	YRG / GURRGU
3' (acceptor) splice site followed by a polypyrimidine stretch	Y_{12}NYAG/N
Branch point located 18-200 bp upstream of the 3' splice site	YNYURAY

Table 1-1: Sequence elements defining major class (GT-AG) introns. Symbols used: Y-pyrimidine, R-purine, N-any nucleotide. Slash (/) indicates the exon/intron border. Invariant nucleotides are underlined.

Another, minor class of introns found in higher eukaryotes are the U12-type introns, which contain AT and AC at their 5' and 3' splice sites, respectively. Their consensus sequences (Table 1-2) are more highly conserved than in U2-type GT-AG introns and they are spliced by a distinct spliceosome (Hall and Padgett, 1994; Tarn and Steitz, 1996; Tarn and Steitz, 1997).

element	sequence
5' (donor) splice site	/AUAUCUU
3' (acceptor) splice site followed by a polypyrimidine stretch	CAC/
Branch point located 18-200 bp upstream of the 3' splice site	UCCUUAAC

Table 1-2: Sequence elements defining minor class (AT-AC) introns. Slash (/) indicates the exon/intron border. Invariant nucleotides are underlined.

By analysis of splice junction pairs extracted from mammalian GenBank annotated genes it was found that 98.71% hold canonical dinucleotides GT-AG, 0.56% non-canonical GC-AG and 0.73% contain other non-canonical splice termini (Burset, Seledtsov *et al.,* 2000; Burset, Seledtsov *et al.,* 2001).

The excision of introns and the following exon re-ligation take place in a two-step transesterification reaction in the spliceosome.

1.1.1 The splicing reaction

The splice reaction is a dynamic process involving formation and disruption of RNA:RNA interactions, RNA:protein interactions and protein:protein interactions. In the first reaction the 3'-5' phosphodiester bond of the intron at the 5' splice site is opened by a nucleophilic attack of the 2'-hydroxyl group of the conserved adenine at the branch point. This generates a 2'-5' phosphodiester bond at the branch point and a free 3' hydroxyl group on the 5' exon and leads to a branched lariat intermediate. In the second reaction the phosphodiester bond of the 3' splice site is opened by a nucleophilic attack of the free 3' hydroxyl group, which leads to release of the intron lariat and ligation of the two flanking exons (see Figure 1-1).
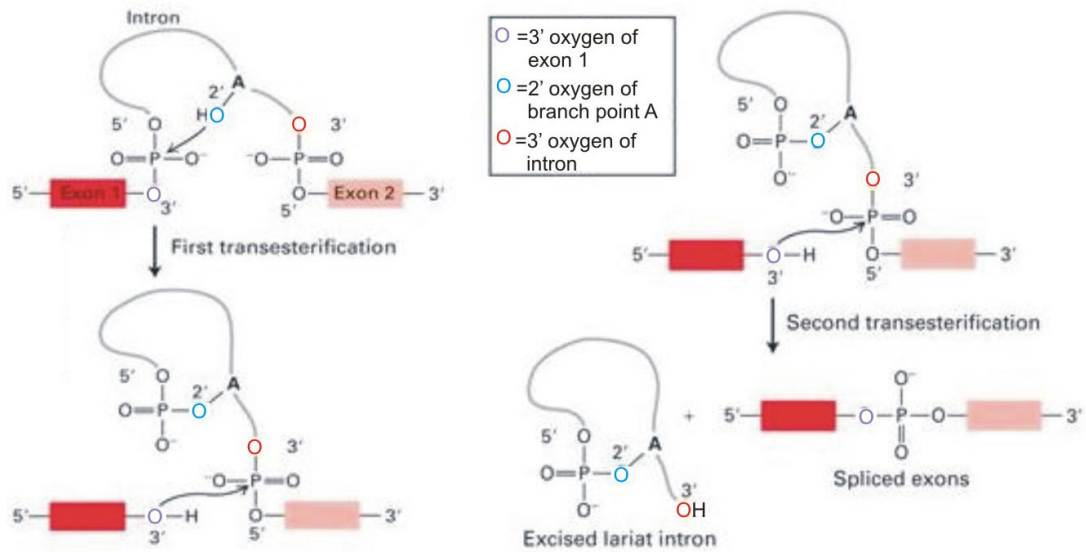
Figure 1-1: pre-mRNA basic splice reaction. In the first transesterification (left) reaction, the ester bond between the 5' phosphorus of the intron and the 3' oxygen (violet) of exon 1 is exchanged for an ester bond with the 2' oxygen (blue) of the branch point A residue. In the second transesterification reaction (right), the ester bond between the 5' phosphorus of exon 2 and the 3' oxygen (red) of the intron is exchanged for an ester bond with the 3' oxygen of exon 1, releasing the intron as a lariat structure and joining the two exons. Arrows show the position where the activated hydroxyl oxygens react with phosphorus atoms. Figure adapted from Lodish, Berk *et al.,* 2003

1.1.2 Spliceosome assembly

The central catalytic core of the major spliceosome is composed of the small nuclear ribonucleoparticles (snRNPs) U1, U2, U4, U5 and U6 (reviewed in Moore, Query *et al.,* 1993; Krämer, 1996; Burge, Tuschl *et al.,* 1999; Will and Lührmann, 2001), whereas the core of the minor spliceosome contains U11, U12, U4atac, U5, U6atac snRNPs (Hall and Padgett, 1994; Tarn and Steitz, 1997). Each snRNP is composed of multiple proteins and a single small nuclear RNA (snRNA), which is complementary to the conserved sequence elements of the pre-mRNA.

Spliceosome assembly is a stepwise process (Figure 1-2), in which complexes E, A, B and C are formed. It begins with formation of the E (early) complex. U1 snRNP binds to the 5' splice site, where U1 snRNA specifically forms base pairs with conserved sequence elements (Zhuang and Weiner, 1986). Binding of U1 snRNP facilitates the recognition of polypyrimidine stretch and 3' splice site by auxiliary factors U2AF65 and U2AF35, respectively (Staknis and Reed, 1994; Wu, Romfo *et al.,* 1999). U2 snRNP binds to the branch site in the following A complex. For stable binding of U2, ATP is hydrolysed and U2AF and other proteins bound in the E complex, like SF1 and SF3, are needed. Next the B1 complex is formed by binding of the U4/U6·U5 triplet snRNP. Afterwards reorganisation starts by several conformational changes: U5 binds to the exon at the 5' splice site and U6

binds to U2. In the next step U1 is released, U5 moves from the exon to the intron and U6 binds the 5' splice site (B2 complex). The two transesterification reactions take place in the next complex (C1 complex). U4 is released, U6/U2 catalyses the transesterification reactions, U5 binds at the 3' splice site of the exon and the 5' splice site is cleaved, forming the lariat structure. Finally the C2 complex forms, where U2/U5/U6 stay bound to the lariat, the 3' splice site is cleaved and the exons are ligated using ATP hydrolysis. The spliced RNA and the lariat are released (reviewed in Hastings and Krainer, 2001; Will and Lührmann, 2001).
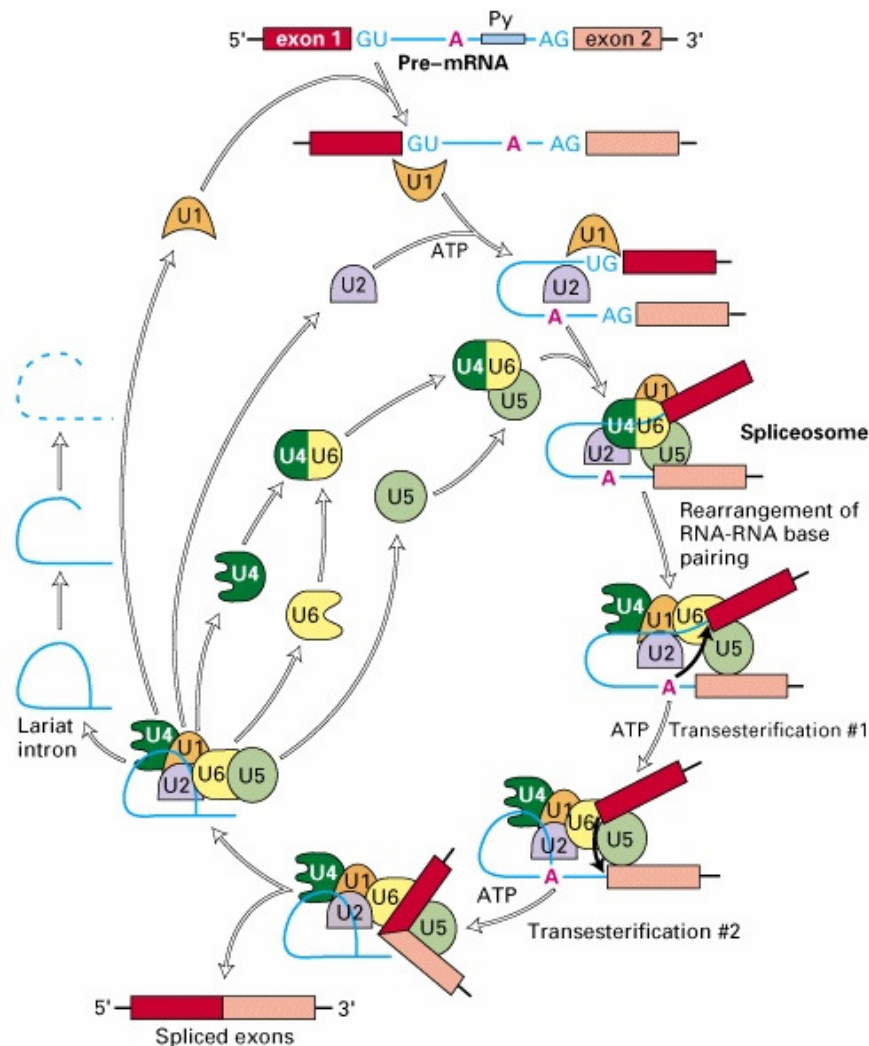
Figure 1-2: Spliceosome association and reorganisation during splicing. Exons are marked in light and dark red, intron in blue, the branch-point "A" in the pre-mRNA is indicated in boldface. Adapted from Moore, Query *et al.,* 1993.

1.2 Alternative splicing

After sequencing of the human genome was complete, it was clear that humans only have about 20,000-25,000 genes (Consortium, 2004), which is much less than previously expected (Lander, Linton *et al.,* 2001; Venter, Adams *et al.,* 2001). Therefore, alternative splicing is the most important mechanism to generate the estimated 250,000 proteins from this

limited number of genes. In the alternative splicing process, several different isoforms of one gene can be created, by either including an exon into the mRNA or excising it as a part of an intron.

Detailed analysis demonstrated that 59% of the 245 genes present on chromosome 22 are alternatively spliced and 1,859 transcripts were found for the 544 genes on chromosome 19 (Lander, Linton *et al.,* 2001). Exon junction microarray experiments showed that at least 74% of all human genes are alternatively spliced (Johnson, Castle *et al.,* 2003).

The increasing number of human diseases that have been described to be caused by missplicing events also shows the importance of alternative splicing.

Alternative splicing is observed in all tissues, but tissue specific splicing is most frequently observed in brain cells (Stamm, Zhu *et al.,* 2000; Xu, Modrek *et al.,* 2002). Cells also can control alternative exon usage depending on the developmental stage.

There are five major patterns into which alternative splicing events can be classified: cassette exons, the use of alternative 5' splice sites and alternative 3' splice sites, mutually exclusive exons and retained introns. Recently, also usage of alternative promoters and poly A sites further contributes to isoform diversity (Figure 1-3).
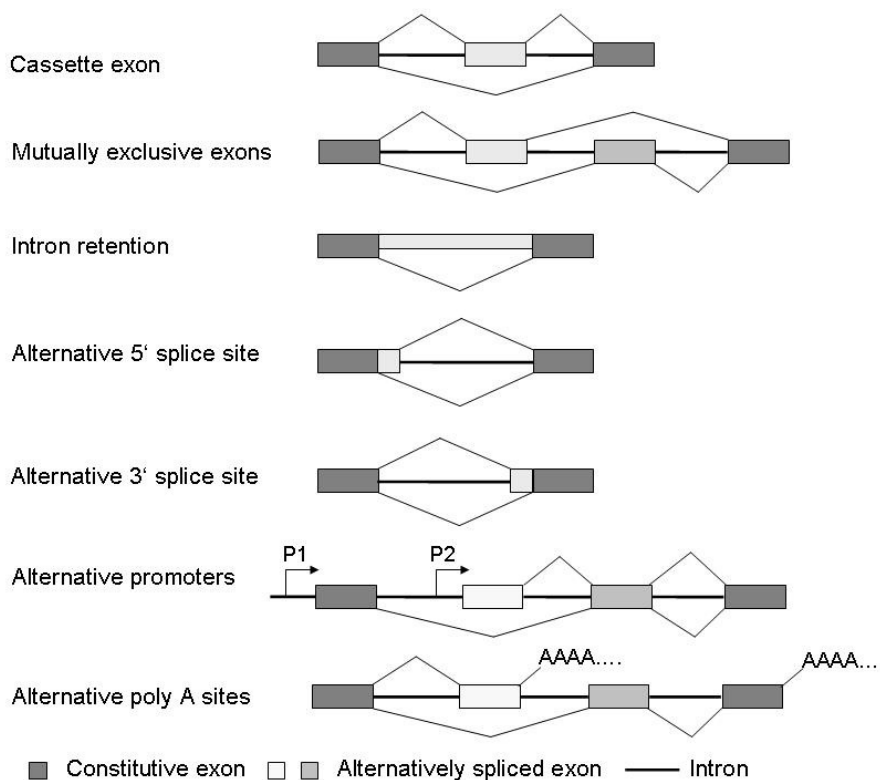
Figure 1-3: Types of alternative splicing. Exons are indicated as boxes, introns as thick lines. Thin lines indicate splicing patters. P1/P2: alternative promoters; AAAA…: poly A sites

Database analysis showed that about 53% of the human alternative exons are cassette exons (Stamm, Zhu *et al.,* 2000). More complicated alternative splicing patterns, combining basic patterns, are also frequently observed, for example the simultaneous skipping of several exons in the CD44 gene (Screaton, Bell *et al.,* 1992) or multiple alternative 3' splice sites in the SC35 gene (Sureau and Perbal, 1994). The majority (about 75%) of alternative splicing events changes the coding region, indicating that alternative splicing is the major mechanism for increasing protein diversity (Kan, Rouchka *et al.,* 2001; Zavolan, Kondo *et al.,* 2003).

A human gene usually generates an average of 2-3 transcripts. Extreme cases exist where genes can form a very high number of isoforms by combinatorial alternative splicing of multiple cassette exons. This is for example the case for neurexin3, which could generate 1,000 different mRNAs from one gene or for *Dscam* (Down syndrome cell adhesion molecule) in drosophila, which can produce over 38,000 different mRNA isoforms, which is 2-3 times the number of drosophila's predicted genes (Graveley, 2001).

1.2.1   Exon recognition and splice site selection

The weakly conserved consensus sequences marking exon/intron borders are loosely defined, very short and degenerated. This is not sufficient for precise exon recognition and intron removal, nevertheless the splicing machinery is able to discriminate small exons and huge introns very accurately. Therefore, several additional splicing elements are needed to define an exon. These cis-acting elements are found within exons or introns and either give way to exon inclusion (exonic/intronic splice enhancers ESE/ISE) or exon skipping (exonic/intronic splice silencers ESS/ISS) (Blencowe, 2000). These elements also have loosely defined, very short (5-8 nt), degenerate consensus sequences. This avoids interference with the coding ability of exons. Several types of enhancer/silencer elements have been identified (reviewed in Zheng, 2004), like purine rich (Dominski and Kole, 1994), pyrimidine rich (Zuccato, Buratti *et al.,* 2004) and A/C-rich elements (Coulter, Landree *et al.,* 1997).

Splice enhancers are found close to the splice sites they activate. Their action on splice sites is position dependent. Altering the location of splicing enhancers changes their dependence on particular trans-acting factors (Tian and Maniatis, 1994) and determines whether they activate 5' or 3' splice sites (Heinrichs, Ryner *et al.,* 1998) or even can convert enhancers into negative regulatory elements (Kanopka, Mühlemann *et al.,* 1996).

Proteins binding to cis-elements can be classified into two major groups: SR and SR-related proteins (Graveley, 2000) (see 1.3) and heterogeneous nuclear

ribonucleoproteins (hnRNPs) (Dreyfuss, Kim *et al.,* 2002) (see 1.4). These proteins usually contain one or more RNA binding motifs and protein:protein interaction domains. As the interaction between the individual splicing factors and the regulatory cis-elements is weak and not highly specific, different factors can act on the same elements and influence the same splice sites. A high specificity for exon recognition is achieved by interaction of several RNA binding proteins with other proteins. Interactions among these proteins and interactions with the catalytic core spliceosome components form a transient complex, which marks the exon (Wu and Maniatis, 1993; Hertel, Lynch *et al.,* 1997; Tacke and Manley, 1999) (Figure 1-4). In this way, exons are recognised with an extraordinary fidelity through combinatorial control (Smith and Valcarcel, 2000).
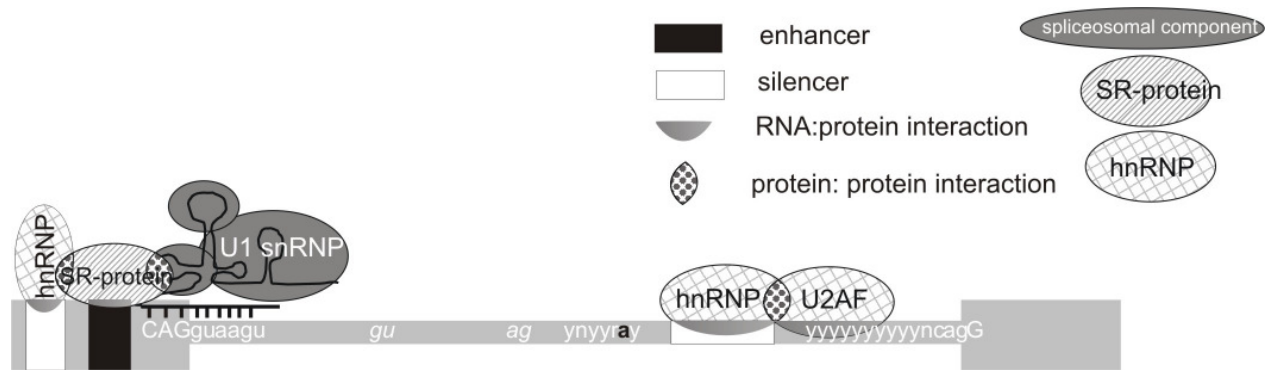
Figure 1-4: Elements involved in splice site selection. The figure shows the proteins that assemble on a pre-mRNA theoretical substrate. Exons are indicated as boxes, the intron as a thick line. Splicing regulatory elements (enhancers or silencers) are shown as white or black boxes. The 5' splice site CAGguaagu and 3' splice site (y)$_{10}$ncagG, as well as the branch point ynyyr**a**y, are indicated (y = c or u, n = a, g, c or u). Upper-case letters refer to nucleotides that remain in the mature RNA. Two major groups of proteins, hnRNPs (checked) and SR or SR-related proteins (striped), bind to splicing regulatory elements; the protein:RNA interaction is shown in grey. This protein complex assembles around an exon enhancer, stabilizing binding of the U1 snRNP close to the 5' splice site, for example because of protein:protein interaction (shown in dots) between an SR protein and the RS domain of U1-70K. This allows hybridisation (thick black line with bars) of the U1 snRNA (black) with the 5' splice site. The formation of the multi-protein:RNA complex allows discrimination between proper splice sites indicated as exon-intron borders and cryptic splice sites (small *gu ag*) that are frequent in pre-mRNA sequences. Factors at the 3' splice site include U2AF, which recognises pyrimidine-rich regions of the 3' splice sites and is antagonised by binding of several hnRNPs (e.g. hnRNP I) to elements of the 3' splice sites. Stripes: SR and SR-related proteins; checks: hnRNPs; grey: protein:RNA interaction; dotted: protein:protein interaction; thick black line with bars: RNA:RNA hybridisation. Adapted from Heinrich, Zhang *et al.,* 2005

The significance of the splicing regulatory elements and their interacting proteins is emphasised by the growing number of pathologies associated with them (reviewed in Stoilov, Meshorer *et al.,* 2002; Faustino and Cooper, 2003; Heinrich, Zhang *et al.,* 2005; Licatalosi and Darnell, 2006; Novoyatleva, Tang *et al.,* 2006) (see 1.5 for more details).

A number of special web-based programs is available for searching ESE/ESS motifs in specific sequences. Table 1-3 gives an overview of some of these tools.

Name	URL	Reference
ESE finder	http://rulai.cshl.edu/cgi-bin/tools/ESE3	Cartegni, Wang *et al.,* 2003; Smith, Zhang *et al.,* 2006
RescueESE	http://genes.mit.edu/burgelab/rescue-ese/	Fairbrother, Yeo *et al.,* 2004
ESRsearch	http://ast.bioinfo.tau.ac.il/ESR.htm	Goren, Ram *et al.,* 2006
PESX	http://cubweb.biology.columbia.edu/pesx/	Zhang, Kangsamaksin *et al.,* 2005

Table 1-3: Tools for searching ESE or ESS sequence elements.

1.2.2 Regulation of alternative splicing

Alternative splicing is highly regulated by transacting factors in a tissue or developmental stage specific manner. As a result, different splicing patterns can occur through tissue or development specific differences in the amount or activities of splicing factors. Some tissue specific splicing factors have been previously described, for example brain specific proteins nPTB (Markovtsov, Nikolic *et al.,* 2000) and Nova-1 (Jensen, Dredge *et al.,* 2000) or brain and testis specific SLM-1 (Stoss, Novoyatleva *et al.,* 2004).

Expression levels of several ubiquitously expressed general splicing factors like SR proteins and hnRNP A vary from one tissue to another, which can affect tissue specific splicing. Besides the expression level of splicing factors or their post-translational modifications also play an important role in the regulation of alternative splicing.

Extracellular stimuli, like cellular stress (Daoud, Mies *et al.,* 2002), hormones (Patel, Apostolatos *et al.,* 2004), growth factors (Cogan, Prince *et al.,* 1997), cytokines (McKay, Hunter *et al.,* 1994; Eissa, Strauss *et al.,* 1996) or changes in neuronal activity (Xie and Black, 2001) can also be reasons for changes in splice site selection. These external signals act through activation of signaling pathways, for instance the MAPK (mitogen activated protein kinase) pathways like Ras-Raf-MEK-ERK or SAPK/JNK that transduce the signal to the nucleus, leading to a wide range of responses which can effectively change activation of splicing factors (Van Der Houven Van Oordt, Diaz-Meco *et al.,* 2000; Matter, Herrlich *et al.,* 2002; Blaustein, Pelisch *et al.,* 2005).

1.2.3 Phosphorylation dependent control of the pre-mRNA splicing machinery

The proteins of the pre-mRNA splicing machinery undergo phosphorylation and dephosphorylation during the splicing process. Reversible phosphorylation of SR proteins marks certain steps in the splicing reaction (Cao, Jamison *et al.,* 1997; Xiao and Manley, 1998; Shin, Feng *et al.,* 2004; Shi, Reddy *et al.,* 2006). Experiments using phosphatase inhibitors also show that dephosphorylation is important for the catalytic splice reaction (Mermoud, Cohen *et al.,* 1992).

Changes in the phosphorylation stage, leading to hyper- or hypophosphorylation of SR proteins, were found to inhibit their splicing activity (Prasad, Colwill *et al.,* 1999). Phosphorylation of serine residues is important for the efficient recruitment of SR proteins from their storage compartments, nuclear speckles, to the sites of active transcription, which leads to diffusion of the speckles (Misteli, Caceres *et al.,* 1998). Changes in the phosphorylation state of splicing factors can influence their ability to interact with other proteins (Hartmann, Nayler *et al.,* 1999) or RNA (Chen, Cote *et al.,* 2001). For example, the interactions between SF2/ASF and other RS domain containing splicing factors such as U1-70K are regulated by phosphorylation (Xiao and Manley, 1997) and binding of SF2/ASF to cytoplasmic mRNA is enhanced by dephosphorylation (Sanford, Ellis *et al.,* 2005b). Phosphorylation can change the active concentration of splicing factors by controlling the release of SR proteins from the storage compartments (Wang, Lin *et al.,* 1998) and causing relocalisation of hnRNP proteins to the cytoplasm (Van Der Houven Van Oordt, Diaz-Meco *et al.,* 2000).

Splicing dependent dephosphorylation of shuttling SR proteins was studied (Huang, Yario *et al.,* 2004; Lai and Tarn, 2004). It was shown that the mRNA export receptor TAP favours binding of the hypophosphorylated forms of shuttling proteins, like SF2/ASF (Lai and Tarn, 2004). So dephosphorylation of SR proteins might be critical for their nuclear export or postsplicing functions.

Consequently, dynamic phosphorylation of SR proteins plays a significant role in several processes like spliceosome assembly, regulation of splice site selection, subcellular localisation of splicing factors and in mRNP maturation and/or export.

Tyrosine phosphorylation of non-SR proteins can also lead to changes of cellular localisation. For example, phosphorylation of the splicing factor YT521-B by specific nuclear non-receptor tyrosine kinases causes diffusion of YT bodies and drives the phosphorylated protein into the insoluble nuclear fraction (Rafalska, Zhang *et al.,* 2004).

Many studies demonstrated that phosphorylation of splicing factors can change splice site selection. It was shown that phosphorylation of specific splicing factors by CDC2-like kinases (CLK) 1-4 promotes exclusion of Tau exon 10 (Hartmann, Rujescu *et al.,* 2001). Exon 10 of human microtubule associated protein Tau is connected to frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17). Mutations in the gene encoding Tau increase exon 10 inclusion and lead to production of Tau protein with additional microtubule binding sites, which result in protein aggregation and formation of

filamentous lesions (Goedert, Crowther *et al.,* 1998; Hutton, Lendon *et al.,* 1998; Poorkaj, Bird *et al.,* 1998; Spillantini and Goedert, 1998; Hasegawa, Smith *et al.,* 1999; Varani, Hasegawa *et al.,* 1999; Jiang, Cote *et al.,* 2000; Kowalska, Hasegawa *et al.,* 2002).

Another case of phosphorylation dependent regulation of splice site selection is the formation of different CD44 isoforms during immune response (Weg-Remers, Ponta *et al.,* 2001). Following T-cell receptor stimulation, the Ras-Raf-MEK-ERK signaling pathway is activated and exon v5 inclusion is promoted. Enhanced ERK-mediated exon v5 inclusion could also be observed after forced expression of Sam 68 and phorbol ester stimulation (Matter, Herrlich *et al.,* 2002). Tyrosine phosphorylation of SLM-1 and SLM-2 (Sam68-like mammalian proteins) leads to inhibition of their RNA-binding activities (Haegebarth, Heap *et al.,* 2004) and influences their splicing properties (Stoss, Novoyatleva *et al.,* 2004).

1.2.4 Function of alternative splicing

Gene regulation through alternative splicing is more versatile than regulation through promoter activity. Similar to transcripts originating from different promoters, transcripts produced by alternative splicing are often tissue and/or developmental specific, resulting in effects only observed in special cells or developmental stages. Changes in promoter activity mainly modify the expression levels of mRNA. In contrast, changes in alternative splicing can alter the structure of the protein by deleting or inserting protein parts and vary transcript expression levels by causing mRNAs to undergo nonsense mediated decay (NMD). The main structural changes are classified in three groups: changes of the protein structure, changes in the 3' or 5' untranslated region and introduction of stop codons. The effects caused by alternative splicing range from a complete loss of function to slight effects that are not easy to detect. Information on functional characteristics of alternative splicing is collected in the Alternative Splicing Database (ASD) at http://www.ebi.ac.uk/asd/ and reviewed in Stamm, Ben-Ari *et al.,* 2005. Several examples of the functional importance of alternative splicing are summarised below.

1.2.4.1 Introduction of stop codons

mRNAs that contain premature stop codons can be degraded by nonsense mediated decay (NMD). During pre-mRNA splicing, the splicing machinery marks exon:exon junctions with a protein complex that influences the subsequent mRNA translation. An essential requirement for NMD to occur is that proteins are translated. If no translation takes place, mRNA is not subjected to NMD, even when premature stop codons accomplish the NMD

criteria (Maquat, 2005). About 25-35% of all alternative exons introduce frameshifts or stop codons into the pre-mRNA (Stamm, Zhu *et al.,* 2000; Lewis, Green *et al.,* 2003). Since roughly 75% of these exons are expected to be subject of NMD, an estimated 18-25% of transcripts are switched off by stop codons caused by alternative splicing and NMD (Lewis, Green *et al.,* 2003). This process which is named RUST (regulated unproductive splicing and translation) presently characterises the function of alternative splicing with the most obvious biological consequences (Figure 1-5).
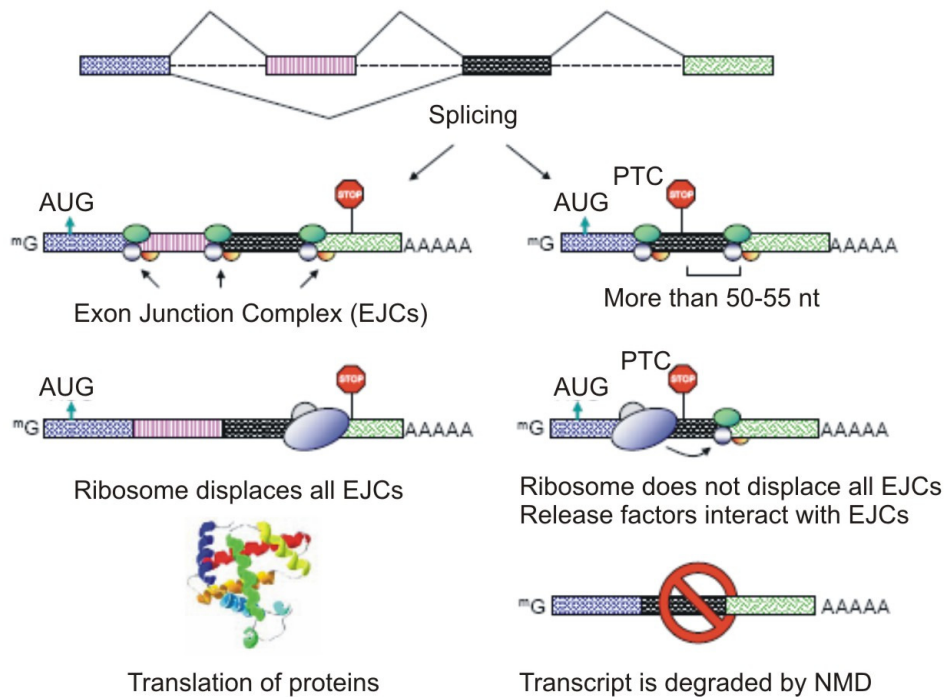
Figure 1-5: A current model for NMD in mammalian cells. A premature termination codon (PTC) 50 nucleotides (nt) upstream of the last exon-exon junction activates NMD. Translation causes the displacement of the exon junction complexes (EJCs) placed upstream of each splice junction. Termination of translation upstream of one or more EJCs triggers NMD by recruitment and activation of factors released by ribosomes. Termination in the last exon results in displacement of all EJCs and a stable mRNA. (Figure adapted from http://compbio.berkeley.edu/people/ed/rust/)

The precise number of genes influenced by RUST is only an approximation, as mRNAs undergoing nonsense mediated decay will be unstable and underrepresented in cDNA libraries, which leads to an underestimation of RUST (Green, Lewis *et al.,* 2003; Lewis, Green *et al.,* 2003). However, recent array data suggest that transcripts undergoing RUST are only minor forms (Pan, Saltzman *et al.,* 2006).

1.2.4.2 Changes of the protein structure

Approximately 75% of alternative splicing events occur in the translated regions of mRNAs and will affect the protein coding region (Okazaki, Furuno *et al.,* 2002; Zavolan, Kondo *et al.,* 2003). Modifications in the protein primary structure can change the binding

properties of proteins, influence their intracellular localisation and alter their enzymatic activity and/or protein stability by diverse mechanisms. One frequently found mechanism is the introduction of protein domains that are caused to undergo posttranslational modification, such as phosphorylation.

1.2.4.2.1 Binding properties

Protein isoforms generated by alternative splicing vary in their binding properties to small molecular weight ligands (e.g. hormones) as well as to macromolecules such as proteins or nucleic acids. Binding activity can be completely abolished due to alternative splicing as it happens for example for the TSH (thyroid stimulating hormone) receptor. Alternative variants of the TSH receptor, occurring in TSH-secreting tumours, are unable to bind TSH and cause insensitivity to TSH (Ando, Sarlis *et al.,* 2001). Alternative splicing can also determine the ligand specificity of a receptor. A well-studied example is the FGFR-2 (fibroblast growth factor receptor) gene, which creates two isoforms that differ by 49 amino acids in the extracellular domain. Depending on the presence of this domain, the receptor binds to both fibroblast and keratinocyte growth factor or only to fibroblast growth factor (Miki, Bottaro *et al.,* 1992). The affinities between the modified protein and its ligand can also be changed. For example, the angiotensin II type receptor isoforms show threefold difference in binding affinity (Martin, Willardson *et al.,* 2001) or binding of GnRH (gonadotrophin releasing hormone) to shorter variants of GnRH receptor is reduced 4-10 fold (Wang, Oh *et al.,* 2001), which abolishes signaling.

In the same way, interactions of transcription factors with DNA can be changed by alternative splicing, which contributes to transcriptional regulation (reviewed by Lopez, 1995). The loss of binding between a transcription factor isoform and DNA can restrain transactivation in a dominant-negative way, if the binding-negative isoform can substitute the binding-competent isoform in the transactivation complex. Alternative splicing often does not directly influence DNA binding, but alters the formation of complexes between different transcription factors, which as a result regulates the affinity between transcription factor complexes and DNA (Kozmik, Kurzbauer *et al.,* 1993; Ormondroyd, De La Luna *et al.,* 1995). The binding properties of RNA binding proteins can be altered by alternative splicing in a similar way, for example of the Staufen RNA binding protein (Monshausen, Putz *et al.,* 2001).

1.2.4.2.2 Intracellular localisation

Alternative splicing can modulate the intracellular localisation of proteins, usually by influencing localisation signals or regulating the interaction of proteins with membranes. Deletion or interruption of transmembrane domains of membranous proteins can lead to their accumulation in the cytoplasm or secretion into the nuclear space. Nonmembrane bound soluble isoforms can lose their ability to transduce signals (Kestler, Agarwal *et al.,* 1995; Tone, Tone *et al.,* 2001) and become less stable (Garrison, Hojgaard *et al.,* 2001). If the soluble isoform keeps the ability to bind a ligand, it can control the concentration and bioactivity of that ligand, which indirectly interferes with the function of the membrane bound form. This type of regulation has for example been described for the IL-4 (interleukin-4) receptor (Blum, Wolf *et al.,* 1996). Alternative splicing can regulate the localisation of proteins in different subcellular sites and organelles. Proteins can be sequestered into compartments, where they carry out no function. This mechanism is widely used for receptor molecules. Alternative splicing can regulate their retention in membrane enclosed compartments. For example, the inclusion of an endoplasmatic reticulum retention signal in the metabotropic glutamate receptor mGluR1 decreases cell surface expression of this receptor and limits its trafficking (Chan, Soloviev *et al.,* 2001). Also sub-localisation of proteins within organelles can be affected by alternative splicing. For example, due to alternative splicing, proteins in the nucleus can be present in different nuclear substructures, such as the nucleoplasm or speckles (Nishizawa, Usukura *et al.,* 2001).

1.2.4.3 Protein and mRNA stability

Inclusion of alternate protein domains can control the half-life of proteins. Protein stability can be altered due to autophosphorylation that signals the degradation of receptor molecules. For example, the autophosphorylation-dependent degradation of interleukin-1 receptor-associated kinase (IRAK) is isoform specific, leading to a molecule that is not down-regulated by its ligand (Jensen and Whitehead, 2001). The effect of alternative splicing dependent protein stability also has been studied for the *fosB* gene, which creates a shorter isoform that is more stable than the full-length protein. This isoform accumulates in a specific brain region in response to many types of chronical behavioural changes (Nestler, Kelz *et al.,* 1999). Some examples have been described where alternative splicing changes properties of mRNA. Alternative splicing events occurring in 5' and 3' UTRs may change the stability of RNA. For example, alternative exons in the 5' UTR of HIV-1 can either promote or inhibit

the nuclear degradation of their mRNA, which regulates HIV-1 gene expression (Krummheuer, Lenz *et al.,* 2001).

1.2.4.4 Posttranslational modifications

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

1.3 SR and SR-related proteins

As already mentioned above, SR and SR-related proteins are essential for the splicing reaction (Manley and Tacke, 1996; Graveley, 2000). They are members of a class of highly conserved proteins in metazoans and are necessary for constitutive splicing as well as for the regulation of alternative splice site selection (Fu, 1995; Graveley, 2000). SR proteins have a characteristic structural organisation (Figure 1-6), containing one or two N-terminal RNA-binding domains (RNA recognition motif, RRM), that interact with pre-mRNA and a C-terminal arginine/serine rich domain of variable length, needed for protein interactions. The serine residues in the RS domain were found to be extensively phosphorylated. This phosphorylation influences protein interactions (Wang, Lin *et al.,* 1998; Xiao and Manley, 1998) as well as the subcellular localisation of SR proteins (Gui, Lane *et al.,* 1994; Colwill, Pawson *et al.,* 1996). Both events can alter their function in splicing. The RS domain of some SR proteins further acts as a nuclear localisation signal mediating the interaction between SR proteins and the nuclear import receptor transportin-SR, which mediates nucleocytoplasmic shuttling of SR proteins (Caceres, Misteli *et al.,* 1997; Kataoka, Bachorik *et al.,* 1999; Lai, Lin *et al.,* 2000). The RS domain can also directly bind to RNA (Shen and Green, 2006).

The SR-related proteins (SRrps) are members of another class of RS domain containing proteins. Most of these proteins also contain RRMs. To this class belong e.g. U1-70K, both subunits of U2AF, splicing coactivators SRm 160/300, RNA helicase hPRP16, as well as alternative splicing regulators like Tra and Tra2, which are involved in splice site selection.

SR and SR-related proteins play a role in splice site selection and spliceosome assembly by interacting with other splicing factors through their RS domain. They are recruiting components of the core spliceosome to the splice sites (Wu and Maniatis, 1993; Tacke and Manley, 1999). It was also shown that the RS domain can directly interact with the pre-mRNA branchpoint as well as the 5' splice site (Shen, Kan *et al.,* 2004). Additionally to pre-mRNA processing, they play a significant role in mRNA transport, stability and translation (reviewed by Sanford, Longman *et al.,* 2003).

A. Human SR Proteins

SRp20 RRM RS

SC35 RRM RS

SRp46 RRM RS

SRp54 RRM RS

SRp30c RRM RRMH RS

SF2/ASF RRM RRMH RS

SRp40 RRM RRMH RS

SRp45 RRM RRMH RS

SRp75 RRM RRMH RS

9G8 RRM Z RS

B. Human SR-related Proteins

U2 Auxiliary Factor

U2AF35 RRM RS

U2AF65 RS RRM RRM RRM

snRNP Components

U1-70K RRM RS RS

U5-100K RS DEXD/H Box

U4/U6 U5-27K RS

hLuc7p Zr Zn RS

Splicing Regulators

hTra2alpha RS RRM RS

hTra2beta RS RRM RS

Splicing Coactivators

SRm160 RS RS

SRm300 RS RS/P

RNA Helicases

hPrp16 RS DEXD/H Box

HRH1 RS DEXD/H Box

Protein Kinases

Clk/Sty RS Kinase

Figure 1-6: Schematic diagram of human SR and SR-related proteins. A) Domain structures of known members of the human SR protein family are depicted. Light red boxes: RRM (RNA recognition motif) and RRMH (RRM homology); green box: Zinc knuckle (Z); dark red boxes: arginine/serine (RS) rich domain. B) Domain structures for some of the human SR-related proteins that participate in pre-mRNA splicing are depicted. Light red boxes: RRM; dark red boxes: RS domain; green: zinc finger (Zn); DEXD/H Box: motif characteristic for RNA helicases. Figure adapted from Graveley, 2000.

One important function of SR and SR-related proteins is to activate suboptimal adjacent splice sites (Blencowe, 2000) in alternative splicing. They are thought to stimulate the recognition of weak upstream 3' splice sites by recruiting U2AF, or to facilitate U1 snRNP binding to the 5' splice site (reviewed in Black, 2003). In addition, several SR proteins have antagonistic effects on alternative splicing, e.g. the regulation of β-tropomyosin through antagonistic effects of SF2/ASF and SC35 (Gallego, Gattoni *et al.,* 1997). SR and SR-related

proteins bind to the cis-acting elements located within exonic or intronic sequences. SR protein binding sites within ESEs have a positive effect on splice site selection. Their binding leads to recruitment and stabilisation of U1 snRNP and U2AF at the 5' and 3' splice site (Robberson, Cote *et al.*, 1990; Boukis, Liu *et al.*, 2004). This process is called exon definition and is schematically shown in Figure 1-7 A. By forming numerous protein:protein interactions across introns, SR proteins bring the 5' and 3' splice sites close to each other in early spliceosome assembly (Figure 1-7 B). As a result, the so-called intron bridging complex is formed, which is mediated by simultaneous interactions of SR proteins with U1 snRNP-associated 70 kDa protein (U1-70K) at the 5' splice site and the 35 kDa subunit of U2AF (U2AF35) at the 3' splice site. After E complex formation SR proteins also help in the recruitment of the U4/U6·U5 tri-snRNP to the pre-spliceosome (Roscigno and Garcia-Blanco, 1995) (Figure 1-7 C). Moreover, SR proteins can compensate for a weak polypyrimidine tract by recruiting U2AF (Figure 1-7 D, upper panel), while interacting with ESEs. By binding to ESEs, SR proteins can antagonise the negative activity of hnRNPs, such as hnRNP A1 (Blencowe, 2000; Hastings and Krainer, 2001) (Figure 1-7 D, lower panel).
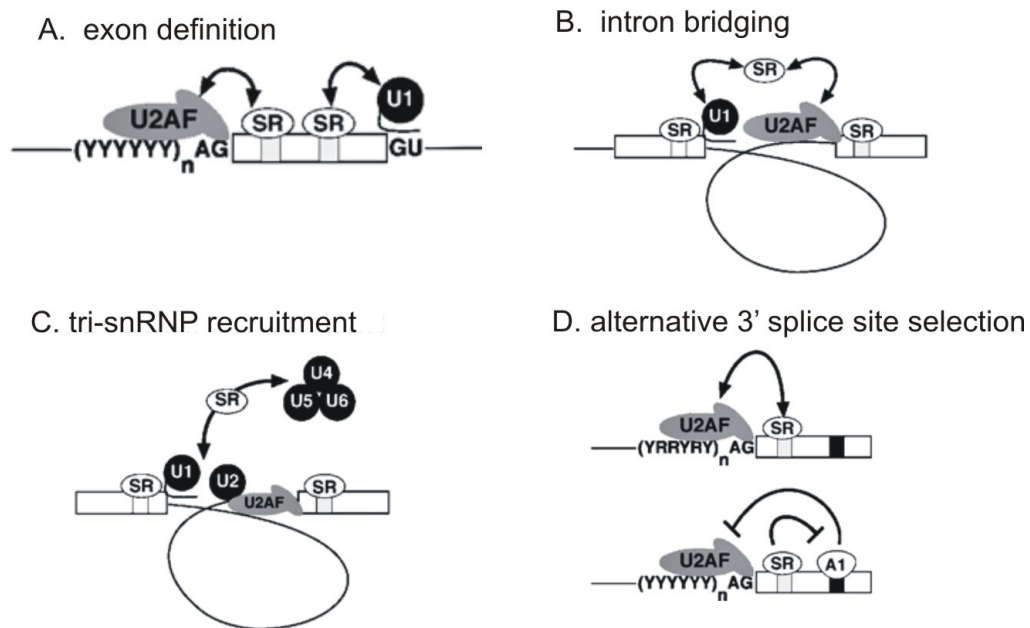
Figure 1-7: Roles of SR proteins in spliceosome assembly. A) U2AF (grey oval) at an upstream 3' splice site and U1 snRNP (black circle) at a downstream 5' splice site. The binding to RNA is facilitated by SR proteins bound to ESEs (light grey boxes). YYYYYY: strong polypyrimidine tract. B) 5' and 3' splice sites can be juxtaposed early in the splicing reaction by intron bridging interactions between SR proteins and the RS domain containing subunits of U1 snRNP and U2AF. C) SR proteins can recruit the U4/U6·U5 tri-snRNP to the pre-spliceosome. D) SR proteins bound to ESEs promote alternative 3' splice site selection by recruiting U2AF to suboptimal 3' splice sites. YRRYRY: weak polypyrimidine tract. Alternatively, exonic splicing silencers (ESS, black boxes) can recruit splicing repressor proteins like hnRNP A1 and block 3' splice site selection by U2AF. Figure adapted from Sanford, Ellis *et al.*, 2005a.

In addition, SR and SR-related proteins can remove factors from the pre-mRNA that inhibit splicing by competing for binding sites on the target RNA (Eperon, Makarova *et al.,* 2000; Zhu, Mayeda *et al.,* 2001). This was shown by *in vitro* studies on substrates that have rather strong 3' splice sites which do not require an RS domain (Zhu and Krainer, 2000).

In summary, exon recognition and splice site selection are achieved by coordinated positive and negative regulation, supplied by SR and SR-related proteins and hnRNP proteins, respectively.

Tra2-beta1

Human Transformer 2-beta (Tra2-beta) is a homologue of the sex determination factor Transformer 2 (Tra2) from *Drosophila melanogaster*. Together with Transformer (Tra), this protein regulates the sex determination in somatic cells through a cascade of alternative splicing events (Dauwalder, Amaya-Manzanares *et al.,* 1996; Nayler, Cap *et al.,* 1998). Another mammalian homologue of *Drosophila* Tra2 is Tra2-alpha (Matsuo, Ogawa *et al.,* 1995; Dauwalder, Amaya-Manzanares *et al.,* 1996; Segade, Hurle *et al.,* 1996; Beil, Screaton *et al.,* 1997). Tra2, Tra2-alpha and Tra2-beta share a similar structure of two RS domains flanking a central RRM. Human Tra2-alpha has regulatory functions in splicing that are conserved between *Drosophila* and humans. hTra2-alpha can partially compensate for the loss of *Drosophila* Tra2, when expressed in flies, which influences both female sexual differentiation and alternative splicing of doublesex dsx pre-mRNA. Tra2-beta was identified via its interactions with splicing factors SC35, SF2/ASF and SRp30c (Amrein, Hedley *et al.,* 1994; Beil, Screaton *et al.,* 1997; Nayler, Cap *et al.,* 1998).

The human tra2-beta gene consists of 10 exons, of which two are alternatively spliced (Figure 1-8). It generates at least five RNA isoforms (tra2-beta1 to beta5) by alternative splicing, alternative polyadenylation and alternative promoter usage. Two of these RNAs are translated to generate the proteins Tra2-beta1 and Tra2-beta3. tra2-beta2 and tra2-beta4 cannot be translated due to the presence of exon two, which inserts an in-frame stop codon. The translation of tra2-beta5 mRNA also gives rise to the Tra2-beta3 protein.
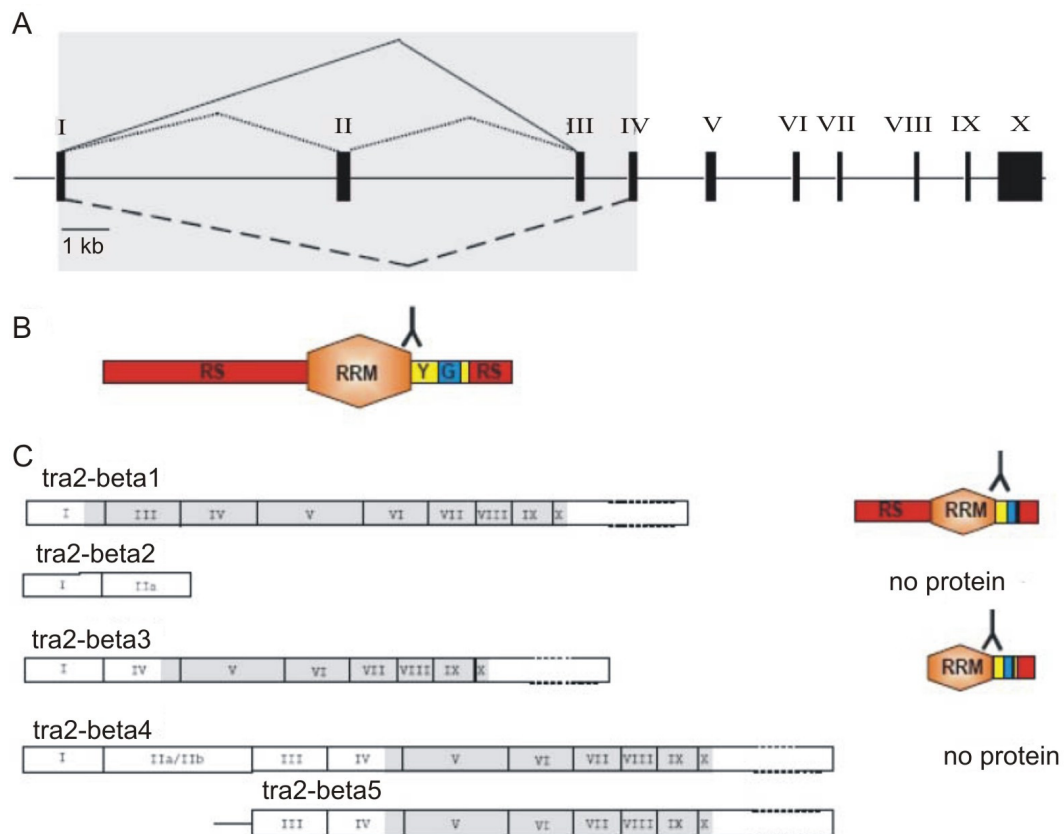
Figure 1-8: The tra2-beta gene structure. A) The exon-intron structure is drawn to scale. Exons are shown as black boxes, introns as lines. The shaped region marks the sequence of Tra2-beta minigene. B) Structure of Tra2-beta1 protein consisting of an RNA recognition motif (RRM), flanked by two SR repeats (red). The protein also has a tyrosine rich (yellow) and glycine rich (blue) stretch, located between the C-terminal SR repeat and the RRM. The position of the epitope in Tra2-beta1 protein recognised by the pan-Tra2 antiserum is shown on top. C) Transcripts derived from the tra2-beta gene. Boxes indicate the individual exons. The shading shows the open reading frame. Right side: proteins encoded by each of the transcripts. The position of the epitope in the proteins recognised by the pan-Tra2 antiserum is shown on top. Figure adapted from Stoilov, Daoud *et al.,* 2004.

The two protein isoforms differ in the presence of the first RS domain (Figure 1-8). The resulting hTra2-beta3 protein is expressed in a number of tissues and has no influence on Tra2-beta splice site selection. The generation of the two RNA isoforms which are not translated to proteins is regulated through alternative splicing by external stimuli, such as T-cell receptor stimulation (Beil, Screaton *et al.,* 1997) and neuronal activity (Daoud, Da Penha Berzaghi *et al.,* 1999).

hTra2-beta1 is a SR-related protein, which localises in speckles in the nucleus and interacts with chromatin organising proteins. It is upregulated in breast cancer and regulates alternative splicing of the CD44 gene (Watermann, Tang *et al.,* 2006).

Tra2-beta1 protein concentration is autoregulated through a negative feedback regulation. Increased Tra2-beta1 concentration changes the splicing of its own pre-mRNA to generate an isoform that does not yield protein. Hyperphosphorylated Tra2-beta1 has reduced

ability to bind to RNA. It was shown that presence of CLK2 kinase inhibits usage of exons 2 and 3, giving rise to htra2-beta3 mRNA (Stoilov, Daoud *et al.,* 2004).

Recently, it was established that hTra2-beta1 binds to the degenerate RNA sequence GHVVGANR. This motif is found more frequently in exons than in introns (Stoilov, Daoud *et al.,* 2004). It was shown that SMN2 exon 7 contains this sequence and Tra2-beta1 can mediate inclusion of this exon (Hofmann, Lorson *et al.,* 2000). Thus, splicing factor Tra2-beta1 usually promotes inclusion of exons by recruiting or stabilising an exon recognition complex after binding to a degenerate RNA element.

1.4   Heterogeneous nuclear ribonucleoproteins

Heterogeneous nuclear ribonucleoproteins (hnRNPs) were first described as a family of proteins binding heterogeneous nuclear RNA in hnRNP complexes. All hnRNPs have some structural motifs in common. Most common is the consensus RNA recognition motif (RRM), also called RNP motif, which usually is found at the N-terminus. Binding studies have shown that hnRNP proteins can bind sequence-specifically to single-stranded RNA or DNA (Dreyfuss, Matunis *et al.,* 1993). The RNP motif contains two consensus sequences RNP1 and RNP2, which are located at a distance of about 30 amino acids to each other in the approximately 90 amino acids long RRM domain (Dreyfuss, Swanson *et al.,* 1988). The RRM of certain hnRNPs was also found to be involved in protein:protein interactions. For example, the interaction of hnRNP A1 with IκBα is mediated by its N-terminal RNA binding domain (Hay, Kemp *et al.,* 2001).

Other domains in hnRNPs are RGG and KH motifs, which are not found throughout all family members. The RGG box is an RG-rich region, which contains a number of arginine/glycine/glycine (RGG) repeats with several aromatic acids in between (Kiledjian and Dreyfuss, 1992). It is involved in RNA binding and also connected to protein:protein interactions, transcriptional activation and nuclear localisation (Dreyfuss, Matunis *et al.,* 1993). Many of the arginine residues within the RGG box are potential methylation sites, which could play a role in regulation of the RNA binding activity. Arginine methylation is a common modification for several hnRNP proteins (Liu and Dreyfuss, 1995; Kzhyshkowska, Schutt *et al.,* 2001). The KH domain was first identified in hnRNP K (Siomi, Matunis *et al.,* 1993) and can also be found e.g. in hnRNP E1 (Leffers, Dejgaard *et al.,* 1995; Ostareck, Ostareck-Lederer *et al.,* 1997). Most KH proteins have more than one copy of the KH motif. hnRNP K e.g. has three copies. The KH domain is 50 amino acids long and contains

conserved octapeptide repeats of Ile-Gly-X-Gly-X-Ile (X being any amino acid), which are essential for RNA binding (Siomi, Matunis *et al.,* 1993).

A number of hnRNP genes produce multiple isoforms through alternative splicing, e.g. hnRNP D. hnRNP D has 10 exons and generates four isoforms by alternative splicing of exons 2 and 7 (Wagner, Demaria *et al.,* 1998). It has also been shown that several hnRNP proteins are able to shuttle between the nucleus and the cytoplasm, e.g. hnRNP A1 (Pinol-Roma and Dreyfuss, 1992).

hnRNP proteins were found to play a role in several processes in the cell, e.g. transcription regulation, telomere-length maintenance, immunoglobulin gene recombination, splicing, pre-rRNA processing, transport, localisation and stability of mRNAs (Dreyfuss, 1986; Dreyfuss, Kim *et al.,* 2002). hnRNP A1, one of the best studied hnRNP proteins, was found to play an antagonistic role to the action of SR proteins that promote distal 5' splice site usage in E1A and β-globin pre-mRNAs (Mayeda and Krainer, 1992; Caceres, Stamm *et al.,* 1994). Another example for negative regulation of splice site selection is the ubiquitously expressed polypyrimidine tract binding protein PTBP1 (hnRNP I), which mediates silencing of exons by binding to a number of intronic splicing silencers of alternatively spliced pre-mRNAs (reviewed by Wagner and Garcia-Blanco, 2001).

Recent studies also showed that some hnRNP members play a role in mediating DNA repair, for example hnRNP B1 (Iwanaga, Sueoka *et al.,* 2005). Other hnRNPs were found to regulate transcription or translation, e.g. addition of hnRNP C1 enhanced translation of c-myc mRNA *in vitro* (Kim, Paek *et al.,* 2003). Based on their functions certain hnRNP family members have also been linked to tumour progression (reviewed in Carpenter, Mackay *et al.,* 2006). A number of hnRNPs was found to play a role in HIV gene expression, amongst them hnRNP E (Woolaway, Asai *et al.,* 2007), hnRNP H (Schaub, Lopez *et al.,* 2007) or hnRNP U (Valente and Goff, 2006).

hnRNP G

hnRNP G, also known as RBMX, is a ubiquitously expressed O-glycosylated nuclear protein, which is encoded by the *RBMX* (RNA-binding motif gene, X chromosome) gene localised on the X chromosome. The hnRNP G protein contains one RNP-consensus RNA binding domain (RBD) at the N-terminus and a domain rich in serine, arginine and glycine residues at the C-terminus. This domain also contains three RGG boxes, surrounded by other RG/GR repeats, similar to an RGG box (Soulard, Valle *et al.,* 1993). hnRNP G has a

paralogue on the Y chromosome, the *RBMY* (RNA-binding motif gene, Y chromosome) gene. It encodes the protein RBM (or RBMY), which is involved in nuclear RNA-processing during spermatogenesis and is expressed only in nuclei of male germ cells. Deletions of *RBMY* are associated with male infertility (Ma, Inglis *et al.,* 1993). The cDNAs of hnRNP G and RBM show about 60% homology (Delbridge, Ma *et al.,* 1998).

Multiple homologous (92-96% homology) *RBMX*-like sequences (RBMXLs) were found dispersed in the human genome. These RBMXLs all lack introns and are probably derived from retroposition events. They are conserved sequences and present in all primates. Some of them show deletions, insertions or stop codons, which probably results in non-functional proteins. RBMX was found subject to X inactivation as it is common for X-partners of X/Y gene pairs, so the expressed RBMXLs might provide compensatory expression (Lingenfelter, Delbridge *et al.,* 2001). Also they could play an important functional role during pachytene of meitosis, since the X and Y chromosomes are condensed into transcriptionally inactive structures (XY body) (Mccarrey and Thomas, 1987) at this time. Because of their high sequence similarity they might encode proteins with the same functions as RBMX and might replace them during prophase (Elliott, 2004). Another RBMX-related gene is hnRNP G-T, which is meiosis-specifically expressed in testis (Elliott, Venables *et al.,* 2000; Venables, Elliott *et al.,* 2000). It also contains no introns, but with only 69% identity on nucleotide level and 73% identity on protein level it shows a lower sequence homology than the RBMXLs (Lingenfelter, Delbridge *et al.,* 2001).

hnRNP G, hnRNP G-T and RBM interact with each other and with ubiquitously expressed proteins involved in splice site selection and signal transduction (e.g. SRp30c, Tra2-beta1, Sam 68, SLM-2) (Venables, Vernet *et al.,* 1999; Elliott, Bourgeois *et al.,* 2000; Venables, Elliott *et al.,* 2000). RBM was found to co-localise with several splicing factors in spermatocytes, amongst them Tra2-beta1 (Elliott, Oghene *et al.,* 1998). Furthermore, it was shown that RBM as well as hnRNP G interact with Tra2beta1 *in vitro* and *in vivo* (Venables, Elliott *et al.,* 2000; Hofmann and Wirth, 2002) and both can promote inclusion of SMN2 exon 7 *in vivo*. SMN2 is an almost identical copy of the SMN1 gene, which is lost in spinal muscular atrophy (SMA) (1.5.2). The inclusion of SMN2 exon 7 is mediated by direct interaction of RBM or hnRNP G with Tra2-beta1 (Hofmann and Wirth, 2002).

Other data demonstrated that hnRNP G also can have an opposite effect to Tra2-beta1 upon the incorporation of several exons, e.g. tropomyosin exon 5 or the dystrophin pseudogene. Tropomyosin exon 5 exists in 2 versions, which differ in skeletal muscle cells

(SK exon) and non-muscle cells (NM exon). NM usage is stimulated by hnRNP G and repressed by Tra2-beta1, while SK usage is stimulated by Tra2-beta1 and repressed by hnRNP G. Also it was shown that hnRNP G inhibits CD44 v5 exon 5 inclusion which is promoted by SRrp86, to which it strongly binds (Li, Hawkins *et al.,* 2003).

A possible target sequence for hnRNP G could be AAGT, comparing the sequences from SK exon and dystrophin pseudo-exon, to which it binds (Nasim, Chernova *et al.,* 2003). These sequences also contain CAA motifs. Interestingly, it was found recently for RBMY that it binds to RNA stem loops with a CA/UCAA loop and a GUC-loop-GAY consensus in a novel mode of interaction, whereas hnRNP G or hnRNP G-T were unable to bind this stem loop structure (Skrisovska, Bourgeois *et al.,* 2007).

Knockdown of *rbmx* in zebrafish embryos, which is highly homologue to human hnRNP G, showed that it plays a key role in brain development (Tsend-Ayush, O'Sullivan *et al.,* 2005). This supports the role it might play in X-linked mental retardation, as its chromosome locus makes it a prime candidate for this disease (Graves, Gecz *et al.,* 2002). Recent studies showed that hnRNP G is associated with the proapoptotic Bax gene in human breast cancer (Martinez-Arribas, Agudo *et al.,* 2006), it has growth inhibitory effects against human oral squamous cell carcinomas (Shin, Kang *et al.,* 2006), and plays a main role in DNA repair in response to p53 (Shin, Kim *et al.,* 2007).

These examples show that hnRNP G might play a role in several mechanisms in the cell. However, its function is still not well understood and needs further investigation.

1.5   Human diseases that are caused by missplicing events

An increasing number of diseases associated with missplicing events demonstrates the physiologic importance of alternative splicing (Stoilov, Meshorer *et al.,* 2002, Faustino and Cooper, 2003; Novoyatleva, Tang *et al.,* 2006). Approximately 10% to 15% of human disease-causing mutations affect splicing (Krawczak, Reiss *et al.,* 1992; Stenson, Ball *et al.,* 2003). The mutations can be subdivided into two types. Type I mutations affect invariant positions of splice sites, usually resulting in severe disease because recognition of the affected exon is destroyed. Type II mutations occur in variant positions of splice sites, in enhancer or silencer regions. These mutations often lead to an altered ratio of alternative exon use and the resulting phenotypes can be more subtle. Often, these mutations are silent, affecting the protein structure only through a change in the mRNA composition (Table 1-4)

Gene	Name	Wild type	Mutant	Ref
Adenosine deaminase	A215T	CTGTCCAC**G**CC	CTGTCCAC**A**CC	Ozsahin, Arredondo-Vega *et al.,* 1997
Adenomatosis polyposis coli	R623R	ACTTACCG**G**AGC	ACTTACCG**T**AGC	Montera, Piaggio *et al.,* 2001
Androgen receptor	S888S	ATGGTGAG**C**GTG	ATGGTGAG**T**GTG	Hellwinkel, Holterhus *et al.,* 2001
Ataxia telangiectasia mutated (ATM)	748C>T	ATT**C**GAGTG	ATT**T**GAGTG	Teraoka, Telatar *et al.,* 1999
	802C>T	ACT**C**AACAT	ACT**T**AACAT	
	3576G>A	AAG**G**TTTTA	AAG**A**TTTTA	
	6154G>A	CTC**G**AAACA	CTC**A**AAACA	
ATPase Cu^{2+} transporting, alpha-polypeptide	C1302R	GAT**G**GAATC	GAT**C/A**GAATC	Das, Levinson *et al.,* 1994
BRCA1	64T>G	ATC**T**TAGAG	ATC**G**TAGAG	Yang, Swaminathan *et al.,* 2003
CD45	77C>G	CACC**C**GCAA	CACC**G**GCAA	Lynch and Weiss, 2001
Fumarylacetoacetate Hydrolase (FAH)	Q279R	AAGC**A**GGAC	AAGC**G**GGAC	Dreumont, Poudrier *et al.,* 2001
FAH	N232N	ATGAA**C**GAC	ATGAA**T**GAC	Ploos Van Amstel, Bergman *et al.,* 1996
Fibrillin 1 FBN1	I2118I	ATCAT**C**GTG	ATCAT**A/T**GTG	Liu, Qian *et al.,* 1997
Fibrillin 2 FBN2	D1114H	ACG**G**ACATC	ACG**C**ACATC	Babcock, Gasner *et al.,* 1998
Hexosaminidase A	P417L	GCGC**C**GGGC	GCGC**T**GGGC	Wakamatsu, Kobayashi *et al.,* 1992
Hypoxanthine phosphoribosyl transferase (HPRT1)	G40R	CAT**G**GACTA	CAT**A**GACTA	Torres, Mateos *et al.,* 2000
	R48H	GAAC**G**TCTT	GAAC**A**TCTT	
	V133M	ATT**G**TGGAA	ATT**A**TGGAA	
Microtubule-associated protein tau (MAPT)	N279K	ATTAA**T**AAG	ATTAA**G**AAG	Clark, Poorkaj *et al.,* 1998
	S305N	GGCA**G**Tgtg	GGCA**A**Tgtg	D'souza, Poorkaj *et al.,* 1999
	L284L	GATCT**T**AGC	GATCT**C**AGC	D'souza, Poorkaj *et al.,* 1999
	N296N	ATAA**T**ATCA	ATAA**C**ATCA	Spillantini, Yoshida *et al.,* 2000
	Δ280K	AAT**AAG**AAG	AATAAG	Rizzu, Van Swieten *et al.,* 1999
MutL homologue 1, MLH1	K461Stop	GAG**A**AGAGA	GAG**T**AGAGA	Stella, Wagner *et al.,* 2001
Neurofibromin I	K354K	CTTAA**G**AAC	CTTAA**A**AAC	Fahsold, Hoffmeyer *et al.,* 2000
Phenylalanine hydroxylase	V399V	AGAAAGT**A**AGG	AGAAAGT**T**AGG	Chao, Hsiao *et al.,* 2001

Table 1-4: Mutations in regulatory elements that cause aberrant splicing. Examples of mutations not located in splice sites are given. A comprehensive and updated list is available at the Alternative Splicing Database (Thanaraj, Stamm *et al.,* 2004) under http://www.ebi.ac.uk/asd/.

A change of alternative splicing without obvious mutations has been observed in several diseases. For example, in schizophrenia, altered isoform ratios of the gamma-aminobutyric acid receptor (Huntsman, Tran *et al.,* 1998), neural cell adhesion molecule (Vawter, Frye *et al.,* 2000), and N-methyl-D-aspartate R1 receptor (NMDAR1) (Le Corre, Harper *et al.,* 2000) have been observed, indicating that the composition of regulatory proteins can affect splice site selection. An interesting observation is that the same mutation-bearing allele shows different alternative splicing patterns depending on the genetic background, which suggests that alternative splicing is a potential genetic modifier (Nissim-Rafinia and Kerem, 2002).

In the following subchapters two well-characterised examples of splicing disorders will be discussed to show the complexity of enhancer arrangements and their regulation through multiple proteins.

1.5.1 Tauopathies

Tauopathies are a group of diverse dementias and movement disorders, to which frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) belongs. FTDP-17 is an autosomal dominant disorder caused by mutations in the *MAPT* gene, which encodes the protein Tau. Symptoms of FTDP-17 are personality changes, reduced speech, and a loss of judgement and insight (Wilhelmsen, 1999). Tau is a microtubule binding protein expressed in the nervous system and involved in the assembly and stabilisation of microtubules (Hong, Zhukareva *et al.,* 1998; Hutton, Lendon *et al.,* 1998). *MAPT* has 16 exons. The four microtubule binding domains at the N-terminus are encoded by exons 9 through 12. By alternative splicing of exons 2, 3 and especially 10, six major tau transcripts are produced in adult human brain (Andreadis, Brown *et al.,* 1992). Exon 10 encodes one of the microtubule binding domains. In healthy individuals, the ratio between tau with three and tau with four microtubule binding domains is approximately equal. Misregulation of splicing can lead to an increased inclusion of exon 10. This results in the overproduction of the tau isoform containing four binding repeats (4R Tau). The 4R Tau isoform is deposited in insoluble aggregates of fibrillar tangles and helical filaments, which leads to neuronal cell death and causes the neurological symptoms of FTDP-17 (Goedert, Crowther *et al.,* 1998; Spillantini, Murrell *et al.,* 1998). Exon 10 splicing is regulated by a set of regulatory elements in, and next to, exon 10. In addition, the 5' splice site is located in a putative stem-loop structure that could prevent binding of the U1 snRNP (Hutton, Lendon *et al.,* 1998; Grover, Houlden *et al.,* 1999) or may bind a splicing inhibitor (D'souza, Poorkaj *et al.,* 1999; Grover,

Houlden *et al.,* 1999; D'souza and Schellenberg, 2000; Wang, Gao *et al.,* 2004). A large number of FTDP-17 mutations in exon 10 regulatory elements have been described (Table 1-4, Figure 1-9). The majority of mutations cause an increase of exon 10. It has been shown that many SR and hnRNP proteins inhibit exon 10 inclusion to some extent. Some of these factors act directly on the exons, whereas others work indirectly through sequestration (Wang, Gao *et al.,* 2004).

Figure 1-9: Regulatory elements in tau exon 10 that play a role in FTDP-17. The exon-intron structure is indicated by boxes and lines, respectively. Regulatory elements (light grey: silencers; dark grey: enhancers) are indicated by numbers: 1, ESE (exonic sequence enhancer) element: AATAAGAAG; 2, ESS (exonic sequence silencer): TTAG; 3, ESE: CAAC; 4, ESS: ATA; 5, stem-loop structure that acts as an ISS (intronic sequence silencer) (Wang, Gao *et al.,* 2004). Figure adapted from Heinrich, Zhang *et al.,* 2005.

1.5.2 Spinal muscular atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive disease with an incidence of 1 in 6000 newborns. In SMA, spinal cord motor neurons are progressively lost, resulting in weakness and wasting of voluntary muscles. SMA is one of the leading genetic causes of infantile death. It is caused by mutations in or loss of the survival of motor neurons gene (SMN1). The encoded gene product functions in the assembly of small ribonucleoproteins and recycling. Deletion of both alleles of SMN1 causes the most severe forms of SMA. This is surprising, because humans possess a second gene, SMN2, which is almost identical to SMN1. SMN2, however, cannot compensate for the loss of SMN1 because the nucleotide at position 6 of exon 7 is mutated from C to U. As a result exon 7 is skipped in SMN2, and the mRNA encodes a truncated SMN2 protein that is less stable and has a reduced ability to oligomerise. For this and other unknown reasons, it cannot compensate for the loss of SMN1.

Several studies have identified a number of proteins that bind to SMN2 exon 7 (Figure 1-10). Exon 7 contains a weak splice site (Lim and Hertel, 2001), leading to its dependency on additional elements. Elegant *in vivo* selection experiments have shown that the exon can be subdivided into three parts: exinct (extended inhibitory context), a conserved tract, and a

3' cluster (Singh, Androphy et al., 2004a; Singh, Androphy et al., 2004b). It has been shown that SF2/ASF (Cartegni and Krainer, 2002), hnRNP A1 (Kashima and Manley, 2003), possibly in an extended inhibitory context (Singh, Androphy et al., 2004b), and Tra2-beta1 (Hofmann, Lorson et al., 2000) bind to the exon.

Figure 1-10: Regulatory elements in SMN1/2 exon 7 that play a role in spinal muscular atrophy. The exon-intron structure is indicated by boxes and lines, respectively. Regulatory elements (light grey: silencers; dark grey: enhancers) are indicated by numbers: 1, ISS (intronic sequence silencer) element: 5'-GUAAAAUGUCUUGUGAAACAAAAUACUUUUUAACAUCCAUAUAAA-3' (Miyajima, Miyaso et al., 2002); 2, ESE (exonic sequence enhancer): AAAGAAGGA, transacting splicing factor Tra2-beta1 (Lorson and Androphy, 2000); 3, ESS (exonic sequence silencer): UAGACAA, transacting splice factor hnRNP A1 (Kashima and Manley, 2003); 4, ISE (intronic sequence enhancer): 5'-GAAAACAAAUGUUUUUGAACA UUUAAAAAGUUCAGAUGUUAGAAAGUUGAAAGGUUAAUGUAAAAC-3' (Miyajima, Miyaso et al., 2002); 5, stem-loop structure that acts as an ISS (Miyaso, Okumura et al., 2003). Figure adapted from Heinrich, Zhang et al., 2005.

1.5.3 Current models for therapeutic approaches

Several experiments have proved the principle that diseases caused by an alteration of splicing can be reversed *in vivo*. Two major approaches are emerging: defective regulatory sequences can be masked with oligonucleotides or the concentration of regulatory factors can be changed. Oligonucleotides have been used to alter missplicing events (Sazani and Kole, 2003). Typically, an oligonucleotide is designed to bind to and block cryptic splice sites generated by a mutation. Oligonucleotides used to regulate alternative splicing should not activate RNaseH, which would destroy the pre-mRNA prior to splicing. Therefore, chemically modified oligonucleotides lacking RNaseH induction are used. They have been shown to revert aberrant splicing in beta-thalassemias (Suwanmanee, Sierakowska et al., 2002), CFTR (Friedman, Kole et al., 1999), FTDP-17 (Kalbfuss, Mabon et al., 2001), spinal muscular atrophy (Lim and Hertel, 2001), Duchenne muscular dystrophy (Mann, Honeyman et al., 2001) and myasthenia gravis associated with missplicing of acetylcholine esterase (Brenner, Hamra-Amitay et al., 2003). Alternative splice site recognition depends on the formation of dynamic regulatory complexes on the pre-mRNA. Because the individual interactions in these complexes are weak, changing the concentration of regulatory factors

influences splice site selection. Therefore, an increase in the concentration of a certain factor can correct missplicing *in vivo*. This concept has been proved in several model systems. For example, overexpression of the splicing factor Tra2-beta1 stimulates exon inclusion of the SMN2 gene, which could then substitute for the SMN1 gene product, missing in spinal muscular atrophy (Hofmann, Lorson *et al.,* 2000). The effect of mutation in tau exon 10 regulatory sequences can be partially overcome by overexpressing factors that bind directly to pre-mRNA or by sequestering proteins that bind to it (Wang, Gao *et al.,* 2004). The formation of regulatory complexes depends on protein:protein interaction, which is often sensitive to phosphorylation. As a consequence, phosphorylation of splicing factors can alter splice site selection *in vivo* (Matter, Herrlich *et al.,* 2002; Rafalska, Zhang *et al.,* 2004; Stoss, Novoyatleva *et al.,* 2004). This can be used as a therapeutic principle, as it has been found that hyperphosphorylation of splicing factors by CLK kinases reverts the missplicing of mutated neurofilament tau *in vivo* (Hartmann, Rujescu *et al.,* 2001). Despite the obvious problems associated with the delivery of cDNAs, these studies prove the principle that alternative missplicing can be corrected *in vivo*. The use of RNA binding substances such as gentamicin, chloramphenicol, and tetracycline clearly demonstrates that drugs can be targeted against RNA and/or RNA binding proteins. It is therefore not surprising that several drugs influence splice site selection. The first group of components probably alters the expression of regulatory splicing factors. For example, alternative splicing of SMN2 is influenced by treatment with sodium butyrate or valproic acid (Chang, Hsieh-Li *et al.,* 2001; Brichta, Hofmann *et al.,* 2003; Sumner, Huynh *et al.,* 2003). The effect of manipulating the phosphorylation levels has been demonstrated by the ability of the protein phosphatase inhibitor vanadate to reverse missplicing of SMN2 exon 7 (Zhang, Lorson *et al.,* 2001), which could be mediated through the phosphorylation state of STAR proteins (Stoss, Novoyatleva *et al.,* 2004). An important function of alternatively spliced exons is the introduction of new stop codons in mRNA isoforms. Misregulation of these exons bearing premature stop codons can cause disease. An estimated 5% of all mutant alleles causing cystic fibrosis carry premature stop codons. Aminoglycoside antibiotics such as gentamicin or tobramycin can suppress premature stop codons *in vivo*, and their administration shows promising effects in individuals experiencing missplicing events (Wilschanski, Yahav *et al.,* 2003). These emerging examples clearly show that the treatment of splicing disorders with drugs might be possible in the future.

2 RESEARCH OVERVIEW

Alternative splicing is one of the main mechanisms in regulating eukaryotic gene expression. Alternative splice site selection involves the synchronised action of a number of protein:RNA and protein:protein complexes. For better understanding of this process, it is important to identify the involved proteins and to find in which way they can influence splice site selection.

In the first part of this research (4.1), it was tested if the RNA binding protein RBM4 can change splice site selection of several minigene constructs in vivo and if binding of WT1 can modulate the influence of RBM4.

The second part of this study (4.2) focuses on Tra2-beta1, an SR-related protein which contains the protein phosphatase 1 binding motif RVDF. We investigated whether dephosphorylation by PP1 affects heteromultimerisation of Tra2-beta1 to its interactor SF2/ASF *in vivo* and *in vitro*. Furthermore, it was examined if reducing PP1 activity either by its inhibitors or by RNAi promotes usage of alternative Tra2-beta1 dependent exons.

In the main part of this work (4.3), the role of heterogeneous nuclear ribonucleoprotein G (hnRNP G) in splicing was studied, since it was found as binding partner of other splicing factors. The goal was to determine whether hnRNP G is a target for tyrosine kinases and if tyrosine phosphorylation has an effect in splice site selection. The tyrosine phosphorylation site of hnRNP G was found and a phosphodeficient mutant created. Furthermore, we were trying to find a possible binding sequence of hnRNP G by SELEX and CLIP analysis, as well as possible target genes by microarray analysis and 2D gel electrophoresis.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Product	Supplier	Product	Supplier
Acetone	Merck	β-Mercaptoethanol	Merck
Acetic acid	Carl Roth GmbH	Methanol	Carl Roth GmbH
30% Acrylamide/bis 37.5:1	Sigma-Aldrich	$[^{35}S]$-Methionine	Amersham
		Ni-NTA agarose	Qiagen
40% Acrylamide/bis 19:1	Carl Roth GmbH	Nonidet P-40/Igepal CA-630	Sigma-Aldrich
Agar (Select Agar)	Sigma-Aldrich	NTPs	Roche
Agarose UltraPure	Invitrogen	dNTPs	Invitrogen, Sigma-Aldrich
Ammoniumpersulfate	Sigma-Aldrich		
Ampicillin	Sigma-Aldrich	Paraformaldehyde	Merck
Aprotinin	Sigma-Aldrich	PEG 3500	Sigma-Aldrich
γ-$[^{32}P]$-ATP	Hartmann Analytics	Pepstatin	Sigma-Aldrich
		Perhydrol 30% H_2O_2	Merck
Bluo-Gal	Sigma-Aldrich	Phenol:Chloroform:Isoamyl alcohol	Sigma-Aldrich
Boric acid	Carl Roth GmbH		
Bradford reagent (BioRad Protein Assay)	BioRad	PMSF	Sigma-Aldrich
		Ponceau S solution	Sigma-Aldrich
Brilliant Blue R 250	Sigma-Aldrich	Potassium acetate	Riedel de Haën
Bromophenol blue	Merck	Potassium chloride	Merck
Calciumchloride	Merck	Potassium dihydrogen phosphate	Merck
Cellfectin	Invitrogen		
Chloramphenicol	Sigma-Aldrich	Protease inhibitor cocktail	Sigma-Aldrich
Chloroform:Isoamyl alcohol	Sigma-Aldrich	Protein A sepharose	Amersham
		PTP 1B inhibitor	Calbiochem
Crystal violet	Merck	RNase inhibitor	Roche
Dextrose	Sigma-Aldrich	SDS	Carl Roth GmbH
DMSO	Sigma-Aldrich	Sepharose CL-4B	Pharmacia
DTT	Merck	Silver nitrate	Merck
Dynabeads Protein A	Dynal Biotech	Sodium acetate	Merck
EDTA	Carl Roth GmbH	Sodium chloride	Carl Roth GmbH
EGTA	Merck	Sodium deoxycholate	Sigma-Aldrich
Ethanol	Carl Roth GmbH	Sodium dihydrogen phosphate	Merck
Ethidium bromide	Sigma-Aldrich		
Ficoll 400	Fluka	Sodium fluoride	Sigma-Aldrich
Formaldehyde	Merck	Sodium hydroxide	Merck
Forskolin	Calbiochem	Sodium orthovanadate	Sigma-Aldrich
Gelatin	Carl Roth GmbH	Sodium pyrophosphate	Merck
Gel/Mount	Biomeda	di-Sodiumhydrogen phosphate	Merck
Gentamycin	Sigma-Aldrich		
Glycerol	Sigma-Aldrich	Sodium thiosulphate	Merck
Glycerol 2-phosphate	Sigma-Aldrich	Sucrose	Carl Roth GmbH
Glycine	Carl Roth GmbH	Superfect	Qiagen
Guanidine hydrochloride	Fluka	Tautomycin	Calbiochem
		TEMED	Sigma-Aldrich
Heparin	Sigma-Aldrich	Trichloro acetic acid	Riedel de Haën
HEPES	Sigma-Aldrich	Tris base	Sigma-Aldrich
HiPerfect	Qiagen	TRIzol	Sigma-Aldrich
Hydrochloride	Merck	Triton X-100	Carl Roth GmbH

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Product	Supplier	Product	Supplier
Imidazole	Carl Roth GmbH	Tryptone	Sigma-Aldrich
Isopropanol	Carl Roth GmbH	Tween 20	Sigma-Aldrich
p-Iodophenol	Sigma-Aldrich	Urea	Merck
Kanamycin	Sigma-Aldrich	α-[^{32}P]-UTP	Hartmann Analytics
Leupeptin	Sigma-Aldrich		
Luminol	Sigma-Aldrich	Yeast extract (select yeast extract)	Sigma-Aldrich
Magnesium chloride	Merck		
Magnesium sulphate	Merck	Xylene cyanole FF	Merck

3.1.2 Commercially available kits

Product	Supplier	Product	Supplier
QIAGEN plasmid maxi kit	Qiagen	QIAprep spin M13 kit	Qiagen
QIAEX II gel extraction kit	Qiagen	Miniquick spin RNA columns	Roche
QIAquick gel extraction kit	Qiagen	JetStar plasmid maxi kit	Genomed
RNeasy mini kit	Qiagen	TNT® T7 coupled reticulocyte lysate system	Promega
TOPO TA cloning kit	Invitrogen		

3.1.3 Enzymes, proteins and standards

Product	Supplier	Product	Supplier
Abl protein tyrosine kinase	New England Biolabs	Restriction endonucleases	New England Biolabs/Fermentas
Antarctic phosphatase	New England Biolabs	RNase A	Roche
		Taq DNA polymerase	Invitrogen, PeqLab
Benzonase	Sigma	T4 DNA ligase	New England Biolabs
Calf intestinal alkaline phosphatase (CIP)	New England Biolabs		
DNase I	Roche	T4 polynucleotide kinase	New England Biolabs
MultiMark® multi-coloured protein standard	Invitrogen	T4 RNA ligase1 (ssRNA ligase)	New England Biolabs
peqGOLD protein-marker IV (Prestained)	PeqLab	T7 DNA polymerase	New England Biolabs
Platinum Pfx polymerase	Invitrogen	T7 RNA polymerase	Roche
Precision Plus protein prestained standards	BioRad	TrackIt™ 100bp DNA ladder	Invitrogen
Protein phosphatase 1	New England Biolabs	TrackIt™ 1kb DNA ladder	Invitrogen
Pwo polymerase	PeqLab	SuperScript II	Invitrogen

3.1.4 Bacterial strains and media

Strain	Genotype	Reference
E.coli 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*IqZΔM15 Tn10 (Tetr)]	Bullock, Fernandez *et al.*, 1987
E.coli CJ 236	F' cat (pCJ105 = pOX38::cat= FΔ(HindIII)::cat [Tra$^+$ Pil$^+$ Cam^R]/ ung-1 relA1 dut-1 thi-1 spoT1	Kunkel, Roberts *et al.*, 1987
One Shot® Top10 Chemically competent *E.coli*	F$^-$*mcr*A Δ(*mrr*-*hsd*RMS-*mcr*BC) Φ80*lac*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

Strain	Genotype	Reference
MAX Efficiency® DH10Bac™	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G /pMON14272 / pMON7124	Invitrogen

LB Medium (1 L)   **LB Agar (1 L)**

10 g   NaCl   10 g   NaCl
10 g   Tryptone   10 g   Tryptone
5 g   Yeast extract   5 g   Yeast extract
                                 20 g   agar

3.1.5 Cell lines and media

Name	Description	ATCC number
Cos-7	African green monkey kidney SV40 transformed	CRL-1651
HEK293	Human embryonic kidney transformed with adenovirus 5 DNA	CRL-1573
HeLa	Human adenocarcinoma epitheloid cells	CCL-2
Neuro-2a	Mouse neuroblastoma	CCL-131
SF9 insect cells	Spodoptera frugiperda (fall armyworm)	CRL-1711

The eukaryotic cell lines were cultured in DMEM supplemented with 10% foetal calf serum (both Invitrogen). For subculturing, 1 x Trypsin/EDTA (Invitrogen) was used.

Insect cells were cultured in TNM-FH (BD Biosciences).

3.1.6 Antibiotics

Antibiotic	Stock concentration	Working concentration	
		Liquid culture	**Agar plates**
Ampicillin	50 mg/ml	100 µg/ml	100 µg/ml
Chloramphenicol	30 mg/ml	15 µg/ml	30 µg/ml
Gentamycin	10 mg/ml	7 µg/ml	10 µg/ml
Kanamycin	20 mg/ml	50 µg/ml	50 µg/ml
Tetracyclin	5 mg/ml	50 µg/ml	50 µg/ml

3.1.7 Antibodies

3.1.7.1 Primary antibodies

Antibody	Organism	Dilution for Western Blot	Supplier
anti-Abl	Mouse	1:2000	Santa Cruz
anti-actin	Mouse	1:2000	Amersham
anti-beta-actin	Rabbit	1:2000	abcam
anti-FLAG M2	Mouse	1:1000	Sigma
anti-GAPDH	Mouse	1.2000	abcam
anti-GFP	Mouse	1:4000	Roche
anti-hnRNP A1	Mouse	1:1000	abcam
anti-hnRNP G	Rabbit	1:2000	custom made[1]

Antibody	Organism	Dilution for Western Blot	Supplier
anti-phospho-hnRNP G	Rabbit	1:1000	custom made[2]
anti-PP1γ1 (C-19)	Goat	1:200	Santa Cruz
anti-pTyr (PY20)	Mouse	1:5000	Santa Cruz
anti-tra2-beta+alpha (ps568)	Rabbit	1:2000	custom made[3]
anti-SF2/ASF	Mouse	1:200	Zymed Laboratories
anti-SC35	Mouse	1:2000	Sigma
anti-Src	Mouse	1:1000	upstate
anti-SLM-1	Rabbit	1:1000	custom made[4]
anti-SLM-2	Rabbit	1:1000	custom made[5]
anti-U1-70K	Mouse	1:600	Synaptic Systems
anti-YT521-B (PK2)	Rabbit	1:3000	custom made[6]
anti-SF3A1 (SF3a120)	Rabbit	1:500	kindly provided by A. Krämer (Krämer, Mulhauser *et al.,* 1995)
anti-SF3A2 (SF3a66)	Rabbit	1:500	kindly provided by A. Krämer (Brosi, Hauri *et al.,* 1993)
anti-SF3A3 (SF3a60)	Rabbit	1:1000	kindly provided by A. Krämer (Krämer, Legrain *et al.,* 1994)

Custom made antibodies were raised against the following peptides (Pineda, Berlin):

[1] hnRNP G: RDDGYSTKD

[2] Phospho hnRNP G: RDDGY^{P}STKD

[3] ps568/Tra peptide: GC(StBu)SITKRPHTPTPGIYMGRPTY (Stoilov, Daoud *et al.,* 2004)

[4] SLM-1: VNEDAYDSYAPEEWTTCG and DQTYEAYDNSYVTPTQSVPECG (Stoss, Novoyatleva *et al.,* 2004)

[5] SLM-2: VVTGKSTLRTRGVTCG and PRARGVPPTGYRPCG (Stoss, Novoyatleva *et al.,* 2004)

[6] YT521-B: RSARSVILIFSVRESGKFQCG and KDGELNVLDDILTEVPEQDDECG (Nayler, Hartmann *et al.,* 2000)

3.1.7.2 Secondary antibodies

Antibody	Organism	Dilution for Western Blot	Supplier
anti-mouse IgG-HRP	Sheep	1:10000	Amersham
anti-rabbit IgG-HRP	Donkey	1:10000	Amersham
anti-goat IgG-HRP	Donkey	1:10000	Santa Cruz
CY3-conjugated anti-rabbit	Mouse	1:500	Dianova
CY3-conjugated anti-mouse	Goat	1:500	Dianova

3.1.8 Plasmids

3.1.8.1 Minigenes

Name	Backbone	Description	Reference
SV9/10L/11	Exontrap	Tau minigene	Gao, Memmott *et al.,* 2000
pCR3.1 MGtra	pCR3.1TA	Tra2-beta minigene	Stoilov, Daoud *et al.,* 2004
pXB (X16)	pCRneo	SRp20 minigene	Jumaa and Nielsen, 1997
pET v5	Exontrap	CD44 v5 minigene	König, Ponta *et al.,* 1998
pSMN2	pCI	SMN2 minigene	Lorson, Hahnen *et al.,* 1999

3.1.8.2 Clones from the lab collection or outside sources

Name	Backbone	Description	Reference
pEGFP-C2	pEGFP-C2	CMV-promoter, Kana^r/Neo^r, f1 ori	Clontech
pRK5-abl	pRK5	c-Abl kinase	O. Nayler
c-Src wt	pcDNA3.1	c-Src kinase	Wong, Besser *et al.,* 1999
Sik-YF	pcDNA3	Constitutively active Sik kinase	Derry, Richard *et al.,* 2000
pRK5-fyn	pRK5	Fyn kinase	O. Nayler
pSVL-Syk	pSVL	Syk kinase	Zhang, Berenstein *et al.,* 1996
CSK	pcDNA3	CSK kinase	O. Nayler
AUG1 (pcDNA3-Rlk)	pcDNA3	Rlk kinase	Debnath, Chamorro *et al.,* 1999
pUHG10-3 (FER)	pUHD10-3	FerH kinase	Hao, Ferris *et al.,* 1991
pEGFP-DYRK-1A	pEGFP-C2	DYRK1A kinase	Sitz, Tigges *et al.,* 2004
HA-ACK2	pCDNA3	HA tagged ACK2 kinase	Yang, Lo *et al.,* 2001
Bcr-abl	pCDNA3	Bcr-abl kinase	Warmuth, Bergmann *et al.,* 1997
RAK	pCIneo	RAK kinase	Craven, Cance *et al.,* 1995
lyn	pCMV-SPORT6	lyn kinase	RZPD, Berlin
LAR	pCMV-SPORT6	phosphotyrosine phosphatase LAR	RZPD, Berlin IMAGp998P2312140Q3
MEG1	pCMV-SPORT6	phosphotyrosine phosphatase MEG1	RZPD, Berlin IMAGp998I199578Q3
MEG2	pCMV-SPORT6	phosphotyrosine phosphatase MEG2	RZPD, Berlin IMAGp998G249687Q3
PTP1B	pCMV-SPORT6	phosphotyrosine phosphatase PTP1B	RZPD, Berlin IMAGp998C079948Q3
PTPIA2β	pCMV-SPORT6	phosphotyrosine phosphatase PTPIA2β	RZPD, Berlin IMAGp998B059626Q3
PTPα	pCMV-SPORT6	phosphotyrosine phosphatase PTPalpha	RZPD, Berlin IMAGp998O079750Q3
SHP2	pCMV-SPORT6	phosphotyrosine phosphatase SHP2	RZPD, Berlin IMAGp998K169625Q3
STEP	pCMV-SPORT6	phosphotyrosine phosphatase STEP	RZPD, Berlin IMAGp998A0212719Q3

Name	Backbone	Description	Reference
pEGFP-hnRNP G-C2	pEGFP-C2	EGFP tagged full length rat hnRNP G	A. Hartmann
pEGFP-hnRNP G-TH-C2	pEGFP-C2	EGFP tagged rat hnRNP G with deletion of first RRM	A. Hartmann
hnRNP G-TH-Flag	pCDNA	Flag tagged rat hnRNP G with deletion of first RRM	A. Hartmann
pEGFP-hTra2-beta	pEGFP-C2	EGFP tagged human Tra2-beta1	Beil, Screaton *et al.,* 1997; Nayler, Cap *et al.,* 1998
pEGFP-hTra2-beta1-RATA	pEGFP-C2	EGFP tagged human Tra2-beta1 with PP1 binding site RVDF mutated to RATA	Novoyatleva, Heinrich *et al.,* in press
pEGFP-hTra2-beta-RSA	pEGFP-C2	EGFP tagged human Tra2-beta1 with all serine residues in the first RS domain mutated to alanine	Y. Tang
pEGFP-hTra2-beta-RSE	pEGFP-C2	EGFP tagged human Tra2-beta1 with all serine residues in the first RS domain mutated to glutamic acid	Y. Tang
hTra2-beta-HA	pFastBac-HTa	Human Tra2-beta1 in *Drosophila* vector for generating bacmid	S. Kishore
EGFP-NIPP-C1	EGFP-C1	EGFP tagged nuclear inhibitor of PP1	Van Eynde, Wera *et al.,* 1995
pEGFP-RBM4	pEGFP-C2	EGFP-tagged full length RBM4 (LARK)	Markus, Heinrich *et al.,* 2006; Markus and Morris, 2006
pCMV RBM4-HA	pCMV-HA	HA-tagged full length RBM4 (LARK)	Markus, Heinrich *et al.,* 2006; Markus and Morris, 2006
pEGFP-M1	pEGFP-C2	EGFP-tagged RBM4 (LARK), C-terminal region deleted	Markus and Morris, 2006
pCMV WT1(+KTS)-myc	pCMV-myc	c-myc-tagged full length WT1	Markus, Heinrich *et al.,* 2006; Markus and Morris, 2006

3.1.8.3 Newly made clones

Name	Backbone	Description
pEGFP-Y211F-C2	pEGFP-C2	EGFP-tagged full length rat hnRNP G mutant Y211F
pEGFP-Y211F-TH-C2	pEGFP-C2	EGFP tagged rat hnRNP G mutant Y211F with deletion of first RRM
hnRNP G-YFP	pFRED15	hnRNP G in shuttling vector
hnRNP G-Y211F-YFP	pFRED15	hnRNP G mutant Y211F in shuttling vector
pFastBac-HT-hnRNP G	pFastBac-HTa	Rat hnRNP G in *Drosophila* vector for generating bacmid; HIS-tag
pFastBac-HT-Y211F	pFastBac-HTa	hnRNP G mutant Y211F in *Drosophila* vector for generating bacmid; HIS-tag
pFastBac-HT-RATA	pFastBac-HTa	Tra2-beta1 RATA mutant in *Drosophila* vector for generating bacmid; HIS-tag

3.1.9 Oligonucleotides

3.1.9.1 Primers used for cloning and sequencing

Name	Orientation	Sequence 5'→3'	Target
Y211F	sense	AGAGATGATGGATTTTCTACAAAAGAC	hnRNP G to create Y211F mutant
hnG-deltaRRM-Eco	sense	CTGAATTCCCATCTTTTGAAAGTGGC	Y211F; cloning of pEGFP-Y211F-TH-C2
hnG-end-Bam	antisense	GTGGATCCCTAGTATCTGCTTCTG	
hnG-start-Eco	sense	TCGAATTCATGGTTGAAGCAGATC	hnRNP G/Y211F; for cloning into pFastBac-HTa
hnG-end-Sal	antisense	GTGTCGACCTAGTATCTGCTTCTG	
hnG-start-SacII	sense	CCGCGGGCCACCATGGTTGAAGCA	hnRNP G/Y211F; for cloning into shuttling vector
hnG-end-NheI	antisense	GTGCTAGCGTATCTGCTTCTGCC	
GST-age-for	sense	GGACCGGTGCCACCATGTCCCCTATACTAGGTTATTGG	GST; cloning of hnRNP G/Y211F-GST (in TOPO)
GST-eco-rev	antisense	CCTCCAAAATCGGATCTGGTTCCGGAATTCCG	
ForEcoR1 TRA (RATA)	sense	GAATTCATGAGCGACAGCGGCGAG	Tra-RATA; for cloning into pFastBac-HTa
Rev Sal1 (RATA) new	antisense	GTCGACTTAATAGCGACGAGGTGACTAT	
mutseq hnRNP G	sense	GAGGACCACCACCACGAA	sequencing of hnRNP G/Y211F
FastBacHT_Seq	sense	CGGTCCGAAACCATGTCGTAC	sequencing of inserts in pFastBac-HTa
T3	sense	ATTAACCCTCACTAAAGGGA	sequencing in TOPO vector
T7	antisense	TAATACGACTCACTATAGGG	sequencing in TOPO vector
M13rev	antisense	CAGGAAACAGCTATGAC	sequencing in TOPO vector

3.1.9.2 Primers used for amplifying SELEX sequences

Name	Orientation	Sequence 5'→3'
T7pro	sense	TAATACGACTCACTATAGGGATCCGAATTCCCGACT
RT	antisense	GCGTCTCGAGAAGCTTCCc

3.1.9.3 Primers used for minigene analysis

Name	Orientation	Sequence 5'→3'	Minigene
pCR3.1 RT revers	antisense	GCCCTCTAGACTCGAGCTCGA	Tra2-beta
MG Tra-Bam	sense	GGGCCAGTTGGGCGACCGGCGCGTCGTGCG	
MG Tra-Xho	antisense	GGGCTCGAGTACCCGATTCCCAACATGACG	
N5 INS	sense	GAGGGATCCGCTTCCTGCCCC	CD44v5
N3 INS	antisense	CTCCCGGGCCACCTCCAGTGCC	
T7	sense	TAATACGACTCACTATAGGG	SRp20
X16R	antisense	CCTGGTCGACACTCTAGATTTCCTTTCATTTGACC	
INS1	sense	CAGCTACAGTCGGAAACCATCAGCAAGCAG	Tau
INS3	antisense	CACCTCCAGTGCCAAGGTCTGAAGGTCACC	

Name	Orientation	Sequence 5'→3'	Minigene
pCl for	sense	GGTGTCCACTCCCAGTTCAA	SMN2
SMNex8 rev	antisense	GCCTCACCACCGTGCTGG	

3.1.9.4 Primers used for Microarray analysis

3.1.9.4.1 *Primers used for Microarray with overexpressed Tra2-beta1*

Name	Orientation	Sequence 5'→3'	Target	Exon
Clk4 for	sense	GTCCGCAGCAGGAGAAGC	CDC-like kinase 4	208.8.3 Intron retention Ref: NM_020666 Var: BX491417
Clk4 rev	antisense	ATGCCATGATCAATGCACTC		
CPSF6 for	sense	CAATCAGGACAAATGTCTGGG	Cleavage and polyadenylation specific factor 6, 68kDa	148.1.1 Novel exon Ref: NM_007007 Var: AL557975
CPSF6 rev	antisense	CACTGGCATCAGACACAGC		
DDX23 for	sense	GAACGTCGGGAACGCAGG	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23	91.1.1 Intron retention Ref: NM_004818 Var: BU174750
DDX23 rev	antisense	GTAGTCAATGGATGTGTCCT		
eIF2B for	sense	GCAGATCTTGTCATAGTTGG	Eukaryotic translation initiation factor 2B, subunit 1 alpha, 26kDa	14.1.1 Exon skipping Ref: NM_001414 Var: AA923281
eIF2B rev	antisense	CTCATCGCTGACTGCTGAG		
Fe65 for	sense	CAGAGCCGTTGCCCCAAG	Amyloid beta (A4) precursor protein-binding, family B, member 1	909.039.002 Exon skipping Ref: NM_001164 Var: BX420711.1
Fe65 rev	antisense	CACTGTCCCGCCCGAC		
FUS for	sense	GACAACAACACCATCTTTGTG	Fusion (involved in t(12;16) in malignant liposarcoma)	93.1.1 Exon skipping Ref: NM_004960 Var: AJ549096
FUS rev	antisense	CCTCCACGACCATTGCCAC		
Fyn for	sense	GAGAGCTGCAGGTCTCTG	FYN oncogene related to SRC, FGR, YES	907.002.002 Novel exon Ref: NM_002037.3 Var: NM_153047.1
Fyn rev	antisense	CTCGGTGACGATGTAGATG		
hnRNP M for	sense	GATGAGAGGGCCTTACCAA	Heterogeneous nuclear ribonucleoprotein M	114.1.1 Exon skipping Ref: NM_005968 Var: AL516884
hnRNP M rev	antisense	CCTGCCCATGTTCATCCCA		
MTMR11 for	sense	GAGACTGAGCGGAAGAAGC	Myotubularin related protein 11	137.1.1 Novel exon Ref: NM_006697 Var: U78556
MTMR11 rev	antisense	CTGACACTGTCATGAAGAGC		

Name	Orientation	Sequence 5'→3'	Target	Exon
NOL5A for	sense	GTGGAGGAGTCTGTGCTC	Nucleolar protein 5A (NOL5A)	127.3.1 Novel exon Ref: NM_006392 Var: BE253695
NOL5A rev	antisense	GCAGGTGGGTCTCCAAGAG		
PPIE for	sense	GTCAGATGATGACTGGTTGAA	Peptidylprolyl isomerase E (cyclophilin E)	117.8.1 Exon skipping Ref: NM_006112 Var: BI821836
PPIE rev	antisense	CATGAGTGCACAGGCAGCG		
PPIL3 for	sense	GAGCTCGCTGTAAGACTGAG	Peptidylprolyl isomerase like protein 3	236.10.1 Alternative splice acceptor Ref: NM_032472 Var: BU195819
PPIL3 rev	antisense	GATATTCACTGTATTCATC		
SFRS4 for	sense	CAGCCATCACTGCCGTTGCC	Splicing factor, arginine/serine-rich 4 (Srp75)	104.9.3 Novel exon Ref: NM_005626 Var: BX447499
SFRS4 rev	antisense	GCGTCCAGAACCGTAACTGC		
SFRS5 for	sense	GAGGATCCAAGGGATGCAGAT	Splicing factor, arginine/serine-rich 5	144.1.3 Partial internal exon deletion Ref: NM_006925 Var: BC018823
SFRS5 rev	antisense	GGCCAGCTGACTCTTGAGGA		
SFRS14 for	sense	CAAGGACTTGGACTTCGCC	Splicing factor, arginine/serine-rich 14	179.1.1 Exon skipping Ref: NM_014884 Var: AI089022
SFRS14 rev	antisense	CTTCTAGGCTTTATCAAGGC		
SRRM1 for	sense	GATGAACGACCCAAGAGATC	Serine/arginine repetitive matrix 1	110.3.1 Novel exon Ref: NM_005839 Var: BE931442
SRRM1 rev	antisense	GGAGACCGTCGCCTTCTG		
WT1 for	sense	CAAATGACATCCCAGCTTGAA	Wilms' tumour 1	222.1.2 Exon skipping Ref: NM_024426 Var: AK093168
WT1 rev	antisense	GACGCAAGAAGAGATGTCAT		

3.1.9.4.2 *Primers used for Microarray with overexpressed hnRNP G in mouse cells*

Name	Orientation	Sequence 5'→3'	Target	Gene ID
mASF/SF2 for	sense	GTGGAAGCTGGCAGGACTTA	Splicing factor, arginine/serine rich 1 (Sfrs1)	Ref: NM_173374
mASF/SF2 rev	antisense	TCACAGTCTGAAGAGCATGGA		
mCasp2 for	sense	TTCTTCATCCAAGCATGTCG	Caspase 2	Ref: NM_007610
mCasp2 rev	antisense	ATGGCTGACAGGAAACAAGC		
mCasp7 for	sense	CCGAGTGCCCACTTATCTGT	Caspase 7	Ref: NM_007611
mCasp7 rev	antisense	GGAACCGTGGAGTAAGCAAA		
mCasp9 for	sense	GATGCTGTCCCCTATCAGGA	Caspase 9	Ref: NM_015733
mCasp9 rev	antisense	GCAGAGAAGCCCTGTTCTTG		

Name	Orientation	Sequence 5'→3'	Target	Gene ID
mhnRNPK for	sense	GGACGTGCACAACCTTATGA	Heterogeneous nuclear ribonucleoprotein K	Ref: NM_025279
mhnRNPK for	antisense	GCAGCAAATACTGTGCGTTC		
mPtbp1 for	sense	GGATGGCCAGAACATCTACAA	Polypyrimidine tract binding protein 1 (PTB-1)	Ref: NM_008956
mPtbp1 rev	antisense	CATCTGCACAAGTGCGTTCT		
mPpih for	sense	GGTGGCAAATTCAAGTCCAG	Peptidyl prolyl isomerase H	Ref: NM_028677
mPpih rev	antisense	AAATTTTCATCCGCAAATGG		
mSC35 for	sense	CTCGAGGTCAAGGTCCAGGT	Splicing factor, arginine/serine rich 2 (Sfrs2)	Ref: NM_011358
mSC35 rev	antisense	TAGCCAGTTGCTTGTTCCAA		
U5-15kD for	sense	AGGTAGACCAGGCCATCCTT	Thioredoxin-like 4 (Txn14)	Ref: NM_025299
U5-15kD rev	antisense	GACAGTGCAGGGGTCATACA		

3.1.9.4.3 *Primers used for Microarray with overexpressed hnRNP G in human cells*

Name	Orientation	Sequence 5'→3'	Target	Exon
CPSF4 for	sense	GCGTGGACCACATCAAGTT	Cleavage and polyadenylation specific factor 4, 30kDa	136.4.1 Novel exon Ref: NM_006693 Var: BM807314
CPSF4 rev	antisense	GAATTCACAGACAGCAGCG		
CPSF6 for	sense	CAATCAGGACAAATGTCTGGG	Cleavage and polyadenylation specific factor 6, 68kDa	148.1.1 Novel exon Ref: NM_007007 Var: AL557975
CPSF6 rev	antisense	CACTGGCATCAGACACAGC		
DDX11 for	sense	GAAATCGCAAACTGGGCGT	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, S. cerevisiae) (DDX11)	224.5.2 Novel exon Ref: NM_030653 Var: BU859051
DDX11 rev	antisense	GAAGTCTTCCTGGATGGAAT		
Fyn for	sense	GAGAGCTGCAGGTCTCTG	FYN oncogene related to SRC, FGR, YES	907.002.002 Novel exon Ref: NM_002037.3 Var: NM_153047.1
Fyn rev	antisense	CTCGGTGACGATGTAGATG		
NOL5A for	sense	GTGGAGGAGTCTGTGCTC	Nucleolar protein 5A (NOL5A)	127.3.1 Novel exon Ref: NM_006392 Var: BE253695
NOL5A rev	antisense	GCAGGTGGGTCTCCAAGAG		

Name	Orientation	Sequence 5'→3'	Target	Exon
SF3A2 for	sense	GACCAAGCAGAGAGACTCG	Splicing factor 3a, subunit 2, 66kDa	150.2.1 Intron retention Ref: NM_007165 Var: BM927190
SF3A2 rev	antisense	GATCCTCTGCTCGTACGCA		
SFRS3 for	sense	GTGGCTGCCGTGTAAGAGT	Splicing factor, arginine/serine-rich 3	39.5.1 Novel exon Ref: NM_003017 Var: BQ643106
SFRS3 rev	antisense	CTGCGAGAGAAGCTTCTCCT		
SFRS6 for	sense	GTACAGAATACAGGCTTATTGT	Splicing factor, arginine/serine-rich 6	122.4.1 Intron retention Ref: NM_006275 Var: BG540891
SFRS6 rev	antisense	CAGAGTAGGAGCGAAACTCAAT		
SRRM1 for	sense	GATGAACGACCCAAGAGATC	Serine/arginine repetitive matrix 1	110.3.1 Novel exon Ref: NM_005839 Var: BE931442
SRRM1 rev	antisense	GGAGACCGTCGCCTTCTG		
TAF 15 for	sense	GAGGCTACGGTGGAGAGAG	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa	247.1.3 Partial internal exon deletion Ref: NM_139215 Var: AB060882
TAF15 rev	antisense	CCTCCATAGCCACCTCGGT		
USP39 for	sense	GTACAAGCAGTACACAAGAAT	Ubiquitin specific protease	134.7.1 Exon skipping Ref: NM_006590 Var: BM803640
USP39 rev	antisense	CTGTGTTCTTATTTACCATCAT		

3.1.9.5 Primers used for siRNA knockdown

Name	Target	Supplier
hnRNP G siRNA	human hnRNP G	Santa Cruz, sc-38274
PP1α siRNA	human PP1α	Santa Cruz, sc-36299
PP1β siRNA	human PP1β	Santa Cruz, sc-36295
PP1γ siRNA	human PP1γ	Santa Cruz, sc-36297

3.1.9.6 Primers used for CLIP

Name	Sequence 5'→3'	Function
RNA-X6aR	CAGACGGGGCACAAAUA	3' RNA linker
TraEx3 RNA	CUGGAAGCAGAACGGGAUU	5' RNA linker
TraEx3 for	AATCCCGTTCTGCTTCCAG	RT, PCR
X6aRfor	CAGACGGGGCACAATA	PCR

3.2 Methods

3.2.1 Plasmid isolation

Plasmid DNA was isolated from bacteria using small-scale (1.5-3 ml) miniprep and large-scale (100-200 ml) maxiprep purifications, which are based on the alkaline lysis protocol with SDS described by Birnboim and Doly, 1979.

3.2.1.1 Miniprep

1.5-3 ml from a bacterial over night culture were centrifuged for 5 min at 10,000 rpm at room temperature to remove the medium. The pellet was resuspended in 200 µl buffer P1. Lysis was carried out in 200 µl buffer P2 for 5 min at room temperature. After adding 200 µl neutralisation buffer P3, the mixture was kept on ice for 20 min. Cell debris was removed by centrifuging for 10 min (14,000 rpm, 4 °C) and the supernatant was transferred to a new tube. This step was repeated once. 500 µl isopropanol were added to the supernatant and incubated on ice for 20 min or at -20 °C for 10 min. To precipitate the DNA, a centrifugation step was carried out at 14,000 rpm at 4 °C for 15 min. Finally, the DNA pellet was washed with 200 µl 70% ethanol and dried. The dry pellet was diluted in TE or dH_2O.

Buffers used for Miniprep:

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

3.2.1.2 Maxiprep

For large-scale purification of plasmid DNA, commercially available kits from Qiagen or Genomed were used. The procedure was carried out according to the manufacturer's protocol. The dry pellet was diluted in TE or dH_2O.

3.2.2 Electrophoresis of DNA

0.7-2% agarose or 5% native polyacrylamid gels in 1 x TBE buffer were used to resolve DNA. Electrophoresis was performed for 60 min at 100 V, or 45 min at 70 V, respectively. The gels were either stained for 20 min in a 0.5 mg/ml ethidium bromide solution or agarose gels were prepared containing 0.05 µg/ml ethidium bromide. The stained DNA was visualised under UV light at $\lambda = 260$ nm wavelength.

1 x TBE

90 mM	tris base
90 mM	boric acid
20 mM	EDTA

6 x gel loading buffer

0.25%	bromophenol blue
0.25%	xylene cyanol FF
15%	ficoll 400

5% native PAA gel (5 ml)

3.34 ml	dH$_2$O
620 µl	40% acrylamide
1 ml	5 x TBE
35 µl	APS
10 µl	TEMED

3.2.3 DNA purification from agarose gels

For visualisation of DNA under normal light, the desired DNA was resolved on 0.7-2% agarose gels in 1 x TBE buffer containing 2 µg/ml crystal violet. The DNA was cut with a clean scalpel and purified using the Qiagen Qiaex II gel extraction kit according to the manufacturer's protocol.

6 x Crystal violet gel loading buffer

0.25%	crystal violet
15%	ficoll 400

3.2.4 Determination of DNA concentration

Concentrations of nucleic acids in buffered solution were determined using a spectrophotometer (Eppendorf BioPhotometer 6131). Absorbance was measured at 260 nm in plastic cuvettes. The concentration was calculated using following formulas:

1 A$_{260}$ = 50 µg/ml double stranded DNA

1 A$_{260}$ = 37 µg/ml single stranded DNA

1 A$_{260}$ = 40 µg/ml RNA

The purity was determined with help of the A260/A280 ratio, which should be in the range of 1.8-2.0 for pure nucleic acids.

3.2.5 Enzymatic reactions with DNA

3.2.5.1 Restriction analysis of DNA

Restriction of DNA was performed with endonucleases using the buffer system suggested by the supplier. Amount of enzyme and incubation time were used depending on the DNA amount and the number of restriction sites in the plasmid at 37 °C if not suggested differently by the supplier. Inactivation of enzymes was performed according to the supplier's

protocol, if possible. The size of the DNA fragments was confirmed by agarose gel electrophoresis or desired bands were purified from the agarose gel.

3.2.5.2 Dephosphorylation of 5' DNA ends

To avoid re-ligation of linearised vectors with compatible ends, phosphate groups at their 5' ends were removed by alkaline phosphatase. The protocol was carried out according to the supplier's instructions.

3.2.5.3 Ligation of DNA fragments

Compatible DNA fragments were covalently linked by T4 DNA ligase. The ligation was carried out according to the supplier's protocol using 200-300 ng vector with insert in a molar ratio of 1:1-1:3. The reaction was incubated over night at 4 °C.

3.2.5.4 DNA amplification by PCR

For PCR amplifications, a standard PCR reaction was set up. 40-80 ng of highly pure plasmid DNA was used as a template for the reaction.

PCR reaction

40-80 ng	template DNA
5-25 pmol	5' oligonucleotide
5-25 pmol	3' oligonucleotide
200 μM	dNTPs
2.5 µl	10 x Taq Polymerase buffer
1 U	Polymerase
up to 25 µl	dH$_2$O

For cloning purposes, a proofreading polymerase (Pwo from PeqLab or Pfx from Invitrogen) was used instead of Taq DNA polymerase. The amplification was carried out in a Perkin Elmer GeneAmp PCR System 9700 thermocycler under the following conditions:

PCR program

initial denaturation	94 °C	2-4 min	
denaturation	94 °C	15-30 sec	25-35 cycles
annealing	Tm of specific primers	30 sec	
extension	72 °C	1 min per 1 kb	
final extension	72 °C	5-10 min	

Annealing temperature depended on the oligonucleotides used. The PCR product was confirmed by agarose gel electrophoresis (3.2.2). If not one single sharp band was visible the amplified fragment was gel-purified (3.2.3). For cloning, the PCR reaction or the purified PCR product were used in a TOPO cloning reaction (Invitrogen) according to the manufacturer's protocol.

3.2.5.5 Site-directed mutagenesis of DNA

Site-directed mutagenesis was performed according to the method described by Kunkel (Kunkel, Roberts *et al.,* 1987).

The DNA of interest was cloned into pEGFP-C2 which carries the f1 phage origin of replication. Therefore, single- and double-stranded DNA could be made from this vector.

The recombinant plasmid was transformed into *E.coli* strain CJ236 deficient in dUTPase (*dut*) and uracil N-glycosylase (*ung*). These mutations result in a number of uracils being substituted for thymine in the nascent DNA. After transformation, bacteria were grown on plates containing chloramphenicol in addition to the plasmid-specific antibiotic, to ensure the presence of the F' episome necessary for production of helper phage. To isolate single-stranded DNA from the plasmid of interest, colonies were grown in 5 ml of LB medium for 180 min and then 5×10^8 pfu of helper phage M13KO7 (New England BioLabs) was added. The culture was grown overnight at 37 °C and single-stranded DNA was isolated with the QIAprep spin M13 kit according to the manufacturer's protocol.

This uracil-containing ssDNA was used as a template in the in vitro mutagenesis reaction. Phosphorylated oligonucleotides containing desired mutations were annealed to the template at a molar ratio of 20:1 in 10 μl of 1 x T7 DNA polymerase buffer. The DNA was denatured for 5 min at 94 °C and then the temperature was gradually decreased from 70 °C to 37 °C at a rate of 1 °C per minute. The extension of the annealed primer was carried out in 20 μl by adding to the same tube 1 μl of 10 x T7 DNA polymerase buffer, 0.8 μl of 10 mM dNTPs, 1.5 μl of 10 mM ATP, 3 U T7 DNA polymerase and 2 U FastLink T4 DNA ligase. The reaction was incubated at 37 °C for 45 min and the ligase was subsequently inactivated by incubation at 65 °C for 20 min. The mutagenesis reaction was transformed into competent XL1Blue *E.coli* cells. Replication of the plasmid in this strain leads to repair of the template strand and consequently to production of plasmid carrying the desired mutation. All mutant plasmids were verified by sequencing.

3.2.6 Preparation of KCM competent *E. coli* cells

A single colony of the desired *E.coli* strain was inoculated in LB medium and cultured overnight. 4 ml of the culture was added into fresh 250 ml LB medium and grown to early logarithmic phase (OD_{600} = 0.3-0.6). The culture was centrifuged for 10 min at 2,500 rpm at 4 °C. The bacterial pellet was resuspended in 1/10 volume of ice cold TSB buffer and

incubated on ice for 10 min. Cells were aliquoted into pre-chilled Eppendorf tubes and frozen in liquid nitrogen. Competent bacterial cells were stored at -80 °C.

TSB buffer

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

3.2.7 Transformation of KCM competent *E.coli* cells

An aliquot of competent cells was thawed on ice. 20 µl 5 x KCM was added to 100 ng-1 µg DNA or a ligation reaction and filled up to 100 µl with dH_2O. This was carefully mixed with 100 µl of cell suspension and kept on ice for 20 min. After heat shock for 10 min at room temperature, cells were kept on ice for another 2 min. Finally, 1 ml LB medium was added to the mixture and incubated for 1 hour at 37 °C.

Afterwards, cells were plated on selective LB Agar plates and incubated overnight at 37 °C.

5 x KCM

500 mM	KCl
150 mM	$CaCl_2$
250 mM	$MgCl_2$
in dH2O	

3.2.8 *In vitro* transcription

The SELEX sequences were amplified from TOPO vector by PCR using primers T7pro and RT for their flanking regions. T7pro contains the T7 promoter. After PCR amplification the cDNA was purified from a 2% agarose gel using QIAquick gel extraction kit (Qiagen) following the manufacturer's protocol. The purified cDNA was eluted with 35 µl dH_2O and used as template in the following radio-labelled transcription reaction:

5-10 µl	cDNA
2.5 µl	10 x transcription buffer
1.0 µl	10mM rATP
1.0 µl	10 mM rCTP
1.0 µl	10 mM rGTP
1.0 µl	10 mM rUTP
2-3 µl	α-[^{32}P]-UTP (400 Ci/mmol)
1 µl	RNase Inhibitor
1 µl	T7 RNA polymerase
	up to 25 µl with dH_2O

The reaction was incubated for 1 hour at 37 °C. Afterwards, 1 µl DNase was added and incubated for 30 min at 37 °C. Finally, the reaction was purified with miniquick spin RNA columns (Roche) following the manufacturer's protocol. The purified RNA product was checked on a PAA gel and used for electrophoretic mobility shift assay (3.2.9).

3.2.9 RNA electrophoretic mobility shift assay

RNA gel shift assay was performed according to the protocols reviewed earlier (Black, Chan *et al.,* 1998; Thomson, Rogers *et al.,* 1999). 1 µg of purified recombinant HIS-tag protein was pre-incubated for 5 min at room temperature in 1 x binding buffer, containing 12.5 mM ATP, 2 µg heparin, 5 U RNase inhibitor and 0.5 mM PMSF in a final volume of 25 µl. After 2-4 µl radiolabeled in vitro transcribed RNA was added, the mixture was incubated for 5-10 minutes more. RNA:protein complexes were separated on 5% native polyacrylamide gels in 0.5 x tris/glycine buffer at 4 °C. The gel was pre-run for 30 min at 4 °C, 200 V before loading the samples. The gel run was continued for 1-2 hours at 200 V.

5 x binding buffer		**5 x tris / glycin buffer (1 L)**		**5% native PAA gel (30 ml)**	
50 mM	HEPES	30.28 g	tris-base	21.03 ml	dH_2O
15 mM	$MgCl_2$	142.7 g	glycine	4.95 ml	30% acrylamide/bis
200 mM	KCl	3.92 g	EDTA	3 ml	5 x tris/glycin buffer
25%	glycerol			750 µl	glycerol
5 mM	DTT			240 µl	APS
				30 µl	TEMED

3.2.10 Freezing, thawing and subculturing eukaryotic cell lines

To freeze eukaryotic cells, 6×10^5 cells per 10 cm dish were seeded and grown to mid logarithmic phase (about 75% confluency). The cells were trypsinised for 1-5 min (1 ml 1 x Trypsin/EDTA per 10 cm dish), 10 ml DMEM was added and centrifuged for 2 min at 1,500 rpm. The supernatant was discarded and the cells were resuspended in 1 ml of the freezing medium (90% FCS and 10% DMSO). The cell suspension was cooled down in cryotubes in a cryo freezing device at 1 °C/min at -80 °C and finally stored in liquid nitrogen.

Frozen cells were thawed at 37 °C, transferred to 10 ml culture medium and centrifuged for 2 min at 1,500 rpm to remove DMSO. The pellet was resuspended in 10 ml culture medium and seeded on a 10 cm dish. Cells were grown in the incubator with 37 °C and 5% CO_2 until they were ready for subculturing.

After reaching confluency, the cell monolayer was detached by adding 1 ml 1 x Trypsin/EDTA and incubation for 1-5 min at 37 °C. 10 ml culture medium was added to the trypsinised cells and they were centrifuged for 2 min at 1,500 rpm. The cell pellet was

resuspended in 10 ml culture medium and 1-2 ml (1 x 10^6 cells) of this solution was added to a new dish containing 7 ml fresh culture medium. The cells were incubated at 37 °C with 5% CO_2.

3.2.11 Transfection of eukaryotic cells

3.2.11.1 Transfection of plasmid DNA

For transfecting plasmid DNA in HEK293 cells the calcium phosphate method was used, which is based on the protocol published by Chen and Okayama, 1987.

3 x 10^5 cells per well in 2 ml growth medium containing 10% FBS were plated in a 6-well format. Cells were incubated at 37 °C and 5% CO_2 for about 24 hours to reach 60-80% confluency. For transfection in one well, 1-5 µg of expression construct were mixed with 25 µl in a final volume of 100 µl. 100 µl of 2 x HBS buffer was added drop wise with constant vortexing on very low speed. The mixture was incubated for 20 min at room temperature to form a precipitate and then added to the growth medium. Cells were grown for additional 16-24 hours at 37 °C and 3% CO_2 to express the transfected plasmids.

2 x HBS

280 mM	NaCl
10 mM	KCl
1.5 mM	$Na_2HPO_4 \cdot 2\ H_2O$
12 mM	Dextrose
50 mM	HEPES
	pH 6.95

For transfection of cos7 cells Superfect reagent (Qiagen) was used according to the supplier's protocol. This method is based on the fusion of a DNA-lipid complex with the cell membrane (Felgner, Gadek *et al.,* 1987).

3.2.11.2 Transfection of siRNA to knock down specific genes

siRNA knockdown of desired genes was performed by transfecting 20 nM siRNA oligos with HiPerfect transfection reagent (Qiagen) and DMEM without FBS using the reverse transfection procedure according to the supplier's protocol in a 24-well format. After 40-48 hours siRNA treatment, RNA was isolated from 6 x 10^5 cells, in parallel 3 x 10^5 cells were lysed in RIPA buffer. 1/10 of the lysates was used for SDS-PAGE (3.2.20) and Western Blot (3.2.23) to confirm the knockdown of the desired genes on protein level.

3.2.12 Fixing attached eukaryotic cells on cover slips

Cells grown on cover slips and transfected with pEGFP-C2 constructs (3.2.11.1) were fixed with 4% paraformaldehyde in 1 x PBS, pH 7.4 for 20 min at 4 °C. Cells were washed three times in PBS before mounting on microscope slides with Gel/Mount. The cells were examined using a confocal laser scanning microscope (Leica).

3.2.13 Immunostaining

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

3.2.14 Immunoprecipitation of proteins

20-24 hours after transfection (3.2.11), cells were washed with 1 x PBS. Cell lysis was performed for 25 min at 4 °C on ice in 200 µl RIPA buffer per well. Lysates were collected in Eppendorf tubes and cleared by centrifugation for 1 min at 12,000 rpm. The supernatant was diluted with 3 volumes of RIPA rescue buffer and an antibody recognising the expressed protein or an attached tag was added. After incubation on a rotating wheel for 2 hours at 4 °C, 50 µl Protein A Sepharose/Sepharose CL-4B (1:1) was added and the incubation continued over night under the same conditions. The Sepharose beads were pelleted for 1 min at 1,000 rpm in a microcentrifuge, followed by 3-5 washes with 400 µl of 1 x HNTG buffer. 20 µl 3 x SDS sample buffer were added to the pellet and boiled for 5 min at 95 °C to denature the proteins. After spinning down shortly, the supernatant was resolved by SDS polyacrylamide gel electrophoresis (3.2.20) and analysed by Western Blot using appropriate antibodies (3.2.23).

Protein A Sepharose / Sepharose CL-4B preparation:

Protein A Sepharose beads were twice washed in 15 ml dH$_2$O and pelleted at 500 rpm for 2 min. Then equal volume of Sepharose CL-4B was added and the beads were washed two more times in RIPA rescue buffer and stored in equal volume of RIPA rescue buffer at 4 °C.

RIPA

1%	NP-40
1%	Na-deoxycholate
0.1%	SDS
150 mM	NaCl
10 mM	Na$_3$PO$_4$, pH 7.2
2 mM	EDTA
5 mM	β-glycerolphosphate

+ freshly added

4 mM	Na$_3$VO$_4$
1 mM	DTT
1 mM	PMSF
20 µg/µl	aprotinin
1µg/µl	pepstatin
1 µg/µl	leupeptin
1 x	PIC
100 U/ml	benzonase

4 x HNTG

200 mM	HEPES, pH 7.5
450 mM	NaCl
4 mM	EDTA
40%	glycerol
0.4%	Triton-X-100

+ Freshly added to 1 x HNTG

2 mM	Na$_3$VO$_4$
100 mM	NaF
1 mM	DTT
1 mM	PMSF
20 µg/µl	aprotinin
1 µg/µl	pepstatin
1 µg/µl	leupeptin
1 x	PIC

RIPA rescue

20 mM	NaCl
10 mM	Na$_3$PO$_4$, pH 7.2
1 mM	NaF
5 mM	β-glycerolphosphate

+ freshly added

2 mM	Na$_3$VO$_4$
1 mM	DTT
1 mM	PMSF
20 µg/µl	aprotinin
1 µg/µl	pepstatin
1 µg/µl	leupeptin
1 x	PIC

3.2.15 Isolation of nuclear protein cell extract and RNA immunoprecipitation

After transfection, cells were washed twice with PBS and collected in 500 µl EDTA-PBS per well. 2 wells were combined in one Eppendorf tube and centrifuged at 3000 rpm for 5 min at 4 °C. The pellet was resuspended in 300 µl harvest buffer and incubated on ice for 10 min. Cells were centrifuged at 1000 rpm for 10 min at 4 °C, the pellet was resuspended in 300 µl buffer A and centrifuged for another 5 min at 1000 rpm and 4 °C. The pellet was resuspended in 550 µl buffer C and vortexed for 5 min at high speed

(25 Hertz) and 10 min at low speed (15 Hertz) at 4 °C, followed by a centrifugation step at 14,000 rpm and 4 °C for 10 min to pellet nuclear debris. Immunoprecipitation with anti-GFP and Protein A Sepharose was performed overnight at 4 °C, followed by 5 washes in cold RIPA buffer. RNA was isolated from sepharose beads using phenol-chloroform extraction after DNase treatment. After ethanol precipitation the RNA pellet was dissolved in 20 µl RNase free water.

Harvest buffer

10 mM	HEPES pH 7.9
50 mM	NaCl
0.5 M	sucrose
0.1 mM	EDTA
0.5%	Triton X-100

+ freshly added

1 mM	DTT
10 mM	sodium pyrophosphate
100 mM	NaF
17.5 mM	β-glycerolphosphate
1 mM	PMSF
4 µg/ml	aprotinin
2 µg/ml	pepstatin

Buffer A

10 mM	HEPES pH 7.9
10 mM	KCl
0.1 mM	EDTA
0.1 mM	EGTA

+ freshly added

1 mM	DTT
1 mM	PMSF
4 µg/ml	aprotinin
2 µg/ml	pepstatin

Buffer C

10 mM	HEPES pH 7.9
500 mM	KCl
0.1 mM	EDTA
0.1 mM	EGTA
0.1%	Igepal

+ freshly added

1 mM	DTT
1 mM	PMSF
4 µg/ml	aprotinin
2 µg/ml	pepstatin

3.2.16 Freezing, thawing and subculturing insect cells

For freezing SF9 insect cells, cells were seeded in 25 cm^2 flasks grown to about 90-100% confluency. Cells were detached by vigorous shaking for 2-3 times and centrifuged at 1,000 rpm for 2 min. The cell pellet was resuspended in 2 ml serum-free TNM-FH medium (Becton-Dickenson) and mixed with 2 ml freezing medium (80% medium and 20% DMSO). 1 ml aliquots were frozen in cryotubes in a cryogenic freezing device at -80 °C and finally stored in liquid nitrogen.

SF9 cells were thawed rapidly at 37 °C and transferred to 4 ml serum-free TNM-FH medium. After 24 h, the medium was changed to remove DMSO.

For maintenance, SF9 insect cells were cultured under serum-free conditions in TNM-FH medium in 25 cm^2 flasks at 28 °C. When 90-100% confluency was reached, cells were subcultured by detaching them by vigorous shaking and diluting 1:5 in fresh culture medium.

3.2.17 Expression of HIS-tagged protein in the Baculovirus system

The Bac-to-Bac® Baculovirus expression system from Invitrogen was used for expression of HIS-tagged proteins. This method is based on site-specific transposition of an

expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli* (Luckow, Lee *et al.,* 1993; Ciccarone, Polayes *et al.,* 1997).

pFastBac vector containing the gene desired for expression was transformed into *E. coli* DH10Bac™. Generating bacmid from these cells was done according to the Bac-to-Bac manual from Invitrogen. The isolated bacmid was checked by PCR with the forward gene specific primer used for cloning into pFastBac-HTa and M13 reverse primer.

Bacmid transfection for generating virus was performed in 6-well plates according to the Bac-to-Bac manual from Invitrogen, using unsupplemented Grace's medium and Cellfectin (both Invitrogen).

When cells showed typical signs of infection (about 72 hours after transfection), the medium was collected from each well (about 2 ml) and transferred into sterile 15 ml falcons. The P1 viral stock was stored at 4 °C, protected from light. For amplification of P1 viral stock, SF9 cells were infected at a multiplicity of infection (MOI) according to the manual.

Cells were harvested about 48 hours after infection and expression of recombinant protein was analysed by SDS-PAGE and Western Blot or protein staining (see 3.2.20-3.2.23).

3.2.18 Purification of HIS-tagged protein in insect cells

48 hours after infection, SF9 cells were centrifuged at 500 g for 10 min. The pellet was resuspended in 1 ml of denaturing lysis buffer. The suspension was lysed with a 19 G hypodermic needle and centrifuged at 14,000 rpm for 25 min in a 5417R centrifuge (Eppendorf). The supernatant was incubated for 1-2 hours at 4 °C with Ni-NTA agarose resin (Qiagen), equilibrated once with dH_2O and once with denaturing bind/wash buffer, pH 7.8. After incubation, the resin was washed twice with denaturing wash buffer, pH 7.8, twice with denaturing wash buffer, pH 6.4, and once with native buffer. Protein elution from the resin was performed with native buffer containing 250 mM imidazol. Fractions of each step were run on SDS-PAGE.

denaturing lysis buffer

6 M	guanidine HCl
20 mM	Na_3PO_4, pH 7.8
500 mM	NaCl

denaturing wash buffer

8 M	urea
20 mM	Na_3PO_4, pH 7.8/pH 6.4
500 mM	NaCl
0.1%	Triton X-100
30 mM	imidazol

denaturing bind/wash buffer

8 M	urea
20 mM	Na_3PO_4, pH 7.8
500 mM	NaCl

native buffer (pH 8.0)

50 mM	NaH_2PO_4
300 mM	NaCl
30 mM	imidazol

3.2.19 Determination of protein concentration

Concentration of proteins was determined using BioRad protein assay kit which is based on the Bradford method (Bradford, 1976).

Protein in 800 µl dH_2O was mixed with 200 µl of 1 x Dye reagent and incubated for 5 min at room temperature. BSA concentrations were used as standard. Absorbance was measured in a spectrophotometer at $\lambda = 595$ nm. Concentration of protein was read from a standard curve where OD_{595} was plotted against concentration of BSA standards.

3.2.20 Phosphorylation dependent pull down assay

For phosphorylation of HIS-Tra2-beta, protein bound to Ni-NTA agarose was incubated in a typical reaction mixture (see below) for 30 min at 30 °C. The samples were then washed once in 1 x PBS and twice in native buffer (see 3.2.18) containing Triton X-100. Half of the protein was dephosphorylated with 2.5 U protein phosphatase 1 (PP1) in PP1 buffer for 60 min at 30 °C. PP1 was removed by stringent washing in native buffer containing Triton X-100. For binding, 0.25 µg GST-SF2/ASF was added to phosphorylated and dephosphorylated HIS-Tra2-beta and incubated in a total volume of 100 µl in native buffer for 2-3 hours at 4 °C on a rotating wheel.

After centrifuging, 1/5 of the supernatant was collected and boiled in SDS sample buffer. The resin was boiled in SDS sample buffer after being washed once with native buffer. The proteins were run on SDS polyacrylamide gels and detected by Coomassie staining and Western Blot.

Phosphorylation reaction

60 µl	HeLa nuclear extract
1 mM	ATP
25 mM	$MgCl_2$
3.3 mM	tris-acetate, pH 7.8
6.6 mM	potassium acetate
1 mM	magnesium acetate
0.5 mM	DTT

3.2.21 Electrophoresis of proteins

Proteins were resolved using denaturing SDS polyacrylamide gel electrophoresis (Laemmli, 1970). For the separating gel, 7.5-15% acrylamide was used depending on the molecular weight of the proteins, for the stacking gel 4% acrylamide was used. The proteins were mixed with sample loading buffer, boiled for 5 min and cooled on ice prior to loading. Electrophoresis was performed at 100-150 V for 2-2.5 hours in SDS gel running buffer.

separating gel (10 ml)		**4% stacking gel (10 ml)**	
2.5 ml	1.5 M Tris-HCl, pH 8.8	2.5 ml	0.5 M Tris-HCl, pH 6.8
100 µl	10% SDS	100 µl	10% SDS
100 µl	10% APS	100 µl	10% APS
10 µl	TEMED	10 µl	TEMED
desired conc.	30% acrylamide/bis	620 µl	30% acrylamide/bis
up to 10 ml	dH$_2$O	up to 10 ml	dH$_2$O

3.2.22 Staining of protein gels

To detect proteins in SDS polyacrylamide gels, Coomassie or silver staining were used. For Coomassie staining, the gel was placed in staining solution for 2-3 hours at room temperature or overnight at 4 °C. Then the gel was washed 2-3 times in 50% Methanol/10% acetic acid and 2-3 times in 20% Methanol/10% acetic acid.

Coomassie staining solution

0.25%	Coomassie Brilliant Blue R250
45%	methanol
10%	acetic acid

For silver staining, Silver Stain Plus (BioRad) solutions were used according to the manufacturer's protocol.

3.2.23 Western Blotting

After gel electrophoresis, SDS polyacrylamide gels were transferred to a nitrocellulose membrane (Protran, Schleicher & Schüll). Before placing the membrane on the gel, gel and membrane were equilibrated shortly in protein transfer buffer. The transfer was performed for 45 min at 120 °C. Afterwards, the membrane was blocked for 1 hour in 1 x NET gelatine at room temperature. Primary antibody diluted in 1 x NET gelatine was added and incubated overnight at 4 °C or 2-4 hours at room temperature. After three washes for 15 min in 1 x NET gelatine, incubation with HRP-coupled secondary antibody, diluted in 1 x NET gelatine followed for 1-2 hours at room temperature. The membrane was washed another three times and detection of antibodies was carried out with the ECL system. The membrane was incubated for 5 min with equal amounts of ECL1 and ECL2 solutions, exposed to an X-ray film (Fuji Super RX) and developed in a Kodak X-omat 1000.

Transfer buffer		**1 x NET gelatine**	
192 mM	glycine	150 mM	NaCl
25 mM	tris base	5 mM	tris-HCL, pH 7.5
20%	methanol	0.05%	Triton X-100
		0.25%	gelatine

ECL1   **ECL2**

4.5 mM	luminol	0.003%	H_2O_2
4.3 mM	p-iodophenol	100 mM	tris-HCL, pH 9.5
100 mM	tris-HCL, pH 9.5		

For reblotting membranes with another antibody, membranes were stripped with stripping buffer for 5-10 min before washing with NET gelatine and incubation in new first antibody.

Stripping buffer

30%	trichloroacetic acid
1%	acetic acid
1%	Ponceau S solution

3.2.24 Isolation of total RNA

Total RNA was isolated from eukaryotic cells grown in 6-well plates using RNeasy mini kit (Qiagen) according to the supplier's protocol. RNA was eluted from the column in 30-40 µl RNase free dH_2O.

3.2.25 RT-PCR

200-500 ng of total RNA was used in the following reverse transcription reaction.

RT reaction   **RT buffer**

200-500 ng	RNA	300 µl	5 x first strand synthesis buffer
5 pmol	3' oligonucleotide	150 µl	0.1 M DTT
5 µl	RT buffer	75 µl	10 mM dNTPs
40 U	SuperScript II		up to 1 ml with dH_2O
4 U	DpnI (optional)		

To avoid later amplification from transfected DNA, DpnI could be added to the RT reaction to digest endogenous DNA. DpnI specifically cuts GATC in double-stranded DNA with methylated adenosine residues, but not non-methylated single-stranded DNA or cDNA.

A control reaction using dH_2O instead of RNA was included. The RT reactions were incubated for 45 min-1 hour at 42 °C.

1-3 µl of the reverse transcription reaction was used for amplification of resulting cDNA in the following reaction.

PCR reaction

1-3 µl	cDNA
5 pmol	5' oligonucleotide
5 pmol	3' oligonucleotide
200 nM	dNTPs
2.5 µl	10 x Taq polymerase buffer
1 U	Taq polymerase
	up to 25 µl with dH$_2$O

The conditions for the PCR program were dependent on the template (see 3.2.26 and 3.2.30).

3.2.26 *In vivo* splicing assay

To determine the influence of a protein on the splicing of selected minigenes, *in vivo* splicing assays were performed as described earlier (Stoss, Stoilov *et al.,* 1999; Tang, Novoyatleva *et al.,* 2004).

Eukaryotic cells were plated in a 6-well format. 24 hours after plating, 1-2 µg of plasmid carrying the desired minigene were cotransfected with a protein expression construct in each well (3.2.11.1). The plasmid for expressing the protein was usually transfected in increasing amounts from 0-3 µg to test for a concentration dependent effect. To ensure a constant amount of transfected DNA, the parental vector of the expression plasmid missing only the gene to be expressed was added in decreasing amounts. After cells were incubated for 14-18 hours at 37 °C and 3% CO$_2$, total RNA was isolated (3.2.24) and RT-PCR was performed (3.2.25)

If the effect of siRNA knockdown on different minigenes was studied, siRNA was transfected shortly after plating cells in a 24-well format (3.2.11.2). After 24-28 hours incubation at 37 °C and 5% CO$_2$, 250 ng minigene were transfected per well using the calcium phosphate method (3.2.11.1). After incubation for 16 hours at 37 °C and 5% CO$_2$, total RNA was isolated (3.2.24) and RT-PCR performed (3.2.25).

The reverse primer used for RT was specific for the vector which carried the minigene to avoid reverse transcription of endogenous RNA.

PCR was carried out in a Perkin Elmer GeneAmp PCR System 9700 thermocycler using minigene specific primers, which amplified alternatively spliced minigene products. A control reaction using RNA instead of DNA was included.

Optimised PCR conditions were used for each minigene:

Tau minigene

94 °C	2 min	
94 °C	1 min	
60 °C	1 min	30 cycles
72 °C	48 sec	
72 °C	10 min	

SMN2 minigene

94 °C	4 min	
94 °C	20 sec	
62 °C	20 sec	25 cycles
72 °C	20 sec	
72 °C	5 min	

Tra minigene

94 °C	2 min	
94 °C	20 sec	
65 °C	20 sec	33 cycles
72 °C	40 sec	
72 °C	2 min	

CD44v5 minigene

94 °C	5 min	
94 °C	20 sec	
72 °C	50 sec	30 cycles
72 °C	7 min	

pXB (X16) minigene

94 °C	5 min	
94 °C	50 sec	
55 °C	50 sec	20 cycles
72 °C	1 min	
72 °C	7 min	

PCR reactions were resolved on a 2% agarose or 5% polyacrylamide gel. The image was analysed with Image J software (http://rsb.info.nih.gov/ij/download.html).

3.2.27 UV-crosslinking and immunoprecipitation (CLIP)

UV-crosslinking and immunoprecipitation (CLIP) was done according to the protocol published by the R. Darnell lab (Ule, Jensen *et al.,* 2003; Ule, Jensen *et al.,* 2005). HEK293 cells were grown in four 10 cm dishes for 2 days. 2 dishes were irradiated at 400 mJ/cm^2 twice. Then lysis was performed in RIPA buffer (3.2.14) in absence of benzonase (400 µl per dish). Cells were harvested and lysates from UV-treated cells and control were treated with high concentrated (50 ng) and low concentrated (1 ng) RNase, respectively. After centrifuging, lysates were immunoprecipitated with 2 µl anti-hnRNP G, 900 µl RIPA rescue including RNase inhibitor and 55 µl dynabeads over night. Dynabeads were prepared before by washing once with 0.1 M Na$_3$PO$_4$, pH 8.1 and twice with RIPA rescue buffer. The following day, the beads were washed twice each with buffer A, buffer B and buffer C, then CIP treatment and 3' RNA linker (TraX3) ligation (over night at 16 °C) were performed on the samples treated with low concentrated RNase. The following day, the beads were washed 3 times with buffer C and all samples were labelled with γ-ATP by T4 polynucleotide kinase. After washing the beads 4 times with buffer C, the samples were boiled for 10 min at 70 °C and SDS-PAGE and Western Blot were performed. The membrane was rinsed with 1 x PBS and exposed to X-ray film over night. The next day, 3 bands which ran above the normal size

of hnRNP G were cut from the UV-treated and low RNase treated sample. To isolate and purify the bound RNA, the membrane pieces were treated with Proteinase K and urea and phenol-chloroform extraction was performed. Next, the 5' RNA-Linker (X6aR) was ligated to the purified RNA over night at 16 °C. After DNase treatment and phenol-chloroform extraction, the RNA was amplified by RT-PCR using specific oligos priming the linker sequences. TOPO cloning was performed and DNA was sequenced (Agowa, Berlin) to find the RNA binding targets.

3.2.28 Systematic evolution of ligands by exponential enrichment (SELEX)

For performing *in vitro* SELEX, an initial PCR was performed to amplify a DNA pool using primers T7pro and RT. The PCR product was cut from an 8% polyacrylamide gel, crashed and 600 µl extraction solution (4 ml ammoniumacetate, 0.8 ml EDTA 0.5 M, pH 8.3, 5.2 ml H_2O) added. The mixture was incubated at 37 °C over night on a rotating wheel. After spinning down at full speed for 5 min, the supernatant was taken to a fresh tube, mixed with 1 ml isopropanol and placed at -80 °C for 3 h. After centrifuging, the pellet was washed with 70% ethanol and dissolved in 10 µl H_2O.

After performing in vitro transcription with 1 µg of the amplified DNA pool in a total of 250 µl for 3 hours at 37 °C, DNAse treatment and phenol-chloroform extraction were carried out and the transcribed RNA precipitated over night at -80 °C. The mixture was centrifuged and washed with 70% ethanol and resuspended in 70 µl binding buffer (10 mM Tris, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 0.1% TritonX-100, 0.1 mg/ml tRNA).

25 µl Ni-NTA agarose resin was washed in binding buffer without tRNA and the RNA was pre-incubated with the resin for 15 min at 4 °C. The resin was removed, 20 µl purified HIS-hnRNP G added and incubated at room temperature for 30 min. 25 µl resin, washed with binding buffer, was added and incubated for 30 min at 4 °C. After washing the resin twice with binding buffer, 50 ml 2x proteinase K buffer (200 mM Tris, pH 7.5 + 25 mM EDTA, 300 mM NaCl + 2% SDS) and 5 µg proteinase K were added and incubated at 37 °C for 30 min.

The RNA was isolated by phenol-chloroform extraction and ethanol precipitation containing glycogen.

For performing reverse transcription reaction, the RNA was resuspended in 1x RT buffer (10 µl first strand buffer, 4 µl RT primer, 5 µl 10 mM dNTPs, 31 µl H2O), incubated for 10 min at 65 °C, spin down and put on ice for 2 min. 0.5 µl DTT (0.1M),

1.0 µl RNase inhibitor and 2 ml Superscript II were added and incubated at 42 °C for 2 hours. The reaction was heated for 15 min at 72 °C and PCR was performed using primers T7pro and RT.

The amplified DNA pool was purified from an 8% polyacrylamide gel as described above and another round of SELEX performed. After 5 rounds, the PCR products of the last SELEX round were cloned into TOPO vector and sequenced.

3.2.29 Preparation of supraspliceosomes

Supraspliceosomes were prepared from HEK293 or HeLa cells using a protocol described previously (Spann, Feinerman *et al.,* 1989; Sperling and Sperling*,* 1998). Briefly nuclear supernatants enriched for supraspliceosomes were prepared from purified nuclei of cells by micro-sonication of the nuclei and precipitation of the chromatin in the presence of tRNA. The nuclear supernatants were fractionated in 10-45% glycerol gradients in 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$ and 2 mM vanadyl ribonucleoside complex. Centrifugations were carried out at 4 °C in a SW41 rotor run at 41,000 rpm for 90 min (or an equivalent ω2t).

3.2.30 Array analysis

Array analysis was performed by Exonhit. 5 µg of total RNA was reverse transcribed in 30 µl total reaction volume with 200 U SuperScript II (Invitrogen), 4 µg random primers (Invitrogen) and dTTP and aminoallyl (aa)-dUTP at 250 µM final concentrations on 1 x first strand buffer (Invitrogen) at 42 °C for 2 h. RNA strands were hydrolysed with 10 µl NaOH (1 N) during 10 min at 65 °C. The reaction mixtures were neutralised with 10 µ HCl (1 N). The resulting aa-cDNAs were precipitated using 6 µl NaAc·3H$_2$O (3M), pH 5.2, 132 µl absolute ethanol, 0.5 µl glycogen and incubated over night at -20 °C. aa-cDNA pellets were resuspended in 2.5 µl RNase free water and labelled with 5 µl sodium bicarbonate (0.1 M), pH 9.0 and 2.5 µl Cy3 or Cy5 (Amersham Biosciences) in DMSO. Each sample was incubated in the dark for 1 h. The reactions were completed by adding 4.5 µl hydroxylamine (4 M) and incubated for 15 min.

Labelled materials were purified using JetQuick PCR purification columns (Genomed) following the manufacturer's instructions and eluted twice with 50 µl RNase free water preheated at 65 °C. The cDNA yields and dye incorporation was quantified by spectrophotometry. Hybridisations were performed using 2.5 µg of Cy3 and Cy5 labelled

targets (for 11K and 22K slides), respectively, along with 20 µg of herring sperm DNA (Invitrogen).

3.2.31 Databases and computational tools

Database / Software	URL	Description	Reference
ASD	http://www.ebi.ac.uk/asd/	alternative splicing database	Thanaraj, Stamm *et al.,* 2004; Stamm, Riethoven *et al.,* 2006
ClustalW	http://www.ebi.ac.uk/clustalw/	multiple sequence alignment program for DNA or proteins	Thompson, Higgins *et al.,* 1994; Chenna, Sugawara *et al.,* 2003
ESE	http://rulai.cshl.edu/tools/ESE	finds putative binding regions for several splice factors	Cartegni, Wang *et al.,* 2003
Human BLAT Search	http://www.genome.ucsc.edu/ cgi-bin/hgBlat	sequence alignment tool similar to BLAST	Kent, 2002
NCBI BLAST	http://www.ncbi.nlm.nih.gov/ BLAST/	finds regions of sequence similarity	Altschul, Gish *et al.,* 1990; Altschul, Madden *et al.,* 1997

4 RESULTS

4.1 Regulation of alternative splice site selection by RBM4 (LARK)

Lark is an RNA binding protein containing an RNA recognition motif (RRM) and is required for embryonic development in *Drosophila* (Newby and Jackson, 1993). In the sequence published for human Lark (Jackson, Banfi *et al.,* 1997) a frame-shift error was discovered, revealing that Lark and RBM4 are the same proteins (Markus and Morris, 2006), a recently described novel splicing regulator. RBM4 shares a nuclear import pathway with SR proteins, being delivered to the nucleus by transportin-SR2 (TRN-SR2), an importin β-like nuclear transporter (Lai, Kuo *et al.,* 2003). RBM4 is able to modulate alternative 5' splice sites and exon selection on an adenovirus E1A reporter *in vivo* (Lai, Kuo *et al.,* 2003) and was shown to directly influence the expression of the skeletal muscle specific α-tropomyosin isoform (Lin and Tarn, 2005). RBM4 contains two consensus RRMs and a retroviral zinc finger (RTZF). In this way, it resembles the SR protein 9G8. Unlike SR proteins, it does not contain an RS rich C-terminal domain, but several alanine stretches termed CAD (C-terminal alanine-rich domain) (Lai, Kuo *et al.,* 2003). Similar to RS domains, the RBM4 CAD region is important for protein:protein interaction as well as for the splicing effect. Furthermore, RBM4 was found to reside in speckles like many other splicing factors (Lai, Kuo *et al.,* 2003; Markus and Morris, 2006).

In yeast-two-hybrid screenings, it was found that RBM4 binds to the tumour suppressor WT1(+KTS) (Wilms' tumour 1) isoform, whereas the other major isoform WT1(-KTS) only showed a possible weak interaction (Markus, Heinrich *et al.,* 2006).

WT1 is involved in multiple molecular processes and has been implicated in 10−15% of Wilms' tumours, a paediatric renal malignancy (Little and Wells, 1997) often seen with the congenital abnormalities WAGR (Wilms' tumour, aniridia, genitourinary abnormalities, mental retardation), Denys-Drash and Frasier syndromes (Pelletier, Bruening *et al.,* 1991; Kohsaka, Tagawa *et al.,* 1999; Guo, Menke *et al.,* 2002). In normal development, WT1 has a prominent role in the differentiation of the metanephric mesenchyme by mediating mesenchyme-to-epithelial transition (reviewed in Hastie, 2001; Scharnhorst, Van Der Eb *et al.,* 2001). WT1 also plays a role in early haematopoiesis, and WT1 expression is associated with certain acute leukaemia, as well as prostate and breast cancers (reviewed in Yang, Han *et al.,* 2007).

Alternative splicing, RNA editing and alternative translation initiation sites generate up to 24 WT1 isoforms, which seem to have overlapping but also distinct functions during embryonic development and maintenance of organ function. In particular, the insertion or deletion at the end of exon 9 of only 9 nucleotides, which code for the three amino acids Lys−Thr−Ser (KTS), results in altered functions for the two isoforms (Hammes, Guo *et al.,* 2001). While WT1(−KTS) has strong DNA binding capacities and acts as a transcriptional regulator (Scharnhorst, Van Der Eb *et al.,* 2001), WT1(+KTS) has been found to be active post-transcriptionally, having an ability to bind RNA (Caricasole, Duarte *et al.,* 1996; Ladomery, Sommerville *et al.,* 2003) and be incorporated into spliceosomes (Davies, Calvio *et al.,* 1998). WT1 is also known to localise within the nucleus in speckles, defined compartments or in a diffuse manner, depending on the protein isoform (Larsson, Charlieu *et al.,* 1995).

To check the influence of RBM4 on splice site selection, an *in vivo* splicing assay was performed. Several reporter minigenes were transfected into HEK293 cells along with increasing amounts of RBM4 expression constructs (Figure 4-1 A).
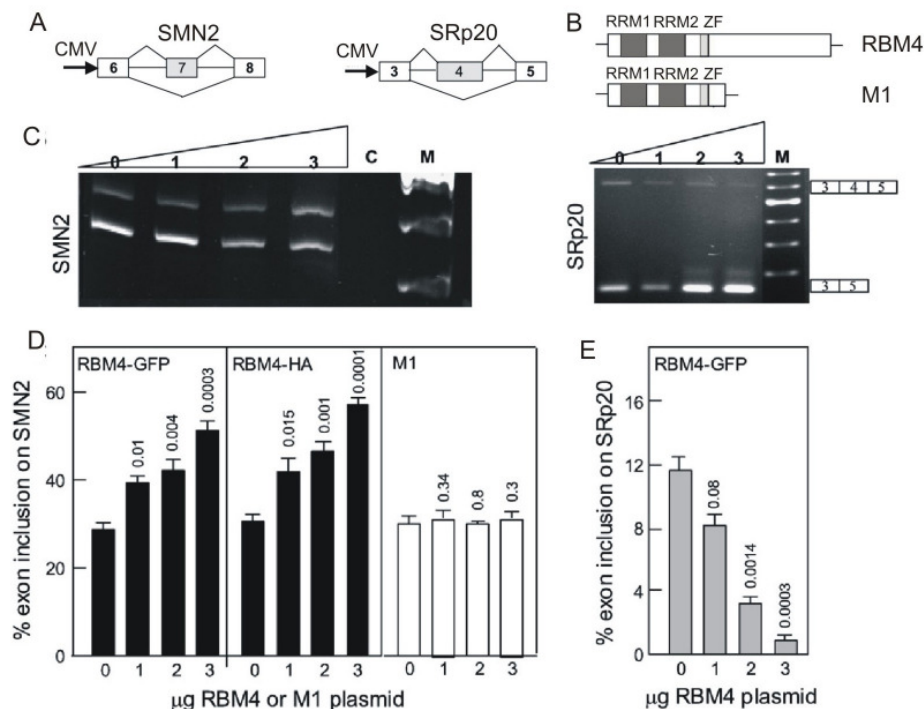
Figure 4-1: RBM4 influences alternative splice site selection in HEK293 cells *in vivo*. A) Structures of SMN2 and SRp20 minigenes. Indicated are alternative exon (grey), intron (horizontal line) and promoter (arrow). Lines connecting the exons indicate the two splicing patterns, either including or omitting the alternative exon. B) RBM4 wild type protein and C-terminus deleted mutant M1. RRMs (dark grey) and zinc finger (light grey) are indicated. C) RBM4 promotes exon 7 inclusion on SMN2 and exon 4 exclusion on SRp20. An increasing amount of GFP-RBM4 or RBM4-HA (0-3 µg) was cotransfected with 1 µg of the minigenes. Parental vector was added to ensure that similar amounts of cDNAs were transfected. The ethidium bromide stained gels are from representative experiments. D) and E) Splicing results of 4 independent experiments using GFP-RBM4, HA-tagged RBM4 or mutant M1 with minigene SMN2 (C), as well as GFP-RBM4 with minigene SRp20 (D). Error bars indicate +/- SE. All p values are from t-test versus 0 µg RBM4 plasmid.

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The mRNA isoforms were detected by RT-PCR using primers directed at sequences located in the flanking constitutive exons. Figure 4-1 C shows results of representative agarose gels for minigenes SMN2 and SRp20.

For SMN2, both GFP- and HA-tagged RBM4 expression clones promoted exon inclusion in a concentration-dependent manner (Figure 4-1 D).

From a baseline of approximately 30%, such exon inclusion was elevated to 60%, which is comparable to the effect of Tra2 beta1 or SRp30c on pre-mRNA from SMN2 (Hofmann, Lorson *et al.,* 2000; Young, Didonato *et al.,* 2002). A deletion mutant M1 lacking the C-terminal region of RBM4 (Figure 4-1 B) was unable to alter splice site selection of SMN2, indicating that the carboxyl terminal region of RBM4 is important for regulation of splice site selection (Figure 4-1 D). RBM4 had the opposite effect on minigene SRp20. It stimulated the skipping of exon 4 (Figure 4-1 E).

A weaker effect was observed on Tra2-beta minigene, with a reduction in exon inclusion from 30% down to 21% (Figure 4-2, left) as well as in CD44v5 minigene with an increase from 10% up to 21% (Figure 4-2, right).
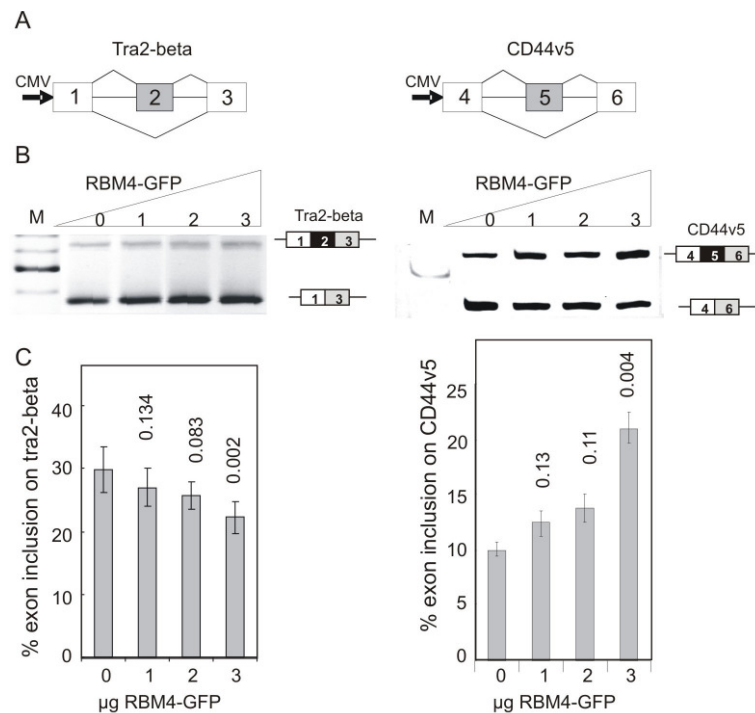
Figure 4-2: RBM4 influences alternative splice site selection on Tra2-beta and CD44v5 minigenes *in vivo*. Panel A shows the structures of SMN2 and SRp20 minigenes. Indicated are: alternative exon (grey), intron (horizontal line) and promoter (arrow). Lines connecting the exons indicate the two splicing patterns, either including or omitting the alternative exon. Panel B shows that RBM4 promotes exon 2 exclusion on tra2-beta and exon 5 inclusion on CD44v5. An increasing amount of GFP-RBM4 (0-3 µg) was cotransfected with 1 µg of the minigenes. Parental vector was added to ensure that similar amounts of cDNAs were transfected. The ethidium bromide stained gels are from representative experiments. Panels C and D show splicing results of 3 independent experiments using GFP-RBM4 with minigenes tra2-beta (left) and CD44v5 (right). Error bars indicate +/- SE. All p values are from t-test vs. 0 µg RBM4 plasmid.

These results accord with earlier findings (Lai, Kuo *et al.,* 2003; Lin and Tarn, 2005) that RBM4 modulates alternative 5' splice site selection and is able to promote or prevent exon inclusion, depending on the minigenes or pre-mRNA used.

Next, the functional significance of the binding of RBM4 to WT1 was examined by testing the ability of WT1 to modulate the influence of RBM4 on splice site selection. An increasing amount of RBM4 (0, 1, 2, 3 µg) was cotransfected into HEK293 cells with a constant amount of WT1(+KTS) expression construct. As shown in Figure 4-3, WT1(+KTS) abolished the influence of RBM4 on splice site selection of SMN2 pre-mRNA (Figure 4-3 A+C) and CD44v5 pre-mRNA (Figure 4-3 H) and significantly reduced the effect on SRp20 (Figure 4-3 D). This was probably caused by sequestration of RBM4 by WT1(+KTS) and demonstrates that the interaction between RBM4 and WT1 occurs *in vivo*. A WT1(-KTS) plasmid, where nine nucleotides at the end of exon 9 were deleted, was not, however able to inhibit the RBM4 splicing effect (Figure 4-3 B), indicating that the KTS coding exon is necessary for the effect of WT1 on RBM4.
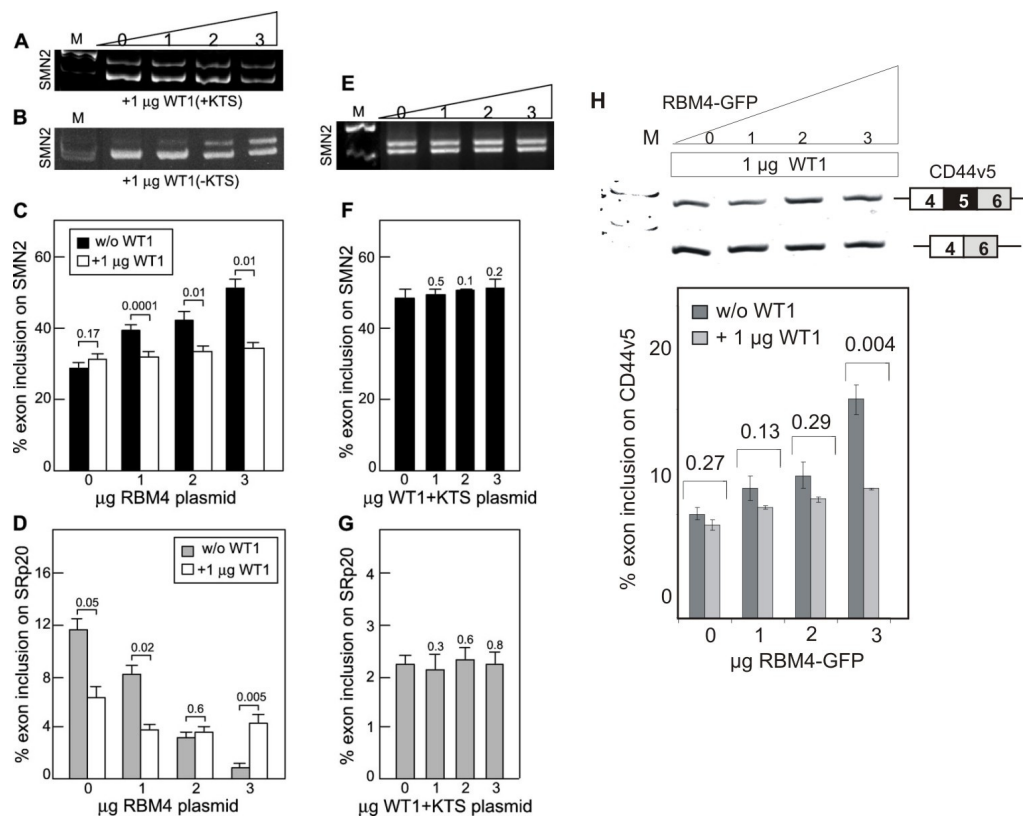
Figure 4-3: WT1 antagonises the RBM4 splicing effect in HEK293 cells. Panels A and C show that WT1(+KTS) antagonises the effect of RBM4 on SMN2, panel H shows that it antagonises the effect of RBM4 on CD44v5. One microgram of WT1(+KTS)-myc was cotransfected with an increasing amount of GFP-RBM4 and SMN2 or CD44v5 minigenes as indicated and compared with transfections without WT1 ('w/o WT1'). Panel C and panel H (bottom) show results from 3 independent experiments. Panel B shows that WT1(-KTS) is not able to inhibit the RBM4 splicing effect. Panel D shows that WT1(+KTS) suppresses the RBM4 splicing effect on SRp20 (n = 3 independent experiments). WT1 alone does not influence the splicing patterns of SMN2 and SRp20 minigenes (E-G).

To exclude the possibility that WT1 has an effect on splice site selection of the tested minigenes independently of RBM4, WT1(+KTS) plasmid alone was transfected and the effect in exon inclusion measured. It was found that WT1(+KTS) had no significant effect on splice site selection with SMN2 or SRp20 (Figure 4-3 E-G), suggesting that the inhibitory effect it has on splice site changes by RBM4 is mediated through its interaction with RBM4.

Together, these results show that RBM4 is able to change splice site selection, which can be influenced by protein:protein interaction with WT1. A possibility is that RBM4 is sequestered to the splice sites by interacting with WT1.

4.2 Regulation of alternative splice site selection by reversible phosphorylation

Reversible protein phosphorylation is a main regulatory mechanism of intracellular signal transduction. A number of kinases and phosphatases participate in several different transduction pathways. Protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), 2B (PP2B - calcineurin) and 2C (PP2C) are four major serine/threonine phosphatases, mediating signaling cascades in eukaryotes. Earlier investigations showed that PP1 is involved in the organisation of pre-mRNA splicing factors in the mammalian cell nucleus (Misteli and Spector, 1996). PP1 (Mermoud, Cohen *et al.,* 1992) and PP2C (Murray, Kobayashi *et al.,* 1999; Allemand, Hastings *et al.,* 2007) were found to associate with complexes formed on pre-mRNA and can be identified in the B complex (Deckert, Hartmuth *et al.,* 2006). Recently, it was demonstrated that PP1 or PP2A mediated dephosphorylation of U2 and U5 snRNP components facilitates the structural rearrangements that are necessary for the transition from the first to the second step of splicing (Shi, Reddy *et al.,* 2006). Reversible protein phosphorylation differently regulates the subcellular localisation and activity of shuttling SR proteins (Sanford, Ellis *et al.,* 2005b). Dephosphorylation of the RS domain of SR proteins participates in a sorting mechanism for mRNP transition from splicing to export (Lin, Xiao *et al.,* 2005).

Human transformer Tra2-beta1 is an SR-related protein, which could be detected in all metazoans except plants. Our group demonstrated that protein phosphatase 1 (PP1) binds to Tra2-beta1 via a phylogenetically conserved RVDF sequence located downstream of the alpha2 helix in the beta4 strand of the RNA recognition motif (RRM) of Tra2-beta1 and dephosphorylates it. The PP1 binding motif is also present in RRMs of other SR proteins (Novoyatleva, Heinrich *et al.,* in press). The RVxF motif was previously shown to be a PP1 binding motif and is present in most PP1 binding partners. This docking motif interacts with a hydrophobic channel of PP1 that is remote from the catalytic site (Egloff, Johnson *et al.,* 1997; Bollen, 2001). As in other PP1 interactors, the RVDF motif of Tra2-beta1 is N-terminally flanked by basic residues that promote the initial binding to PP1 (Meiselbach, Sticht *et al.,* 2006). The RVDF motif is fully conserved in all Tra2-beta1 sequences from vertebrates, except some fish species, and non-vertebrate species, where it is changed to RVDY.

4.2.1 Tra2-beta1 and Tra2-beta1-RATA both bind to RNA

It was found that PP1 can dephosphorylate Tra2-beta1 *in vivo* and *in vitro*, but not the Tra2-beta1 mutant Tra2-beta1-RATA. In this mutant, the RVDF motif was exchanged to RATA, which is known to destroy or severely weaken the ability of PP1 to bind this protein sequence (Wakula, Beullens *et al.,* 2003).

To test whether mutating the RVDF motif into RATA disrupts the RRM, gel retardation assays were performed using recombinant proteins and a purine rich synthetic RNA (GGGCGGGACAGACGCGAGCC). As shown in Figure 4-4, both proteins bound to the RNA, demonstrating that the mutation did not disrupt the overall structure of the RRM.
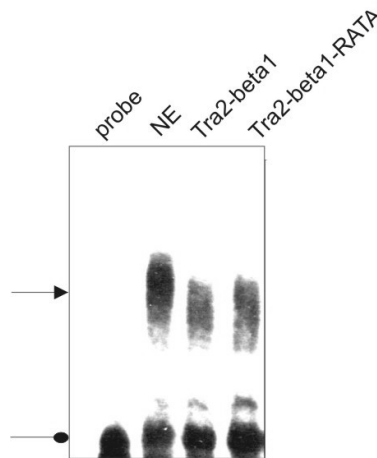
Figure 4-4: Tra2-beta1 and Tra2-beta1-RATA bind to RNA. Gel retardation assay, 1µg of nuclear extract (NE), recombinant Tra2-beta1 and Tra2-beta1-RATA were incubated with a purine-rich RNA probe and analysed by native gel electrophoresis. The pointed arrow indicates the RNA:protein complexes, the round arrow the free probe.

4.2.2 Phosphorylation dependent binding of Tra2-beta1 and SF2/ASF

Tra2-beta1 is known to form homodimers and can also heterodimerise with other SR proteins, such as SF2/ASF (Beil, Screaton *et al.,* 1997; Nayler, Cap *et al.,* 1998). Our group found that PP1 influences the dimerisation. It was shown that in contrast to wild type Tra2-beta1, the Tra2-beta1-RATA mutant no longer homomultimerises with endogenous Tra2-beta1. In addition, it was shown that Tra2-beta1 and PP1 partially colocalise in cells, although both proteins have a dynamic localisation. This colocalisation was found to depend on the presence of the PP1 binding motif (Novoyatleva, Heinrich *et al.,* in press).

Next, we determined whether the known interaction between Tra2-beta1 and SF2/ASF (Nayler, Cap *et al.,* 1998) is dependent on the PP1 binding site of Tra2-beta1 *in vitro* and *in vivo*.

4.2.2.1 Phosphorylation dependent *in vivo* binding of Tra2-beta1 with other proteins

The Tra2-beta1 protein consists of two RS domains (RS1 and RS2), which are flanking the RRM. It was shown previously that the serine residues in these RS domains could be phosphorylated (Cao, Jamison *et al.,* 1997; Xiao and Manley, 1998). To have a tool for studying the influence of phosphorylation of Tra2-beta1, all the serine residues in this RS domain were mutated either to alanine residues (Tra2-beta1-RSA) or to glutamic acid residues (Tra2-beta1-RSE). The phosphorylation of serine residues can be abolished by their mutation to alanines, while mutation to glutamic acid residues mimics a phosphorylation state.

EGFP tagged wild type Tra2-beta1, Tra2-beta1-RATA, Tra2-beta1-RSA and Tra2-beta1-RSE mutants were immunoprecipitated with anti-GFP and the presence of endogenous SF2/ASF in the precipitates was detected by Western Blot. The immunoprecipitations were performed in the presence of benzonase to omit RNA-mediated interactions.

As shown in Figure 4-5 (top row), no multimerisation between SF2/ASF and Tra2-beta1-RATA could be observed. For both Tra2-beta1 mutants RSA and RSE splicing factor SF2/ASF could be detected in the precipitates. For the non-phosphorylated mutant Tra2-beta1-RSA even a stronger band is visible than for the phosphorylated form Tra2-beta1-RSE, which indicates a possible stronger interaction.

It is known that SF2/ASF acts antagonistically to hnRNP A1 to regulate alternative splicing. Increased concentrations of SF2/ASF select proximal 5' splice sites, while high hnRNP A1 levels promote distal 5' splice site selection (Mayeda and Krainer, 1992; Caceres, Stamm *et al.,* 1994). Therefore, a specific antibody was used to detect hnRNP A1 in the immunoprecipitates. For Tra2-beta1-RATA, where no SF2/ASF was found, hnRNP A1 could be detected and for the Tra2-beta1-RSA mutant, where a strong SF2/ASF band was detected, only a very weak band for hnRNP A1 was visible (Figure 4-5, bottom row). This shows that in the interaction with Tra2-beta1 hnRNP A1 depends more on a phsophorylated state of Tra2-beta1 than SF2/ASF.
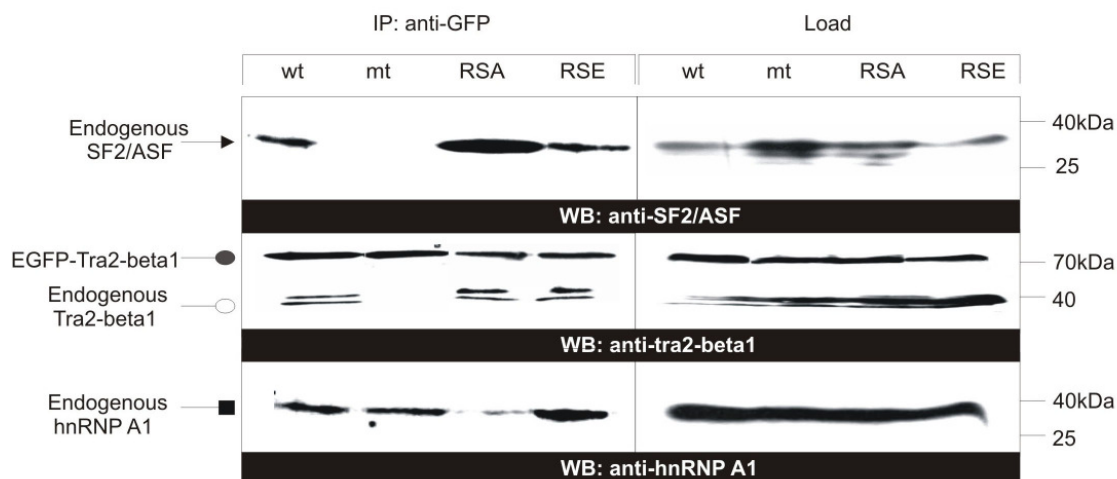
Figure 4-5: PP1 dependent heteromultimerisation of Tra2-beta1. EGFP tagged wild type Tra2-beta1 and EGFP tagged Tra2-beta1-RATA was expressed in HEK293 cells. Protein complexes were recovered by immunoprecipitation with anti-GFP antisera. Top row: endogenous SF2/ASF was identified with anti-SF2/ASF; middle row: reblot with a pan-Tra2-beta1 antiserum, PP1 dependent homomultimerisation of Tra2-beta1 with endogenous Tra2-beta1 is visible. Bottom row: reblot with anti-hnRNP A1 to identify endogenous hnRNP A1. Pointed arrow: endogenous SF2/ASF; black round arrow: EGFP tagged Tra2-beta1; white round arrow: endogenous Tra2-beta1; square arrow: endogenous hnRNP A1.

4.2.2.2 Phosphorylation dependent *in vitro* binding of Tra2-beta1 and SF2/ASF

The phosphorylation dependency of the binding between SF2/ASF and Tra2-beta1 was analysed using recombinant proteins. Baculovirus generated (section 3.2.18) His tagged Tra2-beta1 was immobilised on Ni-NTA agarose and phosphorylated *in vitro* by incubation with nuclear extract and ATP. The nuclear extract contains Clk/Sty kinases that phosphorylate SR proteins (Nayler, Stamm *et al.,* 1997). After removing the nuclear extract and performing stringent wash steps, the phosphorylated Tra2-beta1 was incubated with PP1 from rabbit skeletal muscle. The activity of PP1 was blocked by tautomycin, a cell permeable phosphatase inhibitor that blocks PP1 about four times more potently than protein phosphatase PP2A and other phosphatases. This affinity matrix was then used to determine binding of bacterially expressed GST-SF2/ASF. As shown in Figure 4-6 A, the dephosphorylated Tra2-beta1 protein exhibited a higher affinity towards SF2/ASF. This reflects the *in vivo* situation where Tra2-beta1 wild type binds to SF2/ASF and Tra2-beta1, whereas the Tra2-beta1-RATA mutant shows no interaction (4.2.2.1). After tautomycin treatment, Tra2-beta1 had a lower affinity to SF2/ASF similarly to unphosphorylated Tra2-beta1.
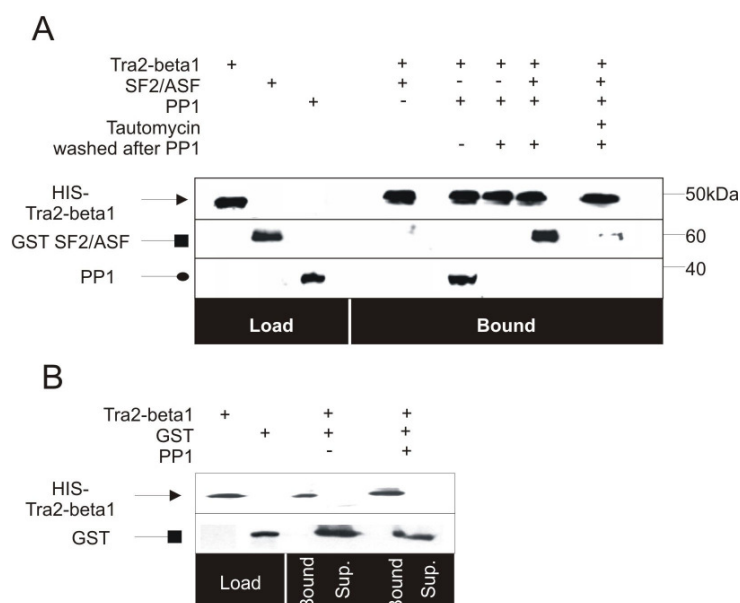
Figure 4-6: Phosphorylation dependent interaction between recombinant Tra2-beta1 and SF2/ASF. A) HIS-Tra2-beta1 was immobilised on Ni-NTA agarose and the binding of recombinant GST-SF2/ASF was determined without or after PP1 treatment or after PP1 and tautomycin treatment, as indicated on top. "washed after PP1" indicates that prior to loading SF2/ASF, the Tra2-beta1 affinity matrix was washed and no PP1 could be detected. The upper row shows that equal amount of HI S-Tra2-beta1 were present on the matrix. B) Control that GST alone does not bind to HIS-Tra2-beta1 on the affinity matrix. HIS-Tra2-beta1 was bound to Ni-NTA agarose and incubated with GST and PP1 as indicated. (Load: Tra2-beta1, SF2/ASF, PP1, loaded on column; Sup.: Supernatant).

Residual PP1 binding was not observed prior to loading SF2/ASF to the Tra2-beta1 affinity matrix, indicating that the effect is phosphorylation mediated and does not reflect a tethering activity of PP1 (Figure 4-6 A, bottom row). Furthermore, we did not observe binding of GST to Tra2-beta1 bound to the matrix under any experimental conditions (Figure 4-6 B).

The data from chapter 4.2.2 indicate that the dephosphorylation of Tra2-beta1 by PP1 promotes the multimerisation between Tra2-beta1 and SF2/ASF, while the interaction with hnRNP A1 is abolished.

4.2.3 PP1 regulates the usage of alternative exons

Tra2-beta1 regulates alternative exons by binding to a characteristic, degenerate, but purine rich sequence that acts as an exonic enhancer (Tacke, Tohyama *et al.,* 1998; Stoilov, Daoud *et al.,* 2004). Therefore, we next studied whether PP1 could regulate such exons. Experimentally, it was shown that in a concentration dependent manner Tra2-beta1 promotes inclusion of its own exon 2, tau exon 10 and SMN2 exon 7 (Stoilov, Daoud *et al.,* 2004). Since the molecular interactions of Tra2-beta1 were influenced by PP1-mediated dephosphorylation, these exons were tested for their dependency on PP1 activity. Established

minigene systems were employed, where each alternative exon is flanked by its constitutive exons in a reporter gene construct. This reporter construct is cotransfected with expression vectors for trans-acting factors and their influence on alternative splicing is determined by RT-PCR (Stoss, Stoilov *et al.*, 1999). NIPP1 (nuclear inhibitor of protein phosphatase 1), is a specific inhibitor of PP1 (Beullens and Bollen, 2002). Therefore, reporter minigenes containing Tra2-beta1 dependent alternative exons were cotransfected together with either PP1cgamma or NIPP1 expression clones into HEK293 cells, which increased or decreased cellular PP1 activity (Beullens and Bollen, 2002). The influence of PP1 activity on alternative splicing was determined by RT-PCR (Stoss, Stoilov *et al.*, 1999). An increase of PP1 expression promoted the exclusion of Tra2-beta1 dependent exons. In contrast, the inhibition of nuclear PP1 by the expression of NIPP1 promoted Tra2-beta1 dependent exon inclusion (Novoyatleva, Heinrich *et al.*, in press). To further rule out non-specific effects, we decreased the concentration of PP1 by performing RNA interference (section 3.2.11.2). The amount of the three endogenous PP1 isoforms, PP1alpha, PP1beta and PP1gamma was reduced by cotransfecting three pairs of siRNA with the reporter minigenes. As shown in Figure 4-7 A-C, decreasing the PP1 concentration with siRNA promoted inclusion of the Tra2-beta1 dependent exons, an effect similar to blocking PP1 activity with NIPP1, whereas control siRNA had no influence on splice site selection. The effect on splice site selection was proportional to the decrease of PP1 that was detected by Western Blot (Figure 4-7 D).
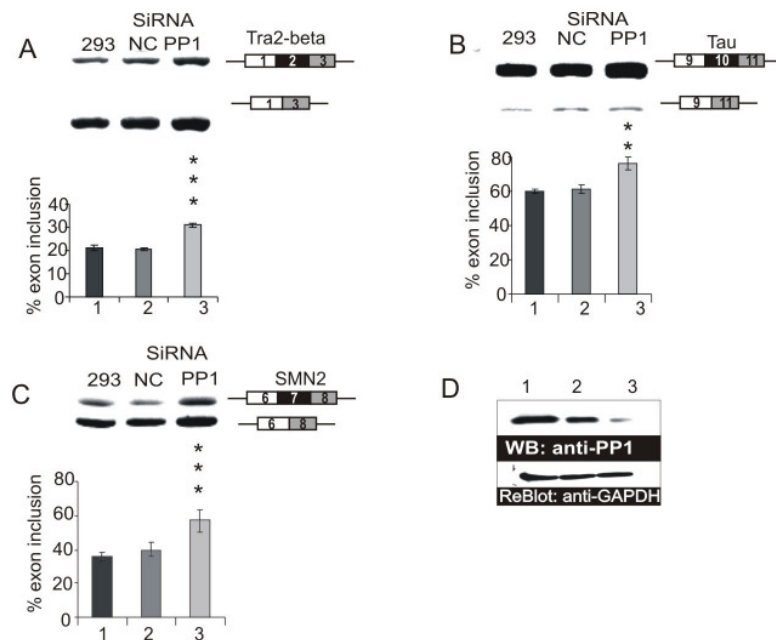
Figure 4-7: PP1 regulation of alternative exon usage. A-C) The expression of PP1 was reduced by cotransfecting siRNA against all PP1 isoforms with the reporter minigenes. 293: cells were treated only with the transfection reagent; NC: non-specific siRNA; PP1: siRNA against all three PP1 isoforms. A: Tra2-beta, exon 2; B: tau, exon 10; C: SMN2, exon 7; stars indicate p-values from student's test, when non-specific siRNA was transfected (lane 2) and siRNA for PP1 transfected cells (lane 3) were compared (Tra2-beta: p = 0.00015; SMN2: p = 0.002; tau: p = 0.0006). D) Western Blot showing the decrease of PP1, using specific primers.

4.2.4 PP1 mediated alternative splice site selection of Tra2-beta1 dependent exons

To test whether blocking PP1 activity has an effect on splice site selection of endogenous genes we constructed an alternative exon specific oligonucleotide array that contained all known alternative splice variants from splicing factors, as well as some well studied model systems. The experiment was performed in collaboration with ExonHit Therapeutics, Paris. For hybridisation, total RNA from untreated HEK293 cells, HEK293 cells transfected with expression constructs for Tra2-beta1, NIPP1 as well as cells treated with 40 nM tautomycin were used. RNA was obtained 18 hours after transfection. The alternative splicing events were detected by a combination of exon junction and exon body probes, as previously described (Fehlbaum, Guihal *et al.,* 2005). Dye swap experiments showed strong probe variation (correlation coefficient: 0.75 and 0.59, Figure 4-8). Therefore, the array was used as an exploration tool and findings verified by RT-PCR (4.2.4.1).
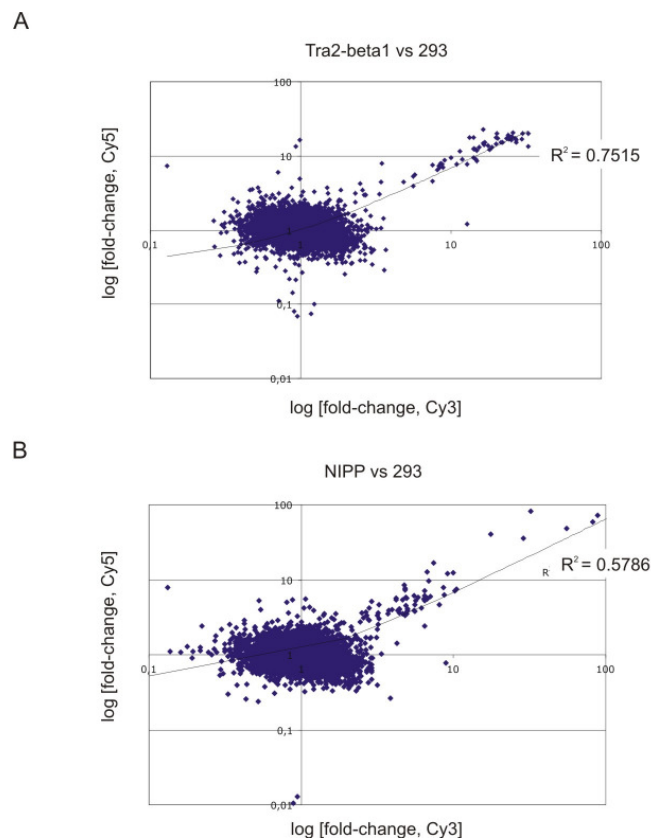
Figure 4-8: Dye swap analysis of the array experiments. A) Dye swap of an experiment comparing HEK293 cells with cells overexpressing Tra2-beta1. The log[fold change Cy3] is compared with log[fold change Cy5] in a scatter plot. B) Dye swap of an experiment comparing HEK293 cells with cells overexpressing NIPP1.

The fold changes in the array analysis indicated that 65 out of a total of 942 alternative splicing events were strongly influenced by Tra2-beta1 concentration.

4.2.4.1 Validation of oligonucleotide array results by RT-PCR

17 of the 65 events were chosen for subsequent validation of the array data by RT-PCR (see summarised in Table 4-1). These genes and their corresponding exons together with their potential function are also indicated in Table 4-3, p. 75-76.

From these 17 events, 15 were confirmed for their dependency on Tra2-beta1 by RT-PCR using primers in the flanking exons. The two events which could not be confirmed are marked in grey in Table 4-1.

Gene name	Exon ID	Splicing event	Reference	Variant
Clk4	208.3.3	Intron retention	NM_020666	BC063116
CPSF6	148.1.1	Novel exon	NM_007007	AL557975
DDX23	91.1.1	Intron retention	NM_004818	BU174750
eIF2B	14.1.1	Exon skipping	NM_001414	AA923281
Fe65	909.039.002	Exon skipping	NM_001164	BX420711.1
FUS	93.1.1	Exon skipping	NM_004960	AJ549096
Fyn	907.002.002	Novel exon	NM_002037	NM_153047.1
hnRNP M	114.1.1	Exon skipping	NM_005968	AL516884
MTMR11	137.1.1	Novel exon	NM_006697	BI833138
NOL5A	127.3.1	Novel exon	NM_006392	BE253695
PPIE	117.8.1	Exon skipping	NM_006112	BI821836
PPIL3	236.10.1	Alternative splice acceptor	NM_032472	BU195819
SFRS4	104.9.3	Novel exon	NM_005626	BX447499
SFRS5	144.1.1	Alternative splice donor	NM_006925	BC018823
SFRS14	179.1.1	Exon skipping	NM_014884	AI089022
SRRM1	110.3.1	Novel exon	NM_005839	BE931442
WT1	222.2.1	Exon skipping	NM_024426	AK093168

Table 4-1: Validated alternative exons and their splicing events. Exon ID indicates the number of the exon in the splice array; reference and variant indicate the number of the corresponding sequences from Entrez Nucleotide (http://www.ncbi.nlm.nih.gov).

With the exception of alternative exons in CPSF6, MTRM11, PPIE and PPIL3, Tra2-beta1 promotes exon inclusion for most of the tested genes (Figure 4-9 and Figure 4-10, middle lane), supporting earlier findings that the protein acts as a general activator of exons (Tacke, Tohyama *et al.,* 1998; Stoilov, Daoud *et al.,* 2004).

Next, overexpression of Tra2-beta1 was compared with the inhibition of PP1 by its specific inhibitor tautomycin in HEK293 cells. HEK293 cells were treated with 40 nM of tautomycin before isolation of total RNA 18 hours later. In most cases (10 from 15, Figure 4-9), blocking PP1 activity with tautomycin and increasing Tra2-beta1 concentration showed a similar effect on alternative splice site selection. Only for CPSF6, MTMR11, PPIE, PPIL3 and SRRM1 blocking PP1 activity with tautomycin had an opposite effect (Figure 4-9).
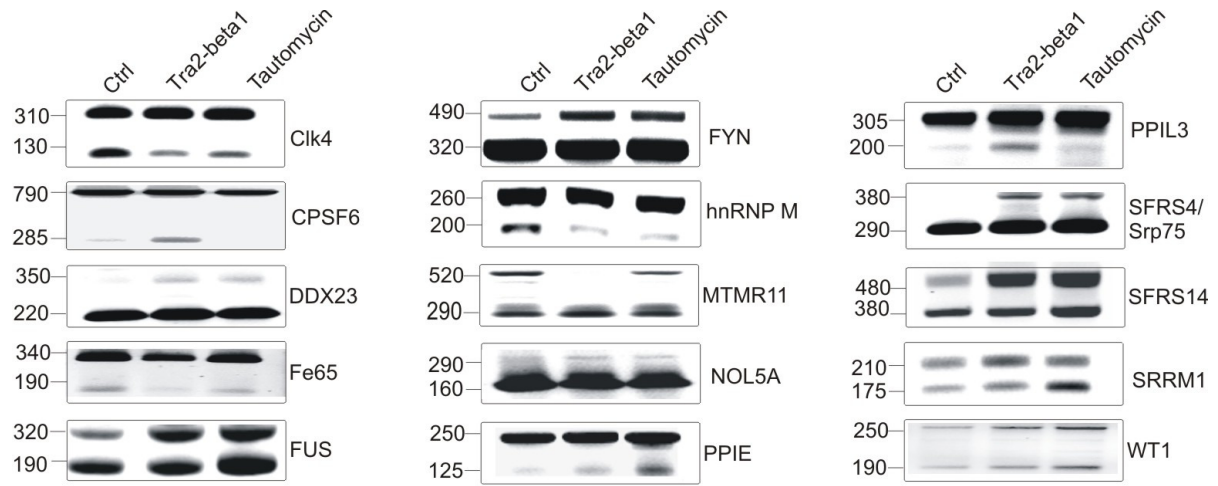
Figure 4-9: Tautomycin treatment changes usage of Tra2-beta1 dependent exons. RNA from untreated HEK293 cells, 293 cells overexpressing EGFP-Tra2-beta1 or 293 cells treated with 40 nM tautomycin was amplified by RT-PCR using specific primers. Ctrl: untreated HEK293 cells.

Next, the overexpression of Tra2-beta1 was compared with inhibition of PP1 by NIPP1 overexpression in HEK293 cells. For most cases for which the array data could be confirmed by RT-PCR, we found that blocking PP1 activity by NIPP1 also has a similar effect on alternative splice site selection as increasing the Tra2-beta1 concentration (Figure 4-10). In some cases (Clk4, DDX23, FUS), NIPP1 overexpression had a stronger effect on exon inclusion than Tra2-beta1 overexpression. Interestingly, likewise to PP1 inhibition by tautomycin, PP1 inhibition with NIPP1 showed an opposite effect than Tra2-beta1 for MTMR11, PPIE and PPIL3, but not for CPSF6 and SRRM1.
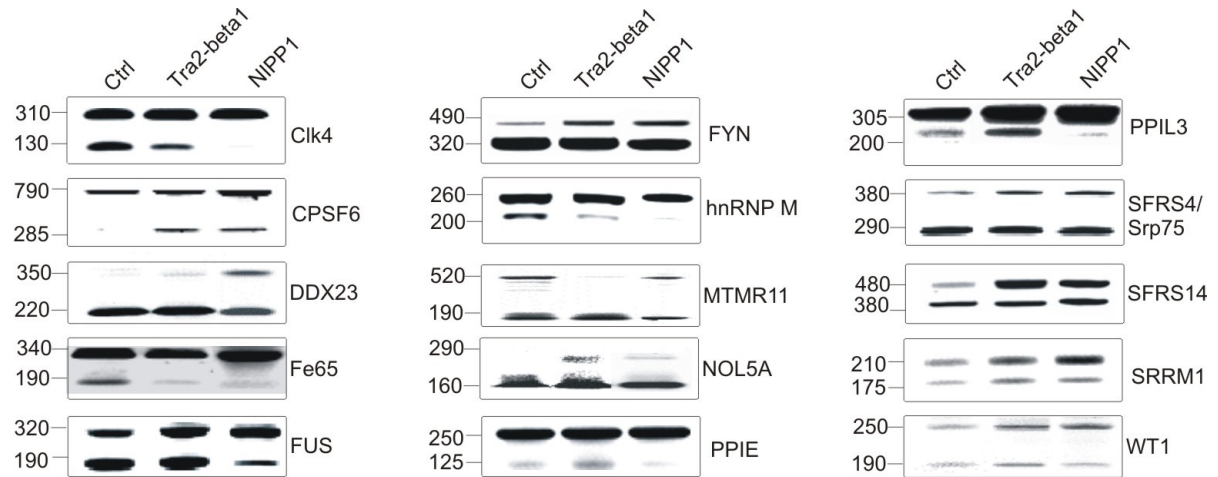
Figure 4-10: NIPP1 overexpression changes the usage of Tra2-beta1 dependent exons. RNA from untreated HEK293 cells, 293 cells overexpressing EGFP-Tra2-beta1 or EGFP-NIPP1 was amplified by RT-PCR using specific primers. Ctrl: untransfected HEK293 cells.

These data show that many, but not all Tra2-beta1 dependent exons are influenced by PP1 mediated dephosphorylation of Tra2-beta1.

To further rule out effects from overexpression, we removed all three PP1 isoforms by siRNA and amplified the RNA with specific primers of some the Tra2-beta1 dependent

exons. As shown in Figure 4-11, siRNA treatment had an effect similar to NIPP1 overexpression, demonstrating that the effect on splice site selection is mediated by PP1.
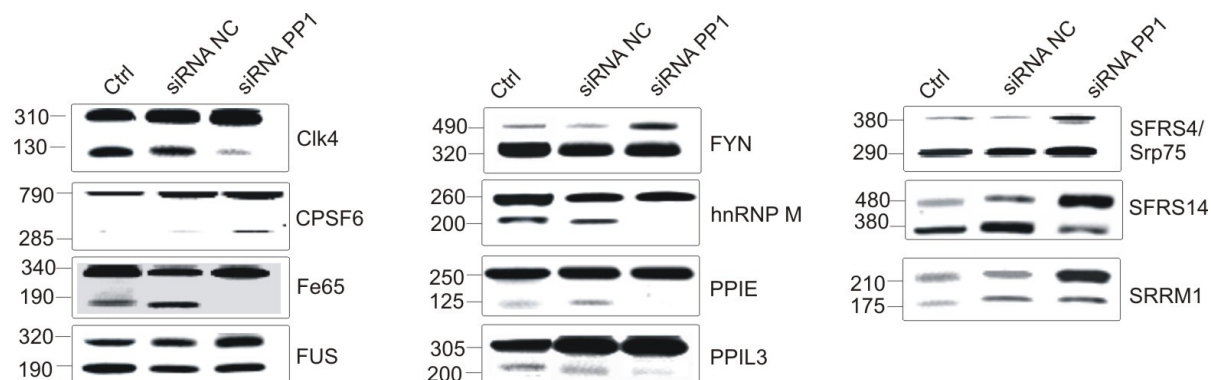
Figure 4-11: PP1 siRNA treatment changes the usage of Tra2-beta1 dependent exons. HEK 293 cells were treated with mock siRNA (siRNA NC) or a mixture of siRNAs against all PP1 isoforms (siRNA PP1). RNA from these cells was amplified by RT-PCR using specific primers. Ctrl: untreated HEK293 cells.

Together these data indicate that PP1 activity, specifically regulated by NIPP1, can influence alternative splice site selection.

4.2.5 Chip gene analysis with forskolin treatment

In neurons the predominant inhibitor of PP1 is DARPP32 (Dopamine and cAMP-regulated phosphoprotein), which binds to and inhibits PP1 after phosphorylation at threonine 34. T34 is phosphorylated by protein kinase A (PKA) (Kwon, Huang *et al.,* 1997).

Forskolin increases the cAMP level and activates PKA, which then leads via DARPP32 to PP1 inhibition. Thus forskolin indirectly acts in the same way as NIPP1 and tautomycin.

Therefore, we investigated the effect of forskolin treatment on Tra2-beta1 dependent exons. We expected a similar effect as inhibition of PP1 with NIPP1 and tautomycin or PP1 removal by siRNA. RNA was isolated from HEK293 cells after 5 nM and 10 nM forskolin treatment and RT-PCR was performed with specific primers. As shown in Figure 4-12, most of the exons show the same effect as for PP1 inhibition or removal. With 10 nM forskolin the effect is stronger in several cases than with 5 nM. Interestingly, for Fe65, PPIL3 and WT1, forskolin treatment did not show the same effect. While PPIL3 and WT1 showed no effect, Fe65 showed exon skipping, which is the opposite effect.
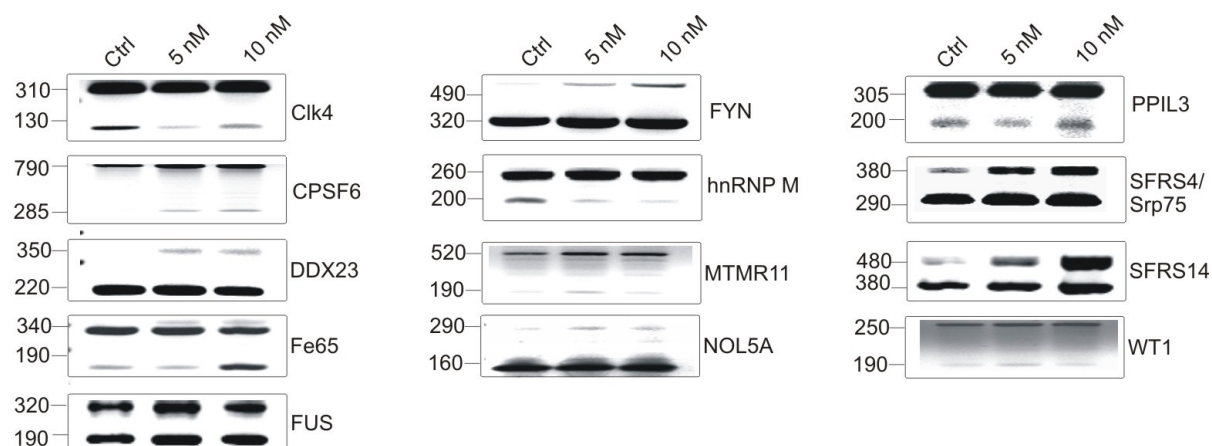
Figure 4-12: Forskolin treatment changes usage of Tra2-beta1 dependent exons. RNA from untreated HEK293 cells and HEK293 cells treated with 5 nM or 10 nM forskolin was amplified by RT-PCR using specific primers. Ctrl: untreated HEK293 cells.

These data show that PKA mediated PP1 inhibition reached by forskolin has the same effect on most of the Tra2-beta1 dependent exons as direct inhibition by PP1 inhibitors or PP1 removal by siRNA. Together this shows that PP1 activity can influence alternative splice site selection and is regulated by cAMP levels.

Table 4-2 shows a summary of all changes observed with Tra2-beta1 and NIPP1 overexpression as well as tautomycin and forskolin treatment.

Gene name	Exon ID	Tra2-beta1	Tautomycin	NIPP1	Forskolin
Clk4	208.3.3	increase	increase	increase	increase
CPSF6	148.1.1	skipping	increase	skipping	skipping
DDX23	91.1.1	increase	increase	increase	increase
Fe65	909.039.002	increase	increase	increase	skipping
FUS	93.1.1	increase	increase	increase	increase
Fyn	907.002.002	increase	increase	increase	increase
hnRNP M	114.1.1	increase	increase	increase	increase
MTMR11	137.1.1	skipping	no change	no change	no change
NOL5A	127.3.1	increase	increase	increase	increase
PPIE	117.8.1	skipping	skipping	increase	ND
PPIL3	236.10.1	skipping	no change	increase	no change
SFRS4	104.9.3	increase	increase	increase	increase
SFRS14	179.1.1	increase	increase	increase	increase
SRRM1	110.3.1	increase	skipping	increase	ND
WT1	222.2.1	increase	increase	increase	no change

Table 4-2: Changes of usage of Tra2-beta1 dependent exons by Tra2-beta1, tautomycin, NIPP1 and forskolin. ND: not determined.

Gene	Exon sequence	Protein	Potential function
Clk4 208.3.3 NM_020666 Homo sapiens CDC-like kinase 4	CCCCGCTGAATGAATTGCGTATTCTGCCTGAATTCACTC TGATATATTGATTGGCTGGACGATCTTGGTGCTGCCCACT TGCCGTTCCAGAAGAGCCACCGAAGGAAAAGATCCAGGAG TATAGAGGATGATGAGGAGGGTCACCTGATCTGTCAAGT GGAGACGTTCTAAGAGCAAGAT	PAE*	Stop Codon; NMD
CPSF6 148.1.1 NM_007007 Homo sapiens cleavage and polyadenylation specific factor 6, 68kDa	GGACAGACTCCACCACGTCCACCCTTAGGTCCTCCAGGCC CACCTGGTCCACCAGTCCTCCACCTCCTGGTCAGTTCT GCCTCCTCCTAGCTGGGCCTCCTAATCGAGGAGATCGC CCTCCACCACCAGTTCTTTTCCTGGACAACCTTTTGGGC AGCCTCCATTGGGTCCACTTCCTCCTGGCCCTCCACCTCC AGTTCCAGGCTACGGCCCCCCTCCACCTCCGGCCCACCTCCA CAACAGGGACCACCTCCACCTCCAGCCCCTTTCCACCTC GTCCACCCGGTCCACTTGGGCCACCCTTACACTAGCTCC TCCTCCGCATCTTCCTGGACACCTCTTTTCCTCCACCAA CCAGCTCCGCATGCTACATCAGATAGCCGAGGTCCACCAA CTAACAGTGGCATGCTACATCAGATAGCCGAGGTCCACC ACCAACAGATCCATATGGGCGACCTCCACCATATGATAGG GGTGACTATGGCCCCCCTGGA	GQTPPRRPPLGPPGPPGPPGPPPPGQV LPPPLAGPPNRGDRPPPPVLFPGQPF GQPPLGPLPPGPPPPVPGYGPPPGPP PPQQGPPPGPGFPPRRPPGPLGPPLT LAPPPHLPGPPPGAPPPAPHVNPAFF PPPTNSGMPTSDSRGPPPTDPYGRPP PYDRGDYGPPG	N-myristoylation site Cell attachment sequence Proline-rich region profile
DDX23 91.1.1 NM_004818 Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 23	GTGCAGGCCTTGGCTCCATCTTCACTTCTCTCCCACACAA AAATGAGGTTTAGTTATTTGGCCATTGTGATCTCTGCATG TGAGAATTGGGCCTCAAAGCTCATGTGCTTTGTGTTCTGT CCCTGGGTAG	VQALAPSSLLSHTKMRFSYLAIVISA CENWASKLMCFVFCPWV	Casein kinase II phosphorylation site
EIF2B1 14.1.1 NM_001414 Homo sapiens eukaryotic translation initiation factor 2B, subunit 1 alpha, 26kD	ATTGGAACCAACCAGATGGCTGTGTGTCCAAAGCACAGA ACAAACCTTTCTATGTGGTTGCAGAAAGTTTCAAGTTTGT CCGGCTCTTTCCACTAAACCAGCAAGACGTCCCAGATAAG TTTAAG	IGTNQMAVCAKAQNKPFYVVAESFKF VRLFPLNQQDVPDKFK	Protein kinase C phosphorylation site
Fe65 909.039.002 NM_001164 Homo sapiens amyloid beta (A4) precursor protein-binding, family B, member 1 (APBB1)	TGTTTCGCCGTGCGCTCCCTAGGCTGGGTAGAGATGACCG AGGAGGAGCTGGCCCCTGGACGCAGCAGTGTGGCAGTCAA CAATTGCATCCGTCAGCTCTCTTACCACAAAAACAAACCTG CATGACCCCATGTCTGGGGCTGGGGGAA	CFAVRSLGWVEMTEEELAPGRSSVAV NNCIRQLSYHKNNLHDPMSGGWGE	Casein kinase II phosphorylation site
FUS 93.1.1 NM_004960 Homo sapiens fusion (involved in t(12;16) in malignant liposarcoma)	ACAAACAAGAAAACGGACAGCCCATGATTAATTTGTACA CAGACAGGAGAAACTGCAAGCTGAAGGAGGAGCAACGT CTCTTTGATGACCCACCTTCAGCTAAAGCAGCTATTGAC TGGTTTGAT	TNKKTGQPMINLYTDRETGKLKGEAT VSFDDPPSAKAAIDWFD	Exon skipping leads to frameshift Protein kinase C phosphorylation site Casein kinase II phosphorylation site
FYN 907.002.002 NM_002037 Homo sapiens FYN oncogene related to SRC, FGR, YES	AAAGCTGATGGTTTGTGTTTAACTTAACTGTGATTGCAT CGAGTTGTACCCCACAAACTTCTGGATTGGCTAAAGATGC TTGGGAAGTTGCACGTCGTTCGTTGTGTCTGGAGAAGAAG CTGGGTCAGGGTGTTTCGCTGAAGTGTGGCTT	KADGLCFNLTVIASSCTPQTSGLAKD AWEVARRSLCLEKKLGQGCFAEVWL	N-myristoylation site N-glycosylation site
hnRNP M 114.1.1 NM_005968 Homo sapiens heterogeneous nuclear ribonucleoprotein M	CATGGCCTTGGTGGTATTGGCATGGGGTTAGGACCAGGAG GGCAACCCATTGATGCCAATCACCTGAATAAAGGCATCGG AATGGGAAACATAGGTCCCGCA	HGLGGIGMGLGPGGQPIDANHLNKGI GMGNIGPA	N-myristoylation site

Table 4-3: Genes and their exon sequences chosen for validation of the microarray data. Continued next page.

Gene	Exon sequence	Protein	Potential function
MTMR11 137.1.1 NM_006697 Homo sapiens myotubularin related protein 11	GGTCTTTGTTTCTCTTCACTTCATGTTGCTTTCTCAATC CTCCTACCCCAAACCTTCCCCCTGTGCCCTCCACTTCT CCTTGCCGTATTCTCTTCTTCATTCTCTTCTGCCCTAGTT TCTTGGACCCCTCTTCTTTGTCCCTCTTCCTCTTTATCA CCCAGAGCGCGGTGATCTGTGATCTCAATGGCCTCCTCT TCACTCGTCCAGCTGCTTTCAGCCCCCGAAGCCCGAACAC TGTTTGGCTTCCAATCACTAGTACAGCGAGAGTGGGTGGC AGCTGGACATCCCTTCCTGACTCGGCTTGGGGGAACTGGG GCCAGTGAAGAG	GLCFLFTSMCFLNPPTPNLSPLCLSL LLAVFLFSFSSALVSWTPLLCPFFLF ITQSAVIVISMASSLHSSSCFQPPKP EHCLASNH*	Changes last exon Leucine zipper pattern
NOL5A 127.3.1 NM_006392 Homo sapiens nucleolar protein 5A (56kDa with KKE/D repeat)	GACCTGAATGCAGTTGTTCCCATGGCTGGGCCAGGCTTTG CAGTGATGACTTGGCGAATCAAATCGTCAATCCCCTGAGT GCAATCACTGATGTCTCCATGTCTCTGAGCAATGCCTGAT GGGGAGGGATCTAG	DLNAVVPMAGPGFAVMTCESNLSIP*	N-glycosylation site Stop codon; NMD
PPIE 117.8.1 NM_006112 Homo sapiens peptidylprolyl isomerase E (cyclophilin E)	GGAGAGCCCATTGCTAAAAAGCCCGCTCAAATCCTCAGG TGTACATGGACATCAAGATTGGGAACAAGCCGGCTGGCCG CATCCAGATGCTCCTGCGTTCTGATGTCGTGCCCATGACA GCAG	GEPIAKKARSNPQVYMDIKIGNKPAG RIQMLLRSDVVPMTA	Exon skipping leads to frameshift N-myristoylation site
PPIL3 236.10.1 NM_032472 Homo sapiens peptidylprolyl isomerase (cyclophilin)-like 3	ATGGAGTCTCGCTGTGTCCCCCAGGCTGGAGTACAATGGC GCGATCTCGGCTCACTGCAACCTCCGCCTCCTGGGTTCAA GCAAGTCTTCTGCCCTCAGCCTCCCGA	MESRCVPQAGVQWRDLGSLQPPPPGF KQVFCLSLP	Unknown
SFRS4(Srp75) 104.9.3 NM_005626 Homo sapiens splicing factor, arginine/serine-rich 4	ACATCCCTAATCCTGACCAGCCATGAGAGGTTCGGTTCT TACAAGGAAAATGAGTGAATGTGGGAATGGCTCACCACAT TGTGAAAACAAAACTCACATCCTGCTGATGGTTGGCTGAG GACTGTCATGTCCACATACAAAGAAAAGCATGTGGCACAG TTTAGGACAAAG	TSLILTSHGEVRFLQGK*	Casein kinase II phosphorylation site Stop codon, NMD
SFRS5 144.1.1 NM_006925 Homo sapiens splicing factor, arginine/serine-rich 5	GTAGGTGAGTGGCTCACTTTGAGGGCAAGCCTTCTCGGAT CGAGGCTTCTTCATGGCCGCTCAGATCGTGAGCGGCCGGG GCTGCTCTCTTTGCGGAGGATGGCGTCTAATGAGCGCAGT TGATTCGAG	VGEWLTLRASLLGSRLLHGRSDRERP GLLSLRRMASNERS*	Protein kinase C phosphorylation site Casein kinase II phosphorylation site Stop codon, NMD
SFRS14 179.1.1 NM_014884 Homo sapiens splicing factor, arginine/serine-rich 14	GGAACCCCTCCGAAGGGAAGGGAGAGGGTTGGGTGCTGACGGGC AGGAGCACAAAGAAGCACACATTCGATGTGTTCCGACAGAG GATGATGCAGATGTACAGACACAAGCCGGGCCAACAAATAG	GTPSEGEGLGADGQEHKEDTFDVFRQ RMMQMYRHKRANK	Casein kinase II phosphorylation site
SRRM1 110.3.1 NM_005839 Homo sapiens serine/arginine repetitive matrix 1	ATGCAGATGGGAAAGCGATGGCAATCGCCAGTGACTTGGT AG	MQMGKRWQSPVTW*	Amidation site Stop codon, NMD
WT1 222.2.1 NM_024426 Homo sapiens Wilms' tumour 1	GTTGCTGCTGGGAGCTCCAGCTCAGTGAAATGGACAGAAG GGCAGAGCAAC	VAAGSSSSVKWTEGQSN	N-myristoylation site Protein kinase C phosphorylation site

Table 4-3 (cont.): Genes and their exon sequences chosen for validation of the microarray data.

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4.3   Properties of hnRNP G and its role in splice site selection

The transcripts of most metazoan protein coding genes are alternatively spliced. The detailed mechanisms involved in the control of splice site selection are still not well understood. Phosphorylation of splicing regulatory proteins is one of the general regulatory mechanisms to change alternative splice site usage. Several heterogeneous nuclear ribonucleoproteins (hnRNPs) are known to be involved in splice site selection.

The heterogeneous nuclear ribonucleoprotein G (hnRNP G) was shown to bind to Tra2-beta1 in yeast two hybrid screens. It was also identified as binding partner of several other known splicing factors and has several tyrosine residues which can possibly be phosphorylated. Therefore, it is a potential substrate for tyrosine kinases. Studies presented in this chapter examine the role of unphosphorylated and tyrosine phosphorylated hnRNP G related to splice site selection and try to discover its possible target genes.

4.3.1   hnRNP G protein structure

The sequence and domain structure of hnRNP G is shown in Figure 4-13. Similar to other hnRNPs, hnRNP G has an RNA recognition motif (RRM) containing two RNP domains. In addition, it also contains RGG motifs, which were also shown to have RNA-binding activity (Kiledjian and Dreyfuss, 1992). Furthermore it contains a proline rich region, which can putatively bind to SH3 (Src homology) domains and a tyrosine rich region, which provides possible targets for tyrosine kinase phosphorylation. Additionally, a serine arginine (SR) rich region was found similar to many proteins involved in splice site selection, as well as arginine/glycine rich residues, which could be methylated by arginine methyl transferases (Cote, Boisvert *et al.,* 2003) and so regulate protein:protein interaction (Bedford, Frankel *et al.,* 2000).
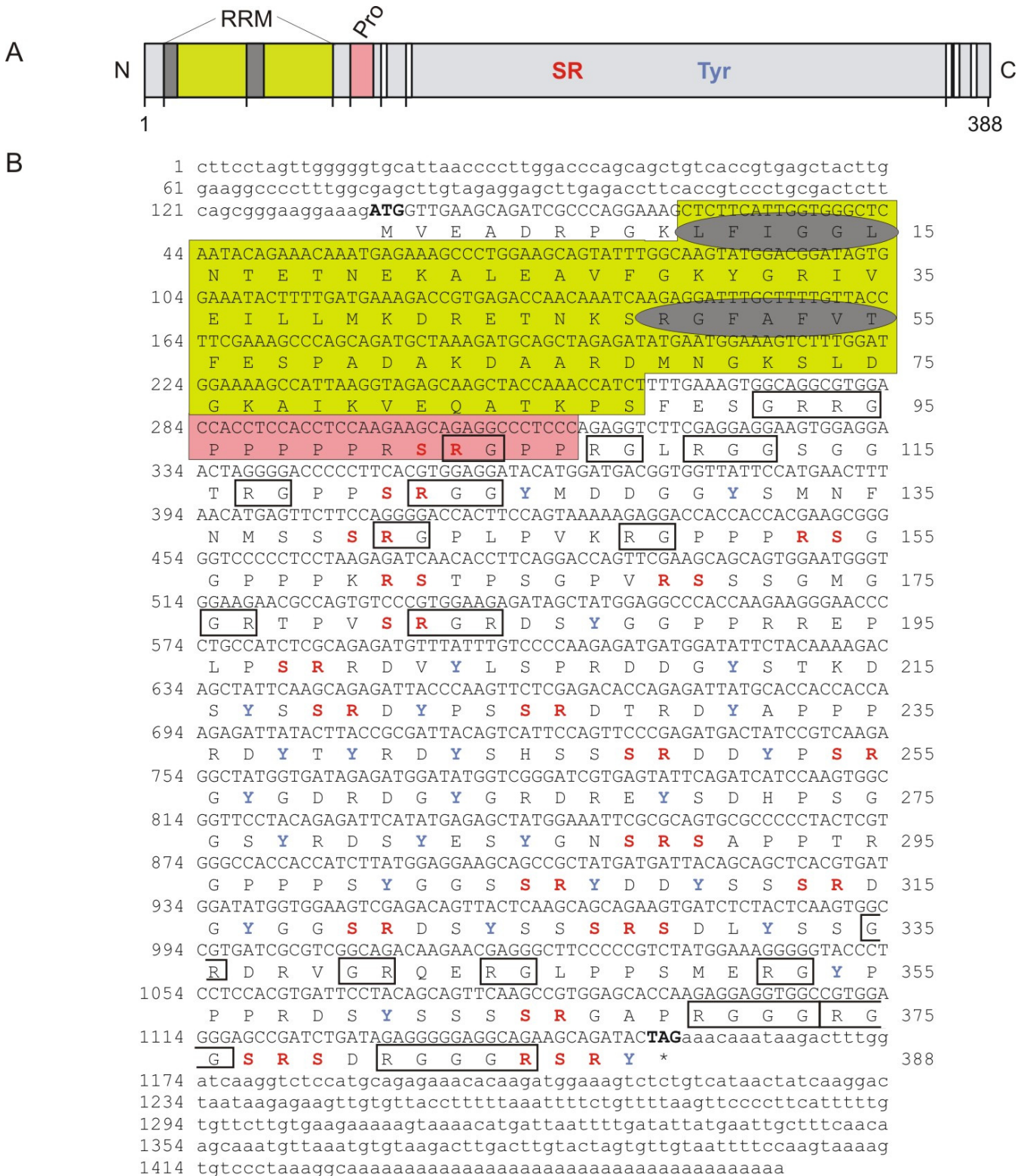
Figure 4-13: Sequence analysis of the hnRNP G protein. A) Domain structure of hnRNP G. The RRM is indicated in green with the two RNP boxes in dark grey. Pro: proline rich region, marked in pink. B) cDNA and protein sequence of human hnRNP G. Start and stop codons are shown in bold. The protein sequence is shown underneath the cDNA sequence in one letter code. The RRM domain is shown as green box with the two RNP boxes as grey ovals. The proline rich region is shown in a pink box. SR residues are indicated in red, tyrosine residues in blue. The RGG and RG motifs are boxed.

4.3.2 Phosphorylation of hnRNP G by several kinases

Tyrosine kinases play an important role in regulating protein:protein interaction involved in splice site selection by phosphorylation of proteins. As hnRNP G was found to interact with several splicing factors, we investigated whether it can as well be phosphorylated by tyrosine kinases. Since hnRNP G is detected in the nucleus, we were testing if non-receptor tyrosine kinases whose expression is often not only restricted to the cell membrane (Pendergast, 1996) can cause its phosphorylation. Humans possess 90 unique tyrosine kinases. 32 of them are non-receptor type kinases, which can be subdivided into 10 subfamilies (Robinson, Wu *et al.,* 2000). In unstimulated cells, the activity of the endogenous kinases can only be detected using phosphorylation specific antibodies against specific target sites. To test the result of kinase activation, we increased their activity by transfecting expression clones of certain kinases. Members of different subfamilies of non-receptor tyrosine kinases were cotransfected with EGFP-hnRNP G. After immunoprecipitation with anti-GFP, tyrosine phosphorylation of hnRNP G was detected by Western Blot using the phosphotyrosine specific antibody PY20. As shown in Figure 4-14, hnRNP G is already in a phosphorylated state in the cell, as a weak band could be detected when no kinase was cotransfected (Figure 4-14, upper panel, last lane "w/o"). It can also be seen that most of the kinases increase the phosphorylation of hnRNP G, especially c-Abl (member of the ABL family), Rlk (TEC family) Syk (SYK family), Fyn and c-Src (both SRC family), while CSK (CSK family), Ferh (FES family) and Sik (FRK family) only show a slight increase compared to the control.
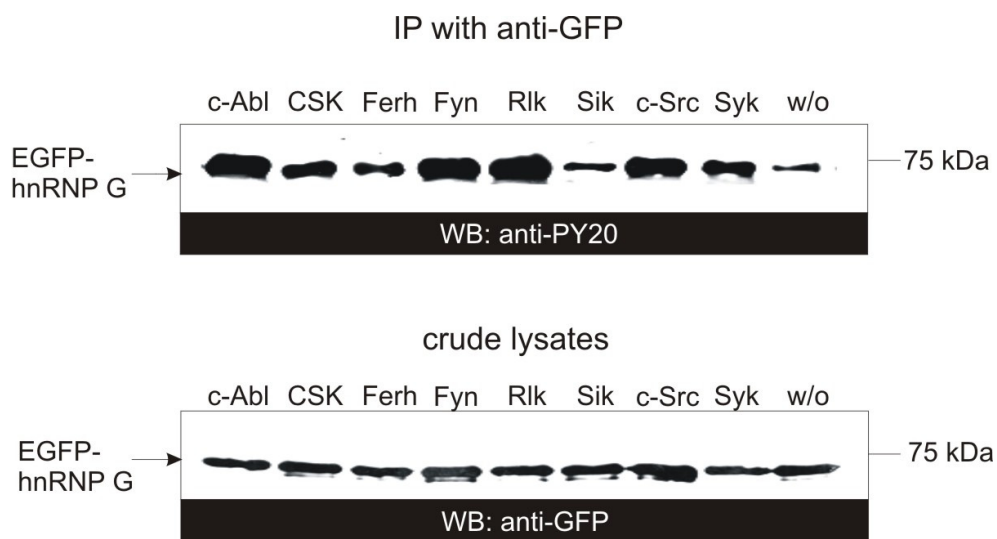
Figure 4-14: Several non-receptor tyrosine kinases phosphorylate hnRNP G. EGFP-hnRNP G was co-expressed in HEK293 cells with the indicated tyrosine kinases. Protein was precipitated with anti-GFP and tyrosine phosphorylation detected with the phosphotyrosine specific antibody anti-PY20 (upper panel). Crude lysates of cells were analysed for EGFP-hnRNP G expression with anti-GFP antibody. IP: Immunoprecipitation.

Western Blot analysis of crude lysates used for immunoprecipitation shows the presence of EGFP-hnRNP G in all experiments (Figure 4-14, lower panel).

These data show that hnRNP G can be phosphorylated by endogenous kinases and the phosphorylation can be increased by specific non-receptor tyrosine kinases.

4.3.3 Phosphorylation on specific tyrosine residue

Next, we wished to determine on which site hnRNP G is phosphorylated. For these experiments, EGFP-hnRNP G was cotransfected together with c-Abl kinase in HEK293 cells, immunoprecipitated with anti-GFP and analysed by mass spectrometry. A phosphoanalysis using precursor ion scanning could clearly identify three different peptides, which were phosphorylated on single residues (Figure 4-15). In these three peptides two tyrosine residues were found, from which the second one is the more probable one from according to the spectrum. This tyrosine is located at position 211 in the protein.
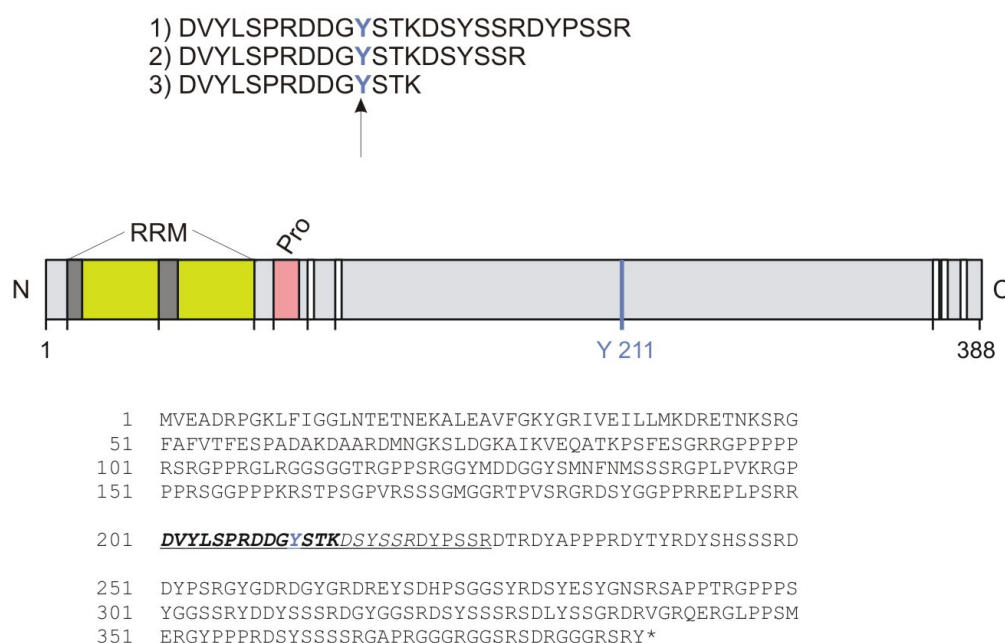
Figure 4-15: Peptides found for hnRNP G by mass spectrometry with phosphorylation site. The arrow indicates the second tyrosine residue in these peptides, which is the most probable phosphorylation site. Underneath, this tyrosine residue is shown at its position 211 in the domain structure and the protein sequence.

4.3.4 Generation of a phosphorylation deficient hnRNP G mutant and specific antisera for hnRNP G

To develop tools for further investigations, sera against the phosphorylation site containing peptide and a phosphorylation deficient Y-F (tyrosine to phenylalanine) mutant were generated.

4.3.4.1 Phosphorylation deficient hnRNP G mutant

The Y-F mutant was cloned by exchanging the tyrosine residue at position 211 to a phenylalanine residue following the Kunkel method for site-directed mutagenesis (section 3.2.5.5). Next, it was tested by immunoprecipitation followed by Western Blot analysis whether the Y211F mutant can still be detected with anti-PY20 antibody. As shown in Figure 4-16 A, no immunoreaction with PY20 could be detected after the mutant was transfected into HEK293 cells. However, the wild type hnRNP G could be detected. The reblot with anti-GFP showed that both proteins were expressed and precipitated in equal amounts. These data show that phosphorylation of hnRNP G by endogenous kinases happens on the same tyrosine residue 211 as phosphorylation by recombinangt kinases.

Furthermore, it was tested whether the phosphorylation deficient mutant changes the localisation in the cell. EGFP-tagged hnRNP G and hnRNP G-Y211F were transfected into cos7 cells, plated on cover slips and fluorescence was visualised by microscopy. In Figure 4-16 B, it is shown that wild type and mutant hnRNP G both localise in the nucleus and the localisation of hnRNP G in the cell does not depend on its phosphorylation state.
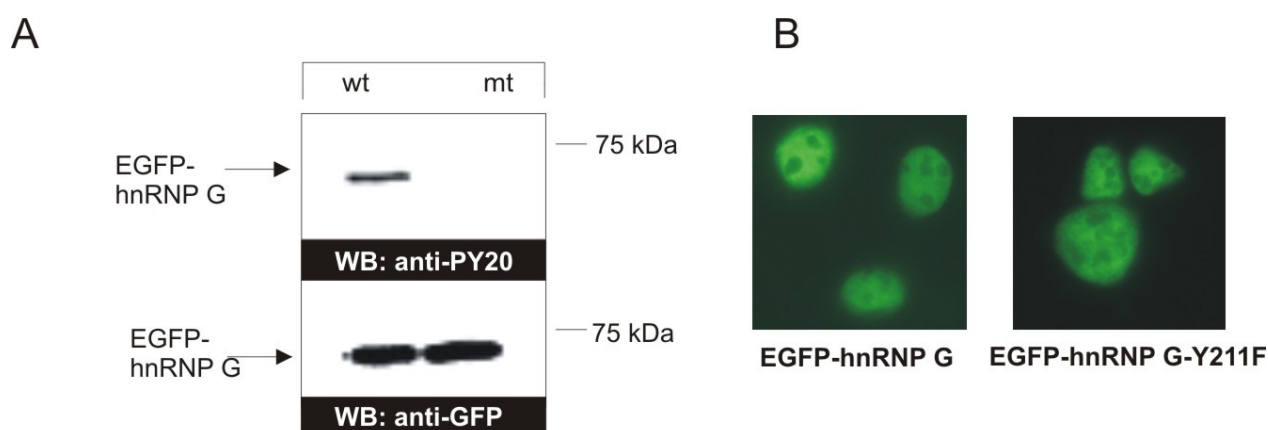
Figure 4-16: The hnRNP G mutant Y211F is not phosphorylated by endogenous kinases and located in the nucleus. A) EGFP-hnRNP G (wt) and EGFP-hnRNP G-Y211F (mt) were transfected into HEK293 cells. Protein was precipitated with anti-GFP and tyrosine phosphorylation detected with anti-PY20 (upper panel). The lower panel shows the reblot of the precipitates with anti-GFP to compare the expression of both proteins. B) EGFP-hnRNP G and EGFP-hnRNP G-Y211F localise both in the nucleus.

4.3.4.2 Specific antisera against hnRNP G

To further analyse phosphorylation, rabbit polyclonal antisera were raised against peptide RDDGYSTKD (anti-hnRNP G) and phosphopeptide RDDGY^{Phos}STKD (anti-phospho-hnRNP G).

To test the obtained serum, EGFP-hnRNP G and EGFP-hnRNP G-Y211F were cotransfected with c-Abl kinase into HEK293 cells. After immunoprecipitation with

anti-GFP, tyrosine phosphorylation of hnRNP G was detected by Western Blot using anti-phospho-hnRNP G. As shown in Figure 4-17 (upper left panel), anti-phospho-hnRNP G can detect phosphorylated wild type hnRNP G, but not the phosphorylation deficient Y211F mutant. However, when the mutant was co-transfected with c-Abl, a very weak band could be detected by the antiserum. To test whether the non-phospho-specific hnRNP G antiserum recognises wild type hnRNP G and its mutant, a reblot was performed with this antiserum. Figure 4-17 (lower left panel) shows that anti-hnRNP G recognises both proteins specifically. Western Blot analysis of crude cell lysates (load) used for immunoprecipitation demonstrates equal amounts of wild type and mutant hnRNP G in all experiments (Figure 4-17, upper right panel). A reblot with serum before immunisation of the rabbits shows that no protein can be detected (Figure 4-17, lower right panel).
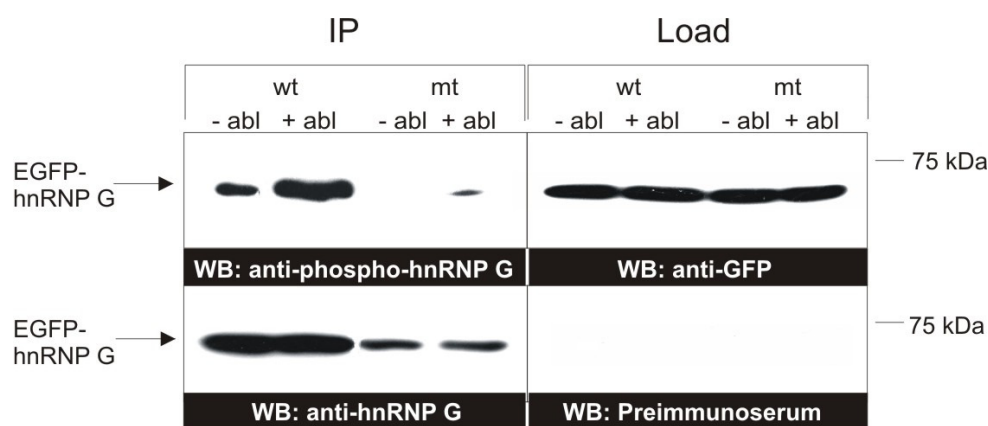
Figure 4-17: Antisera against hnRNP G specifically recognise the protein. Left: EGFP-hnRNP G and EGFP-hnRNP G-Y211F were coexpressed with c-Abl in HEK293 cells. Protein was precipitated with anti-GFP and phosphorylated protein detected with anti-phospho-hnRNP G (upper panel). A reblot showed that anti-hnRNP G recognises both wild type and mutant protein. Right: crude cell lysates were analysed for EGFP-hnRNP G and EGFP-hnRNP G-Y211F expression with anti-GFP antibody (upper panel). A reblot with preimmunoserum showed no detection of the protein (lower panel).

For further analysis, the result of kinase activation on hnRNP G (4.3.2) was repeated with additional kinases and precipitates were checked by Western Blot analysis with the phospho-specific antiserum. In addition to the kinases c-Abl, Rlk, Syk, Ferh, Sik, CSK, Fyn and c-Src, kinases Ack2 (ACK family), lyn (SRC family), RAK (FRK family), DYRK (DYRK family) and Bcr-Abl were cotransfected with EGFP-hnRNP G. Bcr-Abl is a constitutively active tyrosine kinase with transforming capacity for haematopoietic cells. It is a product of fusion between chromosome 9 and 22, called the Philadelphia translocation t(9;22), which is characteristic for chronic myeloid leukaemia (CML) (Ben-Neriah, Daley *et al.,* 1986; Daley, Van Etten *et al.,* 1990). As shown in Figure 4-18, the result obtained by Western Blot with anti-phospho-hnRNP G is comparable to the result obtained with

anti-PY20. hnRNP G is phosphorylated by endogenous kinases and can be further phosphorylated by several kinases of different subfamilies and by Bcr-Abl.
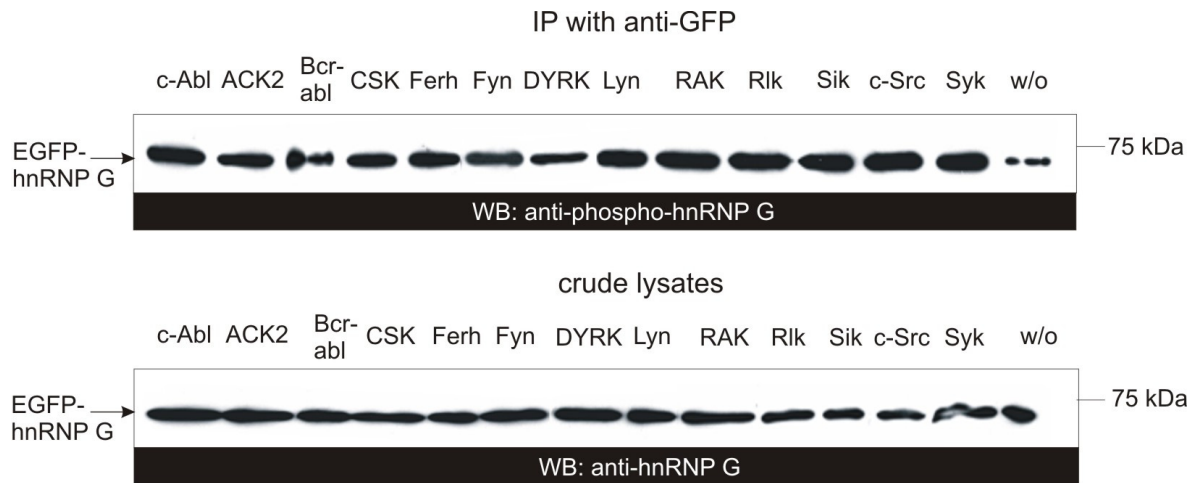
Figure 4-18: Anti-phospho-hnRNP G detects phosphorylation of hnRNP G by several non-receptor tyrosine kinases. EGFP-hnRNP G was co-expressed in HEK293 cells with the indicated tyrosine kinases. Protein was precipitated with anti-GFP and tyrosine phosphorylation detected with the phospho-specific antibody anti-phospho-hnRNP G (upper panel). Crude lysates of cells were analysed for EGFP-hnRNP G expression with anti-hnRNP G antiserum. IP: Immunoprecipitation.

4.3.5 Phosphorylation dependent shuttling of hnRNP G between nucleus and cytoplasm

hnRNP G is phosphorylated by the membrane bound kinase c-Src (Figure 4-14 and Figure 4-18). Since hnRNP G is detected only in the nucleus of cells, a phosphorylation by membrane bound kinases would only be possible during mitosis when the nuclear structure disintegrates or it would require shuttling of hnRNP G between nucleus and cytosol. Shuttling has been reported for several proteins implicated in splice site selection and hnRNPs (Pinol-Roma and Dreyfuss, 1992; Pinol-Roma and Dreyfuss, 1993; Caceres, Screaton *et al.,* 1998). To test these possibilities, we assessed the shuttling capability of hnRNP G-YFP using a previously described cell fusion assay. The experiments were performed in collaboration with Dr. Ruth Brack-Werner from GSF Institute of Molecular Virology in Neuherberg, Germany. The assay monitors accumulation of a fluorescent protein in acceptor nuclei of a newly formed polykaryon (Lee, Neumann *et al.,* 1999; Neumann, Afonina *et al.,* 2001). Transfected HeLa cells expressing hnRNP G-YFP were fused with an excess of untransfected HeLa cells and accumulation of hnRNP G-YFP in acceptor nuclei monitored by time-lapse imaging. As shown in Figure 4-19, hnRNP G-YFP fluorescence is visible in the acceptor nuclei 5 hours after cell fusion.
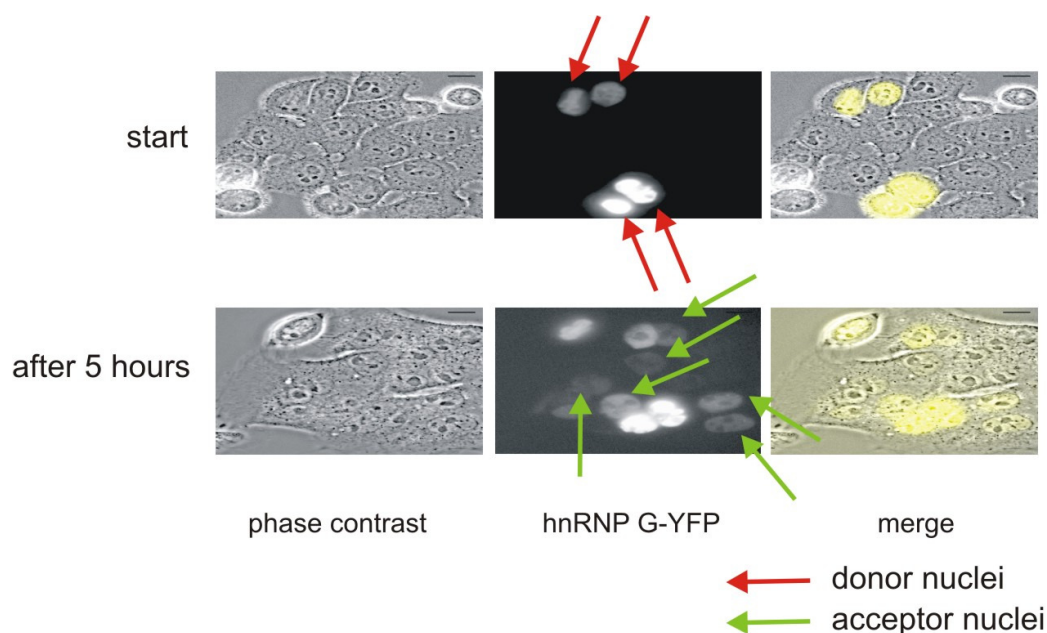
Figure 4-19: hnRNP G shuttles between nucleus and cytosol. HeLa cells transfected with hnRNP G-YFP were fused to an access of untransfected cells. Fluorescence after 5 hours is shown in the lower panel. Red arrows indicate donor nuclei, green arrows indicate acceptor nuclei showing accumulation of hnRNP G-YFP.

These results indicate that hnRNP G shuttles between nucleus and cytosol, where its interaction with membrane bound SRC-family kinases is possible.

Next, we determined whether there is a difference in shuttling behaviour between wild type and mutant hnRNP G. HeLa cells were transfected with hnRNP G-YFP or hnRNP G-Y211F-YFP together with CFP-plasmid and fused with non-transfected HeLa cells. Cell fusion was induced and YFP- and CFP-fluorescence monitored for 250-300 min. New protein synthesis was inhibited by addition of cycloheximide (at a final concentration of 100 µg/ml). Successful fusion is indicated by distribution of CFP from donor to acceptor cells. As shown in Figure 4-20, a strong decrease of wild type hnRNP G fluorescence was visible in donor nuclei, but only a very weak decrease in hnRNP G-Y211F fluorescence.

This effect was independently observed in experiments with or without cycloheximide. With cycloheximide, almost no increase in Y211F fluorescence is observed in acceptor nuclei, but still an increase in wild type hnRNP G fluorescence is detected.
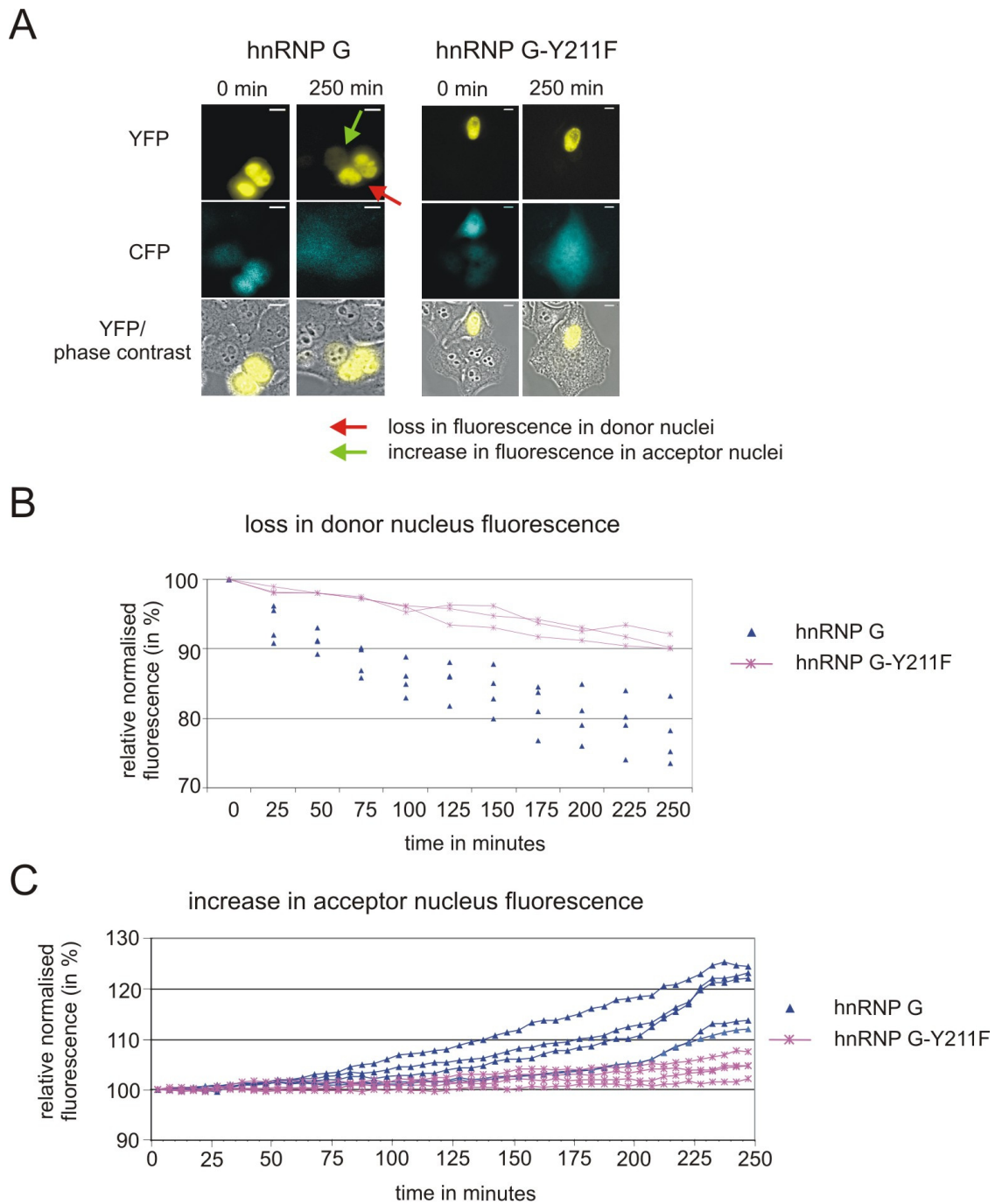
Figure 4-20: Mutant hnRNP G-Y211F does not shuttle any more between nucleus and cytoplasm. A) Cell pictures showing fluorescence at the start point and after 250 minutes. Red arrows indicate a loss of fluorescence in donor nuclei, green arrows indicate an increase in fluorescence in acceptor nuclei. B) and C) Quantitative analysis of fluorescence. Graphs show the loss in donor nucleus fluorescence (B) and the increase in acceptor nucleus fluorescence in wild type hnRNP G (blue triangle) and mutant hnRNP G-Y211F (pink star).

These results show that the hnRNP G-Y211F mutant shuttles significantly less than the wild type hnRNP G, indicating that tyrosine phosphorylation is important for the movement of the protein in the cell.

4.3.6 Interaction of hnRNP G with other proteins

4.3.6.1 hnRNP G binds to c-Src kinase

hnRNP G is phosphorylated by c-Src (Figure 4-14 and Figure 4-18). As this kinase is a membrane bound kinase and hnRNP G was found to shuttle, a direct molecular interaction between hnRNP G and c-Src was investigated. It was tested whether hnRNP G would co-immunoprecipitate with c-Src in an overexpression experiment. EGFP-hnRNP G was co-transfected with c-Src. After immunoprecipitation with anti-GFP, phosphorylated hnRNP G was detected by Western Blot using anti-phospho-hnRNP G. Then, a reblot was done with anti-src antibody to check for the protein. As shown in Figure 4-21, c-Src was detected in the precipitate.
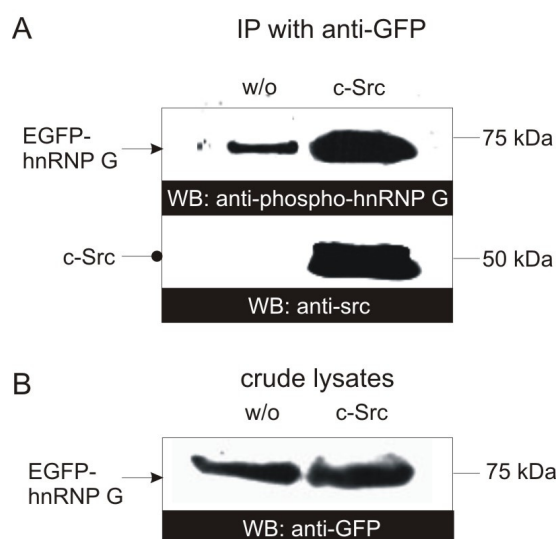
Figure 4-21: hnRNP G interacts with c-Src kinase. A) EGFP-hnRNP G was co-expressed with or without a c-Src expression construct in HEK293 cells. Proteins were immunoprecipitated with anti-GFP. Immunoprecipitates were analysed with antisera against phosphorylated hnRNP G (upper panel) and c-Src (lower panel). B) Crude lysates of cells were analysed for EGFP-hnRNP G expression with anti-GFP antibody. A pointed arrow indicates EGFP-hnRNP G, a round arrow c-Src. IP: Immunoprecipitation.

4.3.6.2 Phosphorylation dependent interaction of hnRNP G with proteins involved in splicing

Next, we investigated whether phosphorylation of hnRNP G is necessary for interaction with other splicing factors. EGFP-hnRNP G and EGFP-hnRNP G-Y211F were transfected into HEK293 cells and immunoprecipitation performed with anti-GFP. The precipitates were analysed by Western Blot with antibodies against specific splicing factors, which detected the endogenous protein. As shown in Figure 4-22, interaction occurs with endogenous Tra2-beta2, Sam68-like mammalian protein 1 (SLM-1), Splicing attachment factor B (Saf-B), YT521-B and with hnRNP G itself. The phosphorylation deficient mutant

EGFP-hnRNP G-Y211F showed the same interaction as the hnRNP G wild type. This indicates that the interaction is not dependent on tyrosine phosphorylation of hnRNP G.
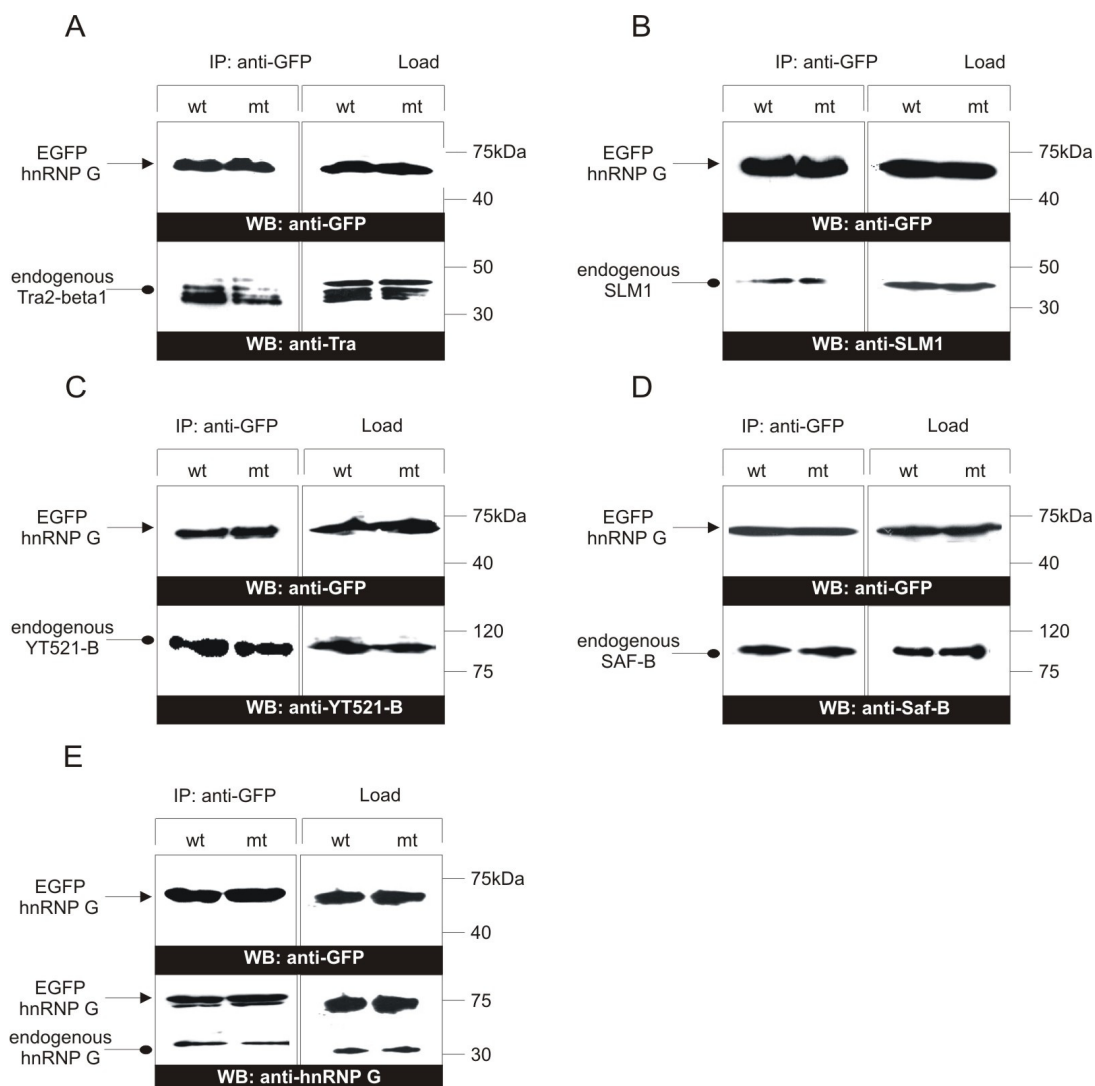
Figure 4-22: Interaction of hnRNP G with several endogenous splicing factors. EGFP-hnRNP G (wt) or EGFP-hnRNP G-Y211F (mt) were expressed in HEK293 cells and precipitated with anti-GFP antibody. Co-immunoprecipitated (IP) endogenous Tra2-beta1 (A), SLM-1 (B), YT521-B (C), Saf-B (D) and hnRNP G (E) in the precipitates and the lysates (load) were detected by Western Blot using their specific antibodies (lower panels). The EGFP-fused wild type and mutant hnRNP G were detected by anti-GFP in the co-immunoprecipitates and the lysates (upper panels).

4.3.7 Dephosphorylation of hnRNP G

Protein phosphorylation is a dynamic process, depending on the antagonistic interplay between protein kinases and phosphatases. Often, it is important that effects of an activated protein are temporary, so cells must be able to dephosphorylate proteins that have been phosphorylated by a kinase. Tyrosine phosphorylation is removed by protein tyrosine phosphatases (PTPs). PTPs can be divided into two major categories: the tyrosine-specific or classical PTPs and the dual specificity phosphatases (DSPs), which can dephosphorylate tyrosine, serine and threonine residues. The human genome has a total of 107 genes encoding

PTPs, of which 38 genes encode classical PTPs (Alonso, Sasin *et al.,* 2004; Andersen, Jansen *et al.,* 2004). Members of the PTPs can be divided structurally into non-transmembrane or transmembrane receptor-like molecules, which can additionally be divided by the amino acid sequence homology of their conserved PTP domains into 17 main subtypes (Andersen, Jansen *et al.,* 2004).

hnRNP G is phosphorylated by several kinases. In resting cells, phosphorylated hnRNP G could be detected without transfection of a kinase. Therefore, it was examined whether hnRNP G could be dephosphorylated by specific protein tyrosine phosphatases.

EGFP-hnRNP G was cotransfected together with expression constructs of PTPs. After immunoprecipitation with anti-GFP, tyrosine phosphorylation of hnRNP G was detected by Western Blot using the phosphotyrosine specific antibody PY20 or the phospho-specific hnRNP G antiserum. As shown in Figure 4-23 (A), the phosphatases PTPIA-2beta (R8 subtype) and MEG1 (NT5 subtype) could slightly dephosphorylate hnRNP G, especially when the phosphorylation was increased by cotransfecting c-Abl. The phosphatase PTP1B (NT1 subtype) was able to dephosphorylate hnRNP G so that phosphorylation could be no longer detected (Figure 4-23, B).
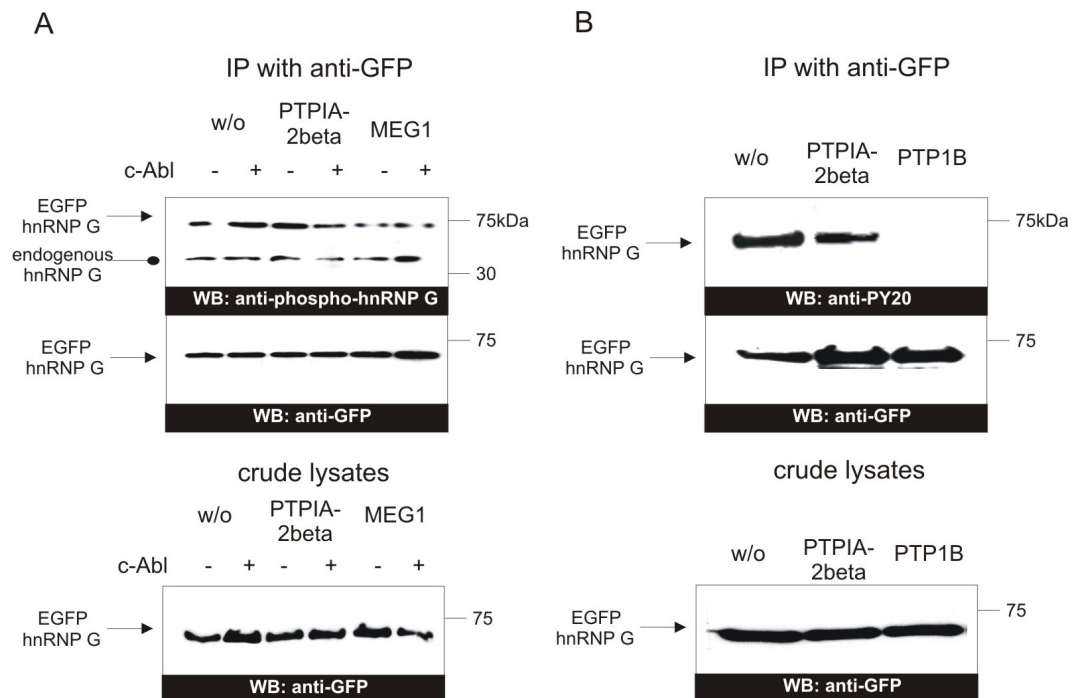
Figure 4-23: hnRNP G can be dephosphorylated by specific protein tyrosine phosphatases. A) Expression constructs of phosphatases PTPIA2beta or MEG1 were cotransfected with EGFP-hnRNP G with or without c-Abl. Phosphorylated hnRNP G was detected using anti-phospho-hnRNP G antiserum (upper panel). A reblot with anti-GFP was performed to test for equal expression in the precipitates. B) EGFP-hnRNP G was cotransfected with expression constructs of phosphatases PTPIA2beta or PTP1B. Phosphorylated hnRNP G was detected using anti-PY20 (upper panel). Reblot with anti-GFP was done to check for equal expression in the precipitates. Below (A and B): Crude lysates of cells were analysed for EGFP-hnRNP G expression with anti-GFP antibody.

The PTPs LAR (R2A subtype), STEP (R7 subtype), SHP1 (NT2 subtype), MEG2 (NT3 subtype) and PTPalpha (R4 subtype) did not have an effect on tyrosine phosphorylation (data not shown).

These data show that tyrosine phosphorylation of hnRNP G can be decreased specifically by PTP1B.

4.3.8 hnRNP G is part of the supraspliceosome

Next, we investigated in collaboration with Dr. Ruth Sperling's lab in Jerusalem whether hnRNP G is a part of the supraspliceosome The supraspliceosome is a large nuclear RNP particle (overall mass about 21MDa), in which endogenous pre-mRNAs are packaged with all five spliceosomal U snRNPs and a number of splicing factors (Müller, Wolpensinger *et al.,* 1998; Raitskin, Angenitzki *et al.,* 2002). In addition, editing enzymes and other RNA processing factors are integral components of the supraspliceosome (Raitskin, Cho *et al.,* 2001). Thus supraspliceosomes might represent the nuclear processing machinery.

Nuclear supernatants enriched with supraspliceosomes were prepared from HEK293 and HeLa cells and were fractionated on a 10-45% glycerol gradient. Supraspliceosomes sediment as 200S complexes in fractions 8-14 with a peak in fractions 10-11. The 200S peak fractions were combined and fractionated in a second glycerol gradient. Aliquots from each fraction were analysed by Western Blot using anti-hnRNP G antiserum. As shown in Figure 4-24 A, hnRNP G could be detected in the supraspliceosomal fractions. The localisation was further tested by immuno-electron microscopy using anti-hnRNP G antibodies and gold-tagged secondary IgG. As seen in Figure 4-24 B, hnRNP G coincides with supraspliceosomal complexes in native gel electrophoresis.
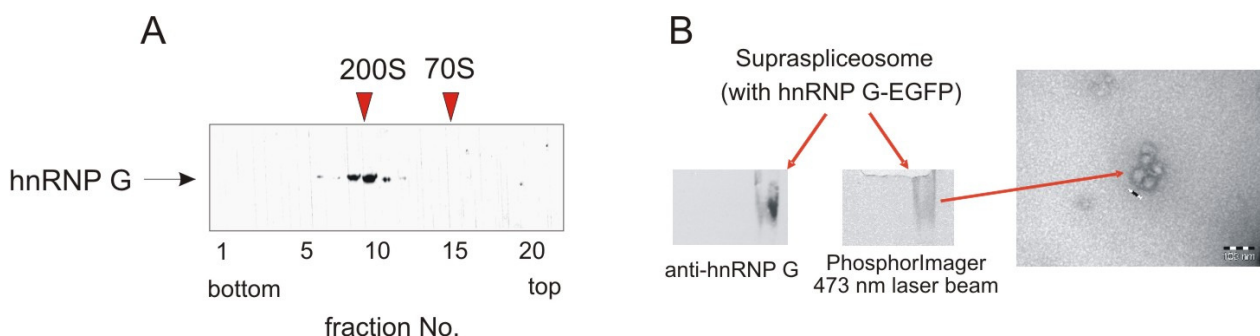
Figure 4-24: hnRNP G is a supraspliceosomal protein. A) Nuclear supernatants enriched in supraspliceosomes prepared from HeLa cells were fractionated in a 10-45% glycerol gradient and collected (bottom to top) in 20 fractions. The 200S peak fractions were combined and fractionated in a second glycerol gradient. Aliquots from each fraction were analysed by Western Blot using anti-hnRNP G. B) Localisation of hnRNP G in supraspliceosome particle by Immuno-EM. Left side: native gel. Bar indicates 100 nm.

Next, we wanted to test what role phosphorylation of hnRNP G plays in its distribution in the supraspliceosome. EGFP-hnRNP G and EGFP-hnRNP G-Y211F were transfected into HEK293 cells and fractionated on the 10-45% glycerol gradient. As shown in Figure 4-25 A, hnRNP G in HEK 293 cells has an equal localisation in the supraspliceosome as in HeLa cells. Also the distribution of EGFP-tagged hnRNP G was comparable to endogenous protein. However, the phosphorylation deficient mutant EGFP-hnRNP G-Y211F showed a broader distribution than the wild type, especially in the lower fraction numbers (Figure 4-25 B), which contain the larger sized supraspliceosome subpopulations.
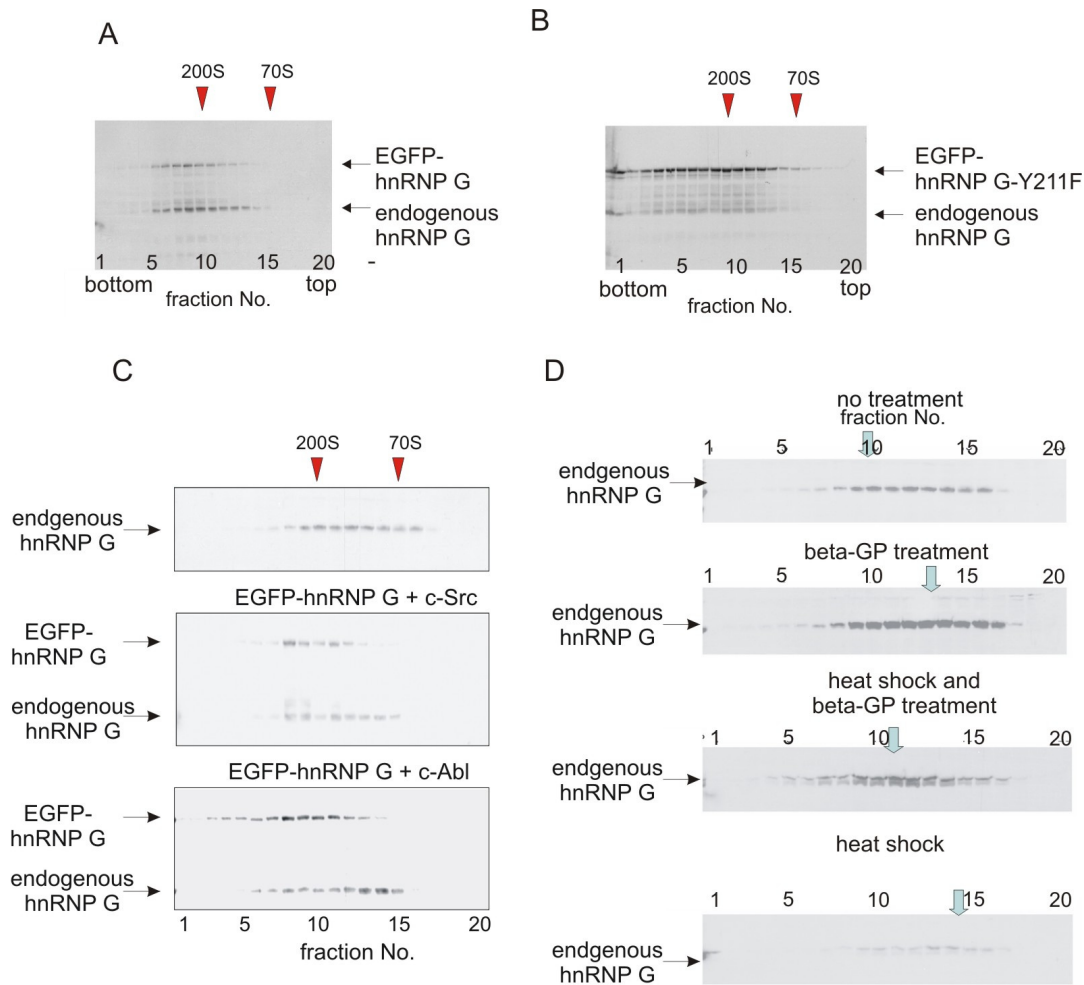
Figure 4-25: The distribution of hnRNP G in the supraspliceosome changes with phosphorylation. A)+B)+C) Nuclear supernatants from HEK293 cells transfected with EGFP-hnRNP G (A), EGFP-hnRNP G-Y211F (B), EGFP-hnRNP G and c-Src or c-Abl (C), were fractionated in a 10-45% glycerol gradient and collected (bottom to top) in 20 fractions. Aliquots from each fraction were analysed by Western Blot using anti-hnRNP G. D) Nuclear supernatants from HeLa cells treated with phosphates inhibitor (beta-GP), heat shock or both were fractionated in a 10-45% glycerol gradient. Aliquots from each fraction were analysed by Western Blot using anti-hnRNP G.

Cotransfection of EGFP-tagged hnRNP G with expression constructs of c-Abl or c-Src also resulted in hnRNP G with altered distribution in the supraspliceosome. This was more pronounced, when c-Abl kinase was used. In this case, endogenous hnRNP G shifted from

large-size to lighter supraspliceosomal subpopulations (Figure 4-25 C). Inhibiting endogenous phosphatases with a general phosphate inhibitor like beta-glycerolphosphate is expected to hyperphosphorylate hnRNP G and to have the same effect as coexpression of kinases. As can be observed in Figure 4-25 D, this also causes a shift to lighter subpopulations of the supraspliceosomal complexes. Stress situations like heat shock has the same effect on hnRNP G as inhibiting dephosphorylation, however if heat shock treatment was applied together with inhibition of phosphatases, hnRNP G is shifted back towards the nuclear pellet fraction.

Next, we wanted to test whether hnRNP G can be found together with its interacting proteins in the supraspliceosome. As an example we used Tra2-beta1. After detecting hnRNP G in the supraspliceosome, a reblot was performed with anti-tra2 antiserum. Figure 4-26 shows that hypophosphorylated Tra2-beta1 appears in different fractions in the supraspliceosome than hnRNP G, but its hyperphosphorylated form shows an overlapping distribution with hnRNP G.
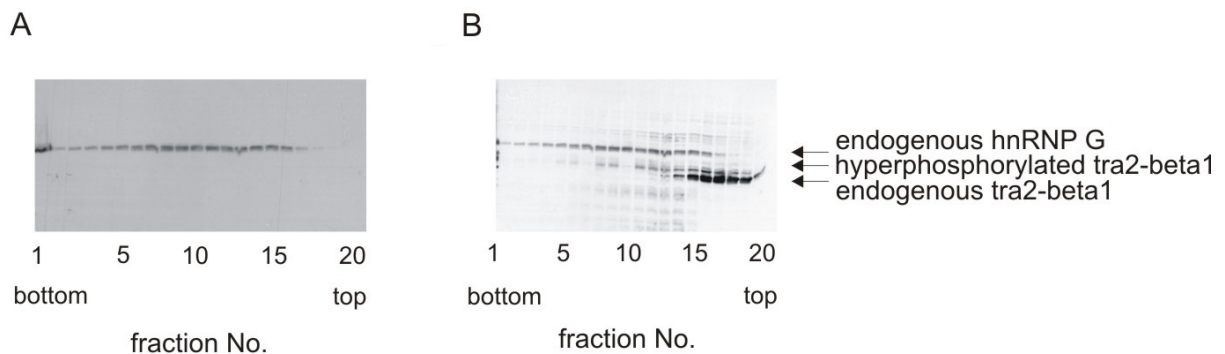
Figure 4-26: hnRNP G and Tra2-beta1 show overlapping distribution in supraspliceosomes. Nuclear supernatants of HeLa cells enriched in supraspliceosomes were fractionated in a 10-45% glycerol gradient and collected (bottom to top) in 20 fractions. Aliquots from each fraction were analysed by Western Blot using anti-phospho-hnRNP G (A) and anti-tra2 (B) antiserums.

In summary, these data show that hnRNP G is present in the supraspliceosome. Its distribution can change according to its phosphorylation status or through stress situations like heat shock and it has partial overlapping distribution with Tra2-beta1, one of its binding partners.

4.3.9 Changes of splice site selection on SMN2 minigene

hnRNP G binds to several proteins involved in splice site selection like Tra2-beta1, YT521-B, SLM-1, Saf-B and to itself. All these proteins contain RNA binding domains. Tra2-beta1, Saf-B and hnRNP G have RRMs (Soulard, Valle *et al.,* 1993; Nayler, Cap *et al.,* 1998; Weighardt, Cobianchi *et al.,* 1999), YT521-B contains the putative acid binding domain

YTH (Zhang, Rafalska *et al.,* submitted; Stoilov, Rafalska *et al.,* 2002) and SLM-1 contains a KH (hnRNP K homology) domain (Di Fruscio, Chen *et al.,* 1999). It was previously shown that hnRNP G could promote inclusion of SMN2 exon 7 (Hofmann and Wirth, 2002). Therefore, we compared hnRNP G proteins in *in vivo* splicing assays on SMN2 minigene and checked whether they show changes in the splicing pattern of SMN2 minigene. The SMN2 minigene consists of alternative exon 7 flanked by constitutive exons. Exon 7 contains a purine-rich exonic enhancer (Lorson, Hahnen *et al.,* 1999; Stoss, Olbrich *et al.,* 2001). Increasing amounts (0-3 µg) of EGFP-hnRNP G or EGFP-hnRNP G-Y211F were cotransfected with 1 µg SMN2 reporter minigene into HEK293 cells. EGFP-C2 empty vector was cotransfected for equal amounts of DNA. After RNA isolation RT-PCR was performed using specific primers. As shown in Figure 4-27, hnRNP G can regulate splice site selection in a concentration-dependent manner. Increasing hnRNP G concentration promotes inclusion of exon 7 as shown before (Hofmann and Wirth, 2002). The phospho-deficient Y211F mutant was also able to promote exon 7 inclusion, but to a significantly less extent than the wild type.
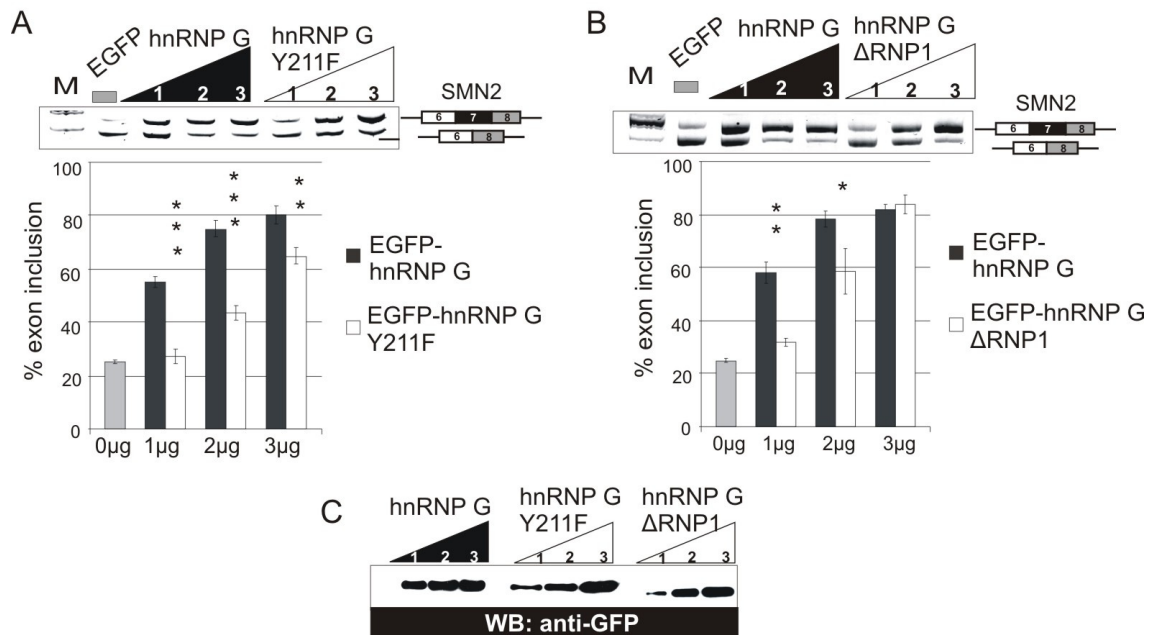
Figure 4-27: hnRNP G changes splice site selection of SMN2 minigene. An increasing amount of EGFP-hnRNP G, EGFP-hnRNP G-Y211F (A) or EGFP-hnRNP G-ΔRNP1 (B) and SMN2 minigene were cotransfected in HEK293 cells. The alternative splicing of SMN2 was determined by RT-PCR using specific primers. A representative ethidium-bromide stained gel of each experiment is shown. The statistical evaluation of four independent experiments is shown below the gels. C) Representative Western Blot showing the expression of the transfected EGFP tagged proteins. Stars indicate p-values from student's t-test comparing wt and mutant hnRNP G: A) 1 µg = 0.00003; 2 µg = 0.0003; 3 µg = 0.01. B) 1 µg = 0.008; 2 µg = 0.03.

In addition, the SMN2 minigene was cotransfected with EGFP-hnRNP G-ΔRNP1, an hnRNP G mutant where the first part of the RRM with the first RNP is deleted. Since the RRM is needed for RNA binding, we expected that the influence on splice site selection should decrease. As shown in Figure 4-27 B, lower concentrations (1 and 2 µg) of

EGFP-hnRNP G-ΔRNP1 showed a reduced exon inclusion compared to wild type hnRNP G. However, increasing the concentration to 3 µg had the same effect on exon inclusion as the wild type. This indicates that hnRNP G acts on SMN2 minigene independently of RNA binding.

Next, we tested whether additional phosphorylation of hnRNP G can change splice site selection towards further inclusion of exon 7 of SMN2 minigene. Increasing amounts of EGFP-hnRNP G were cotransfected with an expression construct of c-Abl and SMN2 minigene into HEK293 cells. As a control EGFP-hnRNP G-Y211F, which cannot be phosphorylated, was cotransfected with c-Abl. As shown in Figure 4-28, the concentration-dependent increase of exon 7 by hnRNP G is partially abolished by induced c-Abl phosphorylation, as c-Abl blocks the increase in exon inclusion promoted by hnRNP G similarly to the phosphorylation deficient mutant. c-Abl expression could not change splice site selection when transfected alone with the minigene or cotransfected with increasing amounts of the mutant.

Figure 4-28: Phosphorylation of hnRNP G changes splice site selection of SMN2 minigene. A) An increasing amount of EGFP-hnRNP G (left) or EGFP-hnRNP G-Y211F (right) was cotransfected with expression constructs of c-Abl and SMN2 minigene in HEK293 cells. The alternative splicing of SMN2 was determined by RT-PCR using specific primers. A representative ethidium bromide stained gel of each experiment is shown. The statistical evaluation of at least three independent experiments is shown below the gels. B) Representative Western Blot showing the expression of the transfected EGFP tagged proteins. Stars indicate p-values from student's t-test comparing data for hnRNP G with and without c-Abl: 1 µg = 0.02; 2 µg = 0.05.

These data show that hnRNP G influences splice site selection of SMN2 minigene. Tyrosine phosphorylation of hnRNP G can change this influence towards the exon skipped

isoform of SMN2. The deletion of the RRM still can promote exon 7 inclusion of SMN2 although RNA binding should theoretically be decreased. This indicates that it acts by sequestration of other splicing factors, for example Tra2-beta1, whose sequestration through proteins with non-functional RRM was shown previously (Stoilov, Daoud *et al.*, 2004).

4.3.10 hnRNP G acts on tau minigene (RNA-IPs)

hnRNP G contains an RRM that binds to RNA and was found as binding partner of several splicing factors. Thus we were interested whether a direct interaction between hnRNP G and RNA of tau minigene could be detected. EGFP-hnRNP G, EGFP-hnRNP G-Y211F and EGFP-hnRNP G-ΔRNP1 were immunoprecipitated in the presence of tau minigene and RNA was detected in the precipitates by RT-PCR (Buckanovich and Darnell, 1997) using tau minigene specific primers. The amplification of tau isoforms demonstrates that hnRNP G associates with this mRNA and thus can possibly regulate it directly (Figure 4-29:). Moreover, it also shows that the hnRNP G mutants Y211F and also the ΔRNP1 mutant can bind to this RNA. The protein immunoprecipitates were tested having equal amounts of each protein.
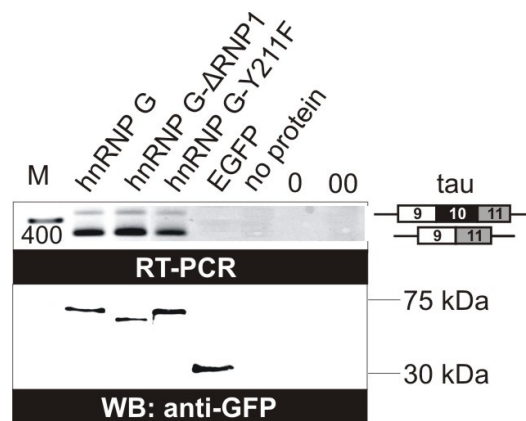
Figure 4-29: Association of hnRNP G with tau pre-mRNA *in vivo*. Tau minigene was cotransfected with EGFP-hnRNP G, EGFP-hnRNP G-Y211F or EGFP-hnRNP G-ΔRNP1. As control the minigene was cotransfected with the parental vector EGFP-C2. Protein-RNA complexes were isolated by immunoprecipitation with anti-GFP in the absence of RNases. Tau RNA was detected by RT-PCR using minigene specific primers. 0: immunoprecipitate without reverse transcriptase, 00: RT-PCR control (no template).

These data show that hnRNP G, hypophosphorylated hnRNP G (Y211F) and also the RRM deletion mutant associate with tau minigene and can possibly regulate it.

4.3.11 Phosphorylation dependent changes of splice site selection on tau minigene

Since we found that hnRNP G can change splice site selection on SMN2 minigene and that it is associated with tau pre-mRNA, we investigated if it also can change splice site

selection of the tau minigene. Increasing amounts of EGFP-tagged expression constructs of hnRNP G, hnRNP G-Y211F and hnRNP G-ΔRNP1 were cotransfected with tau minigene. After RNA isolation RT-PCR was performed using tau specific primers. Figure 4-30 shows that increasing hnRNP G is able to regulate splice site selection of tau minigene in a concentration-dependent manner. Increasing hnRNP G concentration promotes tau exon 10 skipping. In contrast, weak exon 10 skipping can be observed for higher concentrations of the hnRNP G-Y211F mutant, while for lower concentrations exon 10 inclusion is promoted compared to the wild type.
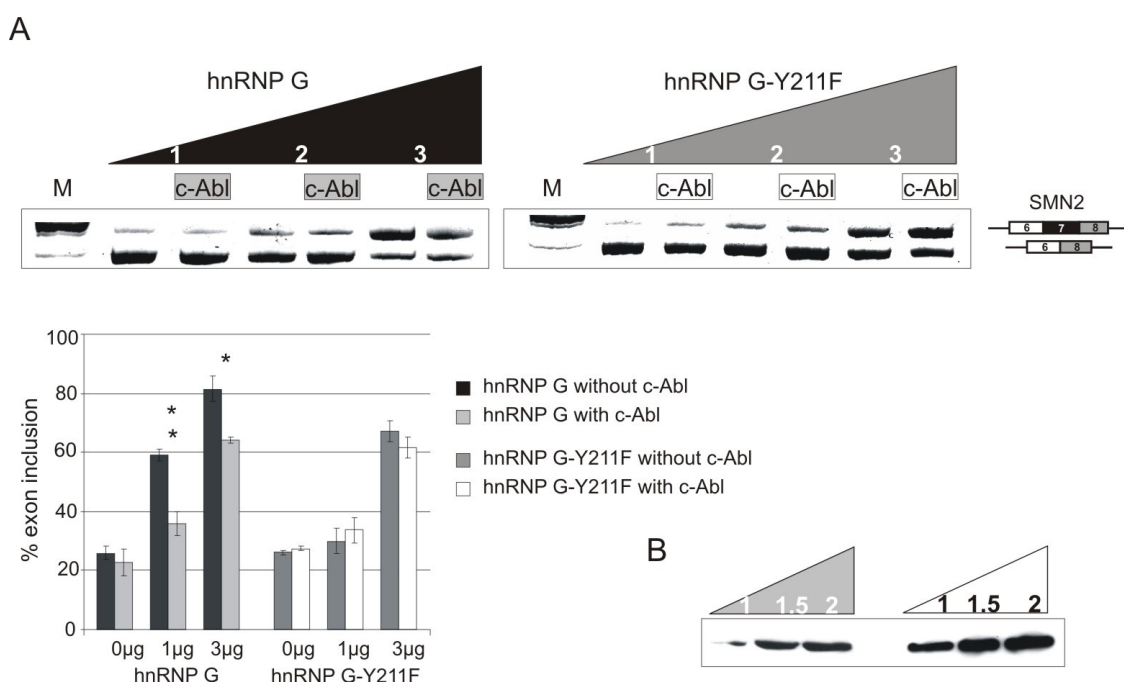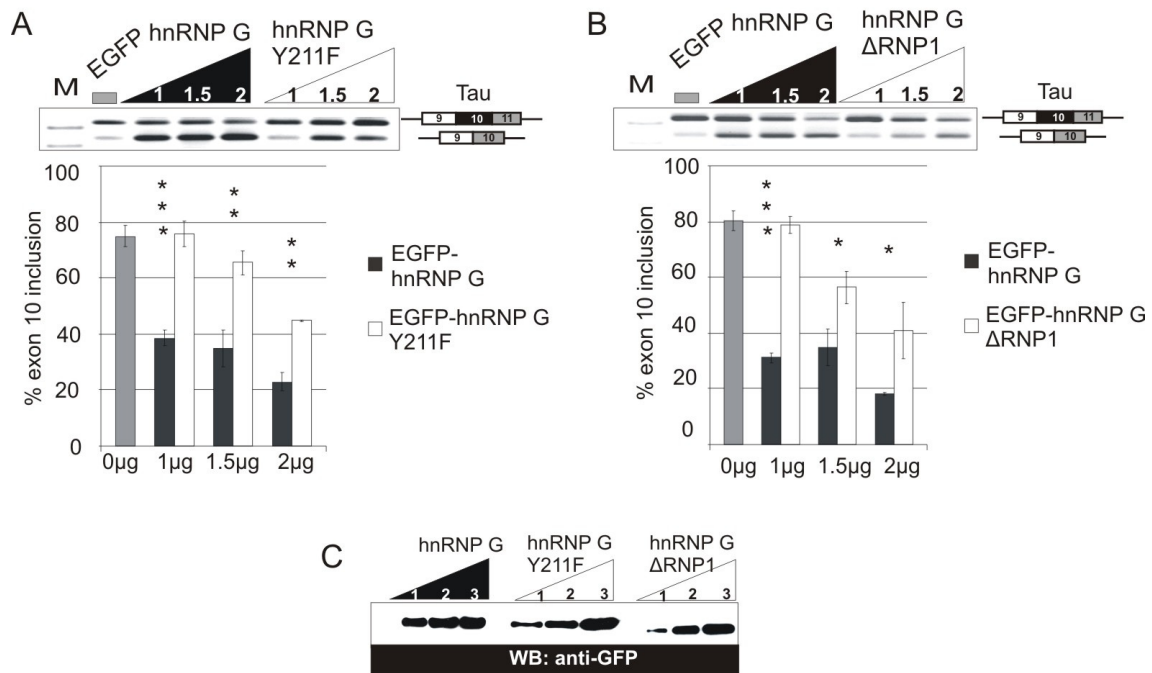

Figure 4-30: hnRNP G changes splice site selection on tau minigene. An increasing amount of EGFP-hnRNP G, EGFP-hnRNP G-Y211F (A) or EGFP-hnRNP G-ΔRNP1 (B) was cotransfected with tau minigene in HEK293 cells. Alternative splicing of tau minigene was determined by RT-PCR using specific primers. A representative ethidium bromide stained gel of each experiment is shown. The statistical evaluation of at least three independent experiments is shown below the gels. C) Representative Western Blot showing the expression of the transfected EGFP-tagged proteins. Stars indicate p-values from student's t-test comparing wt and mutant hnRNP G: A) 1 µg = 0.0002; 1.5 µg = 0.004; 2 µg = 0.0003. B) 1 µg = 0.00002; 1.5 µg = 0.04; 2 µg = 0.03.

Next, we investigated if additional phosphorylation of hnRNP G can change splice site selection of tau minigene. Increasing amounts of EGFP-hnRNP G were cotransfected with an expression construct of c-Abl and tau minigene into HEK293 cells. As a control, EGFP-hnRNP G-Y211F which cannot be phosphorylated was cotransfected with c-Abl. As shown in Figure 4-31, we could not detect a statistical significant change in exon 10 skipping when the mutant was additionally phosphorylated.
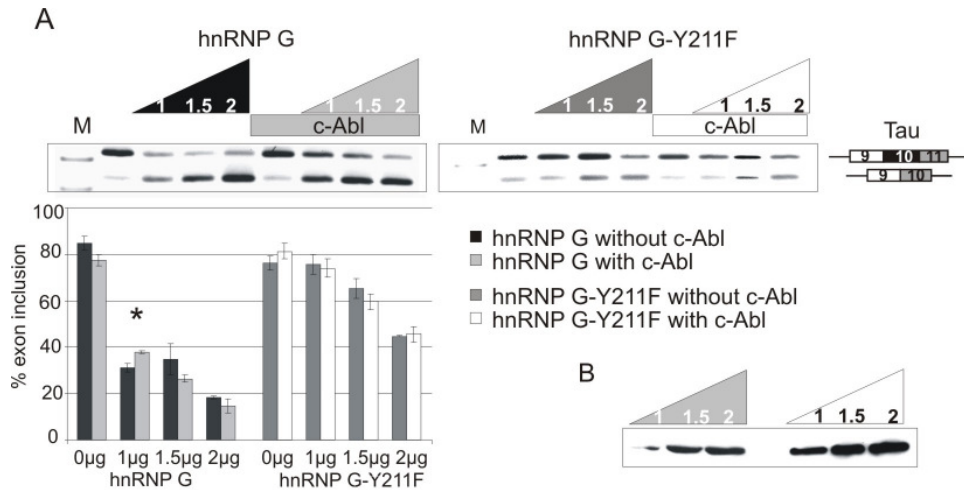
Figure 4-31: Phosphorylation of hnRNP G does not effect splice site selection of tau minigene. A) An increasing amount of EGFP-hnRNP G (left) or EGFP-hnRNP G-Y211F (right) was cotransfected with expression constructs of c-Abl and tau minigene in HEK293 cells. The alternative splicing of tau minigene was determined by RT-PCR using specific primers. A representative ethidium bromide stained gel of each experiment is shown. The statistical evaluation of at least three independent experiments is shown below the gels. B) Representative Western Blot showing the expression of the transfected EGFP tagged proteins. Star indicates the p-value from student's t-test comparing data for hnRNP G with and without c-Abl: 1 µg = 0.02.

4.3.12 siRNA knockdown of hnRNP G changes splice site selection

To analyse the effect of splice site selection when hnRNP G was knocked down, *in vivo* splicing assays with tau minigene were performed. The amount of hnRNP G was reduced by cotransfecting siRNA with the reporter minigene tau. As shown in Figure 4-32 A, decreasing the hnRNP G concentration with siRNA leads to weak inclusion of tau exon 10, whereas control siRNA had no influence on splice site selection.
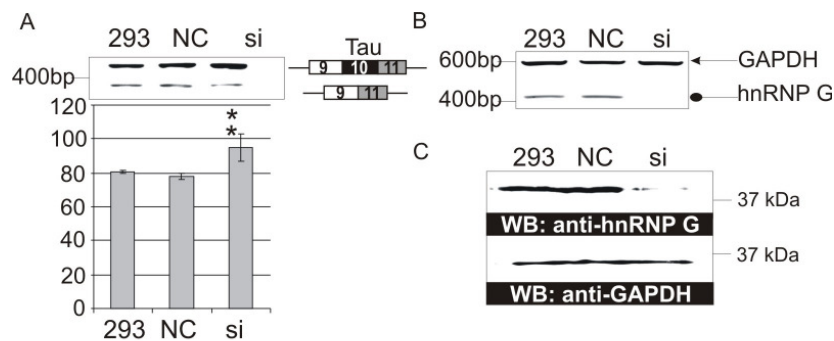
Figure 4-32: hnRNP G knockdown leads to exon 10 inclusion in tau minigene. A-C) The expression of hnRNP G was reduced by cotransfecting siRNA against hnRNP G with tau minigene. 293: cells were treated only with the transfection reagent; NC: non-specific siRNA; si: siRNA against hnRNP G. A) tau minigene; a representative ethidium bromide stained gel is shown. The statistical evaluation of three independent experiments is shown below the gel. Stars indicate p-values from student's t-test, when non-specific siRNA transfected and siRNA against hnRNP G transfected cells were compared (p = 0.003). B) RT-PCR using specific primers for hnRNP G and GAPDH as a control. C) Western Blot analysis of protein expression using specific antibodies.

The effect on splice site selection was proportional to the decrease of hnRNP G that was detected by RT-PCR using specific primers (Figure 4-32 B) and Western Blot (Figure 4-32 C).

4.3.13 Target sequences and target genes of hnRNP G

4.3.13.1 Finding target sequences by SELEX

To find a possible target sequence for hnRNP G, SELEX (systematic evolution of ligands by exponential enrichment) was performed. Previously, SELEX was successfully used to find binding sites for some SR proteins or RBMY, a protein related to hnRNP G (Cavaloc, Bourgeois *et al.,* 1999; Skrisovska, Bourgeois *et al.,* 2007). A pool of DNA was *in vitro* transcribed to generate an RNA pool. Recombinant HIS-tagged hnRNP G protein, generated from Baculovirus, was incubated with this RNA pool. After binding, the protein was digested with Proteinase K and the bound RNA was purified by phenol-chloroform precipitation and amplified by RT-PCR. The resulting cDNA was gel-purified, *in vitro* transcribed and another round of SELEX was performed. Five to six rounds of SELEX were performed before the resulting cDNA was cloned into TOPO vector and sequenced (Agowa, Berlin). The possible target sequences found for hnRNP G are listed in Table 4-4.

Nr	sequence flanked by SELEX primers		
	forward primer T7pro	target	reverse primer RT
1	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	UCCAGAAUUAUCCGUGGGCC	GGAAGCUUCUCGAGACGC
2	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	GACGUGAAUGCCAUAUGCAU	GGAAGCUUCUCGAGACGC
3	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	GCGCCGCUGGUGACCGUCCC	GGAAGCUUCUCGAGACGC
4	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	UGAUGCGAACAUCCACGCGG	GGAAGCUUCUCGAGACGC
5	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	GCGGGAUGUGCUCCCUGCCG	GGAAGCUUCUCGAGACGC
6	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	CUUGCCACGCUGCUCCGAUG	GGAAGCUUCUCGAGACGC
7	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	GAAUCGAUCUAGUACCACGU	GGAAGCUUCUCGAGACGC
8	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	AAGGCAUGAGUGAAGCUGCC	GGAAGCUUCUCGAGACGC
9	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	GCGACGCUCAACGACGCCCG	GGAAGCUUCUCGAGACGC
10	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	UAAACGGAUGUCCCAUCGGU	GGAAGCUUCUCGAGACGC
11	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	AUGCCUUCCUCAACACACCC	GGAAGCUUCUCGAGACGC
12	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	ACCUUGUCCGCGUAUGUGU	GGAAGCUUCUCGAGACGC
13	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	AAGCCUGCAGCGGACGCUGU	GGAAGCUUCUCGAGACGC
14	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	GCGGGCCAGUCAGACGCGUA	GGAAGCUUCUCGAGACGC
15	UAAUACGACUCACUAUAGGGAUCCGAAUUCCCGACU	GCCAUUUACCCCAACCUGCU	GGAAGCUUCUCGAGACGC

Table 4-4: Possible target sequences for hnRNP G found by SELEX. The sequences are flanked by the SELEX primers used for amplification of the DNA pool. Numbers marked in bold: sequences used for gel retardation assay (Figure 4-33). Highlighted in grey are possible binding motifs, underlined is the stem loop binding motif found for RBMY.

Analysing the obtained sequences, we could not find a possible consensus sequence. Work published by Nasim, Chernova *et al.,* 2003 suggested that a possible binding motif for hnRNP G contains a CAA triplet. For the hnRNP G paralogue RBMY, a CA/UCAA loop was identified recently as binding motif. This sequence could possibly also be recognised by hnRNP G (Skrisovska, Bourgeois *et al.,* 2007). Therefore, we checked for similar CAA triplets in our sequences. Indeed CAA was found, however more often similar triplet

nucleotides CCA or CCC were found (highlighted in light grey in Table 4-4). Interestingly, sequences 9 and 11 contained the RBMY stem loop binding motif CUCAA (underlined in Table 4-4)

To analyse if hnRNP G can actually bind to these sequences, RNA gel retardation experiments were performed with available sequences. For PCR-amplification of the desired sequences from TOPO vector, the SELEX primers T7pro and RT were used. The cDNA was *in vitro* transcribed with T7pro, which contains the T7 promoter site and incubated with recombinant HIS-tagged hnRNP G protein, purified from insect cells. Figure 4-33 shows that hnRNP G could strongly bind to SELEX sequences 4, 6 and 9 and more weakly to SELEX sequence 7, but not to sequences 8 and 13. This suggests that hnRNP G is able to bind to CCA repeats, as both sequences 8 and 13 did not contain these triplets.
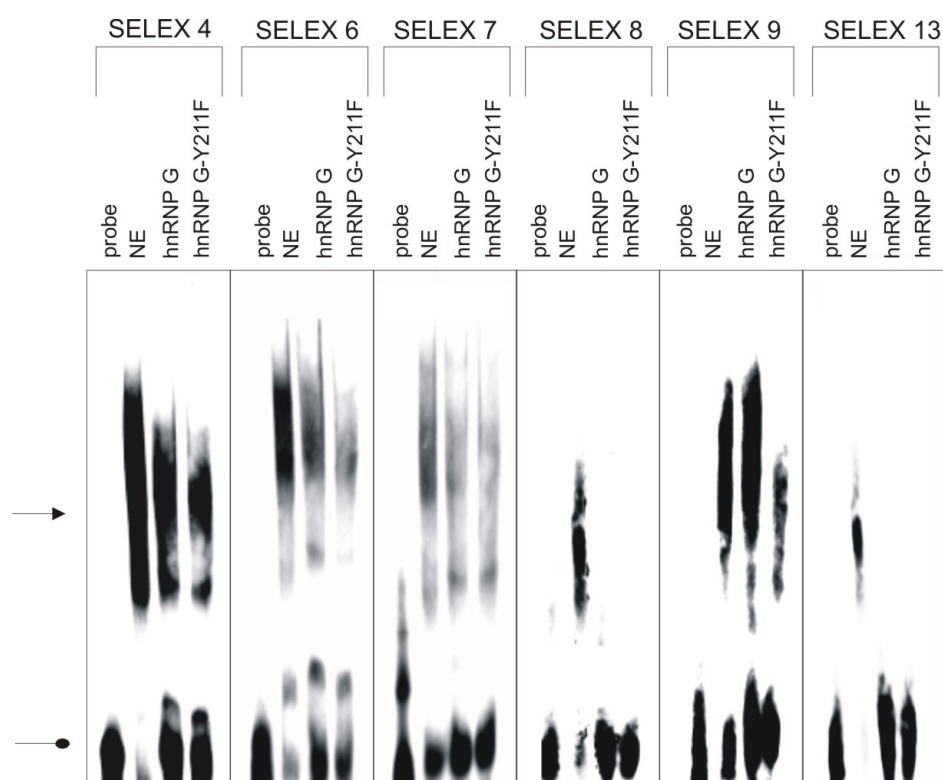
Figure 4-33: Interaction of hnRNP G with SELEX sequences. Gel retardation assay: 1µg of nuclear extract (NE), recombinant hnRNP G and hnRNP G-Y211F were incubated with a number of radioactive probes, whose sequences were found by SELEX, and analysed by native gel electrophoresis. The pointed arrow indicates the RNA:protein complexes, the round arrow the free probes.

hnRNP G-Y211F was used for comparison, to see possible differences between wild type and mutant. A difference in binding could not be observed for most of the sequences, only for sequence 6 it seems a bit weaker. No binding was observed for sequences 8 and 13. Nuclear extract was used instead of recombinant protein as a control for binding activity of the probes.

4.3.13.2 hnRNP G overexpression changes splice site selection in mouse exons

To study changes in alternative exons, a DNA microarray analysis was performed in cooperation with Hermona Soreq in Jerusalem. The microarray contained mainly splicing and apoptosis related genes. EGFP-hnRNP G was overexpressed in Neuro2A cells. Total RNA was isolated 18 hours after transfection and sent for hybridisation. RNA with overexpressed hnRNP G was compared to RNA from cells with overexpressed empty EGFP-C2 vector as a control. DNA chip analysis revealed several genes which are up or down regulated by hnRNP G. Most prominent amongst them were several SR and SR-related proteins, snRNPs and apoptosis genes, but also other hnRNPs or factors involved in mRNA processed.

The genes with the most significant alterations, for which the fold change was above 0.4 or below -0.4 with the oligo located in an alternative exon, are listed in Table 4-5.

Gene	Refseq	Gene's full name	Function group
hnRNPC	NM_016884.	Heterogeneous nuclear ribonucleoprotein C	hnRNPs
hnRNPK	NM_025279	Heterogeneous nuclear ribonucleoprotein K	
Ptbp1	NM_008956	Polypyrimidine tract binding protein 1 (PTB-1)	
SF2/ASF	NM_173374	Splicing factor, arginine/serine rich 1 (Sfrs1)	SR and SR-related proteins
SC35	NM_011358	Splicing factor, arginine/serine rich 2 (Sfrs2)	
SRp75	NM_020587	Splicing factor, arginine/serine rich 2 (Sfrs4)	
Srrm1	NM_016799	Serine/arginine repetitive matrix 1 (SRRM 1)	
U2af1	NM_024187	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF) 1	
SF3a2	NM_013651	splicing factor 3a, subunit 2 (SF3a2)	snRNPs
Snrpa	NM_015782	small nuclear ribonucleoprotein polypeptide A	
Snrpb2	NM_021335	U2 small nuclear ribonucleoprotein B	
Snrpd3	NM_026095	small nuclear ribonucleoprotein D3	
Snrpn	NM_013670	small nuclear ribonucleoprotein N	
U1snrp70	NM_009224	U1 small nuclear ribonucleoprotein polypeptide A	
Ppih	NM_028677	Peptidyl prolyl isomerase H	
U5-15kD	NM_025299	Thioredoxin-like 4 (Txn14)	
Ddx27	NM_153065	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	helicases
Ddx5 (p68)	NM_007840	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	
Cpsf 4	NM_178576	cleavage and polyadenylation specific factor 4	mRNA processing
Pabpc1	NM_008774.	poly A binding protein, cytoplasmic 1	
Pabpn1	NM_019402.	poly A binding protein, nuclear 1	
NIPP1	NM_146154.	protein phosphatase 1, regulatory (inhibitor) subunit 8	Splicing factors phosphorylation
Clk4	NM_007714.	CDC like kinase 4	
Bax_alpha	NM_007527	Bcl2-associated X protein	apoptosis
Bcl2_alpha	NM_009743	Bcl2-like 1 (Bcl2l1)	
Casp2	NM_007610	Caspase 2	
Casp7	NM_007611	Caspase 7	
Casp9	NM_015733.	Caspase 9	

Table 4-5: Mouse genes found by array analysis which were up- or downregulated by overexpressed hnRNP G. Marked in grey are the genes which were tested by RT-PCR.

To confirm data from the microarray analysis, RT-PCR was performed for several genes (marked in grey in Table 4-5) using specific primers in the flanking regions. Overexpressed EGFP-hnRNP G was compared to overexpressed EGFP-hnRNP G-Y211F and EGFP-C2 as a control. The chip results could be proved for all tested genes, except for hnRNP K and Caspase 2, which only showed one band (Figure 4-34). The second isoform could not be detected.
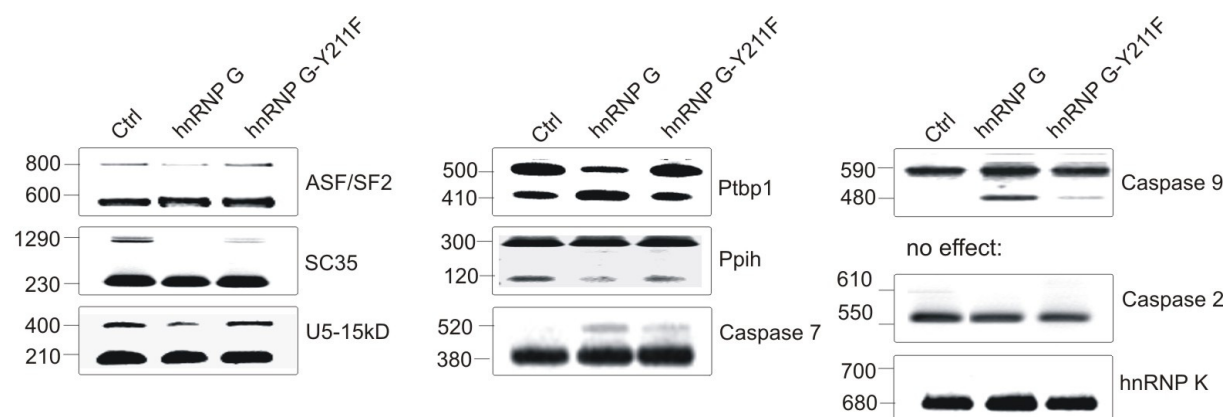
Figure 4-34: hnRNP G overexpression changes splice site selection in mouse genes. RNA from Neuro2A cells transfected with pEGFP-C2 as control (Ctrl), Neuro2A cells overexpressing EGFP-hnRNP G or EGFP-hnRNP G-Y211F was amplified by RT-PCR using specific primers.

Interestingly, for most genes hnRNP G overexpression promoted exon skipping, only for Ppih and Caspase 7 exon inclusion was promoted. This supports earlier findings that hnRNPs bind to splice silencers and function as antagonists to SR proteins (Mayeda and Krainer, 1992; Caceres, Stamm *et al.,* 1994; Rothrock, House *et al.,* 2005). The mutant hnRNP G-Y211F has the same, but clearly weaker effect than hnRNP G, similar to the effect seen on SMN2 and tau minigene (sections 4.3.9 & 4.3.10).

4.3.13.3 hnRNP G overexpression changes splice site selection in human genes

To test whether overexpression of hnRNP G has a similar effect on splice site selection of human genes like on mouse genes, we used oligonucleotide array analysis in cooperation with ExonHit Therapeutics, Paris. Total RNA of HEK293 cells transfected with EGFP-hnRNP G and EGFP-C2 as a control was used for hybridisation. RNA was isolated 18 hours after transfection. The alternative splicing events were detected by a combination of exon junction and exon body probes, as previously described (Fehlbaum, Guihal *et al.,* 2005).

For validation of the array data, RT-PCR was performed for chosen genes using specific primers in the flanking regions. Overexpressed EGFP-hnRNP G was compared to overexpressed EGFP-hnRNP G-Y211F and EGFP-C2 as a control. In Figure 4-35 some genes are shown for which the array data could be confirmed. Although the effect is fairly weak for

some genes, it is clearly visible that hnRNP G overexpression changes the splicing pattern. Interestingly, other than for the mouse exons, for the majority of theses genes exon inclusion is promoted, only in four genes (CPSF6, SFRS6, SFRS3, SF3A2) exon skipping is favoured.
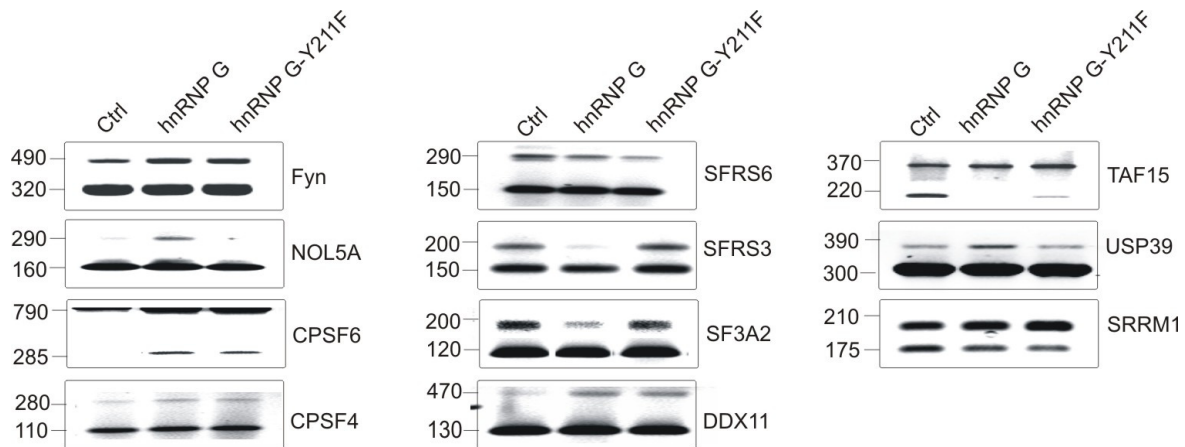
Figure 4-35: hnRNP G overexpression changes splice site selection in human genes. RNA from HEK293 cells transfected with pEGFP-C2 as control (Ctrl), HEK293 cells overexpressing EGFP-hnRNP G or EGFP-hnRNP G-Y211F was amplified by RT-PCR using specific primers.

For five genes (NOL5A, SFRS3, SF3A2, TAF15 and USP39) the effect of the hnRNP G mutant shows a weaker effect than hnRNP G, similar to the changes observed in the mouse exons (section 4.3.13.2) or SMN2 and tau minigene (sections 4.3.9 & 4.3.10). However, for the other genes hnRNP G-Y211F has the same effect as the wild type. This suggests that for regulation of some genes tyrosine phosphorylation of hnRNP G seems to be important to change splice site selection. In these cases the mutant might not be able to sequester other proteins to the splice sites, as a result changing the splicing pattern.

Comparing the mouse genes changed in the array analysis (Table 4-5) with the human genes, it is interesting to notice that a number of them are equivalent genes.

For example, changes in mouse and human cells were found in the microarray data for SRRM1, SF3A2 and CPSF4 and were validated in human cells. In the mouse microarray also changes for the DEAD box polypeptides (DDX) 5 and 27 were found, whereas in human cells changes were confirmed for DDX11. Amongst the genes for which changes were found in both microarrays were also U2AF1 or other hnRNP proteins, e.g. hnRNP C for both organisms, hnRNP K in mouse, hnRNP M in human. However, these results could not be validated by RT-PCR.

Next, we wanted to test the influence of siRNA knocked down hnRNP G on splice site selection. For this purpose, the human exonarray from Affymetrix was used. Total RNA of HEK293 cells transfected with siRNA for hnRNP G and a negative control siRNA was used

for hybridisation. RNA was isolated 18 hours posttransfection. However, only two possible events could be detected, which could not be validated by RT-PCR

4.3.13.4 siRNA knockdown of hnRNP G changes expression of other proteins

To test whether hnRNP G can change the expression of other proteins, hnRNP G was knocked down by siRNA and protein lysates were analysed by two dimensional gel electrophoresis (2-DIGE) (performed by Applied Biomics, California). Cell lysates in which a non-specific siRNA (NC) was transfected were used as control. Changes in proteins between NC and siRNA knockdown of hnRNP G of two experiments were compared. Figure 4-36 shows the knockdown of hnRNP G and the resulting 2D gels from the two experiments.
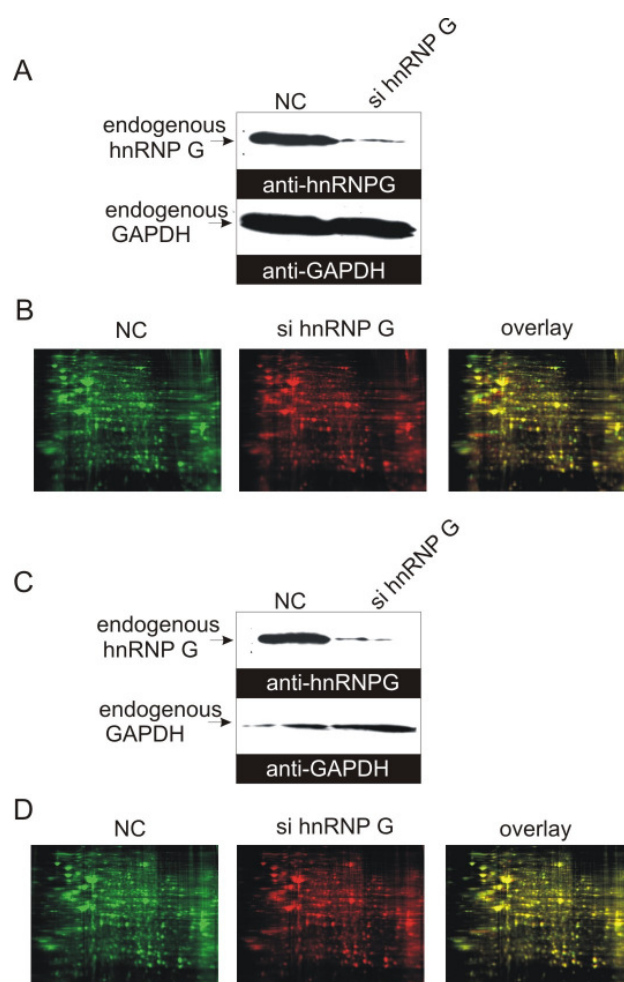
Figure 4-36: 2D gel electrophoresis of HEK293 cell lysates with hnRNP G siRNA knockdown. A) and B) show experiment 1, C) and D) experiment 2. Western Blots were probed with anti-hnRNP G to check the knockdown of the protein and anti-GAPDH as a control (A and C). Proteins in lysates with mock siRNA (NC) were stained in green, while proteins in lysates with knockdown siRNA were stained in red. The overlay shows that some proteins were changed by knockdown of hnRNP G (B and D).

The overlays of both protein gels were compared and spots from proteins showing changes in gels from both experiments were picked and analysed by mass spectrometry. Figure 4-37 shows the overlays of both experiments with the analysed protein spots marked.
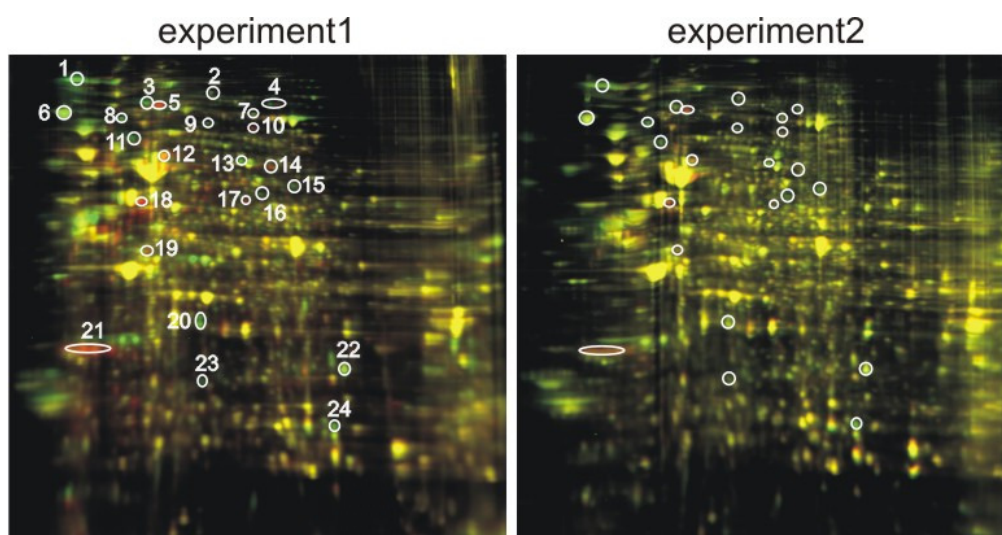
Figure 4-37: Protein spots from 2-DIGE, which were analysed by mass spectrometry. Overlay of the gels from both 2DIGE experiments. The numbered proteins marked with circles were cut. Green: NC, red: siRNA knockdown of hnRNP G.

Table 4-6 shows the proteins found by mass spectrometry of spots from 2-DIGE, which showed differences by siRNA knockdown of hnRNP G compared to control siRNA

Spot	Gene symbol	Protein name	Expression
1	NEFM	neurofilament medium polypeptide	decreased
2	SF3A1	splicing factor SF3a 120K chain	decreased
3	AARS	alanyl-tRNA synthetase variant	decreased
4	GLDC	glycine dehydrogenase (decarboxylating)	decreased
5	UBE1	ubiquitin-activating enzyme E1	increased
6	HSP90B1	tumour rejection antigen (Gp96) 1	decreased
7	MCM4	DNA replication licensing factor MCM4 (CDC21 homolog) (P1-CDC21)	decreased
8	VCP	transitional endoplasmic reticulum ATPase (TER ATPase)	decreased
9	LEPREL2	leprecan-like 2 protein	decreased
10	EEF2	Elongation factor 2 (EF-2).-	increased
11	HSP90AB1	heat shock protein 90kDa alpha	decreased
12	HSPA1A	dnaK-type molecular chaperone precursor, mitochondrial	increased
13	CTPS	CTP synthase	decreased
14	XRCC6	ATP-dependent DNA helicase II 70 kDa subunit	increased
15	STIP1	IEF SSP 3521	decreased
16	PHGDH	D-3-phosphoglycerate dehydrogenase	decreased
17	GRSF1	G-rich sequence factor 1 (GRSF-1)	increased
18	ACTL6A	BAF53A protein	increased
19	RPLP0	acidic ribosomal protein P0	increased
20	LDHB	L-lactate dehydrogenase B chain	decreased
21	YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	increased
22	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	decreased
23	COQ5	Coenzyme Q5 homolog, methyltransferase (S. cerevisiae)	decreased
24	HSD17B10	3-hydroxyacyl- CoA dehydrogenase type II	decreased

Table 4-6: Proteins changed by siRNA knockdown of hnRNP G. Proteins found by mass spectrometry of the proteins which showed changes (up or down shown in last column) in 2-DIGE, when hnRNP G was knocked down by siRNA compared to a control siRNA.

Interestingly, one of the proteins showing changes in the expression between control cells and cells with hnRNP G siRNA knockdown was SF3A1, which is the 120 kDa subunit of SF3A. The 66 kDa subunit of SF3A, SF3A2, showed changes by RT-PCR when hnRNP G was overexpressed (section 4.3.13.3, Figure 4-35).

4.3.13.5 Binding of hnRNP G to SF3A

The expression level of the 120 kDa subunit of SF3A (SF3A1) was found to decrease when expression of hnRNP G was decreased by siRNA (section 4.3.13.4) and the splicing pattern of the 66 kDa subunit of SF3A was changed when hnRNP G was overexpressed (Figure 4-35). SF3A is a complex consisting of the three subunits SF3A1 (SF3a120), SF3A2 (SF3a66) and SF3A3 (SF3a60) and is an essential component of U2 snRNPs. The finding that hnRNP G overexpression is regulating SF3A2 mRNA and changes the expression level of SF3A1 suggests that SF3A is a possible target of hnRNP G.

To confirm the result from 2-DIGE, Western Blot analysis of lysates, in which hnRNP G was knocked down by siRNA, was performed. As seen in Figure 4-38, only the 66 kDa isoform SF3A2 was slightly weaker expressed in the lysates with hnRNP G knocked down compared to the untreated 293 cells and cells transfected with control siRNA. The 120 kDa subunit SF3A1, which was found in 2-DIGE, did not show any changes. The ratio for the change of expression was -1.57 in gel 1 and -1.44 in gel 2. The ratio for the expression level change from some of the other proteins was around +/-3. It is possible that the decrease is too weak to be visible in Western Blot analysis.
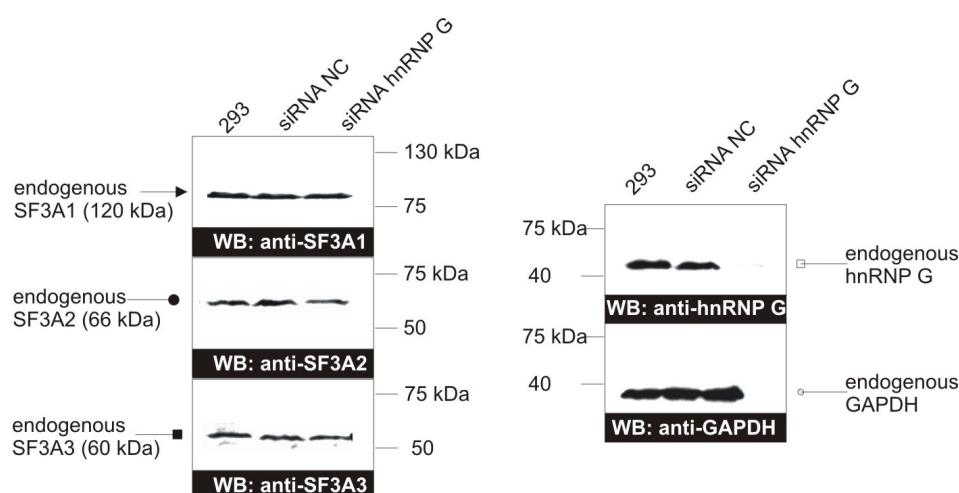
Figure 4-38: Expression level of the SF3A subunits in lysates with siRNA knocked down hnRNP G. 293 cells were transfected with siRNA for hnRNP G and a control siRNA. SF3A subunits were detected in isolated lysates with their specific antibodies (left). The knock down of hnRNP G was detected by anti-hnRNP G (upper right); anti-GAPDH was used as control to show equal amounts of proteins (lower right).

To test whether hnRNP G is binding to any of the SF3A subunits, immunoprecipitation was performed and it was looked for endogenous SF3A proteins. EGFP -hnRNP G was overexpressed in HEK293 cells and immunoprecipitation was done with anti-GFP. The precipitates were analysed by Western Blot with antibodies against the three subunits of SF3A. Figure 4-39 shows that only SF3A3, the 60 kDa unit, could be pulled down together with EGFP-hnRNP G. SF3A1 and SF3A2 which showed changes on protein level and in splicing pattern, respectively, could not be detected in the precipitates. However, all proteins were present in the lysates (load).
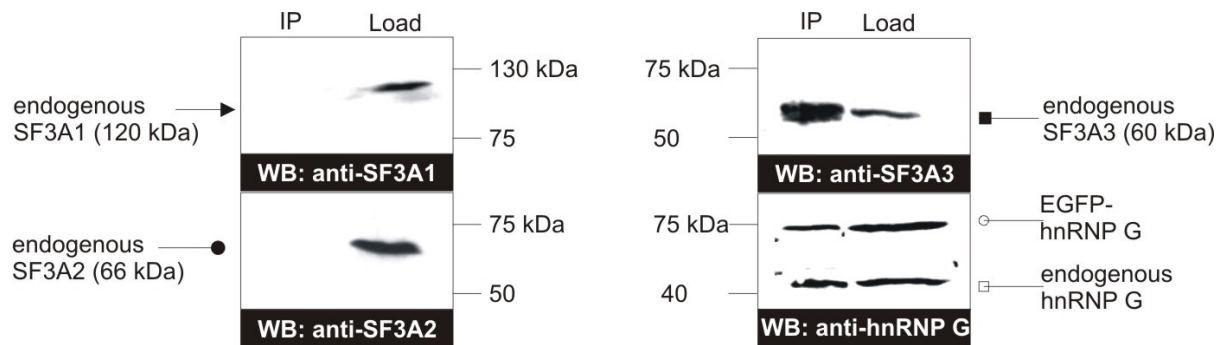
Figure 4-39: Interaction of hnRNP G with the subunits of endogenous SF3A. EGFP-hnRNP G was expressed in HEK293 cells and precipitated with anti-GFP. Co-immunoprecipitated (IP) subunits of endogenous SF3A and lysates (load) were detected by Western Blot using their specific antibodies. Endogenous and EGFP-tagged hnRNP G were detected by anti-hnRNP G.

As a result, hnRNP G does not bind to SF3A1, but the expression level of this subunit decreases when hnRNP G is knocked down by siRNA. It also does not bind to SF3A2, for which hnRNP G causes exon skipping when overexpressed. This is interesting, as the subunits form the SF3A complex and all three should be detected, if one is detected. It seems that hnRNP G does not bind to SF3A3 in the SF3A complex, only to the SF3A3 monomer, but is able to regulate the other two subunits in some way.

4.3.13.6 Finding target genes with CLIP

In another attempt to find target genes for hnRNP G, UV-Crosslinking and immunoprecipitation (CLIP) was performed according to the protocol published by the Darnell lab (Ule, Jensen *et al.,* 2003; Ule, Jensen *et al.,* 2005). With this method, RNA targets of hnRNP G could be found. A list of targets, which were found several times, is shown in Table 4-7.

Sequence 5'→3'	Gene
AAGTGG<mark>CCCA</mark>CTAGGCACTCGCATT<mark>CCA</mark>CG<mark>CCC</mark>GGCT<mark>CCA</mark>CG<mark>CC</mark> <mark>C</mark>AGAGAGCCGGGCTTCTTA<mark>CCCA</mark>TTTAAAGTTTG	NR_003287: Homo sapiens 28S ribosomal RNA AY830084.1: Homo sapiens survivin variant mRNA, complete cds, alternatively spliced
AAGTGG<mark>CCCA</mark>CTAGGCACTCACATT<mark>CCA</mark>CG<mark>CCC</mark>GGCT<mark>CCA</mark>CG<mark>CC</mark> <mark>C</mark>AGCGAGCCGGGCTTCTTA<mark>CCCA</mark>TTTAAAGTTTGAGAATAGGTTG AGATCGTTTCGG<mark>CCCCA</mark>AGACCTCTAATCATTCGCTTTACCGGA TAAAACTGCGTGGCG	NR_003287: Homo sapiens 28S ribosomal RNA AY830084.1: Homo sapiens survivin variant mRNA, complete cds, alternatively spliced
ACTAG<mark>CCC</mark>TGAAAATGGATGGCG	NR_003287: Homo sapiens 28S ribosomal RNA
CT<mark>CCC</mark>TGAGCAGCCTGTGTT<mark>CCA</mark>GATGCCGACTTCA<mark>CAAA</mark>CACC TTCTACTTGCTGAGCTCCTT<mark>CCC</mark>AGTGGAG	NM_031454: Homo sapiens selenoprotein O (SELO)
AAATGA<mark>CCCA</mark>CTAAAAGCTCTGC	NM_032449.1: Homo sapiens coiled-coil and C2 domain containing 1B (CC2D1B)
GGTACTGAGAAGGGCTCAGGGACATCG	NM_000420.2: Homo sapiens Kell blood group, metallo-endopeptidase (KEL)
GTGCGGCGCCGACGTCT<mark>CCACCA</mark>	NM_001004416.1 / NM_173568.2: Homo sapiens uromodulin-like 1 (UMODL1), transcript variant 1/2
GCGTGCTCCTTCTG<mark>CCA</mark>CTCAGACAGGTCTCC	NM_015694.1: Homo sapiens zinc finger protein 777 (ZNF777)

Table 4-7: RNA targets of hnRNP G found by CLIP. The found RNA sequence is shown with the according gene and its refseq number. Highlighted in grey are the possible binding motifs, found in the SELEX sequences.

In these targets also the possible CCA/CAA/CCC binding motifs which were discovered in the SELEX sequences could be found (marked in grey in Table 4-7)

Interestingly, 28S ribosomal RNA was also one of the targets, but the part of its sequence to which hnRNP G binding was detected is equal to survivin mRNA as well. The binding to 28S ribosomal RNA could be a hint that hnRNP G plays a role in translation of proteins.

The localisation of the CLIP sequences in the exons of the genes is shown in Figure 4-40.
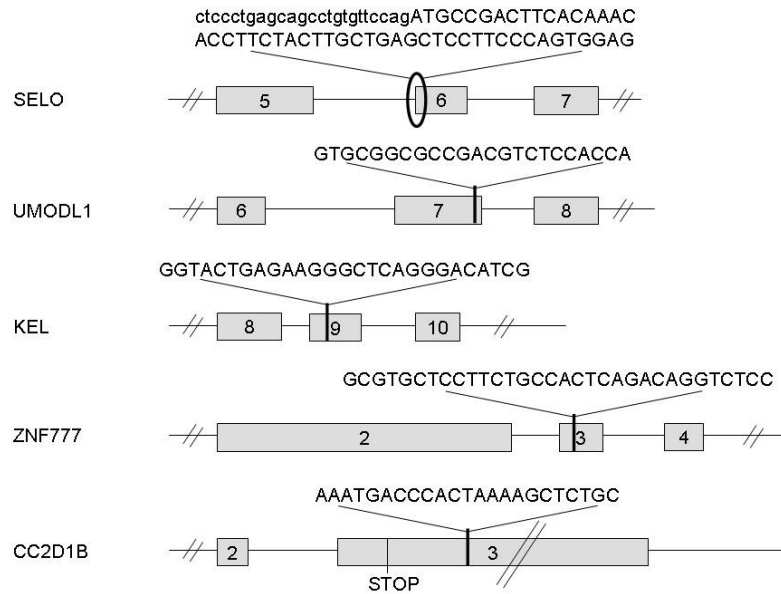
Figure 4-40: Localisation of CLIP sequences for hnRNP G in exons. Exons and flanking exons are shown as grey boxes, introns as black lines.

We could not find isoforms of these genes in which the CLIP target containing exons are alternatively spliced. The sequence found for protein uromodulin-like 1 (UMODL1) exists in two different splice variants. Splice variant 2 contains a longer exon through intron extension. However, the CLIP sequence is several hundred kb upstream of this intronic sequence. For CC2D1B the CLIP target sequence is located in the 3'UTR. The CLIP target sequence for SELO is partially in the intron (indicated in small letters) and the adjacent exon. However, no isoform could be found in which this exon is skipped. SELO has one isoform in which the previous exon is skipped and several isoforms are annotated which start or end in the exon with the CLIP target sequence (e.g. BG769764 or BX363833).

For KEL and ZNF777 the CLIP target sequence is again in exons for which no spliced isoforms could be detected: Also for these genes several isoforms which start or end in this exon are annotated (e.g. KEL: AA11415 or BF995823; ZNF777: CX757003 BG956474).

The fact that shorter isoforms exist for all of these genes could be a hint that hnRNP G binds to their mRNA and regulates their splicing pattern. To check whether the exons in these genes are skipped, further experiments like RT-PCR with exon-specific primers would have to be performed.

5   DISCUSSION

In the first part of this work, it was demonstrated that RBM4 (LARK) is a splicing factor whose interaction with WT1 is important for splice site selection by sequestering other proteins. One of the first models demonstrating regulation of alternative splicing by sequestration is Tra2-beta1 (Stoilov, Daoud *et al.,* 2004).

The second part of the work showed that reversible phosphorylation of Tra2-beta1 by PP1 is important for the interaction with Tra2-beta1 binding proteins and also changes splicing patterns of several Tra2-beta dependent exons.

The third part of the work concentrated on hnRNP G, a major interacting protein of Tra2-beta1. It was shown that hnRNP G is also reversibly phosphorylated, which significantly influences its shuttling ability. Phosphorylation of hnRNP G is regulated by PTP1B. Furthermore, it was shown that hnRNP G is able to change splicing of several exons and can bind to RNA containing CCA motifs.

5.1   Lark is involved in splice site selection

It was found that RBM4 (LARK) modulates alternative splice site selection of SMN2, SRp20, Tra2-beta and CD44v5 minigenes (Figure 4-1 & Figure 4-2). Similar to earlier findings (Lai, Kuo *et al.,* 2003), the carboxyl terminal region of RBM4 (aa 176-364) is necessary for the effect of RBM4 on splicing, as the C-terminus deleted mutant M1 could not alter splice site selection of SMN2. The C-terminal region of RBM4 contains the protein binding site to bind WT1, as it was shown that an RBM4 mutant only containing the C-terminus was sufficient for interaction with WT1 (Markus, Heinrich *et al.,* 2006). It is thus possible that RBM4 modulates splicing by interacting with proteins involved in splice site selection, such as WT1. The ability of RBM4 to alter the splicing pattern of SMN2 and SRp20 pre-mRNAs was suppressed by WT1(+KTS) (Figure 4-3). These results demonstrate that the interaction between RBM4 and WT1 is functional *in vivo* and that WT1(+KTS) is critical for RBM4 splicing. They also support the fact that changes in the ratio of splicing regulators can affect alternative splicing of pre-mRNA *in vivo* (Hanamura, Caceres *et al.,* 1998). Furthermore, it is important to note that only WT1(+KTS), but not WT1(-KTS) was able to inhibit the effect of RBM4 on splicing. This corresponds with other data that point to the +KTS isoform as being an RNA processing factor. WT1(+KTS) may inhibit RBM4 by binding to the C-terminal region of RBM4 and sequestering it. The exact mechanism of inhibition still needs to be determined. Splicing in nuclei of living cells occurs while

pre-mRNA is being packaged in supraspliceosomes (Müller, Wolpensinger *et al.,* 1998; Sperling and Sperling, 1998). Supraspliceosomes have been shown to be active in splicing and are composed of four functional spliceosomes interconnected by the pre-mRNA (Azubel, Habib *et al.,* 2006). This structure should enable efficient communication between the native spliceosomes, which is important to allow the non-sequential removal of introns and for splicing regulation and alternative splicing. RBM4 and WT1 were found to cosediment with supraspliceosomes (Markus, Heinrich *et al.,* 2006). This supports the effect of RBM4 on alternative splicing and also supports their interaction and possible cooperation in RNA processing. In conclusion, the findings show that the WT1 isoform containing amino acids KTS in exon 9 (WT1+KTS) is a direct regulator of the splicing factor RBM4. This adds to the importance of WT1 in post-transcriptional processing. These results also invite further research on the role of RBM4 in Wilms' tumour, Denys-Drash and Frasier syndromes, as well as leukaemia, breast and prostate cancers, as well as sex determination, in which mutated WT1 plays a role. Finding direct targets of RBM4 could help in understanding their mechanisms, since WT1 regulates RBM4.

5.2 Reversible phosphorylation of Tra2-beta1 changes binding to other proteins involved in splice site selection

Previous studies on SR-protein kinases demonstrated the importance of SR-protein phosphorylation for alternative splice site selection (Chalfant, Mischak *et al.,* 1995; Duncan, Stojdl *et al.,* 1997; Du, McGuffin *et al.,* 1998; Muraki, Ohkawara *et al.,* 2004). Phosphorylation of SR proteins is an important step for arrangement of protein:protein and protein:RNA interactions during spliceosome assembly. Phosphorylation influences the affinity between SR-proteins which can regulate the formation of protein-complexes on the pre-mRNA that identify exons (Xiao and Manley, 1997). Furthermore, it was shown that dephosphorylation of SF2/ASF and possibly other SR proteins is necessary for the splicing reaction to occur after spliceosome assembly (Mermoud, Cohen *et al.,* 1994; Cao, Jamison *et al.,* 1997; Murray, Kobayashi *et al.,* 1999), showing that SR-protein function is regulated by an interplay between kinases and phosphatases. However, the binding partners of the phosphatases in the spliceosome were unknown.

This work demonstrated that protein phosphatase 1 (PP1) binds directly to the RVDF docking motif in the RRM of the splicing factor Tra2-beta1, which results in dephosphorylation of Tra2-beta1. Sequence alignment of all available species for Tra2-beta1 revealed the presence of this PP1 binding motif with high evolutionary conservation. Detailed

computational analysis showed that RRMs from nine other SR and SR-related proteins contained the characteristic RVXF motif (Novoyatleva, Heinrich *et al.,* in press).

In agreement with earlier studies investigating hyperphosphorylation of SF2/ASF after kinase treatment, we found that the phosphorylation state of Tra2-beta1 controls its binding to interacting proteins. Dephosphorylation of Tra2-beta1 promotes its RS domain mediated homomultimerisation and binding to SF2/ASF (section 4.2.2). After destruction of the RVDF docking motif, these interactions are abolished, indicating that PP1 dephosphorylates sites in the RS-domains of Tra2-beta1. This was also supported by performing an experiment with a Tra2-beta1-RSA mutant, in which the serine residues in the RS domain are mutated to alanine and cannot be serine phosphorylated and with a Tra2-beta1-RSE mutant, which mimics a phosphorylated protein. The RSA mutant showed a stronger interaction with SF2/ASF, while the RSE mutant can bind SF2/ASF in amounts similar to the wild type. hnRNP A1 acts antagonistically to SF2/ASF and was almost abolished for the Tra2-beta1-RSA mutant, which could bind strongest to SF2/ASF.

We next tested whether PP1 activity can influence splice site selection by performing several *in vivo* splicing assays (section 4.2.3-4.2.5), since the formation of transient protein complexes on exons is necessary for their proper recognition. PP1 activity was decreased either by its specific inhibitors tautomycin or NIPP1, indirectly by forskolin or knocked down by siRNA. NIPP1 is a nuclear protein that specifically inhibits PP1. Tautomycin is a cell permeable phosphatase inhibitor that blocks PP1. Forskolin increases the cAMP level and activates PKA which phosphorylates DARPP32 on threonine 34 and enables it to bind to and inhibit PP1 (Kwon, Huang *et al.,* 1997).

In most cases analysed, we found that a decrease of PP1 activity had a similar effect on alternative exon usage as did an increase of the Tra2-beta1 concentration. However, there are several cases of alternative exons that responded much stronger to NIPP1 or PP1 siRNA knockdown than to Tra2-beta1. This most likely indicates that other splicing factors that are dephosphorylated by PP1, such as SF2/ASF or SRp30c, are part of the protein complexes regulating these exons. The catalytic subunit of PP1 associates with numerous different proteins that can function as subcellular targeting proteins, substrate-specifiers or inhibitors. Cellular signal transduction pathways, for example cAMP-dependent activation of protein kinase A, regulate the binding of PP1 to these proteins. This explains why the subcellular localisation of the regulatory subunits depends on the cell type and is highly dynamic in a given cell (Ceulemans and Bollen, 2004). The association of PP1 with regulatory proteins will

affect its ability to dephosphorylate splicing factors with the PP1 docking motif. This could explain why alternative splice site usage in various cell types is often very different, although the concentration of regulatory factors is quite similar. Our data suggest that the phosphorylation state of splicing factors is as important as their relative concentration and that PP1 activity is a key component in this regulation.

hnRNP G, a binding partner of Tra2-beta1, is another protein which is involved in splicing and shows phosphorylation. The results of the studies with hnRNP G will be discussed in the following chapters.

5.3   hnRNP G is in a phosphorylated state in resting cells and is able to shuttle between nucleus and cytoplasm

Protein phosphorylation is important for regulation of signaling cascades in the cell. Proteins can be activated or deactivated by phosphorylation and dephosphorylation. Also the components of the pre-mRNA splicing machinery undergo phosphorylation and dephosphorylation during the splicing process.

hnRNP G was found to affect splicing (sections 4.3.9 & 4.3.10) and to bind to several splicing factors (Figure 4-22). Furthermore its sequence shows many tyrosine residues, which are possible phosphorylation sites. In unstimulated cells, the activity of endogenous kinases can only be detected using phosphorylation-specific antibodies against specific target sites. To investigate the result of kinase activation, the activity of kinases was increased by transfection of cDNAs expressing kinases. hnRNP G was found to be phosphorylated, even when no kinase was added, using the phospho-specific antibody PY20. This is in contrast with other splicing factors, which did not show a background level of phosphorylation in absence of recombinant kinases. This is for example visible in tyrosine phosphorylation studies of SLM-1 (Stoss, Novoyatleva *et al.,* 2004) or YT521-B (Rafalska, Zhang *et al.,* 2004). This suggests that hnRNP G seems highly phosphorylated in resting cells. In addition, it could be demonstrated that the level of phosphorylation can be increased through overexpression of several kinases e.g. SRC family kinases or c-Abl. Mass spectrometry of hnRNP G coexpressed with c-Abl showed that one predominant phosphorylation site is located on the tyrosine residue 211. A specific tyrosine phospho-antibody against this site could also recognise hnRNP G phosphorylated by endogenous kinases, but not a phosphorylation deficient mutant hnRNP G-Y211F, which emphasises the finding of Y211 as tyrosine phosphorylation site.

The most striking difference between the phosphorylated protein and its non-phosphorylated mutant was observed in protein shuttling between nucleus and cytosol. The mutant was shuttling much slower than the wild type. So, it seems that tyrosine phosphorylation on residue 211 is very important for the ability to shuttle. Numerous SR and SR-related proteins or hnRNPs were shown to shuttle. Some of the shuttling proteins are thought to escort mature mRNAs into the cytoplasm in the form of mRNPs. A subset of hnRNP proteins was found to shuttle while associated with both pre-mRNA and mature mRNA (Pinol-Roma and Dreyfuss, 1992).

Through shuttling, the protein is also able to contact the membrane bound SRC family kinases c-Src and p59[fyn,] by which it is phosphorylated (Figure 4-14 & Figure 4-18). hnRNP G was not only shown to be phosphorylated by c-Src, but also to bind to it in immunoprecipitation (Figure 4-21). It is therefore possible that SRC family kinases can phosphorylate hnRNP G directly while hnRNP G is shuttling. hnRNP G also has a proline rich region in its structure, which is a binding motif for the SH3 homology domain contained in many kinases. c-Src could bind to the proline rich region of hnRNP G and then phosphorylate it.

In this work, we also could demonstrate that PTP1B is able to dephosphorylate hnRNP G (Figure 4-23) while some other phosphatases did not have an effect. c-Src is activated by dephosphorylation at its tyrosine residue 527 by several tyrosine phosphatases, amongst them PTP1B, SHP1 or PTPalpha (Roskoski, 2005). PTP1B was even characterised to be the main phosphatase for c-Src activation in breast cancer cells (Bjorge, Pang *et al.,* 2000). Overexpression of PTP1B leads to dephosphorylation of hnRNP G, but at the same time should activate endogenous c-Src by dephosphorylation, which is one of the kinases demonstrated to phosphorylate hnRNP G. In conclusion, inhibition of PTP1B should lead to phosphorylated hnRNP G again. First tests with specific PTP1B inhibitor showed that hnRNP G could be detected with the phospho-specific antibody (Figure 5-1).
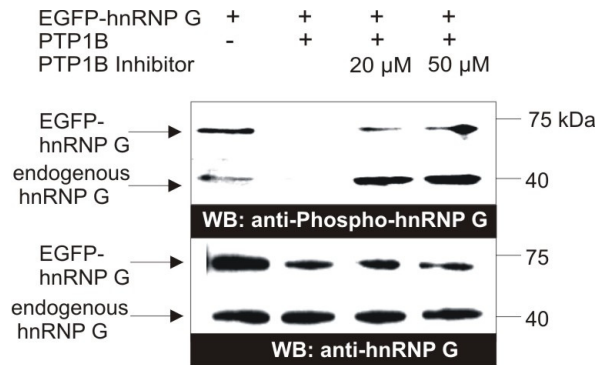
Figure 5-1: Inhibition of PTP1B reverses phosphorylation of hnRNP G. EGFP-hnRNP G was cotransfected with an expression construct of PTP1B in HEK293 cells. Cells were treated with 20 or 50 µM PTP1B inhibitor.

In conclusion, these data show that hnRNP G undergoes reversible tyrosine phosphorylation, which is important for splice site selection (Chalfant, Mischak *et al.,* 1995; Duncan, Stojdl *et al.,* 1997; Du, McGuffin *et al.,* 1998; Muraki, Ohkawara *et al.,* 2004). The phosphorylation of hnRNP G is regulated by PTP1B and facilitates the protein's shuttling between nucleus and cytoplasm. Shuttling of hnRNP G indicates that it seems to have non-nuclear functions, e.g. transport of mature mRNA to the cytoplasm, regulation of mRNA stability or translation. Since in CLIP analysis hnRNP G was found to bind to 28S ribosomal RNA, it might be possible that the protein plays a role in translation. Another indication for a translational function could be our finding from microarray data that several exons in the 5' or 3' untranslated region showed up or down regulation.

Another support for this theory could be data from sucrose gradients, in which hnRNP G was found in the polysomal region (Figure 5-2).
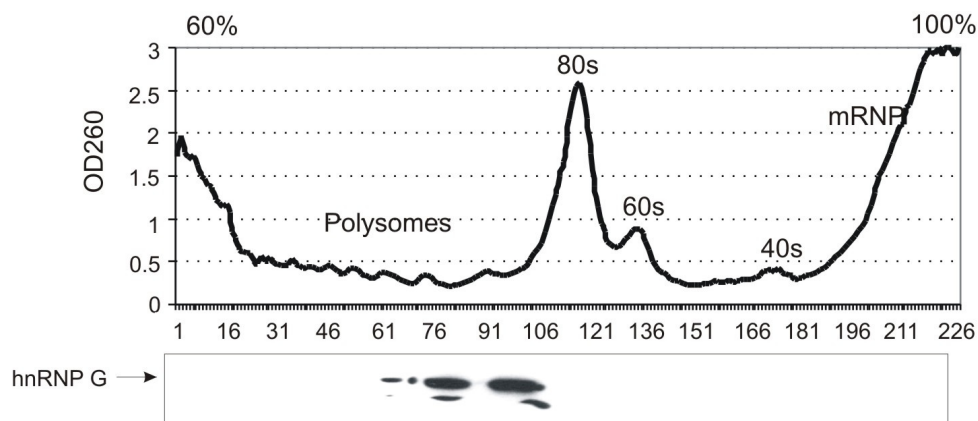
Figure 5-2: hnRNP G localisation in sucrose gradient. Protein lysates were fractionated in a 60-100% sucrose gradient and fractions collected. Western Blot analysis with anti-hnRNP G was performed on the fractions.

It was found previously that for example binding of hnRNP K can silence 15-lipoxygenase translation by binding to a CU-rich sequence motif in the 3'UTR (Ostareck, Ostareck-Lederer *et al.,* 1997). Other hnRNPs like hnRNP I (Hellen, Witherell *et al.,* 1993),

hnRNP L (Hahm, Kim *et al.,* 1998) or hnRNP D (Kiledjian, Demaria *et al.,* 1997) were shown to have functions in translation. Yet, the role of hnRNP G in translational processes remains to be analysed further.

5.4 hnRNP G changes splice site selection phosphorylation dependently

hnRNP G has several features common to splicing factors like the RNA recognition motif (RRM), a serine/arginine (SR) rich region and several tyrosine residues. Additionally, it contains an RGG motif which can also bind to RNA (Kiledjian and Dreyfuss, 1992). Furthermore, hnRNP G was shown to interact with several splicing factors (Figure 4-22) (Venables, Vernet *et al.,* 1999; Elliott, Bourgeois *et al.,* 2000; Venables, Elliott *et al.,* 2000) and demonstrated that it resides in supraspliceosomes (section 4.3.8), large particles, in which pre-mRNA splicing takes place (Müller, Wolpensinger *et al.,* 1998; Sperling and Sperling, 1998). The distribution of hnRNP G in these particles changes according to phosphorylation or to the protein. In the supraspliceosome, it can also have overlapping distribution with other splicing factors, e.g. Tra2-beta1, which is one of its main interactors.

hnRNP G can *in vivo* change the splice site selection of SMN2 minigene from 20% to 80% exon 7 inclusion and of tau minigene from 75% to 20% exon 10 inclusion. Changes in splicing are clearly reduced for both minigenes by the phosphorylation deficient mutant compared to the wild type. This mutant could only change exon 7 inclusion for SMN2 up to 65% and exon 10 inclusion for tau minigene down to 40%.

The mutant with deleted RRM still promotes exon 7 inclusion in SMN2 minigene, although RNA binding should be affected. However, for tau minigene it could not promote exon 10 skipping as efficiently as the wild type, similar to the Y211F mutant.

It seems that the phosphorylation deficient mutant can sequester other proteins to the splice sites less sufficiently than wild type hnRNP G. This is interesting, as comparing wild type and Y211F mutant in co-immunoprecipitation studies, both could bind equally well with other splicing factors (Figure 4-22). However, the effect of hnRNP G on tau minigene was reduced when using the RRM deletion mutant, so binding to RNA will be affected. The changes of splicing for SMN2 do not occur via direct RNA-binding of the RRM, as the RRM deletion mutant can still change splice site selection of SMN2 minigene similar to the wild type. The RRM deletion mutant should still be able to recruit other splicing factors, e.g. Tra2-beta1, which binds to an ESE in SMN2 and promotes exon 7 inclusion (Hofmann,

Lorson *et al.,* 2000). This sequestration of other splicing factors however, is not sufficient for splicing of tau exon 10.

A possibility for the association with tau pre-mRNA is that the RNA is not bound by the RRM, but by the RGG rich region, which can also interact with RNA (Kiledjian and Dreyfuss, 1992). The RRM of some hnRNPs was even found to be involved in protein:protein interactions (Hay, Kemp *et al.,* 2001). Maybe hnRNP G also can interact with certain proteins via its RRM domain. As this interaction is abolished, the RRM deletion mutant might not be able to change splice site selection of tau minigene in the same way as the wild type. However, this would have to be investigated further, for example by binding studies with the RRM deletion mutant or by performing splicing assays with other mutants of hnRNP G. For this experiments, mutants could be used with the full RRM missing or mutants where the RGG motif is mutated.

When hnRNP G was knocked down by siRNA in a cotransfection with tau minigene *in vivo*, a weak, but significant increase in exon 10 inclusion could be observed (Figure 4-32). This shows that the presence of hnRNP G is needed to exclude this exon, which is important for a normal function of Tau. Increased exon 10 inclusion leads to accumulation of abnormal filaments of Tau protein and can cause tauopathies, a group of diverse dementias and movement disorders, amongst which are frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) or Alzheimer's disease.

hnRNP G was also shown to regulate exons of several endogenous genes involved in splicing in mouse and human cells. For several of these exons it was shown that the phosphorylation deficient mutant is unable to change the splicing pattern to the same level as the wild type hnRNP G. This is another evidence for the phosphorylation dependent activity of hnRNP G in splice site selection (Figure 4-34 & Figure 4-35).

Furthermore, the genes found by CLIP analysis all have splice variants, which is another indication that hnRNP G functions as a splicing factor. It might bind directly to the obtained sequences to regulate splicing of these genes.

5.5 hnRNP G binds to CCA-rich motifs

To find possible target sequences of hnRNP G, SELEX was performed. The obtained sequences contained CAA or CCA/CCC triplets. It was shown that hnRNP G is able to bind to these CCA rich sequences by gel shift assay, as it could not bind to sequences not containing such motifs (Figure 4-33). The genes found by CLIP (section 4.3.13.6) also

contain CAA, CCA and CCC triplets (Table 4-7). We generated a weight matrix for our assumed binding site, showing the CCA triplet in the middle, flanked by degenerate sequences (Figure 5-3).

A

```
1                              uUCCAGAAUUAUCCGUGGGCC
2                    GACGUGAAUGCCAUAUGCAU
3          GCGCCGCUGGUGACCGUCCCgg
4               UGAUGCGAACAUCCACGCGG
5               GCGGGAUGUGCUCCCUGCCG
6                          CUUGCCACGCUGCUCCGAUG
7               GAAUCGAUCUAGUACCACGU
9                       GCGACGCUCAACG
9 cont                       ACGCCCGg
10             UAAACGGAUGUCCCAUCGGU
11           AUGCCUUCCUCAACA
11 cont                      CACCCgg
14                       GCGGGCCAGUCAGACGCGUA
15                         uGCCAUUU
15 cont                 ACCCCAACCUGCU
28S a                  ACUAGCCCUGAAAAUGGAUGGCG
CC2D1B               AAAUGACCCACUAAAAGCUCUGC
UMODL1   GUGCGGCGCCGACGUCUCCACCA
ZNF777       GCGUGCUCCUUCUGCCACUCAGACAGGUCUCC
SELO                    CUCCCUGAGCAGCCUGUG
SELO cont1             UUCCAGAUGCCGACUU
SELO cont2             CACAAACACCUUCUACUUG
SELO cont3       CUGAGCUCCUUCCCAGUGGAG
28S b                AAGUGGCCCACUAGGCACUCA
28S b cont1           CAUUCCACGCCCGG
28S b cont3             CUCCACGCCAGCGAGCCGG
28S b cont4       GCUUCUUACCCAUUUAAAGUUUGAGAAUAGGUUGAGAUCG
28S b cont5         UUUCGGCCCCAAGACCUCUAAUCAUUCGCUUUACCGGAUAAAA
```

B

Figure 5-3: RNA motif to which hnRNP G binds determined by SELEX and CLIP data. A) Representative SELEX and CLIP sequences. The common sequence motif is highlighted in bold. B) Weight matrix, generated by WebLogo program (http://weblogo.berkeley.edu/logo.cgi) describes the sequence element present in all sequences.

It can be seen that the triplet containing CCA, CCC or CAA is pronounced in the binding sites. Therefore, we analysed the target genes, which were found by microarray analysis and confirmed by RT-PCR (Figure 4-34 & Figure 4-35) for CCA-rich motifs. Table 5-1 (p. 119-120) and Table 5-2 (p. 120-121) show the mouse genes and the human genes, respectively, with their corresponding exons together with their potential function. The CCA rich motifs are marked in grey. All genes which showed differences in the splicing pattern contain several CAA, CCA or CCC motifs except TAF 15 which has none. This suggests that hnRNP G regulates splice site selection by binding to CCA-rich sequences in specific exons. It does not seem to require double strand RNA sequences as its paralogue RBMY, which

binds to a stem loop structure (Skrisovska, Bourgeois *et al.,* 2007). In this novel mode of binding, the RRM β-sheet surface of human RBMY binds to the RNA loop (CUCAA) in a sequence specific manner and the β2-β3 loop of the RBMY inserts into the major groove of the RNA stem. This second step takes place only for RBMY due to a structural difference to hnRNP G, hnRNP G-T or mouse RBMY, whose RRM is very well conserved. The latter three all have an additional amino acid, which is missing in the RRM of RBMY. RBMY also contains another non-conserved mutated amino acid in the β2-β3 loop. Both mutations are necessary to bind the stem-loop structure (Skrisovska, Bourgeois *et al.,* 2007) (Figure 5-4). Therefore, it is unlikely that hnRNP G needs a stem loop structure to bind, but likely that the RRM can also bind to CAA sequences like RBMY.
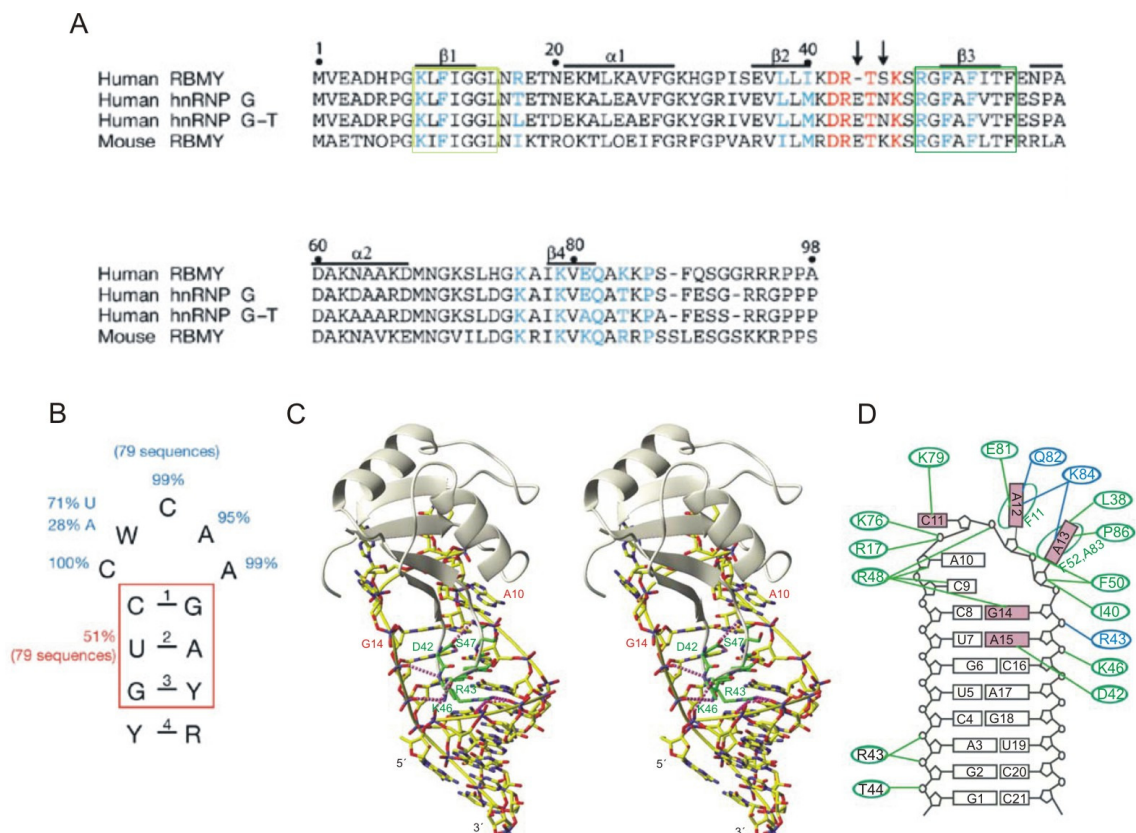
Figure 5-4: Binding of RBMY to RNA stem-loop sequence. A) Sequence alignment of RRMs of human RBMY (hRBMY), and related orthologues or paralogues. The RNP2 and RNP1 motifs are indicated by light green and dark green boxes, respectively. Amino acids of hRBMY found in contact with the RNA loop and stem are indicated in blue and red, respectively. The arrows show the non-conserved mutated amino acids of β2-β3 loop. B) Consensus sequence of the stem-loop structure with the highly conserved CWCAA loop. The red box shows the core consensus sequence of the stem. C) Stereoviews of the contact between the RRM β2-β3 loop and the RNA stem. D) Schematic diagram of interactions. The amino acid residues in contact with the RNA side chain and main chain are shown in green and blue, respectively. The nucleotides shown in red are sequence specifically recognised by human RMBY RRM. Y: C or U; W: U or A; R: G or C. Picture from Skrisovska, Bourgeois *et al.,* 2007.

As hnRNP G was shown to change splice site selection of SMN2 exon 7 and tau exon 10, the sequences of these exons were also searched for CCA rich sequences. Interestingly, in tau exon 10 were found several CAA/CCA triplets, while the proximate

5' and 3' introns have a few CCA and CCC triplets. In SMN2 exon 7 several CCA/CCC triplets were found, while the surrounding introns have a number of CAA triplets.

These findings strengthen the assumption that hnRNP G can bind to exons containing CCA rich sequences and regulate their splicing pattern.

Gene	Exon sequence	Protein	Potential function
SF2/ASF NM_173374 Mus musculus splicing factor, arginine/serine-rich 1	GGAGAAACTGCCTACATCCGGGTTAAAGTTGATGGGCCCA GAAGTCCAAGTTATGGAAGATCTGATCTGAAGCCGTAG TCGTAGCAGAAGCCGTAGCAGAAGCAACAGCAGGAGTCGC AGTTACTCCCCAGGGAGAAGCAGAGGATCAACGCTATT CTCCCGTCATAGCAGATCTCGCTCTCGTACATAA	GETAYIRVKVDGPRSPSYGRSRSRSRSR SRSRSRSNSRSRSYSPRRSRGSPRYSPR HSRSRSRT*	Shorter isoform Protein kinase C phosphorylation site
	GTAATGTCTGCGGGAATCTGAGACACGCCCTAACTCCCGT GGGTATGGAGTAGGTTTTTGAGCCTTGTTAGCATAAAG ACTCCTGGTGCTAAGAGTACTACTGCTCTGAAGTTACTCA GCTTTGGGAGTGATGCTGAAGAGGGGTCTGCAGAGAGGAT TGTGTAAAGCTTAGATAGTAATGGCGTTTCATGCTGTTT GAGACCTATTAATGAAAATGACTATTTCTTGCTGTTTTA TCCAACGTCTGCATTTCCCCCTTTAAAGCTGCGGTCTCC TGTTTATCAAAGAATATTGGCCAGTATTGCAGATTTTAA CTGATTTGGCTGATCCTCCAGGGACCAGTTTCTGTGGGCG TGTATTGGCTGTTGTCTTTAAATGTAAAGATGCAC TATCCTCATAGAGAAAAACATTCAGTGCAACTGTTGTTGT GCTGACTGGGACTTCTTACTCTAATGGATGTGGCAAAGGA ATTGCAATTAAGAAGCAATGAACTTTTGGAACCCCAAAAG AAAGTTACAGGTATTGATCCGCACCGGGTGGGAAAGGAT AGTGTGTCTTAACTCTAAATTGTTGGTCCTATTTTTCT TTTTAAAGGAAAGGCCCTAAGTAGCTGATTAAGAACATC CTCAACTGCCAAACGTTCATTTTCAAACATCTTACAAAG TGTAGACTAATTTCTGATAGCAAGTTTTGTTGGAAACA GCCCCACCCTGCCCCCTACCTTTTTTTCTTTTCTTTCT CCTTGCCTCCTTTTTTTCCCCCTTTTTTTTTCTTTTTCT TTTTTTTTTTTTTTTTTTTGGTCTTGGTTATT TGGCCAAGAATCACATATCCGAACATGCTGGTGGTACC CTTTGGAAAAACTCCCATTTGGGCCTTAAAAAAACTGAT AATGGCAGGTGGAACCTTCTGTAACCTACTTGTTTCACC AGAGCCTAGTAAGTACGTACCTGTGATAAATGAAAACCATGTG CACTTTGGAACCTTTTTTAGTAGTGGAAAGTAAGCTGAACC TCTTTCTTTTCAAACCTAG		
SC35 NM_011358 Mus musculus splicing factor, arginine/serine-rich 2 (Sfrs2)		VMSAGI*	NMD
U5-15kD NM_025299 Mus musculus thioredoxin-like 4 (Txnl4)	CCCCACCCCCTCTCTGGGAGAGAGAAAACCGGATTCAC TGTGTCGCCCAGAGGGACCTTGCACATTCTTCCTTCCTCC CTGCCTCCTCCCAGTCCTCGGGATGAGCCTCAAGCTCTTT AAATGAAGATAGTGGGAGATCTTCCTCATCTTGTATGAG TCCTGTTTTGGGATATCCCAGCTCGTTTTAAA	PPPPLWERRKPDSLCRPEGPCTFFPSPC LLPSPGMSLKLFKWKIVGDLPHLV*	Shorter isoform Protein kinase C phosphorylation site
ptbp1 NM_008956 Mus musculus polypyrimidine tract binding protein 1	GGTGCGCCGGCATAATGTCAGCCTCTCCGTATGCAGGAG CCGGGTTCCCTCCCACCTTTGCCATCCCTCAGGCCGCAG	GAPGIMSASPYAGAGFPPTFAIPQAA	N-myristoylation site
Ppih NM_028677 Mus musculus peptidyl prolyl isomerase H	GAAGTTGGTCGCATGAAAATCGAGCTCTTTGCAGACGTGG TGCCTAAGACGGCAGAGAACTTAGGCAATTCTGCACCGG AGAGTTCAGAAAAGATGCGTTCCGATAGGATACAAAGGA AGGGTCATAAAGAGATTTCATGATTCAGGGTGGAGATTTTG TTAAT	EVGRMKIELFADVVPKTAENFRQFCTGE FRKDGVPIGYKGRVIKDFMIQGGDFVN	N-myristoylation site

Table 5-1: CCA-rich motifs in exons of mouse microarray genes regulated by hnRNP G. Continued next page.

Gene	Exon sequence	Protein	Potential function
Casp7 NM_007611 Mus musculus caspase 7	GGTATGGACGTCCGGAATGGGACGGACGACAAAGATGCAGGGG CCCTCCTTCAAGTGCTTCCAAAACCTGGGTTTGAAGTAAC CGTCCACAATGACTGCTCTTGTGCAAAGATGCAAGATCTG CTTAGAAAAA	GMDVRNGTDKDAGALFKCFQNLGFEVTV HNDCSCAKMQDLLRK	N-glycosylation site Protein kinase C phosphorylation site; Casein kinase II phosphorylation site
Casp9 NM_015733 Mus musculus caspase 9	GGTTTGTCTCCTGGAGGGACAAGAAAAGTGGCTCCTGGT ACATCGAGACCTTGGATGGCATTCTGGAGCAGTGGGCTCG CTCTGAAGACCTGCAGTCCCTCCTTCTCAGG	GFVSWRDKKKSGSWYIETLDGILEQWARS EDLQSLLLR	Protein kinase C phosphorylation site; Casein kinase II phosphorylation site

Table 5-1 (cont.): CCA-rich motifs in exons of mouse microarray genes regulated by hnRNP G. The according protein sequence is shown and a possible function of this exon in the proteins.

Gene	exon ID	Refseq	Exon sequence	Protein	Potential function
CPSF4 Homo sapiens cleavage and polyadenylation specific factor 4, 30kDa (CPSF4)	136.4.1	NM_006693	AGAGACTCTTCCACCTCCACCTCTGAGACCCTCCTCTCTGGA TCCCGGGACGCTCCCCTTGGTTCAGGACTCCTGACTCCA GGCCCCATTTCCACCGTCTGGGGACTCCTTTTACCCTCAC TCGGACTCAGGAGTTCACTGCCTCCGATCTTTGGCCTCTG CGGCTGTTCAAAG	RDSSHSILRPLLDPGTLP LVSGLLTPRPFPFPSGDSF YPHSDSGVHCLGSLALCGC SK	Protein kinase C phosphorylation site
CPSF6 Homo sapiens cleavage and polyadenylation specific factor 6, 68kDa (probe name)	148.1.1	NM_007007	GGACAGACTCCACCACGTCCACCCTTAGGTCCTCCAGCCC ACCTGGTCCACCAGGTCCTCCAATCGAGGAGATCGCCCT CCACCACCAGTTCTTTTCCTGGACAACCTTTTGGGCAGCCT TCCATTGGGTCCACTTCCTCCTGGCCCTCCACCTCCAGTTC CAGGCTACGGCCCCCTCCTGGCCCACCACCTCCACAACAG GGACCACCTCCACCTCCCAGCCCCCTTTCCACCTCGTCCACC CGGTCCACTTGGGCAACCCTTACACTAGCTCCTCCTCCGC ATCTTCCTGGACAGCTTTCTTTCCTCCACCAACTAACAGTGG CATGTGAACCAGTTTCTTTCCTGCCAGCTCCACCAACAGTGG CATGTCAACCATCAGACCGAGGTCCACCAACCAACAGATGG CATATGGCGACCTCCACCATATGATAGGGGTGACTATGGC CCCCCTGGA	GQTPPRPPLGPGPGPGPPG PPPPGQVLPPPLAGPPNRG DRPPPVLFPGQPFGQPPL GPLPPGPPPPVPGYGPPPG PPPPQQGPPPPGPFPPRP PGPLGPPLTLAPPPHLPGP PPGAPPAPHVNPAFFPPP TNSGMPTSDSRGPPPTDPY GRPPPYDRGDYGPPG	N-myristoylation site Cell attachment sequence Proline-rich region profile
DDX11 Homo sapiens DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, S. cerevisiae)	224.5.2	NM_030653	GACTTCACAACGGGATAAAGTGAAGTAGCTTCGGCTTGTGA ATGTGCATTGCAGACGACGTGGGAGAAGAAGCTGCAAAAGT CATTATAAGCAACAACCCTTGATCTTAGGGGGTGCCTCGT GAGAAGAGAGCTCTAAGCCTTTTGTAGAGGCATTAGAAAGG TATCGGAGCCACGGTGAAATGCAGGGGAGAGATTGGGTTTAGG GGCTTCCTGGTCGTCCATTCTGCTACAGCCGTTAAATGCCG CTAGATGGAGTGCGTGATTCTGGTATGGCCTCACGTGACC TGCTGCGAAGGATTGGAGAACCATGGTCTCTGCTTCCCAGA AAAAAGGAGAAATTTGG	DFTTG*	NMD
Fyn Homo sapiens FYN oncogene related to SRC, FGR, YES	907.002.002	NM_002037	AAAGCTGATGGTTTGTGTTTAACTTAACTGTGATTGCATC GAGTTGTACCCCACAAACTTCTGGATTGGCTAAAGATGCTT GGGAAGTTGCACGTCGTTCGTTGTGTCTGGAGAAGAAGCTG GGTCAGGGGTGTTCGCTCGCTGAAGTGTGGCTT	KADGLCFNLTVIASSCTPQ TSGLAKDAWEVARRSLCLE KKLGQGCFAEVWL	N-myristoylation site N-glycosylation site

Table 5-2: CCA-rich motifs in exons of human microarray genes regulated by hnRNP G. Continued next page.

Gene	exon ID	Refseq	Exon sequence	Protein	Potential function
NOL5A Homo sapiens nucleolar protein 5A (56kDa with KKE/D repeat)	127.3.1	NM_006392	GACCTGAATGCAGTTGTTCCCATGGCTGGGCCAGGCTTGC AGTGATGACTTGGCGAATCAAATCTGTCAATCCCCTGAGTGC AATCACTGATGTCTCCATGTCTCCTGAGCAATGCCTGATGGG GAGGGATCTAG	DLNAVVPMAGPGFAVMTCE SNLSIP*	N-glycosylation site Stop codon; NMD
SF3A2 Homo sapiens splicing factor 3a, subunit 2, 66kDa	150.2.1	NM_007165	GTGAGATCAGGGACTTGGGCGTGGGGGCGGCCAAAGGCAC CCAAGAGGCGGCTCTGCCACCCGGCCGTGTCCCTGCA	VRSGTWAWGAAAKGTQEAAL PPGRVPA	N-myristoylation site
SFRS3 Homo sapiens splicing factor, arginine/serine-rich 3	39.5.1	NM_003017	AGTCACCATCATGTCTCTTCTCACCACCCTCTGAATCTGCA T	SHHHVSSHHPLNLH	
SFRS6 Homo sapiens splicing factor, arginine/serine-rich 6 (SFRS6)	122.4.1	NM_006275	GTATGTTTGTAATCAAGATAGAAATGATATGACTAATGC TTTAAAGTAAACTCCTTTTATATTCTTATTTCTGGGAATTT ATTATGGTATATAGATGTTTTAAGATTTCCGAGTCTGACCA ATCTTGTCTTTCAG	VCFVIQDRNDMTNALK*	NMD
SRRM1 Homo sapiens serine/arginine repetitive matrix 1	110.3.1	NM_005839	ATGCAGATGGGAAAGCGATGGCAATCGCCAGTGACTTGGTA G	MQMGKRWQSPVTW*	Amidation site Stop codon, NMD
TAF15 Homo sapiens TAF15 RNA polymerase II, TATA box binding protein (TBP)–associated factor, 68kDa	247.1.3	NM_139215	GCTATGGTGGAGACAGAAGTGGGGGTGGCTATGGTGGAGAC AGAAGCAGCGGTGGCTGGCTACAGCGGAGATAGAAGTGGGGG CGGCTATGGTGGGACAGAAGTGGGGGTGGCTATGGTGGGG ACAGAGGCGGCGGCTATGGTGGGGACAGAGGAGGCGGCTAT GGAGGAGACCGAGGAGGTGGCTATGGAGGAGATCGAGGTGG CTATGGAGGAGACCGAGGTGGAGGCTATGGTGGAGACCGAG GAGGCTATGGAGGAGATCGAGGAGGTTACGGAGGAGATCGA GGAGGTTATGGAGGAGATCGAGGAG	AMVETEVGVAMVETEAAVV ATAEIEVGAAMVETEVGVA MVGTEAAAMVGTEEAAMEE TEEVAMEEIEVAMEETEVE AMVETEEAMEEIEEVTEEI EEVMEEIEE	Casein kinase II phosphorylation site N-myristoylation site
USP39 Homo sapiens ubiquitin specific protease 39	134.7.1	NM_006590	GGGACAGGCAAATGGTATGAATTACAAGACCTCCAGGTGAC TGACATCCTTCCCCAGATGATCACACTGTCAGAGGCTTACA TTCAG	GTGKWYELQDLQVTDILPQ MITLSEAYIQ	Protein kinase C phosphorylation site Casein kinase II phosphorylation site

Table 5-2 (cont.): CCA-rich motifs in exons of human microarray genes regulated by hnRNP G. The according protein sequence is shown and a possible function of this exon in the proteins.

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In conclusion, we suggest a working model for hnRNP G function in the cell (Figure 5-5). In the nucleus, hnRNP G can bind to exons containing CCA motifs and regulate splice site selection. For some exons this was shown to be phosphorylation dependent (e.g. SF3A2, NOL5A or SFRS3). Phosphorylation of hnRNP G controls shuttling of the protein. hnRNP G is tyrosine phosphorylated in the nucleus by nuclear tyrosine kinases, like c-Abl, Sik or RAK. Phosphorylation enables the protein to shuttle to the cytoplasm, probably transporting mRNA outside the nucleus. There it can be dephosphorylated by the protein tyrosine phosphatase PTP1B and interact with the membrane-bound tyrosine kinase c-Src. Shuttling back to the nucleus requires phosphorylation in the cytoplasm, which can be done by c-Src or other kinases. The role hnRNP G plays in the cytoplasm remains still to be investigated. A possible function can be a part in translation which is emphasized by preliminary experiments like CLIP analysis and running sucrose gradients.
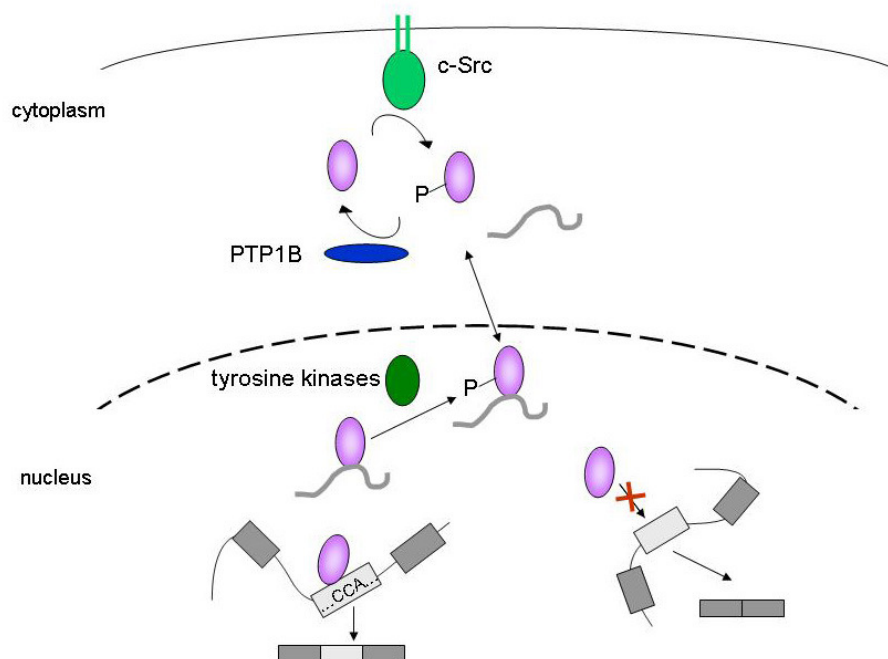
Figure 5-5: Working model for hnRNP G. hnRNP G (violet oval) binds to exons (grey boxes) which contain CCA-rich motifs and is able to regulate their splicing pattern. hnRNP G is phosphorylated in the nucleus by nuclear tyrosine kinases (dark green) and in the cytosol by c-Src (light green). Phosphorylation facilitates nuclear cytosolic shuttling, possibly to transport mRNA (thick grey lines). In the cytosol, it can be dephosphorylated by PTP1B (blue).

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CURRICULUM VITAE

Name: Bettina Heinrich
Date of Birth: 24.02.1975
Place of Birth: Fürth, Germany

Education:

03/2004 – 10/2007   Friedrich-Alexander University Erlangen-Nürnberg, Germany
Graduate student in Prof. Dr. Stefan Stamm's lab

11/1994 – 02/2001   Friedrich-Alexander University Erlangen-Nürnberg, Germany
Master of Biology (Diploma),
Diploma thesis: „Konstruktion eines Selektionssystems in *Saccharomyces cerevisiae* zur Isolierung von Tet Repressoren mit veränderten Eigenschaften"
Advisors: Dr. Christian Berens, Prof. Dr. Wolfgang Hillen

09/1985 – 07/1994   Heinrich-Schliemann-Gymnasium Fürth, Germany
Abitur, July 1994

09/1981 – 07/1985   Grundschule Soldnerstraße, Fürth, Germany

Research Experience

03/2004 – 10/2007   Friedrich-Alexander University Erlangen-Nürnberg, Germany
Research for PhD thesis

10/2004 – 11/2004   The Hebrew University of Jerusalem, Israel
Visiting scientist in Prof. Dr. Ruth Sperling's lab

12/2001 – 02/2004   Friedrich-Alexander University Erlangen-Nürnberg, Germany
Technician in Prof. Dr. Stefan Stamm's lab

05/2000 – 02/2001   Friedrich-Alexander University Erlangen-Nürnberg, Germany
Research for diploma thesis

03/1998 – 09/1999   Friedrich-Alexander University Erlangen-Nürnberg, Germany
Several practical courses as undergraduate student

Scholarships

10/2004-11/2004   Minerva short-term research grant

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