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SWITCH TO THE L ISOFORM OF THE MAP/MICROTUBULE AFFINITY-REGULATING KINASE 4 (MARK4) GENE, MAINLY EXPRESSED IN HUMAN GLIOMA, VIA PRE-mRNA ALTERNATIVE SPLICING MODULATED BY POLYPYRIMIDINE TRACT-BINDING PROTEIN (PTB)

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Abstract

MARK4 (MAP/Microtubule Affinity-Regulating Kinase 4) is a serine-threonine kinase that phosphorylates the Microtubule Associated Proteins (MAPs) taking part in the regulation of microtubule dynamics involved in cell cycle regulation and proliferation. MARK4 is ubiquitously expressed and two different isoforms are produced through alternative splicing: the constitutive MARK4S, consisting of 18 exons, and the alternative MARK4L encoded by a transcript that undergoes skipping of exon 16 and consequent frameshift of the reading frame. The differential expression of the two MARK4 isoforms in human tissues and, particularly in the Central Nervous System, point to their not fully overlapping roles. MARK4S is predominant in normal brain and differentiated neurons and has been associated to cell differentiation, while MARK4L is mainly expressed in neural progenitors and is up-regulated in hepatocarcinoma and glioma, suggesting a function of this isoform in cell proliferation. In glioma and glioblastoma-derived cancer stem cells (GBM CSCs), we pointed out an imbalance between the two MARK4 isoforms, directly proportional to cell de-differentiation and tumour grade. Such imbalance is triggered by decrease in MARK4S expression, associated with overexpression of MARK4L. The MARK4 expression profile observed in glioma matches that observed in mouse neural stem cells (NSCs). Moreover, MARK4L has been found expressed in the embryonic ventricular zone and adult sub-ventricular zone, both well known regions of neurogenesis, suggesting that the balance between the two MARK4 isoforms is critical for neural differentiation and proliferation. Therefore, in glioma a subverted MARK4L/MARK4S ratio may contribute to cellular de-differentiation and proliferation during gliomagenesis.

Having ruled out mutations or copy number loss/gain as cause of deregulation of MARK4 expression in glioma, we hypothesised that alterations at the post-transcriptional level, possibly in alternative splicing, might be at the origin of the observed MARK4 isoforms imbalance. MARK4L total mRNA evaluation by real-time PCR in glioma tissue samples and GBM CSCs, failed to reveal differences in the overall expression of the kinase, even in the presence of significantly different expression levels of the two isoforms, supporting the hypothesis of an alteration in alternative splicing at the root of MARKL/MARK4S imbalance in glioma.

Alternative splicing is the most important mechanism in generating proteomic diversity from a limited number of genes. Alternatively spliced isoforms expression is thinly controlled according to specific cells signalling and developmental stages by a complex interplay between spliceosome and splicing regulatory factors, which can be grouped into the two main classes of SR (serine-arginine rich) and hnRNP (heterogenous nuclear ribonucleoprotein) proteins. The intrinsic plasticity of alternative splicing in proteome modelling, make sthis process a tempting

target for cancer cells to enhance the production of protein isoforms sustaining tumour growth and spread. Protein isoforms produced from exon skipping, like MARK4L, are commonly found enriched in cancer, including glioma, as a consequence of hnRNP protein overexpression. We thus performed bioinformatic analysis to identify putative binding sites for hnRNP proteins in MARK4 pre-mRNA. Among the predicted putative binding sites for hnRNPs, three binding motifs for PTB (polypyrimidine-tract binding protein) appeared of particular interest, given the role of PTB in regulating neural stem cells proliferation and differentiation and its established involvement in glioma, where it is aberrantly overexpressed and induces the expression of protein isoforms produced by exon skipping. We found two putative PTB binding sites in intron 15 (IVS15) and one in intron 16 (IVS16). One of the two identified regulatory sequences in IVS15 is embedded in a polypyrimidine rich context and may represent a high affinity PTB binding site that could favour MARK4 exon 16 skipping by competition with U2AF65 or, together with the other PTB binding sites, by polymerisation or intron looping. A functional role of these sites is also suggested by the high sequence conservation between human and mouse.

Western blot analysis showed a significant overexpression of PTB in our astrocytoma and glioblastoma samples, correlating with MARK4L expression and thus with tumour grade and cell de-differentiation. Moreover, GBM CSCs, both undifferentiated and differentiated, showed high levels of PTB coherently with the nearly exclusive expression of MARK4L. However, while NSCs show undetectable levels of MARK4L upon differentiation, in differentiated GBM CSCs high expression levels of MARK4L are maintained possibly sustained by PTB overexpression.

We then performed minigene splicing assays to identify the specific sequences involved in MARK4 alternative splicing. We focused our attention on the polypyrimidine tract in MARK4 VS15 and constructed a MARK4 splicing minigene by cloning, in a mammalian expression vector, the MARK4 genomic region comprising exons 15, 16 and 17 and IVS15 and IVS16. Sequential deletions of different IVS15 portions revealed that the last 87 nucleotides of intron 15 contain a functional intronic splicing silencer (ISS). However, mutagenesis of the PTB binding site contained in this region did not affect minigene splicing, suggesting that PTB may be involved in MARK4 splicing by binding to a non canonical ISS or by cooperating with other identified PTB binding sites. Moreover, electrophoretic mobility shift assays (EMSA) highlighted a specific shifted band probably due to the interaction of splicing factors with MARK4 pre-mRNA. Mass spectrometry experiments are in progress aiming at identifying the

protein(s) bound to MARK4 IVS15, and seem to confirm the presence of PTB in the shifted band.

The achieved data, suggest that PTB overexpression in glioma may favour MARK4L expression causing the observed imbalance between the two MARK4 isoforms, featuring alternative splicing as an oncogenic mechanism that through the fine tuned regulation of MARK4 isoforms may foster proliferation and de-differentiation in glioma.

Introduction

1.1 Splicing

Pre-mRNA splicing is a fundamental process regulating gene expression in eukaryotes, given the split nature of eukaryotic genes. Intron removal and the consequent exon joining is catalysed by the spliceosome, a ribonucleoprotein complex that assembles on the pre-mRNA in different complexes. Spliceosome conformation and composition are dynamic, allowing accuracy and, at the same time, flexibility of the splicing machinery. In eukaryotes, there are at least two types of spliceosomes, U2-dependent spliceosome and the rare U12-dependent spliceosome, that differ in the regulatory sequences and the proteins involved. Some exons are constitutively spliced being included in every mature mRNA produced from a given pre-mRNA, while many are alternatively spliced to generate, from the same pre-mRNA, multiple splicing isoforms containing a different combination of exons. Data from high-throughput RNA sequencing of transcriptomes indicate that at least 95% of human multi-exon genes undergo alternative splicing (Pan *et al.*, 2008; Wang *et al.*, 2008) and the frequency of alternative splicing increases with cell types and species complexity (Barbosa-Morais *et al.*, 2012; Nilsen and Graveley, 2010). Therefore alternative splicing allows the production of protein isoforms of differing and even opposing functions from a single gene, featuring as the major contributor of proteomic diversity. Indeed, through the alternative splicing mechanism, isoforms expression is thinly regulated during development and according to specific cell signalling. The key role of alternative splicing in different cellular processes is confirmed by the fact that 50% of human genomic diseases arises from mutations in the consensus splice site sequences or in other auxiliary sequence elements. Moreover, the plasticity that characterises the mechanism of alternative splicing and its crucial involvement in proteome remodelling offers the opportunity to cancer cells to subvert the process and induce the expression of proteins that sustain tumour growth and spread.

1.1.1 Core splicing signals and the catalytic steps of splicing

As other mechanisms implicated in the regulation of gene expression, splicing involves both *cis*- and *trans*-acting components represented by specific sequences on the pre-mRNA and ribonucleoprotein factors, respectively. It is often very difficult to make a clear distinction between “constitutive” and “alternative” splicing components and usually the relative functional strengths of splice sites and RNA-protein interaction determine the exon fate. Constitutive splicing concerns short conserved sequences at the 5’ (GU) and 3’ (AG) splice sites and at the branch site (A nucleotide in a specific context YNYUR**A**Y) that provides information for the

identification of intronic sequences that have to be removed from pre-mRNA (Figure 1).

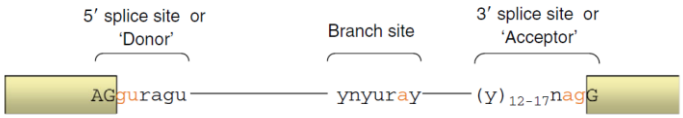


Figure 1. Consensus sequences for 5' splice site (donor), 3' splice site (acceptor) and branch site (Srebrow and Kornblihtt, 2006).

Additional regulatory sequences, defined exonic and intronic splicing enhancers (ESEs and ISEs) or silencers (ESSs and ISSs), modulate both constitutive and alternative splicing through the binding of protein factors that either stimulate or repress the assembly of the spliceosomal complexes at adjacent splice sites (Smith and Valcarcel, 2000). The recognition and assembly of splicing factors to both splice-site recognition and non-splice site sequences (ESE/ISE and ESS/ISS) constitute an integrated splicing code that ensures the high fidelity of the splicing process.

Introns are removed by the spliceosome in two consecutive transesterification reactions (Figure 2A and B). The U2-dependent spliceosome is composed of U1, U2, U5 and U4/U6 small nuclear ribonucleoproteins (snRNPs), in addition to numerous non-snRNP proteins. Each snRNP is formed by small nuclear RNA (snRNA), a common set of seven Sm proteins (B/B', D3, D2, D1, E, F and G) and a variable number of particle-specific proteins (Will and Lührmann, 2006). Nascent RNA transcribed from RNA polymerase II is bound by the earliest spliceosomal complex, the E complex. In particular, the U1 snRNP is recruited to the 5' splice site while SF1/BBP (branch site binding protein) and U2AF, all non-snRNP factors, bind to the branch site and the downstream polypyrimidine tract, respectively. Subsequently, the U2 snRNP associates with the branch site forming the A complex, also defined pre-spliceosome. The U4/U6.U5 tri-snRNP is recruited to form the catalytic B complex. Following RNA-RNA and RNA-protein interaction rearrangements destabilise the U1 and U4 snRNPs giving rise to the activated spliceosome or the B^{act} complex (Figure 2A) (Will and Lührmann, 2011; Smith and Valcárcel, 2000). The activated B complex catalyses the enzymatic reactions that allow intron excision and exons joining. The 2'OH group of the branch adenosine in the intron, through a nucleophilic attack on the 5' splice site, cleaves this site and ligates the 5'-end of the intron to the branch adenosine, resulting in the formation of a lariat structure (Figure 2B). The 3' splice site is then attacked by the 5' end of the exon, leading to the 5' and 3' exons joining and the release of the

intron. At the end of the catalytic process the spliceosome disassembles and other heterogeneous ribonucleoproteins (hnRNP) bind to the mature mRNA to facilitate nucleocytoplasmic export (Will and Lührmann, 2011). Even if proteins that constitute the spliceosomal complex have been always considered the main actors of splicing, it has been recently proposed that pre-mRNA catalysis is at least partially RNA-based with a mechanism similar to that of self splicing introns, with U2 and U6 playing key roles (Wachtel and Manley, 2009).

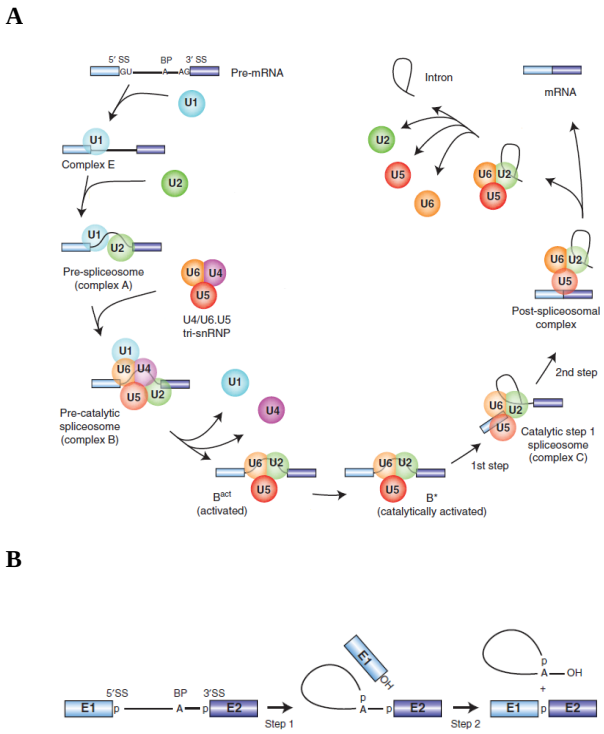


Figure 2. Pre-mRNA splicing catalysed by U2-dependent spliceosome. **A**) Assembly and disassembly of the U2-dependent spliceosome. **B**) Schematic representation of the two transesterification reactions of pre-mRNA splicing (Adapted from Will and Lührmann, 2011).

1.1.2 Alternative splicing

The fidelity of the splicing process mostly resides in the recognition of splice sites. Even if the spliceosome, with the dynamic and thinly controlled assembly of different components, ensures the correct identification of constitutive splice sites, a certain degree of freedom is enabled. The splicing mechanism has actually evolved to increase proteome diversity in the presence of a relative small number of genes. Therefore, alternative splicing generates different mRNAs encoding different protein products, increasing the coding capacity of genes. Weak splice sites, that deviate from the consensus sequence, are the key players of alternative splicing. They show less affinity for the spliceosome since their sequence differs from the canonical one. A second

class of *cis*-acting elements enhances the recognition and use of weak splice sites: enhancers and silencers of splicing. These are short (maximum 10 nucleotides) conserved sequences placed in exons or introns, either isolated or in clusters, that stimulate or inhibit the use of weak splice sites. Exonic/intronic splicing enhancers (ESE/ISE) typically bind members of the Ser/Arg-rich (SR) family of proteins, while splicing silencers (ESS/ISS) are binding sites for heterogeneous nuclear ribonucleoproteins (hnRNPs) (Figure 3).

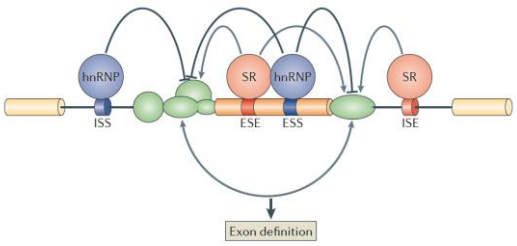


Figure 3. Schematic representation of exon definition by ESE/ISE and ESS/ISS. SR (red) and hnRNP (bleu) proteins bind to enhancer and silencer sequences and target components of the spliceosome (green) associated with the 5' and 3' splice sites of the alternative exon favouring or inhibiting the recognition and use of these weak splice sites (Kornblihtt *et al*., 2013).

These splicing activators and repressors commonly affect the formation of the E complex and result in one of the five observed alternative splicing events (Figure 4): i) cassette exons are single exons that can be included in the mRNA or skipped. They can also be subdivided into "skipped" or "cryptic" exons on the basis of whether the main isoform includes or excludes the exon, respectively; ii) mutually splicing events, instead, implicate the selection of one exon among an array of two or more alternative exons; iii) competing 5' and 3' splice sites are "exon modifications" that result in exons of different sizes. Other rarer alternative splicing events include retained introns, alternative splicing associated with alternative promoters and alternative 3'-end processing (Matlin *et al*., 2005).

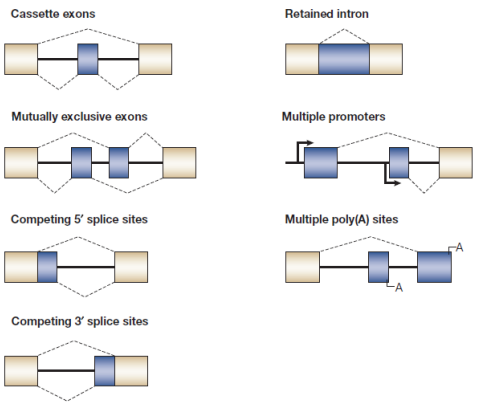


Figure 4. Alternative splicing patterns reported so far (Matlin *et al*., 2005).

1.2 Regulation of alternative splicing by SR and hnRNP proteins

Regulatory SR proteins and hnRNPs usually bind to short degenerate sequences, frequently represented in the genome, and a single binding site is generally sufficient to define splice site use (Singh and Valcárcel, 2005). Splicing enhancers are usually recognized by SR proteins, leading to the recruitment of U1 and U2 snRNPs and U2AF to the pre-mRNA. Thus, SR proteins induce RNA-RNA and RNA-protein interactions that allow splice sites definition and correct positioning of the spliceosome for the catalysis of splicing reactions. Splicing silencers are, instead, recognised by hnRNPs that antagonise the effect of SR proteins but their mechanism of action are less known compared to SR proteins (Busch and Hertel 2012). Modulation of SR and hnRNP proteins expression thus allows developmentally, tissue specific and signalling regulated alternative splicing.

1.2.1 SR proteins

SR proteins were originally discovered in *Drosophila* in the early 1990s (Fu 1995). They have a modular structure composed of one or two RRM (RNA recognition motif) at the N-terminus, that confers to the protein the RNA-binding specificity, and a C-terminal RS domain (serine-arginine rich) that promotes protein-protein interaction needed for spliceosome recruitment (Wu and Maniatis 1993; Kohtz *et al*., 1994). SR proteins function in both constitutive and alternative splicing favouring the inclusion of alternative exons in the mature mRNA (Long and Cáceres, 2009). Three different mechanisms of action have been proposed for SR proteins (Figure 5). In the "recruitment model", SR proteins bound to ESEs recruit and stabilise the interactions of U1 snRNP with the 5' splice site and of U2AF65 with the 3' splice site (Graveley *et al.*, 2001). According to the "inhibitor model", instead, enhancer-bound SR proteins antagonise the negative effect of hnRNPs (Zhu *et al*., 2001). SR proteins may also get organised in a network across introns to juxtapose the 5' and 3' splice sites and loop out the intron (Wu and Maniatis, 1993).
The functions of SR proteins go beyond constitutive and alternative splicing: SR proteins play a role also in mRNA nuclear export, nonsense-mediated decay (NMD) and translation (Huang and Steitz, 2005). SR proteins have a nuclear localisation signal and accumulate in nuclear speckles (Lamond and Spector, 2003). However, a subset of SR proteins shuttle continuously between the nucleus and the cytoplasm and function in the nucleocytoplasmic export of mRNA by interacting with the nuclear export apparatus (Cáceres *et al.*, 1998). SR proteins have also been implicated in the NMD pathway. In particular, overexpression of SF2/ASF, SC35 and SRp40 SR proteins was found to enhance NMD (Zhang and Krainer, 2004). Moreover, a subset of SF2/ASF are

implicated in pre-mRNA splicing of kinases involved in translation, including MNK2 and S6K1 (Karni *et al.*, 2007). SR proteins have also been demonstrated to directly affect translation, by interacting with polyribosomes, and IRES-mediated translation (Sanford *et al.*, 2004; Michlewski *et al.*, 2008).

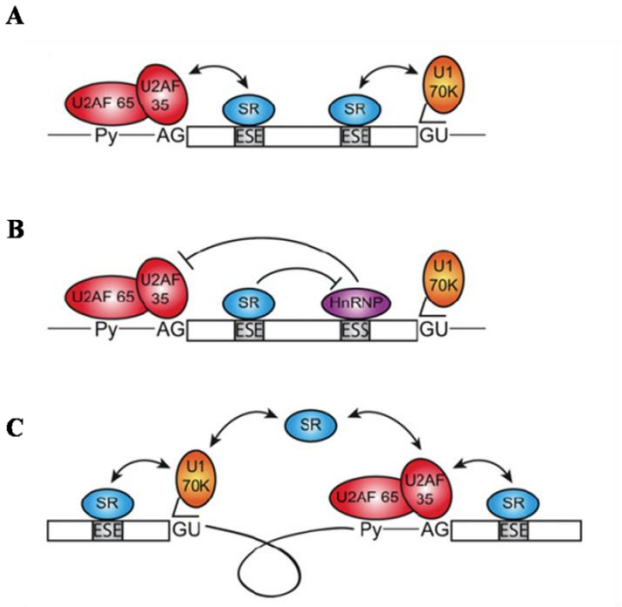


Figure 5. Mechanisms of action of SR proteins. **A**) Recruitment model, **B**) inhibitor model and **C**) network of protein-protein interactions to loop out the intron (Long and Cáceres, 2009).

1.2.2 hnRNP proteins

HnRNPs are modular proteins consisting of different domains connected by a liker domain. Each hnRNP contains at least one RNA-binding motif including an RNA recognition motif (RRM), a hnRNP K homology domain (KH) or an arginine/glycine-rich (RGG) box (Dreyfuss *et al.*, 1993; Krecic and Swanson 1999). HnRNPs localise in the nucleus, but are excluded from the nucleolus, and most of them shuttle between the nucleus and cytoplasm (He *et al.*, 2005). HnRNPs associate to the nascent pre-mRNA by binding to ESSs and ISSs and inhibit the use of 3' splice site or favour the use of more distal 5' splice sites. HnRNPs generally antagonise the function of SR proteins (Martinez-Contreras *et al.*, 2007). Binding of hnRNPs to ESSs or ISSs along the intron-exon junction limits the access of the splicesome to the splice sites and of SR proteins to splicing enhancer sequences (Mayeda and Krainer 1992) (Figure 6A). Binding of hnRNPs sterically blocks the binding ofU2 snRNP and inhibits spliceosome assembly (Tange *et al.*, 2001) (Figure 6B). Moreover, binding of hnRNPs to both introns flanking the alternative

exon allows interaction between hnRNPs and favours the looping out of the exon (Nasim *et al.*, 2002) (Figure 6C).

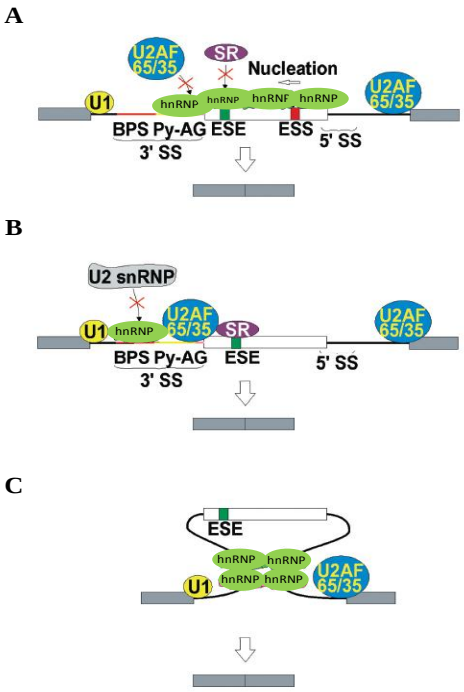


Figure 6. Mechanisms of hnRNP-mediated splicing (adapted from He and Smith, 2009).

In addition to pre-mRNA splicing, other important functions of these proteins include telomerase biogenesis/maintenance (Zhang *et al.*, 2006), transcription (Xia 2005), nuclear import-export (Brumwell *et al.*, 2002), cytoplasmic trafficking of mRNA (Ma *et al.*, 2002), mRNA stability (Hamilton *et al.*, 1999) and translation (Bonnal *et al.*, 2005). As regards the roles in DNA stability hnRNPs seem to destabilise G-quartets structures, that are crucial for DNA replication, transcription and telomere maintenance (Fukuda *et al.*, 2002). HnRNPs also take part in DNA replication. In particular, UP1 activates the DNA polymerase α, that synthesises an RNA-DNA primer and allows the formation of the Okazaki framgments during the synthesis of the lagging DNA strand (Chai *et al.*, 2003). Moreover, hnRNP A1 interacts with DNA topoisomerase I, which cleaves one strand of duplex DNA, relaxing supercoiled DNA and then regulating DNA topology during replication, chromosome condensation and transcription (Gupta et. al, 1995). The role of hnRNPs in DNA metabolism also includes telomeres maintenance as hnRNPs associate with the 3'single-stranded telomeric extension and protect it from nuclease attack (Zhang *et al.*, 2006). HnRNP proteins function as a bridge between the telomeric DNA template and the RNA component of telomerase. Furthermore, hnRNPs play a role in DNA repair.

HnRNP B1 associates with the DNA-dependent protein kinase that mediates DNA double-strand breaks repair (Iwanaga *et al.*, 2005).

Although hnRNPs preferentially bind to RNA, some of them have been demonstrated to associate with promoter sequences and thus regulate transcription. For example, hnRNP A1 binds the promoters of *c-myc*, *APOE*, *thimidyne kinase*, *γ-fibrinogen* and *vitamin D receptor* genes, even if the mechanisms of action are unknown (Takimoto *et al.*, 1993, Campillos *et al.*, 2003, Lau *et al.*, 2000). Similarly to SR proteins, also hnRNPs are involved in the nuclear export of mRNAs, but the molecular mechanisms are not well understood. It has been supposed that hnRNPs may associate with components of the nuclear export complex (Michael *et al.*, 1995).

1.3 Other levels of regulation of alternative splicing

Alternative splicing is one of the most exploited mechanisms underpinning dynamic remodelling of the transcriptome according to specific cell signalling and developmental stages. Different regulatory networks have been identified that affect alternative splicing throughout physiological changes. Different observations support this fundamental role of alternative splicing: i) any modification at the transcriptional level is accompanied by a co-integrated post-transcriptional response; ii) physiological splicing modifications are finely tuned in a spatio-temporal manner; iii) splicing modifications that are associated with specific cell signalling are phylogenetically conserved; iv) genome-wide studies have revealed the presence of a set of genes that are regulated at the transcription level, with modification of mRNA expression without differences in the expression of splice variants, and a second set of genes characterised by changes in the expression of mRNA isoforms without changes in the total expression (Kalsotra and Cooper, 2011). Therefore, the use of alternative exons can be controlled by the cells in a developmental, tissue-specific or pathology-specific stage. Different mechanisms and signal transduction pathways have been linked to the regulation of alternative splicing. In this context alternative splicing, transcription, chromatin structure and transduction pathways are integrated to ensure transcriptome dynamic changes according to cell requirements (Figure 7).

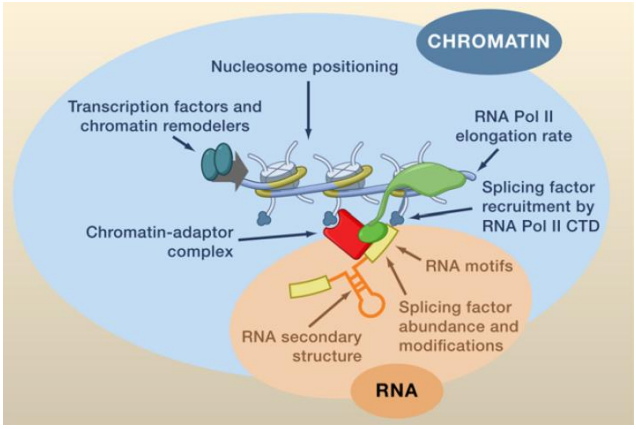


Figure 7. Integrated model for the regulation of alternative splicing (Luco *et al.*, 2011).

1.3.1 Phosphorylation of splicing factors and related underlying transduction pathways

The regulation of the phosphorylation status of splicing factors is crucial for the correct outcome of splicing patterns. Phosphorylation is, in fact, the main mechanism for regulating the activity

and the cellular localisation of SR and hnRNP proteins. Phosphorylation of splicing factors is carried on by different protein kinases, some specific and other involved in common signal transduction pathways that allow modulation of alternative splicing patterns according to external or internal cues. SR-protein specific kinases include SR-protein kinases (SRPKs) which regulate, in the nucleus, the release of SR proteins to the nucleoplasm from the nuclear speckles and, in the cytoplasm, their nuclear import (Yun and Fu, 2000; Wang *et al.*, 1998). Cyclin-dependent like kinases (CLKs) represent another class of SR protein kinases mainly involved in the regulation of SR proteins subcellular localisation (Naro and Sette 2013).

Signal transduction pathways have also been found to regulate alternative splicing according to physiological and pathological stimuli. The Ser-Thr kinase AKT, for example, is a key component of the phosphoinositide-3-kinase-protein-kinase (PI3K) pathway and transduces the signals of growth factors and cytokines. It has been reported that AKT phosphorylates both SR and hnRNP proteins that contain, in the RS domain, multiple AKT phosphorylation consensus sequences (RXRXXS/T) (Obata *et al.*, 2000) and is able to regulate the activity of the SRPKs, achieving the coordinated modulation of both activators and effectors (Zhou *et al.*, 2012).

Mitogen-activated protein kinases (MAPKs) are Ser-Thr kinases that control metabolism, proliferation, survival, differentiation and motility, through modulation of the protein activity and stability, and alternative splicing (Cargnello and Roux, 2011). Indeed, the RAS-RAF-MEK-ERK pathway is involved in the inclusion of the alternative v5 exon in the CD44 mRNA upon T-cell activation. Phosphorylation by ERK of the splicing regulator Sam68 reduces its affinity for U2AF65 and thus the recognition of the 3' splice site (Matter *et al.*, 2002).

cAMP-dependent protein kinase (PKA) is another kinase with a well established role in the regulation of alternative splicing. First evidences on the involvement of PKA in the alternative splicing modulation, came from the observation that a fraction of the active subunit of the kinase translocates to the nucleus, colocalises with SRF2 and phosphorylates several SR proteins (Kvissel *et al.*, 2007). Subsequently, several stimuli that lead to the increase in the amount of intracellular cAMP were shown to affect alternative splicing through phosphorylation of both SR and hnRNP proteins. For example, forskolin, that stimulates the synthesis of cAMP, modulates the inclusion of exon 10 in Tau mRNA (Shi *et al.*, 2011). PKA also modulates alternative splicing of genes that are crucial for neuronal differentiation through the phosphorylation of hnRNP K. Phosphorylation increases the binding activity of hnRNP K to its target mRNAs in competition with the spliceosomal component U2AF65, impairing the recognition of the 3' splice site and leading to the skipping of target exons (Cao *et al.*, 2012).

Some other proteins were found to have an unexpected kinase activity towards splicing factors. Among these proteins DNA topoisomerase I has been shown to phosphorylate several SR proteins, even in the absence of a canonical ATP binding site (Labourier *et al.*, 1998) and the dual activity of the DNA topoisomerase I may allow the coordination of DNA transcription and splicing. Also Aurora kinase A (AURKA) was demonstrated to be involved in the alternative splicing of BCL-X. AURKA positively regulates the expression of the antiapoptotic variant, BCL-X_L through stabilisation of SRF1 splicing factor (Moore *et al.*, 2010).

1.3.2 Coupled transcription and splicing

More than 20 years ago electron microscopy on *Drosophila* embryos provided the first evidence that splicing can occur cotranscriptionally (Beyer and Osheim, 1988). Cotranscriptionality of splicing was then demonstrated also in humans for the dystrophin gene (Tennyson *et al.*, 1995). Cotranscriptionality does not mean that the two events are coupled, but only that splicing is committed or catalysed before transcription termination and release of mRNA. In this context, the transcription and the splicing apparatus interact with each other and one influences the outcome of the other. Efficient coordination of the two mechanisms is mainly achieved through modification of the promoter and by the C-terminal domain (CTD) of RNA polymerase II.

Recent studies have actually highlighted that the type of promoter used to drive transcription by RNA polymerase II may impact alternative splicing (Cramer *et al.*, 1997). Two models have been proposed. In the "recruitment model", changes in the promoter architecture affect the recruitment of splicing factors to the nascent mRNA. Instead, in the "kinetic model", the promoter structure influences RNA polymerase II elongation rate, so that the splicing machinery has more or less time to recognise and splice alternative exons (Kornblihtt, 2007).

Targeted experiments highlighted that the rate of RNA synthesis affects its secondary structure that in turn affects splicing. Experimental data support the hypothesis of the so called "first come, first served" model of splicing (Aebi and Weissmann, 1987). In this model, slow elongation, favours the removal of the upstream intron of an alternative cassette exon before the removal of the downstream intron. In an alternative version of the same model slow elongation allows the recruitment of splicing factors to the upstream intron before the downstream intron is synthesised.

The C-terminal domain (CTD) of RNA polymerase II provides an additional link between transcription and splicing. The CTD can undergo posttranslational modifications by phosphorylation of specific residues and differentially affects transcription and RNA processing (Hsin and Manley, 2012). Affinity chromatography allowed the identification of splicing factors

as CTD-binding proteins and phosphorylated CTD has been demonstrated to stimulate splicing in vitro through the association with the core of spliceosome (Hsin and Manley, 2012; David *et al.*, 2011). These evidences added to the finding that CTD is more efficient in promoting splicing of a substrate with associated exon definition factors, raised the hypothesis that CTD might function as a platform to assist exon definition and commitment complex formation.

1.3.3 Chromatin and histone modifications as regulators of alternative splicing

RNA-binding elements, RNA polymerase II elongation rate and CTD cannot fully explain the regulation of alternative splicing. Recently, a role of chromatin structure and histone modifications have been pointed out (Luco *et al.*, 2011). A first evidence on the involvement of chromatin structure in alternative splicing came from studies that highlighted the physical interaction between the histone acetyltransferase STAGA with U2 snRNP (Martinez *et al.*, 2001) and the histone arginine methyltransferase CARM1 with U1 snRNP (Cheng *et al.*, 2007), suggesting a role for chromatin complexes in facilitating the assembly of spliceosome to the nascent mRNA. Moreover, genome-wide mapping of nucleosome positioning showed that nucleosomes are non-random positioned along genes and are mainly enriched at intron-exon junctions, thus marking exons (Andersson *et al.*, 2009). Interestingly, the average size of a mammalian exon equals the length of the DNA wrapped around a nucleosome, suggesting a protective role of nucleosome (Schwartz *et al.*, 2009). Furthermore, included alternative exons are more enriched in nucleosome than excluded exons (Schwartz *et al.*, 2009) and nucleosome density is higher in correspondence of weak splice sites (Spies *et al.*, 2009). It has been also demonstrated that nucleosomes behave as a barrier that locally modulate RNA polymerase II elongation rate inducing its pausing, so that nucleosomes and RNA polymerase II may cooperate in the regulation of alternative splicing by favouring the recruitment of splicing factors (Hodges *et al.*, 2009a).

Histone modifications are emerging as important regulators of alternative splicing. Genome-wide analysis of histone modifications, revealed that they are non random distributed in the genome with some modifications specifically enriched in exons (Kolasinska-Zwirz *et al.*, 2009). In particular, trimethylated H3K36 (H3K36me3), H3K4me3 and H3K27me2 are higher in alternatively spliced exons while H3K9me3 is depleted (Dhami *et al.*, 2010; Spies *et al.*, 2009). Moreover, the level of histone modifications does not correlate with the transcription activity of genes, supporting a role of histone modifications in alternative splicing (Andersson *et al.*, 2009). Additional evidences come from treatment of cells with the histone deacetylase inhibitor

trichostatin A (TSA) that causes skipping of the alternative exon 33 of *fibronectin* and exon 18 of the *neural cell adhesion molecule* (Nogués *et al.*, 2002). Another clear example of histone modification-guarded alternative splicing is represented by the *human fibroblast growth factor receptor 2* (*FGFR2*), whose exons IIIb and IIIc are mutually exclusive included in the mature mRNA depending on the cell specific type in which the receptor is expressed. In mesenchymal cells, H3K36me3 and H3K4me3 histone modifications are enriched in exon IIIc, that is preferentially included, while in epithelial cells the same histone modifications are enriched in exon IIIb, achieving by this way a tissue-specific isoform expression (Luco *et al.* 2010). Also DNA methylation has been proposed as a mechanism of alternative splicing regulation based on the non random presence of DNA methylation patterns across the genome and on the correlation between the methylation levels and H3K36me3 (Hodges *et al.*, 2009b).

Recently, a direct physical crosstalk between chromatin and splicing machinery has emerged from studies of genes whose alternative splicing is induced by the polypyrimidine-tract binding protein (PTB) splicing factor. Indeed, PTB-spliced genes are enriched in H3K36me3 and depleted in H3K4me3 at the level of alternatively spliced regions (Luco *et al.*, 2010). The mechanism by which H3K36me3 influences alternative splicing is not imputable to the modulation of RNA polymerase II elongation rate, but to the formation of a chromatin platform that facilitates the recruitment of the splicing machinery to the nascent RNA via an adaptor protein, MRG15 (Luco *et al.*, 2010). High levels of the H3K36me3 attract MRG15 that recruits PTB, in this way, to the nascent RNA that in turn causes the skipping of the alternative exon. If levels of H3K36me3 are low, PTB cannot be recruited to the pre-mRNA and thus the PTB-dependent alternative exon is included in the mature mRNA (Luco *et al.*, 2010). Similar mechanisms of action involve other chromatin-splicing adaptor systems. For example, the chromatin-adaptor protein CHD1 seems to play a role in the recruitment of the early spliceosome to the cyclin D1 pre-mRNA via H3K4me3 (Sims *et al.*, 2007). Moreover, mass spectrometry analysis revealed that also HP1α/β acts as a chromatin-adaptor protein by interacting with H3K9me and recruiting SRp20 and ASF/SF2 alternative splicing factors (Loomis *et al.*, 2009).

1.3.4 ncRNA in alternative splicing

Recently ncRNAs have been pointed out as novel modulators of alternative splicing. One of the proposed mechanisms involved the regulation of a key splicing factor by microRNA (miRNA) during development and differentiation. For example, the neuron-specific miR-124 directly regulates the expression of the splicing repressor PTB, triggering a neuronal-specific splicing program essential for differentiation of neural progenitor into mature neurons (Makeyev *et al.*,

2007). In differentiating myoblasts, instead, upregulation of miR-133 leads to downregulation of nPTB and the activation of a muscle-specific differentiation program (Boutz *et al.*, 2007). Apart from microRNA, other ncRNAs have been implicated in the regulation of alternative splicing. The long ncRNA MALAT-1 seems to control the pool of available SR proteins in the nucleoplasm, by binding and sequestering SR proteins in nuclear speckles (Tripathi *et al.*, 2010). In addition to this indirect mechanism, a direct regulation of alternative splicing by ncRNAs has been suggested. The brain-specific small nucleolar RNA (snoRNA) HB-52II is processed in psnoRNAs, smaller variants that are reported to complementary bind to a silencer element in exon Vb of the serotonin receptor 5-HT2C pre-mRNA. By this way psnoRNAs mask the binding site for a splicing repressor thus favouring alternative exon inclusion (Kishore *et al.*, 2010; Kishore and Stamm, 2006).

1.4 Alternative splicing and cancer

The plasticity offered by alternative splicing to model the proteome according to specific cell requirements and developmental stages, makes alternative splicing a tempting target for cancer cells to enhance the production of protein isoforms sustaining tumour growth and spread. Subverted alternative splicing is commonly observed during tumour initiation and progression and accompanies the known and emerging hallmarks of cancer cells including reprogrammed metabolism, evasion of apoptosis, deregulated cell cycle control, chromosomal instability, invasion, metastasis and neoangiogenesis (Venables 2004; Ghigna *et al.*, 2008, Hanahan and Weinberg, 2011). Two main alterations in differentially spliced isoforms in tumours have been identified: the expression of aberrant isoforms or the re-expression of protein isoforms usually thinly associated with distinct developmental stages and donwnregulated in differentiated cells (Figure 8). The first class of alterations are caused by mutations in oncogenes and oncosuppressors leading to aberrantly spliced isoforms that are advantageous to transformed cells and positively selected during tumour progression (Grosso *et al.*, 2008). On the other hand, specific splice variants are reported to be commonly found enriched in cancer tissues where the spliced genes are not mutated, indicating that the defects imply alterations in the mechanism of splice site selection. It has been reported that these alterations mainly consist of *cis*-acting mutations of splice sites or splicing regulatory sequences or deleterious modifications in *trans*-acting factors (Grosso *et al.*, 2008).

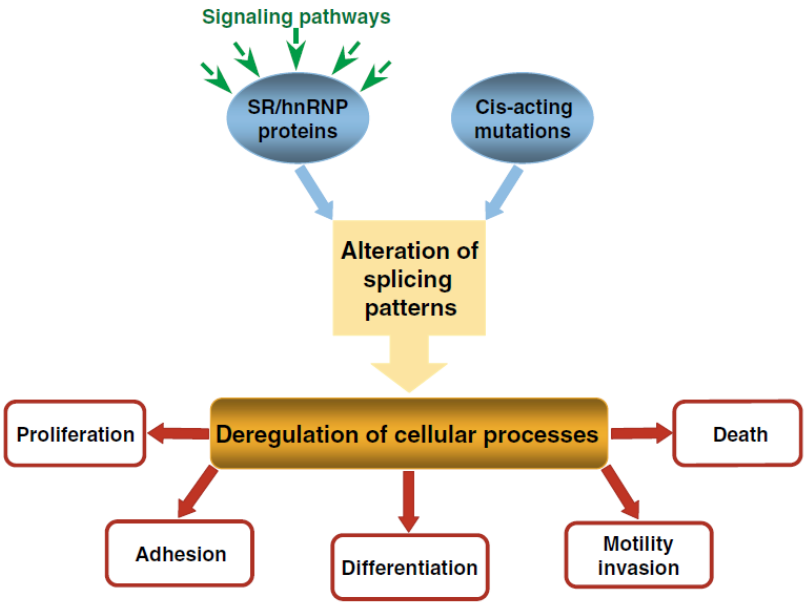


Figure 8. Alterations of splicing patterns commonly found in cancer (Srebrow and Kornblihtt, 2006).

1.4.1 *Cis*-acting mutations

Inherited or somatic mutations affecting splicing process can be subdivided in two subclasses: i) class I, occurring in 60% of cases, includes splicing mutations at the canonical splice sites that completely abolish exon recognition; ii) class II mutations affect either the variable motifs (polypyrimidine tract and regulatory sequences) or are associated with the formation of cryptic donor or acceptor splice sites (Ghigna *et al.*, 2008).

Kruppel-like factor 6. KLF6 is a zinc finger transcription factor that acts as a tumour suppressor by inhibiting cell growth via activation of the cyclin-dependent kinase inhibitor p21. A single nucleotide polymorphism associated to prostate cancer creates a new binding site for the SR protein SRp40 leading to increased expression of the KLF6-SV1 isoform. This alternative isoform results from the use of an alternative 5' splice site and encodes for a protein that lacks the zinc finger DNA-binding domain but retains the activation domain (DiFeo *et al.*, 2009; Narla *et al.*, 2005a). KLF6-SV1 acts as a dominant-negative protein and antagonises the activity of the canonical KLF6, favouring cell proliferation and migration (Narla *et al.*, 2005b).

Cyclin-dependent kinase inhibitor 2A. The *CDKN2A* gene encodes two tumour suppressors, $p14^{ARF}$ and $p16^{INK4a}$ that control the G1/S transition via the Rb and p53 proteins, respectively. Loss of function of these two proteins has been associated to melanoma development (Nelson and Tsao, 2009). A new mutation in the acceptor splice site of intron 1, that promotes skipping of exon 2 in both $p14^{ARF}$ and $p16^{INK4a}$ and consequent protein inactivation, has been found in a family with melanomas, neurofibromas and multiple dysplatic naevi (Petronzelli *et al.*, 2001).

Liver kinase B1. LKB1 is a tumour suppressor gene that encodes for a Ser-Thr kinase involved in different cellular processes (cell cycle arrest, p53-mediated apoptosis, cell polarity etc.). Mutations in LKB1 are associated to the Peutz-Jeghers Syndrome (PJS, OMIM #175200), an autosomal dominant disorder characterised by gastrointestinal polyposis and increased cancer risk. A fraction of PSJ patients carries a mutation at the 5' splice site of intron 2. The mutated splice site is still used but in conjunction with a cryptic 3' splice site close to the canonical one, leading to frameshift and premature termination codon in the aberrantly spliced isoform (Hastings *et al.*, 2005).

Receptor tyrosine kinase KIT. C-kit is aproto-oncogene constitutively activated in multiple cancers and found to be activated by aberrant splicing in some cases of gastrointestinal stromal tumours (GISTs). Patients carrying a deletion of the intron-exon segment that harbours the 3' splice site of intron 10 show a constitutively active aberrant protein isoform produced by a new exonic 3' splice site created within exon 11 (Chen *et al.*, 2005).

Liver intestine cadherin. CDH17 is a cell-cell adhesion protein overexpressed in hepatocellular carcinomas, gastric and pancreatic cancers. Splice variants with skipping of exon 7 have been associated with poor prognosis and a high incidence of tumour recurrence. Skipping of exon 7 is mainly caused by a point mutation that disrupts the branch site in intron 6 and a second point mutation at position 651 in exon 6 which might either generate an ESS or disrupt an ESE (Wang *et al.*, 2005).

Breast cancer type 1 susceptibility protein. BRCA1 is a well-known breast and ovarian cancer predisposing gene. The first splicing mutation identified in this gene was a nonsense mutation in exon 18 that actually disrupts an ESE leading to skipping of exon 18 (Mazoyer *et al.*, 1998). Since the identification of this splicing mutation, a multitude of mutations affecting BRCA1 splicing have been reported and *in silico* analysis of splicing regulatory sequences in BRCA1 revealed that at least 60% of these sequences are target of mutation (Pettigrew *et al.*, 2005).

1.4.2 Alteration in *trans*-acting factors

Most cancer-associated aberrant splicing are not associated to mutations in the affected gene, suggesting alterations in the expression and/or activity of splicing regulatory factors (Vénables 2004). Indeed, alterations in the repertoire of SR and hnRNP proteins are frequently observed in cancer accompanied by alterations in the relative expression of splicing isoforms, which represent an hallmark of cancer cells (Ladomery, 2013). Therefore, specific alternative spliced isoforms are favoured in tumours and appear fundamental to sustain the transformation process (Singh *et al.*, 2004; Zhou *et al.*, 2003).

CD44. The trans-membrane glycoprotein CD44 has been one of the first genes for which changes in alternative splicing were demonstrated to be associated with alterations in growth signalling pathways. CD44 is involved in cell-cell and cell-matrix interactions and different isoforms are produced by inclusion of 10 alternative exons (v1-v10) belonging to its extra-cellular domain (Naor *et al.*, 2002). The CD44 isoform lacking all the alternative exons, is

predominantly expressed in normal tissues, while alternative isoforms, in particular those containing v5, v6 and v7, are overexpressed in tumours where they promote cell invasion and metastasis (Naor*et al.*, 2002). In tumours it has been demonstrated a direct correlation between expression levels of the alternative CD44 isoforms, overexpression of SR proteins and alterations in the expression of other splicing factors, among which hnRNPA1, SRp55, SF2/ASF, Tra-2-beta, YB-1 and Sam68 (Ghigna *et al.*, 1998; Stickeler*et al.*, 1999; Watermann *et al.*, 2006). In particular, Matter and colleagues demonstrated that, in cancer cells, Sam68 (Src-associated in mitosis 68-kDa) favours v5 inclusion as a consequence of activation of the Ras-Raf-Mek-Erk pathway (Matter *et al.*, 2002). They suggested that phosphorylated Sam68 binds exon v5 and blocks the repressive activity of hnRNP A1, confirming the direct connection between a mitogenic signalling pathway and alternative splicing. Afterwards, Cheng and Sharp proved that also SRm160 is another Ras-regulated splicing factor involved in CD44 exon v5 inclusion and correlated this tumour-specific alternative splicing to invasiveness and metastasis (Cheng and Sharp 2006).

Fibronectin. Fibronectin is an extracellular matrix component that has been associated to proliferation, migration, invasion and metastasis in tumours. Indeed, the fibronectin alternative isoform EDA, produced by the inclusion of an additional exon, is expressed at low levels in normal adult tissues, but is detectable in embryos, during wound healing process and in tumours. Growth factors appear to induce EDA exon inclusion through activation of the Ras/PI3-kinase/AKT pathway. Direct phosphorylation of the SR proteins 9G8 and SF2/ASF by AKT promote the recognition of EDA exon by the spliceosome (Blaustein *et al.*, 2005).

Bcl-X. Bcl-X is a member of the Bcl-2 family and is involved in apoptosis. Two alternative isoforms are produced through the use of alternative 5' splice sites in exon 2: Bcl-XL, with pro-apoptotic function and the antiapoptotic Bcl-XS (Akgul *et al.*, 2004). Different splicing factors have been associated to the Bcl-X alternative splicing. Overexpressed Sam68, in particular, has been demonstrated to induce the production of Bcl-XS. The 5' splice site choice appears to be driven by the lipid ceramide pathway (Massiello and Chalfant, 2006). Ceramide is also able to modulate the phosphorylation status of SR proteins, among which SF2/ASF, one of the major regulators of Bcl-X alternative splicing (Massiello *et al.*, 2006). The ratio between Bcl-XL and Bcl-XS is critical to determine the sensitivity of cells to apoptotic signals and alteration of this balance in cancer cells promotes cell survival, drug and chemotherapy resistance (Paronetto *et al.*, 2007).

Ron. The "Recepteur d'origine nantais" (Ron) is a tyrosine kinase receptor for the macrophage stimulating protein (MSP). MSP binding results in tyrosine autophosphorylation of the receptor and, consequently, in the activation of several pathways involved in motility and extracellular matrix invasion (Ghigna *et al.*, 2005; Wagh *et al.*, 2008). Ron alternative splicing is frequently altered in tumours. Till today about six Ron splice variants, called ΔRon, have been reported. All these variants are characterised by deletions that make the receptor constitutively active (Lu *et al.*, 2007). In particular, the ΔRon165 originates from skipping of exon 11, leading to a 49 aminoacids in frame deletion in the extracellular domain (Ghigna *et al.*, 2005). This isoform is expressed in breast and colon cancers, as a result of overexpression of the SR protein SF2/ASF, which binds to an ESE in exon 12 and induces exon 11 skipping. The expression of ΔRon165 leads to the morphological and functional changes characteristic of the epithelial-to-mesenchymal transition in cancer cells (Ghigna *et al.*, 2005).

Rac1. Rac1 is a GTPase that has a fundamental role in cell migration, cell proliferation and in the production of reactive oxygen species (Bosco *et al.*, 2009; Kheradmand *et al.*, 1998). Mutations of Rac proteins are very rare in cancer, but alterations in Rac alternative splicing have been detected in colorectal and breast cancers leading to the production of a Rac1 isoform, called Rac1b, which contains an additional exon of 57 nucleotides (Jordan *et al.*, 1999). Racb1b mediates matrix metalloproteinase-3 (MMP3)-induced malignant transformation (Radisky *et al.*, 2005). By transient overexpression of different splicing regulators Jordan and colleagues found that SRF1 increases Rac1b expression, while SRp20 and 9G8 have the opposite effect. They also demonstrated that inhibition of the Wnt/β-catenin pathway reduces SRF3 levels and increases Rac1b expression (Goncalves *et al.*, 2009).

VEGFA. Vascular endothelial growth factor A is the most important ligand secreted by tumour cells to promote the formation of new vessels in hypoxia conditions. VEGFA mRNA undergoes alternative splicing and the most abundantly expressed isoform in adult tissues is VEGF165b, resulting from the use of a 3' alternative splice site in the last exon. The expression of the canonical VEGF transcript is upregulated in several tumours associated with reduced levels of VEGF165b (Woolard *et al.*, 2004). VEGF165b acts as an inhibitor of angiogenesis, binding to VEGFR but failing to engage the multimeric complex required for the full activation of VEGFR (Kawamura *et al.*, 2008). In tumours, overexpression of SRPK1/2, SRSF1 and SRSF5 SR proteins has been associated with canonical VEGFA increased expression (Nowak *et al.*, 2008).

Cyclin D1. Cyclin D1 is a master regulator of cell cycle by binding to cyclin-dependent kinase 4 or 6. Alternative splicing of Cyclin D1 produces two isoforms: Cyclin D1a, the full-length variant with five exons, and Cyclin D1b which is polyadenylated within intron 4 (Betticher *et al.*, 1995). Even if Cyclin D1b can be detected in non cancerous tissues, it is upregulated in tumour cells, including prostate and breast carcinomas (Burd *et al.*, 2006; Wang *et al.*, 2008). At difference of the D1a isoform, D1b is constitutively localised in the nucleus and by this way it escapes degradation (Lu *et al.*, 2003). The choice between D1a and D1b expression is driven by a competition between splicing and polyadenylation of intron 4. It has been reported that a common polymorphism in the last nucleotide of exon 4, G870A, affects the exon recognition by the splicing machinery and results in polyadenylation of intron 4 and thus in the expression of the D1b isoform. Interestingly, the G870A polymorphism, is associated with an increased risk to develop multiple cancers (Knudsen *et al.*, 2006). In addition, in cancer cells, the expression of D1b is also stimulated by overexpressed Sam68 and SRF1 splicing proteins (Paronetto *et al.*, 2010; Olshavsky *et al.*, 2010).

1.4.3 Splicing as a target of therapy

As discussed above, alterations in alternative splicing determine a splicing signature that appears specific and instrumental for each cancer cell type. The presence of cancer-specific isoforms that are expressed as a consequence of mutations, or the altered ratio of physiologically expressed isoforms as a result of splicing factors alterations, make the alternative splicing a promising field to identify diagnostic and prognostic markers and for design of new therapeutic strategies. One of the mechanism exploited for targeting cancer-associated splice variants involves the use of antibodies conjugated with radioisotopes (Figure 9A).

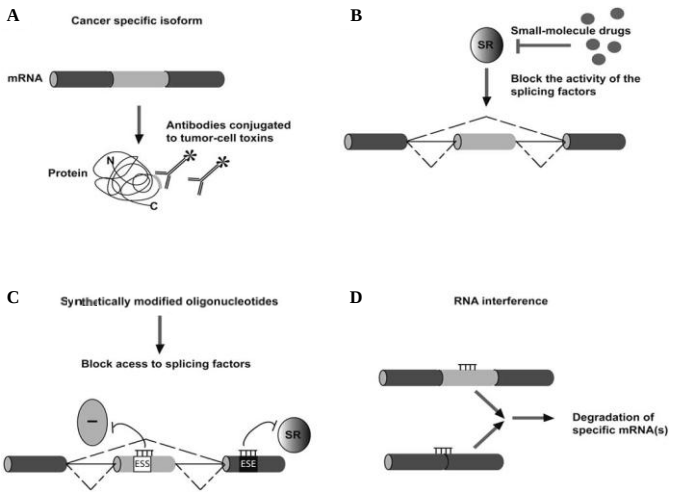


Figure 9. Therapeutic approaches to target alternative splicing in cancer cells (Ghigna *et al.*, 2008).

These antibody-based tumour target strategies are particularly useful for surface receptors involved in cell-cell and cell-matrix interactions that are frequently spliced in a tumour specific manner. For example, radiolabelled antibodies raised against CD44-v6 are promising therapeutic tools and are now in clinical trials for head and neck cancer treatment (Börjesson *et al.*, 2003).
Small molecules have also been tested to modify alternative splicing patterns in cancer (Figure 9B). Since phosphorylation of SR proteins is the best characterised post-translational modification of splicing factors, small molecule kinase inhibitors have been designed against GSK3, one of the most important kinases involve in the activation of SR proteins. Even if these innovative drugs appear to be efficient in restoring alternative splicing events driven by GSK3, a major challenge in their application is represented by the involvement of the target in several pathways, making this therapeutic strategy not very specific (Hernandez *et al.*, 2004). Short antisense oligonucleotides (AONs), complementary to a portion of the target mRNA are in study to reverse or correct aberrant splicing (Figure 9C). The synthetically modified oligonucleotides block the inappropriate exon selection by binding to splice sites or splicing regulatory sequences and have proven efficient in correcting the alternative splicing of the dystrophin gene, in both Duchenne and Becker muscular dystrophies, β-globin, CFTR and tau genes (Sazani and Kole 2003). Finally, small interfering RNAs (siRNAs) have been used to downregulate the expression of SR and/or hnRNP proteins in cancer (Figure 9C). They appear to be effective in inducing apoptosis and increasing chemotherapy sensitivity of cancer cells by downregulation of SRPK1 expression and hence affecting the expression of Bcl2 and Bax proteins (Hayes *et al.*, 2006).

1.5 Alternative splicing in brain

Alternative splicing is particularly exploited in the central nervous system (CNS), where it represents a key mechanism for the fine tuning of protein isoform expression needed for CNS proper development and function maintenance. In the CNS thousands of alternatively spliced isoforms are produced and translated into related protein isoforms that take part in several processes including learning and memory, neuronal cell recognition, neurotransmission, ion channel function, and receptor specificity (Grabowski and Black, 2001). Most mRNA have been demonstrated to undergo different splicing during neuronal development or be alternatively spliced in different brain regions to achieve a high specificity of functions.
Several nervous system diseases are associated with abnormalities in alternative splicing leading to alterations in the abundance, location or timing of isoform expression.

1.5.1 Brain-specific regulation of alternative splicing

Representative examples of CNS-specific alternative splicing concern the genes encoding for $GABA_A$, tyrosine kinases (c-src) and tropomyosin. A common feature of these splicing events is the involvement of the polypyrimidine-tract binding protein (PTB), which is a member of the hnRNP family (Dreyfuss *et al.*, 1993).
As regards splicing enhancement, as previously described (see 1.2.1) it is mainly accomplished by SR proteins binding to ESE or ISE sequences. In the CNS tissue specificity is achieved also by targeted expression of SR proteins or regulatory factors. For example, the SR-specific kinase 2 (SRPK2) is mainly expressed in brain where it is believed to fulfil tissue and developmental-specific alternative splicing events (Wang *et al.*, 1998). It has been reported that the ratio of SR proteins plays a significant role in the regulation of alternative splicing in brain (Hannamura *et al.*, 1998). Generally, this mechanism involves the ratio of splicing proteins with opposing activities, like ASF/SF2 which expression is inversely proportional to the expression of hnRNP A1.
CELF (CUG binding proteins and ETR3 like factors) proteins have been identified as a new class of brain-specific alternative splicing regulators. CELF proteins are expressed with a varying tissue specificity and regulate alternative splicing of CLCB and NMDA favouring inclusion of cassette exons (Zhang *et al.*, 1999).
Different neuron-specific RNA binding proteins have been identified and associated with defined splicing patterns in the CNS. The *elav* protein family, firstly identified in *Drosophila*

melanogaster, comprises different members, exclusively expressed in neurons, that participates in the regulation of mRNA stability and metabolism during neural development (Antic *et al.*, 1999; Atasoy *et al.*, 1998). Moreover, the expression of Nova-1 and Nova-2 is restricted to the nucleus of neurons where these two splicing repressors are involved in the regulation of GlyRα2 and $GABA_A$ receptors (Jensen *et al.*, 2000).

1.5.2 Alterations of alternative splicing in brain diseases

Neurodegeneration. Mutations in the *tau* gene represent a clear example of the effects of mutations in alternative splicing. Tau belongs to the MAP (Microtuble Associated Protein) protein family and is involved in microtubule assembly and stabilisation upon phosphorylation by specific kinases, including MARK4 (MAP/Microtubule Affinity Regulating Kinase 4) (Cleveland *et al.*, 1977). At least six different tau isoforms are produced by alternative splicing, and most of them differ in the numbers of microtubule (MT)-binding motifs. In particular, exon 10 exclusion/inclusion determines the formation of tau proteins with three (Tau3R) or four (Tau4R) MT-binding motifs, respectively. In normal human brain the ratio of Tau4R/Tau3R is approximately 1 (Goedert *et al.*, 1998; Hong *et al.*, 1998; Hutton *et al.*, 1998).

Mutations in the *tau* gene cause the frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), characterised by filamentous aggregates of tau protein in neurons and glial cells, a feature also reported in Alzheimer's disease. Several mutations in FTDP-17 patients are associated with increase in the ratio of Tau4R/Tau3R, suggesting that the proper ratio between the two isoforms is essential for neuronal survival and functions (Spillantini and Goedert, 1998). These splicing mutations include both intronic and exonic mutations. Many intronic mutations in FTDP-17 patients are near to the 5' splice site of intron 11 and result in destabilisation of the stem and loop RNA structure leading to increased interaction of U1 snRNP with the 5' splice site, enhanced exon 10 inclusion and hence increase in the Tau4R/Tau3R ratio (Jiang *et al.*, 2003). Also mutations in exon 10 have been found to affect tau splicing. Indeed, Jiang and colleagues demonstrated the presence of an exonic splicing enhancer (ESE) in exon 10 whose mutations enhance the binding of the splicing regulator Tra2β and thus exon 10 inclusion (Jiang *et al.*, 2003). Further studies also revealed that Tra2β works in competition with another SR protein, SRp54, that, in this case, antagonises exon 10 inclusion (Wu *et al.*, 2006).

Studies on spinal muscular atrophy have highlighted that this motor neurons degenerative disease can be considered a trans-acting splicing disorder. Indeed, the *survival motor neurons* (*SMN*) gene is required for regeneration and recycling of spliceosomal snRNPs (Pellizzoni *et al.*,

1998). Therefore, it has been proposed that mutations in the *SMN* gene reduce the functional protein, impairing pre-mRNA splicing.

Neurological and psychiatric disorders. Mutations in both *cis*-acting and *trans*-actin splicing factors have been associated with neurological diseases. A representative example isprovided by a nonsense mutation in the dystrophin gene leading to exon 31 skipping by activation of an exonic splicing silencer for the hnRNP A1 protein (Disset *et al.*, 2006). Mouse models of type 2 myotonic dystrophy (DM), caused by CCUG expansion in intron 1 of the zinc finger protein 9 (ZNF9) gene (Ranum and Day, 2004), have revealed that actually repeats expansion results in the sequestration and upregulation of MBNL and CUG-BP1 splicing factors, leading to misregulation of alternative splicing in striated muscle and brain of DM patients (Kanadia *et al.*, 2006; Ranum and Cooper, 2006).

Schizophrenia, one of the major psychiatric disorders, is reported to have a causative component inaberrant alternative splicing too. In particular, increase in the γ2L versus the γ2S isoform of $GABA_A$ receptor has been reported in schizophrenic patients with detrimental effects on cortical functions (Huntsman *et al.*, 1998). Alterations in the relative abundance in specific isoforms of the neural cell adhesion molecule (VASE) and the NMDR1 receptor have been pointed out and correlated with compromised neuronal functionality in schizophrenia patients (Vawter *et al.*, 2000; Le Corre *et al.*, 2000).

Glioma. Gliomas are the most common primary brain tumours in adults, accounting for about 70% of all the tumours in the CNS (Ohgaki 2009). According to the updated World Health Organization (WHO) classification (Rousseau *et al.*, 2008), gliomas can be histologically sub-divided in astrocytic tumours, including astrocytoma and glioblastoma, oligodendroglial tumours, mixed gliomas, ependymomas and other neuronal, neuro-glial and neuroepithelial tumours. Gliomas are also classified in four malignancy grades (WHO grade I-IV) according to the presence/absence of nuclear atypia, mitosis, microvascular proliferation and necrosis. Brain tumour stem cells are thought to be responsible for glioma growth, invasiveness and resistance to chemotherapy and radiation (Reya *et al.*, 2001). Different hypotheses have been formulated on the brain tumour stem cell origin which may derive from de-differentiation of mature glial cells, neural progenitors or neural stem cells (Vescovi *et al.*, 2006; Canoll *et al.*, 2008; Germano *et al.*, 2010).

Most brain tumours are sporadic, even if a few familiar syndromes are associated with an increased incidence of brain neoplasia. Alternative splicing can be included among the processes

targeted by oncogenic alterations in glioma. Different splicing factors have been reported to drive an oncogenic splicing switch in glioma. Among them hnRNP H, which is upregulated in glioma, favours the aberrant expression of the anti-apoptotic isoform MADD of the insuloma glucagoma protein 20 (IG20/MADD) and the constitutively active RON isoform in glioma (LeFave *et al.*, 2011). Moreover, hnRNP A2/B1 are considered putative proto-oncogenes in glioma, since their overexpression is sufficient to induce malignant transformation *in vitro* probably through aberrant RON splicing (Golan-Gerstl *et al.*, 2011).

Alternative splicing of FGFR1 (Fibroblast Growth Factor Receptor 1) is also impaired in glioma. Increased skipping of α exon leads to the overexpression of a high affinity isoform that confers a growth advantage to glioma cells. Several intronic splicing repressors have been characterised in introns flanking α exon and are thought to be involved in aberrant FGFR1 splicing (Wang *et al.*, 1995).

1.6 Polypyrimidine-tract binding protein (PTB)

Polypyrimidine-tract binding protein (PTB) is an ubiquitous splicing regulator belonging to the hnRNP protein family. It was originally identified for its ability to induce exon skipping, but it is currently shown to play a role in different cellular processes including polyadenylation, mRNA stability and translational initiation. PTB has aroused particular interest for its involvement in neural development and glioma.

1.6.1 PTB transcription, alternative splicing and protein structure

The *PTB* gene is localised on chromosome 19p13.3 and it is expressed in four differently spliced isoforms. PTB, also known as PTBP1, derives from an mRNA that undergoes skipping of exon 9 (mRNA 3203 base pairs; 531 aminoacid protein). PTBP2 (mRNA 3260 base pairs ; 550 aminoacid protein) and PTBP4 (mRNA 3281 base pairs; 557 aminoacid protein) result from exon 9 inclusion and the use of two alternative 3' splice sites, while PTB-T has been reported to result from alternative splicing of exons 2-10 (Sawicka *et al.*, 2008).

PTB encodes for a 57 kDa protein characterised by four RNA recognition motifs (RRMs) and a conserved N-terminal domain that harbors both nuclear localisation and export signals (NLS and NES). Through the RRMs, PTB binds to the transcript at multiple sites within large polypyrimidine tracts leading to conformational changes suitable to functional mRNA processing (Sawicka *et al.*, 2008). PTB shuttles continuously between the nucleus and the cytoplasm. Cytoplasmic localisation is mainly induced by the PKA-mediated phosphorylation of a specific serine residue(Ser-16) within the NLS and predominantly occurs during cell stress (Sawicka *et al.*, 2008). PTB has also been considered a key component in maintaining the integrity of the perinucleolar compartment, a subnuclear structure predominantly found in transformed cells (Wang *et al.*, 2003).

1.6.2 PTB expression and neuronal differentiation

PTB is ubiquitously expressed, but it is reported to play a major role in guiding alternative splicing in brain. Indeed, a splicing switch from PTB to its highly homolog neuronal PTB (nPTB) drives neuronal differentiation. The expression of PTB and nPTB is mutually exclusive, with PTB expressed in neural precursors and glial cells, while post-mitotic neurons express only nPTB (Boutz *et al.*, 2007). PTB expression is regulated by PTB itself through alternative

splicing. In particular, PTB binds to its mRNA and induces the skipping of exon 11 and the consequent degradation of the resultant frameshift mRNA through non-sense mediated mRNA decay (Wollerton *et al.*, 2004). A regulatory expression loop, that exploits alternative splicing, also is acting between PTB and nPTB (Spellman *et al.*, 2007). By this mechanism PTB downregulation in neuronal-committed neural progenitors is sufficient to induce nPTB expression and changes in the alternative splicing program lead to neuronal differentiation (Boutz *et al.*, 2007). nPTB is highly homologous to PTB and the two proteins are very similar in the structure of their RRMs. They bind to the same regulatory elements but with some differences in affinity (Markovtsov *et al.* 2000; Oberstrass *et al.* 2005). Moreover, as previously reported (see 1.3.4), mir-124 regulates the expression of PTB in non-neuronal cells (Makeyev *et al.*, 2007). Xue and colleagues recently demonstrated that downregulation of PTB is able to induce trans-differentiation of fibroblasts into neuronal-like or even functional neurons *in vitro*. In this context, PTB appears to be a fundamental component of a microRNA program that dismantles multiple components of the REST complex (RE1-silencing transctiption factor) (Xue *et al.*, 2013). This transcriptional complex downregulates the expression of a large array of neuronal specific genes in non-neuronal cells, including mir-124, thus priming an autoregulatory loop during neuronal differentiation. Thus, PTB is not only a target of mir-124, but also negatively regulates the expression of this and other miRNA important for neuronal differentiation and induces the expression of critical transcription factors needed to promote trans-differentiation of mouse embryonic fibroblasts (MEFs) into functional neurons (Xue *et al.* 2013).

Other insights into the function of PTB in neural development come from studies on mouse embryonic stem cell lines (ES). Shibayama and colleagues demonstrated that PTB homozygous mutations cause embryonic lethality shortly after implantation, while $Ptb^{-/-}$ ES show severe defects in cell proliferation, apparently without aberrant differentiation. Further analysis of cell cycle progression and cell synchronisation revealed that $Ptb^{-/-}$ ES are characterised by prolonged G_2/M phase, demonstrating that PTB is essential for early mouse development and ES cell proliferation (Shibayama *et al.*, 2009). In 2011 Suckale and colleagues demonstrated that PTB is expressed in mouse developing organs, in particular in the brain cortex and sub-ventricular zone, well known regions of neurogenesis in both humans and mouse. In contrast with previous studies of Shibayama, they also found that ES lacking PTB not only show impaired proliferation, but also defective differentiation (Suckale *et al.*, 2011).

1.6.3 PTB functions

PTB was originally identified as repressor of alternative splicing (Garcia-Blanco *et al.*, 1989) but Additional roles in mRNA processing, viraltranslation and replicationand IRES-mediated translation have been described(Sawicka *et al.*, 2008).

Regulation of alternative splicing. PTB commonly acts as repressor of alternative splicing favouring skipping of alternative exons. Different models of PTB activity have been proposed (Spellman and Smith, 2006) (Figure 10): i) binding competition with the splicing factor U2AF65 at the 3' splice site of alternative exons; ii) polymerisation of PTB on the alternative exon masking splicing enhancer sequences; and iii) looping out of alternative exons by PTB binding of flanking intronic sequences.

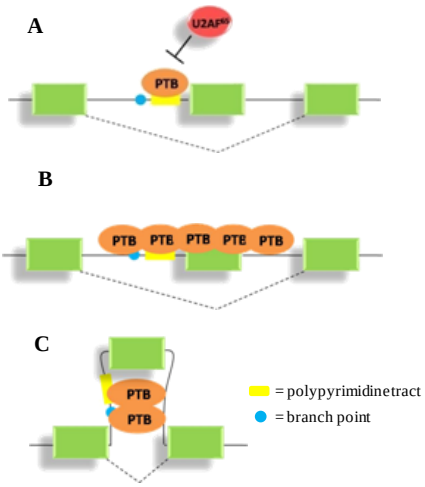


Figure 10. Mechanisms of PTB-derived alternative splicing. **A**) Competition with U2AF^{65} factor. **B**) Propagation: PTB polymerises along the pre-mRNA and masks binding sites for positive regulators of alternative splicing. **C**) Looping: PTB binds to both flanking introns and induces exon excision by looping it (adapted from Spellman and Smith, 2006).

α-tropomiosin, α-actinin, GABAAγ2 (gamma aminobutyric acid Aγ2), c-src and FGFR2 (fibroblast growth factor receptor 2) are known targets of PTB-mediated alternative splicing (Spellman *et al.*, 2005). However, recent studies also reported a role of PTB in exon inclusion depending on the position of its binding sites relative to the target exon. In detail, upon binding to the upstream intron and/or within the exon, PTB represses exon inclusion, while by binding to the downstream intron, it activates exon inclusion, delineating a position-dependent activity. Specific splice site features determine the PTB-mediated repression or inclusion of alternative exons: included exons show weaker 5' splice sites, whereas skipped exons have longer polypyrimidine tracts (Llorian *et al.*, 2010).

3'-end processing. PTB both promotes and inhibits the mRNA 3'-end cleavage required for polyadenylation. PTB may prevent mRNA polyadenylation through competition with the cleavage stimulating factor (CstF), or stimulate polyadenylation by binding to pyrimidine-rich upstream elements(USEs) (Castelo-Branco *et al.*, 2004; Le Sommer *et al.*, 2005). PTB is also involved in alternative polyadenylation, leading to mRNA with variable 3' ends and thus proteins with different C-terminals. In this case PTB has been reported to bind to the same regulatory sequence identified for splicing repression but nearby alternative polyadenylation sites (Hall-Pogar *et al.*, 2007).

mRNA transport. Evidences for a role of PTB in mRNA transport come from experiments in *Xenopus*, where the PTB homologue (VgRBP60) is involved in the localisation of the Vg1 mRNA (Cote *et al.*, 1999). In vertebrates PKA-activated PTB is involved in α-actin mRNA localisation at neurite terminals, a fundamental process for cell motility and neuronal axon growth (Ma *et al.*, 2007). PTB has also been reported to regulate the nuclear export of hepatitis B virus RNAs (Zang *et al.*, 2001).

mRNA stability. PTB increases the stability of specific transcripts by binding to the untranslated regions of mRNA and thus competing with factors involved in mRNA degradation. Glucose-stimulated binding of PTB to the 3'-UTR of rat insulin mRNA has been demonstrated to increase mRNA stability by involving the mTOR pathway (Tillmar *et al.*, 2002a). Also hypoxia may induces PTB binding to insulin mRNA, but without involvement of the mTOR pathway (Tillmar *et al.*, 2002b). PTB, in association with CSD (cold shock domain) Y-box proteins, also controls the stability of VEGF (vascular endothelial growth factor) mRNA. CSD-PTB complexes bind to 3'- and 5'-UTRs and lead to conformational changes that protect mRNA from degradation (Coles *et al.*, 2004). Other mRNAs stabilised by PTB-binding include CD154 and iNOS (Hamilton *et al.*, 2003; Pautz *et al.*, 2006).

Viral translation and replication. PTB acts as an ITAF (IRES -internal ribosomal entry site-*trans*-acting factor) for mRNA translation of virus belonging to the *Picornaviridae* family and lacking cap structure. Indeed, PTB binds to IRES elements containing large polypyrimidine tracts with PTB consensus binding sequences and stabilises or alters the IRES structure to direct ribosome binding to the correct start codon, thus acting as a chaperone (Song *et al.*, 2005). Interestingly, PTB also binds to the HCV mRNA within IRES elements but it is not essential for

IRES activity, even if it stimulates viral replication (Chang and Luo, 2006). These evidences suggest that PTB might be a potential target for anti-HCV therapeutic agents (Xue *et al.*, 2007).

IRES-mediated translation. PTB has been also proposed as a general ITAF that allows IRES-mediated translation under conditions that inhibit cap-dependent translation, such as cell stress, apoptosis and viral infection (Mitchell *et al.*, 2005). Targets of PTB-induced IRES translation include BAG-1 (Bcl-2-associated athanogene 1) and Apaf-1 (apoptotic protease activating factor 1) (Pickering *et al.*, 2004; Mitchell *et al.*, 2001). Cytoplasmic relocalisation of PTB is a critical factor in IRES-mediated translation. This relocalisation has been observed after exposure to chemotoxic agents and appears fundamental in IRES activation during apoptosis (Dobbyn *et al.*, 2008; Bushell *et al.*, 2006).

1.6.4 PTB and human pathologies

Since PTB expression is thinly regulated in tissues, above all in brain, deregulation of its expression has been implicated in different pathologies, including glioma, ovarian cancer and Alzheimer's disease.

Glioma. PTB is aberrantly overexpressed in glioma and its expression levels correlate with glial cell transformation. Increased PTB expression contributes to gliomagenesis by deregulating the alternative splicing of genes involved in cell proliferation and migration, including FGFR-1 (fibroblast growth factor receptor-1), PKM (pyruvate kinase) and USP5 (ubiquitin specific peptidase 5) (McCutcheon *et al.*, 2004; Cheung *et al.*, 2006). In particular, PTB overexpression increases FGFR-1 α-exon skipping that results in the synthesis of a receptor with higher affinity for the fibroblast growth factor thus favouring transformed cell growth (Jin *et al.*, 2000).
In transformed glial cells, PTB overexpression leads to the re-expression of the embryonic pyruvate kinase isoform, PKM2. The switch from PKM1, normally expressed in terminally differentiated cells, to PKM2 is achieved through the PTB-mediated inclusion in the PKM mRNA of exon 10, instead of exon 9. In transformed cells, PKM2 promotes aerobic glycolysis and proliferation. Recently c-Myc overexpression has been associated to upregulation of PTB transcription in transformed glialcells (David *et al.*, 2010).
In glioblastoma, PTB overexpression forces the expression of USP5 isoform 2, a protein involved in ubiquitination. USP5 isoform 2 has a low activity and favours cell growth and migration (Izaguirre *et al.*, 2011).

Ovarian tumour. PTB is overexpressed in the majority of epithelial ovarian tumours and deregulates cell proliferation, anchorage-dependent growth and invasiveness. PTB targets in ovarian transformed cells have not yet been identified (He *et al.*, 2007).

Alzheimer's disease (AD). Recent evidences delineate PTB as a regulator of the amyloid precursor protein (APP) expression in neurons. In particular, PTB altered expression in neuronal cells, likely mediated by miR-124, enhances the expression of APP isoforms including exon 7 and/or 8. These isoforms have been found enriched in AD patients and associated with β-amyloid production (Smith *et al.*,2011).

1.7 MAP/Microtubule Affinity Regulating Kinase 4 (MARK4)

1.7.1 MARK4 gene, alternative splicing and protein structure

MARK4 belongs to the MARK protein family (MARK1-4) and is the less characterised member of the family. The *MARK4* gene was discovered by Kato and colleagues in 2001 and named *MARKL1* (*MARK-Like 1*) on the basis of its homology to *MARK* (Kato *et al.*, 2001).

MARK4 gene maps on chromosome 19q13.2 and consists of 18 exons. Two different isoforms are produced through alternative splicing (Figure 11): MARK4S (short), the constitutive isoform consisting of 18 exons (3609 base pairs), with the stop codon included in the last exon, and encoding a 688 aminoacid long protein; and MARK4L (long) a 752 aminoacid protein translated by a transcript that undergoes skipping of exon 16 and the consequent frameshift of the reading frame.

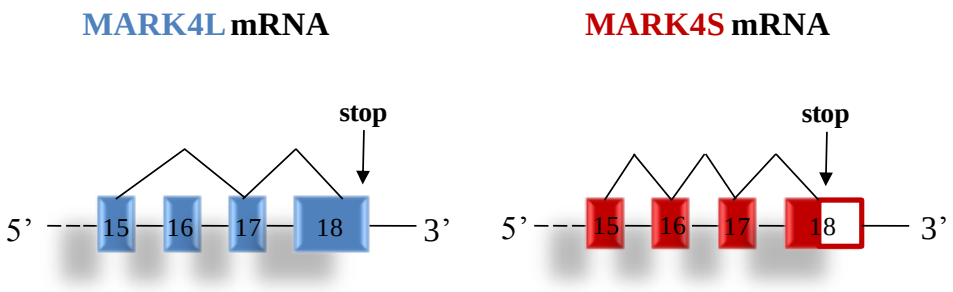


Figure 11. MARK4 alternative splicing.

MARK4 sequence shares near 55% homology with the other MARKs, with the highest homology with MARK3. MARK4 isoforms show the characteristic MARK protein structure and domains, including an N-terminal catalytic domain, a linker domain (common docking domain), an ubiquitin associated (UBA) domain, a spacer domain and a C-terminal tail. The functional kinase domain shares 90% homology with the other MARKs. The MARK4 characteristic T-loop harbours the activating (T214) and the inhibitory (S218) phosphorylation sites. MARK4 alternative splicing leads to the production of two protein isoforms that differ in the C-terminal tail: MARK4L shows the Kinase-Associated 1 (KA1) domain, like the other MARK proteins, whereas MARK4S contains a domain with no homology with any known structure (Espinosa *et al.*, 1998), suggesting non-overlapping functions of the two isoforms. Despite the MARK4 tail

domain shows low sequence homology compared to the other MARKs it folds in a conformation similar to those of MARK1, 2 and 3, thus suggesting an autoinhibitory and interactor binding function also for the MARK4 C-terminal region (Marx *et al.*, 2010).

1.7.2 MARK4 regulation

Given the conserved protein structure compared with the other MARK proteins, the regulation of MARK4 protein function has been inferred from the other family members.

MARK4 is activated by LKB1 (Liver Kinase B1) that phosphorylates the threonine 214 residue inside the T-loop (Lizcano *et al.*, 2004; Brajenovic at al., 2004). It has been recently demonstrated that MARK4 is polyubiquitinated and interacts with USP9X, a deubiquitinating enzyme. MARK4 mutants in USPX9 binding sequence are hyperubiquitinated and not phosphorylated at the T214, thus inhibiting LKB1 activation. Ubiquitin binds the MARK4 UBA domain in the steady state making the T-loop not accessible to LKB1. An alternative model predicts that ubiquitin may mask the T214 site or induce conformational modifications favouring the action of phosphatases (Al-Hakim *et al.*, 2008). MARK4 has also been demonstrated to interact with aPKC (Brajenovic*et al.*, 2004) and thus it may be phosphorylated and inactivated by this kinase, as reported for MARK2 and MARK3 (Hurov *et al.*, 2004).

1.7.3 MARK4 interactors

Tandem Affinity Purification (TAP) and immunoprecipitation experiments allowed the identification of about twenty putative MARK4 interactors (Brajenovic*et al.*, 2004). Among them, PKCλ and Cdc42 are implicated in cell polarity and TGFβIAF (Transforming Growth Factor β-Inducing Anti-apoptotic Factor) is considered an hortologue of Miranda, a protein involved in the asymmetric division of neuroblasts in *Drosophila*. MARK4 also interacts with the 14-3-3η isoform (Brajenovic*et al.*, 2004; Angrand*et al.*, 2006). 14-3-3 proteins have a well established rolein the control of many cellular processes by binding to phosphorylated proteins. 14-3-3ηcould thus regulate MARK4 activity or act as a bridge between different pathways. Other MARK4 interactors are ARHGEF2, a cytoskeleton binding protein, and Phosphatase 2A, a microtubules associated protein that regulates tau (Brajenovic *et al.*, 2004).

MARK4 protein was also found to co-localise and co-immuoprecipitate with α-, β-, and γ-tubulin, myosin and actin (Trinczek *et al.*, 2004; Brajenovic *et al.*, 2004).

1.7.4 Gene expression and protein localisation

Few *MARK4* expression studies are reported in literature not always with concordant results (Kato *et al.*, 2001; Trinczek *et al.*, 2004; Schneider *et al.*, 2004; Moroni *et al.*, 2006). MARK4 is ubiquitously expressed, with relevant expression levels in brain and testis. In particular, MARK4L appeared highly expressed in testis, brain, kidney, liver and lung (Trinczek *et al.*, 2004; Schneider *et al.*, 2004; Moroni *et al.*, 2006), while MARK4S expression is predominant in testis, heart and brain (Kato *et al.*, 2001; Moroni *et al.*, 2006). In brain, MARK4 protein was predominantly observed at the tips of neurite-like processes (Trinczek*et al.*, 2004) while immunohistochemistry on rat cerebral cortex and hippocampus showed MARK4L and MARK4S expression restricted to neurons of the grey matter (Moroni *et al.*, 2006).

Exogenous GFP-conjugated MARK4 protein localises in interphase centrosomes in both CHO (Chinese Hamster Ovary) and neuroblastoma cell lines. Inactive MARK4 has been reported to localise near the nucleus, associated to the endoplasmic reticulum and, once activated, it associates with microtubules in the centrosome to exert its functions (Trinczek *et al.*, 2004). MARK4 protein has been recently demonstrated to localise at interphase centrosomes also in glioma cell lines, and to co-localises with γ-tubulin at all cell cycle phases (Magnani *et al.*, 2009; Magnani *et al.*, 2011). This centrosome association was not abolished by depolymerisation of microtubules induced by nocodazole, suggesting that MARK4L is a core component of centrosomes. Moreover, two novel MARK4L localisation at the nucleolus and the midbody have been highlighted (Magnani *et al.*, 2009).

1.7.5 MARK4 in the central nervous system and tumours

The *MARK4* gene was first identified in hepatocarcinoma cells. Indeed, accumulation of β-catenin in the nucleus, after inactivation of the Wnt/β-catenin pathway, determines increased expression of several genes, including *MARK4*, in hepatocarcinoma cells. These evidences led Kato and colleagues to hypothesise that MARK4 could be a downstream component of the Wnt-signalling pathway (Kato *et al.*, 2001).

MARK4 was also found transiently up-regulated after ischemic events in brain. Among the multitude of gene overexpressed in the injured brain, was also included LKB1 that phosphorylates MARK4. Since cell vitality of hepatocytes was strongly reduced after experimental overexpression of MARK4S, MARK4S up-regulation in the early stages of an ischemic event was then hypothesised to increase neuron death (Schneider *et al.*, 2004).

Analysis of chromosomal rearrangements frequently observed in glioma, led to the identification of an amplified region in 19q13.2. Interestingly, the amplified region was centromeric to the Loss Of Heterozygosity (LOH) area in gliomas (Hartmann *et al.*, 2002) but never deleted in these tumours, suggesting the presence of a key gene for cancer cells. DNA sequence of the amplified region revealed that it harbours the *MARK4* gene (Beghini *et al.*, 2003). Moreover, array-CGH (Comparative Genomic Hybridisation) showed *MARK4* duplication in a glioblastoma cell line (MI-4) (Beghini *et al.*, 2003; Roversi *et al.*, 2006). Further semi-quantitative analysis of MARK4 expression in glioma pointed out a MARK4L up-regulation, directly correlated with malignancy grade, in 8 tissue samples and 26 cell lines (10 I and II grade, 11 III grade and 5 IV grade gliomas). Contrarily, MARK4S appeared highly expressed in normal brain and hardly detectable in glioma samples and human neural progenitors. It was therefore suggested that MARK4L might be a mitogen protein, necessary for proliferation and thus highly enriched in proliferating or undifferentiated cells (Beghini *et al.*, 2003).

Competitive PCR outlined that MARK4S expression levels increase during neuronal differentiation of neural progenitors, while MARK4L expression levels were unchanged in differentiated neurons. MARK4S was therefore suggested to be a neuron-specific marker in the central nervous system (CNS) and to drive neuronal commitment.

Our real-time PCR and immunobloting experiments confirmed the overexpression of MARK4L in glioma tissues, associated with low levels of MARK4S that appeared inversely correlated with tumour grade and cellular de-differentiaton (Figure 12).

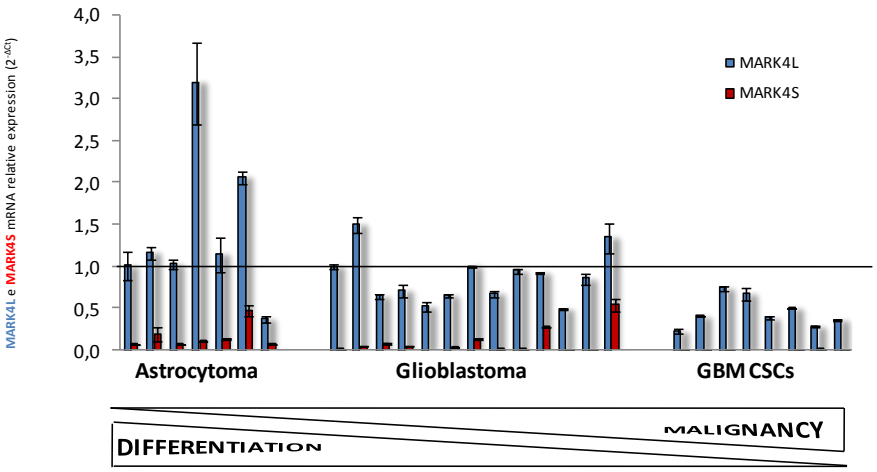


Figure 12. Relative expression of MARK4L (blue bar) and MARK4S (red bar) mRNAs in astrocytomas, glioblastomas and GBM CSCs compared to normal brain (set as 1 and represented as a horizontal black line) (adapted from Magnani *et al.*, 2011).

A high MARK4L/MARK4S ratio also characterised glioblastoma-derived cancer stem cells (GBM CSCs), with a MARK4 expression profile comparable to mouse neural stem cells (NSCs) (Figure 13) (Magnani *et al.*, 2011).

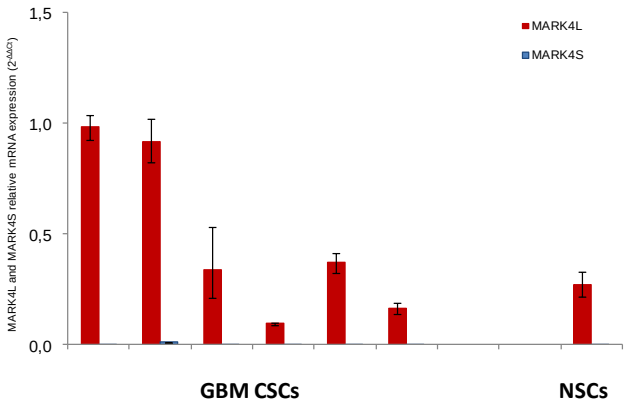


Figure 13. MARK4 mRNA expression profile in GBM CSCs and NSCs.MARK4L (red bar) and MARK4S (blue bar) mRNA expression in human undifferentiated GBM CSCs and mouse undifferentiated NSCs compared to human neural progenitor cells (conventionally set as 1) (adapted from Magnani *et al.*, 2011).

Furthermore, MARK4 immunohistochemistry on both human and mouse adult and embryonic brain, disclosed a peculiar expression of MARK4L in the embryonic ventricular zone and adult sub-ventricular zone, both well known regions of neurogenesis. According to real-time and immunobloting data, MARK4S was undetectable in these germinal zones, but marked post-mitotic neurons (Magnani *et al.*, 2011). Overall these evidences suggest that the balance between the two MARK4 isoforms is critical for neural differentiation and proliferation. MARK4L characterises undifferentiated cells and the expression of MARK4S drives cell differentiation. In glioma, a subverted MARK4L/MARK4S ratio may contribute to cellular de-differentiation and proliferation during gliomagenesis.

MARK4 has also been associated with Alzheimer's disease (AD), on the basis of its kinase activity on tau protein, which is crucial in AD pathomechanism, and for its gene localisation nearby a locus of susceptibility (*ApoE*) to AD (Trinczek *et al.*, 2004). A role of MARK4 in AD was also confirmed by a recent study that highlighted a strong MARK4 overexpression and MARK4-tau interaction in AD brains, correlating with the clinical stages of the disease (Gu *et al.*, 2013).

1.7.6 MARK4 functions

MARK4 phosphorylates MAPs, increasing microtubule dynamics, and thus taking part in cell cycle control (Brajenovic *et al.*, 2004). MARK4L, given its interaction with microtubules in glioma and its localisations at centrosomes and midbody, is suggested to play a role in cell cycle progression and in cytokinesis (Magnani *et al.*, 2009). MARK4L overexpression in tumours also sustains its role in cell proliferation (Kato *et al.*, 2001; Beghini *et al.*, 2003). On the contrary MARK4S may be a neuron-specific marker in the CNS and could drive neuronal commitment (Moroni *et al.*, 2006; Magnani *et al.*, 2011). The observed altered ratio of the two MARK4 isoforms in glioma, suggests that the balance between the two isoforms is carefully guarded during neuronal differentiation and that it may represent a target of tumour transformation in glioma.

Recently, other functions of MARK4 have been pointed out. Tang and colleagues found a peculiar role of MARK4 in the ectoplasmic specialisation (ES), a centrosome-derived structure, during spermatogenesis (Tang *et al.*, 2012). MARK4 was detected at the apical and basal ES with restrictive spatio-temporal expression and found to co-localise with markers of the apical and basal ES. MARK4 expression was profiledas stage-specific during the epithelial cycle of the ES, and MARK4 protein appeared associated with different components of the tubulin cytoskeleton. As downregulation of the kinase leads to apical ES disruption and consequent detachment of spermatids from the epithelium the authors concluded that MARK4 is likely fundamental for the maintenance of the apical ES integrity (Tang *et al.*, 2012).

MARK4 has also been suggested to play a key role in energy metabolism since MARK4 deficient mice show hyperphagia, hyperactivity, and hypermetabolism, possibly being protected by diet-induced obesity and related complications. MARK4 knockdown appeared to enhance insulin-stimulated AKT phosphorylation in metabolic tissues and thus to reduce obesity-associated insulin resistance (Sun *et al.*, 2012).

Supporting evidences on the role of MARK4 in proliferation and tumour come from its association with the mTORC pathway. MARK4 overexpression significantly inhibits TORC1 activation through Rag GTPases and phosphorylation of Raptor, key components of mTORC1 (Li and Guan 2013). However, in this study no discrimination between the two MARK4 isoforms has been made, even if we speculate that, according to other studies in literature, the target of this work was MARK4S, which has been constantly associated with differentiation and reduced proliferation.

MARK4 has also been proposed as critical regulator of the early steps of ciliogenesis. Recent evidence associates the MARK4 protein with the basal body and ciliary axoneme in human and

murine cell lines and an active function at the level of axonemal extension is suggested (Kuhns *et al.*,2013).

Materials and methods

2.1 Tissue samples and cell cultures

2.1.1 Human glioma tissue samples

A total of 15 human glioma tissue biopsies were provided by Professor Lorenzo Bello (Department of Medical Biothecnologies and Translational Medicine, Università degli Studi di Milano, Milano). Biopsies were excised from patients who had received no previous chemotherapy or radiation treatment, in accordance with a protocol approved by the Internal Review Board of the University of Milan's Neurosurgery department. The specimens were stored at -80°C immediately after surgery. Table 1 reports features of the glioma samples under study.

SEX/AGE at surgery	*TISSUE SAMPLE*	*HISTOLOGICAL DIAGNOSIS*	*WHO GRADE*	*PROGNOSIS*	*mMGMT*	*Del1p*	*Del19q*
M/32	1	Protoplasmic astrocytoma	II (III)	alive-no relapses	+	-	-
N.A.	41	Astrocytoma	II	N.A.	N.E.	N.E.	N.E.
M/34	64	Protoplasmic astrocytoma	II	alive-relapse	N.E.	-	-
N.A.	94	Astrocytoma	II	N.A.	N.E.	N.E.	N.E.
F/52	108	Anaplastic astrocytoma	III	dead-32 months	+	-	-
F/35	164	Fibrillary astrocytoma	II	alive-no relapses	+	-	-
M/34	216	Protoplasmatic astrocytoma	II	alive-relapse	+	-	-
F/66	5	Glioblastoma	IV	dead-12 months	+	+	+
F/72	21	Giant cell glioblastoma	IV	dead-5 months	N.E.	N.E.	N.E.
F/65	81	Glioblastoma	IV	dead-11 months	+	+	+
M/68	96	Glioblastoma	IV	dead-18 months	+	N.E.	N.E.
M/63	113	Glioblastoma	IV	dead-15 months	+	-	-
M/44	121	Glioblastoma	IV	dead-3 months	+	-	-
M/51	132	Glioblastoma	IV	dead-26 months	-	-	+
M/50	194	Glioblastoma	IV	alive-no relapses	-	+	-
M/64	207	Glioblastoma	IV	alive-relapse	-	-	+

Table 1. Clinic and pathological features of astrocytomas (n=7) and glioblastomas (n=9). mMGMT = methylation of *MGMT* gene; Del = deletion; N.A. = not available; N.E. = not evaluated.

2.1.2 Human normal brain

Normal brain samples (NB) obtained from two male patients operated for intractable epilepsy and used as normal controls for real-time and immunoblotting experiments; samples were stored at -80°C immediately after surgery. RNA from total human normal brain (MVP Total RNA, Human Brain) was also purchased by Stratagene (La Jolla, CA, USA).

2.1.3 Cell cultures

Human Embryonic Kidney 293T (HEK293T) and the oligoastrocytoma G157 (WHO grade II) cell lines were used for *in vivo* splicing assays. G157 cell line has been selected from a panel of 21 human primary glioma cell lines, obtained from post-surgery specimens and characterised as described elsewhere (Magnani *et al.*, 1994; Perego *et al.*, 1994; Beghini *et al.*, 2003; Roversi *et al.*, 2006).

HEK293T cells were grown in DMEM supplemented with 10% fetal bovin serum (FBS), while G157 were grown in RPMI with 5% FBS, both with 100 U/ml penicillin (Pen) and 100 U/ml streptomicine (Strep) at 37°C in a 5% CO_2 atmosphere. Cells were always collected in the exponential growth phase.

Normal Human Neural Progenitor Cells (NHNP) (Lonza, Walkersville, MD, USA) were grown in Neural progenitor maintenance bulletkit according to the manufacturer's protocol, at 37°Cin a 5% CO_2 atmosphere.

2.1.4 Human glioblastoma-derived cancer stem cell and mouse neural stem cells

Human glioblastoma-derived cancer stem cell lines (GBM CSCs) and a mouse neural stem cell line (NSC), both undifferentiated and differentiated, were kindly provided by Dr. Rossella Galli (Neural stem cell biology unit, Division of regenerative medicine, stem cell and gene therapy, Istituto Scientifico San Raffaele, Milano).

GBM CSCs and NSCs were characterised as reported (Mazzoleni *et al.* 2010; Galli *et al.* 2004; Foroni *et al.* 2007). Briefly, post-surgery tissues from primary glioblastomas or mouse brain tissue were dissected, enzymatically digested and dissociated into a single cell suspension. Viable cells were plated at clonal density in serum-free, stringent and low-density conditions. DMEM/F12 medium added with 20 ng/ml of both EGF and FGF2, was used to allow selection away of differentiated/differentiating cells and enriching for the stem cell component (Reynolds and Rietze 2006). The resulting neurospheres were analysed and validated for being *bona fide* stem cells, by assessing their ability for long-term proliferation, self-renewal (by subcloning experiments), multipotency (by inducing differentiation into the three neural cell lineages) and tumorigenicity (by assessing their capacity to give rise to tumours *in vivo*).

0622 and 070104 GBM CSCs and NSCs were terminally differentiated in the three major neural cell types (astrocytes, oligodendrocytes and neurons) by culturing them in mitogen-free medium supplemented with 2% FBS. Immunofluorescence for neural antigens was performed to assess differentiation.

2.2 Gene expression analysis

We performed real-time quantitative PCR on cDNAs from tissue samples and GBM CSCs to quantify total MARK4 expression levels and from HEK293T and G157 cell lines to outline MARK4L and MARK4S expression profile.

2.2.1 RNA extraction

RNA was extracted from tissues and cells using the TRIreagent (Total RNA Isolation reagent, Sigma, Saint Louis, MI, USA), a phenol-guanidine thiocianate solution that dissolves cell components leaving nucleic acids intact.

Tissue specimens were homogenised in an appropriate volume of TRIreagent (according to their weight) with a ULTRA-TURRAX T25 homogeniser (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany); cells were instead harvested, washed in PBS and resuspended in TRIreagent by repetitive pipetting. After 10 min incubation, the homogenates were supplemented with chloroform, vigorously shaken and centrifuged at 12,000 g for 15 min at 4°C. Following centrifugation the mixture separates into three phases: RNA is in the upper-aqueous phase, DNA is in the interphase, while the lower-organic phase contains proteins; RNA was then extracted according to manufacturer's instructions.

DNase I (RNase-free, New England BioLabs, Inc., Ipswich, MA, USA) was added to RNA to remove residual DNA.

RNA quantity and quality were determined by measuring absorbance at 230, 260 and 280 nm with the ND-1000 Spectrophotometer (NanoDrop products, Waltham, MA, USA, by Thermo Fisher Scientific, Inc.).

2.2.2 Reverse transcription PCR (RT-PCR)

Complementary DNA (cDNA) was obtained from tissues and cell lines by reverse transcription PCR (RT-PCR). 500 ng of total RNA from cell lines and 250 ng of total RNA from tissue samples were processed using the High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), with random examers, according to the manufacturer's protocol.

Each sample was reverse-transcribed in two independent reactions. A PCR amplification of the housekeeping gene GAPDH was then performed to check the efficiency of RT-PCR. PCR conditions and primers used are reported below.

PRIMER	*SEQUENCE*	*Ta*
GAPDH F	5'-ACAACAGCCTCAAGATCATCAG -3'	62°C
MARK4 del87 nt R	5'-GGTCCACCACTGACACGTTG -3'	

2.2.3 Real-time quantitative PCR

Real-time PCR allows cDNA quantification of a target gene with high accuracy and sensitivity. Differently from the traditional PCR reaction, real-time PCR enables the measurement of amplified products as the reaction progresses, through the inclusion in the reaction of a sequence-specific, fluorescently labelled oligonucleotide probe, called TaqMan, that reports an increased in the amount of the target product with a proportional increase in fluorescent signal. Therefore, the measured fluorescence reflects the amount of amplified product in each cycle.

The real-time reaction shows an exponential phase, when the amount of PCR product approximately doubles in each cycle, followed by a plateau phase, when one or more of the components is/are consumed during the reaction and become(s) limiting.

The real-time PCR instrument registers the fluorescent signal in the exponential phase, in particular at the cycle number when enough amplified product accumulates to yield a fluorescent signal that overcomes the background level, called the threshold cycle or C_T. At the C_T the amount of the generated PCR products is directly proportional to the original quantity of the target gene cDNA.

Relative mRNA levels of the target gene may be obtained by relative quantification through data normalisation on reference housekeeping genes.

2.2.3.1 TaqMan assays and real-time PCR

Real-time PCR experiments were performed with the TaqMan Fast Universal PCR Master Mix 2x No AmpErase UNG and specific TaqMan gene expression assays, all from Applied Biosystems. In particular we used inventoried assays for actin β (ID: Hs99999903_m1) and total MARK4 (Hs00230039_m1), a pre-developed assay for GAPDH (ID:4333764F) and custom assays for MARK4S and MARK4L. Features of the TaqMan assays on demand are provided in Table 2:

ASSAY	*PRIMER SEQUENCE*	*PROBE SEQUENCE*
MARK4L	**F**5' CCGAAGGGTCGCAGACGAA 3' **R**5' CCGTTTGATCCCAAGGTAGATG 3'	5' CCTGAGGTCACAAGTT 3'
MARK4S	**F**5' GTTACCCTCGATCCCTCTAAACG 3' **R**5' GTTCGTCTGCGACCTGATCTT 3'	5'CAGAACTCTAACCGCTGTGT 3'

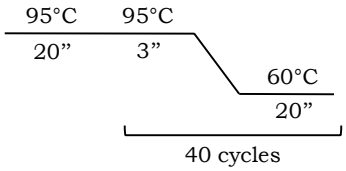
Table 2. Primer and probe sequences for MARK4S and MARK4L custom assays.

Primers and probes for MARK4S and MARK4L assays have been designed to specifically amplify only one isoform and, when possible, at the exon-exon boundary to avoid genomic DNA amplification. In detail, MARK4S forward primer and probe bind to the alternative exon 16, specific of the S isoform, and reverse primer is localised at the exons 16-17 boundary. MARK4L forward primer binds to the exons 15-17 junction, while the probe and reverse primer span exon 18.

A reaction mix was prepared as follow for each sample and assay:

H_20	2.5 µl
Master Mix (2x)	5.0 µl
Assay (20x)	0.5 µl
cDNA (1.5 fold diluted)	1.5 µl

Reactions were run on the StepOne Real-Time PCR System (Applied Biosystems), under the following conditions:



All samples were reverse transcribed in two independent reactions and loaded in triplicate in real-time experiments.

2.2.3.2 Relative quantification analysis

C_T values from each triplicate were recovered from the StepOne Software (v1.2; Applied Biosystems) and then analysed using two different methods: the $2^{-\Delta\Delta C}{}_T$ method (Livak*et al.*, 2001) for cell lines, and the geNorm method ($E^{-\Delta C}{}_T$) (Vandesompele 2002) for tissue samples and GBM CSCs.

In both cases, MARK4S and L expression levels were normalised on housekeeping genes and then referred to a sample chosen as reference (NHNP for cell lines and normal brain for tissue specimens and GBM CSCs), whose expression value was set as 1.

According to Livak method, for each sample the target gene expression level = $2^{-\Delta\Delta C_T}$

where: $\Delta\Delta C_T$ = sample ΔC_T – reference ΔC_T;

sample ΔC_T = [target gene C_T – control gene C_T] in the sample;

reference ΔC_T = [target gene C_T – control gene C_T] in the reference;

(sample and reference ΔC_T correspond to the mean value between the ΔC_Ts of the two independent RT reactions).

In the geNorm method, instead, multiple control genes are used and the amplification efficiency (E, set as 2 in the $2^{-\Delta\Delta C_T}$ method) is calculated for each assay, taking into account the specific efficiency of each assay.

Values with standard deviation exceeding 0.5% or standard error exceeding 0.3% were excluded and experiments repeated. Real-time expression data were expressed as mean ± standard deviation.

2.2.3.3 Statistical analysis

The Kruskal-Wallis and the 1-sample Wilcoxon tests were used for statistical analysis performed with the StatistiXL 1.8 software for Microsoft Excel (www.statistixl.com). Differences between experimental groups were considered significant when $p < 0.05$.

2.2.4 TaqMan assay validation

Amplification efficiency and stability of TaqMan gene expression assays (total MARK4, MARK4L, MARK4S, GAPDH, actin β, RPLP0 and HPRT) were evaluated before relative quantification analysis.

2.2.4.1 Assay amplification efficiency

Assay amplification efficiency is essential to allow an accurate and reproducible quantification of target cDNA. This optimisation was achieved by running serial dilutions of a cDNA pool of the samples under study and using the resulting C_T to generate a standard curve. The standard curve was obtained by plotting logarithmic dilution values of each concentration against the relative C_T value. The equation of the linear regression line was then used to evaluate

amplification efficiency (E). E was calculated according to the $E=10^{-1/S}$ formula, where S is the slope of the regression line. As reported in Table 3 the resulting efficiency was near 2 for all the analysed assays. A 100% amplification efficiency has an E value of 2 since, ideally, PCR product quantity should double at every PCR cycle. Therefore, all tested assays have a good amplification efficiency.

2.2.4.2 Assay stability

To assess assay stability, the ability to amplify the target gene with the same efficiency in a broad range of sample dilutions, we calculated the relative quantity (Q) of each gene in the cDNA serial dilutions, according to the following formula $Q=E^{-\Delta C_T}$ (ΔC_T =sample C_T – reference C_T), and then we sorted the assays on the basis of their stability using the GeNorm software (version 3.5; http://medgen.ugent.be/jvdesomp/genorm/). GAPDH and actin β were chosen as control genes, since they were the most stable and showed an amplification efficiency, in our pool of samples, more similar to that of our target genes. In detail, for cell lines, given their homogenous nature, we used a single reference gene (GAPDH), while GAPDH and actin β were used in combination for tissue samples and GBM CSCs.

ASSAY	*DILUTIONS*	C_T	*S*	*E*	*ΔCt*	*RELATIVE QUANTITY*
	1	16.52	-3.30	**2.01**	0.00	1.00
	1:10	19.73			3.21	0.11
GAPDH	1.100	23.05			6.52	0.01
	1:1000	26.41			9.89	0.00
	1	16.78	-3.65	**1.88**	0.00	1.00
	1:10	20.35			3.57	0.11
ACTB	1.100	24.02			7.25	0.01
	1:1000	27.71			10.93	0.00
	1	17.74	-3.28	**2.02**	0.00	1.00
	1:10	20.85			3.10	0.11
RPLP0	1.100	24.18			6.44	0.01
	1:1000	27.56			9.82	0.00
HPRT	1	21.78	-3.31	**2.00**	0.00	1.00

	1:10	24.95			3.18	0.11
	1.100	28.20			6.43	0.01
	1:1000	31.74			9.96	0.00
	1	26.19	-3.32	**2.00**	0.00	1.00
MARK4	1:10	28.84			2.65	0.11
	1:100	32.30			6.11	0.01
	1:1000	36.11			9.92	0.00
	1	23.75	-3.40	**1.97**	0.00	1.00
	1:10	27.00			3.25	0.11
MARK4L	1.100	30.50			6.75	0.01
	1:1000	33.92			10.17	0.00
	1	28.00	-3.54	**1.92**	0.00	1.00
	1:10	31.26			3.25	0.11
MARK4S	1.100	35.00			6.99	0.01
	1:1000	N.D.			/	/

Table 3. Assay amplification efficiency (E). For each assay, C_T values, corresponding to serial dilutions of a pool of glioma cDNAs, are displayed. S, slope of the interpolating line; N.D., undetermined.

2.3 Bioinformatic prediction of splicing factor binding sites and analysis of conservation

Putative binding sites for splicing factors in MARK4 IVS15 and the proximal region of IV16 were predicted using SFmap (http://sfmap.technion.ac.il/) and SpliceAid (http://193.206.120.249/splicing_tissue.html). The two web resources use different approaches. SFmap predicts splicing factors binding sites by the implementation of the COS(WR) algorithm (Akerman *et al.*, 2009), which analyses similarity scores for a given regulatory motif on the basis of information derived from its sequence environment and its evolutionary conservation. The most important advantages of this algorithm are that it is very accurate in prediction and it calculates standardised scores for any motif, so that the predictions of different binding sites are comparable (Paz *et al.*, 2010). On the other hand, SpliceAid is a database of strictly experimentally assessed RNA sequences bound by splicing factors. To date a total of 680 target sites and 54 proteins have been annotated (Piva *et al.*, 2009).

Conservation analysis of the predicted binding sites for splicing factors was performed with Clustal Omega, a web resource that allows multiple alignments of sequences exploiting the multiple progressive alignment method. Briefly, the program initially starts to align short portions of the query sequences that appear more correlated, then it proceeds aligning the remaining parts of the sequences without changes in the previous preliminary alignment. By this way the program is able to output the alignment with the maximum score (Sievers *et al.*, 2011).

2.4 Protein expression analysis

2.4.1 Protein extraction

Tissue samples were homogenised using an ULTRA-TURRAX T25 homogeniser (Janke & Kunkel) in 400 µl lysis buffer/50 mg, whose composition is reported below.

150 mM NaCl 50 mM Tris pH 7.5 1% NP-40, 0.25% deoxycholic acid, protease inhibitor cocktail (*Complete EDTA-free,* from Roche Diagnostic, Manheim, Germany)

Suspensions were placed on ice for 30 min with occasional inversion to ensure complete lysis. Lysates were then spun at 12,000 g for 30 min at 4°C, to prevent protein degradation, and the supernatant (whole cell lysate) was stored at -20°C.

Protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), according to the manufacturer's protocol, and the ND-1000 Spectrophotometer (NanoDrop products).

2.4.2 Immunoblotting

Immunoblotting enables separation of proteins according to their molecular weight and the identification of the presence of a specific protein, its size and its relative expression level.

For each sample 30 µg of extracted proteins were supplemented with reducing SDS (Sodium Dodecyl Sulphate) loading buffer (Blue loading buffer pack, Cell Signaling Technology, Inc., Beverly, MA, USA) and denatured at 99°C for 3 min.

Proteins were then resolved by SDS PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (4% stacking gel at 100V for 10 min and 10% resolving gel at 130V 100 min). Proteins were then transferred by electroblotting to a PVDF (Polyvinylidene fluoride) membrane (Roche Diagnostic, Manheim, Germany). Mini-PROTEAN Tetra cell and Trans-blot semi-dry electrophoretic transfer cell (both Bio-Rad, Hercules, CA, USA) were respectively used for electrophoresis and electroblotting (10V, 30 min) as instructed by the manufacturer. The Biotinylated protein ladder (Cell Signaling Technology) and ColorBurst electrophoresis marker (Sigma) were used as molecular weight standards.

Membranes were then washed twice in PBS-T 0,3% (PBS ph7.5: 100 mM NaCl, 80 mM HNa_2O_4P, 20 mM NaH_2PO_4 with 0.3% Tween20), and non-specific binding was blocked by incubating the membranes in 5% skimmed milk, PBS-T 0,3% for 1 h at room temperature (RT),

in gently agitation. The membranes were incubated with primary antibodies in PBS-T at 4°C overnight in agitation.

Rabbit anti-MARK4L (Genscript Corporation, Piscataway, NJ, USA)	1: 1,5000
Mouse anti-PTB (ab30317; Abcam, Cambridge, UK)	1: 1,200
Mouse anti-GAPDH (ab8245, Abcam)	1: 10,000

After five washes in PBS-T 0,3%, membranes were incubated with secondary antibodies in PBS-T 0,3% at RT for 1 h in agitation. Secondary antibodies are conjugated to HRP (Horse Radish Peroxidase) and an anti-biotin antibody was added to detect the biotinylated protein ladder.

Goat anti-rabbit IgG-HRP (sc-2004; Santa Cruz Biotechnology)	1: 10,000
Goat anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotechnology)	1: 25,000
Anti-biotin, HRP-linked antibody (Cell Signaling)	1: 2,500

For quantification, the membranes were cut horizontally immediately after the blocking step and incubated with the appropriate antibodies.

After four washes in PBS-T 0,3 % and two washes in PBS, membranes were incubated 5 min with a peroxide/enhancer solution (Westar ηC,Cyanagen, Bologna, Italy) and blots were scanned using the Gbox Chemi XT4 system (Syngene, Cambridge, UK).

2.4.3 Semi-quantitative analysis

To compare PTB and MARK4L protein expression in different tissue samples or cell lines we did a relative quantification analysis, using GAPDH as normaliser. For each sample two immunoblotting experiments were performed maintaining unaltered conditions. For quantification, blot images were acquired at low pixel saturation levels, so that the acquired signal intensity was directly proportional to the emitted chemiluminescent intensity. Images were analyzed with the Gene Tools Gel Analysis software (Syngene). Each lane (including both PTB or MARK4L and GAPDH bands) was selected and lane profile plots were automatically generated for each band in the lane, displaying a two-dimensional graph reporting peaks corresponding to gel bands (on the X-axis) and their relative pixel intensities (on the Y-axis). After automatic subtraction of the background the peaks area was measured in square pixels. For data normalisation, in each sample MARK4L or PTB area value was divided by the respective GAPDH value.

2.5 Splicing minigene assay

Minigene constructs are a useful tool for identification and *in vivo* analysis of *cis*-regulatory elements and *trans*-acting factor involved in alternative splicing regulation. In particular, minigenes are commonly used to : i) identify splice sites implicated in the mechanism of exons recognition and splicing; ii) identify exonic/intronic splicing enhancers and silencers; iii) determine the effect of an allelic variant or a mutation on splicing; iv) identify cell-specific splicing events and the underneath elements; v) identify binding sequence for a specific splicing.

2.5.1 Minigene design

We cloned the genomic fragment corresponding to MARK4 exons 15, 16 and 17 and the intervening sequences (IVS) 15 and 16 in the mammalian expression vector pcDNA3.1 (Invitrogen, Camarillo, CA, USA) (Figure 14). The fragment was PCR amplified from DNA of HEK293T cells, extracted with the QIAmp DNA mini kit (Qiagen S.P.A., Milano, Italy), with specific primers containing unique restriction enzyme sites at their 5'-ends, to allow the insertion of the amplified fragment into the multicloning site of pcDNA3.1 (Figure 14).

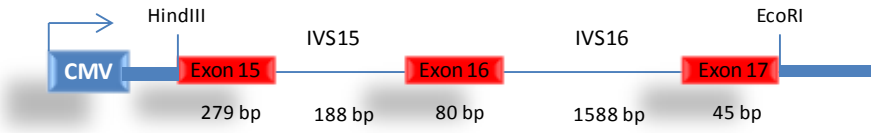


Figure 14. Schematic representation of MARK4 exons 15-17, IVS15 and IVS16 cloning into the mammalian expression vector pcDNA3.1.

In particular, the forward primer contained the restriction site for HindIII (AAGCTT) and the reverse primer the restriction site for EcoRI (GAATTC). Since many restriction enzymes do not cut efficiently when the restriction site is at the end of the molecule, six non-specific nucleotides have been included as the first nucleotides of the primers. These nucleotides are followed by the restriction site and 12 nucleotides complementary to the 5'-end of exon 15 and the 3'-end of exon 17,respectively (Table 4).

PRIMER	***SEQUENCE***
MARK4 cl 15-17 F	5'-**ATAACC**AAGCTTCTCAGGCACCCC-3'
MARK4 cl 15-17 R	5'-**CGCACT**GAATTCCTTGTGACCTCA-3'

Table 4. Sequences of primers used for MARK4 exons 15-17 and interposed introns cloning. Non specific nucleotides are bolded and restriction site sequences are underlined.

The PCR reactions were performed with the high fidelity polymerase Phusion Taq (Finnzymes, Espoo, Finland), to avoid insertion of mutations, and using a specific buffer for GC-rich sequences (GC Buffer) since the MARK4 sequence of interest appeared very difficult to amplify, probably because of the nucleotide composition.

GC Buffer	5 µl
dNTPs (20 mM)	1 µl
DMSO	2 µl
MK4 cl 15-17 F	1,2 µl
MK4 cl 15-17 R	1,2 µl
DNA	80 ng
Taq	0,5 µl
H_2O	to 50 µl

PCR amplicons were then run on a 2% agarose gel to verify their molecular weight. PCR were precipitated by adding 1/10 of volume of sodium acetate 2.5 M pH 5.2 and two volumes of 100% cold ethanol O.N at -20°C. Precipitated PCR were then spun at 13,000 g for 30 min at 4°C and washed twice in 70% cold ethanol.

Both the empty pcDNA3.1 vector and the precipitated MARK4 PCR were cut with HindIII and EcoRI restriction enzymes (Promega, Madison, WI, USA) as follows:

vector/insert	500 ng	
Buffer E	2 µl	
BSA	0.2 µl	1 h 15 min 37°C
EcoRI	0.5 µl	
HindIII	0.5 µl	
H_2O	to 20 µl	

Digested vector and PCR were run on a 1% agarose gel, excise and gel purified with the MinElute Gel Extraction kit (Qiagen) according to the manufacturer's protocol. Eluted bands were then quantify with the ND-1000 Spectrophotometer (NanoDrop products) and ligated with the T4 DNA Ligase (Promega) maintaining a vector:insert molar ratio of 1:3 as reported below:

10X Buffer	2 µl	
pcDNA3.1	60.1 ng	
MK4 cl 15-17	50 ng	1 h 16°C
T4 DNA Ligase	0.5 µl	
H_2O	to 10 µl	

2.5.2 Transformation and plasmid DNA extraction

E. coli TOP10 were transformed with the whole ligation reaction. Cloning and transformation of the selected MARK4 sequence was particularly hard, probably because of the nucleotide composition of the sequence with multiple repeated stretches that favour plasmid recombination. TOP10 were selected among different available *E. coli* strains, after multiple transformation experiments, to obtain a higher transformation efficiency. Briefly, 100 µl of chemically competent TOP10 were incubated on ice with the whole ligation reaction for 20 min. Bacteria were heat shocked at 42°C for 2 min and then, after addition of 1 ml of LB broth, they were grown at 37°C for 1 h with shaking at 230 rpm. Transformed bacteria were pelletted at 6,000 rpm for 5 min at room temperature and then plated on LB agar plates supplemented with 100 µg/ml Ampicillin (Sigma) for O.N growth at 37°C.

The day after three colonies were selected and grown in 3 ml LB broth O.N at 37°C with shaking at 230 rpm. Plasmid DNA was then extracted using the PureYield^{TM} Miniprep System (Promega) and quantify with the ND-1000 Spectrophotometer (NanoDrop products).

To verify the presence of the MARK4 insert, its correct orientation and the absence of mutations the extracted plasmids were PCR amplified and sequenced. GoTaq polymerase and reagents from Promega were used for PCR amplification, while Big DyeTerminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) was used for sequencing.

2.5.3 Transfection and splicing assay

MARK4 wild type minigene was transfected in HEK293T and G157 cells to perform splicing assays. Cells were plated in Petri dishes of 35 mm at a concentration of 500,000 cells and grown in DMEM supplemented with 10% fetal bovin serum (FBS) for HEK293T or in RPMI with 5% FBS for G157, in both cases without antibiotics, at 37°C in a 5% CO_2 atmosphere. After 24 h cells were transfected with the MARK4 minigene splicing vector. The MARK4 minigene (1 µg) was incubated with 6 µl of X-tremeGENE HP DNA Transfection Reagent (Roche) in 200 µl DMEM or RPMI without FBS and antibiotics for 20 min at RT. The transfection reaction was then gently added to the cells after replacing the medium. After 24 h RNA was extracted from transfected cells using TRIreagent (Sigma) and treated with DNaseI (New England Bio-Labs, Inc.) as previously described (see 2.2.1). After quantification by the ND-1000 Spectrophotometer (NanoDrop products), 250 ng of total RNA were reverse transcribed using the High capacity cDNA reverse transcription kit (Applied Biosystems) as previously described (see 2.2.2). To

verify the efficiency of reverse transcription and the absence of genomic DNA contamination a PCR for GAPDH was preformed (see 2.2.2).

Splicing of the MARK4 minigene was analysed by RT-PCR with primers specifically designed to amplify the transcripts derived from the minigene. In particular, the forward primer binds to MARK4 exon 15, while the reverse primer is complementary to a pcDNA3.1 portion that is transcribed, corresponding to the bovine growth hormone (BGH) polyadenylation signal. Primers sequences are reported below:

PRIMER	*SEQUENCE*
MARK4 PSTK10 F	5'-CTGACCTCCAAACTGACCCG-3'
MARK4 BGH R	5'-GGCTGGCAACTAGAAGGCAC-3'

2.5.4 MARK4 splicing minigene mutagenesis

To identify splicing regulatory sequences localised in IVS15 involved in MARK4 alternative splicing, serial deletions were performed in the MARK4 splicing minigene vector. Two different approaches were used to obtained the designed deletions: excision of a portion of the minigene and re-synthesis of the excised portion with the desired deletion using partially complementary primers; alternatively the QuickChange II XL Site-Directed Mutagenesis kit (Agilent, Santa Clara, CA, USA) was used.

The first approach was used to make the deletion of the last 82 nucleotides of MARK4 IVS15. We designed a reverse primer with, starting from the 5'-end, the restriction site for the endonuclease enzyme Bgl2, 7 nucleotides corresponding to the 5'-end of the exon 16, the two last bases of IVS15 and 11 nucleotides corresponding to the MARK4 IVS15 sequence upstream the deletion (Table 5). The forward primer was the same used for the wt MARK4 minigene vector. The desired sequence was PCR amplified from MARK4 wt minigene using the Phusion Taq (Finzymes) and the primers reported in Table 5 and then precipitated by ethanol-sodium acetate as previously reported (see 2.5.1). Both MARK4 wt minigene and the newly synthesised insert were digested with HindIII for 3 h at 37°C, then they were precipitated by ethanol-sodium acetate and digested with Bgl2 endonuclease enzyme for 3 h at 60°C. Precipitated digested vector and insert were then ligated and used in transformation experiments as reported in section 2.5.2.

PRIMER	*SEQUENCE*	*Ta*
MARK4 del87 nt F	5'-**ATAACC**AAGCTTCTCAGGCACCCC-3'	68°C
MARK4 del87 nt R	5'-**CGCACT**GAATTCCTTGTGACCTCA-3'	

Table 5. Primers and annealing temperature used for deletion of 87 nucleotides of IVS15 in MARK4 minigene.

For all other deletions the QuickChange II XL Site-Directed Mutagenesis kit (Agilent) was used following manufacturer's instructions. Primers were designed with the specific QuickChange primer design tool (available online at www.genomics.agilent.com/primerDesignProgram.jsp) (Table 6). Briefly, PCR products were digested with Dpn I to remove template from the reaction and then 2 µl were used for transformation of XL10-Gold ultracompetent *E. Coli* strain (Qiagen). After 30 min incubation of the transformation reaction on ice, cells were heat shocked for 30 seconds at 42°C and then grown in NZY^+ broth for 1 h at 37°C with shaking at 230 rpm. Transformed cells were then plated on LB agar plates with 100 µg/ml Ampicillin and grown O.N. at 37°C. Plasmid extraction and sequencing were performed as previously described in section 2.5.2.

PRIMER	*SEQUENCE*
MARK4 delPTB F	5'-TACCCCAACATTTCC------CTCCTCCTCCTCCTC-3'
MARK4 delPTB R	5'-GAGGAGGAGGAGGAG------GGAAATGTTGGGGTA-3'
MARK4 del15prox F	5'-GTACCCCAACATTTCC------CCTCGTTTCCTCCTCC-3'
MARK4 del15prox R	5'-GGAGGAGGAAACGAGG------GGAAATGTTGGGGTAC-3'
MARK4 del32 nt F	5'-GTACCCCAACATTTCC------TCCTCCTCCTTTCCTC-3'
MARK4 del32 nt R	5'-GAGGAAAGGAGGAGGA------GGAAATGTTGGGGTAC-3

Table 6. Primers used for deletions in IVS15 of MARK4 minigene vector.

All mutated MARK4 minigenes were transfected in both HEK293T and G157 cell lines to perform splicing assays. Splicing of the mutated minigene was evaluated by RT-PCR as previously reported in section 2.5.3.

2.6 Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assays were performed to evaluate *in vitro* interaction of splicing regulators with the MARK4 IVS15. Further mass spectrometry experiments are in progress to identify proteins bound to MARK4 mRNA in the shifted band.

2.6.1 MARK4 intron 15 *in vitro* RNA synthesis

A single strand RNA corresponding to 71 nucleotides of the polypyrimidine tract of IVS15 was in vitro synthesised by means of the T7 Quick High Yield RNA Synthesis Kit (New England BioLabs) using a forward primer containing the T7 promoter sequence at the 5'-end to obtain a single strand molecule of MARK4 IVS15 RNA (Table 7).

PRIMER	*SEQUENCE*
MARK4 EMSA F	5'-TAATACGACTCACTATAGGGTCTCCTGTACCCCAACA-3'
MARK4 EMSA R	5'-GGAAAGGAGGAGGAG-3'

Table 7. Primers sequences used for MARK4 IVS15 RNA in vitro synthesis. The T7 promoter sequence is underlined.

Reactions conditions were as follow:

NTP buffer mix	10 µl	
MARK4 wt minigene	1 µg	37°C O.N.
T7 RNA polymerase mix	2 µl	
H_2O DNAse/RNAse free to	30 µl	

Synthesised RNA was then extracted with phenol-chloroform and precipitated in ethanol according to manufacturer's instructions. Purified RNA was dosed by ND-1000 Spectrophotometer (NanoDrop products).

2.6.2 MARK4 RNA 3'-end biotinylation

MARK4 IVS15 RNA was biotinylated at the 3'-end to performed non-radioactive EMSA. We used the RNA 3' End Biotinylation Kit(Pierce) under the following conditions

10X RNA Ligase Reaction Buffer	3 µl	
RNase Inhibitor	1 µl	
MARK4 IVS15 RNA	50 pmol	
Biotinylated Cytidine (Bis)phosphate	1 µl	16°C O.N
T4 RNA Ligase	2 µl	
30% PEG	15 µl	
Nuclease-free water	to 30 µl	

Biotinylated RNA was then extracted by chloroform:isoamyl alcohol and precipitated in 10µL of 5M NaCl, 1µL of glycogen, and 300µL of ice-cold 100% ethanol at -20°C for 1 h.

2.6.3 EMSA

Non-radioactive EMSA experiments were performed using the LightShift Chemiluminescent RNA EMSA Kit (Pierce). Three binding reactions were set up: 1) only MARK4 biotinylated RNA, used to define the position of unshifted EMSA band; 2) MARK4 biotinylated RNA incubated with cell lysate, to identify possible shifted band by comparison with reaction 1; and 3) MARK4 biotinylated RNA incubated with cell lysate and 200-fold molar excess of unlabeled MARK4 RNA, to understand if the observed shift results from a specific protein-RNA interaction. Reactions were performed as follow:

Reagents	***Reaction***		
	1	**2**	**3**
10X Binding Buffer	2 µl	2 µl	2 µl
tRNA (10 mg/ml)	0.2 µl	0.2 µl	0.2 µl
MARK4 unlabeled RNA	-	-	10 µM
HEK293T protein extract	-	3 µg	3 µg
MARK4 biotinylated RNA	2 nM	2 nM	2 nM
Nuclease-free water	to 20 µl	to 20 µl	to 20 µl

Binding reactions were incubated 30 min at R.T. and then loaded on a 8% native polyacrylamide gel in 0.5X TBE (pre-run for 1 h in 0.5X TBE) after addition of 5 µl 5X Loading Buffer. After 1 h run at 100 V the gel was transferred on a nylon membrane (GE Healthcare) soaked in 0.5X TBE for at least 10 min. Transfer was performed with the Trans-blot semi-dry electrophoretic transfer cell (Bio-Rad) for 30 min at 10 V. The membrane was then crosslinked with a hand-held UV lamp (254 nm) for 5 min at a distance of 0.5 cm. Detection of biotin-labeled RNA was performed according to the manufacturer's instructions and images of the membranes were acquired with the Gbox Chemi XT4 system (Syngene).

Results

3.1 Alternative splicing as regulator of MARK4 expression

The identification of a MARK4L/MARK4S imbalance in glioma, derived from the overexpression of the alternative MARK4L isoform to the detriment of the canonical MARK4S, and not imputable to genomic mutations (Magnani *et al.*, 2011), prompted us to hypothesise an alteration at the post-transcriptional level responsible for the observed MARK4 isoforms altered ratio.

The observation that the MARK4L/MARK4S ratio is carefully guarded during neural differentiation and that a switch from MARK4L expression to MARK4S drives neuronal commitment and terminal differentiation (Moroni *et al.*, 2006; Magnani *et al.*, 2011), put on scene alternative splicing as the physiological mechanism for MARK4 isoform expression regulation according to cell differentiation hinting it might go awry during gliomagenesis. A splicing regulation of isoforms expression implies that no differences in the total expression of mRNA are observed, even if the relative quantity of the single isoforms differ between samples. We thus performed real-time quantification of total MARK4 as starting point to define the role of alternative splicing in the regulation of MARK4 isoforms expression in normal and transformed cells.

3.1.1 Total MARK4 mRNA expression profile in glioma tissues and glioblastoma-derived cancer stem cells

Real-time PCR analysis on 7 astrocytomas (A) (II/III WHO grade), 8 glioblastomas (GBM) (IV grade) and three glioblastoma-derived cancer stem cells (GBM CSCs) compared to human normal brain, did not show, despite the heterogenous expression of total MARK4,significant differences in the expression of total MARK4among the three subgroups ($p = 0.07$ at Kruskal-Wallis test) (Figure 15). Total MARK4 expression levels are comparable to those observed in normal brain ($p > 0.05$ at Wilcoxon test) even if, within the same glioma samples (A, GBM and GBM CSCs), the relative expression of the two MARK4 isoforms was significantly different from normal brain, as reported in Magnani *et al.*, 2011. A slight decrease in MARK4 expression is apparent when considering the mean values of the single subgroups but no correlations between MARK4 expression levels and molecular diagnostic and prognostic markers of glioma samples could be retrieved according to Kruskal-Wallis test.

These evidences suggested that the observed altered ratio in MARK4 isoforms expression is not due to alterations in the *MARK4* gene transcription, but probably in post-transcriptional mechanisms.

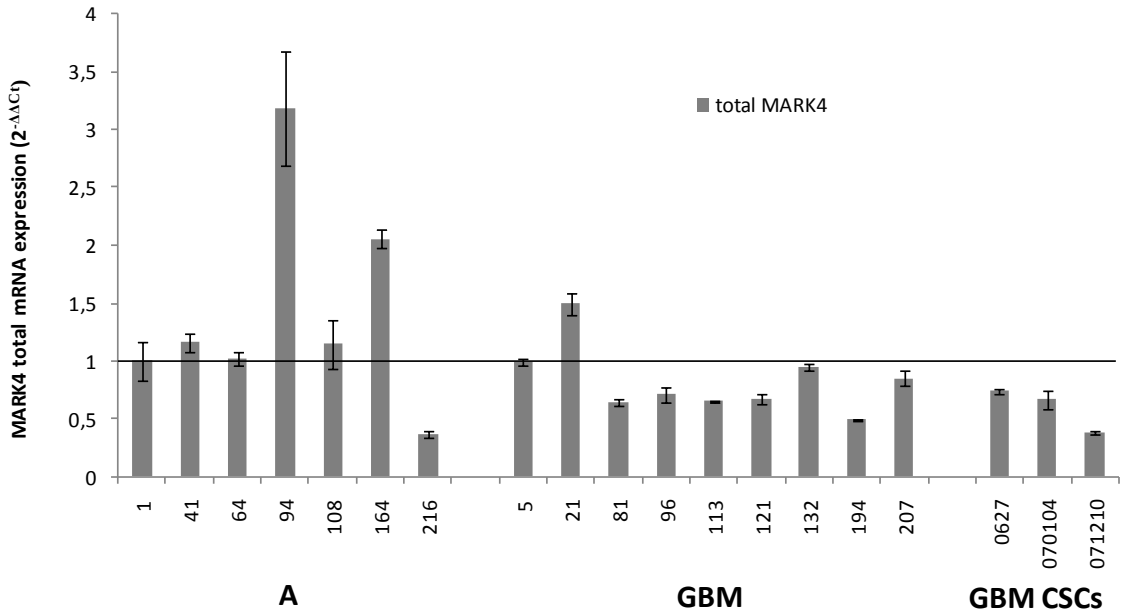


Figure 15. Relative expression levels of MARK4 total mRNA in astrocytomas (A), glioblastomas (GBM) and glioblastoma-derived cancer stem cells (GBM CSCs) compared to human normal brain (conventionally set as one and represented by a horizontal black line). Data are presented as mean ± standard deviation of three independent experiments.

3.2 Bioinformatic identification of putative splicing regulatory sequences in MARK4 mRNA

We thus performed bioinformatic analysis of the MARK4 mRNA region involved in alternative splicing to identify putative regulatory sequences targeted by trans-acting factors, i.e SR or hnRNP proteins.

Since it has been reported that also splicing regulatory sequences, like other regulatory elements, are conserved across divergent species (Sorek and Ast, 2003), we also evaluated the conservation between human and mouse of the regulatory sequences identified by bioinformatic analysis.

3.2.1 Identification of PTB binding sites in MARK4 introns 15 and 16

To identify putative regulatory sequences, in MARK4 mRNA, bound by splicing regulators, we used SFmap (http://sfmap.technion.ac.il/) and SpliceAid (http://193.206.120.249/splicing_tissue.html). These bioinformatic tools were chosen among the free available, because they also search for negative regulators of alternative splicing belonging to the hnRNP family. Indeed, given the mechanism of MARK4 alternative splicing and the observed altered ratio of the two isoforms in glioma, we suspected a role of hnRNP in the regulation of exon 16 skipping in both physiological and pathological conditions. The use of both prediction tools revealed the presence of a multitude of putative regulatory sequences falling in the two main classes of positive and negative splicing regulators (Table 8 and Figure 16). We then selected out of the sites located in the introns flanking the alternative MARK4 exon 16, only those predicted to induce exon skipping and reported to play a role in neural differentiation and gliomagenesis. Three putative binding sites for PTB were indentified in MARK4 IVS15 and IVS16 and appeared of particular interest, given the fundamental role of PTB in the proliferation of glial cells and in tumour transformation (Boutz *et al.*, 2007). In detail, two putative PTB binding sites are located upstream and one downstream the alternative exon 16 (Figure 17). The putative PTB binding sites indentified in both introns flanking exon 16 (highlighted in red) are compatible with all PTB mechanisms of alternative splicing as summarised in the Introduction (see1.6.3). In particular, the intronic sequence just upstream exon 16that includes one of the putative PTB binding sites is a long polypyrimidine tract, that represents the proper sequence environment for PTB activity, and is compatible with PTB mechanism of action by competition. Also the other identified sequences may be target of PTB

binding, allowing skipping of MARK4 exon 16 by propagation or looping, starting from the high affinity binding of PTB at the polypyrimidine tract (see Figure 10 of Introduction).

A

Predictions for sequence: sequence1

Splicing Factor: 9G8, Motif: wggacra, Cutoff: 0.557

Sequence Position	Genomic Coordinate	K-mer	Score
40	chr19:45801252	gggccga	0.609

Splicing Factor: SC35, Motif: gryymcyr, Cutoff: 0.600

Sequence Position	Genomic Coordinate	K-mer	Score
77	chr19:45801289	gucuccug	0.781

Splicing Factor: Tra2alpha, Motif: gaagaggaag, Cutoff: 0.500

Sequence Position	Genomic Coordinate	K-mer	Score
19	chr19:45801231	gcaggggcag	0.575

Splicing Factor: Tra2beta, Motif: ghvvganr, Cutoff: 0.637

Sequence Position	Genomic Coordinate	K-mer	Score
41	chr19:45801253	ggccgaug	0.775

Splicing Factor: SRp20, Motif: cuckucy, Cutoff: 0.557

Sequence Position	Genomic Coordinate	K-mer	Score
60	chr19:45801272	gucuucc	0.799
68	chr19:45801280	cucugcu	0.799
70	chr19:45801282	cugcucu	0.699
79	chr19:45801291	cuccugu	0.701
96	chr19:45801308	uuccucu	0.784
99	chr19:45801311	cucuucc	0.763
102	chr19:45801314	uuccucc	0.698
105	chr19:45801317	cuccucc	0.734
108	chr19:45801320	cuccucc	0.733
111	chr19:45801323	cuccucc	0.733
114	chr19:45801326	cuccucc	0.732
117	chr19:45801329	cuccucg	0.730
120	chr19:45801332	cucguuu	0.730
125	chr19:45801337	uuccucc	0.710
128	chr19:45801340	cuccucc	0.690
131	chr19:45801343	cuccucc	0.648
137	chr19:45801349	cuccucc	0.565
140	chr19:45801352	cuccuuu	0.616
145	chr19:45801357	uuccucc	0.616
148	chr19:45801360	cuccucc	0.683
151	chr19:45801363	cuccccu	0.696
165	chr19:45801377	cucaccu	0.639
167	chr19:45801379	caccucc	0.639
174	chr19:45801386	cuccuca	0.682

Splicing Factor: SRp20, Motif: wcwwc, Cutoff: 0.720

Sequence Position	Genomic Coordinate	K-mer	Score
61	chr19:45801273	ucuuc	0.908
100	chr19:45801312	ucuuc	0.780

Splicing Factor: hnRNPA1, Motif: rgnyag, Cutoff: 0.683

Sequence Position	Genomic Coordinate	K-mer	Score
17	chr19:45801229	uggcag	0.770
23	chr19:45801235	gggcag	0.800

Splicing Factor: hnRNPF, Motif: gukgykg, Cutoff: 0.586

Sequence Position	Genomic Coordinate	K-mer	Score
32	chr19:45801244	ggggcgg	0.766

Splicing Factor: hnRNPF, Motif: gggug, Cutoff: 0.700

Sequence Position	Genomic Coordinate	K-mer	Score
37	chr19:45801249	ggggg	0.792
38	chr19:45801250	ggggg	0.792

Splicing Factor: hnRNPU, Motif: uguauug, Cutoff: 0.500

Sequence Position	Genomic Coordinate	K-mer	Score
59	chr19:45801271	ugucuuc	0.571

Splicing Factor: MBNL, Motif: ygcuky, Cutoff: 0.650

Sequence Position	Genomic Coordinate	K-mer	Score
71	chr19:45801283	ugcucu	0.721

Splicing Factor: NOVA1, Motif: ycay, Cutoff: 0.750

Sequence Position	Genomic Coordinate	K-mer	Score
159	chr19:45801371	ucac	0.875
166	chr19:45801378	ucac	0.875
178	chr19:45801390	ucac	0.875

Splicing Factor: PTB, Motif: cucucu, Cutoff: 0.650

Sequence Position	Genomic Coordinate	K-mer	Score
58	chr19:45801270	cugucu	0.771
66	chr19:45801278	cacucu	0.781
73	chr19:45801285	cucugu	0.781
75	chr19:45801287	cugucu	0.786

Splicing Factor: PTB, Motif: ucuu, Cutoff: 0.750

Sequence Position	Genomic Coordinate	K-mer	Score
61	chr19:45801273	ucuu	0.840
100	chr19:45801312	ucuu	0.852

Splicing Factor: CUG-BP, Motif: ugcug, Cutoff: 0.600

Sequence Position	Genomic Coordinate	K-mer	Score
71	chr19:45801283	ugcuc	0.714
80	chr19:45801292	uccug	0.741

B

Predictions for sequence: sequence1

Splicing Factor: 9G8, Motif: wggacra, Cutoff: 0.557

Sequence Position	Genomic Coordinate	K-mer	Score
308	chr19:45801788	uggacug	0.656

Splicing Factor: SC35, Motif: gryymcyr, Cutoff: 0.600

Sequence Position	Genomic Coordinate	K-mer	Score
268	chr19:45801748	gucuccua	0.752

Splicing Factor: SC35, Motif: ugcygyy, Cutoff: 0.586

Sequence Position	Genomic Coordinate	K-mer	Score
6	chr19:45801486	ucccguc	0.597
35	chr19:45801515	ugccucc	0.662
66	chr19:45801546	ugcgggc	0.602

Splicing Factor: Tra2beta, Motif: aaguguu, Cutoff: 0.500

Sequence Position	Genomic Coordinate	K-mer	Score
187	chr19:45801667	aagugac	0.536
242	chr19:45801722	aaauggu	0.571
243	chr19:45801723	aaugguu	0.607
274	chr19:45801754	uaggguu	0.643
298	chr19:45801778	aagugac	0.571
336	chr19:45801816	aagugcu	0.694
347	chr19:45801827	augugua	0.703

Splicing Factor: SRp20, Motif: cuckucy, Cutoff: 0.557

Sequence Position	Genomic Coordinate	K-mer	Score
7	chr19:45801487	cccgucc	0.760
19	chr19:45801499	cuguucu	0.786
32	chr19:45801512	cucugcc	0.780
180	chr19:45801660	cucugcc	0.635
229	chr19:45801709	uuccucu	0.764
232	chr19:45801712	cucuucu	0.774

Sequence Position	Genomic Coordinate	K-mer	Score
233	chr19:45801713	ucuuc	0.871

Splicing Factor: SRp40, Motif: yywcwsg, Cutoff: 0.643

Sequence Position	Genomic Coordinate	K-mer	Score
21	chr19:45801501	guucucg	0.721
25	chr19:45801505	ucgcugg	0.721
214	chr19:45801694	ucucugg	0.785

Splicing Factor: hnRNPA1, Motif: rgnyag, Cutoff: 0.683

Sequence Position	Genomic Coordinate	K-mer	Score
153	chr19:45801633	agccag	0.762
174	chr19:45801654	uggcag	0.758
198	chr19:45801678	agcaag	0.782

Splicing Factor: hnRNPA2B1, Motif: aggwuhgr, Cutoff: 0.537

Sequence Position	Genomic Coordinate	K-mer	Score
157	chr19:45801637	agacucgg	0.693

Splicing Factor: hnRNPF, Motif: gukgykg, Cutoff: 0.586

Sequence Position	Genomic Coordinate	K-mer	Score
89	chr19:45801569	guggaug	0.727
116	chr19:45801596	cugguug	0.726
119	chr19:45801599	guuguuc	0.726
278	chr19:45801758	guuauug	0.636

Splicing Factor: hnRNPF, Motif: gugkau, Cutoff: 0.583

Sequence Position	Genomic Coordinate	K-mer	Score
61	chr19:45801541	gugugu	0.706
89	chr19:45801569	guggau	0.818
349	chr19:45801829	guguau	0.753

Splicing Factor: hnRNPF, Motif: gggug, Cutoff: 0.700

Sequence Position	Genomic Coordinate	K-mer	Score
61	chr19:45801541	gugug	0.781
76	chr19:45801556	ggggg	0.794
80	chr19:45801560	ggggg	0.794
91	chr19:45801571	ggaug	0.794
99	chr19:45801579	ggguc	0.797
107	chr19:45801587	gcgug	0.788
112	chr19:45801592	ggguc	0.788
118	chr19:45801598	gguug	0.775
165	chr19:45801645	ggguu	0.724

Splicing Factor: hnRNPM, Motif: gguugguu, Cutoff: 0.550

Sequence Position	Genomic Coordinate	K-mer	Score
114	chr19:45801594	gucugguu	0.562
118	chr19:45801598	gguuguuc	0.562

Splicing Factor: hnRNPU, Motif: uguauug, Cutoff: 0.500

Sequence Position	Genomic Coordinate	K-mer	Score
70	chr19:45801550	ggcauug	0.641
327	chr19:45801807	ugccuug	0.598

Splicing Factor: MBNL, Motif: ygcuky, Cutoff: 0.650

Sequence Position	Genomic Coordinate	K-mer	Score
26	chr19:45801506	cgcugg	0.698

Splicing Factor: PTB, Motif: cucucu, Cutoff: 0.650

Sequence Position	Genomic Coordinate	K-mer	Score
24	chr19:45801504	cucgcu	0.727
38	chr19:45801518	cucccu	0.790

Splicing Factor: PTB, Motif: ucuu, Cutoff: 0.750

Sequence Position	Genomic Coordinate	K-mer	Score
233	chr19:45801713	ucuu	0.805

Splicing Factor: CUG-BP, Motif: ugcug, Cutoff: 0.600

Sequence Position	Genomic Coordinate	K-mer	Score
66	chr19:45801546	ugcgg	0.662

Splicing Factor: FOX1, Motif: ugcaug, Cutoff: 0.450

Sequence Position	Genomic Coordinate	K-mer	Score
43	chr19:45801523	ugccug	0.729
47	chr19:45801527	ugcaug	0.740
90	chr19:45801570	uggaug	0.708
106	chr19:45801586	ugcgug	0.708
344	chr19:45801824	uacaug	0.583

Table 8. Putative regulatory elements, including both positive and negative regulators, identified by SFmap in MARK4 IVS15 (**A**) and the 5' portion of IVS16 (**B**). Scores of the probability that the indicated factor is really involved in alternative splicing are reported. Negative regulators are boxed in yellow. Putative PTB binding sequences are boxed in red.

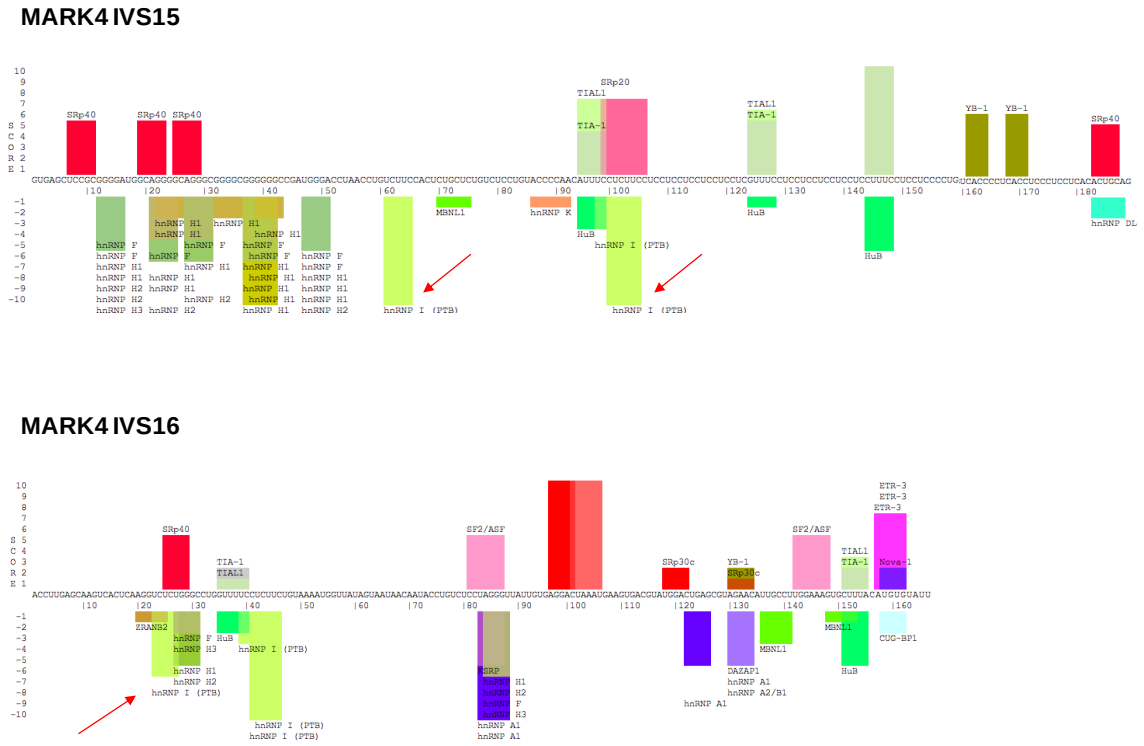


Figure 16. Putative regulatory elements identified by SpliceAid in IVS15 and in the 5' portion of IVS16 of MARK4. The regulatory factors are indicated by colored boxes, with positive splicing regulators upon and negative regulators below the intronic sequence. The height of the boxes is proportional to the probability (score reported on the left) that the factor might be really involved in alternative splicing. The PTB binding sites are pointed by the red arrows.

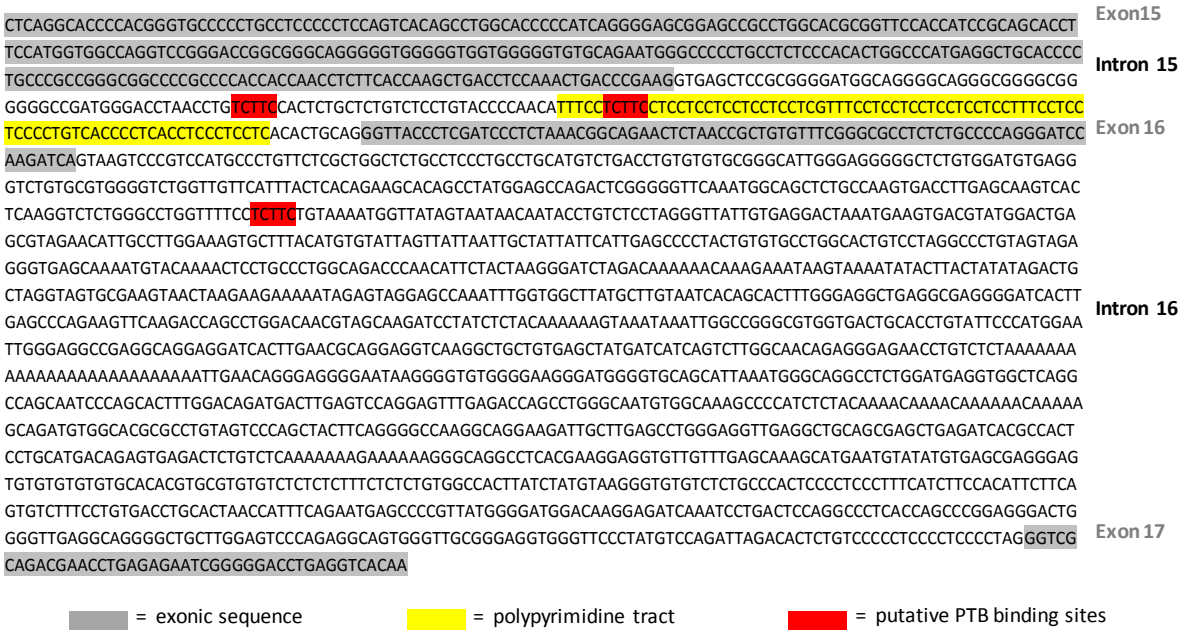


Figure 17. Putative PTB binding sites (red) identified in MARK4 IVS15 and IVS16 flanking the alternative exon 16. Exons sequence is shaded in grey and the polypyrimidine tract in intron 15 is highlighted in yellow.

3.2.2 Conservation analysis of the identified putative PTB binding sites

We then performed the analysis of the evolutionary conservation of the predicted PTB binding sites in MARK4 pre-mRNA by Clustal Omega. As reported in Figure 18, all the putative PTB binding sequences identified show a high conservation, between 60% to 100%, in human and mouse. Conversely, the overall conservation, in human and mouse, of the two introns is very low, supporting an effective functional role for the intron-embedded conserved sequences.

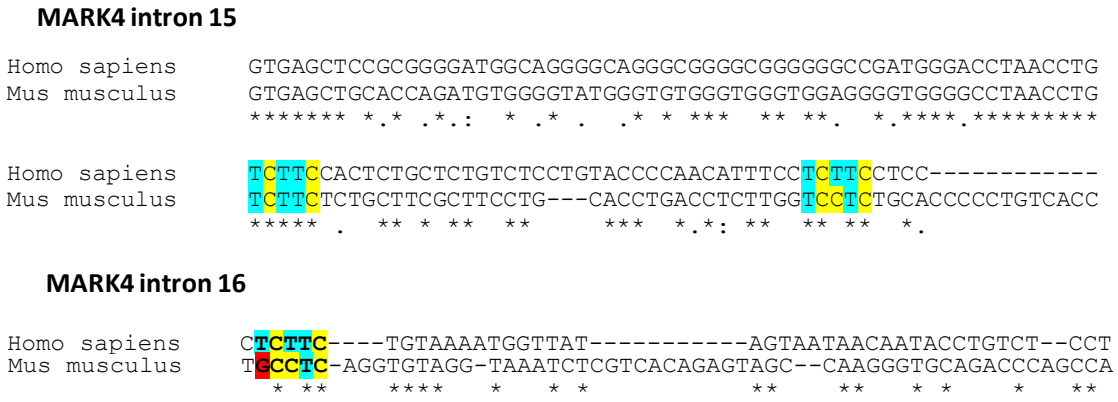


Figure 18. Human-mouse conservation of putative PTB binding sites identified in MARK4 pre-mRNA. Stars indicate 100% conservation while the dashed line represents gaps included by the program to reach the best alignment score of the two sequences.

3.3 Evaluation of PTB protein expression in glioma

PTB belongs to the hnRNP protein family and it is reported to mainly induces skipping of alternative exons: overexpression is thus a prerequisite for its involvement in aberrant splicing in glioma. Indeed, several studies reported upregulation of PTB expression in glioma and the consequent overexpression of protein isoforms that undergo exon skipping (David *et al.*, 2010).
To evaluate PTB expression profile in our panel of glioma samples, in addition to undifferentiated and differentiated GBM CSCs we performed immunoblot experiments.

3.3.1 PTB expression profile in glioma tissues and GBM CSCs

Western blot analysis of PTB expression in the same tissue samples used for total MARK4 (see 3.1.1) and MARK4L and MARK4S mRNA expression evaluation (Magnani *et al.*, 2011), showed a significant overexpression of PTB in glioma samples compared to normal brain ($p < 0.001$ at Wilcoxon test). Figure 19 shows a selection of representative astrocytoma and glioblastoma tissues as compared to normal brain. Moreover, the expression of PTB increases along with tumour grade and cellular de-differentiation, with the highest expression in GBM CSCs (mean values: A = 0.1, GBM = 0.3 and GBM CSCs = 0.7) (Figure 20A). In addition, PTB expression levels correlate with the relative increase of MARK4L expression, inferred from the MARK4L/MARK4S ratio, previously observed in the same samples (Magnani *et al.*, 2011) (Figure 20B).

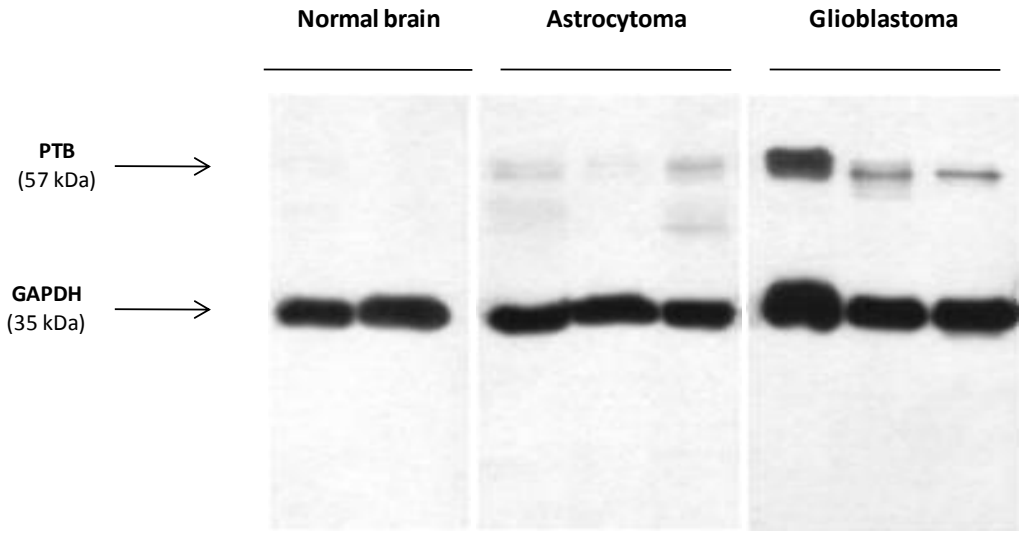


Figure 19. Western blot evaluation of PTB protein expression in human normal brain and three representative astrocytomas (A) and glioblastomas (GBM). The PTB band of 57 kDa is apparent in astocytomas and much more intense in glioblastomas. GAPDH expression was used for normalisation.

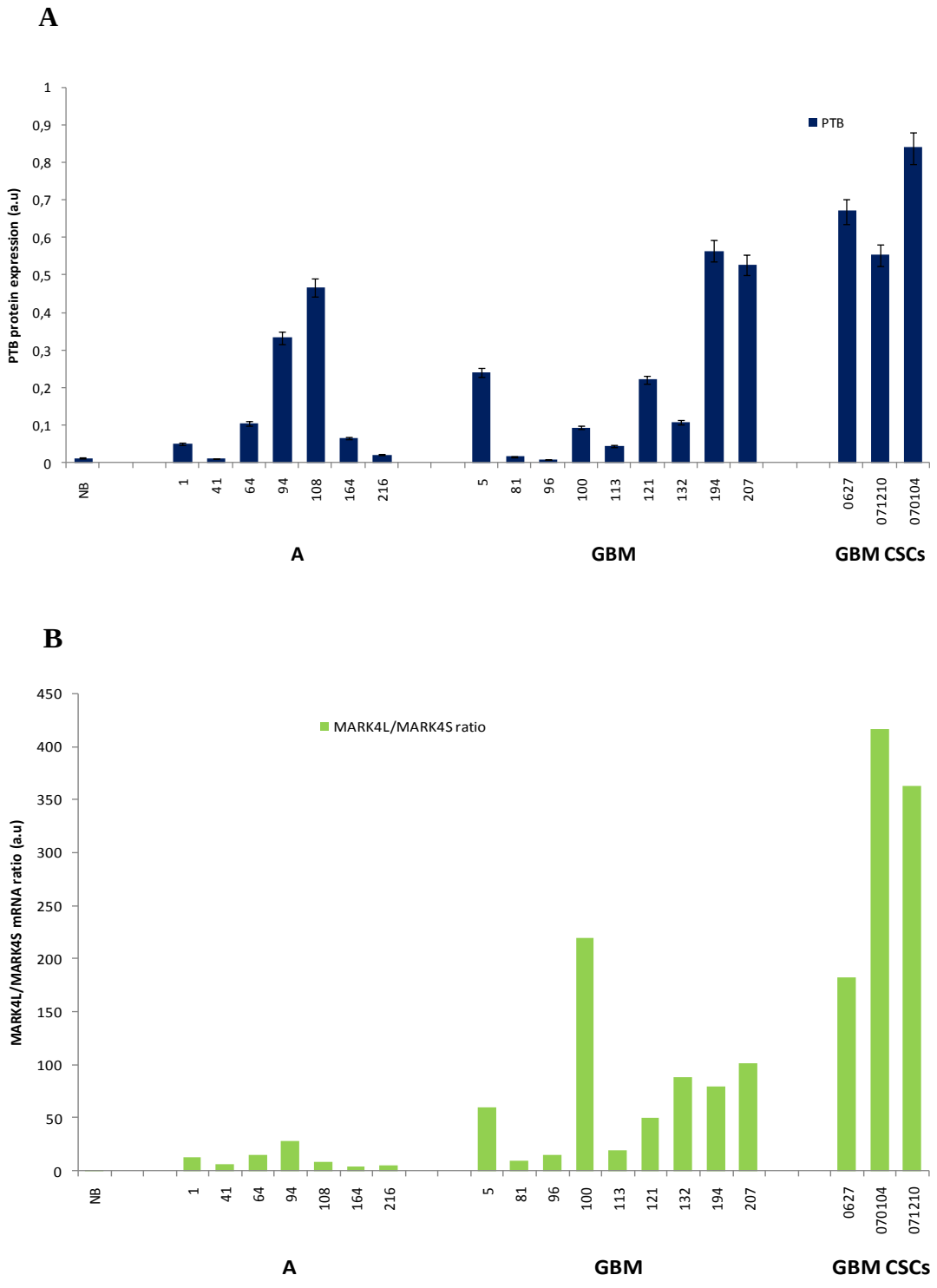


Figure 20. PTB protein expression compared to MARK4L mRNA increment in all the glioma tissues and GBM CSCs investigated in this study. **A**) PTB protein expression in astrocytomas (A), glioblastomas (B) and glioblastoma-derived cancer stem cells (GBM CSCs) compared to normal brain (NB). Data are expressed as mean ± standard deviation of three independent experiments and PTB expression has been normalised on GAPDH protein expression. **B**) MARK4L/MARK4S mRNA ratio in astrocytomas (A), glioblastomas (GBM) and glioblastoma-derived cancer stem cells (GBM CSCs) (adapted from Magnani *et al.*, 2011).

3.3.2 Comparative expression of PTB and MARK4L in undifferentiated and differentiated GBM CSCs

We have previously demonstrated that MARK4L increase in glioma tissues is directly proportional to tumour grade and cellular de-differentiation, and that GBM CSCs show a MARK4 expression profile similar to that of glioblastoma tissues and mouse neural stem cells (NSCs), with high levels of MARK4L and slightly detectable levels of MARK4S (Magnani *et al.*, 2011). These evidences suggested a role for MARK4L in proliferation and for MARK4S in cell differentiation, respectively. To further support this hypothesis we analysed MARK4L protein expression in undifferentiated and differentiated NSCs and GBM CSCs. We highlighted that all subgroups of stem cells show high levels of MARK4L expression (Figure 21A). Moreover, both undifferentiated and differentiated GBM CSCs share similar MARK4L expression levels of MARK4L, while in NSCs MARK4L is undetectable by the 7th day of differentiation (Figure 21A).

We also analysed the expression of PTB in undifferentiated and differentiated GBM CSCs, to evaluate the role of this splicing regulator in gliomagenesis and in tumour cell differentiation. Western blot analysis of PTB revealed that GBM CSCs, both undifferentiated and differentiated, show high levels of PTB in accordance with the nearly exclusive expression of MARK4L (Figure 21B).

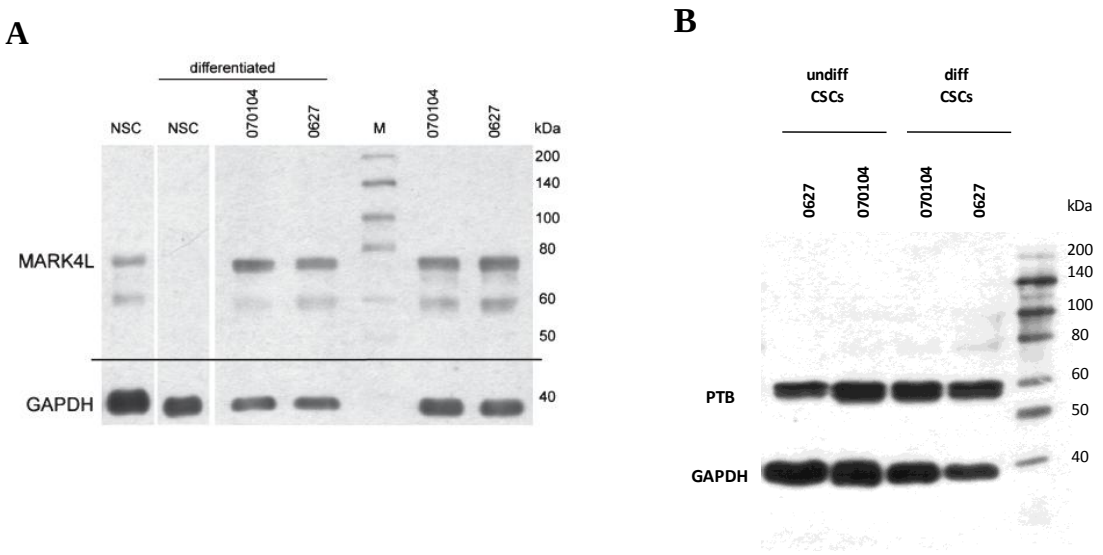


Figure 21. PTB and MARK4L expression in undifferentiated and differentiated stem cells. **A**) MARK4L protein expression in undifferentiated and differentiated NSCs,070104 and 0627GBM CSCs; **B**) PTB protein expression in undifferentiated and differentiated GBM CSCs.

3.4 MARK4 minigene splicing assay

To investigate the presence of functional active splicing regulatory sequences in the MARK4 introns flanking the alternative exon 16, we constructed a splicing minigene. The minigene was realised by cloning in a mammalian expression vector the genomic sequences corresponding to exons 15, 16 and 17 in addition to IVS15 and IVS16. Firstly, we tested the MARK4 splicing minigene in both normal HEK293T and oligoastrocytoma G157 cell lines. Then, we generated serial deletions of the minigene which were transfected in both HEK293T and G157 cell lines aiming at to pointing out alterations in MARK4 splicing caused by the absence, in the minigene, of specific splicing regulatory elements and hence identifying the sequences involved in MARK4 alternative splicing.

3.4.1 MARK4 wild-type minigene splicing assay

We performed splicing minigene assays on the HEK293T and G157 cell lines, that have been selected for their expression of MARK4L and MARK4S. In particular, HEK293T show a MARK4 expression profile similar to that of glioma cell lines, with nearly exclusive expression of MARK4L and slightly detectable levels of MRK4S (Figure 22).

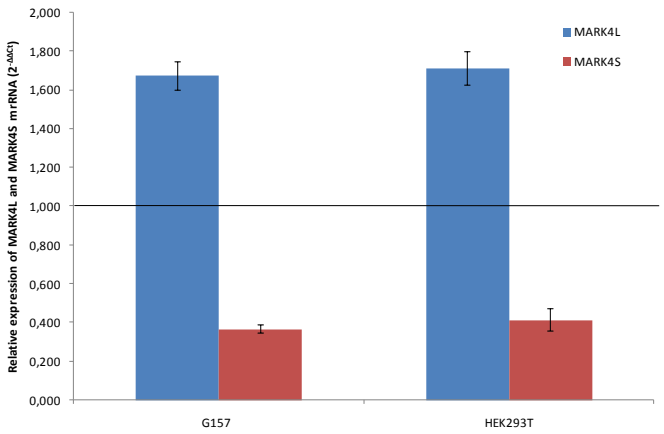


Figure 22. Relative expression of MARK4L and MARK4S mRNA in HEK293T and G157 cell lines compared to human neural progenitor cells (HNPCs) (conventionally set as 1 and represented by an horizontal black line).

This expression profile of MARK4 isoforms mRNA is concordant with PTB protein expression in these cell lines. As reported in Figure 23, both HEK293T and G157 cell lines show high levels of PTB expression, as expected given the embryonic nature of HEK293T and the cancer origin of G157 cells.

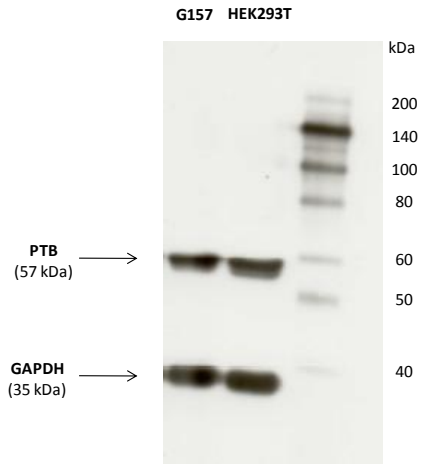
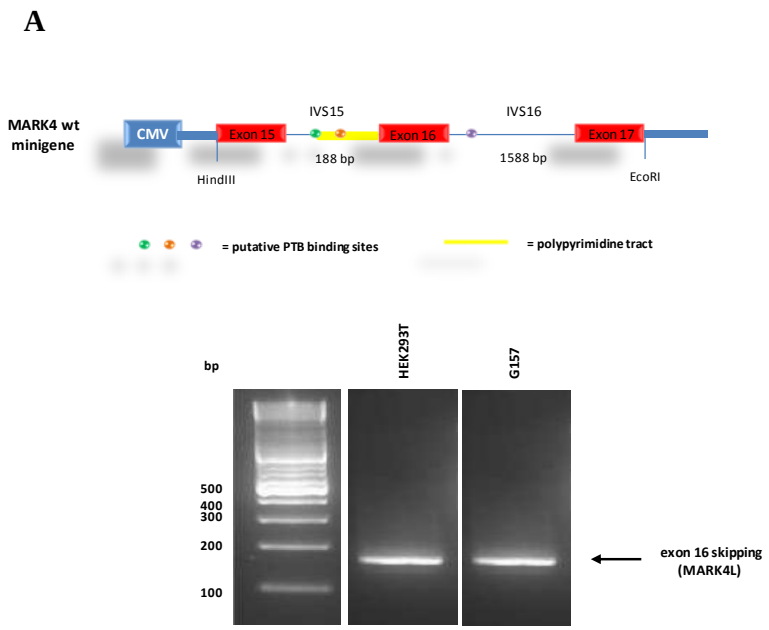


Figure 23. PTB protein expression in HEK293T and G157 cell lines.

Transfection of wild-type MARK4 minigene in HEK293T and G157 cell lines and subsequent RT-PCR to evaluate minigene splicing, confirmed that the vector undergoes splicing that results in a band of 194 base pairs (Figure 24A). Sequencing of the band revealed skipping of exon 16, confirmed by the presence of the exon 15-17 junction, and thus highlighted that the minigene is converted in the MARK4L isoform by alternative splicing (Figure 24B).



B

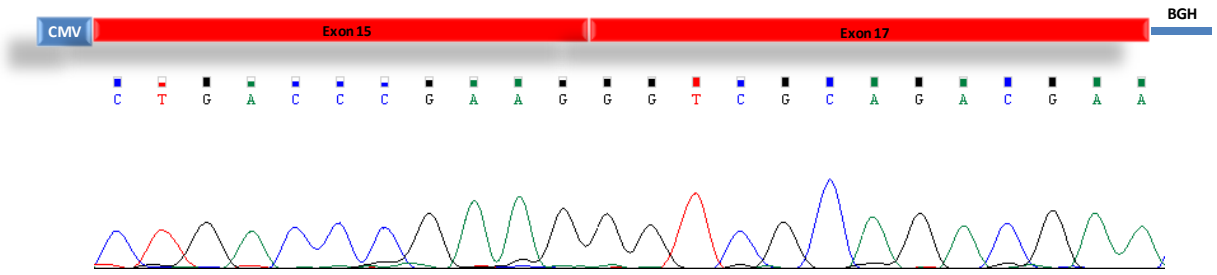


Figure 24. MARK4 wild-type minigene splicing assay. **A**) Schematic representation of MARK4 wild-type minigene construct and RT-PCR of minigene splicing assay in HEK293T and G157 cell lines. **B**) Sequencing of the RT-PCR band of MARK4 wild-type minigene confirms the presence of MARK4 exons 15-17 junction. CMV, cytomegalovirus promoter; BGH, bovine growth hormone binding site; nt, nucleotides; bp, base pairs.

3.4.2 Mutated MARK4 splicing minigene assays

To evaluate the presence of active splicing regulatory elements in MARK4 minigene, we introduced serial deletions in the minigene splicing vector and then evaluated alterations in the splicing of the mutated minigenes. We focused our attention onto IVS15 and, in particular, the polyprymidine tract harbouring one of the putative PTB binding sites identified by bioinformatic analysis. We performed deletions of different portions of the polypyrimidine tract in IVS15 to assess the presence of functional regulatory motifs.

We started deleting 87 nucleotides of IVS15 3'-end comprising the polypyrimidine tract and one of the putative previously identified PTB binding sites (MARK4 del87 nt). We then selectively deleted the PTB binding site included in the polypyrimidine tract (MARK4 delPTB) and the 3' proximal region of intron 15 (MARK4 del IVS15 prox) to determine whether any of the splicing regulatory sequences involved in exon 16 skipping was represented by the canonical identified PTB binding site or by a different splicing motif (Figure 25A).

Deletion of the last 87 nucleotides of MARK4 IVS15 (MARK4 del87 nt) turned out to foster exon 16 inclusion, confirmed by sequence analysis (Figure 25A and B), indicating the presence of a functional intronic splicing silencer (ISS) in this region. By contrast, deletion of the predicted PTB binding site (MARK4 delPTB) contained in this region did not affect minigene splicing, suggesting that PTB may bind to a non-canonical ISS (Figure 25A). To further elucidate the specific sequence involved in MARK4 alternative splicing we also deleted 32 nucleotides (MARK4 del32 nt) comprising the putative PTB binding site in the polypyrimidine

tract of IVS15 and the 27 downstream nucleotides. Also this deletion did not modify the splicing of the MARK4 minigene. Interestingly, deep analyses of the MARK4 splicing minigene derived from deletion of these 32 nucleotides, revealed that the deletion reconstructed a specific sequence, TTTCCTCCTCC, that might be the candidate regulatory sequence involved in MARK4 alternative splicing.

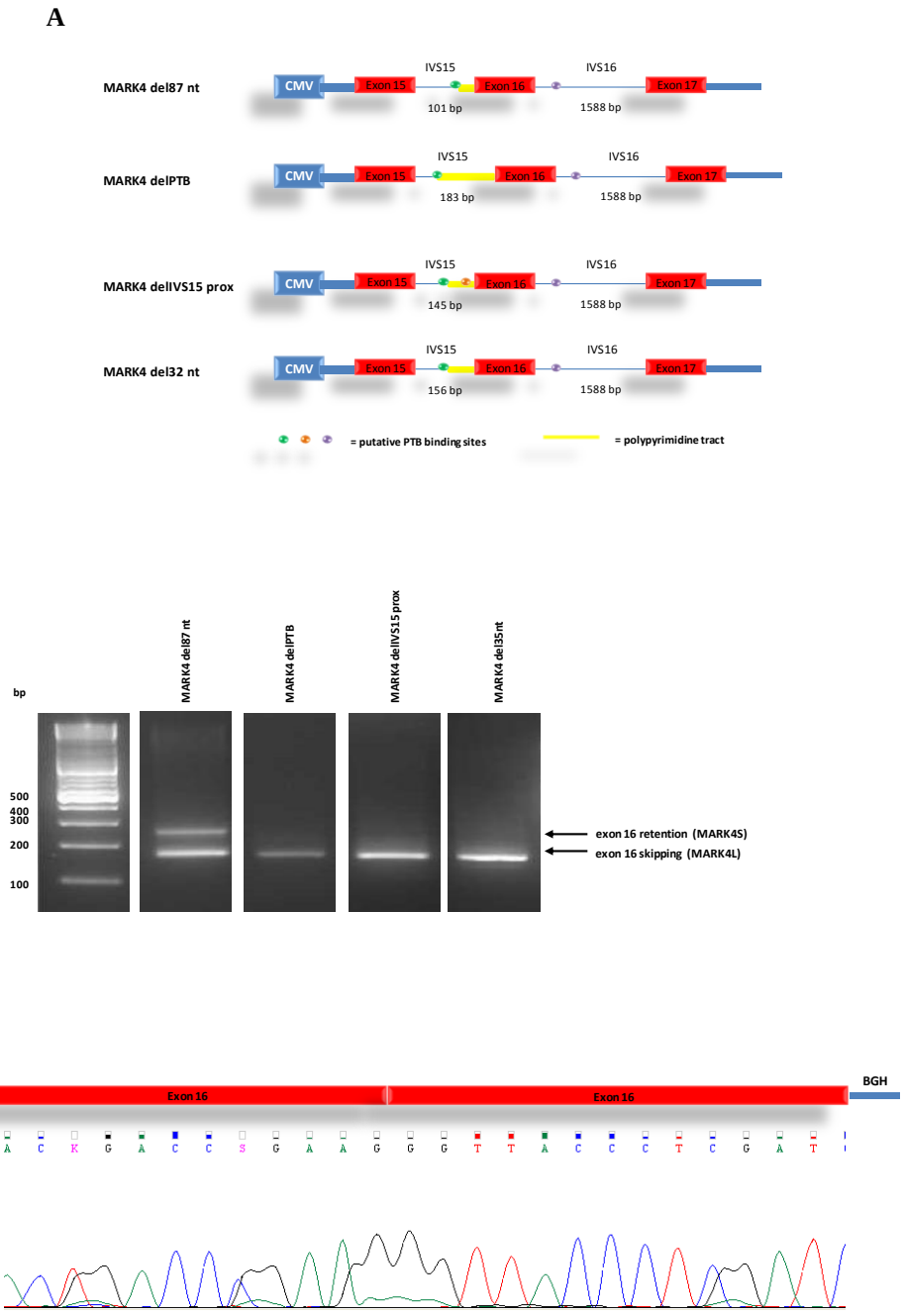


Figure 25. MARK4 deleted minigene splicing assays. **A**) Schematic representation of MARK4 deleted minigene constructs and RT-PCR of minigene splicing assays. **B**) Sequencing of the RT-PCR upper band of MARK4 obtained by MARK4 del87nt minigene splicing assay confirmed the presence of MARK4 exons 15-16 junction. CMV, cytomegalovirus promoter; BGH, bovine growth hormone binding site; nt, nucleotides; bp, base pairs.

3.5 MARK4 electrophorectic mobility shift assay

To identify splicing regulatory factors bound to the 87 nucleotides deleted in the MARK4 del87 nt minigene, we synthesised a biotinylated single strand riboprobe, corresponding to the deleted region, and performed electrophoretic mobility shift assay (EMSA) experiments. Incubation of MARK4 riboprobe with HEK293T total cell lysate, highlighted a shifted band derived from the binding of a splicing factor to the riboprobe (Figure 26, lane 2). As depicted in the Figure 26 in lane 3 the intensity of the shifted band is attenuated after addition of a large amount of non-biotinylated MARK4 riboprobe as binding competitor, confirming that the observed shift derives from a specific RNA-protein interaction. Preliminary mass spectrometry experiments seem to confirm the presence of PTB in the shifted band.

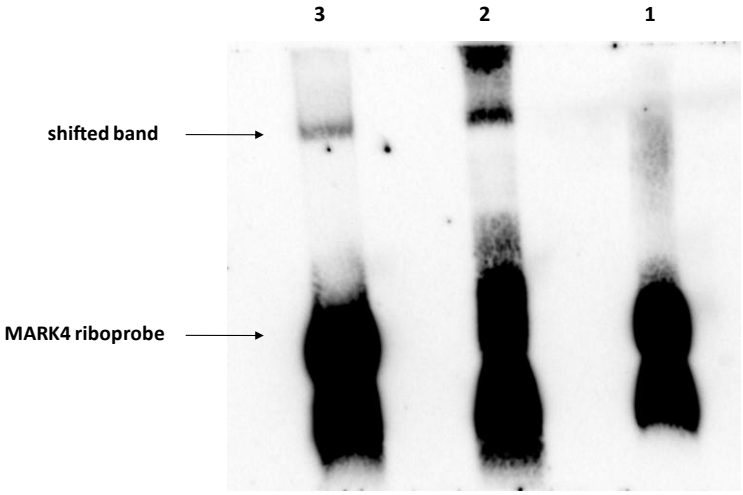


Figure 26. MARK4 EMSA experiments. Lane 1: only MARK4 biotinylated RNA was loaded allowing to establish the position of the unshifted band. Lane 2: MARK4 biotinylated RNA incubated with HEK293T protein extract was loaded. A shifted band is observed attesting the binding to MARK4 mRNA of a putative target protein. Lane 3: MARK4 biotinylated RNA incubated with HEK293T protein extract and 200-fold molar excess of MARK4 unlabeled RNA as binding competitor was loaded. The shifted band is observed, but with lower intensity, demonstrating that the shift results from specific protein-RNA interaction.

Discussion

The differential expression of MARK4L and MARK4S in human normal and transformed tissues, and in particular in the Central Nervous System (CNS), suggests that the expression of the two MARK4 isoforms is tissue-specific and regulated according to distinct developmental stages. This implies that the two isoforms do not have fully overlapping functions, and that isoform-specific activities are required in different cell types and conditions. Indeed, the findings on the predominance of MARK4L in neural progenitors and its upregulation in glioma (Beghini *et al.*, 2003; Magnani *et al.*, 2011), coupled to the observed duplication of the *MARK4* gene in glioma cell lines (Roversi *et al.*, 2006), imply a role for this isoform in cell proliferation. The MARK4S isoform appears instead involved in neural differentiation given its expression in normal brain and terminally differentiated neurons (Moroni *et al.*, 2006). Moreover, glioma tissues are characterised by an altered MARK4L/MARK4S ratio that appeared directly correlated to tumour grade and cell de-differentiation (Magnani *et al.*, 2011). A high MARK4L expression, associated with slightly detectable levels of MARK4S also labelled glioblastoma-derived cancer stem cells (GBM CSCs) and mouse neural stem cells (NSCs), suggesting that the balance between the two MARK4 isoform may be thinly regulated during neural differentiation and might be subverted in gliomagenesis (Magnani *et al.*, 2011).

The demonstrated absence of genomic mutations that could favour the expression of MARK4L to the detriment of MARK4S in glioma, prompted us to hypothesise alterations at the post-transcriptional level that may account for the observed altered ratio of MARK4 isoforms in glioma. A prerequisite to assume the involvement of post-transcriptional alterations is the observation of altered relative expression of the two isoforms in the absence of differences in the overall expression of the gene. We thus performed real-time quantification of MARK4 total mRNA to point out possible differences in gene transcription in the same glioma samples used for MARK4L and MARK4S mRNA quantification. No significant differences in the expression of MARK4 total mRNA have be highlighted in astrocytomas, glioblastomas and GBM CSCs, neither among subgroups nor between tumour samples and normal brain. This recent evidence merges with the previous data on the altered ratio between the two MARK4 isoforms in supporting the working hypothesis of this study on post-transcriptional alterations of MARK4L and MARK4S expression in glioma.

We thought that alternative splicing could be the most likely mechanism responsible for the aberrant MARK4L/MARK4S ratio in glioma. Indeed, alternative splicing is not only the most effective mechanism to generate proteome diversity from a restrict number of genes, but also a frequent target of alterations in cancer (Venables 2004; Ghigna *et al.*, 2008). Alternative splicing is particularly exploited in the CNS where protein isoform expression is fine tuned during

neuronal development and in different brain regions to achieve high specific functions (Grabowski and Black, 2001). The intrinsic flexibility of alternative splicing enables the thinly-regulated expression of specific isoforms, often with divergent functions, according to developmental stages, cell signalling and reprogramming of energetic metabolism of tumor cells. *Cis*- and *trans*-acting factors are the key players in the recognition of weak splice sites and mutations in enhancer and silencer sequences, or alterations in the expression/activation of SR and hnRNP proteins, have been heavily associated with cancer as either causative, modulating or compensatory mechanism (Venables 2004; Ghigna *et al.*, 2008, Hanahan and Weinberg, 2011). Specific splice variants are commonly found enriched in cancer tissues compared to normal ones (Singh *et al.*, 2004; Zhou *et al.*, 2003). In these cases, enriched in aberrantly spliced isoforms, the spliced genes are not mutated, indicating that the defects imply a change in the nuclear environment that regulates splice site choice (Grosso *et al.*, 2008). Several studies reported that the up-regulation of different splicing factors in tumours, including gliomas, affects the splice site selection. As a consequence, the generation of splice variants encoding protein isoforms, which are advantageous to transformed cells, are positively selected during tumour progression (Grosso *et al*., 2008). In particular, the inappropriate expression of isoforms produced by exon skipping, like MARK4L, is generally favoured in cancer as a consequence of overexpression of negative regulators of alternative splicing belonging to the hnRNP protein family (He and Smith, 2009).

We thus performed a bioinformatic analysis of putative binding sites for hnRNP proteins in introns flanking MARK4 alternative exon 16. Several putative hnRNP binding sites have been identified by SFmap and SpliceAid and most were reported to be involved in brain development and related pathologies (Grabowski and Black, 2001). The identified putative binding sites for the Polypyrimidine-Tract Binding protein (PTB) aroused our interest for the position of its binding sites in MARK4 introns and for the well established role of this splicing factor in neural differentiation and glioma (Boutz *et al.*, 2007; David *et al.*, 2010). Indeed, the switch from PTB, manly expressed in neural progenitors and glial cells, to its neuronal homologue nPTB drives the differentiation along the neuronal lineage (Boutz *et al.*, 2007). PTB and nPTB expression profile and behaviour thus appear very similar to that of MARK4L and MARK4S respectively. Moreover, immunohistochemistry on mouse brain revealed a PTB localisation in developing brain overlapping that of MARK4L. The two proteins appear highly expressed in the embryonic ventricular and adult sub-ventricular zones, well known regions of neurogenesis (Suckale *et al.*, 2011; Magnani *et al.*, 2011). We thus hypothesised that PTB may favour the expression of the MARK4L isoform during neural development, setting up a MARK4 splicing profile that

supports cell proliferation and the maintenance of an undifferentiated phenotype. The switch off of PTB expression in committed progenitors may thus address the expression of MARK4S in the context of a nPTB-dependent neuronal-specific splicing pattern. Bioinformatic analysis revealed the presence of three putative PTB binding sites in both introns flanking the alternative exon 16, two in IVS15 and one in IVS16. The position of these binding sites is consistent with all the proposed mechanisms of PTB-induced alternative splicing (Spellman and Smith, 2006). Indeed, the putative PTB binding site in MARK4 intron 15 more proximal to exon 16, is embedded in a polypyrimidine rich sequence, that represents the ideal environment for PTB action. PTB may thus bind to this site with high affinity, in competition with the splicing factor U2AF65, leading to inhibition of the 3' splice site recognition. As regards the other two putative PTB binding sites identified by bioinformatic analysis, they may cooperate with the high affinity binding site in the polypyrimidine tract and induce exon 16 skipping by polymerisation along introns and exon 16 to mask splicing sites and enhancer elements favouring exon inclusion. Alternatively, PTB binding at adjacent introns may force conformational changes in the pre-mRNA that bring in close proximity exons 15 and 17 with concomitant looping out of exon 16.

Additional support on a possible functional role of identified PTB binding sites comes from sequence conservation analysis. It has been reported that 77% of alternatively spliced exons are surrounded by conserved intronic sequences, compared to only 17% of non-alternatively spliced exons. Furthermore, the conservation picks at specific short sequences frequently represented by splicing enhancers and silencers (Sorek and Ast, 2003). The high conservation between human and mouse of the putative PTB binding sites identified in MARK4 IVS15 and IVS16 point to a functional role of these sequences in MARK4 alternative splicing.

PTB has also been implicated in the deregulation of alternative splicing in several pathologies, including glioma. The thinly tuned expression of this splicing regulator is frequently subjected to alterations that lead to the aberrant expression of associated protein isoforms. PTB has been reported to be overexpressed in glioma, with expression levels correlating with tumour grade (McCutcheon *et al.*, 2004; Cheung *et al.*, 2006; Cheung *et al.*, 2009). In our panel of glioma samples, including astrocytomas, glioblastomas and GBM CSCs, PTB is overexpressed, with expression levels directly correlated with MARK4L mRNA expression, and thus with tumour grade and cell de-differentiation (Figure 20). These evidences strengthen the role of PTB in MARK4 alternative splicing: in physiological conditions low levels of PTB may favour the expression of MARK4S and hence direct and maintain the correct differentiation status of neural progenitors and derived cells, while in glioma, PTB overexpression, and the consequent expression of MARK4L to the detriment of MARK4S, may enhance the maintenance of the

undifferentiated phenotype of transformed cells, endowed with a high proliferation rate, needed to support tumour growth and spread. A role of PTB in stem cells proliferation and differentiation has been recently highlighted by knockout experiments on mouse embryonic fibroblasts, in which PTB absence impairs proliferation and differentiation (Shibayama *et al.*, 2013; Suckale *et al.*, 2011). We have demonstrated that PTB overexpression also characterises GBM CSCs and is not downregulated after induction of differentiation, supporting a fundamental role of this splicing factor in cancer cell proliferation. We also pointed out the intriguing coincidence, that also MARK4L is highly expressed in both undifferentiated and differentiated NSCs and GBM CSCs. Furthermore, while in normal stem cells MARK4L expression is switched off since the 7^{th} day of differentiation, in GBM CSCs MARK4L is maintained overexpressed also upon differentiation. Together with the expression of PTB in GBM CSCs, these data let us hypothesise that PTB overexpression is an early event in glial cell transformation and that, in keeping with MARK4L overexpression, it is part of a stemness-expression program that is aberrantly reactivated in glioma.

The definition of *cis*- and *trans*-acting factors involved in alternative splicing of a specific pre-mRNA necessarily passes through the association between the splicing factor and the putative binding sequence. To reach this goal we used minigene splicing assays which are diffuse and reliable tools for the study of alternative splicing. We encountered several difficulties in the construction of a functional splicing minigene, probably due to the presence of long stretches of repeated nucleotides, adenine in particular, in the sequence to be cloned. These repetitive sequences made the vector unstable upon bacteria transformation and in most cases the cloned fragment appeared to be partially or completely excised from the vector. We tried several conditions of transformation and many bacterial strains and we finally succeeded in obtaining a complete and functional MARK4 minigene vector, even if the efficiency was repeatedly low. The wild-type MARK4 minigene appeared to be correctly processed by alternative splicing producing a nearly 100% exon 16 skipping in both HEK293T and oligoastrocytoma G157 cell lines, consistent with the high expression of PTB in these cell types. Indeed, both HEK293T and G157 cell lines show physiologically low levels of MARK4S expression associated with high levels of MARK4L mRNA and PTB protein expression. To identify binding sequences involved in MARK4 alternative splicing we performed serial deletions in the MARK4 minigene, focusing our attention on intron 15 3'-end that harbours the polypyrimidine tract, considered to be a suitable candidate to contain a functional PTB binding motif. Deletions were designed to avoid excision of the branch site, predicted to be localised upstream the polypyrimidine tract, and to maintain the acceptor splice site at the intron 15-exon 16 boundary, as to prevent intron retention

and preserve exon 16 recognition. We identified a regulatory element of MARK4 alternative splicing in the last 87 nucleotides of intron 15, as deletion of this region determines exon 16 inclusion in the minigene splicing assay. As can be noted in Figure 25A, the reversion of alternative splicing of the minigene from MARK4L to MARK4S was not complete, as two RT-PCR bands are visible, suggesting the cooperation of multiple factors in MARK4 alternative splicing. This finding may be accounted by the huge complexity of the splicing process. Since the deletion of the putative PTB binding site, comprised in these 87 nucleotides, did not affect alternative splicing, we are considering the possibility that PTB binds to a non canonical site, as demonstrated for other PTB-dependent alternative splicing (Ladd and Cooper, 2002).

We thus decided to perform electrophoretic mobility shift (EMSA) assay to definitely demonstrate the interaction between MARK4 pre-mRNA and splicing regulatory factors. We demonstrated an interaction between the polypyrimidine tract of IVS15 and protein factors, but EMSA experiments were not conclusive in the definition of the specific splicing factor involved in MARK4 alternative splicing. Mass spectrometry experiments, on the shifted band obtained by EMSA, are ongoing with the aims of: i) assign to PTB a definitive role in MARK4 splicing modulation and/or ii) identify other splicing regulators associated with MARK4 mRNA affecting its alternative splicing. Preliminary results seem to confirm the presence of PTB in the shifted band.

In conclusion by this study we tried to go to the root of the imbalance between the two MARK4 isoforms observed in glioma by studying MARK4 alternative splicing as the causative mechanism. Suggestive bioinformatic evidences have been achieved and pieces of experimental evidence have been obtained, notwithstanding the experimental pathway is not yet closed. Should splicing factors involved in MARK4 alternative splicing be definitely identified, this novel knowledge will also be instrumental to insert the expression of this kinase in a specific pathway of cell signalling, as was the case for pyruvate kinase that has been linked to c-myc and related pathway in glioma (David *et al.*, 2010). Moreover, specific *cis*-acting elements, demonstrated to regulate MARK4 alternative splicing, will be ideally target of molecular therapy and might be exploited to revert the balance between the two MARK4 isoforms in glioma.

Perspectives

In light of the achieved results we are pursuing the following experiments to confirm the role of PTB in MARK4 alternative splicing:

- Mass spectrometry analysis of the shifted band obtained by EMSA experiments.
- Electrophoretic mobility shift assay with MARK4 riboprobe mutated in the hypothetical splicing factor binding site.

We are also planning further experiments aimed at

- Downregulating PTB expression by siRNA and then monitoring MARK4 isoforms expression byminigene splicing.
- Carrying on RNA immunoprecipitation experiments to confirm the interaction between MARK4 pre-mRNA and identified splicing factors.
- Carrying on chromatin immunoprecipitation experiments to identify alterations in histone modifications that may favour PTB-dependent splicing of MARK4 pre-mRNA in glioma.

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