# 2 The Diverse Roles of RNA-Binding Proteins in Glioma Development

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**Abstract**

Post-transcriptional regulation of gene expression is fundamental for all forms of life, as it critically contributes to the composition and quantity of a cell's proteome. These processes encompass splicing, polyadenylation, mRNA decay, mRNA editing and modification and translation and are modulated by a variety of RNA-binding proteins (RBPs). Alterations affecting RBP expression and activity contribute to the development of different types of cancer. In this chapter, we discuss current research shedding light on the role of different RBPs in gliomas. These studies place RBPs as modulators of critical signaling pathways, establish their relevance as prognostic markers and open doors for new therapeutic strategies.

**Keywords**

ADARs · Cancer · Glioblastoma · hnRNP · HuR · IGF2BPs · Musashi1 · PTBP · RNA-binding proteins · Translation initiation

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## Abbreviations

| | |
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| RBPs | RNA-binding proteins |
| MS | Mass spectrometry |
| UTRs | Untranslated regions |
| GBM | Glioblastoma multiforme or Glioblastoma |
| hnRNP | Heterogeneous nuclear ribonucleoproteins |
| PTBP | Polypyrimidine-tract-binding protein |
| ADARs | Adenosine deaminases that act on RNA |
| HuR | Hu antigen R |
| MSI1 | Musashi1 |
| IGF2BPs/IMPs | Insulin-like growth factor II mRNA binding proteins |
| PK | Pyruvate-kinase |

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| EGFR | Epidermal growth factor receptor |
| HK2 | Hexokinase2 |
| GLUT3 | Glucose Transporter 3 |
| IG20 | Insuloma-glucagonoma protein 20 |
| RON | Recepteur d'Origine Nantais |
| EMT | Epithelial-mesenchymal transition |
| PDCD4 | Programmed cell death protein 4 |
| MMP | Matrix metalloproteinases |
| STAT3 | Signal transducer and activator of transcription 3 |
| NPC | Neuronal precursor cells |
| RRMs | RNA recognition motifs |
| FGFR | fibroblast growth factor receptor-1, -2 |
| FBN | fibrilin |
| CASP2 | caspase 2 |
| ABCC1 | ATP binding cassette subfamily C member 1 |
| RTN4 | Reticulon 4 |
| MARK4 | Microtubule affinity-regulating kinase 4 |
| GluR | Glutamate receptors |
| *ELAV* | *Embryonic lethal abnormal visual* |
| *AREs* | *AU-rich elements* |
| VEGF | Vascular endothelial growth factor |
| SIRT1 | Silent mating type information regulation 2 homolog 1 |
| BCL-2 | B-cell lymphoma 2 |
| ProTα | Prothymosin α |
| PCM | pericentriolar matrix |
| SCs | Stem cells |
| GSCs | Glioblastoma stem cells |
| MVD | microvessel density |
| DNA-PKcs | DNA-Protein Kinase Catalytic Subunit |
| PMAs | Pilomyxoid astrocytomas |
| QKI | Quaking |
| INF | Interferon |
| PI3K/MAPK | Phosphatidylinositol 3-Kinase/Mitogen-activated Protein Kinase |

## 2.1 Introduction

The large majority of cancer studies dedicated to the identification of "driving events" and genes contributing to tumor development have focused on processes occurring at the DNA level, such as mutations and chromosomal defects, changes in methylation status, and transcriptional regulation. Recent studies performed in different species and scenarios have shown a poor correlation between mRNA and protein levels for the majority of the transcriptome. These results support the role of "RNA-based" mechanisms as key modulators of gene expression in both, normal and tumorigenic cells [1, 2]. Such regulatory mechanisms are primarily driven by RNA-binding proteins (RBPs). Large-scale quantitative methods, next-generation sequencing, and modern protein mass spectrometry (MS) have been employed recently to expand the RBP catalogue, to identify their protein co-factors and target transcripts. The number of known RBPs in the human genome is over 1500 [3, 4], which represent ~7.5% of human coding genes. RBPs form complexes with pre-mRNA, mRNA, and a variety of ncRNAs (lncRNAs, miRNAs, rRNAs, etc.), to modulate an array of processes that include splicing, polyadenylation, maturation, modification, transport, stability, localization, and translation. RBPs exert their effect by recognizing specific sequences and/or secondary structures present in untranslated regions (UTRs), coding sequences and/or introns [3, 5].

Mutations and alterations in the expression levels of numerous RBPs are observed across tumor tissues and known to impact the expression of large set of genes, contributing to tumor initiation and growth [6–8]. This scenario is observed in gliomas, an heterogenous group of brain tumors encompassing astrocytomas, oligodendrogliomas, oligoastrocytomas and glioblastoma multiforme [9]. Gliomas are responsible for the majority of deaths caused by primary brain tumors. The absence or presence of anaplastic features is used by the World Health Organization (WHO) for assigning grades of malignancy [9, 10]. Among them, glioblastoma multiforme

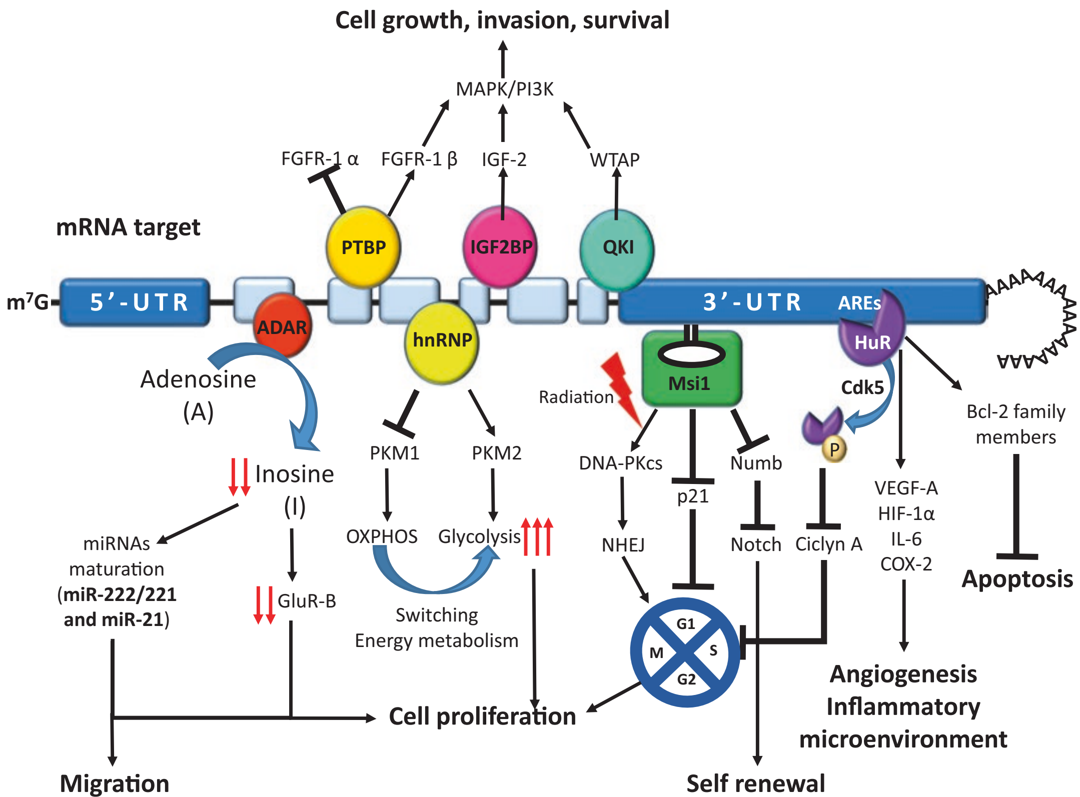


**Fig. 2.1** Schematic representation of the involvement of RNA-binding proteins (RBPs) in glioblastoma development. We show the RBP families discussed in this article, the region of the mRNA where they make target, their most relevant target genes, and both the processes and pathways affected by the described regulation

(GBM, grade IV) is the most aggressive and accounts for 45.2% of all malignant primary brain and CNS tumors, and 54% of all gliomas [11]. The number of well-characterized oncogenic RBPs in gliomas is still relatively small. As indicated by a recent genomic study from our lab, aberrant RBP expression is a common feature in GBMs and dozens of these proteins potentially contribute to the acquisition of cancer phenotypes [12].

In the next sections, we discuss the role of some of the best characterized RBPs in the context of gliomas, namely Heterogeneous nuclear ribonucleoproteins (hnRNP), Polypyrimidine-tract-binding protein (PTBP), Adenosine deaminases that act on RNA (ADARs), Hu antigen R (HuR), Musashi1 (MSI1), Insulin-like growth factor II mRNA binding proteins (IGF2BPs/IMPs), and Quaking (QKI) – In Fig. 2.1 we depict the global biology of these RBPs in glioma development.

## 2.2 Heterogeneous Nuclear Ribonucleoproteins

Heterogeneous nuclear ribonucleoproteins are a family of RBPs that includes approximately 20 genes termed hnRNPs A1-U, which range in size from 34 to 120 kDa [13]. Several hnRNPs have been implicated in the development of various tumor types and their expression levels have been linked to patient survival [14]. HnRNPs regulate different aspects of pre-mRNA processing in gliomas and their expression levels are altered in both low and high grade astrocytomas [15–17].

In gliomas, hnRNPA1/A2 is associated with the regulation of glucose metabolism, where it influences Pyruvate-kinase (PK) splicing and function. PK is a rate-limiting enzyme in glucose metabolism and is encoded by two paralogous genes, PKLR and PKM. Both genes are alternatively spliced; therefore, resulting in four PK isoforms in mammals [18, 19]. The PKM gene consists of 12 exons, of which exons 9 and 10 are alternatively spliced in a mutually exclusive fashion, producing the PKM1 and PKM2 mRNA isoforms, respectively [19]. Interestingly, the expression of hnRNP A1/A2 and PTBP is regulated by the oncogenic transcription factor c-Myc [20]. In cancer cells, hnRNP A1/A2 and PTBP bind to PKM pre-mRNA and repress the inclusion of exon 9. When expression levels of hnRNPA1/A2 and PTBP are reduced, PKM exon 9 inclusion is promoted and more PKM1 transcripts are produced; the end result is a decrease in glucose consumption and lactate production [21]. This is an example of a critical dialogue between RBP-mediated splicing and modulation of metabolism and cell proliferation.

Astrocytomas display mutations in growth-factor-receptor genes, such as epidermal growth factor receptor (EGFR). Alterations in the splicing profile of EGFR are also frequently observed. The most relevant one gives rise to a transcript encoding EGFR variant III (EGFRvIII) [22]; the resulting protein is constitutively active and contributes to a major shift in GBM cell metabolism [23]. Babic et al. (2015) demonstrated that in GBM cells expressing EGFRvIII, the Myc-binding partner Max is alternatively spliced. hnRNPA1 binds upstream of exon 5 of the Max pre-mRNA and facilitates its inclusion, leading to the production of a truncated Max protein referred to as Delta Max. Increased Delta Max production contributes to the expression of the glucose transporter GLUT3 and HK2 and promotes GBM cell proliferation in glucose-containing media [24].

Evasion of programmed cell death, tissue invasion, and metastasis are three important hallmarks of cancer [25]. A number of studies have described hnRNPs as regulators of apoptosis and cell invasion via its impact on RNA processing. For instance, hnRNPH regulates the splicing of Insuloma-glucagonoma protein 20 (IG20) and Recepteur d'Origine Nantais (RON), a death-domain adaptor protein and a tyrosine kinase receptor that participate in apoptosis and cell invasion, respectively. Alternative 5′ splice site usage in exon 13, plus inclusion/skipping of exon 16 generates four main splicing isoforms of IG20 (MADD), which have been described to be aberrantly expressed in tumors [26, 27]. In the case of RON, exon 11 exclusion generates RONΔ11, a transcript that gives rise to a protein missing part of the extracellular domain. This active isoform promotes cell motility and mediates epithelial-mesenchymal transition (EMT) [28] . hnRNPH binds to UGGG elements in the 5′ region of exons 16 and 11 of IG20 and RON pre-mRNAs, respectively, and inhibits its inclusion [29]. Knockdown of hnRNPH promotes inclusion of the exons that are normaly spliced out, and is associated with a decrease in cell viability and migration in gliomas [29]. Binding of hnRNPC to pre-miR-21, which promotes maturation, represents an example of interactions between hnRNPs and miRNAs. Silencing of hnRNPC decreases miR-21 levels, upregulating expression of Programmed cell death protein 4 (PDCD4), thus affecting the proliferative and metastatic potential of GBM cells [30].

## 2.3 Polypyrimidine-Tract-Binding Protein

Polypyrimidine tract-binding proteins (PTBPs) are a family of RNA binding proteins whose most of its members are preferentially expressed in the brain [31, 32]. PTBP2 (also known as neural PTB) for instance is specifically expressed in post-mitotic neurons [33]. PTBP3 is the least studied of the three PTB paralogs. In rat and human it is encoded by the gene *ROD1*. In rats, it is predominantly expressed in hematopoietic cells or organs of embryonic and adult individuals. In humans, *ROD1* overexpression inhibits pharmacologically-induced differentiation of both megakaryocytic and erythroid K562 leukemia cells, supporting the notion that *ROD1* plays

a role in differentiation control in mammalian cells [34]. These RBPs shuttle between the nucleus and cytoplasm, functioning in large and diverse number of cellular processes, including mRNA splicing, polyadenylation, stability and translation. PTBPs preferentially bind to polypyrimidine-rich stretches through its four RNA recognition motifs (RRMs) [35, 36]. As a splicing factor, PTBP1 induces exon skipping in pre-mRNAs encoding proteins involved in proliferation (FGFR1, FGFR2), invasion (CSRC), motility (ACTN, FBN), apoptosis (FAS, CASP2), and multi-drug resistance (ABCC1) [37].

Several studies have reported significant differences in the expression of PTBP1 in gliomas [37, 38]. PTBP1 regulates numerous splicing events relevant to gliomagenesis. Global exon array analysis identified Nogo (also known as RTN4) as one of the main targets of PTBP1. Nogo triggers a rearrangement of actin filament extensions in neighboring cells to inhibit neurite outgrowth [39]. Nogo has multiple mRNA isoforms, but only three characterized protein variants: Nogo-A, Nogo-B and Nogo-C [40]. Cheung *et al*, (2009) showed that Nogo-B is the predominant mRNA isoform expressed in glioma cells, where high levels of PTBP1 ensure that exon 3 is skipped [37]. Functional assays suggest that regulation of Nogo splicing by PTBP1 plays a role in proliferation and migration [37]. Another study identified the FGFR-1 pre-mRNA as PTBP1 target [41]. FGFR1 is implicated in growth and differentiation pathways and precise regulation of its splicing is critical [42]. PTBP1 interacts in a sequence-specific manner with the intronic RNA sequence, termed ISS-1 element, located upstream of the α exon of FGFR-1 pre-mRNA. This interaction induces exon α exclusion and leads to the production of a receptor with enhanced affinity for fibroblast growth factor (FGFR-1β) [41]. Beta form of FGFR-1 is the predominant isoform and has been described to drive tumor progression [42]. Another PTBP1 target is the kinase MARK4, which belongs to the family of AMP protein kinases. The two MARK4 splicing isoforms (MARK4L and MARK4S) differ in relation to their C-terminal end [43]. While MARK4L is upregulated in glioma cells and is expressed at high levels in neural progenitor cells, MARK4S is found predominantly in normal brain tissue and terminally differentiated neurons [44]. Bioinformatic and biochemical approaches identified PTBP1 binding sites in intron 15 of the MARK4 pre-mRNA, indicating that PTBP1 regulates the inclusion of exon 16. This process influences splicing of exon 18 which contains a stop codon; the result are two alternative protein products containing or not the C-terminal kinase 1 domain [45].

## 2.4 Adenosine Deaminases that Act on RNA

RNA editing is an important mechanism in which RNA modifications are used to modulate gene expression and function [46]. Adenosine deaminases that act on RNA (ADARs) are key regulators of RNA editing and changes in ADAR expression levels may lead to the development of different neoplasms, including gliomas. They are responsible for adenosine (A) to inosine (I) conversion in RNAs. These RNA modifications often lead to changes in amino acid incorporation that could ultimately affect protein properties [47]. In vertebrates, three members of the ADAR family have been identified. ADAR1 and ADAR2 are ubiquitously expressed and exhibit catalytic activity, whereas ADAR3 is specifically expressed in the brain and has no catalytic activity. ADAR3 has been revealed to competitively inhibit the deaminase activity of other ADARs by binding to dsRNA [48]. In particular, ADAR2 editing activity is crucial for the function of many proteins expressed in central nervous system and is essential for normal brain development [49].

In mammals, the catalogue of genes affected by RNA editing has been expanding. Among them are the glutamate receptors (GluR) sensitive to AMP (B, C and D subunits) and those sensitive to kainate (5 and 6 subunits). A to I editing causes the substitution of the neutral amino acid glutamine (Q) for an arginine (R), located in the pore domain of GluR. The positively charged Arginine prevents the passage of $Ca^{2+}$. This editing occurs in a majority of AMPA receptors in the central

nervous system and acts as a protective mechanism against excitotoxicity mediated by massive $Ca^{2+}$ influxes [50]. Due to the critical importance of accurate RNA editing in normal brain function, deregulation of editing may influence the progression of pathophysiological processes, such as neuro-degeneration and tumorigenesis [51].

Analyses of GluR-B transcripts in normal brain tissue showed that 100% of Q codons are edited to R codons in both gray and white matter. In contrast, GBM tissue showed a reduction in Q/R-site editing (12–31% decrease). This comes as a result of a decrease in the ADAR2 enzymatic activity [51]. Another study linked a reduction in editing of GluR-B (sites R/G and Y/C) and GluR-6 (sites I/V, Y/C and Q/R) transcripts to grade of malignancy in pediatric astrocytomas [47]. This editing has been attributed to alterations in ADAR2 catalytic activity. It has also been observed that elevated levels of ADAR2 inhibits proliferation and migration of astrocytoma cell lines [47]. Galeano et al. (2012) reported that ADAR2 editing affects proliferation of astrocytoma cells *in vitro* and *in vivo* via cell cycle modulation. Increased ADAR2 expression considerably prolonged survival and significantly inhibited astrocytoma growth *in vivo* [49].

Alternative splicing is also a critical mechanism that regulates ADAR2 activity [49, 50]. A splicing variant of ADAR2, which contains a 47-nucleotide insertion, is increased in gliomas in comparison to normal brain. Furthermore, the presence of this splicing variant correlates with malignancy [52]. Alternative splicing of exon 5a is also an important event in gliomas [53]. Transcripts containing exon 5a encode a protein with ~50% reduced activity that is predominantly expressed in gliomas [53].

ADAR2 is also essential for the editing and modulation of 90 miRNAs in glioblastomas. Rescue of ADAR2 activity in glioblastoma cells recovered the expression of onco-miRNAs and tumor suppressor miRNAs to levels observed in normal human brain. Wild-type ADAR2 activity was shown to inhibit miR-221, miR-222, and miR-21 maturation, causing accumulation of their precursors in different cell lines, impacting cell proliferation and migration [54].

## 2.5 Hu Antigen R

Hu antigen R (HuR) is a member of the *embryonic lethal abnormal visual* (ELAV) family, initially identified in *Drosophila* as a factor involved in neuronal development, plasticity and memory [55, 56]. HuR is ubiquitously expressed, whereas other members of the family (HuB, HuC and HuD) are tissue specific [56]. HuR regulates a variety of target mRNAs associated with processes like inflamation [57], cell cycle [58], angiogenesis [59], cell survival, and apotosis [60]. HuR preferently binds uracil-rich sequences and has been shown to regulate mRNA stability, splicing and translation [61, 62]. Recent studies have also linked HuR activity with microRNA metabolism and function [63, 64].

Angiogenesis is required for glioma development. Vascular endothelial growth factor (VEGF)-A is considered the major mediator of angiogenesis in malignant tumors, including high-grade astrocytomas [59]. HuR regulates VEGF-A expression and, under hypoxic conditions, inhibition of HuR cytoplasmic translocation by leptomycin B reduces VEGF-A upregulation in astrocytic tumor cells [59, 65]. In gliomas, it was shown that HuR also regulates the expression of angiogenic factors via mRNA stability [66, 67].

Several studies support a role of HuR in cell survival. In GBM, HuR binds the 3′UTR of bcl-2 family members and promotes both mRNA stability and translation. Using a mouse model, Filippova et al. (2011) showed that silencing of HuR promotes apoptosis by decreasing bcl-2 family members levels [68].

The development of an inflammatory microenvironment has long been considered important for the initiation and progression of glioblastoma. GBMs display high levels of the *pro-inflammatory cytokines like* IL-1β, IL-6 and IL-8 [69]. HuR increases the stability of IL-6 mRNA, leading to increased *pro-inflammatory cytokine* IL-6 protein production and secretion [57, 67].

Several studies have shown that HuR regulates the cell cycle in a variety of ways. One of them involves HuR phosphorylation at serine 202 by CDK5. Prolonged disruption of the balance between phosphorylated and unphosphorylated

HuR provokes an arrest of cell cycle progression in glioma cells by altered cyclin A levels [70]. Oscillation of cyclin A levels occurs during mitosis and is critical for DNA replication and centrosome duplication [71]. Moreover, SRC and c-Abl kinases regulate HuR sub-cellular trafficking and influence its accumulation in the pericentriolar matrix (PCM) via a growth factor dependent signaling mechanism [72]. Finally, HuR phosphorylation in the nucleus by Pyruvate kinase M2 (PKM2), results in increased glioma cell growth. The loss of the nuclear interaction between PKM2 and HuR leads to cytoplasmic redistribution of HuR and subsequently an increase in cap-independent mRNA translation of cyclin-dependent kinase inhibitor p27 mRNA, resulting in cell cycle arrest [73]. Some of the molecular mechanisms involved in the expression or activity of HuR in gliomas are mediated by growth factors. AKT/HSF1/HuR axis impacts Rictor expression, a component of the mTORC2 complex. EGF and IGF stimulation increases HuR transcription mediated by HSF1. HuR in turn, enhances Rictor mRNA translation which leads to elevated mTORC2 activity, tumor growth and invasion in GBM [74]. HuR is also subject to microRNA regulation. Yang et al. showed that miR-146b-5p overexpression could reverse HuR overexpression in glioma stem cells (GSCs). This regulation affects GSCs viability and cell cycle progression [75].

## 2.6 Musashi1

Musashi1 (MSI1) is a highly conserved RBP that controls the balance between self-renewal and differentiation [76]. MSI1 has been described as a pro-oncogenic factor in multiple tumor types [77]. High levels of MSI1 have been observed in several cancers, including medulloblastoma, hepatocarcinoma, cervical carcinoma, breast cancer and gliomas, and is linked to poor survival [78–82]. MSI1 regulates both translation and mRNA decay by binding to UAG motifs present in stem loops [83].

Uren et al. (2015) identified more than 1000 MSI1 target mRNAs in glioblastoma cells, using individual-nucleotide resolution cross-linking and immunoprecipitation (iCLIP) [83]. These targets are preferentially located in cancer relevant pathways such as focal adhesion, adherens junction, Wnt, JAK/STAT, p53, MAPK, VEGF, and ErbB. Functional assays showed that MSI1 knockdown impairs cell adhesion, migration, invasion, apoptosis, proliferation, and cell cycle regulation [83].

MSI1 has been linked to radio- and chemoresistance. MSI1 expression increases in response to DNA damage in glioblastoma cells [84]. MSI1 knockdown increases radiosensitivity by affecting DNA damage repair through regulation of DNA-activated catalytic polypeptide (DNA-PKcs). DNA-PKcs is a key enzyme involved in the classic nonhomologous end-joining (NHEJ) pathway of DNA double-strand break repair in mammals [84]. In addition, it has been demonstrated that overexpression of MSI1 effectively protects GBM cells from drug-induced apoptosis, like cisplatin, via down-regulation of pro-apoptotic genes [85].

## 2.7 Insulin-Like Growth Factor II mRNA Binding Proteins

The Insulin-like growth factor 2 (IGF2BP/IMPs) family of proteins consists of three members, IMP1 (IGF2BP1), IMP2 (IGF2BP2) and IMP3 (IGF2BP3), which are mainly expressed in early stages of embryogenesis [86]. High expression of IMP proteins has been observed in a broad range of cancer types, including pancreatic, lung, renal cell, ovarian, endometrial, cervical, and glioblastoma [87].

IMP3 is involved in the activation of MAPK and PI3K pathways through the activation of IGF-2, affecting cell proliferation and invasion of GBM cells [88]. Multivariate analysis identified high IMP3 as an unfavorable prognostic factor for pediatric [89] and adult astrocytoma patients [88, 90, 91].

Recent studies have shown that IMPs promote mRNA stability by preventing miRNA-mediated silencing [87]. In gliomas for instance, IMP2 protects mRNAs from let-7-dependent silencing by binding to the corresponding miRNA-binding sites [92].

## 2.8 Quaking

Quaking (QKI) belongs to the signaling transduction and activation of RNA (STAR) family of proteins. QKI pre-mRNA undergoes extensive alternative splicing to generate at least four transcripts producing isoforms termed QKI-5, QKI-6, QKI-7, and QKI-7b. These QKI isoforms share an RNA-binding KH domain, but differ by several amino acids at the C-terminus [93]. Analysis of glioma tumors found a high incidence of expression alterations in the human quaking gene [94]. It has also been reported that lost of the QKI-7 isoform decreases expression of genes involved in interferon (INF) induction, suggesting a role for QKI-7 as a regulator of the inflammatory pathway in glioblastoma [95]. QKI-6 expression correlates positively with glioma grade and promotes migration and invasion of glioblastoma cells via activation of PI3K/AKT and ERK pathways [96]. Recently, an in-frame MYB-QKI gene fusion was identified as hallmark genetic alteration in the majority of angiocentric gliomas [97–99]. The MYB-QKI rearrangement disrupts both MYB and QKI, resulting in hemizygous deletion of 3′ portion of MYB and the 5′ portion of QKI [99]. *In vitro* and *in vivo* studies show that the MYB-QKI fusion promotes tumorigenesis [97].

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