RIBONUCLEOPROTEIN COMPLEXES AND PROTEIN ARGININE METHYLATION : A ROLE IN DISEASES OF THE CENTRAL NERVOUS SYSTEM

by

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ABSTRACT

For the past 45 years, QKI has been studied for its role in the processes of development and central nervous system myelination using the *qk*^{v} mouse. The presence of a single KH domain and the recent identification of a high-affinity binding site in mRNAs, suggests that it can bind to and regulate mRNAs through processes such as stability, splicing and transport. As a member of the STAR RNA binding family of proteins the QKI isoforms may also be involved in cell signaling pathways.

QKI's involvement in all of these processes, lead us to examine both the protein partners and the mRNA targets of the QKI complex in order to identify potentially new pathways regulated by QKI. In doing so, we identified a novel direct protein-protein interaction with PABP and for the first time described the relocalization of QKI to cytoplasmic granules following oxidative stress. In addition, *in vivo* mRNA interaction studies were performed and allowed the identification of approximately 100 new mRNA targets in human glioblastoma cells. One of the targets identified was VEGF mRNA.

Another QKI target mRNA is MBP, a major protein component of the myelin sheath and the candidate auto-antigen in multiple sclerosis (MS). *In vivo* MBP is symmetrically dimethylated on a single arginine residue. To further establish the role of the methylation of MBP in myelination, a methyl-specific antibody and an adenovirus expressing a recombinant protein arginine methyltransferase 5 (PRMT5) was generated. We show that methylated MBP is found in areas of mature myelin and that over-expression of the PRTM5 blocked the differentiation of oligodendrocytes.

Taken together these datas implicate QKI for the first time in the process of human cancer angiogenesis and could explain the vascularization defects observed in some of

the *qkI* mutant mice. In addition, arginine methylation of MBP may prove to have an important role in the process of myelination and in the pathogenesis of demyelination and the autoimmune reaction in diseases such as MS.

SOMMAIRE

Historiquement, les travaux de recherche sur QKI étaient concentrés sur son implication dans les processus de développement et de myélinisation du système nerveux central chez la souris qk^{v}. La présence d'un domaine KH, ainsi que la découverte d'une séquence consensus présente dans plusieurs cibles d'ARNm s'associant à QKI, suggèrent que cette protéine a pour fonction de lier ces ARNm de façon hautement spécifique. Cette association QKI/ARNm permet leur transport, épissage et stabilisation. Compte tenu de son appartenance à la famille de protéines liant l'ARN STAR, il est aussi concevable que les isoformes de QKI soient impliquées dans plusieurs voies de signalisation cellulaire.

L'implication de QKI dans ces différents processus nous a amené à déterminer les protéines et les ARNm associés à QKI afin de la localiser dans de nouvelles voies de signalisation cellulaire. Dans le cadre de cette investigation, nous avons identifié un nouvel intéractant, soit PABP. Nous avons également observé une relocalisation de QKI dans les granules cytoplasmiques suite à un stress oxydatif. De plus, des études in vitro sur des extraits d'ARNm provenant de cellules de glioblastomes humains ont permis l'identification d'une centaine d'ARNm pouvant s'associer à QKI, tel que l'ARNm de VEGF.

Un autre ARNm associé à QKI code pour la protéine MBP laquelle est une composante protéique majeure des feuillets de myéline. Cette protéine est suspectée d'être ciblée par le système immunitaire, ce qui pourrait être la cause d'une maladie telle que la sclérose en plaques (MS). On remarque avec intérêt que la protéine MBP peut être méthylée symétriquement, *in vivo*, sur une arginine par la méthylase PRMT5. Pour étudier la fonction de MBP méthylée, nous avons généré un anticorps methyl-spécifique

ainsi qu'un adénovirus exprimant la méthylase impliquée dans cette réaction, soit PRMT5. Ces outils ont permis de démontrer que MBP est symétriquement méthylée dans les zones myélinisées et que la surexpression de PRMT5 permet de bloquer la différentiation des oligodendrocytes.

Ces résultats ont aussi permis, pour la première fois, d'impliquer QKI dans des processus d'angiogenèse associés au cancer humain en plus d'expliquer les défauts de vascularisation observés chez les souris dont le gène de QKI est muté. De plus, le degré de méthylation de MBP pourrait avoir une implication majeure dans la formation de la myéline et la démyélinisation observée dans certaines pathologies telle que la sclérose en plaques.

PREFACE

This Ph.D. thesis was written in accordance with the Guidelines for Thesis preparation from the Faculty of Graduate Studies and Research of McGill University. I have exercised the option of writing the thesis as a manuscript-based thesis. For this, the guidelines state: "...Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. ....The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory. ...The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary. .... In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. ....In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. ...."

A general introduction and literature review is presented in Chapter 1, whereas a final discussion is included in Chapter 4.

Papers included in this thesis:

Chapter 1 **Chénard, CA** and Richard, S. (**2008**) New Implications for the Quaking RNA binding protein in Human Diseases. *J Neurosci Res.* 86(2):233-242.
- (Sections 1.3.2-1.4.2, 1.4.4-1.5.3; Figures 1.3, 1.4, 1.5; Table 1.2)

Boisvert, FM. **Chénard, CA**. Richard, S. Protein Interfaces in Signaling Regulated by Arginine Methylation. *Sci. STKE,* Vol. 2005, Issue 271. pp. re2, 15 February 2005.
- (Pages 69-70; Fig 1.7)

Chapter 2 **Chénard, CA.** Lacroix, G. Pilotte, J. and Richard, S. The QUAKING RNA binding proteins interact with PABP protein and the VEGF mRNA: Defining the QKI complex. *Manuscript in preparation.*

Chapter 3 **Chénard, CA.** Almazan, G. and Richard, S. Novel Myelin Basic Protein (MBP) Methyl-Specific Antibodies Reveal that Methylation of MBP by PRMT5 is Important for Myelination in the Central Nervous System. *Manuscript in preparation.*

Contribution of Authors:

The candidate performed most of the research presented in this thesis and wrote all of the included manuscripts with support from Dr Stéphane Richard. The contribution of other authors to this work is described below:

In Chapter 2, Geneviève Lacroix performed experiments in Figure 2.1 and performed the immunoprecipitation for Table 2.1. Dr. Julie Pilotte performed

experiments in Figure 2.2. The microarray data was performed in collaboration with Edouardo Hernandez and Dr. John Hiscott at the Lady Davis Institute Core MicroArray Facility.

The different studies were all conducted under the supervision of Dr Stéphane Richard.

Contributions not included in this Thesis:

In addition to the papers included in this thesis, the candidate contributed to the following study, which has been published or submitted:

Boisvert, FM. **Chénard, CA.** Richard, S. Protein Interfaces in Signaling Regulated by Arginine Methylation. *Sci. STKE,* Vol. 2005, Issue 271. pp. re2, 15 February **2005**.

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2D: two dimensions

A2RE: hnRNP A2 response element

A: adenine

AA: astrocytic astrocytoma

aDMA: asymmetric dimethylarginine

Ala: alanine

AS: alternative splicing

Asp: Aspartate

AR-JP: autosomal juvenile Parkinson's disease

BBP: branch point binding protein

BRK: breast tumor kinase

CBC: cap binding complex

cDNA: complementary deoxyribonucleic acid

CDS: coding sequence

CNP: 2',3'-cyclic nucleotide 3'-phosphodiesterase

CNS: central nervous system

C-terminal: carboxy terminal

DEAD box: Asp-Glu-Ala-Asp box

DNA: deoxyribonucleic acid

dsRBD: double stranded RNA binding domain

dsRNA: double stranded RNA

EAE: experimental autoimmune encephalomyelitis

E48G: mutation of glutamic acid to glycine at position 48

EGFR: endothelial growth factor receptor

EGR-2: early growth response gene-2

eIF: eukaryotic initiation factor

ENU: N-ethyl-N-nitrosourea

ER : endoplasmic reticulum

EST : expressed sequence tag

FACS : fluorescence activated cell sorting

FMRP: Fragile X Mental Retardation protein

FPLC: fast protein liquid chromatography

G: guanine

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GAR: glycine arginine rich

GBM: glioblastoma multiforme

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

GLD-1: defective in germ line development

Glu: glutamic acid

Gly: glycine

GSG: GRP-33, Sam68, GLD-1

hGLI: human GLI (zinc finger transciption factor)

HIF1α: hypoxia induced factor 1 alpha

HI-RPB: hypoxia induced RNA protein binding

HIV: human immunodeficiency virus

hnRNP K: human heterologous nuclear ribonucleoprotein K

IgG: Immunoglobulin G

IP: Immunoprecipitation

kb: kilo bases

kDa: kiloDalton

KH: hnRNP K homology

LC/MS/MS: liquid chromatography coupled with tandem mass spectrometry

L-MAG: large MAG

MAG: myelin-associated glycoprotein

MAP1B: Microtubule-associated protein 1B

MBP: myelin basic protein

mGLI: mouse GLI

miRNA: microRNA

MMA: mono-methyl arginine

MOG: myelin oligodendrocyte glycoprotein

mRNA: messenger ribonucleic acid

mRNP: messenger ribonucleoprotein

MS: Mulitple Sclerosis

MTA: methyl thio adenosine

NLS: nuclear localization signal

NMD: nonsense mediated decay

N-terminal: amino terminal

OL: oligodendrocyte

OPC: oligodendrocytes precursor cell

ORF: open reading frame

P0: protein zero

$p27^{KIP1}$: cyclin-dependent kinase inhibitor $p27^{KIP1}$

PAS: peptidylarginine deiminase

PABP: Poly-A binding protein

Pacgr: *Parkin* co-regulated gene

PAGE: polyacrylamide gel electrophoresis

PAIP: PABP-interacting protein

PI: propidium iodide

PRKN: parkin gene

P-body: processing body

PBS: phosphate buffered saline

PCR: polymerase chain reaction

Phe: phenylalanine

PLP: proteolipid protein

PNS: peripheral nervous system

PRMT: protein arginine methyl transferase

QASE: QKI-5 alternative splicing element

QKI: QUAKING (protein)

qkI: quaking gene, class I

qk^{v}: quaking viable

QRE: QKI Recognition Element

RBP: RNA binding protein

RG box: short repeats of arginine and glycine

RISC: RNA induced silencing complex

RNA: ribonucleic acid

RNAi: RNA interference

RRM: RNA recognition motif

SAM : S-adenosylmethionine

Sam68: Src-associated-in-mitosis protein of 68kDa

SBE: STAR Binding Element

SCZ: Schizophrenia

SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis

SELEX: Systematic evolution of ligands by exponential enrichment

SF1: splicing factor 1

SG: stress granule

SH3: Src homology domain 3

shRNA: short hairpin RNA

siRNA: small interfering RNA

S-MAG: small MAG

SMC: smooth muscle cell

STAR: Signal Transduction and Activator of RNA

TCA: trichloroacetic acid

TGE: *tra* GLI element

TCL: total cell lysate

UPF1: regulator of nonsense transcripts

UTR: untranslated region

V157E: mutation of valine to glutamic acid at position 157

VEGF: vascular endothelial growth factor

WB: Western blot

WHO: World Health Organization

Chapter 1

Introduction and Literature Review

1.1 *General Introduction*

RNA binding proteins (RBP) are involved in every step of an RNA molecule's lifecycle. Recent studies, using genetic models and the systematic evolution of ligands by exponential enrichment (SELEX) technique, have identified numerous new RNA binding proteins as well as the *cis*-elements that they bind. There remains a lot to understand about RBPs, how they regulate posttranscriptional gene expression and how their posttranslational modification affects this regulation. The objective of this thesis was to characterize the components of the ribonucleoprotein complex formed by the RBP Quaking (QKI) in an attempt to understand some of its properties and functions. The first Chapter will describe the known characteristics of the QKI protein and its family members, as well as give some insight into the formation of RNPs and their biological roles. Chapter 2 will describe the proteomic and microarray analysis of the QKI RNP. Chapter 3 will describe the use of a novel antibody that recognizes arginine methylated myelin basic protein to investigate its localization in the central nervous system (CNS). Finally, chapter 4 will discuss the importance of the findings presented in this thesis.

1.2 *RNA Binding Proteins*

At every step in its lifecycle RNA is found associated with proteins. Posttranscriptionally, RBPs regulate numerous processes such as splicing, trafficking, stability and decay.

1.2.1 The Ribonucleoprotein complex

Early in the 1970s, it was demonstrated that most mRNAs in the cell are associated with numerous proteins (Greenberg 1972). mRNAs are found associated with RNA binding proteins forming complexes called ribonucleoprotein complexes (RNP). The RNPs regulate gene expression posttranscriptionally and regulate processes such as splicing, stabilization, and degradation. The RNPs are very dynamic and the associated proteins interact with the transcripts via several different RNA binding motifs.

The mRNPs undergo several steps and transformations in order to allow the expression of proteins from their mRNA cargo. The assembly begins during the process of transcription when proteins associate with the RNA being transcribed from the DNA template in order to begin processing the RNA with a 5′ cap, a poly-A tail, splicing and screening the mRNA for quality control problems and if identified, subsequent targeting to nonsense mediated decay. From the nucleus, the transcripts must be transported to the cytoplasm through the nuclear pore and this process requires many different associated RBPs, not only for the transport of the mRNAs, but also for the dissociation of the complex once through the pore. Once in the cytoplasm not all mRNAs are immediately translated and many of the transcripts are sorted into cytoplasmic compartments to await either specific translation cues or specific signals. In addition, the transcripts can also be sorted into specific large RNPs which are discussed below. Transcripts can be shuttled between different mRNPs and compartments depending on the proteins associated with the mRNA, which are often dependent on cellular signals, meaning that the 'quality control' mRNP may help in shuttling or recruiting aberrant mRNAs to the decay machinery. Not much is known about the signals regulating mRNPs or the *cis*-elements

required for their formation, but an attempt will be made in the sections below to summarize the data on some of the major mRNPs.

1.2.2 The polysome

As mentioned above, RBPs play pivotal roles in posttranscriptional regulation of gene expression. There are several RBPs present in mRNP complexes that associate with the translation machinery implicating these proteins in protein translation (Khandjian et al. 1996). Translating mRNAs are found in a large complex termed the polyribosome or polysome. The polysome is a cluster of ribosomes connected by a strand of mRNA and functioning as a unit in protein synthesis (Lodish 2000). mRNA translation can be divided into several steps including pre-initiation, initiation, elongation and termination. Because the pre-initiation complexes are smaller than the 80S ribosome, particles separated by sucrose gradients that are larger than 80S are considered to be associated with the polyribosomes (Zalfa et al. 2006). The role of many of the polyribosome-associated RBPs remains unknown and several of them are both found in slow sedimenting RNPs and associated with polysomes. One example of this is the Fragile X mental retardation protein (FMRP), a KH containing RNA binding protein whose mutations and inappropriate gene methylation are associated with the X-linked mental retardation syndrome, Fragile X syndrome. Several studies showed that FMRP cofractionates with polyribosomes (Khandjian et al. 2004; Stefani et al. 2004). Meanwhile, others have shown that it cofractionates primarily with mRNPs (Siomi et al. 1996) or is equally distributed between the fractions (Feng et al. 1997).

Protein Class	Protein component	Stress granule	P-body
Ribosomes	40S	Kedersha et al., 2002	Unpublished data
	60S	Kedersha et al., 2002	Unpublished data
Translation	eIF2	Kedersha et al., 2002	Unpublished data
	eIF3	Kedersha et al., 2002	Kedersha et al., 2005
	eIF4E	Kedersha et al., 2002	Kedersha et al., 2005
	4E-T	ND	Ferraiuolo et al, 2005
	PABP	Kedersha et al., 1999	Kedersha et al., 2005
	RCK/CGH-1	Wilczynska et al., 2005	Cougot et al., 2004
	TIA-1/R	Kedersha et al., 1999	Kedersha et al., 2005
RNA stability	TTP	Stoecklin et al., 2004	Stoecklin et al., 2004
	HuR/D	Gallouzi et al., 2000	Unpublished data
RNA binding protein	Staufen	Thomas et al., 2005	Unpublished data
	SMN	Hua and Zhou, 2004	ND
	G3BP	Tourriere et al., 2003	Kedersha et al., 2005
Sm/Lsm proteins		Unpublished data	Ingelfinger et al., 2002 van Dijk et al., 2002
Decapping	DCP1/2	Kedersha et al., 2005	Ingelfinger et al., 2002
Exonucleases	XRN1	Kedersha et al., 2005	Bashkirov et al., 1997
siRNA	GW182	Kedersha et al., 2005	Eystathioy et al., 2003
	Argonautes	Unpublished data	Liu et al., 2005

Table 1.1 Comparative components in P-bodies and Stress granules.

Highlighting = absent. Adapted from (Anderson and Kedersha 2006).

There are several hypotheses as to the role of FMRP when it is present in different complexes. One is that the fraction of FMRP present in 'light mRNPs' is involved in translational repression by inhibiting translational intiation (Laggerbauer et al. 2001), perhaps through interaction with the RNA interference silencing complex (RISC) (Caudy et al. 2002). In its polysome associated form, it has been suggested that FMRP acts as a translational repressor in a 'stalling ribosomes' model. This model suggests that some FMRP can become associated with polysomes that have stalled ribosomes and must await a signal to resume translation (Ceman et al. 2003). This could be particularly important at the synapse, where a bolus of locally required protein could be synthesized when needed (Ceman et al. 2003).

1.2.3 P-Bodies

In the cytoplasm mRNAs undergo a quality control surveillance that will either promote mRNA degradation or repress mRNA translation during this period. This can be triggered either by association of RBPs with the mRNAs or by the association of small complimentary RNAs in a process called RNA-mediated gene silencing (Eulalio et al. 2007). There are many proteins and regulatory RNAs that are involved in these processes and in eukaryotic cells these factors colocalize in discrete cytoplasmic granules called mRNA processing bodies (P-bodies).

The initial discovery of P-bodies and the suggestion that they may play a role in mRNA degradation stems from the discovery of the decapping enzyme DCP2, which removes the 5′ cap from mRNA (Table 1.1) (Bashkirov et al. 1997; van Dijk et al. 2002). Since their identification, many other proteins have been shown to localize within P-bodies (Table 1.1) (Eulalio et al. 2007). These bodies range in size from 100nm to 300nm

and their number and size can be increased upon cell stress (Sheth and Parker 2006). In addition to proteins involved in mRNA degradation, identification of proteins involved in mRNA surveillance, RNA interference and translational repression in P-bodies has suggested an important role for P-bodies in the regulation of gene expression (Table 1.1).

The presence of mRNA decay enzymes in P-bodies, raised the question as to whether they represent sites of mRNA degradation. In eukaryotic cells, mRNA degradation occurs by two pathways following deadenylation: 3′-5′- exonucleolytic decay catalyzed by the exosome (Houseley et al. 2006) or 5′-3′ digestion following decapping (Garneau et al. 2007). Several lines of evidence exist to suggest that mRNA decay occurs in the P-bodies. Inhibition of transcription or exposure of cells to ribonuclease A (RNase A) leads to P-body loss (Teixeira et al. 2005). The same is true by blocking the early stages of deadenylation (Sheth and Parker 2006). This indicates that the presence of intact RNA is required for the integrity of the P-bodies. Finally, mRNA decay intermediates are detected within P-bodies (Eulalio et al. 2007).

mRNAs are constantly being surveyed to ensure that only fully processed and error free mRNA is being translated (Fasken and Corbett 2005). If an mRNA contains a premature termination codon, then it is degraded through the nonsense mediated decay (NMD) pathway. Important components of this pathway, such as UPF1-3 (regulator of nonsense transcripts), are found within P-bodies. It is established that the surveillance machinery recruits decay proteins but it is unknown whether the NMD machinery brings the mRNA to the P-body or whether P-body formation is triggered by the accumulation of mRNAs committed to NMD (Eulalio et al. 2007).

In the past five years the number of proteins identified within P-bodies has grown quite quickly and so to have the biological functions attributed to them. An exciting field

connected to P-bodies in this way is the small interfering RNAs (siRNA) and microRNAs (miRNA) that regulate gene expression posttranscriptionally (Bartel 2004; Filipowicz 2005). Although siRNAs and miRNAs differ in their biogenesis (Figure 1.1), the effectors of their regulatory functions are members of the Argonaute family. These proteins promote the translational repression or decay of target mRNAs that are complementary to the siRNA or miRNA. In animals, the miRNA degradation pathway procedes through the general mRNA decay machinery, which is accelerated by deadenylation, a process that requires the Argonaute proteins and several P-body components. Although there are several lines of evidence that show that P-body components are involved in the gene-silencing pathway, recent studies have shown that P-body integrity is not necessary (Chu and Rana 2006) and siRNAs and miRNAs can function in the absence of P-bodies (Eulalio et al. 2007).

1.2.4 Stress Granules

There are several definitions of stress granules, and most are characterized by the localization of a specific protein to these cytoplasmic domains upon cell stress. Changes in the cellular environment require cells to modulate the repertoire of proteins that they produce. This is particularily important during times of cell stress when global repression of translation is required and specific stress-induced transcripts are translated with priority (Kedersha and Anderson 2002). Cell stresses include oxidative stress, heat shock, UV radiation, osmotic shock, endoplasmic reticulum (ER) stress and viral infection in addition to others.

One universal definition is that stress granules (SG) are cytoplasmic subdomains into which mRNAs are dynamically sorted in response to phosphorylation of eukaryotic

intitiation factor (eIF) 2α, which is important for translational initiation (Table 1.1) (Kedersha and Anderson 2002). The phosphorylation of eIF2α prevents its dissociation of eIF2B, the enzyme that regenerates the ternary complex (Met-tRNAi:eIF2:GTP), thus stopping translational initiation (Kedersha and Anderson 2002) and repressing translation. SGs are very dynamic in nature. Upon certain stresses, like arsenite treatment, SGs form within 15 minutes (Kedersha et al. 2000) and continue to grow in size for another ~2 hours. After withdrawl of the stressing agents, the SGs disperse as quickly as they were formed. The ability of certain inhibitors of translational elongation that stabilize polysomes, such as cyclohexamide, to reverse SG assembly suggests that the associated mRNAs are in a sort of equilibrium with polysomes.

The recruitment and translational repression of the bulk of cellular mRNAs upon stress (Kedersha et al. 1999), formation of SGs and their subsequent disassembly requires numerous shuttling proteins, RNA binding proteins and ribosomal subunits. Recruited to the SGs are the small ribosomal subunits eIF3, eIF4E and eIF4G (Kedersha et al. 2002). The RNA binding proteins, poly-A binding protein (PABP), T-cell internal antigen-1 (TIA-1), TIAR (TIA-1 related protein) (Kedersha et al. 2000) and HuR (Brennan and Steitz 2001) are among those found within the SGs, but is by all means not an inclusive list (Table 1.1). After eIF2α phosphorylation there is a decrease in eIF2-GTP-tRNA levels allowing TIA-1 and TIAR to bind to the 48S complex instead of the ternary complex. This promotes polysome disassembly and the transfer of the mRNAs into the SGs.

1.2.5 RNPs and their Role in Translation

After transcription, mRNAs are translated into proteins to be used by the cell and this is a highly coordinated process whereby mRNAs are translated in specific places and times in the cell. The process is regulated by the assembly of protein complexes in the cell that do not allow translation to occur until the proper signals are received. Although many of these complex processes are controled and take place in distinct cytoplasmic domains as discussed above, the assembly of the translation machinery on the mRNA can itself be considered an RNP, as it is a complex of proteins and mRNAs, and it is often this process that is regulated or disrupted in the granules previously discussed.

In order for mRNAs to be translated in the cytoplasm, there must be methylation of the 5' guanosine cap structure in the nucleus, which is recognized by the cap-binding complex (CBC). After translocation of the complex to the cytoplasm, the CBC is replaced by eIF4E, the translation initiation factor for assembly of the translational machinery. Assembly of the translational initiation complex is the rate limiting step of translation and is the primary target of translational control (Gingras et al. 1999). The initiation factor including eIF4E, eIF4G, a scaffolding protein, eIF4A the RNA dependent helicase and eIF4B are then recruited to the mRNA. Activation of eIF4A relaxes the secondary structure upstream of the start site in anticipation of translational activation. The 43S pre-initiation complex, which includes the 40S ribosomal subunit, initiation factors and the initiator tRNA, Met-tRNA-eIF2-GTP, is then recruited to the methyl-cap.

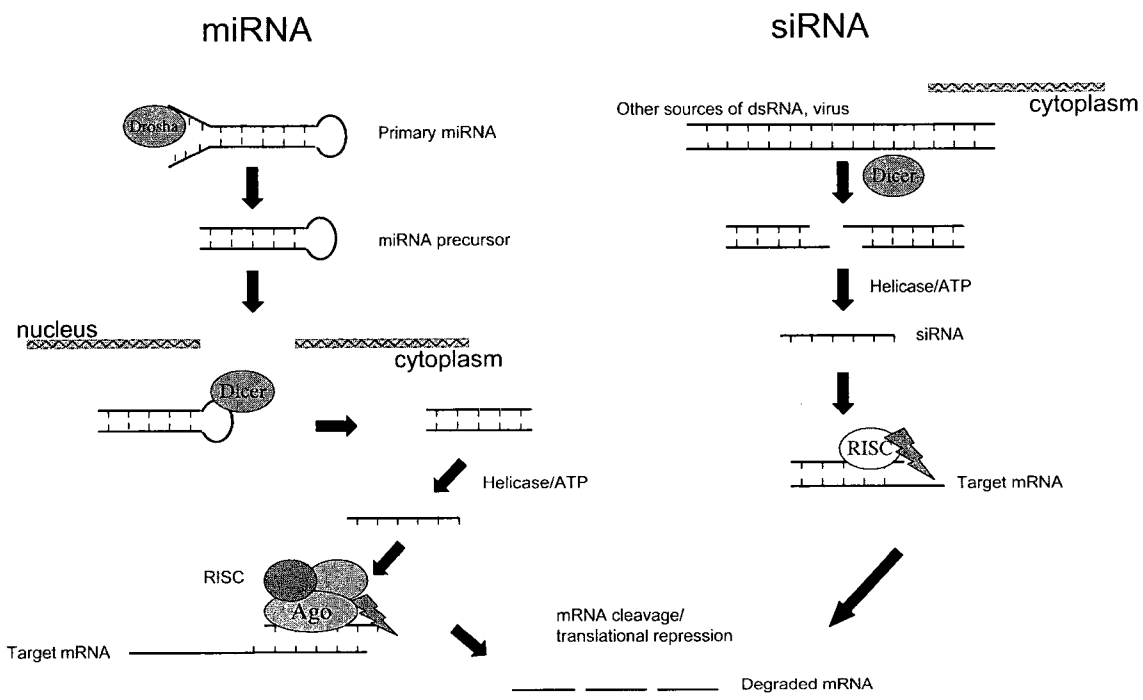

Figure 1.1 Models of RNA silencing pathway.

Two related mechanism for miRNA and RNAi biogenesis (adapted from Mak 2005)

The 43S complex scans the mRNA (5′– 3′) until it identifies the AUG start codon in a process called 'scanning'. Once at the start codon, the initiation factors are released and the 60S ribosomal subunit joins to form the 80S ribosome and elongation of the polypeptide occurs (Gingras et al. 1999).

1.2.5.1 The Poly-A Tail and Translational Control

Translation is not only regulated by proteins binding at the 5′ end of the mRNA. The poly-A tail plays a much larger role than early experiments predicted. In fact, experiments have shown that in many cases, the length of the poly-A tail correlates with the global translational activity of the cell (Gingras et al. 1999). The poly-A tail of mRNAs is coated with many copies of the PABP. PABP is a highly conserved RNA binding protein with four RNA recognition motifs. Key to the establishment of PABPs role in translation was the development of a model whereby the poly-A tail and the cap structure work synergistically to enhance transcription. This model, termed the closed loop, demonstrated that the poly-A tail enhanced recruitement of the 40S subunit binding (Figure 1.2) (Jacobson and Favreau 1983; Tarun and Sachs 1995).

How PABP enhances translation is explained by its ability to bind to eIF4G directly. eIF4G performs a bridging function, bringing together the mRNA and the ribosome (Gingras et al. 1999). PABP binds to eIF4G and circularizes the mRNA, bringing its 3′ end into close proximity to its 5′ end (Figure 1.2). The PABP-eIF4G interaction requires RNA, suggesting that PABP is able to bind eIF4G only once it is bound to the poly-A tail (Tarun and Sachs 1996). The fact that this circularization is essential for cap dependent translation implies important biochemical ramifications for initiation. The exact function of the circularization is not yet clear although there have

been many suggestions as to its purpose. Perhaps it is useful for the recycling of the 40S subunit without disassembly from the mRNA, prevention of translation of shortened or nicked mRNA or involvement in assembly of the initiation complex proteins (Gingras et al. 1999). In addition, blocking the PABP :eIF4G interaction would provide an interesting yet effective mechanism to inhibit translation.

1.2.6 RNPs and their Role in Transport

Although the life of a mRNA molecule begins in the nucleus, its lifecycle does not end there. The pre-mRNAs undergo numerous processes, such as capping, polyadenylation, and splicing. These processes as well as the proteins that associate with the mRNAs serve as a mark to determine whether the mRNA will be exported, translated, stabilized or degraded. In addition, to be exported to the cytoplasm, the export machinery must recognize and distinguish between mature mRNAs and pre-mRNAs. mRNAs are exported from the nucleus through the nuclear pore, but this requires many shuttling RBPs and nuclear export factors to bridge the interaction as well as help the mRNA to cross the pore (Erkmann and Kutay 2004).

Once on the other side of the nuclear pore complex, it is not clear what signals are required for the dissociation of the RNP from the nuclear pore. Once released, the mRNAs can proceed to the next phase of their life cycle. Certain cells, as well as some developmental stages in certain organisms, require that the translation of specific mRNAs be restricted to specific compartments. For example, the RBP Staufen is required for the localization and transport of *oskar* mRNA to the posterior pole of the *Drosophila* oocyte (Micklem et al. 2000).

Some mRNAs need to be transported far away from the nucleus prior to being translated. This is particularly true in oligodendrocytes and neurons. In the CNS, oligodendrocytes (OL) myelinate axons in order to ensure proper conduction of nerve impulses. The oligodendrocytes myelinate the axons by generating the myelin component mRNAs in the nucleus and transporting them to the distal tips of their processes, where the mRNAs are translated and the proteins subsequently incorporated into the growing myelin sheath. Most notably in these cells, myelin basic protein (MBP) mRNA is localized to the myelin compartment (Carson et al. 1998). mRNAs to be transported in oligodendrocytes are packaged into RNPs termed trafficking intermediates, composed of mRNA molecules, proteins and translational machinery (Barbarese et al. 1995). The pathway that each mRNA takes is determined by *cis*-acting sequences within the mRNA and the associated *trans*-acting proteins. For example, MBP mRNA trafficking is mediated by an eleven nucleotide sequence termed the hnRNP A2 response element (A2RE). A2RE containing mRNAs bind to hnRNP A2 and remains bound to it throughout its subsequent trafficking along the microtubule network towards the plus end (Ainger et al. 1997; Carson et al. 1998). The A2RE sequences in MBP and the hnRNP A2 proteins are sufficient and necessary for the trafficking RNP assembly, transport on the microtubules (Carson et al. 1997), and stimulation of translation (Kwon et al. 1999).

In neurons, the situation is very similar. mRNAs assemble into large granules, often associating with their *trans*-acting RBPs in the nucleus and those RBPs have an important impact on the mRNA's localization in the cytoplasm (Hachet and Ephrussi 2004). Proteomics studies of the granules have identified >42 proteins as part of the RNA granule such as Staufen, hnRNP U, PSF that are required for RNA transport (Hirokawa 2006). Modifications to mRNA transport to the dendrites appears to occur upon neuronal

activation. For instance, calcium-calmodulin-dependent kinase II (CamKIIα), a protein essential for memory and long-term potentiation, becomes localized and translated in the synaptic dendrites following depolarization of hippocampal neurons (Rook et al. 2000) as does the β-actin mRNA (Tiruchinapalli et al. 2003).

As a general rule, RNA found within transport granules is translationally repressed (Carson et al. 2001), and there is translational activation at the final destination site. The translation of mRNAs within the proper compartments has been shown to regulate plasticity and synaptic efficacy as well as axon guidance and synaptic formation (Campbell and Holt 2001; Lyles et al. 2006; Ming et al. 2002). Elucidating the function of the locally translated mRNAs as well as the proteins required to get them there and translated is very challenging (Martin and Zukin 2006) and much work remains to be done to identify the key components.

1.2.7 RNPs and their role in mRNA stability and degradation

Until quite recently, it has been assumed that mRNA stability plays a passive and not an active role in the posttranscriptional pathways. But several groups have demonstrated that mRNA stability is a highly and tightly regulated process and is particularily important in response to certain cell stimuli and particularily in embryogenesis and development (Cooperstock and Lipshitz 1997; Macdonald and Smibert 1996). Regulating translation allows the use of mRNAs at different times during oogenesis or embryogenesis. In addition, many cytokines, oncoproteins and signaling molecules are inherently unstable, allowing for rapid changes in the levels in response to environmental, pharmacological or hormonal changes. The control of mRNA stability is ultimately linked to translational control. The best example of this is found in *Drosophila*

where protein gradients are dependent on translational repression of target mRNAs followed by their subsequent degradation (Newbury 2006). The initial step of the patterning restricts translation of *oskar* mRNA to the posterior pole of the oocyte. This is achieved by the RBPs bruno and staufen, which bind to elements within the 3′UTR of *oskar* and are required for *oscar's* localization. Once translated, the oskar protein is required for the translation of *nanos* mRNA at the posterior pole. The cascade is completed by the *nanos* dependent repression of *hunchback* mRNA in the posterior part of the embryo (Macdonald and Smibert 1996). This example of patterning misdirects development if it happens outside the normal context, showing that translation control of each of the genes in the cascade is essential.

Thus far, the various control elements appear to be found primarily in the 3′ UTR of mRNAs. Although since most pathways affecting both mRNA stability and decay interact/interfere with the translation machinery and the mRNA is being held in the 'closed loop' with the 5′ cap in close proximity to the poly-A tail, proteins that specifically repress translation often disrupt the RNP complex holding the 5′ and 3′ ends together. The subsequent degradation of these mRNAs likely require the interaction of the decay machinery with the translation machinery (Newbury 2006). The most common *cis*-acting element in the 3′ UTR of mammalian mRNA is the AU-rich element (ARE) (Khabar 2005; Shaw and Kamen 1986). AREs are defined by their ability to promote rapid deadenylation-dependent mRNA decay, decapping and exosome recruitement (Shim and Karin 2002). The sequence requirement is the nonamer UUAUUUAUU (Chen and Shyu 1995).

The destabilizing activity of the AREs can be modulated by interaction with RBPs. There are at least fourteen RBPs that have been shown to bind AREs, including the well

known heterologous nuclear ribonucleoprotein (hnRNP) D, tristetraprolin (TTP) and HuR. These proteins are well known to modulate the turnover of ARE-containing mRNAs (Brennan and Steitz 2001). Whereas hnRNP D increases mRNA degradation upon binding, HuR, a ubiquitously expressed member of the ELAV family, has the ability to stabilize ARE-containing mRNAs after their deadenylation and its over expression leads to accumulation of mRNAs (Fan and Steitz 1998; Ford et al. 1999). HuR likely acts by interacting with the ARE and disrupting an endonucleolytic site in the mRNA and protecting the it from endonuclease cleavage (Zhao et al. 2000).

1.3 *RNA binding Motifs*

The plethora of posttranscriptional mechanisms discussed above are essential for the expression of genetic information. Recent information has defined the molecular mechanisms by which the RBPs recognize their target mRNA sequences and influence the lifecycle and function of those RNA molecules. The proteins that regulate gene expression contain RNA-binding domains and typically domains that mediate protein-protein interaction and subcellular targetting (Burd and Dreyfuss 1994). There are four major classes of RBPs : those with RNA recognition motifs (RRMs), the double stranded RNA binding domain (dsRBD) class, those with the DEAD-box helicase domain and the hnRNP K (KH) containing proteins (Siomi and Dreyfuss 1997). The most studied of the RBPs are those containing the RNP motif (RRM or RBD). There are currenly 300 known RNP motif proteins (Siomi and Dreyfuss 1997). This family requires two RRMs to bind RNA. The second class of RBPs contain one or more dsRBD. The dsRBD is a 65 amino acid motif that is able to interact with as little as eleven base pairs of dsRNA. Unlike the other classes of RBPs, this binding seems to be dependent on RNA structure and not on

sequence recognition. This family of proteins is particularily important in signaling in response to virus infection (Koromilas et al. 1992) as well as miRNA biogenesis and RNA interference (Forstemann et al. 2005). The DEAD-box protein family is characterized by the presence of the Asp-Glu-Ala-Asp box. DEAD-box proteins use the energy from ATP hydrolysis to rearrange molecular RNA structure or to dissociate RNA-protein complexes. In yeast, one family member, Dbp5, has been shown to remodel the mRNP on the cytoplasmic side of the NPC and help in the recycling of the RNP and allow for unidirectional gene transport (Stewart 2007). The final RNA binding motif, the KH domain is a ~70 amino acid motif and is often found in multiple copies within a protein.

Understanding the activation of RNAs through modulation of RNA binding proteins in many cell systems will be greatly enhanced by our understanding of protein-protein interactions. It will also be important to identify the *cis*-acting elements within the RNAs that mediate the interaction with the RBPs (Siomi and Dreyfuss 1997).

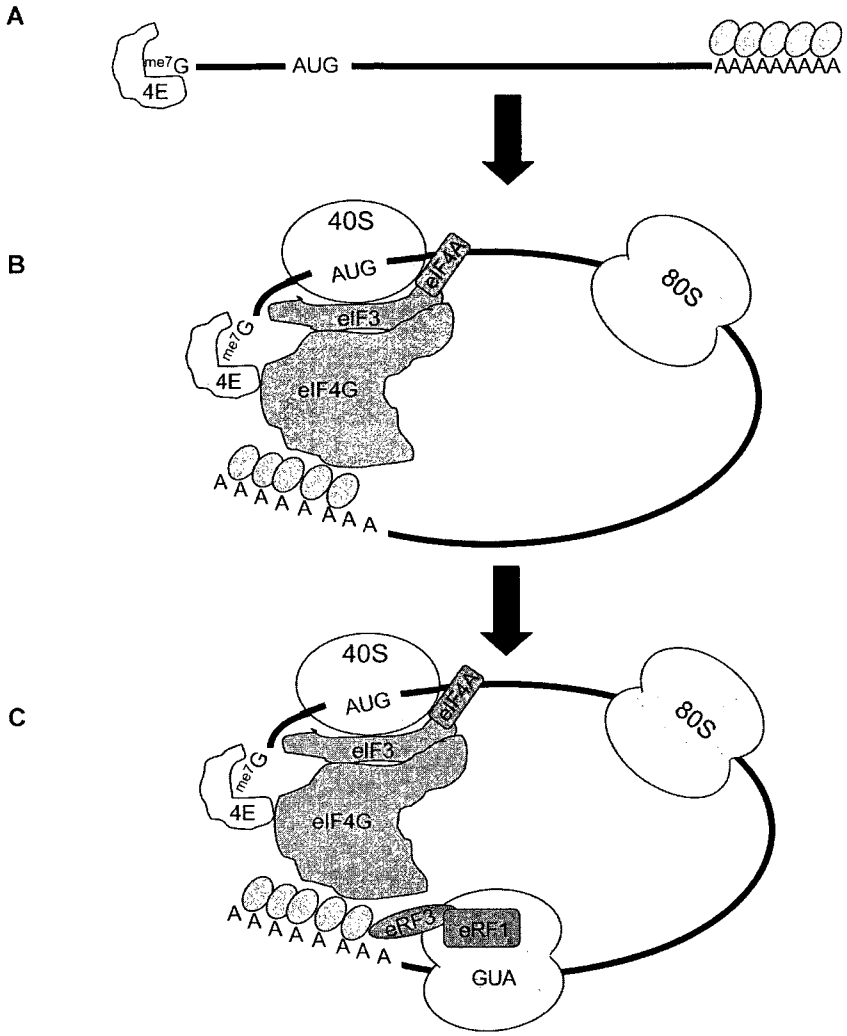

Figure 1.2 Roles of PABP in mRNA translation and stability.

The different stages of a cytoplasmic mRNA 'life cycle', in which distinct roles can be ascribed to PABP ( ). **(A)** Association of PABP with the mRNA poly(A) tail. **(B)** Interaction of PABP with elongation initiation factor eIF4G to promote formation of the 'closed loop', thus initiating translation and antagonizing decapping. **(C)** Interaction of PABP with the termination factor eRF3 and recycling of the ribosome from the 5′ to the 3′ end of the same mRNA. Turquoise ovals represent PABP. Figure modified from (Mangus et al. 2003).

1.3.1 KH domain containing proteins

The second most common RNA binding motif found in proteins is the KH motif (Burd and Dreyfuss 1994). In fact to date, using the sequences of the mouse and human genome there are 28 predicted KH-containing proteins (Lukong and Richard 2003). The KH domain was originally defined in the hnRNP K protein, an important pre-mRNA binding protein, which actually has three KH motifs (Siomi et al. 1993). In many KH-containing proteins the motif is present in multiple copies. For example, human fragile X protein has two copies and chicken vigilin has fifteen copies (Gibson et al. 1993). The presence of multiple KH domains suggests that the proteins bind RNA as a multimer. Within the KH domain, there is a highly conserve tetrapeptide, Gly-X-X-Gly, where X is usually a positively charged amino acid (Lukong and Richard 2003; Siomi and Dreyfuss 1997). The KH domain consists of three antiparallel β sheets that are packed against three α helices and the tetrapeptide is located at the loop between the first two α helices and plays an important role in RNA binding (Siomi and Dreyfuss 1997).

A subclass of KH containing proteins is the signal transduction and activation of RNA (STAR) family. The hallmark of this family is the presence of a single KH domain flanked by conserved C- and N- terminal sequences as well as other signaling motifs. Members of the STAR family include *Artemia salina* GRP33, Sam68, *Caenorhabditis elegans* GLD-1, SF1, *Drosophila* Who/How, *Xenopus* Xqua, mouse Qk1, zebrafish Qk1, *Drosophila* KEP1 and Sam50, and *Drosophila* Qk1-related proteins. These proteins are linked to signaling pathways due to the presence of other protein domains within the coding region such as proline rich domains, a cluster of tyrosines, SH3 binding sites and arginine/glycine (RG) boxes. Proline rich motifs are known binding sites for SH3 and WW domain containing proteins and serve as protein-protein interaction domains.

Tyrosines and phosphotyrosines are attachment sites for SH2 and PTB domain-containing proteins. The RG repeats are targets of arginine methylation and have been shown to regulate function of some STAR family members, such as Sam68 (Cote et al. 2003).

Proteins in this family are involved in cell cycle, apoptosis, cell fate decisions, virus replication and cell migration. Sam68 (**S**rc-**a**ssociated in **m**itosis of 68 kDa) is one of the most studied and well characterized members of this family. It has been shown to be a substrate of several tyrosine kinases such as Breast tumor kinase (BRK/SIK), Fyn and Lck (Derry et al. 2000; Lang et al. 1997; Lukong et al. 2005; Vogel and Fujita 1995) as well as to interact via its SH3 and SH2 domains with many signaling molecules such as c-Src, Grb2 and phosphopipase C γ1 (Richard et al. 1995; Taylor et al. 1995). Sam68 has also been shown to functionally replace Rev function in **h**uman **i**mmunodefiency **v**irus (HIV) infection (Reddy et al. 1999), as well as be involved in tumorigenesis and metastatsis (Richard et al, 2008), demonstrating important roles for STAR family members in numerous biological processes.

As eluded to above, recently KH containing proteins have been shown to be regulators of tumorigenesis. The *C. elegans* STAR family member, GLD-1 acts as a germline tumor suppressor. In the germline, the somatic gonad plays an important role in regulating decisions between mitotic proliferation and meiotic development. Primordial germ cells are set aside early in development and eventually expand and proliferate. In *gld-1* tumor suppressor mutants, the germ cells re-enter the cell cycle and overproliferate

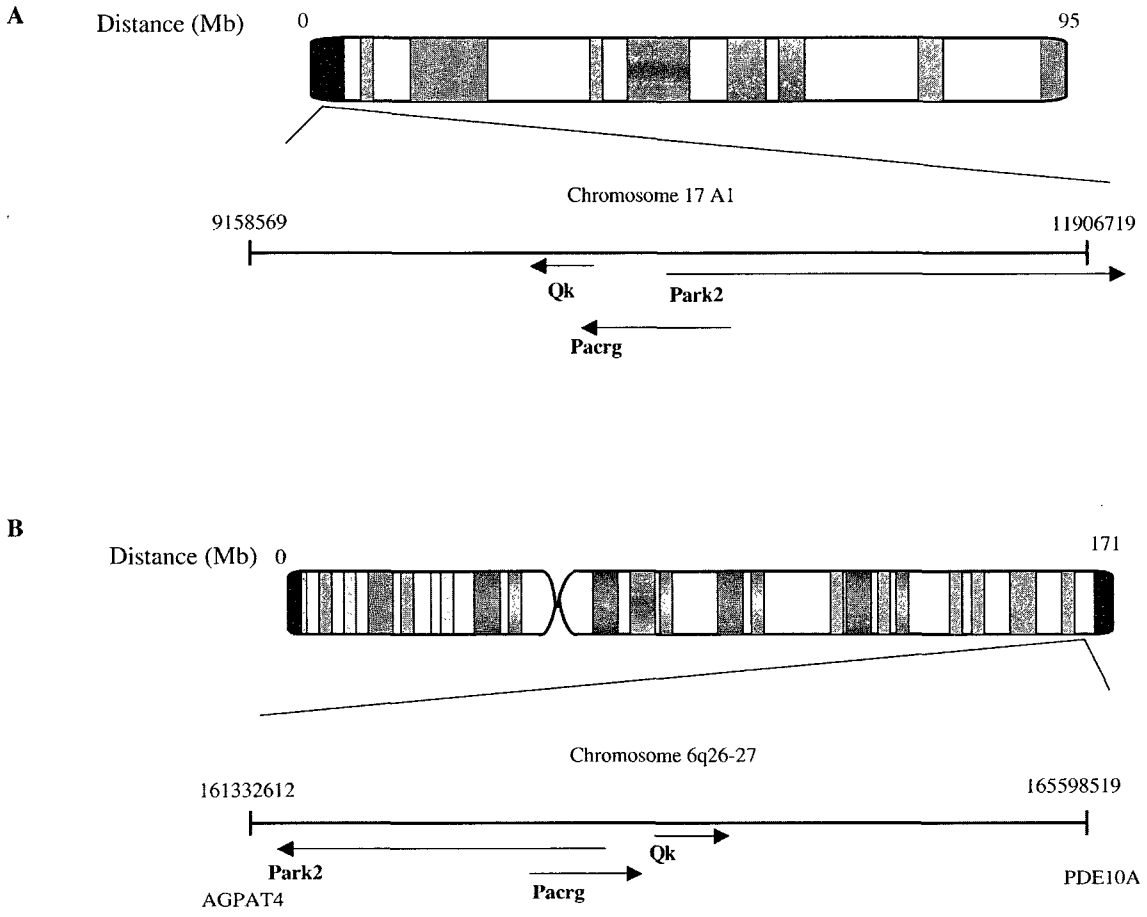

Figure 1.3 Genomic organization of Quaking

The location of *qkI* and surrounding genes on the mouse chromosome 17 **(A)** and human chromosome 6 **(B)**.

in the early stages of oogenesis (Francis et al. 1995). The cells eventually fill the entire organism and it dies early in life. Due to the characteristics of these cells, they most likely closely approximate vertebrate cells early in tumorigenesis (Pinkston et al. 2006). Sam68 deficient mice have delayed breast tumor onset and reduced metastasis suggesting that Sam68 may be a requirement for certain tumors (Richard et al. 2007).

1.3.2 Quaking

The field of neurobiology has been greatly advanced by the availability of mutant mouse models. Several naturally occurring dysmyelination models have aided in the understanding of the process of myelination and the regulation of individual myelin components. These models have allowed the dissection of the essential components of myelin biology and identification of the major structural proteins of the myelin sheath. The quaking viable (*qk*^{v}) mouse was first described in 1964 by Sidman et al., and was so named to distinguish it from other qkI mutants that are embryonic lethal (Sidman et al. 1964). Its phenotype was studied extensively for 30 years before the genetic defect and the gene responsible for the *qk*^{v} phenotype was identified and cloned, during the mid-1990s, (Ebersole et al. 1992; Ebersole et al. 1996). The use and characterization of the *qk*^{v} mouse, as well as several genetic mutants in the past ten years, has proved invaluable to determine the genomic organization and proteomic structure, as well as numerous biological functions, of *qkI*. Recently, numerous studies have examined the role of *qkI* in human diseases and there is now emerging evidence that QKI may play a role in diseases involving the CNS.

1.3.2.1 Gene Organization and Alternative Splicing

The *qkI* gene is found on mouse chromosome 17 and human chromosome 6 with a contig that spans approximately 65 kb of the genome (Figure 1.3) (Kondo et al. 1999). The *qkI* gene is highly conserved in Xenopus (*Xqua*) (Zorn et al. 1997), chicken (*qk*) (Mezquita et al. 1998), zebrafish (*zqk*) (Tanaka et al. 1997) and Drosophila (*how*) (Baehrecke 1997; Lo and Frasch 1997; Zaffran et al. 1997). This mammalian *qkI* gene undergoes a complex pattern of alternative splicing and generates three major transcripts of 5, 6 and 7 kb hence the terminology QKI-5, QKI-6 and QKI-7 (Ebersole et al. 1996). Each of the transcripts encodes a KH domain with its flanking N- and C-terminal regions termed NK (or QUA1) or CK (QUA2) regions (Figure 1.4). The resulting isoforms are alternatively spliced at the C-termini such that the C-terminus of QKI-5 has 30 unique residues, QKI-6 contains 8 and QKI-7 has 14 unique residues (Figure 1.4).

The proximal breakpoint of the qk^{v} mice lies ~1 kb upstream of the *qkI* transcription start site (Kondo et al. 1999) whereas the distal breakpoint is within intron 5 of the *parkin* gene (Lockhart et al. 2004; Lorenzetti et al. 2004a). This deletion results in a complete loss of both Parkin (PRKN) and Parkin co-regulated gene (Parcg) mRNAs and proteins (Lockhart et al. 2004; Lorenzetti et al. 2004a). Deletion or loss-of-function mutations of PRKN lead to autosomal juvenile Parkinson's disease although no such pathology is detected in qk^{v} mice. It is the loss of *parcg* and not the loss of *qkI*, which causes the male sterility observed in the qk^{v} mice, since transgenic expression of *parcg* cDNA in the qk^{v} mice testes restores fertility (Lorenzetti et al. 2004b). The deletion of 5′ upstream elements likely delete a promoter/enhancer element required for isoform-specific protein expression as only the QKI-6 and -7 proteins are absent in oligodendrocytes of the qk^{v} mice (Hardy et al. 1996). Moreover, *qkI* expression remains

unchanged in other non-myelinating glial cells. The loss of specific isoforms may actually be a more of a mosaic phenotype that changes throughout the qk^{v} brain, with the areas that are more highly affected showing a more pronounced reduction in QKI levels (Lu et al. 2003).

1.3.2.2 Functional Domains

The KH domain is an evolutionarily conserved protein module consisting of 70 to 100 amino acids and as mentioned previously is the second most prevalent RNA binding motif (Dreyfuss et al. 2002). The KH domain mediates direct protein-RNA interaction with a three-dimensional $\beta1\alpha1\alpha2\beta2\beta3$ topology (Musco et al. 1996).

A role of QKI in signaling is supported by the presence of other protein motifs within the coding region such as three proline-rich motifs, a cluster of five tyrosines, and two arginine-glycine repeats. Indeed QKI has been shown to be tyrosine phosphorylated by the Src family kinase $p59^{fyn}$ (Zhang et al. 2003) and is arginine methylated (Cote et al. 2003). In addition, the QKI-5 isoform possesses a non-canonical nuclear localization signal within its unique C-terminal 30 amino acids (Figure 1.4). The nuclear localization signal is conserved among other members of the STAR protein family with a consensus sequence of RXHPYQ/GR (Wu et al. 1999).

1.3.2.3 QKI Expression Pattern

Quaking mRNA and proteins are expressed primarily in the brain, heart, lung and testis (Ebersole et al. 1996; Kondo et al. 1999). The transcripts encoding the QKI

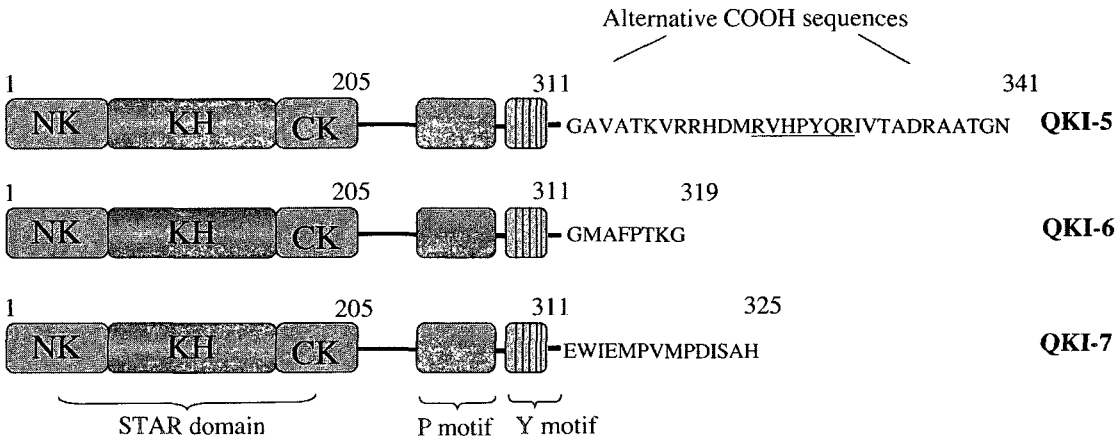

Figure 1.4 The three major QKI isoforms

All isoforms have a single KH domain flanked by the NK and CK domains and the entire region is called the STAR domain. Proline- and tyrosine-rich motifs are indicated (P and Y motifs). The isoforms differ only in their alternatively spliced carboxy-termini and the unique residues are shown. The nuclear localization signal of QKI-5 is underlined.

isoforms are first detected in the neuroepithelium of the head-folds at E7.5, in the nascent brain and heart at E8.5, and in the neural tube at E9.5 (Ebersole et al. 1996). In the CNS, QKI immunoreactivity was found predominantly in oligodendrocytes throughout the brain including the cerebellum, but not in neurons (Hardy 1998a). In addition, high levels of QKI expression have been detected in Bergmann glia of the cerebellum, while moderate levels of QKI have been detected in astrocytes of the cerebellum, hippocampus, cerebral cortex and white matter tracts. In the PNS, QKI is restricted to glia and is highly expressed in myelinating Schwann cells (Hardy et al. 1996).

The cellular localization of the QKI isoforms differs. The presence of an NLS within QKI-5 dictates its nuclear localization (Pilotte et al. 2001; Wu et al. 1999). The localization of QKI-6 is both cytoplasmic and nuclear, whereas QKI-7 is predominantly localized in the cytoplasm. A coiled-coil domain located within the NK region mediates QKI homo- and heterodimerization (Chen and Richard 1998; Pilotte et al. 2001). Interestingly, an ENU mutation E48G disrupts this coiled-coil and may be the molecular mechanism by which this amino acid substitution disrupts QKI function *in vivo* (Chen and Richard 1998). The expression of the QKI isoforms maintains a proper cytoplasmic to nuclear balance. When this balance is disrupted, for example by the overexpression of QKI-5 isoform, QKI mRNA targets are retained in the nucleus as observed with the myelin basic protein mRNAs (Larocque et al. 2002). Indeed, the absence of the QKI-6 and QKI-7 isoforms, as observed in the qk^{v} mice, also results in MBP mRNA nuclear retention (Larocque et al. 2002).

The QKI mRNAs and proteins are temporally expressed. During embryogenesis, QKI-5 is the most abundant isoform and its expression declines postnatally (Ebersole et al. 1996). The expression of QKI-6 and QKI-7 is observed in the multipotential

progenitor cells of the ventricular zone and peaks at postnatal days 14 to 16 and coincides with the time myelination and their expression remains elevated throughout adulthood (Hardy 1998a; Hardy et al. 1996). The presence of QKI-5 is required for embryogenesis, as the qk^{v} mice maintain QKI-5 expression, whereas qkI *null* mice do not.

1.3.2.4 ENU Mutations

In addition to the role of QKI in normal CNS development, the identification of several ENU-induced mutations has allowed the identification of additional roles for the QKI isoforms (Figure 1.5). The fact that four of these ENU alleles were found to be homozygous embryonic lethal at mid-gestation and failed to complement the qk^{v} dysmyelination phenotype suggests that QKI has a role in early embryonic development which is separate from its role in the nervous system. The $qk^{kt3/4}$ allele is an A to G transition in the coding region resulting in an amino acid change from a glutamic acid to a glycine at position 48 (QKI: E→G) in the NK region that abolishes the dimerization of the QKI proteins (Chen and Richard 1998). The qk^{k2} allele represents a T to A transversion, which causes a change from a valine to glutaminc acid (QKI: V→E) in the KH domain, which results in loss of RNA binding but not dimerization (Larocque et al. 2002). The $qk^{l\text{-}1}$ mutant is an A to G transition, which results in the loss of a splice site that is necessary to produce the nuclear QKI-5 isoform (Cox et al. 1999). The final embryonic lethal allele is qk^{kt} and has yet to be fully characterized. A fifth ENU mutation that is homozygous viable has also been isolated (qk^{e5}). These mice have severe CNS

Table 1.2 Quaking mutant mice and their phenotypes (Adapted from Cox et al. 1999)

	Mutant	Mutation	Molecular defect	Phenotype	Survival	Reference
embryonic lethal	*qkk2*	T to A tranversion (QKI:V-E) KH domain	loss of RNA binding	defective yolk sac vascular remodeling enlarged heart, pericardial effusion	E10.0-12.5	Justice and Bode, 1988 Larocque et al.,2002 Noveroske et al., 2002 Bohnsack et al., 2006
	qk kt1	Unknown	Unknown	spontaneous seizures abnormal heart, cranial defects	E9.5	Justice and Bode, 1988 Shedlovsky et al., 1988
	qk kt3/4	A to G transition (QKI: E-G) NK domain (QUA1)	abolishes dimerization	abnormaol somites heart and cranial defects	E9.5	Ebersole et al., 1996 Chen and Richard, 1998
	qk l-1	A to G transition in a QKI-5 specific splice site	failure to express QKI-5	vascular remodeling defects	E8.5-11.5	Shedlovsky et al., 1988 Cox et al., 1999 Bohnsack et al., 2006
	qk null	knock out mouse	failure to express all isoforms	thin vitelline artery connecting yolk sac vessel deficient in smooth musle cells	E8.5-10.5	Li et al., 2003
viable	*qk v*	1 Mb deletion promoter/enhancer deletion of *prkn, parcg*	failure to express QKI-6/7 in oligodendrocytes	dysmyelination tremors progressing to tonic clonic seizures sterility	viable	Sidman et al., 1964
	qk e5	unknown not in coding region	failure to express QKI-6/7 in oligodendrocytes	severe ataxia severe dysmyelination purkinje axonal swelling reduced lifepan	few months 35% survive to 5 months	Noveroske et al., 2005

hypomyelination, early onset seizures, severe ataxia and decreased lifespan (Noveroske et al. 2005). These mice demonstrate that a point mutation in the QKI gene can induce dysmyelination. The qk^{e5} mutation is predicted to lie in an oligodendrocyte-specific enhancer in the *qkI* gene (Table 1.2). The embryonic lethality of the *qkI* ENU mutations prior to neural development does not permit the assessment of the role of the QKI isoforms in gliogenesis using a loss-of-function approach.

1.3.2.5 RNA Binding Specificity

The KH domain adopts a βααββα-fold (Musco et al. 1996) and the maxi-KH domain of STAR proteins adopts βααββααβα-fold (Lewis et al. 2000; Liu et al. 2001; Maguire et al. 2005) necessary to mediate Watson and Crick base pairing with specific RNAs (Lewis et al. 2000). The QKI recognition element (QRE) was defined as direct repeats of hexanucleotide 5′- ACUAAY -3′ and a half site 5′- UAAY -3′ spaced by 1 to 20 nucleotides (Galarneau and Richard 2005; Ryder et al. 2004; Ryder and Williamson 2004). Bioinformatic analysis using the bipartite consensus sequence identified 1433 putative mRNA targets (Galarneau and Richard 2005), which included two of the known QKI targets, myelin basic protein (MBP) (Larocque et al. 2002; Li et al. 2000) and early growth response gene-2 (EGR-2) (Nabel-Rosen et al. 2002). The newly identified potential targets are involved in development, cell adhesion, morphogenesis, organogenesis, transport and cell differentiation, cell growth and/or maintenance and cell communication (Galarneau and Richard 2005).

1.4 *Cellular Processes Regulated by QKI*

The QKI isoforms regulate aspects of RNA metabolism in several cellular processes including myelination and cell fate determination. Moreover, the QKI isoforms are required for embryogenesis, blood vessel development and regulate apoptosis and protein translation.

1.4.1 Embryogenesis

The qk^{v} mice are have pleiotropic defects, as the mutant mice display both embryogenesis and myelination defects (Ebersole et al. 1996). The embryos of four lethal ENU mutations show arrested growth with generalized abnormalities at the 15 to 26 somite stage (Cox et al. 1994; Cox et al. 1993; Justice and Bode 1988). QKI-5 is the most abundant isoform observed during embryogenesis and may in part explain why the qk^{v} mice are viable (Ebersole et al. 1996). Since QKI-5 is mainly nuclear it may fulfill a nuclear role during embryogenesis. Indeed, QKI-5 has been shown to affect the alternative splicing (Wu et al. 2002), but the targets during embryogenesis remain to be defined.

1.4.2 Blood Vessel Development

The ENU-induced QKI mutants as well as the *qkI* null mice die early in embryogenesis due to vascular remodeling defects of the capillary plexus and of the yolk sac as well as pericardial effusion and less compact hearts (Bohnsack et al. 2006; Noveroske et al. 2002). The *qkI* null mice also die at E9.5-E10.5 due to the failure of blood to circulate in the yolk sac. These *qkI* null yolk sacs completely lack the larger vitelline vessels and only have primary vascular plexus. More specifically at E9.5 the

embryos were smaller, and had irregular-shaped omphalomesenteric artery and the vitelline artery was thinned at the point of contact with the yolk sac. At this embryonic stage, the important vitello-embryo connection is achieved by the above-mentioned vasculature (Li et al. 2003). There is also an absence of smooth muscle cells (SMC) lining the arteries in these mice. This lack of SMCs and failure of proper vascular remodeling was due to the inability of the resident endothelial cells to differentiate into mature SMC. This differentiation was not directly due to the lack of QKI expression since QKI null cells can be differentiated into SMC in the proper environment, but is likely that QKI-positive endothelial cells secrete a 'vascular' factor, likely a protein in the vascular endothelial growth factor or the angiopoetin pathway, that can induce the differentiation of endothelial cells into SMCs. These findings are consistent with the hemorrhaging observed in the qk^{k2} embryos, since the vessel integrity would be lost due to the lack of vascular SMCs. In addition to these vascular defects, some other defects observed in the QKI null mice had kinked neural tubes, pericardial effusion, open neural tubes and incomplete embryonic turning (Li et al. 2003).

1.4.3 Alternative Splicing

The data on misregulation of several myelin genes suggests that QKI is important in the localization, stability and translation of its target RNAs. The QRE sequence resembles the splicing factor 1 (SF1) or branch point binding protein (BBP) recognition element UACCAAC (Liu et al. 2001; Peled-Zehavi et al. 2001), which directs the RNA-protein contact. Splicing factor 1 (SF1) binds U2AF, which directs cooperative binding to intron branch site and neighboring polypyrimidine tract. It has been shown that in fact SF1 plays a kinetic role in the splicing process. Some of the principle myelin components

are alternatively spliced and their splicing events seem to be abnormal in *qk*^{v} mice (Wu et al. 2002). One of the alternatively spliced targets is myelin associated glycoprotein (MAG). MAG is a transmembrane protein thought to be important in the initiation and maintenance of the myelin sheath. In fact, it is thought to be one of the limiting proteins in the regeneration of the CNS (Liu et al. 2002). Alternative splicing generates two MAG isoforms by the inclusion or exclusion of exon 12, which contains an in-frame stop codon. In the long isoform (L-), exon 12 is skipped and when it is included the small isoform is produced (S-). The isoforms are regulated developmentally and co-exist in the myelin sheath. The L- isoform is abundant in young mice where the S- isoform is expressed highly in adults. In *qk*^{v} mice there is reduced expression of L-MAG and S-MAG is overexpressed (Fujita et al. 1990). *In vivo*, at postnatal day 14 the *qk*^{v} mice express higher levels of the exon 12 included isoform in the brain compared to normal mice (Wu et al. 2002). *In vitro*, overexpression of QKI-5 caused a repression of MAG exon 12 inclusion. Wu and colleagues then mapped the QKI-5 alternative-splicing element (QASE), which behaves like an intronic splicing silencer, to a 53-nucleotide element in the downstream intron. Deletion of this element increased exon 12 inclusion from 12-73%. MAG does not possess the identified QRE and purified QKI has not been shown to bind even a short purified QASE element. Furthermore, in *qk*^{v} mice, S-MAG is overexpressed whereas there is almost no expression of L-MAG. Since in *qk*^{v} mice, QKI-5 is expressed and should therefore favor the exclusion of exon 12 and hence the generation of the large isoform as is seen in young animals where QKI-5 is overexpressed relative to the other isoforms. QKI-5 may be playing a role in alternative splicing but this may be indirectly through a QKI-5 ribonucleoprotein complex. Early studies on the *qk*^{v} mice indicated that the reduction in the L-MAG levels could be due to selective

endocytosis of the L-MAG from the periaxonal membranes (Bo et al. 1995) and may therefore not be due to alternative splicing defects at all.

1.4.4 Glial Cell Fate Determination

The abundance of immature oligodendrocytes in the brains of adult qk^{v} mice, suggests that the oligodendrocytes progenitors of these mice are unable to complete their differentiation process. The absence of QKI-6 and -7 isoforms in these mice implies a role for these isoforms in oligodendrocyte differentiation. Cellular differentiation requires cell cycle arrest followed by changes in gene expression and subsequent differentiation (Tartaglia and Goeddel 1992). Indeed, either over-expression or *in vivo* ectopic-expression of QKI-6 and QKI-7 in oligodendrocyte progenitors promotes glial cell fate decisions (Larocque et al. 2005). In the beginning of neurogenesis, proliferating neural progenitors are restricted to the ventricular zone and as the neural progenitors differentiate into neurons they migrate towards the outer portion of the neural tube (Rowitch 2004). The glial subtypes also arise from this region but arise after the majority of neurogenesis is completed. At E12.5, there is a dramatic increase in QKI levels in the ventricular zone and by E13.5 these QKI-positive cells migrate away laterally and ventrally into the surrounding parenchyma (Hardy 1998b). Gliogenesis requires both the repression of the neuronal phenotype as well as activation of glial-specific genes (Morrison 2001).

This 'favoring' of glial cell lineage differentiation is achieved, at least in part, through the QKI-6/7 isoforms binding to $p27^{KIP1}$ mRNA and stabilizing the mRNA. $p27^{KIP1}$ is cyclin-dependent kinase (CDK) inhibitor that binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell

cycle progression at G1 (Philipp-Staheli et al. 2001). $p27^{KIP1}$ accumulation is required for oligodendrocyte precursor differentiation (Casaccia-Bonnefil et al. 1997; Durand et al. 1997; Dyer 2003; Friessen et al. 1997). When QKI-6/7 are expressed, the $p27^{KIP1}$ mRNA and subsequently the protein accumulates causing cell cycle arrest and then differentiation (Larocque et al. 2005). In addition to regulating glial cell fate, the ectopic expression of QKI-6 and QKI-7 are able to direct the progenitor cells of the ventricular zone to migrate to areas of high myelination, such as the corpus callosum, and become oligodendrocytes. *In vivo*, progenitor cells also developed into astrocytes, migrated and localized to the border of the corpus callosum into the rostral marginal zone (Larocque et al. 2005). There is also evidence that other KH containing proteins can regulate cell fate decisions such as FMRP (Jin and Warren 2003), Sam68 (Richard et al. 2005), Nova (Darnell 2004) and Mex-3 (Draper et al. 1996).

1.4.5 Apoptosis

Several protein families have been shown to induce apoptosis. When the QKI isoform balance is disrupted in favor of QKI-7 over-expression apoptosis is observed in fibroblasts and primary rat oligodendrocytes (Chen and Richard 1998; Pilotte et al. 2001). A short 14 amino acid sequence in the unique C-terminus of QKI-7 was identified as the killer sequence that could cause cell death when added to heterologous proteins (Pilotte et al. 2001). If the QKI isoform balance is restored by the presence of QKI-5 or QKI-6 or Bcl-2 expression, the cell death mediated by QKI-7 is prevented (Pilotte et al. 2001). The physiological role of the QKI proteins in regulating cell death remains to be determined.

1.4.6 Protein translation

Many RBPs bind their target RNAs and directly influence their levels through regulation of translation. Sex determination in *C. elegans* is regulated by expression of the *tra-2* gene. Translationally, *tra-2* is regulated by two elements in its 3′ UTR termed TGEs (*tra-2* and GLI element). GLD-1 specifically binds these TGEs and translationally represses the RNA. Translational repression of *tra-2* is required for hermaphrodite spermatogenesis (Clifford et al. 2000). In addition, GLD-1 was shown to translationally repress *tra-1*, whose protein directs female development in the soma, in a TGE-dependent manner (Lakiza et al. 2005). In fact, GLD-1 has been shown to translationally repress all of its identified targets (Lee and Schedl 2001; Lee and Schedl 2004). This finding led to the hypothesis that other KH containing proteins, such as QKI, may function through a TGE-like mechanism to regulate target mRNAs. The studies on the qk^{v} mouse have demonstrated a role for QKI in developmental cues. Most of the studies have focused on QKI-6 due to its cytoplasmic localization, which is consistent with a role in translational control (Saccomanno et al. 1999). QKI-6 is able to bind to and exert translational repression on the TGE element (functionally substituting for GLD-1). In addition, QKI-6 is able to bind and repress translation of *gli* mRNA. *Gli* is a family of transcription factors involved in vertebrate development (Lakiza et al. 2005) and is expressed in the brain along with QKI. To date, along with mRNA stability, translational repression may represent the major regulatory mechanism by which QKI isoforms regulate their target RNAs.

1.5 *QKI involvement in human diseases*

The QKI role in myelination and gliogenesis has provided insights that have linked QKI to ataxia, glioblastoma multiforme and schizophrenia (Figure 1.5).

1.5.1 Ataxia

A strategy to gain insight into the role of the proteins in disease is to look systematically at their protein-protein interaction networks (Humbert and Saudou 2006). An extensive study on the function of proteins involved in Purkinje cell degeneration generated an interaction network for proteins involved in 23 inherited ataxias (Lim et al. 2006). Many ataxia-causing proteins interact and share binding partners. The qk^{v} mouse is classified with other autosomal recessive human ataxic diseases even though the cerebellums of the mice are normal. Since both the qk^{v} mouse and the qk^{e5} mouse show Purkinje cell axonal swelling, indicative of neuronal degeneration (Noveroske et al. 2005; Suzuki and Zagoren 1975), mapping the QKI-interactome could generate candidate genes for other cerebellar ataxic diseases whose genetic defects have not been identified. This study suggests that although these ataxic diseases differ in the genes that are mutated, they share protein interactions and pathways. In the ataxia-ome a total of 29 QKI-interacting partners were identified (Lim et al. 2006). In addition, QKI physically interacts with a genetic modifier of spinocerebellar ataxia type 1, suggesting that some

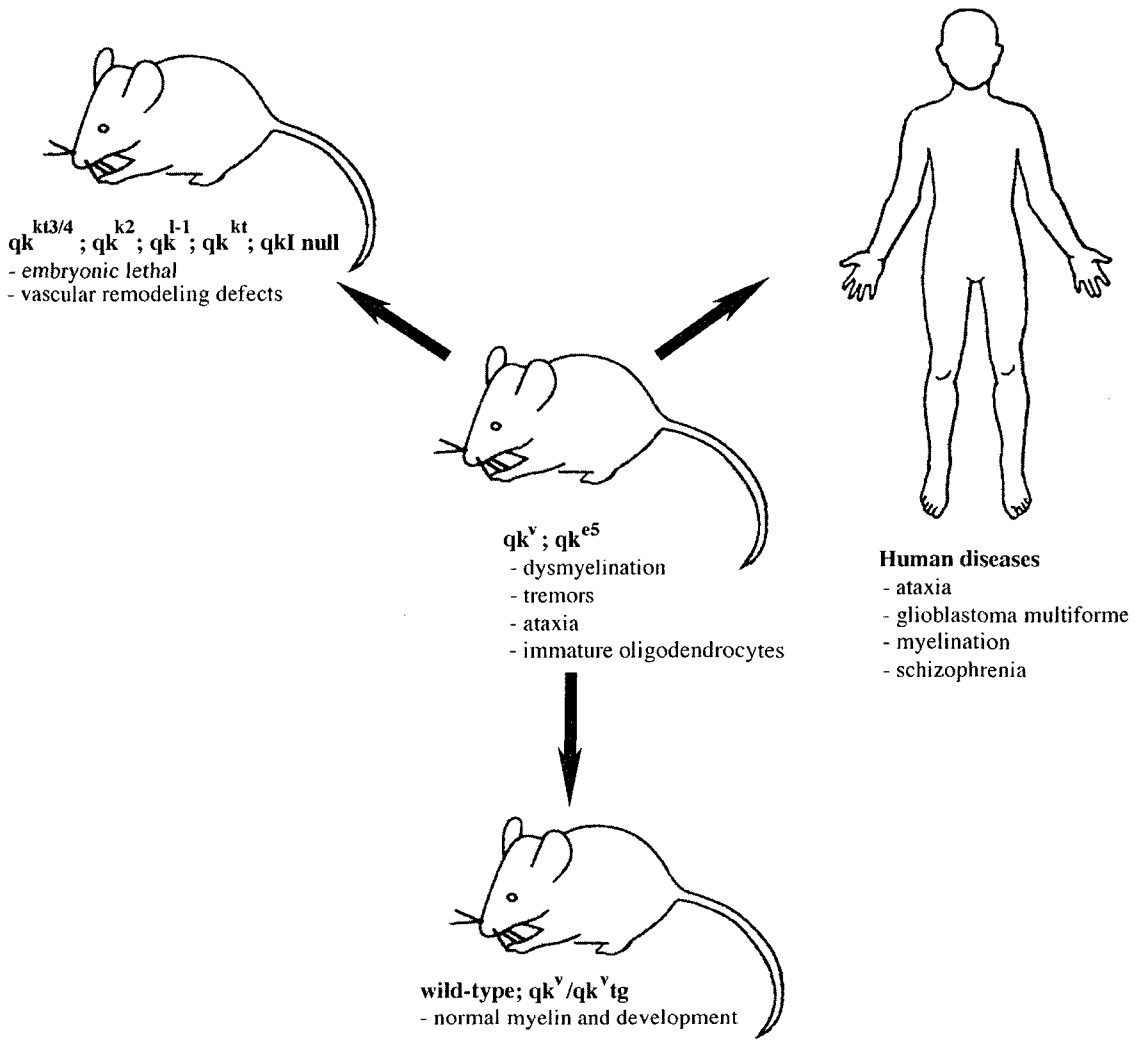

Figure 1.5 Phenotypes and diseases associated with Quaking alterations.

Phenotypes attributed to *qkI* mutations, transgenic mice and alterations in *qkI* expression. (qk^v/qk^vtg; FLAG-QKI-6 transgenic mouse)

genetic modifiers may also modify other diseases and therefore may be in a common pathway (Lim et al. 2006). Mutations in other KH containing proteins have been shown be involved in ataxia-like syndromes, such as Nova (Yang et al. 1998) and FMRP (Hagerman 2006).

1.5.2 Glioblastoma Development

Gliomas are the most common primary malignant type of brain tumor in adults (Sanai et al. 2005). Gliomas originate from cells of glial lineage, primarily astrocytes, oligodendrocytes and ependymal cells. Gliomas of astrocytic origin (astrocytomas) are classified into pilocytic astrocytoma (grade I), astrocytoma (grade II), anaplastic astrocytoma (AA; grade III) and glioblastoma multiforme (GBM) (grade IV; (Belda-Iniesta et al. 2006). Despite current therapies, which include the combination of surgery, radiation and chemotherapy, the average survival time of a patient with GBM is less than one year. It has been demonstrated that there are alterations in QKI isoform expression in approximately 30% (6/20) of human glioblastomas, whereas QKI isoform expression was unchanged in all of the schwannomas and meningiomas tested (Li et al. 2002). Of these primary tumors, two were devoid of the transcripts encoding the 3 major isoforms, two were missing only the *qkI-7* transcript and one was missing only the *qkI-5* transcript. Two subsequent studies have characterized chromosome alterations in AA and GBM. Both studies identified discrete deletion on chromosome 6q25-26 which included both *qkI* and *parcg* genes (Ichimura et al. 2006; Mulholland et al. 2006). The identification of chromosome 6 alterations in 75% of AA and 42% of GBM but not in astrocytomas suggests that genes on chromosome 6 do not play a role in the oncogenesis of astrocytomas and the reduction in the frequency of chromosome 6 alterations in GBM

suggests that other pathways likely play a role distinct from progression through AA (Ichimura et al. 2006). The majority of GBMs also show alterations in genes such as p53 and retinoblastoma (Ichimura et al. 2006), amplifications and/or rearrangements of EGFR (Liu et al. 2000), or loss of wild-type PTEN (Knobbe and Reifenberger 2003) and therefore *qkI* may not be the primary mutation or the so-called 'first hit' in GBMs. QKI isoforms may act as an anti-proliferation factor in normal cells their loss or inactivation may be necessary for the progression to GBMs. Alternatively, loss of QKI may affect a downstream RNA target involved in oncogenesis, transformation or angiogenesis. In addition, the *qkI* gene lies in a chromosomal area that has been identified as a common fragile site with profound genomic instability and that is frequently altered in cancers (Smith et al. 2006).

1.5.3 Schizophrenia

The QKI isoforms have most recently been implicated in schizophrenia (SCZ). Growing evidence exists which suggests that myelin and oligodendrocyte-dysfunction contributes to the developments of the disease (Stewart and Davis 2004). Tight, functioning neural circuits are required in the brain and these are dependent on the proper myelination by oligodendrocytes. Since QKI is important in this process, it is possible that QKI may be altered in diseases of the CNS such as SCZ. It has previously been reported that there is decreased oligodendrocyte density and changes in morphology in the white matter of SCZ patients and many of the alterations observed are very similar to those observed in the qk^{v} mice (Haroutunian et al. 2006). A susceptibility locus was mapped to chromosome 6q25-6q26, more specifically to a region spanning 0.5Mb. *qkI* is found in that region of the chromosome. Several studies have examined expression of

various transcripts in areas of the brain affected by SCZ such as the anterior cingulated cortex and the superior temporal cortex. Several myelin-related genes were decreased in the white matter of SCZ patients. They include 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) (Aberg et al. 2006a; McCullumsmith et al. 2007), myelin-associated glycoprotein (MAG) (Aberg et al. 2006a; McCullumsmith et al. 2007), transferrin (TF) (Stewart and Davis 2004), proteolipid protein 1 (PLP) (Aberg et al. 2006a) and QKI (Aberg et al. 2006a; Aberg et al. 2006b; Haroutunian et al. 2006). Many of these transcripts are identified targets of the QKI proteins and the differences observed can be explained by variation in the relative mRNA levels of QKI. Although both MAG splice variants are affected, the s-MAG difference is less pronounced, therefore the variation of ratio between the two MAG splice variants is significantly altered in SCZ patients (Aberg et al. 2006a).

The role of how each QKI isoforms contributes to the SCZ pathology is still not known. Early evidence suggested that all of the isoforms were decreased, but a more precise analysis has suggested that the QKI-7 and QKI-7b isoforms were significantly reduced in patients relative to total QKI isoforms (Aberg et al. 2006b). These findings suggest that the QKI-7 isoforms significantly contribute to the pathology of SCZ.

The most common treatment for SCZ is antipsychotic medications, which treat a large number, but not all, of the symptoms. The levels of QKI expression are decreased in SCZ patients regardless of their neuroleptic status (Haroutunian et al. 2006). Aberg and colleagues showed that when comparing the relative amounts of the four splice variants (3 major isoforms and QKI-7b) in patients treated with typical neuroleptics, there was twice the levels of QKI mRNA levels in the areas tested than those treated with atypical neuroleptics and patients that had not been treated with drugs (Aberg et al. 2006b). This

may suggest that treatment of patients with neuroleptics undoes some of the molecular damage caused by the disease, but does not result in recovery of QKI isoforms to normal levels.

1.5.4 Multiple Sclerosis

Although QKI has not been directly implicated in multiple sclerosis (MS), the dysmyelination phenotype of the qk^{v} mouse and the identification of myelin basic protein (MBP) as an mRNA target of QKI has led people to hypothesize that by understanding QKI's role in the normal oligodendrocyte physiology, we could perhaps harness QKI to activate the remyelination process. MS is a chronic and often debilitating disease of the CNS. Converging lines of evidence suggest that the disease is caused, at least in part, by a disturbance in immune function. This disturbance permits cells of the immune system to attack myelin, the insulating sheath that surrounds the axons located in the CNS. When myelin is damaged, electrical impulses cannot travel quickly along nerve fiber pathways in the brain and spinal cord. Disruption of electrical conductivity results in fatigue and disturbances of vision, strength, coordination, balance, sensations, and bladder and bowel function (Kurtzke 1983). The brains of MS patients show scarring, called plaques, which contain immune cells, astroglial cells, inflammatory molecules and myelin components.

There is a well-established role of the immune system in the pathogenesis of MS, and although the cause of the disease is unknown, there is evidence that suggests both an environmental component and a genetic component to the disease. The environmental theory proposes that an environmental factor triggers the immune response and subsequently the illness. The observation that MS is diagnosed more frequently in temperate climates than in tropical or subtropical climates supports this theory.

Therefore, the prevalence of MS increases with northern latitude (Center 2008). This theory is supported by observations that people born in areas of high-risk acquire a lower-risk if they relocate to areas of low-risk before the age of 15 (Center 2008). Another theory is that the environmental factor is a virus. MS attacks are more likely to occur in people who experience non-specific viral syndromes, but the virus has not been isolated or identified. Therefore to date, no specific environmental factor has been identified to cause MS in these latitudes.

The genetic theory supposes that there is a genetic susceptibility to the disease. This hypothesis is supported by the fact that MS is more common in Caucasians and rarely found in African blacks, Native Americans and Asians, even if they live in high-risk areas. This suggests that only people that are genetically susceptible will develop the disease. In addition, if a family member (mother, father, sister, brother) is diagnosed with MS, then your risk of developing the disease increases between 2.67 – 26.7% (Center 2008).

1.6 *Properties of myelin and its organization*

Myelin is the fatty material that surrounds axons in tightly packed mutilamellae of the CNS and PNS and facilitates the salutatory conduction of nerve impulses in the vertebrate nervous system (Harauz and Musse 2007). Myelin constitutes approximately 40-50% of the white matter in the CNS based on dry weight. The significance of myelin in human development is highlighted by its involvement in several human diseases, including MS, peripheral neuropathies and leukodystrophies (Ludwin 1997). Microscopic studies as well as X-ray diffraction show that cross-sections of compact myelin appear as consistent concentric layers of ensheathing plasma membrane (Morell and Ousley 1994).

Each myelin segment is approximately 150-200 μm in length. On the axon, two adjacent segments of myelin are separated by a Node of Ranvier, an area not covered by myelin, therefore leaving it exposed to the extracellular milieu (Figure 1.6) (Bunge 1968). The Nodes of Ranvier play an important role in nerve impulse conduction as they allow for fast saltatory conduction and impulse jumping from node to node. The insulating properties of myelin are largely due to its structure, its major protein components and its richness in lipids. Myelin has a high lipid to protein ratio. The specific components of myelin, the glycolipids and proteins, are made by the OLs in the CNS. This requires activation of numerous processes, such as lipid metabolism, production and transport of myelin components to the distal processes of the OLs. Therefore, we could view myelin as a metabolically active membrane. Many of the protein components involved in the structure of the myelin sheath are well characterized, among them CNPase, MBP, MAG, MOG and PLP (Figure 1.6).

CNPase is an enzyme that hydrolyzes 2′ and 3′-cyclic nucleotides into their 2′-derivatives. It represents approximately 4% of myelin proteins. The CNPase knockout mice develop axonal swelling and neurodegeneration, leading to hydrocephalus and early death (Lappe-Siefke et al. 2003). MAG is a minor protein component that was discussed earlier with regards to its alternative splicing. In the CNS, MAG in confined to the periaxonal region of the myelin internodes and is in direct contact with the axons. It contains a membrane spanning domain as well as intra- and extra-cellular regions. It is believed that MAG might be involved in signaling and adhesion between the OLs and neurons (Wang et al. 2002). MOG is another transmembrane glycoprotein located on the outermost lamella of compact myelin. It has been implicated as a cytotoxic T-cell target in myelin autoimmune diseases of the CNS (de Rosbo and Ben-Nun 1998). PLP

represents approximately 50% of myelin proteins. It has four transmembrane regions, with an intracellular domain that accounts for the spacing at the intraperiod line (Nave et al. 1987). Knock-out mice, still show myelinated axons with compact myelin but there is axonal degeneration and reduced stability of the membrane (Griffiths et al. 1998). MBP will be discussed below in section 1.6.2.

1.6.1 Glial cell of the CNS

Glial cells are non-neuronal cells that provide support and nutrition, maintain homeostasis, form myelin and participate in signal tranduction (Zhang 2001). In the CNS, glial cells are derived from the ectodermal tissue of the embryo, more specifically in the neural tube. There are five types of glial cells in the CNS: microglia, radial glia, ependymal cells astrocytes and oligodendrocytes, Microglia are specialized macrophages designed to protect neurons in the CNS and are derived from hematopoetic cells. Radial glia are neuronal progenitors and act as scaffolding in the developing CNS. They can also regulate synatptic plasticity and directional communication with neurons. Ependymal cells line the cavities of the CNS and secrete and circulate the cerebral spinal fluid.

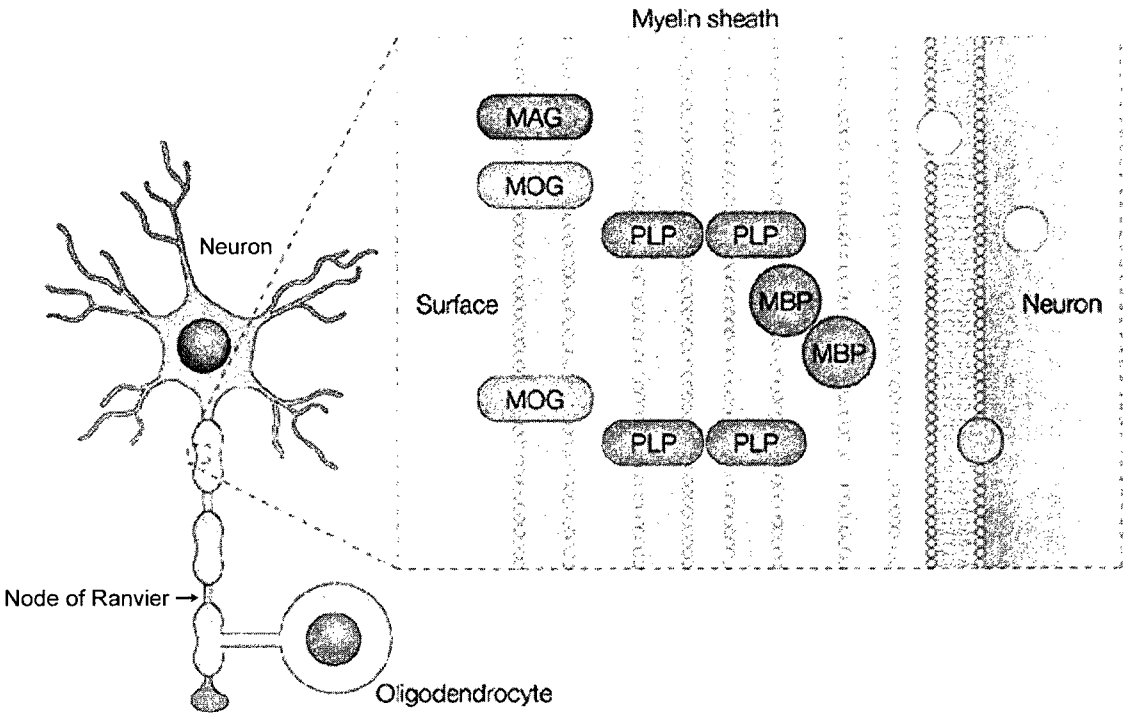

Figure 1.6 Composition of CNS myelin

The major proteins present in myelin membrane are schematized. The oligodendrocyte is the myelinating cell of CNS where it can myelinate several axons. MAG; myelin-associated glycoprotein, MBP; myelin basic protein, MOG; myelin oligodendrocyte glycoprotein, PLP; proteolipid protein. Figure modified from (Hemmer et al. 2002).

Astrocytes are the most abundant glial cells and anchor to the neurons to provide them with their blood supply and regulate their external chemical environment. Finally, OLs coat the neurons and produce the myelin that forms the myelin sheath, which allows the neurons to conduct the electrical impulses more efficiently (Zhang 2001).

QKI is expressed in all glial cells although it is highly expressed in OLs. The generation of each of the cell types in the brain occurs in temporally distinct yet overlapping phases. OLs and astrocytes are derived from a common precursor cell, oligodendrocyte-type-2 astrocyte (O-2A) progenitors (Raff et al. 1983). The individual lineage-differentiation requires transcription factors and growth factor cell signaling. As they differentiate, OLs express the glycolipid galactocerebroside (GalC) (Raff et al. 1979), CNPase (McEwan 1996), MBP, Nestin (Almazan et al. 2001) and MOG (Scolding et al. 1989), which serve as markers for the OL lineage. In contrast, astrocytes express the glial fibrillary acid protein (GFAP) (Ghandour et al. 1979) and downregulate expression of early OL lineage markers. These markers are used to distinguish between the two cells types in the brain. As the cells differentiate, their morphology changes as well. Mature astrocytes are star shaped, whereas OLs have elaborate primary, secondary and tertiary processes.

1.6.2 Myelin Basic Protein

MBPs are major protein components of the CNS comprising 30% of myelin proteins (Harauz and Musse 2007). Murine CNS myelin contains structurally and antigenically related MBP variants of apparent molecular masses of 21.5 kDa, 18.5 kDa, 17.0 kDa, and 14.0 kDa, which are encoded by alternatively spliced mRNAs (de Ferra et al. 1985). Humans have four major MBP isoforms of 21.5, 20.2, 18.5 and 17.2 kDa

(Campagnoni 1988). The MBP gene is composed of seven exons spanning over 30-35 kilobases (kb) of DNA (Pedraza et al. 1997). The 21.5-kDa MBP has peptide sequences encoded by all seven exons. The splicing of exons 2 or 6 yields the mRNAs encoding the 18.5-kDa or 17.0-kDa MBP, respectively. The 14.0-kDa MBP is obtained when both exons 2 and 6 are excised (Pedraza et al. 1997). It was reported that the distribution of 14.0-kDa and 18.5-kDa MBPs was confined to the plasma membrane, and exon 2-containing 21.5-kDa or 17.0-kDa MBP was diffuse in the cytoplasm and the nucleus. In addition, the 14.0-kDa isoform emerges during active myelination and is the most abundant isoform throughout adulthood (Allinquant et al. 1991; Barbarese et al. 1978). These findings indicated that individual MBPs likely play markedly different roles in the elaboration of myelin.

The MBP proteins are synthesized at the distal tip of the oligodendrocyte under tight control, and are directly incorporated into the myelin sheath. Studies of *shiverer* mutant mouse, which has a large deletion of the MBP gene and no MBP expression, provides direct evidence that MBPs play a major role in the compaction of myelin in the CNS (Readhead et al. 1987).

Post-translational modifications of the MBP isoforms add to the microheterogeneity of the family (Harauz and Musse 2007). Modifications include acetylation, N-terminal acylation, deamidation, phosphorylation, arginine methylation, deimination and citrullination (Harauz and Musse 2007; Moscarello et al. 2002). MBP separated by cation-exchange chromatography yield charge isomers called C1-C8, where C1 is the least modified and the most cationic and C8 has the most modifications and is the least cationic (Cheifetz et al. 1984; Chou et al. 1976; Fannon and Moscarello 1991).

One of the modifications that is getting a lot of attention is citrullination, due to its possible implication in the pathogenesis of MS. Citrulline is a non-standard amino acid not generated during protein synthesis or from a specific codon, but during metabolic processes (Curis et al. 2005). It is both polar and uncharged. It is an intermediate in the biosynthesis of arginine from ornithine and is closely related to arginine. The enzyme dimethylarginase converts free monomethylated Arg and free asymmetrically dimethylated Arg to citrulline. In MBP, peptide linked arginine is converted to citrulline by Ca^{2+}-dependent enzymes called peptidylarginine deiminases (PADs) (Harauz and Musse 2007). It is thought that the conversion of the positively charged arginine to citrulline will have significant consequences on the protein's structure and function (Harauz and Musse 2007). In addition, an increase in citrulline-containine MBP has been found in MS patients (Kim et al. 2003).

1.6.2.1 Protein arginine methylation and the implications for MBP

In humans, protein arginine methyltransferases (PRMTs) represent a family of enzymes that use S-adenosylmethionine (SAM) as a methyl donor (McBride and Silver 2001). The characteristics of PRMTs include the presence of a highly conserved methyltransferase domain that oligomerizes into a ring-like structure (Weiss et al. 2000; Zhang and Cheng 2003; Zhang et al. 2000). Little structure-function analysis has been performed on the PRMTs to identify their regulatory domains.

The PRMTs are classified into two groups: type I and type II enzymes (Figure 1.7).

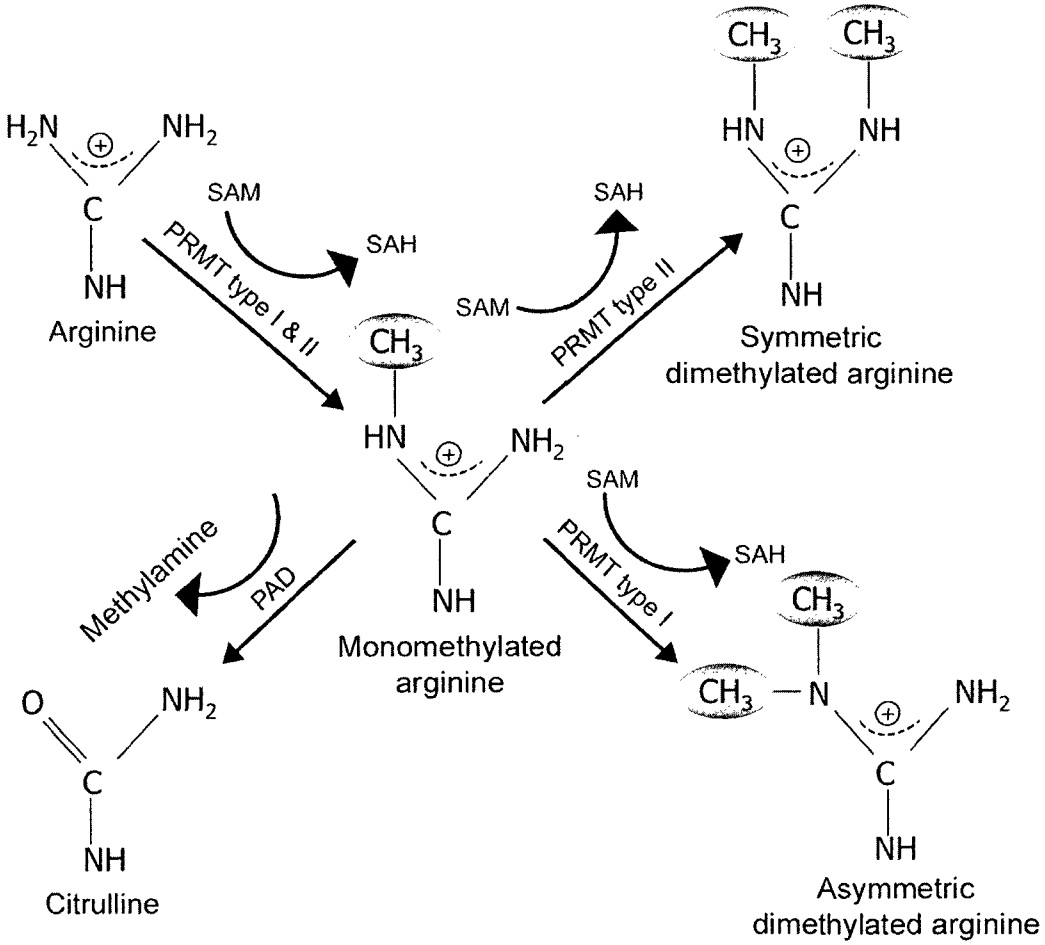

Figure 1.7 Methylation of arginines.

Arginine can be methylated on a guandino nitrogen atom by type I and type II protein arginine methyltransferases (PRMT) to become monomethylated. Symmetrically dimethylated arginines result from the addition of a second methyl group to the opposite nitrogen atom by type II enzymes, whereas asymmetrically dimethylated arginines result from the addition of a second methyl group to the same nitrogen with type I enzymes using SAM as a methyl donor releasing S-adenosylhomocysteine (SAH). Monomethylarginines can also be deimidated by peptidylarginine deiminases (PADs) resulting in the formation of citrulline (Figure modified from Boisvert et al. 2005; page 2).

There are five known type I PRMTs that catalyze the formation of mono-methylarginine (MMA) and asymmetric dimethylarginine (aDMA): PRMT1 (Lin et al. 1996), PRMT2 (Scott et al. 1998), PRMT3 (Tang et al. 1998), PRMT4 or CARM1 for coactivator methyltransferase (Chen et al. 1999), and PRMT6 (Frankel et al. 2002). There are two type II enzymes that catalyze the formation of MMA and symmetric dimethylarginine (sDMA): PRMT5 (Pollack et al. 1999) and PRMT7 (Lee et al. 2005). PRMT7 was originally identified in a genetic screen for targets conferring resistance to a topoisomerase II inhibitor (Gros et al. 2003). PRMT7 catalyzes the formation of MMA (Miranda et al. 2004) and can also catalyze the formation of sDMA (Lee et al. 2005). HRMT1L3 (GenBank accession number AAF91390), herein called PRMT8, has ~80% sequence identity with PRMT1 and is most likely a type I enzyme because of its similarity with PRMT1. Arginine methyltransferase activity was also detected in the Golgi. Q9DD20 is the enzyme proposed to mediate this activity (MacCoss et al. 2003). However, its amino acid sequence diverges from that of the known arginine methyltransferases, and it remains to be demonstrated whether or not it is a bona fide PRMT.

MBP was the first protein identified to be modified by symmetrical arginine dimethylation (Baldwin and Carnegie 1971). As mentioned above, the MBP protein contains 19 arginyl residues, only arginine 107 in humans (arginine 106 - murine) is modified by mono- and symmetrically dimethylation (Kim et al. 2003; Kim et al. 1997). MBP is extensively post-translationally modified. These posttranslational modifications determine the nature and extent of secondary structure and allow the protein to adopt multiple conformations (Kim et al. 2003). The interactions between the positively charged arginines and lysines with the negatively charged phospholipids bilayer are

essential to the structure of compact myelin and any changes in the charge of MBP may decrease the strength of the interactions (Kim et al. 2003). Arginine methylation does not affect the charge of the protein but rather increases its bulkiness, blocks hydrogen bonding, increases its hydrophobicity, and does affects its availability to the PAD enzymes (Boisvert et al. 2005). Of the 19 arginines available for deimination in MBP, only arginine 107 is not converted to a citrulline. This observation led to the hypothesis that arginine methylation of the MBP arginine protects it from citrullination. This has important implications, since methylation of MBP by vitamin B_{12} treatment reversed the meylinolysis observed during vitamin B_{12} deficiency in the spinal cord (Kim et al. 1997). Also, it has been shown that a greater portion of MBP from MS samples was methylated and when combined with other data, it is hypothesized that this increase methylation in MS patients likely reflects attempts at remyelination (Kim et al. 2003; Kim et al. 1997). In addition, methylated MBP is found in compact myelin suggesting that this modification is important for myelin structure (Kim et al. 1997).

Objective and Hypothesis

The main objective of my thesis was to characterize the protein and RNA components of the QKI ribonucleoprotein complex. I hypothesized that determining protein partners and RNA targets will allow for the identification of new roles for the QKI proteins and new pathways regulated by these proteins as well as identification of possible proteins/mRNAs involved in the already described functions of QKI. The major protein complexes that were identified include components required for translation and RNA metabolism. The major categories of mRNAs identified within the RNP include mRNAs involved in cellular transport, cell death and cell growth. At the protein level, we decided to investigate the interaction of QKI and PABP due to the already well-characterized role of PABP in posttranscriptional gene regulation and translation. In addition, I performed the characterization of QKI binding to VEGF mRNA. The phenotypes of the different genetic mutations suggested that QKI was regulating blood vessel development through a previously unidentified secreted factor. Finally, I performed analysis of protein arginine methylation of myelin basic protein, one of the QKI mRNA targets that has been well-characterized at the RNA level, as the final part to this thesis.

Chapter 2

The QUAKING RNA binding proteins interact with PABP protein and the VEGF mRNA: Defining the QKI complex

2.1 *Preface*

Prior to this project, only a few mRNA targets of the QKI proteins had been identified, among them, MBP and Krox20. In addition, no protein partners were known. In order to discover new QKI protein partners, we used a newly generated isoform-specific antibody in a proteomic experiment to determine the QKI protein complex. In addition, we also used this antibody to purifiy mRNAs bound by the QKI complex and used cDNA analysis to identify the ribonucleotide component of the complex. We hypothesized that identification of the ribonucleoprotein complex would give us some understanding of QKI's function. This study led to the identification of numerous QKI-associated proteins and mRNAs and to the identification of new roles for this protein.

2.2 *Abstract*

The *quaking viable* (*qk*^{v}) mouse has several developmental defects that result in rapid tremors and problems of the central nervous system (CNS). The *qkI* locus expresses three major alternatively spliced mRNAs (5, 6 and 7 kb) encoding the QKI-5, QKI-6 and QKI-7 RNA binding proteins that differ in their C-terminal 30 amino acids. Although the QKI isoforms are known to regulate RNA metabolism within oligodendrocytes and several target mRNAs have been identified, little is known about the composition of the ribonucleoprotein (RNP) complex. Here we identify the QKI RNP complex of ~375-400 kDa in U343, a human astrocytic cell line. Interestingly, the protein component of the QKI complex contains several other RNA binding proteins and molecular chaperones. We also identified the mRNAs bound to the QKI complex by array technology, notably the mRNA encoding vascular endothelial growth factor (VEGF). We show the loss of QKI proteins causes an upregulation of secreted VEGF levels. These data provide the ingredients necessary to examine the role of QKI proteins in several processes such as vascularization, mRNA trafficking and signaling in human cells.

2.3 *Intoduction*

The quaking (QKI) proteins have been shown to bind specific mRNAs in the cell regulating alternative splicing, translation and localization (Larocque et al. 2002; Saccomanno et al. 1999; Wu et al. 2002). These proteins are widely expressed in mouse tissues and particularly abundant in the central and peripheral nervous system, specifically in astrocytes and oligodendrocytes (Hardy 1998). In the *quaking viable* (qk^v) mouse, which has an extensive 1 MB enhancer/promoter deletion that causes loss of expression of QKI-6 and QKI-7 specifically in oligodendrocytes, there is nuclear retention of myelin basic protein mRNA, which is one of the targets of the QKI proteins (Larocque et al. 2002). This results in severe dysmyelination and tremors that start approximately two weeks after birth. The loss of *qkI* in mice is embryonic lethal as a result of defects in blood vessel development in the embryonic yolk sac (Li et al. 2003). Studies on several mutant mice have revealed that QKI is important for glial cell fate decisions (Larocque et al. 2002) and that these complex decisions may in part be regulated by QKI's RNA binding functions.

QKI is an RNA-binding protein of the heterogenous nuclear ribonucleoprotein particle K (KH) domain containing family. Proteins of this family are highly conserved among species and include *Artemia salina* GRP33, Sam68, *Caenorhabditis elegans* GLD-1, SF1, *Drosophila* Who/How, *Xenopus* Xqua, mouse QkI, zebrafish QkI and *Drosophila* KEP1 and Sam50. In part, glial cells of the CNS express the three major *qkI* transcripts of 5 kilobases (kb), 6kb, and 7kb which encode a set of alternatively spliced RNA binding proteins: QKI-5, -6, and –7 respectively (Ebersole et al. 1996). In oligodendrocytes, the myelinating cells of the CNS, formation of the myelin sheath requires transport of myelin component mRNA from the nucleus to the distal tips of the processes where it is

translated locally. How the QKI proteins regulate this process is unclear. But the QKI proteins can dimerize and their balanced expression is required for proper myelination through localization of MBP mRNA (Larocque et al. 2002). In addition, the QKI isoforms are localized differently in the cell, QKI-5 being nuclear, whereas QKI-6 and QKI-7 are localized to the perikaryal cytoplasm with lower levels in the cytoplasm (Hardy et al. 1996). It is thought that mRNAs are bound by QKI-5 in the nucleus and transported to the cytoplasm through heterodimerization with QKI-6 and –7 and likely other proteins.

Several post-transcriptional functions have been attributed to the QKI proteins. QKI-6 has been shown to bind and stabilize the mRNA of microtubule associated protein 1B (MAP1B) (Saccomanno et al. 1999) and the cyclin dependent kinase inhibitor, $p27^{KIP1}$ (Larocque et al. 2005). QKI-6 has also been shown to mediate exon inclusion during alternative splicing in the myelin associated glycoprotein (MAG) mRNA. Many of these functions are of ultimate importance during embryogenesis where sequence specific activation of translation is important for oogenesis, organ specification and cellular maturation and in oligodendrocytes where mRNAs are translationally repressed and subsequently translated locally in the distal compartments.

Because of its role in the CNS, in glial cell maturation and in myelination, QKI has now been implicated in several human diseases. Altered expression of QKI isoform occurs in approximately 30% of human glioblastomas, but unchanged in all of the schwannomas and meningiomas tested (Li et al. 2002). Of these primary tumors, two were devoid of the transcripts encoding the 3 major isoforms, two were missing only the

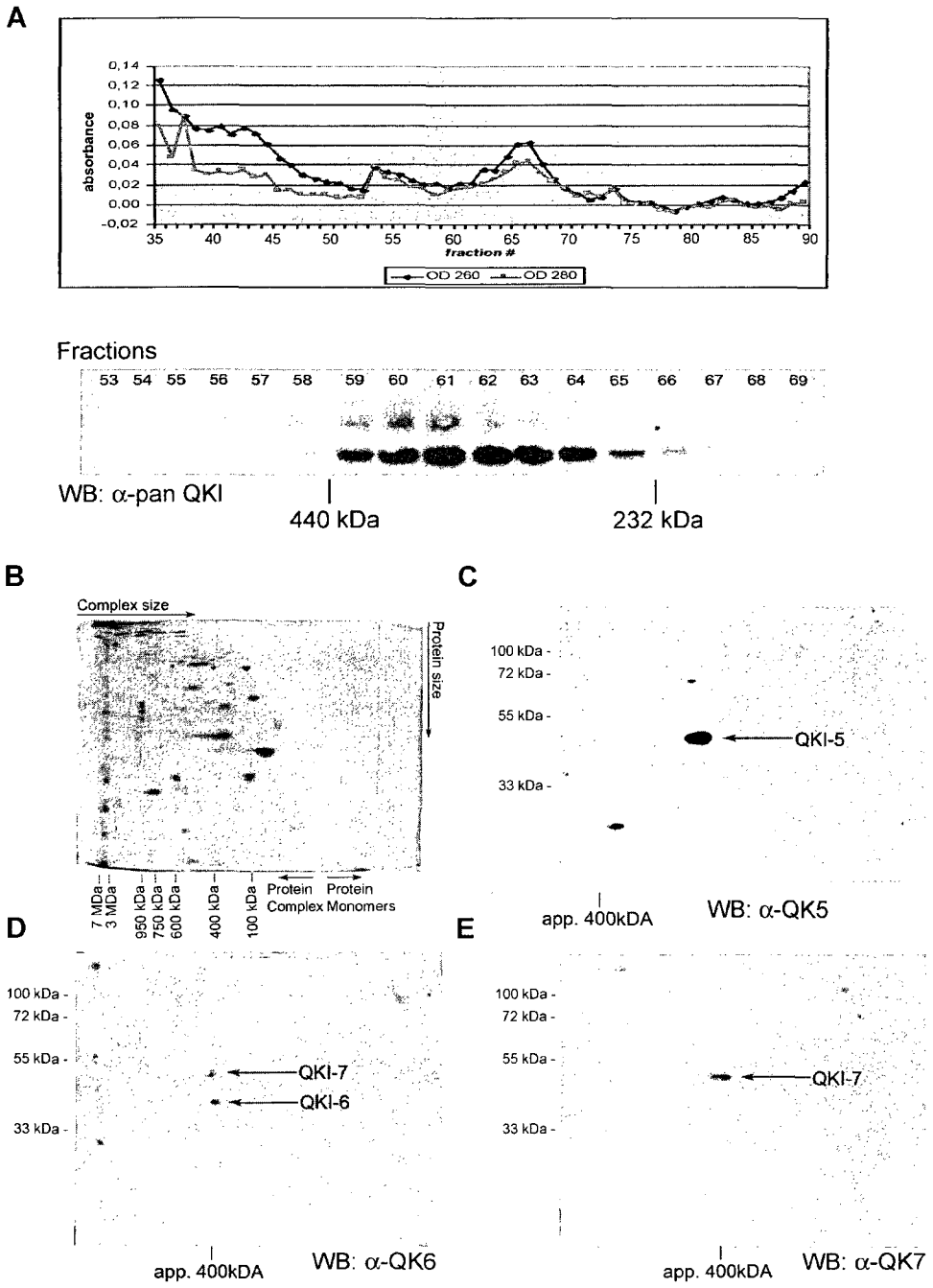

Figure 2.1 The QKI complex migrates ~400kDa in U343 glioblastoma

(A) Cell extracts from U343 were separated on a Superose 6 column and the optical optical density 260 and 280 measured for each fraction. The protein content of each fraction was separated by SDS-PAGE and immunoblotted with the anti-pan-QKI antibody. Fractions 53 to 69 are shown. **(B)** Cell extracts from U343 were separated by Native-PAGE (2-7% gradient) and the gels were silver stained **(B)** or immunoblotted with anti-QKI-5, 6 and 7 specific antibodies **(C, D, E)**.

qkI-7 transcript and one was missing only the *qkI-5* transcript. More recently QKI has been linked to schizophrenia (SCZ) (Aberg et al. 2006a; Aberg et al. 2006b; Haroutunian et al. 2006). A susceptibility locus for SCZ was mapped to chromosome 6q25-6q26, a region spanning 0.5Mb that also contained the *qkI*. Some studies have examined expression of various transcripts in areas of the brain affected by SCZ and several myelin-related genes were downregulated in the white matter of SCZ patients (Aberg et al. 2006a; Aberg et al. 2006b; Haroutunian et al. 2006). They include 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (Aberg et al. 2006a; McCullumsmith et al. 2007), myelin-associated glycoprotein (MAG) (Aberg et al. 2006a; McCullumsmith et al. 2007), transferrin (TF) (Stewart and Davis 2004), proteolipid protein 1 (PLP) (Aberg et al. 2006a) and QKI (Aberg et al. 2006a; Aberg et al. 2006b; Haroutunian et al. 2006). Many of these transcripts are identified targets of the QKI proteins and the differences observed can be explained by variation in the relative mRNA levels of QKI.

We previously applied the systemic evolution of ligands by exponential enrichment (SELEX) strategy to identify a high-affinity binding site called Quaking Response Element (QRE) in new mRNA targets of the QKI proteins (Galarneau and Richard 2005). The QRE is a bipartite motif consisting of a core site followed by a half site separated by one to twenty nucleotides, NACUAAY-N(1-20)-UAAY (Galarneau and Richard 2005). Through bioinformatics scanning, 1430 putative mouse RNA targets were also identified in this study. These mRNAs included previously identified QKI targets such as MBP and Krox20. Many mRNAs encoding proteins implicated in development, cell adhesion, morphogenesis, organogenesis, cell differentiation and cell growth were also identified in this study (Galarneau and Richard 2005).

We hypothesize that QKI proteins exist in a functionally relevant complex and that identifying the components of the complex will improve our understanding of how the QKI proteins regulate RNA metabolism. Here we determine the molecular composition of the QKI complex in human cells. We demonstrate that QKI is present in a small RNP complex of approximately 400 kDa. We perform a proteomic analysis that allowed us to identify 13 proteins that are potentially part of the QKI complex. We show that QKI interacts directly with one of the newly identified protein partners, PABP and map the domains required for their interaction. In addition, we perform cDNA microarray analysis in order to define the RNA composition of the complex and show that QKI binds to and regulates the expression of vascular endothelial mRNA.

2.4 *Materials and Methods*

2.4.1 Antibodies and plasmids

The anti-QKI-5 antibody was generated using the following peptide antigen in rabbits KVRRHDMRVHPYQRIVTADRAATGN. The anti-QKI-6, QKI-7 and pan-QKI antibodies have been previously described (Chen and Richard 1998; Larocque et al. 2005). The monoclonal antibody against α-actin was purchased from Sigma. The HA- and Flag-epitope tagged PABP were a generous gift of Nahum Sonenberg (McGill University, Montreal, Canada) (Imataka et al. 1998).

2.4.2 Gel Filtration and Native PAGE

U343 cells were cultured in five 150 mm tissue culture dish until confluence. Cells were lysed in 0,5% Triton lysis buffer (0,5% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 μg/ml aprotinin, and 0.01% phenylmethanesulfonyl fluoride) at a final volume of 1ml (including the cell pellet). Protein complexes were fractionated on a Superose 6 prep grade packed in a SR column to achieve high-resolution separation of protein complexes. OD 260 and 280 was measured for fractions 35 to 89. All fractions were precipitated using 20 % TCA and separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with α-pan-QKI antibody. The Superose 6 column was calibrated using bovine serum albumin (BSA, 66 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (699 kDa) and blue dextran (2000+ kDa, Sigma).

U343 cells were harvested from a semi-confluent 100 mm tissue culture dish with PBS and centrifuged to a pellet. The cell pellet was lysed for 10 min on ice using buffer A (1% TDOC, 50 mM Bis-Tris pH 7.5 and 5 mM iodoacetamide). After extraction, the

lysate was supplemented with glycerol to a final concentration of 10%, 1 µl of 1% BromophenolBlue and loaded on a 2-7% gradient native gel. Native gel was visualized with Coomassie Blue R according to manufacturer procedures. Strips of stained gels were excised and equilibrated in Laemmli sample buffer for 20-30 minutes and loaded on SDS-PAGE (10% acrylamide) gels. After electrophoresis, gels were silver stained or transferred to nitrocellulose membranes and immunoblotted using the designated primary antibodies. Immunoreactive proteins were visualized using goat α-rabbit antibody conjugated to horseradish peroxidase (ICN Pharmaceuticals) and the chemiluminescence (ECL) detection kit (DuPont).

2.4.3 Mass Spectrometry

Endogenous QKI complexes were immunopurified (IP) from 5 x 10^8 U343 cells and lysed in 1% Triton lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 µg/ml aprotinin, and 0.01% phenylmethanesulfonyl fluoride). Lysates were pre-cleared with 100 µl of 50% protein A-Sepharose slurry at 4 °C for 1 hour with constant end-over-end mixing. Pre-cleared lysates were incubated on ice with 10 µg of the primary antibody α-QKI-5 for 30 min. Then 100 µl of 50% protein A-Sepharose slurry was added and incubated at 4 °C for 2 hours with constant end-over-end mixing. The beads were washed twice with 1% Triton lysis buffer and once with PBS. Immunopurified proteins were analyzed on SDS-PAGE and visualized with Brilliant Blue G Colloidal Coomassie (Sigma) according to manufacturer protocol. The region between ~72kDa to ~100kDa was excised, trypsin digested and analyzed by LC/MS/MS sequencing of peptides (University of Calgary Mass Spectrometry Proteomics Facility).

Table 2.1 QKI-interacting proteins identified by mass spectrometry

Protein	GenPept Accession	Peptides	Score
Heat shock cognate 71 kDa protein	P11142	19	851
hnRNP Q	O60506	9	353
78 kDa glucose-regulated protein precursor	P11021	7	341
Propionyl-Co A carboxylase alpha chain	P05165	10	280
Peroxisomal multifunctional enzyme type 2	P51659	6	205
hnRNP R	O43390	5	182
alpha-actinin	P12814	2	104
Heat shock 70 kDa protein 6	P17066	4	118
Heat shock 70 kDa protein 1L	P34931	3	111
Poly-A binding protein	P11940	3	88
RasGAP-activating-like protein 1	O95294	20	45
Kinesin-like protein	O43896	2	35
Alcohol dehydrogenase 1B	P00325	2	35

The LC-MS/MS analysis for protein identification was performed on either a QSTAR Pulsar iHybrid quadrupole time of flight mass spectrometer (Applied Biosystems/PE sciex) or and Agilent MSD Ion Trap XCT interfaced with an Agilent 1100 series Nano LC.

2.4.4 Protein Expression

For GST pull-down assays, 20 µl of 50% slurry containing 2 mg of GST fusion protein, covalently coupled to beads, per ml was incubated with cell lysate expressing HA-PABP or FLAG-PABP for 1 h with constant mixing. For RNAse treatment, after lysis cell lysates were treated with 120 µg/ml of RNAse A for 1 hr at room temperature. The samples were washed and analyzed. Immunoblotting was performed with anti-myc FLAG or anti-HA. The designated primary antibody was followed by goat anti-mouse or goat anti-rabbit antibodies conjugated to horseradish peroxidase (Organon Teknika-Cappel), and chemiluminescence (Dupont) was used for protein detection.

2.4.5 Microarray analysis

RNA bound to the QKI-5 complex or to the normal rabbit serum (NRS) IP was purified using the TRIzol method as per the manufacturer's instructions (Invitrogen Life Technologies). Concentrations were calculated using the OD_{260} for each sample and RNA quality was determined using the Agilent 2100 Bioanalyzer (Agilent). Ten µg of RNA from each cell line was reverse transcribed, labeled with the appropriate fluorochrome (indocarbocyanine or indodicarbocyanine; PerkinElmer), and hybridized to a DS human 8kv1 chip (University Health Network Microarray Center, Toronto, Canada). Four independent experiments with corresponding reverse labeling were conducted. Data

acquisition was performed using GenePix Pro (4000B; Axon Instruments) at photomultiplier tube gain between 600 and 700. Flagging parameters were set to reject spots with [F532 mean—B532 (Cy3) and F635—B635 (Cy5)] intensity values lower than 200. Data analysis was performed using Iobion Gene Traffic software (version 3.0; Iobion) for a two-class experiment. Data were normalized with Lowess (subgrid) method with background subtraction. Statistical analysis for microarray was applied on spot tables with a *p* value cut off of 0.05 and a differential expression cut off of 2-fold and significant, differentially expressed genes in each class were selected.

2.4.6 RT-PCR

Total RNA was isolated using TRIzol isolation reagent Invitrogen Life Technologies) according to the manufacturer's protocol. RT-PCR was performed on 50 ng of RNA using the One-Step RT-PCR kit (Qiagen) and 0.6 μM of each primer (Supplementary Table 2.4). Cycling conditions were 30 min at 50 °C, 15 min at 95 °C, 25 or 30 cycles of 40 s at 94 °C, 40 s at 60 °C, 30 s at 68 °C, with a final extension of 10 min at 68 °C. RT-PCR products were electrophoresed in 2% agarose gels and ethidium bromide staining visualized using a Kodak digital science IC440 system.

2.4.7 RNA binding assays

Reverse transcription PCR and electrophoretic mobility shift assays were performed as described previously (Galarneau and Richard 2005) using primer sequences in Supplementary Table 2.4 and 2.5. For the *in vitro* RNA binding, substrates were generated by PCR and subsequently ^{32}P-labeled VEGF 3′-UTR RNAs were transcribed *in vitro* with the T7 MegaShortScript (Ambion) (Supplementary Table 2.6) and tested for

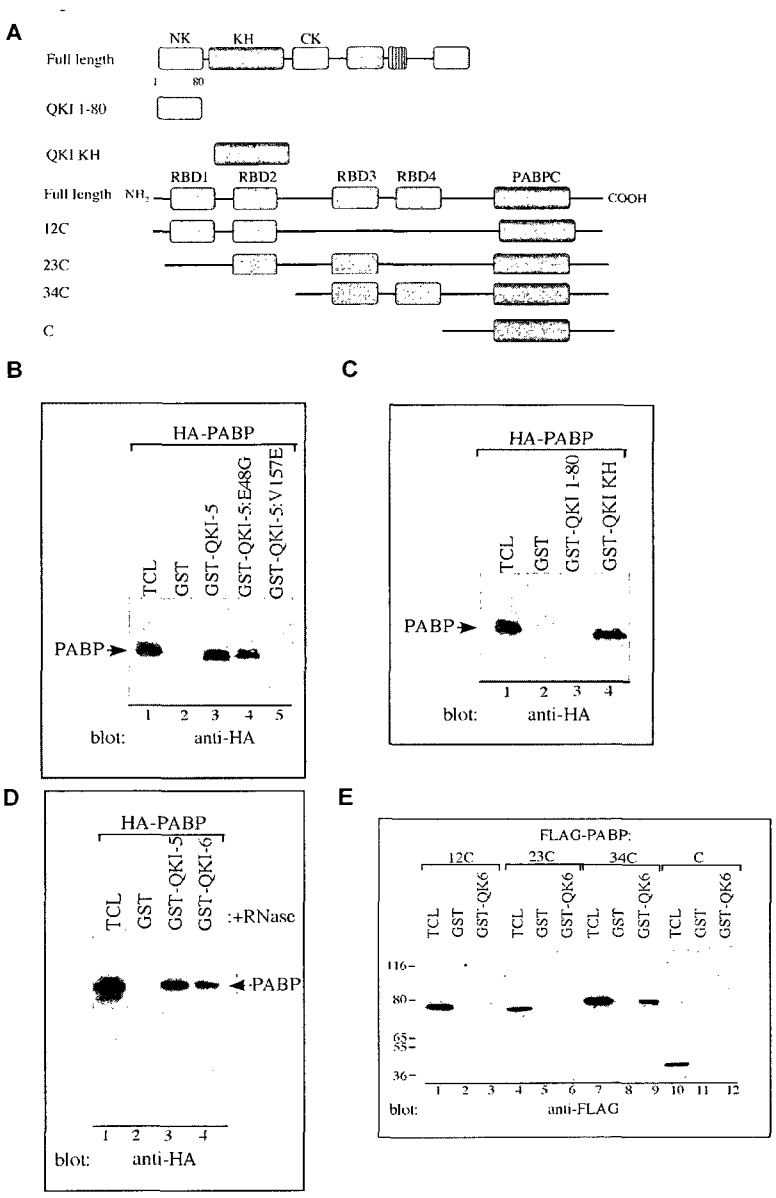

Figure 2.2 Mapping the QKI and PABP interaction

(A) Schematic of the truncated QKI and PABP proteins used in this study. **(B)** HA-PABP was expressed in HEK293 cells and the lysates subjected to GST-pull down with QKI-5 and two mutant forms of the protein (E48G and V157E). **(C)** HA-PABP was transfected into HEK293 cells and the lysates were then subjected to treatment with 120 µg/ml of RNAse A for 1 hr at room temperature. The lysates were then subjected to GST-pull down with GST alone, GST-QKI-5 or GST-QKI-6. **(D)** HEK293 cells were transfected with HA-PABP and the lysates were subjected to GST-pull down with the different domains of the QKI proteins. **(E)** HEK293 cells were transfected with FLAG-tagged constructs encoding the different PABP domains. The lysates were then subjected to GST-pull down with purified GST-QKI-6.

association with recombinant GST-QKI-5 prebound to glutathione beads. Binding was quantified by scintillation counting.

2.4.8 VEGF Enzyme Linked Immunosorbant Assay (ELISA) and Real-time PCR

Levels of human VEGF were measured by ELISA in cell culture supernatant samples (diluted 1:50) from the indicated cell lines. All analyses were performed in triplicate. ELISA for hVEGF was made using a standard quantitative sandwich ELISA (R&D Systems, Lille, France) according to manufacturer's instructions (Majka et al. 2000).

cDNA was generated by reverse transcribing 5 μg of total RNA in 40 μl total reaction volume using random hexamers. The resulting cDNA was diluted to 100 μl and 2μl was used in the real-time PCR reaction. Reactions were set up using TaqMan Fast Universal PCR master mix, No Amp Erase UNG (Applied Biosystem) according to manufacturers instructions exept that the final reaction volume was 10 μl. All samples were analysed in triplicate (Supplementary Table 2.7). The reactions were run using the 7500 Fast Real-Time PCR system (Applied Biosystems). The conditions were as follows for 40 cycles: 95 degrees for 20s, 95 degrees for 3s and 60 degrees for 30s. The results were analyzed by using SDS software.

2.5 *Results*

2.5.1 QKI exists in a low molecular weight complex

The QKI proteins are known to dimerize via their coiled-coil domain in their region N-terminal to the KH domain (Chen et Richard 1998). However, very little is known about their interacting proteins. We first examined the size of the QKI complex by gel-filtration and native 2D-PAGE. The three QKI isoforms are expressed in glial cells and their expression is abundant in the U343 human astrocytic cell line (Supplementary Figure 2.8). U343 cell extracts were fractionated using a Superose 6 column and the proteins in each fraction were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with the 'pan' anti-QKI antibody. QKI was detected in fractions 57 to 67 with an approximate molecular mass of 400 kDa (Figure 2.1A).

The molecular mass of the QKI complex was also confirmed using the two-dimension native SDS-PAGE. Here, lysates are first subjected to non-denaturing conditions using a gradient of 2-7% to separate the complexes according to size. The complexes are then extracted and separated on a second dimension under denaturing conditions to separate the individual protein components of the complex. The proteins are transferred to nitrocellulose and immunoblotted with anti-QKI-5, -6 or -7 antibodies. The proteins in the same complex are thus vertically aligned. We observed that the QKI isoforms are present in an ~400 kDa complex (Figure 2.1, B-E).

2.5.2 Proteome analysis of QKI complex

To identify the protein components found in the QKI complex, an α-QKI-5 immunoprecipitation was performed using the U343 cell. We chose to use the QKI-5

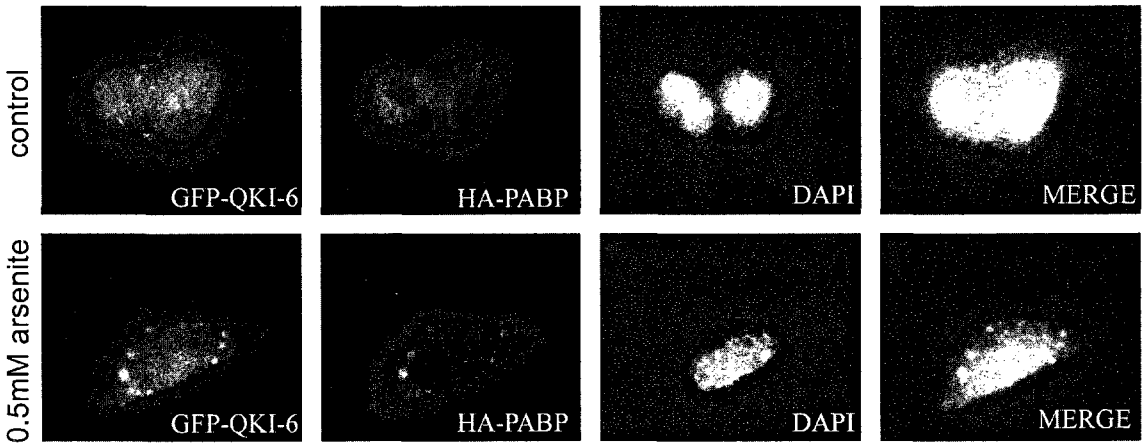

Figure 2.3 QKI-6 and PABP colocalize in cytoplasmic stress granules

GFP-QKI-6 and HA-PABP were transfected in SK-N-SH neuroblastoma cells. 24 hrs post-transfection, the cells were either mock-treated with DMSO or treated with 0.5 mM of sodium arsenite for 30 min, washed and subsequently fixed and labeled for immunofluorescence with anti-HA antibodies and the nucleus labeled with DAPI. The presence of PABP and QKI-6 was visualized by fluorescence microscopy.

antibody because it does not show any non-specific bands. In addition, we have previously shown that QKI-5, QKI-6 and QKI-7 all bind the Quaking Response Element (QRE) within mRNAs with very similar affinities (K_d ~ 99 nM, 107 nM, 114 nM respectively) (Galarneau and Richard 2005) suggesting that mRNAs bound by one isoform may also be targets for the others. A large-scale immunoprecipitation was performed on U343 cells and the proteins separated by SDS-PAGE and visualized with Colloidal Coomassie. The majority of associated proteins were found between 72 kDa and 100 kDa and we selected this for further characterization. Enriched proteins were excised and sent for liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). Proteins that had significant scores of $p<0.05$ were considered as potential interactors. Table 2.1 lists the significant interactors identified. The significance of the data is determined by calculating ion scores, which is -10*Log(P), where P is the probability that the match is a random event. Individual ions scores greater than 34 indicate identity or extensive homology ($p<0.05$). The identified proteins fell within many different functional categories. Interestingly, some of them are well known RNA binding proteins such as polyadenylate binding protein 1 and 5 (PABP1 and PABP5) as well as hnRNP R and hnRNP Q. hnRNP R and Q are expressed in the nervous system and found in a nuclear complex involved in mRNA decay, splicing and transport (Rossoll et al. 2002). In addition to the RNA binding proteins, there were also a large number of proteins in the heat shock family identified. Heat shock proteins are molecular chaperones of the cells and also shuttle proteins from one compartment to another. We identified heat shock 70 kDa protein 1L and 6, heat shock cognate protein 71 kDa, as well as the 78 kDa glucose-related protein precursor (GRP 78) which is also known as heat shock protein 5 (Lee 1992). Another interesting protein partners included

peroxisomal multifunctional enzyme type 2, which is important for normal function of the brain, eyes and testis and loss of the protein causes severe astrogliosis (Huyghe et al. 2006). We also identified proteins such as propionyl-CoA carboxylase alpha chain, Ras GAP-activating-like protein 1, kinesin-like protein, alpha-actinin 1 (brain isoform) and the L-myc-1 proto-oncogene protein, but their functional link to QKI remains unclear. Table 2.1 only represents a short list of potential interacting proteins of molecular weights between 72-100 kDa, it is probable that there are other interacting proteins yet to be identified.

2.5.3 Poly(A)-binding protein and QKI interaction

We chose to extend the characterization of PABP/QKI interaction because of the potential involvement of QKI in translational control. To validate the PABP/QKI interaction, we expressed HA-tagged PABP into HEK293 cells and performed glutathione S-transferase (GST)-pull down assays using recombinant GST-QKI-5. The bound proteins were separated by SDS-PAGE and immunoblotted with anti-HA antibodies. We observed that PABP interacted with recombinant QKI-5, whereas a mutant protein that harbors an amino acid substitution (E48G) that disrupts the coiled-coil domain did not interact with PABP (Figure 2.2B). Interestingly, a mutant harboring a V157E substitution in the KH domain was unable to associate with PABP. This mutation has been shown previously to cause a loss of RNA binding abililty (Larocque et al. 2002). The KH domain alone was sufficient to interact with PABP (Figure 2.2C). The PABP/GST-QKI interaction was not RNA-dependent since high doses of RNase A, that

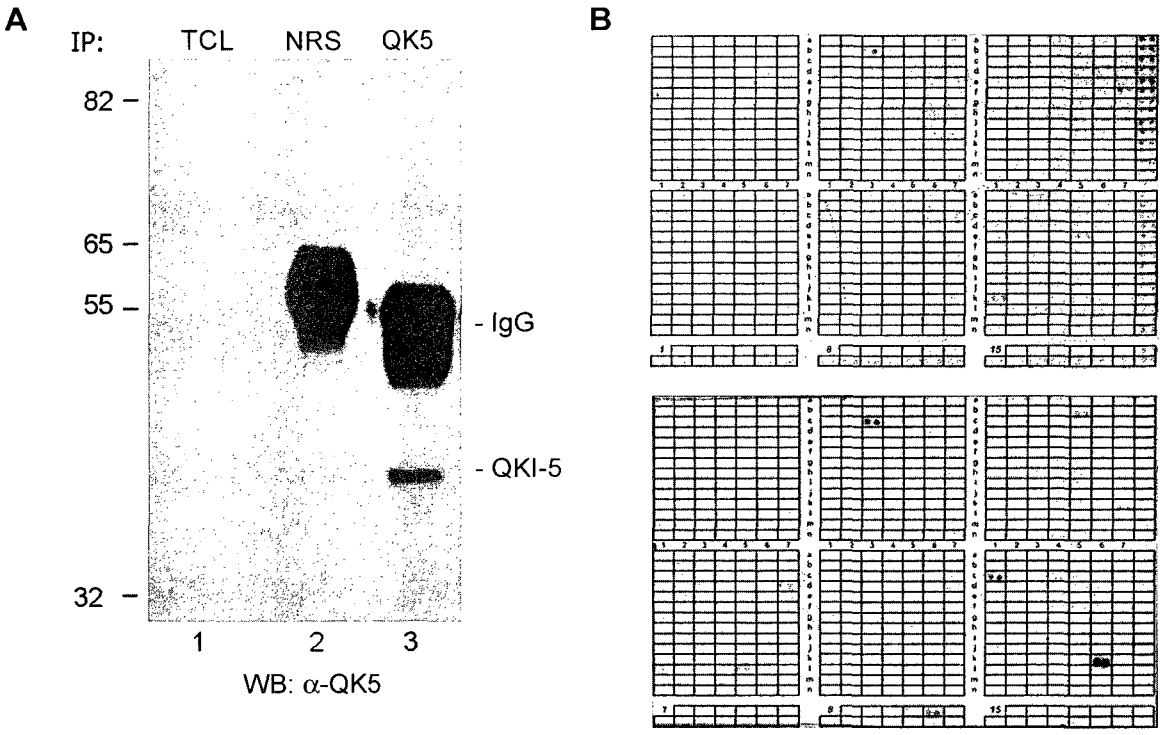

Figure 2.4 VEGF mRNA identified as a QKI RNA target by filter array

(A) U343 cells were immunoprecipitated with control normal rabbit serum (NRS) and with anti-QKI-5 antibodies. The bound proteins were detected after SDS-PAGE by immunoblotting with anti-QKI-5 antibodies. The large bands at ~50kDa represent the IgG heavy chain and QKI-5 migrates ~40kDa. **(B)** mRNAs bound to immunoprecipitated QKI-5 were purified and labeled by reverse-transcription using radiolabeled nucleotides. The labeled purified transcripts were hybridized to the cDNA array for analysis and visualized by autoradiography. Representative arrays for the control (NRS, top panel) and QKI-5 (bottom panel) experiments are shown.

degraded all observable RNA by electrophoresis (data now shown), did not inhibit the interaction (Figure 2.2D). Even though the KH domain of QKI alone is not sufficient for specific RNA binding, however, it is always possible that a small, protected RNA is required.

PABP is a 633 amino acid protein containing four RRM-type RNA binding domains and a C-terminus (PABPC) (Figure 2.2A). RRM 1 and 2 are the major motifs responsible for the poly-A binding activity of PABP (Deardorff and Sachs 1997; Kuhn and Pieler 1996). To map the regions of PABP required for interaction with QKI, we expressed flag-tagged PABP truncated proteins (Figure 2.2A) into HEK293 cells and performed GST-pull down assays using recombinant GST-QKI-6. The bound proteins were separated by SDS-PAGE and immunoblotted with anti-Flag antibodies. We show that QKI-6 interacted with PABP harboring RRM3-RRM4 and the C terminal domain (Figure 2.2E). But the absence of binding to the C terminal domain of PABP alone suggests that the QKI/PABP interaction occurs via RRM3 and RRM4.

These data validate PABP as a QKI-6 interacting protein and demonstrate that the QKI binding interface lies within the RRM3 and RRM4 regions of PABP. It has been reported that the dissociation of polysomes following cellular stress induces the accumulation of RNA binding proteins and certain elements of the translational machinery into perinuclear entities known as stress granules (Anderson and Kedersha 2002a; Anderson and Kedersha 2002b; Kedersha et al. 2000; Mazroui et al. 2002). Arsenic-induced oxidative stress causes chromosome alterations, contributes to DNA damage and also causes protein expression aberrations (Trouba et al. 2002). Oxidative stress also produces a well-characterized response in cells, which mobilizes the translational machinery to cope with the insult (Dolzhanskaya et al. 2006). One of the

proteins known to relocalize to stress granules following cell stress is PABP. To determine if QKI-6 and PABP colocalize to stress granules following cell stress, we co-expressed GFP-QK6 and HA-PABP in the neuroblastoma cell line SK-N-SH. After 24 hours, the cells were exposed to 0.5mM sodium arsenite or DMSO alone for 30 minutes. The cells were then fixed and localization of OABO and QKI-6 analyzed by immunofluorescence microscopy. We observed cells treated with sodium arsenite were laden with cytoplasmic granules that contained both QKI-6 and PABP (Figure 2.3). These data demonstrated that QKI and PABP colocalize in cytoplasmic granules upon cellular stress.

2.5.4 Identification of neuro-specific mRNA targets of the QKI complex using cDNA filter arrays

To identify the mRNAs bound to the QKI complex we first utilized a human neurobiology specific filter array that consists of cDNAs spotted in duplicate on a positively charged nylon filter. Using the human U343 glioblastoma cells as the source for QKI-5 we performed immunoprecipitation experiments (Figure 2.4A). mRNAs extracted from immunoprecipitated pellets showed negligible reactivity with the genomic DNA spots normally used to orient the perimeter of the array membrane, further indicating selectivity for specific mRNAs in this QKI complex. The arrays generated by the immunoprecipitations with the control normal rabbit serum (NRS), displayed virtually no reactivity as expected (Figure 2.4B top panel: NRS). The QKI-5 arrays identified sixteen reproducible spots (Figure 2.4B bottom panel: α-QKI-5 and Table 2.2). The mRNA targets are classified into several functional groups including trafficking, intracellular signaling and growth factors. They include the trafficking proteins lectin and

archain 1, the cellular signaling molecules casein kinase and RAP1B and the growth factors/chemokines vascular endothelial growth factor and thymosin beta (Table 2.2).

2.5.5 Identification of QKI mRNAs by microarray analysis

To further identify the mRNA targets of the QKI complex, RNA co-purified with QKI-5 following an anti-QKI-5 immunoprecipitation was isolated and utilized to generate probes for analysis on a DS human 8kv1 microarray chip. A total of 68 mRNA targets were identified which had at least a 2-fold enrichment with $p < 0.05$ and a spot intensity >2000 (Table 2.3). We analyzed these targets using the DAVID annotation software to determine if the targets were enriched within specific functional categories (Dennis et al. 2003). Indeed, there was enrichment for mRNAs involved in establishment of localization, protein transport, intracellular signalling cascades and cell death/apoptosis (data not shown). These results suggest that the QKI complex participates in many different cellular processes, some of which have not previously been described.

We next proceeded to validate four of the mRNA targets *in vivo* by immunoprecipitation followed by RT-PCR. Using this method we confirmed the association of RBP1, a protein involved in retinol transport (Farias et al. 2005), Rab11a, a member of the small GTPase superfamily (Yu et al. 2007), atrophin, a gene involved in a rare neurodegenerative disorder (Luciano et al. 2007) and parvin, an actin binding protein associated with focal contacts (Zhang et al. 2004), as mRNA targets of the QKI-5 complex (Figure 2.5A). Ferritin mRNA shown to bind non-specifically to the NRS

Table 2.2 QKI bound mRNAs identified by filter array

Location	Gene Name	GenBank#	SwissProt#	Classification
A02d	Lectin	X71661	P49257	Trafficking/targetting proteins
A03c	archain 1	X81197	P48444	Trafficking/targetting proteins
B03c	casein Kinase 1, alpha 1	X80693	P48729	intracellular kinase network
C01d	dual specificity phosphatase 7	X93921	Q16829	intracellular protein phosphatases
C04n	G protein, beta polypeptide 2-like 1	M24194	P25388	Intracellular Transducers/Effectors/Modulators
C05b	calmodulin 3 (phosphorylase kinase, delta	J04046	P02593	Calcium bindin proteins
C07k	RAP1B	X08004	P09526	G-proteins
D01l	RAB5C, member RAS oncogene family	U18420	P51148	General trafficking
D03n	adaptor-related protein complex 2, mu 1	D63475	P20172	other cytoskeleton/motility proteins
D04n	dynein, cytoplasmic, light polypeptide	U32944	Q15701	motor proteins
D05l	opioid receptor, delta 1	U07882	P41143	neurotransmitter receptors; G protein coupled
D07d	corticotropin releading hormone receptor 1	X72304	P34998	Growth factor & chemokine receptor
F01c	syntaxin 1A (brain)	L37792	Q16623	Exocytosis
F03l	laminin, gamma 1 (formerly LAMB2)	J03202	P11047	Extracellular matrix proteins
F04c	vascular endothelial growth factor	M32977	P15692	Growth factors, cytokines, and Chemokines
F06k	thymosin, beta 10	M92381	P13472	Growth factors, cytokines, and Chemokines

control and protein arginine methyltransferase 5 (PRMT5) served as a negative control for the immunoprecipitation (Figure 2.5B).

2.5.6 QKI binds with high affinity to VEGF mRNA

Many interesting target mRNAs were generated in the aforementioned experiments, but we opted to extend characterization of vascular endothelial growth factor (VEGF) mRNA because of its involvement in angiogenesis and vascularization. Furthermore the *qkI* null mice display embryonic lethality attributed to an angiogenic failure (Li et al. 2003). In order to determine where on the VEGF mRNA QKI binds, we performed *in vitro* pull down assays. Previous data from our laboratory suggests that the QRE is more prevalent in the 3′ UTR compared with the 5′ UTR or the coding sequence (Galarneau and Richard 2005). Thus small synthetic radiolabeled RNA generated from the 3′ UTR of VEGF was subjected to a pull-down assay with GST-QKI-5 and the bound RNA by residual radioactivity. A region from nucleotides 3227-3580 (354 bases), in the terminal 3′ portion bound to QKI with high affinity (Figure 2.6A). The area from nucleotide 3227-3580 was analyzed to determine whether or not there was a QRE within the sequence. Although this region does not harbour the 'perfect' QRE, a 'relaxed' sequence with a single nucleotide change was identified (Figure 2.6B). This sequence ACUAAA-N13-UAAUUUUAAU differs from the previously identified QRE in the core sequence where the final pyrimidine is replaced by an adenine (shown in bold). Our data therefore defines a potential QRE within the 3′ UTR of VEGF.

To further validate the VEGF QRE sequence, a short ~65 nucleotide RNA was generated and binding to QKI determined by electromobility shift assay (EMSA). The RNA was labelled with ^{32}P and incubated with buffer, GST alone or increasing

Table 2.3 QKI bound mRNAs identified by microarray

Rank	Name	Accession	Fold Chg.	Rank	Name	Accession	Fold Chg.
1	sim. to XP_518535.1	BX458721	3.8	35	DKFZP434F122 protein	AL080200	2.34
2	sim. to NP_112576.1 SH3 domain binding prot.	CA314843	3.6	36	Rho/rac guanine nucleotide exchange factor 18	AK127045	2.4
3	CDNA clone IMAGE:5271818	BC070115	3.5	37	Immunoglobulin superfamily, member 4B	AF363367	2.3
4	Melanoma antigen, family F, 1	BQ279149	3.5	38	H2A histone family, member X	BM917453	2.3
5	Nucl. factor of • light polypept. gene enh. in B-cells inh., •	NM_0010017163.4		39	Phosphoribosylformylglycinamidine synthase	NM_012393	2.3
6	Myosin XVA	NM_016239	3.3	40	Integrin, beta 2 (antigen CD18 (p95)	AK095992	2.3
7	SCY1-like 1 (S. cerevisiae)	BC069233	3.2	41	Tetratricopeptide repeat domain 10	AK126668	2.3
8	Hypothetical protein FLJ13955	AK025015	3.2	42	Nucleoporin 210kDa	NM_024923	2.3
9	De-etiolated 1	NM_017996	3.1	43	MutS homolog 3 (E. coli)	NM_002439	2.2
10	Catenin, beta like 1	AK091216	3	44	Cadherin 4, type 1, R-cadherin (retinal)	AL834206	2.2
11	Hypothetical protein FLJ20719	AK124686	3	45	Vitamin D (1.25- dihydroxyvitamin D3) receptor	NM_000376	2.2
12	Glutaredoxin (thioltransferase)	AK125636	3	46	Signal-induced proliferation-associated 1 like 1	AK122930	2.2
13	CGI-09 protein	AB032979	3	47	Protein kinase, lysine deficient 2	AB051547	2.2
14	Chromosome X open reading frame 1	NM_004709	3	48	Pantothenate kinase 2	NM_153638	2.1
15	Transcribed locus	BF337208	3	49	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7	NM_019886	2.1
16	Bol, boule-like (Drosophila)	NM_033030	2.8	50	WD repeat domain 6	NM_018031	2.1
17	Capping protein (actin filament) muscle Z-line, α 1	BX648738	2.7	51	Benzodiazapine receptor (peri) associated protein 1	AB014512	2.1
18	Fructose-1,6-bisphosphatase 2	NM_003837	2.7	52	Transmembrane protein 27	NM_020665	2.1
19	Immunoglobulin superfamily, member 10	NM_178822	2.7	53	Chromosome 6 open reading frame 47	BC012950	2.1
20	*LanC lantibiotic synthetase component C-like 2*	NM_018697	2.7	54	Homolog of yeast INO80	NM_017553	2.1
21	Dynein, axonemal, heavy polypeptide 8	AF356519	2.7	55	Myosin VA (heavy polypeptide 12, myoxin)	U90942	2.1
22	FLJ40142 protein	AK094845	2.6	56	MHC class II transactivator	NM_000246	2
23	Hypothetical protein FLJ10884	NM_019079	2.6	57	Chromosome 6 open reading frame 155	AK023251	2
24	Leukemia inhibitory factor receptor	NM_002310	2.6	58	Polymerase (DNA directed), gamma	BC050559	2
25	Peroxisomal biogenesis factor 7	BC031606	2.6	59	Twist homolog 1(Drosophila)	CR619156	2
26	Spermatogenesis associated 2	NM_006038	2.6	60	Hypothetical protein MGC11061	AK098486	2
27	Collagen, type IX, alpha 3	NM_001853	2.5	61	Chromosome 6 open reading frame 201	AK124114	2
28	Transition protein 2	NM_005425	2.5	62	Phosphodiesterase 11A	NM_016953	2
29	Src family associated phosphoprotein 2	NM_003930	2.5	63	Small nuclear ribonucleoprotein polypeptide F	CD388516	2
30	GLI-Kruppel family member GLI4	BM727419	2.5	64	Homo sapiens, clone IMAGE:4826152, mRNA	BM925744	2
31	Ankyrin repeat and SOCS box-containing 6	BX648923	2.4	65	H2A histone family, member V	BF573768	2
32	Guanylate cyclase activator 1A (retina)	BX647537	2.4	66	Upstream regulatory element binding protein 1	NM_031407	2
33	WD repeat and SOCS box-containing 1	NM_134264	2.4	67	plasmolipin	NM_015993	2
34	FGFR1 oncogene partner 2	AK094888	2.4	68	High-mobility group nucleosome binding domain 1	CR616640	2

concentrations of GST-QKI-5. GST-QKI-5 bound the selected RNA and formed slowly-migrating species in native gel electrophoresis (Figure 2.6C). The K_d for QKI-5 binding to the VEGF RNA sequence was ~146 nM, which is consistent with the previously reported relative binding of QKI (Galarneau and Richard 2005). The *in vitro* binding was validated *in vivo* by QKI-5 immunoprecipitation followed by RT-PCR from the U343 cells. Both VEGF and GAPDH mRNA were detected in the total cell lysate (TCL). These data demonstrate the specificity of the QKI-5 and VEGF mRNA interaction. Indeed, QKI-5 immunoprecipitated VEGF mRNA and not GAPDH mRNA control (Figure 2.6D).

2.5.7 Knockdown of QKI protein causes an increase in VEGF mRNA and protein levels

Regulation of VEGF expression occurs at two main levels: transcriptionally through the hypoxia-inducible factor (HIF)-1 or through marked stabilization of the normally labile mRNA (Bardos and Ashcroft 2005). Since VEGF mRNA interacts with the QKI protein complex as described above, we stably knocked down QKI by short hairpin RNA in U343 cells to determine the effect of QKI on VEGF mRNA and protein levels. We were able to achieve between 50-70% knockdown of all three QKI isoforms in the U343shQKI cells. Total RNA was isolated from these cells and reverse transcribed using random hexamers. The QKI and the VEGF mRNA levels in both cell lines were determined by real-time PCR using TaqMan probes. As expected we observed a reduction in the level of QKI mRNA of approximately 50% in the U343shQKI cells compared to the controls (U343pRS), which coincided with the observed decrease in

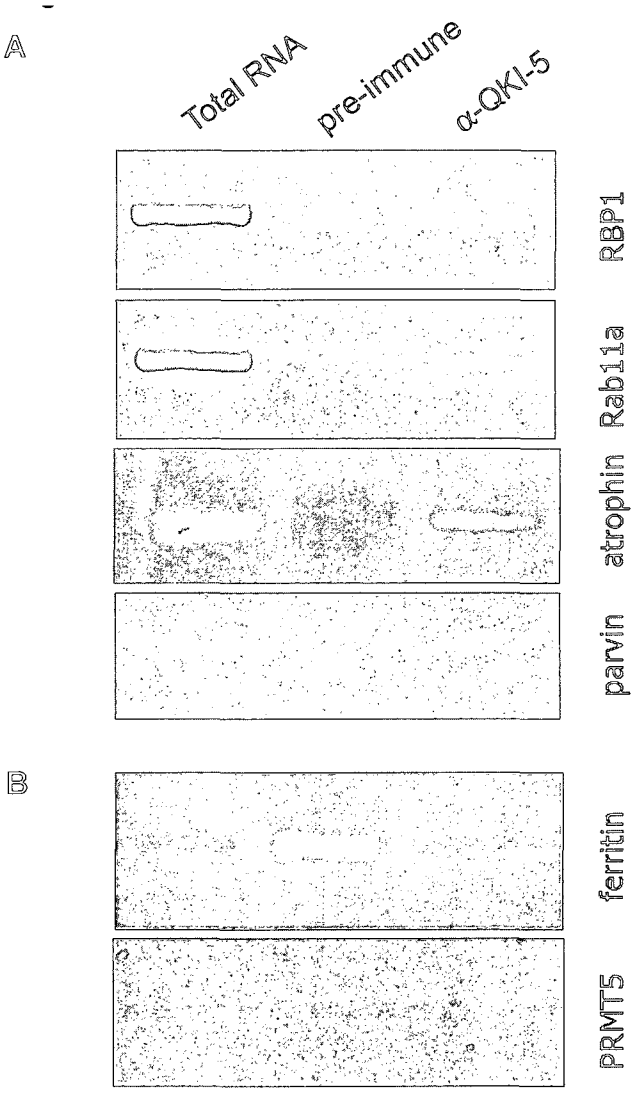

Figure 2.5 Validation of QKI microarray RNA targets

Immunoprecipitation with NRS (control) or a QKI-5 antibody followed by RT-PCR analysis of mRNA targets identified in the microarray analysis. (n>3) (B) Control experiments performed as above with a target identified to bind preferentially to the NRS, ferritin, or a negative control PRMT5.

protein levels (Figure 2.7 and data not shown). VEGF mRNA levels on the other hand showed an 11-fold increased in the U343shQKI cells (Figure 2.7).

Since VEGF plays a role in cell migration and cellular interactions with the endothelium and is a secreted, endothelial cell-specific mitogen (Dibbens et al. 1999), we determined the levels of secreted VEGF in the medium of the U87 knockdown and control cells by ELISA (Enzyme-linked immunosorbant assay). The ELISA kit recognizes the two main isoforms of VEGF [165, 121]. The QKI knockdown cells expressed 2-fold more VEGF protein in their supernatant when compared to the cells expressing the vector alone (Figure 2.7). These data show that QKI acts as a negative regulator of VEGF mRNA and protein expression.

2.6 *Discussion*

In this study, we present several lines of evidence demonstrating that QKI is found in a small mRNP complex. First, the three major isoforms of QKI are found within a complex that is approximately 400 kDa. Second, we identified several proteins within this complex that are involved in RNA metabolism. Thirdly, in addition to the numerous proteins found in the complex, we identified a large population of mRNAs, including VEGF mRNA that may be regulated by the QKI proteins. Finally, we characterized the binding of QKI to VEGF mRNA and show that QKI binds the mRNA in a sequence specific manner to regulate its mRNA and protein levels.

2.6.1 Quaking is present in a 400 kDa complex

We demonstrated both by gel filtration and native 2D-PAGE analysis that the QKI proteins are found in a rather small protein complex. Our proteomic analysis identified many potential components of the complex and this raised the question as to whether all the associated proteins are found within a single complex given the small size of the complex. It is possible that QKI is found in several different complexes at any given time, given that QKI has been shown to participate in several cellular processes. RNPs are generally defined not only by their components, but also by the size of the complex. This being said, QKI is present in a small complex of ~400 kDa and only a very small portion of QKI is found to sediment with the large mRNPs as has been demonstrated for many other KH containing RNA binding proteins such as Sam68 and FMRP (Paronetto et al. 2006; Stefani et al. 2004). RBPs that sediment with the 'light' mRNP fractions are hypothesized to regulate translation of the component mRNAs (Zalfa et al. 2006). Fragile X mental retardation protein (FMRP) for example, has been shown to

associate with both lighter mRNPs as well as with the heavy polyribosomes and mRNA granules (Brown et al. 2001). FMRP forms both hetero- and homo-dimers and has many co-associating proteins, reminiscent of QKI (Bardoni and Mandel 2002). This suggests that there are different QKI- or FMRP containing particles that play distinct roles in mRNA processing. Lin28, is a protein also detected within cytoplasmic granules as well as in the heavy polysome fraction bound to translationally competent mRNAs (Balzer and Moss 2007). In fact the role of RNPs in the movement of RNAs between the translational machinery and cytoplasmic granules has been well documented (Anderson and Kedersha 2006; Eulalio et al. 2007; Kedersha et al. 2005).

The identification of proteins involved in protein synthesis like PABP1, suggests that the QKI complex is involved in posttranscriptional regulation of the bound mRNA. In addition, the presence of RNA binding proteins involved in RNA stability and transport (hnRNP R and Q), and proteins involved in transport of other proteins, maintenance of protein conformation and folding and prevention of unwanted protein aggregation (HSP 70 protein 1L, 6 and 8, and GRP 78) suggests that the QKI complex is likely a dynamic mRNP that regulates several stages of mRNA processing and we believe that QKI can be found in several different complexes of protein and RNA.

2.6.2 Quaking and PABP associate via the KH domain and RRM 3/4

PABP consists of a highly conserved N terminus containing four tandem RNA recognition motifs (RRM) followed by a more variable C terminus (Sachs et al. 1997). The first two RRMs are sufficient for specific polyA-binding (Deardorff and Sachs 1997; Deo et al. 1999; Sachs et al. 1987). In addition, RRM2 promotes the interaction between PABP and eIF4G (Imataka et al. 1998; Kessler and Sachs 1998; Otero et al. 1999).

RRM4 is responsible for most of the nonspecific RNA binding of PABP (Deardorff and Sachs 1997; Kuhn and Pieler 1996) and in yeast the first half of RRM4 is sufficient to confer viability to cells depleted of the normal PABP gene (Sachs et al. 1987). The C-terminal region of PABP was previously reported to be involved in mediating protein-protein interactions (Melo E et al., 2003). Several PABP interacting proteins have been recently identified. Human ataxin-2 is involved in a role in RNA metabolism, the antiproliferative protein Tob that translationally suppresses the expression of IL-2 and DAZL a protein essential protein in gametogenesis, all bind to PABP via the PABPC domain (Collier et al. 2005; Okochi et al. 2005; Ralser et al. 2005). BRCA1 on the other hand, binds PABP via the RRM1-4 (Dizin et al. 2006). Our data demonstrating that PABP associates with QKI through its RRM3/4, is one of the first demonstrating protein-protein interaction for these two domains.

Although the KH domain and its flanking sequences have been shown to be important in RNA binding, there is also evidence that the KH domain of many STAR proteins (Sam68, GLD-1, GRP33 and QKI) is involved in protein-protein interactions. In Sam68 homo-oligomerization requires both the KH domain and cellular RNA (Chen et al. 1997; Lukong and Richard 2003). It is possible that in the case of QKI a short bridging RNA and is not digested by the RNAse treatment, mediating the QKI-PABP interaction. Interestingly, a mutant QKI protein (QKI:E48G) which cannot bind RNA due to its inability to homo-dimerize, was shown to bind the PABP constructs (Fig. 2.2A).

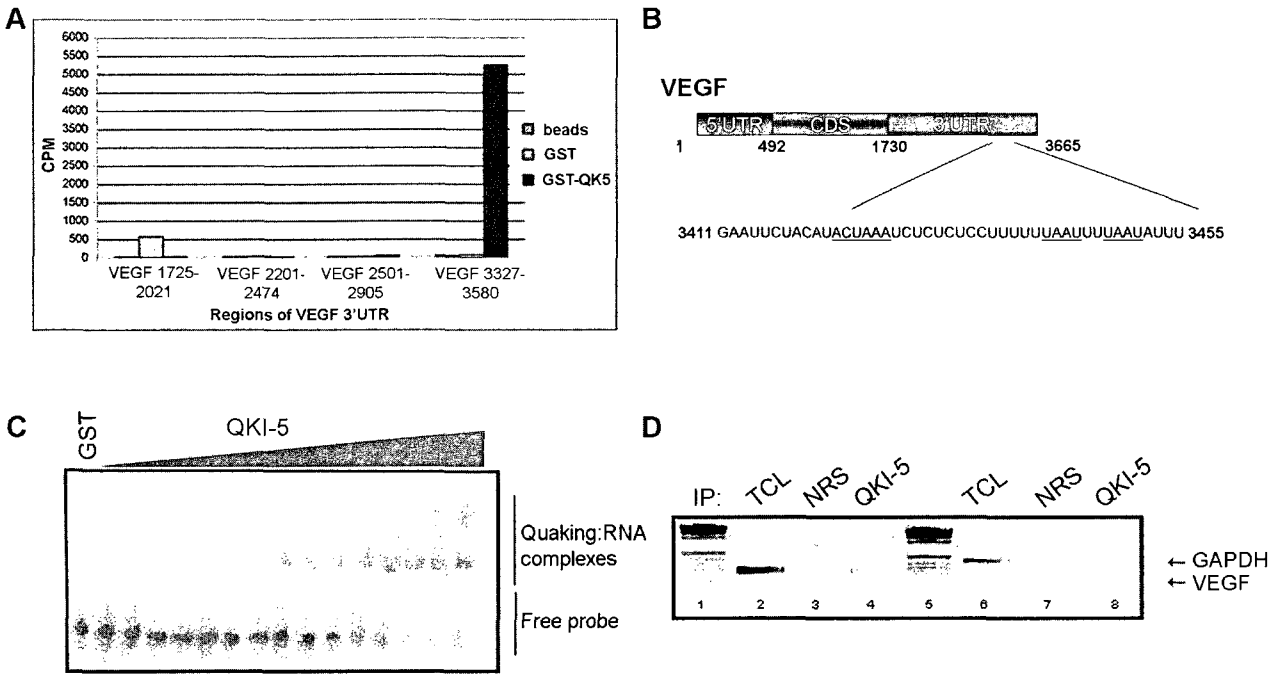

Figure 2.6 The QKI proteins associate with VEGF mRNA *in vitro* and *in vivo*.

(A) GST-tagged QKI-5 was incubated with ^{32}P-labeled RNA representing different regions of the 3′ UTR of VEGF mRNA. The reactions were washed, and the radioactivity associated with the proteins quantified. **(B)** Identification of the possible **Q**uaking **R**esponse **E**lement of VEGF. **(C)** Electromobility shift assay using the sequence VEGF 3227-3580 as a probe, with increasing concentrations of QKI-5 (factor 2; highest concentration 3.58μM). **(D)** Immunoprecipitation using either normal rabbit serum (NRS) or a QKI-5 specific antibody was performed in the U87 cell line. The association of QKI and VEGF mRNA was confirmed by RT-PCR. GAPDH served as a negative control.

Stress granules (SG) are cytoplasmic subdomains into which mRNAs are dynamically sorted in response to phosphorylation of eukaryotic intitiation factor (eIF) 2α, which is important for translational initiation (Kedersha et al. 2002). The recruitment and translational repression of the bulk of cellular mRNAs upon stress (Kedersha et al. 1999), formation of SGs and their subsequent disassembly requires numerous shuttling proteins, RNA binding proteins and ribosomal subunits. A host of RNA binding proteins including PABP, T-cell internal antigen-1 (TIA-1), TIAR (TIA-1 related protein) (Kedersha et al. 2000) and HuR are among those found within the SGs (Brennan and Steitz 2001). In the present study, we identified the QKI and PABP interaction in terminally differentiated, non-stressed cells and subsequently confirmed their colocalization *in vivo* in stress granules (Figure 2.3). In thse cells, PABP is found diffuse in the cytoplasm and subsequently becomes concentrated in SGs following induction of oxidative stress. Similarly, we observe that QKI-6, which is primarily cytoplasmic, also relocalizes from a diffuse cytoplasmic distribution to distinct cytoplasmic foci following arsenite treatment. This overlapping localization suggests that QKI and PABP could interact in the SGs. It is therefore conceivable that both QKI and PABP could be found together on an mRNA molecule in many physiological situations. BRCA1, which interacts with PABP through the RRM 1-4 motifs that play a role in translational regulation, is therefore suggested to participate in the regulation of mRNA translation (Dizin et al. 2006). In addition, it can also be speculated that the effect of BRCA1 on translation may depend on the physiological status of the cell, as defined by induction of the stress response, UV-induced DNA damages, or malignant transformation for example (Dizin et al. 2006).

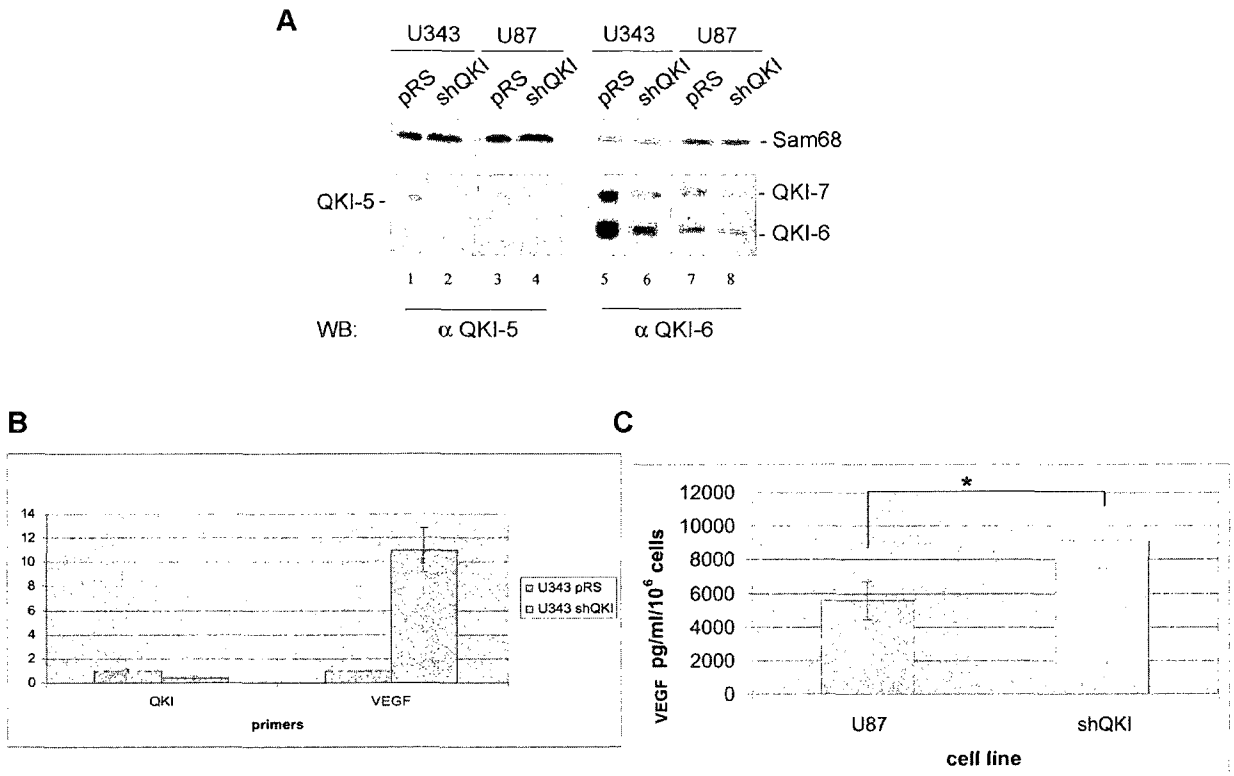

Figure 2.7 Reduced QKI expression upregulates the VEGF mRNA and protein levels.

(A) Stable cell lines were generated by transfecting U87 or U343 cells with either pRS alone or pRS shQKI followed by selection with puromycin. Cell lysates were resolved by SDS-PAGE and immunoblotted with either a polyclonal antibody against QKI-5 or a polyclonal antibody against QKI-6 that cross-reacts with QKI-7. **(B)** Real-time PCR analysis of Quaking and VEGF mRNA levels in the control and shQKI U343 cells. (n=3; primer = TaqMan probe used) **(C)** VEGF levels in the supernatant of U87 control and shQKI cells as measured ELISA (n=3).

2.6.3 QKI's mRNA targets

Our lab previously identified a bipartite consensus sequence of NACUAAY-N(1-20)-UAAY to which QKI binds with high affinity (QRE). A bioinformatics analysis of the mouse genome using this consensus identified ~1400 putative mRNA targets in the mouse (Galarneau and Richard 2005). We analyzed the mRNAs that we identified by microarray for the presence of the identified consensus site. For the purpose of this study, we considered the target to harbour the consensus site if it possessed only the core sequence and NACUAAY. This facilitated our analysis and was also done since we have shown that there is binding of QKI to target mRNAs that differ from the full consensus by 1 or 2 nucleotides (Galarneau and Richard 2005). Of our identified targets, 24% of the microarray targets (Table 2.3) and 20% of the neuroarray targets (Table 2.2) possessed the core consensus site. This suggests there are mRNAs that likely belong to other QKI-associated RNA binding proteins.

The QKI proteins have been extensively studied for their role in myelination and development in the qk^{v} mouse. It has been reported that the blood vessel defects observed in one of the ENU-induced mutants qk^{k2}/qk^{k2} were the cause of the embryonic lethality (Noveroske et al. 2002). Analysis of the *qkI* null mice revealed a lack of blood circulation in the embryonic yolk sac due to a thin vitelline artery lacking smooth muscle cells (SMC). The results from the *qkI* null mice indicate that the endothelial cells may secrete a factor that is required for endothelial cell differentiation into SMCs (Li et al. 2003). VEGF plays an important role in the differentiation and migration of endothelial cells and is expressed almost exclusively during embryogenesis, in wound healing and during reproductive functions in the adult (Ferrara and Davis-Smyth 1997). Early disruption of VEGF signaling through single allele inactivation leads to abnormal blood vessel

development and yolk sac defects. In addition the normal fusion of angioblasts and the spatial organization of the endothelial cells is affected (Carmeliet et al. 1996). The *qkI* null phenotype suggests that a loss of QKI expression would lead to a loss of a factor required for endothelial cell differentiation. This suggests that in terminally differentiated cells such as U343s, QKI may signal through different pathways or have another role of VEGF expression.

Understanding the protein-protein interactions as well as the target mRNAs can provide enormous amounts of information on the functions and pathways influenced by the QKI proteins. Considering there is downregulation of QKI mRNA targets in the cortex of schizophrenia patients and disregulation of *qkI* expression in glioblastoma cells, understanding the function of QKI in relation to its mRNA targets and interacting partners is becoming increasingly important. The data reported here provides necessary elements to examine the molecular basis of QKI involvement in numerous pathways and diseases.

2.7 *Acknowledgements*

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2.9 *Supplementary Information*

Supplementary Table 2.4 Primers used for RT-PCR

Gene	Direction	Sequence (5′ – 3′)
RBP1	Forward Reverse	tgtggacaagaccgaacactac aactgtgtagcagtggctttca
Rab11a	Forward Reverse	atagtccatttcccaggtctga acaggcttccatgatgaaaagt
Atrophin	Forward Reverse	tgcgcactctcagtgaatatg atgtaggggttcccagctcact
Ferritin	Forward Reverse	ctcttgcttcaacagtgtttgg aggtacaaattgaccaggctgt
VEGF	Forward Reverse	gggcagaatcatcacga tggtctgcattcacatttg
PRMT5	Forward Reverse	ggacctcattgtacagcttggag ctctcagtaccagcaggccatc
GAPDH	Forward Reverse	atggtagaaggtcggtgtcaac ttactccttggaggccatg

Supplementary Table 2.5 Substrates used for T7 RNA MegaShortscript

Gene	Substrate
VEGF	aaatattaaaattaaaaaa ggagagag atttagtatgtagaattcctatagtgagtcgtattaaatt

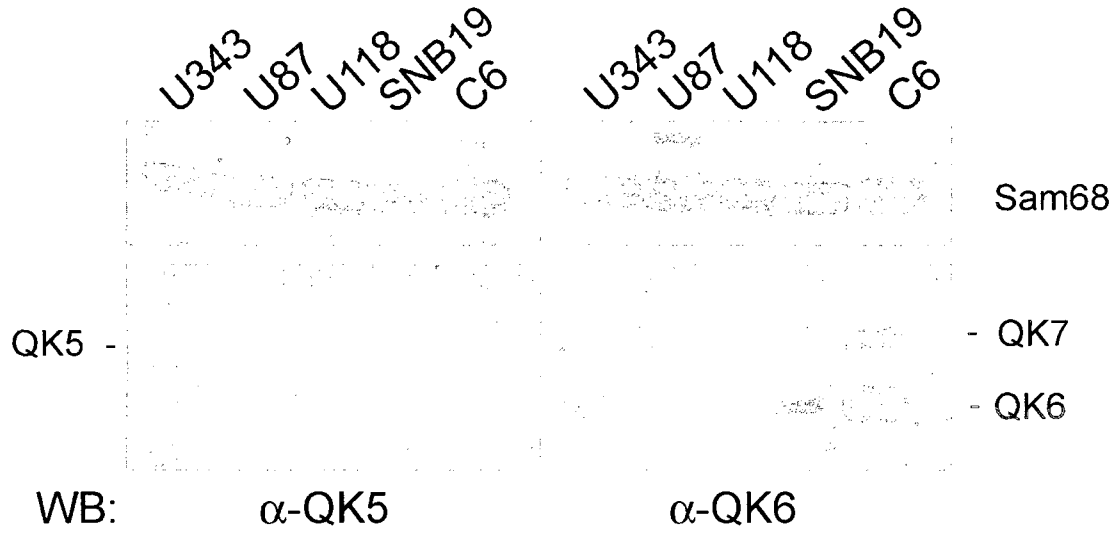
Supplementary Table 2.6 Primers for RNA binding assays

Name	Region	Primer
T7 VEGF	3′UTR 2201-2216	AATTTAATACGACTCACTATAGGGAGGATGTATTTGACTGC (Forward)
T7 VEGF	3′UTR 2474-2495	CCTGGTCAGAGCCGGTGTCCTC (Reverse)
T7 VEGF	3′ UTR 2501-2520	AATTTAATACGACTCACTATAGGGAGGGGAGCTTCAGGACATTG C (Forward)
T7 VEGF	3′ UTR 2905-2926	CTCCACAATGGGCACGTGGATC (Reverse)
T7 VEGF	3′ UTR 2904-2924	AATTTAATACGACTCACTATAGGGAGGATCCACGTGCCCATTGTGG (Forward)

T7 VEGF	3′ UTR 3409-3431	GAGATTTAGTATGTAGAATTCTC (Reverse)
T7 VEGF	3′ UTR 3227-3246	AATTTAATACGACTCACTATAGGGAGGCTCCCCAGCACACATTCC (Forward)
T7 VEGF	3′ UTR 3580-3600	ATCTGTATTTCTTTGTCGTTG (Reverse)

Supplementary Table 2.7 TaqMan real time PCR primers

Gene	**TaqMan number**	**Amplicon length (nt)**
Human QKI	Hs00287641_m1	75
Human VEGF	Hs00900054_m1	60
Human GAPDH	Hs99999905_m1	122



Supplementary Figure 2.8 Expression of the QKI isoforms in glioma cells

(A) Proteins from glioma cell lines were separated by SDS-PAGE and immunoblotted with either an antibody specific to the QKI-5 isoform, the anti-QKI-6 antibody (which recognizes QKI-7 also) or an antibody against Sam68 to serve as a loading control.

2.10 *Chapter Summary: The QUAKING RNA binding proteins interact with PABP protein and the VEGF mRNA: Defining the QKI complex*

- QKI is found in a small complex ranging in size from 232 kDa to 400 kDa. This is larger than simply QKI homo- or hetero-dimers and implies that there are other proteins found within the complex.
- All three major isoforms of QKI are found within the same protein complex as determined by native 2D PAGE analysis as well as FPLC. Previous evidence from our lab demonstrated that the QKI proteins can homo- and hetero-dimerize and that this is important for localization of target mRNAs (Pilotte et al. 2001), therefore it is logical that the isoforms are found in the same complex at one time or another *in vivo*.
- QKI is found in a protein complex with numerous proteins involved in posttranscriptional mRNA processing. These proteins include PABP, heat shock proteins, hnRNPs as well as proteins involved in the microtubule network and the cytoskeletal network.
- QKI and PABP interact with one another in an RNA-independent manner.
- The QKI-PABP interaction is mediated through the KH domain and the RNA binding domain 3 and 4 of QKI and PABP polypeptides respectively. There are few known proteins to interact with PABP through the RRM 3 and 4.
- Following arsenic-induced oxidative stress PABP relocalizes to cytoplasmic granules where it co-localizes with QKI-6. This represents a potential mechanism by which QKI-6 could mediate its translational inhibition, by relocalizing the mRNAs to which it is bound to translationally repressed granules. This

recruitment may perhaps be realized through protein-protein interactions similar to those observed with PABP.

- Microarray analysis identified approximately 68 mRNA targets of the QKI mRNP that were statistically significant ($p<0.05$) and bound >2-fold over the control immunoprecipitation. The microarray analysis allowed the confirmation of four new mRNA targets of QKI-5, Rab11a, RBP1, atrophin and parvin. The mRNAs are classified in several functional categories including intracellular trafficking, cell death and establishment of localization.
- Using a neurobiology filter array, an additional list of sixteen mRNA targets of the QKI-5 mRNP. Many of the identified targets were classified as cytokines, or to be involved in intracellular signaling and trafficking.
- Among the targets identified in the neurobiology array was VEGF mRNA. QKI-5 was shown to bind to VEGF mRNA *in vitro* and *in vivo* at the terminal portion of the 3′ UTR. The calculated K_d ~146nM was similar to the results obtained in previous studies (Galarneau and Richard 2005).
- Biologically, knock down of QKI expression in human glioblastomas causes and 11-fold increase in VEGF mRNA levels and a 2-fold increase in the secreted protein levels.
- The QKI isoforms are differentially expressed in human glioblastoma cell lines. All of the glioblastoma cell lines tested express the three isoforms of QKI but with differing levels.

Chapter 3

Novel Myelin Basic Protein Methyl-Specific Antibodies Reveal that Methylation of MBP by PRMT5 is Important for Myelination in the Central Nervous System

3.1 *Preface*

A role for protein arginine methylation in myelination was up until now largely unstudied due to limited availability of *in vitro* myelination sytems and post-mortem tissue. Our previous study aimed to identify new protein and mRNA partners of the QKI ribonucleotide protein complex. This chapter describes the generation of a methylspecific antibody to MBP and the characterization of methyl-MBP expression in the brain. In addition, we determine the methyltransferase responsible for the *in vivo* methylation of MBP, that has until now remained elusive.

3.2 *Abstract*

Multiple Sclerosis (MS) is a central nervous system (CNS) neurodegenerative disease characterized by the destruction of the myelin sheath. Myelin basic protein (MBP), a major protein component of myelin, represents the major candidate auto-antigen in MS. In the CNS, methylated MBP is found in areas of abundant myelination. *In vivo,* murine MBP contains a symmetrically dimethylated arginine at position 106. Recent studies indicate that there is an increase in the methylation status of MBP in MS patients when compared to unaffected patients and this post-translational modification of MBP may play an important role in the demyelination and autoimmune processes associated with MS. In order to distinguish between methylated and unmethylated MBP, we have developed a novel antibody reacting with the four methylated isoforms of MBP. We show that MBP is methylated by a member of the protein arginine methyltransferase (PRMT) family PRMT5. We observed that PRMT5 is temporally regulated during mouse brain development and that its protein expression coincides with the peak time of myelination. Using an adenovirus expressing recombinant PRMT5 we show that PRMT5 blocks the differentiation of primary rat oligodendrocytes. These results suggest that this post-translational modification plays a role in the development of mature myelin and has an important role in the pathogenesis of demyelinating diseases such as MS.

3.3 *Introduction*

Myelin is a multilamellar structure that covers and protects nerves and allows for efficient conduction of action potential down the axon (Hartline and Colman 2007). Myelin basic protein (MBP) is a structural myelin protein that accounts for approximately 30% of the total myelin protein in the CNS (Norton and Cammer, 1984). Its primary role is the stability and maintenance of the mammalian myelin sheath (Staugaitis, SM Bioessays, 1996). Myelin and its components are produced by oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system (PNS). In the CNS MBP is an essential protein for myelin formation (Moscarello MA, 1997), but its role in the PNS is uncertain most likely due to the presence of other structural proteins in the PNS (Boggs 2006). In mice, the most robust myelination occurs during the first few weeks after birth.

MBP is an extrinsic protein having domains in contact with the hydrophobic interior bilayer of the plasma membrane (Braun, 1984). Because of this, MBP is exclusively located at the cytoplasmic layer, likely as a dimer (Kim et al. 1997). The MBP gene is composed of seven exons (Pedraza et al. 1997). Mice and rats have four major MBP isoforms produced by alternative splicing of exons 2 and 6 with molecular masses of 21.5 kDa, 18.5 kDa, 17.0 kDa and 14.0 kDa. The relative amount of each of the isoforms in mature myelin is 1:10:3.5:35 respectively (Barbarese et al. 1978). In addition, MBP has never been crystallized, due to its myriad of post-translational modifications (Kim et al. 2003). The protein can adopt many different structures depending on the degree of post-translational modification. These post-translational modifications include those that reduce the net positive charge of the proteins such as serine/threonine phosphorylation (Zand et al. 1998), deamination and citrullination (Wood and Moscarello 1989). MBP is also modified by alanine acylation as well as both

mono and symmetrical arginine dimethylation (Baldwin and Carnegie 1971). As its name suggests, MBP is highly basic containing 19 arginyl and 12 lysyl residues. Because the interactions between the positively charged residues (arginine and lysine) of MBP and the negatively charged phosphate groups of the myelin phospholipids bilayer are essential for the structure of the compact myelin, any changes in the positive charge of MBP would affect the strength of this interaction (Cheifetz and Moscarello 1985). The positively charged histidine residues of MBP may also be involved in the modulating of the adhesive interface of the myelin bilayer similar to the trans-acting protein zero (P0) (Luo et al. 2007).

Protein arginine methylation of MBP has not been extensively studied. Although, changes in the level of MBP arginine methylation have been reported upon administration of vitamin B_{12} during treatment of subacute combined degeneration of the spinal cord (Kim et al. 1997) and in patients with multiple sclerosis. Methylation was shown to be upregulated during myelination and MBP from the most compact myelin has been shown to have higher methyl-acceptability (Cruz and Moscarello 1985). The enzymes that catalyze the addition of the methyl groups belong to a family of proteins called protein arginine methyltransferases (PRMTs). The PRMTs are classified into two groups: type I and type II enzymes. The addition of monomethyl arginine (MMA) and asymmetric dimethylarginine (aDMA) is catalyzed by type I family members which include PRMT1, PRMT2, PRMT3, PRMT4, PRMT6 and PRMT8 (Krause et al. 2007). Type II enzymes add MMA or symmetric dimethylarginines (sDMA). Members of this family include PRMT5, PRMT7 and the recently identified PRMT9, which possesses methyltransferase activity but is not related to PRMT1-8 (Krause et al. 2007).

Herein we report the generation of a polyclonal antibody that is able to recognize MBP methylated at R106. We describe the localization of endogenous methylated MBP to areas of high myelination in the brain such as the corpus callosum and the caudate putamen. *In vivo,* PRMT5 is the enzyme responsible for the methyl-MBP (meMBP) epitope. In myelinating glial progenitor cultures, ectopic expression of PRMT5 causes the cells to arrest in an immature state, with limited MBP expression. We also show that methylation of MBP is critical for the formation of mature myelin.

3.4 *Materials and Methods*

3.4.1 Antibodies

Peptides were synthesized at W.M. Keck Biotech Resource Center (New Haven, CT). Polyclonal antibodies were generated using New Zealand White rabbits injected with peptides coupled to keyhole limpet hemocyanin (Sigma-Aldrich, St. Louis, MO). The peptide used to generate antibodies against symmetrically dimethylated arginine 106 of MBP is as follows: peptide meMBP (SRRASVPSQGKGR*GLSLSR) where the R* is the arginine that contains an sDMA. The 9E10 anti-myc monoclonal antibody (mAb) was from the American Type Culture Collection (Manassas, VA). The polyclonal antibody was affinity purified over the antigenic peptide coupled to Affigel beads, eluted with 100 mM glycine pH 2.5, and concentrated by using Centricon columns (Millipore, Bedford, MA). The anti-Sam68 polyclonal antibody AD-1 has been described previously (Chen *et al.*, 1999b). The monoclonal antibody, anti-MBP was purchased from Covance Research Products (formerly Sternberger monoclonals; Berkeley, CA).

3.4.2 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA plates (*Costar*, Cambridge, MA) were coated with the indicated quantity of peptide in 50 µl of 50 mM carbonate buffer, incubated at 37°C for 30 min, and blocked with blocking buffer (1% bovine serum albumin, 5% sucrose in phosphate-buffered saline [PBS]). Primary antibodies were diluted at 1:1000 in dilution buffer (1% bovine serum albumin, 0.5% ovalbumin, 10 mM Tris pH 7.4, 150 mM NaCl) and added to the corresponding well followed by incubation at 37°C for 30 min. The plate was washed extensively with PBS containing 0.1% Tween. Goat anti-rabbit antibodies covalently

coupled to horseradish peroxidase (Cappel Laboratories, Durham, NC) were incubated at 1:1000 in dilution buffer at 37°C for 15 min. The plate was washed and developed using BM Blue POD substrate (Roche Diagnostics, Indianapolis, IN) and quantitated by using spectrophotometry at 405 nm.

3.4.3 In situ Chemical Cross-linking

SKN cells were concentrated into 0.5 ml of PBS, pH 8.0, by centrifugation and incubated at room temperature for 30 minutes with or without 1 mM bis(maleimido)hexane (BMH; Sigma and Pierce) as described elsewhere (Chen et al. 1997). Cells were pelleted by centrifugation and resuspended in 2X sample buffer (60 mM Tris-HCL [pH 8.0], 2% SDS, 2% dithiothreitol, 10% glycerol, 0.01% bromophenol blue, 0.25 mM phenylmethanesulfonyl fluoride and 2 mM sodium vanadate) and loaded onto SDS-13% polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-myc antibody (9E10).

3.4.4 Primary Rat OL Cultures and Adenovirus Infections

Cultures of OL progenitors were generated as described (Almazan et al. 1993). OL progenitors, also termed O-2A progenitors, were plated on 6-well dishes at a density of 15 x 10^3 cells/cm^2. In order to expand their number and prevent differentiation, the cultures were grown in media containing 2.5 nb/ml basic fibroblast growth factor (Peprotec) and platelet-derived growth factor AA for 4 days. Infections were performed as previously described (Larocque et al. 2002), Briefly, the cultures were infected with the indicated adenovirus co-expressing QKI-5 or PRMT5 from the TR5 promoter and GFP from the CMV promoter (Massie et al. 1998; Pilotte et al. 2001) or a control

adenovirus expressing GFP from the TR5 promoter and an adenovirus tTa that expresses the transactivator. A multiplicity of infection (MOI) of 100 was sufficient to infect about 80% of the cells (Larocque et al. 2002). After infection, the cells were grown in differentiation media containing bFGF, PDGF-AA, and T3 at 40 ng/ml. Morphological examination established that the progenitor cultures were essentially homogenous bipolar cells, and acquired ramified processes as they differentiated into mature OLs *in vitro*.

3.4.5 Tissue Processing and Immunolabeling of Brain Cross-Sections

For immunofluorescence studies, 30-day-old mice were treated intraperitoneal with sodium pentobarbital before intracardial perfusion at 40 ml with 0.1 M cold PBS and then 40 ml of ice-cold 4% paraformaldehyde in PBS, pH 7.4. The entire brain was removed and post-fixed in 4% paraformaldehyde at 4°C overnight, and then immersed in 30% sucrose until dehydrated (~ 2-5 days). Tissue blocks were embedded in OCT compound and frozen on dry ice. Serial coronal sections at a thickness of 15 μm were cut in a cryostat, collected on +/+ glass slides (Fisher) pretreated with gelatin-chromium sulfate, and stored at -70°C. Sections were pre-incubated for 30 min in 5% calf serum-1% Triton X-100 in PBS. This was followed by a 24 hr incubation in PBS-0.1% Triton with the MBP antibodies. The monoclonal anti-MBP antibody (1:1000, Covance Research Products) and anti-meMBP (1:200) were used for staining the MBP isoforms in the mouse brain cross-sections. Species-specific Alexa 546 (Molecular Probes) secondary antibodies diluted 1:400 in PBS:0.1%Triton were used for detection.

3.4.6 Immunofluorescence Microscopy

Transfection of SKN cells for immunofluorescence was achieved using LipofectAMINE PLUS (Invitrogen) according to the manufacturer's protocol by using 1 μg of DNA and 100 ng of siRNA. Cells were fixed with 1% paraformaldehyde in 1X PBS at pH 7.4 for 5 min at room temperature (RT) and permeabilized with 0.5% Triton X-100 in PBS for 5 min at RT. The cells were incubated with the primary antibodies at RT for 1 h in PBS. The cells were washed with 0.1% Triton X-100 in PBS and incubated with the appropriate secondary antibodies in PBS. Goat anti-rabbit coupled to Alexa 488 (Molecular Probes, Eugene, OR) and goat anti-mouse coupled to Alexa 546 (Molecular Probes) were used as secondary antibodies. The cells were washed again, mounted onto glass slides, and visualized with an Olympus immunofluorescence microscope.

3.5 *Results*

3.5.1 Specificity of the sDMA MBP antibody

MBP was the first protein identified to contain symmetrically methylated arginines (Baldwin and Carnegie 1971). To examine the *in vivo* methylation status of MBP, we generated an antibody that recognizes a symmetrically dimethylated arginine at position 106 (in humans arginine 107 is the methylated arginine; in mice/rats it is found at position 106). Rabbits were immunized with the peptide N-SRRASVPSQGKGR*GLSLSR where the R* is the arginine that contains an sDMA (Figure 3.1A), covalently coupled to keyhole limpet hemocyanin. The affinity-purified polyclonal antibody, named meMBP, recognized the methylated but not the unmethylated peptide (Figure 3.1B). As predicted by the peptide sequence, the antibody recognized four major murine isoforms separated from whole mouse brain lysates (Figure 3.1C), as did the commercially available monoclonal MBP antibody.

To further characterize the antibody, primary rat oligodendrocyte cultures were used. Oligodendrocyte precursors do not express MBP whereas oligodendrocyte progenitor cultures differentiated for 3 days begin to express MBP (Khorchid et al. 2002). To determine if the antibody was indeed methyl-arginine specific, we induced the oligodendrocyte progenitor cells to differentiate by removing the growth factors and subsequently treated the cells with either the drug vehicle dimethyl sulfoxide (DMSO, Figure 3.1D, lane 1) or with different concentrations of the methyl-thio-adenosine (MTA) methylase inhibitor (Figure 3.1, lanes 2 and 3). Treatment of oligodendrocytes with MTA causes a reduction in MBP methylation as determined by a loss of the meMBP epitope as determined by Western blot analysis (Figure 3.1D). In addition, amino acid substitution

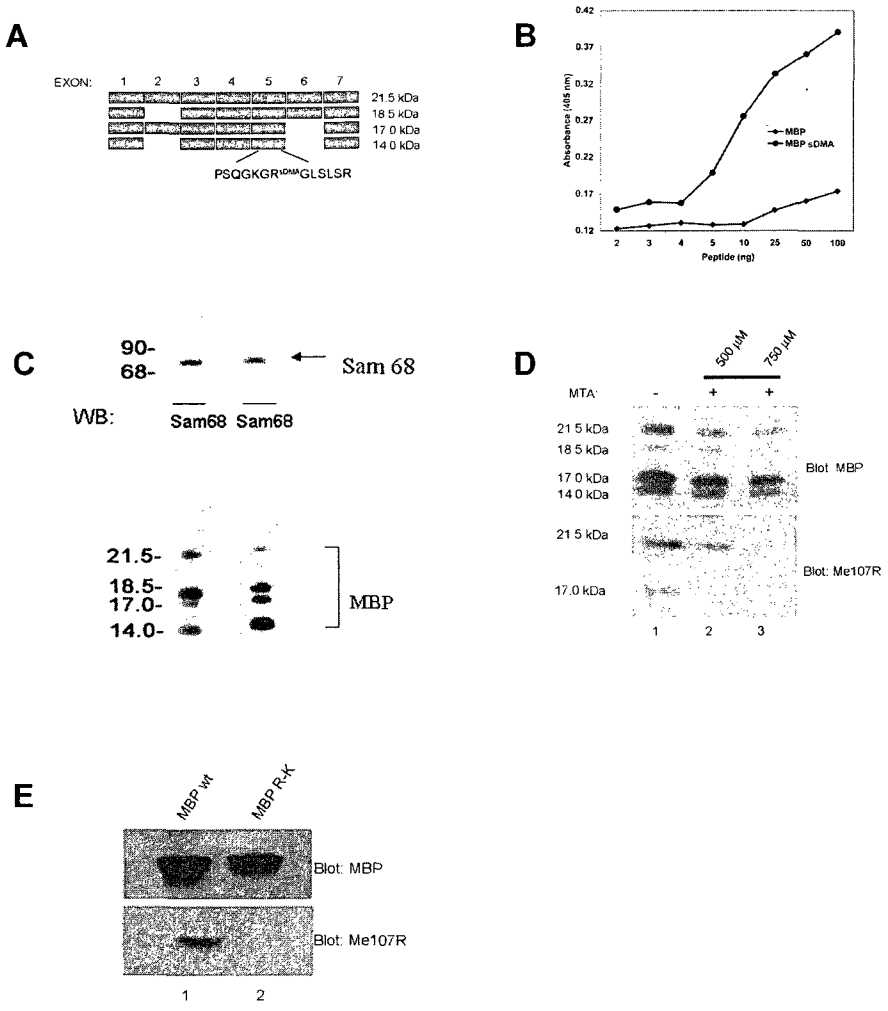


Figure 3.1 The meMBP antibody recognizes the 4 murine MBP isoforms

(A) Aligned sequences of the mouse MBP isoforms. The sequence of the peptide used to generate the methyl-MBP specific polyclonal antibody is shown **(B)** The specificity of the methyl-MBP antibody was examined by ELISA. The quantity of peptide is indicated on the abscissa and the absorbance on the ordinate. Note the purified anti-methyl-MBP antibody is specific for the sDMA methylated peptide and not the non-methylated peptide **(C)** Primary rat oligodendrocytes were grown in culture from the progenitor stage in the presence or absence of the methyltransferase inhibitor MTA. The cells were lysed and proteins separated by SDS-PAGE and immunoblotted using the monoclonal α-MBP (SMI99) or α-meMBP antibodies. **(D)** HEK 293 cells were transfected with either wild type (wt) 21.5 kDa MBP or 21.5 kDa MBP with arginine 106 substituted with a lysine (R-K). The lysates subjected to SDS-PAGE and immunoblotted with α-MBP or α-meMBP antibodies.

at position 106 of the arginine to a lysine in the 21.5 kDa isofom abolishes recognition of the mutant protein by the α-meMBP antibody isofom abolishes recognition of the mutant protein by the α-meMBP antibody (Figure 3.1E). These data therefore validate the specificity of the novel meMBP antibody.

3.5.2 The methylation state of MBP does not affect its ability to self associate

Studies of several RNA-binding proteins have demonstrated that methylation can influence protein-protein interactions (Bedford et al. 2000; Brahms et al. 2001; Friesen et al. 2001). MBP is has been shown to homodimerize and its self-association is proposed to be functionally relevant to the formation of CNS myelin. To examine whether or not methylation of MBP will affect its ability to dimerize, myc-tagged wild-type MBP and the R106K mutant of the 21.5 kDa isoforms were transfected into SKN cells. 24 hours post-transfection, the cells were treated or not with BMH and lysed in sample buffer and complexes separated by SDS-PAGE and immunoblotted with an antibody against the myc-tag. In addition to the 22 kDa band representing monomeric MBP, a band with an apparent molecular weight of 45 kDa was observed in the lysates treated with BMH (Figure 3.2). However, it is possible that the myc-tag is affecting the association, although previous studies using Sam68, have shown that Sam68's self-association using the same technique is not infuenced by the presence of a myc-tag (Chen et al. 1997).

3.5.3 Expression of methylated MBP in the brain and in cultured oligodendrocytes

In order to examine the localization of endogenous methylated MBP *in vivo,* coronal cross sections were made in the brains of 11 day old mice. Both the meMBP

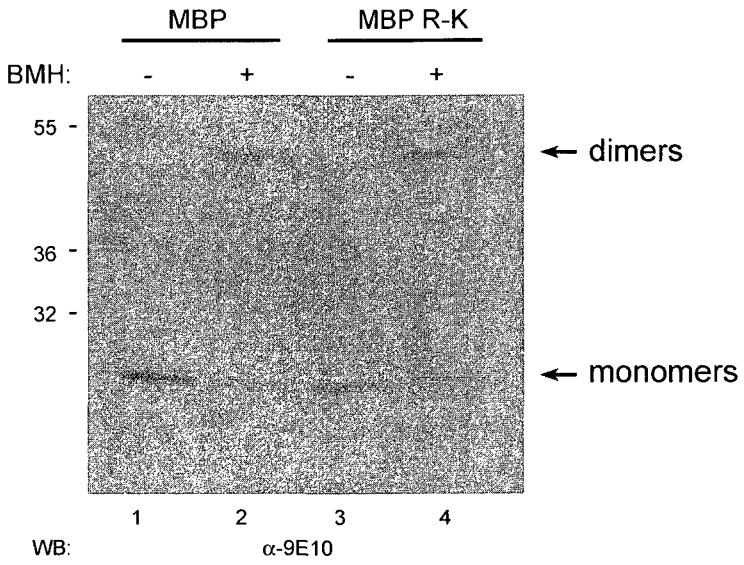


Figure 3.2 Arginine methylation does not affect MBP dimerization

SKN cells are mock treated (lanes 1 and 3) or BMH-treated in situ. The cells were lysed in sample buffer and separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-9E10 antibodies.

antibody and a monoclonal MBP antibody were used. Both antibodies stained myelin tracts of the corpus callosum (Figure 3.3). In addition, the antibody recognized other areas of high myelination such as the caudate putamen (data not shown). The localization of the methyl-MBP to the myelin tracts suggests that modified MBP is incorporated into the mature myelin sheath.

3.5.4 MBP is methylated by PRMT5

The identity of the methyltransferase that is responsible for MBP methylation is not known. It has long been assumed that PRMT5 was the enzyme since until recently it was the only known type II enzyme. In order to determine if PRMT5 was the enzyme responsible for the methylation of MBP at position 106, we applied small interfering RNA (siRNA) technology to knock down endogenous PRMT5 levels in HEK293 cells. The cells were transfected with a MBP expression construct as well as a control siRNA or a siRNA directed specifically to target PRMT5 mRNA. Cell extracts were prepared 72 hours posttransfection and the levels of PRMT5, total MBP and methylated MBP examined by immunoblotting (Figure 4A). Introduction of siRNA against PRMT5 caused a decrease in the levels of PRMT5 protein compared to control siRNA (Figure 3.4A compare lanes 1 and 3 to lanes 2 and 4). In addition, in cells with reduced PRMT5 levels a similar reduction in meMBP reactivity is seen when equivalent total MBP is present (Figure 3.4A compare total MBP to meMBP).

We have previously shown that PRMT5 is responsible for generating a specific pattern of methylated proteins using an antibody generated against symmetrically methylated arginines (SYM10) (Boisvert et al. 2002). When the above cell lysates were probed with SYM10 a striking reduction of the SYM10 signal in cells treated with siRNA

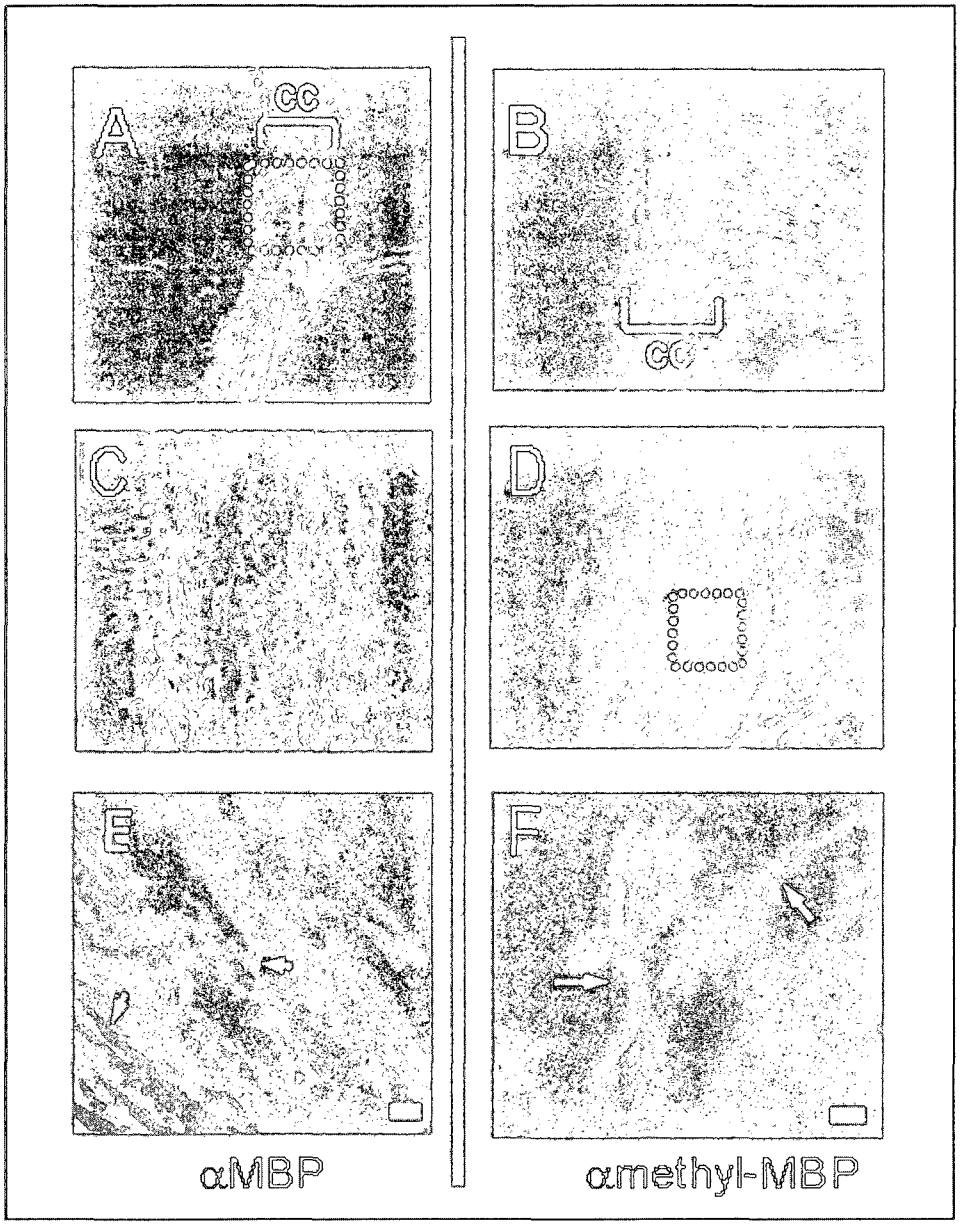


Figure 3.3 Methylated MBP is found in areas of abundant myelination

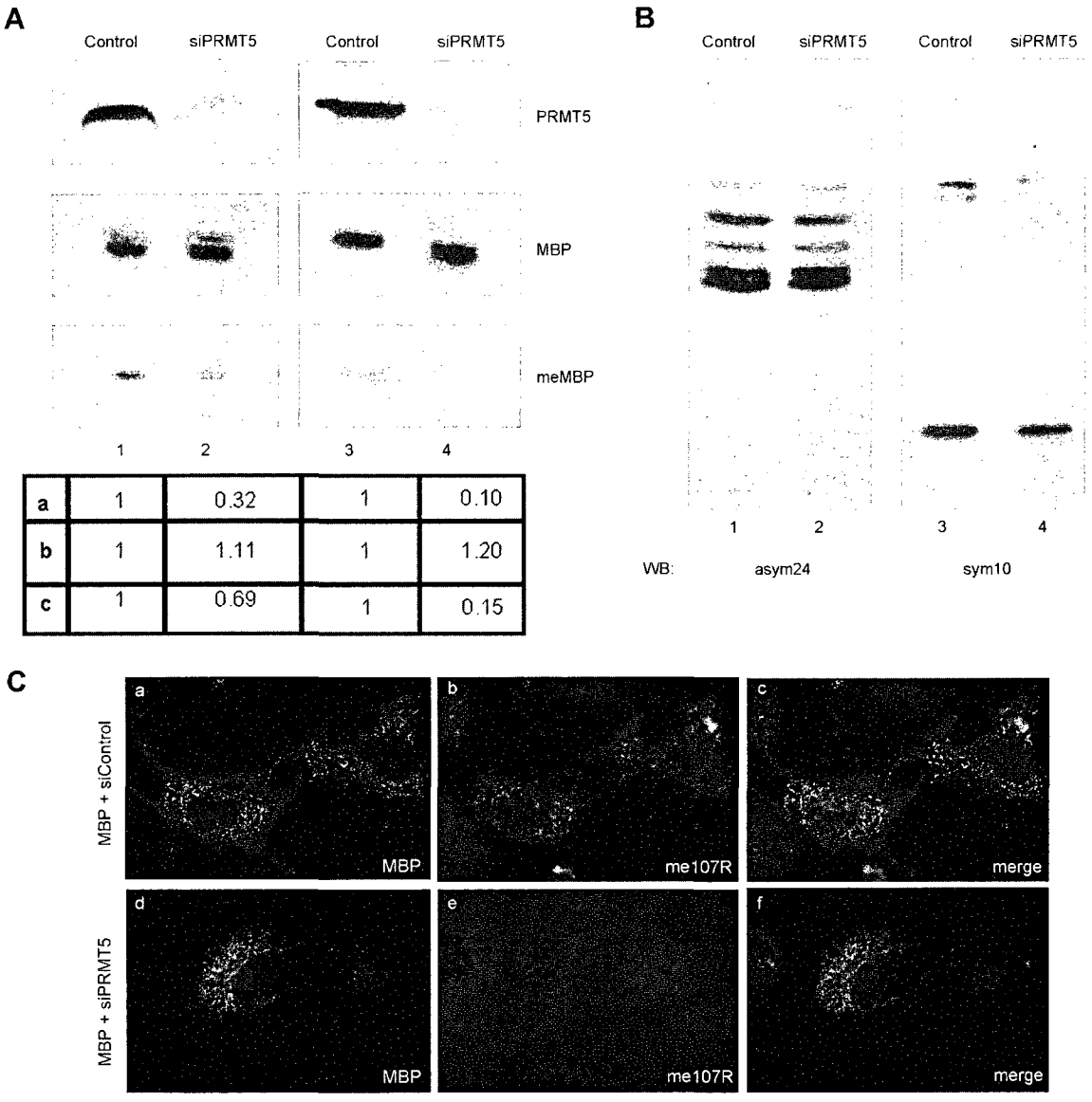
Coronal sections through the corpus callosum (cc) of postnatal day 11 wild-type mice were immunostained with anti-MBP and anti-methyl MBP antibodies followed by a secondary antibody conjugated to rhodamine. The staining was visualized by confocal microscopy (panels A,C,E) or fluorescence microscopy (panels B,D,F). Panel C and F represent amplifications of the regions that are boxed in panels A and D respectively. Panel E represents a high magnification of the corpus callosum. The bar scale represents 10 μm.

against PRMT5 was observed (Figure 3.4B, lanes 3 and 4), compared with an antibody against asymmetrically methylated proteins (ASYM24) (Figure 3.4B lanes, 1 and 2).

Ectopic expression of MBP isoforms in non-myelinating cells has shown that the 21. 5 kDa and 18.5 kDa isoforms are found primarily in the cytosol. Due to the difficulty in transfecting primary oligodendrocyte cultures, we used SKN cells to examine PRMT5 methylation of MBP in cell cuture. We transfected SKN cells with the 21.5 kDa isoform of MBP along with either a control siRNA or an siRNA directed agains PRMT5. When MBP is expressed with the control siRNA, both the monoclonal MBP antibody and the meMBP antibody recognize a granular staining pattern, as previously described (Staugaitis et al. 1990). This is in contrast to the staining observed when MBP is transfected along with the siRNA specific to PRMT5. Although we see a similar staining pattern as the control with the MBP antibody, we see an almost complete loss of the meMBP signal. These data as a whole suggest that PRMT5 is responsible for the symmetrical methylation of MBP in cell culture.

3.5.5 Ectopic expression of PRMT5 in oligodendrocyte precursors causes a block in differentiation

In order to determine the role of PRMT5 in myelination, we generated an inducible adenoviruses that expresses an inducible myc-tagged PRMT5 (AdPRMT5) as well as GFP, which is constitutively expressed from the virus. Initial tests were performed in 293 cells to ensure the inducibility of the protein expression. Induction of a myc-tagged PRMT5 is seen when AdPRMT5 is co-infected with the tetracycline transactivator (tTa) and is not seen in its absence (Figure 3.5A). We also used a previously generated QKI



a	1	0.32	1	0.10
b	1	1.11	1	1.20
c	1	0.69	1	0.15

Figure 3.4 MBP is methylated by PRMT5

(A) MBP along with either a control siRNA or an siRNA directed against PRMT5 was transfected in 293 cells. After 48 hours proteins were separated by SDS-PAGE and subjected to immunoblotting using antibodies against PRMT5, MBP and meMBP. **(B)** The lysates used above were also immunoblotted with anti-sDMA (SYM10) and an anti-aDMA (ASYM25) antibody. **(C)** Control siRNAs (panel A-C) or siRNAs engineered to specifically target PRMT5 mRNAs (panels D-F) were co-transfected with a myc-tagged MBP construct in SKN cells. 72 hours after transfection, cells were fixed, permeabilized and stained for immunofluorescence using an antibody against the myc tag (panel A and D) or methyl-MBP (panel B and D).

adenovirus as a positive control (Pilotte et al. 2001). In addition, myc immunoprecipitations were performed followed by *in vitro* methylation assays to assess the activity of induced PRMT5 (supplementary Figure S1).

To examine the contribution of PRMT5 to the development of mature oligodendrocytes and therefore indirectly to the process of myelination, oligodendroctye precursors (OPC) were co-infected with AdPRMT5 and tTA. MOIs sufficient to transduce about 85% of the cells as shown by the expression of the green fluorescent protein (GFP) were used. The presence of the tTA is necessary to drive the expression of the inducible PRMT5. At the time of infection, the OPCs were changed to differentiation media and allowed to differentiate for 72hrs, and then either fixed in 4% paraformaldehyde and permeabilized for immunofluorescence analysis with an anti-MBP antibody (Figure 3.5B) or lysed in Laemmli buffer for SDS-PAGE analysis (Figure 3.5C). The OPC that were infected with AdPRMT5 and tTa, displayed much less MBP expression as demonstrated by immunofluorescence (Figure 3.5B) and confirmed by western blotting for total MBP expression (Figure 3.5C). This result suggests a defect in OL maturation since OL differentiation is accompanied by expression of major myelin proteins such as MBP and myelin associated glycoprotein (MAG) (Khorchid et al. 2002) as well as enzymes such as 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase). In contrast, the expression of vimentin is high in glial progenitor cells, and decreases upon differentiation into galactocerebroside-positive OLs (Raff et al. 1984). In agreement with the observed decrease in MBP levels and a block in differentiation, we observed a decrease in CNPase and no loss of vimentin expression (Figure 3.5C) in the cells infected with AdPRMT5. These data confirm there is a block in OPC maturation upon ectopic expression of PRMT5 by adenoviral infection.

3.6 *Discussion*

In the current study, we investigated the expression of methylated MBP isoforms using a new arginine methylation specific MBP peptide antibody. The antibody was able to recognize the four murine isoforms isolated from whole brain lysates. In addition, we observed the localization of methylated MBP to areas of high myelination, such as the corpus callosum, the corpus callosum horn and the caudate putamen. We identified PRMT5 as the methyltransferase responsible for the *in vivo* methylation of MBP. The ectopic over-expression of PRMT5 in oligodendrocyte progenitors caused a block in differentiation as observed by the absence of cell markers of mature oligodendrocytes. Our findings are the first to observe the localization of methylated MBP *in vivo* and have resulted in the generation of a tool that will allow the study of methylation of MBP in disease pathology.

The molecular and physiological relevance of protein arginine methylation is poorly understood. One of the major pitfalls in the understanding of protein methylation is the limited knowledge on the regulation of the methyltransferases. Many cellular targets are arginine methyated, including several that are involved in nucleic acid metabolism. Methylation of the transcription factor CBP/p300 can modulate its ability to influence transcription (Xu et al. 2001), methylation of Stat proteins on a conserved arginine residue influences its cellular localization (Mowen et al. 2001) and nucleosome methylation affects promoter assembly and transcription (Chen et al. 1999; Schurter et al. 2001).

Although the role of arginine methylation of MBP in the formation of myelin has yet to be determine, it has long been assumed that arginine methylation of MBP plays an important role in the structural integrity of the myelin sheath. These hypotheses are

supported by studies in mice with subacute combined degeneration (SCD), which is characterized by the degeneration of the posterior and lateral columns of the spinal cord. When the disease is induced, and their diet supplemented with methionine, they are free of any detectable changes in the spinal cord (Scott and Weir 1981). Furthermore, cycloleucine was shown to induce changes indistinguishable from SCD (Jacobson et al. 1973). Cycloleucine is an analogue of methionine that inhibits the biosynthesis of S-adenosyl-L-methionine, the most important biological methyl-donor (Kim et al. 1986). In humans, treatment of cancer with cycloleucine resulted in symptoms of demyelination (Aust and Roux 1965). More recently, studies of MBP samples from MS patient samples show that there is an increase in overall MBP methylation in MS brains and that methylation of MBP was increased prior to the appearance of symptoms (Kim et al. 2003).

MBP plays a role in the adhesion of intracellular surfaces of compact myelin through protein-protein and/or protein-lipid interactions by interacting electrostatically with acid lipids and subsequently causing the aggregation of lipid vesicles (Boggs et al. 1997). MBP is also involved in the compaction of the lamellar structure of CNS myelin by forming the extra-cytoplasmic surfaces that interact to form the intraperiod line. MBP is an extrinsic protein having domains in contact with the hydrophobic interior layer of the bilayer. MBP is located exclusively at the cytoplasmic layer, likely as a dimer, with a head to tail orientation (Kim et al. 1997). Since arginine methylation has been shown to influence protein-protein interactions of many different proteins, we investigated the effect of methylation on the ability of MBP to dimerize. We did not

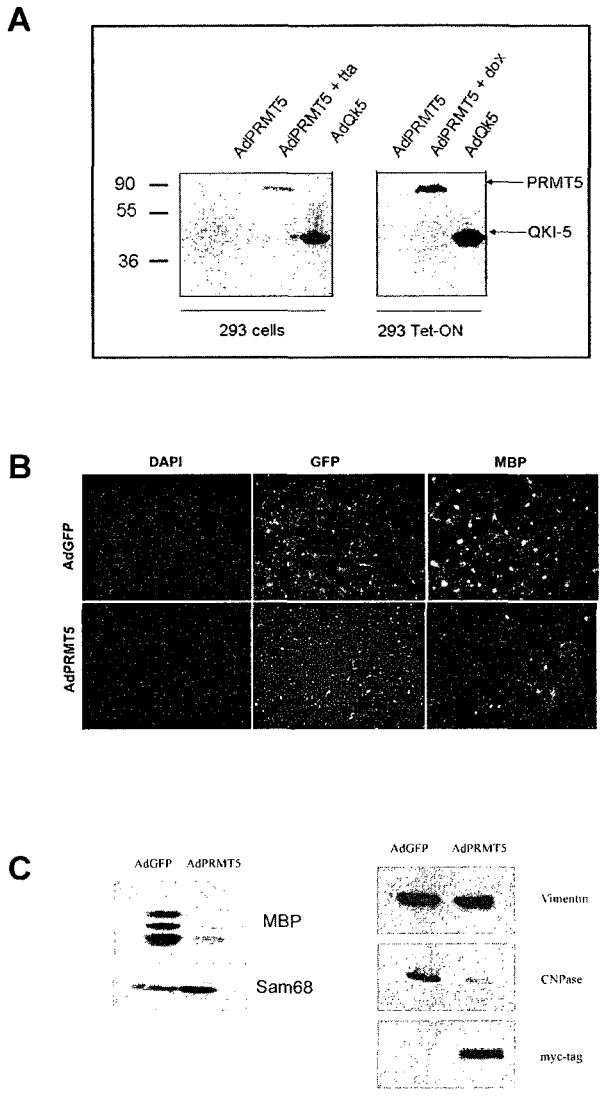


Figure 3.5 Ectopic expression of PRMT5 causes a block in oligodendrocyte differentiation

(A) 293 cells or 293 tet-on cells were infected with either an adenovirus encoding GFP QKI-5 or PRMT5 along with tTa or tetracycline. The proteins were separated by SDS-PAGE and immunoblotted with an antibody against the myc-tag. **(B)** Oligodendrocyte precursors were infected with an adenovirus encoding for PRMT5 or for GFP and allowed to differentiate for 72 hours. Infection of oligodendrocyte precursors with the adenovirus encoding PRMT5 causes a reduction in the amount of MBP as visualized by immunoblotting and by immunofluorescence **(C)** In addition the level of CNPase is also decreased but not the levels of vimentin.

observe any difference in the ability of either wild-type or a mutant form of MBP, which cannot be arginine methylated, to form dimers. In fact, when treated with the cross-linking agent, the majority of both forms of MBP were found in dimers.

In mice, PRMT5 activity in the brain increases during the peak period of myelination, days 15-18 of life (Campagnoni et al. 1984). This observation suggests that methylation is important in the myelination process. Our study shows that methylated MBP is indeed found within mature myelin and supports the hypothesis that methylation of MBP is required for the formation of a compact myelin sheath. Further studies have been hindered by the fact that the PRMT5 knock-out mice are embryonic lethal (Boisvert et al. 2005; Krause et al. 2007).

MBP is an unusual protein in that it belongs to a group of proteins considered to be intrinsically disordered. The protein is able to adopt multiple conformations dependent on to the myriad of post-translational modifications. As an important structural protein component of myelin, the interaction between the positively charged residues with the negatively charged phospholipids bilayer is important for the generation of compact myelin. Changes in the charge of MBP through post-translational modifications could affect the strength of these interactions. Methylation of arginine residues does not change the charge of the protein, but alters its bulkiness, its ability to hydrogen bond and it increases its hydrophobicity (Boisvert et al. 2005). In addition, arginines that are methylated are less able to be cleaved by the PAD enzymes (Pritzker et al. 2000), suggesting that arginine methylation protects MBP from deimination. This has implications both for the biological myelination process and the physiology of demyelinating diseases. These findings suggest that methylation of MBP is required for interactions that are essential for the formation of the myelin sheath and that if these

interactions are blocked, due to a decrease in methylation or an increase in deimination, they can result in loss of myelin stability.

It should be noted that the MBP isoforms have differential expression pattern. The exon-2 containing isoforms, 21.5 kDa and 17.0 kDa are distributed diffusely in the cytoplasm and the nucleus and this resembles the distribution pattern in the early-stages of myelination (Pedraza et al. 1997). In contrast, the 18.5 kDa and 14.0 kDa MBP isoforms are confined to the plasma membrane and resemble the pattern in late myelination (Akiyama et al. 2002). The expression of the predominant isoforms in mature myelin, 18.5 kDa and 14.0 kDa, has been shown to play a role in the maintenance of the structural integrity of myelin (Allinquant et al. 1991). It has been suggested that exon-2 containing isoforms normally appear earlier during myelination and those isoforms are re-expressed in remyelination lesions in MS patients (Akiyama et al. 2002; Capello et al. 1997). Our data shows that during OL differentiation, exon 2 containing isoforms appear to be methylated earlier than the other two isoforms (Figure 3.1C). We suggest that arginine methylation occurs co-translationally (Chenard, CA and Richard, S unpublished data) and that the major 14.0 kDa myelin localized isoform, is methylated at the myelin sheath just prior to being incorporated into the myelin.

Over-expression of PRMT5 causes a dramatic differentiation block in oligodendrocyte progenitor cells and an apparent arrest in cell cycle (Figure 3.5) as previously reported (Krause et al. 2007). Cell cycle exit is required prior to oligodendrocyte differentiation and requires several different proteins including cell cycle inhibitors such as $p27^{kip1}$ (Durand and Raff 2000). This cell cycle block is in contrast to what is observed in yeast, where ectopic expression of Hsl7p promoted cell growth (Fujita et al. 1999). PRMT5 activity requires two other proteins, MEP50 and pICln, and

perhaps the over-expression of one of the components acts as a dominant negative in this case. This artificial block in cell cycle does not allow the coordinated differentiation process to proceed and we do not observe the appearance of the known markers of differentiation, such as MBP and CNPase.

In summary, our findings are the first to examine methylated MBP *in vivo,* using a methyl-specific antibody. We were able to show that MBP is methylated by PRMT5 and that methylated MBP is localized in mature myelin. Over-expression of PRMT5 caused a differentiation block and a cell cycle arrest in oligodendrocytes and further analysis of PRMT5's regulation during myelination is necessary. The tools described in this article will allow the future analysis of samples from different murine models of demyelination, from demyelinating diseases as well as from MS patients.

3.7 *Acknowledgements*

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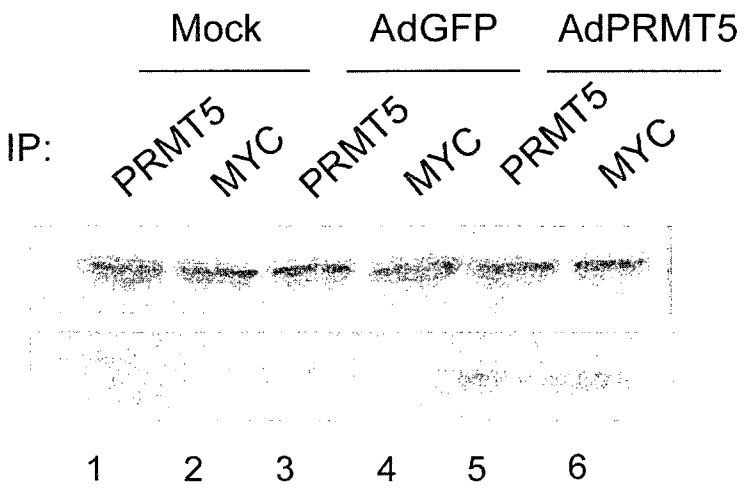
3.9 *Supplementary Data*



Supplementary Figure 3.6 Activity of AdPRMT5

CRL2097 cells were either mock infected (lane 1, 2) or infected with an adenovirus encoding GFP (lane 3. 4) or myc-tagged PRMT5 (lane 5, 6) for 48 hours. Cell lysates were split and immunoprecipitated with an antibody against PRMT5 or against the myc-tag. Immunoprecipitated proteins were incubated with bovind MBP as an exogenous substrate in the presence of (methyl-3H)-SAM. Proteins were resolved by SDS-PAGE, stained with Coomassie blue (top) and visualized by fluography (bottom).

3.10 *Chapter Summary: Study of expression of arginine methylated murine myelin basic proteins (MBPs) using a novel antibody reacting with arginine methylated isoforms*

- A novel peptide antibody against arginine methylated MBP was developed. This antibody recognizes the four murine isoforms of MBP when they are arginine methylated.
- *In vivo,* methylated MBP is found in areas of high myelination such as the corpus callosum and the caudate putamen.
- The methyltransferase responsible for the methylation of MBP *in vivo* was identified to be PRMT5. Biologically, when PRMT5 is knocked down, there is a loss of the meMBP epitope.
- We generated an inducible adenovirus encoding a myc-tagged PRMT5 that also constitutively expresses GFP.
- Over-expression of PRMT5 in oligodendrocyte progenitor cells causes a block in differentiation. This was observed by a decrease in MBP by immunofluorescence, and the presence of bipolar morphology of the cells. In addition, a decrease in MBP and CNPase protein levels was observed by SDS-PAGE, suggesting that the cells were blocked early in the differentiation process.

Chapter 4

General Discussion

4.1 *New roles for the QKI proteins*

For the past 45 years, QKI has been studied for its role in the processes of development and CNS myelination using the *qk*^{v} mouse. The presence of a single KH domain and the recent identification of a QKI high-affinity binding site for QKI in mRNAs suggests that it can bind to and regulate mRNAs through processes such as stability (Larocque et al. 2005), splicing (Wu et al. 2002) and transport (Larocque et al. 2002). As a member of the multifunctional STAR RNA binding family of proteins the QKI isoforms may also be involved in cell signaling pathways. In the oligodendrocytes of the CNS, the QKI proteins bind to myelin component mRNAs, such as MBP, then heterodimerize and shuttle it's target mRNAs from the nucleus to the cytoplasm where these mRNAs are transported to the distal tips for local translation and incorporation into the myelin sheath.

The involvement of QKI in all of these processes led us to examine both the protein partners and the mRNA targets of the QKI complex to identify new potential pathways regulated by QKI. In doing so, we identified a novel direct protein-protein interaction with PABP and for the first time described the relocalization of QKI to cytoplasmic granules following oxidative stress. In addition, *in vivo* mRNA interaction studies were performed and resulted in the identification of approximately 100 new mRNA targets in human glioblastoma cells. One of the identified targets was VEGF mRNA and this not only implicated QKI for the first time in the process of human cancer

angiogenesis, but also provided a rationale for vascularization defects observed in some of the *qkI* mutant mice (Bohnsack et al. 2006; Li et al. 2003).

We also examined the arginine methylation of MBP and its role in myelination. We describe a novel antibody that is able to recognize the MBP isoforms in their methylated state and show that methylated MBP is localized to areas of active myelination. We were also able to show that MBP is methylated by PRMT5 and that ectopic expression of the methyltransferase in primary oligodendrocytes causes an arrest in their differentiation.

4.2 *Involvement of QKI proteins in posttrancriptional gene regulation*

The regulation of gene expression occurs at multiple stages and posttranscriptional regulation involves the recruitment of numerous RNA binding proteins at the various steps. Early embryonic development is particularly dependent on posttranscriptional modifications of RBPs that regulate temporal and spatial gene expression (Lee and Schedl 2004). This regulation could occur at several levels such as during splicing, nuclear export, localization, stability and translation, and involves a host of RBPs including QKI (Lee and Schedl 2004).

4.2.1 Stability and translational control

The stability of mRNAs is tightly regulated and the half lives of diffrent mRNAs varies greatly in eukaryotes (Peltz et al. 1991). In vertebrates, the half-life of messages can range from 20 minutes to over 24 hours and 1000-fold differences in cellular abundance of mRNA species can result from minor differences in half-lives (Ross 1995). Proteins that interact with the messages can either help in the degradation process or

selectively stabilize the mRNA. The regulated control of mRNA stability is achieved through the orchestrated interaction of structural elements of RNAs and specific *cis*- and *trans*-acting factors (Shim and Karin 2002). These elements include the 5′-cap structure, 5′ UTR, the coding region, the 3′ UTR and the 3′ poly-A tail. The bioinformatic analysis with the bipartite motif identified several putative target mRNAs and the location of the QRE varied within the transcripts. The QRE is found within the 5′ UTR (12.7%), the CDS (36.2%) and within the 3′ UTR (51.1%) of transcripts. But how does QKI's interaction with its target mRNAs influence their subsequent processes?

At the protein level, the poly-A tail of mRNAs interacts with PABP and this plays a role in the message's stability (Bernstein and Ross 1989; Sachs 1990) by protecting it from deadenylases and 3′-5′ exonuclease. This is important since poly-A shortening is the rate-limiting step in the turnover of many mRNAs (Brennan and Steitz 2001). In addition, when bound to the poly-A tail, PABP interacts with the translation factor eIF4G at the 5′ end of the RNA (Wells et al. 1998). This results in circularization of the mRNA which stimulates assembly of the translational machinery and increases the rate of translation (Otero et al. 1999; Shim and Karin 2002). The RNA independent association of QKI with PABP has two possible outcomes. First, the interaction of QKI and PABP could lead to release of the constitutive association between PABP and the mRNA leading to degradation of the transcript by the nucleases or perhaps modulate the mRNA circularization leading to reduced or enhanced translation. Second, the QRE may represent a stabilizing factor or may recruit other RBPs involved in stability including PABP.

In the first scenario, the QKI interaction with PABP could be viewed similarly to the PABP-PAIP1/PAIP2 (PABP interacting protein) interaction. PAIP1 is protein that has

sequence similarity to eIF4G and can therefore bind to eIF4A as well as to PABP (Craig et al. 1998). The interaction of PABP with PAIP1 is thought to enhance translation by favoring circularization of the mRNA. In addition, the circularization may aid in the selection of mature mRNAs, may protect the mRNAs against degradation or may help in the reinitiation of terminating ribosomes (Craig et al. 1998). PAIP2 has the opposite effect on translation. When PAIP2 interacts with PABP it promotes PABP's dissociation from the polyA tail. PAIP2 can also interact with eIF4G and can inhibit the PABP-eIF4G interaction, subsequently inhibiting translation (Karim et al. 2006). Taken together, these data on QKI and the work presented in this thesis support a role of QKI both in translational repression and mRNA stability (Figure 2.7 and Figure 4.1) (Lakiza et al. 2005; Larocque et al. 2005; Saccomanno et al. 1999). Therefore, at least in the case of VEGF mRNA, presented in this thesis, one could envision QKI acting in a similar manner to PAIP2 since the presence of QKI would act as translational repressor and loss of QKI causes an increase in VEGF mRNA steady state levels and subsequent protein levels.

4.2.1.1 Hypoxia

Overcoming oxygen deprivation is a common feature of solid tumors. In this condition, the hypoxia-induced signaling pathway involving the oxygen response protein, hypoxia induced factor 1 alpha (HIF1α) is switched on. HIF1α is a transcription factor that regulates a myriad of genes involved in the hypoxic response such as angiogenesis, invasion and metastasis, and apoptotic survival (Brahimi-Horn and Pouyssegur 2006). One of the genes regulated by HIF1α during hypoxia is VEGF, which stimulates new blood vessels to form and migrate into the tumor mass re-establishing nutient and oxygen

homeostasis (Carmeliet 2003). VEGF is also regulated by mRNA stability and its half life is increased 3-8 fold during hypoxia (Levy et al. 1996a; Levy et al. 1996b; Levy et al. 1995). In a search for stability elements in the 3′ UTR of VEGF, an AU-rich stabilizing element was identified and a novel protein complex was shown to bind to this region during hypoxia. Three proteins of approximately 34, 28 and 17 kDa were identified to bind there (Levy et al. 1996a). In addition, five hypoxia-induced RNA protein binding (HI-RBP) sites were identified in the 3′ UTR of VEGF by EMSA analysis (Levy et al. 1997). One of the HI-RPB actually is found directly adjcent to and encompassing the QRE that we have shown to be bound by QKI in VEGF mRNA.

The binding of QKI to the QRE may have several effects. Since the loss of QKI proteins causes either stabilization or translational upregulation, one plausible hypothesis is that under 'normal' conditions QKI is bound to the stability element and then upon induction of hypoxic conditions QKI is released and stabilizing proteins such as HuR are recruited to the site. HuR has already been shown to bind to the VEGF mRNA in the 3′ UTR and regulate its stability (Levy et al. 1998). The steady state of HuR remains constant during hypoxic and normoxic conditions, lending perhaps to changes in the protein-RNA interactions during hypoxia, mediating the changes in interactions and subsequent stability.

4.2.1.2 μORF

An important *cis*-acting elements involved in posttranscriptional gene regulation is upstream open reading frames (μORF) present in the 5′ UTR. μORFs control the translation of downstream ORFs usually by altering the activity of the ribosomes (Morris and Geballe 2000; Sonenberg et al. 2000). The truncated peptides produced by the translation of the μORF interfere with the ribosomes cause stalling. This results in failure

to translate the downstream ORF and this subsequently mRNA stability (Linz et al. 1997; Ruiz-Echevarria et al. 1996). This RNA stability is achieved through nonsense mediated decay (NMD), a surveillance system that prevents the production of truncated proteins by degrading mRNAs harboring premature stop codons (Shyu et al. 2008). GLD-1 has been shown to be both a translational repressor and a mediator of the NMD response and in turn it also affects mRNA stability through its regulation of NMD (Lee and Schedl 2004). There are several ways that GLD-1 could affect its target genes. First, GLD-1 may exert its effect as a general translational repressor of its target mRNAs, regardless of the presence or absence of a μORF. Second, it may protect the messages from NMD by inhibition of translation from all ORFs, including from the μORF. This thereby increases mRNA stability by inhibiting the production of the nascent peptides caused by reducing degradation. Finally, it may physically impede translation initiation by binding near the AUGs (Lee and Schedl 2004). The most likely scenario is probably a combination of the first two where GLD-1 is inhibiting translation in general from all the ORFs including from the μORFs and this in turn would increase mRNA stability by preventing the generation of the truncated peptide. This ability to act on mRNA with μORFs in the 5′ UTR is possible even if the RBP-RNA interaction is mediated through elements in the 3′ UTR. *Lin-45* for example, is bound by GLD-1 only in the 3′ UTR and not in the 5′ UTR like some of its other targets. In this case, GLD-1 is still able to protect the message from NMD (Lee and Schedl 2004; Xu et al. 2001). This mechanism would work in agreement with the multiple functions decribed so far for QKI, that is translational repression and stability. The role of QKI in NMD has not been examined but with a list of putative mRNA targets, sequence analysis can be performed to identify potential targets regulated by this mechanism. Interestingly, $p27^{KIP1}$ mRNA has a μORF in its 5′ UTR (Gopfert et al.

2003) and has been shown to be regulated by QKI (Larocque et al. 2005). This implies that QKI may act in a similar manner as GLD-1.

4.2.2 Localization

Most work on QKI, until recently, has been done on the characterization of mouse QKI and little work has been done on human QKI. These mouse studies revealed that QKI is required for early glial cell fate decisions, myelin component mRNA export from the nucleus and oligodendrocyte differentiation (Chen et al. 2007; Larocque et al. 2005; Larocque et al. 2002). Most, if not all of these processes are regulated by the RNA binding ability of QKI (Chen et al. 2007). The selective distribution of the QKI isoforms in specific cell types, and their temporal expression during development, obviously brings more specificity to their RNA binding capabilities through availability of other RBPs and mRNAs.

Since there has been a comprehensive study identifying the high affinity QRE, as well as *in vivo* interaction studies, the dynamics of the interaction between the proteins and mRNAs in a complex with QKI will need to be further examined. To date, the functional consequence of QKI heterodimerization on mRNA localization has been well studied. Currently, the accepted model is that mRNAs are bound by QKI-5 in the nucleus and then heterodimerize with QKI-6, the nucleocytoplasmic shuttling isoform (Larocque et al. 2002; Larocque and Richard 2005; Wu et al. 1999), before being exported from the nucleus for further regulation. Whether or not the mRNAs are released by QKI in the cytoplasm or whether QKI is involved in their localization from the cytoplasm, has not been determined. In oligodendrocytes, the translational repression and transport of mRNAs in granules to the distal tips of the processes for local translational activation is

well characterized. The association of the mRNAs with hnRNP A2, a member of the hnRNP family involved in regulation of hnRNPA2 response element (A2RE) containing mRNAs, and its subsequent interaction with the cytoskeletal network are required for proper mRNA trafficking (Ainger et al. 1997; Brumwell et al. 2002). Other protein partners interact with this 'basic complex' of mRNA and hnRNP A2 at all stages of trafficking and translation. PABP is a likely component of this complex, at least at early stages after nuclear export and at the final stages of translational activation. QKI could play a role at several stages of this mRNA trafficking. It has already been demonstrated that QKI plays a role in the nuclear export of mRNAs and during trafficking the mRNAs being transported must be translationally repressed and this suggests that QKI could play a role in that process through the mechanisms discussed in section 6.2.1.

4.2.2.1 Stress Granules

Stress granules (SG) are compartments of very dynamic movement. Proteins and mRNAs are continually entering and leaving the granules, with many resident proteins only staying within the granules for 2-6 seconds (Kedersha et al. 2000). In addition, drugs that destabilize polysomes promote SG assembly (Anderson and Kedersha 2006) suggesting that they are not stable storage areas for mRNAs. What are the determinants of the proteins and the mRNAs that regulate their entry and exit from this distinct cellular body?

Unlike other cytoplasmic granules, SGs are not present in cells during favorable growing conditions but are rapidly formed (~15 minutes) in response to stress (Anderson and Kedersha 2006). Cell signaling in response to stress has been shown to be essential for their fomation. A family of stress activated kinases such as protein kinase R and

GCN2 phosphorylates eIF2α, reducing the availability of the eIF2-GTP-$tRNA_i^{Met}$, thereby blocking translation initiation and promoting polysome disassembly and promoting SG assembly (Kedersha et al. 1999). Phosphorylation of conserved C-terminus tyrosines in QKI by *src* protein tyrosine kinases has been shown to regulate its RNA binding capabilities (Zhang et al. 2003). In addition, there are also other tyrosines in the N-terminus that are not targets of the src family of kinases. Signaling through these motifs in times of stress could potentially lead to association with other proteins and induce localization to SGs. In addition, protein-protein interactions could be mediated through the proline-rich SH3 domain or even the KH domain of QKI. Stress signaling creates a new pool of potential protein partners for QKI in numerous ways. One, through posttranslational modification of proteins in the cell, generating new docking/interaction sites. And second, through the relocalization of pools of nuclear proteins to the cytoplasm. Upon relocalization, the now cytoplasmic proteins are free to interact with QKI. Finally, new proteins are produced through the upregulation of transcription,

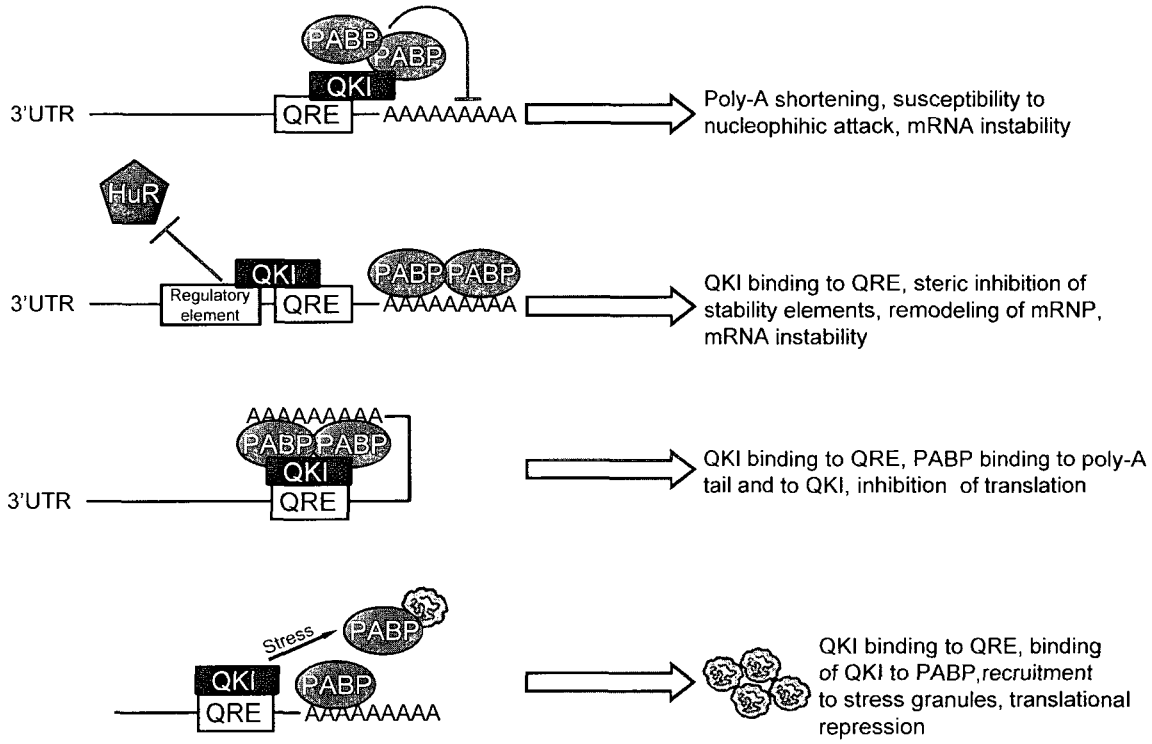


Figure 4.1 Multiple models for QKI involvement in regulation of mRNAs

Quaking binding to the QRE and/or to PABP may affect mRNA regulation at multiple levels such as mRNA stability, translation or localization to specific cellular domains.

translation or stability of specific proteins in times of stress.

It is also important to consider the interaction between SGs and P-bodies (PB) in times of stress. Although they are distinct structures, they do share components (Table 1.1) (Anderson and Kedersha 2006) and PBs are highly motile and can occassionally encounter a SG. They interact via 'docking' mediated by TTP and BRF1. These two destabilizing proteins may then facilitate the transfer of mRNAs from SGs to PBs for decay (Anderson and Kedersha 2006) in a kind of sorting process. Interestingly, results from our lab have shown an interaction between QKI and components of the RNA-induced silencing complex and a component of PBs (G. Lacroix, personal communication).

At this point, we are still left with the age old question, which came first the chicken or the egg? Is it the proteins that coat the mRNA and their interactions that determine the localization of the mRNA in these cytoplasmic granules? Or do the proteins reflect where the mRNA has been and are 'picked up' from these bodies? And what proteins or signals mediate the migration of mRNAs from the polysomes, to SGs to PBs ? Our understanding of this complex circle of mRNA localization requires further elucidation of the key molecular events involved.

4.3 *Defining a role for QKI proteins in cancer development*

Oncogenic transformation occurs when specific genes are aberrantly expressed, mutated or deleted (Parsa and Holland 2004). In order to understand oncogenic transformation, it is essential to define the mechanisms responsible for the changes in gene expression and identify the genes that may regulate tumor development and progression. The first indication that the STAR proteins could participate in

tumorigenesis came from mutational analysis of *gld-1* (Jones and Schedl 1995). More recently, studies on human QKI has shown alterations/loss in *qkI* gene expression in GBMs (Ichimura et al. 2006; Li et al. 2002; Mulholland et al. 2006). In this thesis, we examined the expression of QKI proteins in glioblastoma cell lines (Supplementary Figure 2.8) and interestingly, QKI was expressed in all of the cell lines tested. The reduction of QKI by shRNA causes an upregulation in VEGF levels, which is required for tumor angiogenesis, suggesting that QKI loss somehow contributes to glioblastoma development through increased vascularization.

There is a recurring paradigm in cancer development that there is an increase in transcription after the activation of aberrant growth signals. But transcriptional control is non-existent in certain phases of development, specific stages of embryogenesis and some instances of stress. Therefore, translational control in cancer becomes potentially important and if disrupted may promote malignant transformation. Signaling through the Akt and Ras pathways, by affecting translation, have been shown to be sufficient to convert anaplastic astrocytomas (AA) to glioblastomas (GBM) (Holland et al. 2000; Sonoda et al. 2001). QKI has been shown to bind to and potentially affect numerous mRNAs involved in cell growth and maintenance (Galarneau and Richard 2005) and one can envision that disfunction of these important factors, through loss of QKI or through translational disregulation, could affect cellular growth properties. Many of the gene products that have been shown to be regulated translationally in the transition from AA to GBM have proliferative, invasive and angiogenic properties (Holland et al. 2000; Sonoda et al. 2001). It would be interesting to examine whether or not QKI regulates any of these mRNAs, and if so, what effect it has on the observed growth properties.

4.4 *Regulating QKI expression*

Very little is known about how the QKI isoforms are regulated. Studies using the *qk*^{v} mice suggest that there is an oligodendrocyte-specific enhancer 5′ to the *qkI* gene that allows for the temporal expression of the isoforms during development (Hardy et al. 1996). The requirement for QKI-5 during embryogenesis and the subsequent upregulation of QKI-6 and QKI-7 postnatally, suggests that they may in fact play differing roles in these distinct stages. This spatio/temporal expression will in turn affect mRNAs regulated by each isoform and may influence the developmental process as is seen with many mRNAs in *Drosophila melanogaster* and *C. elegans* (Clifford et al. 2000; Macdonald and Smibert 1996; Newbury 2006). Identifying the upstream genomic elements required for the isoforms expression and the regulation of these elements may in turn help us understand the role of QKI in mRNA metabolism during development. In addition, understanding this expression pattern and the subsequent disregulation of QKI expression in human diseases, such as schizophrenia and glioblastoma multiforme, may allow us to understand how the expression of QKI influences diseases. The analysis of the genomic organization of the *qkI* gene and its regulation is beyond the scope of this thesis but could assist us in our understanding of the role of the QKI isoforms and their target mRNAs in many developmental processes.

4.5 *Future directions for QKI in posttranscriptional gene regulation*

Although we have described a role for QKI in the regulation of VEGF expression likely through posttranscriptional modulation of its mRNA stability and/or translational repression, much work still needs to be done to determine the precise role of QKI in these two processes. First of all, the biochemical mechanism by which QKI regulates its target

mRNAs needs to be determined. GLD-1 translationally represses all of its target mRNAs (Lakiza et al. 2005; Lee and Schedl 2004; Marin and Evans 2003; Schumacher et al. 2005), and GLD-1 exerts this posttranscriptional role in a couple of ways. One through translational repression and second by protecting messages from NMD, which affects stability, and often both of these processes occur on the same mRNA molecule (Lee and Schedl 2004). This is one of the few models that is able to tie together all of the known data on QKI in a cohesive way. Therefore this is likely a good avenue to pursue in the future. One way to do this is by analysis of the elements within the target mRNAs, whether or not they possess a μORF, a stability element, the QRE and then examine what role QKI has on these mRNAs. Ideally, this would be done in QKI knockout cells. Due to the early embryonic death of the knock out mice these cells are not available, therefore knockdown technology would have to be used. Using NMD deficient cells (ex. smg-2 null cells), one could envision knocking down QKI with siRNA and examining the role of QKI in the NMD response in these cells as well as wild-type cells and the NMD deficient cells using real time PCR or in situ RNA analysis.

At the protein level, more interacting partners need to be analyzed and identified to better understand QKI and its numerous roles in various processes, including some of the ones described in this thesis. In addition, how QKI signals in various stages of development would be an interesting topic to investigate. Whether it be through modifications of QKI itself, or through new protein-protein interactions, understanding how QKI is regulated will be extremely important to fully understand its role.

At the cellular level, it will be crucial to elucidate the mechanism regulating QKI localization to different cytoplasmic structures. We have found that a subset of QKI co-localizes with PABP in stress granules, perhaps confirming a role for QKI in translational

repression in response to cellular stress. It will be important to investigate the mechanism regulating QKI localization to these granules. Perhaps QKI contributes directly to the localization in these domains or indirectly through interaction with a resident protein.

Finally, at the genetic level, the contribution of the individual isoforms during development, myelination and cancer development has not been examined due to the lethality of the null mice. The best way to examine this in the future is through the generation of conditional transgenic mouse models. Ideally, a conditional QKI-6, -7 mouse would be generated by targeting the exons specific to the two isoforms. Then by using *cre*-recombination, QKI-6 and QKI–7 can be removed in a cell specific manner. For example, using GFAP-*cre*, the isoforms could be removed in astrocytes and the contributions of the isofoms to astrocytoma/GBM development could be examined. Or by using MBP-*cre* or PLP-*cre* one could analyze QKI's involvement at different stages of myelination.

4.6 *Protein Arginine Methylation*

Posttranslational modification of proteins allow the cell to expand its repertoire beyond the constraints imposed by the 20 amino acids (McBride and Silver 2001). Approximately 200 proteins were identified in a proteomic search for arginine methylated proteins (Boisvert et al. 2003), though bioinformatics studies seems to increase that number to 1000 proteins that contain the target glycine arginine rich (GAR) environment (Boisvert 2005). Although methylation of MBP was identified over 30 years ago, its significance has not yet been truly determined. Its modification by methylation has become increasingly important due to the observation of increased MBP methylation in MS patients as well as the high levels of citrullinated MBP in the disease. It is

interesting to note that the number of identified methyltransferases is relatively small when compared to the number of known kinases, and very few examples of their regulation and specificities have been demonstrated. It is therefore possible that arginine methylation is constitutive and may persist over the majority of the lifetime of the protein. This would suggest that this posttranslational modification does not regulate the function of the protein but rather may play a role in the protein's maturation process as well as the maturation of any protein complexes or interactions it may be involved in.

4.6.1 Localization of methylated MBP to compact myelin

Although the role of arginine methylation of MBP in the formation of myelin has yet to be determined, it has long been assumed that it plays a role in the structural integrity of the myelin sheath. This likely occurs in several ways. Firstly, MBP contributes to the integrity of the myelin membrane by electrostatic and nonpolar interactions with the lipid components (Brostoff and Eylar 1971). These interactions are facilitated by both the basic properties of the protein and its highly open or disordered conformation. The protein is found in an open, double chain structure due to a proline bend just 5′ to the arginine at position 106. This structure provides a backbone for lipid association and may be stabilized by interchain interactions. Therefore the methylarginine may provide sites for stabilization of the double chain conformation, either by interacting with lipid elements or other side chains found in close proximity (Brostoff and Eylar 1971). In addition, MBP is able to bind to two lipid surfaces at once, most likely through dimerization. This causes adhesion and aggregation of lipid vesicles and multibilayer formation, a requirement in the generation of the compact myelin sheath.

4.6.2 Protein-Protein interactions

Posttranslational modifications are known to affect protein-protein interactions. The classical example is the interaction of the src-homology domain-2 (SH2) domain with phosphorylated tyrosines (Pawson 2004). Arginines are particularity important in hydrogen bonding. For example, Sam68 binds to SH2/SH3- and WW-domain containing proteins. Methylation of arginines neighboring the proline motifs in Sam68 prevents its association with SH3 domains (Bedford et al. 2000). There are also examples where arginine methylation increases protein-protein interactions. Methylation of SMN is required for its interaction with the small nuclear ribonucleoproteins SmB/B′, SmD1 and SmD3 (Selenko et al. 2001).

Methylation does not change the overall charge of the residue, but the methylation increases steric hindrance and removes amino hydrogens that might be involved in hydrogen bonding (McBride and Silver 2001). This means that arginine methylation may serve to modulate the intra-and inter-molecular interactions of the target protein. Although we have shown that the methylation of MBP does not affects its ability to form dimers, which are functionally important for the formation of compact myelin (Section 4.6.1), we have not analyzed whether MBP methylation affects its ability to associate with its other known protein partners. MBP binds to polyanions such as actin filaments and microtubules and also binds to proteins such as Ca^{2+}-calmodulin, tropomyosin and clathrin (Boggs 2006). Although these interactions are known to have a low degree of specificity but a very high affinity (Jones et al. 2004), they are functional interaction for the myelin sheath and therefore may have functional consequences. The MBP charge isomer that is most commonly found in chronic MS patients (6 arginines deiminated)

cannot effectively aggregate lipids, are more effectively digested by cathepsin D, have a greater proportion of random secondary structure and therefore are less likely to form compact myelin (Bates et al. 2002).

4.6.3 Citrullination of MBP and the implications for MS

Although the etiology of MS is unknown, genetic, environmental and immune processes all work together in a complex mechanism that leads to demyelination (Cao et al. 1999). In MS patients, the chemical structure of MBP is modified. The level of the citrullinated form of MBP is elevated 2-3 fold in chronic MS (Moscarello et al. 1994), and in a case of fulminating MS more than 90% of the MBP was in the citrullinated form (18 residues of citrulline per mole) (Wood et al. 1996). The deimination of arginines is catalyzed by PAD, which has been shown to work much less efficiently on methylated arginine substrates then on the same non-methylated substrate (Kearney et al. 2005). Therefore, it is assumed that methylation of MBP would serve as a protective mechanism. This is supported by the fact that in the case of fulminating MS, where 18 of the 19 arginine residues are citrullinated, only arginine 107 is not deiminated. In MS samples there is an overall increase in methylation (Kim et al. 2003). It is hypothesized that because methylation is essential for the formation of compact myelin, the increase in methylation in MS likely represents an attempt at remyelination, which ultimately fails in the demyelinating environment (Kim et al. 2003).

Immunoreactive peptides of MBP have been found in the cerebral spinal fluid and in the urine of MS patients (Cao et al. 1999). This peptide is increased in MS and is generated by cathepsin D, a proteinase that cleaves at Phe-Phe linkages (Whitaker and Seyer 1979). The immunodominant epitope 45-89 is unavailable to antibody in the intact

protein but is available as an isolated peptide (Cao et al. 1999), suggesting that it is internalized by secondary structure in normal cases. It has been demonstrated that the greater number of citrulline residues, the greater the susceptibility to enzyme digestion (Cao et al. 1999). So conversely, the increase in methylation could represent increased stability of the protein due to increased resistance to proteolysis.

4.7 *Future perspectives for methylation of MBP*

With the discovery of changes in the levels of posttranslational modifications on the myelin basic protein in human diseases such as MS and subacute combined degeneration, understanding and identifying these modifications becomes much more important. The intricate assembly of the myelin sheath, its role in impulse conduction and MBP's role in the autoimmune reaction in MS underscores MBP's importance. Due to the extremely controled localization and the production of MBP by very specialized cells in both the CNS and PNS, experiments must be carefully designed in order to evaluate the role of Arg 106 and the role of its posttranslational modification.

Shiverer is an autosomal recessive mutation in the mouse that results in an almost total lack of CNS myelin due to a large deletion of the MBP gene. Although small amounts of other myelin proteins are present, the isoforms of MBP are not detectable (Molineaux et al. 1986). In order to truly address the role of Arg 106 of MBP, a transgenic can be created using the *shiverer* mouse. A construct could be generated incorporating 9.5 kb of the MBP promoter, including all of the necessary enhancers and elements (Farhadi et al. 2003), followed by either the wild-type MBP gene or the R-K MBP gene including at least 1kb of the 3′ UTR. This construct could then be used to generate a 'knock-in' in the *shiverer* mouse. Developmental analysis of MBP expression,

in both the wild-type and the R-K mouse, myelin structural analysis, compaction studies and MBP localization studies could then be performed and compared. In addition, lipid aggregation analysis of both types of MBP could be performed, as MBP is known to bind and aggregate acid lipid vesicles (Boggs et al. 1997). The ability of MBP to aggregate the vesicles could regulate the ability of MBP to mediate adhesion between the intracellular surfaces of myelin. Therefore, there could be observable differences between the ability of wild-type MBP, R-K MBP and citrillinated MBP to mediate this adhesion. Using these transgenics, analysis of the MBP charge isoforms in aging mice as well as in the animal model of MS, Experimental Autoimmune Encephalomyelitis (EAE) could be performed. Although EAE is not multiple sclerosis, its different forms resemble the various forms and stages of MS.

In addition, expression of arginine methyltransferases has not been extensively been studied. It would be interesting to analyze the developmental expression of PRMT5, its localization and activity in the CNS, in OLs, as well as its activity in MS brains.

4.8 *Concluding Remarks*

The field of posttranscriptional gene regulation by RNA binding proteins has been rapidly evolving in the last years. A growing number of proteins have since been reported to be involved in a growing number of cellular processes. A better understanding of how RNA binding proteins affect the lifecycle of the each RNA, with respect to their target binding site and other elements affected by binding, will be essential for understanding and identifying new roles for many of these proteins. In the future, characterization of the roles of QKI in these pathways will surely reveal many different aspects of cellular functions regulated by this family of proteins and should underscore

the importance of posttranscriptional regulation in disease. More interestingly, it will be exciting to determine how QKI binding is regulated in the cell, and whether deregulation or different signaling pathways are associated with distinct cellular conditions, such as differentiation, cell cycle progression, transformation, or disease progression. In addition, charaterization of posttranslational modifications, such as arginine methylation, of RNA binding proteins and other important proteins will reveal how arginine methylation is involved in many cellular functions, in the pathways mentioned above and in human diseases.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The present work has focused on the identification and the characterization of the QKI ribonucleoprotein complex. The molecular characterization of QKI binding to VEGF mRNA and the consequences of this binding has been addressed. In addition, I have generated a novel antibody capable of recognizing arginine methylated MBP. These studies have been submitted or are in preparation for submission to peer-reviewed journals. The major contributions of this work to original knowledge are summarized below:

1. A ribonomic profiling and the subsequent identification of over 80 previously unknown mRNAs found in the QKI mRNPs.
2. Identification of QKI as a component of stress granules following arsenic treatment, where it colocalizes with PABP.
3. Characterization and mapping of the binding of QKI to VEGF mRNA as well as the functional characterization of the loss of QKI on VEGF levels.
4. The generation of a novel antibody that recognized arginine methylated myelin basic protein at arginine residue 106.
5. Identification of PRMT5 as the methyltransferase responsible for methylating R106 of MBP.

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Appendix

The appendix contains the following items:

- Permission to the student to use radioactivity
- Permission to the student for the use of biohazard materials
- Proof that the student took and passed the advanced animal workshop
- The approved animal protocol