The Functions of Quaking Proteins in Gliogenesis and Myelination

by

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ABSTRACT

Mutant *quaking* viable mice (qk^V) develop a tremor phenotype in their hind limbs 10 days after birth, likely resulting from severe myelination defects. Genetically, *quaking* viable mice contain a deletion in the promoter/enhancer region of the *quaking* gene. The *quaking* gene is alternatively spliced, producing QUAKING isoforms that differ in their C-terminal amino acid sequence. The QKI-5 isoform is nuclear, whereas the QKI-6 and QKI-7 isoforms are predominantly cytoplasmic. The QKI isoforms contain a single KH RNA-binding domain, which suggests a role in RNA metabolism. Although the dysmyelinating phenotype of the mutant mice suggests a role in myelination, the function of the protein remains largely unknown. The objective of this thesis is to characterize the role of the RNA binding proteins QKI in the differentiation of glial cells and their involvement in the process of myelination. Moreover, this study describes the quaking viable defects and how the isoforms contribute to the observed phenotype.

First, to assess these objectives, we have demonstrated that the mRNA of the myelin basic protein (MBP) in qk^V is retained in the nucleus of oligodendrocytes, and identified myelin basic protein (MBP) mRNA as a target for binding by the QKI proteins. We demonstrated that this interaction occurs at the 3'-UTR of MBP and mapped the region named QRE (Quaking Responsive Element). Moreover, by over-expression of the nuclear isoform QKI-5, we have recreated the nuclear retention of MBP mRNA in primary oligodendrocyte culture. We extended our study to recreating the *quaking* viable phenotype of MBP downregulation by injecting recombinant adenoviruses that express

QKI-5 into the brains of normal mice. These findings suggest that QKI participates in myelination by regulating mRNA metabolism of key protein components in oligodendrocyte physiology.

Thirdly, we analyzed the expression of the three major QKI isoforms during the normal myelination process. We showed that expression of two QKI isoforms, which are normally expressed during myelination, inhibits the proliferation of primary rat oligodendrocytes by post-transcriptionally inducing $p27^{KIP1}$ expression. The QKI isoforms induced the differentiation of oligodendrocytes, as visualized by morphological analysis and staining with anti-myelin basic protein antibody. In addition, co-cultures of Schwann cells with dorsal root ganglia neurons displayed thicker myelin sheaths when transduced with the QKI-6 and QKI-7 isoforms, as visualized by electron microscopy. These data demonstrate that the QKI RNA binding proteins promote myelination by regulating the post-transciptional expression of $p27^{KIP1}$. This thesis provides strong evidence that RNA binding proteins play a key role in development and cell fate, thus providing a better understanding of the development of organs such as the brain.

RÉSUMÉ

Les protéines liant l'ARN jouent un rôle central afin de contrôler l'expression génique, l'épissage alternatif et la localisation des ARN messagers. Pour qu'une cellule se différencie, les gènes doivent être régulés au niveau de leur expression et de la localisation de leurs ARNm. Un des phénomènes physiologique de différentiation au niveau du système nerveux est la myélinisation des axones. En vulgarisant, la gaine de myéline serait l'isolant entourant le fil électrique étant l'axone.

Un des modèles de choix pour l'étude de la myéline est la souris quaking. La souris viable *quaking* possède des anomalies de myélinisation au niveau du système nerveux central et périphérique. Au stade de 10 jours post-natal, les souris *quaking* développent un tremblement au niveau des membres postérieurs. Auparavant, certains auteurs avaient démontrés par des analyses histologiques que la souris *quaking* a un problème de différentiation au niveau des oligodendrocytes et de mauvais compactage de la gaine de myéline du système nerveux central et périphérique. Génétiquement, les souris *quaking* se caractérisent par une délétion de 1.3 Mb de la région promotrice/amplificatrice du gène *quaking*. Le gène *quaking* est sujet à un épissage alternatif résultant à trois isoformes majeurs de la protéine QUAKING (QKI) qui se distinguent de 8 à 30 acides aminés différents dans la région C-terminal. L'isoforme QKI-5 est nucléaire contrairement aux isoformes QKI-6 et QKI-7 qui sont principalement cytoplasmiques. Les isoformes QKI ont tous un domaine KH responsable de lier l'ARN. Au début de cette étude, aucun ARN cible pour les protéines n'avait été découvert.

Tout d'abord, l'objectif était d'identifier un élément d'ARN étant régulé par les protéines QKI. Parmi les ARNm majoritaires présents dans la gaine de myéline, l'ARNm de MBP (protéine basique de la myéline) s'est avéré une cible pour la protéine QKI. Nous avons établi que QUAKING est capable de se lier à une région précise de la séquence d'ARNm non-traduite 3'-UTR de MBP. Cette région a été nommée QRE (Elément Responsable de la liaison de QKI). Nous avons aussi récapitulé le phénotype de la souris viable *quaking* en surexprimant QKI-5 dans le cerveau de souris saines. À l'instar de la souris viable *quaking*, la surexpression de QKI-5 cause une rétention de l'ARN messager de MBP au niveau du noyau des oligodendrocytes. Par conséquent, l'expression de la protéine MBP est diminué drastiquement. Cette étude démontre qu'un mauvais balancement des protéines QKI mène à une faible myélinisation.

Finalement, nous avons tenté d'expliquer le changement d'expression des trois isoformes QKI-5, QKI-6 and QKI-7 lors de la myélinisation et la différentiation des cellules gliales responsables de la synthèse de myéline: les oligodendrocytes et les cellules de Schwann. En effet, l'expression des isoformes cytoplasmiques QKI-6 et QKI-7 est augmentée lorsque les oligodendrocytes myélinisent les axones. De plus, la surexpression des isoformes QKI-6 et QKI-7 dans les cellules gliales progénitrices a démontré un arrêt du cycle cellulaire en G1 et favorise la différentiation des oligodendrocytes en culture. La protéine $p27^{Kip1}$, qui est un joueur clé dans l'arrêt du cycle cellulaire et la différentiation, fut démontré comme étant le facteur augmenté via l'expression de QKI-6 et QKI-7. Des expériences de liaison ont démontré que la

séquence d'ARNm de $p27^{Kip1}$ au niveau de la région 5'UTR est une cible pour les protéines QKI-6 et QKI-7. Afin d'étudier l'effet de QKI sur la myélinisation, un modèle de myélinisation en culture cellulaire mixte utilisant des neurones DRG avec des cellules de Schwann a révélé que les isoformes QKI-6 et QKI-7 permettaient d'obtenir une gaine de myéline plus épaisse. De plus, l'expression de la protéine $p27^{Kip1}$ dans les cellules de Schwann est proximale avec les axones myélinisées. De surcroît, l'expression du régulateur de cellules de Schwann, Krox-20, est augmentée par QKI-6 et QKI-7. En particulier, l'ARN messager de Krox-20 est une cible pour les protéines QKI au niveau de la région 3'-UTR. Ces données réunies démontrent bien que les protéines QKI sont importantes pour la gliogénèse et la myélinisation du système nerveux central et périphérique.

En conclusion, cette thèse démontre, en utilisant l'exemple des protéines QKI, que les protéines liant l'ARN sont activement impliquées dans la différentiation cellulaire par leur métabolisme de certains ARN clés pour la myélinisation. De plus, cette étude ouvre de nouvelles perspectives au niveau de la régulation des gènes via leurs ARNm correspondants. Ainsi, de telles études permettront d'adresser des questions clées dans le domaine fascinant de la neurobiologie.

PREFACE

This Ph.D. thesis was written in accordance with the Guidelines for Thesis Preparation from the Faculty of Graduate Studies and Research at McGill University. It is based on the option of writing the thesis as a manuscript-based thesis. 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. (Reprints of published papers can be included in the appendices at the end of the thesis.)..."

Papers Included in this Thesis:

Chapter 2 Larocque, D., Pilotte, J., Chen, T., Cloutier, F., Massie B., Pedraza, L., Couture, R., Lasko, P., Almazan, G., and Richard, S. (2002). Nuclear retention of MBP mRNAs in the *Quaking Viable* mice. Neuron. 36, 815-829.

Chapter 3 Larocque, D, Galarneau, A., Liu, H.N., Scott, M., Almazan, G. and Richard, S. (2004) $p27^{Kip1}$mRNA protection by QUAKING RNA binding proteins promote oligodendrocyte differentiation. Submitted to Nature neuroscience. July 19, 2004

Chapter 4 Daniel Larocque, Gabriella Fragoso, Martin Loignon, Moulay A. Alaoui-Jamali, Walter E. Mushynski, Stéphane Richard and Guillermina Almazan. QUAKING RNA Binding Proteins promote Schwann Cell Differentiation and Myelination. Manuscript in preparation.

Contributions of Authors:

The candidate performed most of the research. The contributions of other authors are described below:

Chapter 2 was a team effort in which Figures 2-1 and 2-2 were performed by Taiping Chen and Figure 2-1B was done by Stéphane Richard. Julie Pilotte performed Figure 2-3, and the MBP exon II antibody was provided by Liliana Pedraza from the MNI. The high titer adenoviral vectors were produced in Bernard Massie's laboratory with the technical help of Francois Bouthiller at the BRI-NRC. Guillermina Almazan from the Pharmacology Department at McGill University provided her expertise with oligodendrocyte primary cell culture.

The experiments in Chapter 3 were performed by the candidate. André Galarneau performed Figure 3-3b and supplemental figure. Hsueh-Ning Liu and Guillermina Almazan helped us on oligodendrocyte culture and advice on several aspects. Michelle Scott from McGill Center for Bioinformatics performed the supplemental figure.

The experiments in Chapter 4 were performed by the candidate with the help of Dr. Gabriella Fragoso who performed the DRG-Schwann cell co-culture. Walter Mushynski helped during the writing of chapter 4. Guillermina Almazan and Stéphane Richard are corresponding authors for this paper.

All studies were conducted under the supervision of Dr. Stéphane Richard at the Lady-Davis Institute for Medical Research.

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Thank you to Dr. Guillermina Almazan and her laboratory members from the Pharmacology Department at McGill University where I performed all of the oligodendrocyte and Schwann cell work. This was my second lab. I would like to thank the Multiple Sclerosis Society and the Canadian Institute for Health Research (CIHR) for their financial support. I am indebted to Josée Bergeron for her love, encouragement and continuous support.

Thus, after this PhD and all of the parking tickets I amassed around the McIntyre Building, and all of these collaborators in Montreal, I can truly say that Montreal is a great scientific environment that contains many collaborators and friends. After my Postdoctoral training in the USA, I hope to work with all of you again.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF ABBREVIATIONS

a.a	amino acids
ARE	AU-rich element
BMP	bone morphogenetic protein
CaMKII-α	calcium-calmodulin-dependent kinase II α
CBC	cap binding complex
CC	Corpus callosum
CDK	cyclin-dependent kinase
CDKi	cyclin-dependent kinase inhibitor
CK	C-terminal of the KH
CNPase	2'3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CPSF	cleavage and polyadenylation specificity factor
CTD	C-terminal domain
E12.5	embryonic day 12.5 post coitum
EAE	experimental allergic encephalamyelitis
EGF	epidermal growth factor
endoG	endonuclease G
EJC	exon-exon junction complex
ENU	ethyl-N-nitrosourea
EST	expressed sequence tags
FGF	fibroblast growth factor
FMR1	fragile X mental retardation
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GLD-1	germ-line defective
GRPs	glial-restricted precursors
Golli-MBP	gene expressed in the oligodendrocyte lineage-MBP
GSG	GRP33, Sam68, GLD-1
hnRNP K	heterogeneous nuclear ribonucleoprotein K
How	held-out-wings
Hqk	human quaking
Id	Inhibitor of differentiation
IRES	internal ribosomal entry site
kb	kilobases
KH	hnRNP K homology
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MBPexII	MBP containing exon II
MOBP	myelin-associated oligodendrocyte basic protein
MOG	myelin/oligodendrocyte glycoprotein
MOSP	myelin/oligodendrocyte specific protein
mRNPs	messenger RNA ribonucleoproteins
miRNA	micro RNA

MS	multiple sclerosis
NCAM	neural cell adhesion molecule
Ngn	Neurogenin
NK	N-terminal of the KH
NLS	nuclear localization signal
NMD	nonsense-mediated decay
NMR	nuclear magnetic resonance
NPC	nuclear pore complex
NFH	neurofilament heavy isoform
OMgp	oligodendrocyte-myelin glycoprotein
OPC	oligodendrocyte progenitor cell
OSP	oligodendrocyte-specific protein
PAP	poly(A) polymerase
PARN	poly(A) specific ribonuclease
p.c.	post coitum
PDGF	platelet-derived growth factor
PLP	proteolipid protein
PNS	peripheral nervous system
PTB	polypyrimidine tract-binding protein
QKI	Quaking protein
qkI	quaking gene
qk^{v}	quaking viable
QRE	quaking response element
RBPs	RNA binding proteins
RMZ	rostral migratory zone
RNA	ribonucleic acid
RNAi	RNA interference
RNPs	ribonucleoproteins
RRMs	RNA-recognition motifs
RTP	RNA 5' triphosphatase
RTS	RNA trafficking signal
Sam68	Src-associated-in-mitosis
SLM	Sam68-like mammalian proteins
Shh	Sonic hedgehog
SNB	Sam68 nuclear bodies
STAR	signal transduction and activator of RNA
SVZ	subventricular zone
TR5	Tetracyclin response element
TNF-α	tumor necrosis factor-α
TGE	tra-2 and GLI element
UTR	untranslated region
vz	ventricular zone
VLCFA	very long chain fatty acid
Xqua	Xenopus *quaking*

Chapter 1

General Introduction and Literature Review

1.1 General Introduction

The objective of this thesis is to understand the function of QUAKING (QKI) proteins in oligodendrocytes and Schwann cells. This first Chapter will discuss the role of RNA binding proteins such as QKI in the physiology of cells and organisms. Chapter 2 will explore the *quaking* protein's ability to bind to the myelin basic protein mRNA, and will bring further insights into the molecular events leading to the dysmyelination of the *quaking* viable mouse. Chapter 3 will focus on the role of QKI-6 and QKI-7 isoforms in cell cycle progression and oligodendrocyte differentiation. Chapter 4 will explore how the QKI proteins function in Schwann cell differentiation and peripheral nervous system myelination.

The introduction will first cover the background information required for the understanding of the content in Chapters 2, 3 and 4. It will introduce RNA binding proteins and their roles in RNA metabolism (Section 1.2), and will then classify the RNA binding proteins according to the protein domain they harbor (section 1.3). The next section will cover QKI proteins and the QUAKING viable mouse (qk^{V}) (section 1.4). The section 1.5 will discuss about myelination in general and the potential therapeutic role of Quaking proteins in the context of myelin-affected diseases and other brain pathologies. Section 1.6 is dedicated to the development properties of myelinating glial cells, oligodendrocytes and Schwann cell.

1.2 RNA Binding Proteins (RBPs) in General

RNA binding proteins (RBPs) are essential for cell physiology. In fact, after transcription, RBPs must bind nascent RNA molecules for several functions, such as translation, RNA stabilization, RNA trafficking, pre mRNA splicing, micro RNA biogenesis, non-sense mediated decay and RNA interference. Figure 1-1 illustrates the seven essential functions of RBPs.

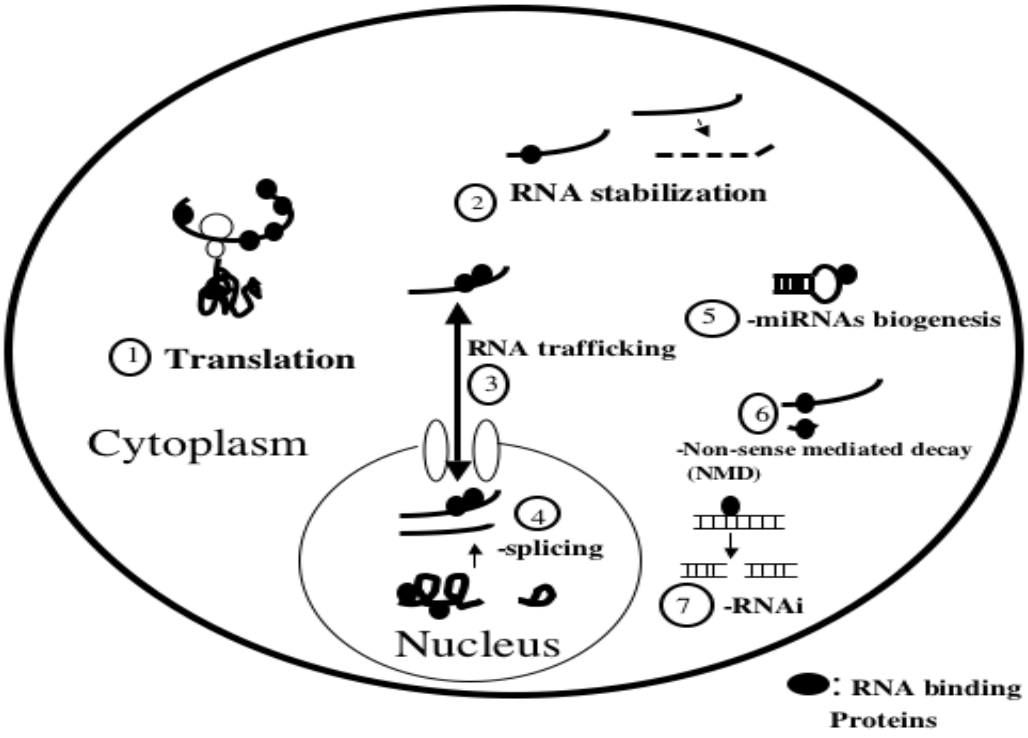

Figure 1-1. RNA binding proteins functions in RNA metabolism

1.2.1 The Roles of RBPs in Translation

Messenger RNAs (mRNAs) in a cell must be translated into proteins at the correct place and time, thus rendering the control of mRNA translation by proteins bound to RNA molecules crucial. The assembly of multi-protein complexes on mRNAs ensures that translation does not occur until the ribosomes, tRNA, the mRNA and translation factors have reached the correct location in the cell. First, modifications of mRNA through the addition of a methylated guanosine cap structure to the 5' end of the pre-mRNA, the 5'UTR, must occur. This cap structure is then recognized by the cap-binding complex (CBC), which is eventually replaced by the translation initiation factor eIF4E when it is translocated to the cytoplasm (Sonenberg and Dever, 2003).

In order for translation to occur, the small 40S subunit is recruited to the mRNA and ribosome scan until it reaches the start codon. Initiation factors, such as eIF4E, which binds the cap binding protein, are recruited to the mRNA. This complex also contains eIF4G, a central scaffolding protein. eIF4A, a RNA-dependent helicase, and eIF4B which are recruited to the mRNA. eIF4B stimulates eIF4A helicase activity, which is thought to relax the secondary structure within the 5'UTR. The 43S pre-initiation complex, which contains the small 40S subunit, the initiator Met-tRNA-eIF2-GTP, as well as additional initiation factors such as eIF3, is located near the methylated cap structure at the 5' end of mRNA. The 43S pre-initiation complex migrates to the start codon by a process called scanning. Finally, initiation factors are released and a large 60S ribosomal subunit joins to form the 80S ribosome. The poly(A) tail of the 3'-UTR is also involved in the 40S subunit recruitment; in fact, the poly(A) binding protein (PABP) is another key player, as

it binds the poly(A) tail and interacts with eIF4G to create a loop that links the 5' end to the 3' end of the mRNA (Gingras et al., 1999). Proteins that bind eIF4E, 4E binding proteins (4EBPs), block its interaction with eIF4E thus regulating the PABP-eIF4G interaction (Pause, 1994). The PABP interacting protein Paip1 provides another way of regulating translation. It has been demonstrated to enhance translation, whereas Paip2 binding to PABP generally represses the translation process (Roy et al., 2004).

The insulin-signaling pathway is a good example of the involvement of RBPs and their translation factors following stimulation by an extracellular signal. In fact, upon insulin stimulation, the translational regulator 4EBPs is phosphorylated; this change leads to the activation of translation due to the formation of the eIF4E-eIF4G bridge (Heesom, 1998). The kinase that phosphorylates 4EBPs is FRAP/mTOR, a member of the PI3K family. Anti-cancer drugs like rapamycin inhibit FRAP/mTOR activity. Thus, the effect of chemo-therapeutic agents on translation as well as the discovery that eIF4E is overexpressed in several human cancers leads us to emerging evidence that translational regulators are potential target molecules for cancer therapy (Mamane et al., 2004; Raught, 2000).

In addition to initiation factors and PABP interacting proteins, there are other pathways that control mRNA translation. One of these mechanisms is the binding of proteins to specific mRNA sequences known as translational control elements. These sequences are often located within the 3'-UTR. Interestingly, the poly(A)-tail length can be regulated by RBPs that lie in the proximity of PABP. By binding to a specific 3'-UTR sequence, RBPs often act as translation silencers by affecting poly(A)-tail length or by affecting the adenylation of the mRNA (Wilkie et al., 2003). Polyadenylation and the

appropriate translation of mRNAs require such proteins as poly(A) polymerase, require the polyadenylation-specificity factors CPSF and CPEB as well as proteins involved in cleavage. In addition, cytoplasmic polyadenylation requires two 3'-UTR sequence elements: hexanucleotide AAUAAA, and a cytoplasmic polyadenylation element (CPE). Interestingly, CPEs have dual roles in translation: activation and repression. The *Xenopus* and *Drosophila cyclin B* mRNA described by the Joel Richter group (University of Massachusetts) is one of the several examples in biology that illustrates this elegant manner of translation control (Groisman et al., 2002).

Cyclin B is a protein that needs to appear and disappear in a short time during mitosis. Frog eggs such as Xenopus oocytes are arrested at the end of first meiotic prophase in G2 of the cell cycle. These giant cells contain many translationally dormant mRNAs, such as the *cyclin B* mRNA, which has a short poly(A) tail. When oocytes are induced to enter in meiosis (i.e. in the M phase), these short poly(A) tails are lengthened and translation occurs. The phosphorylation of CPEB by Aurora, a kinase whose activity oscillates during the cell cycle, mediates polyadenylation. Exit from the M phase requires deadenylation and the subsequent translational repression of *cyclin B* mRNA by Maskin. Maskin prevents eIF4G from binding to eIF4E, thereby playing the role of an adaptor protein that bridges CPEB and eIF4E and causes translational silencing. When cells enter the M phase, Aurora phosphorylates CPEB and causes CPEB to bind to and recruit CPSF and poly(A) polymerase, thus forming a stable poly(A) tail. These events stimulate the translation of *cyclin B* mRNA and prompt the cells entry into the M phase. As soon as cells start to exit the M phase, a phosphatase inactivates CPEB, which leads to the deadenylation of the *cyclin B* mRNA and the loss of PABP, and allows for the re-

association of Maskin with eIF4E. This last event permits the translational silencing. Figure 1-2 illustrates this mechanism.

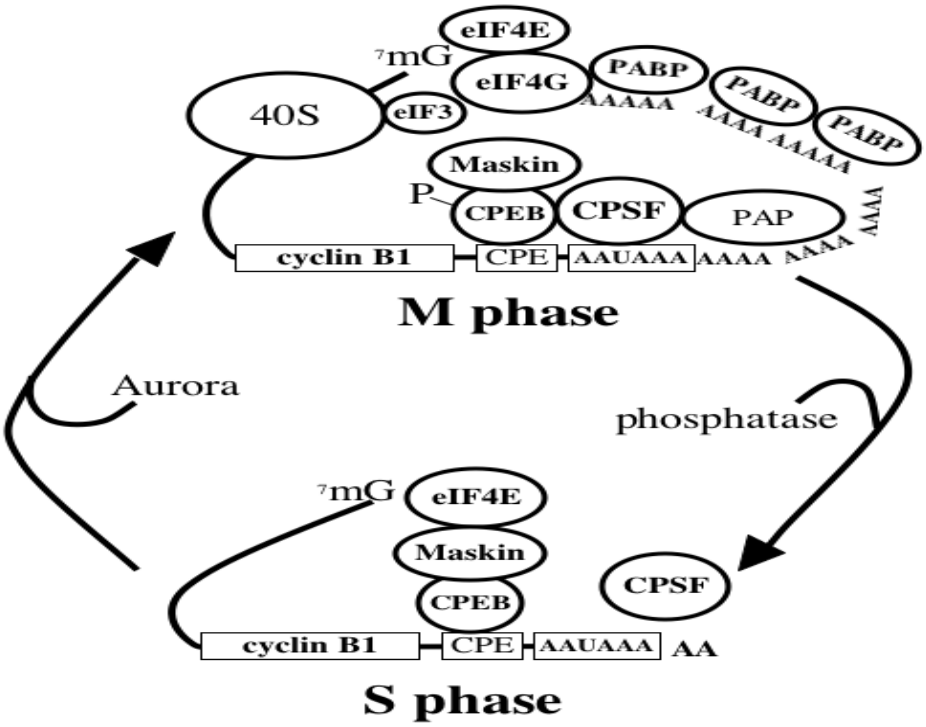

Figure 1-2. Mechanism for translation control of Cyclin B mRNA in *Xenopus* embryos

Cyclin B mRNA translation control is merely one of the many situations in nature in which an organized complex of proteins containing RBPs and initiating factors regulate mRNA translation. Another such example is protamines mRNA being regulated by RBP MSY4 in spermatozoid (Giorgini et al., 2002), and another instance of a gene being regulated at the translation level is hunchback (hb) in *Drosophila* development. Hunchback is a transcription factor that is required for segmentation in the anterior thoracic region of the embryo. Although hunchback mRNA is uniformly distributed, it is translationally repressed in the posterior region of the embryo. Pumilo acts as a sequence-specific RNA binding protein that recruits Nanos (which is highly expressed in the posterior pole). This complex binds hunchback mRNA at the 3'-UTR and leads to translational repression only in the posterior region. Translational regulation in Drosophila development has been broadly studied. These well-designed studies have demonstrated that the localization and translational regulation of mRNA are coupled during development (Lasko, 1999). These mechanisms are only a few of the many examples that prove that translational control is an elegant adaptation, which has developed during the evolution of eukaryotes.

1.2.2 Roles of RBPs in RNA decay and stabilization

The regulation of mRNA stability is one of nature's processes that enable the cell to protect a mRNA transcript without embarking upon the energy-demanding steps of transcription, mRNA export, and processing. mRNA decay is presently considered a major control point in gene expression.

Jay Greenberg in 1972 was the first to demonstrate that most mRNAs in the cell are relatively stable because of the proteins coating it (Greenberg, 1972). In particular,

mRNAs in the cytoplasm are not found in a free state. As illustrated in Figure 1-1, mRNAs are always bound by specific proteins, forming ribonucleoprotein complexes (RNPs). These RNPs regulate gene expression via post-transcriptional mechanisms. The primary mechanism results in the degradation, or protection and stabilization of mRNAs. The degradation of mRNA plays several important roles: it is an essential part of the normal expression of genes, and it causes defective mRNAs to be recognized by the cell and to be rapidly degraded in a process termed mRNA surveillance. mRNA decay is a precise process that is dependent on a variety of specific cis-acting elements and trans-acting factors. Once in the cytoplasm, mRNA translation is not necessarily immediate, as a number of events may still occur prior to the translation of the mRNA and subsequent production of the protein. Key steps such as the binding of protein complexes to the mRNA have, at this point, yet to occur.

mRNAs encoding several oncoproteins, cytokines, and signal transduction proteins are unstable, allowing rapid changes in their levels in response to stimuli. Their instability is particularly determined by A+U-rich elements (ARE), which are found in the 3'-UTR region of certain mRNAs and serve to target the transcripts for rapid degradation. Early in Greenberg's discovery, AREs were thought to be unstable signals that promoted the rapid de-adenylation and degradation of mRNA. This destabilizing activity of the AREs is vital for maintaining a critical level of key differentiation factors and cell cycle regulators thereby preventing cell transformation, and ensuring proper cell development (van der Giessen et al., 2003). AREs were initially discovered in cytokine transcripts (Stoecklin et al., 2001), but they also occur in genes involved in growth control, such as the oncogene c-myc (Jones and Cole, 1987), c-fos (Greenberg et al.,

1990; Grosset et al., 2000; Wilson and Treisman, 1988), and cyclinD1 (Rimokh et al., 1994).

Studies of cancer cell lines have revealed the constitutive stabilization of ARE-containing transcripts by cyclosporin A (Nair et al., 1994). The activation of signal transduction pathways has been shown to stabilize various cytokine mRNAs. Examples include TPA, which stabilizes IL-3 and GM-CSF transcripts in mast cells and macrophages, and the activation of T-cells by anti-CD3 and anti-CD28 antibodies, which stabilizes IL-2, GM-CSF, TNFα, and IFNγ transcripts (Stoecklin et al., 2001). The emerging concept is that, in addition to stimulating transcription and translation, exogenous signals stabilize ARE-containing transcripts via protein phosphorylation, which affects, directly or indirectly, ARE-binding proteins. One ARE-binding protein with established mRNA stabilizing activity is HuR, a ubiquitously expressed member of the ELAV protein family (Fan et al., 1997). Recent evidence indicates that mRNAs can appear in the cell as circular RNP structures linked at the 3' end to polyA binding proteins and linked terminally at the 5'end to components of the translation initiation complex (Wilusz et al., 2001). Thus, ARE-protein complexes may function by controlling the access of specific nucleases. In 1985, the Inder Verma group was the first to discover osteosarcoma in several retrovirus induced bone tumors, exemplified by oncogenic forms of the c-fos gene that had lost the 67 nucleotides AT-rich 3'-UTR sequence that is present in non-oncogenic forms of the gene. This observation led to further investigation of the role of this critical 3'-UTR sequence.

A hallmark of AREs is the pentamer AUUUA that occurs either singularly, or in a reiterated or clustered manner. The binding of RBPs to transcripts bearing an ARE can

have either negative or positive effects on such diverse processes as stability, translation, and the subcellular localization of mRNAs. A good example in mammals of both the positive and negative regulation of mRNA decay controlled by RBPs is found with the intriguing c-*fos* mRNA (Schiavi et al., 1994). There are more AREs containing mRNA discovered in this genomic area. As a common feature, the constitutive expression of most ARE-containing genes is very low, and induction occurs transiently in response to exogenous signals. The ARE has a crucial role in this process. In resting cells, ARE-directed decay contributes to the suppression of mRNA expression and the transient incapacitation of ARE function allows for rapid mRNA accumulation upon cell activation. Subsequently, decay takes over again and ensures the return of the mRNA to basal levels.

As illustrated in Figure 1-3, a destabilizing region within the c-*fos* protein coding region, termed major-coding-region determinant (mCRD), has been identified. (Chen et al., 1992; Grosset et al., 2000). *Cis*-acting elements that modulate transcript stability can also be found in the 5' UTR and coding region of mRNAs. In some cases, these elements act in concert with 3' UTR elements to regulate mRNA decay. For example, the *c-fos* mRNA contains an ARE in both the 3' UTR and the mCRD region.

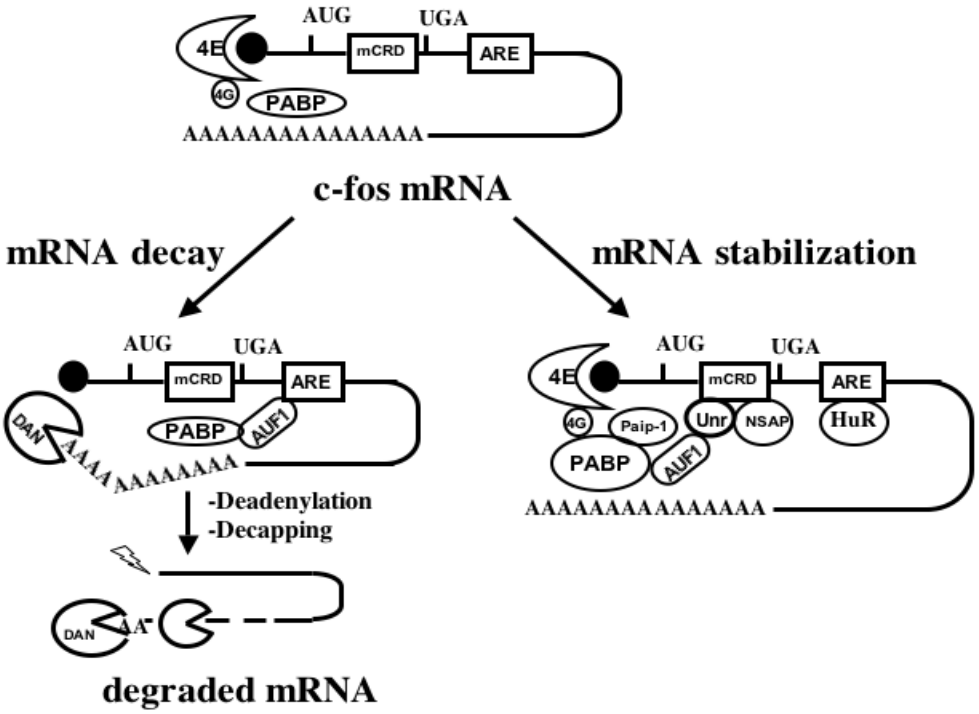

Figure 1-3. Model of c-fos mRNA degradation and stabilization

A complex of five proteins (PABP, PABP-interacting protein, AUF1, NS1-associated protein (Grosset et al., 2000), and Unr (Upstream of N-ras) assembles on the mCRD and this complex stabilizes *c-fos* mRNA by preventing deadenylation. The mCRD needs to be at least 450 nucleotides proximal to the poly(A) tail and requires continuing translation for its destabilizing function. The Shyu group proposes that the transit of ribosomes through the mCRD element disrupts the mCRD complex and triggers the *c-fos* mRNA decay pathway (Grosset et al., 2000). In this case, the mCRD complex might protect untranslated *c-fos* mRNA from the rapid deadenylation-dependent decay that is otherwise promoted by the ARE in its 3' UTR. Moreover, HuR, another general stabilizing factor, binds the ARE region and enhances the binding of PABP to the

poly(A) tail, thus blocking deadenylation and degradation. In the case of *c-fos* mRNA decay, such as that which occurs in resting cells, the interaction of the ARE with the destabilizing factor AUF1 promotes rapid deadenylation by reducing the affinity of the poly(A) binding protein (PABP) for the poly(A) tail. In the final step of mRNA decay, a deadenylating poly(A) nuclease, termed DAN or PARN, degrades mRNA and then decaping enzymes (Dcp1, Pat1, Lsm) finish the *c-fos* mRNA decay process.

It has been shown that the loss of the poly(A) tail profoundly reduces the efficiency of translation initiation. Yet the rapid decay directed by the mCRD requires ribosome transit, of which deadenylation is the necessary first step (Schiavi et al., 1992; Shyu et al., 1991). This finding suggests communication between the mCRD and the poly(A) tail, perhaps via the formation of a unique "bridging" complex. In this interpretation, the bridging complex protects the 3' poly(A) tail from shortening before the message bearing the mCRD is translated. The disruption of the bridging complex follows both translation initiation and the subsequent ribosome transit, and leads to poly(A) tail removal and subsequent decay of the RNA.

1.2.3 Role of RBPs in pre-mRNA Splicing

After transcription, primary transcript RNA molecules are always longer than the final messenger RNA (mRNA). In fact, genes consist of several coding and non-coding segments termed exons (expressed segments) and introns respectively. The average size of a human exon is 150 nucleotides; introns are usually 3500 nucleotides, but can be as large as 500 000 nucleotides (Maniatis and Tasic, 2002). The introns must be removed before translation. This process of splicing allows for the creation of different mRNAs

from a unique pre-mRNA (Berget et al., 1977; Gilbert, 1978). In this way, a single pre-mRNA can produce different proteins. The regulated selection of the exons to be included and joined to generate mature mRNA is defined as alternative splicing. The splicing apparatus recognizes newly synthesized pre-mRNA in the eukaryotic nucleus and includes small nuclear RNAs complexed with a multitude of RNA binding proteins to form small nuclear ribonucleoproteins (snRNPs). These snRNPs associate with the pre-mRNA in an orderly fashion, thus constituting the basal machinery that mediates splicing.

One question remains: What is the function of this non-coding RNA? The answer is becoming much clearer. Although introns can vary in size and in sequence, they maintain several conserved motifs. In their 5'ends, GU dinucleotides encompassed within a larger, less conserved consensus sequence for splice donor sites and at the other end of the intron, the 3'splice site region consist of three conserved sequence elements: the branch point, followed by a polypyrimidine-rich track and a terminal AG at the extreme 3' end of the intron. The components of the splicing machinery recognize all of these motifs: U1 snRNP binds to the 5' splice site (SS); U2 snRNP binds to the branch point adenosine; and U2AF proteins lie on the splice-acceptor sequences. Recognition leads to the recruitment of other components of the spliceosome (U4, U6, and U5 snRNPs), followed by a rearrangement of RNA and the release of the intron.

The mechanism by which exons are chosen to be included during the splicing process remains misunderstood. During the process of alternative splicing, RBPs seem to play a key role in the specific recognition of exons to be excluded or included. Alternative splicing regulation is mediated by the interactions of the splicing machinery

with RBPs that enhance or repress the spliceosome assembly. This process has been studied for the last decade and much remains unknown. There are numerous examples that reveal the clever ways that biological evolution has led to the elaboration of finely regulated mechanisms of alternative splicing. Tissue-specific, positive and negative regulatory factors have recently been shown to regulate neuron-specific alternative splicing in mammalian cells. The Robert Darnell group recently revealed that the mechanism of specific alternative splicing of the GABA receptors is mediated by the RBP Nova-1 (Dredge and Darnell, 2003).

Nova-1 and Nova-2 are closely related RNA binding proteins that were first identified as auto-antigens in patients with paraneoplastic opsoclonus myoclonus ataxia (POMA), a disorder characterized by a loss of the inhibitory control of motor neurons in the spinal cord and brainstem. The expression of Nova-1 is restricted to the brain stem, the diencephalons, and the motor neurons of the ventral spinal cord. In contrast, the expression of Nova-2 is largely complementary to that of Nova-1. High levels of Nova-2 expression are observed in the cerebral cortex, the hippocampus, and the dorsal spinal cord. *Nova-1*-null mice exhibit the apoptotic death of motor neurons. These mice die post-natally and show symptoms similar to those observed in POMA that are consistent with a lack of the inhibitory control of the motor neurons of the ventral spinal cord. Moreover, *Nova-1*-null mice exhibit alternative splicing defects in GlyRα2 and $GABA_A$ receptor γ2 pre-mRNAs (Jensen et al., 2000a).

Compared to their wild-type littermates, knockout mice have a three-fold reduction in the splicing of the GlyRα2 and $GABA_A$ receptor γ2. The $GABA_A$ receptor in particular is the target of choice for treating human diseases such as anxiety and

schizophrenia. The $GABA_A$ receptor is a ligand-gated chloride channel, which is important for synaptic inhibition in the mammalian brain in response to the neurotransmitter GABA (Kandel et al., 2000). Diverse subunits (α, β and γ) and isoforms of the receptor are produced either by the assembly of different combinations of subunits or by alternative splicing. As shown in Figure 4, the alternative splicing of the $GABA_A$ γ2 cassette exon 9 generates two mRNA isoforms: the γ2 Large (γ2L: exon 9 is included), and the γ2 Short (γ2S: exon 9 is skipped). The binding affinity of the GABA and synthetic benzodiazepine agonists is reduced in the γ2L isoform. A preferred RNA binding sequence of Nova-1 (5'-UCAU 3'), is located in intronic regions downstream of exon 9 and plays the role of splicing enhancer for the inclusion of exon 9 and the production of γ2L GABA receptor mRNA.

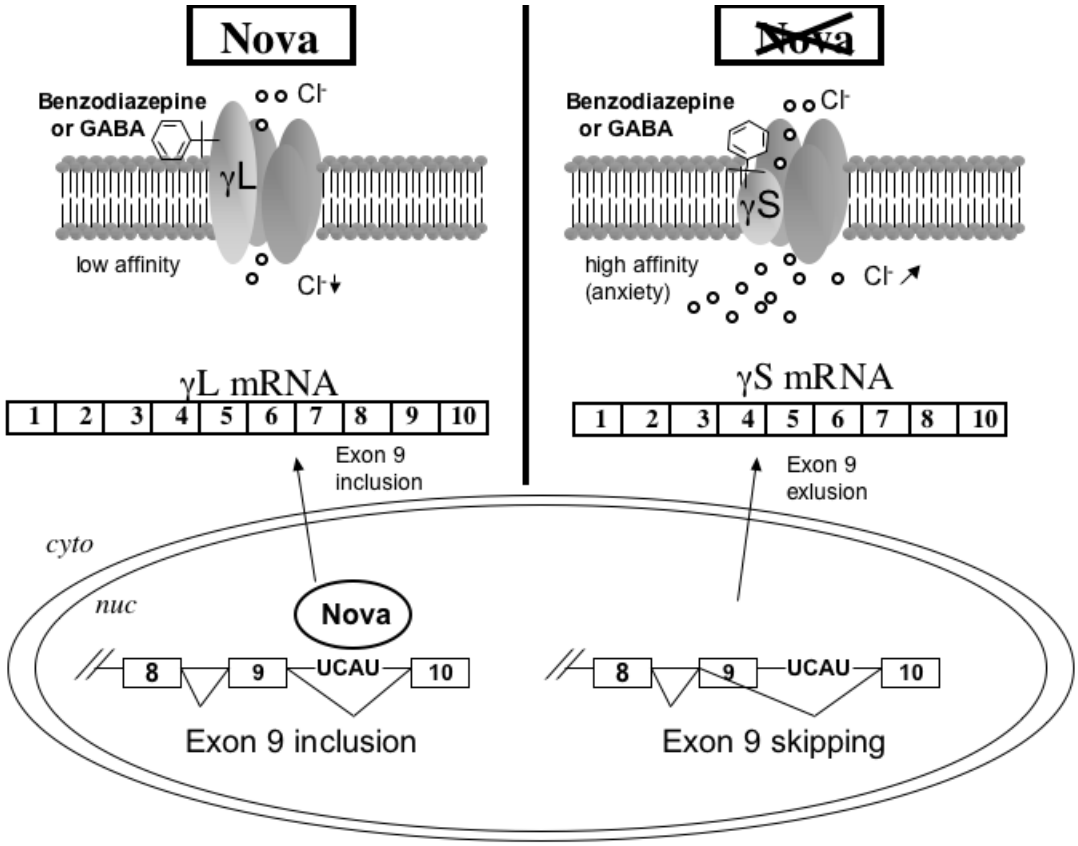

Figure 1-4. Nova proteins function in GABA receptors alternative splicing

This GABA receptor alternative splicing is the first example to demonstrate that a small RNA element is necessary to mediate the action of a neuron-specific mammalian splicing activator. Neural-specific mRNA encoding proteins, such as NCAM (Cote et al., 1999), Neurexins (Ullrich et al., 1995) and Neuroligin (Scheiffele et al., 2000), are among the proteins that have a diversity of spliced isoforms. In the near future, new tools in genomic, proteomic, and ribonomic science will lead us to a better understanding of the RBPs role in biology.

1.2.4 Role of RBPs in mRNA Trafficking

The life of mRNAs begins in the nucleus after transcription by RNA polymerase II. These pre-mRNAs start to undergo a number of processing steps, such as splicing, 5'end capping, and polyadenylation. These modifications distinguish these mRNAs from other RNAs and allow for an improved nuclear export, stabilization, and translation. Like an open pearl necklace, RNA with RBPs form a complex referred to as a ribonucleoparticle (RNP). Electron microscopic analysis has shown that the RNP is the mature form of mRNAs that exits the nucleus. A RNP structure, called a Balbiani ring particle (BR), from the salivary glands of insects has been described (Edstrom et al., 1983; Kiesler et al., 2002). During its transcription, the BR particle is decorated with heterogeneous nuclear ribonucleoparticle (hnRNP) proteins (Lodish, 2004). These observations suggest that mRNA export is coupled with transcription, and that hnRNP proteins coat and follow RNAs from the early formation of nascent RNA transcripts in the nucleus to translation in the cytoplasm (Cullen, 2003).

The process of mRNA export is different from protein export. The protein export machinery uses a nuclear pore complex (NPC), which contains such proteins as karyopherin, cargo, importin, and Ran-GTPase. The RNA export pathway also requires the NPC; however, it does not require the same protein partners. The NPC is a protein complex composed of ~ 50 different proteins that is inserted into the double membrane of the nuclear envelope. Despite some controversy, it is now accepted that mRNA export is more complicated than protein export. Because transcription and processing occur in the nucleus, the mRNA export machinery must be capable of distinguishing between immature pre-mRNA and fully processed mRNA. The NPC recognizes and selectively

transports “export-ready” mature mRNAs. Proteins implicated in mRNA export are transport receptors, such as TAP. Although TAP is able to interact directly with RNA in a sequence-nonspecific fashion through its non-canonical RNP domain, this region of TAP is not essential for mRNA export *in vivo,* thus creating the need for, RNA binding proteins (RBPs) in order to bridge the interaction between TAP and mRNA. RBPs such as hnRNP A1, SR proteins, CBC, and PABP are shuttling proteins; they remain bound to mRNA to facilitate RNA export from the nucleus. Interestingly, SR proteins, which are known to be splicing factors, also have a central role in mRNA export (Huang and Steitz, 2001; Reed, 2003). In fact, the nuclear export complex is formed during the process of splicing at the exon-exon junction complex (EJC). Splicing factors (Y14, Upf3, and SRm160) and other such proteins are implicated in mRNA export. Moreover, adjacent to TAP proteins, RBPs play a key role in the specificity of the mRNA export process. Shuttling proteins such as hnRNPA1, hnRNPA2, HuR, and CRM1 are also important for helping the mRNA to cross the nuclear pore (Erkmann and Kutay, 2004).

One question remains: After mRNAs cross the nuclear membrane, how are they localized inside the cytoplasm? All mRNAs must be translated at a precise site in the cell cytoplasm; they must therefore move within the cell to reach their functional place in the cytoplasm. This dynamic process of mRNA movement is called mRNA trafficking and relies on a complex network of protein interactions, which result in the formation of export-competent mRNP particles. In the cytoplasm, the cytoskeleton is often required for mRNA localization, and the direct visualization of specific mRNAs in living cells has recently proven that cytoskeleton proteins and RBPs transport mRNAs (Cullen, 2003; Gallouzi and Steitz, 2001). RNA localization has been shown to regulate the distribution

of proteins within the cytoplasm of differentiated cells, including cardiomyocytes, neurons, and oligodendrocytes (Belanger et al., 2003; Carson et al., 2001b; Figueroa et al., 2003; van der Giessen et al., 2003).

The mRNAs are often present in structures in the cytoplasm called RNP granules. These structures are thought to contain the necessary components for RNA transport, localization, and translation. Ethidum-bromide staining and *in situ* hybridization procedures have demonstrated that RBPs are present on mRNA in granules. For example, Staufen proteins are localized in RNP granules in the dendrites of mouse neurons (Duchaine et al., 2002; Kiebler and DesGroseillers, 2000). Drosophila Staufen has also been shown to be required for the localization and transport of *oskar* mRNA to the posterior pole of the oocyte (Micklem et al., 2000). Another example is gurken mRNA, whose localization and translation are coupled with an interaction with the translational repressor protein Bruno during oogenesis (Norvell et al., 1999). In the hippocampal neurons of mammals, the α subunit of calcium-calmodulin-dependent kinase II (CaMKII-α) mRNA, which is essential for memory and long-term potentiation, is transported to the dendrites; the mRNA gets translated in the synaptic regions (Mayford et al., 1996; Miller et al., 2002). These results demonstrate that mRNA transport is important for the synaptic delivery of the calmodulin kinase and that its local translation contributes to synaptic and behavioral plasticity (Steward, 2002).

One class of cell that is a model of choice for studying mRNA trafficking is the oligodendrocytes. In the central nervous system (CNS), oligodendrocytes myelinate axons by transporting myelin components to the distal tips of its processes, at which point the plasma membrane wraps around the axons to form myelin sheaths. During normal

oligodendrocyte development, the microtubule network density increases with age (Song et al., 2001). More importantly, this microtubule network with appropriate spacing and branching is essential for the intracellular trafficking of myelin components in oligodendrocytes. Cell fractionation analysis reveals that many proteins are transported along microtubules of the oligodendrocytes from the cell body to the periphery. These include specific myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin-associated glycoprotein (MAG), as well as mRNAs encoding specific myelin proteins such as myelin basic protein (MBP) and myelin oligodendrocytic basic protein (MOBP) (Brophy et al., 1993; Carson et al., 1998; Colman et al., 1982). When these mRNAs reach the periphery, eIF factors with free poly-ribosomes translate the mRNA.

In vivo microscopy of cultured oligodendrocytes microinjected with labeled mRNA has demonstrated that MBP mRNAs are assembled into trafficking intermediates. These granules move bidirectionally along microtubules. The trafficking pathway for each RNP granule is determined by specific *cis*-acting elements in the RNA sequence, and by trans-acting factors such as RBPs transporting the RNA. For example, MBP mRNA trafficking is mediated by an 11 nucleotide *cis*-acting sequence (GCCAAGGAGCC), termed the hnRNP A2 response element (A2RE), and by its cognate *trans*-acting ligand, hnRNP A2 (Hoek et al., 1998). MBP mRNA containing A2RE-sequence at its 3'-UTR is bound to the hnRNP A2 protein. This association is sustained throughout trafficking.

Other sequences in MBP mRNA on 3'-UTR have been identified, one of which is the RNA trafficking signal (RTS). Figure 5 summarizes the model of MBP mRNA

trafficking in oligodendrocytes (Carson et al., 2001a). The MBP mRNA transport hypothesis contains the five following steps: nuclear export, granule assembly, translation repression during the transport process, granule transport, and translation activation. It is noteworthy that translation repression occurs during this mRNA trafficking process. Indeed, RBPs are often implicated in translation inhibition. Despite all of our current knowledge on RNA transport, one question remains: What is the mechanism of translation activation when the mRNA reaches its proper place at the distal tips of the oligodendrocytes?

Moreover, the movement of RNP granules along microtubules requires cytoskeletal proteins such as kinesin, which move granules towards the cell periphery (plus ends). Meanwhile, dynein moves granules towards the cell body (minus ends). In addition, microtubule associated protein Map1B is known to play a central role in the RNA trafficking process in oligodendrocytes (Wu et al., 2001). Because hnRNP A2 is expressed in many different cell types besides oligodendrocytes, it is likely that MBP mRNA transport may also depend on other RBPs that are more oligodendrocyte-specific. These RBP factors are yet unidentified. This thesis will address this question of MBP mRNA transport aided by RBPs.

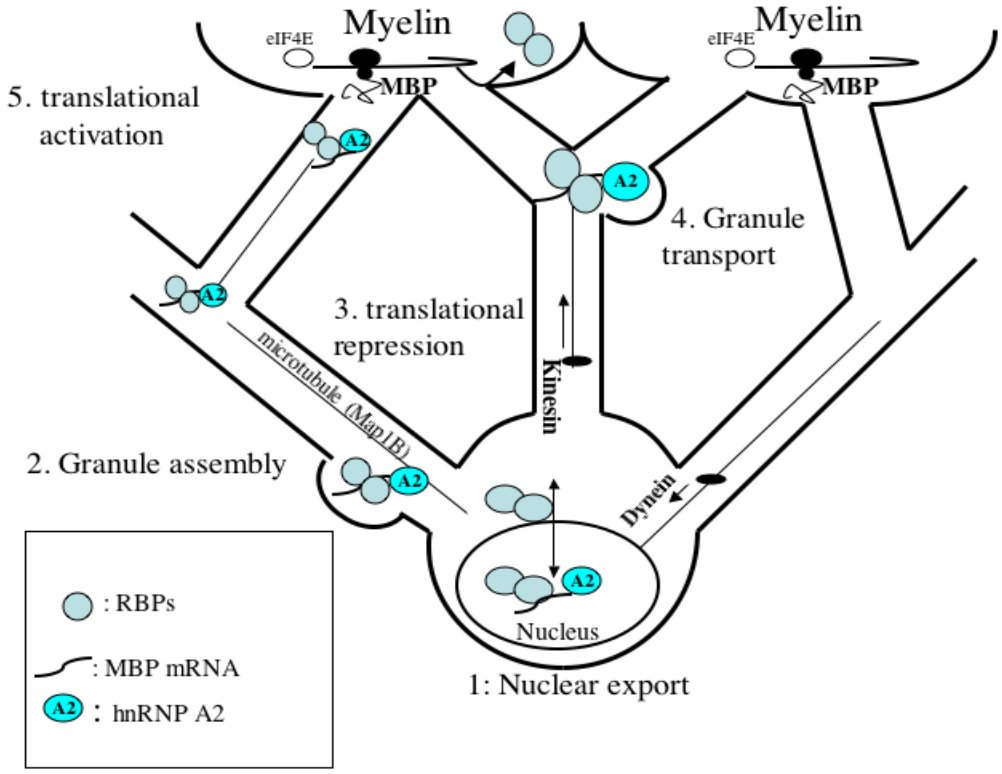

Figure 1-5. Model proposed for MBP mRNA trafficking in oligodendrocytes

1.2.5 Role of RBPs in RNAi and miRNA biogenesis

In the last decade, elegant work using the nematode *Caenorhabditis elegans* formally described that the non-coding RNA molecules could control gene expression. In 1993, Victor Ambros and his colleagues discovered a small RNA (miRNA) called lin-4. This miRNA is antisense to multiple sites in the 3'-UTR of lin-14 (Ambros, 2003). The interaction of lin-4 miRNA with the developmental transcription factor lin-14 suppresses its translation and affects the cell division of the first larval stage of *C. elegans*. More recently, in 1998, a new gene silencing method that used dsRNA was unexpectedly discovered to be more effective than using either the sense or antisense strand alone (Fire

et al., 1998). This methodology was named RNA interference (RNAi). Some distinct differences exist between miRNA and RNAi. In fact, miRNAs are only capable of the hetero-silencing of genes that are unrelated to its own origins. Conversely, RNAi molecules can auto-silence the same or similar genes of its origins.

The total number of miRNAs in humans is unknown, but is estimated to represent ~1% of all human genes (Bartel, 2004). Usually, miRNAs are 19-25 nt in length and function to regulate gene expression and development. As more miRNA targets are described, it becomes apparent that the regulation of development might be a common function of miRNAs. In fact, miRNAs and their targets have been found to affect diverse processes, including developmentally regulated cell proliferation in *Drosophila* (Moberg and Hariharan, 2003), flowering time and leaf patterning in *Arabidopsis*, and neuronal asymmetry in *C. elegans* (Lau et al., 2001). Moreover, miRNA and their associated RNA binding proteins appear to be some of the more abundant ribonucleoproteins complexes in the cell (Bartel, 2004). Nevertheless, methodologies for miRNA cloning are more complicated. A number of algorithms that aim to predict miRNA targets and mammalian miRNAs have therefore been described (Lewis et al., 2003). Despite the identification of >200 miRNAs in plants and animals, very few miRNA targets have been experimentally confirmed. A major challenge for future research will be to elucidate the function of miRNAs and to identify the specific target genes that they regulate.

As illustrated in Figure 1-6, the mechanism of gene silencing by miRNA is known to occur via a highly conserved two-step process. First, the nuclear cleavage of a pre-miRNA produces a 60-70 nt stem loop pre-miRNA precursor. Drosha RNase performs this processing by cleaving both strands of the stem loop. This pre-miRNA is then

transported from the nucleus to the cytoplasm. Afterward, the RNA binding protein Dicer, which also has RNase activity, cuts double-stranded RNA and forms an imperfect RNA duplex. The single-stranded RNA-induced silencing ribonucleoprotein complex (RISC) is formed, and the mature miRNA specifies direct cleavage if the mRNA has sufficient complementarity to the miRNA sequence. Interestingly, accessory RBPs are very important for miRNA biogenesis. The fragile X mental retardation protein (FMRP) has been shown to interact with both miRNAs and the components of the miRNA pathways, including Dicer, Gemin, and Argonaute (Jin et al., 2004). Moreover, the involvement of FMRP in neural development and synaptogenesis was shown to be dependent on its interaction with Argonaute proteins.

The second form of gene regulation initiated by double-stranded RNA is RNAi. The mechanism of gene silencing by RNAi also proceeds via a highly conserved two-step process. First, the ribonuclease Dicer cleaves long dsRNAs by generating small interfering RNAs (siRNAs) that are 21–23 nucleotides in length (Bernstein et al., 2001). Subsequently, the single-stranded antisense siRNA associates with the RISC complex and finally guides the target RNA cleavage (Hannon, 2002; Sharp, 1999). Although many endogenous siRNA have been discovered in *C. elegans*, no endogenous siRNAs seem to regulate gene expression. However, there is no doubt that exogenous synthetic siRNA transfected into cells have the potential effect of down-regulating targeted genes. The siRNAs that form 21-nt double-stranded RNA intermediates have became a powerful tool to knock down specific gene expression in mammalian cells and will potentially be useful for analyzing loss-of-function phenotypes. In conclusion, the discovery of dsRNA

molecules gives new scientific insight and raises new possibilities for the extraordinary potency and efficacy of the therapeutic uses of RNA.

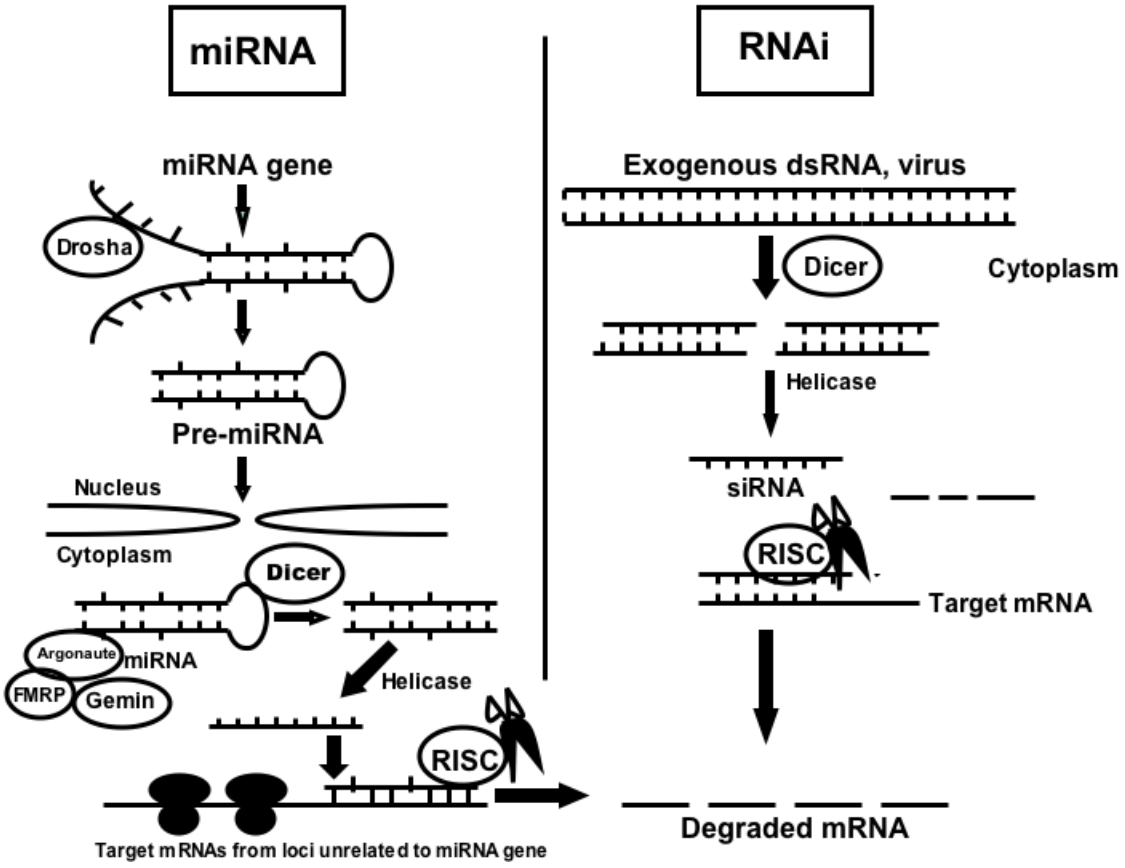

Figure 1-6. Two related mechanism for miRNA and RNAi biogenesis

1.2.6 Role of RBPs in Nonsense-Mediated mRNA Decay (NMD)

NMD is a quality control mechanism that rapidly degrades nonsense transcripts, such as mRNAs harboring premature termination codons and polyadenylation, or mRNAs issued from incorrect splicing (Lykke-Andersen et al., 2000). First discovered in yeast, this quality control mechanism is ubiquitous among eukaryotes. Coupled with the mRNA splicing process at the exon-junction complex (EJC), this pathway results in the degradation of aberrant mRNAs. There is evidence that NMD requires RNA binding proteins such as Upf3, Upf2, Y14, SRm160, and Aly/REF (Singh and Lykke-Andersen, 2003).

The mechanism involves multiple steps. First, abnormal mRNAs that contain premature translation termination codons 50 nucleotides upstream of the last exon-exon junction activate NMD. After splicing, the EJC multi-protein complex is deposited 20-24 nt upstream of each exon-exon junction. Later, although the first translation event removes a major portion of the EJCs from the mRNA, one or more EJCs remain associated with the mRNA. Upf proteins (Upf1, Upf2, Upf3) as well as Y14, SRm160, and Aly/REF are recruited to form an active NMD complex. This event triggers the rapid decay of the mRNA. The degradation of the mRNA begins with the de-capping step, followed by deadenylation, as discussed in Section 1.2.2 of this thesis.

In order to prove the importance of RNA binding proteins in NMD regulation, it is helpful to describe the function of the *C. elegans* GLD-1 protein that is able to repress oocyte production at the distal end of the hermaphrodite germ line. Most importantly, GLD-1 is a translational suppressor, whose activity is dependent upon the presence of target mRNA that GLD-1 binds. In terms of spatio-temporal protein expression in the

worm, GLD-1 is present at the distal end. Its function is to protect the gna-2 mRNAs, one of its RNA targets, from degradation by NMD. In the model recently proposed by Tim Schedl's laboratory, gna-2 RNA has two μORF coding sequences that lead to the degradation of the parental gna-2 mRNA (Lee and Schedl, 2004). The proposed model is that gna-2 mRNA is a naturally occurring mRNA target of nonsense-mediated mRNA decay (NMD), and that the binding of GLD-1 protects gna-2 mRNA from degradation, likely by repressing the translation of the μORFs. This example demonstrates that RBPs play an essential role in RNA biogenesis in animal embryos.

1.3 Classification of RNA Binding Proteins by Domains

Several structural motifs for RNA binding proteins have been described. These motifs serve to classify RNA binding proteins in four major classes: the KH domain containing class, the RRM class, the dsRBD (double strand RNA binding domain) class, and the DEAD-box helicase domain containing class. Table 1 shows this classification and describes a few key examples of proteins for each class.

Table 1. Classes and Characteristics of Representatives Mammalia RNA Binding Proteins

Family	Proteins	Functions	References
KH	SF1	Splicing	Das and Frankel, 2003
	hnRNPK	Translational regulation, Transcription	Dreyfuss et al., 2002
	FMRP	RNA localization in neurons, miRNA	Darnell et al., 2001,Brown et al., 2001; Khandjian, 1999
	Nova-1 and 2	GABA and GlyR receptor splicing, synaptic biology	Dredge and Darnell, 2003
	Sam68	unknown, interacts with Src family tyrosine kinases (Fyn, BRK), HIV RNA export	Lukong and Richard, 2003
	SLM-1 and 2	unknown, SLM-1 interacts with Src	DiFruscio et al., 1999
	QKI	MBP mRNA binding, MAG splicing	Wu et al., 2002; Zhang et al., 200 Chen et al., 1998; This study
RRM	hnRNP A2	RNA transport, MBP mRNA trafficking	Carson et al., 1998
	hnRNP A1	mRNA splicing, Telomere biogenesis, mRNA export	Chabot et al., 2003
	HuR,D,B,C	RNA stabilization, RNA transport Neuronal differentiation (HuD)	Gallouzi and Steitz, 2001 Okano and Darnell, 1997
	Musashi	neuronal differentiation, m-Numb translation repression, activation of Notch signaling	Okano et al., 2002
	Upf3	Nonsense-mediated decay	Lykke-Anderson, 2004
dsRBD	PKR	translation inhibition, interferon response	Saunders and Barber, 2003 Koromilas et al., 1992
	PTBP	mRNA splicing and localization polyadenylation	Chan and Black, 1997
	Staufen-1 Staufen-2	RNA transport RNA granule transport in neurons	Kiebler and DesGroseillers, 2000
	Dicer	RNA interference	Bernstein et al., 2001
DEAD (helicases)	Gemin	splicing, miRNA biogenesis translation initiation,	Dreyfuss et al., 2002
	eIF4A	RNA dependent ATPase, ribosome biogenesis	Sonenberg and Dever, 2003

1.3.1 KH domain containing RNA binding proteins

The first class described in Table 1 is the KH domain containing family, KH denoting heterogeneous nuclear ribonucleoprotein K (hnRNPK) homology. The KH domain was originally studied in hnRNP K (Siomi et al., 1993). The elucidation of the complete sequence of the human and mouse genomes has demonstrated that the KH domain is the second most prevalent RNA binding motif found in proteins (Burd and Dreyfuss, 1994). In fact, the predicted number of KH domain containing proteins in mice and in humans is 28 (Lander et al., 2001; Lukong and Richard, 2003; Venter et al., 2001; Waterston et al., 2002).

The KH domain contains approximately 50 conserved amino acids. In general, hnRNPs containing the KH domain bind RNA, and are involved in pre-mRNA processing and mRNA transport from the nucleus to the cytoplasm. The first three-dimensional structure of the KH domains of the Vigilin protein was clarified by nuclear magnetic resonance (NMR), which showed that the domain consisted of a stable βααββα structure (Musco et al., 1997). It has been suggested that this is a potential RNA binding surface, where two α-helices are connected by a highly conserved glycine-lysine-X-glycine (GKxG) motif (Musco et al., 1996). In addition, the KH motif forms a vise-like structure whose crystal structure has also been elucidated for the Nova-2 protein and for FMRP (Lewis et al., 1999; Musco et al., 1997). For Nova-2, the RNA target sequence consensus is 5'-UCAY(pyrimidine)-3' (Jensen et al., 2000b; Lewis et al., 2000).

One subclass of the KH domain proteins is the STAR proteins; STAR denotes Signal Transduction and Activation of RNA. These proteins, such as Sam68, are linked to signaling pathways and possess proline-rich sequences that are the sites of protein-protein

interactions with SH3 domain-containing proteins (Taylor and Shalloway, 1994). Sam68 is Src-associated in mitosis (Lukong and Richard, 2003), and has been shown to interact via its SH3 and/or SH2 domains with several signaling molecules, including Ras GTPase, phospholipase Cγ-1, Grb2 , PI3K, and IRS-1 (Richard et al., 1995; Sanchez-Margalet et al., 2003; Taylor et al., 1995). Sam68 has also been identified as a substrate for a series of tyrosine kinases from the Src family that includes $p56^{lck}$, $p59^{fyn}$, BRK/SIK, and ZAP70 (Derry et al., 2000; Lang et al., 1997; Richard et al., 1995; Vogel and Fujita, 1995). The association with the SH3 domain proteins and the tyrosine phosphorylation of Sam68 during mitosis has been demonstrated to regulate its RNA binding activity (Chen et al., 1997). Moreover, Sam68 is a ubiquitously expressed nuclear protein. The integrity of the KH domain seems necessary for its nuclear localization (Chen et al., 1999; McBride et al., 1998). Sam68 localizes in novel nuclear structures called SNBs, Sam68 Nuclear Bodies, found mostly in transformed cells (Chen et al., 1999).

Interestingly, because Sam68 is a RNA binding protein, some virologists have tried to elucidate the role of Sam68 in RNA-containing virus replication, such as polioviruses and retroviruses. Upon poliovirus infection, Sam68 interacts with viral RNA polymerase 3D and re-localizes to membranous vesicles at the plasma membrane (McBride et al., 1996). Interestingly, in the case of HIV infection, Sam68 can functionally replace or synergize Rev function and allow for the export of un-spliced HIV RNAs (Reddy et al., 1999). Furthermore, a Sam68 dominant negative form has been described to act with a transdominant negative phenotype in HIV replication (Reddy, 2000). Despite the fact that Sam68 clearly activates Rev, some controversy remains regarding the specificity of Sam68 interaction with RRE (Rev Response Element) (Soros

et al., 2001). In particular, further work on Sam68 in the context of HIV-1 infected lymphocytes cells should address key questions. From a biochemical point of view, Sam68 binds to ribonucleotide homopolymers, such as poly U and poly A (Taylor and Shalloway, 1994). By using recombinant purified Sam68 proteins, a SELEX (systematic evolution of ligands by exponential enrichment) methodology has been performed. Specifically, this assay has shown that Sam68 binds the RNA sequence UAAA with high affinity (Itoh et al., 2002; Lin et al., 1997). However, neither the role of Sam68 in RNA metabolism nor its physiological RNA targets have been identified thus far. Since the discovery of Sam68, we are just beginning to elucidate its RNA target as well as its link to signaling pathways.

1.3.2 QKI, a KH domain-containing RNA binding proteins with a GSG domain

For this thesis in particular, one of the intriguing KH domain-containing proteins among the STAR protein family is QUAKING (QKI) . The gene *qki* and its encoded QKI proteins have been cloned and sequenced by Karen Artzt's laboratory (Ebersole et al., 1996). These *qki* gene generates three major isoforms (QKI-5, QKI-6, and QKI-7), all of which possess the same single KH domain. Interestingly, as described in Sam68, QKI proteins contain an extended KH domain that is flanked by the conserved N- and C-terminal sequences NK (Qua1) and CK (Qua2). Other KH-containing proteins, such as How in Drosophila, and GLD-1 in *C. elegans*, also possess an extended KH domain (Ryder et al., 2004). Figure 1-7 shows the alignment of a QKI-5 protein with most homologous proteins containing a single KH domain.

Figure 1-7. Multiple sequence alignment of QKI protein homologues. Mouse QKI-5, *Drosophila melanogaster* How, *Caenorhabditis elegans* GLD-1 and mouse Sam68 were aligned using ClustalW software. The KH domain is delimited using bars and dashed bars for NK and CK. Conserved amino acids are indicated with a star (*) and similar amino acids are indicated with single (·) and double dots (:).

```
QKI-5          ------------------------------------------------------------
How            MSVCESKAV-VQQQLQQHLQQQAAAAVVAVAQQQQAQAQ---------------------- 38
Gld1           MPSCTTPTYGVSTQLESQSSESPSRSSVMTPTSLDGDNSPRKRFPIIDNVPADRWPSTRR 60
Sam68          MQRRDDPASRLTRSSGRSCSKDPSGAHPSVRLTPSRPSPLPHR----------------- 43

QKI-5          ------------------------------------------------------------
How            ----------------------------AQAQAQAQQQQQAPQVVVP-------------- 57
Gld1           DGWSSVRAPPPARLTLSTNNRHIMSPISSAYSQTPNSLLSPAMFNPKSRSIFSPTLPATP 120
Sam68          --------------------PRGGGGGPRGGARASPATQPPPLLPPSTPGPDATVVG--- 80
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QUA1 (NK)

```
                                    ..................................................
QKI-5          -----------MVGEMETK-EKPKPTPDYLMQLMNDKKLMSSLPNFCGIFNHLERLLDEE 48
How            -------MTPQHLTPQQQQ-QSTQSIADYLAQLLKDRKQLAAFPN---VFTHVERLLDEE 106
Gld1           MSYGKSSMDKSLFSPTATEPIEVEATVEYLADLVKEKKHLTLFPH---MFSNVERLLDDE 177
Sam68          ------SAPTPLLPPSATAAVKMEPENKYLPELMAEKDSLDPS------FTHAMQLLSVE 128
                          .       . :.  .** :*: ::. :        *.:  :**. *
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QUA1 (NK) **KH**

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                                   ..................................................
QKI-5          ISRVRKDMYNDTLNGSTEKRSAELPDAVGPIVQLQEKLYVPVKEYPDFNFVGRILGPRGL 108
How            IARVRASLF--QING-VKKEPLTLPEPEGSVVTMNEKVYVPVREHPDFNFVGRILGPRGM 163
Gld1           IGRVRVALFQ------TEFPRVELPEPAGDMISITEKIYVPKNEYPDYNFVGRILGPRGM 231
Sam68          IEKIQKGESK------KDDEENYLDLFSHKNMKLKERVLIPVKQYPKFNFVRKILGPQGN 182
               * :::           .     *       : : *:: :* .::*.:*** :****:*
```

KH

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               ================================================================
QKI-5          TAKQLEAETGCKIMVRGKGSMRDKKKEEQNR--GKPNWEHLNEDLHVLITVEDAQNRAEI 166
How            TAKQLEQETGCKIMVRGKGSMRDKKKEDANR--GKPNWEHLSDDLHVLITVEDTENRATV 221
Gld1           TAKQLEQDTGCKIMVRGKGSMRDKSKESAHR--GKANWEHLEDDLHVLVQCEDTENRVHI 289
Sam68          TIKRLQEETGAKISVLGKGSMRDKAKEEELRKGGDPKYAHLNMDLHVFIEVFGPPCEAYA 242
               * *:*: :**.** * ******** **.  *  *..:: **. ****::   ..  ..
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QUA2 (CK)

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               ================..................................................
QKI-5          KLKRAVEEVKKLLVPAAEGEDSLKKMQLMELAILNGTYRDANIKSPALAFSLAATAQAAP 226
How            KLAQAVAEVQKLLVPQAEGEDELKKRQLMELAIINGTYRDTTAKSVAVCDEEWRRLVAAS 281
Gld1           KLQAALEQVKKLLIPAPEGTDELKRKQLMELAIINGTYRPMKSPNPARVMTAVPLLSPTP 349
Sam68          LMAHAMEEVKKFLVP--DMMDDICQEQFLELSYLNGVPEPSRGRGVSVRGRGAAPPPPPV 300
                :  *: :*:*:*:*  :  *.: : *::**: :**. .     . :          ..

QKI-5          --RIITGPAPVLPPAALRTPTPAGPTIMPLIR-------QIQTAVMPNGTPHPTAAIVPP 277
How            DSRLLTSTGLPGLAAQIRAP-AAAPLGAPLILN-PRMTVPTTAASILSAQAAPTAAFDQT 339
Gld1           ----LRSSGPVLMSPTPGSGLPSTTFGGSILS-------PTLTASNLLGSNVFDYSLLSP 398
Sam68          P--RGRGVGPPRGALVRGTPVRGSITRGSTVTRGVPPPPTVRGAPTPRARTAGIQRIPLP 358
                     . .    .    :   .     . :           *    .       :  .

QKI-5          GPEAGLIYTPYEYP-YTLAPATSILEYPIEPSG------------VLGAVATKVRRHDMR 324
How            G--HGMIFAPYDYANYAALAGNPLLTEYADHS--------------VGAIKQQRRLATNR 383
Gld1           S--MFDSFSSLQLASDLTFPKYPTTTSFVNSFPG-----------LFTSASSFANQTNTN 445
Sam68          PTPAPETYEDYGYDDTYAEQSYEGYEGYYSQSQGESEYYDYGHGELQDSYEAYGQDDWNG 418
                      :                      .                  :     .

QKI-5          VHPYQRIVTADRAATGN-------- 341
How            EHPYQRATVGVPAKPAGFIEIQ--- 405
Gld1           VSPSGASPSASSVNNTSF------- 463
Sam68          TRPSLKAPPARPVKGAYREHPYGRY 443
                 *      .  .
```

The amino acid sequence within the KH domain is highly conserved. For example, the *Drosophila melanogaster* How protein is 46% similar to QKI proteins. Other KH domain-containing proteins, such as *C. elegans*, GLD-1 (35%), and mouse Sam68 (30%), are similar to QKI-5 in particular in regions outside of KH domain. This entire RNA binding domain is referred to as the GSG (GRP33, Sam68, GLD-1) domain. The acronym "GSG domain" was chosen to describe the first three proteins that were identified as containing this conserved region (Di Fruscio et al., 1998; Jones and Schedl, 1995), and that of the "STAR domain" was created to define the STAR family of proteins (Vernet and Artzt, 1997). Both names are currently used in the literature; however, "GSG domain" is now more widely accepted and is more appropriate, as the "STAR domain" does not contain signaling properties (Lukong and Richard, 2003).

QKI isoforms can homodimerize and heterodimerize (Chen and Richard, 1998; Pilotte et al., 2001; Wu et al., 1999). Mapping experiments using recombinant QKI have shown that the NK region is necessary and sufficient for dimerization (Chen and Richard, 1998; Ryder et al., 2004). QKI putative 3D structure analysis based on other known related KH domains, such as Nova-1 (Lewis et al., 1999), SF-1 (Peled-Zehavi et al., 2001), and FMR-1 (Musco et al., 1997), demonstrated the required structure orientation for RNA binding activity by the QKI dimer (Figure 1-8).

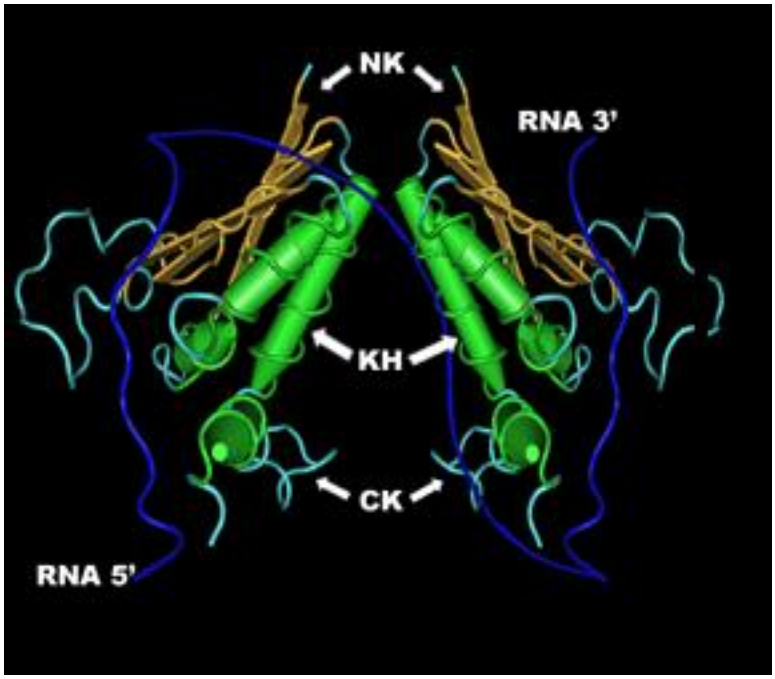


Figure 1-8. Predicted 3D model of the association of QKI dimers with RNA. This putative 3D structure prediction, performed by Cn3D software, is based on other known related KH domains, such as Nova-1, SF-1, and FMR-1. The blue ribbon represents RNA hexanucleotide. The NK region is represented by orange ribbons; the KH region, by green ribbons; and the CK, by light blue.

The QKI structure resembles an open-faced sandwich consisting of two monomers that contain three-stranded anti-parallel beta sheets topped by three alpha helices (Lewis et al., 1999). This structure reveals that the NK region bridges QKI dimers and corroborates mapping experiments that identified the point mutation of glutamic acid 48 to a glycine (E48G) in the NK region that prevents dimerization but not RNA binding activity (Chen and Richard, 1998). The QKI self-association region was mapped to amino acids 18 to 57, a region predicted to form coils. These biochemical results are also consistent with mutant mice harboring the E48G point mutation. In fact, qk^{Kt4} mutant

mice that harbor the E48G mutation and come from chemical mutagenesis using ethylnitrosourea (ENU) are known to be embryonically lethal (Ebersole et al., 1996). The precise reasons for lethality are still unknown; however, the fact that QKI E48G is not able to dimerize suggests that *in vivo* QKI dimerization is crucial.

Moreover, it has been demonstrated that the QKI GSG domain mediates RNA binding activity (Chen and Richard, 1998). In particular, a region spanning the KH and CK regions is required for high-affinity RNA binding (Ryder et al., 2004). Work in our laboratory has recently demonstrated that a RNA element that has the sequence UACUAA(C/A) is the consensus site for QKI binding. Interestingly, structural and functional analysis of *C. elegans* GLD-1 proteins have demonstrated that the hexanucleotide element UACU(C/A)A is the consensus site for the RNA binding affinity by GLD-1 dimers (Ryder et al., 2004). Even though the hexanucleotides found for GLD-1 and QKI are not identical, they are markedly similar. Our laboratory is currently performing further biochemical experiments using *in vitro* RNA retardation assay, SELEX assay, RNA sequence mutants, and immunoprecipitation followed by reverse transcription-PCR to determine the future RNA affinity, the stoichiometry of QKI for RNA sequences, and the *in vivo* RNA targets for QKI proteins.

1.3.3 Other classes of RNA Binding Proteins

Using the human and mouse genomes (Lander et al., 2001; Venter et al., 2001; Waterston et al., 2002), one can attempt to answer the question: How many different RNA binding proteins exist in mammalian organisms? There are fewer annotated RNA binding proteins recorded in the databases for invertebrates, such as *C. elegans* and *D. melanogaster*, than there are for yeast. The value of the recorded RNA binding proteins is

about 2-8% of the total number of known genes. Since the number of human genes is projected to be 32,000, the estimated number of RNA binding proteins encoded in the human genome ranges from as low as 640 (2% of 32,000) to as high as 2,560 (8% of 32,000). Most conservative estimates suggest the number of human RNA binding proteins as being 1,500; however, their distribution across the types of RBPs and their domains remain unknown (Keene, 2001). Moreover, even less is known regarding which of these proteins might interact with mRNAs, pre-mRNAs, small RNAs, or ribosomal RNAs. Although the estimated number of human RNA binding proteins is not clear at this time, the coming years will see ribonomic science elucidate the role of RBPs in the context of all RNA molecules in the cell.

Among the other classes of RBPs described in Table 1, the RRM (RNA Recognition Motif) domain-containing proteins are one of the largest classes of proteins found in the genomic databases (Burd and Dreyfuss, 1994; Kenan et al., 1991). This family requires two RRMs to possess RNA binding activity. Proteins in the Elav/Hu sub-family contain three RRMs, a conserved 80 amino acids region that was first discovered in PABP and splicing factors (Kenan et al., 1991). Hu proteins bind preferentially AU-rich elements (ARE) that are mainly found in the 3'-UTR of specific mRNAs (Gallouzi et al., 2000; Lopez de Silanes et al., 2004). Certain mammalian evidence indicates that the neuronal specific HuD is involved in neuronal differentiation and in the stabilization of the mRNA of GAP-43, a kainic acid-induced gene in mature hippocampal neurons (Perrone-Bizzozero and Bolognani, 2002). Another example of posttranscriptional control is the HuR effect on c-fos mRNA stabilization (see Figure 3, Section 1.2.2). Other similar examples of HuR or HuD action have been described for c-myc mRNA,

neurofilament M, $p21^{Cip1}$, and $p27^{Kip1}$ (Antic et al., 1999; Atasoy et al., 1998; Figueroa et al., 2003; Kullmann et al., 2002; van der Giessen et al., 2003).

The musashi proteins are another interesting example of RRMs being implicated in neurogenesis. Musashi was first isolated in Drosophila and was found to be responsible for the proper asymmetric cell division of sensory organs. Flies lacking the musashi gene possess a double bristle phenotype. The name "musashi" is adapted from this double bristle phenotype. The Musashi was a Japanese samurai warrior who lived about 400 years ago and originated a style of fighting that used two swords simultaneously, while typical samurai used only one sword.

In mammals, Musashi proteins, unlike HuDs, are mainly expressed in neural precursor cells and are gradually down-regulated in the course of neural differentiation (Okano et al., 2002; Sakakibara et al., 2002). Figure 1-9 illustrates the molecular mechanism by which Musashi-1 regulates mRNA translation in neural precursor cells. First, the RNA binding sequences for Musashi-1 were determined by *in vitro* selection using SELEX methodology. A RNA sequence containing repeats of (G/A)U(n)AGU was identified as the RNA sequence bound by Musash-1. These consensus elements were identified in some neural mRNAs, such as mammalian numb (m-numb) mRNA 3'-UTR. This m-numb mRNA encodes m-Numb, a membrane-associated antagonist of Notch signaling. Musashi-1 protein binds *in vitro* and *in vivo* m-numb mRNA in its 3'-UTR. The *m-Numb* and Musashi-1 expression patterns overlap in neuroepithelial cells in the ventricular zone of the neural tube (Sakakibara et al., 2002). In addition, *m-Numb* activates neuronal differentiation (Wakamatsu et al., 1999). Biochemical studies and *in vivo* gain-of-function have confirmed that Musashi-1 translationally represses the

synthesis of m-Numb (Zhong et al., 2000). In effect, the over-expression of Musashi-1 activates Notch1 signaling through a pathway that is dependent on the action of RBP-J (Imai et al., 2001), a transcription factor that forms a functional complex with an intracellular domain of Notch1 proteins within the nucleus. The interaction of RBP-J with the intracellular domain of Notch allows for their translocation to the nucleus and permits the activation of the HES1 promoter. This activation of the HES1 promoter on Notch signaling induces the self-renewal of mammalian neural stem cells (Gaiano et al., 2000).

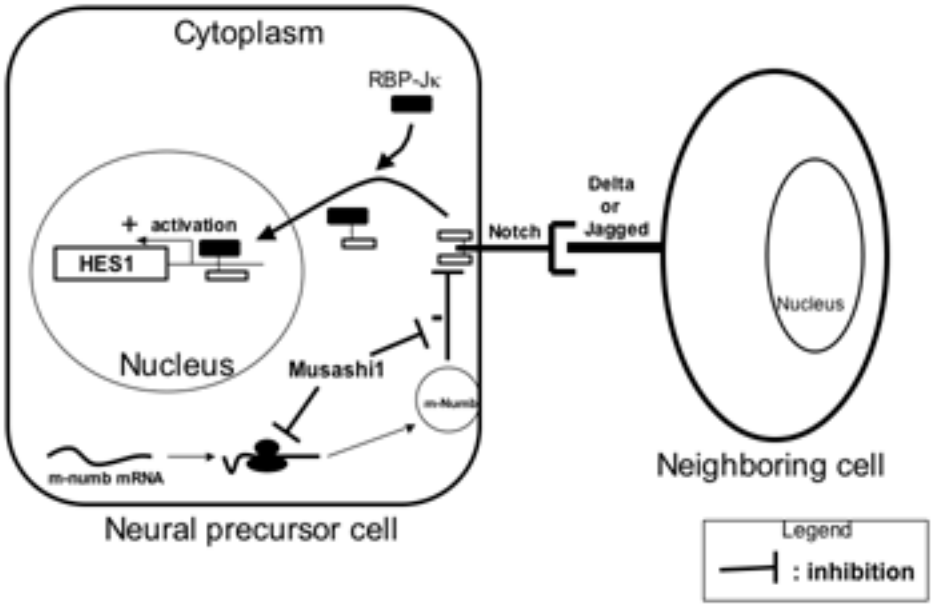


Figure 1-9. A model of Musashi1 function in the regulation of the Notch1 signaling in the development of mammalian neural cells.

Furthermore, the over-expression of Musashi-1 results in the down-regulation of endogenous m-Numb protein expression (Okano et al., 2002). Thus, Notch signaling becomes activated because m-Numb can not exert its repression effect. This high level of Notch helps to maintain a level of immature proliferating neural stem cells in the brain, a very important factor for proper brain function. This example further elucidates the importance of RNA binding proteins in neural development.

The other class of RBPs is the double-stranded RNA binding proteins that contain one or more double-stranded RNA binding domains (dsRBD). The dsRBD is a domain of about 70 amino acids that interacts with the A form of double-stranded RNA (Ryter and Schultz, 1998). There are prokaryotic, viral-encoded, and eukaryotic proteins in this family. Proteins harboring dsRBD are able to interact with as little as 11 bp of dsRNA. Unlike others RBPs, this binding seems to be independent of nucleotide sequence arrangement, but more dependent on RNA structure. Up to twenty double-stranded RNA binding proteins with diverse cell functions have been reported. Examples include the dsRNA-dependent protein kinase PKR that functions in dsRNA, interferon signaling, and host defense against virus infection (Koromilas et al., 1992). An important example is DICER, which is implicated in miRNA biogenesis and RNA interference (RNAi). Another is Staufen, which was originally isolated from *Drosophila* and is important for oocyte development. In Drosophila, Staufen was reported to anchor *bicoid* mRNA at the anterior of the *Drosophila* oocyte and *oskar* mRNA to the posterior of the oocyte (Brendza et al., 2000). In mammals, Staufen-1 and -2 are implicated in RNA transport in fibroblast and neurons (Duchaine et al., 2002; Kiebler and DesGroseillers, 2000). Interestingly, Staufen-1 has also been reported to become encapsidated during retroviral

packaging, which is illustrated in viruses such as HIV-1. This seems to play a role in the retroviral replication cycle (Mouland et al., 2000). Another protein that is implicated in retroviral replication is TRBP (TAR RNA binding protein). TRBP was found in a screen designed to isolate proteins that could interact with the RNA element of HIV-1. TRBP activates HIV-1 replication by assisting HIV-encoded Tat binding to TAR RNA sequences in the long terminal repeat (LTR). Moreover, several studies indicate that TRBP could interact with PKR and inhibit the cell growth function of PKR (Dorin et al., 2003; Gatignol et al., 1993).

Finally, another class of RNA binding proteins that is important in RNA export and processing is DEAD-box helicases (Linder et al., 1989). (Rogers et al., 2002). The Asp-Glu-Ala-Asp-box, or DEAD for the one letter amino acid code, gave the protein class its name. Interestingly, RNA helicases from the DEAD-box are found in bacteria and in mammalian cells. They are also associated with processes ranging from RNA synthesis to RNA degradation (Rocak and Linder, 2004). DEAD-box proteins use the energy from ATP hydrolysis to rearrange inter- or intra-molecular RNA structures or to dissociate RNA-protein complexes. Such structure rearrangements are necessary for the maturation of the RNA and for all of the steps in the life of a RNA molecule.

Over 50 different DEAD-box-protein sequences are present in the human genome. Among them is eIF4AIII, which is important for ribosome biogenesis (Li et al., 1999). Another example is the translation initiation factor eIF4A, which forms part of the cap-binding complex and unwinds RNA-duplex structures at the 5' end of eukaryotic mRNA to prepare it for scanning by the small ribosomal subunit (see Section 1.2.1). Another example of DEAD-box protein is Gemin3, which interacts with the Survival of

Motor Neurons (SMN) protein that is involved in RNA splicing. Moreover, SMN mutations found in some spinal muscular atrophy (SMA) patients strongly reduce this interaction (Charroux et al., 1999).

Each of these examples shows us that RNA binding proteins are crucial for cell function. Their mechanisms of action are complex and demonstrate the importance of protecting the cellular nucleic acids. RNA binding proteins are now considered to play a central role in the signaling networks of a living cell and represent another pathway of regulation of gene expression (Lasko, 2003).

1.4 The Functions of the Wild Type QUAKING Proteins Related in Literature

Section 1.3.2 covered the area of the KH domain containing RBP family to which QUAKING (QKI) proteins belong. The following section will describe the discovery of QKI gene, its mouse model of study, and its function in RNA metabolism and neurobiology, as well as the functional aspect of the *qk* locus and its encoded QKI proteins.

1.4.1 The *quaking* Gene and QKI Proteins

In order to clone the *qkI* locus in the mouse genome, we compared DNA from the qk^v (quaking viable mouse model) with DNA from its wild type (wt) counterpart, the C57BL/6 mouse. Starting from a cosmid library, differential hybridization techniques have mapped a cosmid named Au119. A comparison of the wt mice cosmid library to qk^v chromosomal DNA reveals that the 5' part of the quaking gene has been cloned and identified on chromosome 17 (Ebersole et al., 1992). Consequently, a yeast artificial chromosome (YAC) containing 1.2 Mb of mouse genome was identified as containing

the entire quaking gene (Cox et al., 1994). Moreover, this YAC clone denotes that the deletion in the qk^v mouse spans at least 910 kb in length (Cox et al., 1994). Subsequently, in 1996, Karen Artzt's group mapped and sequenced the quaking cDNA (Ebersole et al., 1996), which led to the discovery that the *qkI* gene possesses six common exons and three different exons spanning a genomic region of 65 kb of DNA. Interestingly, the alternative splicing of the seventh exon produced three major mRNA isoforms that are 5-kb, 6-kb, and 7-kb long, respectively. This alternative splicing gives rise to encoding protein products called QKI-5, QKI-6, and QKI-7 (Figure 1-10).

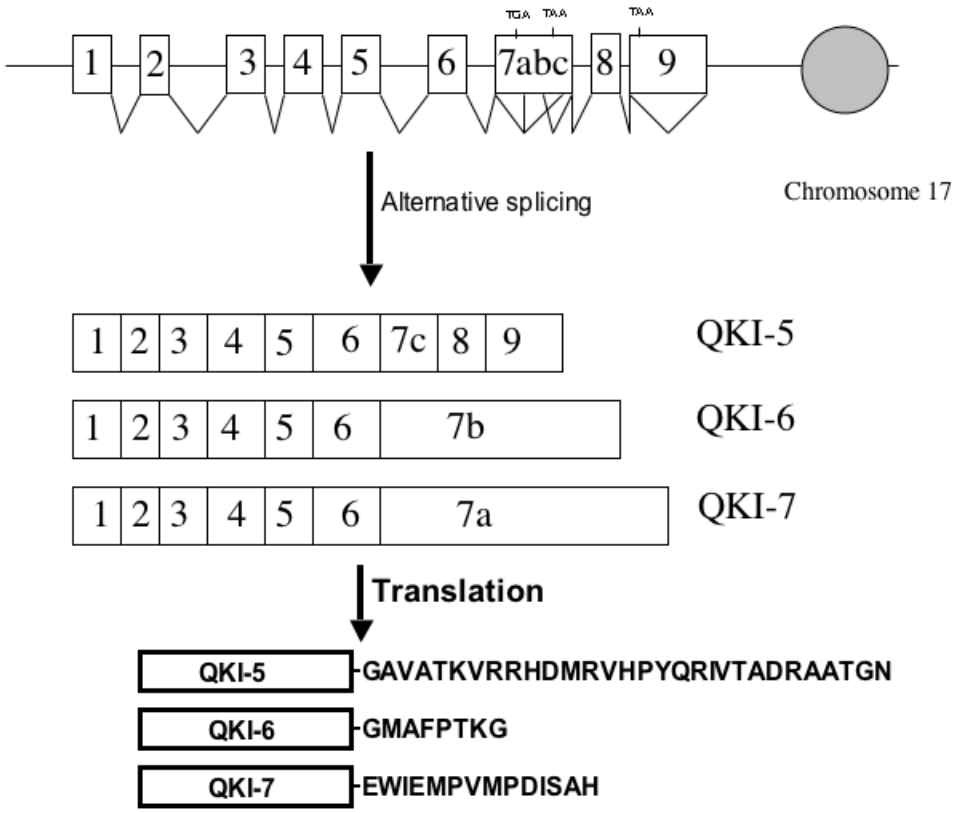


Figure 1-10. Diagram showing the three major alternatively spliced encoded QKI isoforms as well as the resulting QKI-5, QKI-6, and QKI-7 protein products with their distinct C-terminus sequence.

Moreover, the mouse genome project (Accession number: AF467890) reveals that the quaking locus could give rise to six transcripts in total. In addition to the major isoforms QKI-5, QKI-6 and QKI-7, other isoforms such as QKI-5a, QKI-7b, QKI ΔKH and QKI-G also exist even though they are not expressed at the same level as the major transcripts (Cox et al., 1999; Kondo et al., 1999). An important question that remains to be answered is whether *qkI* pre-mRNA is alternatively spliced during brain development?

The genomic arrangement and primary sequence of the human quaking gene is very similar to that of mouse *qkI*. The *Hqk* gene contains 8 exons that span a genomic region of approximately 200 kb and generates at least four alternatively spliced transcripts, *Hqk*-5, *Hqk*-6, *Hqk*-7, and *Hqk*-7B, of which *Hqk*-7 is abundantly expressed in the brain. The human *qkI* gene (*Hqk*) maps to chromosome 6 locus 6q25-q26 (Li et al., 2002). Interestingly, this locus is deleted in a variety of human cancers, such as brain tumors or glioblastoma (Burton et al., 2002; Goussia et al., 2001; Reardon et al., 1999; Thomas and Raffel, 1991). This fact leads us to the hypothesis that QKI proteins could play a role as a tumor suppressor for brain tumors; however, this area requires further research. Experiments that use the glioblastoma mouse model, prompt QKI over-expression in glioblastoma cell lines, and perform gene rescue experiments on human glioblastoma cells that contain the 6q25-26 deletion will address this important question.

In the 1980s, several ENU-induced mutant mouse lines targeting the *qkI* gene were produced (Cox et al., 1999; Justice and Bode, 1988). All of the homozygote mice for each ENU-induced allele are embryonic lethal dying at embryonic days E8.5 to E13.5. The phenotypes of these embryos were very severe displaying problems in closure of the neural tubes, low somite numbers and heart developmental defects. In 2003, a clear

knockout of the *qkI* gene was produced by homologous *qkI* gene replacement with the β-galactosidase-neomycin fusion gene (Li et al., 2003). Once again, homozygous *qkI* null embryos died between E9.5 and E10.5. The observed phenotypes were kinky neural tubes, open neural tubes, incomplete embryonic turning and the failure of blood circulation in the yolk sac resulting in smooth muscle development problems. These pieces of evidence suggest that the *qkI* gene is required for the development of the brain and smooth muscles; however, this knockout methodology targets all of the isoforms of *qkI* mRNA without discriminating between the QKI isoforms. In fact, several studies have shown that the different spliced isoforms of QKI proteins are expressed differently in each organ during the development of mouse embryos (Ebersole et al., 1996; Hardy et al., 1996; Kondo et al., 1999; Noveroske et al., 2002). The QKI-5 isoform in particular is very important at the early stages of development whereas isoforms QKI-6 and QKI-7 become up-regulated in the post-natal stage. In fact, the abundant expression of QKI-6 and QKI-7 is found only in the nervous system. In the heart and smooth muscles, QKI-5 is the predominant isoform, while QKI-6 and QKI-7 are undetectable (Lu et al., 2003).

1.4.2 QKI Localization and importance in neural development

Since *qkI* gene disruption in mice reveals that brain development is altered, a few studies have reported the expression of the three *qkI* messages in the brain and have shown that they are developmentally regulated. Techniques such as whole-mount *in situ* hybridization in embryos revealed that QKI-5 mRNA is first detected in the neuroepithelium of the head folds at E7.5, and that QKI-5 mRNA exhibits strong ventral

expression in the nascent brain and neural tube of E9.5 embryos (Ebersole et al., 1996). Using northern blot analysis, Rebecca Hardy reported that QKI-5 mRNA is highly expressed in the first two postnatal weeks, and decreases dramatically after day 14 (Hardy et al., 1996). At postnatal day 14, QKI-6 and QKI-7 mRNA expression is up-regulated; this timing coincides with active myelination in the brain. Proteins such as MBP are known to be up-regulated between the postnatal day 10 to day 20 in the mouse CNS (Colman et al., 1982; Delassalle et al., 1981). This observation suggests that QKI proteins could play a role in myelination and brain development. Moreover, Kuniya Abe's group reported that additional QKI mRNA are regulated during mouse brain development. Transcripts such as QKI-5b and QKI-ΔKH showed down-regulation during brain development (Kondo et al., 1999).

The mechanisms by which neural progenitors differentiate into the multiple cell types of the mature nervous system are not well understood. Specific antibodies were raised to localize each of the three QKI proteins (i.e. QKI-5, QKI-6 and QKI-7) in order to assess the role of QKI proteins during brain development (Hardy et al., 1996). Immunostaining mouse brain sections at different developmental stages identified QKI positive cells from white matter tracks. At postnatal stages, QKI-6 and QKI-7 proteins are particularly expressed in oligodendrocytes in the corpus callosum and in the optic nerve. In contrast, QKI-5 is mainly expressed in the nucleus of glial precursor cells at embryonic stages. However, some QKI-5 proteins are also detected in the nuclei of astrocytes. Other cells, such as Bergmann glia and Purkinje cells, were also positive for QKI-5. In the peripheral nervous system (PNS), sections of adult sciatic nerves show QKI-6 positive myelin-forming Schwann cells. In contrast, neurons are devoid of any

detectable QKI proteins. Interestingly, both QKI-6 and QKI-7 are mainly localized to the cytoplasm of glial cells, with low levels detected in the nucleus, whereas QKI-5 is restricted to the nucleus. In fact, the carboxyl terminus of QKI-5 contains a nuclear localization sequence (NLS) spanning 7-amino acids (RVHPYQR) (Wu et al., 1999). QKI-5 shuttles between the nucleus and the cytoplasm; furthermore, this was demonstrated using interspecies heterokaryon assays. This observation lead to the hypothesis that QKI-5 might be able to be transported through the nuclear membrane via a rapid dynamic process that is currently unknown.

QKI proteins are believed to function during early glial cell development. Rebecca Hardy's work shows that QKI-5 proteins are expressed in the glial progenitors of the ventricular zone (vz) during murine CNS development, but that QKI-5 expression is down-regulated during oligodendrocyte differentiation and that QKI-6 and QKI-7 expression becomes increased in these white matter oligodendrocytes (Hardy, 1998). These QKI+ cells have characteristics that are consistent with the acquisition of a glial rather than a neuronal fate. In fact, QKI-5 + cells also express the brain stem cell marker nestin and the early glial cell marker PDGFαR. QKI + cells fail to express neuronal markers. Similar QKI-5 + cells are found in the postnatal subventricular zone, a known area of gliogenesis. Furthermore, typical oligodendrocyte early markers, such as the Notch ligand Jagged and PDGFαR, are expressed at the same ventricular zone as QKI-5 + cells in the rodent spinal cord. Figure 1-11 is a schematic of the localization and function of QKI proteins in the neural tube ventricular zone.

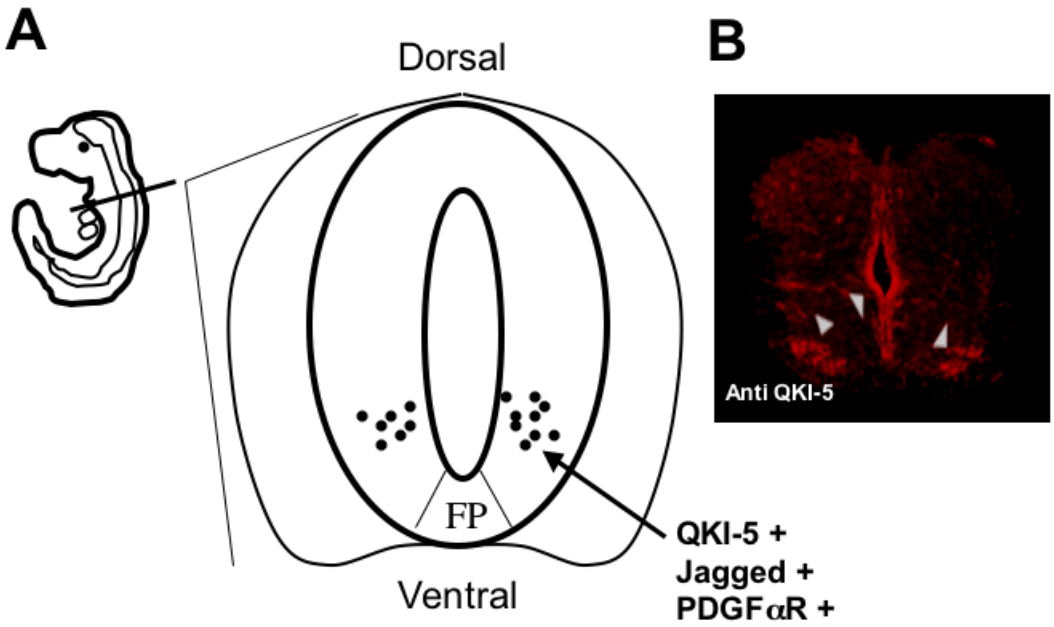


Figure 1-11. Scheme illustrating QKI-5 expression in the E14.5 ventral neural tube.

Illustrates Rebecca Hardy's observation from section through the cervico-thoracic neural tube of a mouse embryo at E14.5 (Hardy, 1998), which shows QKI-5 expression. Early markers of oligodendrocytes such as Jagged and PDGFαR are expressed in the same region of QKI-5 positive cells. In (B), our observations show the expression of QKI-5 by immunostaining using anti-QKI-5 antibodies. Arrowheads point to migrating QKI-5 positive cells in the ventral E11.5 cervico-thoracic section.

More experiments using P19 cells following retinoic acid treatment confirmed that neural progenitor cells also down-regulate QKI-5 expression when they differentiate into neurons, but not when they differentiate into glia. It is therefore tempting to compare the implications of QKI proteins in brain development to another RNA binding protein (Section 1.3.3) implicated in early neural differentiation, mouse Musashi-1. Like QKI proteins, Musashi-1 is expressed in neural precursor cells of the ventricular zone and its expression is down-regulated during neuronal differentiation (Sakakibara et al., 1996). However, the expression of QKI proteins differs from that of Musashi-1. In spite of the fact that QKI and Musashi-1 are expressed in the same subpopulation of the subventricular zone (SVZ) cells, Musashi-1 is down-regulated and QKI is up-regulated as soon as cells commit to become an oligodendrocyte (Hardy, 1998). Nevertheless, Musashi-1 expression is maintained in astrocytes (Sakakibara and Okano, 1997).

Taken together, these observations indicate that QKI-5 expression is a characteristic of glial progenitors. However, this hypothesis remains to be addressed by cell fate experiments using primary glial cells where specific isoforms of QKI will be expressed. This thesis will address this question.

1.4.3 The quaking viable mouse

Glial cells are the major players in myelination and constitute 90% of the cells in the human brain (Rowitch, 2004). Research in this particular area of brain development is exciting and prompts numerous questions. *In vivo* models, such as mutant mice or knockout mice, are particularly important for studying the aspect of genes linked to a disease or a given phenotype. There have been a number of animal models used to study the pathologic processes associated with the myelin lesions (Hogan and Greenfield, 1984). The *quaking* viable mice is an ideal model to study the function of QKI proteins in myelination. The *quaking* viable (qk^v) mouse is a spontaneous dysmyelinating mutant first discovered by Sidman's laboratory (Sidman et al., 1964). A spontaneous mutation in these mice causes a tremor or "quaking" mainly pronounced in the hind limbs around 10 days after birth (Sidman et al., 1964). Adult mice still display tremors and abnormal locomotion, and aging mice have tonic-clonic seizures. Usually, qk^v do not live longer than 9 months. Post-mortem brain pathology examinations revealed hydrocephaly and glioma in a few cases (personal observation). As illustrated in Figure 1-12, the effect on myelination is not caused by a mutation in one of the known components of myelin, but rather by a deletion of the promoter/enhancer region of the *quaking* gene (Ebersole et al., 1996).

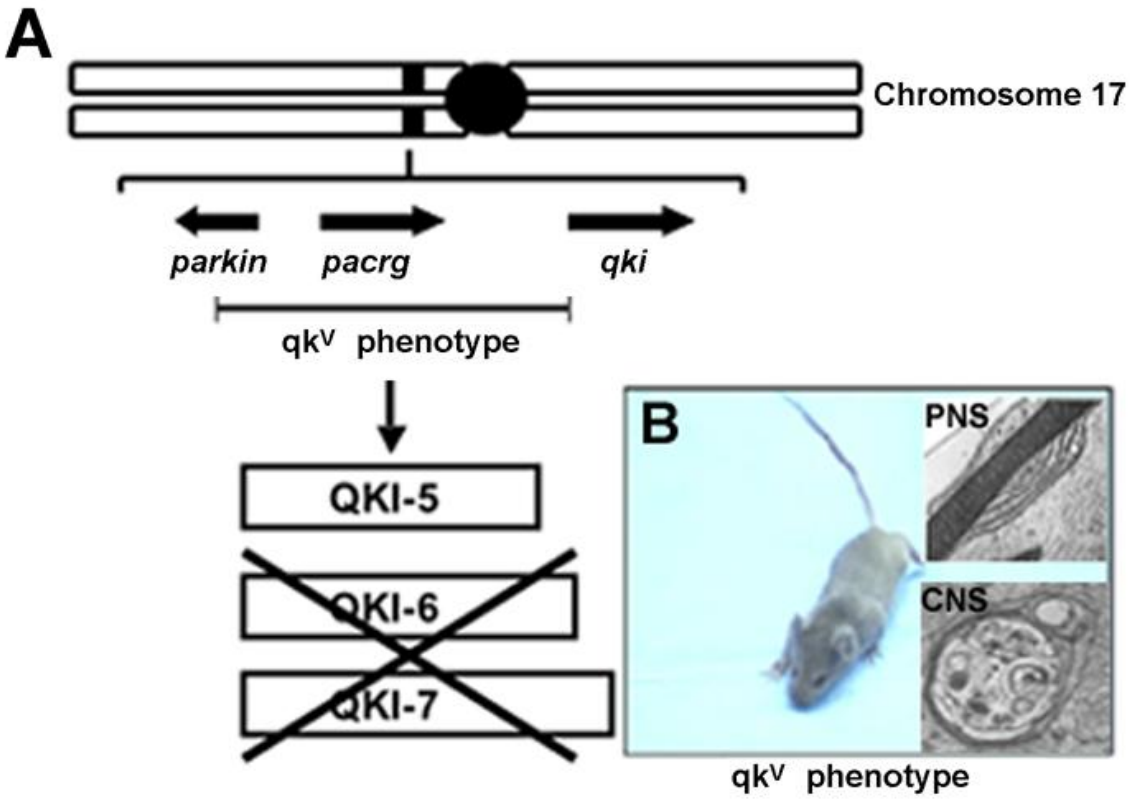


Figure 1-12. The *qk*^{v} mouse genomic organization and its dysmyelination phenotype. (A) illustrates the mouse *quaking* map of the region deleted in the *qk*^{v} mouse chromosome 17. This deletion prevents the expression of the QKI-6 and QKI-7 isoforms. In (B), the picture shows the phenotype of the *qk*^{v} mouse in terms of myelin uncompaction in PNS and CNS (inset). The inset images are from (Suzuki and Zagoren, 1976) and (Watanabe and Bingle, 1972).

The quaking deletion is approximately 1.17 Mb in mouse chromosome 17, resulting in the deletion of a major part of the *qki* gene promoter. This deletion results in the absence of QKI-6 and QKI-7 from myelin-forming cells, such as oligodendrocytes and Schwann cells, whereas the QKI-5 level is comparable to that of normal mice (Hardy et al., 1996). The absence of QKI-6 and QKI-7 expression leads to a defect in myelination; however, the exact molecular link between QKI proteins and myelin deposition remains unanswered. Research presented in this thesis will target this question.

It was recently discovered that the first five coding exons of the *parkin* gene, as well as the *parkin* co-regulated gene (*pacrg*), lie within the qk^{v} deletion (Lockhart et al., 2004; Lorenzetti et al., 2004a). Interestingly, the complete deletion of *pacrg* is the cause of male sterility in the qk^{v} mutant (Lorenzetti et al., 2004b). However, despite the fact that *pacrg* and *parkin* genes encode for a protein which functions in dopaminergic neurons, knockout mouse for the *parkin* and *pacrg* genes do not reveal any myelin problems or any tremor phenotypes. Once again, this observation suggests that the *qki* gene is important for myelination.

The qk^{v} mice have been studied for over thirty years. The CNS of qk^{v} mice contain alterations of oligodendrocyte morphology and differentiation, including cytoplasmic vacuolar inclusion, branching abnormalities, and irregular myelin membranes (Watanabe and Bingle, 1972). In the PNS, sciatic nerve axons and lumbar root axons display a dilation of the periaxonal space of myelinated fibers and a variation of Schmidt-Lanterman incisures (Berger, 1971; Samorajski et al., 1970; Suzuki and Zagoren, 1976; Trapp et al., 1984). However, because many of these phenotypes likely reflect secondary

changes in the glial cell differentiation defect and the results of the hypomyelination in *qk*^{v} mice, the primary mechanism that leads to such a phenotype remains unknown.

Because QKI proteins are RNA binding proteins, myelin RNAs have to be closely examined in *qk*^{v} mice. Aspects such as the RNA of myelin components and their localization are the target of choice for understanding the functions of QKI proteins. In the *qk*^{v} brain, *in situ* hybridization in cultured oligodendrocytes demonstrated that MBP mRNA is mostly retained in the nucleus (Barbarese, 1991; Campagnoni et al., 1991). However, the level of MBP mRNAs is relatively similar in *qk*^{v} mice, when compared to the wild type mouse brain (Carnow et al., 1984; Sorg et al., 1987).

The above observations raise the possibility that QKI proteins may be involved in MBP mRNA trafficking in oligodendrocytes. As discussed and shown in Section 1.2.3 and in Figure 5, RNA binding proteins, such as hnRNA2, are important for MBP mRNA transport during myelination. Since MBP mRNA is known to be retained in the nucleus of oligodendrocytes from *qk*^{v} mice, one of the proposals of this thesis is that QKI proteins could be a specific MBP mRNA transporter, or an important cargo for adequate myelin metabolism.

1.4.4 QKI proteins in apoptosis

Work in our laboratory by Pilotte and collaborators (Pilotte et al., 2001) demonstrated that, when over-expressed, the QKI-7 isoform can induce apoptosis in fibroblasts and primary rat oligodendrocyte progenitors. Moreover, a point mutation in the NK region of QKI, the E48G mutation, prevents the homo- and heterodimerization of all QKI isoforms and causes apoptosis when over-expressed (Chen and Richard, 1998).

The heterodimerization of the QKI-7 isoform with QKI-5 results in the nuclear translocation of QKI-7 and the suppression of apoptosis. This latter observation has proven that the balance between QKI-7 and the QKI isoforms is essential for cell survival. In addition, this study has demonstrated that a short peptide sequence of the C-terminus of QKI-7 could be fused to the GFP protein to induce cell death in fibroblast. Consequently, the unique C-terminal sequences of QKI-7, named "killer sequence," might function as a life-or-death "sensor" that monitors the balance between the alternatively spliced QKI isoforms (Pilotte et al., 2001). Moreover, this finding suggests that nuclear translocation is a novel mechanism for inactivating apoptotic inducers. This study gives rise to a new hypothesis: that RNA binding proteins and their alternative spliced isoforms could regulate cell death and survival. Further *in vivo* evidence must be produced in order to demonstrate the function of QKI-7 in apoptosis and brain development.

1.4.5 *Drosophila* QKI homolog, "How" it works?

The held out wings (how) gene of the fly *Drosophila* encodes a KH domain-containing protein that is very similar to the QKI proteins (Figure 7, Section 1.3.2). *Drosophila* mutant for how genes develop a curved wing defect that extend an angle horizontal from the body axis. This is why these mutant flies are named 'held-out-wings'. How proteins are known to control muscle function as well as tendon cell differentiation (Nabel-Rosen et al., 1999). Two isoforms of How are expressed from alternative spliced transcripts, one encoding a shorter form How(S), which is found in both nucleus and cytoplasm and the other form How(L) encoding specifically a nuclear protein. The major

difference between the two How isoforms is that How(L) possesses a unique 36-amino acid stretch at its C-terminal that includes a nuclear retention signal (NLS). This is another reason why How(L) could be compared to QKI-5 for its cellular distribution.

On the other hand, a major player in tendon cell differentiation in *Drosophila* is the gene *stripe*, which encodes an early growth response (EGR) transcription factor. *Stripe* expression in tendon precursor cells is activated from the Hedgehog and the epidermal growth factor receptor (EGFR) signaling pathways. Both isoforms of How proteins bind the 3' UTR of *stripe* mRNA, but they have a dual effect. How(L) reduces the expression of a GFP reporter construct that carries the *stripe* 3' UTR (Nabel-Rosen et al., 2002). Conversely, How(S) activates the expression of a GFP construct that contains the *stripe* 3' UTR. Furthermore, in an *in vitro* cultured system using Drosophila Schneider cells, these effects seem to be mediated at the level of RNA stability.

This mechanism is also conserved in mammals. In fact, Krox-20 mRNA, the EGR2 homologue that is involved in the terminal differentiation of Schwann cells, could be regulated by QKI proteins. *In vitro* assays have shown that QKI-5 represses gfp-krox20 3'-UTR, whereas QKI-6 and QKI-7 lead to an increase of gfp-krox20 3'-UTR (Nabel-Rosen et al., 2002). Despite these observations, several questions remain: What is the proof that QKI proteins mediate Schwann cells differentiation? Where is the binding site on krox20 mRNA for QKI proteins? The answers to these questions will reveal important aspects of PNS myelination regulated by Schwann cells.

1.4.6 Others possible functions of QKI

The vast amount of literature on the qk^v mice over the past 40 years has described many subtle changes, such as altered myelin protein levels (Fagg, 1979), oscillation of neurotransmitter levels (Hogan and Greenfield, 1984), hyperplasia of the locus coeruleus (Le Saux et al., 2002), and incomplete lipid metabolism (Baumann et al., 1973; Bourre et al., 1986; Dasgupta et al., 2002). In fact, the brains of the quaking mouse mutants are marked by low levels of very long chain fatty acid (VLCFA) due to a reduced fatty acid elongation activity (Suneja et al., 1991). Long chain lipid synthesis, such as C20:0 (Arachidoyl CoA), C22:0 (Behenoyl CoA) (22:0), C24:0 (lignoceric acid), and C24:1 (nervonic acid), were significantly reduced in qk^v mice. However, because many of those effects are observed in other dysmyelinating mouse mutants (shiverer and jimpy mice), they presumably reflect indirect changes in the hypomyelination phenotype. Therefore, other changes in qk^v mice probably do not directly depend on the *qki* gene itself, but rather on the consequence of a lower amount of myelin in the nervous system.

Another subtle change in qk^v mice is the imbalance of myelin-associated glycoprotein (MAG) isoforms (Fujita et al., 1990; Trapp, 1988). In fact, there are two spliced MAG isoforms, the small isoform (S-MAG), and the large (L-MAG) (Arquint et al., 1987). In the qk^v mouse, L-MAG levels are severely reduced, whereas S-MAG levels are increased (Fujita et al., 1990). Moreover, the L-MAG, but not the S-MAG polypeptides can be phosphorylated by tyrosine kinases that are endogenous in qk^v mice myelin (Braun et al., 1990). Both MAG isoforms are hyper-glycosylated in the qk^v mouse (Bartoszewicz et al., 1995). Furthermore, one study revealed that QKI-5 is able to

regulate alternative splicing (Wu et al., 2002). In fact, the over-expression of QKI-5 promoted the L-MAG generated from exon 12 skipping. This study only used QKI-5 isoforms and did not demonstrate whether the alternative splicing of MAG RNA was developmentally regulated or whether this splicing is occurring *in vivo*. Thus, it will be essential to find developmental RNAs regulated by QKI-5 during brain development and to investigate the function of QKI-6 and QKI-7 in alternative splicing in the adult brain.

The well-established development biology system of *Caenorhabditis elegans* was used to elucidate the function of QKI proteins. By using a QKI homologue protein called GLD-1 (germline defective-1), which is known to be necessary for germ-line development, Elizabeth Goodwin's group demonstrated that GLD-1 causes the translational repression of the sex determination gene TRA-2 (Jan et al., 1999). GLD-1 binding to the regulatory RNA sequence element TGE (tra-2 and GLI element) in the 3'-UTR of TRA-2 mRNA caused this translation repression. Mutation in the KH domain caused the loss of GLD-1 binding to the TGE. Furthermore, it was demonstrated *in vitro* and *in vivo*, that QKI-6 functions in the same manner as GLD-1 and can specifically bind to TGE to repress the translation of reporter constructs containing TGE (Saccomanno et al., 1999). This was also confirmed *in vivo* by functionally replacing GLD-1 with QKI-6 in *C. elegans* hermaphrodites (Saccomanno et al., 1999). To prove that RNA levels were not affected, a RNA decay analysis was performed and no changes were observed. The hypothesis is that GLD-1 plays the role of translation repressor by binding RNA on TGE and by masking translational initiation factors as well as poly(A) tail length (Jan et al., 1999).

Recently, GLD-1 has also been shown to protect mRNAs with μORFs (5'-UTR open reading frames) against non-sense mediated decay (Lee and Schedl, 2004). Moreover, immunoprecipitation, substractive hybridization and cloning strategies have identified 15 mRNAs that are putative targets of GLD-1 binding (Lee and Schedl, 2001). Among these targets, the mRNA of *rme-2* yolk receptors is needed for yolk uptake by late-stage oocytes. Other mRNAs, such as the *pumilio* gene as well as the *lin-45* RAF kinase, have been identified using this screening assay. Identification of targets like RAF kinase have lead us to think that both GLD-1 and QKI might be implicated in tumor suppression. Once again, it is tempting to hypothesize that QKI proteins may use a similar process for their function.

1.5 Properties of Myelin and organization

The myelin sheath around axons constitutes the most abundant membrane structure in the vertebrate nervous system. Its biochemical composition and its segmental structure are responsible for the saltatory conduction of nerve impulses that allows the myelin sheath to support rapid electric conduction through the thin fibers and nerves of the vertebrate nervous system. The significance of myelin in human development is highlighted by its involvement in an array of different neurological diseases such as multiple sclerosis (MS), leukodystrophies, and peripheral neuropathies (Ludwin, 1997).

As illustrated in Figure 1-13, myelin is a multi-lamellar membrane that is uniquely found in the nervous system. X-ray diffraction and microscopic studies demonstrate that cross-sections of compact myelin sheaths appear as consistent concentric layers of ensheathing cell plasma membrane (Morell et al., 1994). On one

axon, two adjacent segments of myelin are separated by a Node of Ranvier that is not covered by myelin, thus leaving it directly exposed to the extracellular milieu (Bunge, 1968). In fact, the Nodes of Ranvier play a major role in nerve impulse conduction. They allow for fast saltatory conduction and impulse jumping from Node to Node, as opposed to the slow progress along the axon to which unmyelinated or demyelinated fibers restrict. In myelinated fibers, voltage-gated Na+ channels are concentrated at the Node of Ranvier, whereas K+ channels are localized at the paranodal region. Interestingly, as the following sections will discuss, the insulating properties of the myelin sheath are largely due to its structure, its richness in lipids, and its protein players.

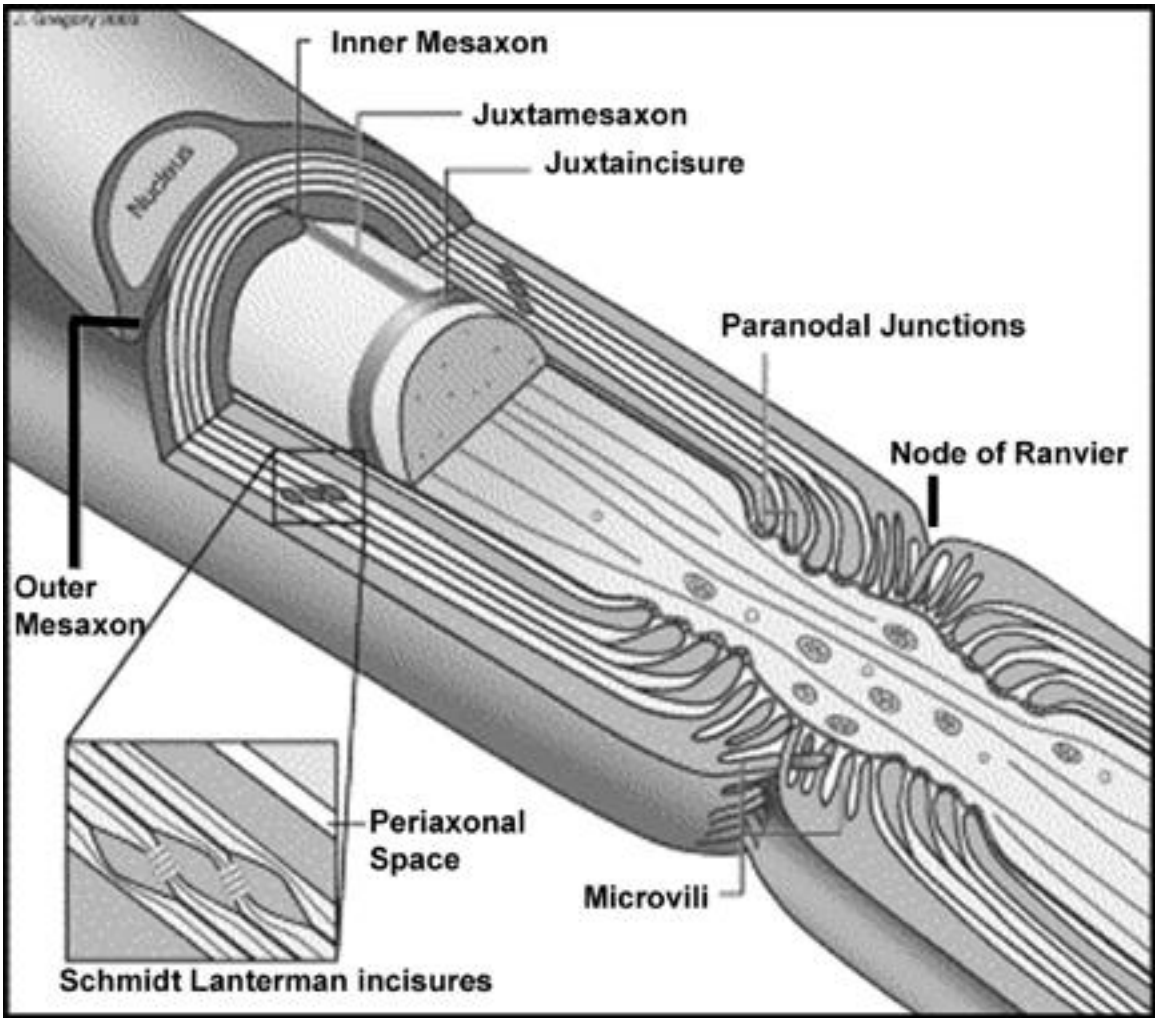


Figure 1-13: The schematic organization of a nerve showing myelin sheaths and their domains in the internode and in the Node of Ranvier. The inset shows the Schmidt Lanterman incisure, which forms gap junctions, after magnification. This figure is modified from Salzer, 2003 (Salzer, 2003).

1.5.1 Myelin: The Reason for Vertebrate Success

In animal evolution, myelin is the structure that distinguishes vertebrates from invertebrates. Without compact myelin, vertebrates simply could not exist (Zalc and Colman, 2000). In fact, invertebrates' axons are ensheathed by supporting cells, but do not have a compact myelin sheath. Consequently, electrical pulses that propagate along invertebrate axons are generally conducted at a speed of about 1 meter per second or less. Vertebrates, on the other hand, require rapid nerve conduction for effective predation or escape maneuvers; they have therefore developed a much more efficient system that creates a compact myelin sheath around their axons, which allows them to conduct potentials with speeds of 50 to 100 meters per second along an axon. The oldest vertebrates that possess myelinated axons are jawed cartilaginous fish, such as rays and sharks, which are fast-reacting and large-bodied predators that require the myelin-mediated conduction of the electric impulse in their nervous system. This evolutionary concept highlights the role of myelin in vertebrate evolution.

1.5.2 Myelination in CNS and PNS

Myelin is the essential component of white matter in the CNS, constituting ~ 40-50% on a dry weight basis. Several structural features characterize myelin (Figure 1-13 and 14). It has a periodic structure, with alternating dense and light layers. The major dense line (dark layer) forms as the cytoplasmic surfaces of the spirally expanding myelinating process of the oligodendrocyte come into close proximity during compaction. The remaining cytoplasm is rich in microtubules, providing a network for the transport of cellular components such as RNA and RNA binding proteins. This is

consistent with the fact that myelin is a metabolically active membrane containing numerous enzymatic activities, voltage-dependent ion transport and G-protein coupled receptors. The two fused outer leaflets (extracellular opposition) of the spirally expanding myelinating process form the intraperiod lines (light layer). Each myelin sheath segment or internode is 150-200 μm in length. The spaces between internodes, where the myelin is lacking, are called Nodes of Ranvier (Kandel et al., 2000; Siegel and Agranoff, 1999). Myelin dry weight consists of 70% lipids and 30% proteins. This high lipid-to-protein ratio is very particuliar to myelin membranes since cellular membranes usually have a high protein-to-lipid ratio. The specific constituents of myelin, glycolipids, and proteins are formed in the oligodendrocyte in the CNS and in the Schwann cells for the PNS: therefore, myelination requires the activation of numerous enzymes for lipid metabolism, to synthesize myelin lipids, and for the production and transport of the specific protein components of myelin to the oligodendrocyte and Schwann cell processes. The best characterized proteins are PLP, MBP, CNPase, MAG and MOG. Figure 1-14 schematizes myelin content and the important proteins implicated in the structure of myelin.

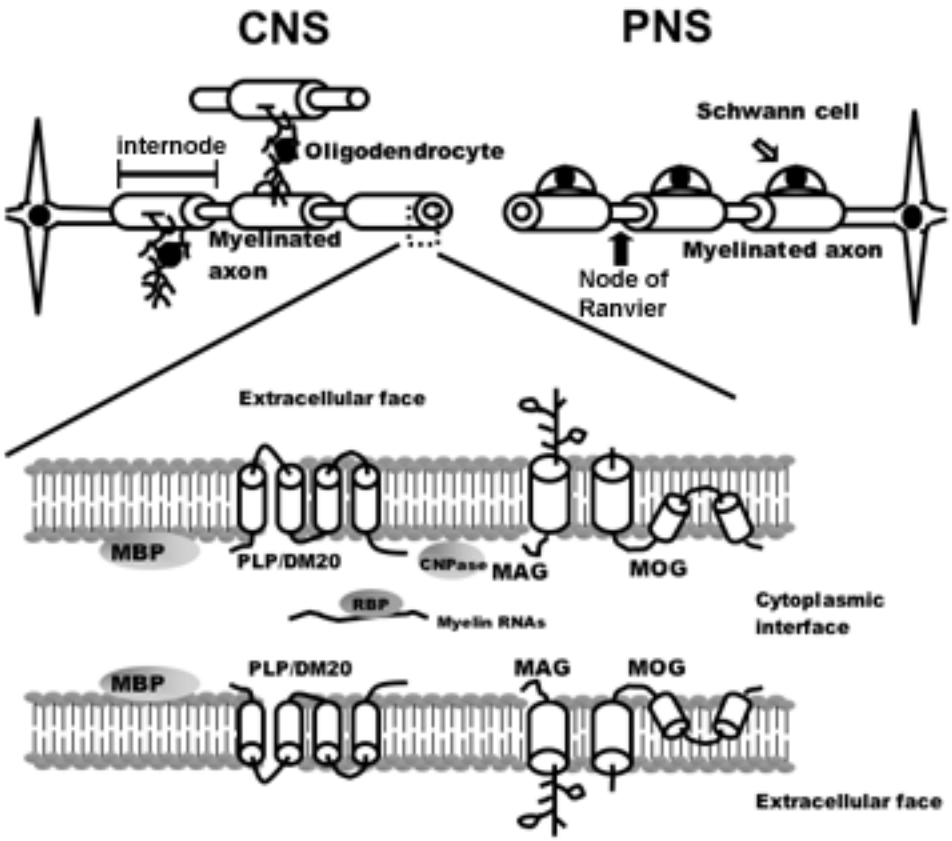


Figure 1-14. Difference in myelin composition in the CNS and the PNS. The major proteins present in myelin membrane are schematized. The oligodendrocyte is the myelinating cell in CNS; it can myelinate several axons. In the PNS, the Schwann cell is the myelinating cell; it can myelinate only one internode.

The PLP protein corresponds to 50% of myelin proteins. Its alternative spliced isoform, DM-20 is 20 kDa. PLP and DM-20 have four trans-membrane regions, with the extracellular domain accounting for the spacing at the intraperiod line (Nave et al., 1987). Both PLP and DM-20 are acetylated containing covalently linked oleic, palmitic, and stearic acids as the major acetyl groups (Agrawal et al., 1987). In knockout mice that do not express PLP/DM-20, oligodendrocytes are still able to myelinate axons and form compact myelin sheaths (Boison and Stoffel, 1994). Nevertheless, the structure of myelin reveals condensation of the intraperiod lines, which results in the axonal degeneration and reduced physical stability of the membrane and axonal degeneration (Griffiths et al., 1998).

MAG proteins are minor constituents that comprise 1% of the total myelin proteins (Baumann and Pham-Dinh, 2001). Section 1.4.6 demonstrated that the two MAG proteins produced by alternative splicing are developmentally regulated. S-MAG transcripts are most abundant in peripheral nerves of both humans and rodents; contrarily, the L-MAG isoform predominates in the adult human brain while the reverse is true for rodents. In contrast to the CNS, where MAG is confined to the periaxonal region of the myelin sheath, the adult rat PNS displays a large MAG distribution in its myelin internodes. MAG has an apparent MW of 100 kDa. However, in the absence of glycosylation, L-MAG is 72 kDa and S-MAG is 67 kDa. MAG has a membrane-spanning domain, an extracellular region that contains five immunoglobulin-like domains with sites for NH2-linked glycosylation, and an intracellular carboxy-terminal. It is localized in the periaxonal membranes of the myelin internodes, and is therefore in direct

contact with axons. Both its location and its membership in the immunoglobulin superfamily suggest that MAG might be involved in adhesion and in signaling between myelin-forming oligodendrocytes and neurons (Wang et al., 2002). Both forms of MAG are phosphorylated *in vivo* and *in vitro*. L-MAG contains a unique phosphorylation site in the cytoplasmic domain that appears to interact with two signal transduction elements: phospholipase C, and p59Fyn, the cytoplasmic tyrosine kinase (Jaramillo et al., 1994). Surprisingly, CNS myelin forms almost normally in MAG-deficient mice (Montag et al., 1994). However, the abnormal formation of the periaxonal cytoplasmic collar that is lacking in most of the internodes is a prominent defect of the mutant myelin sheaths. Another proposed function of MAG is to inhibit neurite outgrowth, i.e. axon regeneration in the CNS after lesioning (McKerracher, 2002). Therapeutic approaches targeting or inactivating MAG might be useful for conditions such as spinal cord injuries.

CNP represents 4% of all myelin proteins. This protein hydrolyzes artificial substrates, and 2'- and 3'-cyclic nucleotides into their 2'-derivatives; however, the biological role of this enzyme activity in the nervous system remains under investigation. The glial CNPase knockout mice develop axonal swelling and neurodegeneration throughout the brain, leading to hydrocephalus and premature death (Lappe-Siefke et al., 2003). However, the ultrastructure, periodicity, and physical stability of myelin are not altered in these mice. Two CNP isoforms (CNP1 and CNP2) are produced by alternative splicing from two transcription start sites (O'Neill et al., 1997). Two translational start sites can be used on the mRNA that encodes the CNP isoform, thus giving rise to CNP1 and CNP2 protein isoforms. Furthermore, Peter Braun's groups here at McGill found that

oligodendrocyte-overexpressing CNPs appear to mature earlier in development (Gravel et al., 1996).

Another protein is MOG, the myelin oligodendrocyte glycoprotein, which is a minor protein of myelin and has a MW of 25 kDa. It is a transmembrane protein that contains a single immunoglobulin-like domain with a single site for glycosylation. MOG glycosylation results in a MW of 26-28 kDa that can form dimers of 52-54 kDa. It is located on the outermost lamella of compact myelin sheaths. This antigenic property has been implicated as a cytotoxic T lymphocyte target in myelin autoimmune diseases of the CNS, such as multiple sclerosis (de Rosbo and Ben-Nun, 1998). Interestingly, the model of multiple sclerosis in mice, which is experimental autoimmune encephalomyelitis (EAE), is produced by immunizing mice with the MOG protein or with a MOG-derived peptide.

1.5.3 Myelin History: In Part, a Montreal story

McGill University researchers played a major role in the discovery of oligodendrocytes and continue to figure prominently in this fascinating field of research. Glial cells constitute the large majority of cells in the nervous system. Despite their number and their role during development, their active participation in the physiology of the brain and the consequences of their dysfunction on the pathology of the nervous system has been emphasized in recent years.

Virchow first discovered that there were cells other than neurons. He thought that it was the connective tissue of the brain, which he called "nervenkitt" (nerve glue), or, neuroglia. The name survived, but the original concept changed radically. Microscopic

studies and the use of metallic impregnation developed by Ramon y Cajal and Rio Hortega, resulted in the characterization of the major glial cell types. Using gold impregnation, Ramon y Cajal identified the astrocyte among neuronal cells, as well as a third element that was not impregnated by this technique (Cajal, 1913). A few years later, using silver carbonate impregnation, Rio Hortega found two other cell types, the oligodendrocyte, first called interfascicular glia, and another cell type, microglia, that he distinguished from the two macroglial cells (Rio Hortega, 1921). In early 1930, Wilder Penfield here at McGill University showed that the oligodendroglia (the old name for oligodendrocytes) that surrounds axons constitutes the major cells in the white matter region of the brain. Based on his discovery, he hypothesized that these cells should be responsible for the synthesis of white matter in the brain (Penfield, 1932). In 1970, McGill University's Dr. Charles Philippe Leblond was the first to perform exhaustive analyses of brain oligodendrocytes by electronic microscopy techniques (Mori and Leblond, 1970).

The morphological characteristics of macroglia have been reviewed. The progress of morphological techniques and the discovery of cellular markers by immunocytochemical techniques suggest the existence of multiple functional macroglial subclasses. Our understanding of the role of glia in CNS function has made important progress during recent years. Glial cells are necessary for correct neuronal development and for the appropriate function of mature neurons. The ability of glial cells to respond to changes in the cellular and extracellular environment is essential for the function of the nervous system. Furthermore, there is now growing recognition that glia, possibly through a glial network, may communicate and complement the neuronal network itself.

The currently growing evidence suggests that the specializations of both neurons and glial cells and their persistent interactions give new insight to the understanding of pathological outcomes. The co-expression of some neuronal and glial neurotransmitters, their transporters, and receptors contributes to the understanding of their functional cooperation. It now seems likely that oligodendrocytes have functions other than those related to myelin formation and maintenance.

1.5.4 Myelin Basic Proteins: the key players

Myelin basic proteins (MBPs) are the major protein of the central nervous system (CNS), comprising 30% of myelin proteins. MBPs are localized on the cytoplasmic face of the myelin membrane, and they facilitate the apposition of the cytoplasmic faces of myelin lamellae. MBPs have four to five major isoforms, the molecular masses of which are 21.5, 18.5, 17, and 14 kDa in the mouse; 21.5, 20.2, 18.5, and 17.2 kDa in humans; and 21.5, 18.5, 17, 16, and 14 kDa in the rat (Akiyama et al., 2002; Campagnoni and Macklin, 1988; Kruger et al., 1999). The expression of these isoforms is developmentally regulated (Campagnoni et al., 1987; Kruger et al., 1999; Pedraza, 1997). Alternative transcripts from the MBP gene, which consists of seven exons, code for these isoforms (de Ferra et al., 1985). Interestingly, exon 2 containing transcripts (coding for the 17 and 21.5-kDa isoforms) appear earlier during oligodendrocyte development in the mouse. These exon 2 containing isoforms re-appear in chronic lesions of MS, and their expression correlates with remyelination (Capello et al., 1997). These isoforms are mainly localized in the cell body of oligodendrocytes, which suggests a regulatory role in myelination for these karyophilic MBPs (Pedraza et al., 1997). As discussed in Section

1.2.3 and shown in Figure 1-5, when myelination is activated in oligodendrocytes, MBP proteins are synthesized at the distal tip of the oligodendrocyte under an activated mechanism where RNA binding proteins are important.

Moreover, posttranslational modification includes acetylation, phosphorylation, methylation, deimination, and citrullination (Moscarello et al., 2002; Pritzker et al., 2000; Seiwa et al., 2000). Indirect evidence suggests that the methylation of MBPs may be important for the compaction of the myelin during myelin maturation.

Studies of the shiverer mutant mouse, which presents a large deletion of the MBP gene and displays inappropriately compacted myelin that leads to the tremor phenotype that is also observed in qk^{v} mice, provide direct evidence that MBPs play a major role in myelin compaction in the CNS. On the other hand, an analysis of the shiverer PNS reveals a threefold increase in the number of Schmidt-Lanterman incisures in sciatic nerves, suggesting a compensation for a defect in Schwann cell-axon communication (Gould et al., 1995). This phenomenon is also observed in qk^{v} mice, which suggests that MBP proteins in qk^{v} mice could be directly affected. In fact, both qk^{v} mice and shiverer mice attempt to overcome their myelination defect by increasing the number of Schmidt-Lanterman incisures. Further experiments must be performed to elucidate the link between MBP proteins and RNA defects within the myelin sheath.

1.5.5 Demyelination and Dysmyelination Diseases

With advances in the technology of genomic science, it will be possible to sequence, in a short time, human genomes of patients who are inflicted with a rare disease, to allow for the identification of specific genes and enable the verification of whether these genes are mutated, deleted, or improperly expressed. Thus far, myelin-

related diseases such as leukodystrophy, Pelizaeus-Merzbacher disease (PMD), Charcot-Marie-Tooth neuropathies, X-linked spastic paraplegia, Cockayne's syndrome, and multiple sclerosis have been studied for linkage to particular genes.

Some genes have been identified for particular diseases. For example, the duplication of the PMP22 gene causes one form of Charcot-Marie-Tooth neuropathy, a disease that results from the increased production of PMP22 and prompts decreased conduction in the peripheral nerves and in the cycles of demyelination and remyelination. Moreover, mutations in the connexin-32 (Cx32) gene seem to lead to Charcot-Marie-Tooth neuropathies as well (Bergoffen et al., 1993). Another key example of a mutation causing myelin problems is the leukodystrophy diseases, such as Krabbe's disease, Alexander's disease, or Canvan's disease, a family of inherited myelin diseases in humans that result from dysmyelination, hypomyelination, or demyelination. We know that the Pelizaeus-Merzbacher disease (PMD) results from a PLP mutation in the membrane-spanning domain of PLP (Nave, 1995; Saugier-Veber et al., 1994). However, for many neurological diseases that have a myelin defect, no gene so far has been identified to be the main cause of these diseases.

In the near future, mutations or deletions in the *qki* gene might be discovered to lead to other dysmyelinating diseases, such as one type of leukodystrophy. Therefore, RNA binding proteins must be thoroughly investigated.

One of the most important and well studied demyelinating disease is multiple sclerosis (MS). It affects 50,000 Canadians, 350,000 individuals in North America, and more than 1.1 million people worldwide. MS appears mainly in young adults between the ages of 20-40. Women are affected at least twice as often as men are. It is an autoimmune

CNS demyelinating disease that causes relapsing and chronic CNS inflammation. Pathological changes that contribute to MS include inflammation, demyelination, oligodendrocyte death, and axonal degeneration (Lassmann, 1983; Steinman, 2000). Axonal loss also occurs in MS, probably due to demyelination.

Apart from oligodendrocytes, which produce the myelin sheath, MS lesions consist of axons and cells that provide neuronal support and protection, that is, astrocytes and microglia. In addition, inflammatory cells, such as Th1 T cells and macrophages, contribute to lesion formation by using small blood vessels to invade the CNS, where they attack myelin and oligodendrocytes. In chronic lesions, the lost axons and myelin are replaced by dense, 'sclerotic' astrocytic scar tissue, hence the name multiple sclerosis. Spontaneous remyelination does occur in MS lesions, but it cannot prevent irreversible axonal damage in the long term (Keirstead and Blakemore, 1997; Raine and Wu, 1993). The reason why the endogenous oligodendrocyte progenitor cells (OPCs) can not differentiate and myelinate is that inflammatory cells and astrocytes create an environment in which surviving oligodendrocyte precursors fail to mature (John et al., 2002). In fact, Brosnan's group demonstrated that the binding of Jagged-1 ligand secreted from the astrocytic scar activates the Notch pathway in OPCs. Therefore, the finding of Notch inhibitory factors and others oligodendrocyte promoting proteins will lead to the development of new therapies for MS. This is why the importance of research on myelin biology and on oligodendrocytes differentiation biology for finding a cure to the neurological disorders implicating myelin.

1.5.6 Remyelination: an ultimate goal for finding another Lorenzo's oil

The 1992 film Lorenzo's Oil, starring Susan Sarandon, Nick Nolte, and Peter Ustinov, is based on the true story of Lorenzo Odone and his parents. Lorenzo suffers from adrenoleukodystrophy (ALD) disease. In the hopes of finding a way to restore the myelin sheath, Lorenzo's father, Augusto Odone, developed a special type of oil containing very long chain fatty acids (VLCFA), which normalizes the level of myelin lipids in the brain to a certain point. Then, an international research consortium called "The Myelin Project" was founded that aims at finding a way to restore the myelin sheath, which is destroyed in ALD and a host of other myelin diseases, such as MS. This project is ongoing and helps to fund research in the field of myelin biology and therapeutics.

In fact, remyelination is possible. Bunge and collaborators first observed the process of spontaneous remyelination (Bunge et al., 1961) after an experimental lesion in the feline spinal cord. However, remyelination is often incomplete such is the case in MS, leukodystrophy, and spinal cord injury (Raine and Wu, 1993). This incomplete remyelination is one of the main reasons for clinical deficits in CNS demyelinating diseases; however, it has been shown that the PNS possesses the ability to remyelinate within 1-2 weeks after injury (Jeffery and Blakemore, 1997). The absence of astrocytes in the PNS might facilitate the remyelination process in PNS. More recent studies have shown that remyelination is possible in the CNS through stem cell implantation (Butt et al., 1999; Pluchino et al., 2003; Scolding et al., 1995; Yandava et al., 1999). Hence, the main focus of research has been directed towards oligodendrocyte progenitor cells

(OPCs) that have been identified in the adult CNS of mice (Dubois-Dalcq, 1987; Ffrench-Constant and Raff, 1986; Sim et al., 2002; Watanabe et al., 2002; Wolswijk et al., 1990; Wren et al., 1992) and humans (Armstrong et al., 1992; Chang et al., 2000; Scolding et al., 1995), as well as in human MS lesions (Chang et al., 2002; Wolswijk, 2002). As discussed in Section 1.5.5, the discovery of factors helping the differentiation of oligodendrocytes will provide good tools for treating myelin-related diseases. Moreover, technology such as OPC transplantation, the transfer of myelin-promoting genes, or the intra-cerebro-ventricular infusion of soluble factors should be improved.

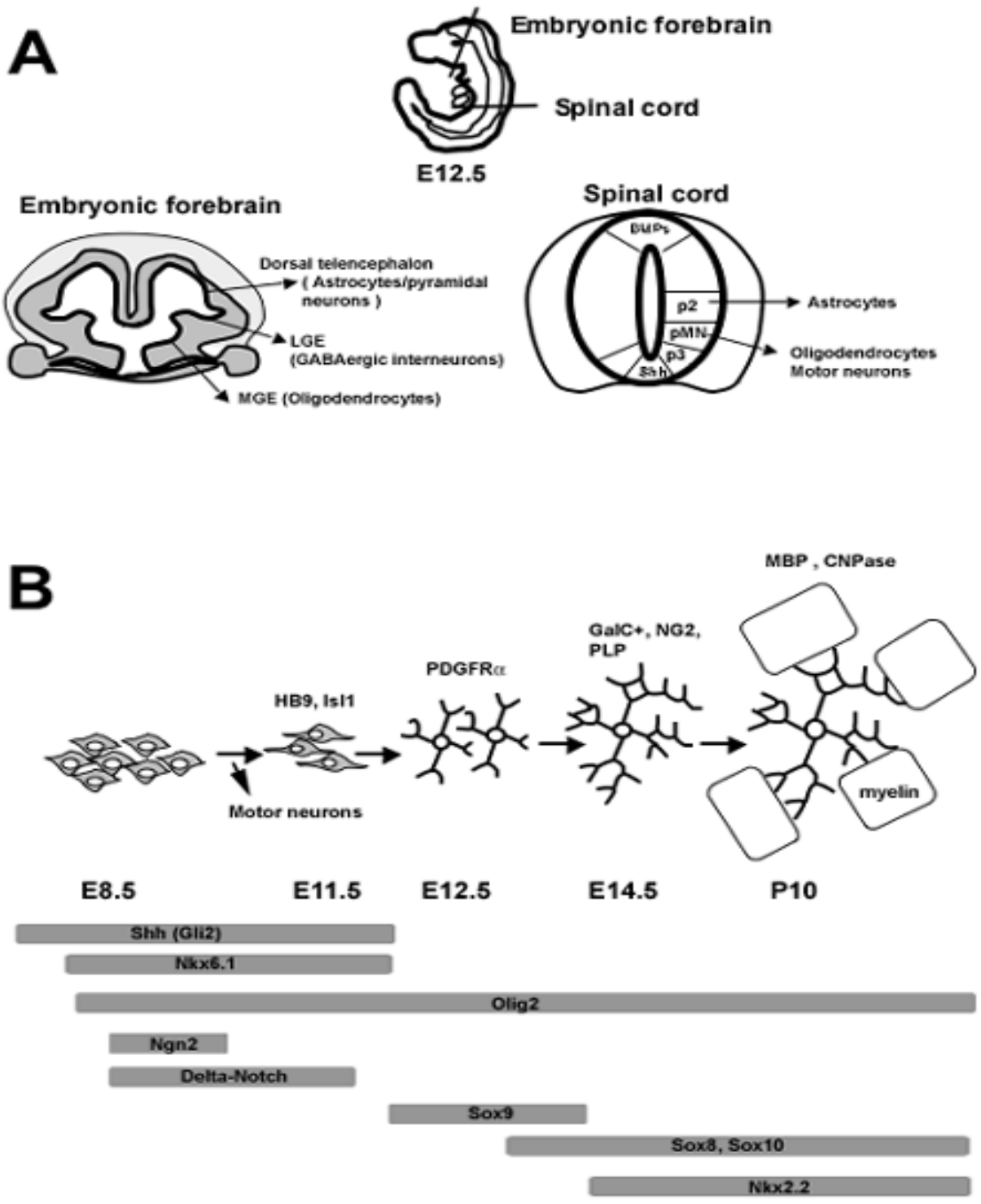
1.6 Oligodendrocytes and Schwann cell development

1.6.1 Glial cell development in CNS

Many studies using neural tube progenitor cells in cultures, and mice, chick, and Drosophila embryos, demonstrated that oligodendrocytes emerge from localized regions of the neural tube, and require specific factors such as transcription factors. In the brain, the generation of all cell types occurs in temporally distinct, albeit overlapping, phases. Figure 1-15 illustrates the neural and glial cell development steps in the CNS. Neurons are first generated at E9.5-E10.5, followed by astrocytes at E11.5 and then oligodendrocytes at E12.5 (Ross et al., 2003). Oligodendrocyte precursor cells (OPCs) develop primarily from ventral zone of the telencephalon. This process requires sonic hedgehog (Shh) signaling, transcription factors containing basic helix-loop-helix domains (bHLH), and extrinsic factors such as growth factors (PDGF and TH). Shh is a secreted protein that is essential for organizing the structures of the ventral midline, such as the notochord and the floor plate (Tekki-Kessaris et al., 2001). Shh induces the emergence of *PDGFR*+ *Sox10*-expressing OPCs from the medial ganglionic eminence (MGE), which is most closely correlated with the expression of a bHLH-containing transcription factor called Olig1. This is later required for oligodendrocyte production in the forebrain and hindbrain (Rowitch et al., 2002). This discovery indicates that OPCs might develop from a subset of cells in the MGE in which the expression of other transcription factors, such as Dlx*2*, Mash1, Sox9/10, and Nkx2.2, overlap. However, more work is needed to clarify the function of these transcription factors. Moreover, the posttranscriptional modification of the mRNAs coming from transcriptional activation by bHLH-containing factors will

need to be addressed. Perhaps the function of RNA binding proteins such as QKI, Musashi, and others will address the key questions in the differentiation of glial cells in the telencephalon.

Figure 1-15. Diagram of the Developing Forebrain Telencephalon and Spinal Cord. In (A), illustration of the origin of cells in the CNS. Oligodendrocytes in the embryonic forebrain arise from the ventral proliferative zone called medial ganglionic eminence (MGE). Astrocytes and neurons are generated in the dorsal telencephalon regions. In mouse spinal cord, oligodendrocyte progenitor cells (OPCs) emerge from the pMN domain in a proximal source of Shh at embryonic day E12.5. Astrocytes emerge from the adjacent dorsal p2 domain. Motor neurons are generated in the pMN domain at early stage (from about E9.5-E10.5). In (B), diagram shows the development of pMN-derived cells to oligodendrocytes during spinal cord development. Since pMN is competent to produce motor neurons and oligodendrocytes, a neuron-glial switch has to occur and this process involves Shh expression from the floorplate, downregulation of Ngn2 and up-regulation of Olig2 expression. Others differentiation factors such as Sox9, Sox10 and Nkx2.2 are important for oligodendrocyte cell fate until they reach the myelinating phenotype at postnatal stage.


A
Embryonic forebrain
Spinal cord
E12.5
Embryonic forebrain
Dorsal telencephalon
(Astrocytes/pyramidal
neurons)
LGE
(GABAergic interneurons)
MGE (Oligodendrocytes)
Spinal cord
BMPs
p2
pMN
p3
Shh
Astrocytes
Oligodendrocytes
Motor neurons
B
MBP , CNPase
GalC+, NG2,
PLP
PDGFRα
HB9, Isl1
Motor neurons
myelin
E8.5
E11.5
E12.5
E14.5
P10
Shh (Gli2)
Nkx6.1
Olig2
Ngn2
Delta-Notch
Sox9
Sox8, Sox10
Nkx2.2

Where does oligodendrocyte differentiation occur in the adult brain? In the adult brain, oligodendrocytes are derived from the migratory progenitor cells that emerge from the dorsolateral subventricular zone (SVZ) to colonize the corpus callosum and the adjacent grey and white matter (Hardy and Friedrich, 1996; Miller, 2002; Reynolds and Hardy, 1997; Weiss et al., 1996). In fact, the SVZ is the germinal matrix area that is adjacent to the cerebral ventricles. It enlarges during the peak of gliogenesis, between P5 and P20, and then shrinks but persists into adulthood. Cell lineage studies of postnatal SVZ cells demonstrate that the majority of progenitors within this germinal matrix are glial precursors that generate astrocytes and oligodendrocytes. Although the majority of cells give rise to homogeneous progeny, some SVZ cells give rise to both oligodendrocytes and astrocytes, and rare cells develop into both neurons and glia. In the neonatal rat cerebrum, oligodendrocytes arise post-natally from the SVZ of the lateral ventricle. Oligodendrocyte progenitors then migrate away from the SVZ along the rostral migratory zone (RMZ) to various regions of the brain, where they develop into mature oligodendrocytes. Interestingly, since oligodendrocytes differentiate late in the CNS, their development may be influenced by signals derived from other neural cells, such as astrocytes and neurons (Baumann and Pham-Dinh, 2001).

Another kind of oligodendrocyte–astrocyte precursor has been identified in cell culture assays from brain or optic nerve cells. Depending on the *in vitro* culture condition, these precursors generate oligodendrocytes, type-1 astrocytes, or type-2 astrocytes, but do not generate neurons. For this reason, these cells were originally called oligodendrocyte–type-2 astrocyte (O–2A) progenitors (Raff et al., 1983). Type-2 astrocytes differ from more common type-1 astrocytes in their expression of A2B5

immunoreactivity (Raff et al., 1984). Nonetheless, type-2 astrocytes have proven to be elusive *in vivo*, indicating that O–2A progenitors do not normally encounter conditions that promote astrocyte differentiation (Fulton et al., 1991). Today, the O-2A term has been replaced by glial-restricted precursors (GRPs). Moreover, in dissociated cell cultures, purified oligodendrocyte progenitors are able to recapitulate myelination. They express the glycolipid galactocerebroside (GalC), the enzyme CNPase, and the three structural myelin proteins: MBPs, PLP, and MAG. In addition, at the tip of their processes, these oligodendrocytes extend large unfolded myelin-like membranes.

Spinal cord development has been studied more because of its well-defined zones. Mouse embryo spinal cords, or neural tubes as they are named at that stage, have regionally-restricted domains in which oligodendrocytes, astrocytes, and motor neurons are formed. For example, the p0 to p3 domains give rise to interneurons and astrocytes, whereas the pMN domain is the source of motor neurons as well as oligodendrocytes (Figure 15). Each segment expresses a specific set of transcription factors. A Shh gradient mainly regulates the initial expression of the transcription factors in the ventral neural tube. Transcription factors such as Nkx2.2, Olig2, Irx3, and Pax6 are involved in neural tube gliogenesis (Panchision and McKay, 2002; Ross et al., 2003; Sauvageot and Stiles, 2002). In fact, the formation of oligodendrocytes in the neural tube at E12.5 is triggered by bHLH-containing factors, such as Olig2 and Nkx2.2. This is coupled with a decrease of other factors, such as Id and Hes transcription factors. Biochemical assays demonstrated that the Olig2 protein forms a complex with the homeodomain transcription factor Nkx2.2 protein (Sun et al., 2003). This interaction occurs at two distinct stages: during neuronogenesis at E9.5, a cross-repressive interaction appears to establish the

precise boundary between the p3 and the pMN domains; at stage E12.5, a cooperative interaction of Olig2 with Nkx2.2 is noted because Nkx2.2 promotes the maturation of oligodendrocyte progenitor cells specified by expression of Olig2. However, it was demonstrated that the formation of an Olig2-Nkx2.2 physical complex is not itself sufficient to induce the formation of OPCs in a developing spinal cord.

Shh is another important player in spinal cord development. The activation of the Shh pathway is antagonized by several repressor molecules, including Gli3, patched, and the bone morphogenic protein (BMP) produced from the roof plate. These two molecules establish a "gradient" of Shh–BMP that confers positional identity along the dorsoventral axis. Regulating the expression of present transcription factors into the ventral neural tube is an important function of Shh signaling. However, we still do not know all of the genes implicated in the ventral neural tube. The discovery of other regulatory proteins, such as transcription factors and RNA binding proteins, will help to elucidate the role of gene regulation in oligodendrocyte development.

It is thought that two distinct regions of the brain generate astrocytes. First, astrocytes derive from the neuroepithelium cells in the dorsal telencephalon where radial glia are located (Gaiano and Fishell, 2002). Regions such as lateral ganglionic eminence (LGE) and dorsal neo-cortex region also give rise to astrocytes. Indeed, LGE generate neurons first and then develop astrocytes.

Ngn2+ cells are the main source of astrocytes. During embryogenesis as well as in the adult stage in the brain, astrocytes emerge from Ngn2+ cells in the dorsolateral SVZ to colonize the adjacent areas in the grey and white matter (Rowitch, 2004). In the embryo spinal cord, astrocyte formation depends mainly on astrogliogenic factors, such

as BMP and the leukaemia inhibitor factor (LIF), secreted from the roof plate. GFAP (Glial fibrillary acidic protein) the typical marker of astrocytes, is up-regulated after BMP and LIF are secreted from the roof plate and promote neuroepithelial differentiation at the p2 region of the neural tube. More precisely, the formation of a complex containing Stat1/3, Smad1, and the co-activators p300/CBP activates the GFAP gene regulatory sequences in the promoter region (Mehler et al., 2000). Interestingly, oligodendrocyte-promoting factors are generally inhibitors for astrocyte differentiation. In fact, Olig2 can antagonize astrocyte differentiation. The ectopic expression of Olig2 inhibited astrocyte differentiation, even in the presence of LIF (Fukuda et al., 2004). The binding of Olig2 to the p300/CBP co-activators, inhibited both complex formation with Stat3 and reporter gene expression driven by GFAP regulatory sequences. Contrarily, the ectopic expression of the negative HLH proteins Id4, Id2, and Hes1 promotes the formation of astrocytes over oligodendrocytes, and down-regulates Olig genes (Gabay et al., 2003; Wu et al., 2003).

Other key factors in gliogenesis are the components of the Notch signaling pathway. The pathway was discussed in Section 1.3.3 and was illustrated in Figure 1-9. In fact, the Notch plays an instructive role in promoting the number of glial-restricted precursor cells in the CNS (Gaiano et al., 2000; Wang et al., 1998). Studies using zebrafish which lack the genes that encode the Delta ligand (a Notch ligand protein), demonstrate an increased number of neurons and a low number of glial cells (Park and Appel, 2003). In addition, such studies also show that Notch signaling is necessary to maintain a proportion of *Olig2*+ precursor cells until the gliogenic phase of development in the pMN region of the neural tube. Interestingly, despite the forced expression of the

Notch receptor, the timing of oligodendrocyte production was unchanged, indicating that the role of Notch for glial cell fate acquisition is permissive rather than instructive (Scheer et al., 2001).

In conclusion, advances in glial cell biology are transforming our understanding of vertebrate development. There is growing evidence that proteins such as bHLH transcription factors, Shh, BMP, and Notch play a role, but that they are not, in and of themselves, sufficient for oligodendrocyte differentiation. The discovery of other proteins related to the main pathway of gliogenesis will have important implications on our understanding of the developmental mechanisms of the CNS.

1.6.2 Schwann Cell Development

The vertebrate peripheral nervous system (PNS) is the essential connection of motor axons, sensory, sympathetic, and parasympathetic nerves to the central nervous system (CNS). Peripheral nerves consist of Schwann cells that establish close contact with axons. Sensory neurons with their cell bodies in the dorsal root ganglia (DRG) transmit signals from the periphery to the spinal cord. Motor neurons extend axons from the spinal cord to the muscles. More importantly, these neurons depend on Schwann cell functions, myelination and the regulation of nerve development (Jessen and Mirsky, 1999). There are two types of Schwann cells: the myelinating and non-myelinating ones. In 1976, McGill University's Albert Aguayo was the first to find that the caliber of the axons is dependent on the presence of myelinating Schwann cells (Aguayo et al., 1976).

Indeed, interactions between Schwann cells and their associated axons underlie the development and regeneration of the PNS. The major function of the Schwann cells is the

formation of the myelin sheath that surrounds the majority of axons and allows for the rapid saltatory propagation of electric impulses. As illustrated in Figure 16, Schwann cells are embryonically derived from the neural crest cells. At embryonic day 9 (E9.5) in the mouse, neural crest stem cells (NCSCs) detach from the dorsal neural tube and migrate along defined pathways to peripheral nerves. These multipotent cells give rise to PNS neurons and Schwann cells, but also to a variety of other differentiated cell types, such as smooth muscle, cartilage, bone, and melanocytes. At E10.5, NCSCs emigrate and colonize the somites and aggregate to form dorsal root ganglia (DRG). The DRG is the main region where progenitor cells differentiate, first into sensory neurons, and later into Schwann cells (Jessen and Mirsky, 1999; Jessen and Mirsky, 2002; Le Douarin and Ziller, 1993). At E12.5, neural crest stem cells make contact with sensory axons sprouting from the DRG and with CNS motor axons, and differentiate into Schwann precursor cells. These cells proliferate during their migration along the nerves. By postnatal day 1 (P1), they have differentiated into early or immature Schwann cells. During this newborn development stage, Schwann cells invade the axons, sitting on them at regular intervals to form internodes. Each internode is myelinated by one Schwann cell. Shortly after birth, some of the Schwann cells begin to form myelin sheaths, whereas others remain non-myelinating Schwann cells (Lobsiger et al., 2002). Figure 16 illustrates the major steps of Schwann cell development and differentiation.

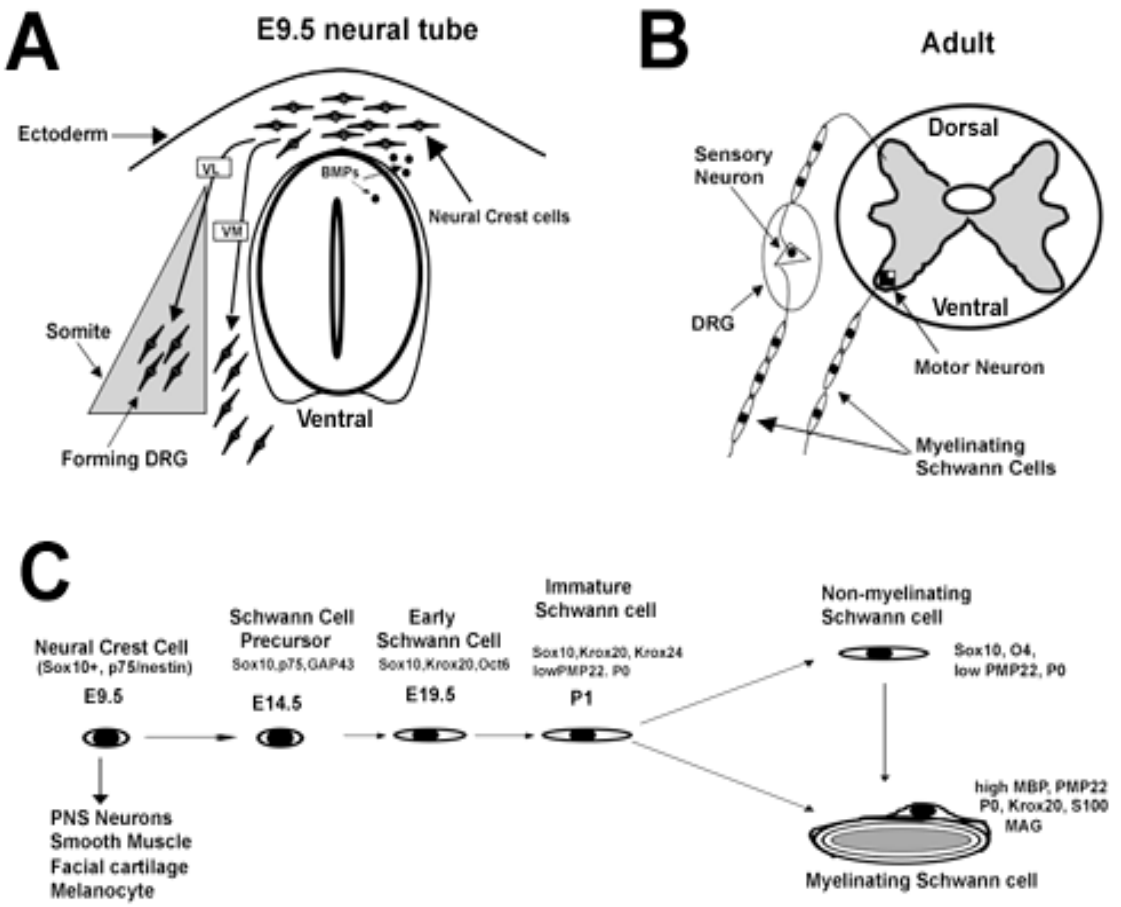


Figure 1-16: Schematic Diagram showing Schwann cell origins until its differentiation in a mouse. In (A), the origin of neural crest cells comes from the roof of the closing neural tube at E9.5 (mid-gestation). The ectoderm secretes BMPs, which triggers the emigration of neural crest cells. Two pathways are possible for neural crest cells: the ventromedial (VM) path, and the ventrolateral (VL) path into the somite to form the dorsal root ganglion (DRG)-containing Schwann cell precursors and peripheral neurons. (B) The diagram illustrates a schematic representation of the adult PNS. Sensory neuron cell bodies are in the DRG. With their cell bodies, the motor neurons start from the ventral horn of the spinal cord and project to axons and the muscles. Axons are surrounded by a myelin sheath deposited by Schwann cells. (C) The diagram illustrates Schwann cell lineage development. The initial step, the formation of Schwann cell precursors until mature post-natal myelinating or non-myelinating cells, is characterized by the appearance of markers such as Krox-20, Oct-6, MBPs, and others.

What are the genes or proteins implicated in Schwann cell development? Three transcription factors, Sox-10, Oct-6, and Krox-20, have been shown to play key roles in Schwann cell lineage (Jaegle et al., 2003). Oct-6 is transiently up-regulated during early development of Schwann cells (Arroyo et al., 1998). At the postnatal stage in PNS, Oct-6 expression is down-regulated, and Krox-20 becomes up-regulated. Krox-20 (also known as Egr-2) is a zinc finger protein that regulates the expression of an array of myelin genes (Nagarajan et al., 2001). In Krox-20 null mice, myelin sheaths do not form and Schwann cells continue to proliferate and remain susceptible to apoptosis (Parkinson et al., 2004; Parkinson et al., 2003). *In vitro* gene transfer experiments show that Krox-20 activates the expression of myelin genes such as P0, periaxin, PMP22, Cx32, and MBP (Nagarajan et al., 2001; Parkinson et al., 2004). In this way, Krox-20 has the ability to promote a large number of phenotypic changes in immature Schwann cells that characterize the transition of these cells to myelinating cells.

Interestingly, cis-acting sequences have been found in the 3'-UTR of Krox-20 mRNA (Ghislain et al., 2002; Giudicelli et al., 2001). The presence of this sequence in the 3'-UTR seems to down-regulate the expression of Krox-20 at the embryonic level [P. Gilardi-Hebenstreit, personal communication]. We suggest that posttranscriptional control by RNA binding proteins could regulate the expression of Krox-20. Schwann cell-specific proteins such as QKI might be important for Krox-20 expression and Schwann cell differentiation. In part, one of the objectives of this thesis will be to investigate this question.

1.6.3 Cell Cycle and Glial Cell Differentiation

A glial precursor cell has to make three fundamental steps in order to generate a differentiated glial cell. First, the cell has to be at the right position in the neural tube or telencephalon. Second, the environment (growth factors, hormones, ligand) sends the cell a signal to decide whether to self-renew or to undergo mitotic arrest. Third, the cell must interpret mitotic arrest using inherited or externally derived information that directs it to a particular fate. The last two steps are an integration of signals from the environment, which occur during the G1 phase of the cell cycle. During the G1/S transition checkpoint, the oligodendrocyte progenitor cell (OPC) responds to extracellular signals and chooses between several possibilities. In the presence of mitogens, the cell decides to duplicate its DNA content in the S-phase and enter another round of cell division. In the absence of mitogens, or in the presence of anti-mitogenic signals, the cell withdraws either transiently or permanently from the cell cycle at the G1 phase and may start the differentiation program (Raff et al., 2001).

The end-point of differentiation for an oligodendrocyte is the branching process and the formation of myelin sheaths around multiple axons. Those defined phenotypes are the main reason why oligodendrocytes are the model of choice for cell differentiation. Moreover, oligodendrocytes are able to myelinate *in vitro* without the presence of axons, unlike Schwann cells, which are not multiple branching cells and are only able to myelinate axons with the presence of axons. Nonetheless, Schwann cells are able to myelinate axons *in vitro* and *in vivo* to produce 16 to 20 layers of myelin sheaths per axon (Fragoso et al., 2003). Compared to Schwann cells, of which the molecular events

of differentiation are poorly understood, the molecular events involved in glial cell differentiation in oligodendrocytes have been well studied.

The mechanism involved in the control of oligodendrocyte proliferation and differentiation remains dependent on cell-cycle machinery (Figure 1-17). In fact, progression through the cell cycle is promoted by holoenzymes composed of regulatory cyclin and catalytic CDK. Proteins such as the nuclear tumor suppressor Rb are phosphorylated during the G1 phase, mainly by the CDK4/6-cyclin D complex. The catalytic activity of CDKs is negatively regulated by cyclin-kinase inhibitors (CKIs), such as p18Ink4C, $p21^{Cip1}$, $p57^{Kip2}$,and $p27^{Kip1}$. In fact, CKIs have been identified as blocking the kinase activity of CDK-cyclin holoenzymes. Thus, CKIs ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) can form stable complexes with either cyclin D–CDK4/6 or cyclin E–CDK2 complexes. According to one model, the up-regulation of CKIs like $p27^{Kip1}$ in a mitotic cell might lead to the disruption of a cyclin D–CDK4/6 complex, which prompts cyclin E–CDK2 inactivation by the newly released Cip/Kip molecule.

Consistent with the role of $p27^{Kip1}$ in the development of all organs, $p27^{Kip1}$-deficient mice display a general enhanced growth (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). In the CNS, the loss of $p27^{Kip1}$ results in an increase in the number of Muller glia (Dyer and Cepko, 2001a), and an increased number of transit amplifying progenitor cells in the adult brain at the SVZ region (Doetsch et al., 2002). In the oligodendrocyte lineage, the loss of $p27^{Kip1}$ results in the increased proliferation and impaired differentiation of cultured oligodendrocyte progenitors (Casaccia-Bonnefil et al., 1997; Durand et al., 1998). *In vivo*, the loss of $p27^{Kip1}$ function also results in increased proliferation and impaired differentiation (Casaccia-Bonnefil et al., 1999), whereas the

loss of $p21^{Cip1}$ does not affect proliferation but instead selectively impairs the differentiation program of progenitor cells (Zezula et al., 2001). Interestingly, an increased number of oligodendrocytes were observed in the brain and spinal cord of $p27^{Kip1}$-null mice. All these facts demonstrate that $p27^{Kip1}$ is a key player in glial cell differentiation (Figure 1-17).

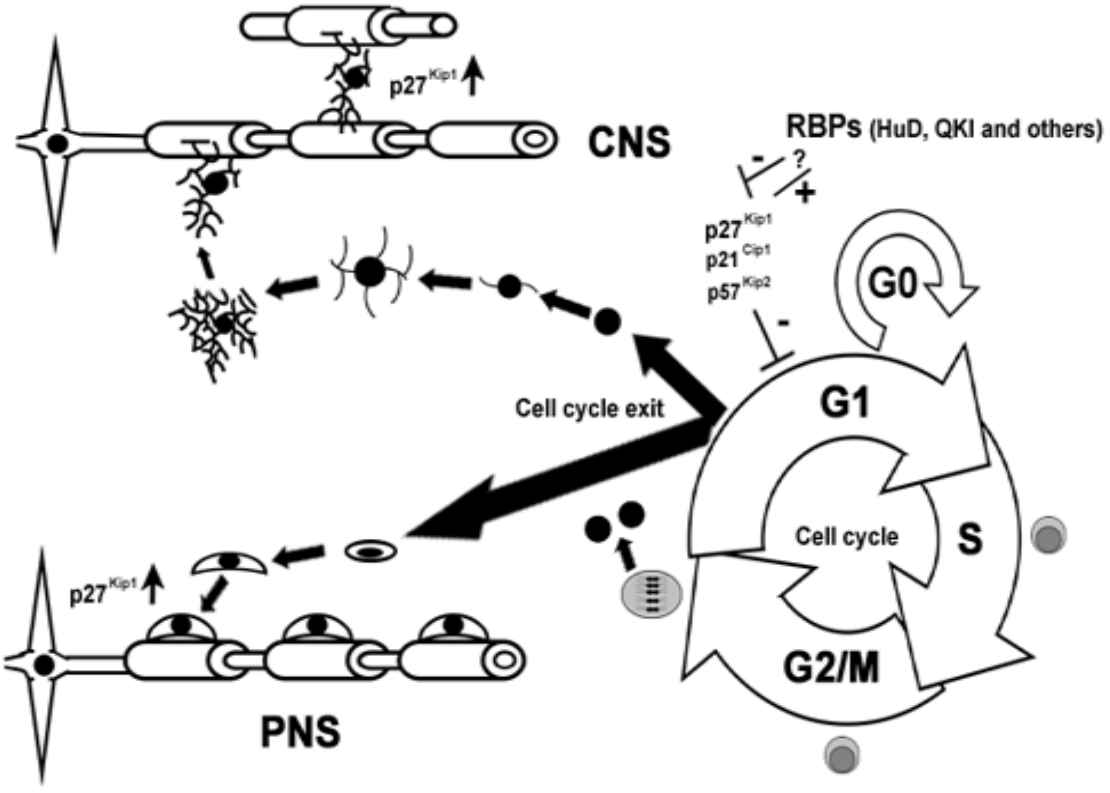


Figure 1-17. The schematic representation of our current knowledge on the link between cell cycle, oligodendrocyte differentiation in CNS, and Schwann cell differentiation in PNS. Cyclin-kinase inhibitors (CKIs), such as p18Ink4C, $p21^{Cip1}$, $p57^{Kip2}$, and $p27^{Kip1}$, play an important role in cell cycle exit at the G1/S phase transition. As shown by (**?**), this model predicts that CKI proteins might be regulated by the actions of RNA binding proteins (RBPs).

Furthermore, the level of $p27^{Kip1}$ protein is known to vary during the cell cycle and differentiation (Hengst and Reed, 1996). There is strong evidence that the $p27^{Kip1}$ oscillation level is the result of posttranscriptional mechanisms, such as protein degradation that is dependant on CDK2 and SKP2 [Pagano, 1995 #95] and translational control (Kullmann et al., 2002). In fact, some studies have shown that the $p27^{Kip1}$ 5'UTR sequence contains an internal ribosomal entry site (IRES) (Kullmann et al., 2002; Millard et al., 2000; Miskimins et al., 2001). RNA binding proteins, such as the ELAV protein HuD or HuR, and hnRNP C1 or C2, negatively regulate the translation of $p27^{Kip1}$. However, no up-regulation of $p27^{Kip1}$ expression level caused by RNA binding proteins has been reported so far, which leads us to think that RNA binding proteins such as QKI proteins or others might result in increased levels of $p27^{Kip1}$ proteins.

Moreover, the over-expression of $p27^{Kip1}$ efficiently inhibits oligodendrocyte precursor cell division, but this cell cycle arrest is not sufficient to induce the differentiation of dividing oligodendrocyte precursors (Tang et al., 1999). Other yet unknown factors might be implicated in making the link between $p27^{Kip1}$-dependent cell cycle arrest and oligodendrocyte differentiation. Modulators of RNA such as QKI proteins must be investigated in order to address the question posed by Raff's group (Tokumoto et al., 2002): Why is $p27^{Kip1}$ mRNA increased during oligodendrocyte differentiation, and which factors contribute to this posttranscriptional regulation? This research project seeks an answer this fascinating question.

REFERENCES: See page 250.

Chapter 2

NUCLEAR RETENTION OF MBP mRNAS IN THE *QUAKING VIABLE* MICE

by

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2.1 Preface

Before beginning this project, many studies were concentrated on the qk^v mice phenotype; however, a few studies tried to explain the main reason for dysmyelination in those mice. We started by performing RNA binding assays using QKI proteins and mRNAs encoding myelin proteins, which are affected in qk^v mice. The RNA with the strongest affinity was the MBP mRNA. This observation led us to study the localization of MBP mRNA in the oligodendrocytes of qk^v mice. This Chapter will demonstrate and prove that a change in the equilibrium of QKI protein isoforms is the main reason for dysmyelination in qk^v mice.

2.2 Summary

Quaking viable (qk^v) mice fail to properly compact myelin in their central nervous systems. Although the defect in the qk^v mice involves a mutation affecting the expression of the alternatively spliced *qk* gene products, their roles in myelination are unknown. We show that the QKI RNA binding proteins regulate the nuclear export of MBP mRNAs. Disruption of the QKI nucleocytoplasmic equilibrium in oligodendrocytes results in nuclear and perikaryal retention of the MBP mRNAs and lack of export to cytoplasmic processes, as it occurs in qk^v mice. MBP mRNA export defect leads to a reduction in the MBP levels and their improper cellular targeting to the periphery. Our findings suggest that QKI participates in myelination by regulating the mRNA export of key protein components.

2.3 Introduction

Quaking viable (qk^v) mice contain a spontaneous mutation resulting in hypomyelination of the central and peripheral nervous systems (Sidman et al., 1964). These animals have been studied for over thirty years and represent an animal model for dysmyelination (Hogan and Greenfield, 1984). These mutant mice develop normally until postnatal day 10 when they display rapid tremors or "quaking" that is especially pronounced in the hindlimbs and experience convulsive tonic-clonic seizures as they mature (Hogan and Greenfield, 1984). Studies using the qk^v mice have identified that oligodendrocyte (OL) function was impaired and that the intracellular transport of myelin components might be the underlying defect (Hogan and Greenfield, 1984). The mutation in qk^v mice has been genetically identified and does not involve a component of myelin. The qk^v mutation consists of a one megabase deletion that includes the promoter and enhancer regions of the *qk* gene, which encodes a family of alternatively spliced RNA binding proteins (Ebersole et al., 1996). Oligodendrocytes (OLs) of normal mice express three major *qk* mRNAs of 5, 6, and 7 kb encoding QKI-5, QKI-6, and QKI-7, respectively (Ebersole et al., 1996). The promoter deletion observed in qk^v mice prevents the expression of alternatively spliced QKI-6 and QKI-7 isoforms in OLs, as analyzed by immunocytochemistry (Hardy et al., 1996b). These observations suggest that the balance between the different QKI RNA binding isoforms may control myelination. The other class of recessive *qk* mutations are ethylnitrosourea-induced recessive mutations, which cause embryonic lethality (Bode, 1984; Justice and Bode, 1988; Shedlovsky et al., 1988). One such allele, qk^{kt4}, was found to alter QKI glutamic acid 48 to glycine (Ebersole et al.,

1996). This amino acid substitution abrogates QKI dimerization mediated by a coiled-coil region in the N-terminal region of QKI and may be the molecular defect of qk^{kt4} (Chen and Richard, 1998). Another allele, qk^{k2}, altering QKI valine 157 to a glutamic acid, remains uncharacterized (Cox et al., 1999).

The dysmyelination phenotype of the qk^{v} mice implicates QKI proteins in the process of myelination. Defects in mRNA processing, mRNA localization, and protein expression of the major protein components of myelin have been observed in the qk^{v} mice (Vernet and Artzt, 1997; Hardy, 1998). The expression and the incorporation of the MBPs in myelin are decreased in the brain of qk^{v} mice (Brostoff et al., 1977; Delassalle et al., 1981; Carnow et al., 1984; Sorg et al., 1986). OLs from qk^{v} mice in culture and in vivo are unable to specifically translocate MBP mRNAs to the periphery (Campagnoni et al., 1990; Barbarese, 1991), where they are locally translated by polyribosomes (Colman et al., 1982; Verity and Campagnoni, 1988). The rate of synthesis of MBPs is unaffected (Brostoff et al., 1977), but the levels of MBP mRNAs in qk^{v} mice are reduced (Li et al., 2000). The level of the alternatively spliced mRNAs and proteins for the small and large isoforms of myelin-associated glycoprotein are also altered in qk^{v} mice (Frail and Braun, 1985; Braun et al., 1990; Fujita et al., 1990; Bartoszewicz et al., 1995; Trapp et al., 1984; Bo et al., 1995). The levels of proteolipid protein mRNA have also been shown to be reduced in qk^{v} (Sorg et al., 1986, 1987). These observations raise the possibility that the QKI proteins are directly involved in the regulation of RNA metabolism of myelin components.

The QKI proteins contain a hnRNP *K H*omology (KH) domain embedded in a larger domain called the GSG (*G*RP33, *S*am68, *G*LD-1) domain (Jones and Schedl,

1995). These proteins are often referred to as STAR (*S*ignal *T*ransduction *A*ctivators of *R*NA) proteins because of their potential link to signal transduction pathways (Vernet and Artzt, 1997). The QKI GSG domain has been shown to be necessary and sufficient for RNA binding and dimerization (Chen and Richard, 1998). The C-terminal QKI sequences that vary from 8 to 30 amino acids due to alternative splicing are involved in targeting the QKI proteins to different cellular compartments (Hardy et al., 1996b). The unique C-terminal sequences of QKI-5 harbor a nuclear localization signal and target QKI-5 to the nucleus (Wu et al., 1999). QKI-6 and QKI-7 are predominantly cytoplasmic. The QKI isoforms are thought to shuttle between the nucleus and the cytoplasm as homo- and heterodimers (Wu et al., 1999; Pilotte et al., 2001). QKI orthologs have been identified from *Xenopus*, *Drosophila*, chicken, and zebrafish, suggesting that the *qk* gene may have a fundamental cellular function (Baehrecke, 1997; Tanaka et al., 1997; Zaffran et al., 1997; Zorn et al., 1997; Mezquita et al., 1998). Recent evidence has indicated the involvement of several QKI orthologs in RNA metabolism. For instance, *Drosophila H*eld-*O*ut-*W*ings (How) has been shown to function in the mRNA export of the transcription factor Stripe during tendon development (Nabel-Rosen et al., 1999). Although the QKI proteins have been shown to control cell survival and cell death (Pilotte et al., 2001), their molecular function in RNA metabolism and their physiological mRNA targets remain unknown.

We now show that the QKI RNA binding proteins bind a short element in the MBP 3' untranslated region. The balance between the nuclear and cytoplasmic isoforms of QKI, which are derived from alternative splicing, controls the nuclear export of MBP mRNAs and the cellular localization of exon II MBP isoform 17 and 21.5. By using OL

cultures overexpressing QKI-5, we have re-created the MBP defects observed in *qk*^{v} mice. Our data provide evidence that the QKI proteins are involved in myelination.

2.4 Materials and methods

2.4.1 Primary rat OL cultures and adenovirus infections.

Cultures of OL progenitors were generated as described (Almazan et al., 1993). OL progenitors, also termed O-2 A progenitors for their ability to generate OLs and type 2 astrocytes in vitro, were plated on 6-well dishes at a density of 15 x 10^3 cells/cm^2. In order to expand their numbers and prevent differentiation, the cultures were grown in media containing 2.5 ng/ml basic fibroblast growth factor (Peprotec) and platelet-derived growth factor AA for 4 days. The cultures were infected with the indicated adenovirus co-expressing QKI-5 from the TR5 promoter and GFP from the CMV promoter (AdTR5-QKI-5; Massie et al., 1998b; Pilotte et al., 2001) or control adenovirus (AdTR5) expressing GFP from the TR5 promoter and an adenovirus AdCMV-tTA that expresses the tTA (Massie et al., 1998a). A multiplicity of infection (MOI) of 100 was sufficient to infect about 80% of the cells as judged by GFP-positive cells. 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 homogeneous bipolar cells, and acquired ramified processes as they differentiated into mature OLs in vitro.

For stereotactic adenovirus injection, 1 µl of 1 x 10^7 pfu/µl adenovirus was injected using a 31-gauge needle in the corpus callosum of postnatal day 1 inbred C57BL/6 mice at the following localization: bregma -1 mm; lateral 0.5 mm; depth of 1 mm. The survival rate was >95% with over 20 pups. The pups were returned to the

mother and monitored daily. At postnatal day 10, the pups were sacrificed and their brains processed for GFP and MBP protein levels by immunofluorescence.

2.4.2 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 for 2 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 minutes in 5% calf serum-1% Triton X-100 in PBS. This was followed by a 24 hr incubation in PBS-0.1% Triton with a mix of two monoclonal MBP antibodies. The monoclonal anti-MBP antibody (1:2500, Sternberger LTD) and mAb 387 (1:1000, Chemicon International) were used for staining the MBP isoforms in the mouse brain cross-sections. Affinity-purified anti-MBPexII was used as described previously (Pedraza et al., 1997). Species-specific Alexa 546 (Molecular Probes) secondary antibodies diluted 1:400 in PBS:0.1%Triton were used for detection.

2.4.3 DNA constructs.

The constructs encoding HA-QKI-7, myc-QKI-5, myc-QKI-6, myc-QKI-7, myc-QKI-7:E48G, glutathione S-transferase (GST)-QKI-7, and GST-QKI-7:E48G were

described previously (Chen and Richard, 1998; Pilotte et al., 2001). Full-length MBP cDNAs for mouse 14, 17, 18.5, and 21.5 kDa were obtained from the Laboratory for Genome Exploration Research Group RIKEN Genomic Sciences Center (GSC), RIKEN Yokohama Institute Japan. Myc-QKI-7:V157E was constructed by inverse PCR with myc-QKI-7 and MBP3'UTR and MBP full-length were generated by PCR.

2.4.4 Preparation of mouse OLs.

OLs progenitor cells were purified from postnatal day 11 mouse brain. At that age, the *qk*^{v} phenotype is clearly visible by a typical tremor in the hindlimbs. Five wild-type C57BL/6 females and five *qk*^{v} females were used for the mouse OL culture experiments. Purification and culture of OL progenitor cells were performed according to the percoll gradient procedure (Lubetzki et al., 1991). These OL progenitor cells were plated on poly-D-lysine-coated glass coverslips. Cultures were incubated for 10 days in OL differentiation media containing T3 hormone as described (Almazan et al., 1993).

For the endogenous MBPexII proteins, analysis from brain crude extract, entire brains were lysed by sonication in 1x Laemmli buffer containing 8 M urea without bromphenol blue. The quantity of total protein in each sample was estimated by the Bradford assay (Bio-Rad, Hercules, California).

2.4.5 Protein analysis.

Myc-QKI-7 and HA-QKI-7 were transfected in HeLa cells, lysed, immunoprecipitated, and analyzed as previously described (Chen and Richard, 1998).

Samples were immunoblotted with anti-myc (9E10), anti-HA (12CA5), anti-MBP (Dako Diagnostics), or anti-actin (Chemicon) antibodies followed by a goat anti-mouse antibody conjugated to horse radish peroxidase (ICN). Chemiluminescence was used for protein detection (Dupont).

2.4.6 In vitro transcription.

^{32}P-labeled MBP RNA fragments were generated by in vitro transcription using the T7 RNA polymerase following the protocols recommended by the manufacturer (Promega). The DNA templates used for in vitro transcription were PCR fragments of different regions of the MBP cDNA with an engineered 5' T7 promoter. The oligonucleotide sequences used to generate the MBP RNAs will be given upon request.

2.4.7 RNA binding assays.

RNA binding of immunoprecipitated QKI was performed as previously described (Chen and Richard, 1998). For EMSA, ^{32}P-labeled RNA (10^5 cpm) was incubated at RT for 30 minutes with GST or GST-QKI fusion proteins (0.5–2.0 μg) in PBS supplemented with 1% Triton X-100, 1 mg/ml of heparin, and 250 μg/ml of tRNA. The samples were electrophoresed on a 4% native polyacrylamide gel (acrylamide:bis, 60:1) in 0.5 x TBE and visualized by autoradiography. For the analysis of QKI-MBP mRNA association in vivo, astrocytes and OLs (OLs) isolated from newborn rats were lysed with lysis buffer supplemented with the RNase inhibitor RNAguard (Pharmacia), and the cell lysates were immunoprecipitated with anti-QKI antibody (Chen and Richard, 1998) or normal rabbit

serum (NRS). Associating MBP mRNAs were amplified by RT-PCR and Southern blot analysis. For UV crosslinking, a synthetic ^{32}P-labeled RNA corresponding to nucleotides 626–885 of the MBP mRNA was prepared by in vitro transcription using ^{32}P-CTP.

2.4.8 In situ hybridization and fluorescence staining.

COS cells were plated on glass coverslips in 6-well dishes and transfected by using Lipofectamine Plus (Canadian Life). Twelve hours after transfection, the cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS. If the cells were transfected with GFP constructs, the coverslips were mounted onto glass slides with Immuno-Mount (Shandon Inc.) containing DAPI or To-Pro3 (Molecular Probes) to stain the nuclei. For Myc and MBP immunostaining, the permeabilized cells were blocked with 10% calf serum in PBS for 30 minutes, incubated with 9E10 (1:1000) or the monoclonal anti-MBP antibody (1:2500, Sternberger LTD) in PBS containing 3% BSA for 1 hour, and then incubated with rhodamine- (1:200) or FITC-conjugated (1:400) goat anti-mouse secondary antibodies (Jackson Laboratories) for 20 minutes. The cells were visualized with a Leitz (Wetzlar, Germany) Aristoplan fluorescence microscope or by confocal microscopy using Zeiss LSM-510 system.

A DNA fragment encompassing nucleotides 35–190 of the 14 kDa MBP isoform was amplified by PCR and in situ hybridization was carried out as described (Lawrence and Singer, 1986). Digoxigenin-labeled RNA was detected with the anti-digoxigenin antibody conjugated to rhodamine in COS cells, whereas the fluorescent antibody enhancement set for DIG detection (Roche) was used on OL slides and visualized as describe above.

2.5 Results

2.5.1 Identification of a 110 Nucleotide QKI recognition element in the 3'-UTR of the MBP mRNAs.

The myelination defect observed in the qk^v mice prompted us to investigate whether the QKI RNA binding proteins bound the mRNAs of the major protein components of myelin. One such component is the myelin basic protein (MBP). Mice contain four alternatively spliced isoforms of MBP (14, 17, 18.5, and 21.5 kDa) that differ in their coding sequence, but not in the untranslated regions of their mRNAs (de Ferra et al., 1985). To investigate whether the QKI proteins bound the mRNAs encoding the MBPs, synthetic RNA transcripts corresponding to portions of the longest mouse MBP mRNA (21.5 kDa, Figure 3-1A) were ^{32}P-labeled and examined for their ability to associate with the QKI proteins. Transfected HeLa cells expressing myc-epitope-tagged QKI-7 were immunoprecipitated with control (IgG) or anti-myc antibodies followed by protein A-Sepharose beads. The control and myc-QKI-7 bound beads were incubated with equal quantities of ^{32}P-labeled MBP transcripts, washed, and the bound RNAs quantitated by scintillation counting. The 3'-UTR of the MBP mRNAs bound immunoprecipitated myc-QKI-7 approximately 30-fold over control immunoprecipitations (Figure 3-1A, compare bars representing IgG with myc for 626-2111). RNA transcripts corresponding to the 5'-UTR (1-49) and the coding region (1-625) of the 21.5 kDa MBP did not associate with QKI-7 (Figure 2-1A). Shorter RNA segments of the 3'-UTR of the MBPs were examined for QKI binding in order to map a short recognition element. The majority of QKI binding resided from nucleotides 626 to 885 in the 3'-UTR of MBP, and a minor binding site was also observed from nucleotides

1441 to 1770 (Figure 2-1A). QKI-5 and QKI-6 also bound the MBP 3'-UTR spanning nucleotides 626 to 790, demonstrating that MBP mRNA binding was not QKI isoform specific (Figure 2-1A). These findings suggest that the mRNAs encoding the MBPs contain one major *Q*KI *R*ecognition *E*lement (QRE) residing from nucleotides 626 to 790.

To confirm that the QKI-7 binding to the MBP mRNAs was a direct interaction, an ultraviolet light (UV)-crosslinking assay was performed. Myc-QKI-7 transfected or untransfected HeLa cells were lysed, and cell lysates were incubated with a ^{32}P-labeled synthetic RNA transcript encompassing nucleotides 626 to 885 and irradiated with UV light. The cell lysates were RNase treated to remove unprotected RNA, immunoprecipitated using control (IgG) or anti-myc antibodies, and the bound proteins separated on SDS 10%-polyacrylamide gels and visualized by autoradiography. A ^{32}P-labeled protein of ~45 kDa corresponding to the size of QKI-7 was present in Myc immunoprecipitates from cells transfected with QKI-7, but not untransfected cells (Figure 2-1B). This experiment provides strong evidence that the interaction between the QKI proteins and the MBP QRE is direct. To further confirm a direct interaction, electrophoretic mobility shift assays (EMSA) were performed using recombinant QKI proteins and MBP ^{32}P-labeled RNA transcripts. Recombinant glutathione-S-transferase (GST) QKI-7 fusion protein and the control GST fusion partner were incubated with a ^{32}P-labeled MBP synthetic RNA corresponding to nucleotides 626 to 885. The QKI-RNA complexes and unbound RNAs were separated on native 4% polyacrylamide gels and visualized by autoradiography. A slow migrating complex that corresponded to a QKI-RNA complex was observed with GST-QKI-7, but not with GST or phosphate-buffered

saline (PBS) alone (Figure 2-1C). QKI bound to a shorter RNA transcript encompassing nucleotides 626 to 790 but not nucleotides 626 to 710 (Figure 2-1C, lanes 6 and 9). Two 5'-deletions were performed and tested for QKI binding. The removal of nucleotides 626 to 679 did not affect QKI binding (lanes 10–15), but the deletion of nucleotides 626 to 719 abrogated QKI binding (lanes 16–21). Thus, the shortest region identified resides from nucleotides 680 to 790 in the mouse MBP 3'-UTR (Figure 2-1C, lanes 13–15) and this area was designated the minimal QRE. The interaction between endogenous QKI proteins and MBP mRNAs was verified in primary rat OLs. The OLs were lysed and immunoprecipitated with control normal rabbit serum (NRS) or anti-QKI antibodies, known to immunoprecipitate the QKI proteins (Chen and Richard, 1998). The presence of coimmunoprecipitating MBP mRNAs was verified by using reverse transcription (RT)-PCR analysis with MBP-specific primers. A DNA fragment of the correct size was observed in the anti-QKI antibody immunoprecipitates only in the presence of the reverse transcriptase, suggesting that the DNA fragment was amplified from mRNA and not genomic DNA (Figure 2-1D, lanes 1–5). To confirm that the DNA fragment was indeed from the MBP mRNAs, a southern blot was performed using an MBP-specific DNA ^{32}P-labeled probe. The DNA fragment amplified from the anti-QKI antibody immunoprecipitation hybridized with the MBP-specific probe (Figure 2-1D, lanes 6–10). These findings demonstrate that the QKI proteins associate with the MBP mRNAs in vivo.

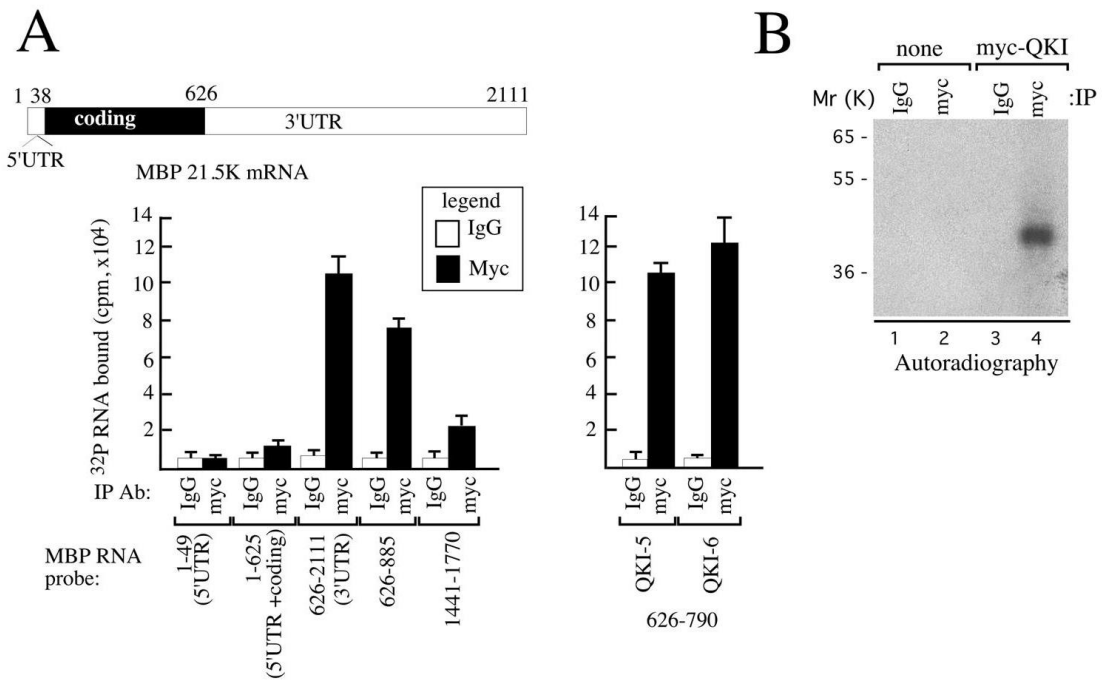

Figure 2-1. QKI proteins bind an RNA element in the MBP 3'-UTR. (A) A schematic diagram representing different regions of the 21.5 K MBP mRNA is shown. QKI-5, -6, or -7 were expressed in HeLa cells and immunoprecipitated with control or myc antibodies. The immunoprecipitates were incubated with ^{32}P-labeled RNA MBP fragments as indicated and quantitated. Each bar represents the mean ± standard deviation of data from n > 6. (B) QKI binds the 3'-UTR of MBP directly. HeLa (none) or QKI-7 transfected HeLa cells were lysed and incubated with a ^{32}P-labeled MBP RNA 626–885 and UV crosslinked. The unbound RNAs were digested and the QKI protein immunoprecipitated with control (IgG) or anti-myc antibodies. The bound proteins were separated by SDS-PAGE and visualized by autoradiography.

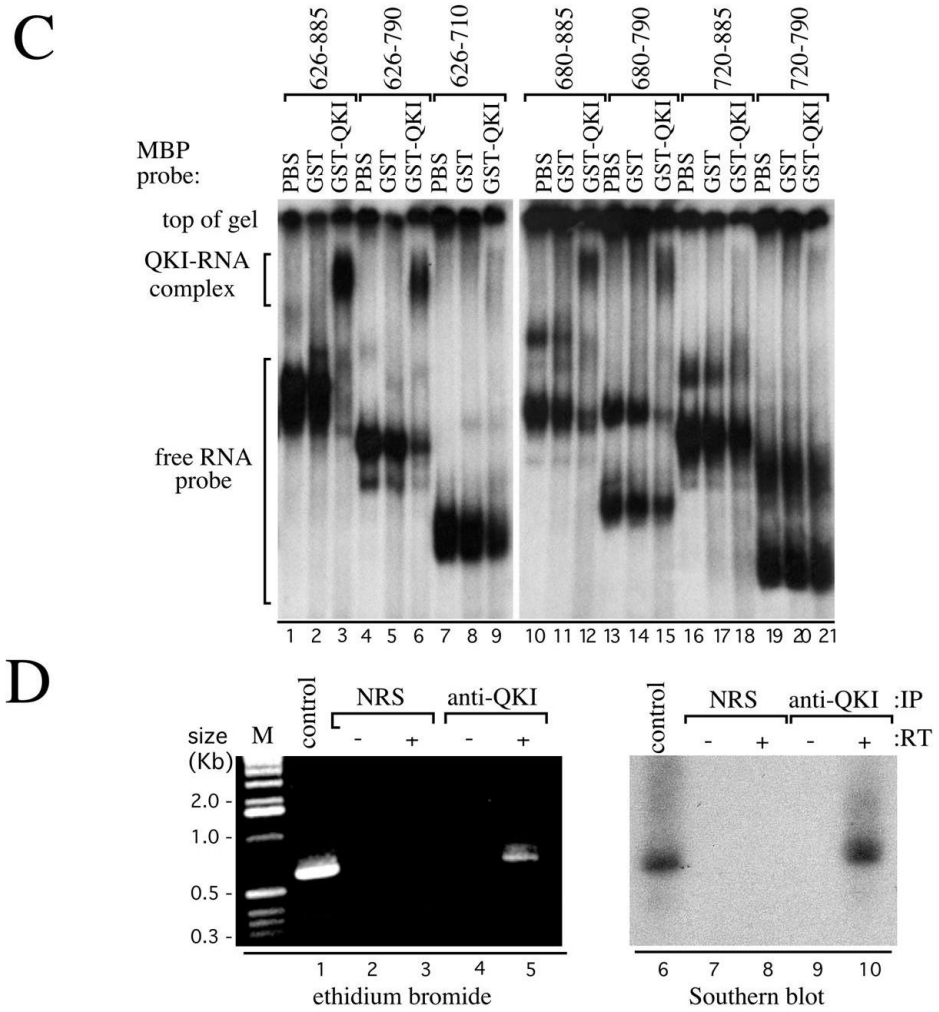

Figure 2-1 cont'd. QKI Proteins bind an RNA element in the MBP 3'-UTR. (C) The QKI recognition element (QRE) is located within nucleotides 680–790 of MBP mRNA. ^{32}P-labeled RNA probes corresponding to different portions of the MBP mRNA were incubated with PBS, GST (1.0 μg), or GST-QKI-7 (1.0 μg), and the reactions were analyzed with native acrylamide gel electrophoresis and autoradiography. The migration of QKI-RNA complexes and free RNA probes is indicated. (D) Mixed rat astrocytes and OLs were homogenized in lysis buffer and immunoprecipitated with normal rabbit serum (NRS) or anti-QKI antibodies. RNAs were extracted from the immunoprecipitates and the presence of MBP mRNA was detected by RT-PCR. For positive control, MBP cDNA was used as a template (control) and for negative control, reverse transcriptase (RT) was omitted. The PCR products were first visualized by staining and verified by Southern blot analysis with an MBP-specific probe.

2.5.2 QKI embryonic lethal point mutations disrupt RNA binding activity.

The identification of a physiological RNA target (MBP) for QKI permitted us to examine whether the two embryonic lethal point mutations identified in the QKI GSG domain altered specific RNA binding. Recombinant GST-QKI-7 bound the MBP QRE in a dose- dependent manner, as visualized by electrophoretic mobility shift assays (Figure 2-2A, lanes 5–7). The substitution of valine 157 to glutamic acid (V157E) or the substitution ofglutamic acid 48 to glycine (E48G) in QKI abolished the ability of the recombinant proteins to bind the QRE (Figure 2-2A, lanes 8–13). We have shown in earlier studies that the substitution of QKI E48G prevents homodimerization by disrupting predicted coiled-coil interactions (Chen and Richard, 1998). Therefore, we wanted to examine whether the inability of the V157E amino acid substitution was a failure to dimerize. HeLa cells were transfected with myc- and HA-epitope-tagged QKI proteins containing the amino acid substitutions. The cells were lysed and immunoprecipitated with control (IgG) or anti-HA antibodies, the bound proteins were separated by SDS-PAGE and immunoblotted with anti-myc antibodies. HA immunoprecipitates of wild-type QKI coimmunoprecipitated myc-epitope-tagged QKI and QKI:V157E, but not QKI:E48G (Figure 2-2B, lanes 1–9). These findings suggest that the V157E mutation prevents RNA binding and that the E48G substitution prevents dimerization. Since the substitution of E48G is unable to bind RNA, this suggests that dimer formation may be essential for MBP RNA binding.

2.5.3 Nuclear export of the MBP mRNAs is controlled by the balance of the QKI isoforms.

The relationship between the localization of the MBP mRNAs and the QKI proteins was examined in COS cells. Since COS cells do not endogenously express the QKI isoforms (Chen and Richard, 1998), the contribution of the different QKI isoforms in MBP mRNA localization can be assessed separately or collectively using transfected genes. COS cells were transfected with an expression vector containing a full-length 14 kDa mouse MBP cDNA. The cells were fixed and the MBP mRNA was detected by using in situ hybridization. The MBP mRNA was localized predominantly in the cytoplasm of COS cells, as expected for mRNA (Figure 2-3A, panel A).

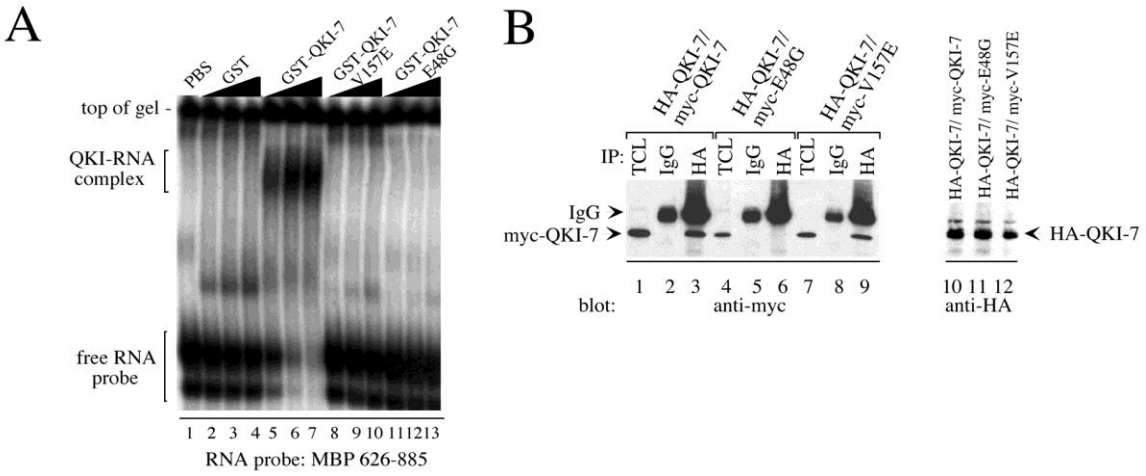

Figure 2-2. The *qk* lethal point mutations abrogate RNA binding. (A) V157E or E48G mutations abolish MBP binding. PBS or an increasing amount (0.5, 1.0, and 2.0 μg) of GST, GST-QKI-7, GST-QKI-7:V157E, and GST-QKI-7:E48G were incubated with a ^{32}P-labeled MBP RNA fragment containing the QRE. The reactions were analyzed with native PAGE and autoradiography. (B) V157E substitution has no effect on QKI dimerization. HA-tagged QKI-7 was cotransfected in HeLa cells with myc-tagged QKI-7, QKI-7:E48G, or QKI-7:V157E as indicated. Total cell lysates (TCL), IgG, and anti-HA immunoprecipitates were analyzed by immunoblotting with anti-myc antibodies (lanes 1–9). TCL were also immunoblotted with anti-HA antibodies to verify the expression (lanes 10–12).

The co-transfection of myc-epitope-tagged QKI-5 with an MBP expression vector containing the untranslated regions caused the nuclear retention of the MBP mRNA (Figure 2-3A, panelD). MBP mRNA without the 3'-UTR (MBP3'UTR) exited the nucleus and failed to be retained by QKI-5 (Figure 2-3A, panel G). Similar results were observed with the MBP 21 kDa isoform (data not shown). These findings suggest that the 3'-UTR of the MBP mRNA is required for QKI-5-mediated nuclear retention.

The heterodimerization of the QKI isoforms (Chen and Richard, 1998) and their nucleocytoplasmic shuttling ability (Wu et al., 1999) suggest that QKI dimers may function in the nuclear export of the MBP mRNAs. If this is indeed the case, the presence of the cytoplasmic QKI-6 and QKI-7 isoforms might relieve the QKI-5-mediated MBP mRNA nuclear retention. COS cells were co-transfected with expression vectors encoding the 14 kDa MBP and various combinations of QKI isoforms. The presence of QKI-6 or QKI-7 relieved part of the QKI-5-mediated nuclear retention of the MBP mRNA. However, a significant portion of MBP mRNA was unable to exit the nucleus (Figure 2-3A, panels J and M). The presence of all three QKI isoforms greatly restored the ability of the MBP mRNA to fully exit the nucleus (Figure 2-3A, panel P). Reverse transcription (RT)-PCR was performed on RNA isolated from cells transfected with or without QKI-5. The presence of QKI-5 did not affect the overall levels of MBP mRNA (Figure 2-3B, compare lanes 3–5 to 7–9 and lanes 11–13 to 15–17). Immunoblotting confirmed that the expression of QKI-5 remained equivalent with the expression of QKI-6 and/or QKI-7 isoforms (Figure 2-3C). These findings demonstrate that the balance of the QKI isoforms regulates the retention and release of the MBP mRNAs from the nucleus.

2.5.4 QKI-5 retains MBP mRNAs in the nucleus of OLs.

The ability of QKI proteins to control the nuclear export of MBP mRNAs was examined in OLs, a cell type where QKI has a physiological role. OLs of qk^v mice only express the QKI-5 isoform (Hardy et al., 1996b), implying that the dysmyelination could be the result of mRNA nuclear export defects of key myelin components. To test this possibility and to mimic the situation in qk^v mice, QKI-5 was overexpressed in OL cultures. OLs were co-infected with adenoviruses expressing QKI-5 (AdTR5-QKI-5) and the tetracycline transactivator (tTA) at MOIs sufficient to transduce about 80% of the cells as shown by the expression of the green fluorescent protein (GFP) which is constitutively coexpressed from the QKI-5 virus. The presence of the tTA is necessary to drive the expression of the inducible QKI-5. The distribution of the QKI-5 protein was predominantly nuclear as viewed by indirect immunofluorescence with an anti-myc epitope antibody 3 days post-infection (Pilotte et al., 2001). OLs infected with AdTR5-QKI-5 were fixed, permeabilized, and MBP mRNAs detected by using in situ hybridization. The "green" QKI-5 overexpressing OLs displayed mainly a nuclear accumulation of MBP mRNAs with only some staining in the perikaryon, as visualized by confocal microscopy (Figure 2-4, panel E). Longer exposure of the MBP in situ hybridization demonstrates that little to no MBP mRNAs are in the OL processes and the periphery (Figure 2-4, panel F). In contrast, OLs infected with a control adenovirus (AdTR5) expressing only GFP localized the MBP mRNAs in the perikaryon and processes with little to no staining in the nucleus (Figure 2-4, panel B). Longer exposure clearly demonstrates that the MBP mRNAs extend into the processes and reach the periphery (Figure 2-4, panel C). Our findings

suggest that a balance toward elevated QKI-5 causes nuclear export defects of MBP mRNAs in OLs.

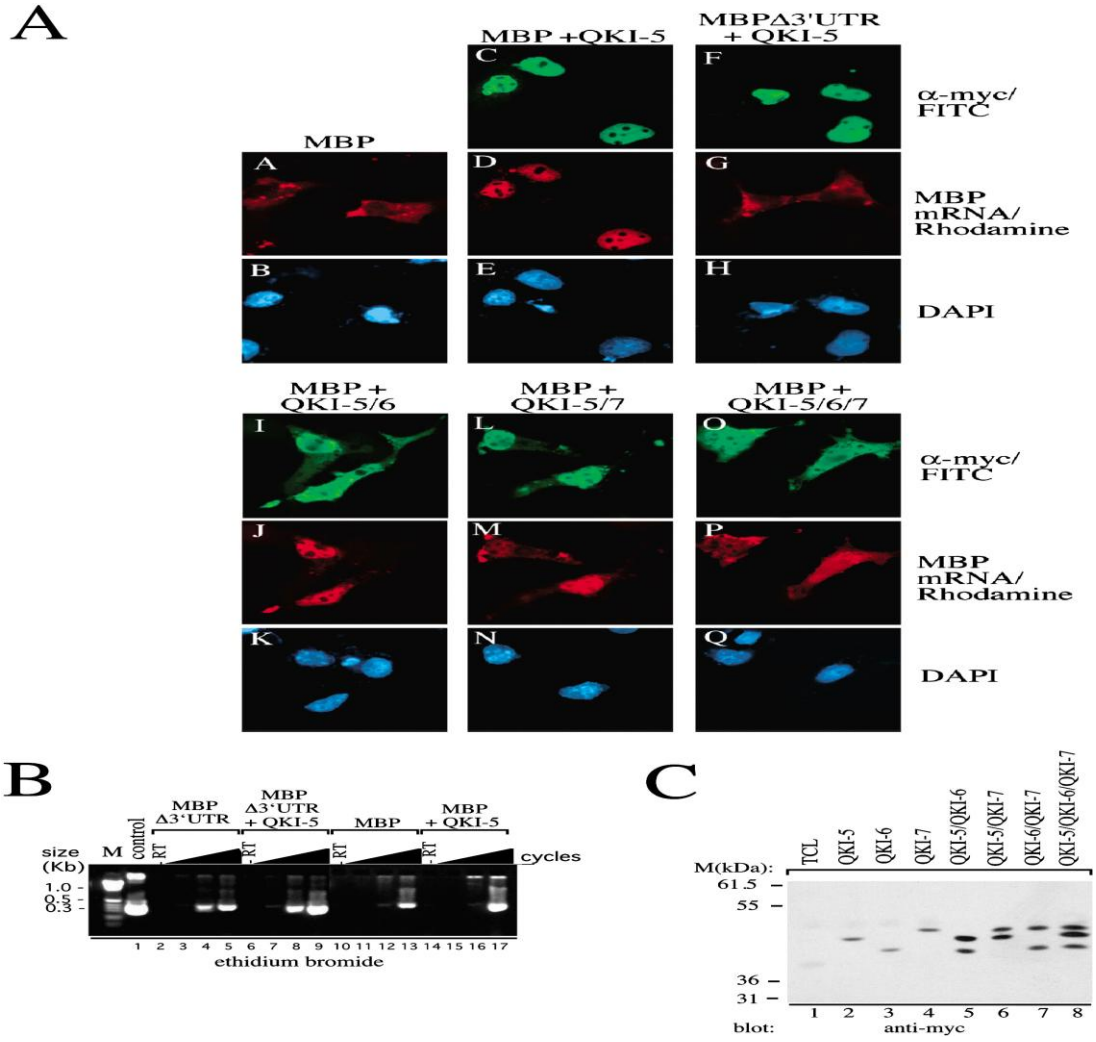

Figure 2-3. The QKI isoforms control the nuclear export of the MBP mRNAs. (A) COS cells were transfected with the plasmids encoding the 14 K MBP or MBP 3'-UTR in combination with the myc-QKI isoforms. After 12 hour, the cells were fixed, permeabilized, and in situ hybridization and immunofluorescence were performed. A digoxigenin-labeled RNA probe was used followed by an anti-digoxigenin rhodamine-conjugated antibody, whereas an anti-myc antibody followed by a FITC-conjugated secondary antibody was used to detect the myc-QKI. The cells were mounted onto a glass slide in the presence of the nuclear stain DAPI and visualized by fluorescence microscopy. (B) The stability of MBP mRNA is unaffected by QKI-5 expression. The 14 K MBP or MBP 3'-UTR were transfected alone or in combination with QKI-5 in COS cells. The RNA was isolated with Trizol, treated with DNase, and subjected to RT-PCR

with MBP-specific primers. DNA fragments were separated on agarose gels and visualized by staining. The MBP cDNA was used as a positive control, whereas RT was omitted (-) during reverse transcription as a negative control. (C) QKI-5 expression is unaltered by the presence of the other QKI isoforms. COS cells were transfected with either myc-tagged QKI-5, -6, or -7 alone and in different combinations. The cell lysates were analyzed by immunoblotting with anti-myc antibodies.

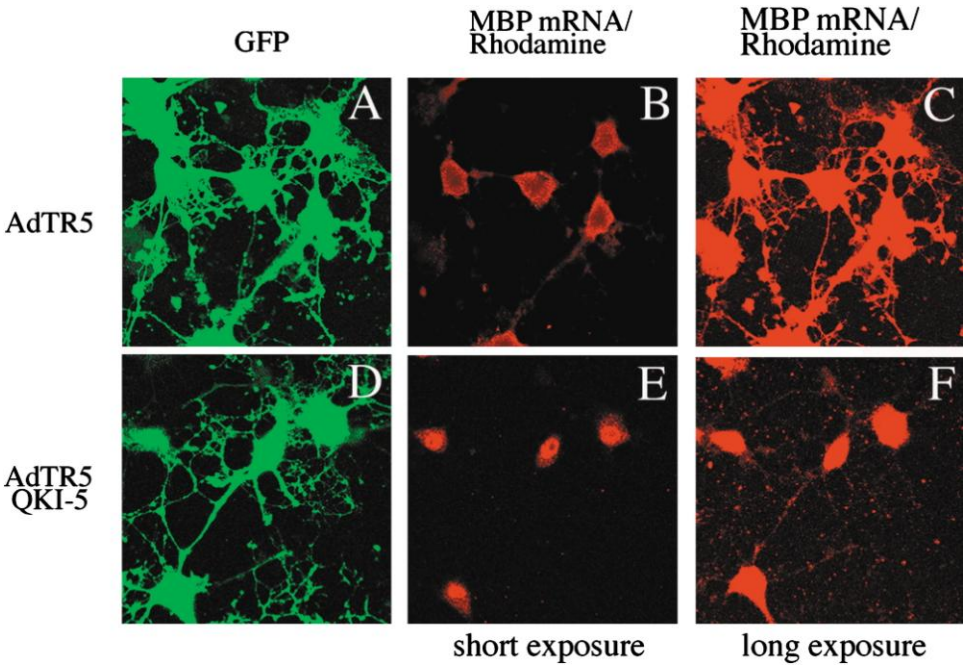

Figure 2-4. Nuclear retention of MBP mRNAs in QKI-5 overexpressing OLs. Primary rat OLs were co-infected with adenoviruses that constitutively expresses the tTA and a tetracycline-inducible adenovirus expressing GFP alone (A, B, and C) or GFP with myc-QKI-5 (D, E, and F). After 3 days, the cells were fixed, and permeabilized to perform in situ hybridization with a digoxigenin-labeled RNA probe. The cells were visualized by confocal microscopy.

The interaction of QKI with the 3'-UTR of the MBPs could have several functional consequences ultimately affecting the protein levels of the MBPs. The effects of retaining the MBP mRNAs in the nucleus and the perikaryon of OLs was verified at the MBP protein level. QKI-5-infected OLs were immunolabeled with a monoclonal anti-MBP antibody and visualized by immunofluorescence. QKI-5-infected OLs had a visibly weaker staining for the MBPs (Figure 2-5A, compare panels b and d) and this was confirmed by Western blot analysis. Thus the five MBP isoforms normally expressed in rat OLs were proportionally reduced (Figure 2-5B). Another observation was that in theQKI-5-infected OLs, MBPs were mostly localized in the perikarya. The failure of MBPs to migrate to the distal branching sites was visualized by immunofluorescence using a conventional (Figure 2-5A, panel d) or a confocal microscope (Figure 2-5C, compare panels b and d). In control cultures infected with the GFP construct alone, the MBPs were localized at the periphery (Figures 5-5A and 5-5C, panels b), demonstrating that adenovirus infection per se did not alter the normal localization of the MBPs in OLs. These data suggest that the nuclear export defect induced by the overexpression of QKI-5 ultimately translates into lower levels of expression of the myelin basic proteins as well as their mislocalization.

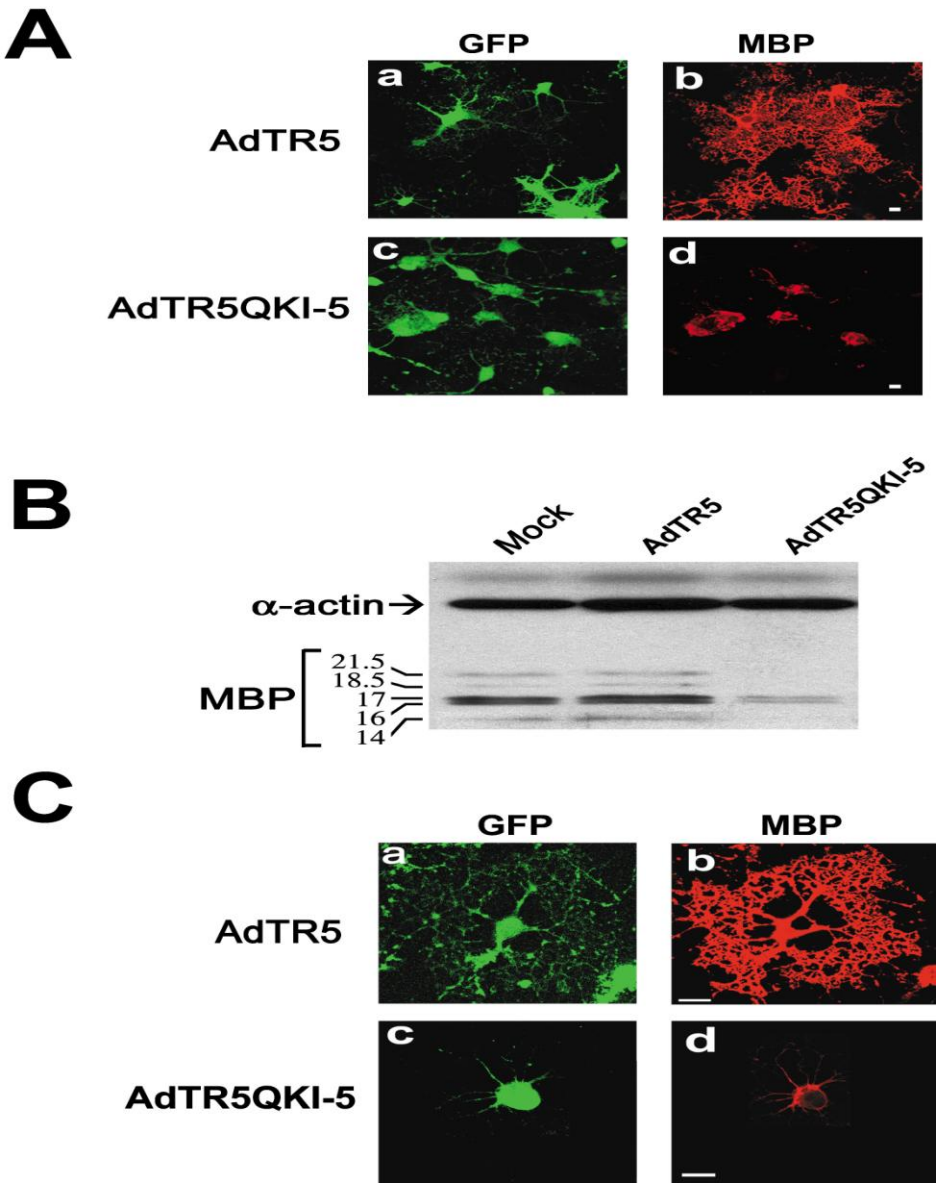

Figure 2-5. The overexpression of QKI-5 suppresses the expression of MBPs and alters their proper localization in OLs. (A) QKI-5 protein expression causes retention of the MBPs in the perikarya of OLs. OLs progenitors were infected with adenoviruses coding for QKI-5 (panels c and d) and GFP only expressing control AdTR5 (panels a and b) and allowed to differentiate. On day 3 after QKI-5 expression, the MBPs were immunostained with anti-MBP antibodies (red) and visualized by fluorescence microscopy (panels b and d). GFP (green) positive cells denote the infected cells in this experiment. Bar scale represents 10 μm. (B) Expression of the MBPs decreases in the presence of QKI-5. OL progenitors infected as in (A) were lysed, separated by SDS-PAGE, and immunoblotted with anti-MBP and -actin antibodies, n = 3. (C) The localization of MBPs was examined by confocal microscopy. Cells infected as in (A) were immunostained with anti-MBP antibodies (red, panels b and d). The GFP (green) is a marker of infection. Bar scale represents 10 μm.

2.5.5 Nuclear retention of MBP mRNAs in *qk*^{v} mice.

MBP mRNAs are concentrated in the cell body of *qk*^{v} OLs, as assessed in 1991 by in situ hybridization using a ^{35}S-labeled MBP probe (Barbarese, 1991). The resolution of this assay did not permit distinction between the cell body, the perinuclear compartments, or the nucleus of the OLs. To better define the compartment in which MBP mRNAs are located in *qk*^{v} mice, we performed in situ hybridization using a digoxigenin-labeled probe and confocal microscopy. Brain slices from the cortex of normal mice showed that MBP mRNAs were located along axons and this was not observed in *qk*^{v} mice (Figure 2-6A). Primary OL cultures from postnatal day 11 wild-type and *qk*^{v} mice were generated and the localization of the MBP mRNAs was visualized. The mRNAs for the MBPs were observed to be located at the periphery of OLs from wild-type mice (Figure 2-6B, panel B). The mRNAs for the MBPs were localized in the nuclei and the perinuclear regions of OLs from *qk*^{v} mice, as visualized by confocal microscopy (Figure 2-6B, panel F). The OLs from *qk*^{v} mice appeared immature and bipolar compared to the OLs from wild-type mice (Figure 2-6B, panels C and G). The bright foci observed with the Hoechst dye do not represent cell death and are characteristic of mice cells (Figure 2-6B, panels A and E). These findings suggest that OLs from *qk*^{v} mice are defective in the nuclear export of the MBP mRNAs.

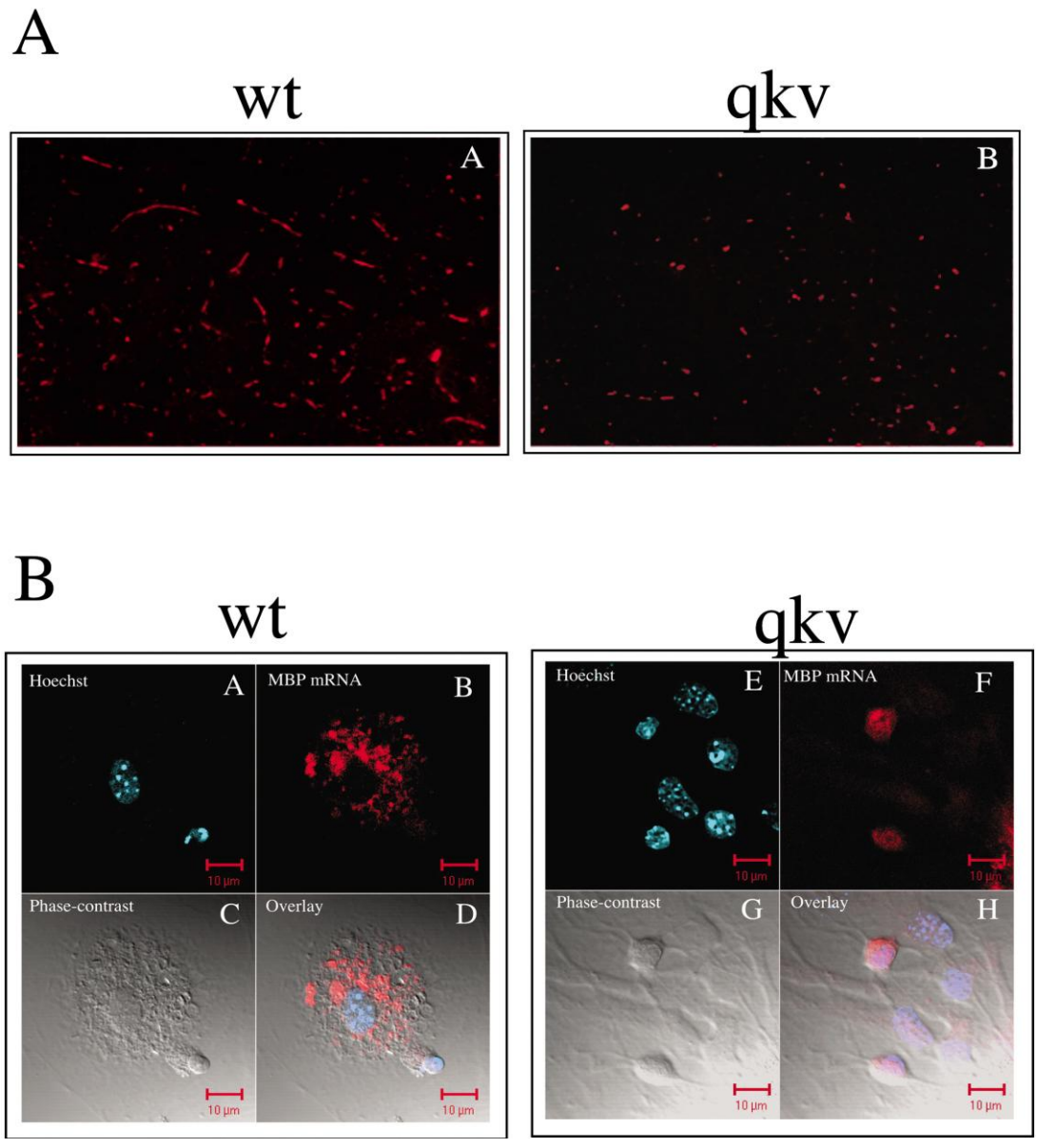

Figure 2-6. mRNAs encoding the MBPs are nuclear in OLs from *qk*^{v} mice. (A) Fifteen micrometer slices from 30-day-old C57BL/6 or *qk*^{v} mouse brains were fixed, permeabilized, and hybridized with a DIG-labeled MBP RNA probe. The RNA was detected with an anti-DIG antibody conjugated to rhodamine, and the brain slices were visualized by fluorescence microscopy. The panels shown represent the cortex region of the mouse brain. (B) MBP mRNAs are retained in the nuclei of OLs in *qk*^{v} mice. Primary mouse OLs were isolated from postnatal day 11 wild-type (wt) or *qk*^{v} mice. The cells were fixed, permeabilized, and subjected to in situ hybridization with a DIG-labeled MBP RNA probe. The cells were visualized by using a Zeiss confocal microscope. Each panel represents the same field of cells as visualized under blue (Hoechst) and red (rhodamine) and phase contrast filters. The overlay represents the merge of the three fields.

2.5.6 The localization of MBPexII isoforms in *quaking* viable mice.

The exon II containing MBP isoforms 17 and 21.5 are nuclear in young OLs, and their presence is thought to regulate mRNA movement to the periphery during myelination (Allinquant et al., 1991; Pedraza et al., 1997). The defect in MBP mRNA export might be explained by the abundance of MBPexII in the OL nuclei in qk^v mice. Although we did not see nuclear retention of MBP in OL culture with QKI-5 (Figure 2-5C, panel d), we wanted to confirm if MBPexII localization was affected in qk^v mutants. Brain slices containing the corpus callosum, a region rich in myelinating OLs, from qk^v mutant and wild-type mice were examined for the MBPexII distribution by using an anti-MBPexII-specific antibody (Pedraza et al., 1997). A cell body staining was observed from the corpus callosum in the wild-type brain sections (Figure 2-7A). In qk^v mice, MBPexII isoforms were clearly localized in the cytoplasm and primary branches of OLs, consistent with the presence of young OLs (Figure 2-7A, panels b and d). The presence of bipolar cells suggests that a maturation defect or delay is observed in qk^v OLs. OL cultures from wild-type and qk^v mice that were matured for 10 days in vitro localized MBPexII proteins in the perinuclear and cytoplasmic regions (Figure 2-7A, panels e and f). Moreover, the ratio of MBP isoforms 17 and 21.5 was not altered between wild-type and qk^v mice, suggesting that the QKI proteins may not regulate inclusion/exclusion of MBP exon II (Figure 2-7B), as recently suggested (Wu et al., 2002). These observations suggest that the MBP mRNA nuclear retention in qk^v mice is not caused by an excess of MBPexII isoforms in the nucleus, but that qk^v OLs clearly have maturation defects.

To examine the role of the QKI proteins on the cellular localization of exon II containing MBP isoforms, primary rat OLs were infected with QKI-5, -6, and –7.

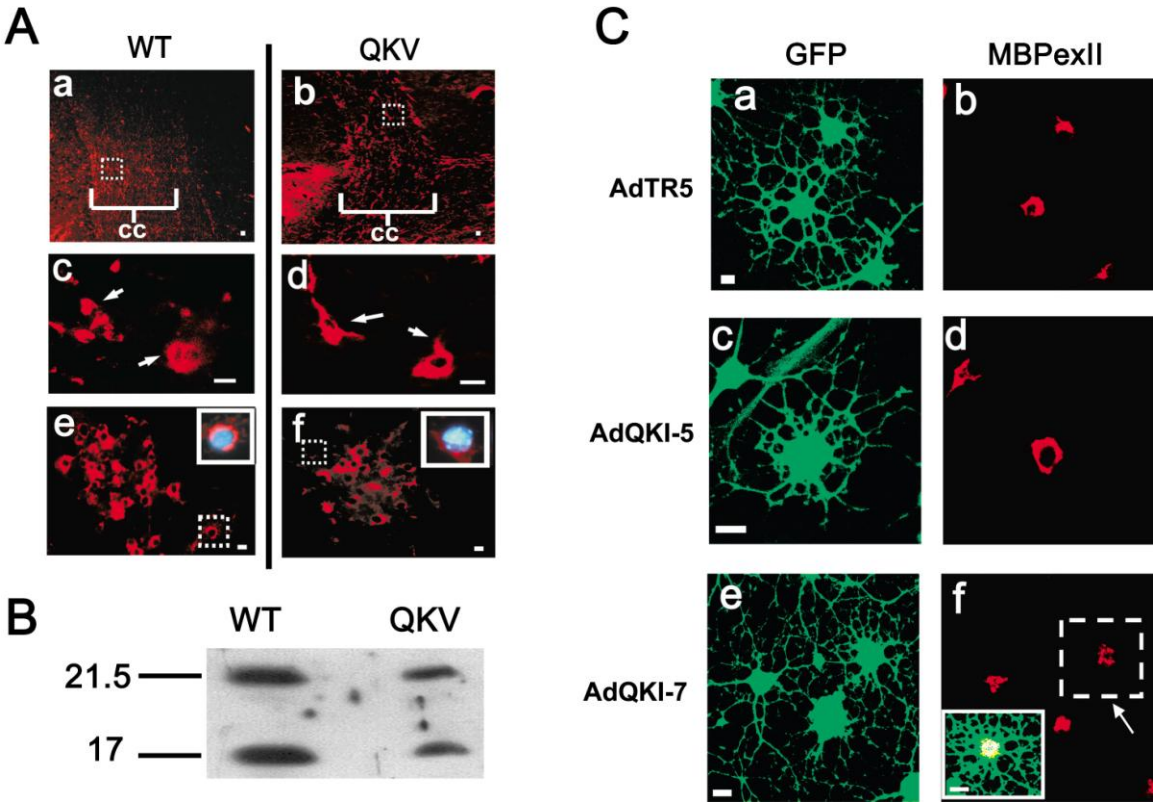

Figure 2-7. Abnormal distribution of MBP exon II isoforms in OLs from qk^v. (A) Coronal sections through the corpus callosum of postnatal day 30 wild-type (wt) and qk^v mice were immunostained with anti-MBPexII antibodies. A secondary antibody conjugated to rhodamine was used and the staining was visualized by confocal microscopy. Panels c and d represent amplifications of the regions that are boxed in panels a and b. The bar scale represents 10 μm. Panels e and f, OL culture from wild-type and qk^v mice, respectively. The inset is an image from the merge of MBPexII and DAPI staining. (B) Protein extracts from 30-day-old wild-type and qk^v mice were separated by SDS-PAGE and immunoblotted with the anti-MBPexII antibody. The positions of mouse MBP isoforms 17 and 21.5 are indicated. (C) The overexpression of QKI-7 causes abnormal distribution of MBP exon II isoforms in OLs. Rat OL progenitors were infected with adenoviruses coding for QKI-5 (panels c and d), QKI-7 (panels e and f), and control AdTR5 (panels a and b) and allowed to differentiate. On day 3, after infection, the cells were immunostained with anti-MBP exon II antibodies (red) and visualized by fluorescence microscopy. The green cells denote the infected cells. An example of nuclear staining is shown in the boxed region of panel f. The inset is an image from a merge of To-Pro3+GFP+MBPexII staining. The white staining denotes the nucleus. The bar scale represents 10 μm.

adenoviruses. Control infected OLs localized MBPexII to the cytoplasm and the perinuclear compartment as detected by immunofluorescence (Figure 2-7C, panels a and b). The presence of QKI-5 (panels c and d), or QKI-6 (data not shown) did not affect MBPexII localization. The overexpression of QKI-7 exclusively localized MBPexII isoforms to the nuclei of OLs (Figure 2-7C, panels e and f). Although we have shown that QKI-7 overexpression causes cell death in OLs (Pilotte et al., 2001), MBPexII localization was assessed at a pre-apoptotic stage. Although wild-type OLs (Figure 2-7C, panel b) contain QKI-7, it is the imbalance in the QKI isoforms that causes the MBPexII retention (Figure 2-7C, panel f). These findings suggest that the absence of QKI-7 in qk^{v} mice prevents the nuclear localization of MBP exon II isoforms.

2.5.7 QKI-5 downregulates the expression of the MBPs in vivo.

To confirm that QKI-5 downregulates the expression of the MBPs in vivo, the corpus callosum of postnatal day 1 mice was injected with a control or QKI-5-expressing adenovirus. The corpus callosum was chosen as the site of injection because it is myelin rich and adenovirus injections are known to spread along the white matter tracts (Kuo et al., 1995). The body weight of QKI-5-injected mice was consistently less than those of control-injected mice at postnatal day 10. The QKI-5 adenovirus-injected mice displayed a reduced weight (3.88 g ± 0.12, n = 6) compared to the control-injected adenovirus (4.30 g ± 0.14, n = 6), and the reason for this is unknown. Brain sections were prepared and stained with anti-MBP antibodies (red, Figures 2-8C to 2-8F) and visualized by confocal microscopy. A single injection was sufficient to spread the GFP-positive adenovirus throughout the corpus callosum, as well as the lateral ventricle (Figure 2-8B). Sections

from two independent injections with the control adenovirus AdTR5 demonstrated that the tracts in the corpus callosum stained positive for both GFP and MBP (Figures 2-8C and 2-8E). In panel C, a single OL can be observed by the arrow where its cell body is green (GFP) and its primary processes are red (MBP), indicating that adenovirus-infected cells are able to produce the MBPs. In contrast, brain sections from two separate mice injected with QKI-5 adenoviruses demonstrated that the tracts in the corpus callosum that were GFP positive (i.e., expressing QKI-5) were MBP negative (Figures 2-8D and 2-8F). The brain section in panel F is quite striking and in addition depicts an area of degeneration. These results provide strong evidence that disturbing the balance of the QKI equilibrium toward nuclear QKI isoform suppresses the expression of the myelin basic proteins.

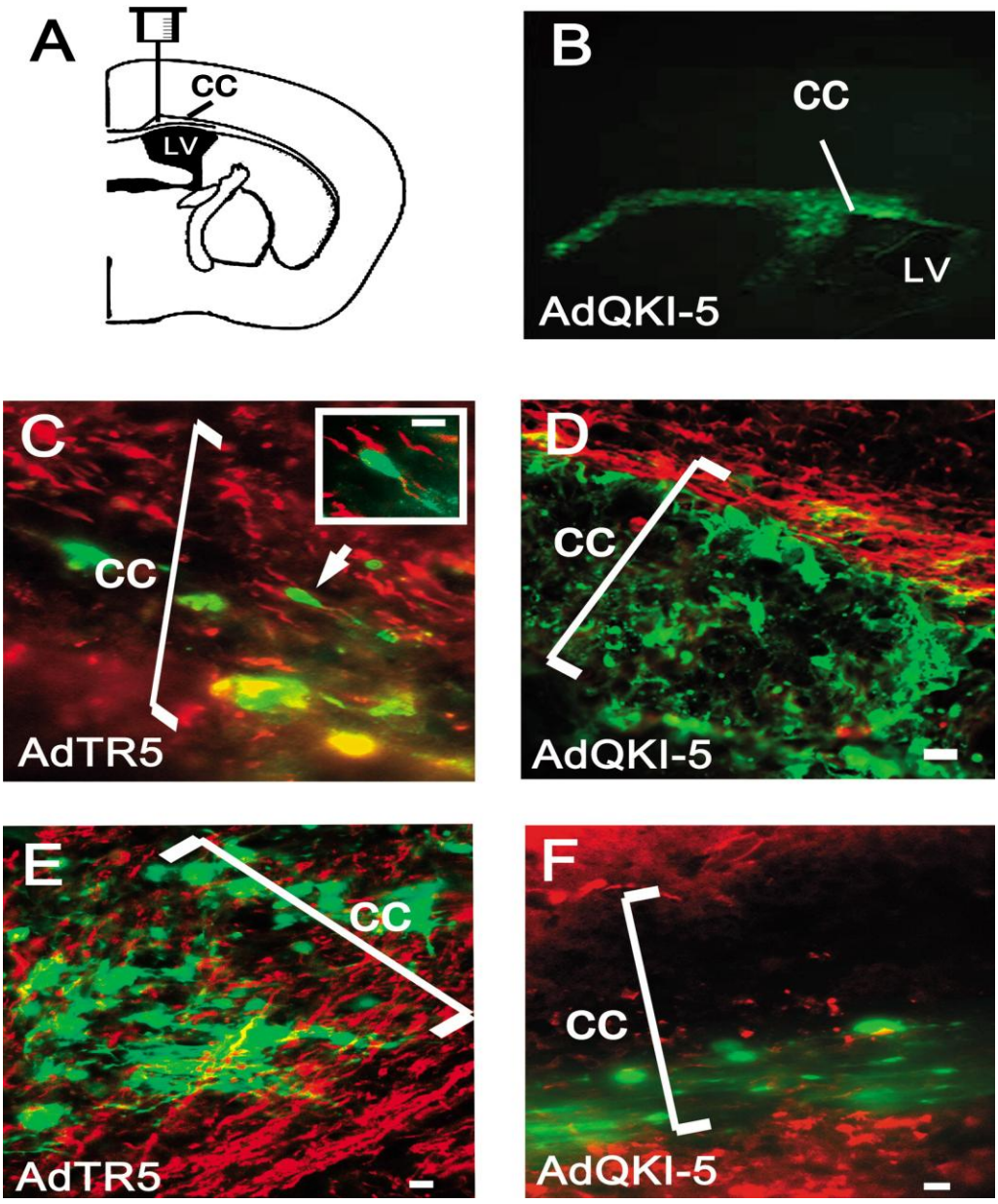

Figure 2-8. QKI-5 causes loss of MBP staining in vivo in the corpus callosum. (A) A schematic diagram of the site of injection is shown. (B) A coronal section of a QKI-5-injected mouse brain. The GFP was visualized by confocal microscopy. The corpus callosum of new born mice were injected with control AdTR5 adenovirus (C and E) or AdTR5 expressing QKI-5 (D and F). Ten days later, the mice were sacrificed and brain sections were stained with anti-MBP antibodies. The visualization of GFP (green) and MBP (red) or both (yellow) was observed by confocal microscopy. The corpus callosum (cc) is denoted by the white brackets. LV denotes the lateral ventricle. The bar scale represents 10 μm. The arrow in (C) points to an infected MBP-positive OL that has been enlarged in the inset.

2.6 Discussion

In this study, we show that MBP mRNA is a target of the QKI isoforms, specifically through a recognition element of ~100 nucleotides situated at position 680–790 of the mouse 3'-untranslated region. We have named this region the QKI recognition element or QRE. The QKI proteins bind the QRE as dimers since an amino acid substitution that prevents dimerization abrogates the ability to bind the QRE. The overexpression of QKI-5 in OLs disrupts the QKI nucleocytoplasmic equilibrium and causes the retention of the MBP mRNAs in the nucleus and perikaryon. The nuclear accumulation of MBP mRNA was further confirmed in OLs of qk^{v} mice. The injection of QKI-5-expressing adenovirus in new born C57BL/6 mice caused a reduction in MBP staining. Our data implicate the QKI RNA binding proteins as regulators of nuclear export of the MBP mRNAs in OLs and provide evidence that the QKI proteins are implicated in myelination.

QKI has been shown to associate with the *tra2* and *gli* element (TGE) bound by another STAR protein *C. elegans* GLD-1 (Jones and Schedl, 1995; Jan et al., 1999; Saccomanno et al., 1999). Although QKI associates with the TGE, the physiological relevance of this interaction is unknown. Schedl and coworkers identified several mRNA targets for GLD-1 and were unable to identify a consensus RNA binding site (Lee and Schedl, 2001). Inspection of the QRE and computer searches have also failed to identify similar sequences in other mRNAs. QKI and GLD-1 may bind a short sequence within a given secondary structure making computer searches erroneous. The QRE, however, is predicted to form several short stem-loop structures (data not shown). During the course of our studies, a QKI RNA binding site was identified in the 3'-UTR of MBP by Feng

and coworkers (Zhang and Feng, 2001). The identification of the nucleotides and RNA secondary structures bound by QKI will require further mapping with RNA footprinting techniques as recently performed for the product of the fragile X syndrome protein (Darnell et al., 2001). The major domain required to bind RNA is the QKI GSG domain (Chen and Richard, 1998), and this is indeed supported by the fact that the V157E amino acid substitution in the KH domain abrogates RNA binding.

The identification of genetic point mutations in the *qk* gene has helped understand the properties of the QKI proteins. We showed previously that the qk^{kt4} allele altering QKI glutamic acid 48 to glycine prevents dimerization by disrupting a predicted coiled-coil (Chen and Richard, 1998). However, we were unable to see a reduction in binding to total cellular mRNAs (Chen and Richard, 1998). Here we extend our studies to show that QKI E48G amino acid substitution cannot bind the QRE in the MBP mRNA. These findings suggest that the QKI proteins bind as dimers to specific mRNA targets. The other amino acid substitution known to cause embryonic lethality in mice is QKI valine 157 to glutamic acid (Cox et al., 1999). This point mutation localizes in the KH domain that resides ~100 amino acids C-terminal to the coiled-coil region and is not predicted to influence dimerization. We observed that QKI V157E abolishes RNA binding, but not dimerization. Thus the molecular defect of the qk^{k2} allele may be the inability of QKI-5 to retain certain mRNAs during development.

QKI-5 is the only isoform expressed during mouse embryogenesis (Ebersole et al., 1996). Expression of QKI-6 and QKI-7 peaks at postnatal day 16 coinciding with the onset of myelination (Ebersole et al., 1996). The mechanism that triggers the expression of QKI-6 and QKI-7 is unknown. Our data suggest that the expression of QKI-6 and

QKI-7 is essential for the "release" or the derepression of the MBP mRNAs imposed by the nuclear QKI-5. The fact that the QKI proteins dimerize (Chen and Richard, 1998) and shuttle between the nucleus and the cytoplasm (Wu et al., 1999) suggest that they function as heterodimers to export the MBP mRNAs. QKI proteins may be directly involved in nuclear export mechanics or may be required to "coat" the MBP mRNAs into mature messenger ribonucleoprotein particles (mRNPs) to be recognized by the export machinery. QKI-5 has been shown to shuttle in fibroblasts, as assessed by heterokaryon assays (Wu et al., 1999). We confirmed that QKI-5 indeed has the ability to shuttle but to a lesser extent than hnRNPK, a well-known shuttling protein (Michael et al., 1997; data not shown). This leads to the question of how an RNA binding protein with shuttling abilities can retain mRNAs in the nucleus. Our data clearly demonstrate that MBP mRNAs are retained in nuclei of OLs and COS cells by QKI-5 and that only in the presence of QKI-6 and QKI-7 are they released in the cytoplasm. This leads us to propose that MBP mRNAs may prevent QKI-5 shuttling, or alternatively the model we favor, that QKI-6 and QKI-7 are involved in the release of mRNAs from QKI-5 by competition for the QRE.

The presence of mRNAs encoding the MBPs in myelin (Colman et al., 1982) and of soluble polyribosomes in OLs (Verity and Campagnoni, 1988) led to the proposal that MBP mRNAs are transported along processes to the periphery, where they are locally translated and incorporated into myelin. The proteins and RNA elements responsible for this journey are beginning to be identified (Kiebler and DesGroseillers, 2000). Microinjection of labeled MBP mRNAs in OLs and the formation of mRNP granules that are transported down the processes has led to the identification of the RNA trafficking

signal (RTS) (Ainger et al., 1993, 1997) . Other RTS found in mRNAs transported along processes include those for myelin OL basic protein (MOBP), -CAMKII, tau, and amyloid precursor protein (APP) (Barbarese et al., 2000; Carson et al., 2001). The RTS is localized from nucleotides 794 to 814 of rat MBP mRNAs or nucleotides 999 to 1019 of mouse MBP (Ainger et al., 1997). Thus the RTS is localized ~200 nucleotides downstream of the QRE in the MBP mRNA. The RTS is sufficient to permit transport of the MBP mRNAs to the processes and is recognized by hnRNP A2 (Ainger et al., 1997; Hoek et al., 1998). Since hnRNP A2 is predominantly nuclear, it is thought that hnRNP A2 binds the RTS of the MBP mRNAs and permits their nuclear export and is implicated in the mRNA transport along the cytoskeletal network in the OL processes (Carson et al., 2001). Our data demonstrate that the QKI proteins are necessary to allow the MBP mRNAs out of the nucleus. Since the QKI and hnRNP A2 bind different elements, they may colocalize in mRNPs and function cooperatively in the nuclear export of the MBP mRNAs. The QRE is also distinct from other RNA elements in the untranslated region of MBP including the Y element (Han et al., 1995; Wu and Hecht, 2000) and the RNA localization region (Ainger et al., 1997). The QKI-6 and QKI-7 isoforms have been shown to localize in the processes of OLs (Hardy et al., 1996b; Wu et al., 2001), but their function in these processes remains unknown. It is tempting to speculate that QKI-6 and QKI-7 may be involved in the translational suppression of the MBPs during the journey along the processes especially since the QKI proteins have been shown to functionally substitute for GLD-1, a translational suppressor (Saccomanno et al., 1999).

OLs from *qk*^{v} have been proposed to have maturation defects (Hogan and Greenfield, 1984). An elevated presence of bipolar OLs that stained with MBPexII

antibodies was observed in the corpus callosum of qk^v mice. Moreover, much fewer primary OLs were obtained from qk^v mice and the cells generally exhibited a bipolar morphology (see Figure 2-6B, panel G and Figure 2-7A, panel d). Thus, OLs of qk^v mice have a delay in OL maturation compared with wild-type OLs at the same developmental stage. It is currently unknown whether the absence of QKI-6 and QKI-7 is the cause or the effect of the maturation defects. The presence of the cytoplasmic QKI-7 permits cultured rat OLs to localize MBPexII within the nucleus. These studies suggest that cytoplasmic QKI isoforms may allow MBPexII nuclear import that will properly initiate the maturation process of the OLs. Thus the QKI isoforms regulate the early events of myelination. It is unknown whether QKI-7 is directly involved in the mechanism by which MBPexII are transported to the nucleus. For example, QKI-7 may remove an exon II NLS masking protein. Alternatively, QKI-7 may have a global effect on the cell that triggers it to myelinate. It is unclear whether this effect is separate from its ability to induce cell death. The brain injection of the QKI-5 isoform in new born inbred C57BL/6 mice resulted in disruption of the MBP-positive tracts in the corpus callosum. These experiments further demonstrate that the disrupted balance toward the nuclear isoform, QKI-5, is sufficient to induce MBP defects in vivo. Thus it may be possible to induce re-myelination by displacing the equilibrium toward the cytoplasmic QKI isoforms.

Drosophila tendon cell differentiation has been shown to involve the opposing activities of two QKI homologs, How(L) and How(S) (Baehrecke, 1997; Zaffran et al., 1997). These How isoforms have been shown to regulate the mRNA export and the degradation rate of the Stripe mRNA (Nabel-Rosen et al., 1999, 2002) . We have also shown that a similar balance between the mammalian QKI isoforms controls cell survival and cell

death (Pilotte et al., 2001). Although the mRNA retention of MBP by QKI-5 parallels the observations of Stripe and How(L) (Nabel-Rosen et al., 1999), QKI-5 is not involved in mRNA stability, unlike How(L) (Figure 2-3B and data not shown). It has been postulated that QKI-5 may have such activity toward the mRNA of Krox-20 (Nabel-Rosen et al., 2002), but QKI-5 was expressed in insect cells and it is unclear whether QKI-5 regulates protein translation, mRNA stability, or mRNA export in that system.

While this paper was under revision, QKI-5 was shown to regulate alternative splicing (Wu et al., 2002). This is most likely an embryonic function because the other QKI isoforms are absent during embryogenesis. Our working model is that during myelination, the elevated presence of QKI-6 and QKI-7 (Ebersole et al., 1996) most likely suppresses the nuclear function of QKI-5 (i.e., alternative splicing) and permits the participation of QKI-5 in the mRNA export with QKI-6 and QKI-7. Thus the presence of splicing defects in qk^v mice is most likely caused by the QKI-5 embryonic function that cannot be suppressed by QKI-6 and QKI-7 because of their absence in OLs from qk^v mice. It will be important to identify the developmental genes regulated by QKI-5 during embryogenesis and to investigate the alternative splicing function of QKI-5 in the presence of QKI-6 and QKI-7 isoforms.

In conclusion, we have shown that the MBP mRNAs are physiological targets of the QKI family of proteins and that the QKI proteins function in the regulation of mRNA export out of the nucleus. The elucidation of the role of the QKI proteins during myelination will only be achieved by studying the isoforms together in OLs. The present manuscript introduces the various QKI isoforms in OLs and recreates a molecular defect observed in qk^v mice. The qk^v phenotype is complicated because some areas of the brain

maintain QKI-5 expression and others are completely devoid of QKI proteins (Hardy et al., 1996a). Our studies show that QKI-5 overexpression, which is predicted to disrupt the nuclear to cytoplasmic ratio of QKI proteins (Pilotte et al., 2001), reproduces in OLs the nuclear retention of the MBP mRNAs. These findings imply that the absence of QKI-6 and QKI-7 in OLs of the *qk*^{v} mice is sufficient to cause myelination defects.

2.7 Ackownledgement

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Chapter 3

$p27^{Kip1}$ mRNA protection by QUAKING RNA binding proteins promote oligodendrocyte differentiation

By

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3.1 Preface

Interestingly, from the study in Chapter 2, we observed that the over-expression of QKI-6 and QKI-7 isoforms in rat oligodendrocytes were causing cell cycle arrest and a high network of cellular branching that was indicative of differentiating oligodendrocytes. After these observations, we decided to examine the molecular mechanism and the *in vivo* significance behind this glial cell differentiation change caused by QKI-6 and QKI-7 proteins. Chapter 3 will explain the change caused by QKI proteins in oligodendrocytes and will reveal new RNA targets for QKI proteins. This may clarify the posttranscriptional mechanisms used to regulate the differentiation of glial cell lineages.

3.2 Summary

The *quaking* locus expresses an RNA binding protein family, and the expression of several alternatively spliced isoforms coincides with the development of oligodendrocytes and the onset of myelination. *Quaking viable* mice harboring an autosomal recessive mutation in this locus, have uncompacted myelin in the central nervous system due to the inability of oligodendrocytes to properly mature. Herein we show that the expression of two QKI isoforms, absent in oligodendrocytes of *quaking viable* mice, induces cell cycle arrest of primary rat oligodendrocyte progenitor cells and differentiation into oligodendrocytes. Injection of QKI-expressing retroviruses in the telencephalon of mouse embryos induced differentiation and migration of multipotential neural progenitor cells into mature oligodendrocytes localized in the corpus callosum. The mRNA encoding the CDK-inhibitor $p27^{Kip1}$ was bound and stabilized by the QKI

leading to an increased accumulation of $p27^{Kip1}$ protein in oligodendrocytes. Our findings demonstrate that QKI is upstream of $p27^{Kip1}$ during oligodendrocyte differentiation.

3.3 Introduction

Quaking viable (qk^v) is an autosomal recessive mutation in mice characterized by uncompact myelin of the central nervous system (CNS)[1]. These mutants develop normally until postnatal day 10 when they display rapid tremors or "quaking" that is most pronounced in the hind limbs and experience convulsive tonic-clonic seizures in adults[2]. Qk^v mice have an oligodendrocyte (OL) dysfunction that is characterized by maturation abnormalities and several defects in RNA metabolism of myelin components[3-6]. The qk^v mutation consists of a megabase deletion that includes the promoter region[7]. Another mutation, qk^{e5}, produced by using ethyl-N-nitrosourea, develop a similar dysmyelination phenotype and thus provide additional genetic evidence implicating *qkI* in OL function and myelination[8]. OLs of normal mice express 3 major *qkI* mRNAs of 5, 6 and 7 kb which encode a family of alternatively spliced RNA binding proteins QKI-5, QKI-6, and QKI-7, respectively[7]. The promoter deletion observed in qk^v mice prevents the expression of QKI-6 and QKI-7 isoforms in OLs, as analyzed by immunocytochemistry[9]. The QKI isoforms dimerize [10] and their balanced expression is required for proper myelination[4]. The over-expression of QKI-5 leads to immature OLs with RNA defects similar to those observed in qk^v mice[4]. QKI-6 and QKI-7 are expressed during embryogenesis[11] but the fact that they are up-regulated during myelination suggest that these QKI isoforms promote OL development.

Glial and neuronal cells in the CNS originate from multipotential neural progenitors in the ventricular zone[12,13]. Transcription factors including members of the basic helix-loop-helix family have been shown to be required for neural progenitor cell fate decisions[14]. However, the posttranscriptional regulators that control cell fate specifications are less understood [15]. Neuronal cell fate has been shown to be regulated posttranscriptionally by RNA binding proteins including Musashi 1 and 2, the fragile X mental retardation protein, HuD and Nova-1 [15-19]. But less is known about the RNA binding proteins that regulate glial cell fate. One key cell differentiation component is the $p27^{Kip1}$ cyclin-dependent kinase (CDK) inhibitor [20]. Accumulation of $p27^{Kip1}$ is required for OL precursor differentiation [21-24] and has been shown to be regulated posttranscriptionally in OLs[25], but the mechanism remains unknown.

The presence of bipolar and immature OLs in qk^{v} mice brain tissue and in primary cultures derived from them [2,4,26], led us to hypothesize that the missing isoforms (QKI-6 and QKI-7) may be implicated in OL differentiation. Herein, we report that the QKI-6 and QKI-7 isoforms promote cell cycle arrest and differentiation of primary rat OL precursors cells in culture. The mRNA encoding the CDK inhibitor $p27^{Kip1}$ was bound and protected by the QKI isoforms leading to an increased accumulation of $p27^{Kip1}$ protein in OLs. *In vivo* we show that gene delivery of the QKI-6 and QKI-7 isoforms into embryonic neural progenitors promotes glial cell fate specification. The majority of transduced progenitors developed into mature OLs localized to the corpus callosum and a smaller number into astrocytes. Our findings identify the QKI RNA binding proteins as being upstream of $p27^{Kip1}$ in the regulation of glial cell fate.

3.4 RESULTS

QKI-6 and QKI-7 induce cell cycle arrest and differentiation of oligodendrocyte precursor cells

Qk^{v} mice lack the expression of isoforms QKI-6 and QKI-7 in OLs[9]. To examine the contribution of these isoforms in OL proliferation, primary rat oligodendrocyte progenitor cells (OPC) were infected for 24 hr with control (AdGFP), QKI-6 (AdQKI-6) and QKI-7 (AdQKI-7) adenoviruses. Approximately 95% of the OPCs expressed the green fluorescent protein (GFP) serving as a marker of infection (Fig. 3-1a). Cell proliferation was examined by bromodeoxyuridine (BrdU) incorporation and analyzed by immunofluorescence staining. Fewer OPCs transduced with AdQKI-6 or AdQKI-7 incorporated BrdU than AdGFP transduced OPCs (Fig. 3-1a,b), suggesting that the QKI isoforms prevent S-phase cell cycle progression. To examine the cell cycle profile of OPC cultures over-expressing QKI-6 and QKI-7, flow cytometry was performed using propidium iodide and BrdU labeling. Control infected cultures contained ~70 % of the cells in G_0/G_1-phase compared to ~90% for QKI-6 and QKI-7 infected cells (Fig. 3-1c). In contrast, the percentage of cells in S-phase decreased from 19% in controls to 8-9% in the QKI infected cultures, consistent with the immunolabeling result in Fig. 3-1a. These findings demonstrate that the expression of the QKI-6 and QKI-7 isoforms arrests OPCs in the G_0/G_1-phase of the cell cycle.

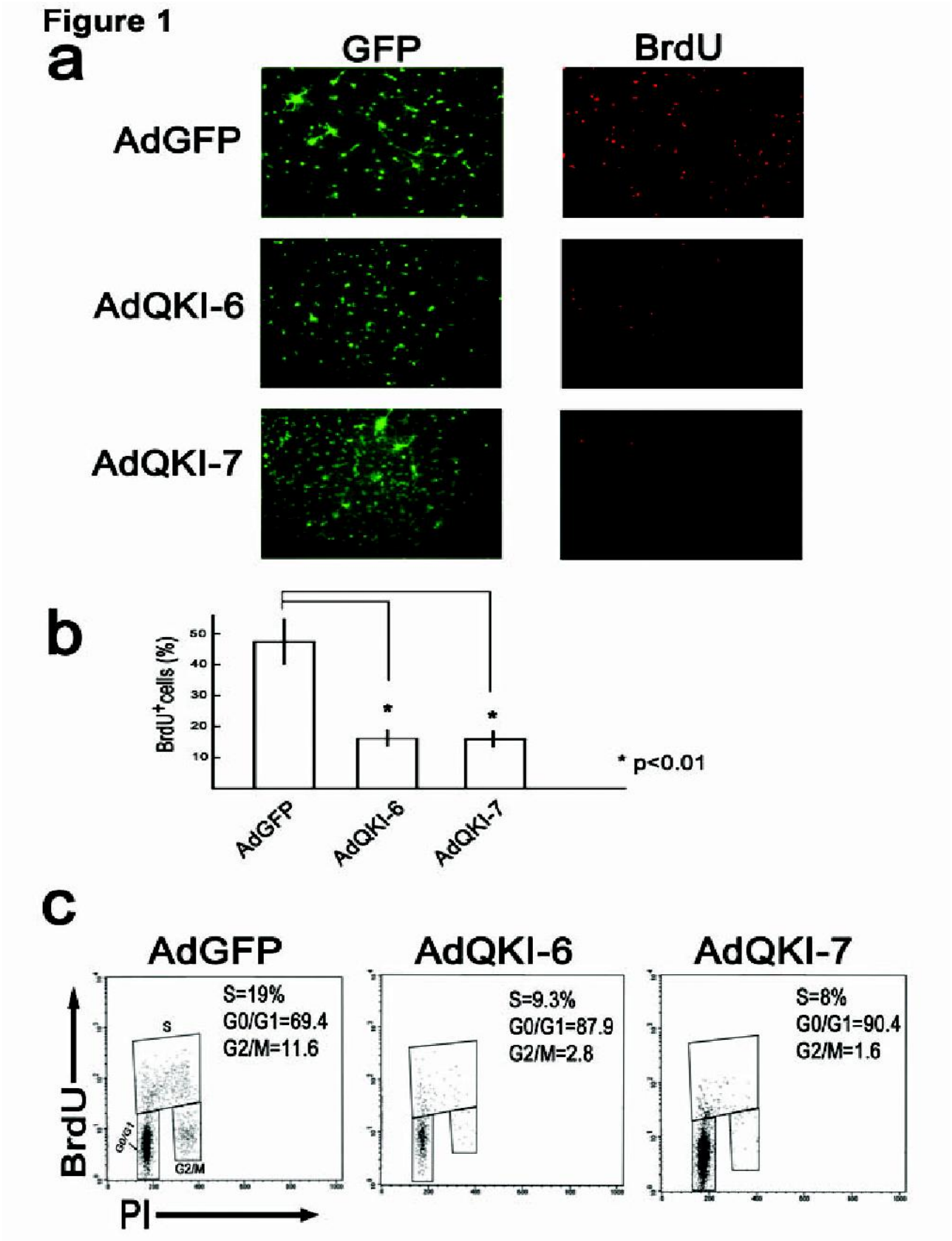
Fig. 3-1. QKI-6 and QKI-7 cause a G_0/G_1 cell cycle arrest in primary rat oligodendrocyte progenitors.

(**a**) OPCs were transduced with the indicated adenoviruses for 24 hr and incubated with BrdU for 8 hr. Incorporation of BrdU was detected with an anti-BrdU antibody followed by a goat anti-mouse conjugated to Alexa 546 (in red).

(b) The percentage of GFP positive cells that incorporated BrdU was quantitated from *n* > 400 cells is expressed as mean +/- standard deviation of the mean. The asterisks denotes $p < 0.01$, as determined by the student *t* test.

(**c**) Cell cycle analysis was performed using flow cytometry. A total of 10 000 cells (events) were selected in each set of experiments. The DNA of the AdGFP, AdQKI-6 or AdQKI-7 infected cells was stained with anti-BrdU antibodies followed by a Cy5-labeled anti-mouse secondary antibody (x axis) and propidium iodide (y axis). Cells in G_0/G_1, S phase and G_2/M were counted and expressed as a percentage using a cytometer.

Figure 1


a
GFP
BrdU
AdGFP
AdQKI-6
AdQKI-7
b
BrdU+cells (%)
50
40
30
20
10
*
*
* p<0.01
AdGFP
AdQKI-6
AdQKI-7
c
AdGFP
AdQKI-6
AdQKI-7
S=19%
G0/G1=69.4
G2/M=11.6
S
G0/G1
G2/M
S=9.3%
G0/G1=87.9
G2/M=2.8
S=8%
G0/G1=90.4
G2/M=1.6
BrdU
PI

The observed inhibition in cell proliferation suggested that OL differentiation might be induced by the QKI isoforms. To test this possibility, OPCs infected with the indicated adenoviruses were differentiated for 6 days and the expression of galactocerebroside C (GalC) and myelin basic protein (MBP), two markers of differentiation, was detected by immunofluorescence. Morphological differentiation was assessed after GalC staining by counting the numbers of secondary and tertiary branching essentially as described [27]. The expression of QKI-6 or QKI-7 isoforms individually or in combination resulted in a statistically significant higher number of OLs (cells expressing GalC/MBP) with secondary and tertiary branches than those expressing GFP alone (Fig. 3-2a-d, 2e). These findings suggest that the QKI expressing OLs are more mature than the control cultures. The presence of more secondary and tertiary branches correlated with higher levels of MBP isoforms, as visualized by immunofluorescence and immunoblotting (Fig. 3-2f, g). Densitometric analysis of the MBP immunoblot reveals a 28-54% increase in MBP expression with the QKI isoforms, correlating with the tertiary branches observed (Fig. 3-2g). These findings demonstrate that QKI-6 and QKI-7 enhance OL differentiation and maturation *in vitro*.

Fig. 3-2. Differentiation of OPCs following expression of QKI-6 and QKI-7 isoforms. Primary rat OPCs were infected with AdGFP (**a**), AdQKI-6 (**b**), AdQKI-7 (**c**) or the combination of AdQKI-6/7 (panel **d**). The transduced cells were allowed to differentiate for 6 days in culture and were immunostained for GalC (red: Alexa 546) as a marker of OL differentiation. The GFP (green) serves as marker of infection. The immunofluorescence staining was detected by confocal microscopy. (**e**) The primary, secondary and tertiary processes of $n>400$ cells were counted for each adenovirus treatment and expressed as the number of processes. * denotes P value of $p<0.01$ using the student *t* test. (**f**) Infected cultures as in panel (**a**) were immunostained with anti-MBP antibodies. The images represent a merging (yellow) of the GFP (green) and MBP (red). (**g**) OPC cultures infected as in panel (**a**) were lysed, the proteins separated were by SDS-PAGE and immunoblotted with anti-MBP and anti-β-actin antibodies. The migration of β-actin and the MBP isoforms is indicated.

Figure 2

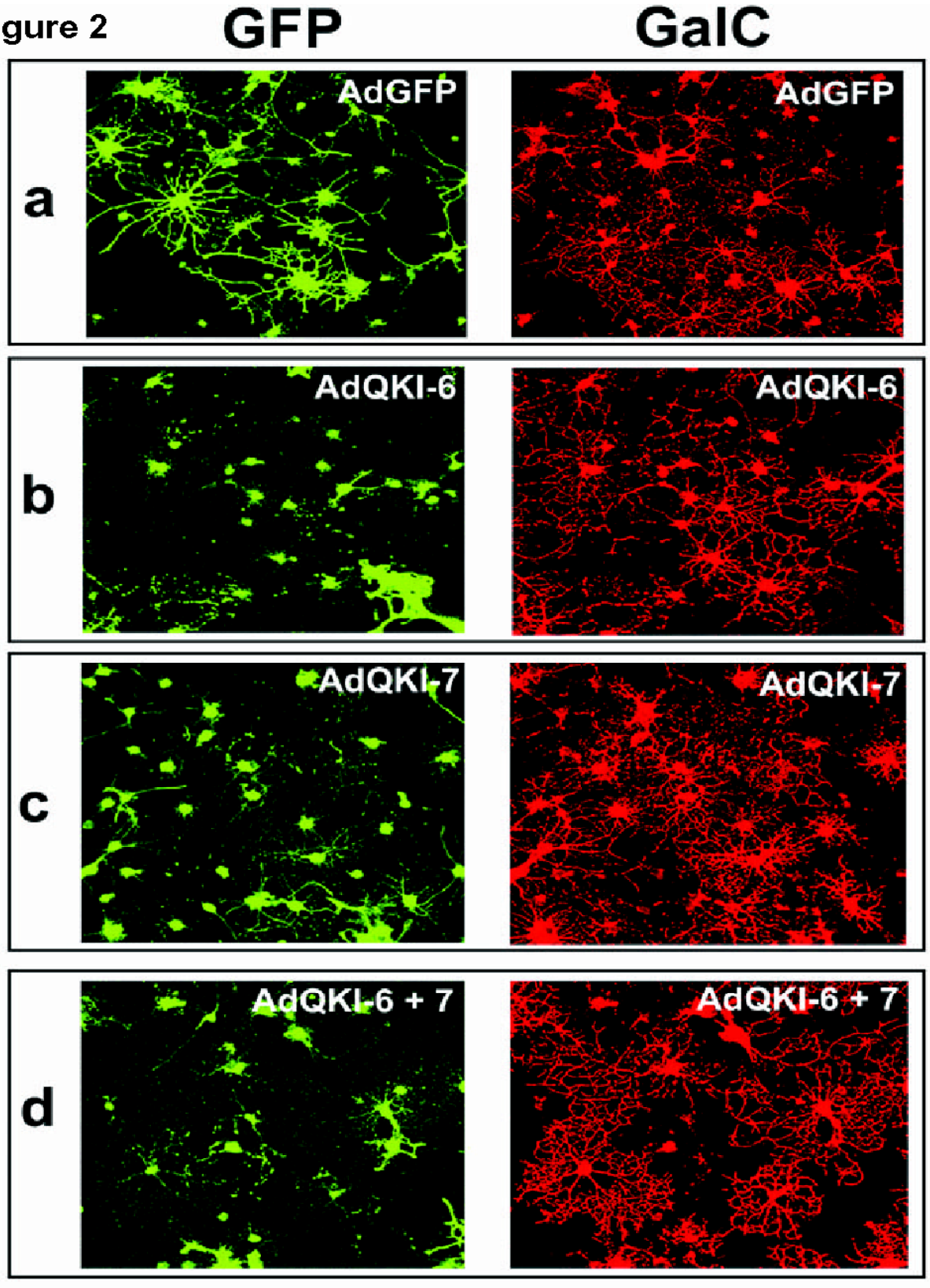
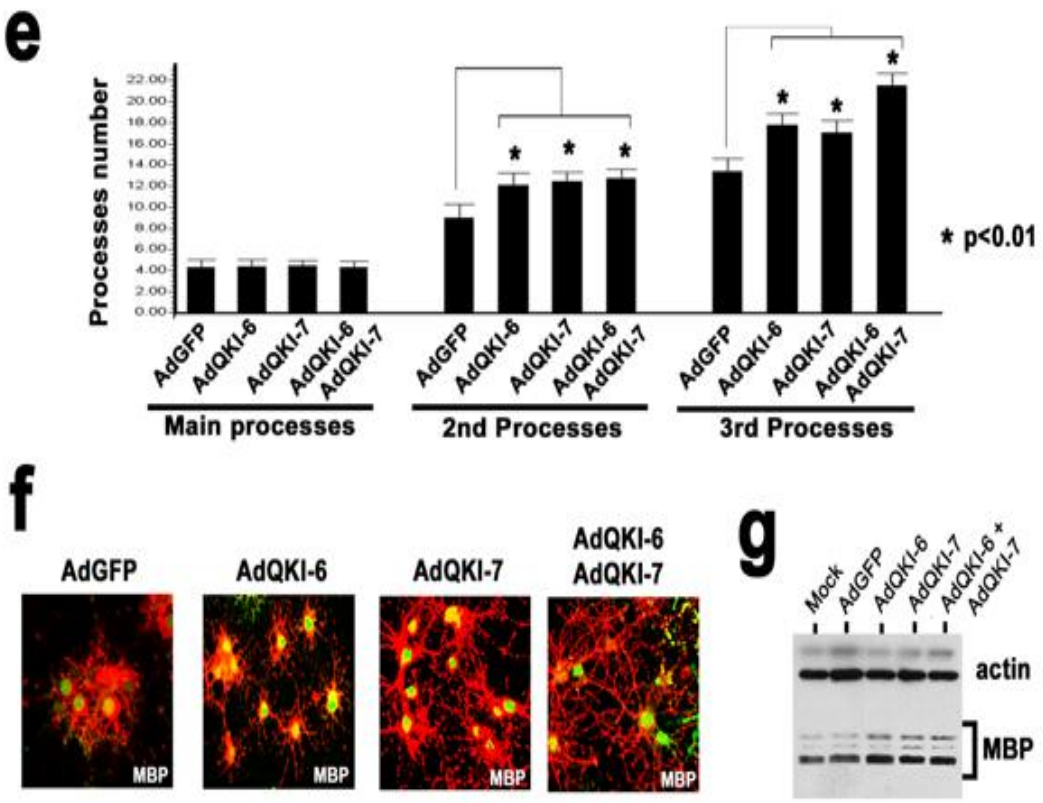

GFP
GalC
a
AdGFP
AdGFP
b
AdQKI-6
AdQKI-6
c
AdQKI-7
AdQKI-7
d
AdQKI-6 + 7
AdQKI-6 + 7

Figure 3-2 suite



QKI-6 and QKI-7 isoforms bind and stabilize the $p27^{Kip1}$ mRNA

To identify QKI mRNA targets responsible for OL differentiation, we performed a bioinformatics analysis (Supplementary Fig. 1) using a QKI RNA binding consensus that we identified by systematic evolution of ligands by exponential enrichment (A. Galarneau and S. Richard, in preparation). A total of 222 mRNAs were identified from mouse cDNA databases with the QKI consensus including a core ACUAAY motif. We identified two putative sequences located in the CDK inhibitor $p27^{Kip1}$ mRNA (Fig. 3a). The mRNAs encoding the CDK inhibitors $p21^{Cip1}$ and $p57^{Kip2}$ were devoid of such sequences. Since $p27^{Kip1}$ is a well-known OL cell differentiation factor[21-24,28] and contains the QKI RNA binding consensus sequence, we investigated whether QKI-6 and QKI-7 bound and regulated the $p27^{Kip1}$ mRNA. Synthetic ^{32}P-labeled RNAs from the $p27^{Kip1}$ regions were generated and tested for their ability to bind recombinant glutathione-S-transferase (GST)-QKI-6 and QKI-7 proteins by electrophoretic mobility shift assays. The synthetic RNA harboring nucleotides 618 to 647 of mouse $p27^{Kip1}$ (accession number AK047669) bound to recombinant GST-QKI-6 and GST-QKI-7 (Fig. 3-3b, lanes 1-10), as slow migrating QKI/RNA complexes were observed with increasing concentration of GST-QKI and not GST alone. The region from 521-550 was not significantly bound by GST-QKI-6 nor GST-QKI-7 (Fig. 3-3b, lanes 11-20). Our results demonstrate that a QRE resides between nucleotides 618-647 of the mouse $p27^{Kip1}$ mRNA.

We next examined whether endogenous and adenovirus expressed QKI-6 and QKI-7 associated with the $p27^{Kip1}$ mRNA in cells. OPCs infected with control, AdQKI-6 or AdQKI-7 were lysed and anti-myc immunoprecipitations were performed to capture the myc-epitope tagged QKI-6 or QKI-7. The bound mRNAs were isolated and the detection of the $p27^{Kip1}$ mRNA was performed by reverse transcription polymerase chain reaction (RT-PCR). A $p27^{Kip1}$ cDNA fragment was amplified from anti-myc immunoprecipitates of AdQKI-6 and AdQKI-7 (Fig. 3-3c, lanes 3 and 4). The absence of a $p27^{Kip1}$ cDNA fragment in AdGFP transduced OPC cultures demonstrates that $p27^{Kip1}$ mRNA binding was specific and mediated by the QKI isoforms (Fig. 3-3c, lane 2). To examine whether endogenous QKI-6 and QKI-7 associated with $p27^{Kip1}$ mRNA, we generated anti-QKI-6 and QKI-7 peptide antibodies. The anti-QKI-6 antibody recognized both QKI-6 and QKI-7 because of the overlapping epitope utilized, whereas the anti-QKI-7 antibody was QKI-7-specific (data not shown). Immunoprecipitations performed with either QKI antibody bound $p27^{Kip1}$ mRNA (Fig. 3-3c, lanes 8-10) in contrast to the negative control serum (NRS, lane 7). The absence of reverse transcriptase abrogated the $p27^{Kip1}$ cDNA signal (lane 5) while the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was not detected in anti-QKI immunoprecipitates (lane 11), further demonstrating the specificity of the interaction between QKI-6/7 and the $p27^{Kip1}$ mRNA.

The presence of a QRE within the $p27^{Kip1}$ mRNA suggested that the QKI isoforms either increased $p27^{Kip1}$ translation or stabilized its mRNA. The fact that the QKI- homolog in *C. elegans,* GLD-1, is a translational suppressor[29,30] and QKI-6 was shown to functionally substitute for GLD-1 as a translational suppressor [31] suggest that

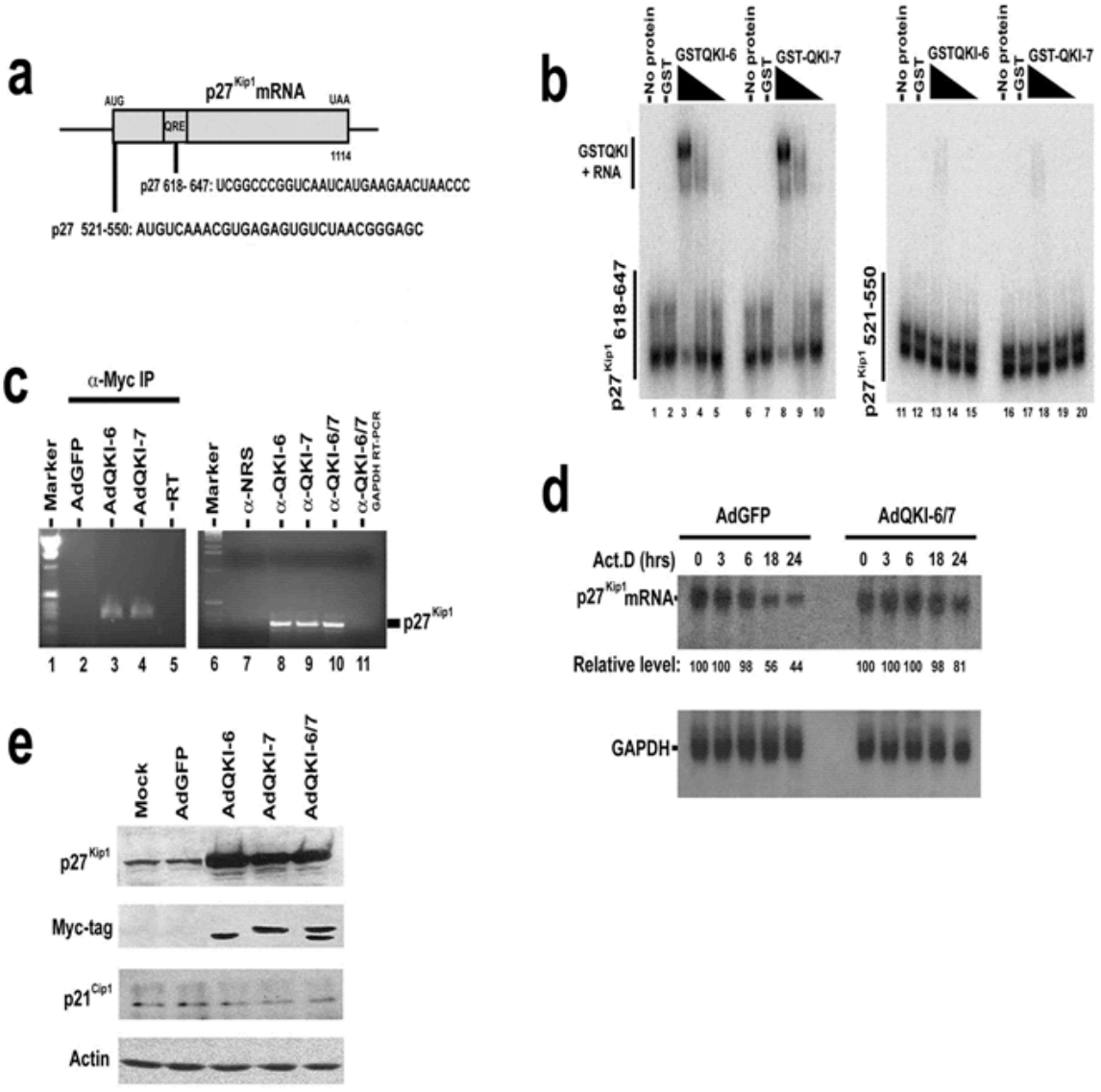
QKI may not up-regulate $p27^{Kip1}$ protein translation *per se*. Thus we tested whether the QKI-6 and QKI-7 isoforms influenced the half-life of the $p27^{Kip1}$ mRNA. OLs, transduced with either control AdGFP or AdQKI-6 and AdQKI-7, were treated with 5 □g/ml actinomycin D to inhibit transcription. RNAs were isolated at various times after actinomycin D treatment, and $p27^{Kip1}$ or control GAPDH mRNAs quantitated by Northern blot analysis. Similar levels of $p27^{Kip1}$ mRNA were detected in control and QKI-6/7 expressing OLs at times 0, 3, and 6 hrs (Fig. 3-3d). These findings are consistent with previous studies reporting that the steady state of the $p27^{Kip1}$ mRNA does not change during OL differentiation[25]. The QKI-6/7 transduced OL cultures maintained 98% and 81% of the $p27^{Kip1}$ mRNA compared to 56% and 44% in the AdGFP-treated cells at 18 and 24 hr post-actinomycin D treatment (Fig. 3-3d). These data demonstrate that the $p27^{Kip1}$ mRNA decayed 6 hr after actinomycin D treatment in AdGFP-expressing cells, but not in QKI-6/7 transduced cells. A similar time course of $p27^{Kip1}$ mRNA decay was observed by using semi-quantitative RT-PCR (data not shown). As a control for equal RNA loading, the GAPDH mRNA did not decay during the time course tested (Fig. 3-3d). These data suggest that QKI-6 and QKI-7 stabilize or protect the $p27^{Kip1}$ mRNA in OLs.

If QKI-6 and QKI-7 stabilize $p27^{Kip1}$ mRNA then there should be a resulting increase in $p27^{Kip1}$ protien levels. To investigate this possibility, total cell extracts of OPCs transduced with control AdGFP, AdQKI-6 and/or AdQKI-7 were immunoblotted for $p27^{Kip1}$ (Fig. 3-3e). The expression of $p27^{Kip1}$ was induced 5-fold by the over-expression of QKI-6 and QKI-7, while the expression of $p21^{Cip1}$ remained unchanged

(Fig. 3-3e), as predicted from the bioinformatics analysis. Anti-myc and anti-□–actin antibodies were used to confirm the myc-epitope tagged QKI expression and equivalent loading, respectively. Confocal immunofluorescence confirmed that $p27^{Kip1}$ was increased in the nucleus of OLs over-expressing QKI-6 and QKI-7 (data not shown). Our findings suggest the $p27^{Kip1}$ mRNA represents a *bona fide* target for the QKI isoforms in OLs.

Fig. 3-3. QKI isoforms induce $p27^{Kip1}$ upregulation by stabilizing its mRNA.

(a) The sequence of the two putative QKI mRNA binding sites identified in $p27^{Kip1}$. **(b)** The identification of an RNA binding site in $p27^{Kip1}$ mRNA was identified by electrophoretic mobility gel shift analysis. Two different regions spanning nucleotides 618-647 (lanes 1-10) and 521-550 (lanes 11-20) of $p27^{Kip1}$ mRNA were identified as putative QREs. Synthetic ^{32}P-labeled RNA was generated and incubated with decreasing concentrations of GST-QKI-6 or QKI-7 (750 nM, 75 nM, 7.5 nM). GST alone (750 nM) or no protein were utilized as controls. Protein/RNA complexes were separated on 8%-acrylamide native gels and visualized by autoradiography. (**c**) *In vivo* binding of $p27^{Kip1}$ mRNA by QKI proteins in rat OLs. Rat OLs transduced with AdGFP, AdQKI-6 or AdQKI-7 were immunoprecipitated with an anti-myc antibody that recognizes the adenovirus produced QKI-6 and QKI-7 myc-epitope tagged. The bound RNAs were extracted from the immunoprecipitates and the presence of $p27^{Kip1}$ mRNA was detected by RT- PCR, separated by agarose gel electrophoresis and visualized by ethidium bromide staining (lanes 2-5). The 1kb ladder (Gibco) was used to estimate the size of the amplified DNA fragment (lane 1 and 6). Primary Rat OL were immunoprecipitated with normal rabbit serum control (NRS)(lane 7), or antibodies specific to isoforms QKI-6 and QKI-7 (lanes 8-10). The bound $p27^{Kip1}$ was determined by RT-PCR. The (–RT) lane denotes the absence of reverse transcriptase. Lane 11 represents an RT-PCR reaction with GAPDH as a negative control. (**d)** Effect of QKI-6 and QKI-7 on $p27^{Kip1}$ RNA stability. Mature OLs were infected for 48 hrs with a combination of QKI-6 and QKI-7 adenoviruses or control adenovirus (AdGFP). Addition of actinomycin D was used to block *de novo* transcription. Total RNA was collected at the indicated times and the levels of $p27^{Kip1}$ or control GAPDH mRNA were detected by Northern blot analysis and quantitated by using a phosphoimager. (**e**) $p27^{Kip1}$ protein expression, but not $p21^{Cip1}$, is induced by QKI. OPCs left untreated (mock) or transduced with AdGFP, AdQKI-6, -7 and -6/7 were lysed and the proteins separated by SDS-PAGE and immunoblotted with anti-$p27^{KIP1}$, anti-$p21^{Cip1}$, anti-9E10 (myc) and anti-β-actin antibodies.


a
p27Kip1mRNA
AUG
UAA
QRE
1114
p27 618- 647: UCGGCCCGGUCAAUCAUGAAGAACUAACCC
p27 521-550: AUGUCAAACGUGAGAGUGUCUAACGGGAGC
b
-No protein
-GST
GSTQKI-6
GST-QKI-7
GSTQKI + RNA
p27Kip1 618-647
p27Kip1 521-550
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
c
α-Myc IP
Marker
AdGFP
AdQKI-6
AdQKI-7
-RT
α-NRS
α-QKI-6
α-QKI-7
α-QKI-6/7
GAPDH RT-PCR
p27Kip1
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AdGFP
AdQKI-6/7
Act.D (hrs) 0 3 6 18 24 0 3 6 18 24
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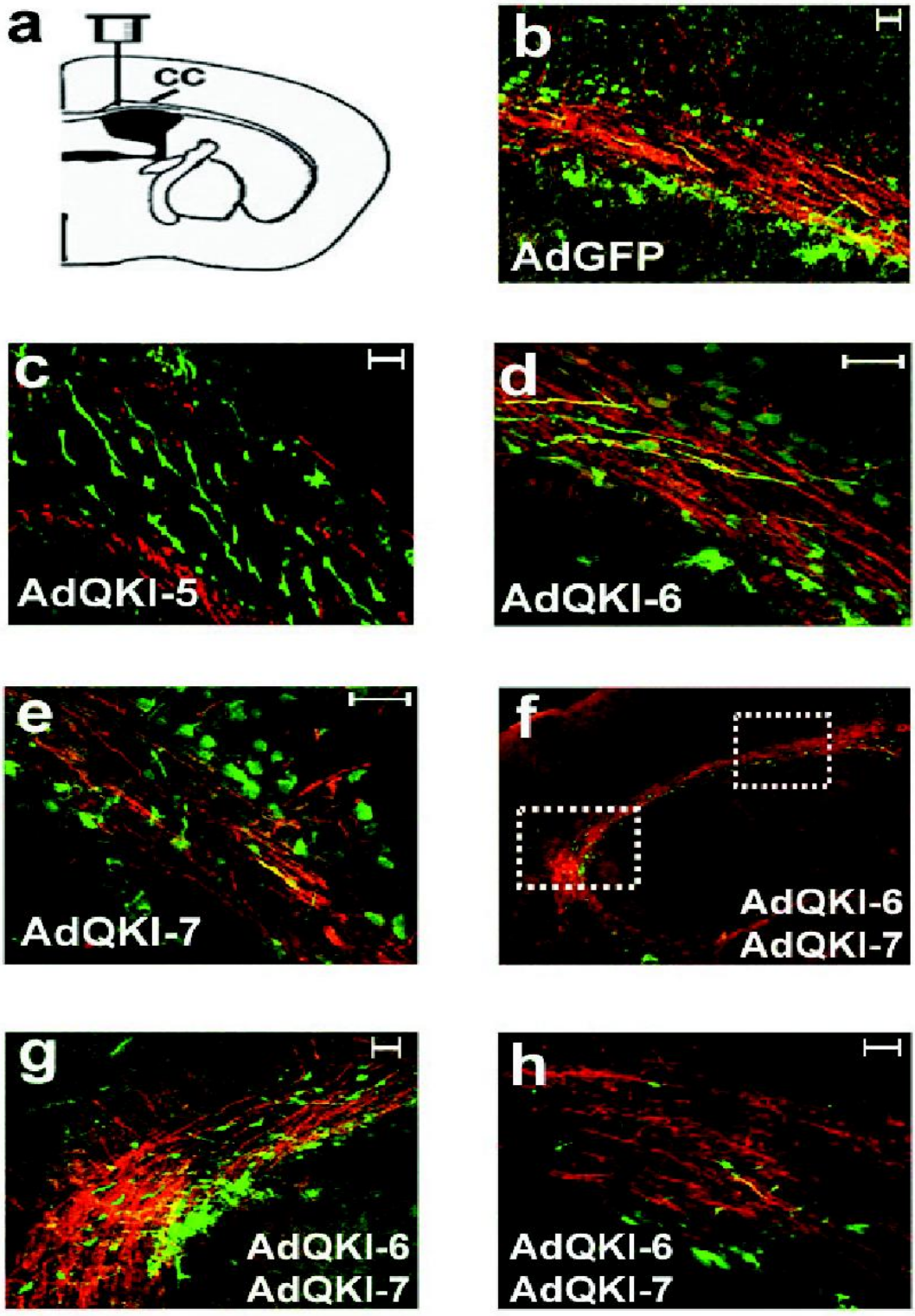
Relative level: 100 100 98 56 44 100 100 100 98 81
GAPDH
e
Mock
AdGFP
AdQKI-6
AdQKI-7
AdQKI-6/7
p27Kip1
Myc-tag
p21Cip1
Actin

QKI-6 and QKI-7 isoforms induce MBP expression *in vivo*

We next examined whether QKI-6 and QKI-7 affect oligodendrocyte maturation *in vivo* by staining with anti-MBP antibodies. The corpus callosum of post-natal day 1 (P1) mice was injected with a control or QKI expressing adenovirus as illustrated (Fig. 3-4). The adenoviruses express independently the specified QKI isoform and GFP, the latter serves as a marker for infection[4]. Coronal brain sections were prepared at P10 and stained with an anti-MBP antibody (red) and visualized by confocal microscopy. The corpus callosum was chosen as the site of injection because it is a myelin-rich region and a single injection was sufficient to spread the GFP and QKI adenoviruses throughout it[4]. Control AdGFP injections demonstrated that the neural tracts in the corpus callosum stained positively for both GFP and MBP (Fig. 3-4b). Thus the adenoviruses *per se* did not cause demyelination. In contrast, brain injections of the QKI-5 adenovirus showed GFP-positive neural tracts (i.e. expressing QKI-5) but the MBP staining was barely detectable in the surrounding area (Fig. 3-4c), as reported previously [4]. Brain cross-sections from AdQKI-6 (Fig. 3-4d), AdQKI-7 (Fig. 3-4e) or both AdQKI6/7 (Fig. 3-4f-h) infected mice showed strong MBP immunoreactivity and the QKI-6/7 over-expressing regions in the corpus callosum typically had stronger local MBP staining than observed in AdQKI-5 injected mice. These results suggest that the QKI-6 and QKI-7 isoforms promote OL maturation *in vivo*.

Fig. 3-4. The areas of the corpus callosum injected with AdQKI-6 and AdQKI-7 contain elevated MBP expression.

(**a**) A schematic diagram illustrating the site of adenovirus injection in the corpus callosum (cc) at post-natal day 1 is shown. Coronal cross-sections from post-natal day 10 mice were immunostained with anti-MBP antibodies (red) and the green cells represent the GFP positive transduced cells. Representative coronal sections of the corpus callosum of AdGFP-(**b**), AdQKI-5- (**c**), AdQKI-6- (**d**), AdQKI-7- (**e**) and AdQKI-6/7(**f**)- injected mice are shown. (**g**) (**h**) Higher magnification of the 2 boxed regions in panel (**f**) are shown. Scale bar represents 50 μm.


a
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AdQKI-7
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AdQKI-6
AdQKI-7

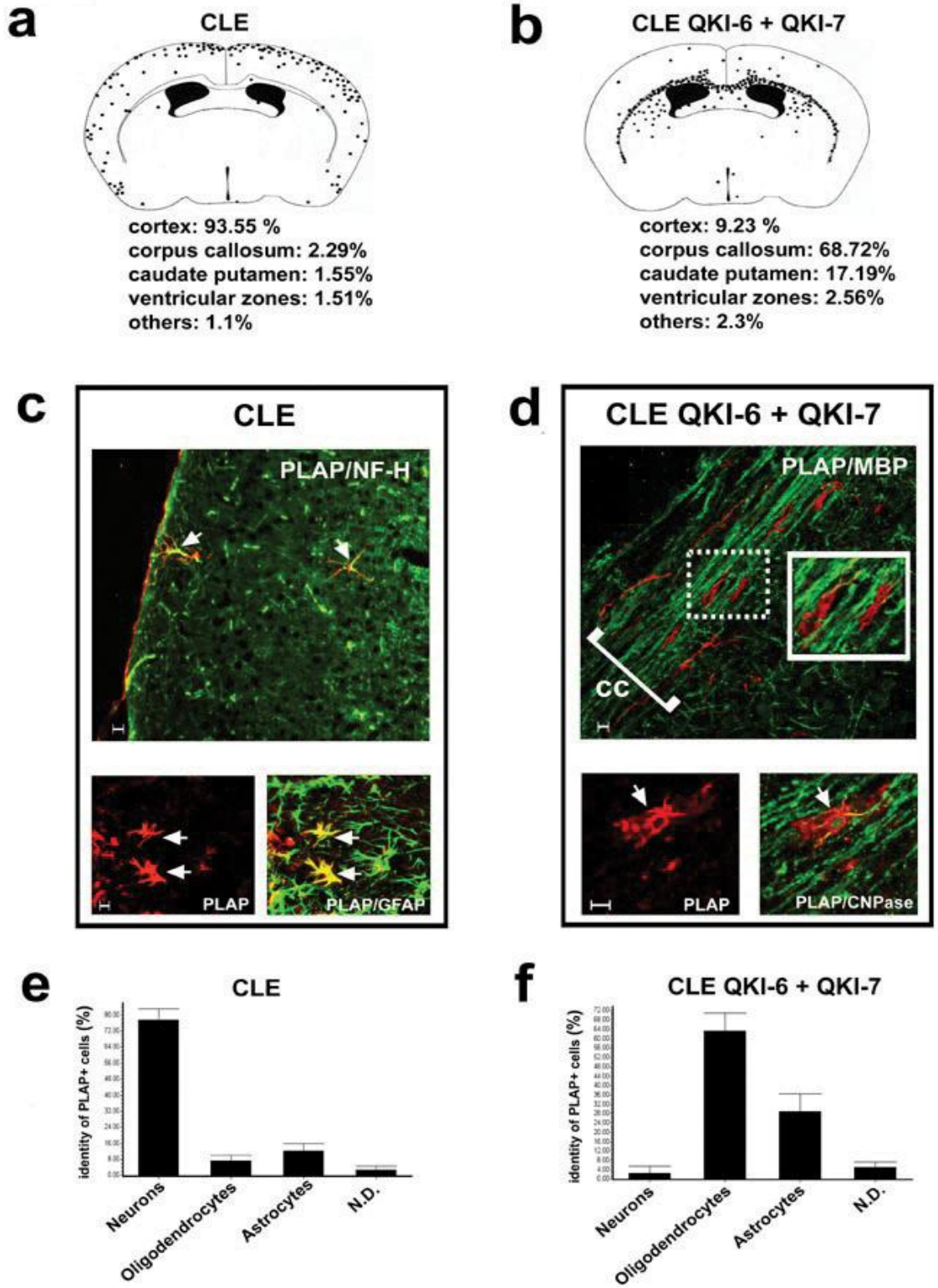
The QKI-6 and QKI-7 isoforms regulate the migration and cell fate of neural progenitors.

Our strategy to use adenoviruses and newborn mice in Fig. 3-4 did not permit us to assess the role of the QKI isoforms in cell fate decisions. The *qkI*-deficient mice die at E9.5 and neuronal and glial cell fate information could not be obtained from these animals[32]. We tested the ability of the QKI isoforms to regulate neural progenitors *in vivo* by using a retroviral gain-of-function approach [33,34]. Retroviruses expressing control, QKI-6 and QKI-7 were injected *in utero* in the telencephalic region of mouse E11.5 embryos. This permitted the targeting of multipotential neural progenitor cells in the ventricular zone and to follow their subsequent positioning and cell fate by staining for the placental alkaline phosphatase (PLAP) marker expressed by the retroviral vector. At P11, brain cross-sections of control-injected mice were examined for PLAP expression and reactivity was found predominantly in the cortex (Fig. 3-5a), as observed previously with the CLE retrovirus[34]. In contrast, mice injected with both QKI-6 and QKI-7 retroviruses, expressed PLAP predominantly in the corpus callosum and the caudate putamen consistent with the differentiation into glia (Fig. 3-5b). The cell fate of the PLAP expressing cells was determined by cellular morphology and double immunostaining with anti-PLAP antibodies and a cell-type specific marker including anti-neurofilament H (NF-H) for neurons, anti-glial fibrillary acidic protein (GFAP) for astrocytes and anti-2',3',-cyclic nucleotide-3'-phosphodiesterase (CNPase) or anti-MBP antibodies for OLs (Fig 5c,d). The cortex of control CLE injected mice stained readily with anti-PLAP antibodies (red) and these cells often co-stained (merge is yellow) with anti-NF-H antibodies (green, Fig. 3-5c). A small percentage of anti-PLAP positive cells co-

immunostained (yellow) with anti-GFAP antibodies being identified as astrocytes (Fig. 3-5c). The majority of CLE injected cells developed into neurons (77.68% $\pm$ 5.4, Fig. 3-5e) with a small portion developing into astrocytes (12.18% $\pm$ 3.75, Fig. 3-5e). Similar co-staining studies were performed in the corpus callosum of QKI-6/7 injected mice. Anti-PLAP antibody (red) positive cells were co-immunostained with anti-MBP and anti-CNPase antibodies (green, Fig. 3-5d). In addition, QKI-6 and QKI-7 transduced cells also developed into anti-GFAP antibody positive astrocytes that localized at the border of corpus callosum into the rostral marginal zone (data not shown). The majority of infected progenitors developed into mature OLs with QKI-6 and QKI-7 ~ 63.37% $\pm$ 7.7 while 28.7% $\pm$ 7.5 of progenitors developed into astrocytes (Fig. 3-5f). Taken together these data demonstrate that QKI-6 and QKI-7 isoforms promote glial cell fate specification.

Fig. 3-5. Ectopic expression of QKI-6 and QKI-7 promotes OL glial cell fate specification and migration to the corpus callosum and caudate putamen *in vivo*.

Control CLE or QKI-6 and QKI-7 retroviruses were injected in the telencephalon of mouse embryos at day 11.5. Brain cross-sections were performed at P11 and stained for the CLE PLAP marker. A schematic diagram illustrating the location of PLAP positive cells is shown for the control CLE retrovirus (**a**) and QKI-6-QKI-7 retrovirus combination (**b**) Double immunofluorescence staining was performed. Anti-PLAP antibodies (red) were utilized to detect the transduced cells and anti- NF-H (**c**), anti-GFAP (**c**), anti-MBP (**d**) or anti-CNPase (**d**) are shown in green and are markers for neurons, astrocytes and OL, respectively. Arrowheads in (**c**) identify PLAP and NF-H positives neurons in the cortex. The inset in (**d**) show that PLAP+ cells have OLs morphology and are in the vicinity of myelinated axons (MBP+ in green) in the corpus callosum (cc). Scale bar represents 20 □m. (**e** and **f**) Histograms are shown comparing the percentage of PLAP+ neurons, OLs and astrocytes in brains after infection with control CLE retrovirus and QKI-6 and QKI-7 retroviruses (n>200 cells counted). N.D. depicts not identified.


a
CLE
cortex: 93.55 %
corpus callosum: 2.29%
caudate putamen: 1.55%
ventricular zones: 1.51%
others: 1.1%
b
CLE QKI-6 + QKI-7
cortex: 9.23 %
corpus callosum: 68.72%
caudate putamen: 17.19%
ventricular zones: 2.56%
others: 2.3%
c
CLE
PLAP/NF-H
PLAP
PLAP/GFAP
d
CLE QKI-6 + QKI-7
PLAP/MBP
CC
PLAP
PLAP/CNPase
e
CLE
identity of PLAP+ cells (%)
Neurons
Oligodendrocytes
Astrocytes
N.D.
f
CLE QKI-6 + QKI-7
identity of PLAP+ cells (%)
Neurons
Oligodendrocytes
Astrocytes
N.D.

Supplementary Fig. 1

Mouse cDNA databases were searched for the QKI RNA binding core ACUAAY consensus identified by SELEX. The target match, accession#, the sequence identified and the location are indicated and classified in groups as indicated.

Target match	Accession #	Sequence	Region
Cell Cycle (Process and Regulation)			
cullin 1 (Cul1),	NM_012042	actaatggagaactagctgtaac	5'UTR
cyclin A1 (Ccna1),	NM_007628	actaacgtcagttgtacataac	3'UTR
cyclin B1 (Ccnb1),	NM_172301	tttaacagtggatccaactaat	3'UTR
cyclin C (Ccnc),	NM_016746	actaatactggtttttgattagataat	3'UTR
cyclin G1 (Ccng1),	NM_009831	actaattgagtcggcccatgataat	CDS
cyclin K (Ccnk),	NM_009832	gttaatagcttcagtaaggagtgactaac	3'UTR
cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) (Cdkn1b),	NM_009875	**ccggtcaatcatgaagaactaac**	CDS
retinoblastoma binding protein 4 (Rbbp4),	NM_009030	actaactgtgtaagtgcttataat	3'UTR
dystonin (Dst),	NM_134448	tgtaacccgcgcaactaac	CDS
sulfatase 1 (Sulf1),	NM_172294	cttaatcactaac	CDS
cell division cycle 37 homolog (S. cerevisiae)-like (Cdc37l),	NM_025950	tataataaaatgtctgactaat	3'UTR
fyn-related kinase (Frk),	NM_010237	ggtaacagggactaac	3'UTR
neuroblastoma myc-related oncogene 1 (Nmyc1),	NM_008709	gttaatctgttatgtactgtactaat	3'UTR
cell division cycle 27 homolog (S. cerevisiae) (Cdc27),	NM_145436	actaatacaccttctgtaat	CDS
transcriptional regulator, SIN3B (yeast) (Sin3b),	NM_009188	ggtaaccactaac	3'UTR
G-protein coupled receptor protein signaling pathway			
adrenergic receptor, beta 2 (Adrb2),	NM_007420	actaaccagactatttaac	3'UTR
anthrax toxin receptor 1 (Antxr1),	NM_054041	tttaatgaaactaac	CDS
cholecystokinin B receptor (Cckbr),	NM_007627	actaatggcttcctgttaat	3'UTR
cysteinyl leukotriene receptor 1 (Cysltr1),	NM_021476	tataactgtgaactaac	3'UTR
endothelial differentiation, lysophosphatidic acid	NM_010336	actaattgaaccttaat	3'UTR
endothelin 1 (Edn1),	NM_010104	actaatcacacaataac	5'UTR
G protein-coupled receptor partial family^{A},	NM_146187	actaataggggtgcagtaat	3'UTR
growth hormone secretagogue receptor (Ghsr),	NM_177330	actaattacaagcctttaat	3'UTR
MAS1 oncogene (Mas1),	NM_008552	actaactgactaac	5'UTR
melatonin receptor 1A (Mtnr1a),	NM_008639	actaatacccaataat	CDS
oligodendrocyte myelin glycoprotein (Omg),	NM_019409	actaatgacttttttttttaat	3'UTR
regulator of G-protein signaling 13 (Rgs13),	NM_153171	tataacatttttgctatagtaggcaactaat	3'UTR
rhodopsin (Rho),	NM_145383	actaatataat	3'UTR
adenylate cyclase 2 (Adcy2),	NM_153534	actaaccatggctgtgtttaac	CDS
arrestin, beta 1 (Arrb1), transcript variant b,	NM_178220	actaacctctcctgtcccccatccttaac	3'UTR
Apoptosis (Process and Regulation)			
Baculoviral IAP repeat-containing 6 (Birc6),	NM_007566	actaattatgtaat	3'UTR
B-cell receptor-associated protein 29 (Bcap29),	NM_007530	actaactttataaaagaagactgtaat	3'UTR
BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP2 (Bnip2),	NM_016787	actaattatattgtaat	3'UTR
BH3 interacting domain death agonist (Bid),	NM_007544	actaacaaactgagtttaat	3'UTR
CASP8 and FADD-like apoptosis regulator (Cflar),	NM_009805	actaatctagaccagtttcttctataac	3'UTR
nucleolar protein 3 (apoptosis repressor with CARD domain) (Nol3),	NM_030152	actaatcctggcctgaacgtgggataac	3'UTR
phosphoprotein enriched in astrocytes 15 (Pea15),	NM_008556	actaacctgccctaat	3'UTR
programmed cell death 6 (Pdcd6),	NM_011051	actaattgtgccatgagacctaat	3'UTR
sphingosine-1-phosphate phosphatase 1 (Sgpp1),	NM_030750	actaacagaagtaaatggcccataac	3'UTR
sulfatase 1 (Sulf1),	NM_172294	actaacgagagcattaat	CDS
thioredoxin-like (Txnl),	NM_016792	actaataacttgtaat	3'UTR
tumor necrosis factor receptor superfamily, member 11b	NM_008764	actaattttatttcttacattaac	3'UTR
tumor necrosis factor receptor superfamily, member 21 (Tnfrsf21),	NM_178589	actaatttattaat	3'UTR
Fas apoptotic inhibitory molecule (Faim),	NM_011810	actaatataaagtgtttctaat	3'UTR
transformation related protein 53 inducible nuclear protein 1	NM_021897	actaacacaagcattaac	3'UTR
Signal Transduction and Intracellular cascade			
G-protein signalling modulator 2 (AGS3-like, C. elegans) (Gpsm2),	NM_029522	tttaacctcagatagtcacactaat	3'UTR
phospholipase C, beta 3 (Plcb3),	NM_008874	actaatactttgggttttttttaac	3'UTR
RAP2C, member of RAS oncogene family (Rap2c),	NM_172413	actaatagtttaaagcaatatttgttaat	3'UTR
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation	NM_011739	tttaatagccaatgcaactaat	CDS
bone morphogenetic protein receptor, type 1B (Bmpr1b),	NM_007560	tttaacagaactgactgttagagaaactaat	3'UTR
transforming growth factor, beta receptor I (Tgfbr1),	NM_009370	tataatttttcaagatcttaaactaac	3'UTR
v-ral simian leukemia viral oncogene homolog A (ras related)	NM_019491	actaataaatataat	3'UTR
disabled homolog 2 (Drosophila) (Dab2),	NM_023118	tttaataaaatgaaagcaagactaac	3'UTR
glutamate receptor interacting protein 1 (Grip1),	NM_133442	ggtaatctagagacacgagaacccactaac	CDS
membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)	NM_019939	actaatatataat	3'UTR
neural precursor cell expressed, developmentally down-regulated	NM_008682	tgtaaccactaat	CDS
periaxin (Prx), transcript variant 2,	NM_019412	actaacccgacactaat	3'UTR
RAS guanyl releasing protein 1 (Rasgrp1),	NM_011246	actaacttgtaat	3'UTR
solute carrier family 6 (neurotransmitter transporter, glycine),	NM_148931	gttaattgtaacctgcactaac	CDS
syndecan binding protein (Sdcbp),	NM_016807	actaatcttaac	3'UTR
Cellular adhesion			
breast cancer anti-estrogen resistance 1 (Bcar1),	NM_009954	actaatagtctacatttaat	3'UTR
cadherin 8 (Cdh8),	NM_007667	actaatgtaac	CDS
catenin alpha 2 (Catna2),	NM_145732	cttaactgaggagaaactaac	3'UTR
CEA-related cell adhesion molecule 2 (Ceacam2),	NM_007543	tgtaacaggcactaat	CDS
desmocollin 1 (Dsc1),	NM_013504	cttaatgaagcccagttcactaac	CDS
extra cellular link domain-containing 1 (Xlkd1),	NM_053247	actaatttttttgttgctgagaataat	3'UTR
killer cell lectin-like receptor, subfamily A, member 5 (Klra5),	NM_008463	actaactgtaat	CDS

neurexin I (Nrxn1),	NM_020252	actaattctgtataac	CDS
neuroligin 1 (Nlgn1),	NM_138666	actaactgctgtaac	3'UTR
neuroligin 1 (Nlgn1),	NM_138666	gataatgcaaaggactttctctgactaac	3'UTR
parvin, alpha (Parva),	NM_020606	actaacccagtgaactaat	3'UTR
protocadherin partial family[A],	NM_023115	actaactcatagagtagattgaccttaat	3'UTR
tumor necrosis factor alpha induced protein 6 (Tnfaip6),	NM_009398	cataattgtactacacagaaataactaat	3'UTR
vascular cell adhesion molecule 1 (Vcam1),	NM_011693	tgtaatgactaac	3'UTR
gap junction membrane channel protein beta 1 (Gjb1),	NM_008124	actaatcttaac	3'UTR
guanylate cyclase activator 1B (Guca1b),	NM_146079	tttaactgggagatgtggggatcctactaat	3'UTR
LIM and senescent cell antigen-like domains 1 (Lims1),	NM_026148	cttaacatacttgtaactactaat	3'UTR
Immune and Inflammatory Response			
CD28 antigen (Cd28),	NM_007642	actaatctgcaatggctattttaat	3'UTR
interferon, alpha-inducible protein (G1p2),	NM_015783	ggtaacaatttcctggtgtctgtgactaac	CDS
interleukin 7 (Il7),	NM_008371	tataactttgttaagagagaaaacactaat	3'UTR
pericentriolar material 1 (Pcm1),	NM_023662	tttaacctgcctggatttactaac	CDS
dual specificity phosphatase 16 (Dusp16),	NM_181320	tgtaatctcaacatgaactgcagactaac	3'UTR
gamma-glutamyltransferase-like activity 1 (Ggtla1),	NM_011820	actaattgggttctaagaggaacttaat	3'UTR
CD86 antigen (Cd86),	NM_019388	actaattcaactaat	CDS
histocompatibility 2, T region locus 9 (H2-T9),	NM_010399	actaatagagataggg tttaat	3'UTR
phosphoglucomutase 3 (Pgm3),	NM_028352	actaataggtaac	3'UTR
leucine rich repeat containing 16 (Lrrc16),	NM_026825	actaataaataat	3'UTR
phosphofructokinase, muscle (Pfkm),	NM_021514	tgtaactacactaat	3'UTR
asparaginase like 1 (Asrgl1),	NM_025610	tttaacgtgccactaat	3'UTR
phytanoyl-CoA hydroxylase (Phyh),	NM_010726	actaacctagggtgtaac	3'UTR
stearoyl-Coenzyme A desaturase 1 (Scd1),	NM_009127	tttaatattctgttgattaactaac	3'UTR
carnitine palmitoyltransferase 1a, liver (Cpt1a),	NM_013495	tgtaacacactaat	3'UTR
mRNA processing			
cleavage stimulation factor, 3' pre-RNA subunit 2 (Cstf2),	NM_133196	cttaataattattaagtttactagcactaat	3'UTR
poly A binding protein, cytoplasmic 2 (Pabpc2),	NM_011033	actaacacatcagcacagataac	CDS
LSM8 homolog, U6 small nuclear RNA associated (S. cerevisiae)	NM_133939	actaatgcaaataat	3'UTR
splicing factor 3b, subunit 1 (Sf3b1),	NM_031179	tttaatgttttgggtcaactaat	3'UTR
splicing factor, arginine/serine-rich 1 (ASF/SF2) (Sfrs1),	NM_173374	actaatgtgggaagaatggctaat	3'UTR
nucleolar protein 3 (apoptosis repressor with CARD domain) (Nol3),	NM_030152	cttaaccagctctgtactaat	3'UTR
splicing factor, arginine/serine-rich 5 (SRp40, HRS) (Sfrs5),	NM_009159	gttaactcaagattagtttaattaaactaac	3'UTR
Mki67 (FHA domain) interacting nucleolar phosphoprotein (Mki67ip),	NM_026472	actaatttgggtttcttgaatataac	3'UTR
Sjogren syndrome antigen A2 (Ssa2),	NM_013835	actaattttcatatttttctaat	3'UTR
Sjogren syndrome antigen B (Ssb),	NM_009278	actaacctgctaat	CDS
U2 small nuclear ribonucleoprotein auxiliary factor (U2AF) 1,	NM_178794	actaatgagagtggttttttttaac	3'UTR
heterogeneous nuclear ribonucleoprotein A1 (Hnrpa1),	NM_010447	actaattgtataac	3'UTR
testis nuclear RNA binding protein (Tenr),	NM_009350	actaatttctaat	CDS
hydrocephalus inducing (Hydin),	NM_172916	tataaccattcacaatcgcactaat	CDS
fibrillarin (Fbl),	NM_007991	actaacattattcctgtaat	CDS
Transcription			
embryonic large molecule derived from yolk sac (Elys),	NM_026375	actaactactaat	CDS
hairy/enhancer-of-split related with YRPW motif-like (Heyl),	NM_013905	actaatctttattacatacttgttaat	3'UTR
ankyrin repeat domain 1 (cardiac muscle) (Ankrd1),	NM_013468	tataataaatgcaaactaat	3'UTR
AT motif binding factor 1 (Atbf1),	NM_007496	actaactgcaattccaaagcttctaac	3'UTR
basic helix-loop-helix domain containing, class B5 (Bhlhb5),	NM_021560	actaatcctactaac	3'UTR
BTB and CNC homology 1 (Bach1),	NM_007520	ggtaataatctcctagagttcaaactaac	3'UTR
E74-like factor 5 (Elf5),	NM_010125	actaataagtctcttaac	3'UTR
forkhead box O1 (Foxo1),	NM_019739	actaatcgtaaaccattgtaat	3'UTR
homeodomain leucine zipper-encoding gene (Homez),	NM_183174	gataatgtctgactcgatttgtcactaat	3'UTR
jumonji domain containing 2B (Jmjd2b),	NM_172132	tttaactgcgctgagtccactaac	CDS
kelch-like ECH-associated protein 1 (Keap1),	NM_016679	actaaccggcttaac	CDS
metal response element binding transcription factor 1 (Mtf1),	NM_008636	actaataatcccaccataac	CDS
myotrophin (Mtpn),	NM_008098	actaacttgagattttaat	3'UTR
nuclear receptor subfamily 0, group B, member 1 (Nr0b1),	NM_007430	tttaataaaatttaaggtactaac	3'UTR
nuclear receptor-binding SET-domain protein 1 (Nsd1),	NM_008739	actaattcccatgcagaccatttaat	CDS
POU domain, class 2, transcription factor 1 (Pou2f1), transcript	NM_011137	tttaaccagtgctgctgtgactaat	CDS
SNF2 histone linker PHD RING helicase (Shprh),	NM_172937	tataatcatactaat	3'UTR
T-box brain gene 1 (Tbr1),	NM_009322	tgtaatgagtaatctactaat	3'UTR
TGFB-induced factor 2 (Tgif2),	NM_173396	actaatcctagatttgtattaac	3'UTR
YY1 transcription factor (Yy1),	NM_009537	actaacctgaaatctcacatcttaac	CDS
nuclear DNA binding protein (C1d),	NM_020558	actaacttttaat	3'UTR
transcription elongation factor A (SII) 1 (Tcea1),	NM_011541	actaattttgtaat	3'UTR
MAX gene associated (Mga),	NM_013720	actaataacactacccttgtaac	CDS
Msx-interacting-zinc finger (Miz1),	NM_008602	actaacaacaacttctgtttaat	3'UTR
Cellular Transport (Protein, Neurotransmitter and Ion)			
calcium channel, voltage-dependent, gamma subunit 5 (Cacng5),	NM_080644	actaacctgggctgcatgcgactaac	3'UTR
fatty acid synthase (Fasn),	NM_007988	ggtaatgctggccaaactaac	CDS
FXYD domain-containing ion transport regulator 6 (Fxyd6),	NM_022004	actaaccccagtcctatattaat	3'UTR
gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 1	NM_010252	gttaacacaaaaatgttcatatgtactaac	3'UTR
glutamate receptor, ionotropic, AMPA4 (alpha 4) (Gria4),	NM_019691	actaacatgtgataggaaaataat	3'UTR
inositol 1,4,5-triphosphate receptor 3 (Itpr3),	NM_080553	actaatgtgttaat	3'UTR

nucleoporin 210 (Nup210),	NM_018815	cataattcagtcctcaattttgccactaac	CDS
potassium voltage-gated channel, Isk-related subfamily, gene 4	NM_021342	actaatgtccccatgaggggttaac	3'UTR
puromycin-sensitive aminopeptidase (Psa),	NM_008942	cttaatatccagactaat	CDS
aquaporin 7 (Aqp7),	NM_007473	ggtaatatccataactaac	3'UTR
ATP-binding cassette, sub-family C (CFTR/MRP), member 2 (Abcc2),	NM_013806	actaaccctatctaac	CDS
contactin 4 (Cntn4),	NM_173004	actaataaatgtctaac	3'UTR
fatty acid binding protein 4, adipocyte (Fabp4),	NM_024406	actaataagcaagcaattaat	3'UTR
high mobility group box 1 (Hmgb1),	NM_010439	actaataaactaat	3'UTR
mitochondrial folate transporter/carrier (Mftc),	NM_172402	actaatactaac	3'UTR
nucleoporin 160 (Nup160),	NM_021512	actaactgattctggtgccttaat	CDS
protein kinase C, iota (Prkci),	NM_008857	gataatttcgattctcagtttactaat	CDS
macrophage scavenger receptor 1 (Msr1),	NM_031195	actaattcatttaac	3'UTR
sortilin 1 (Sort1),	NM_019972	actaacttaat	3'UTR
retinol binding protein 3, interstitial (Rbp3),	NM_015745	tgtaacataaattaaatccttactaat	3'UTR
solute carrier organic anion transporter family, member 2b1	NM_175316	tttaaccagtgcctgagcctacactaat	3'UTR
synaptotagmin 15 (Syt15), transcript variant a,	NM_181529	tttaacacatactaat	3'UTR
vacuolar protein sorting 4b (yeast) (Vps4b),	NM_009190	actaatgtctcattacataat	3'UTR
coatomer protein complex, subunit beta 1 (Copb1),	NM_033370	actaatttataaggtctgtcatgctaat	CDS
RAB12, member RAS oncogene family (Rab12),	NM_024448	tgtaacccaagtcagctatacactaac	3'UTR
synaptobrevin like 1 (Sybl1),	NM_011515	actaattcttcaatcatcctaat	3'UTR
synaptosomal-associated protein (Snap29),	NM_023348	actaaccgttactgacgtgtaac	3'UTR
Protein Modification (Phosphorylation, dephosphorylation, Prenylation, Ubiquitination, Proteolysis)			
TCDD-inducible poly(ADP-ribose) polymerase (Tiparp),	NM_178892	tttaattgtttactaat	3'UTR
X-linked myotubular myopathy gene 1 (Mtm1),	NM_019926	actaatactaat	3'UTR
activin receptor IIA (Acvr2),	NM_007396	cttaatgtctgtcagaagacactaat	3'UTR
casein kinase II, alpha 1 polypeptide (Csnk2a1),	NM_007788	actaacctaac	3'UTR
Eph receptor A5 (Epha5),	NM_007937	actaattagtttatttaaacagctaat	3'UTR
FMS-like tyrosine kinase 1 (Flt1),	NM_010228	actaacaagaatgtaac	3'UTR
hepatitis A virus cellular receptor 2 (Havcr2),	NM_134250	actaatctcaaatgttttaaagtaat	3'UTR
Hus1 homolog (S. pombe) (Hus1),	NM_008316	tttaattaaactaat	3'UTR
kinase interacting with leukemia-associated gene (stathmin) (Kist),	NM_010633	tttaatcctactaat	3'UTR
mitogen activated protein kinase 9 (Mapk9),	NM_016961	actaatggatgctaac	CDS
mitogen-activated protein kinase kinase kinase kinase 5 (Map4k5),	NM_024275	cataattttgtcaaaatagcactaac	CDS
nuclear receptor coactivator 3 (Ncoa3),	NM_008679	gttaatggagtttcttggactaat	CDS
olfactory receptor 1384 (Olfr1384),	NM_146472	tgtaattttggtctgtcctgtagcactaat	CDS
protein kinase C, theta (Prkcq),	NM_008859	actaatgacatcatccctaat	3'UTR
PTEN induced putative kinase 1 (Pink1),	NM_026880	tgtaatgactaac	3'UTR
retinitis pigmentosa 2 homolog (human) (Rp2h),	NM_133669	actaatgctataat	3'UTR
a disintegrin and metalloproteinase domain 30 (Adam30),	NM_027665	actaatgaatgaatgaaaaataat	3'UTR
Bloom syndrome homolog (human) (Blm),	NM_007550	cataattttagaactaat	CDS
ribosomal protein S6 kinase, polypeptide 5 (Rps6ka5),	NM_153587	actaaccaagcatttgctgtcaaaataat	CDS
transferrin receptor (Tfrc),	NM_011638	actaacaactgattttcataat	CDS
type 1 tumor necrosis factor receptor shedding aminopeptidase	NM_030711	actaacaaaggacaatattaat	3'UTR
huntingtin interacting protein 2 (Hip2),	NM_016786	actaactaggataac	3'UTR
F-box only protein 8 (Fbxo8),	NM_015791	actaattttacagaactttttcttaat	3'UTR
proteasome (prosome, macropain) subunit, alpha type 4 (Psma4),	NM_011966	cataacatctgatgctaacgttctgactaac	CDS
ubiquitin carboxyl-terminal esterase L4 (Uchl4),	NM_033607	tttaatattttcattaacttgactaat	3'UTR
Development and cellular differentiation			
nuclear receptor coactivator 6 (Ncoa6), mRNA	NM_019825	actaatcctcctaac	CDS
peroxisome proliferator activated receptor binding protein	NM_013634	actaataataat	CDS
unc-5 homolog C (C. elegans) (Unc5c), mRNA	NM_009472	tataaccaatactaat	3'UTR
Guanine nucleotide binding protein, alpha 13 (Gna13), mRNA	NM_010303	actaacttaat	3'UTR
four and a half LIM domains 1 (Fhl1), mRNA	NM_010211	cttaacaagactaac	3'UTR
ecotropic viral integration site 2a (Evi2a), mRNA	NM_010161	actaatagaaataac	3'UTR
RAS p21 protein activator 1 (Rasa1), mRNA	NM_145452	actaatccatattgtaac	CDS
fragile X mental retardation syndrome 1 homolog (Fmr1), mRNA	NM_008031	actaacttctaaaactgtaat	3'UTR
smoothened homolog (Drosophila) (Smo), mRNA	NM_176996	actaacctaat	CDS
attractin (Atrn), mRNA	NM_009730	actaactggctcttctggatttgtaac	CDS
cadherin EGF LAG seven-pass G-type receptor 1 (Celsr1), mRNA	NM_009886	cgtaactgtcagtgacactaac	CDS
eyes absent 4 homolog (Drosophila) (Eya4), mRNA	NM_010167	actaacataac	CDS
homeo box C5 (Hoxc5), mRNA	NM_175730	actaaccttgtaac	3'UTR
male enhanced antigen 1 (Mea1), mRNA	NM_010787	actaacaactctggtcttaac	3'UTR
seven in absentia 1 (Siah1), mRNA	NM_009172	actaatatatttaaaaataat	3'UTR
sine oculis-related homeobox 6 homolog (Drosophila) (Six6), mRNA	NM_011384	cgtaattgctttgtgactaat	3'UTR
transcription factor EC (Tcfec), mRNA	NM_031198	actaacaaatttggtgataat	3'UTR
wingless related MMTV integration site 2b (Wnt2b), mRNA	NM_009520	actaatatttgtgtaac	3'UTR
paired box gene 9 (Pax9), mRNA	NM_011041	tttaattcaccattaggaaactaat	3'UTR
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	NM_029094	actaattttgttaat	3'UTR
COP9 (constitutive photomorphogenic) homolog, subunit 3	NM_011991	actaacccccatcttgaggtaac	3'UTR
cysteine knot superfamily 1, BMP antagonist 1 (Cktsf1b1), mRNA	NM_011824	actaatgggggaggtaac	3'UTR
hephaestin (Heph), mRNA	NM_181273	actaataagtaac	3'UTR
kit ligand (Kitl), mRNA	NM_013598	actaactcaattcttatagtaat	3'UTR
ribosomal protein S6 kinase, polypeptide 1 (Rps6kb1), mRNA	NM_028259	tataataaattttaactaat	3'UTR

endothelin receptor type B (Ednrb), mRNA	NM_007904	actaatctgattgtaac	3'UTR
early growth response 2 (Egr2), mRNA	NM_010118	actaactggagttcagataat	3'UTR
quaking (Qk), mRNA	NM_021881	actaatgtttaat	3'UTR
UDP-glucuronosyltransferase 8 (Ugt8), mRNA	NM_011674	actaactggatgattaagcataagttaat	3'UTR
sirtuin 1 ((silent mating type information regulation 2, homolog) 1	NM_019812	ggtaatgtccaaacaggcccctgagactaat	3'UTR
Ras homolog gene family, member A (Rhoa), mRNA	NM_016802	tgtaactactttataactaac	3'UTR
osteocrin (Ostn), mRNA	NM_198112	actaataaaggatagtataat	3'UTR
glia maturation factor, beta (Gmfb), mRNA	NM_022023	actaatttctataat	3'UTR
nucleoporin 50 (Nup50), mRNA	NM_016714	actaacctaagtgattcttaat	3'UTR
RAB23, member RAS oncogene family (Rab23), mRNA	NM_008999	actaactacatcggtaac	3'UTR
sema domain, immunoglobulin domain (Ig), and GPI membrane anchor,	NM_011352	tttaataatgtaacatattactaat	3'UTR
single-minded 1 (Sim1), mRNA	NM_011376	tttaatttgaaaaaaaactaat	3'UTR
myelin basic protein (Mbp), mRNA	NM_010777	actaacctcggtggaaaaataac	3'UTR
neurofibromatosis 1 (Nf1), mRNA	NM_010897	actaatattttgtaac	3'UTR

3.5 DISCUSSION

We present several lines of evidence that QKI proteins are determinants of glial cell fate. First, the QKI-6 and QKI-7 isoforms induce cell cycle arrest and enhance the maturation of OL precursor cells. Second, retroviruses expressing QKI-6 and QKI-7 induce the development of OL and astrocyte cell lineage *in vivo*. Third, the injection of QKI-6 and QKI-7 adenoviruses in the corpus callosum enhance the local expression of MBP. Lastly, the mRNA half-life and protein levels of the CDK inhibitor $p27^{Kip1}$, a well known OL differentiation factor[35], are up-regulated by QKI-6 and QKI-7 expression. Our findings in addition to genetic evidence of dysmyelination in the qk^{v} mouse, and the biochemical evidence that QKI regulates RNA metabolism of key myelin components [3-6], make a strong case that the QKI proteins are key components for OL development and myelination. Thus, it is becoming clear that the nuclear QKI-5 isoform maintains OLs in an undifferentiated state [4], while the expression of the cytoplasmic QKI-6 and QKI-7 isoforms promotes the differentiation of OLs.

The $p27^{Kip1}$ protein accumulation has been shown to regulate OL differentiation [21-24] and the steady state levels of $p27^{Kip1}$ mRNA remains constant during this process [25]. Our findings extend these observations, as we show that the half-life of the $p27^{Kip1}$ mRNA is stabilized and/or protected during QKI-mediated OL differentiation. The presence of stabilized $p27^{Kip1}$ mRNA would allow increased production of $p27^{Kip1}$ protein, as observed in our experiments. $p27^{Kip1}$ may function in the nucleus as a CDK inhibitor [20] or may regulate actin dynamics and migration[36,37]. $p27^{Kip1}$ is known to be

regulated by protein sequestration, translational control, and ubiquitin-dependent proteolysis[38] and it is possible that these pathways also contribute to $p27^{Kip1}$ protein accumulation in OLs. Recently, the QKI homolog in *C. elegans*, GLD-1, has been shown to protect mRNAs with μORFs (5'-UTR open reading frames) against non-sense mediated decay[39]. The presence of an μORF in the 5'-UTR of the $p27^{Kip1}$ mRNA that controls cell cycle-dependent translation[40] suggests that the QKI-6 and QKI-7 function in a similar manner to GLD-1.

The expression of QKI-6 and QKI-7 by multipotential progenitor cells of the ventricular zone[11], suggest that QKI participate in cell fate determination during mouse embryogenesis. Our studies with the overexpression of QKI-6 and QKI-7 most likely reflect the normal role for these proteins in glial cell fate specification because QKI expressing progenitor cells failed to express neuronal markers as they migrated away from the ventricular zone, consistent with the acquisition of a glial rather than neuronal fate [11]. The phenomenon of cell migration to the corpus callosum after QKI-6 and QKI-7 ectopic expression was striking. Similar *in utero* retroviral experiments were performed with Olig1 and Sonic Hedgehog, leading to the development of OLs [41,42]. However, Olig1 positive cells migrated to the cortical region while Sonic hedgehog positive cells migrated to the corpus callosum.

The presence of $p27^{Kip1}$ in the ventricular zone of mouse embryos at E12.5[43], supports our model that QKI-6 and QKI-7 favor OL differentiation via $p27^{Kip1}$. Since $p27^{Kip1}$ expression is sufficient to promote OL cell cycle arrest, but not OL differentiation[22,23,44], QKI-6 and QKI-7 are most likely also regulating other mRNAs during OL differentiation including those shown in Supplementary Figure 1. The fact that

the QKI proteins are tyrosine phosphorylated[45] and belong to the family of signal transduction and activator of RNA metabolism (STAR) proteins[3] suggest that signaling cascades induced by growth factors and mitogens may also regulate this pathway. The characterization of the link between cell signaling and RNA metabolism in OLs, will provide a better understanding of glial cell fate specification and myelination.

3.6 METHODS

Antibodies.

The mouse monoclonal antibodies anti-GalC and anti-CNPase were from Chemicon (Temecula, CA). The monoclonal anti-$p27^{Kip1}$ was purchased Upstate Biotech (Upstate, NY). $p21^{Cip1}$ was from Santa Cruz Biotech. (Santa Cruz, CA). The mouse monoclonal anti-MBP and anti-neurofilament H (NfH) were from Sternberger Monoclonals Inc. (Lutherville, Maryland). The anti-β-actin, anti-GFAP, anti-BrdU, anti-PLAP were from Sigma-Aldrich (St. Louis, MO). Specific anti-QKI-6 and anti-QKI-7 were generated by immunizing rabbits with KLH conjugated KEYPIEPSGVLGMAFPTKG for anti-QKI-6
and KGGPIEPSGVLEWIEMPVMPDISAH for anti-QKI-7 antibodies. The pan anti-QKI antibody has been reported previously[10]. The goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to Alex546 and Alexa488 were from Molecular Probes.

Primary rat oligodendrocyte cultures and adenovirus infections.

Cultures of rat OPCs were generated as described[46]. OPCs were plated on 6-well dishes at a density of 15 x 10^3 cells/cm^2. In order to expand their numbers and prevent

differentiation, the cultures were grown in media containing 2.5 ng/mL basic fibroblast growth factor (Peprotec) and platelet-derived growth factor AA for 4 days. The cultures were infected with the indicated adenoviruses as described previously [47]. A multiplicity of infection (MOI) of 10 was used for all adenovirus infection and was sufficient to infect about 95% of the cells as judged by GFP positive cells. After infection, the cells were grown in differentiation media containing T3 at 40 ng/ml for 3 (Fig. 3-3) or 6 (Fig. 3-2) days. Oligodendrocytes processes analysis was performed as described [27] and for statistical analysis, 30 cells from three coverslips of each adenovirus transduction experiment were randomly selected. All data were expressed as mean +/- standard deviation of the mean. The student's *t* test was performed to determine the statistical significance of the difference between groups.

Gain-of-function gene expression *in vivo.*

New born mice were injected with adenoviruses as described previously[4]. Retroviral injections were performed with CD1-timed pregnant mice that were obtained from Charles River Laboratory. Virus preparation was performed as previously described[34]. Stock of CLE retrovirus (control), or CLE-QKI-6 and CLE-QKI-7 were injected in the E11.5 embryo mouse brain through the uterine horn of the pregnant mother in accordance with a protocol approved by the Animal Care Committee at McGill University. A more detailed protocol is available on-line from Connie L. Cepko (http://genetics.med.harvard.edu/~cepko/lineage/). A titer of 1 x 10^7 pfu/ml was injected. At postnatal day 11 (P11), brains were processed for 20 μm cryosections after PBS and 4% paraformaldehyde intracardial perfusion, as described previously[4]. Quantification of cell types in CLE and CLE-QKI transduced brains was determined by morphology and

immunostaining by using specific antibodies against PLAP, NF-H, MBP, GFAP and CNPase. In each case, approximately 200 cells from 4 brains with each retrovirus were scored for the presence of OLs, astrocytes and neurons.

Electrophoretic mobility shift assay.

Complexes between recombinant QKI-6 and QKI-7 with segments of the $p27^{Kip1}$ mRNA were monitored using a gel mobility shift assay. A constant concentration of ^{32}P-labeled $p27^{Kip1}$ RNA (100 pmoles) was incubated with various concentrations of GST-QKI-6 or GST-QKI-7 in 20 mM HEPES (pH 7.6), 330 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg/ml tRNA, 5 mg/ml heparin and 0.01% NP-40 (Sigma). The reactions of 20 μl were incubated at RT for 1 hr prior to adding 2.2 μl of RNA loading dye (glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol). A portion (10 μl) of each sample was separated on a 8% native acrylamide gel.

RNA preparation and purification.

Synthetic RNAs corresponding to sequences in the $p27^{Kip1}$ mRNA were prepared by runoff *in vitro* transcription in the presence of ^{32}P-α-UTP using T7 MegaShortscript (Ambion) according to the manufacturer's protocol. Synthetic ^{32}P -labeled RNAs were gel purified before use. Substrates for T7 RNA polymerase were : T7-521-550 5'GCT CCC GTT AGA CAC TCT CAC GTT T GA CAT CCT ATA GTG AGT CGT ATT AAA TT'3, T7-618-647 5'GGG TTA GTT CTT CAT GAT TGA CCG GGC CGA CCT ATA GTG AGT CGT ATT AAA TT'3 and T7-864-893 5'GAT GAG TTA ACG TCG TAG TTA CGC TGC GTC CCC TAT AGT GAG TCG TAT TAA ATT'3.

Assessment of mRNA stability.

Primary rat OLs transduced with AdGFP control or AdQKI-6/AdQKI-7 for 48 hr were allowed to differentiate for 3 days in differentiation media. Upon the addition of actinomycin D (5 μg/ml), total RNA was extracted from OLs at different time points (0, 3, 6, 18, 24 and 28 hrs) and subjected to Northern blot analysis as described previously[48]. The ^{32}P-probes utilized for Northern blot analysis were prepared from the mouse $p27^{Kip1}$ cDNA (gift from Tony Hunter, Salk Institute) and the rat GAPDH DNA fragment was amplified by PCR from rat OPCs.

In vivo RNA binding assay.

Untreated primary rat OLs or primary rat OLs transduced with AdQKI-6 and AdQKI-7 for 48 hr and allowed to differentiate for 3 days in differentiation media were harvested in lysis buffer consisting of 0.1% Triton X 100, 150 mM NaCl, 50 mM Tris HCl (pH 7.4), 1 mg/ml heparin (Sigma), and 0.5U/μl RNasin (Promega). The cell lysates were immunoprecipitated with either normal rabbit serum (NRS) as a negative control, anti-QKI-6, QKI-7, or anti-myc 9E10 antibody. The bound RNAs were extracted and purified using TRIZOL (Invitrogen) and the detection of the $p27^{Kip1}$ mRNA was performed by using RT-PCR. DNA oligonucleotides used for $p27^{Kip1}$ detection were 5'-AGC TTG CCC GAG TTC TAC-3' and 5'-TCC ACA GTG CCA GCA TTC-3' and for GAPDH detection were 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3'.

Cell cycle analysis of OPCs.

Proliferating OPCs were infected for 24 hr with control AdGFP, AdQKI-6 and AdQKI-7, and the cells were either left untreated or pulsed for 14 hr with 10μM BrdU. The BrdU-labeled cells were collected, fixed in 70% ethanol for 20 min, pelleted, denatured with 1N HCl for 30 min at RT and neutralized with $NaBO_4$ (0.1M). The cells were pelleted by centrifugation resuspended in PBS/BSA/Tween 20, and incubated for 1h with a monoclonal anti-BrdU antibody (Chemicon Inc.). This was followed by a Cy5-labeled anti-mouse secondary antibody (1:500, Molecular Probes), resuspended in PBS containing 10 □g/ml propidium iodide and analyzed by flow cytometry. The cells were analyzed using a FACScalibur flow cytometer (Becton Dickinson). For BrdU labeling on OPC cultured on coverslips, infected OPCs were pulsed for 8 hr with BrdU and fixed with 4% paraformaldehyde in phosphate-buffer saline and visualized by immunofluorescence.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Chapter 4

QUAKING RNA Binding Proteins promote Schwann Cell Differentiation and Myelination

By

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4.1 Preface

The role of RNA binding proteins in the process of myelination is unknown. The *quaking* locus expresses a family of RNA binding proteins containing several spliced isoforms that are not expressed in the *quaking viable* mice. *Quaking viable* mice have uncompacted myelin in their peripheral nervous system. Here we show that the expression of two QKI isoforms, normally expressed during myelination, inhibit the proliferation of Schwann cells by inducing $p27^{Kip1}$, a cell cycle inhibitor implicated in glial cells differentiation. In addition, QKI isoforms modulate the protein levels of Krox-20, a transcription factor important for myelination. Moreover, the QKI-6 and QKI-7 isoforms induce the differentiation of Schwann cells by promoting myelination of DRG-neurons in culture. Furthermore, morphological analysis of the myelin sheaths by electronic and confocal microscopy revealed an increased number of peripheral myelinated fibers as well as an increased number of myelin layers. These data demonstrate that the QKI RNA proteins affect myelination by regulating the mRNA of $p27^{Kip1}$ and Krox-20

4.2 INTRODUCTION

Mutant *quaking* viable mice (qk^V) develop a tremor phenotype in their hind limbs 10 days after birth, resulting from severe myelination defects. Genetically, *quaking* viable mice contain a deletion in the promoter/enhancer region of the *quaking* gene. The *quaking* gene is alternatively spliced, producing QUAKING isoforms that differ in their C-terminal amino acid sequence. Oligodendrocytes and Schwann cells of normal mice express 3 major *qkI* mRNAs of 5, 6 and 7 kb which encode a family of alternatively spliced RNA binding proteins QKI-5, QKI-6, and QKI-7. The promoter deletion observed in qk^v mice prevents the expression of QKI-6 and QKI-7 isoforms in oligodendrocytes and Schwann cells resulting in hypomyelination (Hogan and Greenfield, 1984; Hardy, 1998). The QKI isoforms dimerize (Chen et al., 1997; Chen and Richard, 1998) and their balanced expression is required for proper myelination (Larocque et al., 2002). The QKI-5 isoform is nuclear, whereas the QKI-6 and QKI-7 isoforms are predominantly cytoplasmic in location (Wu et al., 1999). The QKI isoforms contain a single KH RNA-binding domain, which suggests a role in RNA metabolism. Although the dysmyelinating phenotype of the mutant mice suggests a role in myelination, the function of the QKI proteins in myelination remains to be more characterized. The qk^v mice were identified forty years ago (Sidman et al., 1964), and later studies have characterized the CNS hypomyelinating defect (Barbarese, 1991; Larocque et al., 2002; Lu et al., 2003). In particular, QKI has been shown to selectively interact with the 3' untranslated region (3'-UTR) of the mRNA encoding the myelin basic protein (MBP) (Li et al., 2000; Larocque et al., 2002). Previous studies showed that QKI-5 overexpression disrupt the nuclear to cytoplasmic ratio of QKI proteins in oligodendrocytes, leading to the nuclear retention of the MBP mRNAs and

oligodendrocytes maturation defect (Larocque et al., 2002). Studying the function of QKI-5 expression provides the biochemical explanation of the qk^v mice dysmyelinating phenotype in the CNS. However, the reduction of *qkI* mRNA in the qk^v mouse's peripheral nervous system (PNS) is even more pronounced than that observed in the CNS (Lu et al., 2003).

The vertebrate PNS is the essential connection of motor axons, sensory, sympathetic, and parasympathetic nerves to the central nervous system (CNS). Peripheral nerves consist of Schwann cells that establish close contact with axons. Neural crest-derived cells associate with the sensory axons growing out of the dorsal root ganglia (DRG) and with the CNS motor axons, and differentiate into Schwann cells precursor cells. These cells proliferate during their migration along the nerves and then escape from the cell cycle followed by up-regulation of transcription factors and myelin proteins, forming compact myelin around the peripheral nerves. Peripheral myelination in mice starts around birth. Later stages of myelinogenesis, such as Schwann cell differentiation and the axonal wrapping by Schwann cells are less understood. During the (PNS) development, both transcriptional and posttranscriptional mechanisms may be used to regulate the differentiation of neural and glial cell lineages (Ross et al., 2003). Posttranscriptional control of gene expression is mainly regulated by RNA binding proteins, which bind to the nascent mRNA, in the 5 and 3' untranslated region (UTR). The interaction of these proteins on RNA affect RNA metabolic processes, including translation, RNA transport, splicing and RNA stability (Darnell, 2002). A typical KH containing protein is the RNA-binding protein QKI.

In the PNS of the *qk*^{v} mouse, sciatic nerve axons exhibit a separation of intraperiod lines in the myelin sheath, and a variation of Schmidt-Lanterman incisures (Samorajski et al., 1970; Berger, 1971) (Suzuki and Zagoren, 1976). Ventral root axons display a dilation of the periaxonal space of myelinated fibers and failure of Schwann cells to convert mesaxon membranes to compact myelin (Trapp et al., 1984; Trapp, 1988). These observations allow us to hypothesize that QKI proteins might be implicated in Schwann cell differentiation. Despite these observations, the exact mechanism by which QKI proteins participate in the peripheral nervous system (PNS) remains unknown.

The fact that *qk*^{v} mice peripheral nerves display abnormal myelin sheaths led us to hypothesize that the missing isoforms (QKI-6 and QKI-7) or the imbalance of QKI proteins may be implicated in Schwann cell differentiation. In this study we report that the cytoplasmic QKI isoforms QKI-6 and QKI-7 induce Schwann cell maturation as observed by p27^{Kip1}, MBP and Krox-20 immunoblotting. Electron microscopic analysis demonstrated that the QKI-6 and QKI-7-transduced Schwann cells promoted thicker myelin. Interestingly, the nuclear QKI-5 isoform was a negative regulator of myelination and prevented Schwann cell maturation. In addition, QKI-5 transduced Schwann cells had thinner myelin sheath. These data demonstrate that the QKI proteins are regulators of myelination.

4.3 MATERIALS AND METHODS

DRG-Schwann cells co-cultures

Purified DRGN/SC co-cultures were prepared using methods described previously (Giasson and Mushynski, 1996). DRGs were obtained from Sprague-Dawley rat embryos at 15-16 days gestation from Charles River Laboratories (St. Constant, QC, Canada). Embryos were collected in Leibovitz's (L-15) medium, the spinal column was dissected from each embryo and DRGs were plucked from the spinal cords and collected in fresh L-15 medium. DRGs were dissociated with trypsin (0.025 % in Hank's BSS) at 37 ^{o}C for 15 min, followed by treatment with soybean trypsin inhibitor (5 mg/ml in L-15). The dissociated cells were resuspended for plating in defined medium consisting of DMEM/F12 containing N1 supplement, 0.09% BSA, 12 ng/ml 2.5 S NGF and antibiotics (penicillin/streptomycin) and cells from 3 DRGs were plated onto rat tail collagen-coated 24-well plates. Cell cultures were maintained in serum-free N1 medium until their use after 21 days in culture. Myelination was initiated by adding fresh media containing 50 μg/ml of ascorbic acid. At the same time, co-cultures were infected with recombinant adenoviruses as previously described (Larocque et al., 2002) at m.o.i of 50 and this was sufficient to infect about 90% of the cells as judged by GFP positive cells. Five days after infections, cells were used for immunostaining experiments and/or immunoblot experiments. In each adenovirus treatment, 3 different wells were used by experiment. Antibodies used were for MBPs (Rabbit anti-MBP; 1:500), $p27^{Kip1}$ (Mouse anti-p27,

1:1000, Chemicon) (Rabbit Krox-20, 1:200, Santa Cruz Inc.) and for β–actin (Mouse anti-β-actin, 1:500, Chemicon). Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to Alex546 (Molecular Probe) and Cy5 (Jackson Immunoresearch) were used for double-labeling experiments. Confocal analysis was carried out on a Zeiss microscope. The experimental protocol for preparation of primary cell cultures was approved by the McGill University Animal Care Committee and meets the guidelines of the Canadian Council on Animal Care.

Adenovirus Construction and Infection

The co-cultures were infected with the indicated adenovirus co-expressing QKI-5, QKI-6 and QKI-7 driven by the TR5 (Tet-Off) promoter and GFP is under the control of the CMV promoter (Pilotte et al., 2001). The control adenovirus (AdGFP) is a non-insert adenovirus expressing only the GFP from the CMV promoter. Expression was induced by using the adenovirus AdCMV-tTA that expresses the transactivator tTA (Massie et al., 1998a). A multiplicity of infection (MOI) of 50 was sufficient to infect about 90% of the cells as judged by GFP positive cells.

Electron Microscopy

Adenoviruses treated co-culture were rinsed in PBS, fixed in 2.5% gluteraldehyde in 15mM sodium cacodylate (pH 7.4) for 60 min, and postfixed for 1 hr in 1% osmium tetroxide and embedded for EM. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and examined by transmission electron microscopy (McGill University, Anatomy and Cell biology department). A population of 30 axons by treatment from 3 replicates experimental sets were analyzed for the myelin layers

analysis. The statistical significance of myelin lamellae for each QKI adenoviruses treatment versus the control AdGFP was tested by two-samples t test ANOVA for assumption test and Tukey-Kramer Multiple Comparisons Test for P value of 0.001. InStat3 software (GraphPad Inc.) was used for graph and statistic analysis.

Bromodeoxyuridine incorporation and cell cycle analysis

For analysis of BrdU incorporation using immunofluorescence staining, coculture containing Schwann cells and DRG neurons cells were infected for 24 hours with adenoviruses (m.o.i:50) expressing the different QK isoforms or GFP control. Culture progenitors were given a 16 hours pulse with 10μM of 5-bromo-2'-deoxyuridine (BrdU) and then fixed in 4% paraformaldehyde. Cells were permeabilized with ice cold acetone/methanol (1:1). After 30 min in 1N HCl, cells were incubated with monoclonal anti-BrdU antibody (1:100, Chemicon International) in PBS + 0.1%Triton + 5% goat serum for 3 hours. Rabit Alexa 546 (Molecular Probes) secondary antibodies diluted 1:1000 in PBS;0.1% Triton were used for detection.

4.4 RESULTS

Cell cycle arrest promoted by the QKI isoforms

Previous reports have demonstrated that *qk*^{v} mice have a dramatic reduction of QKI-6 and QKI-7 spliced isoforms in Schwann cells (Hardy et al., 1996; Lu et al., 2003). To investigate the functional significance of QKI proteins on peripheral myelination, we used an *in vitro* rat cell culture model consisting of Schwann cells co-cultured with neurons from the dorsal root ganglia (DRG). The co-culture system reflects the real context of glial cells in the proximity of neurons, as occurs *in vivo* (Fragoso et al., 2003).

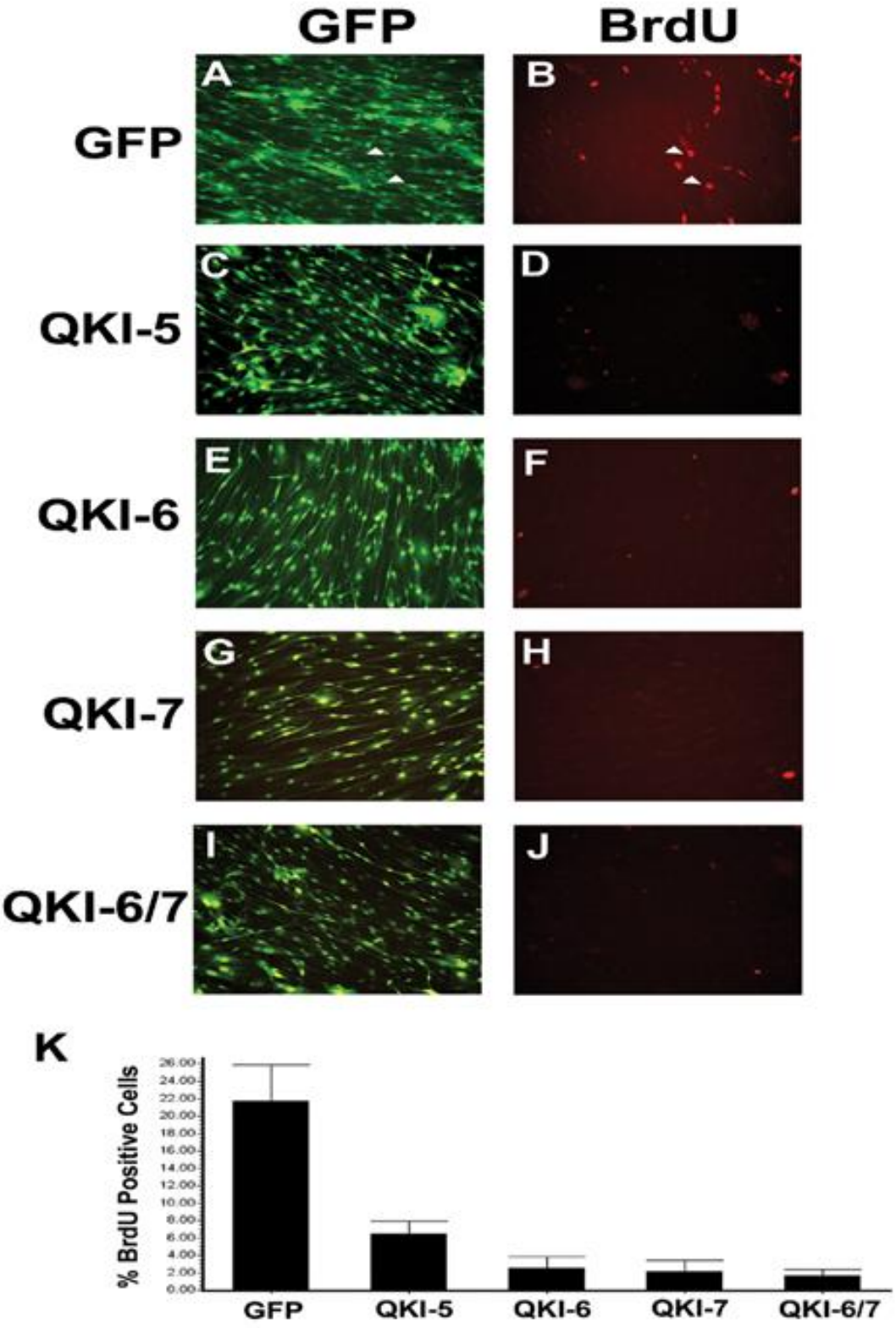
In the mouse, the formation of peripheral myelin is initiated around the time of birth by Schwann cells. At E12.5, neural crest stem cells make contact with sensory axons sprouting from the dorsal root ganglia (DRG) and with motor axons, and differentiate into Schwann cell precursors. These cells proliferate during their migration along the nerves. After the migration, Schwann cells exit from the cell cycle and start their differentiation process to generate the myelin sheath which enwrapps the axons to insulate the axons (Lobsiger et al., 2002). Schwann cell precursors have to progress through three fundamental stages in order to generate a differentiated and myelinating cell. First, the cell has to be properly positioned on the peripheral nerve. Second, the environment (growth factors, hormones, ligand) has to send the cell a signal to decide whether to self-renew or to undergo mitotic arrest. Third, the cell must express certain genes which will promote cell cycle withdrawal and the differentiation.

To determine whether the QKI isoforms influence cell cycle progression, the co-cultures were transduced with control AdGFP or AdQKI-5, AdQKI-6 and AdQKI-7 adenoviruses. The adenoviruses constitutively express GFP, which serves as a marker for infection. Approximately 95% of the Schwann cells were transduced as visualized by the presence of GFP. The proliferation of the Schwann cells was assessed using a 16hr bromodeoxyuridine (BrdU) pulse and subsequent staining with anti-BrdU antibodies. The Schwann cells transduced with the QKI isoforms had a visibly lower number of proliferative cells compared with the AdGFP-infected cells (Figure 1, panel B). In parallel, Schwann cells overexpressing QKI-5 displayed more proliferating cells as compared to those overexpressing QKI-6 and QKI-7, demonstrating that the nuclear QKI-5 isoform may regulate Schwann cells differently than the cytoplasmic QKI-6 and QKI-7 isoforms (Figure 1F, H, J). Quantitative analysis of the BrdU positive cells from three separate experiments confirmed that QKI-overexpressing cells were decreased in their proliferation capacity comparing to the control AdGFP(Figure 1K). The percentage of mitotic Schwann cells decreased to 6% for QKI-5, 2% for QKI-6 and QKI-7, and 1.7% for the combination of QKI-6 and QKI-7 infected Schwann cells.

Figure 4-1. QKI Proteins Inhibit Schwann Cell Proliferation.

Co-culture of non-myelinating Schwann cells and DRG-neurons were infected for 48 hr with adenoviruses coding for QKI-5 (panels C and D), QKI-6 (panels E and F), QKI-7 (panels G and H), the combination of QKI-6/7 (panels I and J), control GFP adenovirus (panels A and B), and incubated with BrdU for 16 hrs. The green cells denote the infected cells (A, C, E, G and I). Incorporation of BrdU was detected with an anti-BrdU antibody followed by a goat anti-mouse conjugated to Alexa 546 (in red). Arrowheads denote the higher number of GFP positive Schwann cells that are also BrdU positive in the controls infected with GFP-adenovirus.

(K) The overexpression of each QKI isoform causes inhibition of Schwann cell proliferation. The graph shows percentage of GFP positive cells that incorporated BrdU. The proliferation was quantitated from $n > 500$ cells from three different experiments (*$p<0.001$, ANOVA). The error bars represent +/- standard deviation of the mean.

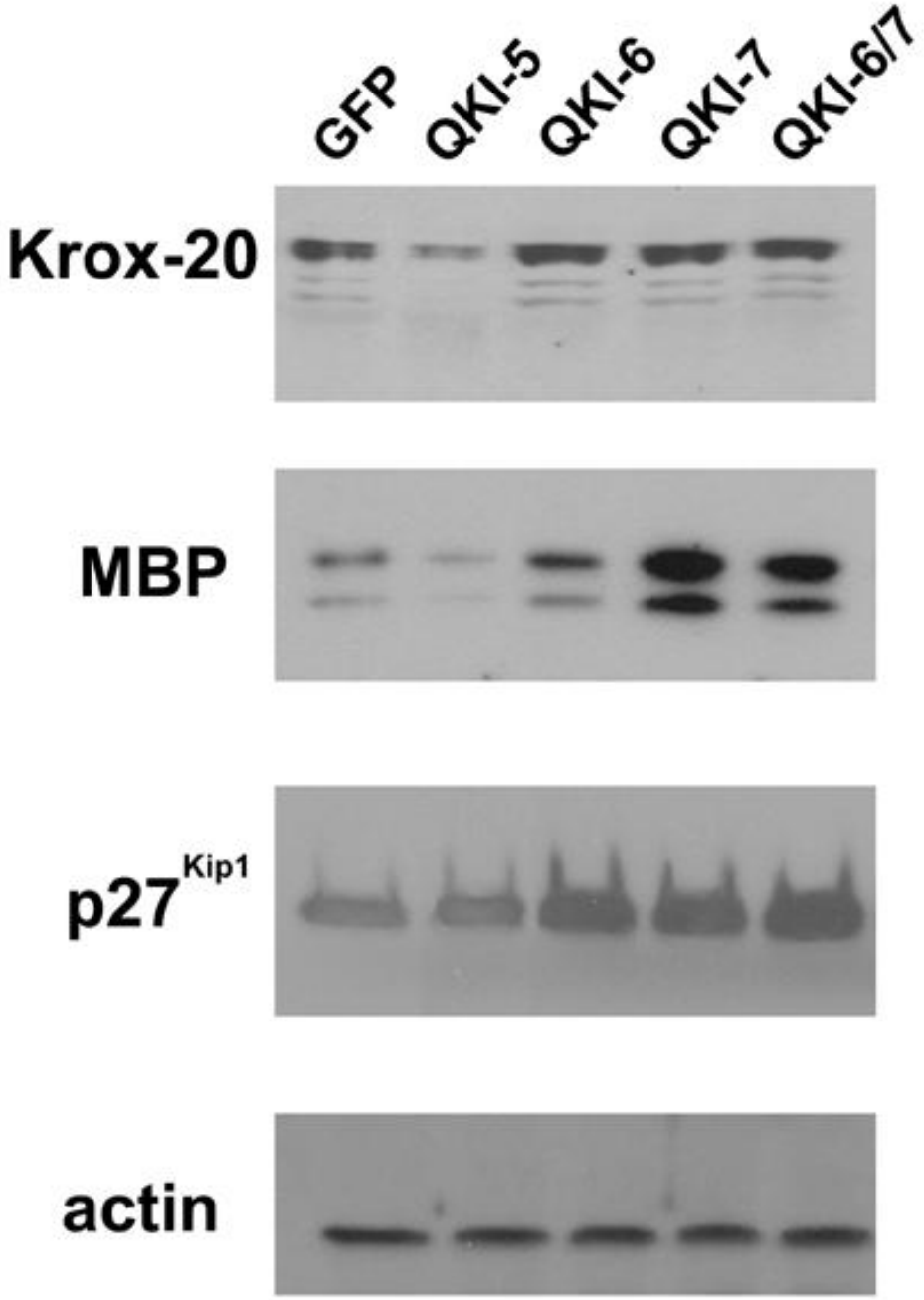

GFP
BrdU
A
B
GFP
C
D
QKI-5
E
F
QKI-6
G
H
QKI-7
I
J
QKI-6/7
K
% BrdU Positive Cells
26.00
24.00
22.00
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
GFP
QKI-5
QKI-6
QKI-7
QKI-6/7

QKI-6 and QKI-7 promote Schwann cell differentiation factors

There are very few factors which are transiently up-regulated in Schwann cells during early stages of myelination. First, Schwann cells require proliferation arrest and one key factor is the cyclin dependant kinase inhibitor $p27^{Kip1}$ (Tikoo et al., 2000; Wei et al., 2003). Second, transcription factors have to be induced for an appropriate expression of myelin-specific genes, and this expression must occur at a specific time during Schwann cell differentiation. Krox-20 is a zinc finger transcription factor known to be a key player in Schwann cell differentiation and peripheral myelination (Topilko et al., 1994). Krox-20 also regulates the expression of the cell cycle inhibitor $p27^{Kip1}$ (Tikoo et al., 2000; Parkinson et al., 2004) and other myelin genes in Schwann cells (Nagarajan et al., 2001; Jessen and Mirsky, 2002). Myelin Basic Protein is among those myelin gene induced by Krox-20. To gain further insight as to whether the proteins levels were affected in Schwann cells by the expression of the QKI isoforms, we performed immunoblotting analyses using anti-Krox-20, $p27^{Kip1}$ and myelin basic protein (MBP) antibodies (Figure 4-2).

QK5 overexpression induced a down-regulation of MBP and Krox-20 protein levels in Schwann cells. The levels of $p27^{Kip1}$ were not increased with the expression of QKI-5, whereas QKI-6 and QKI-7 caused a significant upregulation of Krox-20. These results suggest that QKI-5 is a negative regulator of PNS myelination. Our earlier work also supports this conclusion (Larocque et al., 2002). In contrast, co-cultures transduced with QKI-6, QKI-7 or with the combination of QKI-6/7 had elevated levels of Krox-20, $p27^{Kip1}$ and MBP. These results provide evidence that after QKI-6 and QKI-7 expression, Schwann cells begin to differentiate towards a myelinating phenotype.

Figure 4-2. Krox-20, MBP and $p27^{Kip1}$ proteins expression are downregulated by QKI-5 in Schwann cells and DRG-neurons co-cultures. Cultures were allowed to myelinate for 5 days with ascorbic acid after infection, lysed and the proteins separated by SDS-PAGE, Followed by immunoblotting with anti-Krox-20, anti-MBP, anti-$p27^{Kip1}$ and anti-β-actin antibodies.


GFP
QKI-5
QKI-6
QKI-7
QKI-6/7
Krox-20
MBP
p27Kip1
actin

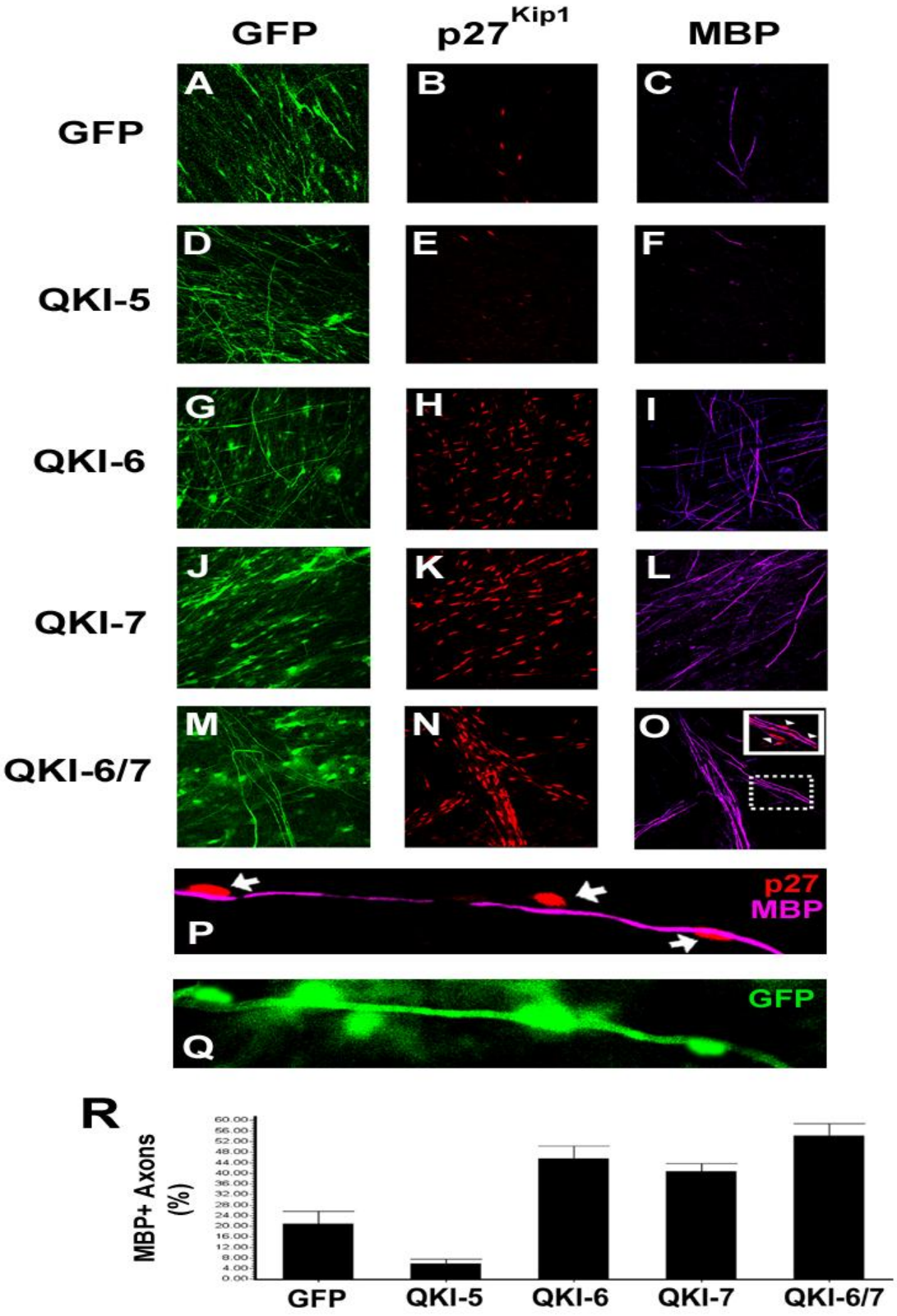
QKI-6 and QKI-7 induce p27^{Kip1} expression in myelinating Schwann cells.

We have recently shown that QKI-6 and QKI-7 isoforms increase the expression of p27^{Kip1} in myelinating oligodendrocytes by stabilizing its transcript (Larocque et al., 2004). However, the role of p27^{Kip1} in Schwann cell myelination is unknown. To assess whether the QKI-6 and QKI-7 isoforms induce p27^{Kip1} in a myelination context, we performed double immunostaining to follow myelinating axons as well as p27^{Kip1} in the nucleus of Schwann cells in culture with DRG neurons (Figure 3). Co-cultures were transduced with control, QKI-5, QKI-6, QKI-7 and QKI-6/7 adenoviruses and myelination was initiated by addition of ascorbic acid, a standard method of inducing myelination in the co-cultures (Fragoso et al., 2003). Five days later, the co-cultures were doubly stained for MBP and the p27^{Kip1}, using specific antibodies and analyzed by confocal microscopy. The number of MBP positive axons was elevated in the QKI-6 and QKI-7 transduced cultures (Figure 3, panels I, L, O) as compared to those transduced with the control GFP adenovirus or QKI-5 (Figure 3, panel C). Interestingly, the myelinating Schwann cells that were wrapped around axons stained positively for p27^{Kip1}, demonstrating for the first time that p27^{Kip1} is a key player in PNS myelination. We also noticed that the QKI-6/7 expression produced more organized myelinated nerve tracts with a directionality (Figure 3, panel N). Confocal microscopy confirmed that p27^{Kip1} positive Schwann cells are in contact with myelinated axons (Figure 3, panel O, P). Quantitatively, the number of the myelinated axons (Figure 3, panel R) was greater in the QKI-6 (45.7% +/- 4.6), QKI-7 (40.8%+/-3.0) and in the combination of QKI-6/7 (54.3%+/-4.4) cultures in comparison to control GFP infected cultures (20.9%+/-4.7). In contrast, the number of MBP positive-axons was low after QKI-5 overexpression

(5.9%+/-1.7), demonstrating that QKI-5-overexpressing Schwann cells are not able to differentiate and myelinate properly, consistent with our previous observations that QKI-5 is a negative regulator of myelination (Larocque et al., 2002).

Figure 4-3. QKI-6 and QKI-7 Overexpression Induce $p27^{Kip1}$ in Schwann Cells, Associated with Myelinated (MBP Positive) Axons.

Co-cultures of Schwann cells and neurons from DRG were infected with adenoviruses coding for QKI-5 (D, E and F), QKI-6 (G, H and I), QKI-7 (J, K and L), combination of QKI-6 and QKI-7 (M, N and O), and control AdGFP (A, B and C). After infection, co-cultures were allowed to myelinate for 5 days with ascorbic acid. Cells were immunostained with anti-$p27^{Kip1}$ antibodies (red), anti-MBP antibodies (purple) and visualized by fluorescence confocal microscopy. The green cells denote infected Schwann cells as well as infected axons. Ectopic expression of QKI-6 (H), QKI-7 (K) and QKI-6/7 combination (N) activate the expression of $p27^{Kip1}$ in the nucleus of Schwann cells. Schwann cells are in the vicinity of MBP+ axons fibers (I, L and O). The inset (O) is an image showing a merge from $p27^{Kip1}$ + Schwann cells and MBP+ axons (arrowheads). (P) Shown is the double staining for $p27^{Kip1}$ (red) and MBP (purple) from a combined infection by QKI-6 and QKI-7 adenoviruses, and demonstrating that $p27^{Kip1}$ Schwann cells are associated with MBP+ axonal internodes (arrows). (Q) GFP+ cells denote the infection by adenoviruses expressing QKI-6 and QKI-7. (R) Graph showing a higher percentage of MBP+ axons after QKI-6 and QKI-7 overexpression. These data are representative images from three experiments. The error bars represent +/- standard deviation of the mean.


GFP
p27Kip1
MBP
GFP
QKI-5
QKI-6
QKI-7
QKI-6/7
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
p27
MBP
Q
GFP
R
MBP+ Axons (%)
60.00
56.00
52.00
48.00
44.00
40.00
36.00
32.00
28.00
24.00
20.00
16.00
12.00
8.00
4.00
0.00
GFP
QKI-5
QKI-6
QKI-7
QKI-6/7

The transcription factor Krox-20 is expressed early during differentiation of Schwann cells (Jaegle et al., 2003), and regulates the expression of an array of myelin genes (Nagarajan et al., 2001). Krox-20 null mice do not form myelin and Schwann cells continue to proliferate (Parkinson et al., 2003; Parkinson et al., 2004). Interestingly, few studies observed the presence of cis-acting sequences in the 3'-UTR of Krox-20 mRNA (Giudicelli et al., 2001; Ghislain et al., 2002). The presence of this 3'-UTR element maintains Krox-20 at low level during embryonic development. Afterward, another study described that How, the *Drosophila* counterpart of the QKI protein, was involved in regulating the tendon cell differentiation. In particular, the isoform How(S) is able to positively regulate the Stripe mRNA stability, and thereby the Stripe protein level. Thus, when the How(S) level is high, the Stripe protein is increased, and the tendon precursor cells become differentiated into mature muscle-bound tendon cells (Nabel-Rosen et al., 2002). This last study leads us to put forward the hypothesis that QKI proteins might use a similar action as the mammalian Stripe homologue, Krox-20. In the literature, there is evidence that Krox-20 is regulated by posttranscriptional mechanisms involving its 3'-UTR and QKI proteins (Nabel-Rosen et al., 2002).

To examine the underlying molecular mechanisms of posttranscriptional control by QKI proteins, recombinant purified QKI proteins were used to perform a SELEX (systematic evolution of ligands by exponential enrichment) experiment (Larocque et al., 2004). Specifically, we established that QKI proteins bind the RNA sequence 5'-CUAAC-3'. This RNA sequence has been found in the 3'-UTR of MBP (Larocque et al., 2002; Ryder and Williamson, 2004), as well as the coding region of $p27^{Kip1}$ mRNA (Larocque et al., 2004). We identified two putative Quaking Response Elements (QRE)

sequences into the 3'-UTR of Krox-20

(5'CUAUUCUAACAUAAAAAAACCACUAACUG-3').

These QRE sequences are distal from the Krox-20 stop codon and are located 1.1 kb downstream of the stop codon (accession number NM_010118).

The QKI-6 and QKI-7 induce thicker myelin in co-cultures.

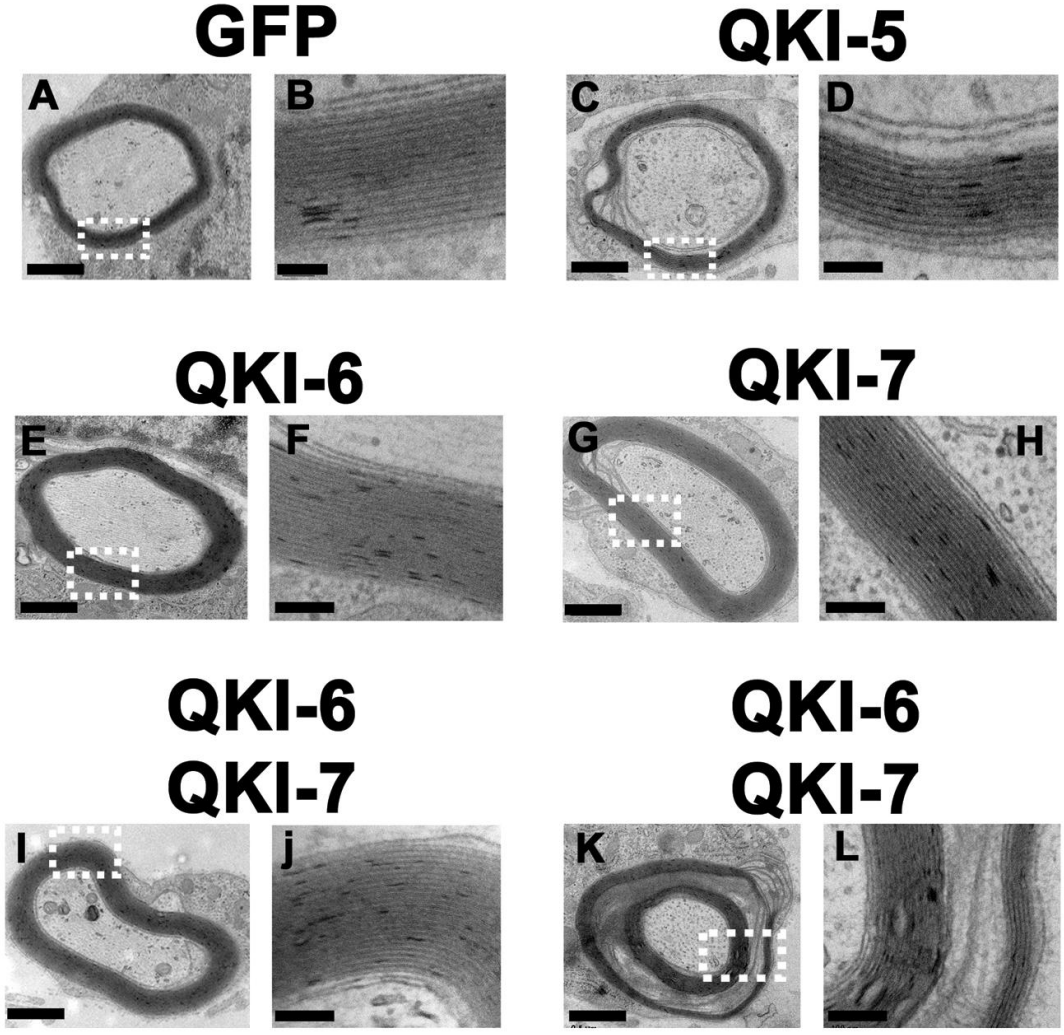
If QKI-6 and QKI-7 promote Schwann cell maturation, then an effect on the myelin sheath should be visible. To further investigate the role of QKI proteins in peripheral myelination, we expressed different QKI isoforms in Schwann cells and examined their ability to myelinate dorsal root ganglion neurons in culture. QKI-6 and QKI-7 expression induces myelin-differentiation genes such as Krox-20, MBP and $p27^{Kip1}$ in Schwann cells. This late induction of Krox-20 permits Schwann cells to develop a promyelinating phenotype. As expected, QKI-5 led to a downregulation of Krox-20 and MBP protein level, suggesting again that QKI-5 is a repressor of Schwann cells differentiation. Co-culture experiments revealed thicker myelin sheaths after QKI-6 and QKI-7 over-expression, as visualized by electron microscopy. Schwann cell / DRG co-cultures were infected with GFP control or QKI adenoviruses for five days and the cells were fixed and analyzed by transmission electron microscopy (Figure 4). The AdGFP control infected cultures contained compact myelin with wraps separated by interperiod lines. For each treatment, 30 different myelinating axons were analysed. On average, 16$\pm$ 1 lamellae were observed with control GFP adenovirus, whereas in the QKI-6 (20+/-1.6), QKI-7 (18+/-1.3) and the QKI-6/7 combination (20 +/-1) contains significantly more lamellae that were wrapped around the axons.

Although compact myelin was observed in QKI-5 infected cultures, the number of myelin lamellae was always lower than14 (average of 11+/-1.5 lamellae). This result is accordance to our previous results showing QKI-5 causing myelin basic protein downregulation *in vivo* and *in vitro* (Larocque et al., 2002). After QKI-6/QKI-7 overexpression, ~21% of the axons contained Schmidt Lanterman incisures and Schwann cell cytoplasm inclusions in interperiodal space (Figure 4, panels K-L)

Figure 4-4. Overexpression of QKI-5 Causes Hypomyelination whereas QKI-6 and QKI-7 Promote Schwann cell Myelination.

Co-cultures of Schwann cells with neurons from DRG were infected with adenoviruses coding for QKI-5 (C and D), QKI-6 (E and F), QKI-7 (G and H), combination of QKI-6 and QKI-7 (I-L), and control adenovirus coding for GFP alone (A and B). Myelin layers were analyzed using electron microscopy. Each picture is a representative image from 30 different axons for each treatment showing a detailed view of the myelin sheath. A control infected cultures show that there are many myelinated axons (A-B). Myelinating sheaths from QKI-5 presented several aberrations (C), and higher magnification (from square inset) reveals uncompacted myelin and smaller number of lamellae (D). Myelin from cultures overexpressing QKI-6 (E-F) or QKI-7 (G-H) was compacted and contained a higher number of wraps (~22). The combination of QKI-6 and QKI-7 produced compact myelin with an increased number of wraps (as high as 28) (I-J). In addition, 21% of the myelinated internodes displayed various abnormalities (K-L), including cytoplasm inclusion in the intraperiodal space. The graph (M) shows the number of lamellae quantified from 30 different myelinated axons in the different group. The average numbers were: 16$\pm$1 for control GFP adenovirus, 11+/-1.5 for QKI-5, 20+/-1.6 for QKI-6, 18+/-1.3 for QKI-7 and 20 +/-1 for the combination of QKI-6 and QKI-7.

The scale bars in A-C-E-G-I-K is 0.5 μm and in B-D-F-H-J-L is 100 nm.


GFP
A
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C
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j
QKI-6
QKI-7
K
L
M
Myelin lamellae number
22.00
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
p<0.001
p<0.001
p<0.001
GFP
QKI-5
QKI-6
QKI-7
QKI-6/7

Surprisingly, the over-expression of QKI-5 reproduced the defect in myelin compaction as seen in qk^{v} mice. In this model, QKI-6 and QKI-7 also promoted an increase protein level of $p27^{Kip1}$ in Schwann cells. Confocal, as well as electron microscopy demonstrate that these $p27^{Kip1}$ positive Schwann cells myelinate the internodes of DRG axons,. Thus, it is becoming clear that the nuclear QKI-5 isoform maintains Schwann cells in an undifferentiated state, while the expression of the cytoplasmic QKI-6 and QKI-7 isoforms promotes the differentiation of Schwann cells.

4.5 DISCUSSION

The role of the QKI isoforms has been studied in CNS myelination, but less is known about their function in PNS myelination. In this paper, we sought to investigate whether the three major isoforms of the RNA-binding protein QKI are involved in the initiation of growth arrest and the differentiation of myelinating Schwann cells. Several pieces of evidence show that QKI proteins are determinants of Schwann cell differentiation. First, we show that the expression of two QKI isoforms (QKI-6 and QKI-7) that are normally expressed during myelination, reduce the proliferation of Schwann cells by inducing $p27^{Kip1}$ and Krox-20. This is in line with the ability of Krox-20 to induce growth arrest (Parkinson et al., 2003).

Second, the QKI-6 and QKI-7 isoforms induce the differentiation of Schwann cells, as indicated by their ability to myelinate DRG-neurons in cultures. Furthermore, morphological analysis of the myelin sheaths by electronic and confocal microscopy revealed an increased number of myelinated fibers and myelin layers when QKI-6 and

QKI-7 are overexpressed. This data demonstrate that the QKI RNA binding proteins affect myelination by regulating the mRNA of $p27^{Kip1}$ and Krox-20

It is noteworthy that Krox-20 null mice have a similar phenotype as qk^{V} mice, both exhibit a tremor phenotype (Schneider-Maunoury et al., 1993). In Krox-20 null mice, the defect is characterized by immature Schwann cells that have become arrested prior to differentiation after having formed a one-to-one relationship with axons and express very low levels of MBP (Topilko et al., 1994; Zorick et al., 1999). A knockout of the *qkI* gene has been produced by the homologous *qkI* gene replacement to β-galactosidase-neomycin fusion gene (Li et al., 2003). The *qkI*-deficient mice die at E9.5 and have open neural tube, thus neuronal and glial cell fate information could not be obtained from these animals. The neural tube defect is similar as that exhibited by the Krox-20 null mice phenotype. Krox-20 deficient mice have severely defective myelination of peripheral nerves. Furthermore, overexpression of Krox-20 in Schwann cells cause a block in Schwann cell proliferation (Parkinson et al., 2004). Our data have shown that QKI-6 and QKI-7 induce cell cycle arrest and an up-regulation of Krox-20. This evidence suggests that the QKI proteins might be linked to Krox-20 regulation.

These observations suggest that QKI-6 and QKI-7 are also implicated in the PNS by promoting Schwann cell differentiation into mature myelinating cells (Figure 4-5). This finding implies that the absence of QKI-6 and QKI-7 and the imbalance in the levels of the QKI isoforms in the Schwann cells of qk^{v} mice is the main cause of the myelination defects seen in the peripheral nerves of these mice. Proteins factors regulating peripheral myelin formation during development remain largely unknown.

Among these factors, QKI-6 and QKI-7 may be downstream elements in the signaling cascade promoting Schwann cells to myelinate axons. Identification of other RNA targets of the QKI proteins and their physiological functions will provide further evidence for QKI's role in myelination. Taken together, the finding that QKI proteins regulate central factors in gliogenesis, such as $p27^{Kip1}$ and Krox-20 mRNAs, suggests that QKI proteins are important for maintaining an appropriate pool of mRNA molecules involved in glial cell development, maintenance and function.

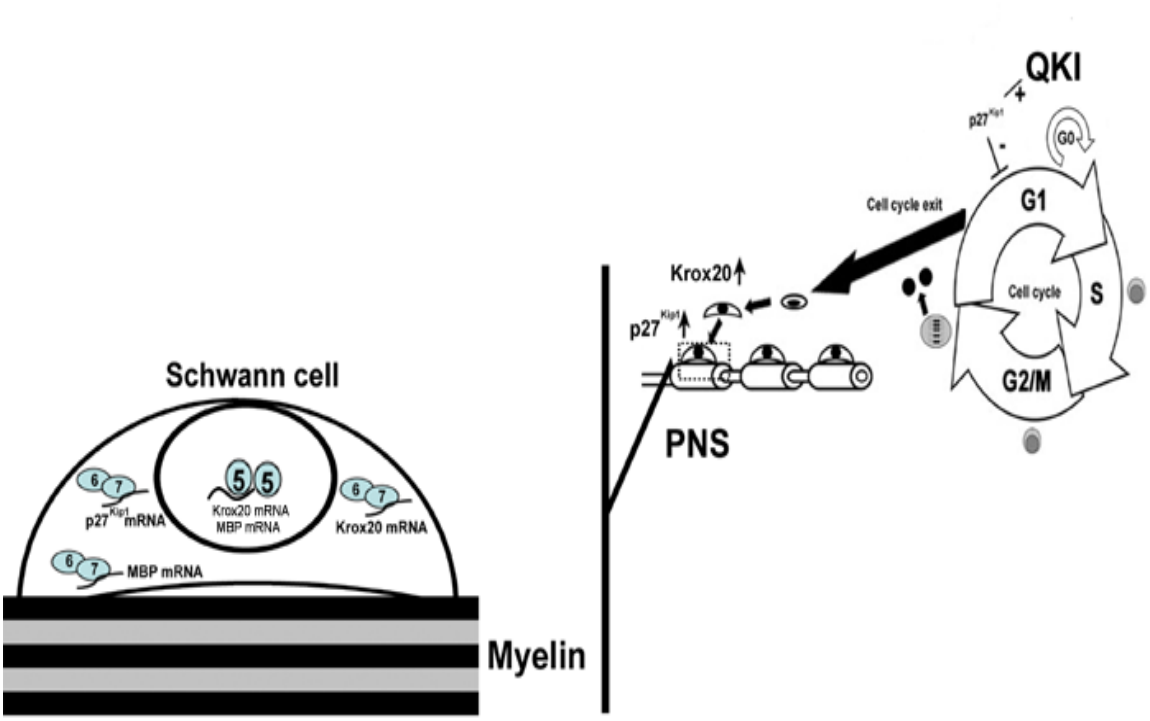

Figure 4-5. Proposed Model for QKI proteins Functions in the PNS.

QKI-6 and QKI-7 activate the expression of $p27^{Kip1}$ and Krox-20. Hence, promoting the cell cycle exit for Schwann cell progenitors. After QKI-6 and QKI-7 expression, Schwann cells become differentiated and synthesize myelin on internode.

Our data demonstrates that the QKI-6 and QKI-7 isoforms promote myelination. In fact, the myelin sheath was thicker but Schmidt-Lanterman incisures were frequently observed in co-cultures transduced with both QKI-6 and QKI-7. The majority of the axons myelinated by QKI-6/7- transduced Schwann cells contained from 16 to 20 layers of myelin sheath. These results demonstrate that QKI-6 and QKI-7 induce myelination, but that an imbalance of the QKI isoforms can lead to defective myelination. We also investigated the ability of the QKI-5 isoform to induce myelination, which is known to prevent oligodendrocyte maturation . We observed that the myelinated axons of nerves in contact with Schwann cells overexpressing QKI-5 contained thinner myelin sheaths as visualized by electron microscopy.

In summary, we have shown a role of QKI proteins in the process of myelination. Within Schwann cells, QKI-6 and QKI-7 are required for the progression of these glial cells into a myelinating cells. The specific role of RNA binding proteins identified in this study opens new areas of research on RNA and proteins interactions regulating Schwann cells development and their interaction with neurons. These findings have important implications not only for understanding how oligodendrocytes and Schwann cells myelinate axons, but also for understanding the mechanism of nerve regeneration after nerve injury and remyelination in demyelination diseases such as multiple sclerosis.

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Chapter 5
GENERAL DISCUSSION

5.1 The Mystery of *qk*^{v} Mice Revealed

The main objective of this thesis was to characterize the function of QKI protein isoforms in glial cells. Moreover, this study of the QKI proteins allows us to explain biochemically the Quaking viable (*qk*^{v}) mice dysmyelinating phenotype. Before this study, the literature describing the *qk*^{v} phenotypes was quite exhaustive. In fact, *qk*^{v} has been studied for over forty years and represents one of the main animal models used for the study of glial cell differentiation and myelination. The *qk*^{v} mouse is known to have thin myelin sheaths in its CNS and PNS. In addition, this mouse does not express the QKI-6 and QKI-7 isoforms in myelinating cells, such as oligodendrocytes and Schwann cells. Anomalies such as variation of Schmidt-Lanterman incisures in PNS myelin, the presence of the Schwann cell periaxonal cytoplasmic collar, the retention of MBP mRNA in the cell body of oligodendrocytes, the low level and mislocalization of MBP proteins and other phenotypes were reported (Barbarese, 1991; Campagnoni et al., 1987; Trapp et al., 1984; Watanabe and Bingle, 1972). In the *qk*^{v} mice, the QKI-6 and QKI-7 isoforms were specifically diminished in oligodendrocytes and Schwann cells (Hardy et al., 1996). It is not clear why QKI-6 and QKI-7 expression is downregulated in oligodendrocytes and Schwann cells of *qk*^{v}, when the expression of QKI-5 is not significantly changed (Hardy et al., 1996). Still, the reduced *qkI* expression is a specific consequence of the *qk*^{v} lesion that is not observed in other hypomyelination mutants. Furthermore, no abnormal

qkI expression was found in the hearts, testes, or astrocytes of the qk^v mice, which suggests that the reduction of *qkI* mRNAs occurred specifically in the myelin-producing cells of the nervous system (Lu et al., 2003).

One hypothesis could be that diminished *qkI* expression results from the deletion of an enhancer that promotes *qkI* transcription specifically in the myelinating glia during active myelinogenesis. In fact, the qk^v deletion break point is located 913 base pairs upstream of the single transcription initiation site of the *qkI* gene promoter (Kondo et al., 1999). Thus, the 913 base pair region may contain a minimal promoter element for the transcription of QKI-5, but not for QKI-6 and QKI-7. Interestingly, there is a potential binding site for Gtx (oligodendrocyte-specific homeodomain protein) downstream of this break point (Awatramani et al., 1997; Kondo et al., 1999). This promoter region is deleted in the qk^v mice genome. One possibility is that an oligodendrocyte or Schwann cell-specific factor, such as Gtx or others, becomes unable to bind to the *qkI* promoter and thus specifically block the expression of QKI-6 and QKI-7 in qk^v mice. Another possibility is that an enhancer sequence for the alternative splicing of the *qkI* gene is missing from the qk^v deletion, and this defect is compensated for in non-glial cells. More work will need to be performed to elucidate the control of *qkI* gene expression and its alternative splicing.

Overall, the qk^v mice dysmyelinating phenotype leads us to hypothesize that QKI proteins are implicated in the control of mRNA homeostasis during myelinogenesis, and that the deficiency of QKI results in the misregulation of its RNA targets, which in turn leads to dysmyelination. This thesis demonstrates that QKI-6 and QKI-7 are important for the release of the MBP mRNAs from the nucleus that are being sequestered by QKI-

5. We confirmed that QKI-6 and QKI-7 can heterodimerize with QKI-5 to export MBP mRNAs maintaining the nuclear-cytoplasmic equilibrium (Chen and Richard, 1998; Larocque et al., 2002). This last hypothesis was investigated in Chapter 2 by changing the balance of QKI isoforms in the cell. The over-expression of QKI-5 in normal oligodendrocytes caused a clear down-regulation of MBP protein levels and MBP mRNA nuclear retention, as displayed in qk^v mice. Two fragments of the 3'-UTR of MBP mRNA were shown to bind QKI. To address the physiological relevance of QKI-MBP mRNA interaction in oligodendrocytes, we reported that oligodendrocyte differentiation is affected in qk^v mice, resulting from the nuclear retention of MBP mRNA. We demonstrated that the overexpression of QKI-5 in normal oligodendrocytes causes the disruption of the nucleo-cytoplasmic equilibrium, and results in the nuclear and perikaryal retention of the MBP mRNAs. This lack of MBP mRNA export to the cytoplasmic processes is one explanation for the qk^v phenotype. We have recreated the nuclear retention of MBP mRNA in primary oligodendrocyte culture obtained from rat brain cerebral hemispheres. *In vivo*, we provided further evidence of the involvement of the QKI proteins in myelination by injecting adenoviruses encoding QKI-5 into the corpus callosum region of the brains of newborn mice. This demonstrated that the over-expression of QKI-5 *in vivo* down-regulates the expression of MBP isoforms. Moreover, we showed that the MBP mRNA export defect leads to a reduction in the MBP protein levels and to improper cellular targeting to the periphery. This finding suggests that QKI participates in myelination by regulating the export of MBP mRNA.

5.2 QKI Proteins are Involved in Gliogenesis

The observation that *qkI*-deficient mice die at E9.5, a stage at which gliogenesis is beginning, suggests that QKI proteins are essential for glial cell fate (Li et al., 2003). During CNS development, both transcriptional and posttranscriptional mechanisms may be used to regulate the differentiation of neural and glial cell lineages. In particular, the expression of a large number of genes is regulated posttranscriptionally during neural development via the interaction of their encoded mRNAs with specific RNA binding proteins (Darnell, 2002; Keene and Tenenbaum, 2002). One of these mRNAs that is tightly regulated is $p27^{Kip1}$. By using RNA binding assay, we established that QKI-6 and QKI-7 bind the RNA sequence 5'-ACUAAC-3' present within the coding region of mammalian $p27^{KIP1}$. Interestingly, this RNA sequence is also found in the MBP 3'-UTR (Larocque et al., 2002; Ryder and Williamson, 2004). This sequence is similar to the consensus hexanucleotide sequence (5'-UACU(C/A)A-3') recognized by the another STAR protein GLD-1 from *C. elegans*, (Ryder et al., 2004). The only difference seems to be that QKI displays a preference for the double adenosine residue after the central uridine. To investigate the physiological relevance of the QKI:$p27^{Kip1}$ mRNA interaction in glial cells, we used adenoviruses expressing QKI-6 and QKI-7 to efficiently transduce primary oligodendrocyte progenitor cells (OPCs). The increased expression of the spliced isoforms QKI-6 and QKI-7 specifically mediates oligodendrocyte differentiation and up-regulates the CDK inhibitor $p27^{Kip1}$. In particular, overexpression of the isoforms QKI-6 and QKI-7 stabilize $p27^{Kip1}$ mRNA, and hence triggers cell cycle arrest in G1 as illustrated in glial progenitors cells.

These observations were in agreement with previous studies showing that the differentiation of oligodendrocytes is $p27^{Kip1}$ dependent. One of these studies was done by Moses Chao and co-workers in New York (Casaccia-Bonnefil et al., 1999) observed that the oligodendrocyte progenitors in $p27^{Kip1}$ -/- mice were also QKI negative when compared to their wild type $p27^{Kip1}$ +/+ counterpart, which stained positive for QKI proteins. This data suggests that QKI proteins may play an important role when the cell cycle inhibitor $p27^{Kip1}$ is present in glial progenitors. Moreover, another study from Martin Raff and co-workers demonstrated that $p27^{Kip1}$ is up-regulated during cell cycle withdrawal and is a result of posttranscriptional mechanisms (Tokumoto et al., 2002). These experiments have led to the hypothesis that QKI proteins may play a role in the posttranscriptional control of $p27^{Kip1}$ expression, thus affecting glial cell differentiation. This was confirmed by *in vivo* by injecting QKI-expressing retroviruses into the telencephalon of mouse embryos. We observed that injection of the QKI-6 and QKI-7, induced the differentiation and migration of multipotential neural progenitor cells into mature oligodendrocytes localized in the corpus callosum.

Another observation that was not described in this thesis is the apoptosis triggered by QKI-7 overexpression in oligodendrocyte precursors cells (Pilotte et al., 2001). It is well established that gliogenesis is dependent on programmed cell death. Growth factor deprivation activates a death signal in glial progenitor cells (Dyer and Cepko, 2001b). When glial cell proliferation becomes deregulated, apoptosis or programmed cell death can eliminate the extra or undifferentiated cells that attempt to re-initiate the cell cycle. These cells undergo apoptosis because they are most probably receiving conflicting

signals to differentiate and proliferate. This could explain why QKI-7 overexpression caused oligodendrocyte differentiation, as well as the apoptosis in a subset of glial cells. The end result in oligodendrocyte cell culture was the majority of cells that received an appropriate level of QKI-7 demonstrated a differentiated phenotype, whereas cells undergoing apoptosis were detached from the cell culture most likely due to an aberrant expression of QKI-7.

5.3 The Role of QKI Proteins in the Peripheral Nervous System

Before this study, little was known about myelin defects in the peripheral nervous systems (PNS) of qk^v mice. In the PNS of the qk^v mouse, sciatic nerve axons exhibit a separation of intraperiod lines in the myelin sheath, and a variation of Schmidt-Lanterman incisures (Berger, 1971; Samorajski et al., 1970; Suzuki and Zagoren, 1976). Ventral root axons display a dilation of the periaxonal space of myelinated fibers and failure of Schwann cells to convert mesaxon membranes to compact myelin (Trapp, 1988; Trapp et al., 1984). These observations allow us to hypothesize that QKI proteins might be implicated in Schwann cell differentiation. Despite these observations, the exact mechanism by which QKI proteins participate in the peripheral nervous system (PNS) remains unknown. Interestingly, the reduction of *qkI* mRNA in the qk^v mouse's PNS is even more pronounced than that observed in the CNS (Lu et al., 2003). Afterward, an interesting publication described that How, the *Drosophila* counterpart of the QKI protein, was involved in regulating the tendon cell differentiation. In particular, the isoform How(S) is able to positively regulate Stripe mRNA stability, and thereby the Stripe protein level. Thus, when the How(S) level is high, the Stripe protein is increased,

and the tendon precursor cells become differentiated into mature muscle-bound tendon cells (Nabel-Rosen et al., 2002). This last study leads us to put forward the hypothesis that QKI proteins might use a similar action as the mammalian Stripe homologue, Krox-20. Interestingly, we have evidence that the mRNA of the typical Schwann cell differentiation factor Krox-20 was shown to bind QKI proteins. The expression of the QKI-6 and QKI-7 in co-culture of Schwann cells and neurons results in an up-regulation of Krox-20 protein level. In addition, co-culture experiments revealed thicker myelin sheaths after QKI-6 and QKI-7 over-expression, as visualized by electron microscopy. Surprisingly, the over-expression of QKI-5 reproduced the reduced myelin compaction as it occurs in qk^{v} mice. In this model, QKI-6 and QKI-7 also promoted an increase level of $p27^{Kip1}$ proteins in Schwann cells. Confocal as well as electron microscopy demonstrate that these $p27^{Kip1}$ positive Schwann cells myelinate the internodes of DRG axons, whereas QKI-5 isoforms downregulate myelination. These observations suggest that QKI-6 and QKI-7 are also implicated in the PNS by promoting Schwann cell differentiation into mature myelinating cells. This finding implies that the absence of QKI-6 and QKI-7, or an imbalance in the level of the QKI isoforms in the Schwann cells of qk^{v} mice is the main cause of the myelination defect in the peripheral nerves of these mice. Protein factors regulating peripheral myelin formation during development remain largely unknown. Among these factors, QKI-6 and QKI-7 might be downstream elements in the signaling cascade helping Schwann cells to myelinate axons. Identification of other RNA targets of the QKI proteins and their physiological functions will provide further evidence for QKI's role in myelination. Taken together, the finding that QKI proteins regulate central factors in gliogenesis, such as $p27^{Kip1}$ and Krox-20 mRNAs, suggests that

QKI proteins are important for maintaining an appropriate pool of mRNA molecules involved in glial cell development, maintenance and function.

5.4 Future Experiments

Future work must be undertaken to elucidate the function of QKI proteins in glial cells. This thesis did not particularly address the role of other QKI isoforms (QKI-5a, QKI-7b, QKI ΔKH and QKI-G). To achieve this, we should perform a similar set of experiments using recombinant adenoviral vectors to over-express these minor isoforms. So far, there is no ideal method to ectopically express a gene in each cell *in vivo*. Still, viral vectors such as adenoviruses or retroviruses remain the best choice to deliver and express transgenes in the targeted cells. However, the spreading and delivery of viral vectors does not reach every cell in the targeted organ (Lowenstein and Castro, 2002). This could be one reason why the injection of the QKI-5 recombinant adenoviruses in the mouse brain did not recreate a *quaking* phenotype. Similarly, the injection of QKI-6 and QKI-7 into the qk^{v} mouse brain did not rescue the *quaking* phenotype (data not shown). Notwithstanding, a transgenic approach should be used to confirm the role of QKI proteins in a particular cell type in the mouse organism. Indeed, the importance of QKI proteins in embryogenesis during the neural tube formation renders the simple gene disruption of the *qkI* locus lethal at mid-gestation (Li et al., 2003). It will be possible to overcome this problem by employing the Cre-lox system, in which Cre recombinase is driven by an MBP promoter, to create oligodendrocyte or Schwann cell-specific *qkI* knockout. Furthermore, the identification of mRNA targets for each QKI isoform at various glial developmental stages may eventually clarify the function of QKI in

regulating myelin development. We believe that other mRNAs that are important for the myelin metabolism are bound by QKI. By using recombinant purified QKI, a SELEX (systematic evolution of ligands by exponential enrichment) methodology has demonstrated that QKI binds the RNA motif UACUAA(C/A) (Chapter 3). A bioinformatic search using the consensus RNA sequence revealed that 222 mRNAs in mouse cDNA databases contained the QKI consensus. Among this exhaustive list, mRNAs such as MBP 3'-UTR, $p27^{KIP1}$ mRNA, and Krox-20 (Egr2) 3'-UTR were confirmed (Chapter 3). Moreover, the list contains a few interesting mRNA targets: the glial maturation factor (GMFB) (Nishiwaki et al., 2001), the POU transcription factor (Jaegle et al., 2003), the RhoA, the homeobox HoxC5, and the QKI mRNA itself. These factors have been described for their implications in glial cell differentiation. In the near future, experiments have to be performed to confirm whether QKI binds those RNA targets *in vivo*. QKI's presence on the list is noteworthy. In fact, immunoprecipitation assays using anti-QKI antibodies and rat oligodendrocytes have shown that QKI binds its own RNA (data not shown). The function of this binding is still unknown; however, an auto-regulation mechanism might be expected.

Another way to regulate the affinity between QKI and its RNA target is by phosphorylation. Interestingly, the QKI proteins are among the STAR (Signal Transduction Activators of RNA) proteins because of their potential link to signal transduction pathways. As illustrated in Figure 1-7, QKI proteins contain a cluster containing five tyrosines (Y285, 288, 290, 292 and 303). Yue Feng and co-workers demonstrated that the interaction between QKI and MBP mRNA was negatively regulated by phosphorylation of QKI proteins (Zhang et al., 2003). Tyrosine kinases such

as Src and $p59^{fyn}$ were found to phosphorylate QKI during early myelin development. Tyrosine phosphorylation of QKI then decreases in the developing myelin, thereby allowing the enhanced interactions between QKI and MBP mRNA and accelerated myelin production. In the near future, it would be interesting to determine the effect of the QKI phosphorylation on different aspects of cell physiology, such as the association of other signaling molecules under growth factor induction, MBP mRNA trafficking, myelin sheath assembly, as well as oligodendrocyte and Schwann cell differentiation.

Another interesting point for future experiments is the role of QKI proteins on glial cell tumors. In fact, the most common primary tumors of the brain are thought to be of glial cell origin (Reifenberger and Louis, 2003). Interestingly, as previously discussed (Section 1.4.1), one study demonstrated that the expression of QKI isoforms is altered in certain human brain tumors (Li et al., 2002). Therefore, since the human *qkI* gene maps to the chromosome 6 locus 6q25-q26 (Li et al., 2002), and this locus is deleted in a variety of human cancers such as glioblastoma it will be interesting to determine the role of QKI in these tumors (Burton et al., 2002). This theory raised the question of whether the *qkI* gene is deleted or mutated in glioma such as oligodendroglioma, schwannoma, or astrocytoma. The ectopic expression of the QKI gene can be envisaged to block the division of such cancer cells due to its involvement in the cell cycle. In the next few years or so, more experiments will have to be performed. Experiments such as using glioblastoma mouse models, QKI over-expression in glioblastoma cell lines as well as gene rescue experiments on human glioblastoma cells containing the 6q25-26 deletion will help to address this important question. We have already performed cell cycle analysis using human glioma, and found that QKI over-expression led to cell cycle arrest

in G1. It would be interesting to look at whether the link between $p27^{Kip1}$ and QKI is also important in this cell growth control.

Overall, the ultimate goal for experimental medicine is remyelination in diseases such as leukodystrophy, Charcot-Marie-Tooth disease and multiple sclerosis (MS). In fact, premyelinating oligodendrocytes are present in the chronic lesions of multiple sclerosis (Chang et al., 2002). Nevertheless, remyelination is limited by the failure of oligodendrocyte progenitor cells to generate mature oligodendrocytes in MS lesion. One useful role of QKI-6 and QKI-7 could be to enhance the differentiation of oligodendrocytes, thereby increasing remyelination. Before moving forward towards this therapeutic approach, we will need to verify whether the oligodendrocytes are QKI positive or not in a MS mouse model, such as the EAE model. Moreover, we will need to know which QKI isoforms are expressed in progenitor cells in the demyelinating lesions. Understanding the cellular function of RNA binding proteins including QKI will lead to effective strategies for enhancing remyelination.

5.5 Proposed Model and Hypothesis

In general, the work presented in this thesis demonstrates that the QKI proteins are implicated in myelination and glial cell differentiation, through the binding of key RNA molecules in glial cells such as MBP, $p27^{Kip1}$, and Krox-20 mRNAs. Taken together, these results provide evidence that RNA binding proteins could play a key role in neural development and glial cell fate, thus helping us gain a better understanding of final cellular differentiation steps like myelination.

The results of experiments described in this thesis present a tentative scheme of the function of QKI proteins in the CNS and the PNS (Figure 5-1). First, during glial cell development in the CNS and the PNS, QKI-6 and QKI-7 proteins stabilize the $p27^{Kip1}$ mRNA and allow the cells to withdraw from the cell cycle. Glial progenitors are arrested in G1 and start the differentiation processes. Interestingly, transcription factors, such as Krox-20 in PNS, become upregulated helping the presence of QKI-6 and QKI-7. In the CNS, at the early stage of oligodendrocyte differentiation, QKI-5 keeps the MBP mRNA in the nucleus until the activation of QKI-6 and QKI-7 expression. The nuclear-cytoplasmic equilibrium of the QKI isoforms allow QKI-6 and QKI-7 to regulate the export of the MBP mRNA out of the nucleus, where it will presumably allow the interaction with other mRNP factors, such as hnRNPA2. This mRNP complex travels down the microtubules to the periphery of the oligodendrocytes. Once they arrive at their destination, the terminal oligodendrocyte processes, the QKI proteins are probably dislodged from the polyribosomes, where the MBP messenger is subsequently translated and integrated into the newly forming myelin sheaths.

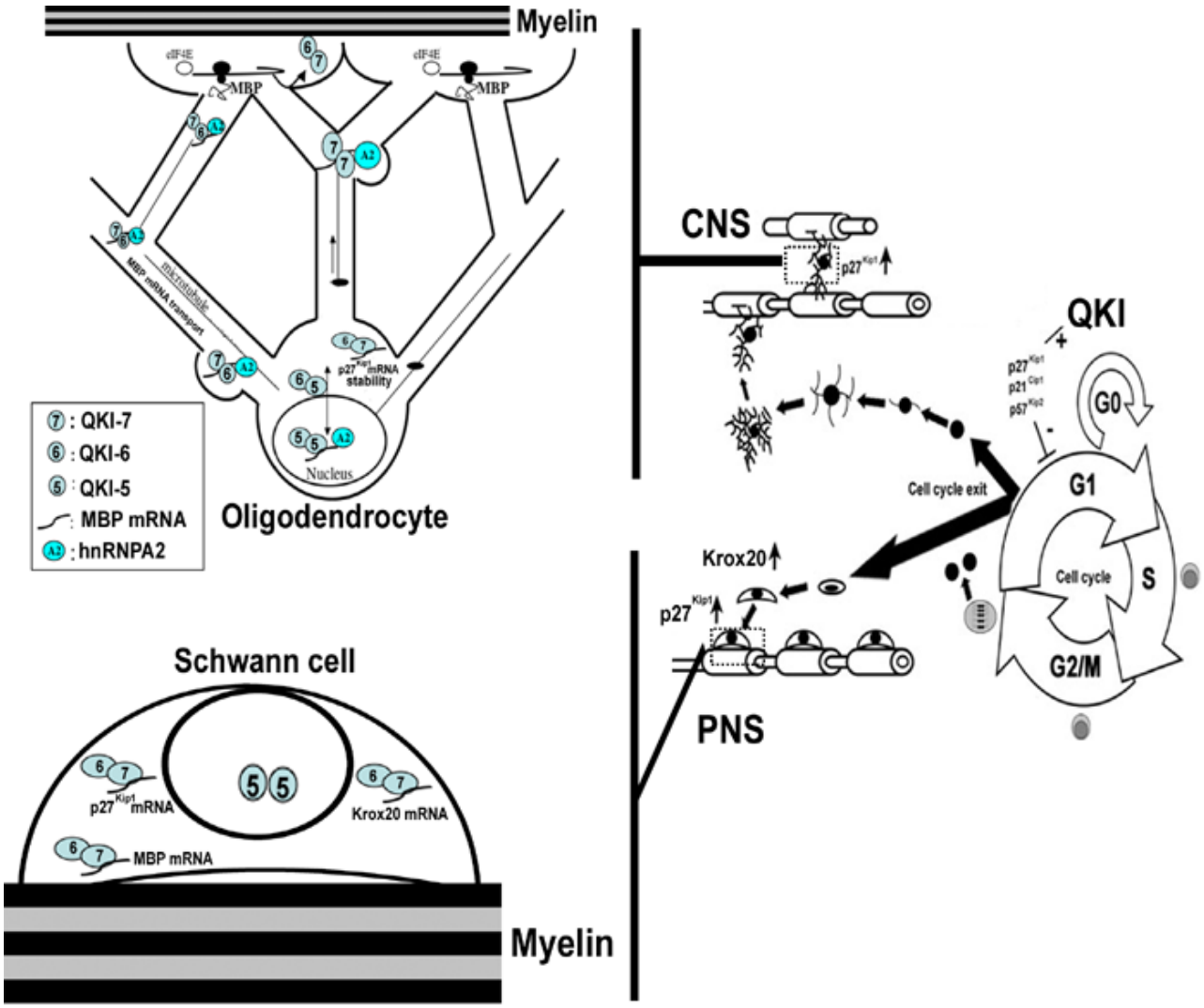

Figure 5-1. Proposed model for QKI protein function in the CNS and PNS. QKI proteins stabilize the mRNA of $p27^{Kip1}$ and induce glial progenitors cells to exit the cell cycle. In the CNS, after QKI-6 and QKI-7 expression, oligodendrocyte progenitor cells become differentiated, acquire branching and synthesize myelin. MBP mRNAs are exported from the nucleus, transported along the oligodendrocyte processes, and translated to the myelin compartment. In the PNS, QKI-6 and QKI-7 promote the differentiation of Schwann cells by up-regulating $p27^{Kip1}$ and Krox-20.

5.6 Concluding Remarks

In conclusion, the work presented in this thesis demonstrates that *qk*^{v} mice fail to properly export the MBP mRNAs, due to the disruption of the QKI isoforms' equilibrium in oligodendrocytes. Moreover, this study illustrates that QKI proteins regulate the export of MBP mRNA, mediate glial cell differentiation by binding p27^{Kip1} and Krox-20 mRNA, and affect myelination.

While many questions remain to be addressed in order to understand the function of QKI in different aspects of RNA metabolism, these findings have important implications not only for the understanding of oligodendrocyte and Schwann cell differentiation and initiation of myelin formation, but also for a better understanding of brain development.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

The following findings presented in this thesis represent original contributions to knowledge:

1- This thesis reviewed for the first time the function of RNA binding proteins and their roles in the RNA metabolism of glial cells, such as oligodendrocytes and Schwann cells.

2 QKI proteins are involved in the nuclear export of the MBP mRNA via interaction with its 3'UTR, and it is demonstrated that mRNA export is affected in *quaking* mice.

3 The overexpression of the spliced isoforms QKI-6 and QKI-7 specifically mediates the oligodendrocyte differentiation and up-regulates the CDK inhibitor $p27^{Kip1}$ by binding and stabilizing its mRNA was reported here for the first time.

4 QKI proteins affect myelination in the CNS and PNS. In particular, QKI-6 and QKI-7 isoforms induces increased layer formation in the myelin sheaths is an original observation.